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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

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Athanasios Bethanis

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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2021
2022

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www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

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Functional characterisation of IgD$^-$CD27$^-$ double negative (DN) B cells in multiple sclerosis pathology*

Bethanis A.[1,2], Beckers L.[1,2], Somers V.[1,2] and Fraussen J.[1,2]

[1]University MS Center (UMSC), Hasselt-Pelt, Belgium
[2]Department of Immunology and Infection, Biomedical Research Institute, Hasselt University, Hasselt, Belgium

*Running title: The role of IgD$^-$CD27$^-$ B cells in MS pathology

To whom correspondence should be addressed: dr. Judith Fraussen
Tel: +3211269270, Email: judith.fraussen@uhasselt.be

Keywords: multiple sclerosis; B cells; autoimmune disease; double negative; activation

ABSTRACT

In multiple sclerosis (MS), an inflammatory autoimmune disease affecting the central nervous system (CNS), IgD$^-$CD27$^-$ double negative (DN) B cells were recently found elevated in a portion of MS patients. This study aimed to investigate their activation potential and migration capacity towards the CNS in MS. CD19$^+$ or CD27$^-$ and CD27$^+$ B cells were isolated from blood of MS patients. The latter (n=3) were stimulated with CD40ligand(L)/ interleukin(IL)-4, CpG/IL-2 or CpG/IL-21/ interferon(IFN)-γ and compared against unstimulated cells to evaluate their activation potential (CD80/CD86), proliferation and proinflammatory markers (C-X-C chemokine receptor 3 (CXCR3)/T-bet). CD19$^+$ cells (n=3) were used in a transwell system to evaluate their migration capacity through an inflamed endothelial layer (hCMEC/d3) representing an *in vitro* blood-brain-barrier (BBB) model.

DN B cells of MS patients had a higher migration capacity when compared to HC, while also showing a more activated state with increased CXCR3 expression. DN B cells could also be activated by all stimulations, seen by their increased frequency of CD80$^+$ and CD86$^+$ cells and increased levels of proinflammatory markers T-bet and CXCR3 following stimulation. Proliferation of DN B cells was furthermore induced by CpG/IL-2 and CpG/IL-21/IFN-γ.

Our results showed activation and proliferation of DN B cells with increased expression of proinflammatory markers upon different *in vitro* stimulations. Moreover, DN B cells proved to be capable of homing over an *in vitro* BBB model, further indicating their potential involvement in MS.

INTRODUCTION

Disease prevenance - Multiple Sclerosis (MS) is an inflammatory autoimmune disease that affects the central nervous system (CNS), leading to loss of myelin and increasing neurodegeneration. For every 10,000 people, 50 – 300 are estimated to be affected by MS worldwide, with a total of 2.8 million affected around the world (1). Disease distribution globally is uneven, with some countries having low and some increased prevalence of the disease. Belgium is a high-risk country for MS development, with an estimated 13,000 people currently living with MS (2). The disease has a three times higher incidence in women over men, and usually manifests in early adult life (20 – 60 years old). Depending on how the disease progresses, MS is classified into different types. Most often, in 70 – 80% of cases, there are attacks where patients exhibit new symptoms or worsening of already present symptoms. These are then followed by a period of stability, where the symptoms diminish or even completely disappear(3). This type of MS is termed relapsing-remitting (RRMS) (4). Other types exist, where after initial onset of symptoms, either only deterioration of neurological functions without

relapses occurs (primary-progressing MS (PPMS)) or after a time of a relapsing-remitting course, the disease transitions to continuous worsening (secondary-progressive MS (SPMS)). Around 20% of RRMS patients transition into SPMS, while a smaller proportion of patients (15%) develop PPMS after onset of symptoms (5). The exact aetiology leading to MS development is unclear, however research has identified several risk factors having a significant contribution to the occurrence of the disease. These include environmental factors, such as vitamin D deficiency, obesity in early life and smoking, as well as genetic factors (6). The first and most studied factor is the MHC class II HLA-DRB1 gene, specifically its allele HLA-DRB1*15:01, which is considered to infer a 3-fold increase in risk for developing MS (7). Genetic susceptibility might also be impacted by the aforementioned environmental factors, as it is well known that the environment can influence gene expression (8–10).

Role of B cells in MS - Why the immune system mounts, and maintains a reaction against CNS antigens in MS is unclear. However, there are two hypotheses behind how these immune responses are initiated (1). One hypothesis of MS aetiology, termed the intrinsic model, suggests that the initial event (e.g. damage of myelin sheaths) takes place within the CNS. It is then followed by release, either via lymph node drainage or actively carried by antigen presenting cells, of potential antigens to the periphery, which cause an autoimmune response that targets back to the CNS. The extrinsic model implies that the initial event occurs in the periphery (e.g. a viral infection), which then leads to an aberrant immune response aimed toward the CNS (11). T cells have long been considered to be the key players that lead to MS pathology via their recruitment to the CNS by certain target (self)antigens. However, although several candidate antigens have been suggested, none have been confirmed to be responsible (1). Recent studies showcase B cells also to be involved in the pathology of MS. Evidence include their identification in damaged tissue and neuronal lesions as well as their presence in the cerebrospinal fluid (CSF). Their populations in the CSF have additionally been clonally linked to populations in the periphery (12,13). They reside in distinct compartments in the CNS but also occupy secondary lymphoid organs that drain the CNS (14). Furthermore, oligoclonal immunoglobulin (Ig) bands, one of the main diagnostic confirmations for MS pathology, have been attributed to CSF B cells as evidenced by the close overlapping of Ig B cell transcriptomes with Ig proteomes (15). Further evidence of B cell association with MS is the improvement of disease symptoms upon B cell depletion therapies, which has been evidenced for both RRMS and PPMS (16,17).

B cell classification - As a result, B cells are increasingly investigated in MS research (18–20). B cell development stems from precursor cells in the bone marrow (precursor B cells), from where they enter the circulation becoming transitional B cells and eventually will mature into naïve B cells (21). Naïve B cells are distinguished from memory B cells based on their expression of the surface markers CD27 and IgD, which distinguishes them into naive B cells, class-switched memory (CSM) B cells and non class-switched memory (NCSM) B cells. In naïve B cells the first Ig to be present during their evolution is IgD characterising this subset with high IgD levels (IgD^+ $CD27^-$). From the bone marrow they find their way into secondary lymphoid organs, such as the spleen and lymph nodes, while recirculating in the body, until they come into contact with antigens and initiate adaptive immune responses. In response to antigen challenges, most naïve B cells undergo class-switching (IgA^+/IgG^+/IgE^+) in germinal centres (GCs) downregulating IgD expression (IgD^- $CD27^+$) while upregulating expression of CD21, CD40, CD80, CD86, and CD95 markers becoming CSM B cells (22). However, in GCs some memory cells will remain non class-switched (NCSM). It is also worth noting that naïve B cells can become NCSM B cell outside GCs in a T-independent pathway (23). Additionally, once activated, a proportion of memory cell will instead rapidly expand, and proliferate into antibody-secreting plasma cells or plasmablasts, which comprise the first rapid line of antigen-specific cells (24). These cells only live for a short period of time, whereas the other memory B cells persist much longer after antigen exposure (25). Usually, memory B cells are characterised by the presence of CD27, although exceptions of $CD27^-$ memory B cells have also been seen (26).

DN B cells - Another B cell subset that is negative for both CD27 and IgD termed double negative (DN) B cells, has also been identified. However, DN B cells are often termed atypical memory B cells as their exact origin remains unknown (27). Some studies have suggested they originate from $CD27^+$ memory B cells, while others consider them as exhausted memory B cells (28). Under normal circumstances, these DN B cells are observed in healthy aged individuals (> 60 years old) as part of immune ageing, where a progressive increase of constant, low-grade systemic inflammation (inflammaging) is seen (29). One of the main driving forces of inflammaging is considered to be the chronic stimulation of the immune system by viruses that are carried for life, such as cytomegalovirus, which promotes a constant pro-inflammatory cytokine production (30). In addition, this IgD^-CD27^- DN B cell subset has also been found to be elevated in several autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, where their frequency was linked to disease severity, corroborating their potential involvement in disease pathogenesis (31–33). HIV infected individuals have also been seen with elevated DN B cell levels (34), as well as people living in malaria-prone regions (35) They have also been linked with recent viral infections from the SARS-CoV-2 virus and disease severity of infected individuals (36).

Their levels have also been previously seen to be higher in certain MS patients, but their association with the disease remains unclear (37). However, in our previous work, we found characteristics of an aged immune system, particularly DN B cell expansion, in a proportion (19.5%) of MS patients younger than 60 years old (32,37). Potential contributions to their expansion were theorised to be the repetitive inflammation present in MS patients, or the repeated antigen challenges during MS relapses (37). The exact mechanism of their expansion remains, however, elusive. Coupled with their increased frequencies in the CSF of MS patients compared with peripheral blood (32) it corroborates their potential involvement in MS pathology.

