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Research Article

Transparent Substrates Prepared from Different Amorphous Polymers can Directly Modulate Primary Human B cell functions

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Abstract: Manipulation of B cell functions such as antibody and cytokine secretion, is of clinical and biotechnological interest and can be achieved by soluble ligands activating cell surface receptors. Alternatively, the exposure to suitable solid substrates would offer the possibility to transiently induced cell signaling, since the signaling is interrupted when the cells are removed from the substrate.
Cell/substrate interactions are mediated by physical valences such as, hydrogen bonds or hydrophobic forces on the substrate surface. Therefore, in this study B cells were cultivated on polymeric substrates, differing in their chemical composition and thus their capacity to undergo physical interactions.
Activated B cells cultivated on polystyrene (PS) showed an altered cytokine response indicated by increased IL-10 and decreased IL-6 secretion. Interestingly, B cells cultivated on polyetherurethane (PEU), which has among all tested polymers the highest potential to form strong hydrogen bonds showed an impaired activation, which could be restored by re-cultivation on tissue culture polystyrene.
The results indicate that B cell behavior can transiently be manipulated solely by interacting with polymeric surface, which could be explained by receptor activation mediated by physical interaction with the substrate or by altering the availability of the soluble stimulatory reagents by adsorption processes.

Keywords: B cells, biomaterials, cell culture devices, cytokines, polymeric substrates

Abbreviations:
7AAD, 7-Aminoactinomycin; AFM, atomic force microscopy; ANOVA, analysis of variance; APC, allophycocyanin; BCR, B cell receptor; CD, cluster of differentiation; CD40L, CD40Ligand; DAPI, 4',6-diamidino-2-phenylindole; FI, fluorescence intensities; FSC-A, forward scatter-area; human serum albumin (HSA), H12MDI, methylene bis(*p*-cyclohexyl isocyanate); Ig, immunoglobulin; IL, interleukin; LAL, *Limulus amebocyte* lysate; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PC, polycarbonate; PE, Phycoerythrin; PEI, poly(ether imide); PEU, polyetherurethane; PS, polystyrene; PSAN, poly(styrene-co-

acrylonitrile); SSC-A, sideward scatter-area; TCP, tissue culture polystyrene; TNF-α, tumor necrosis factor- alpha; TLR, toll-like receptor.

1. Introduction

B cells are non-adherent immune cells, which play a major role in establishing protective long term immunity by secretion of neutralizing antibodies [1]. Additionally, B cells can also present antigens to T cells and regulate immune reactions by the secretion of cytokines [2]. For example, B cells can suppress autoimmune reactions and immune reactions against pathogens by provision of IL-10 or IL-35 [3]. The option to expand B cells and instruct them to differentiate into immune stimulating or regulating phenotypes by the culture substrates, would be an attractive approach for biomedical applications, since the transfer of, for example, tolerance inducing B cells seems to be a feasible approach to suppress ongoing auto-immune reactions [4].

Furthermore, *in vitro* cultivation of B cells is challenging, since they only survive for a short time period in artificial environments [5]. Therefore, biotechnological application including antibody-production requires the generation of immortal B cell hybridomas to produce large quantities of monoclonal antibodies [6].

A prolonged B cell survival linked with enhanced production of antigen-specific antibodies may improve the efficiency of hybridoma generation [7]. The manipulation of such B cell functions can be achieved by soluble ligands, genetic engineering, or alternatively by the transient exposure to the solid substrates guiding B cell behavior.

For adherent cell types including stem and stroma cells, it is accepted that physical substrate properties such as topography and elasticity can influence and guide cells behavior [8]. For example, alteration of substrate elasticity can direct stem cells into specific lineage differentiation [9]. In addition to physical signals, adherent cells will in most cases also receive biochemical signals from proteins coated onto the substrate [10]. Such coatings typically contain extracellular matrix (ECM) proteins and are required for cell adhesion, which is mediated by integrin interactions with the RGD sequence of ECM proteins [11].

Intuitively, for non-adherent cell types including most of the circulating immune cells, the influence of the substrate seems to be negligible. However, non-adherent cells cultured under static conditions will sediment to the substrate bottom, directly contacting the polymeric surface and possibly interacting with their cell surface molecules via physical valences, which are defined by the chemical structure of the

polymer. Physical valences include interactions between π-systems, hydrogen bonds, dipole-dipole interactions and hydrophobic or van der Waals (vdW) forces. Particularly, cell surface receptors could interact with the polymer surface by such physical valences. After steric matching the most ligands interact with their receptor by hydrogen bonds or ionic interaction leading to conformational changes or crosslinking, which subsequently induces intracellular signaling [12].

Unlike stroma cells, B cells express receptors to sense foreign structure by their B cell receptor (BCR) that can bind protein antigens or toll-like receptors (TLR), which are engaged by pathogen associated molecular pattern (PAMP) [1]. Activation of those receptors requires the interactions with a specific ligand by physical valences. Since substrate coating is not required for the cultivation of non-adherent cells, the cells may receive signals directly from the substrate surface. Accordingly, depending on the chemical structure certain polymers may also be able to activate receptors in an unspecific fashion, which may also explain the often unpredictable *in vivo* behavior of polymer-based implants [13]. Free physical valences can also lead to adsorption of certain molecules, such as soluble factors used to activate cells, from cell culture medium. For example, immunoglobulins or PAMP, which are frequently used to specifically activate B cell surface receptors such as the BCR or TLR, can adsorb to polymeric surfaces with a high number of free valences [14].

Such adsorption could, dependent on the arrangement and orientation of the molecules, increase or decrease their stimulatory capacity of those reagents. Accordingly, it can be hypothesized that polymers differing in their valences for the interaction with cell surface molecules or factors present in cell culture medium can substantially influence the behavior of untreated or stimulated B cells regarding their proliferative response and their capacity to secrete antibodies and cytokines.

To prove this hypothesis B cell behavior was investigated on polystyrene (PS), poly(styrene-co-acrylonitrile) (PSAN), polycarbonate (PC), poly(ether imide) (PEI), and polyetherurethane (PEU), which differ systematically in their capacity to form physical interactions via free valences. The roughness levels of the different polymers were in the same range: all with R_q below 1 µm indicating a smooth surface. Therefore, it can be assumed that the roughness can be neglected and should not influence the B cell behavior. Substrate elasticity can have substantial effects on cellular behavior, for example, soft substrates can induce stem cell neurogenic differentiation, while harder substrates mediate osteogenic differentiation

[15]. However, the polymeric substrates used in this study showed elasticities above 6 GPa, which is higher than elasticities found in soft tissue but fits the macroscopic properties of bone [16]. Furthermore, standard cell culture substrates exhibit elasticities in the same range as the substrates used in this study. Therefore it can be assumed that the elasticities of the polymers do not substantially influence the B cell behavior.

All investigated polymers are easily processible and transparent to enable microscopic observation. It could be shown that the substrates exhibit no cytotoxic effects on L929 fibroblasts and mesenchymal stem cells, indicating that during the manufacturing and sterilization process no cell toxic substances are generated or introduced [17]. The substrates were fabricated as cell culture inserts fitting into individual wells of a 24-well cell culture plate to restrict cell contacts exclusively to the polymeric sample [17b]. PS as conventional cell culture vessel material generates π–π interactions resulting from the aromatic groups and has the possibility to interact by dispersive forces such as van der Waals interaction mediated by the carbon hydrate backbone.

PC and PSAN, as copolymer of styrene and acrylonitrile, have a proven durability and biocompatibility [17a, 18]. PSAN also exhibits π–π interactions and can additionally form dipole-dipole interactions by the nitrile group. Likewise PC can also form π–π interactions and the carbonyl groups have the capacity to develop dipole-dipole interactions. PEI as rod-like polymer has multiple valences allowing the formation of strong π–π interactions due to the high number of aromatic rings, hydrophobic interaction by the methyl groups, and dipole-dipole or hydrogen interactions by imide moieties as well as the ether groups are also possible. Additionally, PEI surfaces are suitable for chemical modification or functionalization with biomolecules [19].

