2011
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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Study on the antigen presenting function of B cells in multiple sclerosis

Promotor :
dr. Judith FRAUSSEN

Copromotor :
Prof. dr. Veerle SOMERS

Nathalie Stakenborg

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

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Abbreviations

7-AAD: 7-Aminoactinomycin D

Ab: antibody

Ag: antigen

APC : antigen presenting cell

BBB: blood brain barrier

BSA : bovine serum albumin

CD : cluster of differentiation

CFSE : carboxy fluorescein succinimidyl diacetate ester

CLEC16A: c-type lectin domain family 16 member A

CSF: cerebrospinal fluid

CNS : central nervous system

CMV: cytomegalovirus

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EAE : experimental autoimmune encephalomyelitis

EBV: Epstein-Barr virus

EDTA : ethylene diamine tetraacetate acid

ELISA: enzyme-linked immuno sorbent assay

FACS : fluorescence activated cell sorter

FDA: food and drug administration

FITC : fluoresceine isothiocyanaat

FCS : fetal bovine serum

GC: germinal center

GWAS: genome-wide association studies

HC : healthy control

HLA : human leukocyte antigen

IFN-γ: interferon-γ

Ig: immunoglobulin

IL: interleukin

MAG: myelin-associated glycoprotein

MBP : myelin basic protein

MHC : major histocompatibility complex

MOG : myelin oligodendrocyte protein

MRI: magnetic resonance imaging

mRNA: messenger ribonucleid acid

MS : multiple sclerosis

OCB: oligoclonal bands

OCT: optimal cutting temperature

PB: peripheral blood

PBMC : peripheral blood mononuclear cells

PBS : phosphate buffered saline

PE : phycoerythrine

PerCP : peridinine- chlorophyll protein

PLP: proteolipid protein

PPMS : primary progressive MS

RA: rheumatoid arthritis

RR-MS : relapsing-remitting multiple sclerosis

SP-MS : secondary progressive multiple sclerosis

TCR : T cell receptor

TLR: Toll-like receptor

TNF-α : tumor necrosis factor alpha

Treg : CD4+CD25+ regulatory T cell

TT : Tetanus Toxin

Preface

After five years of hard labor our university experience will shortly come to a close. Soon, we will be able to call ourselves Masters in the Clinical Molecular Sciences. Naturally, I could not have reached this milestone alone, so I would like to praise some special persons.

First off, I would like to thank my co-promoter prof. dr. Veerle Somers, for giving me the opportunity to complete my final internship at the Biomedical Research Institute and prof. dr. Niels Hellings for critically evaluating my thesis.

I especially want to thank my promoter and supervisor, dr. Judith Fraussen for sharing her B cell knowledge. Judith, your guidance, critical view and continuous support in and outside the lab have influenced me in becoming a more critical and skilled scientist. Thank you for everything!

Furthermore, I would like to thank Igna Rutten, Anne Bogaers, Veronique Pousset and Bart Vanwijmeersch for the collection of the blood samples and the FACS people, Bieke Broux and Christel Bocken, for the technical assistance with some FACS experiments.

Next in line to receive some credit are my fellow students with whom I have been huddled up in our student room. Thanks for all those more than pleasant moments over there. You know who you are!

Last but definitely not least, a special thank you goes out to my parents and sister for giving me the opportunity to reach the place where I am standing today and off course my best friends Ann, Dorien, Katrien and Ruben.

Summary

Background: B cells have several established functions, including antibody production and antigen presentation. In B cell multiple sclerosis (MS) research, investigators have mainly focused on antibody-dependent B cell functions, since a definite hallmark of this disease is an increased intrathecal immunoglobulin (Ig) production. The advent of Rituximab has, however, shown that B cells may also play a role as antigen presenting cells (APC) in MS. This B cell depleting agent, namely, caused a profound reduction of the T cell number as well as the MS brain lesions in MS patients, but did not alter the Ig levels. These findings showed that B cells do not only contribute to MS pathogenesis by Ig production. To our knowledge little research has investigated the capability of B cells to participate in MS pathogenesis by (auto)antigen presentation.

Objectives & results: We evaluated whether MS B cells have a higher expression of antigen presentation molecules caused by the deregulated immune response in the peripheral blood (PB), cerebrospinal fluid (CSF) and post-mortem brain tissue. Indeed, the percentage and mean fluorescence intensity (MFI) of peripheral $CD80^+$ B cells and CSF $CD86^+$ B cells was increased in MS patients in comparison to healthy individuals (HC). CSF B cells expressing CD80 also tended to be elevated when compared to PB. Furthermore, $CD80^+$ B cells were detected in MS brains, whereas no infiltrates were found in healthy brain tissue. These observations indicate that costimulatory molecules on B cells increase the antigen presentation capacity of B cells in MS.

In addition, we evaluated the proliferative B cell response against tetanus toxoid (TT), myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), proteolipid protein (PLP) and cytomegalovirus (CMV). As expected, the TT reactivity of the B cells was similar in HC and MS patients. Interestingly, MS patients showed a proliferative response to myelin antigens, while none of the HC were responsive. Moreover, three out of 4 MS patients versus 1 HC were CMV-reactive. However, no significant differences were found between HC and MS patients due to the low number of subjects currently included in our study. The expression of APC and (co)stimulatory molecules on B cells also did not significantly differ after antigen-specific B cell stimulation. In future experiments, we will further evaluate the B cell antigen reactivity and expression of APC and (co)stimulatory molecules after antigen-specific stimulation. Furthermore, we will determine whether B cells of MS patients cause a higher T cell proliferation in response to myelin and viral antigen presentation than HCs. This subsequent T cell response will also be characterized on gene, protein and cellular level.

Samenvatting

Achtergrond: B cellen hebben meerdere functies, waaronder antilichaamproductie en antigeen presentatie. In B cel multiple sclerose (MS) onderzoek hebben wetenschappers zich door de jaren vooral gefocust op antilichaam (Ab) afhankelijke B celfuncties, omdat een verhoogde intrathecale Ab productie een kenmerk is van deze ziekte. De komst van Rituximab heeft aangetoond dat B cellen ook een rol spelen in antigeen presentatie in MS. Dit B cel depletie Ab zorgde namelijk voor een reductie van het totale T cel aantal en vermindering van het aantal MS laesies bij MS patiënten, terwijl Ab levels nagenoeg hetzelfde bleven. Deze bevindingen geven aan dat B cellen niet alleen bijdragen aan MS door middel van Ab productie. Zover geweten is er nog maar weinig onderzoek gedaan naar de bekwaamheid van B cellen om deel uit te maken van de MS pathogenese door middel van (auto)antigeen presentatie. Verder is er ook weinig gekend over de antigeen reactiviteit van B cellen tegen lichaamseigen en lichaamsvreemde antigenen.

Doel & resultaten: We zijn nagegaan of MS B cellen een verhoogde expressie van antigeen presenterende moleculen hebben door een verstoorde immuunrespons in het bloed, cerebrospinaal vocht (CSV) en post-mortem hersenweefsel. Het percentage en de gemiddelde fluorescentie intensiteit (MFI) van perifere $CD80^+$ B cellen en CSV $CD86^+$ B cellen was verhoogd in MS patiënten in vergelijking met gezonde personen. In het CSF was ook een stijgende trend van $CD80^+$ B cellen te zien. Bovendien werden er $CD80^+$ B cellen gedetecteerd in MS hersenweefsel, terwijl geen infiltraten werden gezien in gezond hersenweefsel. Deze bevindingen wijzen erop dat costimulatoire moleculen de antigeen presenterende functie van B cellen verhogen in MS. Verder hebben we de proliferatieve respons van B cellen bepaald voor tetanus toxoïd (TT), myeline oligodendrocyt proteïne (MOG), proteolipide proteïne (PLP) en het cytomegalovirus (CMV). De TT reactiviteit was vergelijkbaar in HC en MS patiënten. MS patiënten vertoonden een proliferatieve respons tegen myeline antigenen, terwijl geen gezonde personen B cel reactiviteit hadden tegen deze antigenen. Drie van de 4 MS patiënten versus 1 HC waren ook CMV-reactief. Er waren echter geen significante verschillen te zien tussen gezonde individuen en MS patiënten, wat mogelijk te wijten is aan het lage aantal personen momenteel opgenomen in deze studie. De expressie van APC en (co)stimulatoire moleculen op B cellen is tot nog toe ook niet verschillend na antigeen-specifieke B cel stimulatie. In toekomstige experimenten zullen we de B cel antigeen reactiviteit en expressie van APC en (co)stimulatoire moleculen na antigeen-specifieke stimulatie verder bestuderen. Bovendien zullen we nagaan of B cellen van MS patiënten zorgen voor een verhoogde T cel proliferatie na myeline en virale antigeen presentatie. Deze daaropvolgende T cel respons zal ook worden gekarakteriseerd op cellulair, gen- en proteïne niveau.