Additional heterogeneity within the DN population has also been observed in autoimmune diseases like SLE, but no consensus classification criteria exist for the DN B cell subsets, with papers often using different gating strategies to differentiate between them. Sanz *et al.* defined two DN subsets, DN1 and DN2 via CD19, CD21 and CXCR5 expression (38), although they have also been defined based on their CD21 and CD11c expression alone (39). In SLE, DN1 cells are elevated in healthy or quiescent SLE, whereas DN2 comprise most of the DN subset in the active form of the disease (40). DN2 cells also show high CD11c and T-box transcription factor T-bet expression, which is why the latter is often used as a marker to distinguish between the subsets, while lacking CXCR5 expression.

Expression of T-bet has been associated with pathogenic responses in B cells as well as seen in autoimmunity, while it is known to be induced in systemic infections (41,42). $T\text{-}bet^+$ B cells have also been found in previous studies in relation to MS (43,44) while B cells have been seen to be induced by interferon (IFN)-γ to express T-bet (43).

In preliminary *in vitro* experiments we observed that DN B cells are able to migrate towards C-X-C chemokine ligand (CXCL) 10 and CXCL13 chemokines, which in turn recognise and bind to proinflammatory receptors C-X-C chemokine receptor (CXCR) 3 and CXCR5. Data already point to CXCR3 leading B cells to site of inflammation, while being expressed on DN B cells suggesting that DN B cells can migrate towards sites of inflammation (45,46).

Following on this, this study will attempt to examine the functional properties of this DN B cell subset in MS patients in conjunction with its migration capacity in an *in vitro* model of endothelial layers mimicking migration from the periphery to the CNS through a blood brainer barrier (BBB). This is particularly important, since currently no other studies have been able to identify the exact aetiology that leads to MS development, and DN B cells seem a promising culprit. Finding more concrete evidence on the activation potential of these cells, given the current inconsistences on their fate in MS, could help develop future treatment options for patients.

We, therefore, hypothesise that DN B cells of MS patients **can be activated *in vitro* by different stimulatory conditions**, and that these **activated B cells can migrate** from the periphery **into the CNS** in *in vitro* models, homing towards certain chemokines like CXCL10 and CXCL13.

EXPERIMENTAL PROCEDURES

Study Subjects – The study was approved by the Medical Ethical Committee of Hasselt University and written informed consent was obtained from all MS patients and healthy controls (HC). MS patients were recruited from either Rehabilitation & MS-Center (Pelt, Belgium) or Zuyderland Medical Center (Sittard-Geleen, The Netherlands) and were diagnosed according to the McDonald criteria (47). HC were recruited from Hasselt University (Hasselt, Belgium). Whole blood and CSF from 9 patients and whole blood from 4 HC was collected, of which two were age- and gender-matched to MS patients (Table 1).

Cell Isolation – Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll density gradient centrifugation (Lympholyte; Cedarlane Laboratories, SanBio B.V., Uden, The Netherlands) to be used in flow cytometric analysis or for further isolation. $CD19^+$ B cells were isolated from PBMC using negative magnetic selection according to manufacturer's instructions (EasySep™ Human B cell Isolation Kit, StemCell Technologies). $CD27^+$ and $CD27^-$ B cells were isolated from PBMC using immunomagnetic positive and negative selection according to manufacturer's instructions, respectively (EasySep™ Human Memory B Cell Isolation Kit, StemCell Technologies). Counting of both PBMC and B cell subtypes was performed using a MOXI Z Mini Automated Cell Counter (Orflo Technologies, United States). For either type of isolation, purity was determined using flow cytometry and was at a minimum >99% for $CD19^+$, >88% for $CD27^-$ and >94% for $CD27^+$ B cells.

CSF vs PBMC Screening - For PBMC screening of MS patients or HC, 1 x 10^6 cells were surface stained using anti-human monoclonal antibodies: IgD PE-Cy7 (clone IA6-2), CD19 Brilliant Violet (BV) 421 (clone HIB19), IgM PerCP-Cy5.5 (clone MHM-88) (all from BioLegend, London, United Kingdom), CD27 APC (clone M-T271) (BD Biosciences, Belgium), IgG FITC (clone G18-145) (BD Biosciences), IgA PE (clone IS118E10) (Miltenyi Biotec, The Netherlands) for 15 min at room temperature (RT). Cells were then washed twice with 1x fluorescence-activated cell sorting (FACS) buffer (5% foetal bovine serum (FBS) (Gilbco, Thermofisher), 0.1% sodium azide in 1x PBS) before acquiring.

CSF cells were isolated by centrifuging samples (10 min; 350 x g) at 4°C. Cells were counted using a hemacytometer and used immediately for flow cytometric analysis. Samples with red blood cell contamination or with no more than 2,500 cells were excluded from analyses. All recovered cells were stained with anti-human CD19 BV421 (clone HIB19), CD268 FITC (clone 11C1), CD11a PE (clone HI111), CD49d PerCP-Cy5.5 (clone 9F10), CD185 APC-Cy7 (clone J252D4), CD80 PE-Dazzle594 (clone 2D10), CD183 BV711 (clone G025H7), IgM BV605 (clone MHM-88), IgD PE-Cy7 (clone IAG-2) (all from BioLegend), CD166 BV786 (clone 3A6), CD27 APC (clone M-T271), IgG AF700 (clone G18-145) (all from BD Biosciences) for 30 min at 4°C. Paired PBMC were stained with the same aforementioned antibodies for 15 min at RT.

Activation assay – Isolated $CD27^+$ and $CD27^-$ B cells were labelled with 1µM Carboxyfluorescein succinimidyl ester (CFSE) dye in a concentration of 10 x 10^6 cell/mL (20 min at 37°C) before being seeded in 96-well U-bottom plates (Greiner Bio-one, Vilvoorde, Belgium) at 1 x 10^5 cells per well in RPMI1640 (Lonza, Belgium), supplemented with 10% FBS, 1/% non-essential amino acids, 1% sodium pyruvate, 50 µg/mL streptomycin and 50 U/mL penicillin (all from Sigma-Aldrich, Germany). Cells were subsequently stimulated for 72 h by one of the following: (i) 1 µg/mL CpG ODN 2006 (ThermoFisher) with 50 U/mL interleukin (IL)-2 (Sigma-Aldrich); (ii) 1 µg/mL CD40ligand (CD40L) (BioLegend) with 10 ng/mL IL-4 (R&D systems); (iii) 1 µg/mL CpG ODN 2006 (ThermoFisher) together with 10 ng/mL IL-21 and 50 ng/mL IFN-γ (BioLegend). As a negative control, unstimulated cells were used.

On day 3, PBMC, $CD27^+$ and $CD27^-$ B cells were first stained with Fixable Viability Dye (FVD) eFluor 780 (eBioscience, San Diego, USA) in 1x phosphate buffer saline (PBS) for 30 min at 4°C to evaluate viability. After a wash with 1x FACS buffer, cells were resuspended in 1x FACS buffer and stained using anti-human IgM PerCP-Cy5.5 (clone MHM-88), CD27 APC (clone M-T271), CD80 BV421 (clone 2D10), CXCR3 BV605 (clone G025H7), CD19 BV650 (clone HIB19), CD11c BV711 (clone 3.9), CD86 BV785 (clone IT2.2), CD21 PE-Dazzle594 (clone Bu32), IgD PE-Cy7 (clone IA6-2) (all from BioLegend), IgG Alexa Fluor (AF)700 (clone G18-145) (BD Biosciences)

and IgA VioGreen (clone IS11-8E10) (Miltenyi Biotec) for 15 min at RT. For intracellular staining, cells were first washed and fixated using the Foxp3/Transcription Factor Staining Buffer kit following manufacturer's instructions (eBioscience™, ThermoFisher Scientific, Waltham, MA, USA) and stained with anti-human T-bet PE (clone 4B10) (BioLegend) for 30 min at RT. Cells were washed twice with the kit's permeabilisation buffer before resuspended in FACS buffer for acquiring. To evaluate proliferation of cells a proliferation fraction (ΔPF) was calculated, which was defined as the difference between proliferation due to stimulation and background unstimulated cells.

Transmigration assay – Human brain endothelial cells (hCMEC/d3) were cultivated in collagen-coated flasks (75 µg/mL of collagen) using the EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (CC-3202, Lonza). When cells were almost confluent, after 3 days, they were detached using 1x PBS-diluted trypsin (2mM) with EDTA (2µM) and harvested. Then, 1.65 x 10^5 cells/mL were added to collagen-coated (75 µg/mL) 3 µm pore filter inserts in transwell plates (Greiner bio-one) (day 0).