PEU as biocompatible elastic thermoplastic material allows the tailoring of mechanical properties by variation of the hard to soft segment ratio [20]. PEU is unable to form π–π interactions due to the absence of aromatic rings, but has a high potential to interact with other cell surface molecules via H-bonds formed by the urethane group and is capable of forming hydrophobic interactions via the carbon hydride backbone. An indicator for the strength of intra-molecular interactions and potentially for the strength of interactions with cell surface molecules is the glass transition temperature T_g. PEU has the lowest T_g followed by PSAN, PS, and PC. PEI

showed the highest T_g ($T_{g,PEU}$ = 56 ± 2 °C; $T_{g,PS}$ = 109 ± 2 °C; $T_{g,PSAN}$ = 108 ± 2 °C; $T_{g,PC}$ = 149 ± 2 °C $T_{g,PEI}$ = 215 ± 2 °C as determined by Differential Scanning Calorimetry (DSC) [17a]).

In order to correlate the considerably different valences and subsequently the potential interactions with cell surface molecules, the response of primary human B cells regarding their survival, proliferation, antibody secretion and cytokine release was investigated after cultivation on the different polymers. The behavior of untreated B cells and B cells activated via their TLR9, CD40, and BCR cultivated on the different polymers was compared to explore whether the polymers can directly activate the B cells or act synergistically or antagonistically with the B cell activating reagents.

2. Experimental Section

2.1 Materials, Processing, and Surface Characterization of the Polymeric Inserts

The following five transparent polymers were processed into cell culture inserts via injection molding as previously described, polystyrene (PS, Type158K, BASF, Germany), polycarbonate (PC, Makrolon® 2805, Bayer, Germany), poly(ether imide) (PEI, ULTEM® 1000, General Electric, USA), polyetherurethane (PEU, Tecoflex® MG8020, Lubrizol, USA) and poly(styrene-*co*-acrylonitrile) (PSAN, Luran HD-20, BASF, Germany) [17a]. The polymers were processed by an injection molding automat (Alrounder 270U, Arburg Corp., Münsingen, Switzerland) equipped with a custom made mold (Dreuco Formenbau GmbH, Berlin, Germany), using previously described parameters [17a]. The chemical composition of the different polymeric insert surfaces (PS, PSAN, PC, PEI, and PEU) and the related valences available for physical interactions with the cells could be confirmed by X-ray photoelectron spectroscopy analysis (data not shown).

Prior to surface characterization and biological experiments the PS, PC, PEU, and PSAN inserts were gas sterilized and the PEI inserts were steam sterilized. To exclude residual ethylene oxide the sterilization phase of 3 hours was followed by a desorption phase of at least 6 hours. This desorption phase includes 5 min cycles of air addition followed by vacuum generation (-0.8 bar) for 1 min. At ambient temperature all inserts exhibited surface roughness levels R_q below 1µm determined by optical profilometry and advancing water contact angles (θ_{adv}) between 84° ± 7°

and 99° ± 5°. The Young's modulus was in the GPa range between 6 ± 1 GPa and 24 ± 5 GPa as determined by atomic force microscopy (AFM) based nanoindentation in the dry state at ambient temperature [17a]. Sterile, pyrogen free, and corona discharged treated TCP, which was used as reference substrate, was purchased from TPP® (Trasadingen, Switzerland). TCP had an advancing water contact angle (θ_{adv}) of 22 ± 1° and a surface roughness R_q of 0.13 ± 0.05 µm determined by optical profilometry. The endotoxin levels of the inserts were determined by the LAL-test (Lonza, Cologne, Germany), which was performed from sample eluates prepared according to ISO 10993/12. All polymeric cell culture inserts showed endotoxin levels below 0.06 EU·mL^{-1}, which is well below the limit stipulated by the U.S. Food and Drug Administration for eluates of medical devices.

2.2 B Cell Isolation and Cultivation

Peripheral blood mononuclear cells (PBMC) were isolated from Buffy coats of healthy donors obtained from the DRK Blutspendedienst Nord-Ost gGmbH Institut (Berlin, Germany) as previously described by density gradient centrifugation using Biocoll™ (Biochrom AG, Berlin, Germany) [21]. To isolate CD19 positive B cells, CD19 negative cells were depleted by magnetic cell sorting using B cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) performed according to manufacturer's instructions using the autoMACS® Pro Separator (Miltenyi Biotec). The purity of the CD19 positive B cells was determined by flow cytometry (MACSQuant®, Miltenyi Biotec) after staining with CD19-FITC (clone LT19, Miltenyi Biotec) as B cell marker and CD3-PE (clone BW264/56, Miltenyi Biotec) and CD14-PC7 (clone M5E2, BD Pharmingen, Heidelberg, Germany) to check for T cell and macrophage contaminations, respectively. Routinely, isolated B cells had a purity of above 95% with a viability of above 98% as defined as 4',6-diamidino-2-phenylindole (DAPI) negative cells. To determine proliferation rates, B cells were labeled according to manufacturer's instruction with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Invitrogen/Live Technologies GmbH, Darmstadt, Germany). 5×10^5 unlabeled or labeled B cells were seeded in 500 µl VLE-RPMI Medium supplemented with 10% FBS (Biochrom AG, Germany) into the different polymeric cell culture inserts to determine their viability and their proliferative response, respectively. B cells were left untreated or were activated with CpG ODN2006 (2 µg·mL^{-1}; Axxora, Enzo Life Sciences, Lörrach, Germany) to stimulate Toll-like receptor (TLR)-9, $F(ab)_2$ (2 µg·ml^{-1}, goat anti-human IgG+IgM (H+L)

(Jackson ImmunoResearch Europe Ltd., Newmarket, UK)) to crosslink the B cell receptor (BCR), and with CD40-Ligand (CD40Lmix; 2.5 µg·mL^{-1}, Miltenyi Biotec) to stimulate CD40. For the re-stimulation experiments 5×10^5 B cells were initially primed in 24 well TCP or PEU for 24 hours, washed and then re-cultivated in a 96 well TCP at 1×10^5 B cells in 200 µl, so that the cell density was identical to 24 well TCP.

2.3 B cell Viability and Proliferation Assessment

The viability of B cells was determined after two days of culture at 37 °C, 5% CO_2 by flow cytometry using the Annexin V-PE/7-AAD Kit (BD Pharmingen) according to manufacturer's instruction.

After five days of cell culture, CFSE dilution in proliferated B cells was analyzed by flow cytometry. For viability and proliferation analysis, B cells were stained with anti-CD19-APC-vio770 (clone LT19, Miltenyi Biotec), to exclude contaminating cells. Prior antibody staining, cells were treated with human FcR-blocking reagent (Miltenyi Biotec) to avoid unspecific antibody binding to Fc-receptors. To discriminate live and dead cells, DAPI (1 µg·mL^{-1}, Roth, Karlsruhe, Germany) was added immediately prior to analysis, while cells were kept at 4 °C to minimize metabolic activity and active uptake pathways of living cells. Acquired data were analyzed with the Flowjo software (Tree Star Inc., Ashland, Oregon, USA).

2.4 Ca^{2+} Influx Detection in B cells

To measure the Ca^{2+} influx in activated B cells the Fluo-4 Direct™ (Life Technologies™, Darmstadt, Germany) calcium assay kit was used according to manufacturer's instructions for flow cytometry and microscopy. In brief, purified B cells were incubated with the Fluo-4 Direct™ (30 min, 37 °C, 5% CO_2) calcium reagent loading solution. Labeled B cells were activated with CpG ODN2006 (2 µg·mL^{-1}), F(ab)$_2$ (2 µg·mL^{-1}), and CD40-Ligand (CD40Lmix; 2.5 µg·mL^{-1}) and immediately flow cytometrically or microscopically analyzed.

