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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Functional characterization of IgD$^-$CD27$^-$ double negative B cells in multiple sclerosis pathology

Serina Rubio

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

dr. Judith FRAUSSEN

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Functional characterization of IgD$^-$CD27$^-$ double negative B cells in multiple sclerosis pathology*

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*Running title: *Functional characterization of DN B cells in MS*

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ABSTRACT

Abnormalities in specific B cell subsets, such as IgD$^-$CD27$^-$ double negative (DN) B cells, have been increasingly identified in multiple sclerosis (MS). DN B cells are abnormally elevated in MS patients and possess pro-inflammatory characteristics. Little or contradictory evidence is available on their activation and migration potential. Therefore, this study functionally characterized DN B cells from MS patients and healthy controls (HCs). To answer this question, CD27$^-$ and CD27$^+$ B cells were isolated from peripheral blood and *in vitro* stimulated with CpG oligonucleotides and interleukin (IL)-2, and CD40 ligand and IL-4. Subsequent analysis was performed using flow cytometry. Additionally, the viability staining for the *in vitro* migration assay was optimized. In this assay, CD27$^-$ and CD27$^+$ B cell migration across inflamed human cerebral microvascular endothelial cells will be measured. For HCs, our results show that after *in vitro* stimulation, increased frequencies of activated CD80$^+$ and CD86$^+$ DN B cells were measured, as well as increased frequencies of DN B cells expressing the chemokine receptor CXCR3. Additionally, DN B cells increasingly proliferated after *in vitro* stimulation. These results indicate that DN B cells can be activated after *in vitro* stimulation. Further investigating DN B cell activation and migration in MS patients will provide more insight into whether DN B cells can play a role in the pathology of MS. These findings may lead to the development of more specialized and targeted therapies for MS patients in the future.

INTRODUCTION

Epidemiology, clinical forms and diagnosis of MS

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), characterized by autoimmune inflammation, demyelination and neurodegeneration (1). This disease greatly impacts a person's quality of life. MS patients can display various clinical symptoms such as fatigue, pain, visual problems, depression, cognitive impairment, muscle-related problems, sexual dysfunction, and bladder and bowel dysfunction (1). With a prevalence of 50 to 300 per 100,000 people, approximately 2.3 million people are affected by MS worldwide. This prevalence is higher in countries that lie further from the equator (2). Additionally, the disease is more common in women with a 3:1 female to male ratio (3). Both environmental and genetic risk factors can predispose people to develop MS. Environmental risk factors for MS are very diverse, including vitamin D deficiency, obesity in early life, cigarette smoking and Epstein-Barr virus infection (4-7). The human leukocyte antigen (HLA) region of chromosome 6, and more specifically, the HLA-DRB1*15:01 allele, is highly implicated in genetic predisposition for MS (8). However, non-HLA regions are also involved in MS development, such as genetic variants of the interleukin (IL)-2 and IL-7 receptor alpha genes (9). Interactions between environmental and genetic risk factors further increase the risk of developing this autoimmune disease (10).

Diagnosis is done using the McDonald Criteria and requires dissemination of brain lesions in space

and time, as detected by magnetic resonance imaging (MRI) (11). For this, respectively, lesions are detected in MS-specific areas in the CNS, and new lesions are compared to old ones on previous MRI images (11). Furthermore, the cerebrospinal fluid (CSF) is screened for the presence of oligoclonal immunoglobulin G (IgG) bands (OCBs). These are IgG protein bands detected by agarose gel electrophoresis with isoelectric focusing, immunoblotting or immunofixation (11).

Several clinical forms of MS can be discriminated (1, 12, 13). Most patients (80-85%) have a relapsing-remitting MS (RRMS) disease pattern, characterized by episodes or relapses, alternated by periods of remission (1, 12, 13). Approximately 15-30% of RRMS patients develop secondary progressive MS (SPMS) with progressive disability (1, 12, 13). Lastly, 15% of all MS patients develop a progressive disability from the onset of the disease and are diagnosed with primary progressive MS (PPMS) (1, 12, 13).

Pathogenesis and B cell involvement in MS

The pathogenesis of MS is mainly dominated by cells of the adaptive immune system (14). More specifically, autoreactive lymphocytes attack CNS-specific antigens (Ags), such as myelin basic protein (MBP) (14, 15). How this process initiates is unclear; hence two hypotheses are proposed (1). The first one, the CNS intrinsic model, suggests that the initial event takes place in the CNS, leading to the release of CNS Ags into the periphery and induction of an autoimmune response. On the other hand, the CNS extrinsic model proposes that the initial event occurs outside the CNS and leads to an abnormal autoimmune reaction against the CNS (1). Nevertheless, both models result in the same pathogenic pathway.

First, autoreactive T cells are activated by antigen-presenting cells (APCs) in the periphery and infiltrate the CNS by crossing the blood-brain barrier (BBB). Here, they are reactivated by APCs present in the CNS. This results in the production of pro-inflammatory cytokines and chemokines by T cells and microglia. Subsequently, autoreactive B cells and macrophages are recruited to the inflammatory sites. Eventually, the inflammatory reaction induces damage to the myelin sheath and axons. This results in the release of more auto-Ags, leading to the infiltration of more autoreactive lymphocytes. Ultimately, this viscous cycle culminates in the hallmarks of MS, which are demyelination and neurodegeneration (14).

Initially, it was believed that T cells were the leading players in MS pathogenesis, as they are the first cells to infiltrate the CNS. Recently, the involvement of B cells in MS was highlighted. First, B cells produce autoantibodies (autoAbs) that target CNS Ags, such as the previously mentioned MBP, and cause demyelination and axonal damage (15). Second, autoreactive B cells form ectopic lymphoid follicles at the site of inflammation in the CNS. These are germinal center-like structures that harbor autoreactive B cells and autoAbs to maintain the inflammatory response locally inside the CNS (16). Next, B cells function as APCs to stimulate Ag-specific T cell activation and expansion (17). Lastly, they are capable of producing several pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and lymphotoxin-alpha (LT-α), to support the inflammatory reaction (18).

In addition to these effector functions, B cell contribution was also proven by genetic studies that showed a strong association between the HLA-DRB1*15:01 allele and Ag-presentation by B cells (19). Next, the high effectiveness of B cell depleting therapy, targeting $CD20^+$ B cells, showed additional proof of B cell involvement in MS (20-22). One of those therapies, ocrelizumab, uses humanized monoclonal Abs (MAbs) against CD20, a transmembrane protein found on all B cells, except on stem cells, pro-B cells and differentiated plasma cells (21, 22). Ocrelizumab showed a rapid reduction in disease activity in MS patients, and as a result, is approved by both the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for both RRMS and early PPMS (23-25).

IgD^-CD27^- double negative B cells

Since the general contribution of B cells in MS pathology has already been demonstrated, the current hot topic in MS research is investigating specific B cell subsets. The main subsets are IgD^+CD27^- naive B cells, IgD^-CD27^+ class-switched memory (CSM) B cells, IgD^+CD27^+ non-class-switched memory (NCSM) B cells and plasma cells (Fig. 1). During B cell development and maturation, naive B cells are activated when they encounter their specific Ag and differentiate into short-lived plasma cells that produce Abs or further mature into memory B cells in a germinal

center (GC) reaction. Here, they can mature into NCSM B cells that keep their IgD expression or into CSM B cells that lose their IgD expression after Ig isotype switching. However, NCSM B cells can also be formed independent of the GCs. Lastly, upon stimulation, memory B cells can differentiate into long-lived plasma cells and produce Abs (14). Recently, a new B cell subset was discovered, namely $IgD^{-}CD27^{-}$ double negative (DN) B cells.

Frequencies and correlations with clinical characteristics of DN B cells – DN B cells were first reported in aging. Colonna-Romano *et al.* identified a new B cell subpopulation lacking both IgD and CD27, which was significantly increased in the elderly (26). In human immunodeficiency virus (HIV) patients, Moir *et al.* defined this population as $CD20^{high}CD27^{-}CD21^{low}$ tissue-like memory B cells (27). In addition, Rinaldi *et al.* showed significantly increased frequencies of DN B cells in young HIV patients (< 40 years) compared to young healthy controls (HCs), as well as in old HCs (≥ 60 years) compared to young HCs (28). Extensive research has been performed on DN B cells in systemic lupus erythematosus (SLE). Increased levels of DN B cells were found in the peripheral blood of SLE patients compared to HCs (29, 30). Jenks *et al*. further divided these cells into two subsets, namely $CD19^{int}CXCR5^{+}$ DN1 cells and $CD19^{high}CXCR5^{-}CD21^{-}$ DN2 cells. In HCs, DN1 cells accounted for most DN B cells, in contrast to SLE, where DN2 cells represented the majority (30). DN B cells were mainly IgG^{+} in the elderly, and in SLE, DN B cells resembled the surface phenotype of memory B cells, especially for IgG and IgM expression levels (26, 29). Also, DN B cells displayed a similar level of somatic hypermutation as CSM B cells, which suggests that they are Ag-experienced (29). Transcriptome analysis indicated a high similarity between DN1 and CSM B cells, with only 22 differentially-expressed genes (30). Lastly, positive clinical correlations between DN B cell frequencies and disease activity, as well as the occurrence of lupus nephritis, were found in SLE (29, 30). Patients with higher DN B cell levels also showed increased autoAb titers for anti-dsDNA, anti-RNA, anti-ribonucleoprotein and anti-Smith Abs (29, 30).

