RESEARCH ARTICLE

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Cultivation of Encapsulated Primary Human B Lymphocytes: A First Step toward a Bioartificial Germinal Center

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Polyelectrolyte microcapsules based on sodium cellulose sulfate (SCS) and poly-diallyl-dimethyl-ammonium chloride (PDADMAC) have previously been proposed as a suitable ex vivo microenvironment for the cultivation and differentiation of primary human T lymphocytes. Here, the same system is investigated for the cultivation of human primary B cells derived from adult tonsillar tissue. Proliferation and differentiation into subtypes are followed and compared to suspension cultures of B cells from the same pool performed in parallel. Total cell expansion is somewhat lower in the capsules than in the suspension cultures. More importantly, however, the differentiation of the initially mainly memory B cells into various subtypes, in particular into plasma cell (PC), shows significant differences. Clearly, the microenvironment provided by the microcapsules is beneficial for an accelerated induction of a germinal center-like B cell phenotype and afterward supports the long-term survival of the PC cells. Then, varying the encapsulation conditions (i.e., presence of human serum and dedicated cytokines in the capsule core) provides a tool for finetuning the B cell response. Hence, this methodology is suggested to pave the way toward ex vivo development of human immune organoids.

1. Introduction

Microencapsulation has the capability of mimicking complex body niches, thereby bringing ex vivo cultivation closer to conditions found in the human body.[1] Providing such an engineered environment is particularly promising for the ex vivo proliferation and differentiation of hematopoietic stem cells including those of the immune system. The development of B cells, for instance, starts in vivo with the maturation of precursor cells in the bone marrow. Afterward, the B cells exit the bone marrow and migrate with the blood to the secondary lymphoid organs, where they are brought into contact with a steady supply of antigens through the circulating lymph.[2–4] Within the secondary lymphoid organs, the germinal centers (GCs) are highly relevant transient microanatomical structures,[5] where somatic hypermutation and clonal selection take place, yielding inter alia high-affinity B cell clones that form the memory components of the humoral immunity.[2,6] B cell maturation and proliferation are driven by T cells presenting the CD40 ligand (CD40L). These cells interact also with the B cells via antigen presentation, which eventually leads to the formation of B cells capable of producing and secreting a specific antibody. Antibody-secreting plasma cells (PC) and plasmablasts (PB) can be distinguished from GC and memory B cells by characteristic patterns of surface receptors, such as CD20, CD27, and CD38, while always including the B cell receptor CD19.[7–10]

Ex vivo expansion and differentiation of naïve primary B cells open new pathways for in vitro studies of the immune response, but also for the production of specific antisera. Immature B cells can be derived from blood and secondary lymphoid organs for ex vivo proliferation and further differentiation. In case of mice, the spleen is an excellent source of large numbers of primary B cells.[11] In humans, tonsils may serve a similar purpose, allowing also the collection of juvenile cells.[12] Ex vivo cultivation of B cells is typically performed in suspension in culture media containing sera supplements (both human and bovine). A serum-free B cell culture medium has been suggested, containing human albumin, low-density lipoprotein, α-tocopherol (antioxidant), and a mixture of chemically defined lipids.[13]

Differentiation and proliferation of B cells can be stimulated by contact with dissolved or immobilized CD40L and fine-tuned via a cocktail of cytokines.[14] While IL-4 drives proliferation, IL-21 promotes differentiation into PCs, and BAFF (B cell activating factor) supports PB survival.[15–17] Soluble CD40L molecules were shown to be endocytosed by B cells after interaction with their receptor.[18,19] In consequence, any dissolved CD40L is steadily consumed by the B cells in culture and needs to be

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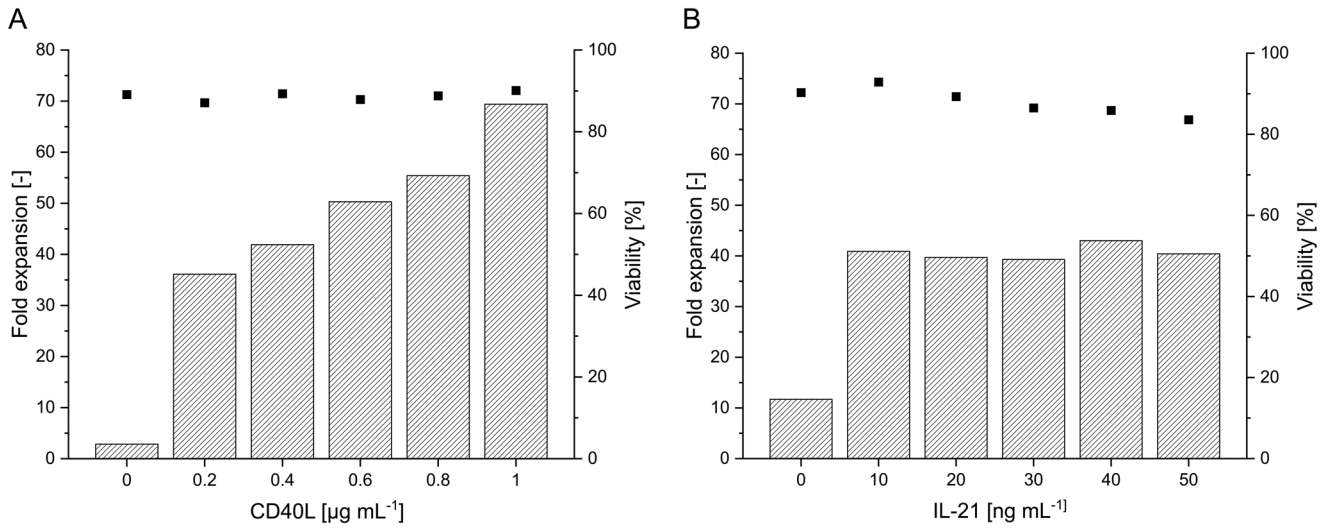

Figure 1. Influence of CD40L- and IL-21 on B cell expansion and viability after seven days of suspension cultivation. 0.1×10^6 B cells were seeded in 24-well plates in growth medium supplemented with A) the indicated amounts of CD40L and 20 ng mL^{-1} IL-21 and B) the indicated amounts of IL-21 and 0.4 µg mL^{-1} CD40L. Shown are the expansion factor (bars) and the viability (■).

regularly replaced to keep the absolute number of signaling molecules per cell constant, something that has been shown to be important for the physiological responses of the cells.[14]

However, cultivation in suspension is incapable of emulating the complex 3D microenvironment guiding B cell maturation in vivo. Alternative approaches have been proposed, where the B cells instead were placed in a surrogate extracellular matrix (ECM).[20,21] For primary human T cells, microencapsulation in sodium cellulose sulfate (SCS) and poly-diallyl-dimethylammonium chloride (PDADMAC) capsules has been proposed and shown to support cellular growth and differentiation.[22] SCS/PDADMAC microcapsules are composed of a liquid-core of unreacted SCS polyelectrolyte enveloped by a mechanically stable semipermeable membrane made of the polyelectrolyte complex formed by the two types of polyion.[23,24] The porous membrane of such polyelectrolyte microcapsules has been shown to have a molecular weight cut-off (MWCO) below 10 kDa,[25] which assures that most cytokines produced or required by the cells are trapped inside the capsules, while low molecular weight nutrients and metabolites can pass. This creates a biomimetic microenvironment that can be self-conditioned by the encapsulated immune cells, the principle of which is illustrated in Figure S1, Supporting Information. Furthermore, cellulose sulfate shares some structural similarities with heparan sulfate (HS), a highly abundant component of the ECM.[26] Binding studies showed that HS-binding motifs are present on many cytokines (e.g., IL-2, IL-4, IL-10, CD40L).[27–30] HS immobilizes these cytokines leading to a range of cellular reactions, as recently reviewed.[31] In the above-cited paper, HS-like binding properties and effector function of SCS/PDADMAC microcapsules were demonstrated for IL-2.[24]

Since cytokines known to be relevant for B cell development such as IL-4 and CD40L have been described to bind to HS as well, here we tested the hypothesis that SCS/PDADMAC-capsules provide a suitable microenvironment for the ex vivo proliferation and differentiation of human primary B cells.