2.5 Multiplex Cytokine and Antibody Profiling of B cells

Cell culture supernatants from B cell cultures were harvested after five days and analyzed for cytokine content using the multiplex suspension assay (Bio-Plex200®, BioRad, München, Germany) according to the manufacturer's instructions. The following factors were determined: IL-6, IL-10, TNF-α, IL-17A, all purchased from BioRad. To calculate the concentration an eight times 4-fold serial dilution was

prepared as standard with the starting concentration of 25,160 pg·mL^{-1} (IL-6), 26,094 pg·mL^{-1} (IL-10), 107,172 pg·mL^{-1} (TNF-α) and 29,157 pg·mL^{-1} (IL-17A). After eight 4-fold dilutions the lowest standard concentration were 1.5 pg·mL^{-1}, 1.6 pg·mL^{-1}, 6.5 pg·mL^{-1}, and 1.7 pg·mL^{-1} for IL-6, IL-10, TNF-α, IL-17A, respectively. To analyze the amount of secreted total IgG, IgM, IgA, and IgE, the Isotyping Panel Kit (BioRad) was used according to manufacturer's instructions.

2.6 Statistical Analysis

Graphical representation and statistical analysis were performed using GraphPad Prism version 6.02 for Windows, (GraphPad Software, La Jolla, California, USA). Graphs show mean values and standard error of mean for all six samples measured. Unless otherwise indicated statistical analysis of the data was performed using one-way analysis of variance (ANOVA) of repeated measurements. To determine statistical significances One-way ANOVA with a Dunnet post-test to compare either all untreated samples with the untreated TCP control or all activated samples with the activated TCP control was performed. (* for $p<0.5$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$.) The mean with error bar indicating standard deviation (SD) is shown.

3. Results

3.1 Viability of B cells After Cultivation in Different Cell Culture Inserts

In order to determine whether polymeric cell culture inserts with different chemical structures and consequently different capacities to interact with cell surface molecules [17a] can influence the B cell behavior, primary human B cells were purified from buffy coats using magnetically associated cell sorting in which all non-B cell were magnetically labelled and separated from the non-labeled B cells. The resulting B cell purity was analyzed by flow cytometry routinely above 95% with less than 5% dead cells (**Figure 1A**). The highest amount of contaminating cell population observed were about 0.5% of CD14 positive monocytes, about 1.2% CD3 positive T cells and about 1.3% of cells negative for CD14, CD3, CD19, and CD20 (data not shown).

The purified B cells were subsequently cultivated for two days in the different culture vessels (Figure 1B). Commercially available polystyrene-based TCP served as reference material. The microscopic assessment of the B cell culture using low level

magnifications provides insights about the activation status of the B cells, while the single cell morphology even when observed with higher magnification will not lead to additional insights, since the single cell morphology will not substantially change after two days in culture. When untreated B cells were cultivated in the different cell culture inserts neither morphological differences of the cell culture nor spontaneous aggregation of the cell could be observed, indicating that the different polymers themselves did not directly activate the B cells (Figure 1B). To induce an optimal B cell proliferation and differentiation into antibody secreting cells BCR, TLR, and CD40 stimulation is required [22]. B cells stimulated for two days on TCP showed large cell aggregates, indicating the induction of a proliferative response (Figure 1B). On PS, PEI, PC, and PSAN the aggregates of activated B cells appeared to be of similar size and number when compared to TCP. Interestingly, B cells cultured on PEU only showed very small cell aggregates, indicating a suppressed activation or a decreased survival of the cells (Figure1B).

In order to analyze the viability of untreated and activated B cells after cultivation in the different substrates, B cells were stained with Annexin V and 7AAD to discriminate apoptotic from dead and necrotic cells by flow cytometry (Fig. 2A). Live B cells were negative for Annexin V and 7AAD, while apoptotic B cells were defined as Annexin V positive and 7AAD negative cells. A double positive staining for Annexin V and 7AAD is indicative for late apoptotic and necrotic B cells (Fig. 2A). After two days of activation on TCP the rate of viable B cells slightly decreased compared to untreated B cells and accordingly the amount of dead/necrotic cells increased (Fig. 2A, middle and right). The rate of apoptotic B cells cultured on TCP is not affected by activation (Fig. 2A). In order to quantify the survival of B cells after contact to different polymeric cell culture inserts, the viability of B cells isolated from six individual donors was analyzed. When untreated B cells were cultured on PS and PEI inserts, their 2-day viability was significantly increased compared to TCP, while survival of B cells cultivated in PC and PSAN inserts was not altered. Furthermore, cultivation of B cells on PEU resulted in a significantly decreased viability (Fig. 2B). The fraction of apoptotic B cells, which have been cultivated in the different cell culture inserts, did not significantly differ from B cells cultured in TCP (Fig. 2C).