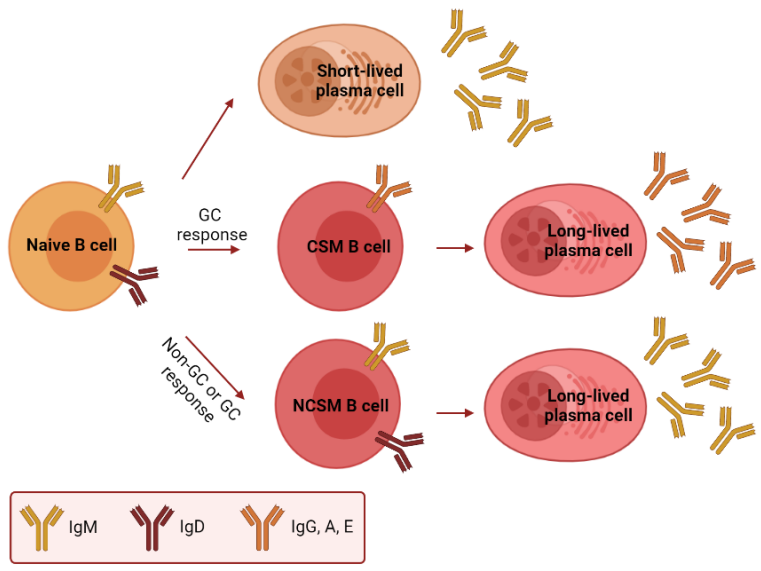

Fig. 1 – Overview of the main B cell subsets. Naive B cells are activated when they encounter their specific Ag. This is followed by differentiation into short-lived plasma cells that produce Abs or further maturation into memory B cells. In a GC response, the cells mature into NCSM B cells that keep their IgD expression or into CSM B cells that lose their IgD expression after Ig isotype switching. However, NCSM B cells can also be formed in a non-GC response. Lastly, upon stimulation, memory B cells can differentiate into long-lived plasma cells and produce Abs. Image created using Biorender. Ab, antibody. Ag, antigen. CSM B cells, class-switched memory B cells. GC, germinal center. Ig, immunoglobulin. NCSM B cells, non-class-switched memory B cells.

Activation potential of DN B cells – When comparing $CD27^{+}$ with $CD27^{-}$ B cells in the elderly, decreased expression of the antigen presentation molecule HLA-DR and costimulatory molecules CD80 and CD40 was evidenced in the $CD27^{-}$ B cell population (26). When further dividing these into DN and naive B cells, DN B cells showed decreased expression of these markers compared to naive B

cells (26). This contrasts with SLE, where DN2 cells showed increased expression of HLA-DR and the costimulatory molecule CD86 compared to naive B cells (30). DN2 cells were highly responsive to stimulation with Toll-like receptor (TLR)7 ligand, IL-21 and IL-10, which are inducers of plasma cell differentiation. Additionally, TLR7 stimulation of DN2 cells induced the expression of the activation marker CD25, whereas CD40 ligand (CD40L) stimulation did not affect CD25 expression (30). After *in vitro* stimulation using anti-human IgG/A/M, CD40L or the TLR9 ligand CpG type B, tissue-like memory B cells of HIV patients showed equal proliferative capacity as memory B cells and decreased proliferative capacity compared to naive B cells (27). On the contrary, in SLE, DN B cells showed increased proliferation after stimulation with only CpG2006 compared to naive B cells, which require additional B cell receptor (BCR) stimulation with anti-human Ig (29). Lastly, in the elderly, DN B cells produced granzyme B after *in vitro* stimulation with anti-human IgG and IL-21 (31).

Migration capacity of DN B cells – In aging, the chemokine receptor C-X-C motif chemokine receptor (CXCR) 3 was significantly increased on DN B cells from young donors compared to elderly donors (31). This receptor is involved in migration towards inflammatory sites in response to C-X-C motif chemokine ligand (CXCL) 10. Additionally, low expression of CXCR5, involved in migration towards lymph nodes in response to CXCL13, was observed for both young and aged groups (31). Accordingly, in HIV, tissue-like memory B cells showed significantly increased levels of CXCR3 and decreased levels of CXCR5 compared to memory and naive B cells (27).

Based on the decreased expression of activation markers (HLA-DR, CD80, CD40) on DN B cells in aging and their phenotypic resemblance with CSM B cells, DN B cells were considered to be exhausted and senescent memory cells that lost expression of CD27 (26). Despite of this, DN B cells could produce proteases (granzyme B) after *in vitro* stimulation (31). In addition, other contradictory results were found in SLE, as DN B cells showed increased expression of activation markers (HLA-DR, CD86, CD25) and increased proliferation potential (29, 30). These results suggest that DN B cells might display other phenotypes and functions in different immune-compromising disorders.

IgD$^-$CD27$^-$ double negative B cells in MS

Our research group is currently investigating DN B cells in the pathogenesis of MS. About one-third of MS patients under the age of 60 years showed elevated DN B cell levels (> 7% of CD19$^+$ B cells) in the peripheral blood compared to age-matched HCs (32). Additionally, DN B cell frequencies were significantly increased in the CSF compared to paired peripheral blood of MS patients. DN B cells had a memory phenotype with about 45% IgG$^+$ cells and about 10% IgM$^+$ cells (32). This was also shown by clonal analysis of DN and memory B cells by Wu *et al.*, revealing a close relationship between both B cell subsets (33). Our research group also performed phenotypic and Ig repertoire analyses and revealed that DN B cells resemble CSM B cells more closely than naive B cells (34). DN B cells also showed similar (in MS patients) and increased (in HCs) expression of HLA-DR/DP/DQ compared with CSM B cells, and intermediate expression of CD80 and CD86 between naive and CSM B cells (32). Further, DN B cells producing TNF-α, LT-α and/or granzyme B were increased or equal to CSM B cells after distinct conditions of stimulation (CD40L, anti-human IgG/IgM, CpG2006 and/or IL-21) (32). Additionally, further research suggests that DN B cells are capable of migration. Preliminary data from our research group shows that DN B cells express the adhesion molecules very late antigen-4 (VLA-4), lymphocyte function-associated antigen-1 (LFA-1) and activated leukocyte cell adhesion molecule (ALCAM) necessary for binding to the endothelium. Further, DN B cells express CXCR3 and CXCR5. Our preliminary data also suggests that CXCR3$^+$ DN B cells are increased in the CSF compared to paired peripheral blood of MS patients. As CXCR3 and CXCR5 are chemokine receptors for CXCL10 and CXCL13, respectively, our data shows that DN B cells can migrate towards these chemokines. Interestingly, both CXCL10 and CXCL13 are elevated in the CSF of MS patients (35, 36).

Our previous results concerning the activation potential of DN B cells seem to align with the results found in SLE. However, the contradictory evidence from other studies makes it difficult to conclude on their activation potential.

Additionally, few data are available on their migratory phenotype, let alone on their capacity to migrate across the BBB. As our published results and preliminary data suggest, DN B cells have functional properties that could be relevant in MS pathology. However, their exact function and contribution to MS pathology remain unclear. Hence, this study aimed to functionally characterize DN B cells in the peripheral blood of MS patients and HCs. This functional characterization consisted of *in vitro* activation and migration assays.

Studying their functional characteristics will provide us with more insight into the pathogenesis of MS. It will clarify to what extent DN B cells are activated in MS, contribute to the disease pathogenesis, and generate information on how these cells migrate towards the CNS. Lastly, in the future, this study can pave the way towards creating more specialized and targeted treatments for the millions of MS patients worldwide.

EXPERIMENTAL PROCEDURES

Study subjects – MS patients were recruited at the Rehabilitation and MS-Center (Pelt, Belgium) and the Academic MS Center Limburg, Zuyderland Medical Center (Sittard, The Netherlands) and were diagnosed according to the McDonald criteria (11). HCs were recruited at Hasselt University (Hasselt, Belgium). The study was approved by the Medical Ethical Committee of Hasselt University and written informed consent was obtained from all participants. Peripheral blood was collected from 26 (suspected) MS patients and 8 HCs. Paired peripheral blood and CSF was collected from 15 (suspected) MS patients. Biological samples were stored in the University Biobank Limburg (UBiLim). Demographic and clinical data of MS patients and HCs are shown in Table 1.

Cell isolation from peripheral blood and CSF – PBMCs were isolated from whole peripheral blood using Ficoll density gradient centrifugation. Briefly, plasma was removed from peripheral blood by centrifugation at 400 g, without brake, for 10 min at room temperature (RT). Next, the remaining blood was added to a Lympholyte® solution (Cedarlane Laboratories, SanBio B.V., Uden, the Netherlands) and centrifuged for 20 min at 800 g, without brake, at RT. PBMCs were collected and washed twice with blank medium (1xPBS (Lonza, Basel, Switzerland); 0.4% 0.5 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen, ThermoFisher, Waltham, MA)) by centrifugation for 10 min at RT at 400 g and 300 g, respectively. Lastly, purified PBMCs were resuspended in 1xPBS or culture medium (RPMI-1640 (Lonza); 10% fetal bovine serum (FBS; Gibco™, ThermoFisher); 1% non-essential amino acids; 1% sodium pyruvate; 50 U/ml penicillin and 50 µg/ml streptomycin (all from Sigma-Aldrich, Germany)).

CSF cells were isolated from the CSF by centrifugation for 10 min at 500g at 4°C. The CSF cells were then resuspended in cold 1xPBS with 2% FBS and immediately analyzed as explained below. CSF samples contaminated with red blood cells were excluded from the analyses.

Screening of PBMCs and CSF cells for elevated DN B cell frequencies and phenotype – PBMCs were analyzed using anti-human CD19 Brilliant Violet (BV) 421 (clone HIB19), IgD phycoerythrin-cyanine 7 (PE-Cy7) (clone IA6-2), IgM peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5) (clone MHM-88) (all from BioLegend, London, U.K.), IgA PE (clone IS11-8E10) (Miltenyi Biotec, Leiden, the Netherlands), IgG fluorescein isothiocyanate (FITC) (clone G18-145) and CD27 allophycocyanin (APC) (clone M-T271) (both from BD Biosciences, Erembodegem, Belgium) (supplementary Table 1).