2. Results and Discussion

2.1. Suspension Cultures of Primary Human B Cells

In order to reduce donor-based heterogenicity, all experiments discussed below were carried out using several cryovials of cells all having been derived from the tissue of a single adult donor. In the pertinent literature, CD40L and IL-21 have been reported as relevant factors for B cell activation and differentiation, respectively. Here, the response of the human adult tonsillar cells to varying concentrations of CD40L and IL-21 in growth medium was analyzed by cultivating 0.1×10^6 cells per well in 24-well plates (V_{total}: 1 mL). First, the CD40L concentration was varied from 0 to 1 µg mL^{-1} while the concentration of IL-21 was kept constant (20 ng mL^{-1}), that is, in the range suggested in the pertinent literature.[14] In the absence of CD40L (0 µg mL^{-1}), a 2.9-fold expansion of the cells was observed after seven days (**Figure 1**A). In the presence of CD40L, expansion increased in a concentration-dependent manner, reaching a 69.4-fold expansion at 1 µg mL^{-1} CD40L. Next, the IL-21 concentration was varied from 0 to 50 ng mL^{-1} while CD40L was kept constant at 0.4 µg mL^{-1}, again to be well within the range reported by Néron et al.[14] (Figure 1B). In absence of IL-21, the cells expanded 11.7-fold within 7 days. Supplementing the medium with 10 ng mL^{-1} IL-21 resulted in a 40.9-fold expansion. A further increase in the IL-21 concentration did not further improve proliferation. In the subsequent cultivation experiments, we nevertheless set the IL-21 concentration to 20 ng mL^{-1}, to avoid the region with rapid change of proliferative outcome between 0 and 10 ng mL^{-1}. In all experiments, the cells viability was >80%.

To verify the reproducibility of the results, four individual cultures (seeding density always: 0.1×10^6 cells mL^{-1}), issuing from one cryovial each, were carried out in growth medium containing 0.4 µg mL^{-1} CD40L (i.e., 4 µg CD40L per 10^6 cells) and 20 ng mL^{-1} IL-21 (i.e., 0.2 µg IL-21 per 10^6 cells), keeping experimental conditions as identical as possible. After thawing,

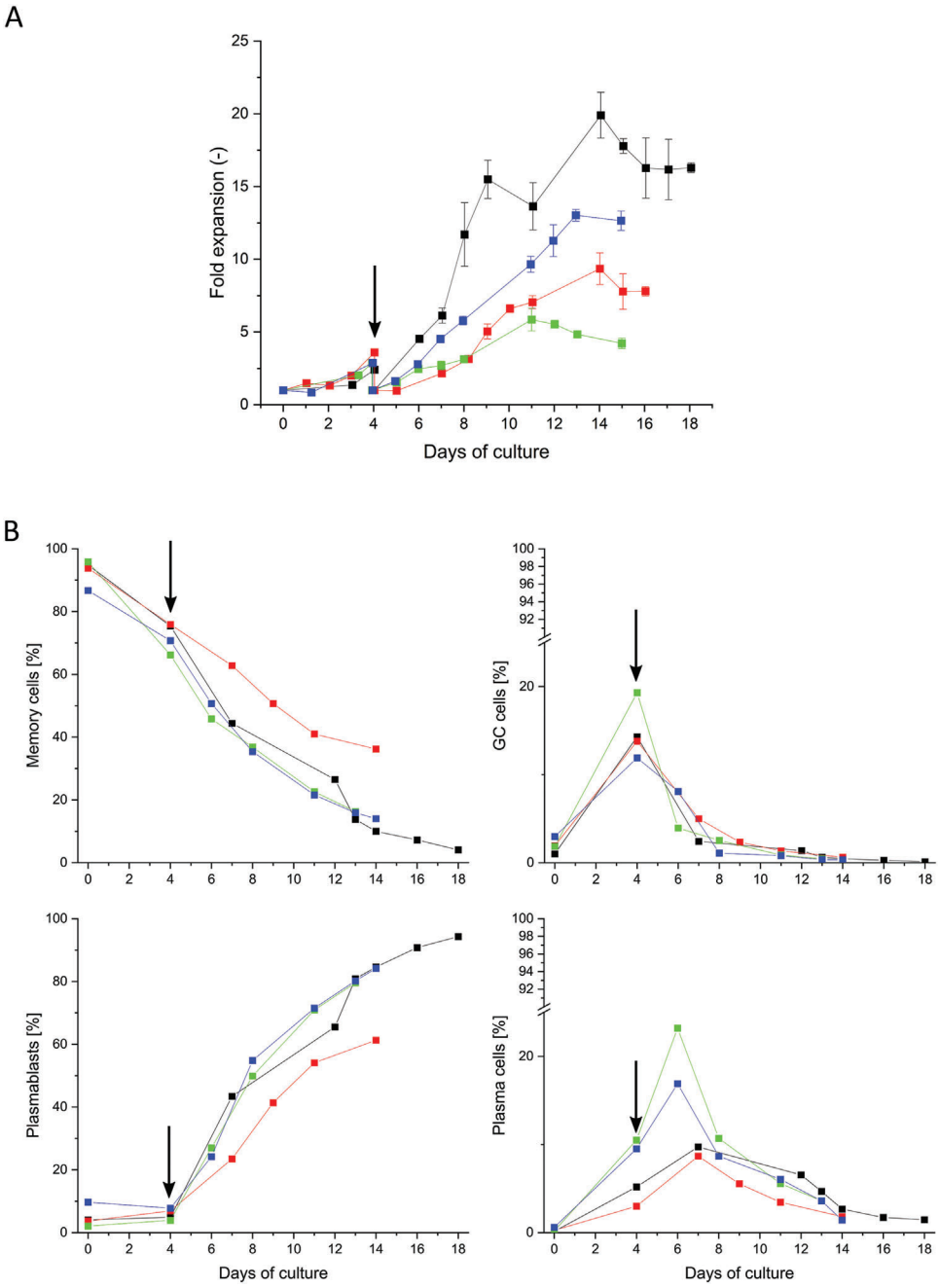

Figure 2. Development of four individual suspension cultures derived from independent cryovials (identical donor). On day 4, cells were reseeded at 0.1×10^6 cells mL^{-1} in fresh growth medium (arrow). In all cases, the viability was consistently above 90%. A) B cell expansion over culture time. Results are presented as mean values with standard deviation, $n = 3$. B) Development of the four B cell subsets (memory cells, GC cells, PB, PC) analyzed by flow cytometry. Subsets were first identified by CD27 versus CD38 dot plots and subsequently analyzed for CD20 expression in the identified subpopulations. Cells were assigned to subsets as follows: Memory cells: $CD19^+/CD20^+/CD27^+/CD38^-$; Germinal center (GC) cells: $CD19^+/CD20^+/CD27^+/CD38^+$; Plasmablasts: $CD19^+/CD20^-/CD27^{++}/CD38^-$; Plasma cells: $CD19^+/CD20^-/CD27^{++}/CD38^{++}$.

cells were initially allowed to recover in growth medium for 4 days. Then, they were reseeded at 0.1×10^6 cells mL^{-1} in fresh medium. Expansion, as well as differentiation into cellular subsets, were followed for up to 18 days (**Figure 2**).

Cells in all cultures entered the exponential growth phase within four days after initiation of the culture and reached their maximum expansion after 11–14 days (Figure 2A). Afterward, cell densities decreased in all cultivations. Despite this, the cell viability remained stably above 90%. The fact that we never observed any accumulation of dead cells may be an indication that cells undergo apoptosis rather than necrosis.[32,33] Although trends were similar and all experiments were conducted with

cells from a single donation and under standardized cultivation conditions, non-negligible differences were observed. The maximum expansion varied up to threefold between experiments. Temperature fluctuations during freezing, as well as handling variations in the thawing process, are known to influence cellular behavior.[34] The likelihood of such interexperimental variations should be taken into consideration when interpreting experimental results.

