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Maastricht University

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

Masterthesis

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

Lien Beckers

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :
Prof. dr. Veerle SOMERS

CO-SUPERVISOR :
dr. Judith FRAUSSEN

MENTOR :
Mevrouw Gwendoline MONTES DIAZ

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Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

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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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Preface

This senior internship offered by Hasselt University in collaboration with Maastricht University contributes to the last part of the master Clinical Molecular Sciences. Being 30 weeks, it gave me ample opportunity to gain practical experience on working in a professional lab environment, developing experimental setups and protocols, planning experiments, interpreting data, communicating results and problem-solving thinking.

I am very grateful for all the skills that I have learned, since they helped me in becoming a critical biomedical scientist. Therefore, I would like to thank my supervisor Prof. dr. Veerle Somers and her research group at the Biomedical Research Institute (BIOMED) of Hasselt University. After I did my junior internship there, they welcomed me back with open arms for this senior internship. In particular, I would like to thank my cosupervisor dr. Judith Fraussen and my daily supervisor drs. Gwendoline Montes Diaz for their support, patience, and trust in me. I could always contact them with all my questions. Thank you for everything, Judith and Gwendoline! Furthermore, I would like to thank the other members of the research group, Dana and Ruth, for staying later in the lab, allowing me to finish my experiments when my supervisors were absent. I would also like to thank Kim Ulenaers for providing us the blood samples and clinical characteristics of the patients needed for our research. More specifically, I would like to thank Prof. dr. Somers for the opportunities she gave me, such as applying for a doctoral grant.

Next, I would also want to thank my fellow students Elissia and Inge with whom I experienced leisurely times in the student room at BIOMED. They were always ready for a small talk and together we helped each other if required. I wish you both good luck in, this often being touched upon, finding a nice job.

In addition, I would like to thank my parents and brother for all their support. Although I often had long days in the lab, they were always ready to listen to my stories. Thanks to my parents, I have reached the place I am today! Finally, I would also like to thank Jente. He was always there for me, not only by listening to me, but also by proofreading my thesis.

I am glad that I have had the opportunity to perform this educational internship, of which I can now share the results with you.

Lien Beckers

Table of contents

List of abbreviations

ABC: age-associated B cell
ALCAM: activated leukocyte cell adhesion molecule
BCR: B cell receptor
CI: chemotactic index
CIS: clinically isolated syndrome
CNS: central nervous system
CSF: cerebrospinal fluid
CSM: class-switched memory
DC: dendritic cells
DN: double negative
EAE: experimental autoimmune encephalomyelitis
EBV: Epstein-Barr virus
HC: healthy control
HLA: human leukocyte antigen
Ig: immunoglobulin
ICAM-1: intracellular adhesion molecule 1
IL: interleukin
LFA-1: lymphocyte function-associated antigen 1
MBP: myelin basic protein
MFI: mean fluorescence intensity
MOG: myelin oligodendrocyte glycoprotein
MS: multiple sclerosis
NCSM: non class-switched memory
PBMC: peripheral blood mononuclear cell
PLP: proteolipid protein
PPMS: primary progressive multiple sclerosis
RA: rheumatoid arthritis
RRMS: relapsing-remitting multiple sclerosis
SPMS: secondary progressive multiple sclerosis
SLE: systemic lupus erythematosus
TLR: toll-like receptor
VLA-4: very late antigen 4

Abstract

Key words: Multiple sclerosis, $IgD^{-}CD27^{-}$ double negative B cells, T-bet, autoreactivity, migration capacity

Introduction: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), characterized by inflammation and demyelination. B cells are central players in MS pathogenesis. Recently, our research group showed an increased population of $IgD^{-}CD27^{-}$ double negative (DN) B cells with pro-inflammatory characteristics in about 20% of MS patients. However, their exact function in MS remains unknown. Additionally, novel findings in different mouse models suggested a role for T-bet^{+} B cells, with similar characteristics as DN B cells, in autoimmune responses. Therefore, we hypothesized that DN B cells contribute to neuroinflammation in MS patients through autoreactive and pro-inflammatory functions regulated via T-bet expression.

Material and methods: Using flow cytometry, the expression of the transcription factor T-bet as well as the chemokine receptors CXCR3 and CXCR5 were investigated in DN B cells from untreated relapsing-remitting (RR) MS patients and secondary progressive (SP) MS patients. The migration capacity of DN, naive and memory B cells from untreated RRMS patients towards the pro-inflammatory chemokines CXCL13 and CXCL10 was analyzed using an *in vitro* chemotaxis assay. The chemotactic index (CI) for each B cell subset was calculated as follows: (number of migrated subtype cells with chemokine – number of migrated subtype cells without chemokine) / (original number of subtype cells in upper chamber). To examine the activation status of DN B cells after CpG2006 stimulation, an *in vitro* B cell subtype proliferation assay was performed to analyze the proliferation index. Thereafter, flow cytometry was performed to assess the activation markers CD80 and CD25.

Results: T-bet^{+} DN B cells can be found in untreated RRMS patients, SPMS patients and HC, with a statistical significant difference between the different study groups. Moreover, expression of T-bet (mean fluorescence intensity; MFI) in DN B cells is the highest compared to naive, NCSM and CSM B cells. Furthermore, DN B cells of untreated RRMS patients can migrate towards CXCL13 and CXCL10 *in vitro* and show a higher migration capacity for CXCL13 compared to naive and NCSM B cells. The percentage of $CD80^{+}$ and $CD25^{+}$ DN B cells was strongly increased following CpG2006 stimulation compared to the memory B cell populations. Stimulating DN B cells with CpG2006 resulted in the highest CD80 and CD25 expression (MFI), in comparison with naive, NCSM and CSM B cells.

Discussion and conclusions: We demonstrated that DN B cells of untreated RRMS patients express T-bet, indicating their possible involvement in autoimmune disease. Additionally, the migration of DN B cells towards chemokines involved in B cell recruitment into the CNS of MS patients further suggests that DN B cells contribute to MS pathology. Furthermore, DN B cells were strongly activated following CpG2006 stimulation, indicating that they are no unresponsive, terminally differentiated cells. Future research into DN B cells will result in better insights into MS pathogenesis and discovery of new therapeutic targets.

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease that is characterized by infiltration of autoreactive immune cells in the central nervous system (CNS), causing demyelination and axonal degeneration. Destruction of the myelin sheet surrounding axons will result in an impaired nerve conduction and consequently neurological disability (1). MS affects 2.3 million people worldwide and 12,000 people in Belgium. In 2013, MS had a global prevalence of 33 cases per 100,000 people. However, the prevalence varies among different countries, with the highest in North America and Europe (2). Women are more frequently affected than men, with an average female-to-male ratio of 2:1. The majority of MS patients show a peak age of disease onset between 25 and 35 years (2, 3).

1.1.1 Clinical aspects

Different clinically-defined subtypes of MS are described. In 85% of MS patients, disease emerges as a clinically isolated syndrome (CIS), characterized by one white matter lesion that may cause an acute or subacute episode of neurological disturbance. Patients with CIS have not met the criteria of MS. However, 30-70% of CIS patients will develop the disease after several years (4). In the majority of patients (85-90%), MS manifests as relapsing-remitting (RR) MS, in which periods of clinical relapses are followed by remission. Over time, the recovery from each relapse is decreased, resulting in the development of secondary progressive (SP) MS in 60-65% of RRMS patients. Primary progressive (PP) MS is diagnosed in 10-20% of MS patients that show progressive disability from disease onset without relapsing periods, causing irreversible damage (1).

The clinical symptoms that MS patients experience depend on the size and location of the CNS lesions and can include impairment of visual, motor and sensory functions. Although symptom presentation varies in patients, some symptoms are more commonly observed such as fatigue, chronic pain and muscle weakness (5). The diagnosis of MS is based on these clinical manifestations as well as neurological examinations. Lesions and abnormalities in the white matter can be visualized by magnetic resonance imaging. Furthermore, cerebrospinal fluid (CSF) analysis shows elevated oligoclonal immunoglobulin G (IgG) bands in more than 90% of MS patients (6). Additionally, recording evoked potentials in afferent and efferent pathways of the CNS allows to evaluate demyelination and axonal loss (7). According to the 2017 revisions of the McDonald criteria, clinically definite MS is diagnosed in patients that have 2 or more clinical attacks, together with dissemination in time and space of CNS lesions. Due to the revisions, the presence of oligoclonal bands could confirm the diagnosis of MS in CIS patients demonstrating dissemination in space only. Furthermore, a pivotal role for MRI was shown, as symptomatic and asymptomatic MRI lesions could be used to indicate dissemination in space or time (8).

1.1.2 Etiology

Although the exact cause of MS is still unknown, it is assumed that both genetic and environmental factors contribute to the development of the disease. About 20% of MS patients have at least one affected relative (9). Genome-wide association studies have determined more than 200 risk genes for the development of MS. One of the most important alleles with the strongest association with MS is the human leukocyte antigen (HLA) class II haplotype HLA-DRB 1501 gene. The HLA class II genes code for the HLA class II molecules that are present on antigen presenting cells and are involved in T cell antigen presentation (10). In addition, genes encoding interleukin (IL)-7 and IL-2 receptors as well as genes associated with costimulatory molecules (e.g. CD80, CD86) are identified as MS susceptibility genes (11, 12). However, the low concordance in monozygotic twins demonstrates that genetic susceptibility alone is not sufficient to develop MS.

Major environmental risk factors include vitamin D deficiency, since a higher level of 25-hydroxyvitamin D is related to a decreased risk of developing MS (13). Several studies have also suggested an association between viral infections and MS. Almost all MS patients are seropositive for the Epstein-Barr virus (EBV), in contrast to healthy controls (HC), whereby high titers of anti-EBV antibodies are associated with an increased susceptibility to MS (14, 15). Moreover, a positive correlation has been demonstrated between cigarette smoking and MS (16).

1.1.3 Immunopathogenesis

The role of autoimmunity in MS pathogenesis is supported by the experimental autoimmune encephalomyelitis (EAE) animal model of MS. EAE can be induced in rodents and rhesus monkeys by immunizing them with myelin antigens of the CNS (e.g. myelin oligodendrocyte glycoprotein (MOG)) in complete Freund's adjuvant or by an adoptive transfer of activated myelin-specific $CD4^+$ T cells. Although Th1 and Th17 seem to be the most pathogenic cell types in EAE, a role for B cells is also described. Immunization of mice with recombinant MOG protein results in the production of MOG-specific antibodies that exacerbate the disease (17).

Since MS is considered to be a complex heterogeneous disease, there is debate about whether the disease is triggered in the periphery (extrinsic) or in the CNS (intrinsic). In both hypotheses, autoreactive T cells that have escaped the tolerance checkpoints migrate through the disrupted blood-brain barrier into the CNS, where they are reactivated by myelin antigens, such as MOG, myelin basic protein (MBP) and proteolipid protein (PLP). As a result, reactivated T cells start to secrete pro-inflammatory cytokines and to activate macrophages and microglia, leading to further influx of immune cells and eventually demyelination (18). In the extrinsic hypothesis, MS pathogenesis starts when autoreactive $CD4^+$ T cells leave the thymus and enter the periphery. This is possible due to the disrupted central tolerance checkpoint in the thymus and peripheral tolerance checkpoint that are observed in MS patients. This allows the cells to bypass these checkpoints, which promotes their survival. In order to be activated, T lymphocytes interact with activated innate immune cells, such as dendritic cells (DCs), in the lymph nodes. Autoreactive $CD4^+$ T cells are activated most likely via molecular mimicry, a process that is characterized by the presence of pathogen-derived epitopes that are cross-reactive with endogenous proteins.

Alternatively, chronic viral infections can stimulate autoreactive lymphocytes by a mechanism termed bystander activation (Fig. 1). During this process, chronic infection will result in tissue damage that releases self-antigens that will trigger autoreactive T cells (19). Additionally, DCs secrete cytokines which will stimulate the differentiation of T cells to a specific effector subtype. The intrinsic hypothesis, on the other hand, suggests that the pathological processes start in the CNS with activation and infiltration of autoreactive lymphocytes as a secondary event (18).

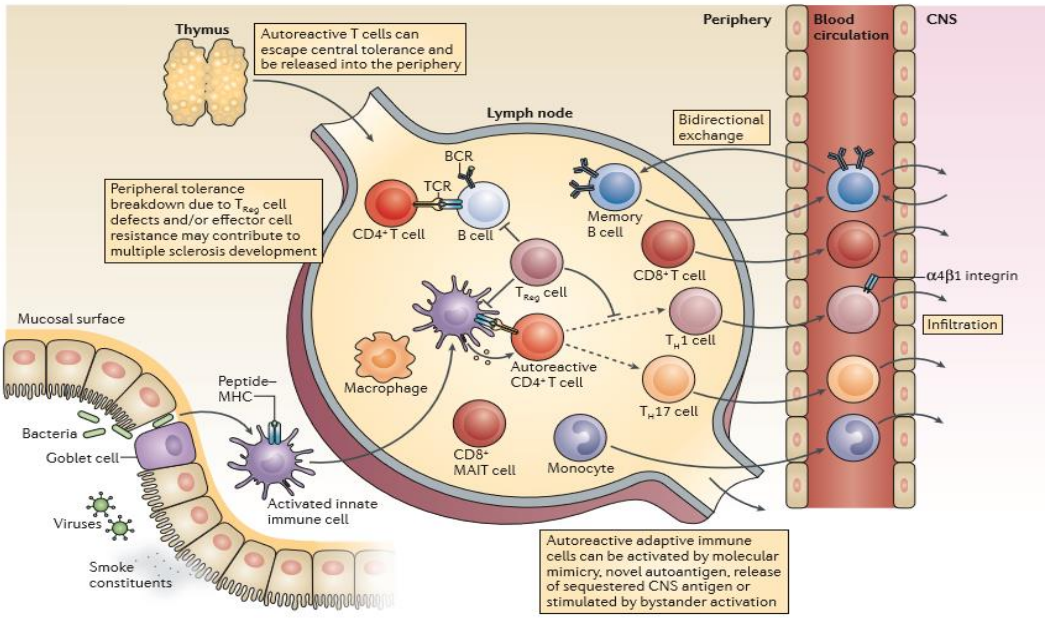


Figure 1. Immunopathogenesis of MS. During T cell development, the central tolerance in the thymus and peripheral tolerance checkpoint regulate the removal of autoreactive T and B cells. However, these checkpoints are defected in MS pathology, which will lead to the survival of these autoreactive cells. After entering the lymph nodes, autoreactive T cells are activated through molecular mimicry or bystander activation and will differentiate into specific effector subtypes. Once activated, T cells, B cells and innate immune cells can infiltrate the CNS. B cells can traffick in a bidirectional way whereby B cells migrating out of the CNS can undergo affinity maturation in the lymph nodes before re-entering the CNS. (Dendrou CA *et al.*, Nat Rev Immunol. 2015;15(9):545-58.)

1.2 B cells as central players in MS pathogenesis

1.2.1 Proof of B cell involvement in MS

B cells are important contributors to the chronic inflammatory processes in MS pathogenesis. The detection of oligoclonal IgG bands in the CSF of MS patients was one of the first indications of B cell involvement in MS and has a diagnostic value, however it is not specific for MS. Oligoclonal IgG bands result from intrathecal plasma cells that produce autoantibodies, although their antigenic targets remain unknown (20, 21). IgG antibodies can cause neuronal degeneration in MS patients by opsonisation of myelin for phagocytosis or by activation of the complement system (22). A critical role for B cells in MS pathology is also defined by their presence at sites of tissue injury in the CNS, where they can form ectopic B cell follicles that are associated with increased meningeal inflammation and related tissue damage (23, 24). Furthermore, clonally expanded B cells are also found in the meninges, parenchyma and CSF of MS patients (25).

Moreover, peripheral B cells show a clonal overlap with B cells present in the CSF, suggesting a bidirectional B cell trafficking that allows B cells to leave the CNS and re-enter the periphery (26). Recent advances in B cell depletion therapy, including the anti-CD20 monoclonal antibodies rituximab and ocrelizumab, in RRMS and even PPMS patients, have indicated an antibody-independent role for B cells. Although antibody-secreting plasma cells do not express CD20 and therefore cannot be targeted by these therapies, clinical trials showed reduced brain lesions and clinical relapses (27-29).These positive outcomes were not the result of decreased antibody titers, but suggested other functions for B cells in MS. B cells can influence antigen-specific T cell responses by antigen presentation, costimulation, and production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), lymphotoxin alpha (LT-α) and interleukin (IL)-6, and the anti-inflammatory cytokines IL-10 and IL-35 (22, 30).

1.2.2 B cell development

Different B cell subsets can contribute to MS pathology. After development in the bone marrow, $CD19^+IgD^+CD27^-CD38^+$ or $CD19^+CD38^{++}CD24^{++}$ transitional B cells are released in the circulation and mature into $CD19^+IgD^+IgM^+CD27^-$ naive B cells. Following antigen encountering in the secondary lymphoid organs, they differentiate into $CD19^+CD138^{++}$ or $CD19^+CD27^+CD38^{++}$ short-lived plasma blasts and $CD38^+CD138^+$ plasma cells, that secrete myelin-specific autoantibodies for a short period. On the other hand, B cells can mature in germinal centers into memory $CD19^+CD27^+$ B cells. These memory B cells can be divided into non class-switched memory (NCSM) B cells that remain IgD^+CD27^+ and class-switched memory (CSM) B cells that will undergo isotype switching leading to the loss of IgD expression (IgD^-CD27^+) and the acquisition of IgG, IgA or IgE expression. However, a small number of memory B cells retain IgM expression and are known as IgM only memory B cells (22, 31). Additionally, a novel population of memory B cells that are characterized by the absence of both IgD and CD27 (IgD^-CD27^- double negative (DN) B cells) was recently described. Within this population, a distinction can also be made between isotype switched DN B cells (IgD^-IgG^+) and non isotype switched DN B cells (IgD^-IgM^+) (32). Memory B cells can also further differentiate into plasma blasts and long-lived plasma cells (22).

1.2.3 B cell migration to the site of inflammation

Several chemokines such as C-X-C motif (CXC) ligand 13 (CXCL13), CXCL10, CXCL12, C-C motif (CC) ligand 20 (CCL20) and CCL19 are involved in B cell migration to the CNS through binding to their chemokine receptors CXC receptor 5 (CXCR5), CXCR3, CXCR4, CC receptor 6 (CCR6) and CCR7, respectively. CSF levels of CXCL13 are increased in active RRMS patients and correlate with the number of total B cells and even more specifically the number of CSM B cells in the CSF (33). Therefore, this chemokine seem to play a central role in B cell recruitment to the CNS. In addition, the inflammatory chemokine CXCL10 contribute to lesion development in MS and its concentration is also significantly increased in MS patients (34). B cell trafficking depends not only on the response to chemokines, as adhesion molecules are also necessary to cross the BBB. Very late antigen (VLA)-4 has a major role in B cell migration since blocking this adhesion molecule *in vitro* inhibits trafficking of B cells across human adult brain-derived endothelial cells (35).

