**Fibroblast-like synoviocytes preferentially induce terminal differentiation of IgD+ memory B cells instead of naïve B cells**

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# Abstract

Rheumatoid arthritis (RA) is a systemic autoimmune disease driven by highly active autoantibody producing B cells. These B cells can be supported within ectopic germinal centers found in afflicted joints. Fibroblast like synoviocytes (FLS) present in inflamed joints support B cell survival, activation and differentiation. CD27+ memory B cells and naïve B cells show very distinct reactions to activation, particularly by CD40 ligand (CD40L). We show that FLS dependent activation of human B cells is dependent on interleukin 6 (IL-6) and CD40L. FLS have been shown to activate naïve as well as memory B cells. If the activatory potential of FLS is different for naïve and memory B cells had not yet been investigated. Our results suggest that FLS-induced activation of B cells is dependent on IL-6 and CD40L. While FLS are capable of inducing differentiation, isotype switching and antibody production in memory B cells, FLS capability to activate naive B cells is significantly lower.

# Introduction

Rheumatoid arthritis (RA) is a chronic auto-inflammatory disease characterized by progressive degradation of the affected joints. Even though RA is a systemic disease, the focus of disease lies within the synovium of involved joints. Progression of the disease is driven by activated, autoreactive B cells (1–4). In germinal centers (GC) T cell derived CD40 ligand (CD40L) and interleukin 6 (IL-6) supplied by follicular dendritic cells (FDC) are required for B cell activation, differentiation and isotype switching (5,6). In the inflamed joint, fibroblast-like synoviocytes (FLS) increase B cell survival, differentiation and activation induced deaminase (AID) dependent class switch recombination (CSR) through secreted and membrane bound interleukins and tumor necrosis factor superfamily ligands such as B cell activating factor of the TNF family (BAFF) and a proliferation inducing ligand (APRIL) (8,10–13). Ectopic lymphoid structures (ELS) arise within the synovial membrane of about 40 % of RA patients and provide support to autoreactive B cells, inducing CSR, driving plasma cell (PC) differentiation and subsequently causing an increase in systemic autoantibody levels (14,15). FLS have been implicated to play a role in the establishment and maintenance of ELS, as FLS support plasma cells in an IL-36 dependent manner (16). Memory B cells and naïve B cells show very distinct reactions to CD40L stimulation and during acute response. CD40L causes memory B cells to differentiate into antibody secreting cells (ASC), while differentiation of naïve B cells is inhibited by continuous CD40L stimulation (17). Similar effects are shown in acute responses, as memory B cell populations give rise to plasmablasts, while naïve B cell populations predominantly develop resting B cell phenotypes (18). Activation of naïve B cells by FLS has been demonstrated, as CSR and IgG production were induced in naïve B cells in FLS – B cell co-cultures in vitro in a BAFF, APRIL and Toll-like receptor 3 (TLR-3) dependent manner (11,19). In co-cultures of heterogeneous B cell populations, containing memory B cells and PCs, vascular cellular adhesion molecule 1 (VCAM-1), expressed by FLS was identified to be responsible to support FLS dependent B cell survival (20). As expression of CD40L and IL-6 by FLS have been demonstrated (10), our goal was to investigate if FLS induced B cell activation is dependent on CD40L and IL-6 and if memory B cells and naïve B cells show distinct reactions to activation by FLS. IgD targeted direct magnetic activated cell sorting (MACS) was used to isolate IgD+ B cells (DBC). DBC populations contain naïve B cells as well as IgD+CD27+ B cells, offering the opportunity to determine differences between naïve B cells and memory B cells after activation by FLS.

# Materials and Methods

## Cells

FLS were isolated from synovial tissue from osteoarthritis (OA) and RA patients undergoing joint replacement surgery at the orthopedics unit at St. Elisabeth Hospital Meerbusch Lank (Table 1).