1 Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), affecting approximately 1 in 1000 persons in Europe and Northern America.[1, 2] The disease remains a leading cause of neurological disability in the Caucasian population and mainly affects individuals in early adulthood (i.e. 20-40 years). Women are affected twice as much as males.[1, 3]

1.1 Multiple sclerosis

1.1.1 Clinical features

The clinical disease course of MS is profoundly heterogeneous since different regions of the CNS can be affected.[4] However, common symptoms include sensory, cognitive and visual impairment, paralysis and other neurological deficits.[3, 4] MS mainly occurs in three forms: relapsing-remitting (RR), primary progressive (PP) and secondary progressive (SP) MS. The most common subtype of MS (85-90%) is RRMS, which is characterized by intermittent episodes of relapse alongside periods of remission with a partial or full recovery. Over time, 40% of the RRMS patients develop SPMS, in which the patients experience a persistent progression of the disease without any remission. About 15-20% of the patients present with PPMS, which is characterized by a steady progression without acute attacks or remissions.[1, 3, 4]

1.1.2 Etiology

Although the true etiology of MS remains unknown, both genetic and environmental factors contribute to MS susceptibility according to epidemiological studies.[1, 5] Family and twin studies have revealed a higher clinical concordance in monozygotic (30%) twins in comparison to dizygotic twins (5%).[3, 4, 6] The partial concordance implies that additional factors (i.e. environment and epigenetics) also attribute to the disease susceptibility.[4, 5] However, the increased inheritability and the decline in risk following the degree of family relation do indicate a strong genetic component for MS.[7, 8]

Linkage studies have shown that the human leukocyte antigen (HLA) class II allele DRB1 (HLA-DRB1*1501) is clearly associated with MS.[2, 6-10] This HLA region encodes molecules that participate in T cell antigen recognition.[8] Recently, more powerful and efficient genome-wide screening tools, such as genome-wide association studies (GWAS), have confirmed the strong HLA association in MS.[2, 7, 8] In addition, new non-HLA susceptibility genes have been identified and replicated using GWAS studies, including the interleukin 2 receptor (IL2RA), the IL-7 receptor (IL7RA) and the c-type lectin domain family 16 member A (CLEC16A) genes.[7, 8, 11] The IL2RA and IL7RA play an important role in the expansion and survival of B and T cells. CLEC16A, most likely, has a function in immunity, since the C-type lectin receptor family provides signal transduction for tolerance or immunity.[7, 12] Even though

genetic susceptibility explains the increased occurrence of MS within certain families, it does not account for the unequal distribution throughout the world. The prevalence of MS, namely, follows a latitudinal gradient, which is strongly inversely correlated with the intensity of sunlight, ultraviolet B (UVB) radiation and vitamin D concentrations.[5, 11, 13]

Over the past years, infectious pathogens have gained much attention as causal agents for MS. In general, two hypotheses have been proposed: the *poliomyelitis* and the *prevalence* theory.[14] The former states that acquiring a common virus in adolescence or adulthood enhances the risk for MS, whereas it confers protection when acquired in infancy. The latter theory by Kurtzke postulates that MS is caused by pathogens, which are common in high frequency MS regions.[5, 14] Among the numerous candidates, viruses that persist as a latent infection throughout life have gained the most interest for their involvement in the development of MS.[5] These viruses namely generate long-lived memory B and T cells, which may reactivate after encountering cross-reactive self-antigens.[11, 15] To date, the leading viral candidates are Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6). EBV is a human deoxyribonucleic acid (DNA) herpes virus, which infects more than 90% of the world's population.[16] During infancy, a primary infection mostly occurs asymptomatic, but in adolescents and adults it frequently manifests as infectious mononucleosis (i.e. 30-50%).[14, 17] Nearly all MS patients (99,5%) are EBV-seropositive in comparison to 90% of the healthy controls (HC).[14, 18] Seronegative EBV individuals have an extreme low risk of developing MS, but an EBV infection elevates this risk several folds. Additionally, a history of infectious mononucleosis further increases the risk for MS.[14, 16-19] These findings suggest that EBV infection predisposes individuals to MS development. However, EBV infection is not essential for MS development, since a lot of EBV seropositive individuals never develop MS and a considerable fraction of pediatric MS patients is EBV-seronegative.[17]

Besides EBV, HHV-6 also is a human herpes virus, which has been implicated in the development of MS. This virus has a seroprevelance of nearly 100% and is known to establish latency within the CNS.[20] In addition, HHV-6 infects T cells and thereby modulates the immune response. Several studies have demonstrated the presence of HHV-6 within MS lesions, serum and cerebrospinal fluid (CSF) of MS patients, but others have not been able to confirm these findings.[17] The cytomegalovirus (CMV) is another herpes virus which infects 40-90% of the world's population. Some CMV-specific cluster of differentiation (CD)8^+ T cells have been isolated from MS lesions.[11] Additionally, experimental autoimmune encephalomyelitis (EAE) studies with nonhuman rhesus monkeys demonstrated that autoreactivity of myelin oligodendrocyte protein $(MOG)_{34-56}$-specific T cells may arise from CMV-specific memory B cells in the immune repertoire.[21] 't Hart and coworkers argued that the former T cell population is reactivated by encountering CMV-antigen presenting cells (APC) in the lymph nodes.[11] Other viruses and bacteria, such as Chlamydia pneumonia, have also been

implicated in MS pathogenesis, but causal evidence is currently lacking.[22] To summarize, viruses and bacteria most likely do not lead to the development of MS, but they can increase the risk for genetically-predisposed individuals.

1.1.3 Immunopathogenesis: current hypothesis

The current hypothesis of MS pathogenesis is that resting myelin-specific T cells are activated in the periphery (Figure 1). Several mechanisms have been implicated in the activation of autoreactive T cells.[15]

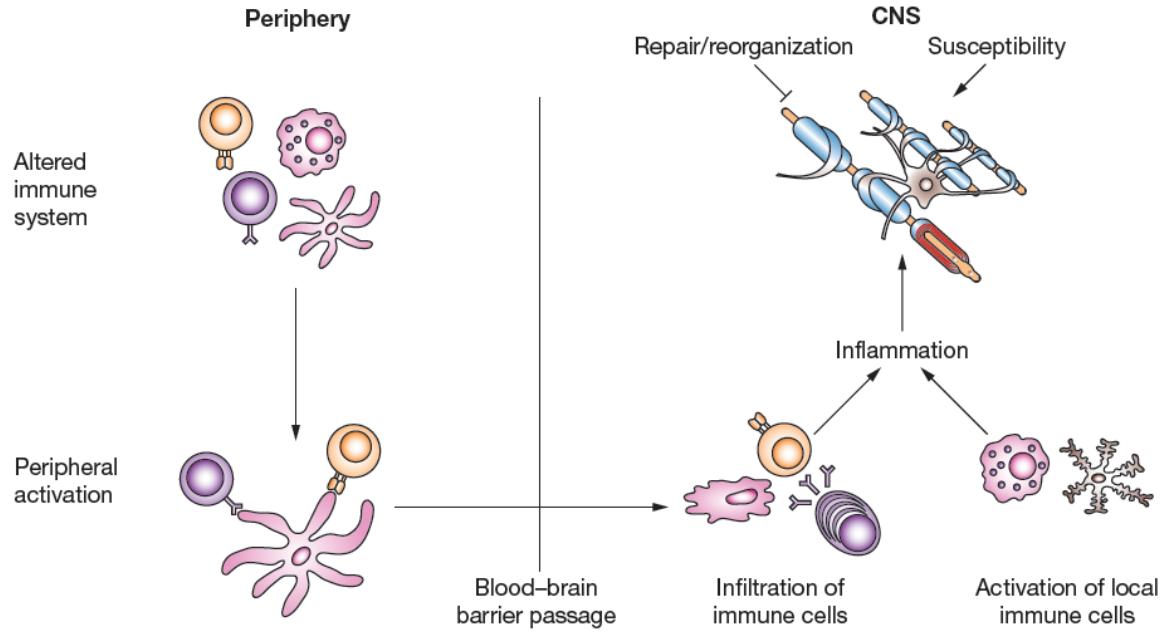

Figure 1. The current hypothesis for the development of MS. Due to genetic and environmental predisposition, MS patients have an altered immune response. Therefore, B cells and T cells may crossreact with infectious agents, which have similarities with self antigens (i.e. molecular mimicry). The subsequent activation of these immune cells in the periphery enables them to cross the blood brain barrier (BBB) and target CNS self antigens causing inflammatory damage and demyelination. Over time, autoreactivity has been shown against additional myelin components (i.e. epitope spreading). Moreover, autoreactive immune cells can be activated nonspecifically by superantigens, molecular patterns or inflammatory cytokines.

Molecular mimicry is one possible culprit for the autoimmune response against myelin antigens in MS. According to this unproven theory, autoreactive T cells can be activated through cross-reactivity with an infectious agent (e.g. CMV and EBV), which resembles certain myelin antigens (e.g. MOG and myelin basic protein [MBP]).[23] In the initial stages of the disease, a single myelin antigen is thought to trigger MS. As the disease progresses, autoreactivity has been demonstrated against multiple myelin antigens (i.e. *epitope spreading*).[1] This phenomenon is most likely due to continuous demyelination, which causes the release of previously unavailable (auto)antigens. Another hypothesis states that T cell receptor (TCR) independent activation of autoreactive T cells may be caused by superantigens, molecular pattern recognition (e.g. Toll-like receptor [TLR]) or inflammatory cytokines, which activate

autoreactive T cells in a non-specific manner (i.e. *bystander activation*).[3, 4] Once activated, the autoreactive T cells cross the blood brain barrier (BBB). In the CNS, resident APCs such as microglial cells and macrophages reactivate the T cells by presenting certain myelin antigens. The activated T cells will secrete inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), leading to the upregulation of adhesion molecules on the BBB endothelium. This will further promote the influx of T cells, macrophages and B cells, expanding the inflammatory response. The end result of this detrimental autoimmune cascade will be myelin destruction (Figure 1).[4, 24]

1.1.4 Pathology

The pathological hallmark of MS is the sharply demarcated lesion within the CNS white matter with a predilection to the periventricular regions, optic nerves, brain stem and spinal cord. These lesions, or sclerotic plaques, are usually infiltrated by T cells, B cells, plasma cells and macrophages.[10, 25, 26] The aberrant immune response is directed against the myelin sheath, which normally provides insulation for optimal electrical conduction between the nerve cells. The destruction of myelin reduces its insulating capacity, resulting in an impaired nerve impulse transmission. Initially, remyelination frequently occurs by oligodendrocytes. Nevertheless, the oligodendrocytes will become irreversibly damaged during progression of the disease, leading to axonal degradation.[4, 5, 7, 9, 10]