The relevance of this adhesion molecule is also illustrated by the monoclonal antibody therapy Natalizumab that is directed against VLA-4. Natalizumab showed reduced migration capacity of PBMCs in MS patients and moreover, a decrease in the rate of clinical relapses was observed in MS patients (36, 37). A role for lymphocyte function-associated antigen (LFA)-1 expression in B cell migration was demonstrated by inhibiting its counterpart intracellular adhesion molecule 1 (ICAM-1) (35). Additionally, most of the B cells express the activated leukocyte cell adhesion molecule (ALCAM), for which contribution to B cell recruitment into the CSN was also demonstrated by blocking this molecule (38).

1.2.4 Age-associated B cells in MS

A proportion of MS patients display characteristics of premature immune aging (39). During aging, the immune system undergoes changes that lead to immune remodeling and deregulation. In elderly, decreased numbers of naive T and B cells are found in the circulation due to a reduced output of both the bone marrow and thymus (40). For T cells, a process known as thymic involution will replace the epithelial space of the thymus by fatty tissue causing the thymus to lose its ability to produce new antigen-inexperienced T cells (39). A decline in the bone marrow density, changes in vascularization and impairments in hematopoietic stem cells will result in decreased numbers of the early B cell populations (41). However, the total number of circulating T and B cells is not impaired as proliferation of memory and effector antigen-experienced T and B cells provides a continuous filling of the immunological space. Nevertheless, reduced antigen-inexperienced T and B cell numbers will generate an inadequate immune response against newly encountered antigens. As a result, bacterial and viral infections increase whereas vaccine efficacy reduces. In autoimmune diseases, it is thought that chronic inflammation (i.e. repeated autoantigen stimulation) leads to similar alterations in immune cell phenotype and function. There is evidence that the humoral immune system is affected by immune aging, since a decreased production of B cells results in reduced protective antibody responses but an increased autoantibody generation (42). B cell receptor (BCR) diversity is decreased while clonal B cell expansion occurs, contributing to a restricted B cell repertoire (43). Moreover, BCR mutations accumulate during aging and lead to an alternation in the selection of memory B cells (44). Furthermore, $IgD^{-}CD27^{-}$ DN B cells, represents a B cell subtype that has been identified as an age-associated B cell (ABC) population (45).

Increased frequencies of DN B cells have been described in healthy elderly (46, 47), subjects infected with rotavirus (48) or human immunodeficiency virus (HIV) (49), and patients suffering from autoimmune diseases such as systemic lupus erythematosus (SLE) (32) and rheumatoid arthritis (RA) (50). In SLE patients, an increased frequency of DN B cells was significantly associated with disease activity and disease-specific autoantibodies, such as anti-double stranded DNA (32), indicating that they may contribute to autoimmune pathology. While DN B cells are negative for the memory marker CD27, they show similarities with $CD27^{+}$ CSM B cells. The majority of DN B cells are IgG^{+} and express mutated Ig genes, which suggests that they were selected by an antigen-driven process similar to $CD27^{+}IgG^{+}$ memory B cells (32, 47).

Furthermore, their shortened telomeres and the low expression of the transmembrane protein ATP-binding-cassette-B1 (ABCB1) transporter indicate that they behave as $CD27^+$ memory B cells, as the ABCB1 transporter is only present in $CD27^-$ naive B cells and absent in IgD^-CD27^+ CSM B cells (47, 51). Moreover it is suggested in HC that a clonal relationship between DN and $CD27^+$ memory B cells exists (52).

DN B cells show similarities with ABCs found in aged and autoimmune-prone mice (53, 54). These ABCs accumulate with age and are positive for CD11c, but lack the expression of CD21 and CD23 (54). Additionally, the majority of these cells are characterized by the expression of the transcription factor T-bet (T-Box Expressed in T cells). T-bet, encoded by the *Tbx21* gene, was first described as a transcriptional regulator of T helper 1 (Th1) cell differentiation. Later on, T-bet expression was also shown in other cell types such as $CD8^+$ T cells, natural killer cells, dendritic cells and B cells (55). T-bet expression in B cells plays an important role during anti-viral immunity, as T-bet^+ B cells are elevated during antiviral responses and secrete IgG2a antibodies for clearing pathogens (56). Moreover, it is shown in mice that T-bet expression induced by interferon (IFN)-γ and toll-like receptor (TLR) 9/TLR7 stimulation is required for class switching to IgG2a and the production of pathogenic autoantibodies in B cells (55). In addition, the formation of autoimmune germinal centers in lupus-prone mice is driven by IFN-γ–mediated T-bet expression in B cells (57, 58). A conditional deletion of T-bet from B cells in lupus-prone mice demonstrated reduced kidney pathology and titers of disease-specific autoantibodies. Furthermore, T-bet-deficient mice were protected from EAE development after immunization with MOG. Increased frequencies of T-bet^+ B cells were associated with Crohn's disease activity (59). T-bet expressing B cells were also found in RRMS patients, although there was no significant difference with healthy donors (60).These data suggests that T-bet expressing B cells play a role in the onset and development of autoimmunity (61). T-Bet^+ ABCs were already found in autoimmune-prone mice, in which they secreted high titers of autoantibodies following *in vitro* stimulation and displayed antigen-presenting features (62). Further, IgD^-CD27^- DN B cells of healthy donors showed T-bet expression that seemed to be higher than in memory and naive B cells (63). T-bet^+ $CD21^{low}$ ABCs have also been described in human autoimmune diseases such as SLE, CVID and RA (53).

However, literature is limited on the role of DN B cells in MS pathogenesis. In MS patients, it has been shown that peripheral Ig class-switched DN B cells were clonally related to intrathecal Ig repertoires, suggesting the involvement of DN B cells in MS pathology (64). In addition, the trafficking phenotype of DN B cells was already investigated. The research group of Colonna-Romano found that DN B cells of healthy elderly (78-90 years) showed increased expression of C-C motif chemokine receptor (CCR)7 and CCR6 in comparison with healthy donors. On the other hand, CXCR3 expression on DN B cells was higher in young donors (25-40 years) and a low CXCR5 expression was observed in both age groups. These data suggested that DN B cells could migrate to inflammatory sites (65).

Our research group recently reported increased expansions of DN B cells with pro-inflammatory characteristics in a proportion of MS patients (46). A higher proportion of MS patients < 60 years (20% of patients) showed peripheral expansions of DN B cells compared to age-matched HC (3% of donors). Furthermore, the majority of DN B cells had an IgG^+ memory phenotype. Increased frequencies of DN B cells in the CSF of MS patients compared to paired peripheral blood samples indicated their presence inside the CNS. After *ex vivo* stimulation, DN B cells produced pro-inflammatory (TNF-α and LT-α) and cytotoxic (granzyme B) cytokines. Moreover, similar expression of antigen presentation molecules as CSM B cells and costimulatory molecule (CD80/CD86) expression intermediate between naive and CSM B cells indicating the potential of DN B cells to induce T cell responses. Further, DN B cells showed a lower CD21 expression in MS patients when compared with HC, suggesting an autoreactive behavior since $CD21^{low}$ B cells seems to be anergic cells that produce autoreactive antibodies (66).

1.3 Hypothesis and aims

Recent literature and published data from our research group demonstrate the possible involvement of DN B cells in the pathological processes of MS and underline the importance of studying their function in human subjects. Therefore, the general aim of this study was to determine how DN B cells could contribute to the pathogenesis of MS.

We thereby hypothesized that IgD^-CD27^- DN B cells contribute to neuroinflammation in a proportion of MS patients through autoreactive and pro-inflammatory functions regulated via T-bet expression. The first objective of this project was to investigate the migration capacity of IgD^+CD27^- naive, IgD^-CD27^- DN, IgD^-CD27^+ CSM and IgD^+CD27^+ NCSM B cells towards pro-inflammatory sites *in vitro*. Accordingly, an *in vitro* transwell assay was performed in untreated RRMS patients and HC to investigate the migration capacity of these four B cell subsets towards the pro-inflammatory chemokines CXCL13 and CXCL10. For the second objective, the autoreactivity of the naive, DN, CSM and NCSM B cells towards different autoantigens that are related to MS pathogenesis was examined via an *in vitro* B cell proliferation assay. In addition, the activation status of these B cell subsets was analyzed after CpG stimulation. Finally, as a third objective, the frequency and phenotype of DN B cells from untreated RRMS and SPMS patients was determined using flow cytometry. More specifically, expression of the transcription factor T-bet was analyzed as well as the chemokine receptors CXCR3 and CXCR5.

Since details about the function of DN B cells in pathological processes of MS remain to be elucidated, further investigation of these cells contributes to a better understanding of MS pathogenesis. Moreover it could have added value in discovering new therapeutic targets that can also be beneficial for other autoimmune diseases in which ABCs are implicated, such as SLE and RA.

2 Materials and methods

2.1 Human samples

Peripheral blood samples were collected from 39 HC, 18 untreated RRMS patients and 5 untreated SPMS patients, all diagnosed according to the revised McDonald criteria (8). MS patients were recruited at the Rehabilitation & MS Center (Overpelt, Belgium) and Zuyderland Medical Center (Sittard, The Netherlands) and HC were recruited at the Biomedical Research Institute (BIOMED, Diepenbeek, Belgium). This study was approved by the local Medical Ethical Committee and written informed consent was obtained from all participants. Samples were stored in the University Biobank Limburg (Diepenbeek, Belgium). Optimization of $CD27^-$ and $CD27^+$ B cell isolation was performed with 14 HC samples. Phenotypic analysis of DN B cells was done using 5 SPMS patients, 10 RRMS patients and 20 age- and gender-matched HC. A total of 8 RRMS patients were used for chemotaxis assays and 5 HC were used for a B cell proliferation assay. Clinical data of the study subjects are summarized in Table 1.

Table 1. Clinical characteristics of MS patients and HC.

Study subjects	Number	Age[a]	Gender F:M (%F)[b]
B cell isolation			
Healthy donors	14	31 ± 7.0	9:5 (64.3%)
Flow cytometric analysis			
Healthy donors 1[c]	15	42.5 ± 12.5	13:2 (86.6%)
Healthy donors 2	5	50.2 ± 7.3	4:1 (80%)
Relapsing-remitting MS	10	42 ± 11.2	8:2 (80%)
Secondary progressive MS	5	50.4 ± 7.5	4:1 (80%)
Chemotaxis assay			
RRMS patients	8	39.8 ± 10.3	6:2 (75%)
B cell proliferation assay			
Healthy donors	5	28.5 ± 5.3	5:0 (100%)

[a] In years, mean ± SD.
[b] F: female, M: male.
[c] Healthy donors matched with relapsing-remitting MS patients
[d] Healthy donors matched with secondary progressive MS patients

2.2 Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient centrifugation (Lympholyte, Cedarlane laboratories, Sanbio B.V., Uden, The Netherlands) B cells were purified using three different negative magnetic selection kits (EasySep™ Human B Cell Enrichment Kit, STEMCELL™ Technologies SARL, Grenoble, France; B-CLL Cell Isolation Kit, Miltenyi Biotec B.V., Leiden, The Netherlands or MojoSort™ Human B Cell ($CD43^-$) Isolation Kit, BioLegend®, ImTec Diagnostics N.V., Antwerp, Belgium) according to the manufacturer's instructions. Subsequently, $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells were purified by magnetic beads (anti-CD27 microbeads or anti-allophycocyanin (APC)-conjugated CD27 microbeads (Miltenyi Biotec B.V)) or isolated via fluorescence-activated cell sorting (FACS) using the FACSAria II flow cytometer with the BD FACSDiva™ software (BD Biosciences).

Alternatively, $CD19^+CD27^+$ and $CD19^+CD27^-$ B cells were directly isolated from PBMCs by positive and negative magnetic selection, respectively (EasySep™ Human Memory B cell Isolation Kit, STEMCELL™ Technologies SARL) according to the manufacturer's instructions. Purity of the isolated $CD19^+$ B cells, $CD19^+CD27^-$ naive B cells and $CD19^+CD27^+$ memory B cells was confirmed on a LSRFortessa™ flow cytometer using BD FACSDiva™ software (BD Biosciences, Erembodegem, Belgium) and was routinely above 94%, 97% and 90%, respectively. The percentage of yield from the different isolation methods was calculated as follows:

$$\%\, Yield = \frac{Number\ of\ isolated\ cells}{The\ expected\ number\ of\ cells} \times 100$$

The number of isolated cells was counted using an automated cell counter and the expected number of cells was based on the percentage of cells in the population, measured via flow cytometry.

2.3 Phenotypic analysis of DN B cells

To phenotype DN B cells, PBMCs (1 x 10^6 cells) were stained with the following anti-human monoclonal antibodies: anti-human brilliant violet (BV) 421-conjugated CD19, phycoerythrin (PE)-Cy7-conjugated IgD, peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated IgM, BV605-connjugated IgM, PE-Dazzle 594-conjugated CD95, BV605-conjugated CD5, BV711-conjugated CD38, APC-Cy7-conjugated CD20, BV711-conjugated CD183 (CXCR3), PE-Dazzle 594-conjugated CD80, APC-Cy7-conjugated CD185 (CXCR5), PerCP-Cy5.5-conjugated CD49d (very late antigen 4, VLA-4), PE-conjugated CD11a (lymphocyte function-associated antigen 1, LFA-1) and FITC-conjugated CD268 (B-cell activating factor receptor, BAFF-R) (all from BioLegend), APC-conjugated CD27, fluorescein isothiocyanate (FITC)-conjugated IgG, Alexa fluor (AF) 700-conjugated IgG, BV786-conjugated CD10, BV786-conjugated CD166 (activated leukocyte cell adhesion molecule, ALCAM) (all from BD Biosciences) and PE-conjugated IgA (Miltenyi Biotec B.V.).

DN, naive and memory B cells were analyzed for T-bet expression by staining PBMCs (2 x 10^6 cells) with anti-human PE-Cy7-conjugated CD19 (BioLegend), APC-conjugated CD27, PE-CF594-conjugated IgD, FITC-conjugated IgG (all from BD Biosciences) and BV605-conjugated IgM (BioLegend). For the detection of the intracellular transcription factor T-bet, PBMCs were washed, fixed and permeabilized according to the protocol of the Foxp3/Transcription Factor Staining Buffer Set (eBioscience™, San Diego, CA). Then the cells were stained with PerCP-Cy5.5-conjugated anti-T-bet antibody (eBioscience™).

PBMCs were incubated for 30 min at 4°C with the fixable viability dye eFluor 506 (1:1000 in PBS; eBioscience™) or for 15 min at room temperature with 7-aminoactinomycin D (7-AAD;eBioscience™) to gate for viable lymphocytes. The cut-off to identify donors with expanded DN B cells was 7% and was determined by the mean percentage DN B cells from HC < 60 y plus two times the standard deviation (46). Fluorescence minus one (FMO) controls were used to determine the setting of the gates. All flow cytometric analyses were performed on a LSRFortessa™ flow cytometer and analysed with BD FACSDiva™ software (BD Biosciences).

2.4 Chemotaxis assay

Unstimulated $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells (1 x 10^5 cells) were transferred in duplo into the upper chamber of a 6.5 mm diameter, 5 µm pore-sized *in vitro* transwell system (Corning®, Lasnel, Belgium). Chemotaxis buffer (Roswell Park Mepmorial Institute (RPMI)-1640 medium (Lonza, Verviers, Belgium) supplemented with 0.5% bovin serum albumin (BSA; Biowest, Nuaillé, France) together with 1000 ng/ml of CXCL13 (n = 7) or CXCL10 (n = 3) (both from PeproTech, Neuilly-Sur-Seine, France)) was added to the lower chamber. After 24 h of incubation at 37°C and 5% CO_2, the number of cells that migrated to the lower compartment was counted. Using flow cytometry the percentages of IgD^+CD27^- naive, IgD^-CD27^- DN, IgD^-CD27^+ CSM and IgD^+CD27^+ NCSM B cells were determined. The chemotactic index (CI) for each subset was defined as:

$$CI = \frac{number\ of\ migrated\ subtype\ cells\ with\ chemokine - number\ of\ migrated\ subtype\ cells\ without\ chemokine}{original\ number\ of\ subtype\ cells\ in\ upper\ chamber}$$

2.5 B cell proliferation assay

Purified $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells were labeled with 5 µM Tag-it Violet™ at a density of 10 x 10^6 cells/ml (BioLegend). After incubating for 20 min at 37°C, cells were washed and incubated with culture medium (RPMI-1640 medium (Lonza) supplemented with 10% foetal bovine serum (FBS, Life Technologies, Gent, Belgium), 1% sodium pyruvate (NaPyr), 1% nonessential amino acids (neAA), 50 U/ml penicillin and 50 µg/ml streptomycin (all from Sigma-Aldrich, Diegem, Belgium)) for 10 min at 37°C. More than 98% of the $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells were labeled with Tag-it Violet™ as determined by analysis on the LSRFortessa™ flow cytometer using BD FACSDiva™ software (BD Biosciences).

Tag-it Violet™-labeled $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells were cultured in a 96-well round-bottom plate (Greiner BioOne, Vilvoorde, Belgium) in triplicate at 1 x 10^5 cells with autologous irradiated (8275 rad) PBMCs (1:1 ratio). Cells were stimulated with 2,5 limit of flocculation (Lf)/ml tetanus toxoid (TT; RIVM, Bilthoven, The Netherlands), 1 µg/ml cytomegalovirus (CMV; BD Biosciences), 40 µg/ml human MBP, purified as described (67), or 10 µg/ml recombinant human MOG protein 1-125 (Eurogentec, Seraing, Belgium). Stimulation with 1 µg/ml CpG2006 (Invivogen, Toulouse, France) and 50 U/ml IL-2 (Sigma-Aldrich) was used as a positive control, whereas unstimulated $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells were a negative control to define background proliferation. After 10 days, cells were harvested to assess activation, cell proliferation (Tag-it Violet signal) and viability (eFluor 506 dye) by flow cytometry using anti-human PE-Cy7-conjugated CD19 (BioLegend), APC-conjugated CD27, PE-CF594-conjugated IgD, PE-conjugated CD80 (all from BD Biosciences) and APC-Cy7-conjugated CD25 (Biolegend). The Δ proliferation fraction (ΔPF) was determined by subtracting the mean percentage of background proliferation (in the absence of antigenic stimulation) from the mean percentage of proliferation in response to antigen. A significant antigen-specific proliferative response was noticed when ΔPF ≥ 2%, similar to thresholds set for positive proliferation documented by our research group previously (68). Analysis was done on the LSRFortessa™ flow cytometer using the BD FACSDiva™ software (BD Biosciences).

2.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 6.01. Due to the small number of patients, nonparametric tests were used. Comparison of two paired groups was done using the Wilcoxon matched-pairs signed rank test. Multiple paired groups were analyzed by the Friedman test, followed by the post hoc Dunn's multiple comparison test. Two-way ANOVA was used to compare two independent variables between groups. A p value of less than 0.05 was considered statistically significant. Results are expressed as mean value + standard error of the mean (SEM) or + standard deviation (SD).

3 Results

3.1 DN B cells of MS patients express the transcription factor T-bet

IgD^-CD27^- DN B cells show similarities with T-bet^+ ABCs found in autoimmune-prone mice and human autoimmune diseases such as SLE, RA and CVID (53, 55). In HC, a proportion of DN B cells showed T-bet expression that seemed to be higher compared to memory and naive B cells (63). Moreover, T-bet^+ B cells are also described in RRMS patients (60). Expression of the transcription factor T-bet in B cells has been associated with class-switching to IgG2a antibodies for antiviral immunity, production of autoantibodies and antigen-presenting functions in ABCs from autoimmune-prone mice (55, 62). These novel findings put forward a role for T-bet in functional processes of ABCs and might therefore also contribute to DN B cell functioning. For this reason, T-bet expression was evaluated via intracellular flow cytometry in different B cell subtypes (naive, DN, NCSM and CSM B cells) of untreated RRMS patients (n = 9), SPMS patients (n = 5) and HC (n = 15; Fig. 2A).