CSF cells were analyzed with anti-human CD19 BV421 (clone HIB19), IgD PE-Cy7 (clone IA6-2), IgM BV605 (clone MHM-88), CD80 PE-Dazzle594 (clone 2D10), CXCR3 BV711 (clone G025H7), CXCR5 APC-Cy7 (clone J252D4), B-cell activating factor receptor (BAFF-R) FITC (clone 11C1), LFA-1 PE (clone HI111), VLA-4 PerCP-Cy5.5 (clone 9F10) (all from BioLegend), CD27 APC (clone M-T271), IgG Alexa Fluor (AF) 700 (clone G18-145) and ALCAM BV786 (clone 3A6) (all from BD Biosciences) (supplementary Table 2). Flow cytometry was performed on an LSRFortessa flow cytometer (BD Biosciences) and analysis was executed using FlowJo software (FlowJo, Ashland, OR).

$CD27^-$ and $CD27^+$ B cell isolation – $CD27^-$ and $CD27^+$ B cells were isolated from PBMCs using the EasySep™ Human Memory B Cell Isolation kit and EasySep™ magnet (STEMCELL Technologies SARL, Grenoble, France) according to the

manufacturer's instructions. Purity of the isolated $CD27^-$ and $CD27^+$ B cells was confirmed by flow cytometry using CD19 PE-Cy7 (clone HIB19; from BioLegend), CD27 APC (clone M-T271) and IgD PE-CF594 (clone IA6-2) (both from BD Biosciences) (supplementary Table 3).

In vitro activation assay – $CD27^-$ and $CD27^+$ B cells were labeled with the proliferation dye carboxyfluorescein diacetate succinimidyl ester (CFSE; BioLegend). $CD27^-$ and $CD27^+$ B cells ($10x10^6$ cells/ml) were incubated for 20 min at 37°C with 1 µM CFSE, washed with culture medium for 10 min at 300 g at RT and resuspended in culture medium. Next, CFSE-labelled $CD27^-$ or $CD27^+$ B cells were seeded at 200.000 cells/well in a 96-well plate (U-bottom; Greiner Bio-one, Vilvoorde, Belgium). Subsequently, several stimulations were added: CpG oligonucleotides (1µg/ml, ODN 2006; Invivogen, Toulouse, France) and IL-2 (50 U/ml; Sigma-Aldrich), and CD40L (1 µg/ml; BioLegend) and IL-4 (10 ng/ml; R&D systems, Minneapolis, MN). The cells were incubated with the stimulations for three days at 37°C.

After three days, the stimulated $CD27^-$ and $CD27^+$ B cells were analyzed with Fixable Viability Dye (FVD) eFluor780 (eBioscience, ThermoFisher), anti-human CD19 BV650 (clone HIB19), CD27 APC (clone M-T271), IgD PE-Cy7 (clone IA6-2), CD80 BV421 (clone 2D10), CD86 BV785 (clone IT2.2), CXCR3 BV605 (clone G025H7), IgM PerCP-Cy5.5 (clone MHM-88) (all from BioLegend), IgG AF700 (clone G18-145; from BD BioSciences) and IgA VioGreen (clone IS11-8E10; from Miltenyi) (supplementary Table 4). Flow cytometry was performed on an LSRFortessa flow cytometer (BD Biosciences) and analysis was executed using FlowJo software (FlowJo). The delta proliferation fraction (ΔPF) was determined as: $\Delta PF = \%\ proliferation_{stimulated} - \%\ proliferation_{unstimulated}$. A cut-off of 2% was used to identify increased proliferation.

Optimization viability staining for in vitro migration assay – PBMCs were analyzed using different viability dyes (FVD eFluor506, 7-aminoactinomycin D (7-AAD); both from eBioscience, ThermoFisher), staining conditions (1xPBS vs. 2% FACS buffer (1xPBS, 2% FBS, 0.1% sodium-azide (VWR, Leuven, Belgium)) vs. 5% FACS buffer (5% FBS); with vs. without Fc receptor blocker (5%; Human TruStain FcX™, BioLegend); with vs. without fixation of PBMCs) (supplementary Table 5) and CD19 PE-Cy7 (clone HIB19), IgM BV605 (clone MHM-88) (both from BioLegend), CD27 APC (clone M-T271), IgD PE-CF594 (clone IA6-2), CD86 FITC (clone 2331) and IgG PE (clone G18-145) (all from BD Biosciences) (supplementary Table 6).

Statistical analysis – Statistical analysis was performed using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA). Normality testing was performed using a Shapiro-Wilk test. For analysis of non-paired data, two groups were compared with an unpaired t-test. For analysis of paired data, two groups were compared with a paired t-test and multiple groups with a repeated measures one-way ANOVA test with Tukey's post hoc testing or a Friedman test with Dunn's post hoc testing. A p value < 0.05 was considered significant.

Table 1 – Demographic and clinical data of MS patients and HCs for flow cytometry.

	Number	Type of MS	Age[a]	Gender, % F	Previous Treatment[b]	EDSS[c]	Disease duration[d]
HC	8	NA	39.1 ± 15	43%	NA	NA	NA
MS	26	RRMS: 9 Suspected MS: 15 UK: 2	42.2 ± 18.3	100%	UT: 12; TF: 1; UK: 13	2.35 ± 1.5	140.5 ± 236.5

[a]In years, presented as mean ± SD. [b]All previous treatments are shown. [c]Presented as mean ± SD. [d]In months, presented as mean ± SD. EDSS, expanded disability status scale. F, female. NA, not applicable. RRMS, relapsing-remitting multiple sclerosis. TF, teriflunomide. UK, unknown. UT, untreated.

RESULTS

In vitro activation assay

Fresh samples are preferable for studying DN B cell activation – To test whether the *in vitro* activation assay could be performed using our existing biobank collection of frozen PBMCs of MS patients and HCs, we compared fresh and frozen PBMC samples for $CD27^-$ and $CD27^+$ B cell isolation and subsequent *in vitro* stimulation. CpG oligonucleotides and IL-2 were used to mimic stimulatory signals of the innate immune system, whereas CD40L and IL-4 were used to resemble costimulation by T cells. We isolated PBMCs from the peripheral blood of two HCs. Half of the PBMCs were frozen for about one week, whereas the other half was immediately used for the *in vitro* activation assay. First, we determined the expression of the activation markers CD80 and CD86 on DN B cells following *in vitro* stimulation. The frequency of $CD80^+$ DN B cells was decreased in frozen samples compared to fresh samples, both after CpG2006 and IL-2 stimulation (fresh: 62.1% ± 5.2% vs. frozen: 48.2% ± 6.7%), and CD40L and IL-4 stimulation (fresh: 66.1% ± 7.2% vs. frozen: 13.7% ± 3.9%) (Fig. 2A). $CD86^+$ DN B cell frequencies were similar between fresh and frozen samples for both stimulations (CpG2006 + IL-2: 26.2% ± 7.5% (fresh) vs 30.4% ± 4.3% (frozen); and CD40L + IL-4: 21.2% ± 4.7% (fresh) vs. 20.7% ± 4.5% (frozen)) (Fig. 2B). Additionally, the frequency of proliferated DN B cells was decreased in frozen samples in comparison to fresh samples for both stimulations (CpG2006 + IL-2: 47.5% (fresh) vs 35.6% (frozen); and CD40L + IL-4: 14.7% (fresh) vs. 12.2% (frozen)) (Fig. 2D-F). Lastly, we determined the frequencies of the different Ig isotypes (IgG^+, IgM^+ and IgA^+ DN B cells) and $CXCR3^+$ DN B cells. We observed a decrease in the frequency of IgG^+ DN B cells in frozen samples compared to fresh samples, both after CpG2006 and IL-2 stimulation (fresh: 32% ± 10.4% vs. frozen: 13.6% ± 8.7%), and CD40L and IL-4 stimulation (fresh: 51.7% ± 17.6% vs. frozen: 20.1% ± 13.1%) (Fig. 2C). No differences were seen for the other Ig isotypes, as well as for $CXCR3^+$ DN B cells (supplementary Fig. 1). As the frequency of activated $CD80^+$, proliferating and IgG^+ DN B cells was higher when using fresh samples, fresh samples are favored over frozen samples for the *in vitro* activation assay.

DN B cells are activated by both CpG2006 and IL-2, and CD40L and IL-4 stimulation – $CD27^-$ and $CD27^+$ B cells were isolated from the peripheral blood of four HCs and subsequently stimulated *in vitro* with CpG2006 and IL-2, or CD40L and IL-4 for three days. Next, we determined the expression of activation markers (CD80, CD86) and chemokine receptors (CXCR3) on DN B cells, as well as the percentage of proliferation, before (baseline) and after stimulation. The gating strategy for this flow cytometric analysis is shown in Fig. 3. Significantly increased frequencies of $CD80^+$ DN B cells were measured after both CpG2006 and IL-2, and CD40L and IL-4 stimulation (91.6% ± 4.4% and 92.5% ± 4%, respectively) compared to the baseline (23.5% ± 15%; $p = 0.0166$ and $p = 0.0126$, respectively) and unstimulated conditions (28.1% ± 9.1%; $p = 0.0012$ and $p = 0.0011$, respectively) (Fig. 4A). The frequency of $CD86^+$ DN B cells was significantly increased after CD40L and IL-4 stimulation (75.4% ± 7.5%) compared to the baseline expression (PBMC: 1.3% ± 0.7%; $p = 0.0061$) (Fig. 4B). Although not statistically significant, $CD86^+$ DN B cell frequencies were increased after CpG2006 and IL-2 stimulation (43% ± 5.8%) versus the baseline (PBMC: 1.3% ± 0.7%; $p = 0.1708$) and unstimulated conditions (10.8% ± 6.2%; $p > 0.9999$), and increased after CD40L and IL-4 stimulation (75.4% ± 7.5%) versus the CpG2006 and IL-2 stimulation (43% ± 5.8%; $p > 0.9999$) (Fig. 4B). Next, a significantly increased frequency of $CXCR3^+$ DN B cells was seen after CpG2006 and IL-2 stimulation (87.2% ± 12.3%; $p = 0.0061$), and CD40L and IL-4 stimulation (87.4% ± 13.4%; $p = 0.0062$) in comparison to the baseline value (PBMC: 23.7% ± 1.3%) (Fig. 4C). Additionally, the frequency of $CXCR3^+$ DN B cells appeared to be increased, but was not statistically significant, after CpG2006 and IL-2 stimulation (87.2% ± 12.3%) compared to the unstimulated condition (50.6% ± 29.8%; $p = 0.1662$) (Fig. 4C).