In regards to the development of the B cell subclasses (Figure 2B) all cultures followed a similar trend again. Whereas memory cells constantly decreased, GC cells peaked at day 4, PCs reached a maximum on day 6 to 7 and then decreased, while PBs increased steadily from day 4 onward. The short-lived burst of PC observed in B cell culturing systems is a well-known phenomenon during B cell expansion in the presence of IL-21.[15] This emulated the development in vivo, where the majority of the PC arising in the GC are short-lived, produce antibodies rapidly, and then die.[16,35,36]

2.2. Production and Characterization of the Capsules as well as the Capsule Materials

The produced capsules were characterized with regards to their size and diffusion characteristics (Figure S2, Supporting Information). The size of the capsules was 1223 ± 47 µm (n = 39). Based on diffusion experiments performed with two small molecules (resorufin: 0.229 kDa; vitamin B12: 1.35 kDa), we concluded that the capsules have a similar permeability as previously reported including an MWCO between 1.35 and 10 kDa.[25] These results also show the suitability of the 3D-printed encapsulation device and underscore the ability of 3D-printing technology to produce such devices.

To the best of our knowledge, B cells have never been encapsulated in SCS and PDADMAC. Therefore, the biocompatibility of the chemicals involved in the encapsulation and release processes was determined. Cells were incubated with 1.8% w/v SCS or 0.85% w/v PDADMAC to mimic concentrations used during encapsulation, as well as with 1% w/v cellulase, that is, the enzyme with which cells come into contact during release from the capsules. In order to investigate the putative toxicity of intact capsules, B cells were also incubated with empty capsules. Cell survival (i.e., viability) and cell fitness (i.e., metabolic activity) were analyzed by live/dead staining and MTT assay, respectively.

Cells are well able to withstand a contact with SCS or cellulase as demonstrated by the remaining high percentage of viable (SCS: 89 ± 2%; cellulase: 89 ± 3%) and metabolically active (SCS: 86 ± 1%; cellulase: 89 ± 1%) cells compared to the respective negative controls. PDADMAC, on the other hand, induced a significant adverse effect on both the viability (59 ± 4%) as well as the metabolic activity (16 ± 1% compared to the control). However, B cells only come into contact with PDADMAC briefly during encapsulation and that while ensconced in the SCS matrix. The capsules are washed immediately following their formation with phosphate-buffered saline (PBS) to remove the surplus PDADMAC from the system. The effectiveness of the washing step is proven by the excellent survival of the cells after incubation with empty PBS-washed capsules (viability: 90 ± 1%; metabolic activity: 91 ± 1% compared to the control).

2.3. Proliferation and Differentiation of Encapsulated B Cells

Subsequently, B cells were encapsulated in SCS/PDADMAC. To allow reliable cell count and phenotyping postrelease from the capsules, the cell density for encapsulation was tenfold higher (1×10^6 cells $mL_{capsule}^{-1}$) than for suspension culture. This posed a question in regard to the comparability between suspension and encapsulated B cell cultures since either the volumetric concentration of the important signal molecules or their amounts per cell would be similar. Initially, the volumetric concentration was chosen to be identical in the capsules' core and the supernatant of the suspension cultures, accepting that this would reduce the amount of cytokine available per cell inside the capsules. As most cytokines have molar masses > 10 kDa (e.g., CD40L 23 kDa, IL-4 15.1 kDa, IL-21 15.5 kDa, BAFF 17 kDa), it was presumed that these molecules, when supplemented during encapsulation, would not be able to leave the capsules. The same would incidentally apply to most signal molecules produced by the cells. The standard protocol for human T cell encapsulation with SCS/PDADMAC, developed by our group, calls for a wash of the cells with human serum (referred to as "serum-coating" in the past) followed by re-suspension in SCS for the actual encapsulation procedure. The serum-coating is presumed to protect the cells against shear stress in the encapsulation unit.[24,25] Since no serum is added to the SCS this procedure is referred to as "cells/capsules$_{withoutserum}$." In case of the B cells, besides this standard procedure and in an attempt to further emulate the environment experienced by B cells in the suspension culture, we also encapsulated the cells in the presence of 10% v/v serum in the SCS and in consequence in the capsule core ("cells/capsules$_{withserum}$").

Since thawing is stressful to the cells and the biomass is low, cells were encapsulated after cultivation in growth medium (i.e., medium supplemented with 10% v/v human AB serum and the full cytokine cocktail) long enough to allow the culture to enter the exponential growth phase (typically after 4 days, vide Figure 2). Microscopic observation of the capsules post-encapsulation (**Figure 3**A) revealed an overall homogenous distribution of the cells in the capsules indicating that the mixing of the cells with the SCS solution before encapsulation led to a homogenous spreading of the cells in the viscous solution. Immediately after encapsulation, aliquots of the capsules were recovered and the cells were released in order to estimate the encapsulation efficiency. For this purpose, the cells released from the capsules were counted as described in the materials and methods section (i.e., live/dead staining) and their number compared to the number of cells initially suspended in the SCS solution. On average, the cell viable density in the capsule was $0.95 \pm 0.07 \times 10^6$ cells $mg_{capsules}^{-1}$ corresponding to an encapsulation efficiency of 95%. The cell density in the capsule thus determined on the day of encapsulation was then used for the calculation of the expansion factor.

The encapsulated cells were cultivated in basal medium containing 10% v/v human AB serum. Cytokines were not added to the medium used for cultivation of the encapsulated cells, since they were presumed to be too big to diffuse into the capsules. Note that all cytokines were instead incorporated into the capsule core during the encapsulation process via the prepared SCS solution. This incidentally reduced the cost of the cultivation medium. The serum, on the other hand, was added to the

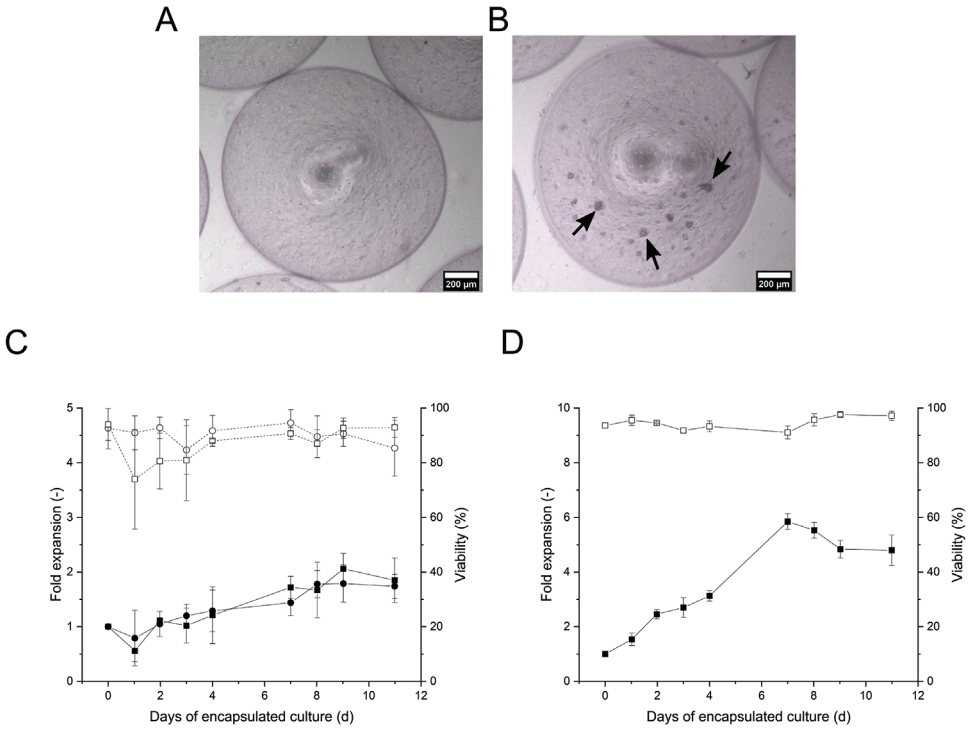

Figure 3. B cell cultures in SCS/PDADMAC capsules and in free suspension. A) Representative capsules directly after encapsulation and B) after 11 days of cultivation (with arrows pointing to aggregates). All images were taken at 10× magnification. C) Expansion and viabilities of encapsulated B cells. B cells were encapsulated after rinsing with human serum (cells/capsules$_{withoutserum}$) (■ expansion, □ viability) or in the presence of 10% v/v human serum in the SCS and hence the capsules' core (cells/capsules$_{withserum}$) (● expansion, ○ viability). Volumetric cytokine concentrations inside the capsules were the same as in the B cell suspension cultures, that is, in growth medium. Cells had been cultivated for four days in growth medium prior to encapsulation (data not shown, day 0 is the day of encapsulation). D) B cell culture in free suspension performed with the same cell batch (i.e., cultivated for four days in growth medium prior to inoculation, data not shown) in growth medium, starting cell concentration: 0.1×10^6 cells mL^{-1}. Results are presented as mean values with standard deviation, $n = 3$ (technical).