A proportion of DN B cells from RRMS patients (20.1%), SPMS patients (24.7%) and HC (23.1%) were T-bet^+ (Fig. 2B). The percentage of T-bet^+ DN B cells was increased compared to naive B cells in RRMS patients ($p < 0.001$) and HC ($p < 0.0001$; Fig. 2B). A significant difference between T-bet^+ DN B cells and T-bet^+ NCSM B cells was also found in HC (Fig. 2B). T-bet expression (MFI) in DN B cells was the highest and was significantly increased compared to naive B cells in all three study groups ($p < 0.05$ for MS patients and < 0.001 for HC; Fig. 2C). Moreover, T-bet was expressed at higher levels in DN B cells compared to NCSM B cells ($p < 0.05$; Fig. 2C).

T-bet expression (% and MFI) in DN B cells was also compared between RRMS patients, SPMS patients and HC, with the HC 1 group being age- and gender matched to RRMS and the HC 2 group being age- and gender matched to SPMS. No significant difference was found for the frequency of T-bet^+ cells in the total B cell population between RRMS patients, SPMS patients and HC. There was also no difference observed for the percentage of T-bet^+ DN B cells between RRMS patients, SPMS patients and HC (Fig. 2D). However, expression levels of T-bet (MFI) in DN B cells were elevated in RRMS and SPMS patients compared with HC 1 ($p < 0.001$) and HC 2 ($p < 0.01$), respectively (Fig. 2E). T-bet expression (MFI) in DN B cells of RRMS patients was also higher in comparison with SPMS patients ($p < 0.05$; Fig. 2E). In addition, T-bet expression in IgG^+ DN B cells was compared to IgM^+ DN B cells, but no difference was found for the frequency of T-bet^+IgG^+ and T-bet^+IgM^+ DN B cells. However, T-bet expression levels for IgG^+ DN B cells were significantly higher in RRMS patients compared to HC (Supplemental Fig. 1).

Together, these data indicate that T-bet^+ DN B cells can be found in untreated RRMS patients, SPMS patients and HC, with a statistical significant difference between the different study groups. Moreover, expression of T-bet (MFI) in DN B cells is the highest compared to naive, NCSM and CSM B cells. By expressing T-bet, DN B cells have a common characteristic with T-bet^+ ABCs found in mice, which also underline the age-associated nature of DN B cells in MS.

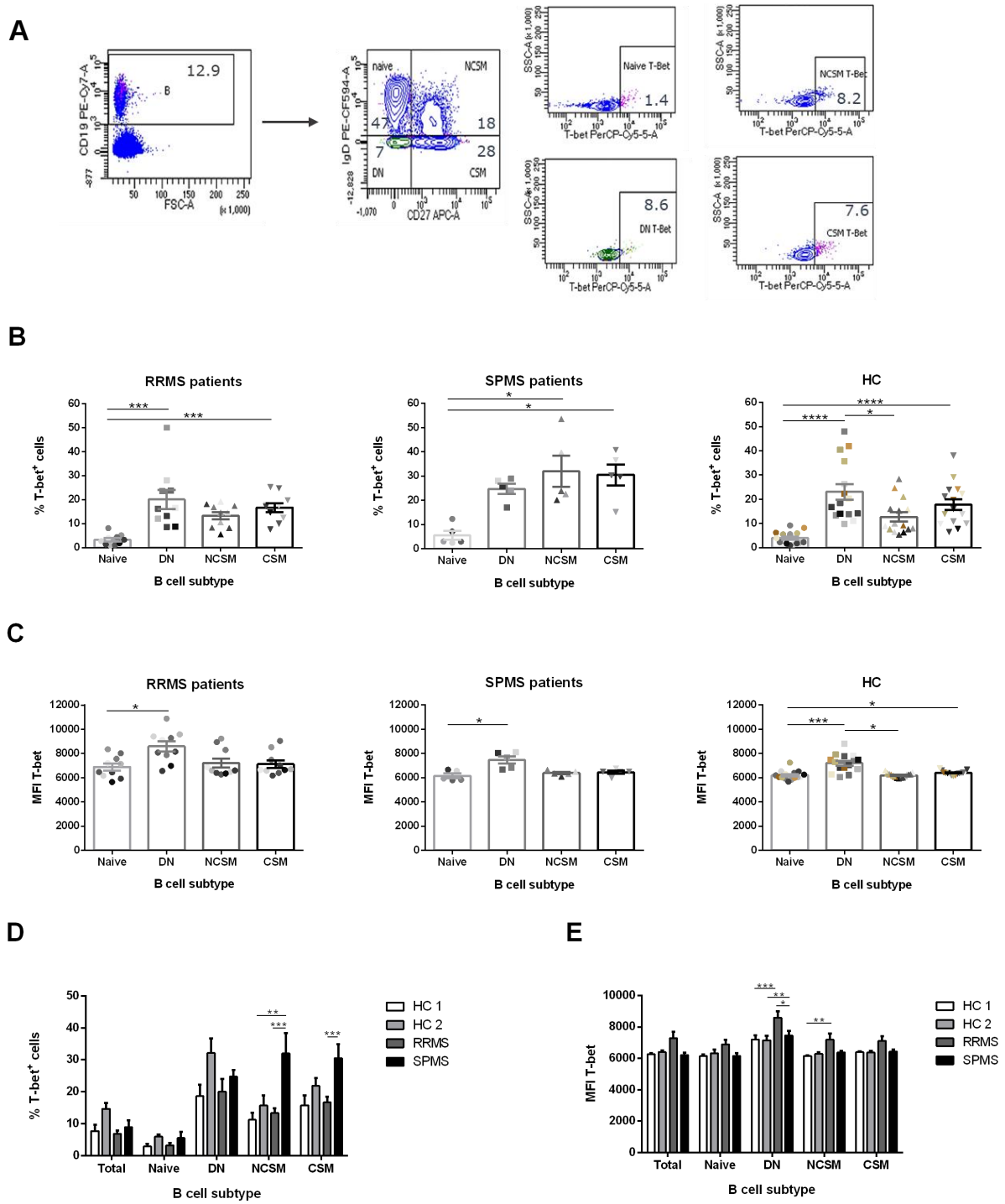


Figure 2. T-bet expression in B cell subsets from MS patients and HC. T-bet expression was measured in peripheral blood B cell subsets (naive, DN, NCSM and CSM B cells) via intracellular flow cytometry. (A) Representative plots of 1 RRMS patient indicating the gating strategy to evaluate T-bet expression. The percentage of T-bet$^+$ cells (B) or MFI within T-bet$^+$ cells (C) in naive, DN, NCSM and CSM B cells from RRMS patients (n = 9), SPMS patients (n = 5) and HC (n = 15). Each dot represents an individual patient sample. The percentage (D) or MFI (E) within T-bet$^+$ naive, DN, NCSM and CSM B cells was compared between RRMS patients, SPMS patients and HC 1 and 2 that were age- and gender matched with RRMS and SPMS patients, respectively. Mean levels + SEM are shown. The Friedman test with post hoc Dunn's multiple comparison test and Two-way ANOVA were used for statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MFI: mean fluorescence intensity, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, HC: healthy control, DN: double negative, NCSM: non class-switched memory, CSM: class-switched memory.

3.2 DN B cells express the chemokine receptors CXCR3 and CXCR5

DN B cells of young HC have shown an elevated expression of CXCR3 compared to naive and CSM B cells and a low expression of CXCR5 (65). CXCR3 and CXCR5 are chemokine receptors to which the corresponding chemokines CXCL10 and CXCL13 can bind, respectively. These chemokines are involved in B cell trafficking to the CNS as the concentration of both chemokines is increased in the CSF of MS patients (34, 69). In addition, our research group recently found increased frequencies of DN B cells in the CSF of MS patient compared with paired peripheral blood samples (46). The presence of DN B cells inside the CNS of MS patients suggests that DN B cells of MS patients express CXCR3 and CXCR5 as well. Using flow cytometry, CXCR3 and CXCR5 expression on the surface of DN B cells was assessed in DN B cells as well as naive and CSM B cells of untreated RRMS patients ($n = 10$), SPMS patients ($n = 5$) and HC ($n = 20$; Fig. 3A).

A percentage of DN B cells from RRMS patients (21.7%), SPMS patients (12.8%) and HC (34%) were positive for CXCR3. Nonetheless, the proportion of $CXCR5^+$ DN B cells was higher in RRMS patients (71.3%), SPMS patients (59.4%) and HC (67.4%; Fig. 3B). The percentage of $CXCR3^+$ DN B cells was significantly elevated compared to naive B cells in HC ($p < 0.001$), while the percentage of $CXCR5^+$ DN B cells was decreased compared to naive and CSM B cells in RRMS patients ($p < 0.001$ and $p < 0.05$, respectively) and HC ($p < 0.0001$ and $p < 0.001$, respectively; Fig. 3B). Expression levels of CXCR3 (mean fluorescence intensity, MFI) on DN B cells were similar to naive B cells in RRMS and SPMS patients, but they were significantly increased compared to naive B cells in HC ($p < 0.01$; Fig. 3C). CXCR5 expression (MFI) was the highest in naive B cells compared to DN and CSM B cells in RRMS patients ($p < 0.0001$ and $p < 0.05$, respectively) and HC ($p < 0.0001$ and $p < 0.001$, respectively), and DN B cells in SPMS patients ($p < 0.05$; Fig. 3C). Moreover, DN B cells showed the lowest CXCR5 expression in all three study groups (Fig. 3C).

The expression of CXCR3 and CXCR5 (% and MFI) on naive, DN and CSM B cells was also compared between RRMS patients, SPMS patients and HC, with the HC 1 group being age- and gender matched to RRMS and the HC 2 group being age- and gender matched to SPMS patients. The percentage of $CXCR3^+$ and $CXCR5^+$ cells in the total B cell population was significantly higher in RRMS patients compared to SPMS patients ($p < 0.05$; Fig. 4A). $CXCR3^+$ DN B cells were increased in RRMS patients compared to SPMS patients ($p < 0.01$; Fig. 4A). However, $CXCR3^+$ DN B cells in HC 1 and HC 2 were elevated compared to RRMS patients and SPMS patients, respectively ($p < 0.01$ and $p < 0.0001$, respectively; Fig. 4A). No significant difference was found for CXCR3 and CXCR5 expression (MFI) on DN B cells between RRMS patients, SPMS patients and HC (Fig. 4B).

The above results indicate that DN B cells of RRMS patients, SPMS patients and HC express the chemokine receptor CXCR3 and to a greater extent also CXCR5. However, no elevated expression levels (% and MFI) of CXCR3 and CXCR5 on DN B cells could be observed in RRMS and SPMS patients. Additionally, a similar expression (MFI) of CXCR3 and CXCR5 on DN B cells of RRMS, SPMS and HC was found, suggesting that these receptors are mainly involved in the CSF instead of the periphery in MS.

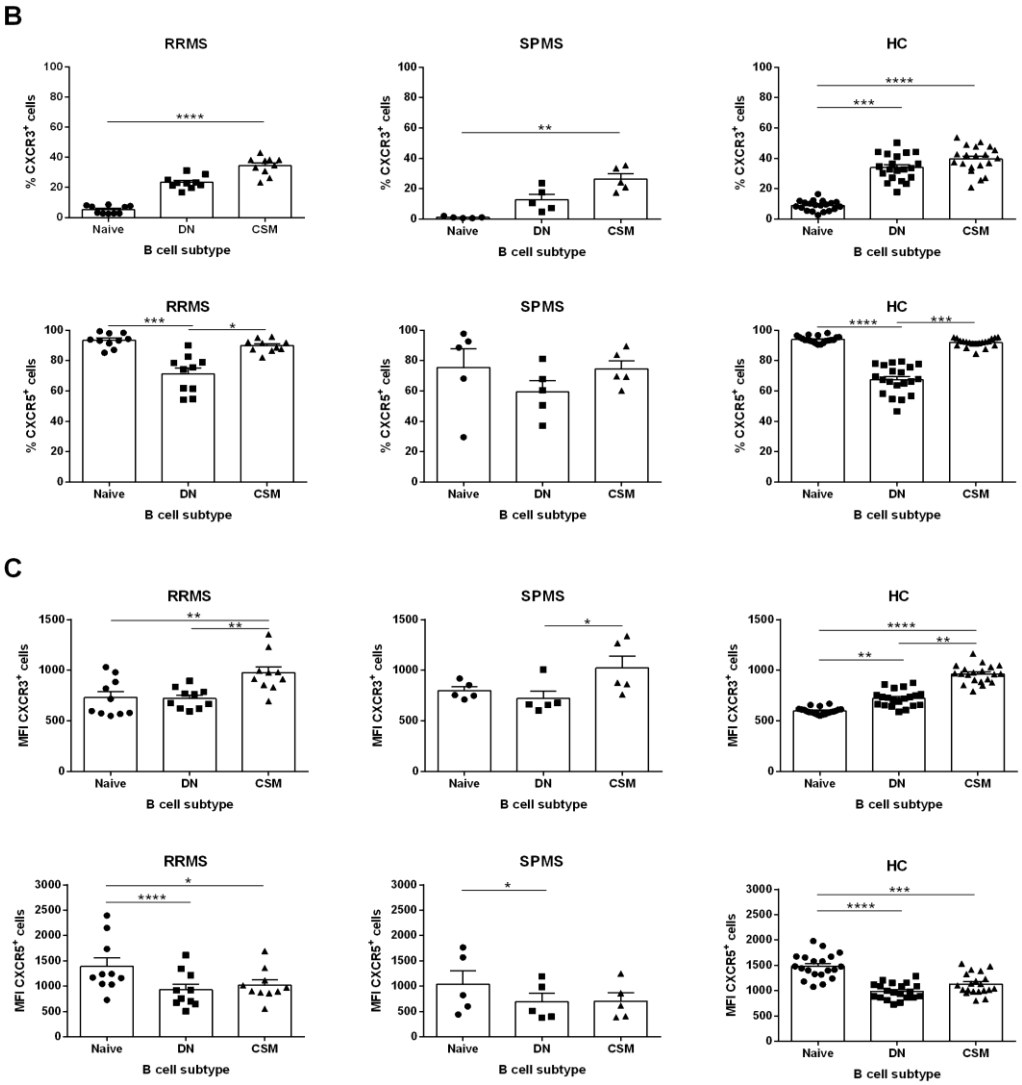


Figure 3. Expression of chemokine receptors CXCR3 and CXCR5 on different B cell subsets from MS patients and HC. Using flow cytometry, CXCR3 and CXCR5 expression was measured in peripheral blood B cell subsets (naive, DN and CSM B cells). (A) Gating strategy of 1 representative RRMS patient to analyze the CXCR3 and CXCR5 expression. The percentage of $CXCR3^+$ and $CXCR5^+$ cells (B) or MFI within $CXCR3^+$ and $CXCR5^+$ cells (C) in naive, DN and CSM B cells from RRMS patients (n = 10), SPMS patients (n = 5) and HC (n = 15). Each symbol represents an individual patient. Mean levels + SEM are shown. The Friedman test with post hoc Dunn's multiple comparison test was used for statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MFI: mean fluorescence intensity, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, HC: healthy control, DN: double negative, NCSM: non class-switched memory, CSM: class-switched memory.

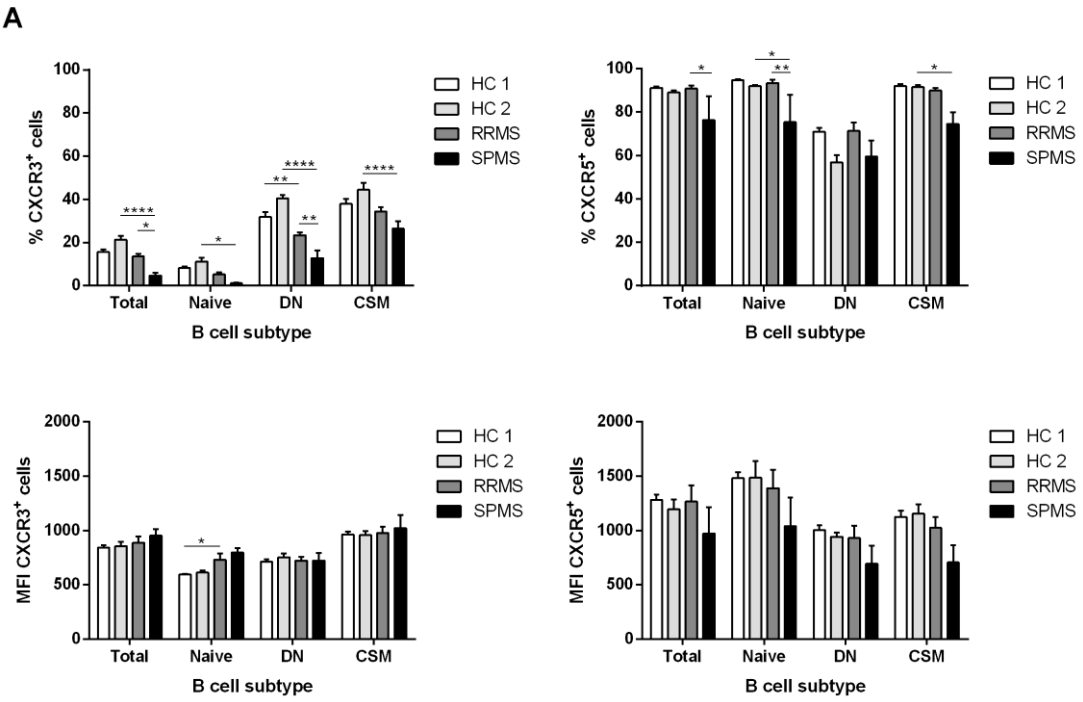


Figure 4. CXCR3 and CXCR5 expression on B cell subsets from MS patients and HC. The percentage (D) or MFI (E) of CXCR3$^+$ and CXCR5$^+$ naive, DN and CSM B cells was compared between RRMS patients, SPMS patients and HC 1 and 2 that were age- and gender matched with RRMS and SPMS patients, respectively. Mean + SEM is shown. The Friedman test with post hoc Dunn's multiple comparison test and Two-way ANOVA were used for statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MFI: mean fluorescence intensity, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, HC: healthy control, DN: double negative, NCSM: non class-switched memory, CSM: class-switched memory.

3.3 Optimization of $CD27^-$ and $CD27^+$ B cell isolation

In order to investigate the functional properties of IgD^-CD27^- DN B cells, and to compare them with IgD^+CD27^- naive and IgD^-CD27^+ memory B cells, these cells must be purified from PBMCs. The most appropriate isolation strategy is to isolate each subtype separately using FACS sorting. Nevertheless, the low number of DN B cells in the blood circulation and the low cell yield after FACS sorting making this method not feasible. On the other hand, the total B cell population can be used for the experiments, whereafter the B cell subtypes can be distinguished via flow cytometry. However, memory B cells lose their CD27 expression after stimulation or incubation, making it difficult to distinguish DN B cells from memory B cells. Therefore, the $CD27^-$ (naive and DN B cells) and $CD27^+$ (CSM and NCSM B cells) B cell populations should be used for the assays, after which flow cytometry can be used to count and characterize each B cell subtype. Different kits were tested to find an optimal isolation method (i.e. high purity, high viability, low activation status and sufficient yield) for purifying $CD27^-$ and $CD27^+$ B cells.

