OPTIMIZATION OF HUMAN B CELL CULTURE CONDITIONS FOR EXPANSION OF ACTIVATED OR DIFFERENTIATED B CELLS

Sokolova SR, Grigorova IL✉

Pirogov Russian National Research Medical University, Moscow, Russia

In vitro B cell cutures are important for fundamental and translational research and can be used to study antigenic specificity of B and T cells, as well as to produce monoclonal antibodies and other biopharmaceuticals. That is why the development of an optimal protocol for culturing of activated B cells, antibody-secreting cells (ASCs), and germinal center (GC) B cells *in vitro* remains an important task. The study aimed to find the conditions ensuring the following: high B cell expansion and survival rates, ASC accumulation, GC B cell production and accumulation. For that the $CD27^-$ and/or $CD27^+$ B cells from human peripheral blood were cultured in the presence of the feeder 3T3-hCD40L line, various combinations of cytokines (IL21, IL4, BAFF), human serum components or under the control conditions throughout 7 days. Flow cytometry analysis of B cell cultures showed that the combined presence of CD40L and IL21 was essential for achieving high B cell expansion, survival, and differentiation with the production of the $CD27^{high}CD38^{high}$ ASCs and $CD95^{high}Bcl\text{-}6^+$ GC-like B cells. The highest expansion was observed in the cultures of CD27- naïve cells in the presence of human serum components. The IL4 supplementation moderately increased the share of GC-like B cells. The maximum ASC accumulation was observed in the cultures of $CD27^+$ memory B cells. The approach developed made it possible to find the optimal conditions for *in vitro* B cell culturing and clearly demonstrated the impact of both individual IL-21, IL-4, BAFF cytokines and their combinations on the B cell cultures of various subpopulations.

Keywords: B cells, antibody-secreting cells (ASCs), germinal center (GC) B cells, human lymphocytes cultures *in vitro*, 3T3-hCD40L

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Pirogov Russian National Research Medical University (protocol No. 240 date 23 May 2024). All subjects signed the informed consent for participation in the study.

✉ **Correspondence should be addressed:** Irina L. Grigorova
Ostrovityanova, 1, Moscow, 117997, Russia; grig76@gmail.com

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ОПТИМИЗАЦИЯ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ В-КЛЕТОК ЧЕЛОВЕКА ДЛЯ ЭКСПАНСИИ АКТИВИРОВАННЫХ ИЛИ ДИФФЕРЕНЦИРОВАННЫХ В-КЛЕТОК

С. Р. Соколова, И. Л. Григорова ✉

Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия

Культуры В-клеток *in vitro* имеют важное значение для фундаментальной и прикладной науки: они могут быть использованы для изучения антигенной специфичности В- и Т-клеток, а также для получения моноклональных антител и других биопрепаратов. Поэтому создание оптимальных протоколов культивирования активированных В-клеток, антитело-секретирующих клеток (АСК) и В-клеток герминативных центров (ГЦ) *in vitro* остается актуальной задачей. Целью работы было подобрать условия, обеспечивающие: высокий уровень экспансии и выживаемости В-клеток, накопление АСК, образование и накопление В-клеток ГЦ. Для этого В-клетки $CD27^-$ *и/или* $CD27^+$ из периферической крови человека культивировали в присутствии фидерной линии 3T3-hCD40L, различных комбинаций цитокинов (IL21, IL4, BAFF), компонентов человеческой сыворотки или в контрольных условиях в течение 7 дней. Цитофлуориметрический анализ В-клеточных культур показал, что совместное присутствие CD40L и IL21 необходимо для достижения высокой экспансии, выживаемости и дифференцировки В-клеток с образованием $CD27^{high}CD38^{high}$ АСК и $CD95^{high}Bcl\text{-}6^+$ ГЦ-подобных клеток. Наибольшая экспансия наблюдалась в культурах из CD27- наивных клеток в присутствии компонентов человеческой сыворотки, добавление IL4 умеренно повышало долю ГЦ-подобных клеток. Максимальное накопление АСК наблюдалось в культурах из $CD27^+$-В-клетки памяти. Разработанный подход позволил подобрать оптимальные условия для культивирования В-клеток *in vitro*, а также наглядно продемонстрировал влияние как отдельных цитокинов IL-21, IL-4, BAFF, так и их комбинаций на В-клеточные культуры из различных субпопуляций.

Ключевые слова: В-клетки, антитело-секретирующие клетки, АСК, В-клетки герминативных центров, ГЦ, культуры лимфоцитов человека *in vitro*, 3T3-CD40L

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✉ **Для корреспонденции:** Ирина Леонидовна Григорова
ул. Островитянова, д. 1, г. Москва, 117997, Россия; grig76@gmail.com

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B cells and the antibodies they produce provide an effective immune response to pathogens and vaccines. Through antigen-dependent differentiation, naive B cells differentiate to antibody-secreting cells (ASCs) and memory cells [1]. In addition to providing a humoral immune response, B cells can function as antigen-presenting cells (APCs) [2]. Upon binding of antigen to B cell receptor (BCR), the complex is internalized by B cells and the processed peptides are then presented on their surface in a complex with MHCII for subsequent interaction with $CD4^+$ T helper (Th) cells [3]. Key signals to B cells from cellular cooperation with Th cells include CD40-CD40L interaction as well as secreted cytokines IL-21 and IL-4 [4]. Upon acquisition of T cell help B cells undergo proliferation and differentiate either into short-lived ASCs or germinal center (GC) B cells (a fate driven by master transcription factor BCL6) [5, 6].

The unique properties of B cells make the *in vitro* cell cultivation critical for various applications such as obtaining APCs for analyzing antigen-specific T-cell responses as well as ASCs for antibody production [7, 8], and creating cell models for studying lymphomas [9]. Numerous studies have investigated how CD40L, T-cell cytokines and microenvironment factors such as BAFF influence B-cell cultures to promote proliferation, class switching, and antibody-secreting cell (ASC) formation [10–12]. However, studies on *in vitro* $BCL6^+$ GC B cell formation from human B cells are still limited. The aim of the work was to find optimal conditions to expand B cells and to efficiently turn them into antibody-secreting cells (ASCs) or germinal center (GC) cells *in vitro*.

In our study, we analyzed human peripheral blood naive B cells and $CD27^+$ memory B cells cultured with T-cell signals (CD40L, IL-21, IL-4), BAFF, and additional medium components. We also assessed B-cell expansion, and accumulation of ASC and $BCL6^+$ B cells.