medium in case of the encapsulated cells as well, since it presumably supplies small molecules (e.g., vitamins) supportive of growth. Throughout cultivation, the B cell density inside capsules increased visibly, with small cell aggregates having formed by day 11 of cultivation (Figure 3B). The expansion fold for cells encapsulated with (cells/capsules$_{withserum}$) or without human serum (cells/capsules$_{withoutserum}$) present in the SCS and hence the capsule core (Figure 3C) was compared to a suspension culture performed in parallel (Figure 3D).

The viability of the encapsulated cells remained at a constant high level, only briefly dropping below 80% 24 h post-encapsulation for cells/capsules$_{withserum}$, then recovering and staying above 85% through the remainder of the experiment. This shift could be an indication of lingering stresses of encapsulation. In comparison, the viability of the cells in suspension cultures was >90% throughout. The experimental variation in the suspension cultures is lower than for the encapsulated cells. The encapsulation process and release from the capsules are additional steps that can cause variation between technical replicates but in our opinion are still acceptable in terms of allowing the interpretation of the obtained results. In terms of expansion, capsules obtained via both encapsulation procedures (with/without serum), led to a twofold expansion after eight days of cultivation. Therefore, a presence of human serum in the capsules' core did not provide any additional benefit for cell proliferation.

In comparison, the control suspension culture (Figure 3D) showed a threefold higher expansion over the course of the cultivation. For encapsulated cultures, some limitations cannot be excluded, as molecules larger than the MWCO of the capsule membrane (e.g., hormones, growth factors (GF), proteins, lipids) cannot be replenished from the outside via the cell culture medium and thus may be gradually depleted inside the capsules during the cultivation time. Further, while the volumetric concentrations, for example, of the cytokines, were identical, as already discussed, the amount of co-encapsulated cytokines per 10^6 cells was tenfold lower inside the capsules compared to the suspension culture.

Next, the subclass development of the encapsulated B cells was analyzed throughout the cultivation, **Figure 4**.

In contradiction to the development in the suspension cultures performed in parallel, the PC phenotype ($CD19^+/CD20^-/CD27^{++}/CD38^{++}$) was not detected among the released encapsulated cells (Figure S3, Supporting Information). Further analysis revealed that the cellulase treatment, used for cell release, prevented the detection of the $CD27^{++}$ subtype (Figure S4, Supporting Information). CD27 is a glycosylated molecule.[37] If the glycosylation site is involved in antibody binding, cellulase treatment could presumably interfere with this binding. Therefore, CD27 expression was not considered further when analyzing the subpopulation of the encapsulated

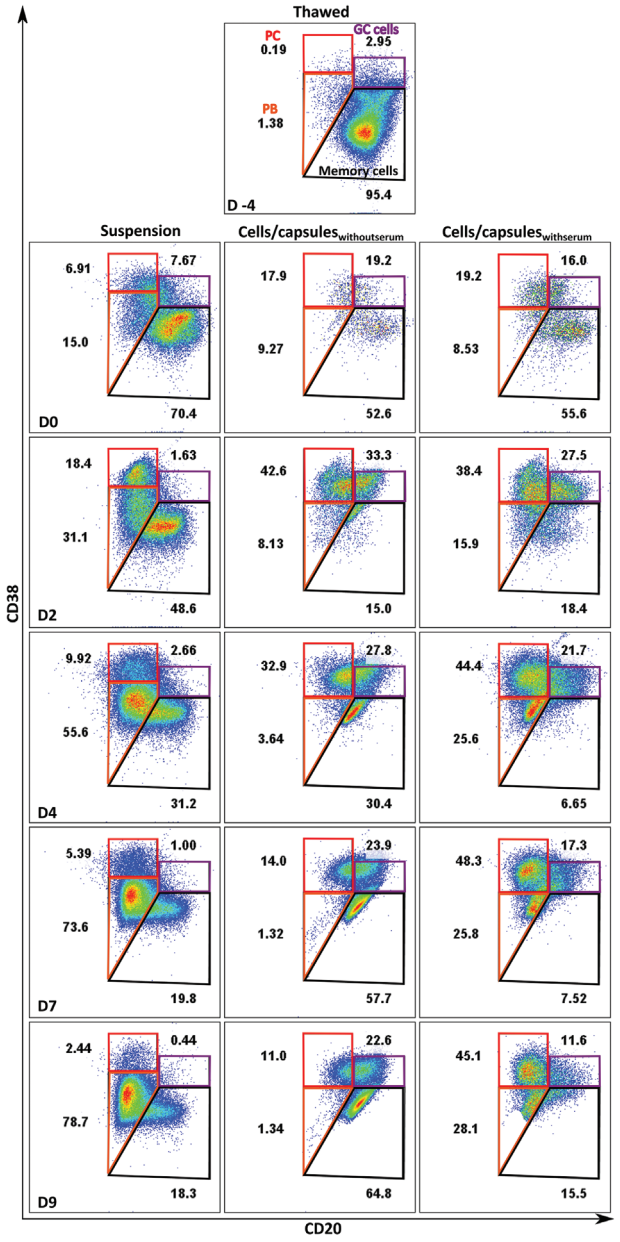

Figure 4. Development of the B cell subpopulations in case of encapsulated cells compared to those cultivated in suspension. "D -4" refers to the day of thawing. "D0" to "D9" refer to the days of cultivation post-encapsulation or post-inoculation at 0.1×10^6 cells mL^{-1} for the suspension culture. For each measurement, either one well of suspension culture of a 24-well plate was harvested or capsules were drawn and cells released by cellulase treatment. For flow cytometry measurements, 80 000 events were analyzed. The gating strategy is shown in Figure S3, Supporting Information.

B cells, which were only identified within the $CD19^+$ population on the basis of the level of their expression of the CD20 and CD38 surfaces markers.

While the presence of 10% v/v human serum in the capsule core had made no difference in survival/expansion of the encapsulated B cells, an interesting influence on subtype development was seen. On the day of encapsulation, the cell pool contained 70.4% memory cells, 7.7% GC cells, 15% PB, and 6.9% PC. In the following days, the suspension culture performed in parallel developed as expected, for trends the reader is referred to Figure 2B. Encapsulated cells showed different behavior. Comparison of all B cell subsets on day 0 shows that encapsulated B cells (D0 "cells/capsules$_{withoutserum}$" and "cells/capsules$_{withserum}$") differed from cells before encapsulation (D0 "Suspension"). Notably, memory cell and PB content was decreased, while that of PC and GC cells increased. This shift could be an indication of lingering stresses of encapsulation. Thereafter, the capsule cultures showed differences in the development of the subpopulation depending on whether serum was present or not in the capsule core. For encapsulated B cells, that had solely been rinsed with human serum (cells/capsules$_{withoutserum}$), PC and GC cell contents peaked on day 2 of cultivation and decreased afterward. At the end of the culture (D9), approximately 65% of the cells had preserved their memory cells phenotype, 22.6% of the cells were GC cells and 11% of the cells could be classified as PCs. The amount of PB was very low throughout (<10%). In case of the cells encapsulated with 10% v/v human serum in the capsule core (cells/capsules$_{withserum}$), the amount of memory and GC cells decreased steadily after day 2, reaching 15.5% and 11.6%, respectively, at the end of the cultivation. Concomitantly, the amount of PC and PB increased about 4-fold (PC) or 20-fold (PB) higher than for the pre-coated cells (cells/capsules$_{withoutserum}$) at the end of the cultivation.