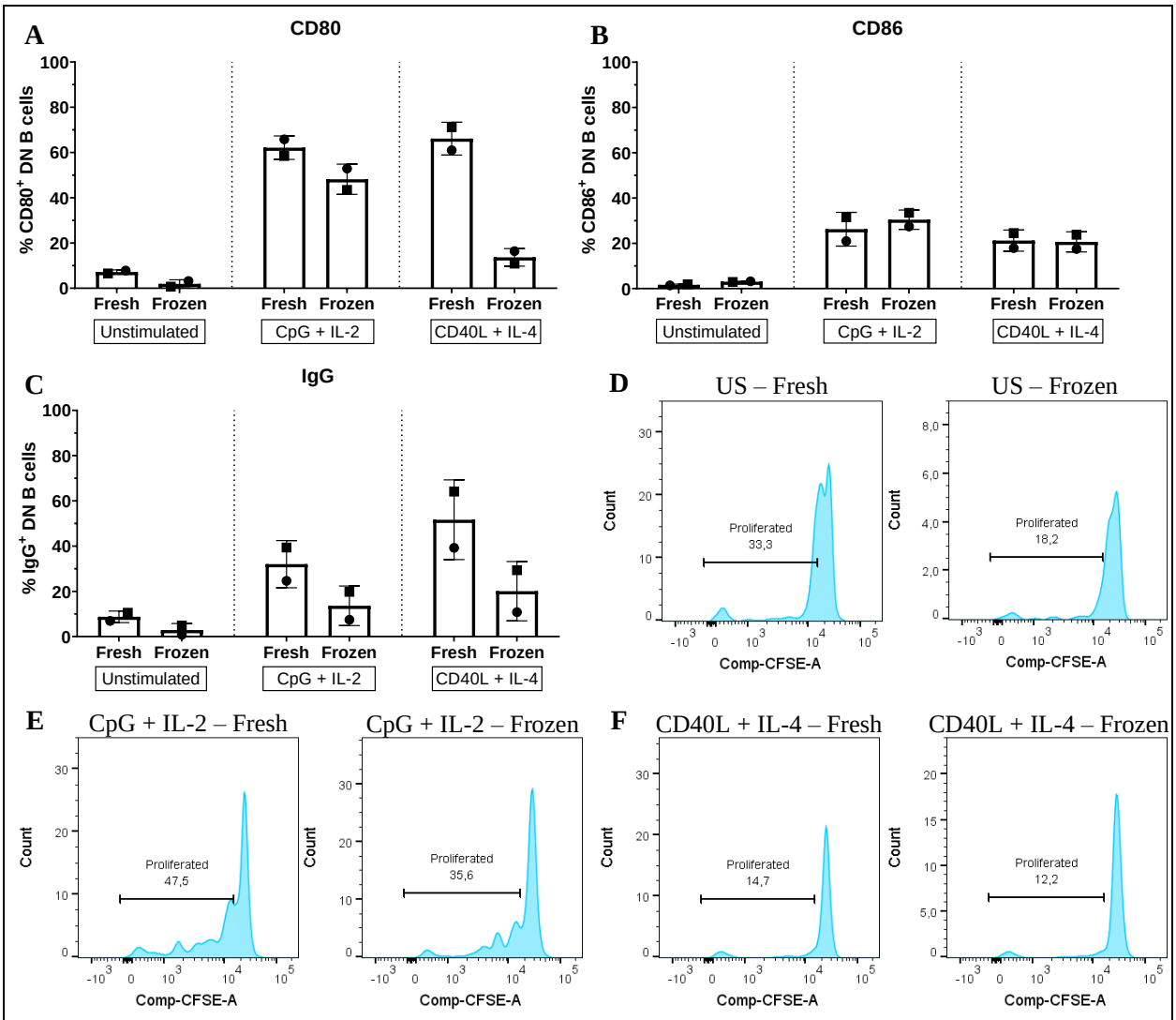

Fig. 2 – Frequencies of activated and proliferating DN B cells in fresh and frozen samples. Fresh and frozen PBMC samples were used for $CD27^-$ and $CD27^+$ B cell isolation, and subsequent *in vitro* stimulation with CpG + IL-2 or CD40L + IL-4, for three days (only DN B cells from $CD27^-$ B cell population are shown). The percentages of (**A**) $CD80^+$, (**B**) $CD86^+$ and (**C**) IgG^+ DN B cells are shown between fresh and frozen samples for the different stimulatory conditions. The percentages of proliferated DN B cells after (**D**) no stimulation, (**E**) CpG + IL-2, and (**F**) CD40L + IL-4 stimulation are shown between fresh and frozen samples. (**A-C**) Mean (bars) ± SD is shown. (**D-F**) Histograms of one representative HC are shown. (n = 2). CD40L, CD40 ligand. CFSE, carboxyfluorescein diacetate succinimidyl ester. DN B cells, IgD^-CD27^- double negative B cells. Ig, immunoglobulin. IL, interleukin. US, unstimulated.

A trend towards increased frequencies of $CXCR3^+$ DN B cells was found after CD40L and IL-4 stimulation (87.4% ± 13.4%) compared to no stimulation (50.6% ± 29.8%; $p = 0.0694$) (Fig. 4C). No difference in CXCR3 expression was seen between the two stimulations ($p > 0.9999$) (Fig. 4C). Proliferation of DN B cells was significantly increased after CpG2006 and IL-2 stimulation (45.2% ± 22.5%) compared to stimulation with CD40L and IL-4 (8% ± 8.8%; $p = 0.0386$) (Fig. 4D). Stimulation of DN B cells induced increased proliferation compared to the unstimulated condition as the ΔPF was higher than the cut-off value (> 2%) for both stimulatory conditions (Fig. 4D). In conclusion, DN B cells can be activated by both CpG2006 and IL-2 stimulation, and CD40L

and IL-4 stimulation, indicated by significantly increased expression of activation markers (CD80, CD86), inflammatory chemokine receptors (CXCR3) and increased proliferation.

Screening of peripheral blood and CSF for DN B cell frequencies and phenotype

DN B cells in CSF have distinct phenotypes from DN B cells in paired peripheral blood – First, we determined the frequency of DN B cells in the peripheral blood of 11 confirmed MS patients and 15 suspected MS patients that underwent a lumbar puncture for diagnostic purposes. No differences were seen in DN B cell frequencies in peripheral blood between suspected and confirmed MS patients (3% ± 1.4% vs. 3.7% ± 1.6%, respectively; p = 0.2055) (Fig. 5A). In paired peripheral blood and CSF of one confirmed and two suspected MS cases, DN B cell frequencies were higher in the CSF for each patient. However, no statistically significant differences were measured in DN B cell frequencies between peripheral blood and CSF (2.5% ± 1.4% vs. 5.8% ± 1.9%, respectively; p = 0.1531) (Fig. 5B).

Next, we performed a phenotypical comparison of DN B cells isolated from paired peripheral blood and CSF samples. Expression of Ig isotypes (IgG, IgM), activation (CD80) and survival (BAFF-R) markers, chemokine receptors (CXCR3, CXCR5), and cell adhesion molecules (LFA-1, VLA-4, ALCAM) was measured (Fig. 6). A trend towards increased frequencies of IgG^+ DN B cells was seen in peripheral blood (53.5% ± 4.6%) compared to CSF (16.2% ± 15.7%; p = 0.0652) (Fig. 6A). The frequency of IgM^+ DN B cells was elevated, although not statistically significant, in peripheral blood (25.9% ± 14.9%) compared to CSF (12.1% ± 11%; p = 0.1897) (Fig. 6B). Further, DN B cells from CSF showed increased expression of CD80 (45.6% ± 26.6%) and similar expression of BAFF-R (91.2% ± 3.9%) in comparison to peripheral blood (CD80: 6.9% ± 4.7%; BAFF-R: 92.1% ± 1.1%), which was not statistically significant (CD80: p = 0.1492; BAFF-R: p = 0.7186) (Fig. 6C-D). The frequency of $CXCR3^+$ DN B cells in CSF (46.7% ± 31.6%) was increased compared to peripheral blood (11.5% ± 8.7%), whereas the frequency of $CXCR5^+$ DN B cells in CSF samples (37.8% ± 23%) was decreased compared to peripheral blood samples (82.7% ± 4.3%) (Fig. 6E-F). However, both were not statistically significant (CXCR3: p = 0.1750; CXCR5: p = 0.0882). No differences were seen in the frequency of LFA-1 expressing DN B cells between peripheral blood and CSF (96.1% ± 3.2% vs. 89% ± 16%, respectively; p = 0.4412) (Fig. 6G). A statistically significant increase in the frequency of $VLA\text{-}4^+$ DN B cells was found in peripheral blood compared to CSF (95.3% ± 3% vs. 90.4% ± 2.3%, respectively; p = 0.0467) (Fig. 6H). Lastly, the frequency of $ALCAM^+$ DN B cells was decreased in the CSF (70.2% ± 30.3%) compared to peripheral blood (91.8% ± 0.9%), although not statistically significant (p = 0.3382) (Fig. 6I). As most of these differences were not statistically significant, the results are not conclusive and our patient cohort needs to be expanded with additional patient samples. However, these primary findings indicate that DN B cells in CSF might have distinct phenotypes from DN B cells in peripheral blood, particularly for the expression of cell adhesion molecules (VLA-4).