1.2 B cells: the new player in MS pathogenesis

Traditionally, autoreactive $CD4^+$ T cells were considered as the main effectors cells in MS pathogenesis due to the findings of activated T cells in MS lesions and the induction of EAE via passive transfer of myelin-specific T cells.[27] Recent studies have, however, demonstrated that B cells also play a role in MS development.[28]

1.2.1 Developmental stages of B cells

B cell development initiates in the bone marrow. The earliest committed B cell is called the pro-B cell, which is characterized by the presence of the B cell marker CD19 (Figure 2).[29] During the pro-and pre-B cell stage, gene rearrangement of diversity (D), joining (J) and variable (V) segments of the immunoglobulin (Ig) heavy chain occurs, followed by the genetic recombination of V and J segments of the light chain. Both chains eventually pair and appear on the cell surface as an IgM molecule (i.e. B cell receptor [BCR]), constituting the transition to naïve immature B cells.[24, 29] The immature B cells express CD19 and are devoid of the surface markers CD27 and CD38.[30] During the developmental stages in the bone marrow, B cells undergo a negative selection process to ensure that their antigen receptor does not possess autoreactivity against self antigens.[24] After selection, naïve B cells migrate to peripheral lymphoid organs. Upon antigen encounter, naïve B cells upregulate the costimulatory

surface markers CD40, CD80 and CD86 and migrate to the T cell rich zone in the lymphoid follicles. At this site, B:T cell interactions trigger T cell-dependent B cell activation, which enables the antigen-primed B cells to commit to the plasma cell pathway and differentiate into short-lived $CD19^{+}CD27^{+}CD38^{+}$ plasma blasts, which produce low affinity IgM during a limited timespan .[24, 30]

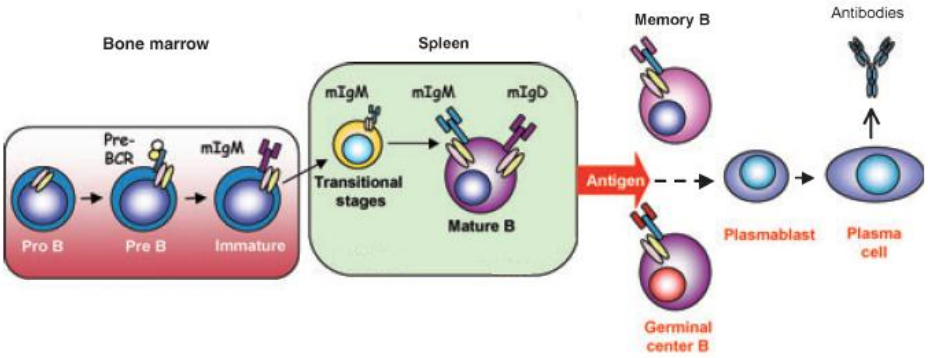

Figure 2. The developmental stages of B cells. During the pro-B cell and the pre-B cell stage, heavy and light chain gene rearrangements occur, respectively. The appearance of BCR constitutes the transition to immature B cells. In the bone marrow, negative selection occurs to eliminate autoreactive B cells. Immature naïve B cells migrate to the peripheral lymphoid organs. Upon antigen exposure, the B cell either commits to the plasma cell pathway or the germinal center reaction, which eventually leads to the production of memory B cells.

In parallel, some naïve B cells enter the germinal center (GC) reaction within the lymphoid organs, in which they undergo affinity maturation and somatic hypermutation of their Ig V genes. High-affinity B cells that recognize their antigen on the follicular dendritic cells are selected to survive and become memory B cells, which are characterized by the expression of CD19 and the memory marker CD27.[30, 31] Upon secondary exposure to the antigen, memory B cells differentiate into plasma cells, which secrete high affinity immunoglobulin G (IgG) and express the following surface markers: CD27, CD38 and the plasma cell marker CD138.[24, 30, 31]

1.2.2 Antibody-dependent functions of B cells in MS

The B cells' main function is the production of antibodies (Abs) whose effector functions include complement activation and Ab-dependent cellular toxicity. Complement is activated by antigen (Ag)-Ab immune complexes. The triggering of the complement cascade ultimately leads to the formation of a membrane-attack complex, which kills the target cell. Ab-dependent cellular toxicity, on the other hand, is a process in which natural killer cells and other leukocytes bind to Ab-coated cells and opsonize the target cell.[24, 29]

In the CSF of most MS patients (i.e. 90%) an intrathecal Ig production is observed. After electrophoretic separation, the CSF Ig fraction of MS patients appears as an oligoclonal band (OCB) pattern.[6, 27] Until recently, the occurrence of OCBs was considered a hallmark in the diagnosis of MS supporting clinical and magnetic resonance imaging (MRI) findings.[27] However, myelin-specific Abs

have rarely been identified within OCBs, suggesting a bystander response.[27] Besides the CSF, the brain compartment provides further evidence for the role of B cells in MS pathology. The most frequent MS lesion pattern (i.e. 70%) is characterized by abundant deposition of Abs and complement activation (i.e. pattern II lesion) in regions of active myelin breakdown, suggesting an Ab-dependent demyelination.[27, 30] Histological studies also demonstrated that B cells, plasma cells and Igs are present in chronic MS plaques as well as acute lesions.[27, 32] Furthermore, B cells isolated from both the CSF and MS lesions show signs of restricted clonal expansion and somatic hypermutation, suggesting an antigen-driven B cell response within the CNS.[27, 28, 30, 32-34]

For many years, investigators have searched for the MS target antigen of the humoral immune response. Since MS lesions are primarily found in the CNS white matter, suspected targets of the B cells and autoAbs are lipid and protein structures of the myelin sheath.[27] Several studies have reported Ab reactivity against the myelin antigens MBP, MOG and proteolipid protein (PLP) in CSF and MS lesions.[30] Furthermore, humoral responses against the infectious agents EBV, CMV and HHV-6 have been detected. However, the aforementioned Ab reactivity is not solely confined to MS, since virus and myelin-specific Abs have also been identified in other neurological disorders and even in healthy individuals.[32-35] Various reasons could explain the inability to identify a specific antigen target in MS patients. The Ab reactivity may not be detected due to shortcomings in the current assays (e.g. enzyme-linked immunosorbent assay [ELISA] and Western blot) or the detection of Ab responses may be limited in the CSF of MS patients, because relevant autoAbs are bound to their CNS tissue targets.[27, 30, 33]

1.2.3 Antibody-independent functions of B cells

Besides the production of (auto)Abs, B cells exert other functions in MS pathogenesis, namely cytokine production that may regulate the local immune environment (i); ectopic lymphoneogenesis (ii), antigen presentation (iii) and T cell stimulation (iv).[30, 34]

Activated B cells produce distinct effector cytokines, depending on the type of stimulation. The crosslinking of BCR and stimulation through CD40 are required to induce B cell secretion of the proinflammatory cytokines lymphotoxin (LT) and tumor necrosis factor (TNF). These cytokines drive a more vigorous immune response.[36, 37] Bar-Or and colleagues demonstrated that MS B cells secreted increased levels of LT and TNF in response to TLR9 ligand CpG and T helper cell (Th1) cytokine IFN-γ in comparison to HCs. Naïve B cells, on the other hand, produce the anti-inflammatory cytokine IL-10, which is protective in EAE. In MS patients, IL-10 producing B cells have been shown to be decreased when compared to HC.[36] In health, B cell cytokines also support the formation and maintenance of

GCs in lymphoid follicles, which are necessary for adaptive immune responses. In disease, however, ectopic follicle structures can form within target sites, promoting a local immune response.[29, 37]

Three main findings have emphasized the involvement of Ab-independent B cell functions in MS.[30, 38] First, ectopic B cell follicles were identified in the meninges of MS patients, indicating a microenvironment within the CNS that may promote B cell proliferation and Ab production.[27, 39] Secondly, a significant proportion of B cells present in the ectopic B cell follicles and MS lesions appeared to be infected with EBV,[40] although other research groups were not able to replicate this finding.[41] Thirdly, positive results have been obtained in phase II/III clinical trials with the anti-CD20 depleting agent Rituximab, which are discussed below.[30]

Rituximab is a genetically engineered chimeric anti-CD20 monoclonal Ab that effectively depletes $CD20^+$ B cells. Possible mechanisms for the B cell lysis induced by Rituximab include Ab-dependent cellular toxicity and complement mediated cytotoxicity.[27] Originally, Rituximab was only Food & Drug Administration (FDA) approved for the treatment of non-Hodgkin B cell lymphomas. In recent years, the possible benefits of Rituximab have been investigated in several autoimmune disorders including rheumatoid arthritis (RA) and MS.[27, 37, 42, 43] In a phase II clinical trial (double-blind; 48 weeks) with Rituximab, the occurrence of exacerbations and the number of new T1 gadolinium-enhancing lesions was significantly reduced in RRMS patients.[44] Similar results were obtained in a comparable longer trial (72 weeks).[45] As Rituximab had no effect on Ab titers, the beneficial results of these studies are not associated to Ab production. This is due to the absence of CD20 on plasma cells. Moreover, the T cell number was significantly decreased in the CSF of RRMS patients 6 months after Rituximab treatment, indicating the importance of B:T cell interactions in MS.[42, 46, 47]

