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Optimized protocols for in vitro T cell-dependent and T cell-independent activation for B cell differentiation studies using limited cells

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Abstract

Background/methods: For mechanistic studies, *in vitro* human B cell differentiation and generation of plasma cells are invaluable techniques. However, the heterogeneity of both T cell-dependent (TD) and T cell-independent (TI) stimuli and the disparity of culture conditions used in existing protocols makes interpretation of results challenging. The aim of the present study was to achieve the most optimal B cell differentiation conditions using isolated $CD19^+$ B cells and PBMC cultures. We addressed multiple seeding densities, different durations of culturing and various combinations of TD stimuli and TI stimuli including B cell receptor (BCR) triggering. B cell expansion, proliferation and differentiation was analyzed after 6 and 9 days by measuring B cell proliferation and expansion, plasmablast and plasma cell formation and immunoglobulin (Ig) secretion. In addition, these conditions were extrapolated using cryopreserved cells and differentiation potential was compared.

Results: This study demonstrates improved differentiation efficiency after 9 days of culturing for both B cell and PBMC cultures using CD40L and IL-21 as TD stimuli and 6 days for CpG and IL-2 as TI stimuli. We arrived at optimized protocols requiring 2500 and 25.000 B cells per culture well for TD and TI assays, respectively. The results of the PBMC cultures were highly comparable to the B cell cultures, which allows dismissal of additional B cell isolation steps prior to culturing. In these optimized TD conditions, the addition of anti-BCR showed little effect on phenotypic B cell differentiation, however it interferes with Ig secretion measurements. Addition of IL-4 to the TD stimuli showed significantly lower Ig secretion. The addition of BAFF to optimized TI conditions showed enhanced B cell differentiation and Ig secretion in B cell but not in PBMC cultures. With this approach, efficient B cell differentiation and Ig secretion was accomplished when starting from fresh or cryopreserved samples.

Conclusion: Our methodology demonstrates optimized TD and TI stimulation protocols for more indepth analysis of B cell differentiation in primary human B cell and PBMC cultures while requiring low amounts of B cells, making them ideally suited for future clinical and research studies on B cell differentiation of patient samples from different cohorts of B cell-mediated diseases.

Introduction

B cells are an essential arm of the adaptive immunity as their differentiation in response to foreign antigen generates protective antibodies and immunological memory (1). The process of B cell differentiation into plasmablasts and plasma cells involves profound molecular changes in morphology, phenotype and gene expression, enabling the cells to produce and secrete large amounts of immunoglobulins (Igs). B cell differentiation is initiated by activation of B cells by exposure to antigen. Classically, B cell responses are categorized in two different B cell responses dependent on the type of antigen, known as T cell-dependent (TD) and T cell-independent (TI) responses (1, 2).

In TD B cell responses, B cells are usually activated by proteinaceous antigens in the secondary lymphoid organs through recognition of their cognate antigen by the B cell receptor (BCR). Differentiation of B cells in these circumstances require T cell help in the form of CD40-CD40L costimulation with T cell-derived cytokines such as IL-4 and IL-21 (3-5). Initially, this process results in the generation of memory B cells, which can rapidly differentiate into high-affinity antibody

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producing plasma cells during secondary antigen exposure (6, 7). Secondly, long-lived plasma cells are generated that move to bone-marrow niches from where they secrete high affinity antibodies (8). These two compartments of humoral immunological memory are hallmarks of many vaccination strategies.

In TI B cell responses, B cells are activated without T cell help (9). TI antigens include multimeric antigens, like bacterial capsule polysaccharides (PS) and bacterial DNA, which can activate B cells through binding of the BCR and engagement of specific Toll like receptors (TLRs) such as TLR-4 and TLR-9 (10-13). In addition to this, multiple different cytokines, produced by multiple immune cells, can interact with their respective receptor expressed on B cells and could potentially modulate the response. TI B cell responses are short-lived and do not result in selection of affinity matured antibodies. However, these TI B cell responses have been shown to result in long-lived antibody production in specific cases (14). Although antibodies are of fundamental importance in the protection against pathogens, aberrant B cell differentiation may lead to autoimmune diseases when tolerance governed by immunological checkpoints is lost (15).

A major hurdle in the study of *in vitro* human B cell differentiation consists of the various methods described to generate *in vitro* plasmablasts and plasma cells; often TD and TI stimuli are combined while this does not closely mimic *in vivo* responses **(Table 1).** Optimal conditions are still elusive and there are many determinant factors. Thus, this study was designed to investigate the most optimal B cell differentiation conditions with regards to several essential factors, i.e. using isolated $CD19^+$ B cells or PBMC cultures, multiple seeding densities, different durations of culturing and various combinations of TD stimuli or TI stimuli. B cell expansion, proliferation and differentiation was analyzed by flow cytometry after 6 and 9 days by measuring B cell numbers, Cell Trace Yellow (CTY) dilution, $CD27^+CD38^+$plasmablast and $CD27^+CD38^+CD138^+$ plasma cell formation and immunoglobulin (Ig) secretion in culture supernatants by Enzyme Linked Immunosorbent Assay (ELISA). In addition, these conditions were extrapolated using cryopreserved cells and differentiation potential of cryopreserved and freshly isolated cells were compared. Resulting protocols are 1-step and minimalistic, ensuring that results from different labs are comparable.

Materials & Methods

Literature review

In order to identify stimuli described in previously reported B cell differentiation protocols, a literature review was carried out using the following search terms: B cell culture, B cell expansion, B cell stimulation, B cell activation, human B cell differentiation, human plasma cell differentiation using the NCBI Pubmed database (https://www.ncbi.nlm.nih.giv/pubmed). The results of this literature review is summarized in Table 1.

Cell Lines

NIH3T3 fibroblasts expressing human CD40L ($3T3\text{-}CD40L^+$) (16) were cultured in IMDM (Lonza,Basel 4002, Switzerland) containing 10% FCS (Serana, 14641 Pessin, Germany), 100 U/mL penicillin (Invitrogen, through Thermo Fisher, 2665 NN Bleiswijk, The Netherlands), 100 µg/mL streptomycin (Invitrogen), 2 mM l-glutamine (Invitrogen), 50 µM β-mercaptoethanol (Sigma Aldrich, 3330 AA, Zwijndrecht, The Netherlands) and 500 µg/mL G418 (Life Technologies, through Thermo Fisher).

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Isolation of PBMCs and B Cells from Human Healthy Donors

Buffy coats of healthy human donors were obtained from Sanquin Blood Supply. All the healthy donors provided written informed consent in accordance with the protocol of the local institutional review board, the Medical Ethics Committee of Sanquin Blood Supply, and the study conformed to the principles of the Declaration of Helsinki. Peripheral blood mononucleated cells (PBMCs) were isolated from buffy coats using a Lymphoprep (Axis-Shield PoC AS, Dundee DD2 1XA, Scotland) density gradient. Afterwards, from half of the fraction of PBMCs, $CD19^+$ B cells were isolated using magnetic Dynabeads (Invitrogen) and DETACHaBEAD (Invitrogen) according to the manufacturer's instructions. Excess cells were resuspended to $20\text{-}50*10^6$ cells per ml in culture medium and slowly cold freezing medium (80% DMSO / 20% FCS, Thermo Fisher) was added in a 1:1 ratio. The cell suspension was resuspended and divided over cryo-vials. Cells were frozen overnight to -80 ^oC in a Mr. Frosty and transferred to cryo-storage the next morning.

In Vitro PBMC and B Cell Stimulation Cultures

$3T3\text{-}CD40L^+$ were harvested, irradiated with 30 Gy and seeded in B cell medium (RPMI 1640 (Gibco, through Thermo Fisher) without phenol red containing 5% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol and 20 µg/mL human apo-transferrin (Sigma Aldrich; depleted for human IgG with protein G sepharose (GE Healthcare, 3871MV, Hoevelaken, The Netherlands) on 96-well flat-bottom plates (Nunc through Thermo Fisher) to allow adherence overnight. $3T3\text{-}CD40L^+$ were seeded at 10,000 cells per well. In some experiments PMBCs were thawed from cryo-storage and washed with B cell medium. PBMCs or B cells were rested at 37 ˚C for 1h before counting. Then, 250, 2500 or 25.000 $CD19^+$ B cells were co-cultured in duplicate in the presence or absence of PBMCs with the irradiated $3T3\text{-}CD40L^+$ fibroblasts in TD settings or in 96well U-bottom plates for TI settings. Stimuli were added as indicated: $F(ab')_2$ fragment Goat AntiHuman IgA/G/M (5 µg/mL; Jackson Immunoresearch, Ely CB7 4EZ, UK), IL-4 (25 ng/mL; Cellgenix, 79107 Freiburg, Germany), IL-21 (50 ng/mL; Peprotech, London W6 8LL, UK), CpG ODN 2006 (1 µM, Invivogen), IL-2 (50 ng/ml, Miltenyi Biotec) and BAFF (100 ng/ml R&D) for up to 9 days. After adding the B cells to the wells, the plate was centrifuged for 1 min at 400x g to force all the cells onto the $3T3\text{-}CD40L^+$ layer. Cryopreserved cells were thawed by agitating the tubes gently in a 37 ^oC waterbath until only a small ice clump was left. The cells were transferred to a 50 ml tube and cold B cell medium was added drop-wise while the tube was constantly agitated.