In vitro migration assay

Optimization of the viability staining – For the *in vitro* migration assay, $CD27^-$ and $CD27^+$ B cells will be isolated from the peripheral blood of HCs and MS patients. These cells will then be added to transwell inserts carrying an inflamed human cerebral microvascular endothelial cell layer, representing the inflamed BBB seen in MS. Afterwards, the cells that have migrated across this layer will be analyzed by flow cytometry. Here, we optimized the viability staining for the flow cytometric read-out of this *in vitro* migration assay. Due to the COVID-19 measures, all human PBMCs must be fixated before use. In a previously optimized *in vitro* chemotaxis assay in our research group, a 7-AAD viability dye was used. This is a nucleic acid staining that is less suitable for cell fixation (37). Hence, the effect of cell fixation on 7-AAD was determined here. Additionally, we tested another viability dye, FVD eFluor506, which is a primary amine staining suited for cell fixation (37).

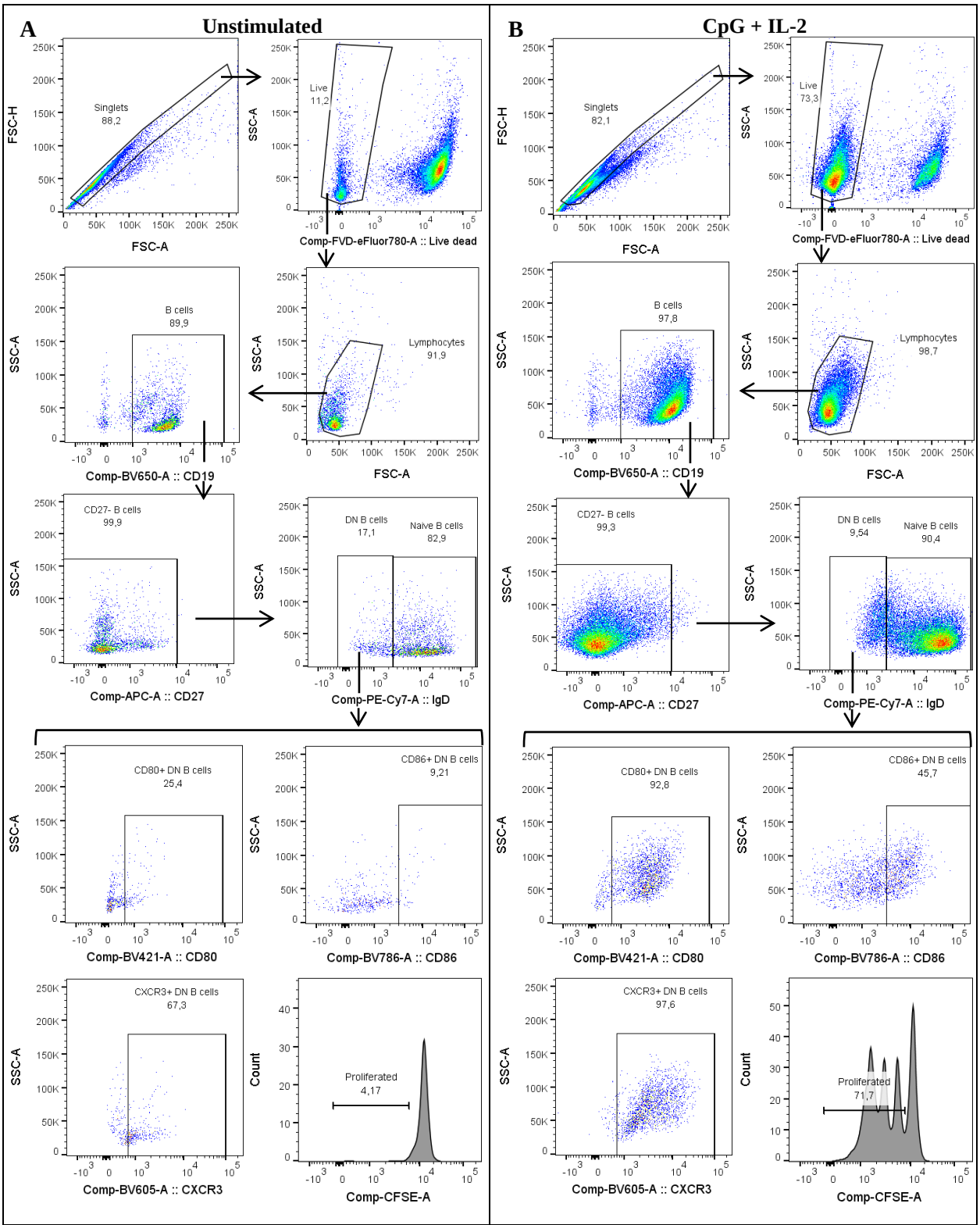

Fig. 3 – Gating strategy of the *in vitro* activation assay. A visual representation of the flow cytometric gating strategy used for the *in vitro* activation assay, for which (**A**) the unstimulated condition and (**B**) the CpG + IL-2 stimulation are shown. Dot plots and histograms of one representative HC are shown. CFSE, carboxyfluorescein diacetate succinimidyl ester. CXCR3, C-X-C motif chemokine receptor 3. DN B cells, $IgD^{-}CD27^{-}$ double negative B cells. FSC, forward scatter. Ig, immunoglobulin. IL, interleukin. SSC, side scatter.

However, for this dye, additional washing steps are required, increasing the loss of cells during the staining process. Therefore, several staining conditions were tested, including different staining buffers and simultaneous instead of sequential incubation with the other antibodies (supplementary Table 5). Our results show that fixation of PBMCs stained with 7-AAD negatively affects the percentage of living cells compared to no cell fixation (Fig. 7A-B). Additionally, the dot plots and histograms show an additional cell population in the living cell population, which is usually not present (Fig. 7A-B). Next, after staining with FVD eFluor506 for 30 min in FACS buffer (2% FBS), the percentage of living cells (95.2% ± 0.1%) was similar to the standard staining method, namely 30 min in 1xPBS (93.9 ± 0.1%) (Fig. 7C-D). Lastly, the two populations (live vs. dead) were still clearly distinguishable for the staining condition using FACS buffer (2% FBS) (Fig. 7D). The other FVD eFluor506 staining conditions gave divergent results compared to the standard staining method (results not shown). Therefore, we optimized the use of the FVD eFluor506 staining for 30 min in FACS buffer (2% FBS) as this gave similar results compared to the standard staining method and as cell fixation cannot be used with the 7-AAD viability dye.

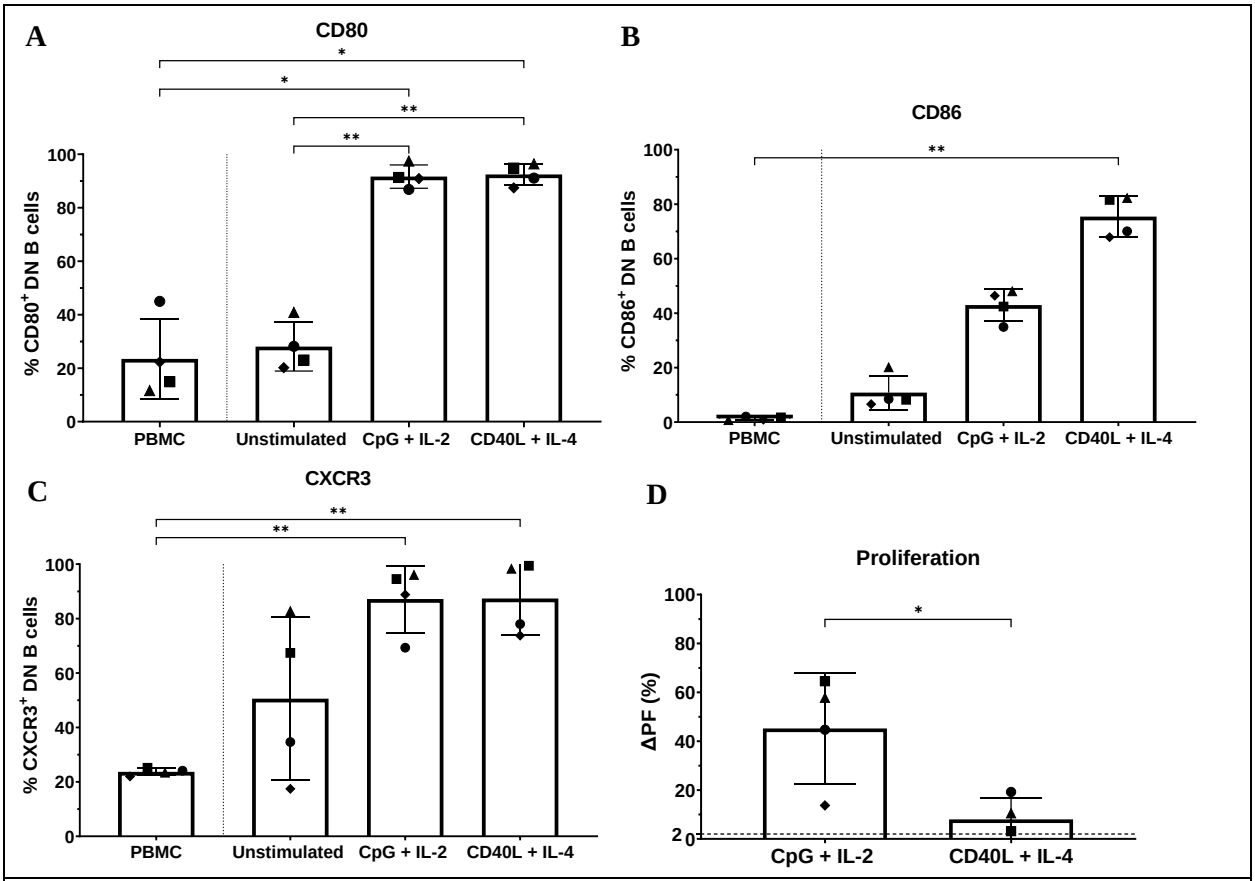

Fig. 4 – Frequencies of activated and proliferating DN B cells after *in vitro* stimulation. $CD27^-$ and $CD27^+$ B cells were isolated from peripheral blood and subsequently *in vitro* stimulated with CpG + IL-2 or CD40L + IL-4, for three days (only DN B cells from $CD27^-$ B cell population are shown). The percentages of (**A**) $CD80^+$, (**B**) $CD86^+$ and (**C**) $CXCR3^+$ DN B cells before stimulation (PBMC, baseline) and after three days of stimulation are shown. (**D**) The percentage of proliferation is shown as the delta proliferation fraction (ΔPF), which is calculated by subtracting the percentage of proliferation in the unstimulated condition from the percentage of proliferation in the stimulated condition. The black dashed line represents the cut-off (2%) and is used to identify increased proliferation. Mean (bars) ± SD is shown. (n = 4). Repeated measures one-way ANOVA test with Tukey's post hoc testing, Friedman test with Dunn's post hoc testing or paired t-test were used. (data passed normality testing,