Synovial tissue was dissociated using Liberase TL Research Grade (Roche Diagnostics GmbH, 68298 Mannheim, Germany). FLS were taken into cell culture flasks in RPMI 1640 (Sigma-Aldrich Chemie GmbH, Munich, Germany) containing 10% Heat inactivated FBS 10500 (Thermo Fisher Scientific, Waltham, MA, USA), 1% Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), 1% Penicillin/Streptomycin (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 2.5% HEPES (Sigma-Aldrich Chemie GmbH, Munich, Germany). FLS were grown until 90 – 100 % confluence. 2.5 \* 10^5 cells per well were seeded into 24 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) or onto microscopy slides using ProPlate Multi-Array Slide System gaskets (Grace Bio Labs, Bend, Oregon, USA) to be used in co-cultures.

B cells were isolated from buffy coats from healthy donors obtained from the Institute of Transplantation Diagnostics and Cell Therapeutics (ITZ) at the University Hospital Düsseldorf. B cells were isolated via PBMC separation through Lymphoprep gradient (PROGEN Biotechnik GmbH, Heidelberg, Germany) and subsequent MACS using IgD+ B cell isolation (DBC) and Naïve B cell isolation II kits (NBC) (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). DBC populations were composed of 86.2% (65.7% min to 94.5% max) naïve B cells (CD27-) as well as 13.8% (5.5% min to 34.3% max) IgD+ memory B cells (CD27+) while indirectly isolated naïve B cell (NBC) populations contained significantly less CD27+ B cells (Fig. 1). 1 \* 10^6 B cells were added to the FLS cultures or kept separately in medium containing 10% FCS w/o 10 ng/mL IL-4 (PeproTech, Rocky Hill, NJ, USA), 2 µg/mL of anti-CD154 (CD40 Ligand inhibiting) Monoclonal Antibody, Functional Grade (Thermo Fisher Scientific, Waltham, MA, USA) or 10 µg/mL of anti-IL-6 antibody (Miltenyi Biotech, Bergisch Gladbach, Germany). All material was collected after informed consent was given and the study was approved by the ethics committee of the HHU under study number: 2018-87\_1-KfogU.

## Flow cytometry

At 0, and after 3 days in co-culture, B cells were separated from the FLS and labeled with anti-IgD-FITC, anti-IgG-APC-Vio, anti-IgM-PE, anti-CD19-VioBlue, anti-CD138-PE-Vio770, anti-CD27-APC and propidium iodide (PI) (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany) and measured on a MACS-Quant analyzer 10 (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). Cell properties and subpopulations were determined using FlowLogic software (Inivai Technologies, Mentone, Victoria, Australia). Gating strategy is depicted in Fig. 1.

## ELISA

Supernatants were collected after 8 days and IgM and IgG ELISAs were performed using goat anti human IgM, goat anti human IgM HRP, goat anti human IgG (H+L), goat anti human IgG (H+L) HRP, (Jackson ImmunoResearch Europe Ltd, Cambridge House, United Kingdom) with IgM and IgG from human serum as standards (Sigma-Aldrich Chemie GmbH, Munich, Germany). IgD ELISA was performed using recombinant rabbit monoclonal anti human IgD antibody, goat anti human IgD heavy chain HRP and native human IgD protein as standard (Abcam, Cambridge, MA, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used to develop the ELISAs.

## RT qPCR

RNeasy Plus micro kit (QIAGEN GmbH, Hilden, Germany) was used to isolate mRNA from B cells after 3 days in culture. 1 ng – 5 µg of RNA were transcribed using FastGene Scriptase II (NIPPON Genetics EUROPE GmbH, Düren, Germany). Expression levels of AID were determined using the qPCR BIO SyGreen Mix Lo-ROX (NIPPON Genetics EUROPE GmbH, Düren, Germany) and 5’-GCATCCTTTTGCCCCTGTA-3’ as forward primer and 5’-CAGAGAAGACTTGAAGGACTGTT-3’ as reverse primer, with GAPDH as housekeeper using 5’-CTTAGCACCCCTGGCCAAGG-3’ as forward primer and 5’-CTTACTCCTTGGAGGCCATG-3’ as reverse primer.