The typical short-lived PC burst followed by an immediate decline in numbers was observed in the suspension culture and to a lesser extent also for "cells/capsules$_{withoutserum}$." However, this was not the case when 10% v/v human serum was present in the capsules' core (cells/capsules$_{withserum}$). Therefore, the presence or absence of human serum within the capsule core had a steering effect on subtype development. A simple rinse with human serum prior to encapsulation seemed to prevent the encapsulated B cells from maturing fully into the PB and PC subclasses. The content of memory cells remained high, while a decrease of the CD20 expression (i.e., differentiation into antibody-secreting cells) in the $CD38^{++}$ population seemed to be hampered, indicating impaired PB and PC development. A high GC cells content was observed, but the concomitant maintenance of a high level of memory cells phenotype rather indicates developmental arrest. The presence of human serum in the capsules core (cells/capsules$_{withserum}$) led instead to an accumulation of PB and PC cells and a decrease of GC and memory cells, causing distinct differences. It is thus tempting to conclude that B cell maturation in capsules is influenced by some high molecular weight components found in human AB serum.

Interestingly, both encapsulation strategies promote the development of GC-type cells, which was not the case when the cells were cultivated in suspension even though identical volumetric concentrations of the relevant cytokines (i.e., CD40L, IL-21, IL-4, and BAFF) were used. This indicates the existence of so far unidentified cell stimulatory mechanisms provided inside the SCS capsules. Autocrine conditioning of their microenvironment by the B cells and/or matrix interactions are possible contributions. For instance, high expression of Syndecan 1 (CD138) is characteristic of PC. This surface marker is upregulated during differentiation from PB into PC[38] and was shown to play a direct role in PC survival in vivo via cell–matrix interaction.[39] HS,

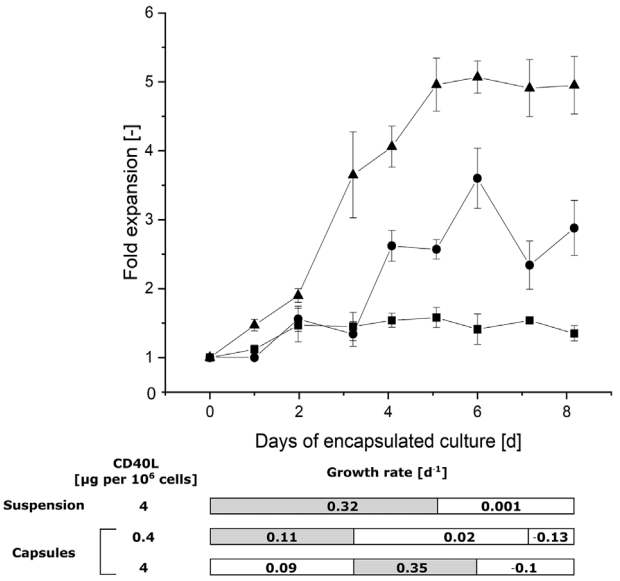

Figure 5. Expansion of B cells after encapsulation. B cells were encapsulated at 1×10^6 cells $mg_{capsules}^{-1}$ with 10% v/v human serum in the capsules' core (cells/capsules$_{withserum}$). The standard volumetric CD40L concentration inside capsules (0.4 µg $mg_{capsules}^{-1}$, i.e., 0.4 µg CD40L per 10^6 cells, ■) was compared to a tenfold increase of the CD40L concentration inside capsules (4 µg $mg_{capsules}^{-1}$, i.e., 4 µg CD40L per 10^6 cells, ●). Other cytokines supplemented in the growth medium were kept at standard concentrations. Encapsulated cells were cultivated in basal medium. In parallel, cells were also cultivated in suspension in growth medium (▲) (seeding density: 0.1×10^6 cells mL^{-1}, 4 µg CD40L per 10^6 cells). Viability of B cells was monitored throughout the experiment and was always above 80%. Data presented as mean values with standard deviation, $n = 3$ (technical).

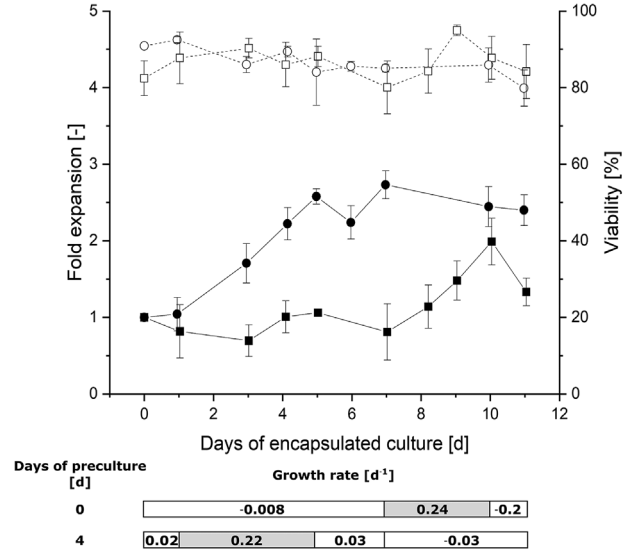

Figure 6. Expansion of encapsulated B cells with and without pre-culture after thawing. B cells were encapsulated at 1×10^6 cells $mg_{capsules}^{-1}$ with standard cytokines concentrations (0.4 µg $mg_{capsules}^{-1}$ and 0.4 µg CD40L per 10^6 cells) as well as 10% v/v human serum (cells/capsules$_{withserum}$) in the capsule core. B cells were either encapsulated according to standard procedures after four days pre-cultures post-thawing (●) or directly after thawing (■). Expansion is represented as solid lines and viability as dotted lines. As B cells were encapsulated on different days of overall culture, cells encapsulated after pre-culture are shown at the same culture time inside capsules as cells encapsulated immediately after thawing . Results are presented as mean values with standard deviation, $n = 3$ (technical).

which has chemical similarities with the cellulose sulfate used here for encapsulation, plays a major role in this process.[26] It stands to to assume that the steady increase in PC content, which we observed in the capsules, is promoted by the capsule material binding to PC and supporting their survival in vitro.

Finally, in an attempt to improve the proliferation of the encapsulated cells, the CD40L concentration inside the capsules was increased by tenfold to reach 4 µg CD40L per 10^6 cells, thereby approaching the conditions in the suspension cultures (**Figure 5**).

Up to day 3, no differences between the two encapsulated cultures were observed. However, afterward, cells encapsulated in the presence of 4 µg CD40L per 10^6 cells continued to divide actively, whereas the cells encapsulated with only 0.4 µg CD40L per 10^6 cells reached a plateau. Suspension cells grew exponentially before plateauing on day 5. B cells encapsulated at standard volumetric CD40L concentration reached a maximum concentration of 1.6×10^6 cells $mg_{capsules}^{-1}$ five days after encapsulation, while a maximal cell density of 3.6×10^6 cells $mg_{capsules}^{-1}$ was reached on day 6 post-encapsulation in the presence of 4 µg CD40L per 10^6 cells. During the intense growth phase of cells encapsulated with 4 µg CD40L per 10^6 cells, the growth rate was comparable with the one of control cells grown in suspension (0.35 and 0.32 d^{-1}, respectively), though the length of maximal growth phase was shorter for encapsulated cells (three days vs five days in suspension). Therefore, with a total of 3.6-fold expansion, the encapsulated culture with 4 µg CD40L per 10^6 cells still did not reach the growth of the suspension cultivation (5.6-fold expansion). This might reflect that the amount of the other cytokines (IL-4, IL-21, and BAFF) might also need to be increased during the encapsulation process. Further, as discussed above, additional factors, with an MW larger than 10 kDa, cannot diffuse through the capsule membrane and their lack might also impair cellular proliferation. Subclass analysis of B cells encapsulated with 4 µg CD40L per 10^6 cells was performed as well. The increased CD40L stimulus did not have an observable effect on B cell differentiation. Detailed data can be found in Figure S5, Supporting Information.