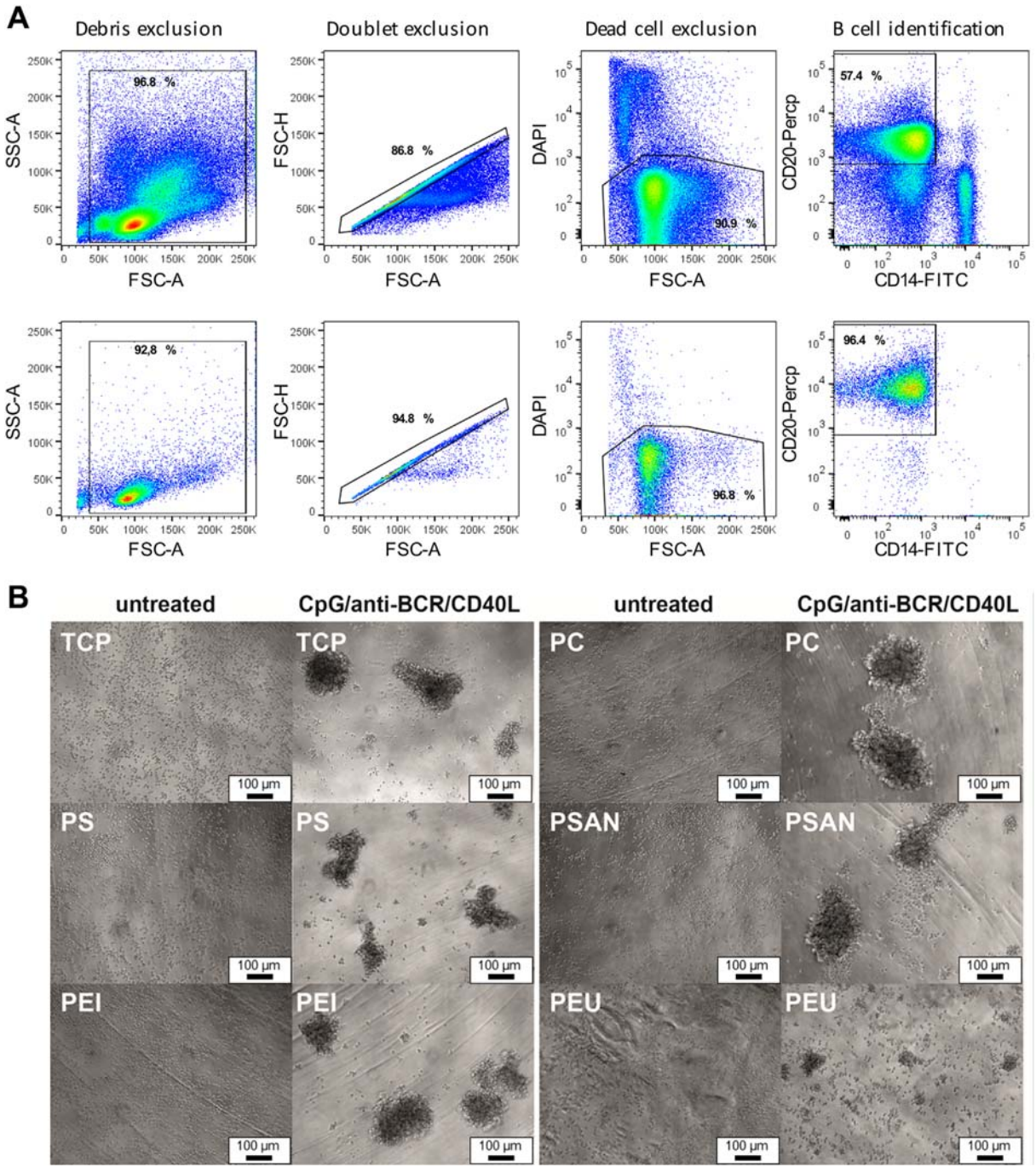

Fig.1: B cell purification and macroscopic culture morphology. (A) Quality assessment of the B cell purification. Flow cytometric analyses of PBMC (top row) and of purified B cells (bottom row). From left to right: Scatter plot, which distinguished cells from debris; FSC-H plotted against FSC-A to exclude cell doublets [53]; DAPI versus FSC-A was plotted to discriminate between live and dead cells; CD20 versus CD14 identified B cells and monocytes. Dot plots are representative of six individual donors analyzed. (B) Images of untreated and activated B cells cultivated in PS, PEI, PC, PSAN and PEU inserts. B cells were cultivated for two days and images were taken by a phase contrast microscope. Images

are representative for cells isolated from six individual donors analyzed in two independent experiments (n = 6).

In line with the increased number of viable B cells the fraction of dead/necrotic B cells was significantly reduced after cultivation in PEI-based inserts, while a significant increase of dead/necrotic B cells was observed after cultivation in PEU-based inserts (Fig. 2D).

B cells activated by CpG/anti-BCR/CD40L showed an impaired viability after two days of cultivation when compared to naïve, untreated B cells. This could be a physiological apoptosis of B cells or antibody-producing, short-lived plasma blasts to avoid undesired immune reactions (Fig. 2B). Cell culture inserts fabricated from PS, PEI, PC, and PSAN did not alter the viability of activated B cells. However, activated B cells cultured on PEU showed significantly more live cells compared to TCP (Fig. 2B). After activation, the fraction of apoptotic cells was significantly increased only when B cells had been cultivated in PEI inserts (Fig. 2C). The fraction of dead/necrotic B cells was decreased significantly on PEI, PC, and PEU, which corresponds to the increased number of live and/or apoptotic cells (Fig. 2D).

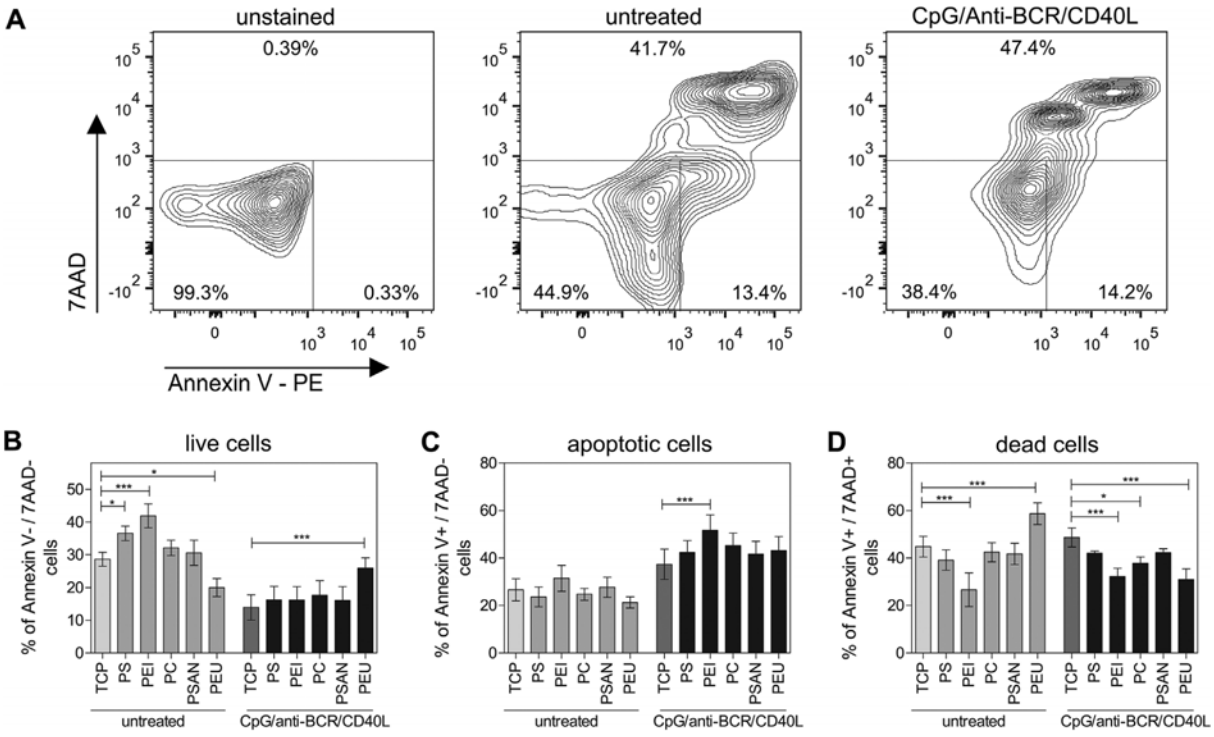

Fig. 2: Quantification of apoptotic cells by flow cytometry. Viability of B cells was determined after two days of incubation. B cells were left untreated or were activated with CpG, anti-BCR, and CD40L. (A) Flow cytometric discrimination of live, apoptotic, and necrotic B cells cultivated on TCP. Gates were set according to the unstained control (left), Annexin V

binding, and 7AAD incorporation were determined in untreated (middle) and CpG-, anti-BCR-, and CD40L-activated B cells (right). (B) Quantification of the live frequencies (C), apoptotic cell frequencies (D), and dead cells frequencies from six individual donors analyzed in two independent experiments (n = 6).

3.2 Proliferation of B Cells Cultivated on Different Polymeric Substrates

The rate of expansion of different cell types can be influenced by the culture substrate used [17a, 23]. Given the transient nature of any interaction of suspension cells merely settling on a substrate surface, it is valid to ask if this holds true for the B cells analyzed here. In order to determine the proliferative response of B cells towards the different cell culture inserts, CFSE-labeled primary human B cells were cultivated for five days in the culture vessels and were subsequently analyzed by flow cytometry for CFSE dilution. To identify proliferated B cells and to quantify their proliferation rate, the gating strategy shown in Figure 3 A-C was applied to every sample. To determine the percentage of B cells that had undergone cell division(s), histogram overlays of unstimulated and activated B cells were used, in which the gate was set according to resting, untreated B cells (Fig. 3D). Untreated B cells did not show proliferation, whereas after activation of TLR-9, the BCR, and CD40 between 75 and 98% of the B cells proliferated after five days in culture on TCP, indicated by declining CFSE signals (Fig. 3E and F). Activated B cells, which were cultivated on PS, PEI, PC, or PSAN showed a similar proliferation compared to TCP. Remarkably, when B cell activation was performed on PEU, the proliferative response was completely abolished (Fig. 3E and F), in line with the non-quantitative microscopic observations presented in Fig. 1B.

Activation of B cells leads to an increase of intracellular Ca^{2+} concentrations, which is a crucial event for their cell cycle entry [24]. Therefore, it was investigated whether the initial activation of B cells is inhibited through their contact with PEU, which could account for their lack of proliferation. Cultures of untreated B cells seeded on either TCP or PEU showed in both cases only few Ca^{2+} cells (fluorescent cells in Fig. 4A, left). B cells activated for 45 minutes and cultivated on TCP showed substantial Ca^{2+} influx, indicated by a high number of fluorescent cells (Fig. 4A, right).