METHODS

pcDNA-hCD40LG Plasmid Design

Human CD40LG gene sequence (NM_000074.3) was obtained using polymerase chain reaction (PCR) from total cDNA of human peripheral blood mononuclear cells (MC) using forward 5'-ATATGGATCCGCCACCATGATCGAAACATACAACCA-3' and reverse 5'-ATATGAATTCACACTGTTCAGAGTTTGAGT AAGCC-3' primers. The primer sequences included BamHI and EcoRI restriction sites at their 5' and 3' ends of CD40LG gene, respectively, as well as Kozak sequence (GCCACCATG) at the 5' end of the gene. After digestion of PCR product and pcDNA3.1+ vector (Thermo Fisher Scientific, USA, Cat. No. V79020) using restriction endonucleases BamHI-HF and EcoRI-HF (New England Biolabs, USA, Cat. Nos. R3136 and R3101), they were ligated using the Quick Ligation™ Kit (New England Biolabs, USA, Cat. No. M2200) and transformed into *E. coli* (strain NEB Stable, New England Biolabs, USA, Cat. No. C3040H). The plasmid was purified and isolated using the Plasmid Miniprep 2.0 kit (Evrogen, Russia, Cat. No. BC221) for further transfection.

Generation of a Mouse Fibroblast Feeder Line Expressing Human CD40L (3T3-hCD40L)

3T3-hCD40L feeder line was generated from NIH 3T3 mouse fibroblast cell line from the cell culture collection of the D. I. Ivanovsky Institute of Virology. NIH 3T3 cells were cultured in DMEM (PanEco, Russia, Cat. No. C415p) supplemented with 10% fetal bovine serum (FBS, STEMCELL Technologies, Canada, Cat. No. 06472) and 1× penicillin/streptomycin antibiotic mixture (PanEco, Russia, Cat. No. A065p). Cells were passaged every 3-4 days. To generate NIH 3T3 clones stably expressing hCD40L, cells were transfected with pcDNA-hCD40LG plasmid using Lipofectamine® 3000 reagent (Thermo Fisher Scientific, USA, Cat. No. L3000015) according to manufacturer's instructions, followed by clone selection in DMEM medium supplemented with geneticin G418 (0.5 mg/ml; Thermo Fisher Scientific, USA, Cat. No. 10131027). Monoclonal cell lines were generated by limiting dilutions. CD40L expression was assessed by flow cytometry using anti-CD154-FITC antibodies (1:20; clone TRAP1, BD Bioscience, USA, Cat. No. 561721). The monoclonal cell line with the highest CD40L expression level was used in the study (Fig. 1A).

Isolation of Mononuclear Cells (MC) from the Peripheral Blood of Healthy Donors

The study was conducted in accordance with the 2013 Declaration of Helsinki. Patient exclusion criteria were (a) infectious diseases in the acute or recovering stage; (b) autoimmune or chronic diseases. Peripheral blood from healthy donors was collected in vacuum tubes containing K3EDTA as anticoagulant. A total of four independent, unrelated donors, aged 21 to 65 years (median 39 years), participated in the study. The male to female ratio was 1:1. MCs were isolated on a Ficoll gradient (PanEco, Russia, Cat. No. P050E) in accordance with the protocol [13]. The obtained cells were stored in cooled cell sorting buffer (0.5% FBS in Dulbecco's phosphate buffered saline (DPBS)). Cells were counted using a CytoSMART Cell Counter (Corning, USA) using trypan blue. Cell viability was > 95%.

B cell Staining for Flow Cytometry Analysis (FACS)

B cell analysis was performed by flow cytometry using antibodies to the cell surface markers CD19, CD27, CD38, CD95 (Fas) as well as the intracellular transcription factor BCL6. Cells were stained and stored at 4 °C. For sorting of all B cells ($CD3^-CD19^+CD20^+$), 20 × 10^6 MCs were pre-incubated in blocking solution with addition of 10 μg/ml Human Fc-block (clone K112-91, BD Biosciences, USA, cat. no. 564220) for 10 min on ice, after which they were stained with a mixture of antibodies anti-CD3-R718 (1 : 100, clone SK7; BD Biosciences, USA, cat. no. 751978), anti-CD19-BV510 (1 : 100, clone SJ25C1; BD Biosciences, USA, cat. no. 562947), anti-CD20-FITC (1:20, clone L27; BD Biosciences, USA, cat. 347673) MCs were incubated for 30 min on ice in the dark. To sort $CD3^-CD19^+CD20^+CD27^-$ naive B cells and $CD3^-CD19^+CD20^+CD27^+$ memory B cells, MCs were additionally stained with anti-CD27-PerCP-Cy5.5 (1:50, clone M-T271; BioLegend, USA, cat. no. 356408). After the staining, the cells were resuspended in sorting buffer. To assess the phenotype of B cell cultures on day 7, the supernatant with B cells was collected from the wells without subsequent trypsinization. The selected cells were resuspended in a blocking buffer and then stained with the following antibody mixture: anti-CD19-BV510 (1:100), anti-CD27-PerCP-Cy5.5 (1:50), anti-CD38-APC-R700 (1:50, clone HIT2; BD Biosciences, USA, cat. no. 564979), anti-CD95-PE (1:100, clone DX2; BioLegend, USA, cat. no. 305608).

Prior to flow cytometry analysis, the cells were stained with Helix NP Blue at a concentration of 25 nM (Biolegend, USA, cat. no. 425305) to exclude dead cells. Intracellular staining of cells was performed using the True-Nuclear™ Transcription Factor Buffer Set (BioLegend, USA, cat. No. 424401) and anti-BCL-6-AlexaFluor488 antibodies (1:20, clone: K112-91;