3.3.1 Sorting $CD27^-$ and $CD27^+$ B cells results in a low cell yield

First, FACS sorting to isolate $CD27^+$ and $CD27^-$ B cells from purified total B cells was tested. $CD27^-$ and $CD27^+$ B cells from two HC were sorted from 99.9% pure B cells (EasySep™ Human B Cell Enrichment Kit). The purity of the $CD27^-$ and $CD27^+$ B cells was 98.6% and 92.8%, respectively. The percentage of $7\text{-}AAD^-$ living cells was 99.8% for $CD27^-$ B cells and 99.6% for $CD27^+$B cells. Furthermore, the $CD27^-$ and $CD27^+$ B cell populations consisted of 0.75% and 2.5% $CD25^+$ cells, 3.6% and 38.8% $CD80^+$ cells and 0.6% and 1.15% $CD86^+$ cells, respectively. However, the yield was only 10.9% for $CD27^-$ B cells and 3.8% for $CD27^+$ B cells. These findings indicate that FACS sorting results in a pure population of viable $CD27^-$ and $CD27^+$ B cells with a low activation status, however, it leads to a lot of cell loss.

3.3.2 Magnetic $CD27^+$ B cell isolation increases yield but reduces purity

Next, magnetic isolation of $CD27^+$ and $CD27^-$ B cells from purified total B cells was tested. First, three different kits for the negative magnetic isolation of total B cells were tested in HC in order to select the one with the highest yield and purity (Fig. 5). B cells were isolated using the EasySep™ Human B Cell Enrichment Kit (n = 6), B-CLL Cell Isolation Kit (n = 8), or MojoSort™ Human B Cell ($CD43^-$) Isolation Kit (n = 3). The purity of the enriched B cells was higher after the Easysep (99.4%) and Mojosort Kit (97.7%) in comparison with the B-CLL kit (90.4%; Fig. 6A). Furthermore, the Mojosort Kit offered the best yield (89.5%; Fig. 6B). A low activation status of the B cells was shown after isolation with the Mojosort Kit with an average of 2.8% $CD25^+$ B cells, 8.7% $CD80^+$ B cells and 0.8% $CD86^+$ B cells (Fig. 6C-E).

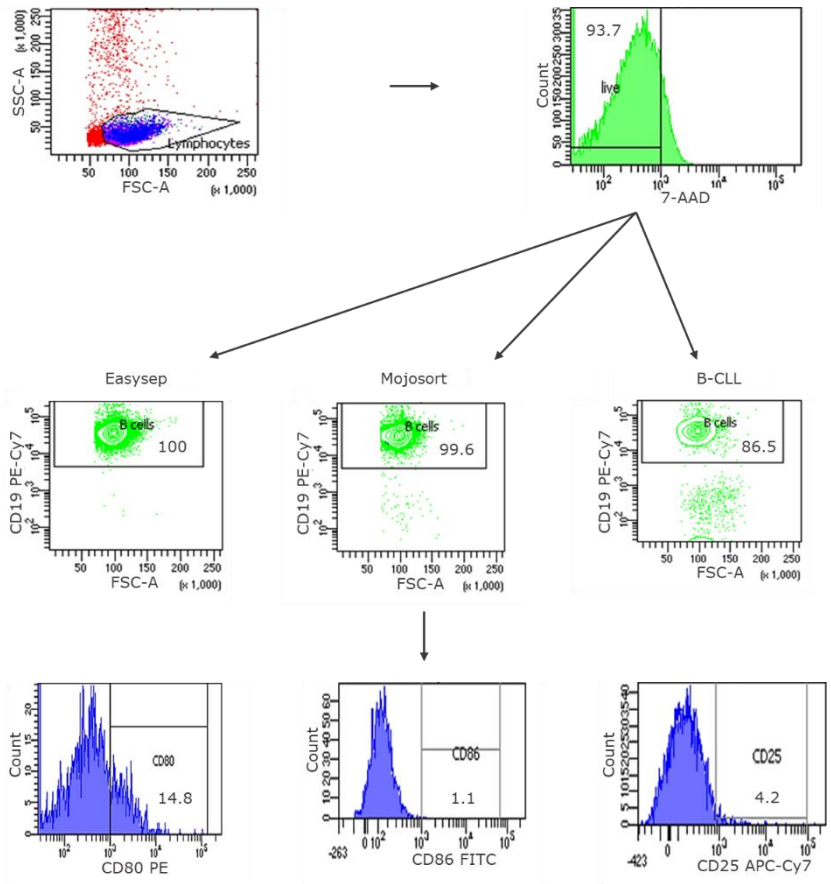


Figure 5. Representative gating strategy to isolate CD19⁺ B cells from 1 HC. CD19⁺ B cells were isolated from PBMC using three different negative magnetic isolation methods (EasySep™ Human B Cell Enrichment Kit, B-CLL Cell Isolation Kit or MojoSort™ Human B Cell (CD43-) Isolation Kit). Subsequently, cell counting and flow cytometric analysis were performed to define the purity, viability and activation status of the isolated B cell population. Therefore, CD19⁺ B cells were stained with anti-human CD19-PE-Cy7, 7-AAD, CD80-PE, CD86-FITC and CD25-APC-Cy7.

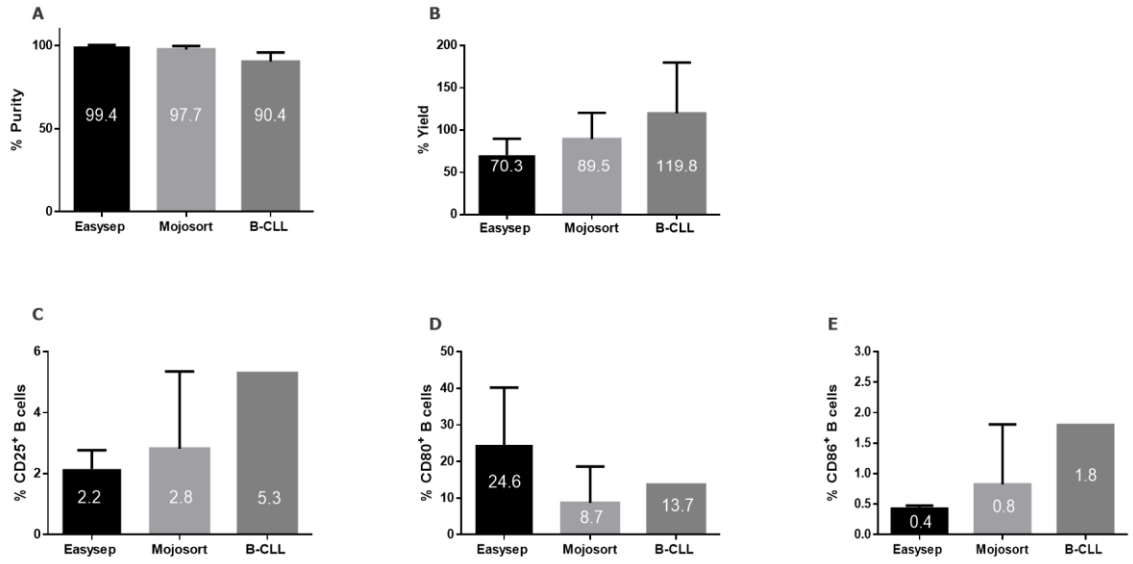


Figure 6. Analysis of three B cell isolation methods. The EasySep™ Human B Cell Enrichment Kit (n = 6), B-CLL Cell Isolation Kit (n = 3), and MojoSort™ Human B Cell (CD43-) Isolation Kit (n = 8) were tested to isolate CD19⁺ B cells from PBMCs. Percentage of (A) pure CD19⁺ B cells after isolation, (B) yield of CD19⁺ B cells after isolation, (C) CD25⁺ B cells, (D) CD80⁺ B cells and (E) CD86⁺ B cells. Values are represented as mean + SEM.

$CD27^-$ and $CD27^+$ B cells were then isolated from the enriched B cells using MACS positive magnetic CD27 microbeads or MACS anti-APC-conjugated CD27 microbeads. The purity of the $CD27^-$ and $CD27^+$ B cells after isolation with MACS positive magnetic CD27 microbeads (n = 2) was 98.7% and 55.4%, respectively. Therefore, this isolation method was further optimized by testing the type of MACS column (LS versus MS), repeating the magnetic separation procedure, and staining the cells with anti-human APC-conjugated CD27 antibody before the isolation (n = 6). Using the LS column resulted in a higher yield of $CD27^-$ B cells compared to the MS column (59% and 44.1% $CD27^-$ B cells, respectively; Fig. 7A). However, no difference was found for the $CD27^+$ B cells (Fig. 7C). Furthermore, the purity of the $CD27^-$ and $CD27^+$ B cells was not significantly different between the LS and MS column (Fig. 7B-D). To increase the purity of the $CD27^+$ B cell population, the magnetic separation was repeated over a second new column. However, this method did not enhance the purity of the $CD27^+$ B cells (Fig. 7D). Anti-CD27 APC staining during the isolation procedure using another clone of the anti-human CD27 APC antibody (clone REA499, Miltenyi Biotec B.V) increased the yield ($CD27^-$: 81.0% and $CD27^+$: 67.5%; Fig. 7A-C) and also provided a high purity ($CD27^-$: 98.7% and $CD27^+$: 73.5%; Fig. 7B-D). Repeating the magnetic separation procedure over a second column after anti-CD27 APC staining, increased the purity of the $CD27^+$ B cells with 10% (Fig. 7D). Although the purity was increased after the anti-CD27 APC staining, the $CD27^+$ B cell population was not sufficiently pure.

The indirect magnetic labeling of $CD27^-$ and $CD27^+$ B cells with anti-APC-conjugated CD27 microbeads (n = 3) showed a lower yield (70.2% and 40.3%, respectively; Fig. 7A-C) for both cell populations compared to the anti-CD27 microbeads. Using the MS column instead of the LS column did not increase the yield (Fig. 7A-C). No significant increase in the purity of the $CD27^-$ and $CD27^+$ B cell populations was observed after isolation with anti-APC-conjugated CD27 microbeads (Fig. 7B-D).

Taken together, these data demonstrate that the MojoSort™ Human B Cell ($CD43^-$) Isolation Kit has a purity that is comparable with the EasySep™ Human B Cell Enrichment Kit and can yield a sufficient number of B cells. Furthermore, isolating $CD27^-$ and $CD27^+$ B cells from enriched B cells with anti-CD27 microbeads gives a higher yield than sorting the cells (75.9% and 69.9% versus 10.9% and 3.8%, respectively). However, after the different optimizations of the protocol for the anti-CD27 microbeads, the $CD27^+$ B cell population was not completely pure.

3.3.3 Direct purification of $CD19^+CD27^+$ and $CD19^+CD27^-$ B cells from PBMCs with the Easysep™ Human Memory B cell Isolation kit shows the highest purity

Since the $CD27^+$ B cell population was not pure using the previous isolation methods, $CD19^+CD27^+$ and $CD19^+CD27^-$ B cells were directly isolated from PBMCs using the EasySep™ Human Memory B cell Isolation Kit (n = 5). Purity of the $CD27^+$ B cells (95.6%) was higher in comparison with the previous methods (Fig 7D), whereas the percentage of pure $CD27^-$ B cells was comparable (Fig 7B).

Although this memory B cell isolation kit yielded a low percentage of CD27$^-$ (42.8%; Fig. 7A) and CD27$^+$ B cells (33.4%; Fig. 7C), the number of isolated CD27$^-$ and CD27$^+$ B cells was usually sufficient to use for the different assays. Due to the high purity of the CD27$^+$ and CD27$^-$ B cell population, this isolation method was further used.

Together, these results show that the purity of the CD27$^+$ B cells was the determining factor to find an optimal isolation technique as the CD27$^-$ B cells were pure after each isolation method (except for the anti-APC-conjugated CD27 microbeads with the MS column).Although FACS sorting resulted in the highest purity for both CD27$^-$ and CD27$^+$ B cells, the Easysep™ Human Memory B cell Isolation kit was the most optimal method as the yield of the CD27$^+$ B cells was 10 times higher than after FACS sorting.

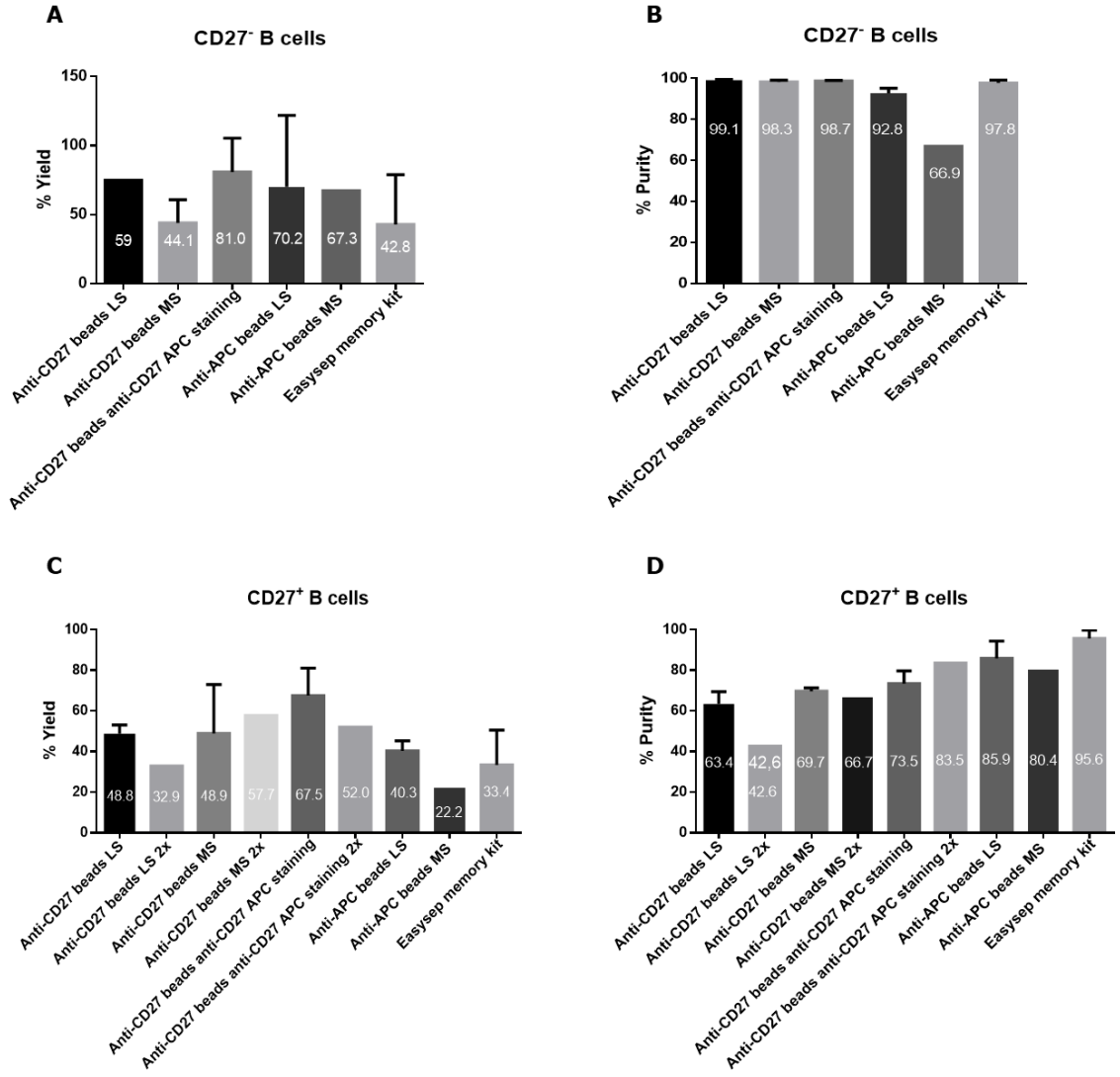


Figure 7. Comparison of isolation methods for CD27$^-$ and CD27$^+$ B cells in HC. CD27$^-$ and CD27$^+$ B cells were isolated from CD19$^+$ B cells using anti-CD27 microbeads (n = 6) or anti-APC-conjugated microbeads (n = 3), or were directly purified from PBMCs using the EasySep™ Human Memory B cell Isolation Kit (n = 5). Anti-CD27 microbeads were optimized by testing the type of MACS column (LS versus MS), repeating the magnetic separation procedure (2x) and anti-human CD27-APC staining before the isolation. Percentage of yield of (A) CD27$^-$ B cells and (B) CD27$^+$ B cells. Percentage of purity of (C) CD27$^-$ B cells and (D) CD27$^+$ B cells. Values are represented as mean + SD.

3.4 DN B cells migrate towards CXCL13 and CXCL10 *in vitro*

In MS patients, B cells can migrate towards pro-inflammatory sites. More specifically, a previous study reported that peripheral Ig class-switched DN B cells are clonally related to intrathecal DN B cells suggesting that these cells can be involved in providing a connection between the periphery and the CNS in MS patients (64). In addition, DN B cells of HC express the chemokine receptors CXCR3 and CXCR5 of the corresponding pro-inflammatory chemokines CXCL10 and CXCL13, respectively (65). Our research group has also shown increased percentages of DN B cells in the CSF of MS patients compared to the peripheral blood (46). These data suggest that DN B cells might have the capacity to migrate towards inflammatory sites. Therefore, an *in vitro* chemotaxis assay was performed to examine the migration capacity of naive, DN, NCSM, and CSM B cells from untreated RRMS patients towards CXCL13 (n = 8) and CXCL10 (n = 5). This assay was firstly optimized.

3.4.1. B cell stimulation does not affect the migration capacity or chemokine receptor expression

In order to optimize this assay, the effect of B cell stimulation on the migration capacity of $CD27^-$ and $CD27^+$ B cells towards CXCL13 was investigated in HC, as well as the effect on the expression of the chemokine receptors CXCR3 and CXCR5 (n = 2). Unstimulated $CD27^-$ and $CD27^+$ B cells or cells stimulated with CpG2006 + IL-4 or CD40L + IL-4 were added to a transwell system for 4, 8, or 24 hours. The chemotactic index (CI) for the $CD27^-$ and $CD27^+$ B cells was calculated as the ratio of the number of migrated cells in the presence of CXCL13 and in the absence of CXCL13. After 4 hours of incubation, $CD27^-$ B cells barely migrated in the presence of CXCL13, in contrast with $CD27^+$ B cells (Fig. 8A-B). However, the CI of $CD27^-$ B cells was the highest after 24 hours of incubation in all three conditions (Fig. 8A). For both $CD27^-$ and $CD27^+$ B cells, the CI after 24 hours was higher in the unstimulated condition (CI: 5.0 and 2.3, respectively), compared to CD40L + IL-4 (CI: 2.1 for $CD27^-$ B cells) or CpG + IL-4 stimulation (CI: 3.9 and 0.6, respectively; Fig. 8A-B). Furthermore, the expression (MFI) of the chemokine receptors CXCR3 and CXCR5 on naive, DN, CSM, and NCSM B cells was measured via flow cytometry. Stimulating the cells did not increase the MFI of CXCR3 and CXCR5 for the different B cells subtypes, but showed values that were comparable to the unstimulated condition, except for the NCSM B cells (Fig. 8C). Moreover, the incubation time had no significant effect on the CXCR3 and CXCR5 expression, with the exception of CXCR5 expression on CSM and NCSM B cells. In these B cells, the MFI was increased after 24 hours (Fig. 8C). Taken together, these results show that stimulating the cells is not necessary, since this will neither influence the migration capacity of $CD27^-$ and $CD27^+$ B cells nor the chemokine receptor expression on the naive, DN, CSM, and NCSM B cells.