Anti-CD20-mediated B cell depletion was also studied in the EAE model. Matsushita et al. demonstrated that B cell depletion prior to EAE induction via MOG_{35-55} immunization exacerbated the disease severity.[48] This disease aggravation was associated with the decrease in interleukin (IL)-10 producing regulatory B cells. The effect of anti-CD20 therapy on the disease course was related to the time of B cell depletion, since the depletion of B cells after EAE onset significantly reduced disease severity.[42, 48] In a similar study by Weber and colleagues, the immunological effects of anti-CD20 treatment were evaluated in 2 related EAE models. In the recombinant MOG (rMOG)-induced EAE model, B cells are required to internalize and process antigens for presentation to encephalitogenic T cells. The depletion of B cells prior to EAE induction prevented rMOG-induced EAE. Similarly, anti-CD20 treatment after EAE onset reversed paralysis. This outcome is attributed to the depletion of rMOG-presenting B cells causing a reduction in MOG-specific proinflammatory Th1 and Th17 cells. In the second model, the MOG_{35-55} peptide was used to induce EAE. The peptide

directly binds to the major histocompatibility complex (MHC) class II molecules on APCs without processing, and causes peripheral T cell activation. The depletion of B cells prior to MOG_{35-55}-mediated EAE induction or after EAE onset exacerbated EAE severity.[48, 49] This outcome is explained by the inefficiency of the MOG_{35-55} peptide to activate B cells, which polarize encephalitogenic MOG-specific Th1 and Th17 cells.[42, 49] In each EAE model used, B cell depletion did cause a reduction in the regulatory T cell frequency and an increase in the proinflammatory function of other competent APCs.[49] In contrast to the results of Matsushita et al., Weber and colleagues suggest that the clinical outcome after B cell depletion is the result of the antigen-specificity and activation status of the B cells instead of the timing of treatment initiation.[42, 49] Both studies do demonstrate that B cells may have a dual role in EAE, with regulatory B cells ameliorating the disease severity in the early stages of EAE and an inflammatory population enhancing the disease progression at later stages.[42, 49]

1.2.4 B cells as antigen-presenting cells

The significant reduction of new gadolinium-enhancing lesions as well as the T cell number in RRMS patients undergoing Rituximab treatment, suggest that a pathogenic B cell population may drive MS progression, since no alterations are observed in the level of Ab titers.[27, 42]

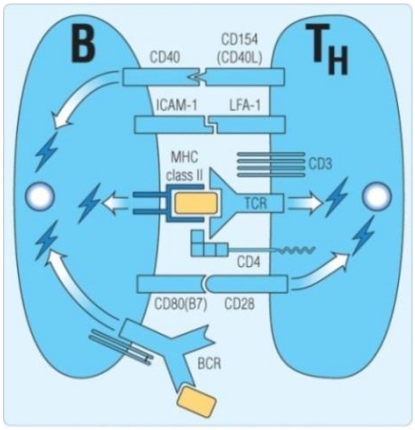

Figure 3. The essential B-T cell interactions for T cell activation and proliferation. B cells present certain peptide complexes on MHC class II molecules, which are recognized by specific $CD4^+$ T cells. Subsequently, these T cells secrete stimulatory cytokines and deliver costimulatory signals through the receptor-ligand pair CD40-CD40L in order to activate the B cell. The activated B cell will upregulate CD80, which interacts with CD28 in order to promote T cell proliferation.

A possible mechanism by which B cells could enhance disease progression is through antigen presentation. B cells are competent APCs that cause T cell activation through essential (co)stimulatory molecules. First, the T cell receptor (TCR) engages with the Ag-MHC complex present on the B cells. Secondly, the (co)stimulatory receptor-ligand pairs CD40-CD154 (CD40L) and CD80/CD86-CD28 interact to enable T cell activation and proliferation (Figure 3). The CD40L-CD40 interaction stimulates the B cells to produce high-affinity IgG. On the other hand, the CD80-CD28 interaction along with the TCR signal initiate T cell proliferation and IL-2 secretion.[24, 31, 50] Genç et al.

reported that peripheral $CD80^+$ B cells are significantly elevated in RRMS patients during exacerbation of disease, but are unchanged in remission in comparison to HC indicating that B-T cell interplay is important in disease progression.[47, 50] A recent study by Harp and colleagues further investigated the B-T cell interactions in MS patients. The authors showed that T cell-dependent B cell activation, unlike bacterial stimulation via the Toll-like receptor, enabled B cells to activate T cells in a MBP-specific manner. However, little is still known about the importance of B cells as APCs in MS pathogenesis. In addition, the mechanisms of this B-T cell interplay remain largely unknown.[47]

1.3 Study aims

Until recently, B cell research mainly focused on Ab-dependent B cell functions, since increased intrathecal Ig production has longtime been considered a hallmark of MS. The advent of Rituximab has, however, demonstrated that B cells may also function as APC in MS. Therefore, this study aims to investigate whether B cells play an important role as APCs in the immunopathogenesis of MS.

First, we will evaluate whether B cells have a higher expression of antigen presentation and (co)stimulatory molecules caused by the deregulated immune response in MS patients. Therefore, the expression of MHC class II (HLA-DR/DP/DQ), MHC class I (HLA-A/B/C) and (co)stimulatory (CD80, CD86, CD40) molecules is directly assessed *ex vivo* on peripheral and CSF B cells from healthy donors (HC), RRMS and SPMS patients. On the other hand, the presence of B cells and their expression levels of (co)stimulatory (CD80) and MHC molecules (HLA-DR) will be determined in post-mortem brain sections of MS patients and HC.

Our second objective is to determine the expression of antigen presentation and (co)stimulatory molecules on B cells after polyclonal and antigen-specific stimulation through flow cytometry. Furthermore, the B cell reactivity against certain myelin and viral targets will be assessed using a carboxy fluorescein succinimidyl diacetate ester (CFSE) assay.

This study will provide further insights into the underlying disease mechanism of MS. A more in-depth knowledge of the B-T cell interplay could be important in the development of a new therapeutic approach for MS, which would target its disease mechanism rather than merely the symptoms.

2 Material & methods

2.1 Study population

Samples from MS patients were obtained from the Revalidation & MS center, the Maria Hospital (Overpelt) and the Orbis Hospital (Sittard). Peripheral blood (PB) was collected from 36 HC and 46 MS patients, of which 14 were RRMS and 11 were SPMS . In parallel, cerebrospinal fluid (CSF) was collected from 9 MS patients for *ex vivo* flow cytometric analysis. The HC and RRMS patients had a mean age of 39, while the SPMS patients had a mean age of 55. The mean age of the MS patients was 47. In addition, PB was collected from 4 MS patients (mean age: 39,25±22,45) and 5 healthy donors (mean age: 32±9,86) for the CFSE assay. The general characteristics of the study groups are stated in Table 1. Two brain biopsies with chronic active lesions were also obtained from the Dutch brain bank for immunohistochemistry. MS was diagnosed according to the 2010 McDonald criteria.[51] An informed consent was obtained from all the donors. The study was also approved by the local and national central ethics committees.

Table 1. General characteristics of MS patients and healthy donors

	HC	MS[a,b]	RRMS	SPMS
Blood samples	36	46	14	11
Male	13	15	5	2
Female	23	31	9	9
Mean age	39,47±14,05	47±13,45	39,83±8,66	55±9,72

[a] Disease status: 14 RRMS, 11 SPMS and 21 unknown
[b] Treatment status: 8 untreated, 4 Avonex, 3 Gilenya , 3 Tysabri, 5 Rebif22 and 23 unknown

2.2 Cell culture

2.2.1 Isolation of peripheral blood mononuclear cells (PBMC) and CD19$^+$ B cells

Peripheral blood mononuclear cells were isolated from the PB by Ficoll density gradient centrifugation (Cederlane, Burlington, Ontario, Canada). The obtained cell population was washed with RPMI 1640 and subsequently resuspended in culture medium (1 mM sodium pyruvate, 1mM nonessential amino acids, 50 U/ml penicillin, 50 mg/ml streptomycin, 10% heat-inactivated fetal bovine serum [FBS]). Three methods for B cell isolation were tested, namely the Easysep CD19 magnetic positive selection and Easysep B cell enrichment kit following instructions of the manufacturer (EasySep® Stem cell, Grenoble, France) and fluorescence-activated cell sorting (FACS). In the latter case, CD19$^+$ B cells were stained with Peridin Chlorophyll Protein (PerCP) Cy 5.5-labeled CD19 Abs (BD biosciences, Erembodegem, Belgium) for 30 min at 4°C. The CD19$^+$ cells were enriched

using a FACSAria™ II (BD Biosciences, Erembodegem, Belgium). The purity of the sorted B cells was routinely > 98%, whereas negative B cell selection achieved a purity of >99%. After sorting and magnetic B cell selection, the B cells were collected and directly used for culture.

Mononuclear cells were isolated from the CSF (± 5 ml) by centrifugation for 12 min at 4°C within 2 hours of the lumbar puncture and resuspended in cold phosphate buffered saline (PBS) supplemented with 2% FBS. When the CSF contained red blood cells (RBCs) or the percentage of B cells in the sample did not exceed 1%, the results were excluded from further analysis.

2.2.2 Cell culture

Peripheral mononuclear cells were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 1 mM sodium pyruvate, 1mM nonessential amino acids, 50 U/ml penicillin, 50 mg/ml streptomycin (all from Invitrogen, Merelbeke, Belgium) and 10% heat-inactivated fetal bovine serum (Gibco, Ghent, Belgium). The cell cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2.

The 3T6 CD40L cell line (a kind gift from 't Hart et al.[11]) was cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, 50 U/ml penicillin, 50 mg/ml streptomycin (all from Invitrogen, Merelbeke, Belgium), $2{,}228 \times 10^{-6}$ g/ml β-mercapto-ethanol (Sigma, Bornem, Belgium) and 10% heat-inactivated FBS (Gibco, Ghent, Belgium). The cell line was incubated at 37°C and 5% CO_2, and passaged twice a week to ensure a continuous growth. Therefore, the cells were detached from the culture flasks with 0,5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. The cell suspension was centrifuged for 10 min at 1400 rpm, where after the cells were further cultured in a 1:10 dilution. In addition, 10 µl/ml geneticin G418 (Invivogen, San Diego, CA, US) was added to select for the 3T6 CD40 ligand transfected cells.