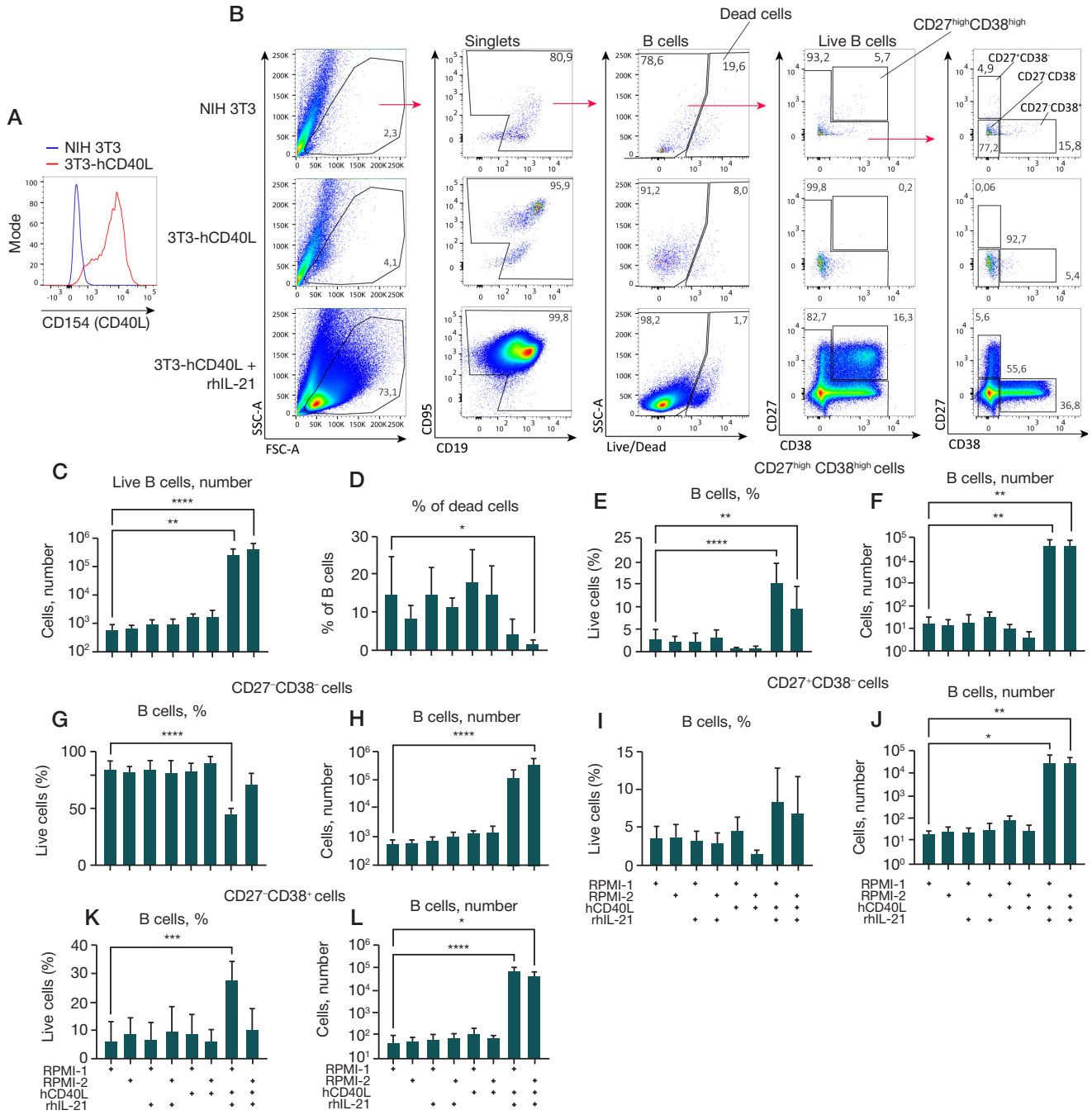

Fig. 1. Analysis of effect of a feeder line expressing rhCD40L, cytokine IL-21, and culture medium composition on the growth of B cell culture. The feeder line 3T3-hCD40L was obtained by transfecting the NIH 3T3 mouse fibroblast line with the pcDNA3.1-hCD40L plasmid to obtain a cell line stably expressing the transgene and subsequent selection of a monoclone with the highest level of hCD40L. 1.5 × 10^4 ($CD19^+$ $CD20^+$) B cells obtained as a result of fluorescence sorting from human peripheral blood PBMCs were cultured on the feeder line 3T3-hCD40L with/without the addition of rhIL-21 (50 ng/ml) in two different culture media compositions (RPMI-1 and RPMI-2) for 7 days. Comparison was performed relative to B cells cultured in the presence of the untransfected NIH 3T3 feeder line. B cell cultures were analyzed by flow cytometry. **A**. Histogram of the distribution of the hCD40L (CD154) expression level on the surface of 3T3-hCD40L feeder cells (marked with a *red line*) compared with control untransfected NIH 3T3 fibroblasts (marked with a *blue line*). **B**. Representative graphs of the cytometric analysis. The gating strategy is indicated by arrows. **C**, **D**. The number of live B cells (**C**) and the percentage of dead B cells (**D**) in the cultures. **E**–**L**. Quantitative analysis of the percentage of live B cells and the total number of live B cells phenotypically divided by surface expression of CD27 and CD38 into $CD27^{high}$ $CD38^{high}$ — plasma cells and plasmablasts (**E**, **F**), $CD27^-CD38^-$ (**G**, **H**), $CD27^+CD38^-$ (**I**, **J**), and $CD27^-CD38^+$- B cells (**K**, **L**). The data is presented as the mean ± standard deviation (SD) for four independent experiments (n = 4), each experiment was performed in duplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons, *, **, ***, **** — $p \leq 0.05$; 0.001; 0.0001; 0.0001, respectively

BD Biosciences, USA, cat. No. 561524) for 12 h. The following subpopulations were identified using cytometric analysis of cultures: $CD19^+CD27^{high}CD38^{high}$ (defined as ASC), $CD19^+CD38^-CD27^-$, $CD19^+CD38^-CD27^+$, $CD19^+CD38^+CD27^-$ B cells, $CD19^+CD95^{high}$ B cells (corresponding to activated B cells), $CD19^+CD95^{high}BCL6^+$ B cells (defined as GC B cells). B-cell sorting and flow cytometry (FACS) analysis of B-cell cultures were performed on a BD FACSAria™ III cell sorter using FACSDiva™ software. FlowJo version 10.8.1 was used for data processing.

Co-cultivation of B cells with Feeder Cells

3T3-hCD40L fibroblasts and control non-transfected cells were inactivated in DMEM with mitomycin C (5 μg/ml; Sigma Aldrich, USA, cat. no. 50-07-7) for 2 h, washed with DPBS (PanEco,

Table 1. Experimental design for optimizing $CD19^+CD20^+$ B cell culture conditions

3T3	3T3-hCD40L	3T3	3T3-hCD40L	3T3	3T3-hCD40L	3T3	3T3-hCD40L
RPMI-1		RPMI-2		RPMI-1		RPMI-2	
–				+ IL-21			

Table 2. Experimental design for studying cultures of $CD20^+CD27^-$ naive B cells and $CD20^+CD27^+$ memory B cells

3T3-hCD40L							
RPMI-2 + IL21							
$CD19^-CD20^-CD27^-$				$CD19^-CD20^-CD27^+$			
–	+IL-4	–	+IL-4	–	+IL-4	–	+IL-4
–	–	+BAFF	+BAFF	–	–	+BAFF	+BAFF