On day 3 the medium in both the top and bottom chambers was replenished with EBM-2 medium supplemented with 2.5% FBS, 1 mg/ml amphotericin, 10 mg/ml gentamycin, 1.4 µM hydrocortisone, 5 ng/ml hFGF-B (hereafter referred to as replenishing medium). On day 5 medium was exchanged again with replenishing medium. On day 6 medium was changed to a hydrocortisone-free replenishing medium and inflammatory cytokines tumour necrosis factor-alpha (TNF-α) (100ng/mL) and INF-γ (10ng/mL) (both from PeproTech, Thermo Fisher Scientific) were added to the top chamber of the filter inserts.

Freshly isolated B cells (4 x 10^6 cell/mL) from PBMC of MS patients or HC were gently added to the top chamber of the hCMEC/d3 layers grown on the insert filters (day 7). Cells were left to transmigrate to the bottom chamber for 24 h. When indicated, chemokines CXCL10 and CXCL13 (1 µg/mL each, PeproTech) were added to the bottom chamber. Each condition was tested in duplicates. On day 8, B cells from the insert filters and potentially migrated B cells from the bottom wells were collected separately for each condition, counted using a MOXI Z Mini Automated Cell Counter, washed with 1x PBS and resuspended in 1x FACS buffer supplemented with 2% FBS. Duplicates of each condition were pooled for staining. Using the cell counts, the percent migrated cells was calculated, which was defined as the difference between the mean number of cells (of each duplicate) in the bottom and top chamber divided by the number of cells placed in the top chamber on day 7 expressed as a percentage.

All cells were stained with anti-human CXCR5 APC-Cy7 (clone J252D4), CD19 BV421 (clone HIB19), CXCR3 BV605 (clone G025H7), CD11c BV711 (clone 3.9), CD86 BV785 (clone IT2.2), IgD PE-Cy7 (clone IA6-2), CD27 APC (clone M-T271), IgM PerCP-Cy5.5 (clone MHM-88) (all from BioLegend), IgG FITC (clone G18-145) (BD Biosciences) and FVD eFluor506 (eBiosciences) for 30 min at 4°C. Cells were then washed twice with FACS buffer before acquiring.

Flow Cytometry Analysis – Fluorescence minus one (FMO) controls were used for gating. The gating strategy for each flow cytometry panel is depicted in Suppl. Figures 1 – 3. All samples were acquired on a LSRFortessa flow cytometer (BD Biosciences), and data were analysed using FlowJo software (FlowJo, LLC, Oregon, USA). *Statistical analyses* –

Statistical analyses were performed using GraphPad Prism (version 9.3.1). For non-paired data, each B cell subset was compared between MS patient and HC or between a stimulation and the unstimulated condition using Mann-Whitney U tests. For analysis of paired data, all B cell subsets were compared against each other with a Friedman test using Dunn's multiple comparison post hoc test. P values < 0.05 were considered as significant.

Table 1 – Characteristics of MS patients and health donors

Donors	n	Age[a]	Gender, % F	MS Type[b]	Treatment	Disease Duration	EDSS[d]
CSF vs PBMC Screening							
MS	3	31.7 ± 6.1	33.3	RRMS: 1; CIS: 1; sMS: 1	FLT: 1; UT: 2	1.5	1.5
Migration Assay							
MS	3	40.7 ± 15.5	100	RRMS: 1; CIS: 1; SPMS: 1	FLT: 1; UT: 2	7 ± 9.5	2 ± 1.4
HC	2	24.5 ± 3.5	100				N/A
Activation Assay[c]							
MS	3	48 ± 11.3	100	RRMS: 3	FLT: 1; UT:2	10 ± 2.8	1.7 ± 0.6
HC	2	43.7 ± 14.8	100				N/A

[a]In years, presented as mean ± SD,
[b]SPMS patient is transitioning from RRMS,
[c]HC were age- and gender-matched to MS patients,
[d]*P*resented as mean ± SD;
F, female; CIS, clinically isolated syndrome; sMS, suspected MS; FLT, first line therapy; UT, untreated; EDSS, expanded disability status scale

RESULTS

DN B cells of MS patients show elevated CXCR3 expression of MS patients – First, the phenotype of DN B cells in the CSF versus the peripheral blood of MS patients (n = 3) was analysed. Due to the low number of patient samples no statistics analysis was possible. The frequency of DN B cells was higher in the patients' CSF (5.1 ± 1.5%) compared to peripheral blood (2.5 ± 1.2%) (Fig. 1). Additionally, DN B cells had a higher percentage of the adhesion molecule CXCR3 in CSF (55.7 ± 14%) than in the peripheral blood (25.6 ± 9.7%). While other markers investigated did not show any differences, several exhibited high interdonor variability; primarily CXCR5, CD80 and IgG (SD > 16). Overall, we see DN B cells with higher CXCR3 expression once they have migrated in the CSF suggesting either coming to contact with

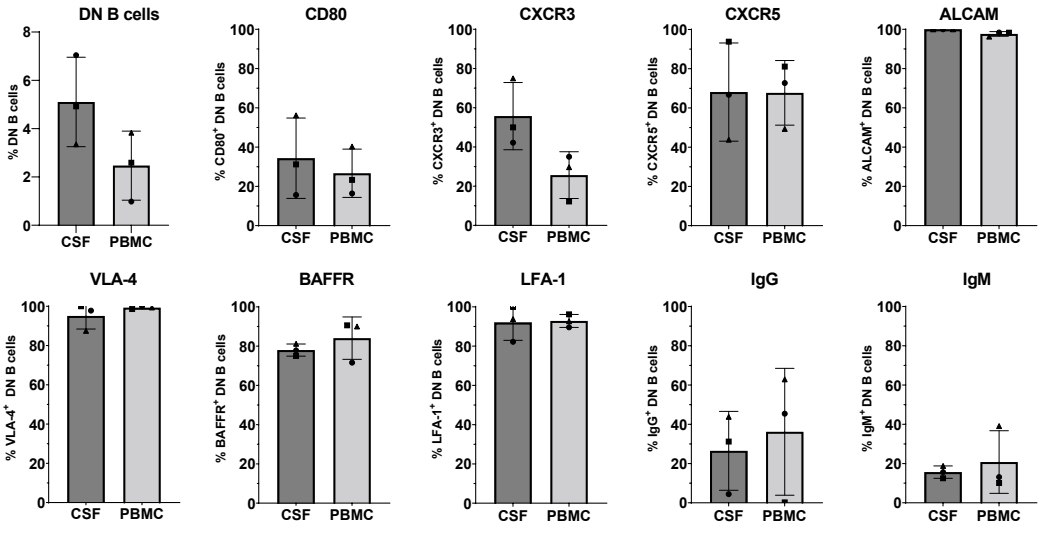

Figure 1. Phenotype of DN B cells in the peripheral blood and CSF of MS patients. Percentages of DN B cells in CSF and peripheral blood (PBMC) of MS patients (n = 3). Percentages of $CD80^+$, $CXCR3^+$, $CXCR5^+$, $ALCAM^+$, $VLA\text{-}4^+$, $BAFFR^+$, $LFA\text{-}1^+$, IgG^+ and IgM^+ cells within the DN B cell subset in peripheral blood and CSF are shown. Mean ± SD is depicted.

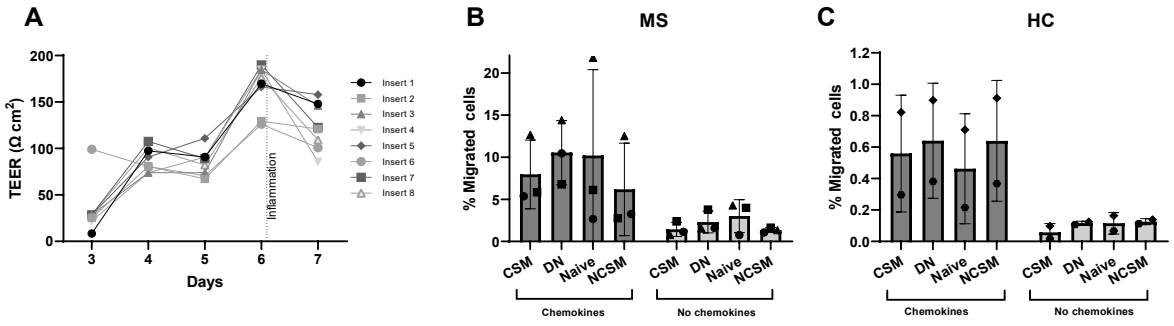

Figure 2. B cells have an increased migration capacity in the presence of CXCL10 and CXCL13 chemokines. **(A)** Representative TEER measurements of 8 transmembrane inserts from days 3 to 7 of experiment. **(B-C)** Percentage of migrated naïve, DN, NCSM and CSM B cells towards CXCL10 and CXCL13 chemokines compared with no chemokines present for MS patients (n = 3) and HC (n = 2). Mean + SD depicted. Percentage of migrated cells was defined as the difference between the number of cells in the bottom and top chambers divided by the number of cells of each subset placed in the top chamber on day 7 expressed as a percentage.

the BBB alters their phenotype or once in the CSF CXCL10 is present in higher amount. However, generally a higher patient number would be required to better understand how other marker might differ.