CelltraceYellow labeling

$CD19^+$ B cells or PBMCs were washed with 10 ml PBS/0.1% bovine serum albumin (BSA, Sigma Aldrich) and resuspended to a concentration of $2x10^7$ cells/ml in PBS/0.1%BSA. Cells and 10 µM CellTrace Yellow (Thermo Fisher Scientific) were mixed at a 1:1 ratio and incubated 20 minutes in a 37^oC waterbath in the dark, vortexing the tube every 5 minutes to ensure uniform staining. Cells were washed twice using a 10 times volume of cold culture medium to end labeling. Thereafter, B cells were cultured according to the protocol described above.

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Flow cytometry

Wells were resuspended and transferred to 96-well V-bottom plates (Nunc). Cells were centrifuged 2 min at 600x g, supernatant was transferred to V-bottom plates, sealed with an ELISA sticker and stored at -20°C. Samples were washed twice with 150 ul PBS/0.1%BSA. Cells were stained in a 25 µL staining mix with 1:1000 LIVE/DEAD Fixable Near-IR Dead cell stain kit (Invitrogen) and antibodies diluted in PBS/0.1% BSA for 20 minutes at RT in the dark. The samples were wash 2x with 150 ul PBS/0.1%BSA. Finally, the samples were resuspended in a volume of 140 µl, of which 90 µl was measured on a LSRII or FACSymphony flow cytometer. Samples were measured on LSRII or Symphony and analyzed using Flowjo software.

Ab	Conjugate	Manufacturer	Clone	Catalog No.	Dilution*
CD19	BV510	BD	SJ25-C1	562947	1:100
CD20	PerCP-Cy5.5	BD	L27	332781	1:25
CD27	PE-Cy7	eBioscience	0323	25-0279-42	1:50
CD38	V450	BD	HB7	646851	1:100
CD138	FITC	BD	MI15	561703	1:50
IgG	BUV395	BD	G17-145	564229	1:100
IgM	APC	Biolegend	MHM-88	314510	1:100
CD3	PerCP	BD	SK7	345766	1:20
LIVE/DEAD	APC-Cy7	Invitrogen		L34976	1:1000

*Optimal antibody dilutions as defined for the method and staining procedure used in this paper. As the staining conditions and flow cytometer settings may differ per lab, it is advised that these dilutions are taken as guidelines and that these are validated within each individual lab.

ELISA of culture supernatants

Supernatants of eligible conditions were tested for secreted IgG, IgA and IgM with a sandwich ELISA using polyclonal rabbit anti-human IgG, IgA and IgM reagents and a serum protein calibrator (X0908, Dako, Glostrup) all from Dako (Glostrup; productnumbers A0423, Q0332 and A0425 respectively). The polyclonal rabbit anti-human IgG, IgA and IgM were diluted in coating buffer to a concentration

of 5 µg/ml, then 100 µl was used to coat Nunc MaxiSorp flat bottom 96 well plates (Thermo Fisher) overnight at 4°C. Plates were washed with PBS/0.05% Tween- 20 and blocked with 100 µl PBS/1% bovine serum albumin (BSA) (Sigma Aldrich) for 1 hour at room temperature (RT). Plates were then washed and 100 µl of serum protein calibrator (X0908, Dako, Glostrup) or culture supernatant diluted in HPE buffer (M1940, Sanquin reagents) (1:25 for IgG and IgA and 1:30 for IgM) was added to each well and incubated for 1.5 hour at RT. Human serum protein low control (X0939, Dako, Glostrup) was used as reference sample on each plate. Following incubation and washing, 100 µl of detection antibody diluted in blocking buffer was added: poly rabbit anti-human IgG/HRP (1.3 g/L, 1:15,000), IgA/HRP (1.3 g/L, 1:15,000), and IgM/HRP (1.3 g/L, 1:10,000) (Dako, Glostrup; product numbers: P0214, P0215, P0216 respectively). Plates were washed and developed using TMB (00-4201-56, Invitrogen by Thermofisher), stopped using 1M H_2SO_4 stopping solution and read using the Biotek microplate reader (450-540nm) (Synergy HT, Biotek) and IgM, IgA and IgG concentrations were calculated relative to a titration curve of the serum protein calibrator.

Interference ELISA

The interference ELISA assay was developed as described in the sandwich ELISA above. Serial dilutions of $F(ab')^2$ fragment Goat Anti-Human IgA/G/M (5, 2.5, 1.25 and 0.625 µg/mL; Jackson Immunoresearch, Ely CB7 4EZ, UK) in HPE buffer (M1940, Sanquin reagents) were incubated (60 min, RT) with the standard curve dilutions of the serum protein calibrator (X0908, Dako, Glostrup). The results were plotted as titration curves.

Graphics

Schematic overviews were created using images from Servier Medical Art, which are licensed under a Creative Commons Attribution 3.0 Unported License (http://smart.servier.com).

Statistics

Statistical analysis was performed using GraphPad Prism (version 8; Graphpad Software). Data were analyzed using a t tests, Repeated Measures one-way ANOVA or Repeated Measures two-way ANOVA where appropriate. Results were considered significant at $p<0.05$. Significance was depicted as * ($p<0.05$) or ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$).

Results

Frequently reported B cell differentiation stimuli for human naïve and/or memory B cells in literature

The first step in establishing optimized *in vitro* protocols for TD and TI stimulation of B cells to induce B cell differentiation was to identify frequently used and reported culture conditions by literature review **(Table 1)**. Following identification of a wide range of stimuli, together with consortium partner labs, standard TD and TI combinations were chosen using concentrations reflective of the publications obtained through literature review or by previous experimental work. For TD stimuli, the combination of CD40L and IL-21 was selected, a combination frequently used to mimic $CD4^+$ T cell help (5). Although multiple methods of CD40L stimulation are reported (either soluble or using feeder cells), here a monolayer of feeder cells consisting of 3T3 mouse fibroblast expressing high levels of human CD40L was selected. For TI the combination of CpG, a well-known ligand of TLR-9, together with

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IL-2, a B cell survival factor, was set up (17, 18). These combinations of stimuli were either constantly or most frequently used and therefore found to be essential.

Following the identification of CD40L and IL-21 as TD stimuli and CpG and IL-2 as TI stimuli, the effect of **(1)** culture duration and **(2)** different seeding densities (or starting B cell numbers) were determined. These experiments were performed with cells from healthy donors either using **(3)** purified $CD19^+$ B cells or **(4)** PBMC cultures corrected for B cell count, comprising of B cells and other PBMCs (mainly T cells and small fractions of monocytes and NK cells) since such cultures do not require B cell isolation and are thus more practical for routine use. For this, PBMCs from the same healthy donors were used to avoid donor variability. Additionally, the augmenting effect of **(5)** additional stimuli, i.e. anti-BCR and IL-4 for TD, anti-BCR and BAFF for TI, was investigated and **(6)** the effect of cryopreservation on B cell differentiation potential was checked. All conditions were cultured in duplicate.

Efficient *in vitro* B cell differentiation after 9 days using T cell-dependent stimulation with CD40L and IL-21 using 2500 starting B cells

In the TD assay either 25.000, 2500 or 250 starting B cells were co-cultured with CD40L-feeder cells and IL-21 enabling 3 conditions, from now on referred to as condition I, II and III **(Fig.1A)**. Due to these settings, different ratios of B cell to feeder cell (1:0.4, 1:4 and 1:40 respectively) were created, resulting in different availability of CD40L during culturing. For each condition, B cell expansion and proliferation was assessed by flow cytometry after 6 and 9 days as well as plasmablast ($CD27^+CD38^+$) and plasma cell ($CD27^+CD38^+CD138^+$) formation. Additionally, the IgG, IgA and IgM secretion was measured by ELISA in culture supernatants, which acts as a second readout for B cell differentiation as B cells differentiate from surface Ig-expressing cells to Ig-secreting cells.