2.2.3 B cell activation

The freshly purified B cells are stimulated either by CpG 2006 in combination with IL-2 or CD40L together with IL-4. For T cell-dependent B cell stimulation, 3T6 murine fibroblasts constitutively expressing human CD40L were irradiated at 8275 rad in a ^{137}Cesium source (CIS Biointernational, Gif-sur-Yvette Cedex, France) to inhibit cell proliferation. The irradiated cells were plated at 5000 cells per cm^2 in a 96 well flat bottom culture plate (Greiner, Wemmel, Belgium) in the appropriate culture medium. After an overnight culture at 37°C and 5% CO_2, the cells were adherent and could be used for B cell activation. Prior to adding the purified B cells, the 3T6 CD40L cell line medium was removed. 1×10^5 B cells were plated on the human CD40L feeder cells and were additionally stimulated with 10 ng/ml recombinant IL-4 (R&D Systems, Minneapolis, MN, USA) for 5 days. In

parallel, $1x10^5$ isolated B cells were stimulated with 1 µg/ml CpG 2006 (ODN2006, 50-tcgtcgttttgtcgttttgtcgtt-30, InvivoGen, San Diego, CA, USA) and 50 U/ml IL-2 (Roche Diagnostics, Brussels, Belgium) for 5 days in a 96 well round bottom culture plate (Greiner, Wemmel, Belgium). The viability and expression of APC and (co)stimulatory molecules on the B cells was determined using Abs directed against HLA-DR/DP/DQ, 7-Aminoactinomycin D (7-AAD) and CD80 (all from BD Biosciences, Erembodegem, Belgium) using flow cytometry on a FACS Calibur™ (BD Biosciences, Erembodegem, Belgium).

2.2.4 CFSE assay

The B cell antigen reactivity and expression of APC and (co)stimulatory molecules following antigen-specific stimulation was determined using a CFSE assay. Therefore, B cells were labeled with the fluorescent molecule CFSE and cultured in the presence of several (auto)antigens, namely tetanus toxoid (TT), MOG, MBP, PLP and CMV. The proliferating (i.e. reactive) fraction of B cells was assessed by the dilution of CFSE and the decrease of the fluorescent signal using flow cytometric analysis.

For CFSE labeling, purified B cells were resuspended in 0,1% bovine serum albumin (BSA) in PBS at a density of $20x10^6$ cells/ml and incubated for 7 min at 37°C with 1 µM CFSE (Invitrogen, Merelbeke, Belgium). Next, B cells were washed and incubated with culture medium for 15 min at 37°C. After the last washing step, the B cells were resuspended in culture medium. CFSE labeling was verified by flow cytometry. Subsequently, $1x10^5$ CFSE labeled B cells were cultured in the presence of irradiated autologous feeder cells (8275 rad) at a ratio of 1:1 in a 96 round bottom culture plate (Greiner, Wemmel, Belgium). Five conditions of antigen-specific stimulation were tested, namely 2,5 limes flocculation units per milliliter (Lf/ml) TT (RIVM, Bilthoven, Nederland), 10 µg/ml PLP mixture of different lengths (Severn Biotech), Kidderminster, UK), 40 µg/ml MBP (Biomed, Diepenbeek, Belgium), 10 µg/ml MOG mixture of different lengths (Severn Biotech, Kidderminster, UK) and 1 µg/ml CMV (50 µg/ml; Miltenyi, Auburn, CA, USA). Culture medium was used as a negative control to assess background proliferation. The cultured cells were collected at day 13 to assess cell proliferation (i.e. CFSE dilution) and viability (i.e. 7-AAD) using flow cytometry. The proliferating fraction (ΔPF) was determined by deducting the percentage of proliferated B cells in the absence of antigen from the percentage of proliferative B cells in response to a certain antigen. The proliferative response was considered significant with ΔPF exceeding 2, similar to thresholds set for positive proliferation in CFSE based assays documented previously.[52] Simultaneously, the expression of APC and (co)stimulatory markers HLA-A/B/C (Biolegend, San Diego, CA, USA), HLA-DR and CD80 (all from BD Biosciences, Erembodegem, Belgium) was determined using the FACS Aria™ II (BD Biosciences,

Erembodegem, Belgium) and Diva software. Mouse $IgG_{1,\kappa}$ and $IgG_{2,\kappa}$ Abs were used as isotype controls (all from BD Biosciences, Erembodegem, Belgium).

2.2.5 The *ex vivo* expression of HLA and (co)stimulatory molecules on B cells

For flow cytometric analysis, PBMCs were stained directly *ex vivo* with fluorescently-labeled monoclonal antibodies (mAb) specific for HLA and (co)stimulatory molecules, including fluorescein isothiocyanate (FITC)-labeled HLA-DR/DP/DQ, Phycoerythrin (PE)-labeled HLA-A/B/C and PerCP Cy5.5-labeled CD19 (1); FITC-labeled CD86, PE-labeled CD80 and PerCP-Cy5.5-labeled CD19 (2); FITC-labeled CD40, PE-labeled CD27 and PerCP Cy5.5-labeled CD19 (3) (all from BD Biosciences, Erembodegem, Belgium) for 30 minutes at 4°C. After the fluorescent Ab staining, the PBMCs were washed 2 times with FACS buffer (10x PBS, 1% FBS, 0,1% sodium azide). The analysis was performed by flow cytometry using FACSCalibur™ (Becton Dickinson, San Diego, California, USA) and the Cellquest® software.

2.3 Immunohistochemistry

Brain tissue of MS patients and HC was obtained from the Dutch Brain Bank. The brain specimens were embedded in optimal cutting temperature (OCT) compound Tissue-Tek (Sakura Finetek, Berchem, Belgium) and frozen for cryosectioning (-80°C) and immunohistochemistry (-20°C). The brain tissue was cryosectioned in 10 µm thick sections using the cryostat (Leica, Groot-Bijgaarden, Belgium).

2.3.1 DAB staining

The sections were air dried, fixated in 100% aceton (-20°C) for 10 min and rehydrated with 1x PBS. To quench the endogenous peroxidase activity, the brain sections were incubated with 0,3% H_2O_2 in 100% methanol for 10 min followed by incubation with the primary Ab (Table 2) diluted in 0,1% BSA dissolved in PBS for 1 hour at room temperature (RT). MS plaques within the brain sections were detected with PLP Abs (Table 2). In addition, the sections were stained with anti-HLA-DR (Dako, Gent, Belgium) to differentiate between active and chronic inactive lesions. The former is characterized by perivascular and parenchymal inflammatory cell infiltration, while the latter is hypocellular and devoid of infiltrating immune cells within the lesion. The presence of myelin and B cell surface markers (Table 2) was visualized with the Envision™ Dual Link System-HRP (Dako, Enschede, The Nederlands) using DAB (Dako, Enschede, The Nederlands) as the substrate following manufacturer's instructions. All sections were counterstained with haematoxylin for 40 seconds and mounted with Depex Polystyrene (DPX). Between all the incubation steps , the sections were washed with PBS (3x 5 min), pH 7,4. The negative control included the omission of the primary Ab.

Table 2. The antibody details for the DAB and double fluorescent stainings

Antigen	Cell specificity	Clone	Dilution	Source
CD20	B cells	L26	1:100	Dako
MHC class II	Antigen presenting cells		1:100	Dako
CD80	Antigen presenting cells		1:200	Abcam
PLP	Myelin	LN3	1:500	Serotec

2.3.2 Fluorescent double stainings

The brain sections were air-dried, fixated in 100% aceton for 10 min and rehydrated in 1x PBS, followed by preincubation with 10% goat serum diluted in PBS for 30 min. Then, the sections were incubated with anti-CD80 and anti-CD20 for 1 hour at RT, respectively. CD80 and CD20 immunopositivity was visualized by incubating the sections with goat anti-mouse IgG1 $F(ab')_2$ and goat anti-mouse IgG2a $F(ab')_2$ (1:400; all from Invitrogen, Merelbeke, Belgium) for 30 min at RT, respectively. All primary and secondary Abs were diluted in PBS containing 0,1% BSA. Next, the autofluorescence was blocked with 0,1% Sudan Black diluted in 70% ethanol for 15 min. The sections were mounted with aqueous fluorescent mounting medium (Dako, Enschede, The Nederlands). Between all incubation steps, the sections were washed three times for 5 min with PBS. The negative control included the omission of both primary Abs.

2.4 Statistical methods

Statistical analysis was performed using GraphPad Prism 4 software. When deviations from the normal distribution were observed by the Kolmogorov-Smirnoff test, nonparametric tests were used. The unpaired student t test or Mann-Whitney U test were performed to compare to 2 independent groups, while the paired t test and Wilcoxon matched pair test were used to compare 2 dependent groups. Statistical differences between more than 2 groups were determined by one-way analysis of variance (ANOVA) or Kruskal-Wallis test followed by the post hoc Dunn multiple comparison test. Two way ANOVA was performed when there were two or more variables and more than 2 groups. Values are given as mean $\pm$ standard deviation. Differences were considered to be significantly different at *=p<0,05, **=p<0,01 and ***=p<0,001

3 Results

3.1 *Ex vivo* expression of APC and (co)stimulatory molecules on B cells of MS patients and HC

In order to evaluate the antigen presentation capacity of B cells in MS and HC, the expression of APC (i.e. HLA-DR/DP/DQ and HLA-A/B/C) and (co)stimulatory (i.e. CD86, CD80, CD40) molecules on peripheral B cells was determined through flow cytometry in 36 HCs and 46 MS patients, of which 14 were RRMS and 11 were SPMS. B cells from the CSF of 9 MS patients were also analyzed. Additionally, the expression of these molecules was compared between different treatment groups of MS patients. Representative graphs for the gating of B cells (Figure 4B) and expression of APC and (co)stimulatory molecules are represented in Figure 4.