Optimisation of FBS concentration for transmigration assay – hCMEC/d3 cells were consistently grown on inserts containing 3 µm pore membranes for 7 days. Based on literature, transmigration assays are usually performed under reduced FBS conditions (0.25%) (48,49). However, such low concentrations of FBS are not optimal for B cell survival, as seen by the low percentage of living cells (Suppl. Fig. 4) as well as absolute cell count following a transmigration assay. Absolute B cell count was on average 416,380 and 10,220 cells with 0.25% FBS, and 618,233 and 24,923 cells with 2.5% FBS in the insert and bottom chambers, respectively. Absolute cell count was defined as the mean number of cells counted (in duplicates) multiplied by the percentage of B cells based on flow cytometry in each chamber. Given the 1.5-fold and 2.4-fold increase in absolute cell count, 2.5% FBS was used in the transmigration assay. Moreover, changes of transendothelial electrical resistance (TEER) were recorded as these are directly related to measures for paracellular tightness (50) and therefore allow for consistent monitoring of endothelial growth (Fig. 2A). This further allowed for choosing inserts with similar endothelial growth for pairing of the experimental duplicates.

DN B cells of MS patients show higher migration capacity than HC – DN B cells of MS patients were able to migrate *in vitro* through the BBB model based on their migrated cell percentage (10.5%) (Fig. 2B). Naïve, CSM and NCSM B cells also had similar levels of migration as DN cells. B cells of MS patients also showed a higher migration capacity when compared to B cells of healthy donors, of which no more than 1% or 0.2% was able to migrate with or without chemokines, respectively (Fig. 2B-C). All subsets also migrated better when CXCL10 and CXCL13 chemokines were present at the bottom chamber, therefore we continued with the addition of the chemokines for our following experiments.

Migration of B cells through an in vitro BBB model leads to increased activation potential – Next, we compared the phenotype of B cells that had migrated versus those that had not migrated within the different B cell subsets. We observed higher levels of $CD86^+$ cells, in all subsets, after they migrated through the endothelial layer in MS patients, while in HC there was almost no difference. An increased frequency of $CD86^+$ cells after migration was also visible when comparing each subset between MS patients and HC (Fig. 4A). A similar but less prominent trend can also be seen for $CXCR3^+$ B cells, primarily in the CSM and DN B cell subsets of MS patients, although arguably present due to the hight interdonor variability (Fig 3B). $CXCR5^+$ B cells remained mostly unaffected

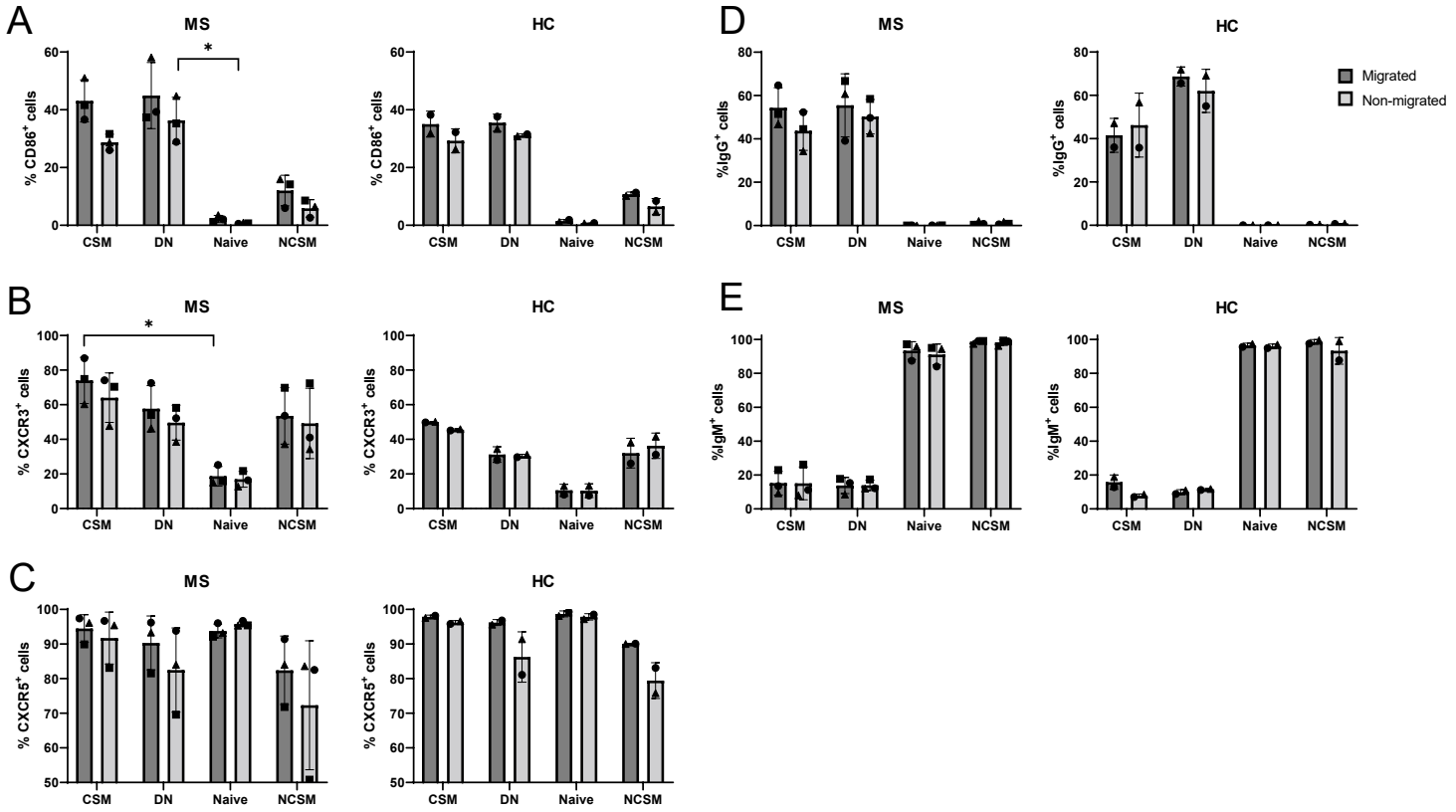

Figure 3. Phenotype of migrated and non-migrated B cell subsets after in the transmigration assay. **(A-E)** Percentages of $CD86^+$, $CXCR3^+$, $CXCR5^+$, IgG^+ and IgM^+ cells within the CSM, DN, naïve and NCSM B cell subsets for MS patients (n = 3) and HC (n = 2). Mean ± SD is depicted. *$p < 0.05$

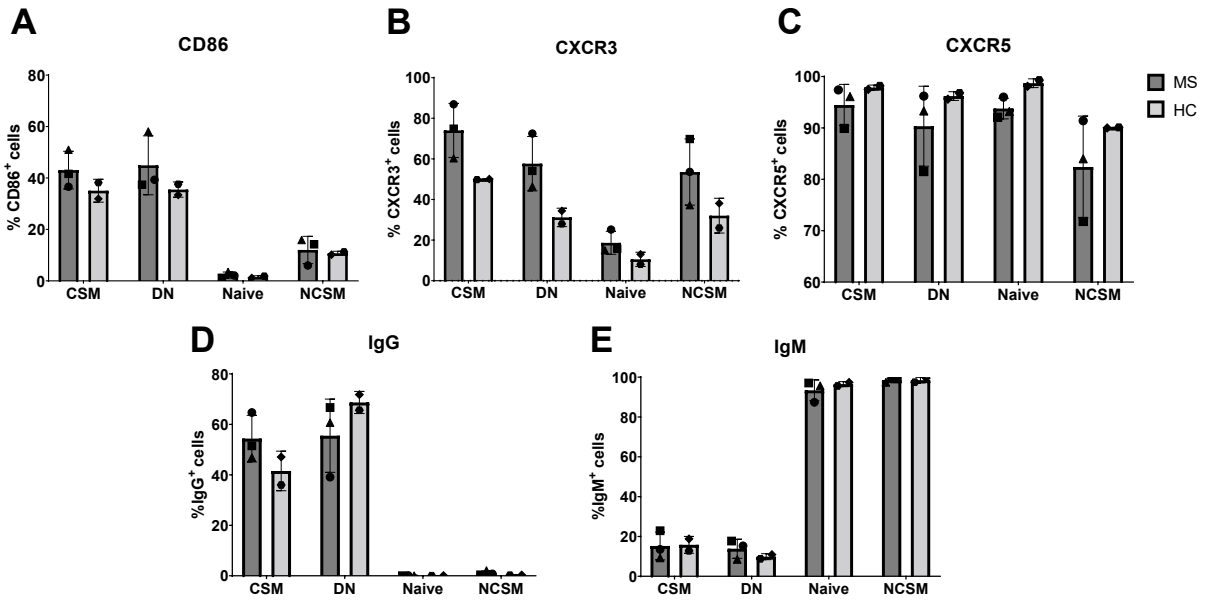

Figure 4. Phenotype of migrated B cell subsets after 24 h incubation in an *in vitro* BBB model. **(A-E)** Percentages of $CD86^+$, $CXCR3^+$, $CXCR5^+$, IgG^+ and IgM^+ cells within the CSM, DN, naïve and NCSM B cell subsets for MS patients (n = 3) and HC (n = 2). Mean ± SD is depicted. *$p < 0.05$