Continued from Fig. 4 - except for (**B**) $CD86^+$ DN B cells). *$p < 0.05$, **$p < 0.01$. CD40L, CD40 ligand. CXCR3, C-X-C motif chemokine receptor 3. DN B cells, IgD^-CD27^- double negative B cells. Ig, immunoglobulin. IL, interleukin. PBMC, peripheral blood mononuclear cells. ΔPF, delta proliferation fraction.

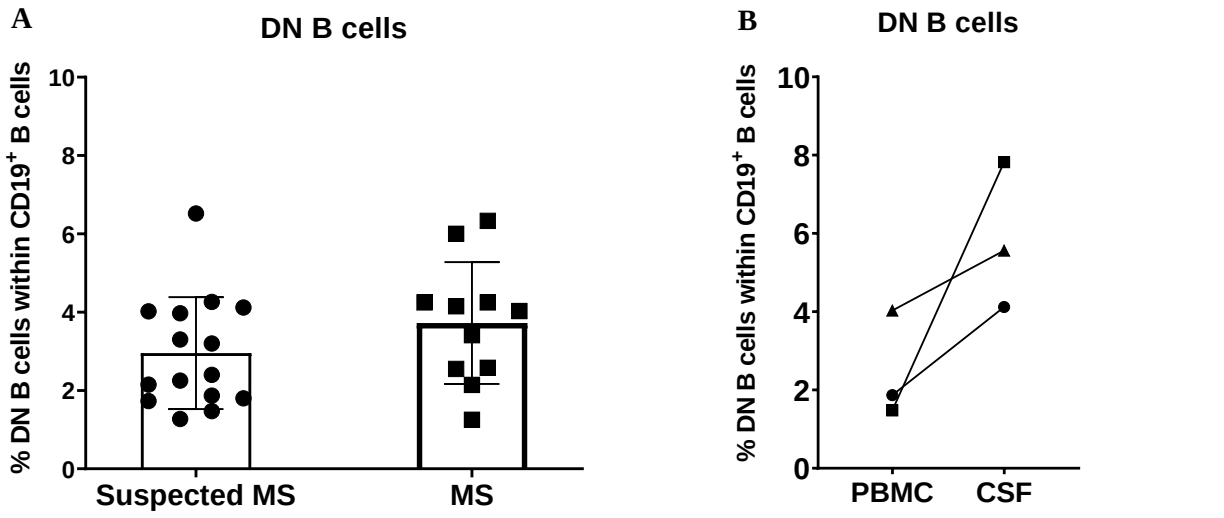

Fig. 5 – Frequencies of DN B cells in peripheral blood and CSF of MS patients. (**A**) PBMCs were isolated from peripheral blood of patients with suspected MS (n = 15) and confirmed MS patients (n = 11). The frequencies of DN B cells were determined with flow cytometric analyses. Mean (bars) ± SD is shown. (**B**) PBMCs and CSF cells were isolated from paired peripheral blood and CSF, respectively, of one MS patient and two patients with suspected MS. The frequencies of DN B cells were determined with flow cytometric analyses. (n = 3). Unpaired or paired t-tests were used (data passed normality testing). CSF, cerebrospinal fluid. DN B cells, IgD^-CD27^- double negative B cells. HC, healthy control. MS, multiple sclerosis. PBMC, peripheral blood mononuclear cells. RRMS, relapsing-remitting multiple sclerosis. PPMS, primary progressive multiple sclerosis.

DISCUSSION

As little or contradictory evidence is available on the activation and migration potential of DN B cells in MS, this study aimed to functionally characterize DN B cells from MS patients and HCs. Our findings demonstrate that DN B cells can be activated *in vitro*. We showed that stimulation with innate immune system and T cell-costimulatory signals resulted in increased expression of activation markers and upregulation of chemokine receptors needed for migration towards inflammatory sites. Additionally, this *in vitro* stimulation resulted in increased proliferation of DN B cells. Lastly, CSF DN B cells showed distinct phenotypes compared to paired peripheral blood DN B cells, mainly based on expression of cell adhesion molecules.

As previously stated, different studies found contradictory evidence on the activation potential of DN B cells. We showed increased frequencies of DN B cells expressing activation markers (CD80, CD86) and chemokine receptors (CXCR3) on DN B cells after stimulations mimicking the innate immune system (CpG2006 + IL-2) and costimulation by T cells (CD40L + IL-4). To some extent, this confirms the results of Jenks *et al*. that DN B cells in SLE can be activated by signals from the innate immune system (30). In contrast to our results, Jenks *et al*. did not find increased activation when using stimulations that mimic T cell-costimulation. This could be explained by the use of shorter incubation times (overnight stimulation) and the use of total B cells in their set-up (30), while we isolated $CD27^-$ and $CD27^+$ B cell populations before the three days-*in vitro* stimulation. Therefore, this could indicate that innate immune system stimulatory signals have a rapid function, but smaller timeframe in which they induce DN B

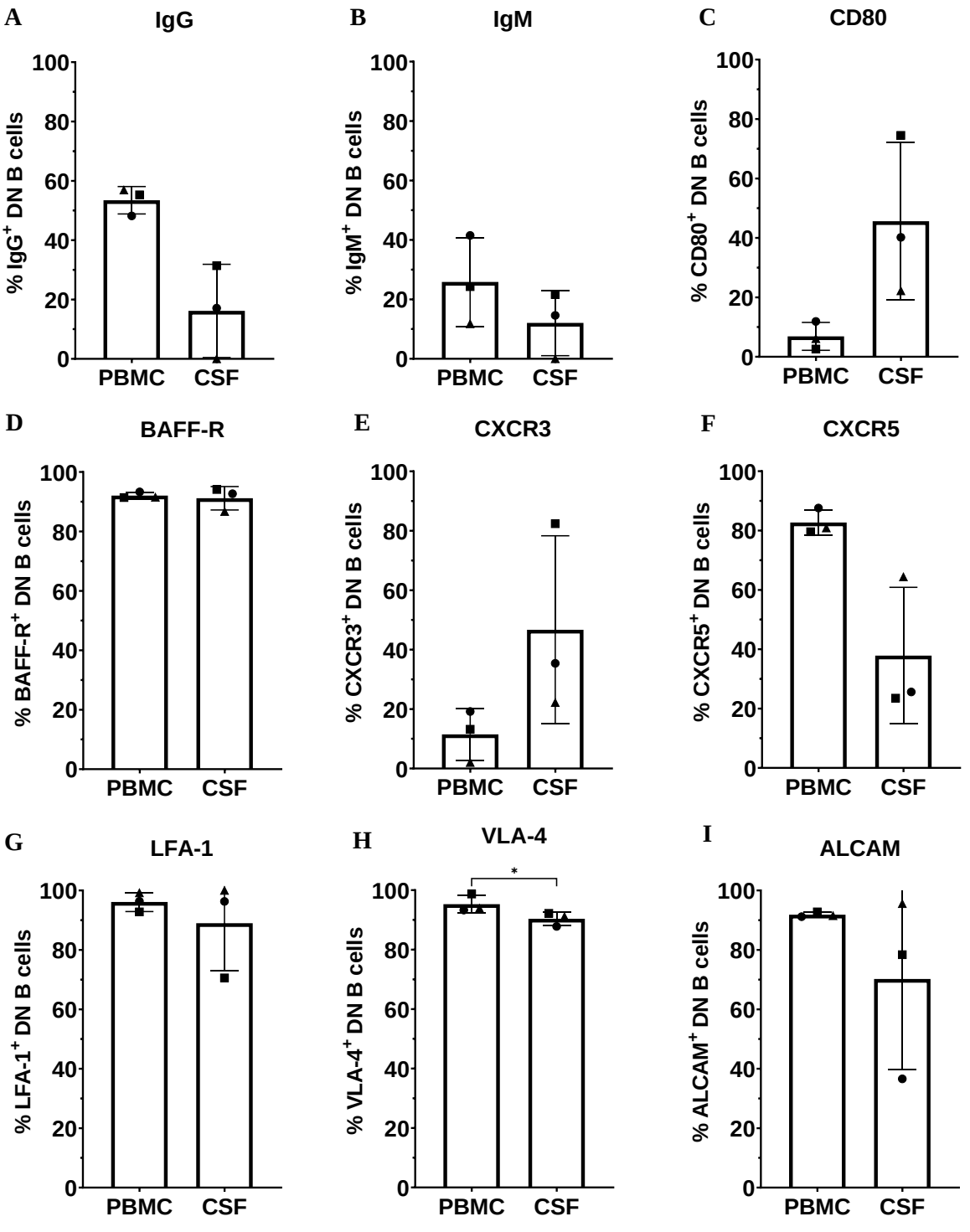

Fig. 6 – Phenotype of DN B cells in peripheral blood and CSF. PBMCs and CSF cells were isolated from paired peripheral blood and CSF, respectively, of one MS patient and two patients with suspected MS. The frequencies of DN B cells (within the $CD19^+$ B cell population) expressing (**A**) IgG, (**B**) IgM, (**C**) CD80, (**D**) BAFF-R, (**E**) CXCR3, (**F**) CXCR5, (**G**) LFA-1, (**H**) VLA-4 and (**I**) ALCAM were determined with flow cytometric analyses. Mean (bars) ± SD is shown. (n = 3). Paired t-test was used (data passed normality testing). $*p < 0.05$. ALCAM, activated leukocyte cell adhesion molecule.