## PCR

RNA isolation and RNA transcription were performed as described above. PCR with subsequent gel electrophoresis was performed to detect cyclic switch products. For IgG switch cycles primers were used from (21). 5′-GTTGCCGTTGGGGTGCTGGAC-3′ was used for Cµ as reverse primer, 5′-GGGCTTCCAAGCCAACAGGGCAGGACA-3′ for Sγ1/2, 5′-AGGTGGGCAGGCTTCAGGCACCGAT-3′ for Sγ3 and 5′-TTGTCCAGGCCGGCAGCATCACCAGA-3′ for Sγ4 as forward primers. For IgD switch cycles, 5′-AGACGAGGGGGAAAAGGGTT-3′ was used for Cµ as reverse primer and 5’- CCTGTCTTCAGCACTATCTGACT-3’ as forward primer for σδ. 34 cycles of PCR were performed at 70 °C annealing temperature.

## In-situ PCR

Fluorescence-labeled in situ-PCR (FLI-PCR) was performed in co-cultures grown on microscopy slides. Culture supernatants were removed and cells were fixed in 4% Paraformaldehyde (PFA) for 4 hours. Fixed cells were permeabilized using 6 µg/mL Proteinase K for 5 – 10 minutes. Scriptase II was used to transcribe mRNAs and High fidelity PCR labeling kit (Jena Bioscience, Jena, Germany) was used to label beta actin (ActB) with ATTO 488 labeled dUTP using 5’-CCTCGCCTTTGCCGATCC-3’ as forward primer and 5’-TGAAGGTCTCAAACATGATCTGG-3’ as reverse primer, AID with ATTO 594 labeled dUTP using the primers mentioned for RT-qPCR and Recombination activating gene 1 (RAG1) with Alexa Fluor (AF) 647 labled dUTP using 5’-GAGAGAGCAGAGAACACACT-3’ as forward primer and 5’-GCTGAGTTGGGACTGGCTTCTGAC-3’ as reverse primer. Each primer pair was combined with the respectively labeled dUTP, supernatants were collected and remaining nucleotides and primers were washed away after each PCR run. In-situ images were captured on Axio Observer 7 microscope (Carl Zeiss AG, Oberkochem, Germany) and PCR products in FLI-PCR supernatants were visualized using the ChemiDoc MP imaging System (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

# Results

## Effects of IL-6 blockade on FLS dependent B cell activation

By blocking IL-6 – IL-6 receptor (IL6R) interactions using IL-6 neutralizing antibodies in DBC monocultures and DBC – FLS co-cultures we found that , FLS induced DBC survival was partly dependent on IL-6. DBC viability was significantly elevated in the presence of FLS (**Fig. 3**). After neutralization of IL-6 overall DBC viability was significantly reduced in FLS co-cultures. In the presence of IL-4, anti-IL-6 effects were attenuated. IL-6 appeared to inhibit FLS dependent support of memory B cells in the presence of IL-4 as memory B cell percentages were increased when IL-6 was neutralized in B cell – FLS co-cultures. Secretion of IgM, IgD and IgG was determined by ELISA and in order to show FLS dependent antibody secretion, immunoglobulin (Ig) concentration values in DBC monoculture supernatants were subtracted from the values in DBC – FLS co-culture supernatants. Relative Ig concentrations were referred to as ΔIg values. FLS dependent IgM and IgG secretion were not affected by blocking IL-6 – IL-6R interactions, while FLS dependent IgD secretion was partly dependent on IL-6 as ΔIgD levels were reduced in the presence of IL-6 neutralizing antibody. Induction of AID expression by FLS was also dependent on IL-6, as Blocking IL-6 – IL-6R interactions caused a reduction in FLS induced AID expression in DBCs in the presence of IL-4.