2.4. Encapsulation of Freshly Thawed B Cells

A cryopreserved B cell pool is the closest one can get to the original tissue. The pre-culture proposed above prior to encapsulation, is beneficial, as it increases the biomass, but it also directs the B cells toward the PC burst typical for suspension cultures (Figures 2 and 4). To avoid any differentiation of B cells during the pre-culture, we tested the option to encapsulate the cells directly after thawing and compared their behavior to that of cells encapsulated after pre-culture (**Figure 6**).

In comparison to the cells encapsulated after the pre-culture, cells encapsulated directly after thawing exhibited a very long lag phase before entering the exponential growth phase. However, one has to keep in mind that cells encapsulated after pre-culture already had experienced a three days lag phase followed by one day of expansion immediately before encapsulation on day 4 (data not shown). Hence, they were already in the exponential

phase on the day of encapsulation. The stress of encapsulation occurring during the cells' vulnerable state, that is, directly after thawing, may also have contributed to the observed elongated lag phase and the somewhat higher cell mortality on the day of encapsulation in case of the directly encapsulated cells. Moreover, B cells encapsulated after pre-culture are supplied with fresh cytokines (i.e., cytokines which are incorporated in the capsule core) on the day of encapsulation, which could be considered as a re-stimulation of the cells (i.e., cells were challenged twice with cytokines at four days interval). Cells directly encapsulated are not (i.e., cells came in contact with the cytokines on the day of encapsulation only). Interestingly, cells encapsulated with or without pre-culture exhibit similar specific growth rates (μ_{max}), also leading to maximal cell densities in the same range (direct encapsulation: 2.7×10^6 cells $mg_{capsules}^{-1}$; post-pre-culture encapsulation: 2×10^6 cells $mg_{capsules}^{-1}$).

Figure 7 shows the subpopulation development for encapsulated B cells with and without pre-culture post-thawing.

The distribution of the memory and GC cells shows similar trends in both cases. The differences in terms of number of cells in each population are most probably related to differentiation occurring during the four days of pre-culture, as shown above for non-encapsulated cells (Figure 2B). After 10 days of cultivation, the memory cell content was notably reduced in both cultures (<20%) and the GC cells represented 20% to 25% of the overall B cell population. Most pronounced differences were detected for the PB and PC subsets. Both cultures started with low PB and PC numbers. As to be expected, the pre-cultured cells exhibited a somewhat higher starting content (10–15% vs <5%) for both subpopulations. Whereas direct encapsulation drove B cells toward the PB phenotype, pre-cultivation post-thawing induced a clear accumulation of PC in the capsules. This may indicate a methodological option for directing B cell differentiation in capsules. Pre-cultures would guide B cells toward PC development, while immediate encapsulation can promote the development of PB and GC cells.

3. Conclusion

Tonsils provide many cell types involved in immunity, making them an excellent source of cells for the development of artificial lymphoid tissues. In this paper, we showed that human primary tonsillar B cells can be cultured in SCS/PDADMAC capsules, as previously only shown for T cells, and most importantly that such a cultivation system allows controlling the development of B cell subsets in vitro as summarized in **Figure 8** for cultivation in suspension versus capsules.

In line with the tissue of origin used for cell isolation (i.e., tonsils), memory cells were most abundant after thawing and steadily decreased independently of the cultivation types. GC cells were only observed in encapsulated cultures and tended to be more abundant when the cells were encapsulated directly after thawing. Whereas PB accumulated in the suspension cultures, their count stays moderate in the capsules. The most important aspect in this context is the PC subset development. Whereas the B cell suspension cultures in our hands only showed the typical transient increase of PC (i.e., a "PC burst" for 24 h), cultivation in SCS/PDADMAC capsules was accompanied by a preservation of the PC subset for several days, in particular when the cells

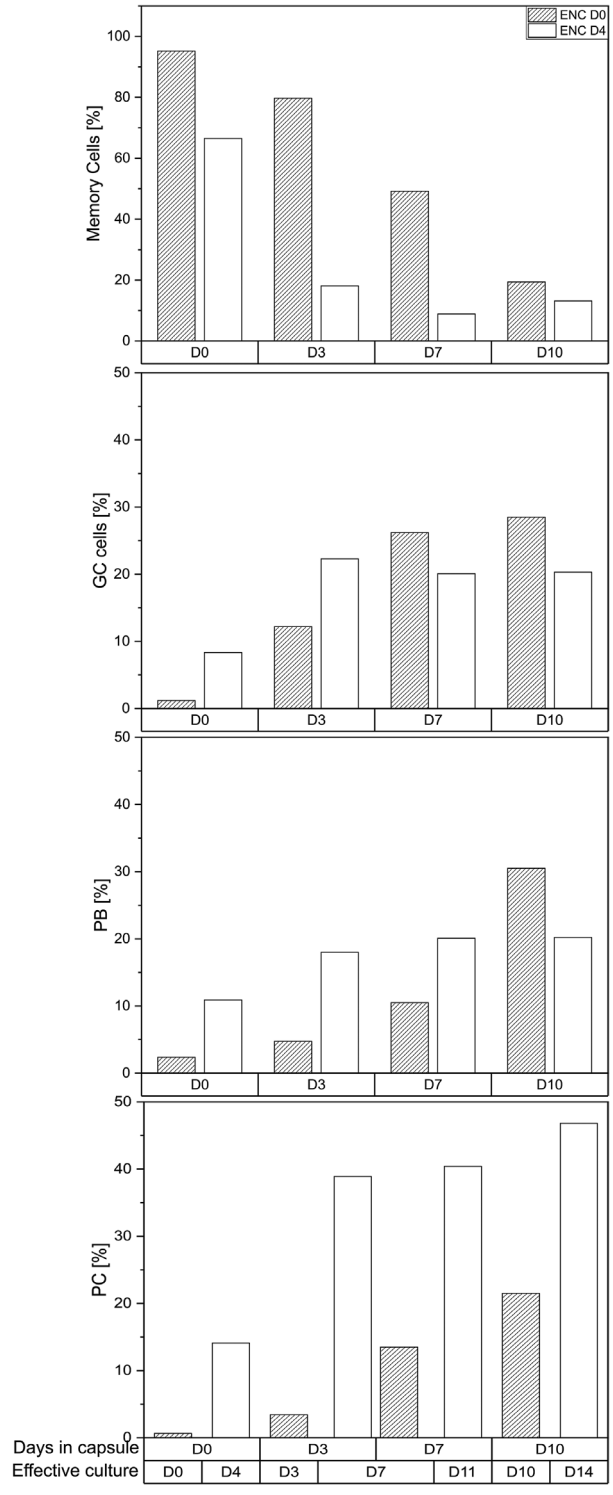

Figure 7. Bar charts representing B cell subpopulations over 10 days in capsule culture with and without four days pre-culture prior to encapsulation. B cells were encapsulated at 1×10^6 cells $mg_{capsules}^{-1}$ with standard GF concentrations (0.4 µg $mg_{capsules}^{-1}$ and 0.4 µg CD40L per 10^6 cells) as well as 10% v/v human serum (cells/$capsules_{withserum}$) in the capsule core. All cultivations were performed in basal medium. The effective culturing time represents the overall duration of B cell culture after thawing. Days in capsule represents the actual culturing time B cells were cultured inside capsules.