with only one of the three MS patients showing an increase after having migrated (Fig. 3C). Interestingly, neither $CXCR3^+$ nor $CXCR5^+$ B cell subsets had any change for HC (Fig. 3B-C). The frequency of IgG^+ and IgM^+ B cells was overall mostly unaffected with some interdonor differences within the IgG^+ CSM and DN B cell subsets (Fig. 3D-E). When comparing B cell subsets of MS patients and HC, $CXCR3^+$ cells of the former were overall increased (Fig. 4B). No difference was observed for $CD86^+$ B cells with the exception of one MS patient showing higher activation of CSM and DN B cells (Fig 4A). No increased frequencies were noted for $CXCR5^+$ migrated B cells for HC and MS patients, however one of the three MS patients generally showed lower levels affecting the mean frequency (Fig. 4C). Similar levels of IgM^+ B cells were observed for both MS patients and HC (Fig. 4E). Together, these data demonstrate that the migration of DN B cells of MS patients through an endothelial layer resembling an *in vitro* BBB model results in their further activation, while increasing their CXCR3 expression. DN B cells also seem to resemble CSM B cells in regard to their activation and CXCR3 expression.

activation markers CD80 and CD86 (Fig. 5). DN B cells alone also showed higher CD80 and CD86 expression after 3 days, but when stimulated there was an almost 2-fold and 5-fold increase in CD80 and CD86, respectively. However, only CpG + IL-2 was able to simultaneously increase DN B cell proliferation. CD40L + IL-2 attributed no more proliferation than unstimulated cells showed after 3 days, based on ΔPF. In addition, when comparing the effect of each stimulation between the different B cell subsets, CD40L + IL-4 was able to mainly activate DN B cells of MS patients and HC based on CD80 expression. To a lesser extend CSM and naïve B cells of MS patients were also activated (Fig. 6A). CpG + IL-2 activated all B cell subsets apart from CSM and NCSM of HC, which showed no changes in CD80 expression. CD86 expression was increased in DN, naïve and CSM B cells in both stimulations for MS patients and HC. Naïve B cells, in particular, exhibited higher CD86 expression in HC than in MS patients in either stimulation (Fig. 6A). Either stimulation also increased $CXCR3^+$ DN and naïve B cells of MS patients, albeit a higher interdonor variability. Of note, $CXCR3^+$ DN and naïve B cells of HC decreased after stimulation with CD40L + IL-4.

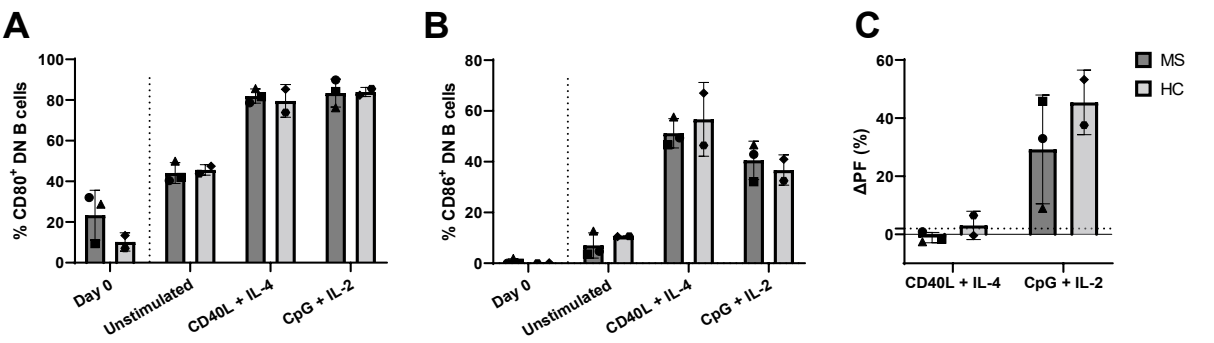

Figure 5. CD40L + IL2 and CpG + IL-2 stimulations are capable of activating DN B cells of both MS patients (n = 3) and HC (n = 2) while only the latter increases their proliferation capacity. **(A-B)** Percentages of $CD80^+$ and $CD86^+$ DN B cells on day 0 and after 3 days of either unstimulated or stimulated conditions. **(C)** Proliferation capacity of DN B cells of MS patients and HC after 3 days of stimulation. Proliferation fraction (ΔPF) defined as the difference between proliferation due to stimulation and background unstimulated cells. Mean ± SD is depicted.

DN B cells are activated following in vitro stimulation – To assess if DN B cells have the potential to be activated, we tested two stimulations on B cells from MS patients (n = 3) and HC (n = 2). After 3 days, both CpG + IL-2 and CD40L + IL-4 were able to activate DN B cells in similar levels for both MS patients and HC, based on the Lastly, the aforementioned stimulation barely offered any proliferation (< 6.2% average), whereas CpG + IL-2 had a better outcome (>12% average) (Fig. 6B).

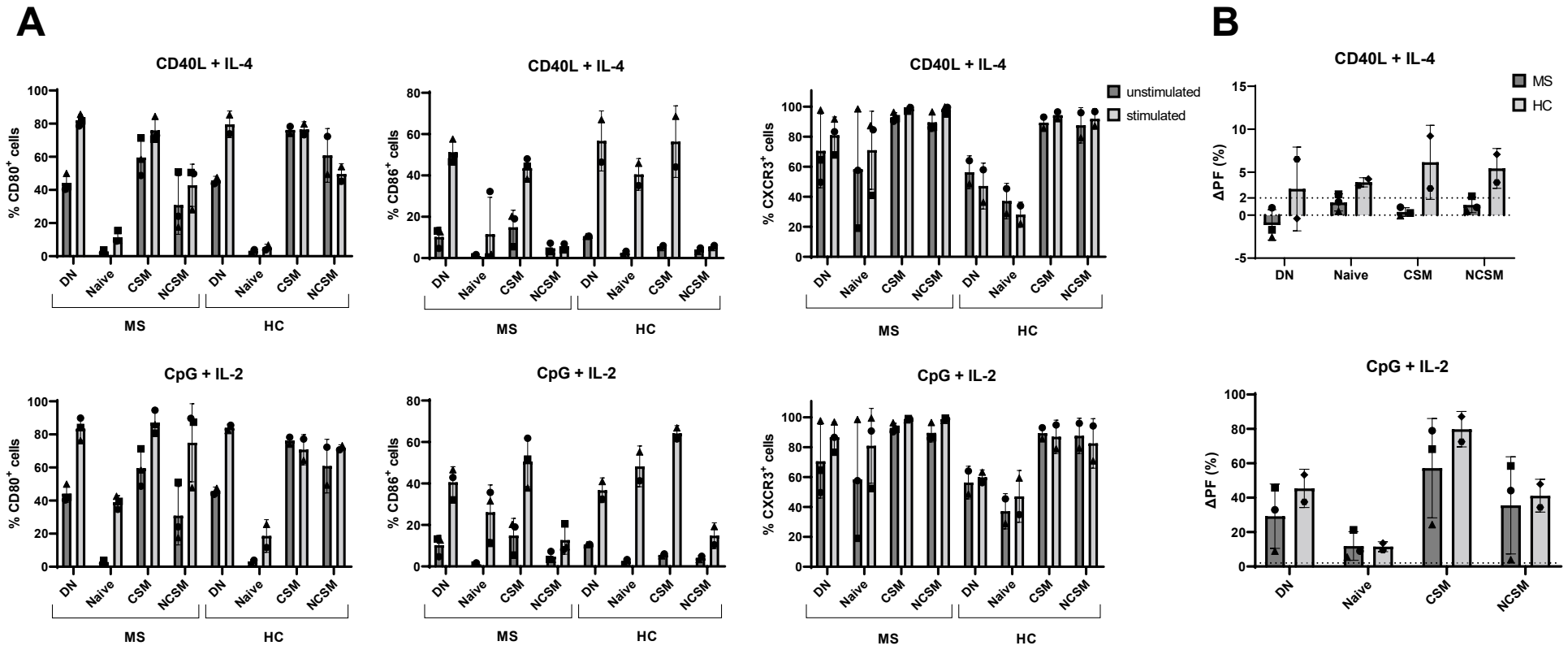

Figure 6. CD40L + IL4 and CpG + IL-2 capable of activating B cells of MS patients and HC **(A)** Percent $CD80^+$, $CD86^+$, $CXCR3^+$ DN, naïve, CSM and NCSM B cell subsets of MS patients (n = 3) and HC (n = 2) compared to unstimulated levels for each stimulation. **(B)** Proliferation capacity for MS patients and HC for each stimulation. Proliferation fraction (ΔPF) defined as the difference between proliferation due to stimulation and background unstimulated cells. Dotted lined represents cut-off for positive proliferation (ΔPF>2). Mean ± SD.