3.4.2. 1000 ng/ml CXCL13 or CXCL10 is the optimal concentration to trigger B cell migration

Based on literature, the concentration of CXCL13 that was used in the previous experiment was 500 ng/ml. However, the optimal concentration of CXCL13 and CXCL10 to be added to the lower chamber of the transwell system had to be determined. Therefore, 100 ng/ml, 500 ng/ml and 1000 ng/ml was tested using the total B cell population of 1 HC.

The CI for the different concentrations was determined as previously mentioned. For both CXCL13 and CXCL10, the migration capacity of the B cells was the highest at 1000 ng/ml, whereas no difference was observed between 100 ng/ml and 500 ng/ml (Fig. 9).

However, for CXCL10, the CI of the B cells was extremely elevated at 1000 ng/ml, compared to 500 ng/ml (Fig. 9B). Therefore, this experiment was repeated in naive, DN, NCSM and CSM B cells from 1 RRMS patient. In the presence of 500 ng/ml CXCL13, the CI of naive, DN, NCSM and CSM B cells was lower compared to adding 1000 ng/ml CXCL13 (Fig. 9C). Furthermore, naive, DN and NCSM B cells migrated more in the presence of 1000 ng/ml CXCL10 (Fig. 9D). Consequently, a concentration of 1000 ng/ml for both chemokines was further used.

3.4.3. No proliferation of B cells after incubation for 24 hours

To exclude potential proliferation of the four different B cell subsets (naive, DN, NCSM and CSM) after 24 hours of incubation, $CD27^-$ and $CD27^+$ B cells from 1 MS patient were labeled with 1 µM of the cell proliferation dye 5,6-carboxy fluorescein diacetate succinimidyl ester (CFSE). The cells were incubated with 1000 ng/ml CXCL13 or CXCL10 in a 96-well plate for 24 hours and then analyzed via flow cytometry to distinguish the proliferative response of the four B cells subtypes. No proliferation of DN, naive, NCMS and CSM B cells is found on the corresponding plots, since the percentage of cells in the proliferated fraction is around 0 - 0.5% (Supplemental Fig. 2).

3.4.4. Migration of DN B cells in MS patients

After optimization, the migration capacity of naive, DN, NCSM and CSM B cells from untreated RRMS patients towards CXCL13 (n = 8) and CXCL10 (n = 5) was investigated. In order to take the original number of cells added to the upper chamber into account, the CI was recalculated for this experiment and was defined for each B cell subtype as: (number of migrated cells in the presence of a chemokine - number of migrated cells in the absence of a chemokine)/(original number of cells in the upper chamber). DN B cells of untreated RRMS patients are able to migrate towards CXCL13 (CI: 16.7 ± 2.5) and CXCL10 (CI: 3.2 ± 1.2; Fig. 10A-B). Migration of DN B cells towards CXCL13 was significantly higher compared to naive (CI: 4.8 ± 1.3; $p < 0.01$) and NCSM B cells (CI: 6.3 ± 1.5; $p < 0.05$). Additionally, CSM B cells showed a higher CI for CXCL13 (15.8 ± 1.8) in comparison with naive B cells ($p < 0.05$; Fig. 10A). For both CXCL13 and CXCL10, most of the migrated DN B cells were IgG^+ (Fig. 10C-D). These results indicate that DN B cells of untreated RRMS patients can migrate towards CXCL13 and CXCL10 *in vitro* and show a higher migration capacity for CXCL13 compared to naive and NCSM B cells.

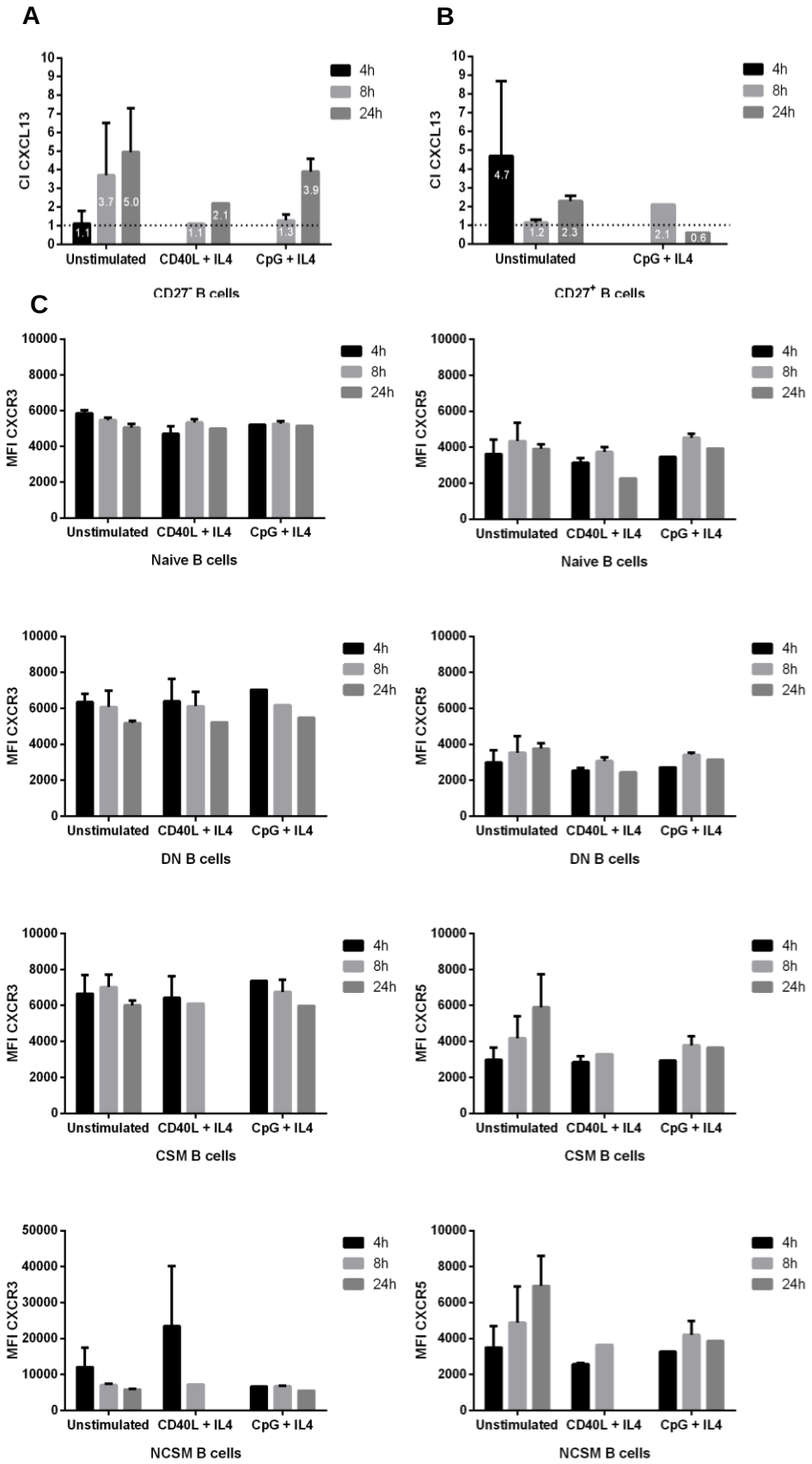


Figure 8. Influence of B cell stimulation on migration capacity of CD27⁻ and CD27⁺ B cells towards CXCL13. Isolated CD27⁻ (A) or CD27⁺ (B) B cells from HC with or without CD40L + IL-4 or CpG2006 + IL-4 stimulation were added to a transwell system for 4, 8 or 24 hours (n = 2). The chemotactic index was calculated as follows: (number of migrated cells with CXC13)/(number of migrated cells without CXCL13). (C) Using flow cytometry, the expression of the chemokine receptors CXCR3 and CXCR5 on stimulated or unstimulated naive, DN, CSM and NCSM B cells from HC was determined (n = 2). Values are represented as mean + SEM. CI: chemotactic index; MFI: mean fluorescence intensity; DN: double negative; CSM: class-switched memory; NCSM: non class-switched memory.

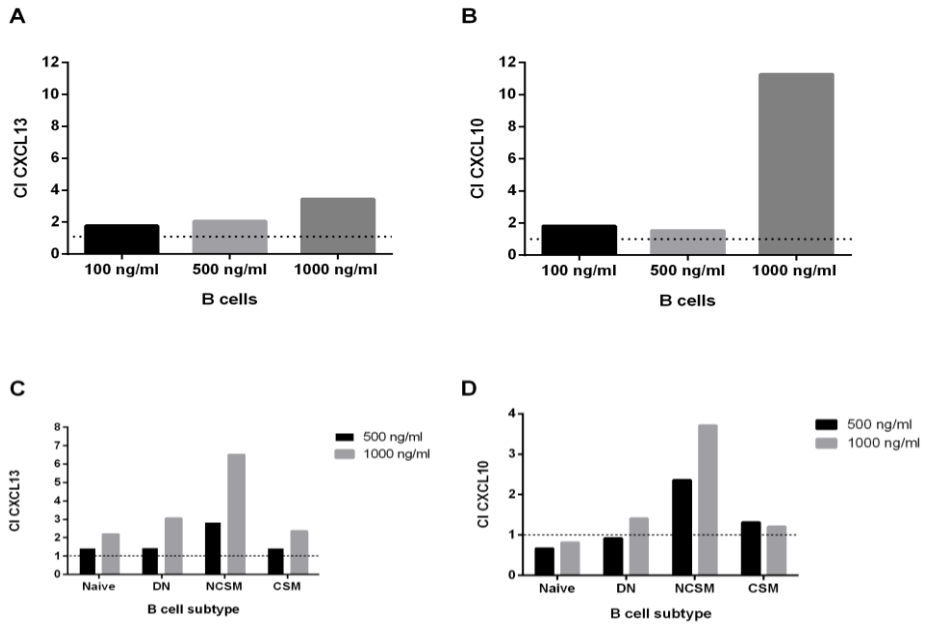


Figure 9. Determination of the optimal concentration of CXCL13 and CXCL10 for an *in vitro* chemotaxis assay. Pure B cells of 1 HC were added to the upper chamber of a transwell system and a concentration of 100 ng/ml, 500 ng/ml or 1000 ng/ml (A) CXCL13 or (B) CXCL10 was added to the lower chamber. Subsequently, isolated $CD27^-$ and $CD27^+$ B cells of 1 untreated RRMS patient were used to test a concentration of 500 ng/ml and 1000 ng/ml (C) CXCL13 and (D) CXCL10. The chemotactic index was calculated as previously described. CI: chemotactic index; DN: double negative; CSM: class-switched memory; NCSM: non class-switched memory.

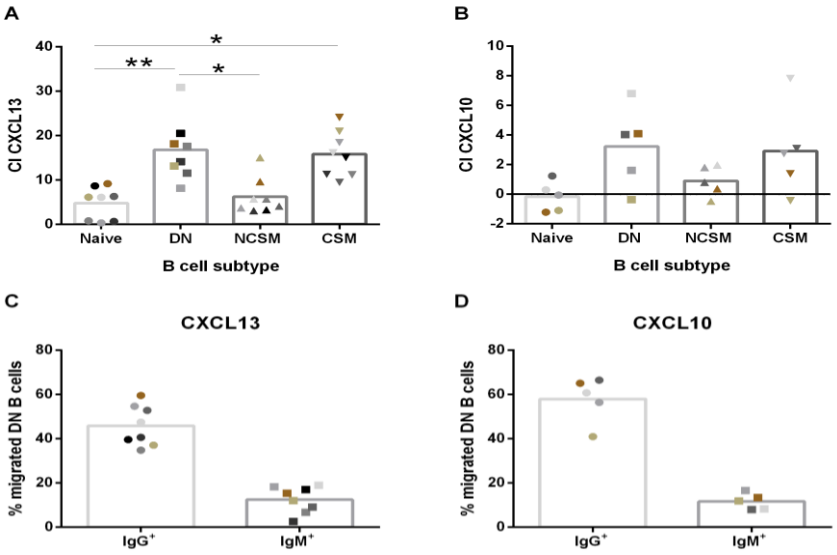


Figure 10. Migration capacity of naive, DN, NCSM and CSM B cells from untreated RRMS patients *in vitro*. Purified $CD27^-$ and $CD27^+$ B cells of untreated RRMS patients were added to the upper chamber of a transwell system in the presence or absence of (A) CXCL13 and (B) CXCL10 in the lower chamber (n = 8 and 5, respectively). The chemotactic index was calculated as follows: (number of migrated cells in the presence of a chemokine - number of migrated cells in the absence of a chemokine)/(original number of cells in the upper chamber).The percentage of IgG^+ and IgM^+ DN B cells was determined in the migrated fraction after migration towards (C) CXCL13 and (D) CXCL10 (n = 8 and 5, respectively). Each dot represents an individual patient sample. Statistical analysis was done using the Friedman test, followed by the post hoc Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$. CI: chemotactic index; DN: double negative; CSM: class-switched memory; NCSM: non class-switched memory.

3.5 B cell subtype proliferation and activation assay after different stimulations

B cells are well known for their role as (auto)antibody-producing plasma blasts and plasma cells in MS pathology (22). T-bet$^+$ ABCs of autoimmune-prone mice also show the ability to secrete autoantibodies after TLR7 stimulation (70). In addition, our research group has demonstrated that DN B cells of MS patients displayed a decreased CD21 expression compared to HC, suggesting an autoreactive nature since CD21^{low} B cells are described as unresponsive autoreactive B cells (66). Therefore, autoreactivity of DN B cells from MS patients was investigated by examining their proliferative response towards the myelin antigens MBP, MOG and bacterial or viral antigens TT and CMV. MOG and MBP are two major antigens located in the myelin sheath (71), whereas CMV is a herpes virus that also has been associated with MS as it shares a mimicry motif with MOG peptides in an EAE model of MS (72). Furthermore, since contradictory results were obtained on the activation potential of DN B cells, their activation status was analyzed after CpG2006 stimulation (32, 47, 73).

3.5.1 Incubation for 10 days with Tag-it Violet labeled cells is the most appropriate approach

First, this assay was optimized in two HC whereby the cell proliferation dye (CFSE or Tag-it Violet) and culture duration (7, 10 or 13 days) were determined. CD27$^-$ and CD27$^+$ B cells labeled with CFSE or Tag-it Violet were stimulated with TT (i.e. positive control) and incubated with irradiated PBMCs for 7, 10 or 13 days. CFSE labeled cells were compared to cells labeled with Tag-it Violet concerning cell viability, proliferation fraction (ΔPF) and activation status using flow cytometry (Supplemental Fig. 3). For both cell proliferation dyes, the number of viable CD27$^-$ and CD27$^+$ B cells in culture medium without stimulation (i.e. unstimulated condition) decreased with time resulting in the lowest cell number after 13 days (Fig. 11). However, there were more Tag-it Violet labeled CD27$^-$ B cells on day 7, 10 and 13 compared to CFSE labeled CD27$^-$ B cells (Fig. 11A). For the CD27$^+$ B cells, no significant differences were observed (Fig. 11B).

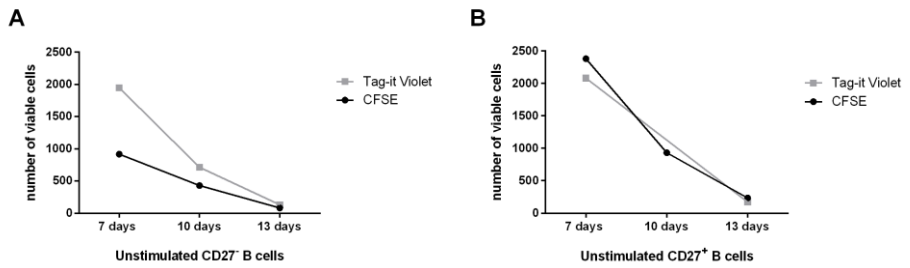


Figure 11. Viability of CFSE or Tag-it Violet labeled CD27$^-$ and CD27$^+$ B cells after 7, 10 or 13 days without antigenic stimulation. Unstimulated CD27$^-$ and CD27$^+$ B cells, isolated from PBMCs of HC (n = 2), were labeled with CFSE or Tag-it and incubated for 7, 10 or 13 days in culture medium. The viability of (A) CD27$^-$ B cells or (B) CD27$^+$ B cells is determined as the number of eFluor 506$^-$ CD19$^+$ B cells. Due to the insufficient number of B cells, not all conditions could be included. Mean levels are shown.

To determine the proliferative response of naive, DN, NCSM and CSM B cells towards TT, the ΔPF was determined for each B cell subset. In contrast to the NCSM and CSM B cells, the percentage of proliferating naive and DN B cells was higher in the Tag-it Violet labeled cells than the CFSE labeled cells (Fig. 12).

Furthermore, the proliferative response of DN B cells towards TT was the highest after 10 days of incubation and exceeded the threshold value (Fig. 12B), whereas naive and CSM B cells had a peak at 13 days (Fig. 12A and 12C).

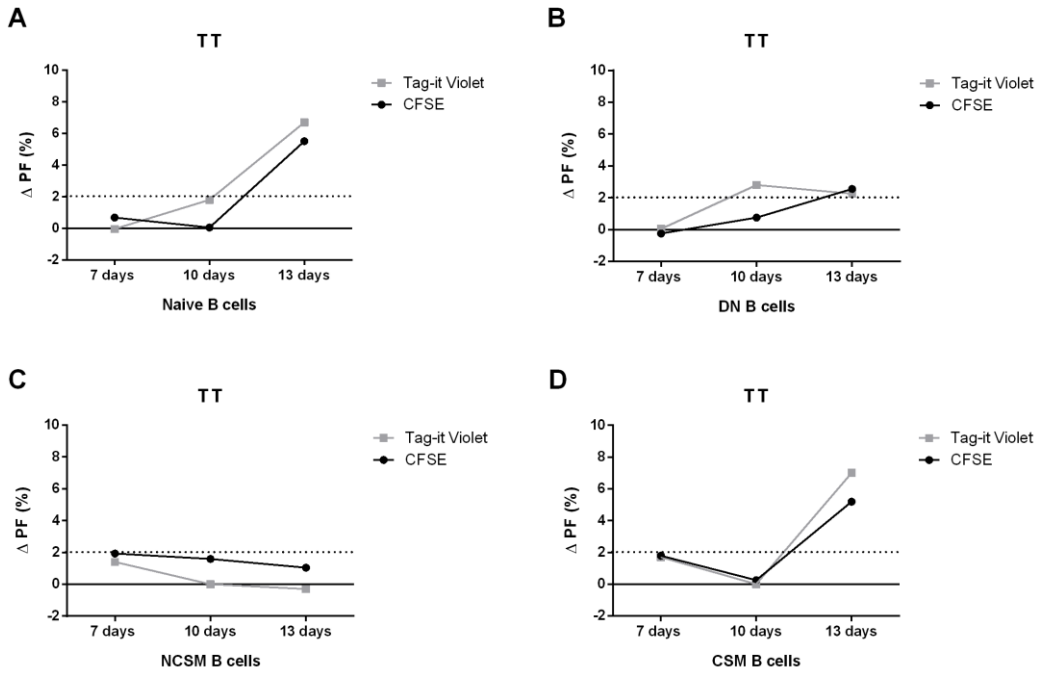


Figure 12. Proliferation of naive, DN, NCSM and CSM B cells towards the viral antigen TT after 7, 10 of 13 days of incubation. CFSE or Tag-it Violet labeled $CD27^-$ and $CD27^+$ B cells of HC (n = 2) were stimulated with TT and cultured for 7, 10 or 13 days. The percentage of proliferating (A) naive B cells (B) DN B cells, (C) NCSM B cells and (D) CSM B cells following TT stimulation (ΔPF) is shown and corrected for background proliferation (i.e. proliferation without antigenic stimulation). The cut-off for positive antigen-specific proliferation was set at ≥ 2% and indicated by the dashed line. Mean levels are shown, but not all conditions could be included for both donors due to the insufficient B cell number. DN: double negative; NCSM: non class-switched memory; CSM: class-switched memory; TT: tetanus toxoid.