## Effects of CD40L blockade on FLS dependent B cell activation

As observed in the context of IL-6, FLS dependent DBC viability was partly dependent on CD40L as DBC viability was reduced after CD40L neutralization (Fig. 4). Memory B cell populations were unaffected by blockade of CD40L – CD40 interactions while an inhibitory effect of CD40L – CD40 interaction was observed for the support of ASCs, as ASC percentages were increased in the presence of FLS only when CD40L was neutralized. FLS dependent production of IgM and IgD was dependent on CD40L as secretion was reduced when CD40L- CD40 interactions were blocked. No effect was observed on IgG secretion. IL-4 dependent, FLS induced AID expression was also dependent on CD40L as AID expression was reduced when CD40L – CD40 interactions were blocked.

## FLS dependent activation of naïve B cells was reduced compared to populations containing IgD+CD27+ B cells

While CD40L – CD40 interactions are necessary for activated B cells to become memory B cells, CD40L – CD40 interactions have also been shown to partially hinder naïve B cell differentiation, while memory B cell differentiation was much less affected by presence of CD40L (17). As FLS induced B cell activation and differentiation of DBCs was dependent on CD40L, we investigated if activation of exclusively isolated naïve B cells (NBC) was more impeded in the presence of FLS compared to DBCs.

DBC viability was FLS dependent while NBC viability was reduced in the presence of FLS (Fig. 5). In the presence of IL-4, FLS effects on B cell viability were reduced and differences between DBC and NBC cultures were diminished. CD27+ B cell percentage was significantly increased in DBC – FLS co-cultures relative to monoculture controls in the presence of IL-4, while CD27+ B cell percentage was not significantly affected by FLS in NBC cultures. FLS dependent CD27+CD138+ ASC percentages were significantly higher in DBC cultures compared to NBC cultures with or without IL-4 present. FLS had no effect on IgM secretion from DBCs and NBCs without IL-4 present. In the presence of IL-4, IgM secretion was induced in DBC monocultures (Fig. S3). Presence of FLS caused significant reduction in IgM secretion compared to DBC monocultures. Significantly less IgM secretion was induced in NBC monocultures (Fig. S3). IgD secretion was induced by FLS in DBC cultures, not in NBC cultures. Without IL-4 present there was no significant difference in FLS induced IgG secretion between DBC and NBC cultures. In the presence of IL-4, IgG concentrations were reduced in DBC cultures in the presence of FLS. In contrast, IgG levels were significantly elevated in NBC cultures when FLS were present. IL-4 stimulation was required to induce FLS dependent AID expression in DBC cultures, while no FLS dependent AID expression was detectable in NBC cultures.

PCR with primers targeting Cµ - Sγ1/2, Cµ - Sγ3, Cµ - Sγ4 and Cµ - σδ was performed to demonstrate CSR towards IgG1/2, IgG3, IgG4 and IgD. Gel electrophoresis revealed that switching to IgD was only induced infrequently (Fig. 5, E). Cµ - σδ PCR products (~ 200 bp) were only detected in DBC – OAFLS co-cultures (lane 24) and DBC monocultures (lane 29). No Cµ - σδ PCR products were detected in NBC cultures. DBC cultures predominantly switched towards IgG4 (358 bp) in the presence of FLS (lanes 17 – 29) (Fig. 5, F). Cµ - Sγ4 PCR products were detected in DBC –FLS co-cultures as well as in DBC monocultures. In NBC cultures Cµ - Sγ4 PCR recombination was not induced in monocultures, Cµ - Sγ4 PCR products were only detected in the presence of FLS (lanes 10 and 13).