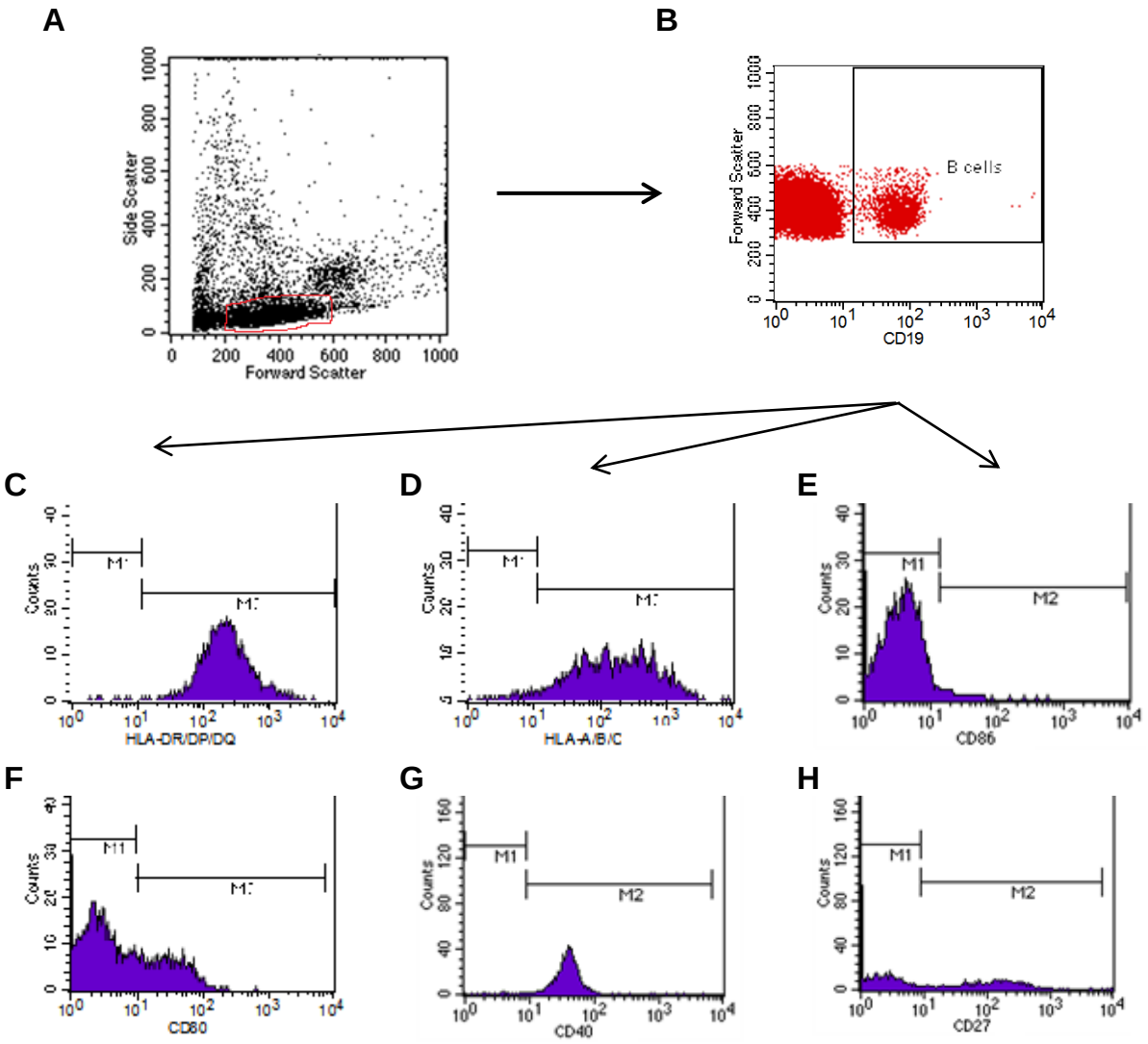

Figure 4. Expression of APC and (co)stimulatory molecules on peripheral B cells. B cells were selected from the lymphocyte population by CD19 expression (A-B). Representative graphs from 1 MS patient are shown for the expression of HLA-DR/DP/DQ (C), HLA-A/B/C (D), CD86 (E), CD80 (F), CD40 (G) and CD27 (H) on peripheral B cells.

In the PB, the percentage of B cells did not differ between MS patients and HC (Figure 5A). The percentage of B cells expressing HLA-DR/DP/DQ and CD40 was reduced in MS patients when compared to HC, in all subpopulations ($p<0,001$; Figure 5B and F). The percentage of HLA-A/B/C$^+$ B cells was also decreased in MS and SPMS patients ($p<0,05$; Figure 5C). The percentage of memory B cells expressing CD27 was greater in HC than in RRMS and MS patients ($p<0,01$; Figure 5G). Interestingly, the percentage of CD80$^+$ B cells was increased in MS patients in comparison to HC ($p<0,05$; Figure 5E). The percentage of CD86$^+$ B cells also tended to be elevated in RRMS ($p=0,09$) and total MS patients ($p= 0,06$; Figure 5D).

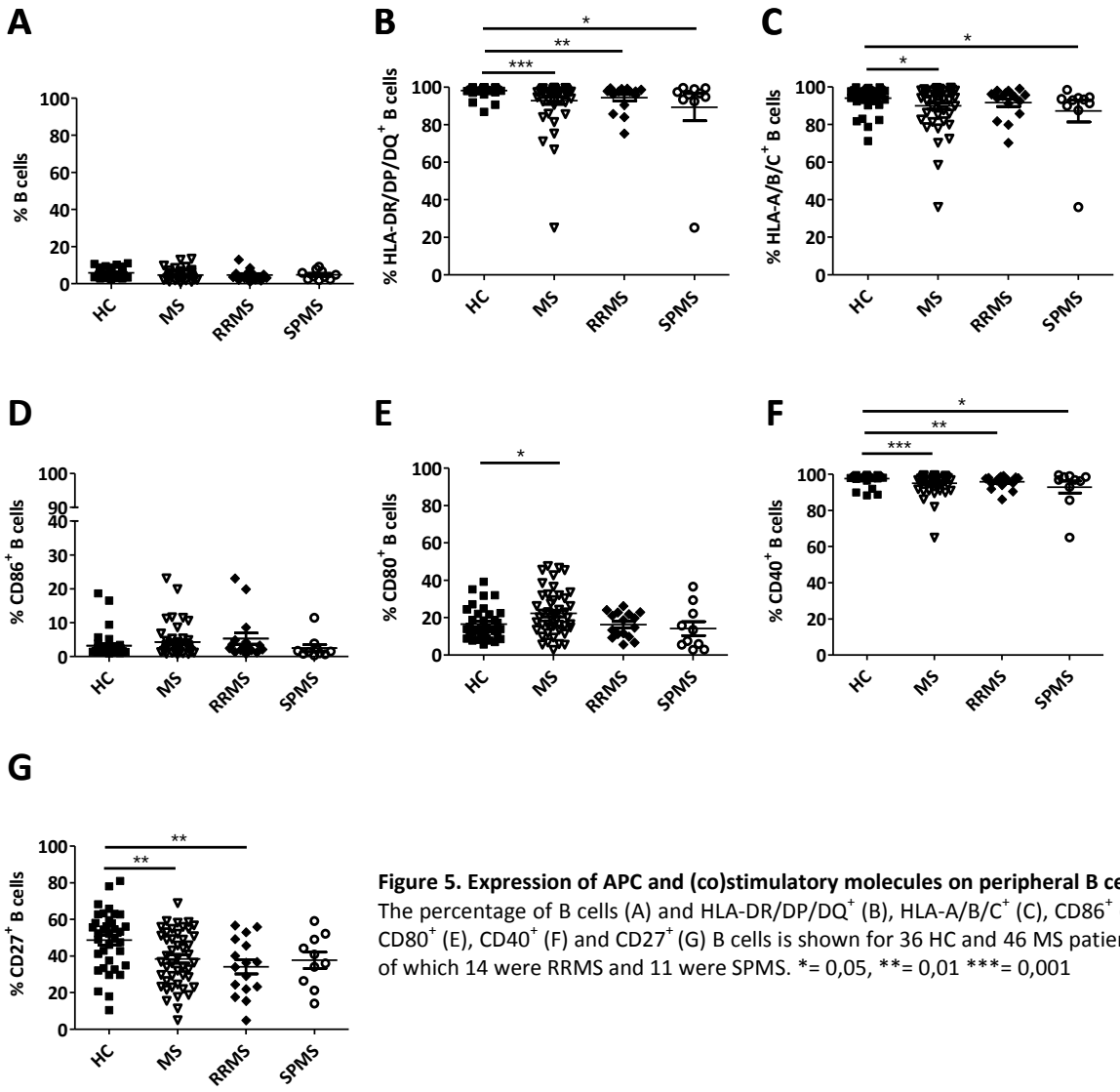

Figure 5. Expression of APC and (co)stimulatory molecules on peripheral B cells. The percentage of B cells (A) and HLA-DR/DP/DQ$^+$ (B), HLA-A/B/C$^+$ (C), CD86$^+$ (D), CD80$^+$ (E), CD40$^+$ (F) and CD27$^+$ (G) B cells is shown for 36 HC and 46 MS patients, of which 14 were RRMS and 11 were SPMS. *= 0,05, **= 0,01 ***= 0,001

In order to analyze the amount of APC and (co)stimulatory molecules on the B cells of HC and MS patients, the mean fluorescence intensity (MFI) was measured. Here, there was no significant difference observed between B cells expressing HLA-DR/DP/DQ (Figure 6A), HLA-A/B/C (Figure 6B),

CD86 (Figure 6C), CD40 (Figure 6E) and CD27 (Figure 6F) in MS patients and HC. Of interest, in addition to the percentage of CD80$^+$ B cells, the MFI of B cells expressing CD80 was also increased in MS patients in comparison to HC (p=0,0152; Figure 6D).