The activation status of CFSE or Tag-it labeled $CD27^-$ and $CD27^+$ B cells was determined by analyzing the expression of CD80 and CD25 (both percentage positive cells and MFI). No significant differences could be found between CFSE and Tag-it labeled naive, DN, NCSM and CSM B cells concerning the activation status of the cells (Supplemental Fig. 4).

Together, these findings showed that Tag-it Violet labeling of $CD27^-$ B cells resulted in a higher viability and proliferative response against TT, compared to CFSE labeling. Taking into account that the number of viable $CD27^-$ and $CD27^+$ B cells was significantly decreased after 13 days and the best proliferative response of DN B cells towards TT was obtained at day 10, the optimal culture duration for DN B cells was determined to be 10 days.

3.5.2 DN B cells of HC do not proliferate in response to MS-related antigens

The B cell proliferation assay was further optimized by including the myelin antigens MOG, MBP, and the viral antigen CMV. Tag-it Violet labeled $CD27^-$ and $CD27^+$ B cells of HC (n = 3) were stimulated with CpG, TT, MOG, MBP and CMV or cultured alone in culture medium for 10 days.

DN and NCSM B cells of the three HC showed a clear proliferative response against TT (Fig. 13A) and 1/3 HC against MBP (Fig. 13B) and CMV (Fig. 13D), whereas no response could be observed towards the MOG autoantigen (Fig. 13C). Naive B cells of 1 HC responded towards TT (Fig. 13A), but for none of the three HC, naive B cells reacted against MBP, MOG or CMV (Fig. 13B-D). Furthermore, CSM B cells did not show a positive proliferative response to any antigenic stimulation (Fig. 13A-D). Although the percentage of proliferating DN B cells in HC usually did not exceed the threshold value for a positive proliferative response towards MS-related antigens, they showed mostly the highest ΔPF compared to naive, NCSM and CSM B cells.

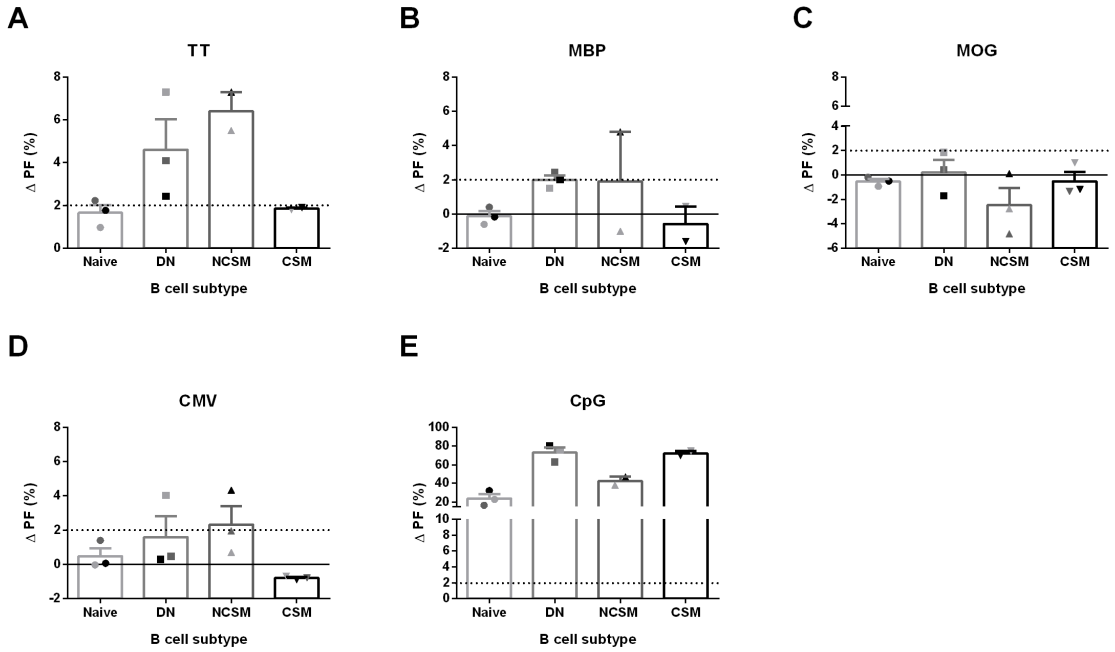


Figure 13. The proliferative response of naive, DN, NCSM and CSM B cells towards the MS-related antigens MBP, MOG and CMV. Tag-it Violet labeled $CD27^-$ and $CD27^+$ B cells of HC (n = 3) were stimulated with TT and the MS-related antigens MOG, MBP and CMV and cultured for 10 days in culture medium together with irradiated PBMCs. The percentage of proliferating naive, DN, NCSM and CSM B cells (ΔPF) following (A) TT stimulation, (B) MBP stimulation, (C) MOG stimulation and (D) CMV stimulation is depicted. The ΔPF was corrected for background proliferation (i.e. proliferation in the absence of antigenic stimulation). The cut-off for positive antigen-specific proliferation was set at ≥ 2% and indicated by the dashed line. Each symbol represents a HC sample. Mean levels + SEM are represented. Due to the insufficient B cell number, not all conditions for all HC could be included. DN: double negative; NCSM: non class-switched memory; CSM: class-switched memory; TT: tetanus toxoid; MBP: myelin basis protein; MOG: myelin oligodendrocyte glycoprotein; CMV: cytomegalovirus.

3.5.3 DN B cells of HC are activated following CpG stimulation

Although DN B cells of HC (n = 3) did not show a positive proliferative response towards the myelin antigens MBP, MOG, and the viral antigen CMV, they proliferated strongly after CpG2006 stimulation (Fig. 13E). Furthermore, the percentage of $CD80^+$ and $CD25^+$ DN B cells was strongly increased following CpG2006 stimulation compared to the memory B cell populations (Fig. 14A). Stimulating DN B cells with CpG2006 resulted in the highest CD80 and CD25 expression (MFI), in comparison with naive, NCSM and CSM B cells (Fig. 14B). Together, the above results not only demonstrate that DN B cells of HC have the ability to proliferate and be activated in response to CpG2006 stimulation,

but also that they are most strongly activated by CpG2006 stimulation as they showed the most positive proliferative response and highest expression (MFI) of the activation markers CD80 and C25.

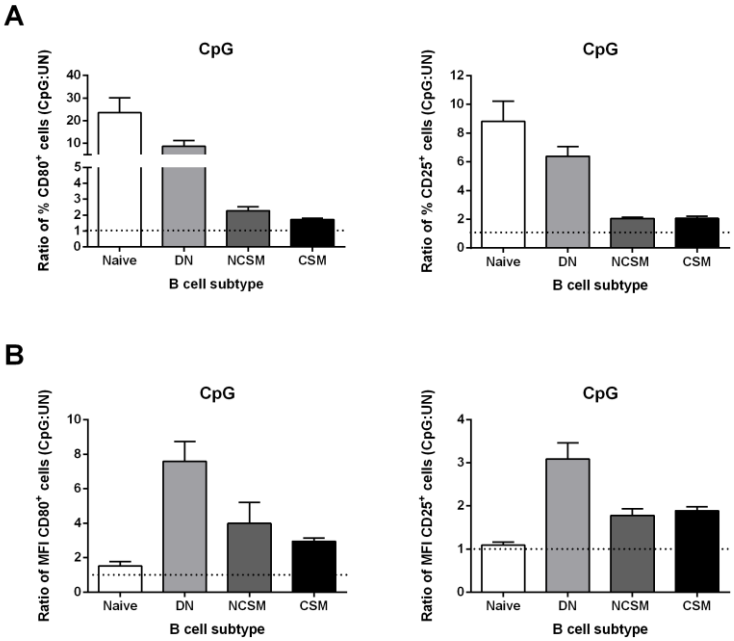


Figure 14. Activation potential of naive, DN, NCSM and CSM B cells after CpG stimulation. $CD27^-$ and $CD27^+$ B cells of HC (n = 3) were stimulated with CpG2006 and cultured for 10 days in culture medium together with irradiated PBMC. Expression levels of activation markers were assessed via flow cytometry. The ratios of (A) the percentage or (B) MFI within $CD80^+$ and $CD25^+$ naive, DN, NCSM and CSM B cells are shown in which the expression in the unstimulated condition is set at 1. Mean levels + SEM are depicted. Due to the insufficient B cell number, not all conditions for all HC could be included. DN: double negative; NCSM: non class-switched memory; CSM: class-switched memory.

4 Discussion

B cells have become increasingly recognized as central players in MS pathogenesis, both by production of (auto)antibodies and by antibody-independent functions, including the stimulation of pro-inflammatory T cell responses via cytokine production, costimulation and antigen presentation (22). Premature immune aging has been evidenced in a proportion of MS patients and is thought to contribute to MS pathogenesis and progression (39). Moreover, it is shown that the humoral immune system is affected by immune aging. Consequently, $IgD^{-}CD27^{-}$ DN B cells were identified as a B cell subtype that is related to an aged immune system (45). Furthermore, DN B cells have also been described during viral infections (48, 49) and in autoimmune diseases (32, 50). Novel findings in different mouse models and autoimmune diseases also suggested a role for T-bet^{+} B cells, with similar characteristics as DN B cells, in autoimmune responses (55). Studies of Palanichamy *et al.* and Colonna-Romano *et al.* suggested that DN B cells could migrate towards inflammatory sites as a clonal relation was described between peripheral and intrathecal DN B cells and expression of inflammatory chemokine receptors on DN B cells from HC was evidenced (64, 65). However, literature is limited on the role of DN B cells in MS pathogenesis. Therefore our research group recently investigated this ABC subtype in more detail (46). An increased proportion of young MS patients (< 60 years) showed peripheral expansions of DN B cells compared with age-matched HC . These cells were further increased in the CSF of MS patients and displayed pro-inflammatory characteristics.

In this study, we have demonstrated via flow cytometry that a proportion of DN B cells from HC, RRMS patients and SPMS patients express the transcription factor T-bet, whereby the expression levels were higher compared to naive and NCSM B cells. Besides, surface expression of the chemokine receptors CXCR3 and CXCR5 was observed on DN B cells from HC, RRMS patients and SPMS patients. We also indicated the migration capacity of DN B cells from untreated RRMS patients towards the pro-inflammatory chemokines CXCL13 and CXCL10 *in vitro* by performing a functional *in vitro* chemotaxis assay. Moreover, in comparison with the other B cell subtypes, DN B cells showed a higher migration capacity. Although DN B cells of HC did not show a positive proliferative response towards the myelin antigens MBP and MOG and the viral antigen CMV, we found that they have the ability to proliferate strongly following CpG2006 stimulation and to be activated as well.

To exclude any type of bias from our results, only untreated patients were included to avoid direct or indirect effects of the therapy. If patients were already treated, only patients who were previouosly treated with a first-line therapy with a wash-out period of one month were eligible for this study. Although a gender bias was present in the patient and HC groups, it was shown by a previous study from our research group that no differences were present in DN B cell frequencies of male and female subjects (46).This could be confirmed in our study population, in which also no differences were found (data not shown).

Our results related to the expression of T-bet in different B cell subsets, have shown that DN B cells from untreated RRMS patients (20.1%), SPMS patients (24.7%) and HC (23.1%) were T-bet$^+$ and that their expression levels were the highest and significantly increased compared to naive and NCSM B cells in all three study groups. A significant difference in T-bet expression (MFI) was also found between the RRMS patients, SPMS patients and HC. Since T-bet expression has already been described as a characteristic of ABCs in aged and autoimmune-prone mice as well as in autoimmune diseases (53, 62), the high expression of T-bet in DN B cells is an additional proof that DN B cells are an aged B cell population. The present data also suggest that this transcriptional regulator may have an important role in DN B cell functioning, as it is shown in mice that T-bet contributes to functional processes in B cells such as class-switching to IgG2a antibodies, secretion of autoantibodies and antigen-presenting functions (55, 62). Therefore, the high T-bet expression (MFI) that was observed in RRMS patients indicates a possible role for DN B cells in autoantibody production in MS pathogenesis as ABCs in mice can produce autoantibodies after TLR9 and IFN-γ stimulation and depletion of ABCs in autoimmune-prone mice causes reduces autoantibody production (53, 55) In addition, DN B cells may have antigen-presenting features as this has already been demonstrated in mouse models (62). Moreover, our research group has shown on DN B cells expression of antigen presentation molecules (HLA-DR/DP/DQ) that was similar to CSM B cells and costimulatory molecules (CD80/CD86) that was intermediate between naive and CSM B cells (46).

A recent study by Frasca *et al.* has analyzed T-bet expression in distinct B cell subtypes from human HC, including IgD$^-$CD27$^-$ DN, IgD$^+$CD27$^-$ naive and IgD$^-$CD27$^+$ CSM B cells (63). In accordance with our data, they have demonstrated an average percentage T-bet$^+$ DN B cells of 20% in HC that was higher compared to naive and CSM B cells. However, we did not observe a significant difference in T-bet expression between DN and CSM B cells. The significant difference indicated by Frasca *et al.* is probably due to the small sample number (n = 4), while we have included 15 HC in total. Another previous study by Knox *et al.* has examined the expression of T-bet in PBMC from human HC (74). They have focused on naive (IgD$^+$CD27$^-$), memory (IgD$^-$CD10$^-$CD38$^{lo/-}$), transitional (IgD$^+$, CD10$^+$CD38$^+$CD27$^-$) B cells and plasmablasts (IgD$^-$CD10$^-$CD38^{hi}CD27$^+$). Similar to this paper, we found in the total B cell population of HC 10% of T-bet$^+$ cells (n = 10). Furthermore, they also observed the highest T-bet expression levels in the memory B cell subset, that consisted in their study of both DN and CSM B cells as they did not make a distinction based on CD27 expression. More than 15% of memory B cells expressed T-bet and a significant reduced expression was found in the other B cell subtypes. In RRMS patients, expression levels of T-bet in CD4$^+$ and CD8$^+$ T cells are elevated during relapses compared to patients in remission and HC (60). However, not many studies have investigated T-bet expression in circulating B cells of MS patients. Frisullo *et al.* has evaluated the expression of T-bet (MFI) in total B cells of RRMS patients with and without relapses (n = 15 for each group) and compared the expression with age- and gender matched HC (n = 20) (60). Similarly to our data, they could not find a difference in MFI values within the total T-bet$^+$ B cell population between RRMS patients and HC. Based on these previous studies and the current knowledge about the role of T-bet in autoimmunity, an increased expression of T-bet in DN B cells from MS patients was expected. In mice, it is shown that B cells express high levels of T-bet via TLR9 or IFN-γ signaling.

Therefore, it is tempting to speculate that T-bet expression may be triggered in DN B cells of MS patients as MS is associated with viral pathogens that can bind to TLR9 and the pathology is characterized by autoreactive Th1 cells that are important producers of IFN-γ (56, 75, 76).

Using flow cytometry, we have shown that DN B cells from RRMS patients, SPMS patients and HC express the chemokine receptors CXCR3 and CXCR5. Bulati *et al.* has also studied the trafficking phenotype of $IgD^{-}CD27^{-}$ DN B cells via flow cytometry and compared that to the $IgD^{+}CD27^{-}$ naive, $IgD^{+}CD27^{+}$ NCSM and $IgD^{-}CD27^{+}$ CSM B cells in young (25-40 years) and aged (78-90 years) HC (65). They have shown that NCSM and DN B cells from young HC have an increased expression of CXCR3 compared to the other B cell subtypes. We have not included NCSM B cells in our analysis, but we demonstrated that the percentage of $CXCR3^{+}$ DN B cells was significantly increased compared to naive B cells in HC. Furthermore, Bulati *et al.* reported that DN B cells from both young and aged HC expressed CXCR5, although it was very low (65).In agreement with these results, we also observed reduced CXCR5 expression (% and MFI) on DN B cells compared to naive and CSM B cells, although the percentage of $CXCR5^{+}$ DN cells and MFI within the $CXCR5^{+}$ DN B cells were still higher than for CXCR3. Together, these data suggests a role for the chemokines CXCL10 and CXCL13 in the migration of DN B cells towards the CNS. Therefore, the next step was to functionally test the migration capacity of DN B cells towards these chemokines.

In order to perform functional assays, an appropriate isolated method to purify $CD27^{-}$ and $CD27^{+}$ B cells from PBMC was needed. Therefore, different isolation techniques were tested and compared with each other concerning purity, viability, activation status and yield. Since DN B cells usually make up < 10% of all B cells, FACS sorting of $CD27^{-}$ and $CD27^{+}$ B cells could not be used as it resulted in a lot of cell loss. Another approach consisting of a negative magnetic B cell isolation kit followed by positive magnetic CD27 microbeads showed a higher yield but a reduced purity compared to FACS sorting. Subsequently, a new, recent developed memory B cell isolation kit from STEMCELL™ Technologies was examined. This method provided pure $CD27^{-}$ and $CD27^{+}$ B cell populations by combining negative and positive magnetic selection in 1 protocol, although the yield was decreased. However, we could increase the number of $CD27^{-}$ and $CD27^{+}$ B cells by starting with a larger amount of PBMC by requesting more blood per donor. Although the threshold value for the starting material was 100 million PBMC, the isolation method could be repeated to increase the yield. Additionally, only donors with high percentages of B cells in their blood circulation were included to increase the chance of sufficient yield. Thus, since there were some options to increase the yield, the memory B cell isolation kit from STEMCELL™ Technologies was further used to isolate $CD27^{-}$ and $CD27^{+}$ B cells in our experiments.

Functional analysis of the migration capacity of naive, DN, NCSM and CSM B cells via an *in vitro* chemotaxis assay showed that DN B cells of untreated RRMS patients had a significantly higher migration capacity towards CXCL13 compared to naive and NCSM B cells. Migration of DN B cells towards CXCL10 was also higher than that of naive and NCSM B cells, nonetheless more patients need to be studied in order to reach statistical significance. These findings suggest that DN B cells of RRMS patients have the capacity to migrate towards pro-inflammatory sites where high levels of CXCL10 and CXCL13 are present.