Russia, cat. no. P060E) at least 3 times, and plated onto a six-well plate (Wuxi NEST, cat. no. 703002) at a density of 3×10^5 cells/well. The following day, 1.5×10^4 sorted $CD19^-CD20^-$ B cells were added. Cultivation was performed in the following media: RPMI-1 based on RPMI-1640 (PanEco, Russia, cat. no. C330p) with the inclusion of 10% FBS, 1× sodium pyruvate (PanEco, Russia, cat. no. F023), 1× GlutaMAX™ (Thermo Fisher Scientific, USA, cat. no. 35050061), 1× antibiotic mixture and RPMI-2 based on the richRPMI (BioinnLabs, cat. no. bn-3A3R), containing human transferrin, insulin, albumin, as well as glutathione and additional microelements and vitamins, with the inclusion of 10% FBS, 1× GlutaMAX™, 1× antibiotics. Recombinant human IL-21 (rhIL-21, SCI-store, cat. #PSG260) was added to the media at a concentration of 50 ng/ml. $CD20^-CD27^-$ and $CD20^-CD27^-$ B cells were cultured in RPMI-2 supplemented with IL-21 ± IL-4 (SCI-store, cat. #PSG040, 10 ng/ml) and ± BAFF (BioLegend, cat. #559604, 100 ng/ml). Cultivation was carried out for 7 days with medium replacement on day 3 and day 5. The experimental schemes for culturing all $CD19^-CD20^-$ B cells, as well as $CD19^-CD20^-CD27^-$ naive and $CD19^-CD20^-CD27^+$ memory B cells under the test and control conditions are presented in Tables 1 and 2.

Statistical Analysis

For the independent experiments described in the study, we used MCs from the blood of four genetically unrelated adult donors. Statistical analysis was performed using GraphPad Prism version 9.5.1 (GraphPad Software Inc, USA). The statistical methods are specified in the figure legends. Statistically insignificant differences between groups are not indicated on the graphs. Outliers are not excluded from the analysis.

RESULTS

In the first stage of the study, we determined how the presence of CD40L, the cytokine IL-21, or specialized additives in the cell medium impacts B-cell survival and expansion as well as the formation of ASCs and germinal center B-cells (Fig. 1, 2). A transgenic NIH 3T3 murine fibroblast cell line stably expressing hCD40L (3T3-hCD40L) was generated for long-term co-culture of B cells with human CD40L (hCD40L) (Fig. 1A).

B cells ($CD19^+CD20^+$) were obtained from human peripheral blood MCs by fluorescence sorting (with a population purity of > 99%). 1.5×10^4 B cells were cultured for 7 days in a six-well plate in the presence of 3T3-hCD40L feeders or control untransfected 3T3 cells. Two media were used for cultivation: standard RPMI-1640 supplemented with 10% fetal bovine serum, sodium pyruvate, and glutamine (designated as RPMI-1) or the same medium enriched with human serum supplements including recombinant insulin, transferrin, lipid-rich albumin, glutathione as well as additional trace elements and vitamins (RPMI-2). IL-21 was added at a concentration of 50 ng/ml (Table 1).

Flow cytometry analysis of the resulting B-cell cultures (Fig. 1B) showed that the combined action of hCD40L and IL-21 resulted in the highest level of B-cell expansion (Fig. 1C) and increased B-cell survival, especially when supplemented with recombinant proteins from human serum (Fig. 1D).

Cultures with hCD40L and IL-21 also showed the highest accumulation of $CD27^{high}CD38^{high}$ ASC (Fig. 1E, F), an increase in the number (but not percentage) of $CD27^-CD38^-$ B cells (Fig. 1G, H), and accumulation of $CD27^+$ and $CD38^+$ B cells (Fig. 1 I-L), with the percentage of $CD27^-CD38^+$ B cells being significantly higher when cultured in RPMI-1 (Fig. 1K).

GC B cells are characterized by increased surface expression of CD95 (Fas) and the presence of the intracellular transcription factor BCL6 [5]. CD95 expression also increases on activated B cells [15]. Based on this, we assessed CD95 expression on the surface of B cells in culture as a marker of cellular activation (Fig. 2A).

Based on the data analysis, up to 97% of B cells in the culture increased their surface expression of CD95 in the presence of 3T3-hCD40L-cells (Fig. 2A-C) while the addition of IL-21 did not significantly affect the percentage of $CD95^{high}$ B cells (Fig. 2B). Moreover, up to 80% of all $CD95^{high}$ B cells had the $CD27^-CD38^-$phenotype (Fig. 2D, E). The number of $CD27^+CD38^-CD95^{high}$ and $CD27^-CD38^+CD95^{high}$ B cells in cultures increased with the combined addition of hCD40L and IL-21 (Fig. 2G, I) while their proportion did not change (Fig. 2F, H).

To assess the accumulation of $CD95^{high}$ $BCL6^+$ (GC-like) B cells, we fixed surface-stained B cells with further intracellular staining with antibodies to BCL6 (Fig. 2J). Quantitative analysis revealed a low representation of this B cell subpopulation in the cultures (< 2%) (Fig. 2K). The most noticeable accumulation of GC-like B cells was observed in the RPMI-2 culture supplemented with 3T3-hCD40L feeders and IL21 (Fig. 2L). In the RPMI-2 medium, more than 50% of GC-like B cells had the $CD27^-CD38^-$ phenotype which is significantly higher than in cultures with RPMI-1 (Fig. 2M, N). $CD27^+CD38^-CD95^{high}$ $BCL6^+$ B cells were equally abundant in cultures with RPMI-1 and RPMI-2 (Fig. 2O, P) while the proportion of $CD27^-CD38^+$ GC-like B cells was significantly increased in cultures with RPMI-1 (Fig. 2Q, R).

Thus, co-cultivation of B cells with 3T3-hCD40L feeders and IL-21 allows for the greatest expansion and survival of B cells. Under these conditions, on day 7 of culture in RPMI-1, approximately 15% of living cells were ASCs and 0.2% were GC-like B cells, with 10% and 0.4% for RPMI-2, respectively. RPMI-2 was chosen for further work as it was compositionally richer for B cells.