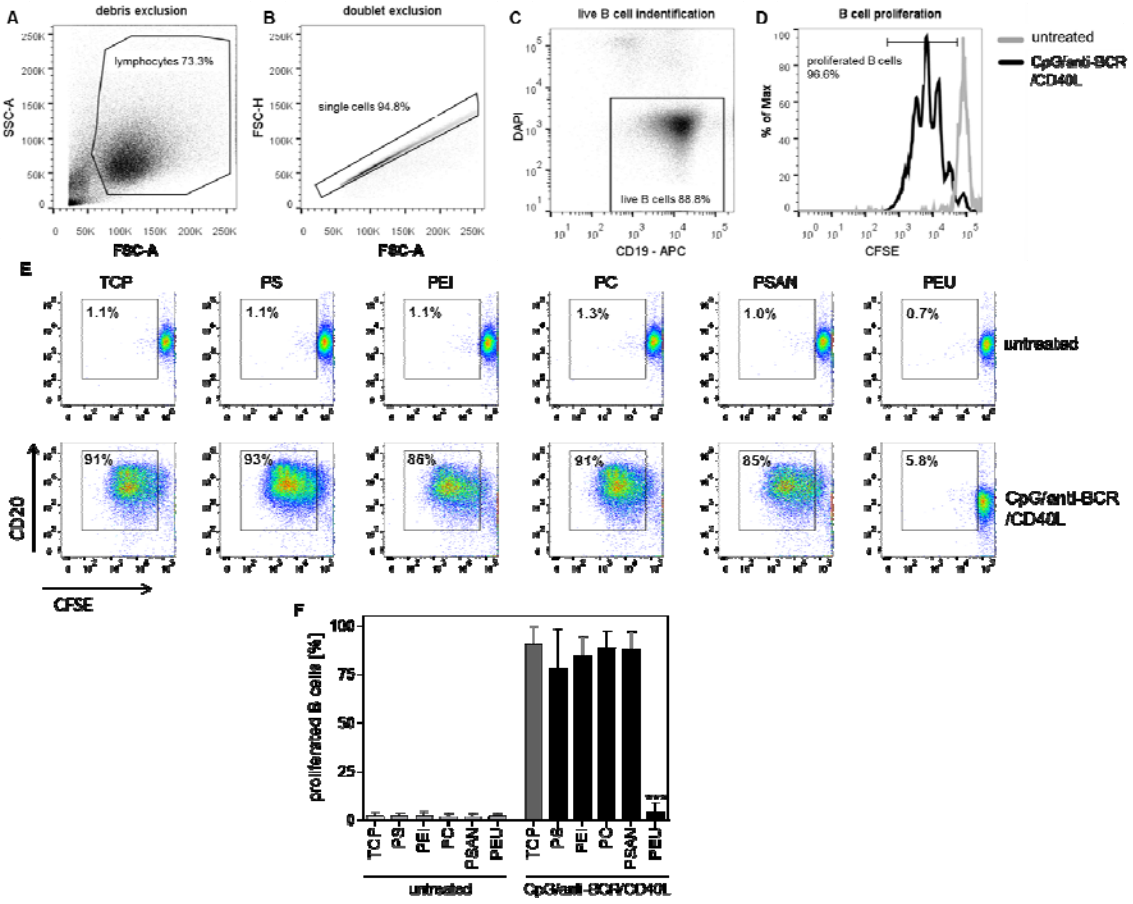

Fig. 3: Quantification of the B cell proliferation after cultivation on the different substrates for five days. To quantify B cell proliferation the following gating strategy was applied: (A) Debris was discriminated from cells according to forward (FSC) and sideward (SSC) scatter parameters. (B) Cell aggregates were excluded by plotting the FSC-area (A) versus FSC height (FSC-H). (C) Live B cells were defined as CD19 positive and DAPI negative. (D) Proliferation of B cells was monitored by CFSE dilution, the gate was set according to untreated B cells. (E) Representative dot plots from one individual donor showing the proliferation of untreated and activated B cells cultivated on the different polymers. (F) Average frequencies of the B cell proliferation pooled from six individual donors analyzed in two independent experiments each with three donors (n = 6).

In contrast, activated B cells cultivated on PEU did not show Ca^{2+} influx, since the number of fluorescent cells did not increase compared to the cultures with untreated B cell in PEU (Fig. 4A). These data indicate that the initial activation of B cells is blocked by PEU. To investigate whether the Ca^{2+} influx inhibition depends on the direct contact with the PEU, untreated B cells were cultivated on TCP and PEU for 24 h, harvested, left untreated or were stimulated with CpG/anti-BCR/CD40L followed by an immediate flow cytometric analysis of the Ca^{2+} influx. As expected, the analysis of

untreated B cells did not show a Ca^{2+} influx (Fig. 4B). B cells harvested from TCP and treated with CpG/anti-BCR/CD40L showed a substantial Ca^{2+} influx. Interestingly, also for B cells, which were harvested from PEU, a moderate increase of intracellular Ca^{2+} levels could be observed, which was higher than that detected for untreated B cells (Fig. 4B). The quantification of the Ca^{2+} influx from three individual donors confirmed that more B cells harvested from PEU and activated with CpG/anti-BCR/CD40L stain positive for Ca^{2+} than in the case of untreated B cells (Fig. 4B). This endpoint quantification of intracellular Ca^{2+} mobilization in B cells revealed that about 60% of the B cells could be activated on TCP, while among untreated B cells only about 10% showed elevated Ca^{2+} levels. After cultivation on PEU more than 20% of B cells could be activated within the three minutes required for flow cytometric analysis (Fig. 4C). These data indicate that at least a fraction of B cells maintain their sensitivity for activation via TLR9, BCR, and CD40 even after priming on PEU for 24 hours. Next, it was investigated whether the B cells response on PEU is permanently blocked or whether the activation is only transiently inhibited. More precisely, can B cells activation be restored upon re-cultivation on TCP? To answer the question whether the proliferative response of PEU-primed B cells can be restored, TCP and PEU cultured B cells were activated and harvested after 24 hours of incubation (Fig. 4D). After washing, B cells were re-cultivated on TCP and either left untreated or re-activated with CpG, anti-BCR, and CD40L followed by another four days of incubation (Fig. 4D). The proliferation of B cells was then analyzed by flow cytometry comparing the re-cultivated B cell with B cells cultivated solely for five days on TCP or PEU. Activated B cells initially cultivated on TCP continued to proliferate after transfer into a new well, but showed a decreased proliferation rate compared to B cells left for the whole five days on TCP (Fig. 4E and F). After re-activation a proliferation profile comparable to B cells activated for five days was observed (Fig. 4E and F). As described above (Fig. 3), the proliferative response of B cells activated on PEU was almost completely inhibited. Interestingly, even PEU-primed B cells, which were not re-activated with CpG/anti-BCR/CD40L started to proliferate after they have been re-cultivated on TCP (Fig. 4E and F). After re-activation, more than 90% of the PEU-primed B cells proliferated on TCP. These data indicate that B cell proliferation is only arrested when B cells are in direct physical contact to PEU, since re-cultivating on TCP activated B cells and restored their proliferative response.