To assess expansion of B cells during culturing, the number of $CD19^+$ live B cells were determined. The conditions II and III showed significant more $CD19^+$ live B cells compared to its specific starting B cell numbers at day 6 and day 9 whilst a significant decline in $CD19^+$ live B cells in condition I was observed **(Fig.1B).** Condition II showed a 4-fold amplification (± 0.6; n=4) on day 6 and a 4-fold amplification (± 1.3; n=4) on day 9 compared to its starting B cell number **(Table 2)**. Condition III showed a 7-fold amplification (± 0.9; n=4) on day 6 and a 27-fold amplification (± 5.2; n=4) on day 9. Interestingly, in all three conditions a similar yield of $CD19^+$ live B cells was detected at day 9, but not at day 6, whilst the starting B cell numbers was different, i.e. 10-100-fold. As shown before, this suggests that the amount of available CD40L critically influences B cell survival and/or expansion during culture (19). For further FACS analysis, we set a cut-off value at a minimum of 1000 events of $CD19^+$ live B cells. To assess proliferation, B cells were labeled with Cell Trace Yellow (CTY) prior to culturing. Proliferation was observed in all conditions at day 6 **(Fig.1C)**, however conditions II and III showed a significant higher dilution of CTY in accordance with the amplification in cell numbers observed. A significant increase in the percentage of $CD27^+CD38^+$ cells between day 6 and day 9 in each condition was observed, suggesting that a 9-day culture period induced higher levels of differentiation **(Fig.1D).** However, we did not observe differences in plasma cell formation between day 6 and day 9 **(Fig.1E)** and there was no significant difference between the 3 culture conditions (statistics not shown). Measurement of secreted IgG, IgA and IgM showed a significant increase between day 6 and day 9, confirming a 9-day culture period induces higher levels of differentiation and Ig secretion **(Fig.1F).** Notably, although the yield of $CD19^+$ live B cells and phenotypically differentiated plasmablasts and plasma cells was comparable in each condition at day 9, different Ig secretion patterns between the 3 conditions was observed. When we compared the 3

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conditions in the flow cytometry analysis, significantly higher percentages of activated $CD27^{-}CD38^{+}$ plasmablast were observed in condition II and III **(Suppl.Fig.1A-B).**

In parallel experiments, PBMCs (corrected for 250, 2500 and 25.000 B cells) were cultured in similar conditions, which created conditions I.2, II.2 and III.2 **(Suppl.Fig.2A)**. Here, we observed again significantly more B cell expansion and proliferation in condition II.2 and III.2 with higher CD40L availability **(Suppl.Fig.2B-C, Table 2)**. The number of $CD19^{+}$ live cells and dilution of CTY showed similar results as in condition I, II and III suggesting limited effect of the presence of PBMCs with CD40L and IL-21 stimulation. Proliferation analysis of $CD3^{+}$ T cells did not show any increased proliferation compared to unstimulated controls, indicating that the used stimuli did not activate T cells which would influence B cell differentiation (data not shown). Again, there was no significant difference between the 3 conditions (statistics not shown) **(Suppl.Fig.2D).** No effect was observed of the presence of PBMCs on the efficacy of plasmablasts or plasma cell induction **(Fig.1D-E, Suppl.Fig.2D-E).** Ig measurements in the supernatants of the PBMC cultures showed a significant increase in IgA and IgM secretion between day 6 and 9, indicating that a 9-day culture period induces higher levels of differentiation and Ig secretion **(Suppl.Fig.2F).**

Taken together, condition I, II and III with 250, 2500, 25.000 starting B cells respectively were all suitable for generating $CD27^{+}CD38^{+}$ plasmablasts and IgG secretion at day 9, two important hallmarks of B cell differentiation. The optimal differentiation conditions for TD stimulation with limited numbers of B cells was defined here as condition II and II.2, being 2500 $CD19^{+}$ cells per 96well with or without other PBMCs, which were used for further experiments.

CD40L and IL-21 stimulation in combination with anti-BCR and/or IL-4 does not increase B cell differentiation and immunoglobulin secretion

In an attempt to drive differentiation and expansion even further in our 1-step *in vitro* B cell differentiation assay, the effect of additional stimuli in our culture conditions was tested. For this purpose, the reference stimuli CD40L and IL-21 were combined with or without $F(ab)_2$ fragments targeting IgM, IgG and IgA to induce BCR signaling (also referred to as anti-BCR). Secondly, we tested whether the addition of IL-4, a cytokine important for naïve B cells during the GC reactions, can augment *in vitro* B cell differentiation induced by CD40L and IL-21. In these cultures, 2500 freshly isolated $CD19^{+}$ B cells (condition II) or PBMCs corrected for B cell number (2500 B cells; condition II.2) were used from the same donors shown in the previous experiments. Flow cytometry was performed on day 6 and day 9 to classify $CD19^{+}$ cells as $CD27^{+}CD38^{+}$ plasmablasts and IgG, IgA and IgM secretion was measured in culture supernatants. In condition II we observed a significant increase in plasmablasts upon adding anti-BCR both on day 6 as on day 9 compared to CD40L and IL-21 alone **(Fig.2A).** In condition II.2 no combination of stimuli was superior to CD40L and IL-21 **(Fig.2B).** Prolonged culture to 9 days allowed a significant increase in plasmablasts in all 4 combinations of stimuli both in condition II and II.2 (statistics not shown). As use of the mixture of $F(ab)_2$ fragments in our cultures might interfere with the IgG, IgA and IgM ELISA assay, an interference ELISA was performed. Indeed, we observed a decrease in measured IgG, IgA and IgM when $F(ab)_2$ fragments in different concentrations were added to the standard curve **(Suppl.Fig.3A-C).** Therefore, samples containing anti-BCR stimulation were excluded for further analysis of secreted Ig. Although the percentages of plasmablasts on day 9 were similar (or higher) upon addition of IL-4, we observed significant lower secreted IgG, IgA and IgM in culture supernatants in the conditions where IL-4 was

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added **(Fig.2C-D).** In conclusion, an augmenting effect of anti-BCR on TD induced B cell differentiation was found but its use prevents Ig secretion analysis. Notably, although we observed no significant effect on plasmablast differentiation, IL-4 reduced Ig secretion under all conditions tested.

Efficient *in vitro* B cell differentiation after 6 days using T cell-independent (TI) stimulation with CpG and IL-2 with 25.000 starting B cells

In the TI assay the effect of (1) culture duration and (2) different seeding densities (or starting B cell numbers) were also determined. Again 25.000, 2500 or 250 $CD19^+$ B cells were cultured, enabling condition IV, V and VI **(Fig.3A)**. We assessed B cell differentiation by flow cytometry analysis and measurements of Ig secretion on day 6 and day 9. Culturing $CD19^+$ B cells with TI stimuli resulted in a decline in $CD19^+$ B cells **(Fig.3B, Table 2).** Flow cytometry analysis of condition V and VI showed less than 1000 events on day 6 and day 9 and these conditions were therefore excluded from further analysis. In condition IV, a significant decline in $CD19^+$ live cells was observed on day 9 compared to day 6, with two out of 4 donors not meeting the cut-off of 1000 events, thus longer culture periods under TI conditions results in lower B cell survival and/or expansion. Samples eligible for further flow cytometry analysis showed sufficient proliferation on day 6 **(Fig.3C).** There was no significant difference between day 6 and day 9 in terms of $CD27^+CD38^+$ plasmablasts, but a significant increase in $CD27^+CD38^+CD138^+$ plasma cells on day 9 **(Fig.3D-E).** Accordingly, a small increase of secreted IgG, IgA and IgM in culture supernatants was observed on day 9 **(Fig.3F).**

Extrapolation of the TI conditions to PBMC cultures enabled conditions IV.2, V.2 and VI.2 **(Suppl.Fig.4A)**. Consistent with condition IV, we observed a significant decrease in $CD19^+$ live B cells condition IV.2 **(Suppl.Fig.4B)**. Interestingly, we observed a 1.0 and 1.5-fold (± 0.8; n=3, ± 0.4; n=3) amplification of B cell numbers on day 6 and 9 in condition V.2, which provided sufficient B cell numbers for further analysis (in contrast to condition V) **(Suppl.Fig.4B, Table 2)**. As shown before, this suggests an additional pro-survival effect of PBMCs in these culture (17). Condition VI.2 did not meet de cut off of 1000 events and was also excluded from further analysis. Proliferation analysis by CTY dilution showed a significant difference in proliferation of $CD19^+$ live B cells at day 6 between conditions IV.2 and V.2 **(Suppl.Fig.4C)**. Proliferation analysis of $CD3^+$ T cells showed minimal proliferation compared to unstimulated controls in the conditions IV.2 and V.2, which suggests that the used stimuli (likely IL-2) could activate the T cells in these PBMC cultures possibly influencing B cell differentiation **(Suppl.Fig.4D)**. Further analysis showed no significant difference between day 6 and day 9 in terms of plasmablasts and plasma cells **(Suppl.Fig.4E-F)**. Despite the lack of increase in the percentages of $CD27^+CD38^+$ plasmablasts and $CD27^+CD38^+$ $CD138^+$ plasma cells between day 6

and day 9, a small increase of secreted IgG, IgA and IgM was observed in culture supernatants on day 9 in condition IV.2 and V.2 **(Suppl.Fig.4G)**. These experiments identified condition IV and condition IV.2, being 25.000 $CD19^+$ cells per well, as most suitable for the assessment of B cell differentiation using TI stimulation.