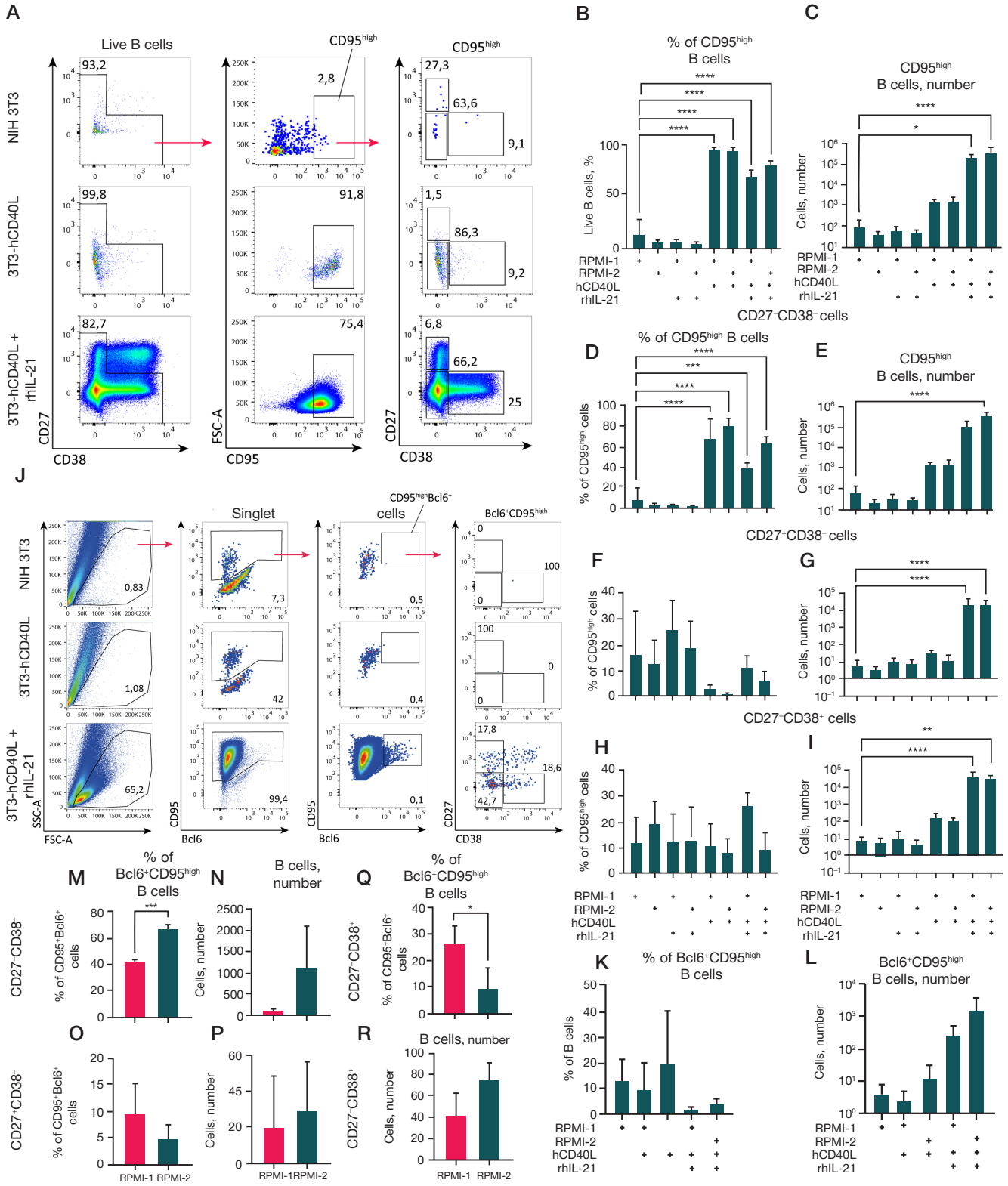

Fig. 2. Analysis of the effect of CD40L, IL-21, and cell culture medium composition on the surface expression of CD95 and intracellular expression of the transcription factor BCL6 in B-cell cultures. **A–I**. Analysis of the surface expression of CD95 and co-expression of CD27 and CD38 on B cells in cultures (excluding $CD27^{high}$ $CD38^{high}$ — plasma cells). Representative graphs of flow cytometry analysis. The gating strategy is indicated by arrows (**A**). Quantitative analysis of the percentage of (**B**) and absolute number (**C**) of $CD95^{high}$ -B cells in cultures. Quantitative analysis of the percentage of B cells and the absolute number of live $CD95^{high}$ -B cells for $CD27^{-}CD38^{-}$ (**D**, **E**), $CD27^{+}CD38^{-}$ (**F**, **G**), and $CD27^{-}CD38^{+}$ (**H**, **I**) B cells. **J–R**. B cells cultured in the presence of NIH 3T3 feeder line expressing hCD40L with or without rhIL-21 (50 ng/ml) in RPMI-1 or RPMI-2 were fixed and permeabilized for staining of the intracellular transcription factor BCL6. Comparison was made relative to B cells cultured in the presence of the untransfected NIH 3T3 line. Representative graphs of flow cytometry analysis of fixed B cells from the cultures. The gating strategy is indicated by arrows (**J**). Quantitative analysis of the $BCL6^{+}CD95^{high}$ -B cells percentage (**K**) and absolute number (**L**) of the total number of B cells. Quantitative analysis of the percentage and absolute number of $CD27^{-}CD38^{-}$ (**M**, **N**), $CD27^{+}CD38^{-}$ (**O**, **P**) and $CD27^{-}CD38^{+}$ (**Q**, **R**) $BCL6^{+}CD95^{high}$ B cells. The graphs present the data as the mean ± standard deviation (SD) for four independent experiments (n = 4), each experiment was performed in duplicate. Statistical analysis of $CD95^{high}$ B cell (**B–I**) and $BCL6^{+}CD95^{high}$ B cell (**K**, **L**) subpopulations was performed by one-way analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons. Statistical analysis of $BCL6^{+}CD95^{high}$ B cell (**M–R**) subpopulations was performed by unpaired T-test. *, **, ***, **** — $p \leq 0.05$; 0.001; 0.0001; 0.0001 respectively