Fluorescence-labeled in situ-PCR (FLI-PCR) was used in order to obtain information on spatial distribution of AID expressing B cells in DBC – and NBC – RAFLS co-cultures in the presence of IL-4 (Fig. 6). In addition to AID, RAG1 expression was visualized. AID and RAG1 expression have not been shown in FLS, inducible expression in B cells has been demonstrated numerous times (11,19,22,23), therefore AID and RAG1 expressing cells were presumed to be B cells. ActB expression was detected in DBC and NBC cultures by ATTO488 signals. AID expression, identified by ATTO594 signal, and RAG1 expression, identified by AF647 signal were visible in DBC and NBC cultures. Both were reduced in NBC cultures compared to DBC cultures. ActB, AID and RAG1 were co-expressed in DBCs and NBCs. DBC cultures contain B cells expressing either AID or RAG1 while expression of both was consistently co-localized in NBC cultures. Gel electrophoresis images of FLI-PCR supernatants can be found in Fig. S4.

# Discussion

IL-6 has been described to be involved in B cell activation, plasma cell differentiation (24,25) and induction of antibody production (26,27). In our study, FLS dependent B cell survival was partly dependent on IL-6, as blockade of IL-6 caused a significant reduction in B cell viability in FLS – B cell co-cultures. Contrary to findings in other studies (24,25), FLS induced B cell differentiation appeared not to be dependent on IL-6 in DBC cultures. DBC IgM and IgG production were also unaffected by IL-6 blockade. While the lack of an effect on IgM production by IL-6 has been previously described (28), a supportive effect on IgG production by IL-6 has been shown previously (26,27). In DBC cultures IL-6 appeared to support IgD production instead of IgG production. This is possibly due to the fact, that in our cultures, in addition to naïve B cells, IgD+ Memory B cells became activated, but IgD- Memory B cells were absent from the initial population. FLS induction of AID expression was also supported by IL-6. Apparently DBC B cells switched towards IgD as well as towards IgG, while NBC B cells exclusively switched towards IgG. AID induced Cµ - σδ recombination of the Immunoglobulin heavy chain locus instead of Cµ - Sγ, Cµ - Sα or Cµ - Sε have been described (29–31). Cµ - σδ recombined B cells are more autoreactive than B cells switched towards other isotypes (29), IgD levels are elevated in cigarette smokers (32) and IgD has been implicated to play a role in the pathophysiology of autoimmune diseases such as SLE and RA (33,34). In sum IL-6 supported FLS dependent B cell survival, IgD production and AID expression, but not differentiation or IgM and IgG production in our co-culture model.

CD40L is a key factor for B cell activation in GCs (5,35) and as CD40L expression has been described on FLS (10), we showed for the first time that FLS dependent B cell survival is significantly reduced when CD40L is blocked by neutralizing antibodies. The supportive effect of CD40L on B cell survival has been described numerous times as increased expression of CD40L enhances B cell survival in vivo (36), survival of autoreactive B cells that would otherwise be subject to deletion (37) and CD40L enhances resistance to apoptosis by induction of survival genes in B cell leukemia (38). A role of CD40L in B cell memory formation has been observed (39), as well as a supportive effect of CD40L on PC differentiation (5,40). In our model, however, blockade of CD40L had no significant effect on FLS dependent CD27+ memory population and FLS effects on CD27+CD138+ ASC differentiation were increased in the presence of CD40L neutralizing antibody. This might indicate an inhibitory effect of CD40L exposition on B cell differentiation. A CD40L – dose dependent reduction of B cell differentiation and accompanying reduction of IgG, IgM and IgA secretion has previously been shown (17). Conversely, FLS dependent IgM and IgD secretion appeared to be enhanced by CD40L. Induction of IgM (40) and IgD (41) secretion by engaging CD40 have previously been demonstrated. In our study, CD40L inhibitory effects apparently were stronger on differentiation of DBCs than on IgD secretion from DBCs. One possible explanation could be that those cells that overcome the threshold posed by CD40L against differentiation require ongoing CD40L stimulation for IgD secretion. IL-4 induced AID expression was dependent on FLS and CD40L. However, As Cµ - Iγ4 CSR products were found in DBC – FLS co-cultures and DBC monocultures, Ig-isotype switching toward IgG and IgG secretion were not entirely dependent on FLS in DBC cultures. In summary, CD40L supported FLS dependent survival and IgD production of DBCs, while induction of ASC differentiation was apparently inhibited by CD40L.