CpG and IL-2 induced B cell differentiation can be amplified by anti-BCR and BAFF stimulation

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To assess potential further assay optimization, the effect of additional stimuli to augment TI induced differentiation was investigated. For this purpose, anti-BCR stimulation with or without B cell activating factor (BAFF), another well-known survival factor and differentiation signal for B cells, were supplemented to the reference stimuli CpG and IL-2. Studies primarily done *in vitro* have shown that BAFF can be expressed by different immune cell types (including monocytes, macrophages, follicular dendritic cells), which BAFF-producing cells contribute to specific B cell responses *in vivo* is not yet understood (20-22). In these cultures, 25.000 freshly isolated $CD19^+$ B cells (condition IV) or PBMCs corrected for B cell number (25.000 B cells; condition IV.2) were used from the same donors shown in the previous experiments. We observed a small increase in plasmablasts upon addition of anti-BCR and/or BAFF both on day 6 and day 9 in condition IV but not in IV.2 suggesting that antiBCR, BAFF or a combination of both can amplify plasmablast formation in the cultures without the presence of other PBMCs **(Fig.4A-B).** Prolonged culture to 9 days did not result in higher percentages of plasmablasts in any 4 combinations of stimuli both in condition IV and IV.2 (statistics not shown). Analysis of samples without anti-BCR stimulation showed that in condition IV the addition of BAFF resulted in significant higher secreted IgG, IgA and IgM in culture supernatants, while this effect was not present in condition IV.2 where other PBMCs were present **(Fig.4C-D).** Taken together, the addition of BAFF stimulation to CpG and IL-2 augments TI induced differentiation and Ig secretion upon culturing purified B cells, but this effect is absent in PBMC cultures.

Preserved *in vitro* B cell differentiation in cryopreserved PBMCs

The decision to use fresh PBMCs or cryopreserved PBMCs for an assay or study will depend on the assay itself as well as the logistics of handling of samples. Collection of patient samples often involves freezing of samples, therefore the effect of freezing and thawing was assessed on the B cell differentiation potential in our optimized TD and TI assays. For this purpose, the B cell differentiation experiments were repeated on frozen samples of previously used healthy donors, either total PBMCs or isolated $CD19^+$ B cells from thawed PBMCs, and assessed their B cell differentiation potential by plasmablast formation using FACS and Ig secretion. Using the culturing conditions II and II.2 described above with CD40L and IL-21 stimulation, with or without IL-4, we detected a tendency to generate less $CD27^+CD38^+$ plasmablasts and subsequently lower secretion of IgG, IgA and IgM in supernatants of frozen samples after 6 and 9 days of culturing in all conditions tested, although we found no significant difference using the preferred CD40L and IL-21 stimulation **(Fig.5A-B, Suppl.Fig.5A-B).** Using the aforementioned conditions IV and IV.2 starting with 25.000 B cells and CpG and IL-2 stimulation supplemented with or without BAFF, again, we observed lower percentages of $CD27^+CD38^+$ plasmablasts and IgG, IgA and IgM secretion in culture supernatants after 6 and 9 days of culturing when compared to their matched fresh sample **(Fig.5C-D, Suppl.Fig.5C-D).** Thus, B cells obtained from cryopreserved PBMCs retain their ability to differentiate after *in vitro* culturing using TD and TI stimulation though we observed a small decrease in their differentiation potential.

Discussion

In this study we report optimized and efficient protocols for *in vitro* B cell differentiation using both TD and TI stimulation while requiring very low numbers of B cells. This has been accomplished by comparing several factors essential for optimal expansion, proliferation and differentiation of B cells, including stimulation duration, seeding density and combinations of activating stimuli reported in various publications for *in vitro* B cell differentiation. Here, we provide a 1-step culture system starting with isolated $CD19^+$ B cells or PBMCs corrected for B cell counts. We demonstrate successful generation of plasmablasts and plasma cells by measuring different parameters, including phenotypic markers (CD27, CD38 and CD138) combined with functional characteristics (IgG, IgA and IgM secretion). Despite the small decrease in differentiation efficiency when using cryopreserved samples, there are numerous reasons why using frozen PBMCs is favored over fresh samples. The two main reasons being that patient sampling is often done in outpatient clinics that are not in close proximity to laboratory facilities where cellular assays are performed. Secondly, patient cohorts are often sampled longitudinally and to prevent assay-to-assay variation, samples are stored for prolonged periods of time and thawed simultaneously. The loss in assay sensitivity in regards to differentiation may be minimized by narrowing down the time span in which all samples are handled or by taking along a known control. However, it should be noted that controls are preferentially also frozen PBMCs and handled in a comparable manner as the patient samples. The low number of required B cells determined here is ideal as patient samples are scarce and have value for multiple immunological assays. We believe that the conclusions and recommendations from this study will provide a base for optimized protocols that can be used to study patient related differences amongst patient cohorts of B cell mediated diseases and to screen compounds that target B cell differentiation.

To date, a plethora of different conditions for inducing B cell differentiation have been published **(summarized in Table 1).** The strength of the current study is the inclusion and comparison of many variables and different stimuli. However, due to study size limitations it was not possible to include and compare all previously reported stimuli. The chosen reference stimuli for the TD assay, CD40L and IL-21, mimics the *in vivo* activation and differentiation in germinal centers (GCs), where B cells interact with CD40L and IL-21 expressing follicular T helper cells (1, 23). In our experience, CD40L-expressing fibroblasts are the strongest activators of B cells by providing sufficient CD40 binding and crosslinking (data not shown). Interestingly, in both B cell and PBMC assay different kinetics of B cell expansion, proliferation and Ig secretion were observed when the ratio of starting B cell to 3T3-CD40L feeder cell was increased. Specifically, in condition III and III.2, starting with only 250 B cells, very high levels of IgM (but limited IgG) were observed on day 9, coinciding with an increased percentage of B cells with a $CD27^-CD38^+$ activated phenotype. It is important to note that comparing the three conditions in terms of absolute Ig production during the 6- and 9-day culture period has its limitations as the number of B cells during these culture periods differed to great extent and this could influence the height of Ig secretion **(Fig.1B, Suppl.Fig.2B).** In our data higher availability of CD40L resulted in increased expansion of B cells and, despite cell number differences, a higher ratio of secreted IgM compared to IgA and IgG. As many facets of B cell differentiation are linked to cell division, it is possible that the timing of isotype switching or the outgrowth of specific B cell subsets occurs differently in these culture settings. Since we studied B cell differentiation in bulk B cells and PBMCs, the specific effects of CD40L on naïve B cells, IgM^+- or isotype switched-memory cells

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cannot be distinguished. These subsets however have been shown to have different requirements for stimulation with regard to differentiation into antibody-secreting cells (24, 25). Although we cannot make firm conclusions from our data, it suggests that CD40 co-stimulation together with IL-21 regulates B cell differentiation and Ig production and that this is driven by CD40L availability. Concluding from our data, we choose 2500 B cells as the optimal starting number to not preferentially outgrow specific subpopulations or isotypes. In order to mimic the *in vivo* response to cognate antigen more closely, we added an anti-BCR trigger (26). However, we show that this hampers Ig detection. This has to be taken into account when these assays are applied for specific research questions where omitting an anti-BCR trigger is not desirable. The same holds true for adding IL-4 to the assays. Previously, we have shown that IL-4 addition is beneficial for B cell differentiation of naïve B cells but only in circumstances with low CD40L stimulation (19). In accordance with other studies using total $CD19^+$ B cells, we show that continuous IL-4 in our assay hampers Ig secretion compared to CD40L and IL-21 alone, indicating a lack of commitment to antibody secretion (5). We do not recommend using IL-4, however, for specific questions regarding pre-GC B cell priming some studies might want to use IL-4 nonetheless.

To mimic *in vivo* TI responses, the most commonly used stimulation is TLR-9 activation through CpG, mimicing antigen activation **(Table 1)**. Activation with CpG induces proliferation of both human naïve and memory B cells (data not shown), whilst the differentiation of naïve B cells is only observed in cultures where PBMCs are present or T cell derived cytokines such as IL-2 are supplemented (17, 27). Adding to this, *in vivo* TI stimulation has been shown to result in long-lived plasma cell generation (14). This together indicates that though direct T-B interactions may not be required, a supportive microenvironment may be crucial to gain plasmablast fate and sustain plasma cell generation in TI responses *in vitro*. Condition IV with 25.000 starting B cells was identified as a minimum when stimulating isolated $CD19^+$ B cells with CpG and IL-2 due to limited B cell survival in this culture. Considering the significant decrease of $CD19^+$ B cells between day 6 and day 9 in condition IV, we do not recommend culturing longer than 6 days, although higher amounts of immunoglobulins can be measured with a longer culture period. Re-stimulation of cells can be opted for, but this was not investigated in this study. Finally, compared to isolated B cell cultures, PBMC TI cultures showed better survival of B cells (condition IV and V versus IV.2 and V.2 **(Table 2)**. Although the microenvironment provided by PBMCs may support survival there was no observation of increased differentiation.