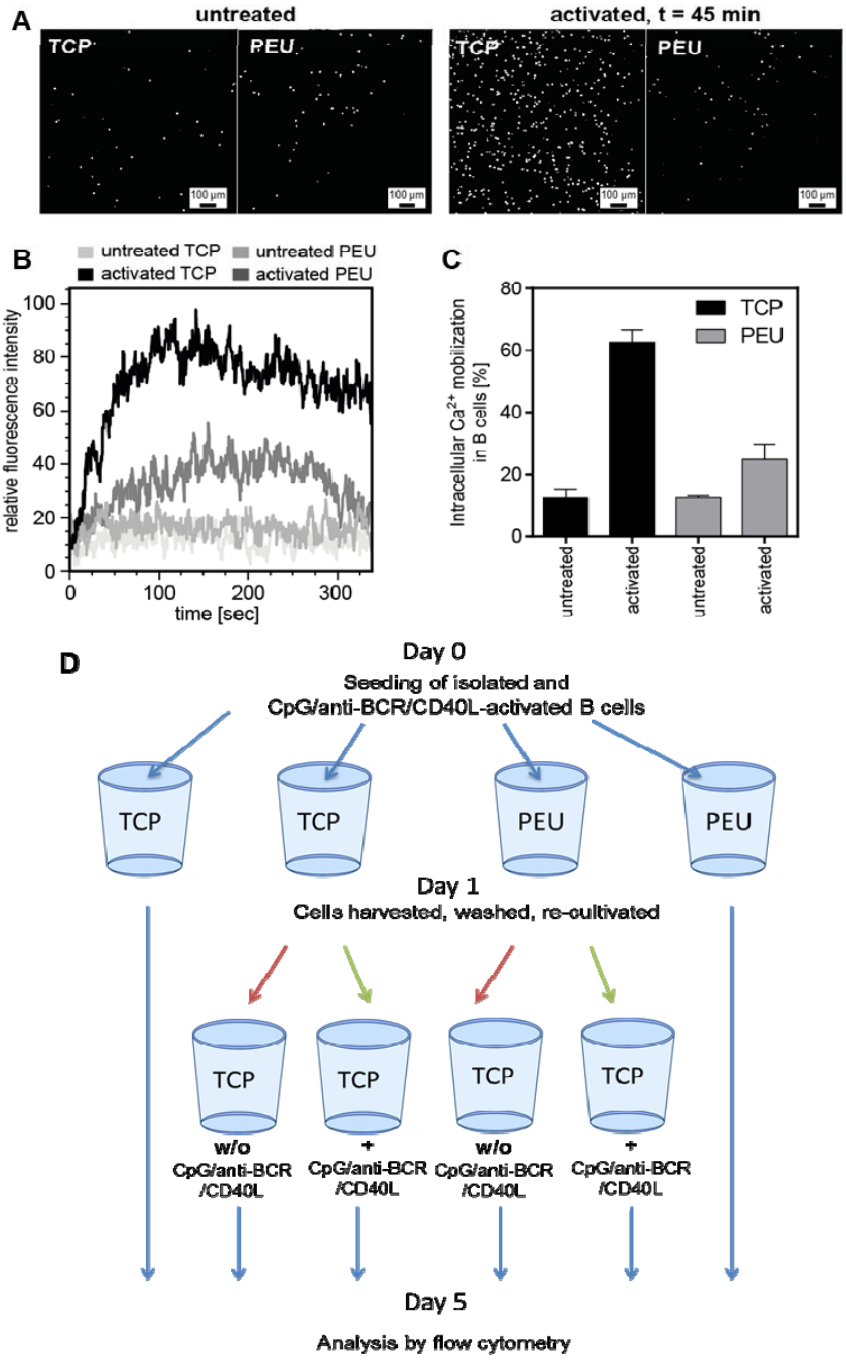
A
untreated
activated, t = 45 min
TCP
PEU
100 µm
B
untreated TCP
untreated PEU
activated TCP
activated PEU
relative fluorescence intensity
time [sec]
C
TCP
PEU
Intracellular Ca2+ mobilization in B cells [%]
untreated
activated
D
Day 0
Seeding of isolated and
CpG/anti-BCR/CD40L-activated B cells
Day 1
Cells harvested, washed, re-cultivated
w/o CpG/anti-BCR /CD40L
+ CpG/anti-BCR /CD40L
Day 5
Analysis by flow cytometry
E
TCP
TCP-primed untreated
TCP-primed activated
PEU
PEU-primed untreated
PEU-primed activated
fluorescence intensity
F
proliferated B cells [%]
ns
5 days culture
primed on TCP
primed on PEU
re-cultivated on TCP

Fig. 4: Activation of B cells is transiently blocked by PEU. (A) Untreated or CpG/anti BCR/CD40L activated B cells were cultivated on TCP or PEU and Ca^{2+} influx was detected microscopically after 45 minutes. Images are representative for two independent experiments. (B) Untreated B cells were cultivated for 24 hours on TCP or PEU. The harvested B cells were activated with CpG/anti BCR/CD40L and immediately analyzed for the Ca^{2+} influx by flow cytometry. (C) Endpoint Ca^{2+} influx quantification from B cells of individual donors (n = 3). (D) Flow chart of the B cell re-cultivation experiment. CpG/anti-BCR/CD40L treated B cells were initially cultivated on TCP or PEU, harvested after 24 hours, washed, and re-cultivated on TCP for another four days. The proliferation was compared to activated B cells left for the whole five days in TCP and PEU. (E) Flow cytometric proliferation analysis of B cells cultivated and re-cultivated as described in (D). Histogram overlays from one representative donor. (F) Quantification of B cell proliferation using cells from three individual donors (n = 3). For statistical analysis One-way ANOVA with a Sidak's multiple comparisons test to compare the two re-cultivated untreated samples with each other and the two activated samples with each other was performed (mean ± SD).

3.3 Antibody and Cytokine Production by B Cells Cultivated on Different Polymeric Substrates

Activated B cells can differentiate into antibody secreting plasma cells, which can produce antibodies of different immunoglobulin (Ig) isotypes such as IgM, IgG, IgA, IgD, or IgE. This Ig secretion capacity is essential for antigen neutralization and immunity in general, but, if dysregulated, can also result in undesired *in vivo* reactions such as allergies [25]. *In vitro*-stimulated B cells can also secrete antibodies, particularly IgM and IgA [26]. Therefore, after cultivation for five days in the different cell culture inserts, the antibody levels of untreated and activated B cells were determined. Similar secretion of IgM and IgA was induced in activated B cells cultivated in TCP, PS, PEI, PC, or PSAN. When B cells were cultured on PEU neither IgM nor IgA antibodies were detectable in the supernatant (Fig. 5A and B). The secretion of IgA seemed to be diminished for TCP cultured B cells compared to B cells cultured on PS, PEI, PC, and PSAN. This effect, however, was not significant, due to high variations within the group of donors. The lack of antibody secretion by PEU is in line with the cell cycle suppression and indicates a temporary blockade of activation (Fig. 5A and B). Activated B cells can regulate immune responses by secretion of cytokines [3b]. Thus, the influence of polymeric substrates on the cytokine production of B cells was investigated. Therefore, supernatants of untreated and activated B cells cultured on TCP and in the different polymeric cell culture inserts were analyzed for IL-6, IL-10, IL-17, and TNF-α expression. The secretion of

the pro-inflammatory cytokine IL-6 was reduced on all inserts but only significantly when B cells were cultured in PS, PSAN, and PEU-based inserts (Fig. 5C). Interestingly, the secretion of the anti-inflammatory IL-10 was significantly increased compared to TCP when B cells were cultivated on PS or PEI inserts (Fig. 5D). B cells cultivated on PEU produce very little amounts of IL-10 (Fig. 5D). Recently, it was described that B cells can produce IL-17, which was found to be required for an optimal response against *Trypanosoma cruzi in vivo* [27]. Here, untreated and activated B cell produced very little amounts of IL-17, independent of the different polymeric substrates used (Fig. 5E). TNF-α is another very potent pro-inflammatory cytokine. After activation B cells produce TNF-α when cultivated on TCP, which was not altered by PS, PEI, PC, and PSAN, but significantly reduced on PEU (Fig. 5F).