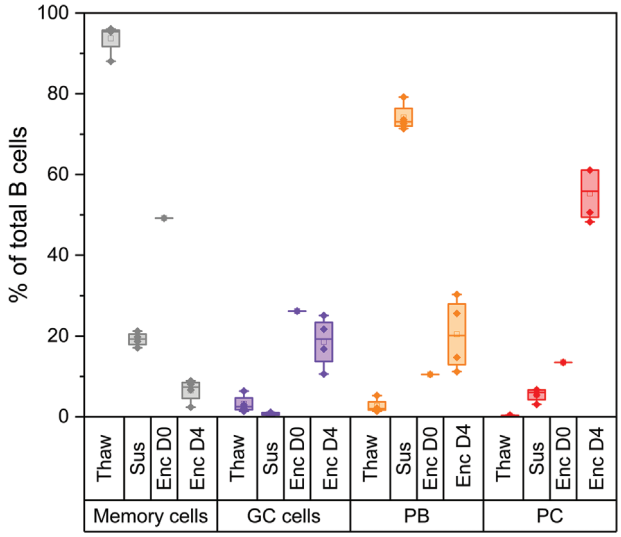

Figure 8. Summary of B cell subsets development in suspension and encapsulated cultures. Grouped boxplots comparing subpopulations of B cells straight after thawing ("Thaw"), on day 7 of encapsulated culture with ("Enc D4") or without pre-culture ("Enc D0") and suspension culture ("Sus"). Whiskers show the interquartile range. □: mean; -: median; ◆: count for a specific experiment.

were pre-cultured in suspension to induce their differentiation before encapsulation. Whether these PC maintain their status after release from the capsules and in particular their capability for antibody production and secretion is an important question that would need to be investigated in the future.

Cultivation in the proposed polyelectrolytes-based capsule system thus might help to conduct controlled B differentiation in in vitro culture without the necessity of adding specific costly signaling molecules. In future, such microcapsules could be used to induce improved organotypic GC development, supporting more tissue-like features than most other reported in vitro systems. Sufficient nutrient supply, conditioning of the microenvironment, and mimicking of ECM are key features of this system. Finally, such an approach paves the way for co-cultivation of multiple cell types involved in adaptive immunity. To conclude, microencapsulation of B cells is a promising method for improved GC reaction in vitro, bringing us one step closer to ex vivo immunization of human patients.

4. Experimental Section

Materials: If not otherwise indicated, Greiner Bio-One (Frickenhausen, Germany) was used as supplier for cell culture materials, while Sigma-Aldrich (Taufkirchen, Germany) was used as supplier for chemicals. Penicillin, streptomycin, amphotericin B, Hanks Balanced Salt Solution (HBSS), and Dulbecco's Phosphate-Buffered Saline (DPBS) were from Biochrom (Berlin, Germany). PBS (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KH_2PO_4, 10% v/v H_3PO_4, pH 6.3) was prepared in-house and sterilized by autoclaving (121 °C; 20 min; 2.05 bar). Fetal calf serum (FCS) and Iscove's Modified Dulbecco's Medium (IMDM) were from Biochrom or Biowest (Nuaillé, France). Human AB serum was from Sigma-Aldrich (lot: SLCF0679). Roswell Park Memorial Institute 1640 (RPMI1640) medium and UltraGlutamine were from Lonza (Visp, Switzerland). ITS-G (100×) (Insulin-Transferrin-Selenium supplement: 1 mg mL^{-1} insulin, 550 µg mL^{-1} rh-transferrin, and 670 ng mL^{-1} selenium) was from ThermoFisher Scientific (Schwerte, Germany). Molecular biology grade bovine serum albumin (BSA) (≥98%) was from Carl Roth (Karlsruhe, Germany). PDADMAC (100–200 kDa) was obtained as a 20% w/v aqueous solution from Sigma-Aldrich. SCS was from SykesTek GmbH (Nuremberg, Germany; charge ME45, degree of sulfation 0.34–0.36). Cellulase Cellulysin, from *Trichoderma viride* (EC 3.2.1.4; 1 U mg^{-1}) was from Merck (Darmstadt, Germany). Thiazolyl blue tetrazolium bromide, 98% (MTT) was from ThermoFisher Scientific. Recombinant human CD40L (23 kDa), IL-4 (15.1 kDa), IL-21 (15.5 kDa), and BAFF (17 kDa) were from Miltenyi Biotec (Bergisch Gladbach, Germany). Cyclosporin A (CSA) was obtained as powder from Sigma-Aldrich. Tonsillar tissue was obtained during routine surgeries (Gemeinschaftspraxis Gollner, Kulmbach, Germany). To reduce donor-derived variations between experiments, all B cells used in the experiments described hereafter stemmed from one particular donation from an adult donor. Written consent for utilizing the cells was obtained from the donor, after verbal and written information about research goals, as approved by the ethical review committee from the University of Bayreuth, Germany (written approval #O 1305/1-GB, 2021).

Isolation and Cryopreservation of Human Tonsillar B Cells: B cells were recovered as previously described.[12] Briefly, directly after removal, the tonsillar tissue was placed in Buffer 1 (HBSS—100 U mL^{-1}, penicillin—100 µg mL^{-1}, streptomycin—2.5 µg mL^{-1}, amphotericin B—2 mM, Ethylenediaminetetraacetic acid (EDTA)—0.5% w/v BSA) and transported in less than 1 h on ice to the laboratory. Thereafter, the tissue was immediately placed in RPMI1640 culture medium and cut into small pieces. The pieces were transferred into a 70 µm cell strainer (Greiner Bio-One), placed on a 50 mL centrifuge tube and the material was pushed through the mesh with help of a syringe piston. Remaining erythrocytes were lysed by incubation in erylysis buffer (155 mM NH_4Cl–10 mM $KHCO_3$–0.1 mM EDTA) for 5 min. Cell debris and any remaining red cells were removed by density gradient centrifugation (Ficoll LSM 1077; PAA Laboratories GmbH, Pasching, Austria) according to the supplier´s instructions. Mononuclear cells were collected and resuspended in buffer 2 (HBSS—10% v/v heat-inactivated FCS). A maximum of 4×10^8 cells in 4 mL buffer 1 were applied to a sterilized 20 mL syringe column (B. Braun, Melsungen, Germany) packed with 1 g sterile nylon wool (Polysciences Inc., Hirschberg an der Bergstrasse, Germany) and incubated upright for 1 h in the cell culture incubator. Afterward, the T cells were eluted by gently rinsing the wool twice with one column volume of buffer 2. Thereafter, the B cells were collected by filling the column with fresh buffer 2, followed by mechanical agitation to detach the cells. B cells were recovered by centrifugation (300 × *g*, 5 min) and resuspended in cryomedium (90% FCS, 10% dimethyl sulfoxide) before cryopreservation.

B Cell Cultivation and Stimulation: B cells were cultivated in IMDM containing 10% v/v human AB serum, 2 mM UltraGlutamine, ITS-G 1×, 1 µg mL^{-1} CSA, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. This medium was referred to as "basal medium." Further, the basal medium was supplemented with a mixture of "growth factors" (GF), namely 4 ng mL^{-1} BAFF, 10 ng mL^{-1} IL-4, 20 ng mL^{-1} IL-21, and 0.4 µg mL^{-1} CD40L, to generate the "growth medium."

After thawing the cryopreserved B cells, 1 mL cell suspension was diluted with 9 mL DPBS and cells were recovered by centrifugation (400 × *g*, 10 min). The supernatant was discarded, and mechanical agitation was used to dislocate the cell pellet. For standard cultivation ("suspension culture"), the B cells were seeded at a cell density of 0.1×10^6 mL^{-1} in growth medium. Suspension cultures were carried out in 24-well tissue culture plates with 1 mL medium per well. For medium change, B cells were recovered via centrifugation (400 × *g*, 10 min) and again resuspended in fresh growth medium at a cell density of 0.1×10^6 mL^{-1}. Encapsulated cells (3 mg of capsules) were cultured in 10 mL basal medium in 10 cm tissue culture plates. All cultures were performed in a cell culture incubator (37 °C, 95% humidity, 5% CO_2).