These results were in agreement with our expectations as we demonstrated the expression of the chemokine receptors CXCR5 and CXCR3 on DN B cells to which CXCL13 and CXCL10 can bind. However, the expression levels of CXCR5 and CXCR3 on the different B cell subsets in RRMS patients did not correlate with their migration capacity towards CXCL10 and CXCL13. Although there was no significant difference between the CXCR3 expression (% and MFI) on naive and DN B cells, we showed a higher CI for DN B cells towards CXCL10. This means that despite similar expression levels, DN B cells had a higher migration capacity compared to naive B cells. On the other hand, CXCR5 expression (% and MFI) on naive B cells was significantly increased compared to DN B cells in RRMS patients, which suggested a higher migration capacity of naive B cells towards CXCL13. Nevertheless, we showed the opposite. However, patient samples that were used for the chemotaxis assay were not the same as those for the flow cytometric analysis. Therefore, the samples used in the chemotaxis assay will still be analyzed for CXCR3 and CXCR5 expression in order to compare the CI and chemokine receptor expression levels with each other. In addition, we reported that most of the migrated DN B cells were IgG^+. Since we did not measure the IgG and IgM levels in the original $CD27^-$ or $CD27^+$ B cell population that was added to the upper chamber, we could not calculate the CI for IgG^+ and IgM^+ DN B cells. This finding was therefore based on the percentage of IgG^+ and IgM^+ DN B cells in the migration fraction, demonstrating that IgG^+ DN B cells are prominently present in the migrated cells. A study from Haas *et al.* compared the distribution of different B cell subsets (naive, DN, NCSM, CSM, plasma blasts and plasma cells) in the peripheral blood and CSF of RRMS patients (33). Additionally, they performed a chemotaxis assay in order to assess the migration capacity of these cell populations towards CSF samples in which high levels of CXCL13 are present. Their results indicated that the expansion of CSM B cells in the CSF of RRMS patients was linked to elevated CXCL13 levels in CSF, by analyzing the CI of the different B cell subtypes towards the CSF whereby CSM B cells showed the highest CI. Nonetheless, the CI was thereby defined as the ratio of percentages of the distinct B cell subpopulations in the migrated PBMC and original PBMC, which differs from our calculation. We have also taken into account the background migration (i.e. the number of cells that migrate without chemokine). However, in our first definition (CI: number of migrated cells in the presence of CXCL13 and in the absence of CXCL13) that was used for the optimization steps, we also did not include the original number of each B cell subtype. Consequently, the CI of naive and NCSM B cells was higher than expected as memory B cells migrated more in the absence of chemokine, which resulted in a lower CI. Therefore, to avoid misinterpreted results, the CI was recalculated to: number of migrated subtype cells in the presence of a chemokine - number of migrated subtype cells in the absence of a chemokine)/(original number of subtype cells in the upper chamber). Together, the expression of CXCR3 and CXCR5 on DN B cells, the *in vitro* migration capacity of DN B cells towards CXCL13 and CXCL10 in RRMS patients and the elevated frequencies of DN B cells in the CSF of MS patients (46) suggest that DN B cells can migrate towards the CSF and thus could exert their pro-inflammatory functions very locally. We could confirm this speculation in the future by performing a chemotaxis assay with CSF instead of medium supplemented with chemokines, which is similar to the experiment from Haas *et al.*, although a more correct CI would be used.

Results from the *in vitro* B cell subtype proliferation assay showed for DN and NCSM B cells a positive proliferative response to TT (3/3 HC), MBP (1/3 HC) and CMV (1/3 HC). In contrast, naive B cells responded only towards TT (1/3) and CSM B cells did not show a positive proliferative response to any antigenic stimulation. Since TT was included in this assay as a positive control, we expected for the CSM B cells a positive proliferative response towards TT as well. CSM B cells are antigen-experienced memory cells that can exert an enhanced response after re-encountering the pathogen. To increase the chance of detecting a proliferative response to TT, only HC that received a tetanus vaccination in the last 8 years were included for this study. Therefore, we expected that some CSM B cells would recognize the antigen. Nevertheless, no response to TT was observed after 10 days. A possible problem in the experimental setup could be the low number of cells that were used (100,000 cells per well), because the frequency of TT-encountered CSM B cells could be very low and therefore below the detection limit of our assay. On the other hand, during the optimization of the culture duration, we demonstrated that the optimal culture duration for DN B cells was 10 days. However, the proliferative response of CSM B cells exceeded the threshold value only after 13 days. As a result, a longer culture duration could be necessar for CSM B cells to detect a positive response. Yet, if the culture duration would be extended, the number of viable $CD27^-$ and $CD27^+$ B cells would decline significantly, making it more difficult to reach the detection limit. Therefore, this experiment must be repeated for $CD27^-$ and $CD27^+$ B cells that are cultured with CpG, TT, MOG, MBP or CMV for 13 days. Alternatively, CSM B cells might not be involved in the TT response. Since we have shown that CSM B cells of MS patients express T-bet, which can be induced via TLR9 signaling, it is expected that these cells can be activated via pathogens (76). The role of T-bet expression in anti-viral immunity was shown in mice, in which T-bet^+ ABCs were expanded and produced high levels of IgG2a antibodies following viral infections (56). Therefore, it seems unlikely that CSM B cells will not respond towards TT or other viral antigens.

Stimulating DN B cells with CpG2006 for 10 days resulted in the strongest proliferative response and highest expression (MFI) of the activation markers CD80 and CD25 compared to naive, NCSM and CSM B cells; although more HC and MS patients need to be tested to reach statistical significance. Nevertheless, these results are in agreement with findings of T-bet^+ ABCs in mice. Liu *et al.* has already shown that CpG2006 induced T-bet expression in mice (76). Therefore, as we have demonstrated that DN B cells of MS patients express T-bet, a positive response towards CpG2006 stimulation was expected. In spite of that, some research groups have reported that DN B cells are not activated via CpG2006 stimulation. Colonna-Romano *et al.* stimulated anti-Ki6 labeled PBMC with CpG2006 for 5 days and showed that there was no significant proliferation of CpG2006 stimulated DN B cells in HC (47). They indicated that BCR costimulation was needed to have a positive proliferative response. However, they used another cell proliferation dye and the whole PBMC population, which is different from our experimental setup. Another study from the research group of Colonna-Romano have reported the proliferation of purified anti-Ki67 labeled B cells after CpG2006 stimulation for 3 days (73). In this paper, they found that DN B cells were strongly activated in young HC (25-40 years old), which is in agreement with our data. Nevertheless, they used 3 µg/ml CpG2006, while we added 2 µg/ml CpG2006. Wei *et al.* cultured CFSE labeled $CD10^-CD27^-IgD^-$ DN B cells with CpG2006 for 4 days (32). Similarly to our results, they indicated a positive proliferative response of DN B cells after CpG2006 stimulation, which was similar to memory B cells.

In addition, Isnardi *et al.* have investigated $CD21^{low}$ B cells in RA patients and indicated that these cells are mostly unresponsive clones that had a decreased expression of CD25 after CpG2006 stimulation (66). Our research group previously reported that DN B cells of MS patients have a low CD21 expression (46). Nevertheless, our data do not indicate that DN of MS patients are unresponsive clones. Even though there are contradictory results concerning the activation potential of DN B cells, we showed a strong activation of DN B cells following CpG2006 stimulation. These finding indicates that DN B cells are no terminally differentiated cells.

5 Conclusion and future research

This study aimed to elucidate how DN B cells could contribute to the pathogenesis of MS. We thereby hypothesized that $IgD^{-}CD27^{-}$ DN B cells contribute to neuroinflammation in a proportion of MS patients through autoreactive and pro-inflammatory functions regulated via T-bet expression. First the expression of the transcription factor T-bet was examined in $IgD^{+}CD27^{-}$ naive, $IgD^{-}CD27^{-}$ DN, $IgD^{-}CD27^{+}$ CSM and $IgD^{+}CD27^{+}$ NCSM B cells using intracellular flow cytometry. Results of this assay showed that T-bet^{+} DN B cells can be found in untreated RRMS patients, SPMS patients and HC, with a statistical significant difference between the different study groups. Moreover, expression of T-bet (MFI) in DN B cells is the highest compared to naive, NCSM and CSM B cells. Further research should be focused on investigating the expression of T-bet via real-time PCR.

Furthermore, the expression of the chemokine receptors CXCR3 and CXCR5 was studied on DN, naive, NCSM and CSM B cells. We found that DN B cells of RRMS patients, SPMS patients and HC express the chemokine receptor CXCR3 and to a greater extent also CXCR5. However, no elevated expression levels (% and MFI) of CXCR3 and CXCR5 on DN B cells could be observed in RRMS and SPMS patients. These results suggest a role for the chemokines CXCL10 and CXCL13 in the migration of DN B cells towards the CNS. Therefore, the next step was to functionally test the migration capacity of DN B cells towards these chemokines. An *in vitro* chemotaxis assay was performed to investigate the migration capacity of DN, naive, NCSM and CSM B cells from untreated RRMS patients. Results of these assay indicated that DN B cells of untreated RRMS patients can migrate towards CXCL13 and CXCL10 *in vitro* and show a higher migration capacity for CXCL13 compared to naive and NCSM B cells. In a next step, we want to include age- and gender matched HC to compare them with the results already found in RRMS patients.

Finally, the autoreactivity of the naive, DN, CSM and NCSM B cells towards different autoantigens that are related to MS pathogenesis was examined via an *in vitro* B cell subtype proliferation assay. In addition, the activation status of these B cell subsets was analyzed after CpG stimulation. From these experiments, we could conclude that although DN B cells of HC did not show a positive proliferative response towards the myelin antigens MBP, MOG, and the viral antigen CMV, they proliferated strongly after CpG2006 stimulation. Furthermore, the percentage of $CD80^{+}$ and $CD25^{+}$ DN B cells was strongly increased following CpG2006 stimulation compared to the memory B cell populations.

In conclusion, the achieved results in this study can only partly confirm our hypothesis, as we have shown that DN B cells can express T-bet and migrate towards pro-inflammatory sites. Nevertheless, the B cell subtype proliferation assays need some more optimization. Therefore, more research is needed.

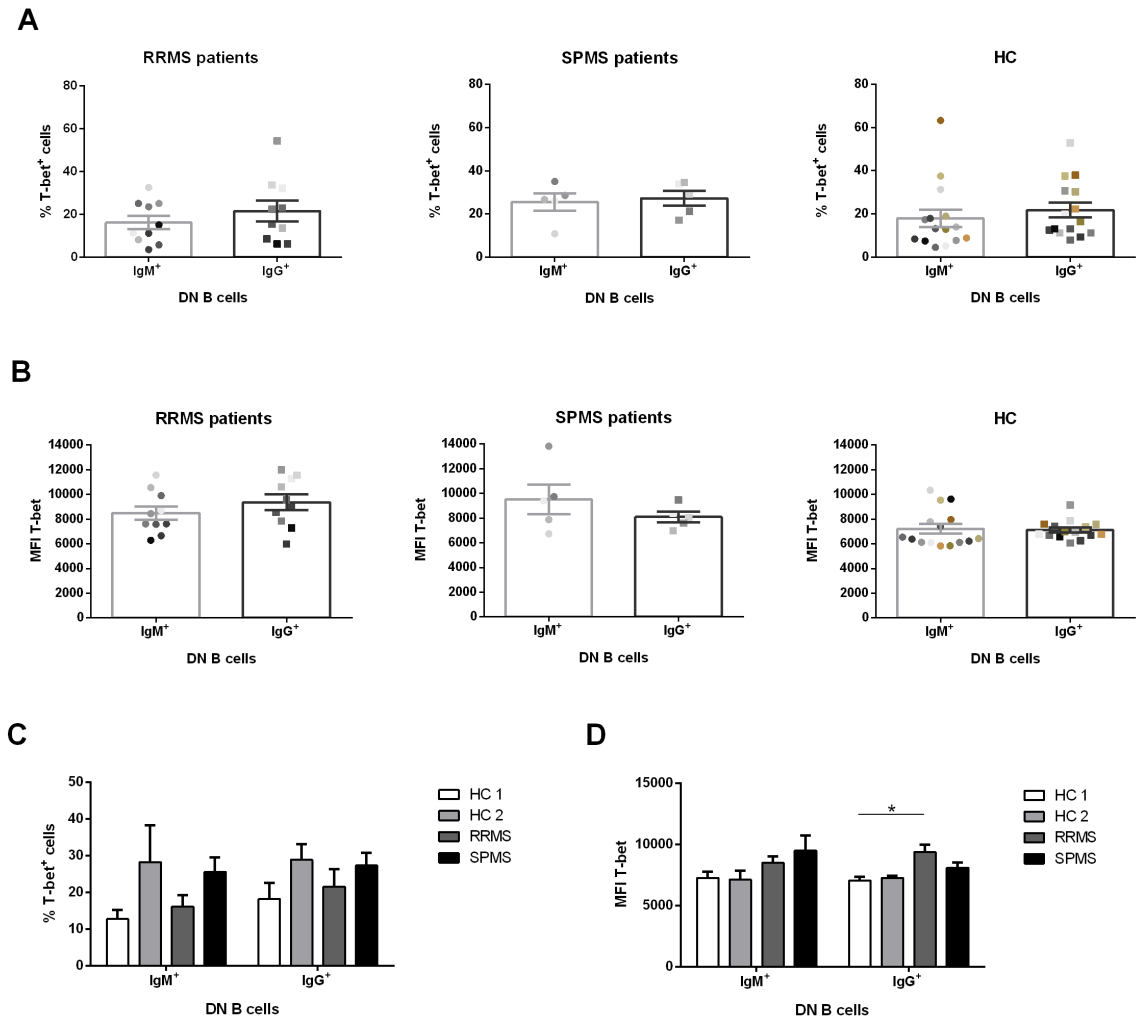
References

1. Høglund RA, Maghazachi AA. Multiple sclerosis and the role of immune cells. World J Exp Med. 2014;4(3):27-37.
2. Federation MSI. Atlas of MS London, United Kingdom2013 [Available from: https://www.msif.org/about-us/advocacy/atlas/.
3. Ascherio A, Munger KL. Epidemiology of Multiple Sclerosis: From Risk Factors to Prevention-An Update. Semin Neurol. 2016;36(2):103-14.
4. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. Lancet Neurol. 2005;4(5):281-8.
5. Bishop M, Rumrill PD. Multiple sclerosis: Etiology, symptoms, incidence and prevalence, and implications for community living and employment. Work. 2015;52(4):725-34.
6. Hunter SF. Overview and diagnosis of multiple sclerosis. Am J Manag Care. 2016;22(6 Suppl):s141-50.
7. Giffroy X, Maes N, Albert A, Maquet P, Crielaard JM, Dive D. Multimodal evoked potentials for functional quantification and prognosis in multiple sclerosis. BMC Neurol. 2016;16:83.
8. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 2018;17(2):162-73.
9. Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-17.
10. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. J Autoimmun. 2015;64:13-25.
11. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med. 2007;357(9):851-62.
12. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214-9.
13. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. JAMA. 2006;296(23):2832-8.
14. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol. 2007;61(4):288-99.
15. Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA. 2005;293(20):2496-500.
16. Hawkes CH. Smoking is a risk factor for multiple sclerosis: a metanalysis. Mult Scler. 2007;13(5):610-5.
17. Glatigny S, Bettelli E. Experimental Autoimmune Encephalomyelitis (EAE) as Animal Models of Multiple Sclerosis (MS). Cold Spring Harb Perspect Med. 2018.
18. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nat Rev Immunol. 2015;15(9):545-58.
19. Chastain EM, Miller SD. Molecular mimicry as an inducing trigger for CNS autoimmune demyelinating disease. Immunol Rev. 2012;245(1):227-38.
20. Cole SR, Beck RW, Moke PS, Kaufman DI, Tourtellotte WW. The predictive value of CSF oligoclonal banding for MS 5 years after optic neuritis. Optic Neuritis Study Group. Neurology. 1998;51(3):885-7.
21. Awad A, Hemmer B, Hartung HP, Kieseier B, Bennett JL, Stuve O. Analyses of cerebrospinal fluid in the diagnosis and monitoring of multiple sclerosis. J Neuroimmunol. 2010;219(1-2):1-7.
22. Claes N, Fraussen J, Stinissen P, Hupperts R, Somers V. B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions. Front Immunol. 2015;6:642.
23. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, Gentleman SM, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain. 2011;134(Pt 9):2755-71.
24. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain. 2007;130(Pt 4):1089-104.
25. Michel L, Touil H, Pikor NB, Gommerman JL, Prat A, Bar-Or A. B Cells in the Multiple Sclerosis Central Nervous System: Trafficking and Contribution to CNS-Compartmentalized Inflammation. Front Immunol. 2015;6:636.
26. Blauth K, Owens GP, Bennett JL. The Ins and Outs of B Cells in Multiple Sclerosis. Front Immunol. 2015;6:565.