BAFF protein is expressed by myeloid lineage cells and acts as both cell surface–associated and soluble forms (28, 29). BAFF has been shown to activate class switch recombination in human B cells, which can be enhanced by BCR crosslinking (30). Ever since, research groups have used BAFF in B cell differentiation assays but most frequently in combinatorial use with CD40 stimulation, preventing the dissection of their individual effects. In the current study, limited effects of BAFF, in addition to anti-BCR stimulation, were found on plasmablasts formation. Interestingly, a donordependent effect of BAFF stimulation on the Ig secretion was observed. Healthy donors, and patients, possibly differ in their expression of BAFF-responding receptors at baseline. Furthermore, because activated monocytes and T cells can also express BAFF-responding receptors, it raises the possibility that in the PBMC cultures the addition of BAFF will stimulate the rest of the PBMCs rather than the B cells. Finally, as monocytes are known to increase BAFF secretion upon TLR-9 stimulation, it is possible that these cells were already supplying sufficient BAFF to the B cells within the PBMC TI cultures (20). Altogether, although no detrimental effects of BAFF on B cell differentiation was observed in our assay, using

BAFF should be complemented with appropriate analysis of its compliant receptors at baseline and throughout the assay.

The data presented here shows that for the TD condition stimulating as little as 2500 $CD19^+$ B cells with CD40L and IL-21 results in significant expansion, differentiation and secretion of IgM, IgA and IgG. For specific purposes, even lower cell numbers can be used. Interestingly, IL-4 did not affect differentiation but did significantly reduce antibody secretion. For studying TI responses, stimulating 25.000 $CD19^+$ B cells with CpG and IL-2 results in proliferation, differentiation and IgM, IgA and IgG production. We do not recommend using lower cell numbers for this condition. Interestingly, addition of BAFF resulted in significant increases of IgM, IgA and IgG production in TI $CD19^+$ B cell cultures. However, this effect is absent in PBMC cultures. Furthermore, we show that both these protocols can be performed with PBMC cultures, omitting the need for B cell isolation and thus making them highly suitable for clinical research. We do however recommend that B cell numbers are corrected using measured B cell percentages, after thawing, as these percentages are variable between donors.

In conclusion, it is still an active area of investigation to define how autonomous factors control TD and TI responses in healthy donors or patients with B cell mediated diseases. Future research is needed to define these autonomous factors and address signaling pathways involved in both beneficial and unwanted plasma cell development. Comparing patients and healthy donors in optimized cultures and assays that detect gene expression and post-translational modifications such as phosphorylation or ubiquitination by intracellular staining methods (31) may aid in these research questions. The TD and TI assay described here in condition II (and II.2), being 2500 $CD19^+$ B cells stimulated with CD40L and IL-21, and condition IV (and IV.2), being 25.000 $CD19^+$ B cells stimulated with CpG, IL-2 and possibly BAFF, supports efficient differentiation of human primary B cells into plasma cells, with warranted B cell expansion, proliferation and quantifiable production of IgG, IgA and IgM. Due to the minimalistic nature of the protocols, results from different labs and facilities will be highly comparable. These assays will allow in-depth dissection of B cell differentiation pathways in B cells of healthy individuals and patients.

Authorship contributions:

J.K., C.M., D.V. designed experiments. C.M., D.V. performed experiments and analyzed data. T.R. and A.T.B. critically revised the manuscript. S.M.H. and T.W.K. devised the concept, supervised data interpretation and critically revised the manuscript. The manuscript was revised and approved by all authors.

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Conflicts-of-interest disclosure:

The authors declare that this research was conducted in the absence of commercial or financial relationships that could be construed as a potential conflict of interest.

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633

634 **TABLES**

635 **Table 1. Frequently reported B cell differentiation stimuli for human naïve and/or memory B cells in literature**

636

Stimuli	Target(s)	Concentrations	Reference
Anti-IgM anti-IgG/IgM F(ab')2 anti-IgG/IgA/IgM F(ab')2	BCR	2, **5**, 10 µg/ml	(5), (17), (19), (27), (32), (33), (34), (35)
BAFF	BAFF-R. BMCA. TACI	75, **100** ng/ml	(32), (36)
aCD40, CD40L	CD40	50, 500 ng/ml 1, 5 µg/ml	(5), (17), (27), (34), (35), (37), (38), (39)
CD40L expressing L cells 3T3-CD40L fibroblasts	CD40	Various ratios of B cell : feeder cell	(3), (19), (32), (33)
CpG-ODN 2006	TLR9	1.0, 2.5, 3.2, 6.0, 10 µg/ml 0.35, **1.0** µM	(17), (27), (32), (34), (35), (36), (37), (38), (39)
IFNα	IFNAR	100, 500 U/ml	(33), (37)
IL-2	IL-2R	20, 50, 100 U/ml 25, **50** ng/ml	(3), (5), (33), (34), (35), (37), (38)
IL-4	IL-4R	10, **25**, 50, 100, 200 ng/ml	(3), (5), (17), (19), (32), (34), (37), (39)
IL-6	IL-6R	10, 50 ng/ml	(33), (37)
IL-10	IL-10R	25, 50, 200 ng/ml	(5), (32), (34), (37), (39)
IL-15	IL-15R	10 ng/ml	(32), (37)

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IL-21	IL-21R	2, 20, **50**,100 ng/ml	(5), (19), (27), (32), (33), (35), (36), (38), (39)
Abbreviations: BAFF-R; B cell activating factor, BCMA; B cell maturation antigen; IFN; interferon, IL; interleukin, ODN; oligodeoxynucleotide, TACI (TNFRSF13B); transmembrane activator and CAML interactor, TLR; toll like receptor. The used concentrations in this study are depicted in **bold**.			

637

638 **Table 2. B cell survival and proliferation during different B cell differentiation conditions**

Isolated CD19⁺ B cells (TD)						
	Condition I		**Condition II**		**Condition III**	
Starting B cell number	25.000		2500		250	
3T3-CD40L cell	10.000		10.000		10.000	
Ratio B cell : 3T3-CD40L cell	1 : 0.4		1 : 4		1 : 40	
Cytokines	IL-21		IL-21		IL-21	
Mean CD19⁺ live cells						
Day 0	25.000		2500		250	
Day 6	12438	± 3120; n=4	9736	± 1566; n=4	1656	± 224; n=4
Day 9	8012	± 2643; n=4	8899	± 3196; n=4	6769	± 1305; n=4
Mean amplification (compared to starting B cell number)						
Day 6	0.5	± 0.1; n=4	3.9	± 0.6; n=4	6.6	± 0.9; n=4
Day 9	0.3	± 0.1; n=4	3.6	± 1.3; n=4	27.1	± 5.2; n=4
PBMCs (TD)						
	Condition I.2		**Condition II.2**		**Condition III.2**	
Starting B cell number	25.000		2500		250	
3T3-CD40L cell	10.000		10.000		10.000	
Ratio B cell : 3T3-CD40L cell	1 : 0.4		1 : 4		1 : 40	
Cytokines	IL-21		IL-21		IL-21	
Mean CD19⁺ live cells						

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Day 0	25.000		2500		250	
Day 6	8601	± 1397; n=3	14487	± 4320; n=3	4139	± 311; n=3
Day 9	6019	± 695; n=3	7819	± 2713; n=3	12826	± 3750; n=3
Mean amplification (compared to starting B cell number)						
Day 6	0.3	± 0.1; n=3	5.8	± 1.7; n=3	16.6	-
Day 9	0.2	± 0.1; n=3	4.2	± 1.1; n=3	51.3	-
Isolated CD19⁺ B cells (TI)						
	Condition IV		**Condition V**		**Condition VI**	
Starting B cell number	25.000		2500		250	
TLR ligand	CpG		CpG		CpG	
Cytokines	IL-2		IL-2		IL-2	
Mean CD19⁺ live cells						
Day 0	25.000		2500		250	
Day 6	4751	± 1397; n=4	85	± 22.7; n=4	17	± 2.3; n=4
Day 9	1571	± 695; n=4	29	± 13.8; n=4	2	± 0.8; n=4
Mean amplification (compared to starting B cell number)						
Day 6	0.2	± 0.1; n=4	-	-	-	-
Day 9	0.1	± 0.0; n=4	-	-	-	-
PBMCs (TI)						
	Condition IV.2		**Condition V.2**		**Condition VI.2**	
Starting B cell number	25.000		2500		250	
TLR ligand	CpG		CpG		CpG	
Cytokines	IL-2		IL-2		IL-2	
Mean CD19⁺ live cells						
Day 0	25.000		2500		250	
Day 6	8978	± 1985; n=3	3823	± 1983; n=3	58	± 18; n=3
Day 9	7841	± 2127; n=3	2463	± 1055; n=3	47	± 26; n=3
Mean amplification (compared to starting B cell number)						
Day 6	0.4	± 0.1; n=3	1.5	± 0.8; n=3	-	-

Day 9	0.3	± 0.1; n=3	1.0	± 0.4; n=3	-	-

Purified B cells or non-purified B cells (PBMC cultures) were cultured using a 1-step culture system for 6 and 9 days with either TD (CD40L + IL-21) or TI (CpG + IL-2) stimuli. On day 6 and day 9 cell counts and viability were determined using flow cytometry with fluorochrome-conjugated Live/Dead and anti-CD19. Results are shown as the mean ± SEM of n = 4 (B cell cultures) or n = 3 (PBMC cultures) donors. – indicates no further analysis due to <1000 $CD19^+$ live events.