DBC cultures containing on average 14% CD27+ memory B cells were dependent on FLS for survival, while survival of NBC cultures consisting of 99% CD27- naïve B cells was reduced in the presence of FLS. Supportive effects on survival of naïve B cells (11), memory B cells and PCs have been shown (20), however these have never been compared. The difference in FLS dependent survival of DBCs and NBCs is possibly explained by findings showing that memory B cell activation is less restrained by CD40L than activation of naïve B cells (17). Continuous CD40L stimulation was also shown to inhibit PC differentiation of naïve B cells while PC differentiation of memory B cells was significantly less limited by CD40L stimulus (17). Removal of CD40L stimulus after memory differentiation is required for PC differentiation of GC B cells (42). FLS dependent memory differentiation was not significantly different between DBC and NBC cultures. FLS dependent ASC formation was significantly reduced in NBC cultures compared to DBC cultures, concurring with afore mentioned findings that memory B cell terminal differentiation is less inhibited by presence of CD40L than terminal differentiation of naïve B cells.

IL-4 was sufficient to induce IgM production in DBC cultures, not NBC cultures. This is consistent with findings showing that IgM production is induced by IL-4 in previously activated B cells such as lymphocytic leukemia B cells or Epstein-Barr virus activated B cells (43,44). FLS induced significantly higher levels of IgD production in DBC cultures compared to NBC cultures. DBC cultures contained IgD+ memory B cells which were driven towards terminal differentiation by FLS, while naïve B cell terminal differentiation was limited in FLS co-cultures. Naïve B cells were not driven towards IgD production, but towards IgG production by FLS, as has previously been shown (11,19).

As described above, switching towards IgD was induced in DBC cultures, not in NBC cultures. Also, switching towards IgG4 was dependent on FLS in NBC cultures, while Cµ - Sγ4 recombination was also detected in DBC monocultures. Low expression of AID in NBC – FLS co-cultures was contradictory to the induction of IgG secretion and detection of Cµ - Sγ4 CSR products induced by FLS. In order to gain spatial information on AID expression, FLI-PCR was established. In-situ AID expression was detected in both DBC and NBC – FLS co-cultures. Induction of AID expression by FLS in naïve B cells has been demonstrated previously (11,19). RAG1 expression was also observed in FLI-PCR images. RAG expression in B cells is dependent on IL-6 and has also been shown to be induced by FLS (22,23,45).

Homing of CD27+ B cells to the inflamed synovium is enhanced as CD27+ B cells express chemokine receptors at higher frequencies than CD27- B cells (46). Additionally it has been shown, that Memory B cell populations are enriched within the synovial tissue in RA patients (47–49) and by analyzing V-region gene mutations, it was shown, that PCs originated from memory B cells within the synovium rather than naïve B cells (50). One possible interpretation of our results is that FLS are involved in driving CD27+ B cells and not naïve B cells towards terminal differentiation within the synovium. This is possibly due to the distinct effect of CD40L stimulation on memory B cells compared to naïve B cells.

These findings underline the rationale for B cell depletion treatment (51) and anti-IL-6 therapy (52,53). Since B cell depletion and anti-IL-6 therapy also increase the patients’ risk of infections (54,55), more targeted approaches to stop recruitment of CD27+ B cells to the synovium or the differentiation of CD27+ B cells to ASCs within the synovium are needed. CD27 targeted systemic depletion might be beneficial and compared to common variable immune deficiency (CVID) patients, where a reduction of CD27+ B cells causes increased risk of infections (56), non-autoreactive B cell memory can be reformed after depletion, reducing the risk of infections long term. There is also reasoning for targeting CD40L – CD40 interactions and a number of studies are currently ongoing, others, however were discontinued due to severe side effects (57). The interactions between synovial cells, particularly FLS, and B cells need to be studied further in order to identify more selective targets to develop more efficient therapies for rheumatoid arthritis.