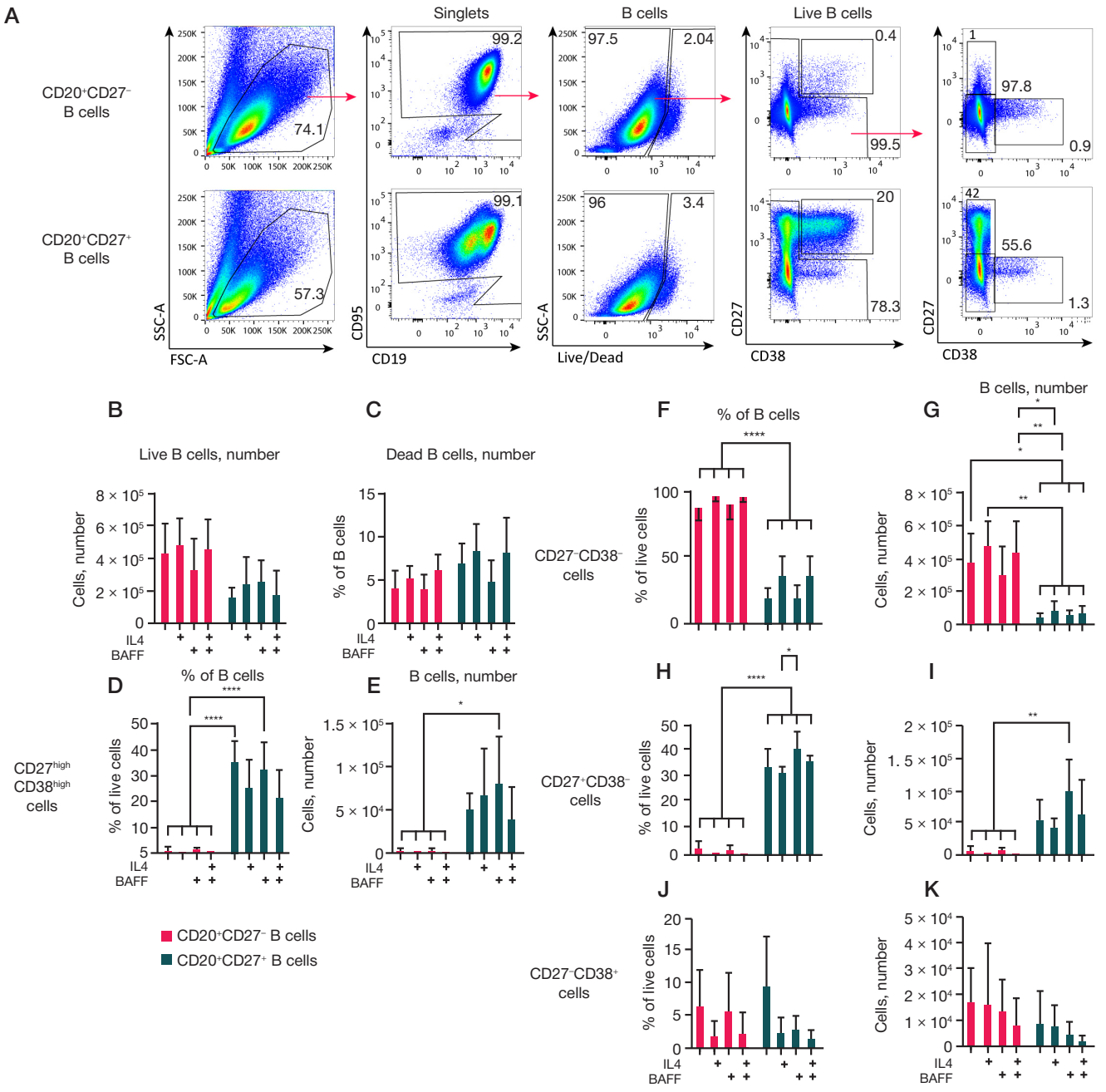

Fig. 3. Analysis of the effect of different cytokine compositions on CD27⁻ and CD27⁺ memory B cell cultures. Predominantly naive B cells (CD19⁺CD20⁺CD27⁻) and memory B cells (CD19⁺CD20⁺CD27⁺) obtained from blood PBMC by FACS, at a concentration of 1.5 × 10^4 were cultured on 3T3 feeder cells expressing hCD40L in RPMI-2 culture medium in the presence of rhIL-21 (50 ng/ml), with or without the addition of rhIL-4 (10 ng/ml) and rhBAFF (100 ng/ml) cytokines for 7 days. Cell cultures were analyzed by flow cytometry. **A**. Representative graphs of flow cytometry analysis. Arrows indicate the gating strategy. The number of live B cells (**B**) and the percentage of dead B cells (**C**) in cultures. Quantitative analysis of the percentage of live B cells and the absolute number of $CD27^{high}CD38^{high}$ — plasma cells and plasmablasts (**D**, **E**), CD27⁻CD38⁻ (**F**, **G**), CD27⁺CD38⁻ (**H**, **I**), and CD27⁻CD38⁺ (**J**, **K**) B cells. The graphs present the data as the mean ± standard deviation (SD) for four independent experiments (n = 4), each experiment was performed in duplicate. Statistical analysis was performed by two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons, *, **, ***, **** — $p \leq 0.05$; 0.001; 0.0001; 0.0001, respectively

In the next series of experiments, we investigated the effect of different cytokine compositions (IL-4, BAFF) on cell cultures of CD20⁺CD27⁻ B cells which were predominantly naive B cells, and CD20⁺CD27⁺ memory B cells. Individual B cell subpopulations were obtained from peripheral blood MCs by fluorescence sorting (with a population purity of > 95%). 1.5 × 10^4 B cells were cultured in RPMI-2 medium in the presence of 3T3 hCD40L cells supplemented with IL-21 (50 ng/ml) and the cytokines IL-4 (10 ng/ml) and/or BAFF (100 ng/ml) (Table 2).

Flow cytometric analysis of cell cultures on day 7 (Fig. 3A) showed that various cytokine compositions had no significant effect on B-cell expansion or survival (Fig. 3B, C). For all cytokine combinations, a trend toward increased expansion and a decreased proportion of dead B cells was observed in cultures of CD27⁻ naive B cells compared with CD27⁺ memory B cells.

Importantly, cultures of CD27⁺ memory B cells showed a significant accumulation of $CD27^{high}CD38^{high}$ ASC (20–35% of all B cells) (Fig. 3D, E). In the cultures of predominantly naive CD27⁻ B cells, the proportion of ASC was less than 2% (Fig. 3D, E) while the proportion of CD27⁻CD38⁻ B cells (Fig. 3F, G) was significantly higher compared to the cultures of CD27⁺ B cells. CD27⁺CD38⁻ B cells were abundant in memory B cell cultures, and CD27⁻CD38⁺ B cells were present in all cases