4. Discussion

Specific receptor/ligand interactions are typically mediated by non-covalent bonding such as hydrogen bonds and electrostatic interaction of the ligand to the receptor [28]. To obtain defined cell response, soluble factors including small molecule, cytokines or antibodies specifically activating surface molecules are added to cell cultures. Alternatively, cell culture substrates could provide biochemical signals, which are often mediated by proteins coated on the substrate, to enhance for example cell adhesion. The bare substrate interphase could also induce cellular responses by structural mimicking receptor ligands, which is of particular interest for non-adherent cells, since protein coatings are not required for their cultivation [29]. In this study, we investigated the interaction of human primary B cells with five polymeric cell culture inserts differing in their chemical composition, while having similar macroscopic interface properties. All inserts exhibited similar surface geometries, wettability, and elasticities [17a]. Due to the similarity of these parameters it is assumed that they have no substantial effects on the B cell behavior. Nevertheless, it cannot be excluded that the combination of the different physical parameters and a certain chemical structure could affect cellular responses.

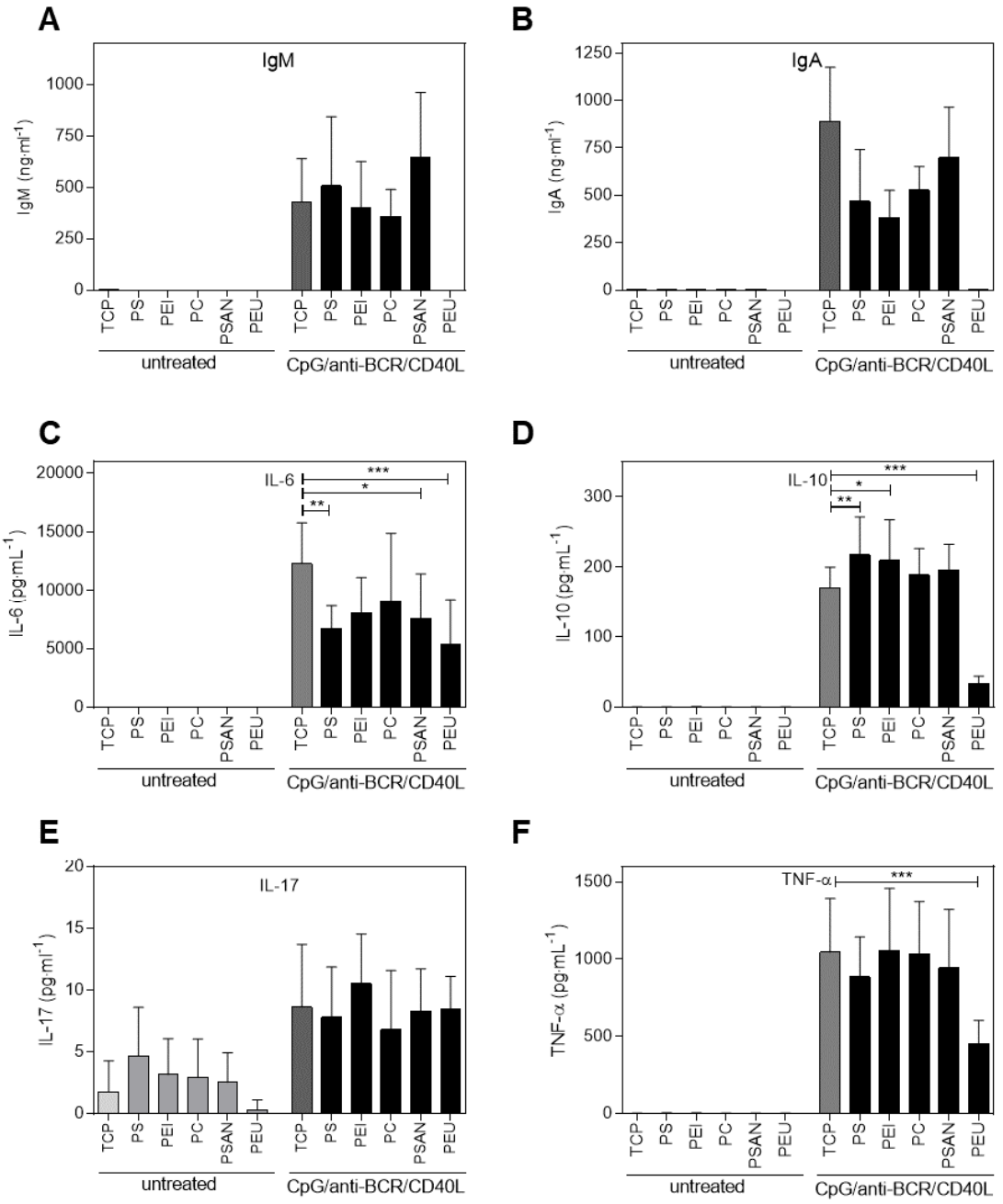

Fig. 5: Antibody and cytokine release by B cells cultivated on the different substrates. Supernatants of untreated and CpG/anti-BCR/CD40L-activated B cells were analyzed for levels of secreted IgM (A) and IgA (B) after five days in culture, as well as for the cytokines IL-6 (C), IL-10 (D) IL-17 (E), and TNF-α (F). Analysis was performed by a multiplex technology (Bioplex). Data were pooled from two independent experiments each with three individual donors (n = 6).

Due to their limited availability primary human or murine B cells are usually cultivated in polystyrene-based multi-well plates of different sizes. In contrast, B cell lines or B

cell hybridomas are generally cultivated in tissue culture flasks commonly fabricated from polystyrene. While polystyrene-based disposable cell culture ware shows adequate performance for most cell culture applications, its surface is difficult to functionalize chemically in a defined way [30]. Thus, a defined substrate mediated cell guidance is difficult to achieve with polystyrene. The difference in the chemical composition of the polymers used in this study correlates with the presence of available valences, by which the substrates could interact with the cell surface. Commercially available TCP served as reference material to validate the results obtained from B cell cultures in PS, PEI, PC, PSAN, and PEU cell culture inserts. TCP is usually functionalized by a corona treatment process leading to highly energetic oxygen ions, which graft onto the surface polystyrene chains resulting in an increased surface hydrophilicity [31]. Therefore, compared to the other five substrates, TCP is the only polymer used showing a hydrophilic surface, which facilitates adhesion of adherent cells, but may on B cell have different effects since these cells do not require substrate adhesion for their functions.

The analyses of the B cell proliferation revealed a similar expansion rate when B cells were cultivated on TCP, PS, PEI, PC, and PSAN, while B cells cultivated on PEU showed a strongly diminished cell growth. A possible explanation for this effect could be a substantially increased apoptosis or necrosis rate of B cells cultivated on PEU. However, the determination of the apoptosis/necrosis rate did not confirm this possibility. The fraction of live of activated B cells cultivated on PEU was even higher than in control cultures. The observed inhibition of B cell activation on PEU also indicates that the B cells may enter a state of permanent unresponsiveness such as anergy [32]. Upon antigen stimulation, but also after TLR engagement, anergic B cells fail to proliferate and to differentiate into antibody secreting cells [33]. B cell anergy is thought to prevent autoimmunity by silencing auto-reactive B cells and the loss of anergy is associated with the development of autoimmune diseases, including systemic lupus erythematosus and type 1 diabetes [32, 34]. Anergy requires constant binding of antigen to the BCR, indicating that a chronic signaling via BCR leads to induction of inhibitory signals [32]. Since it is possible to restore the proliferative response of PEU-primed B cells simply by re-cultivation on TCP, induction of anergy by PEU is unlikely. However, whether PEU is inducing such inhibitory signals via the BCR or other receptors remains unknown and will be elucidated in the future. Interestingly, a similar effect was observed when human $CD4^+$ and $CD8^+$ T cells were

co-cultivated on PEU. Here, the expansion of activated $CD4^+$ T cells was significantly reduced whereas the number of $CD8^+$ T cells remained constant [35]. Together with our observations, these data indicate that B cells and $CD4^+$ T cells share cellular properties, which makes them susceptible for a PEU induced inhibition of activation.