Phenotype of proliferated DN B cells differs from that of non-proliferated DN B cells – Proliferated DN B cells exhibited a higher activation state based on CD86 expression for MS patients and HC, whereas $CD80^+$ DN B cells had no major differences (Fig. 7). A slight increase in $CXCR3^+$ DN B cells was also seen in both cohorts compared to non-proliferated cells. IgG^+ DN B cells generally decreased when proliferating, whereas IgM^+ cells increased, especially when stimulated with CpG + IL-2. IgM^+ DN B cells had a similar trend for both MS patients and HC, whereas IgG^+ cells had a higher frequency in MS patients. IgA^+B cells had a slight overall increase in frequency. (Fig. 7).

Phenotype of T-bet$^+$ DN B cells after stimulation – Lastly, B cells stimulated with CpG + IL-21 + IFN-γ, a T-bet inducing stimulation, showed consistent increase of T-bet expression within DN, naïve, CSM and NCSM B cells (on average 46.4, 74.6, 75.5 and 80.3 %, respectively) of MS patients compared to unstimulated cells (<24.3% overall) (Suppl. Fig. 5). Similar, but slightly decreased, T-bet expression was also seen with the HC (Suppl. Fig. 5). Then, looking at differences between T-bet$^+$ and T-bet$^-$ DN B cells specifically after stimulation, the former had higher $CD80^+$ frequencies in MS patients (Fig. 8). But $CD86^+$ frequencies remained unchanged for both groups. CXCR3 expression increased for MS patients and HC in T-bet$^+$ DN B cells. IgG was increased 3-fold for T-bet$^+$ cells of MS patients, while hardly altered in HC. In contrast, IgM increased in HC and remained unaltered in T-bet$^+$ cells of MS patients. Apart from one MS patient, IgA remained at the same levels for either T-bet$^+$ or T-bet$^-$ DN B cells. Similarly, there was marginally no difference in proliferation of the two groups (Fig. 8).

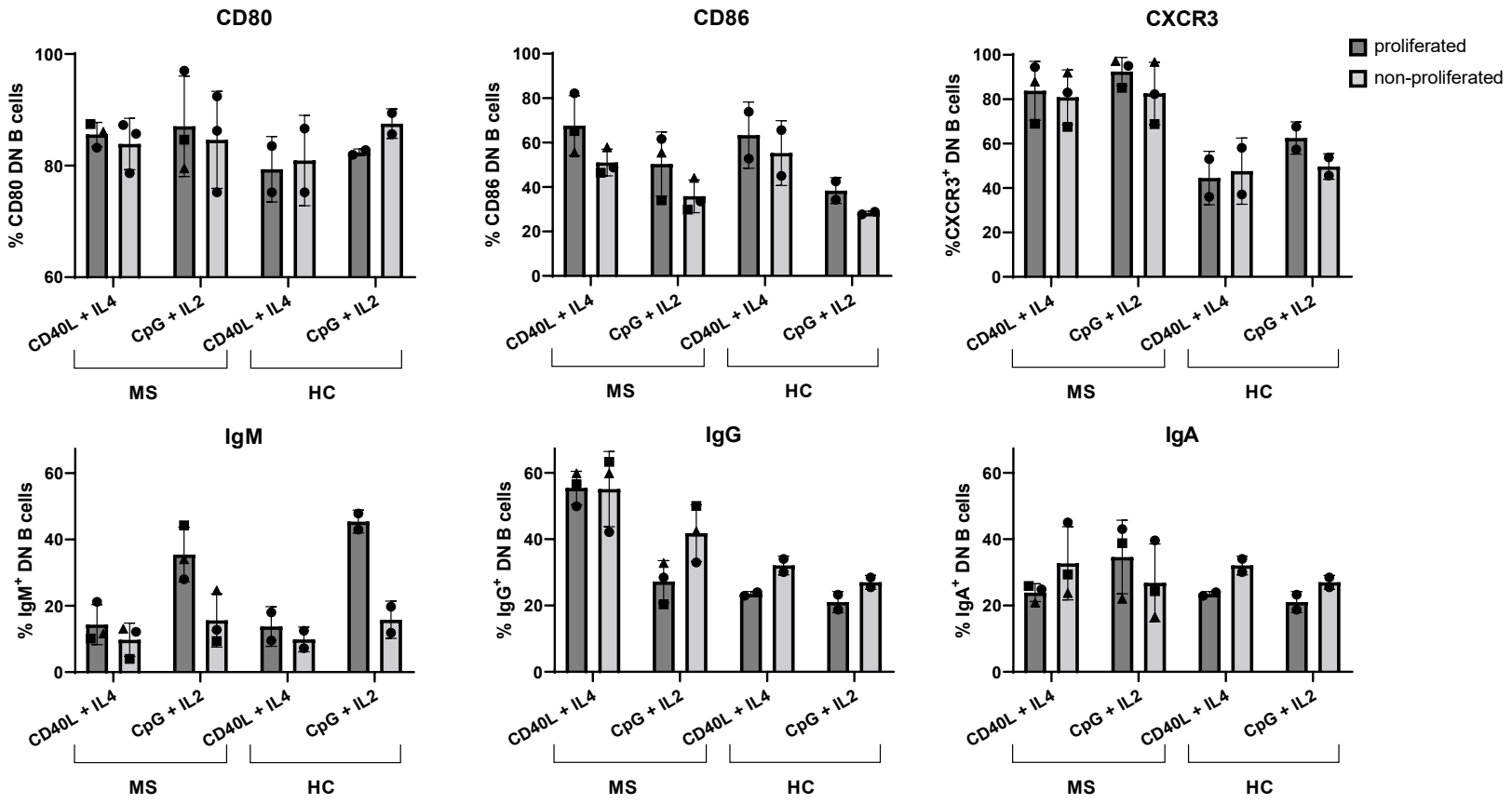

Figure 7 – Phenotype of proliferated and non-proliferated DN B cells based on expression of CD80, CD86, CXCR3, IgA, IgG and IgM for MS patients and HC. Mean ± SD depicted.