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# Figure legends

Fig. 1: IgD+ isolated B cell populations contain significantly higher levels of CD27+ B cells than negatively isolated naïve B cell populations. There was no difference in B cell viability immediately after isolation (A). CD27+ memory B cell presence was significantly elevated in DBCs at 13.8% versus 0.5% in NBCs (pDBC v NBC= 0.027) (B). B cells were isolated from 5 donors. Statistical significances were calculated by ANOVA and Tukey post-hoc analysis (\*: p≤ 0.05; \*\*: p≤ 0.01; \*\*\*: p≤ 0.001; \*\*\*\*: p≤ 0.0001).

Fig. 2: Gating strategy for CD27+ and CD138+ B cells. Cell doublets and aggregates were excluded by gating FSC-H versus FSC-A. B cells were gated by morphology in SSC-A versus FSC-A. Viable B cells were gated by selecting PI low cells. Quadrants for gating CD27 versus CD138 were set according to isotype controls.

Fig. 3: IL-6 blockade affects FLS dependent B cell viability, differentiation and antibody production. (A) B cell viability was determined by PI stain in flow cytometry. FLS dependent B cell viability was significantly reduced in the presence of anti-IL-6 antibody. CD27+ memory B cell percentage was determined by flow cytometry. FLS dependent CD27+ B cell percentage was only elevated in the presence of IL-4 and anti-IL-6 antibody. CD27+CD138+ antibody secreting cell (ASC) percentages were determined by flow cytometry. FLS dependent ASC percentage was only elevated in the presence of IL-4 and anti-IL-6. (B) IgM levels in culture supernatants were determined by ELISA. IL-6 blockade had no effect on IgM secretion. In the presence of IL-4, OAFLS caused a reduction in IgM secretion. IgD levels in culture supernatants were determined by ELISA. FLS dependent IgD secretion was reduced in the presence of anti-IL-6 antibody. IgG levels in culture supernatants were determined by ELISA. IgG secretion was not affected by FLS or IL-6 blockade. (C) Quadrant gates for CD27 and CD138 gating. (D) AID expression was significantly elevated in the presence of FLS and significantly reduced when IL-6 was blocked. B cells were isolated from 4 donors and FLS from 4 OA and 4 RA patients were used for the experiment. Flow cytometry and ELISA were performed after 8 days in culture. AID expression was determined after 3 days in culture. Experiments were performed in duplicates and measurements were performed in duplicates. Statistical significances were calculated by ANOVA and Tukey post-hoc analysis for blocking effects and students T-test for FLS effects (\*: p≤ 0.05; \*\*: p≤ 0.01; \*\*\*: p≤ 0.001; \*\*\*\*: p≤ 0.0001).

Fig. 4: CD40L blockade affects B cell viability, differentiation and antibody production. (A) B cell viability was determined by PI stain in flow cytometry. FLS dependent B cell viability was significantly reduced in the presence of anti-CD40L antibody. CD27+ memory B cell percentage was determined by flow cytometry. FLS dependent CD27+ B cell percentage was only elevated in the presence of IL-4 and not dependent on CD40L. CD27+CD138+ ASC percentages were determined by flow cytometry. FLS dependent ASC percentage was only elevated when CD40L – CD40 interactions were blocked and in the presence of IL-4. (B) IgM levels in culture supernatants were determined by ELISA. IgM secretion was significantly reduced in the presence of anti-CD40L antibody without IL-4 present. In the presence of IL-4, CD40L blockade had no significant effect on IgM secretion. IgD levels in culture supernatants were determined by ELISA. FLS dependent IgD secretion was reduced in the presence of anti-CD40L antibody. IgG levels in culture supernatants were determined by ELISA. IgG secretion was not affected by FLS or CD40L blockade. (C) Quadrant gates for CD27 and CD138 gating. (D) AID expression was significantly elevated in the presence of FLS and IL-4. It was significantly reduced when CD40L – CD40 interactions were blocked. B cells were isolated from 4 donors and FLS from 4 OA and 4 RA patients were used for the experiment. Flow cytometry and ELISA were performed after 8 days in culture. AID expression was determined after 3 days in culture. Experiments were performed in duplicates and measurements were performed in duplicates. Statistical significances were calculated by ANOVA and Tukey post-hoc analysis for blocking effects and students T-test for FLS effects (\*: p≤ 0.05; \*\*: p≤ 0.01; \*\*\*: p≤ 0.001; \*\*\*\*: p≤ 0.0001).