Continued from Fig. 6 - BAFF-R, B-cell activating factor receptor. CSF, cerebrospinal fluid. CXCR, C-X-C motif chemokine receptor. DN B cells, $IgD^{-}CD27^{-}$ double negative B cells. Ig, immunoglobulin. LFA-1, lymphocyte function-associated antigen-1. MS, multiple sclerosis. PBMC, peripheral blood mononuclear cells. VLA-4, very late antigen-4.

cell activation, in comparison to T cell-costimulation requiring longer periods of stimulation for inducing activation.

Next, our results showed that DN B cells proliferated after *in vitro* stimulation. This is consistent with Wei *et al.* who found significantly increased proliferation of DN B cells after stimulation with innate immune system signals (CpG2006) in SLE (29). Different from our study, they stimulated DN B cells for four days with higher CpG2006 concentrations (29). On the contrary, other studies, in elderly and HIV patients, found a low proliferation capacity for DN B cells (26, 27). In elderly, this was determined by measuring Ki67 expression, after stimulating total PBMCs with CpG oligonucleotides for five days (26). In HIV patients, proliferation was studied by measuring thymidine incorporation, after different stimulations for three days followed by a 16 hours-pulsation with thymidine (27). These divergent results might be due to different experimental set-ups, distinct measurements of proliferation or potentially disease-related factors.

The second part of this study was to acquire more information on the migratory phenotype of DN B cells and their capacity to migrate across the BBB. Our findings showed that the DN B cell frequencies were higher in the CSF compared to peripheral blood at the level of the individual patients and suggests that DN B cells do have the capacity to migrate towards the CNS. We also demonstrated that DN B cells in CSF have a distinct phenotype from DN B cells in peripheral blood. We found increased expression of activation markers (CD80) and chemokine receptors needed for migration towards inflammatory sites (CXCR3) in CSF DN B cells, whereas the expression of cell adhesion molecules (VLA-4, ALCAM) and chemokine receptors for migration towards lymph nodes (CXCR5) was decreased. At first, the expression of chemokine receptors seems consistent with other studies that showed increased $CXCR3^{+}$ and decreased $CXCR5^{+}$ DN B cell levels (27, 31). In aging, these levels were compared between young and elderly donors (31), whereas in HIV, DN B cells were compared with naive and CSM B cells (27). However, they performed the phenotypical characterization on total B cells isolated from PBMCs, whereas we used PBMCs and CSF cells. Thus, our results do not agree with Bulati *et al.* and Moir *et al.* (27, 31), as we found lower CXCR3 and higher CXCR5 expression in peripheral blood samples. These findings combined imply that, in MS, DN B cells can cross the BBB, as they express cell adhesion molecules, and subsequently migrate towards the inflammatory sites in the CNS, by increased expression of inflammatory chemokine receptors. They can then be locally activated, indicated by increased frequencies of DN B cells expressing activation markers in the CSF.

Additionally, we optimized the FVD eFluor506 viability staining for the flow cytometric read-out of the *in vitro* migration assay. As previously mentioned, a simultaneous instead of sequential incubation of the FVD viability dye and the other antibodies was preferred to preserve as many migrated cells as possible after the complete staining process. However, staining with a FVD viability dye is usually performed in protein-free 1xPBS, whereas incubation of surface antibodies is performed in serum-containing FACS buffer. That is because FVD dyes cross-link with cellular proteins, which are present in FBS, and staining in FACS buffer may lead to decreased staining intensity of the dead cell population and/or increased background staining in the live cell population (38). However, our data shows that the FVD eFluor506 staining in FACS buffer (2% FBS) can be used in our *in vitro* migration assay, as similar results were found using the standard staining method.

Although this study already shows promising results, the *in vitro* activation assay was currently performed on peripheral blood samples of HCs. At the moment, acquiring peripheral blood samples from MS patients is still challenging due to the COVID-19 pandemic and as we have concluded that fresh samples are preferable over frozen samples, we could not use our existing biobank collection of MS patients. Additionally, it was not yet possible to perform our *in vitro* migration assay as this is currently being optimized.

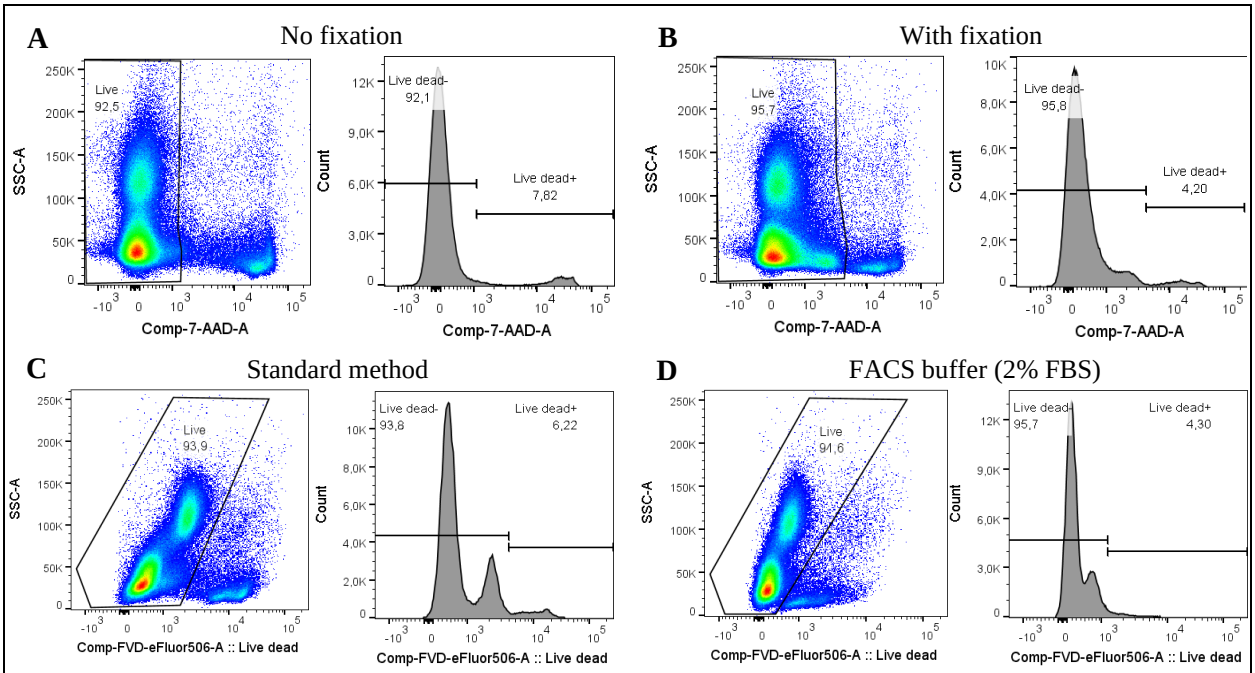

Fig. 7 – Frequency of living cells after 7-AAD and FVD eFluor506 staining. PBMCs were isolated from peripheral blood of two HCs and stained with 7-AAD (**A**) without and (**B**) with fixation or with FVD eFluor506 for 30 min (**C**) in 1xPBS and (**D**) in FACS buffer (2% FBS). Flow cytometry dot plots and histograms are shown for one representative HC. (n = 2). 7-AAD, 7-aminoactinomycin D. FBS, fetal bovine serum. FVD, fixable viability dye. HCs, healthy controls. PBMCs, peripheral blood mononuclear cells. SSC, side scatter.

Another limitation of our study is the lower number of patient samples that were used for our phenotypical comparison of DN B cells in paired peripheral blood and CSF. Therefore, we will include more MS patients in our future patient population in order to perform the correct statistical analyses and to make a definitive conclusion. Lastly, the other B cell subsets were also incorporated in our *in vitro* activation assay in order to compare the activation potential of DN B cells with naive, NCSM and CSM B cells. When looking at our first results, it appears that DN B cells have an activation potential that is intermediate between CSM B cells, and naive and NCSM B cells. However, a thorough and complete analysis of the data still has to be performed.

Taken together, our findings show that DN B cells are not exhausted and senescent B cells, as was concluded by Colonna-Romano *et al.* (26). They have the capacity to respond to different types of stimulations, demonstrated by increased frequencies of DN B cells expressing activation markers and inflammatory chemokine receptors, as well as increased proliferation. Additionally, DN B cell levels were increased in the CSF and these cells displayed distinct phenotypes compared to peripheral blood DN B cells. This strongly suggests that DN B cells can migrate towards the CNS, become activated and play a role in the pathology of MS.

CONCLUSION

In conclusion, our study indicates that DN B cells can be activated by *in vitro* stimulations mimicking both innate and adaptive immune system stimulatory signals. In a follow-up study, the functional characterization will be further complemented by including MS patients in the *in vitro* activation assay and with findings from the *in vitro* migration assay, for which optimization was already initiated in this study. Combining the results of the *in vitro* activation and migration assays will clarify whether DN B cells play a role in the disease pathology of MS. This could potentially lead to the development of more personalized and specific therapies for MS patients in the future.

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Author contributions – Prof. Veerle Somers, Judith Fraussen and Lien Beckers conceived and designed the research. Serina Rubio and Lien Beckers performed the experiments and data analysis. Serina Rubio wrote the paper and Judith Fraussen edited the paper.