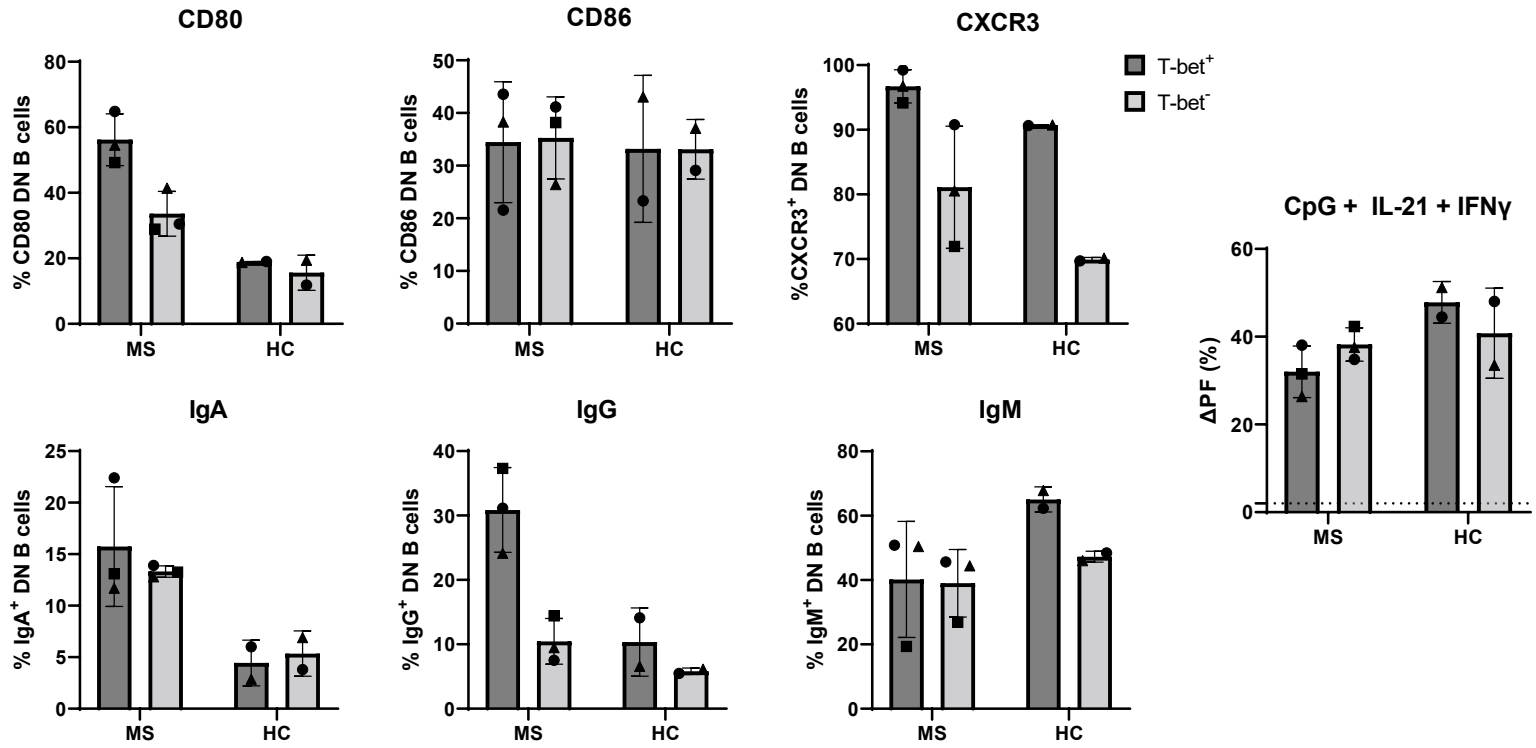

Figure 8. Phenotype of T-bet$^+$ and T-bet$^-$ DN B cells of MS patients (n = 3) and HC (n = 2) after stimulation with CpG + IL-21 + IFN-γ based on frequency of CD80$^+$, CD86$^+$, CXCR3$^+$, IgA$^+$, IgG$^+$ and IgM$^+$ DN B cells. Proliferation fraction (ΔPF) defined as the difference between proliferation due to stimulation and background unstimulated cells. Dotted lined represents cut-off for positive proliferation (ΔPF>2). Mean ± SD depicted.

DISCUSSION

B cell have become increasingly more studied in the last few years as more and more studies showcase their potential environment in MS pathogenesis. Yet, no clear conclusion has been made about the exact role B cells play, as well as the role of DN B cells in particular. They are linked to immune aging in HC, which has been shown to an extend in some MS patients, although is considered premature given the age range MS is developed (51). However, different studies have link DN B cells to viral infections or other autoimmune disease, suggesting DN B cells do not all have the same characteristics in all individuals.

In this study, we observed higher frequencies of DN B cells in CSF of MS patients compared to the peripheral blood, which was in line with previous published data (32,37), further corroborating their potential involvement in MS pathology. The migrated DN B cells in the CSF also showed increased CXCR3 expression. In *in vitro* migration experiments resembling the BBB, DN B cells exhibited higher migration capacity for MS patients compared to HC, with even higher migration when CXCL10 and CXCL13 where present. Migration through the endothelial layers also lead to DN B cells having a more activated state while increasing their CXCR3 expression. DN B cells resembled more with CSM B cells in regards to their activation and CXCR3 expression. Migrated cells also appeared more activated based on CD86 expression compared to the non-migrated cells, indicating that their contact with the BBB model played an important role. This could be attributed to the inflammatory markers released by the endothelial layers that B cells came in contact due to their inflammation stage. B cells are directed into the CNS by an array of chemokines signalling (52), and CXCL10 and CXCL13 have already been studied in relation to MS, as they are known for their role in attracting T and B cells in sites of inflammation. CXCL13 has also specifically been found elevated in the CSF of RRMS and SPMS patients (53). It was therefore expected that the presence of these chemokines would increase the migration capacity of B cells. Interestingly however, the percentage of migrated B cell subsets was several times higher for MS patients than HC clearly showing that B cells of MS patients differ from HC cells. Phenotypically, migrated DN B cells resembled CSM B cells regarding their CD86 expression that was increased for MS patients, while remaining unaffected for HC. Although CXCR3 expression was also increased for migrated B cell subsets of MS patients, it is of note that the frequency of $CXCR3^+$ B cells in general was higher when compared against HC (Fig 4A-B).

Given the higher CD86 levels of migrated B cells, we sought to examine the ability of B cell subsets to be activated and whether they differed between MS patients and HC. As seen in literature already (54), B cells from MS patients and HC were stimulated to mimic either a T-dependent (CD40L + IL-4) or a T-independent (CpG + IL-2) B cell activation. Based on expression of CD80 and CD86 activation markers, both stimulations could activate DN B cells, however only CpG + IL-2 could in parallel lead to proliferation of DN B cells (Fig. 5).

T-dependent signals activated overall DN, naïve and CSM subsets both for MS patients and HC with barely any proliferation. T-independent signals however activated all B cell subsets of MS patients and only DN and naïve B cell subsets of HC. Interestingly for either stimulation, naïve B cells exhibited higher expression of $CD86^+$ for HC than MS patients (Fig. 6). The proliferation levels of CpG + IL-2 also were observed as increased compared to CD40L + IL-4.

In conclusion, we were able to show that DN B cells of MS patients have a higher migration capacity than HC, while having an increased activation state once migrated. In addition, DN B cells can be activated by different stimulations, but not all infer the same level of activation and proliferation to the cells. Unfortunately, the sample size of each assay performed was limiting in order to be able to make strong conclusions based on significance of statistical analyses, however these preliminary data are still valuable as a steppingstone to further continue investigating DN B cells and increase the sample pool appropriately. Due to the limited samples available, several MS patients analysed were also under treatment rather than untreated, however they were only being treated with first line therapies, which previous data of our group has showed not to affect DN B cell percentages (32).

This study is important as having insight in the triggers that lead to activation of DN B cells, could

offer opportunities for blocking inflammatory chemokines that might be responsible or blocking the pro-inflammatory and/or cytotoxic cytokines they, or other cells, might produce. These cells could also offer diagnostic possibilities if in the future a strong correlation is found between certain phenotypic characteristics that DN B cells exhibit and disease severity. At the moment, it still remains unclear what the exact role of DN B cells is in relation to MS pathology, but the further characterisation of the subset is a step towards a better understanding

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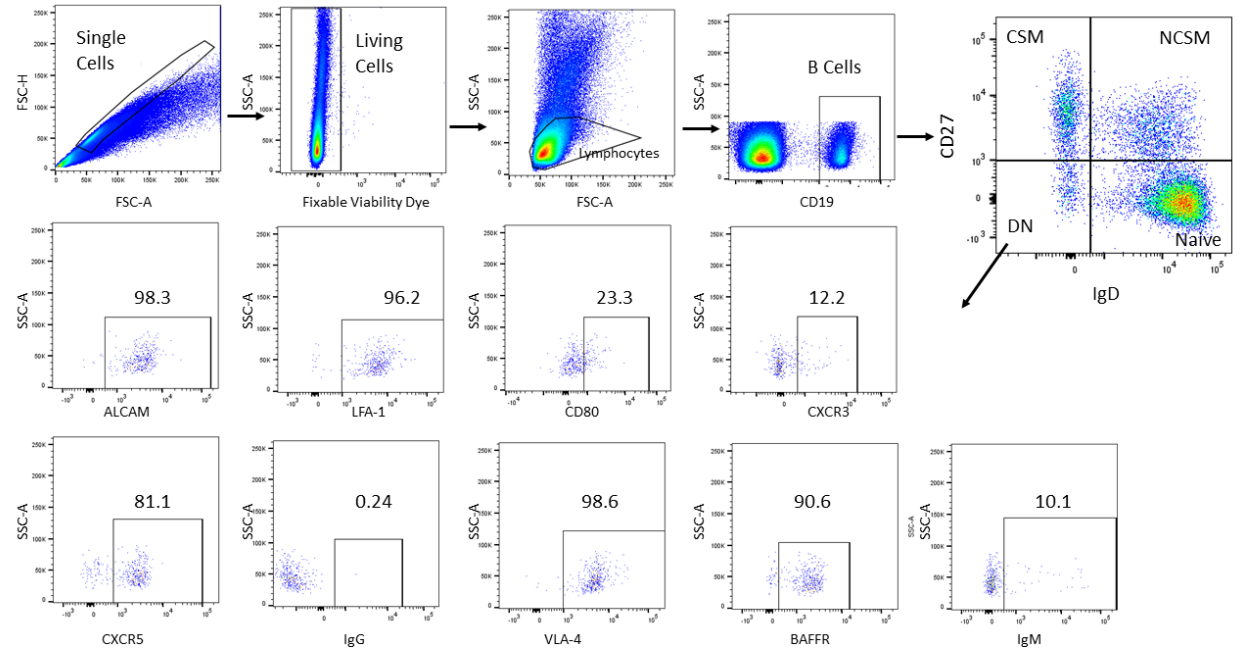
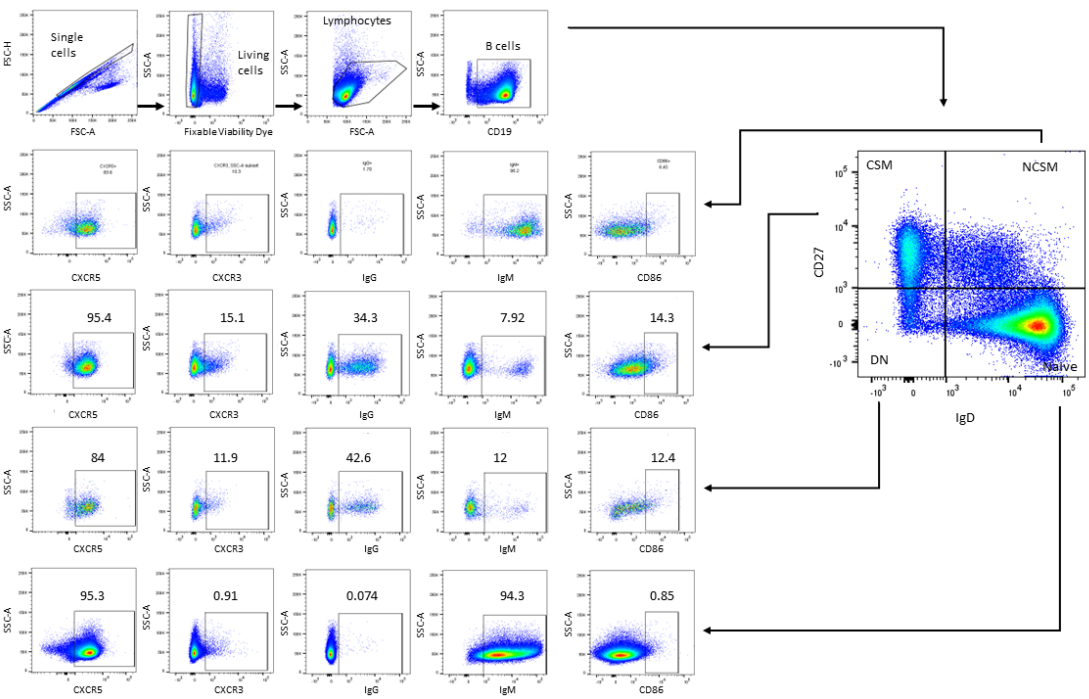
Acknowledgements – I would like to thank dr. Fraussen for giving me the opportunity to be part of Prof. Somer's group during my senior internship, which allowed me to really expand my knowledge on the topic of multiple sclerosis and my research experience as a whole. I'm also very thankful for dr, Fraussen's valuable input during the writing of this manuscript. I would also like to separately thank my daily supervisor Lien Beckers for her daily input, help and understanding throughout the entirety of my internship as well as for her help all the nights we had to spend doing experiments.