Fig. 5: Comparison of NBC and DBC – FLS co-cultures. (A) B cell survival was determined by PI stain in flow cytometry. After 8 days in culture, FLS dependent B cell survival was significantly higher in DBC cultures compared to NBC cultures in untreated conditions (pDBC v NBC< 0.001). DBC survival was significantly elevated in the presence of OAFLS (pm v OA= 0.028) and FLS overall (pm v FLS= 0.003). NBC survival was not dependent on FLS. In the presence of IL-4, FLS effects were diminished. After 8 days, CD27+ B cell frequencies were significantly elevated in DBC – OAFLS and RAFLS co-cultures in the presence of IL-4 (pm v OA= 0.014; pm v RA= 0.037). CD27+CD138+ B cell frequencies were elevated in DBC cultures compared to NBC cultures after 8 days in the presence of IL-4 (pDBC v NBC= 0.029) and without additional stimulation (pDBC v NBC= 0.005). (B) In the presence of IL-4, FLS cause a significant reduction in DBC IgM production (pm v OA= 0.008; pm v RA= 0.038), there was no such effect in NBC cultures (pDBC v NBC= 0.001). FLS dependent IgD production was significantly higher in DBC cultures compared to NBC cultures (pDBC v NBC= 0.001, pm v OA= 0.031; pm v RA= 0.034) in untreated conditions. In the presence of IL-4 these differences were diminished. FLS dependent IgG production was significantly elevated in NBC cultures compared to DBC cultures after 8 days (pDBC v NBC= 0.04). (C) Quadrant gates for CD27 and CD138 gating. (D) AID mRNA expression was significantly higher in DBC cultures compared to NBC cultures in the presence of IL-4 (pDBC v NBC< 0.001). (E) Cyclic switch products for Cµ - σδ recombination were detected in DBC – OAFLS co-cultures and in DBC monocultures in the presence of IL-4. (F) Cµ - Sγ4 recombination was detected in NBC – OAFLS co-cultures and in DBC – OAFLS and RAFLS co-cultures as well as in DBC monocultures. B cells were isolated from 6 donors and FLS from 6 OA and 5 RA patients were used for the experiment. Flow cytometry and ELISA were performed after 8 days in culture. AID expression was determined after 3 days in culture. Experiments were performed in duplicates and measurements were performed in duplicates. Statistical significances were calculated by ANOVA and Tukey post-hoc analysis for blocking effects and students T-test for FLS effects (\*: p≤ 0.05; \*\*: p≤ 0.01; \*\*\*: p≤ 0.001; \*\*\*\*: p≤ 0.0001).

Fig. 6: Fluorescence labeled in-situ PCR (FLI-PCR) of DBC and NBC – FLS co-cultures. (A) Beta Actin, AID and RAG1 expression were detected in DBC – FLS co-cultures. ActB, RAG1 and AID triple positive B cells are indicated by red arrows, FLS nuclei are indicated by white arrows. (B) Beta Actin, reduced RAG1 expression and reduced AID expression were detected in NBC – FLS co-cultures. ActB, RAG1 and AID triple positive B cells are indicated by red arrows, FLS nuclei are indicated by white arrows.

# Conflict of Interest

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

# Author Contributions

DB, GP and MS contributed to conception and design of the study. DB and KL generated the data, organized the database and performed the statistical analysis. DB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

# Data Availability Statement

The datasets generated for this study can be found at figshare under: <https://doi.org/10.6084/m9.figshare.21755876>