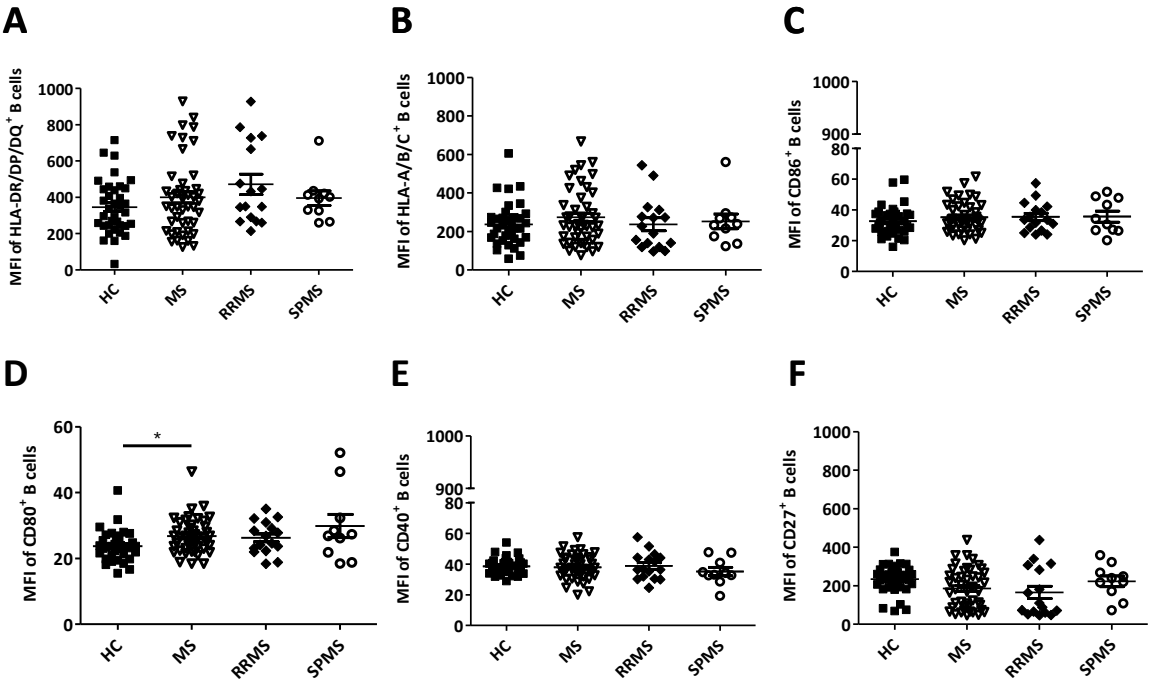

Figure 6. MFI of APC and (co)stimulatory molecules on peripheral B cells. The MFI of HLA-DR/DP/DQ$^+$ (A), HLA-A/B/C$^+$ (B), CD86$^+$ (C), CD80$^+$ (D), CD40$^+$ (E) and CD27$^+$ (F) B cells is shown for 36 HC and 46 MS patients, of which 14 were RRMS and 11 were SPMS. *= 0,05

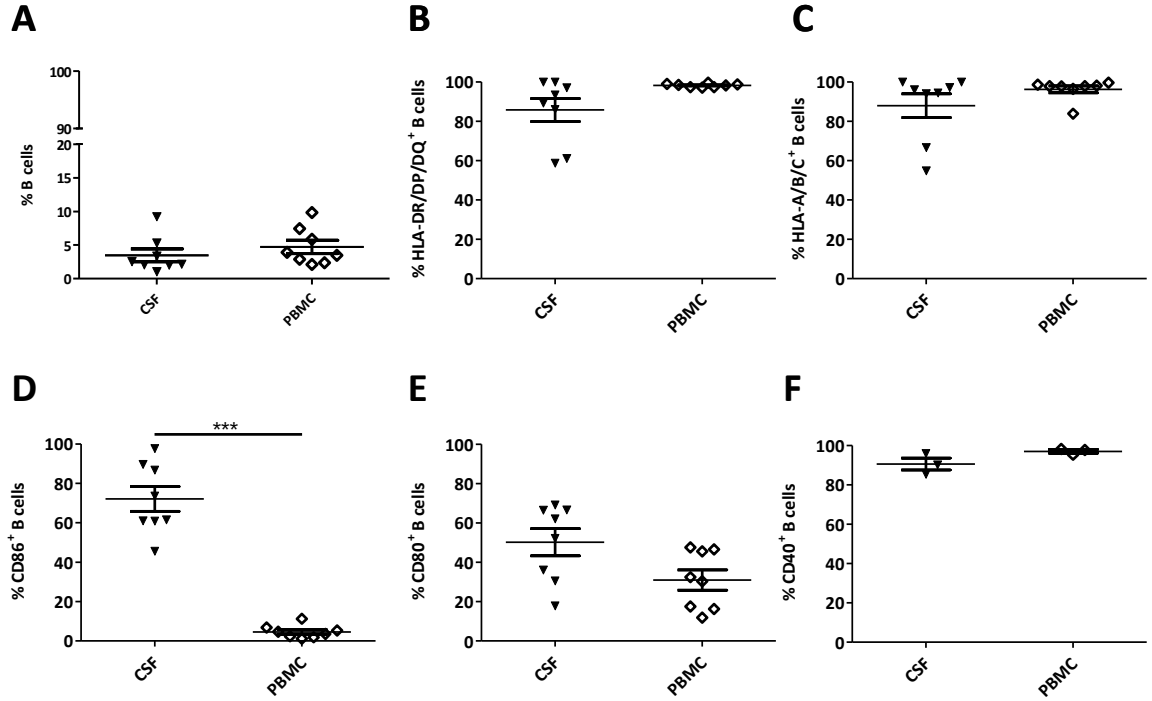

Figure 7. Comparison of APC and (co)stimulatory molecule expression on B cells from PB and CSF of MS patients. The percentage of B cells (A) and B cells expressing HLA-DR/DP/DQ (B) HLA-A/B/C (C), CD86 (D), CD80 (E) and CD40 (F) is shown in the PB and CSF of 9 patients. ***=0,001

We also compared the B cell expression of HLA and (co)stimulatory molecules in the PB and CSF. Therefore, paired PB and CSF was collected from 9 possible MS patients, of which 3 were already diagnosed with clinically definite MS. An equal percentage of B cells was found in the PB and CSF (Figure 7A). In addition, there was no significant difference in the percentage and MFI of HLA-DR/DP/DQ$^+$ (Figure 7B and 8A), HLA-A/B/C$^+$ (Figure 7C and 8B) and CD40$^+$ B cells (Figure 7F and 8E). The decreased presence of HLA-DR/DP/DQ$^+$ and CD86$^+$ B cells in the CSF of some patients, that causes a large standard deviation, is probably due to β-interferon treatment. The percentage of CD80$^+$ B cells tended to be increased in the CSF (Figure 7E; p=0,0921), but no significant differences were found in the MFI of CD80$^+$ B cells between PB and CSF (Figure 8D). However, the percentage and MFI of CD86$^+$ B cells was highly elevated in the CSF when compared to PB (Figure 7D and 8C; p<0,0001).

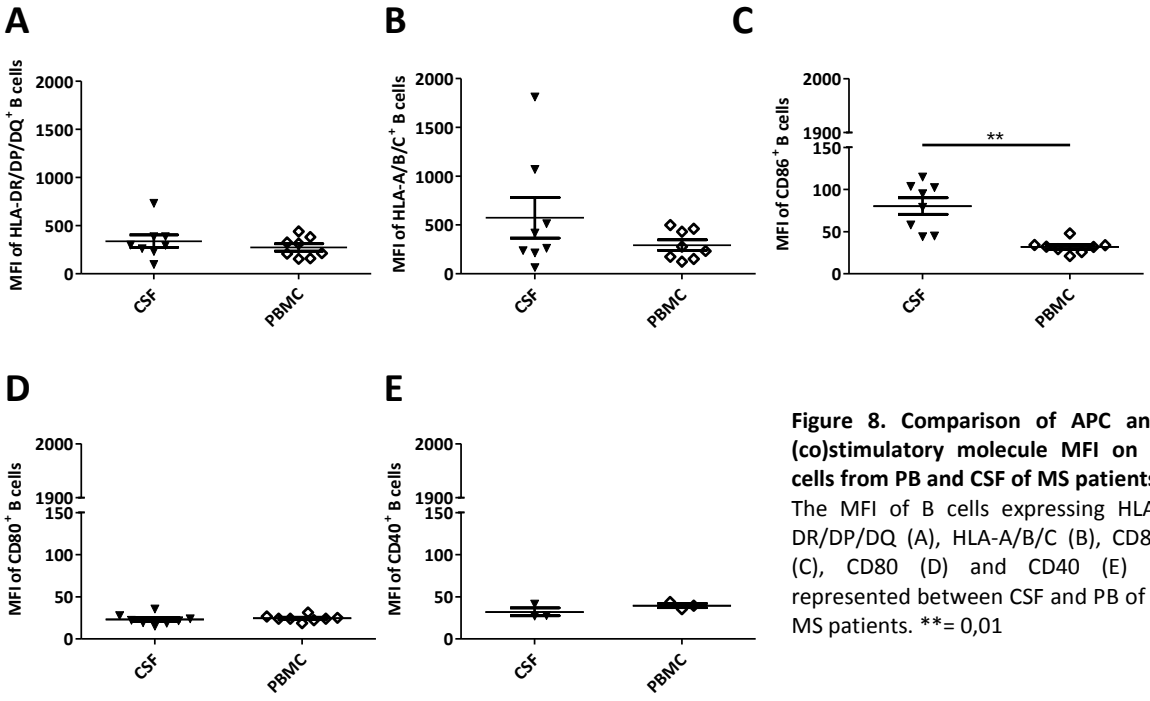

Figure 8. Comparison of APC and (co)stimulatory molecule MFI on B cells from PB and CSF of MS patients. The MFI of B cells expressing HLA-DR/DP/DQ (A), HLA-A/B/C (B), CD86 (C), CD80 (D) and CD40 (E) is represented between CSF and PB of 9 MS patients. **= 0,01