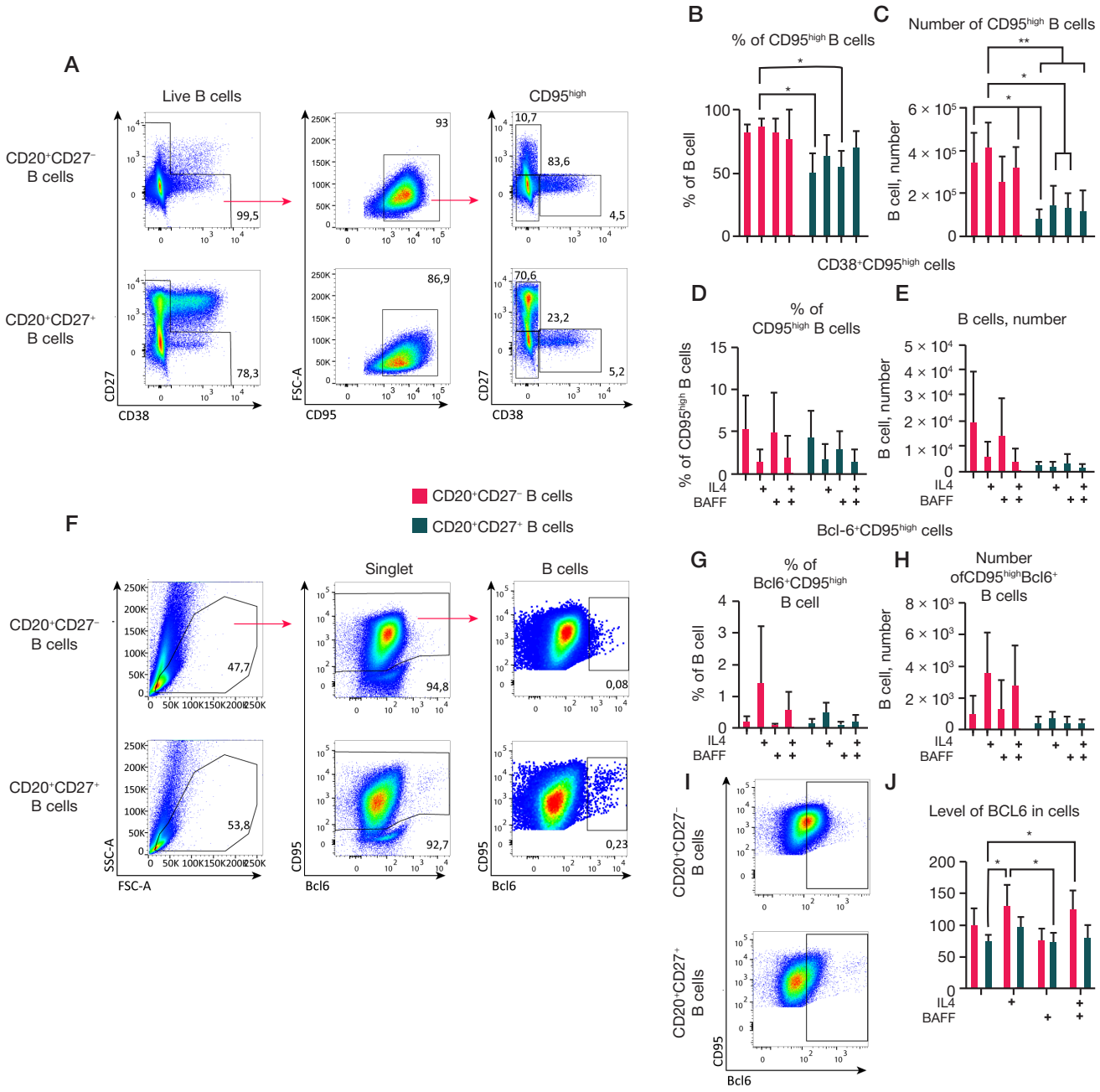

Fig. 4. Analysis of the effect of different cytokine compositions on the surface expression of CD95 and intracellular expression of the transcription factor BCL6 on cultures of CD27⁻ B cells and CD27⁺ memory B cells. 1.5 × 10⁴ CD19⁺CD20⁺CD27⁻ and CD19⁺CD20⁺CD27⁻ B cells were cultured on 3T3 feeder cells expressing hCD40L in RPMI-2 culture medium in the presence of rhIL-21 (50 ng/ml), with or without the addition of the cytokines rhIL-4 (10 ng/ml) and rhBAFF (100 ng/ml) for 7 days. **A**–**E**. Analysis of CD95 surface expression and CD27 and CD38 co-expression on B cells in cultures (excluding CD27^{high} CD38^{high} plasma cells). Representative graphs of flow cytometry analysis of CD95 surface expression. The gating strategy is indicated by arrows (**A**). Quantitative analysis of the percentage of live B cells (**B**) and the absolute number (**C**) of live CD95^{high} B cells in cultures. Quantitative analysis of the percentage of CD95^{high} B cells (**D**) and the absolute number of live CD38⁺CD95^{high} B cells (**E**). **F**–**J**. Analysis of intracellular BCL6 expression in cell cultures. For this, cells from the cultures were fixed and permeabilized for staining of the intracellular transcription factor BCL6. Representative graphs of flow cytometry analysis (**F**). The gating strategy is indicated by arrows. Percentage (**G**) of B cells and absolute number (**H**) of CD95^{high} BCL6⁺ cells. Representative panels of cytometric analysis illustrating the distribution of BCL6 in cell cultures of CD20⁺CD27⁺ and CD20⁺CD27⁻ B cells (**I**). Mean fluorescence intensity (MFI) of the transcription factor BCL6 in cells from cultures (**J**). Quantitative analysis data are presented as the mean ± standard deviation (SD) for four independent experiments (n = 4), each experiment was performed in duplicate. Statistical analysis was performed by two-way analysis of variance (ANOVA) using Tukey's multiple comparison test, *, **, ***, **** — $p \leq 0.05$; 0.001; 0.0001; 0.0001, respectively

without significant differences (Fig. 3H–K). Regardless of the starting population, adding IL-4 decreased the proportion of ASC, CD27⁺CD38⁻, and CD27⁻CD38⁺ cells (Fig. 3D, E, H–K).

Regardless of the conditions and starting cells, the majority of cells had CD95^{high} phenotype. Moreover, the proportion and number of CD95^{high} B cells were slightly increased in CD27⁻ naive B cell cultures (Fig. 4A-C). Notably, a trend toward a decrease in the CD27⁻CD38⁺CD95^{high} B cell subpopulation was observed in the presence of IL4 under all other tested conditions (Fig. 4D, E).

Analysis of the number and proportion of CD95^{high}BCL6⁺ B cells (Fig. 4F) in cultures from CD27⁻ and CD27⁺ B cells revealed no statistically significant differences (Fig. 4G, H). However, the addition of IL-4 was shown to increase the proportion of GC-like B cells (especially in the case of CD27⁻ B cells). Notably, a trend toward increased BCL6 levels in CD27⁻ B-cell cultures compared to CD27⁺ B-cells was observed under all tested conditions except the addition of BAFF without IL-4 (Fig. 4I, J).

When cultured in RPMI-2 medium with 3T3-hCD40L feeders and IL-21, a significantly greater expansion of the naive

CD27⁻ B-cell cultures with minimal ASC accumulation was observed as compared to the CD27⁺ memory B-cell cultures where ASC reached 30%. No statistically significant differences in the accumulation of GC-like B-cells were detected. The addition of BAFF had no significant effect on B-cell cultures while IL-4 caused a slight decrease in the proportion of ASC and an increase in GC-like B-cells, especially in CD27⁻ B-cell cultures.

DISCUSSION

In vitro cultivation of human B cells is of fundamental importance for solving a wide range of biotechnological problems, in particular, obtaining ASCs for antibody production, using B cells as antigen-presenting cells for T cell activation, and testing transgenic immunotherapy products [16]. Although there are numerous studies on B cell cultivation conditions [11–13], developing effective protocols for specific applications still remains important.