Author contributions – VS, FJ and BL conceived and designed the research. BA performed experiments. BA and BL performed data analysis. BA wrote the paper and FJ edited the manuscript.

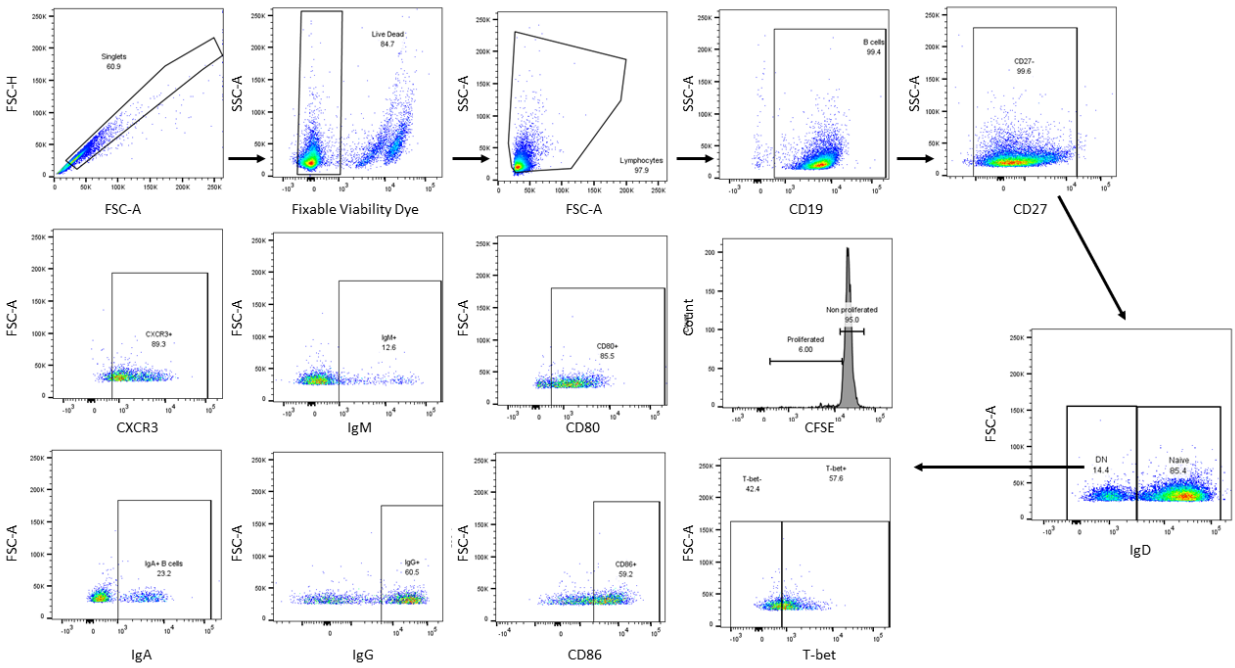
Supplementary Material



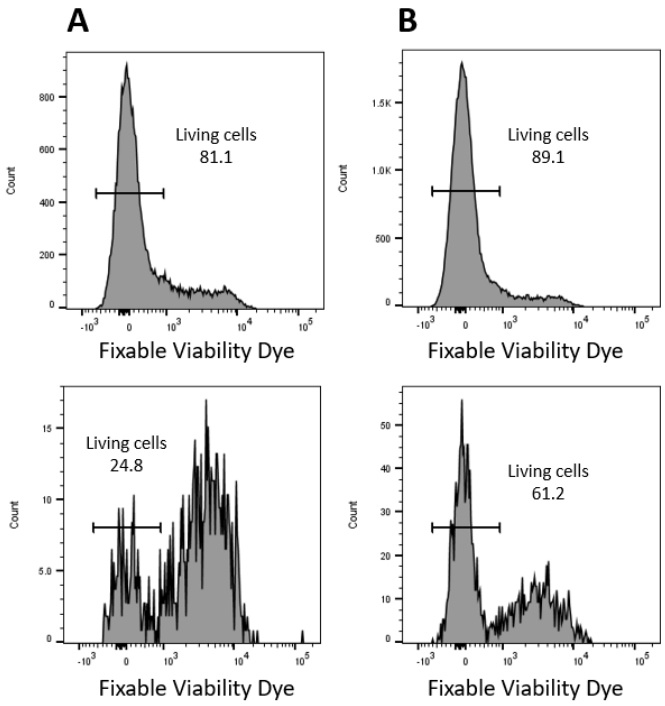
Supplementary Figure 1. Gating strategy of CSF-PBMC paired samples



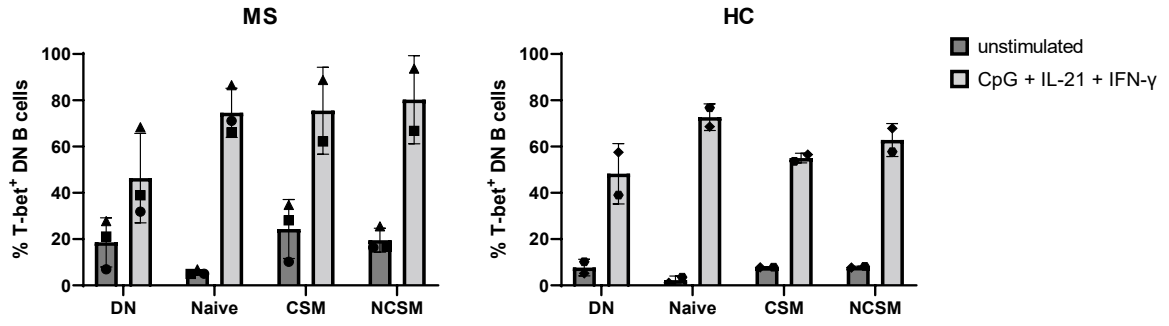
Supplementary Figure 2. Gating strategy of transmigration assay



Supplementary Figure 3. Gating strategy of activation assay



Supplementary Figure 4. Incubation with reduced FBS in the insert lowers B cell survival in the transmigration assay. **(A)** Percentage of living cells in top chamber (upper graph) and bottom chamber (lower graph) after 24 h in medium containing 0.25% FBS **(B)** Percent of living cells in top chamber (upper graph) and bottom chamber (lower graph) after 24 h in medium containing 2.5% FBS.



Supplementary Figure 5. CpG + IL-21 + IFN-γ stimulation is able to stimulate T-bet$^+$ DN, naïve, CSM and NCSM B cell subsets of MS patients compared to unstimulated cells. Mean ± SD