SUPPLEMENTARY TABLES

Supplementary table 1 – Flow cytometry antibodies for screening of peripheral blood.

Marker	Fluorophore	Clone	Company	Dilution	Incubation	Temp.	Buffer
CD19	BV421	HIB19	BioLegend	1/100	15 min	RT	FACS, 2% FBS
CD27	APC	M-T271	BD Biosciences	1/20	15 min	RT	FACS, 2% FBS
IgD	PE-Cy7	IA6-2	BioLegend	1/100	15 min	RT	FACS, 2% FBS
IgG	FITC	G18-145	BD Biosciences	1/100	15 min	RT	FACS, 2% FBS
IgM	PerCP-Cy5.5	MHM-88	BioLegend	1/100	15 min	RT	FACS, 2% FBS
IgA	PE	IS11-8E10	Miltenyi	1/200	15 min	RT	FACS, 2% FBS

Supplementary table 2 – Flow cytometry antibodies for screening of CSF.

Marker	Fluorophore	Clone	Company	Dilution	Incubation	Temp.	Buffer
CD19	BV421	HIB19	BioLegend	1/100	30 min	4°C	FACS, 2% FBS
CD27	APC	M-T271	BD Biosciences	1/20	30 min	4°C	FACS, 2% FBS
IgD	PE-Cy7	IA6-2	BioLegend	1/100	30 min	4°C	FACS, 2% FBS
IgG	AF700	G18-145	BD Biosciences	1/20	30 min	4°C	FACS, 2% FBS
IgM	BV605	MHM-88	BioLegend	1/50	30 min	4°C	FACS, 2% FBS
CD80	PE-Dazzle594	2D10	BioLegend	1/20	30 min	4°C	FACS, 2% FBS
CXCR3	BV711	G025H7	BioLegend	1/50	30 min	4°C	FACS, 2% FBS
CXCR5	APC-Cy7	J252D4	BioLegend	1/50	30 min	4°C	FACS, 2% FBS
BAFF-R	FITC	11C1	BioLegend	1/200	30 min	4°C	FACS, 2% FBS
LFA-1	PE	HI111	BioLegend	1/200	30 min	4°C	FACS, 2% FBS
VLA-4	PerCP-Cy5.5	9F10	BioLegend	1/20	30 min	4°C	FACS, 2% FBS
ALCAM	BV786	3A6	BD Biosciences	1/20	30 min	4°C	FACS, 2% FBS

Supplementary table 3 – Flow cytometry antibodies for $CD27^-$ and $CD27^+$ purity confirmation.

Marker	Fluorophore	Clone	Company	Dilution	Incubation	Temp.	Buffer
CD19	PE-Cy7	HIB19	BioLegend	1/100	15 min	RT	FACS, 2% FBS
CD27	APC	M-T271	BD Biosciences	1/20	15 min	RT	FACS, 2% FBS

IgD	PE-CF594	IA6-2	BD Biosciences	1/100	15 min	RT	FACS, 2% FBS

Supplementary table 4 – Flow cytometry antibodies for the *in vitro* activation assay.

Marker	Fluorophore	Clone	Company	Dilution	Incubation	Temp.	Buffer
CD19	BV650	HIB19	BioLegend	1/50	15 min	RT	FACS, 2% FBS
CD27	APC	M-T271	BioLegend	1/100	15 min	RT	FACS, 2% FBS
IgD	PE-Cy7	IA6-2	BioLegend	1/100	15 min	RT	FACS, 2% FBS
IgG	AF700	G18-145	BD Biosciences	1/20	15 min	RT	FACS, 2% FBS
IgM	PerCP-Cy5.5	MHM-88	BioLegend	1/100	15 min	RT	FACS, 2% FBS
IgA	VioGreen	IS11-8E10	Miltenyi	1/100	15 min	RT	FACS, 2% FBS
CD80	BV421	2D10	BioLegend	1/20	15 min	RT	FACS, 2% FBS
CD86	BV785	IT2.2	BioLegend	1/50	15 min	RT	FACS, 2% FBS
CXCR3	BV605	G025H7	BioLegend	1/33	15 min	RT	FACS, 2% FBS
CFSE cell division tracker kit	CFSE	/	BioLegend	1 µM	20 min	37°C	1xPBS
Live/dead	FVD eFluor780	/	eBioscience	1/1000	30 min	4°C	1xPBS

Supplementary table 5 – Staining conditions and live/dead flow cytometry antibodies for the *in vitro* migration assay.

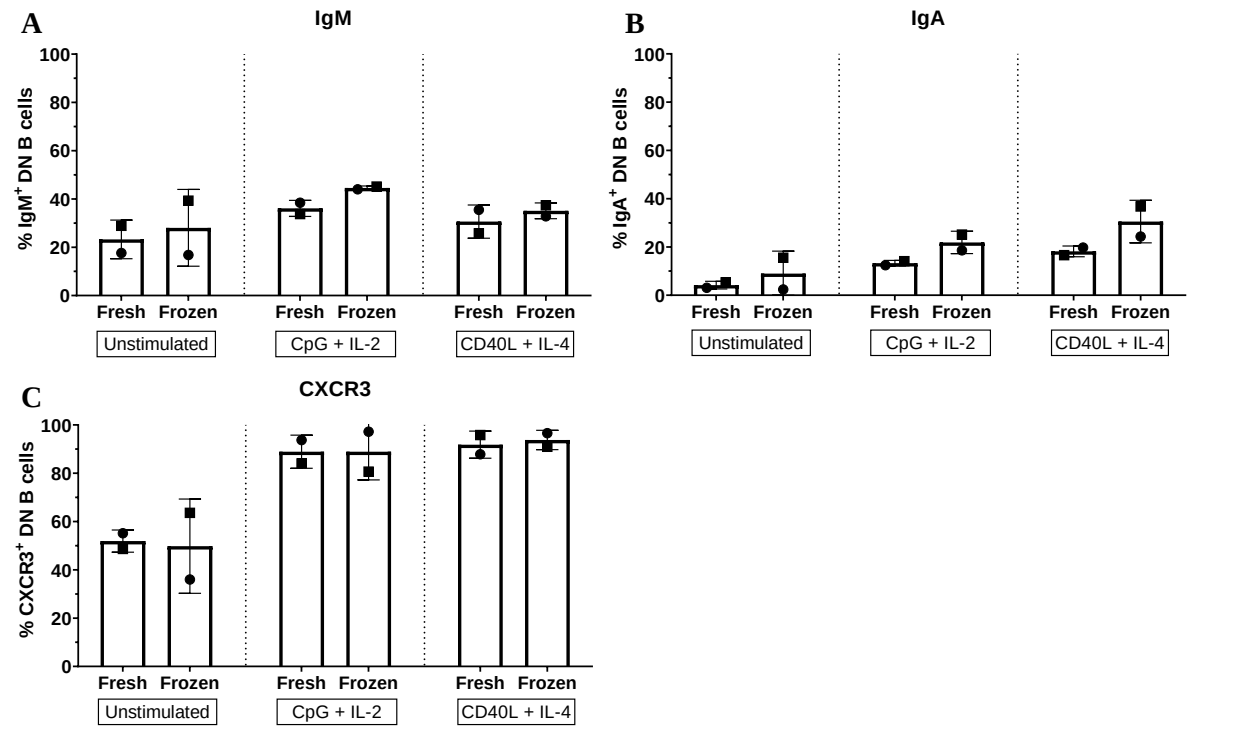
Condition	Live/dead	Surface staining	Incubation	Buffer
1	FVD eFluor506	No	30 min	1xPBS
2	FVD eFluor506	Yes	30 min	1xPBS
3	FVD eFluor506	Yes	30 min	1xPBS, With FcR blocker
4	FVD eFluor506	Yes	30 min	FACS, 2% FBS
5	FVD eFluor506	Yes	30 min	FACS, 5% FBS
6	7-AAD	Yes	15 min	FACS, 5% FBS With fixation
7	7-AAD	Yes	15 min	FACS, 5% FBS No fixation

Marker	Fluorophore	Clone	Company	Dilution
Live/dead	FVD eFluor506	/	eBioscience	1/1000
Live/dead	7-AAD	/	eBioscience	1/40

Supplementary table 6 – Flow cytometry antibodies for the *in vitro* migration assay.

Marker	Fluorophore	Clone	Company	Dilution	Incubation	Temp.	Buffer
CD19	PE-Cy7	HIB19	BioLegend	1/100	15 min	RT	FACS, 2% FBS
CD27	APC	M-T271	BD Biosciences	1/20	15 min	RT	FACS, 2% FBS
IgD	PE-CF594	IA6-2	BD Biosciences	1/100	15 min	RT	FACS, 2% FBS
IgG	PE	G18-145	BD Biosciences	1/100	15 min	RT	FACS, 2% FBS
IgM	BV605	MHM-88	BioLegend	1/50	15 min	RT	FACS, 2% FBS
CD86	FITC	2331	BD Biosciences	1/100	15 min	RT	FACS, 2% FBS

SUPPLEMENTARY FIGURES



Supplementary Fig. 1 – Frequencies of DN B cell Ig isotypes and CXCR3$^+$ DN B cells in fresh and frozen samples. Fresh and frozen PBMC samples were used for CD27$^-$ and CD27$^+$ B cell isolation and subsequent *in vitro* stimulation with CpG + IL-2 or CD40L + IL-4, for three days (only DN B cells from CD27$^-$ B cell population are shown). The percentages of (**A**) IgM$^+$, (**B**) IgA$^+$ and (**C**) CXCR3$^+$ DN B cells are shown between fresh and frozen samples for the different stimulatory conditions. Mean (bars) ± SD is shown. (n = 2). CD40L, CD40 ligand. CXCR3, C-X-C motif chemokine receptor 3. DN B cells, IgD$^-$CD27$^-$ double negative B cells. Ig, immunoglobulin. IL, interleukin. US, unstimulated.