Figure Legends

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Figure 1

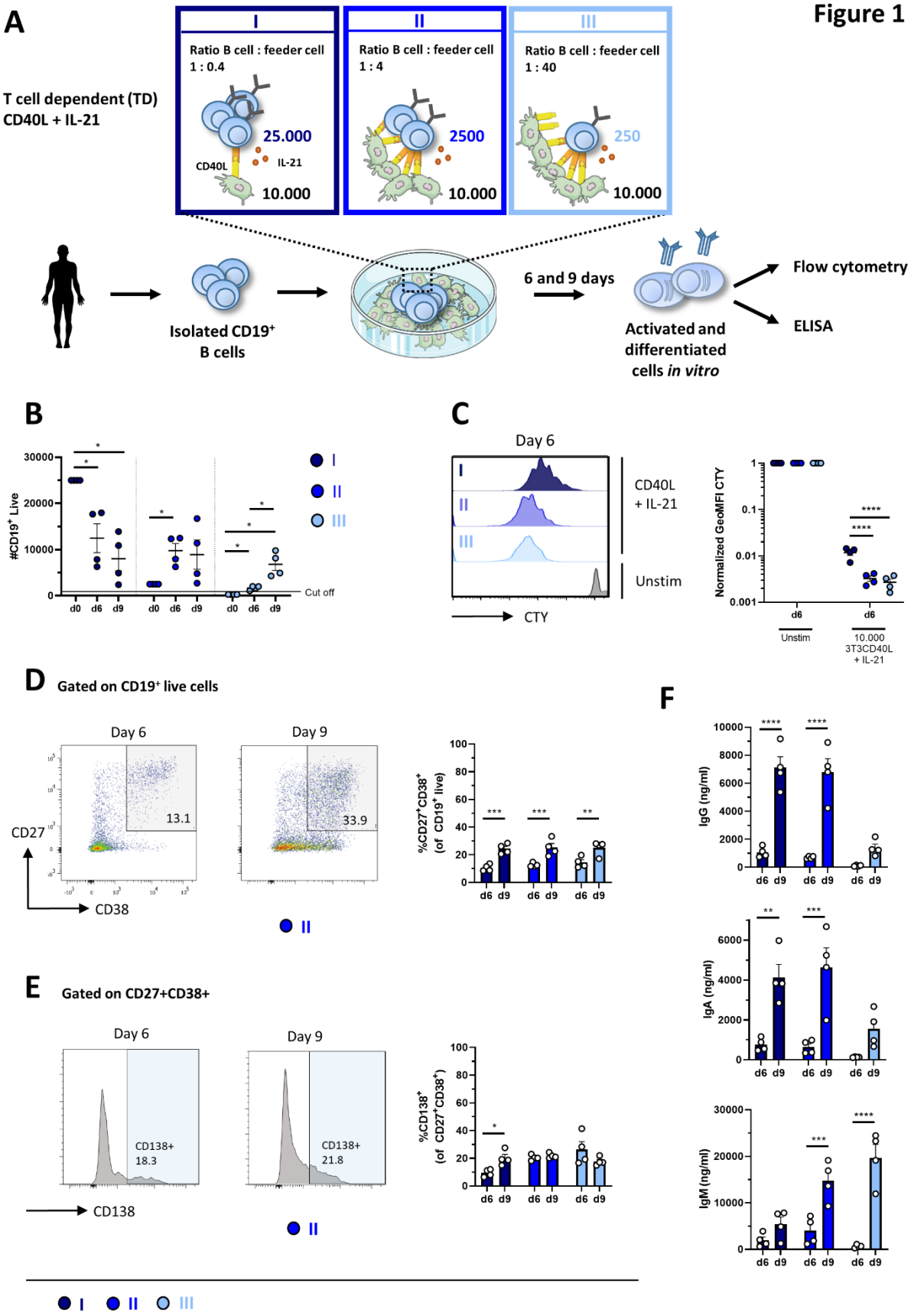

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Figure 1. Proliferation, differentiation and antibody production after T cell dependent in vitro stimulation and culturing of low numbers of primary human CD19$^+$ B cells.

(A) Schematic overview of the T cell dependent (TD) culture system to induce B cell differentiation. A total of 250, 2500 or 25000 CD19$^+$ human B cells (n = 4) were stimulated with a human-CD40Lexpressing 3T3 feeder layer and recombinant IL-21 (50 ng/mL) enabling condition I (dark blue), II (cobalt blue) and III (light blue). Cells were analyzed at day 6 and day 9 by flow cytometry to evaluate **(B)** number of live CD19$^+$ events, (**C**) amount of proliferation by CTY dilution and frequency of **(D)** plasmablast (CD27$^+$CD38$^+$ B cells) and **(E)** plasma cell (CD27$^+$CD38$^+$CD138$^+$ B cells). A cut off of 1000 events was used to proceed with further analysis. (**F**) The supernatant was collected at day 6 and day 9 to evaluate IgG, IgA and IgM production by ELISA (n = 4). Each data point represents the mean of an individual donor with duplicate culture measurements. Mean values are represented by bars and the error bars depict SEM. P values were calculated using two-way ANOVA with Sidak's multiple comparison test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