The observed B cell silencing could be attributed to the chemical structure of PEU. Compared to the other polymers used in the study PEU is the only one lacking aromatic groups allowing its interaction with surface molecule solely by hydrogen bond or dipole-dipole interactions. Additionally, due to the relatively low T_g, PEU has the highest chain mobility at 37 °C [17a]. Therefore, the cell interaction with the polymer chains could be facilitated and the formation of hydrogen bonds particularly between the urethane bond of the PEU chains and the surface receptors could be more pronounced. Consequently, the polymer chains could compete with the natural ligand for the receptor binding and mediate agonistic or antagonistic actions. For example, the polymer chains could interact with the BCR or TLR9, which were triggered in this study. The antigen binding to the BCR as well as the engagement of TLR by PAMP is mediated by hydrogen bond formation between the receptor and the ligand [36]. Accordingly, the role of TLR in biomaterial mediated immune cells activations has been demonstrated for dendritic cells and macrophages [37].

An alternative mechanism, leading to the inhibited B cell response mediated by PEU could be the elimination of the stimulatory factors such as CpG, the anti-BCR $F(ab)_2$ fragment, and CD40L by adsorption onto the PEU surface [14]. Alternatively, the PEU chains could interact with other receptors such as Fc receptors that can induce inhibitory signals by activating the immunoreceptor tyrosine-based inhibition motifs [38].

Furthermore, cells could mediate the disintegration of the polymeric substrate by releasing enzymes or reactive oxygen species (ROS). In homochain (carbon-based) polymers the C-C bond is susceptible for free radical degradation, whereas in polyethers or polyesters, the C – O bond would preferentially be cleaved. [39]. For example, in the oligoether units of PEU, C-H bonds neighboring to the ether group convert to hydroperoxide groups by oxidative degradation, which subsequently convert into reactive radical species potentially undergoing manifold reactions, including homolysis or beta-scission. The reactivity of those C-H bonds is less in aromatic structures than in aliphatic. Polymer chain degradation can lead to the formation of additional valences potentially interacting with the cell surface.

All other polymers investigated in this study are also susceptible for reactive oxygen mediated degradation processes, but to a lesser extent than PEU [40]. Polymeric degradation products could directly bind to cell surface receptors for example by mimicking PAMP leading to the activation of TLR. However, B cell mediated polymer degradation seem to be unlikely since, although in principle possible, their capacity to secrete ROS or degrading enzymes is rather limited [41]. Altogether, PEU appears to be unsuitable as substrate for standard B cell application. However, the possibility to arrest B cell activation on PEU might be of interest for inhibition studies. Whether alteration of surface properties by functionalizing the PEU could restore the B cell activation, is currently not known.

The sole interaction of B cells with polymer surfaces did not lead to the activation of certain cell surface receptors, since untreated B cells cultivated on PS, PEI, PC, and PSAN behaved similar to TCP. However, the response of activated B cells was altered by certain polymers indicating a synergistic or antagonistic action of the polymers. The complete inertness of PS may explain the increased IL-10. On PS the TLR9 could be most efficiently activated, since there may be no competition with CpG. In fact, it could be shown that TLR stimulation can enhance IL-10 expression by B cells [42]. For PEI a similar trend as observed for PS was monitored, IL-10 was increased and IL-6, although not significant, was deceased. Both polymers have been shown to interact with DNA molecules, which was attributed to interaction of purines and pyrimidines with the π-system of the polymers [43]. Here, CpG as hypermethylated DNA, mimicking bacterial genome sequences, was used to activate TLR. The binding of CpG either by its DNA backbone or by the nucleotides to the polymer surface could enhance or inhibit the action of CpG. It needs to be noted that contaminating monocytes present in the B cell preparation can also contribute to the cytokine release detected in the cell culture supernatants. At the end of the cultivation period on day five, no contaminating cells negative for the B cell markers CD19 and CD20 could be detected (data not shown), indicating that the contaminating cells did not proliferate and died during the cultivation period.

As mostly with orthogonal cell/synthetic polymer interactions, the exact mode of B cells interactions with specific chemical groups is currently not known. Elucidation of respective cell surface interaction partners as well as a comparative analysis of downstream signaling triggered would require polymer systems, in which the end groups can be systematically altered quantitatively and qualitatively.

The altered cytokine secretion of activated B cells induced by the different polymers could be of biomedical interest. IL-6 is a pro-inflammatory cytokine, which can be released at high levels by various cell types and induce the differentiation of B cells into antibody-producing plasma cells [44]. B cell-derived IL-6 can also support autoimmune reaction in mice and B cells from patients suffering from multiple sclerosis (MS) secrete higher levels of IL-6 [45]. In contrast, IL-10-producing B cells can suppress autoimmune reactions and MS patients show reduced levels of IL-10-producing B cells [46]. IL-10 can suppress inflammations by inhibition of dendritic cell or macrophage activity [47]. The observation that B cells cultivated on PEI or PS produce elevated levels of IL-10, but reduced levels of IL-6, indicates that B cells may differentiate into a phenotype with regulatory features in a substrate dependent manner. Interestingly, although not proliferating, B cells cultivated on PEU secreted substantial amounts of IL-6 indicating that the proliferative response and the IL-6 secretion are regulated by different signaling pathways. The higher hydrophobicity of PS and PEI compared to the TCP surface could be another reason for the altered cytokine levels. Hydrophobic surfaces generally adsorb higher protein amounts and may induce protein denaturation [48]. In fact, using human serum albumin (HSA) and immunoglobulin G (IgG), it was recently demonstrated that the highest amount of adsorbed HSA and IgG was found on PEI, while PS showed the lowest [49]. These data indicate that the used polymers have different protein adsorption capacities. However, whether these results are of relevance for the more complex adsorption behavior of cell culture media supplemented with fetal bovine serum, antibiotics, and proteins as well as peptide-based growth factors or stimulatory agents remains to be elucidated requiring highly sophisticated protein analytics.

B cells might be activated by denatured proteins adsorbed on the material interface via their BCR or pattern recognition receptors [50]. However, data showing, which receptor and B cell signaling pathways are in involved in such a reaction, are not available. The enhanced IL-10 levels could be explained by a selective expansion of IL-10-producing B cells on PS and PEI. Although a subset of IL-10-producing B cells has not been identified in humans so far [51], future studies will investigate how different B cell subsets respond towards the different polymeric substrates. Such systematic studies may also help to clarify the role of B cells during formation of foreign body-induced fibrosis [52].

5. Conclusion

This study investigated the effects of polymeric substrates differing in their chemical composition on primary human B cells. The different chemical structures correlate with available valences such as π-π interaction, hydrogen bonds, and dipole-dipole interactions that can interact with the cell surface. It could be demonstrated that the investigated polymeric substrates can modulate the B cell behavior. For instance, PS and PEI altered the cytokine pattern released by activated B cells, which renders both polymers for studying induction and expansion of human B cells with immune modulatory capacities. The B cell activation on PEU was completely suppressed - indicated by abolished proliferative, cytokine, and antibody response, which might be attributed to its high capacity to interact with cell surface receptors by forming strong hydrogen bonds. Therefore, PEU might be used to model and investigate B cell transient inhibition of the cell activation. The results could be explained by synergistic or antagonist interaction of the polymer surface with the distinct cell surface molecules of activated B cells, or with a decreased or enhanced availability of stimulatory factors such as CpG or the anti-BCR $F(ab)_2$ fragment due to binding onto the substrate.

Conclusively, our study indicates that polymeric substrates could instruct cell behavior by interaction with available valences, which could facilitate the rationale design of substrates that can steer specific functions of non-adherent cells.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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