Determination of Cell Number and Viability: A LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea) was used to determine the number and viability of the cells. For this purpose, cells were stained with an Acridine Orange (AO)/Propidium Iodide (PI) solution (Logos Biosystems, Gyeonggi-do, South Korea) for live/dead staining according to the supplier's instructions. In case of the encapsulated

cells, the cells were first released from the capsules by cellulase treatment as described below prior to staining with the AO/PI solution. This allowed a statistically relevant estimation of the cell number and viability, which an in situ staining inside the capsules could not provide. Cellular expansion was calculated as the total amount of living cells measured on a given day divided by the number of living cells on starting day of culture or encapsulation, as specified for each individual experiment.

Cytotoxicity Test of Encapsulation and Cell Release Materials: For cytotoxicity testing, the B cells were incubated for 24 h in 1 mL of the substance (1% w/v cellulase, 0.85% w/v PDADMAC, 1.8% w/v SCS, 500 µg washed empty capsules) dissolved/ suspended in DPBS. Unexposed cells served as control. Thereafter, the MTT reagent (1 mg mL^{-1}) was added as described previously.[25] After 4 h of incubation at 37 °C, the cells were recovered and resuspended in 2 mL lysis buffer (40 mM HCl, 3% w/v sodium dodecyl sulfate in isopropanol). The formazan concentration was determined optically at 580 nm in a GENios Pro plate reader (TECAN Group, Männedorf, Switzerland).

Encapsulation and Release of B Cells: Procedures for encapsulation and release of the cells from the SCS/PDADMAC capsules were as reported previously,[24] albeit using a homemade, 3D-printed encapsulation device. Briefly, for encapsulation, a 1.8% w/v SCS solution was prepared in PBS and stirred overnight at room temperature. The solution was sterilized by autoclaving (5 min, 121 °C, 2.05 bar) and allowed to cool to room temperature for 2 h under constant stirring (150 rpm). GF such as IL-4, IL-21, BAFF, and CD40L were added to the SCS solution used to suspend the cells at the same concentrations as in the growth medium unless otherwise indicated. The PDADMAC stock solution was diluted to 0.85% w/v in PBS and sterilized by autoclaving (20 min, 121 °C, 2.05 bar). The B cells (viability > 85%), pre-cultured for 4 days after thawing in growth medium (i.e., culture medium, containing cytokines at the indicated concentrations and 10% v/v human AB serum), were collected by centrifugation (400 × *g*, 10 min). The supernatant was discarded and the cells were resuspended in human AB serum. Thereafter, the serum-coated cells were either i) recovered by centrifugation and subsequently resuspended at a concentration of 1×10^6 cells mL^{-1} in 3 mL of the SCS solution containing the GF at the indicated concentrations ("cells/capsules$_{withoutserum}$") or ii) diluted 1:10 with the SCS solution (final cell concentration also 1×10^6 cells mL^{-1}). In the latter case, the final SCS suspension and in consequence the capsule core still contained 10% v/v human AB serum ("cells/capsules$_{withserum}$"). In case of encapsulation of the B cells straight after thawing, the thawed cells were recovered by centrifugation (400 × *g*, 10 min), resuspended in human AB serum and subsequently mixed with SCS (containing GF at the indicated concentrations). Here also, the final cell density was 1×10^6 cells mL^{-1} and the capsule core contained yet 10% v/v serum. In all cases, the prepared cell/SCS solution was gently stirred for 5 min (50 rpm) and then pumped at 40 mL h^{-1} through the nozzle of the encapsulation device (Figure S6, Supporting Information), where droplets formed in a coaxial N_2 stream (≈4.5 L min^{-1}). A detailed description of the encapsulation nozzle construction is provided in the Supporting Information.

The polyelectrolyte capsules formed instantaneously when the droplets entered the well-agitated (300 rpm) PDADMAC hardening bath (50 mL). Afterward, the capsules were stirred for another 2 min in the hardening bath before adding 50 mL PBS. After 10 min stirring, the PDADMAC/PBS solution was replaced by 100 mL fresh PBS and stirred for another 10 min. This was repeated once more. Afterward, capsules were transferred to a 10 cm tissue culture dish containing 10 mL basal medium for cultivation. Immediately after encapsulation, aliquots of the capsules were recovered, and the cells were released from the capsules as described below. Subsequently, these cells were counted via live/dead staining with acridine orange/propidium iodide. The encapsulation efficiency was calculated from the initial living cell concentration (i.e., cells in the 1.8% SCS solution) before encapsulation compared to the concentration of living cells released from the capsules. The concentration of living cells released from the capsules on the day of encapsulation was subsequently also used for the calculation of the expansion factor during the cultivation. Detailed information on capsule properties as well as characterization methods are presented in the Supporting Information (Figure S2, Supporting Information).

For cell release, aliquots of the capsules were taken and the medium was aspirated as thoroughly as possible. The wet weight of the capsules was determined and 1 mg of capsule material was assumed to be the equivalent of 1 mL. A tenfold surplus (by weight) of a 1% w/v cellulase solution in DPBS was added. The mixture was incubated for 1 h at 37 °C under constant agitation (200 rpm) in a lab shaker (Kuehne, Basel, Switzerland). The brittle capsules were then broken by repeated up and down pipetting with a P1000 pipette (Eppendorf, Hamburg, Germany). To remove residual capsule material, suspensions were passed through a 70 µm mesh cell strainer (BD Biosciences, New Jersey, USA) before further analysis.

Flow Cytometry Analysis and Phenotyping: For phenotyping, characteristic cell surface markers for B cell subsets, CD19, CD20, CD27, and CD38 were assessed by flow cytometry (Cytomics FC500; dual laser (488 nm, 635 nm); Beckman Coulter, Krefeld, Germany) after staining the cells with CD-specific murine antibodies (anti-CD19-APC, #302212; anti-CD20-PE, #302306; anti-CD27-PE-Cy7, #356412; anti-CD38-FITC, #356 610; all from BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. For this, 0.5×10^6 cells were washed twice in 1 mL DPBS (400 × *g*, 5 min) and resuspended in 100 µL DPBS before incubation with the antibodies on ice for 30 min. Subsequently, cells were washed twice with 1 mL DPBS (400 × *g*, 5 min) and resuspended in 500 µL DPBS prior to analysis. Flow cytometric measurements were set to 80 000 events in total. Control cells (cells subjected to mock immunostaining) were used to set the measurement parameters. Forward scatter (FCS), side scatter (SSC), and fluorescence intensity (FITC emission 525 nm, PE emission 575 nm, APC emission 655 nm, PE-Cy7 emission 750 nm) were recorded. For analysis, the population was gated to identify single, viable, non-apoptotic cells using scattering properties (FSC/SSC) (Gate: "Lymphocytes"). Dead cells (low FSC and high SSC) and cell aggregates (high FSC) were excluded from the analysis. The "lymphocytes" population was further analyzed for the presence of surface markers. Within the "lymphocytes" population, a gate "B cells" was defined as containing the $CD19^+$ fraction. The cells, in the "B cells" gate, were then further analyzed for CD20, CD27, and CD38 expression (Figure S3, Supporting Information). In case of the B cells released from capsules, CD27 expression analysis was not possible (Figure S4, Supporting Information). Flow cytometry data were evaluated using FlowJo software v10.7.1 (Tree Star, Stanford University, Stanford, CA, USA, 2016).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.H. and S.B.H. contributed equally to this work. Conceptualization: M.H., S.B.H., V.J., and R.F. Methodology: M.H., S.B.H., K.G., and U.G. Analysis: M.H., S.B.H., and V.J. Investigation: K.G. and U.G. performed the surgery and isolated the tonsils, M.H. approached the research topic

from the biological side, S.B.H. approached the topic from the material science side. Data curation: V.J., and R.F. Writing—Original Draft preparation: M.H., S.B.H., V.J., and R.F. Writing—Review and editing: V.J., and R.F. Visualization: M.H., S.B.H., and V.J. Supervision: V.J. and R.F. Project administration: R.F.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

biomimetic microenvironment, differentiation, encapsulation, germinal center, human primary B lymphocytes

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