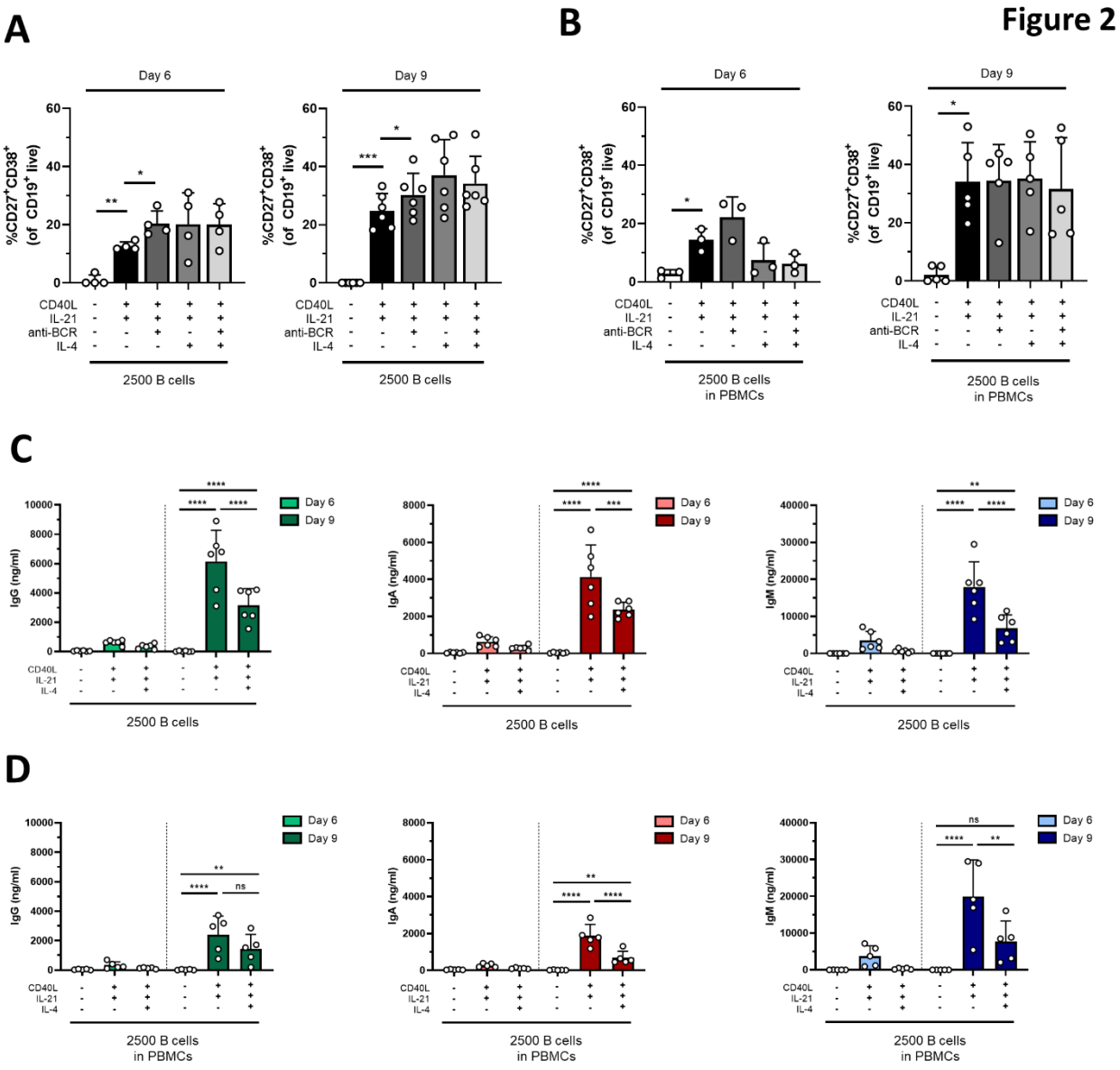

Figure 2. Addition of anti-BCR stimuli in a T cell dependent stimulation results in a modest increase of B cell differentiation while IL-4 severely inhibits antibody production

Human primary B cells obtained from healthy donors were stimulated under conditions described in Figure 1A (condition II) and Suppl. Fig. 2A (condition II.2, PBMC cultures) with or without antiBCR (anti-Ig F(ab)2 mix (5 µg/mL) targeting IgM, IgG and IgA) and/or recombinant IL-4 (25 ng/mL). Frequencies of $CD27^{+}CD38^{+}$ B cells on day 6 and day 9 in **(A)** condition II and **(B)** condition II.2 (n = 4-6). **(C-D)** Total secretion of IgG, IgA and IgM measured in culture supernatants of eligible conditions after 6 and 9 days **(C)** without PBMCs (condition II) and **(D)** within PBMC cultures (condition II.2). Each data point represents the mean of an individual donor with duplicate culture

measurements. Mean values are represented by bars and the error bars depict SEM. P values were calculated using one-way ANOVA with Dunnett's multiple comparison test (A-B) or two-way ANOVA with Sidak's multiple comparison test (C-D). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

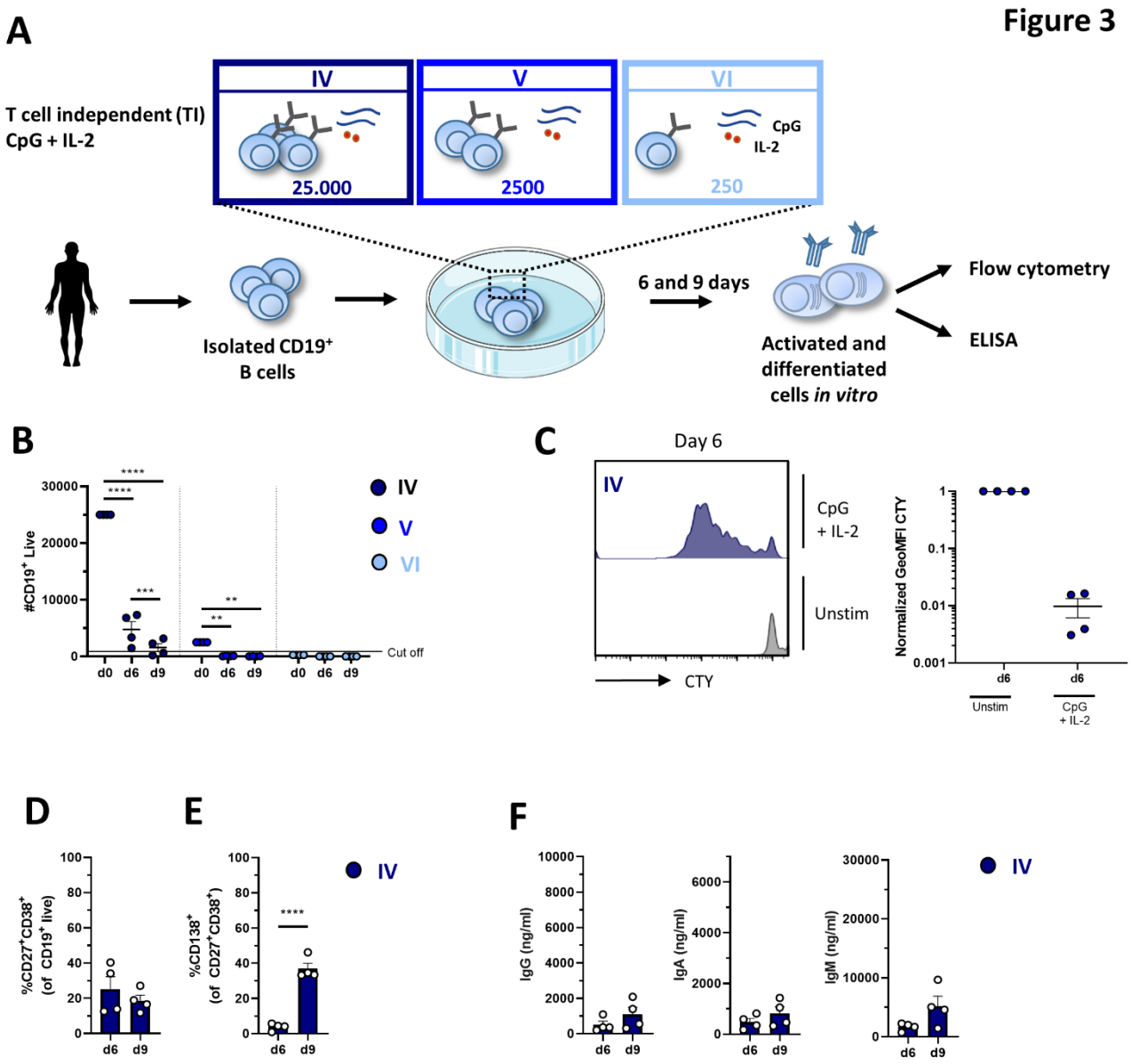

Figure 3. Proliferation, differentiation and antibody production after T cell independent in vitro stimulation and culturing of low numbers of primary human CD19+ B cells.

(A) Schematic overview of the T cell independent (TI) culture system to induce B cell differentiation.

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A total of 250, 2500 or 25000 $CD19^+$ human B cells (n = 4) were stimulated with CpG (1 µM) and IL-2 (50 ng/ml) enabling condition IV (dark blue), V (cobalt blue) and VI (light blue). Cells were analyzed at day 6 and day 9 by flow cytometry to evaluate (**B**) number of live $CD19^+$ events, (**C**) amount of proliferation by CTY dilution and frequency of **(D)** plasmablast ($CD27^+CD38^+$) and (**E**) plasma cell ($CD27^+CD38^+CD138^+$) generation. A cut off of 1000 events was used to proceed with further analysis. (**F**) The supernatant was collected at day 6 and day 9 to evaluate IgG, IgA and IgM production by ELISA (n = 4). Each data point represents the mean of an individual donor with duplicate culture measurements. Mean values are represented by bars and the error bars depict SEM. P values were calculated using two-way ANOVA with Sidak's multiple comparison test (B) or unpaired t test (D-F). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 4