The aim of this study was to find an optimal combinations of conditions for (a) efficient B cell expansion in cell culture; (b) maximal accumulation of ASC population; (c) BCL6-expressing B cells similar to GC B cells. We comprehensively explored the impact of baseline B-cell status based on CD27 marker (CD27⁻ and CD27⁺ subsets), a combination of proteins and supplements replacing human serum, the cytokine BAFF that is critical for B-cell survival, and also the stimuli that mimic T-cell support (CD40L, IL-21, IL-4), on the efficiency of cell culture protocols.

Expansion and Maintenance of Activated B-Cell Viability

Cytometric analysis of B-cell cultures on day 7 of co-culture demonstrated that the highest expansion and survival rates were achieved using a transgenic feeder line expressing human hCD40L in combination with the cytokine IL-21. These data are consistent with previously described results [17, 18]. At the same time, the addition of IL-4 and BAFF cytokines to the culture at concentrations of 10 and 100 ng/ml, respectively, did not have a statistically significant effect on cell proliferation or viability.

Optimization of the cell culture medium composition showed that adding recombinant human insulin, transferrin, and albumin into a base medium containing 10% FBS resulted in the greatest reduction in percentage of dead cells.

Based on the comparative analysis, cultures initiated from CD27⁻ B cells were, on average, 1.5 times more abundant than cultures derived from CD27⁺ cells and also exhibited a lower percentage of cell death as has been previously described [19].

Thus, it is advisable to use CD27⁻ cells as the initial population for optimal expansion and maintenance of activated B cells viability *in vitro*. The most effective protocol involves co-cultivation with feeder cells expressing hCD40L in a medium enriched with IL-21, supplemented with a combination of serum proteins (including insulin, transferrin, and albumin).

Accumulation of ASC in B-cell Cultures

The next task was to determine the cell culture conditions that promote accumulation of $CD27^{high}CD38^{high}$ ASC in B-cell cultures. It was shown that in the presence of both hCD40L and IL-21, $CD27^{high}CD38^{high}$ cells accumulated in the cultures reaching 10-15% of the total cell pool. This is consistent with the previously published results [20, 21, 22]. The addition of recombinant serum proteins to the culture medium resulted in a decrease in the proportion of ASC in the cultures. Significant accumulation of $CD27^{high}CD38^{high}$ B cells was observed in the cultures of memory CD27⁺ B cells (20–35% of all living cells), consistently with previous studies [21, 23] and the rapid recruitment of memory B cells to the ASC response upon repeated exposure to antigen and T-cell help [24]. In contrast to the memory B cell cultures, in the cultures from predominantly naive CD27⁻ B cells the proportion and number of ASC were significantly reduced (1–2% of the total cell number).

It was also demonstrated that in the presence of the cytokine BAFF, the number of $CD27^{high}CD38^{high}$ and $CD27^{+}CD38^{-}$ B cells in the cultures of CD27⁺ B cells was maximal, consistent with other data available [25, 26]. Conversely, upon the addition of IL-4, abundance and proportion of ASC and $CD27^{+}CD38^{-}$ cells tended to decrease while the proportion of $CD27^{-}CD38^{-}$ B cells tended to increase.

Our findings suggest that for ASC abundance in B cell culture, it is preferable to use CD27⁺ memory B cells in combination with hCD40L and IL-21, along with the addition of BAFF. It should be noted that the analysis was performed without considering the levels of ASC antibody secretion which can vary significantly depending on the level of ASC maturation and multiple additional factors, and requires separate studies to optimize antibody production in cell cultures.

Accumulation of GC B Cells in Cell Cultures

As mentioned earlier, the signal through the BCR as well as the Th cell "help" in the form of CD40L and IL-21 secretion induces the appearance of GC B cells. A key marker of GC B cells is the expression of BCL6 transcription factor [5]. Phenotypic analysis of lymphocytes from human secondary lymphoid organs identified $CD95^{high}BCL6^{+}CD38^{+}CD27^{+/-}$ B cells as GC B cells [27]. In our study, we were able to identify GC-like B cells in cultures by a combination of the surface marker CD95 and the intracellular transcription factor BCL6.

Accumulation of $CD95^{high}BCL6^{+}$ B cells occurred in cultures with hCD40L and IL-21, consistently with *in vivo* studies [28]. The initial cell populations of CD27⁻ and CD27⁺ B cells did not significantly affect the proportion and number of $CD95^{high}BCL6^{+}$ in the final B cell cultures. However, in cultures from CD27⁻ B cells, BCL6 level tended to increase. The addition of recombinant serum proteins and IL4 contributed to an increase in the proportion of GC-like B cells and a decrease in ASC. This is consistent with the results of studies suggesting that the combined action of IL-21 and IL-4 secreted by Th increases and stabilizes BCL6 expression in activated B cells [29], promoting their differentiation into GC B cells [5]. Moreover, BCL6 is an antagonist of the transcription factor Blimp-1 and inhibits further differentiation of B cells into ASC [24].

Thus, we were able to demonstrate that culturing B cells in the presence of hCD40L and IL-21 also allows for the formation of GC-like B cells to be observed through increased BCL6 expression. Moreover, a slightly higher accumulation of GC-like B cells was observed in a culture of CD27⁻ naive B cells when IL-4 and human serum substitutes were included in the medium.

However, it should be noted that none of the components used in this study resulted in the accumulation of more than 1% GC-like B cells. GC B cells are highly sensitive to apoptosis in the absence of support from Th and specialized cells — follicular dendritic cells (FDCs) [30]. It was shown that FDC-based cell lines is likely to be preferable for culturing GC B cells [10]. Based on our data and the results of previous studies, we suggest that maintaining GC B-cell cultures *in vitro* requires

using more complex co-cultivation systems with the inclusion of FDC-like B-cell lines.

CONCLUSIONS

The study helped to identify optimal conditions for the effective expansion of B cells from human peripheral blood *in vitro* as well as for obtaining ASC-enriched cell culture. The presence of hCD40L-expressing feeder cells and IL-21 in the cultures was shown to be necessary for both proliferation and survival as well as for B cell differentiation *in vitro*. For optimal B cell culture expansion, it is preferable to use $CD27^-$ naive B cells and a medium supplemented with a combination of human serum proteins (including insulin, transferrin, and albumin). Meanwhile, using $CD27^+$ memory B cells and adding BAFF to medium are preferable for making ASC more abundant. In this study, we were able to assess the accumulation of $CD95^{high}BCL6^+$ GC-like B cells. However, the combination of conditions used was insufficient to significantly increase the abundance of GC-like B cells in cultures. Presumably, the generation of GC-like B cell culture from peripheral blood B cells may require using FDC-like feeder cells and additional soluble factors.

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