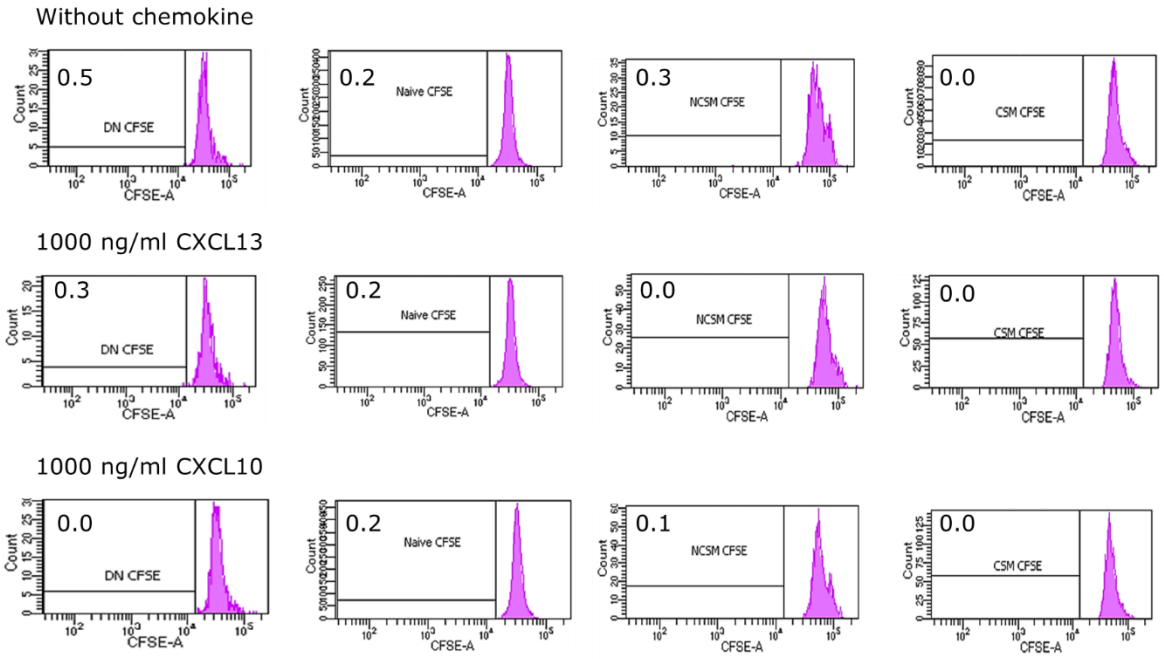
27. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008;358(7):676-88.
28. Hauser SL, Bar-Or A, Comi G, Giovannoni G, Hartung HP, Hemmer B, et al. Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. N Engl J Med. 2017;376(3):221-34.
29. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. N Engl J Med. 2017;376(3):209-20.
30. Fraussen J, Claes N, Van Wijmeersch B, van Horssen J, Stinissen P, Hupperts R, et al. B cells of multiple sclerosis patients induce autoreactive proinflammatory T cell responses. Clin Immunol. 2016;173:124-32.
31. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. J Allergy Clin Immunol. 2013;131(4):959-71.
32. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. J Immunol. 2007;178(10):6624-33.
33. Haas J, Bekeredjian-Ding I, Milkova M, Balint B, Schwarz A, Korporal M, et al. B cells undergo unique compartmentalized redistribution in multiple sclerosis. J Autoimmun. 2011;37(4):289-99.
34. Mahad DJ, Howell SJ, Woodroofe MN. Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis. J Neurol Neurosurg Psychiatry. 2002;72(4):498-502.
35. Alter A, Duddy M, Hebert S, Biernacki K, Prat A, Antel JP, et al. Determinants of human B cell migration across brain endothelial cells. J Immunol. 2003;170(9):4497-505.
36. Niino M, Bodner C, Simard ML, Alatab S, Gano D, Kim HJ, et al. Natalizumab effects on immune cell responses in multiple sclerosis. Ann Neurol. 2006;59(5):748-54.
37. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011;69(2):292-302.
38. Cayrol R, Wosik K, Berard JL, Dodelet-Devillers A, Ifergan I, Kebir H, et al. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. Nat Immunol. 2008;9(2):137-45.
39. Thewissen M, Linsen L, Somers V, Geusens P, Raus J, Stinissen P. Premature immunosenescence in rheumatoid arthritis and multiple sclerosis patients. Ann N Y Acad Sci. 2005;1051:255-62.
40. Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, Pellicanò M, et al. B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans. Ageing Res Rev. 2011;10(2):274-84.
41. Bulati M, Caruso C, Colonna-Romano G. From lymphopoiesis to plasma cells differentiation, the age-related modifications of B cell compartment are influenced by "inflamm-ageing". Ageing Res Rev. 2017;36:125-36.
42. Hakim FT, Gress RE. Immunosenescence: deficits in adaptive immunity in the elderly. Tissue Antigens. 2007;70(3):179-89.
43. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, et al. B-cell diversity decreases in old age and is correlated with poor health status. Aging Cell. 2009;8(1):18-25.
44. Chong Y, Ikematsu H, Yamaji K, Nishimura M, Kashiwagi S, Hayashi J. Age-related accumulation of Ig V(H) gene somatic mutations in peripheral B cells from aged humans. Clin Exp Immunol. 2003;133(1):59-66.
45. Montoya-Ortiz G. Immunosenescence, aging, and systemic lupus erythematous. Autoimmune Dis. 2013;2013:267078.
46. Claes N, Fraussen J, Vanheusden M, Hellings N, Stinissen P, Van Wijmeersch B, et al. Age-Associated B Cells with Proinflammatory Characteristics Are Expanded in a Proportion of Multiple Sclerosis Patients. J Immunol. 2016;197(12):4576-83.
47. Colonna-Romano G, Bulati M, Aquino A, Pellicanò M, Vitello S, Lio D, et al. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. Mech Ageing Dev. 2009;130(10):681-90.
48. Rojas OL, Narváez CF, Greenberg HB, Angel J, Franco MA. Characterization of rotavirus specific B cells and their relation with serological memory. Virology. 2008;380(2):234-42.
49. Rinaldi S, Pallikkuth S, George VK, de Armas LR, Pahwa R, Sanchez CM, et al. Paradoxical aging in HIV: immune senescence of B Cells is most prominent in young age. Aging (Albany NY). 2017;9(4):1307-25.
50. Mahmood Z, Muhammad K, Schmalzing M, Roll P, Dörner T, Tony HP. CD27-IgD- memory B cells are modulated by in vivo interleukin-6 receptor (IL-6R) blockade in rheumatoid arthritis. Arthritis Res Ther. 2015;17:61.
51. Wirths S, Lanzavecchia A. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. Eur J Immunol. 2005;35(12):3433-41.
52. Wu YC, Kipling D, Dunn-Walters DK. The relationship between CD27 negative and positive B cell populations in human peripheral blood. Front Immunol. 2011;2:81.

53. Rubtsov AV, Rubtsova K, Fischer A, Meehan RT, Gillis JZ, Kappler JW, et al. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c^{+} B-cell population is important for the development of autoimmunity. Blood. 2011;118(5):1305-15.
54. Hao Y, O'Neill P, Naradikian MS, Scholz JL, Cancro MP. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. Blood. 2011;118(5):1294-304.
55. Rubtsov AV, Marrack P, Rubtsova K. T-bet expressing B cells - Novel target for autoimmune therapies? Cell Immunol. 2017;321:35-9.
56. Rubtsova K, Rubtsov AV, van Dyk LF, Kappler JW, Marrack P. T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. Proc Natl Acad Sci U S A. 2013;110(34):E3216-24.
57. Domeier PP, Chodisetti SB, Soni C, Schell SL, Elias MJ, Wong EB, et al. IFN-γ receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity. J Exp Med. 2016;213(5):715-32.
58. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. Proc Natl Acad Sci U S A. 2002;99(8):5545-50.
59. Wang Z, Wang J, Diao Y, Qian X, Zhu N. T-bet-Expressing B Cells Are Positively Associated with Crohn's Disease Activity and Support Th1 Inflammation. DNA Cell Biol. 2016;35(10):628-35.
60. Frisullo G, Nociti V, Iorio R, Patanella AK, Marti A, Cammarota G, et al. Increased expression of T-bet in circulating B cells from a patient with multiple sclerosis and celiac disease. Hum Immunol. 2008;69(12):837-9.
61. Rubtsova K, Rubtsov AV, Thurman JM, Mennona JM, Kappler JW, Marrack P. B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. J Clin Invest. 2017;127(4):1392-404.
62. Rubtsov AV, Rubtsova K, Kappler JW, Jacobelli J, Friedman RS, Marrack P. CD11c-Expressing B Cells Are Located at the T Cell/B Cell Border in Spleen and Are Potent APCs. J Immunol. 2015;195(1):71-9.
63. Frasca D, Diaz A, Romero M, D'Eramo F, Blomberg BB. Aging effects on T-bet expression in human B cell subsets. Cell Immunol. 2017;321:68-73.
64. Palanichamy A, Apeltsin L, Kuo TC, Sirota M, Wang S, Pitts SJ, et al. Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis. Sci Transl Med. 2014;6(248):248ra106.
65. Bulati M, Buffa S, Martorana A, Candore G, Lio D, Caruso C, et al. Trafficking phenotype and production of granzyme B by double negative B cells (IgG(+)IgD(-)CD27(-)) in the elderly. Exp Gerontol. 2014;54:123-9.
66. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood. 2010;115(24):5026-36.
67. Deibler GE, Martenson RE, Kies MW. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. Prep Biochem. 1972;2(2):139-65.
68. Fraussen J *ea*. B cells of multiple sclerosis patients induce autoreactive proinflammatory T cell responses. Clin Immunol. 2016;173:124-32.
69. Kowarik MC, Cepok S, Sellner J, Grummel V, Weber MS, Korn T, et al. CXCL13 is the major determinant for B cell recruitment to the CSF during neuroinflammation. J Neuroinflammation. 2012;9:93.
70. Rubtsov AV, Rubtsova K, Kappler JW, Marrack P. TLR7 drives accumulation of ABCs and autoantibody production in autoimmune-prone mice. Immunol Res. 2013;55(1-3):210-6.
71. Fraussen J, Claes N, de Bock L, Somers V. Targets of the humoral autoimmune response in multiple sclerosis. Autoimmun Rev. 2014;13(11):1126-37.
72. Brok HP, Boven L, van Meurs M, Kerlero de Rosbo N, Celebi-Paul L, Kap YS, et al. The human CMV-UL86 peptide 981-1003 shares a crossreactive T-cell epitope with the encephalitogenic MOG peptide 34-56, but lacks the capacity to induce EAE in rhesus monkeys. J Neuroimmunol. 2007;182(1-2):135-52.
73. Martorana A, Balistreri CR, Bulati M, Buffa S, Azzarello DM, Camarda C, et al. Double negative (CD19+IgG+IgD-CD27-) B lymphocytes: a new insight from telomerase in healthy elderly, in centenarian offspring and in Alzheimer's disease patients. Immunol Lett. 2014;162(1 Pt B):303-9.
74. Knox JJ, Cosma GL, Betts MR, McLane LM. Corrigendum: Characterization of T-bet and Eomes in Peripheral Human Immune Cells. Front Immunol. 2016;7:337.
75. Myles A, Gearhart PJ, Cancro MP. Signals that drive T-bet expression in B cells. Cell Immunol. 2017.
76. Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. Nat Immunol. 2003;4(7):687-93.

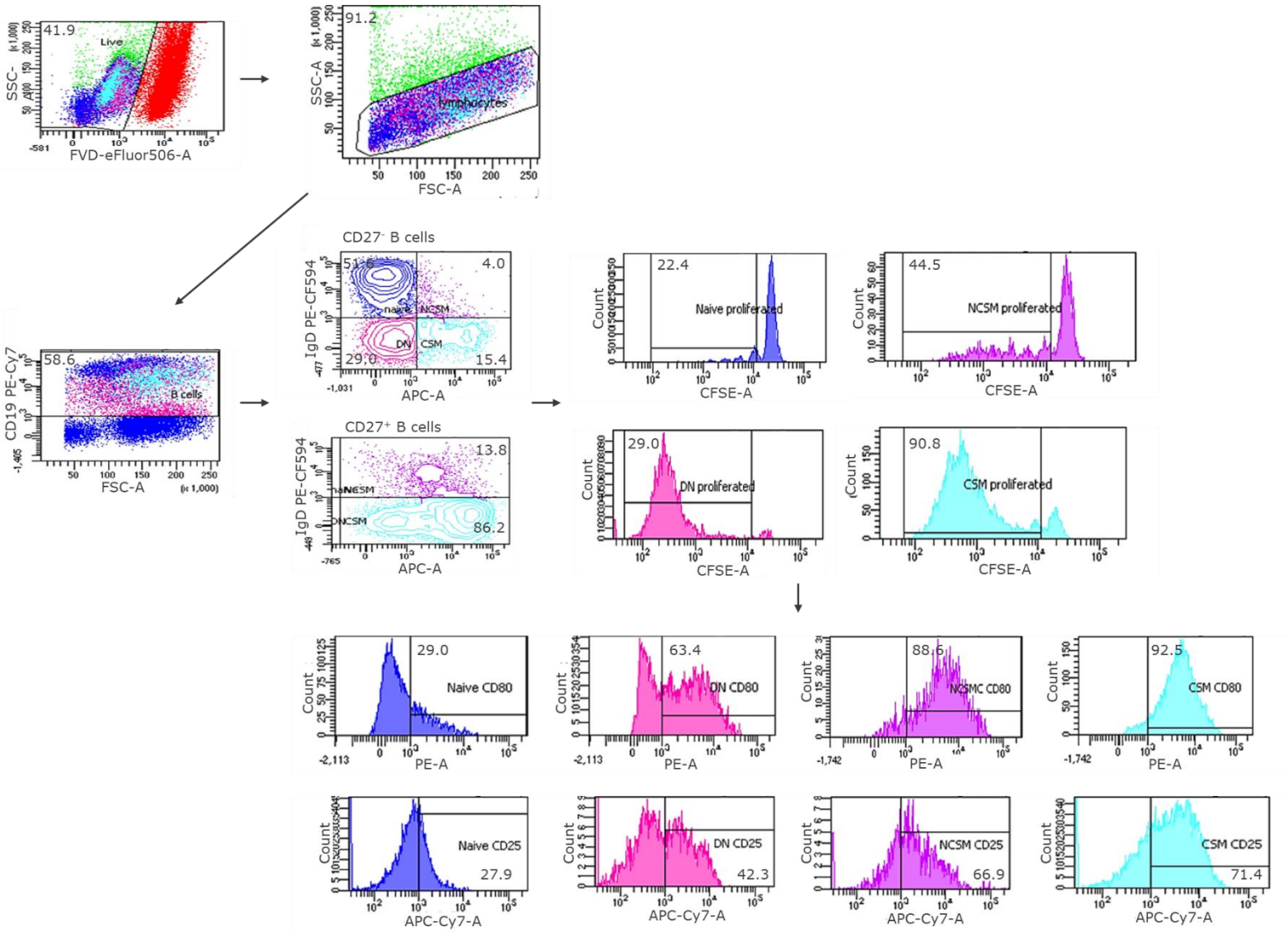
Supplemental data



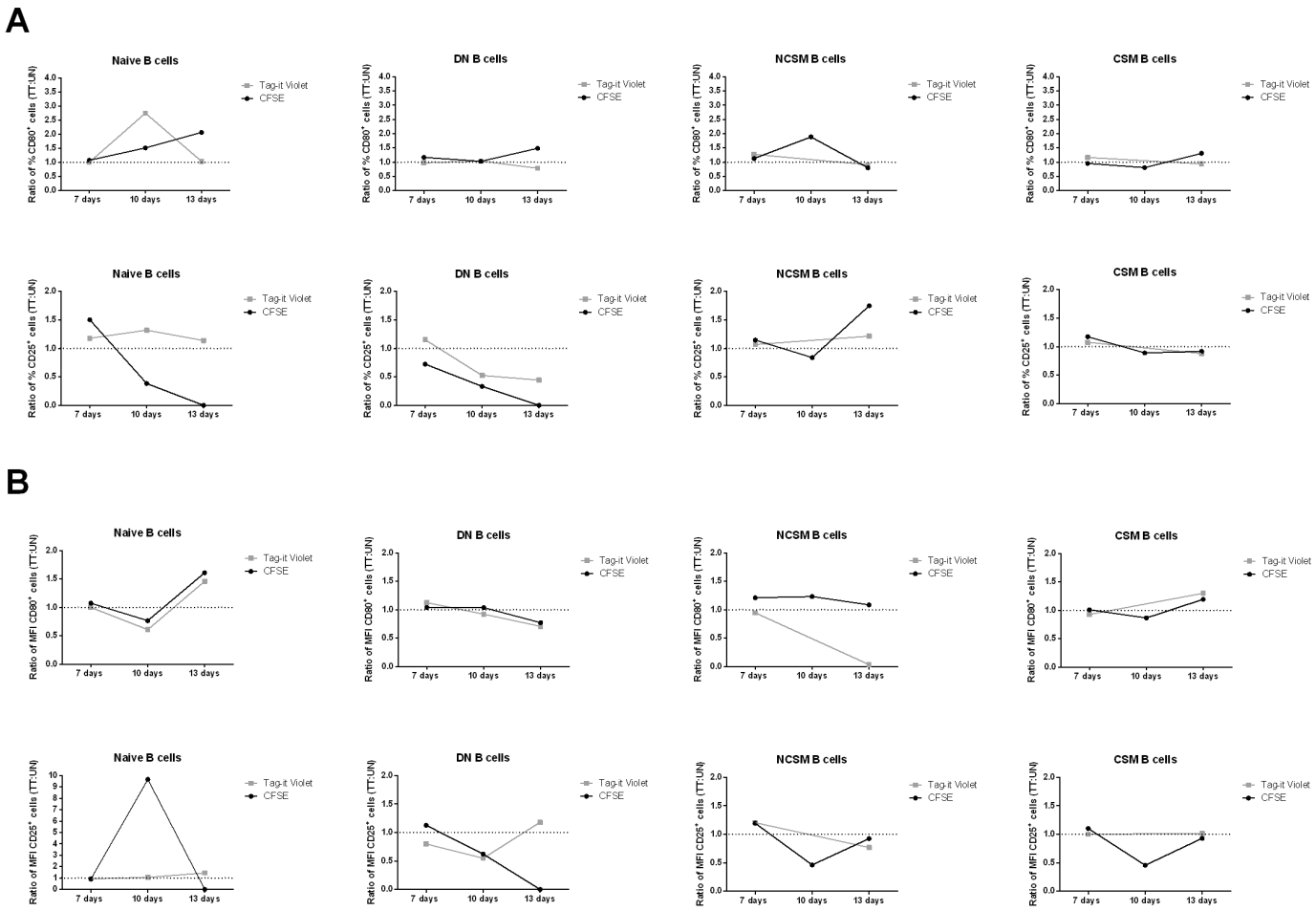
Supplemental figure 1. T-bet expression in IgG$^+$ and IgM$^+$ DN B cells from MS patients and HC. T-bet expression was measured in peripheral blood IgG$^+$ and IgM$^+$ DN B cells via intracellular flow cytometry. The percentage (A) or MFI (B) of T-bet expression in IgG$^+$ and IgM$^+$ DN B cells from RRMS patients (n = 10), SPMS patients (n = 5) and HC group (n = 15). The percentage (D) or MFI (E) of T-bet$^+$ IgG$^+$ and IgM$^+$ DN B cells was compared between RRMS patients, SPMS patients and HC 1 and 2 that are age- and gender matched with RRMS and SPMS patients, respectively. Mean levels + SEM are shown. * $p < 0.05$. MFI: mean fluorescence intensity, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, HC: healthy control, DN: double negative.



Supplemental figure 2. Proliferation of DN, naive, NCSM and CSM B cells after 24 hours in the presence of CXCL13 or CXCL10. CFSE labeled $CD27^-$ and $CD27^+$ B cells of 1 MS patient were incubated for 24 hours in the presence or absence of 1000 ng/ml CXCL13 and 1000 ng/ml CXCL10. Flow cytometry was performed to determine the proliferative response of the four B cell subtypes. Representative plots indicating the non-proliferated and proliferated fraction are shown. The position of the gates is based on the CFSE check on day 0. DN: double negative; CSM: class-switched memory; NCSM: non class-switched memory.



Supplemental figure 3. Representative gating strategy to analyze the viability, proliferative response and activation status of CD27⁻ and CD27⁺ B cells. Purified CD27⁻ and CD27⁺ B cells from 1 HC were labeled with CFSE, stimulated with CpG2006 and incubated with autologous irradiated PBMCs for 7 days. Subsequently, flow cytometric analysis was performed to examine the viability, proliferative response and activation status of the cells via staining them with anti-human CD19-PE-Cy7, CD27-APC, IgD-PE-CF594, CD80-PE, CD25-APC-Cy7 and eFluor 506.



Supplemental figure 4. Activation status of B cell subsets labeled with CFSE or Tag-it Violet following 7, 10 or 13 days with TT stimulation. CFSE or Tag-it Violet labeled $CD27^-$ and $CD27^+$ B cells of HC (n = 2) were stimulated with TT and cultured for 7, 10 or 13 days with irradiated PBMC. The expression of activation markers was analyzed via flow cytometry. Ratios of (A) the percentage of or (B) MFI within $CD80^+$ and $CD25^+$ naive, DN, NCSM and CSM B cells in which the unstimulated condition is set at 1. Mean levels + SEM are depicted. Due to the insufficient B cell number, not all conditions for all HC could be included. DN: double negative; NCSM: non class-switched memory; CSM: class-switched memory; TT: tetanus toxoid.

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Datum: **18/06/2018**