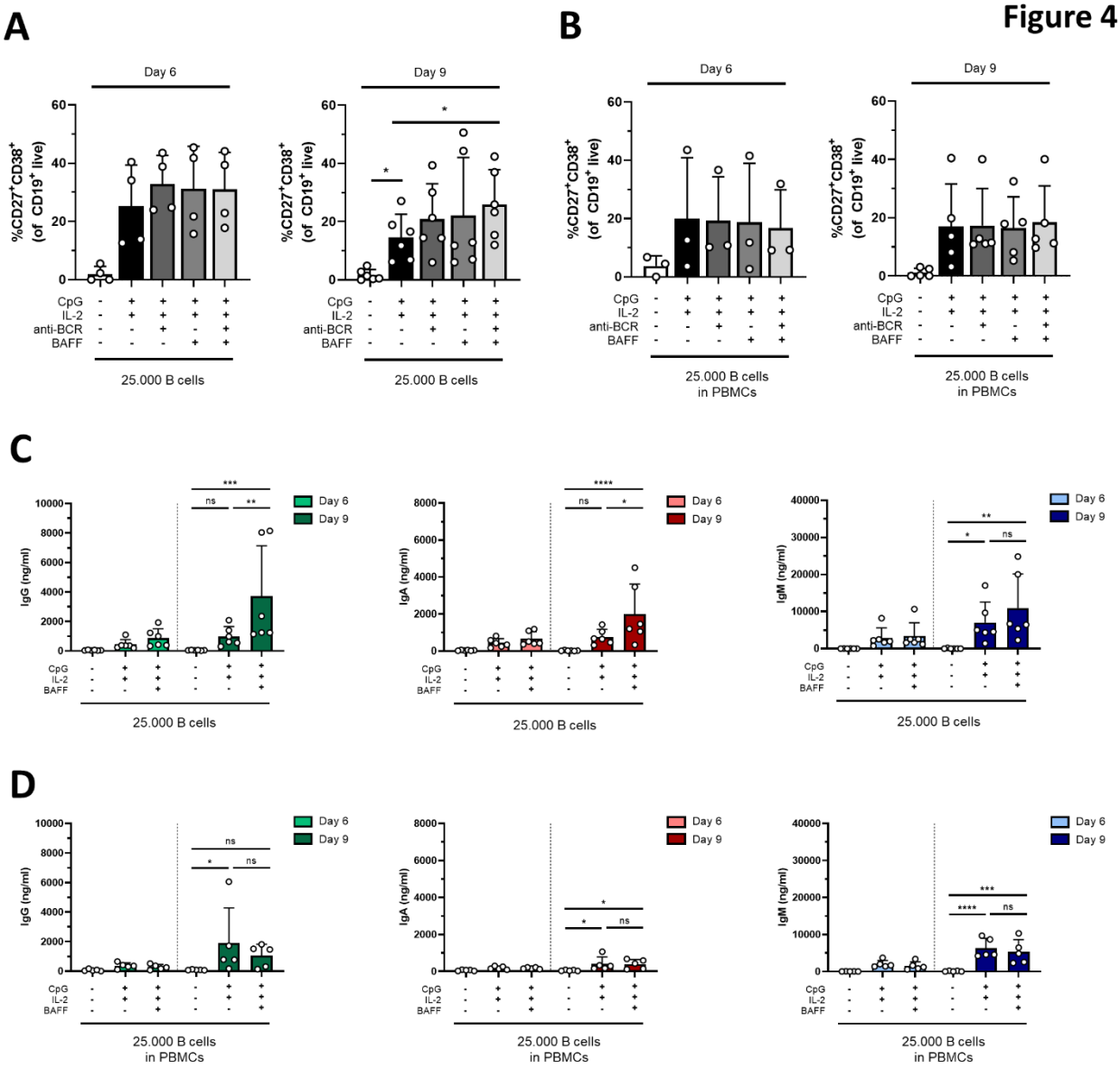

Figure 4. Addition of BAFF in a T cell independent stimulation results in increased IgG and IgA production in isolated B cell cultures

Human primary B cells obtained from healthy donors were stimulated under conditions described in Figure 3A (condition IV) and Suppl. Fig. 4A (condition IV.2, PBMC cultures) with or without antiBCR (anti-Ig F(ab)2 mix (5 µg/mL) targeting IgM, IgG and IgA) and/or BAFF (100 ng/mL). Frequencies of $CD27^{+}CD38^{+}$ B cells on day 6 and day 9 in **(A)** condition IV and **(B)** condition IV.2 (n = 3-5). **(C-D)** Total secretion of IgG, IgA and IgM measured in culture supernatants of eligible conditions after 6 and 9 days **(C)** without PBMCs (condition IV) and **(D)** as PBMC culture (condition IV.2). Each data point represents the mean of an individual donor with duplicate culture measurements. Mean values are represented by bars and the error bars depict SEM. P values were calculated using one-way ANOVA with Dunnett's multiple comparison test (A-B) or two-way ANOVA with Sidak's multiple comparison test (C-D). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

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Figure 5

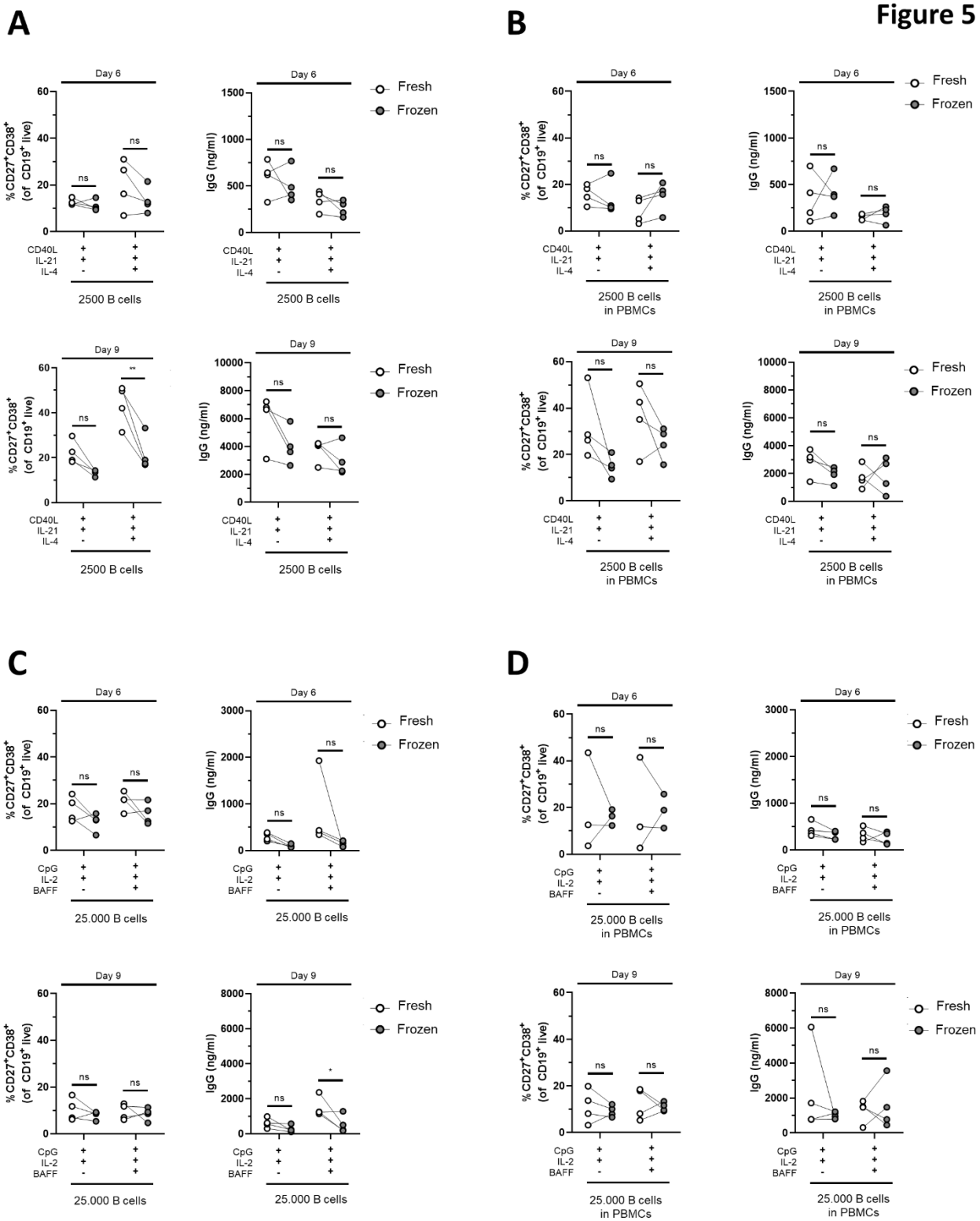

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Figure 5. Cryopreserved B cells respond similarly to freshly isolated cells in T cell dependent and independent assays

Total human B cells were isolated from fresh PBMCs (indicated in white) or frozen PBMCs (indicated in gray) and cultured for 6 and 9 days (n=4). **(A-B)** Using T cell dependent (TD) stimuli (CD40L and IL-21 with/without IL-4) 2500 B cells (fresh and frozen) were cultured under conditions described previously **(A)** without PBMCs (condition II) and **(B)** as PBMC culture (condition II.2). Frequencies of $CD27^{+}CD38^{+}$ B cells (left panel) and IgG production (right panel) on day 6 (upper graphs) and day 9 (lower graphs) are shown. **(C-D)** Using T cell independent (TI) stimuli (CpG and IL-2 with/without BAFF) 25000 B cells (fresh and frozen) were cultured under conditions described previously **(C)** without PBMCs (condition IV) and **(D)** with PBMCs (condition IV.2). Frequencies of $CD27^{+}CD38^{+}$ B cells (left panel) and IgG production (right panel) on day 6 (upper graphs) and day 9 (lower graphs) are shown. Each data point represents the mean of an individual donor with duplicate culture measurements. Mean values are represented by bars and the error bars depict SEM. P values were calculated using two-way ANOVA with Sidak's multiple comparison test. * $P \leq 0.05$.

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