Of importance, the percentage and MFI of B cells expressing the costimulatory molecules CD80 (Figure 5E and 6D) and CD86 (Figure 7E and 8D) were elevated in the PB and CSF of MS patients, respectively. In addition, the percentage of B cells expressing HLA-DR/DP/DQ (Figure 5B), HLA-A/B/C (Figure 5C), CD40 (Figure 5F) and CD27 (Figure 5G) was decreased in the PB of MS patients in comparison to HC. No differences were observed between different treatment groups of MS due to low subject numbers. These results indicate the increased potential of MS B cells, both in PB and CSF, to give costimulatory signals to (autoreactive) T cells. The decreased expression of HLA molecules on MS B cells could be due to therapy, and/or to the migration of disease-relevant B cells into the CNS.

3.2 The presence of B cells and (co)stimulatory molecules in the MS and HC brain

Besides the peripheral and CSF compartment, the presence of CD80$^+$ and HLA-DR/DP/DQ$^+$ B cells was determined in MS and healthy brain tissue. Initially, the lesions were identified with PLP Abs, in which loss of PLP staining clearly indicated demyelinated regions (Figure 9A).

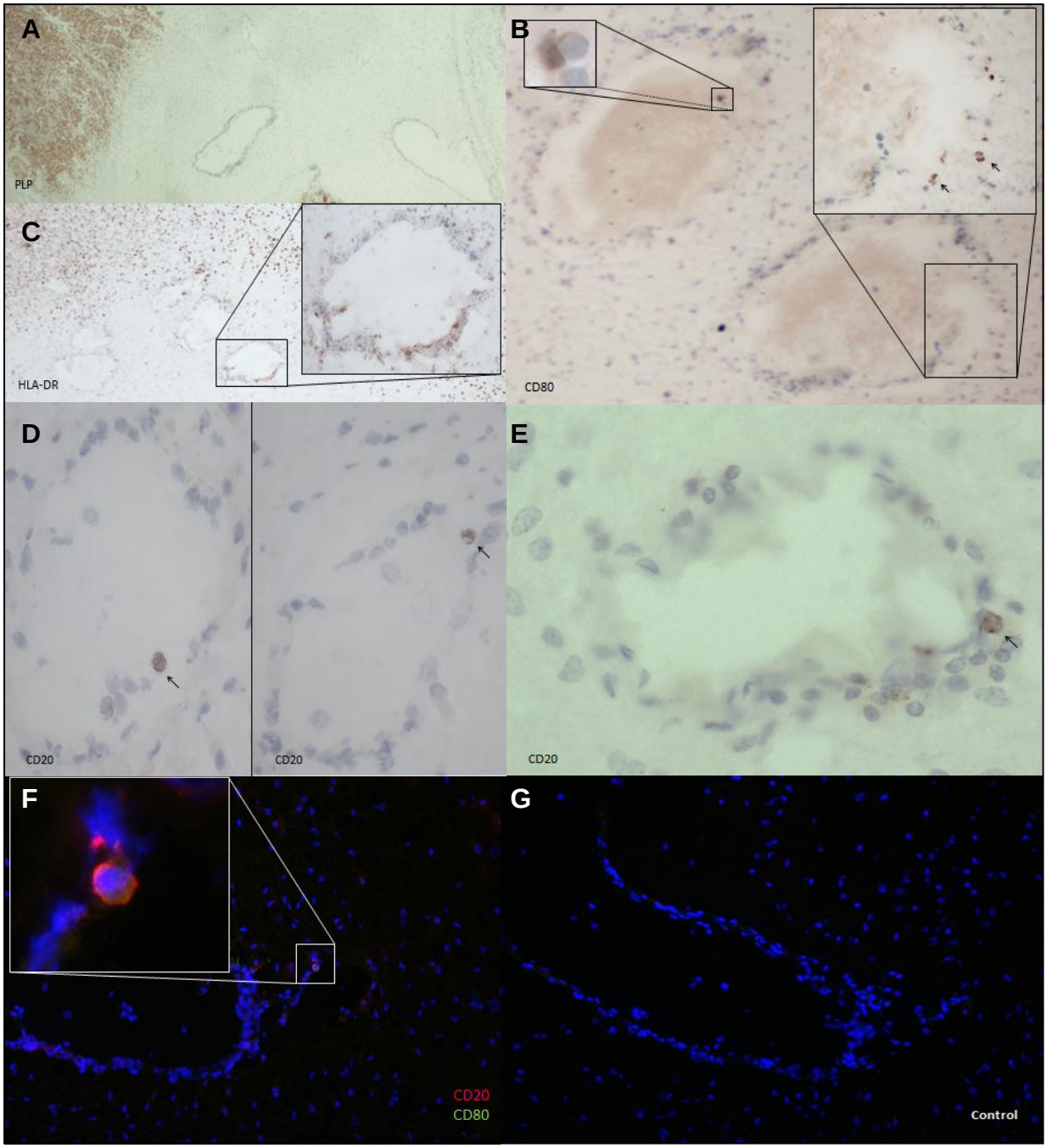

Figure 9. The presence of CD80$^+$ B cells in the MS brain. The lesions were detected with PLP Ab (100x; A). HLA-DR$^+$ (100x, inlet 400x; C) and CD80$^+$ (100x; inlet 400x; B) cells are shown for 1 MS patient using DAB staining. CD20$^+$ B cells were identified in two 2 MS patients (400x; D-E). In healthy brains, inflammatory infiltrates were not identified (data not shown). In 1 MS patient, a CD80$^+$ B cell was identified (100x; inlet 400x; F) using double fluorescent staining. In the negative control (i.e. no secondary Abs) no staining was observed (100x; G)

First, $CD20^+$, HLA-DR^+ and $CD80^+$ cells were identified using DAB in the perivascular cuffs surrounding blood vessels in an active lesion in 2 MS patients. (Figure 9B-E). No $CD20^+$ B cells, HLA-DR^+ and $CD80^+$ cells were found in healthy brain tissue (data not shown). Next, a fluorescent staining for CD20 and CD80 was performed in order to investigate the expression of CD80 on CNS infiltrated B cells. A $CD20^+CD80^+$ B cell was found in an infiltrate in a chronic active MS lesion. To our knowledge, this was the first time that $CD80^+$ B cells were demonstrated in the MS brain (Figure 9F). Aspecific staining of the two secondary Abs was not observed (Figure 9G) .

3.3 Expression of APC and (co)stimulatory molecules on B cells after T cell- and TLR-dependent activation

In order to evaluate the expression of HLA and (co)stimulatory molecules on B cells after TLR9-dependent and T cell-dependent B cell activation, sorted $CD19^+$ B cells were cultured with CpG 2006 and IL-2, or CD40L in combination with IL-4 for 5 days. The CpG 2006+IL-2 system acts as a mimic for T cell independent activation. CpG ODN 2006 is a synthetic oligonucleotide that contains unmethylated CpG motifs. These motifs stimulate B cells through TLR9 ligation leading towards an enhanced immune response,[30] whereas IL-2 is a pro-inflammatory cytokine that acts as a growth factor for activated B cells.[53] The CD40L+IL-4 system mimics a T cell-dependent B cell activation. IL-4 is a T cell product promoting B cell activation and proliferation.[54] CD40L is a cell surface marker on the T cell that also stimulates B cell activation.[31] The expression of HLA and (co)stimulatory molecules on B cells following activation was assessed by flow cytometry using fluorescently labeled Abs against HLA-DR/DP/DQ and CD80.

Both activation systems caused an increase in the percentage and MFI of HLA-DR/DP/DQ^+ (Figure 10A-B) and $CD80^+$ (Figure 10C-D) B cells. T cell-dependent B cell activation (i.e. CD40L + IL-4) led to a higher upregulation in the percentage of $CD80^+$ B cells on day 3 in comparison to TLR-dependent B cell activation (i.e. CpG 2006 + IL-2) ($p<0,01$; Figure 10C). In addition, T cell-dependent B cell activation was more efficient in upregulating the MFI of HLA-DR/DP/DQ^+ (Figure 10B) and $CD80^+$ ($p<0,05$; Figure 10D) B cells after 4-5 days of culture. Normalization was performed by setting the culture medium condition at 100% and adjusting the values of the stimulation conditions accordingly.

Briefly, polyclonal B cell stimulation via TLR-dependent and T cell-dependent mechanisms caused an increased expression of HLA-DR/DP/DQ and CD80 on B cells. The effect of T cell-dependent B cell activation was later but more pronounced. In the following experiments, the expression of APC and (co)stimulatory molecules on B cells was determined following antigen-specific B cell activation.

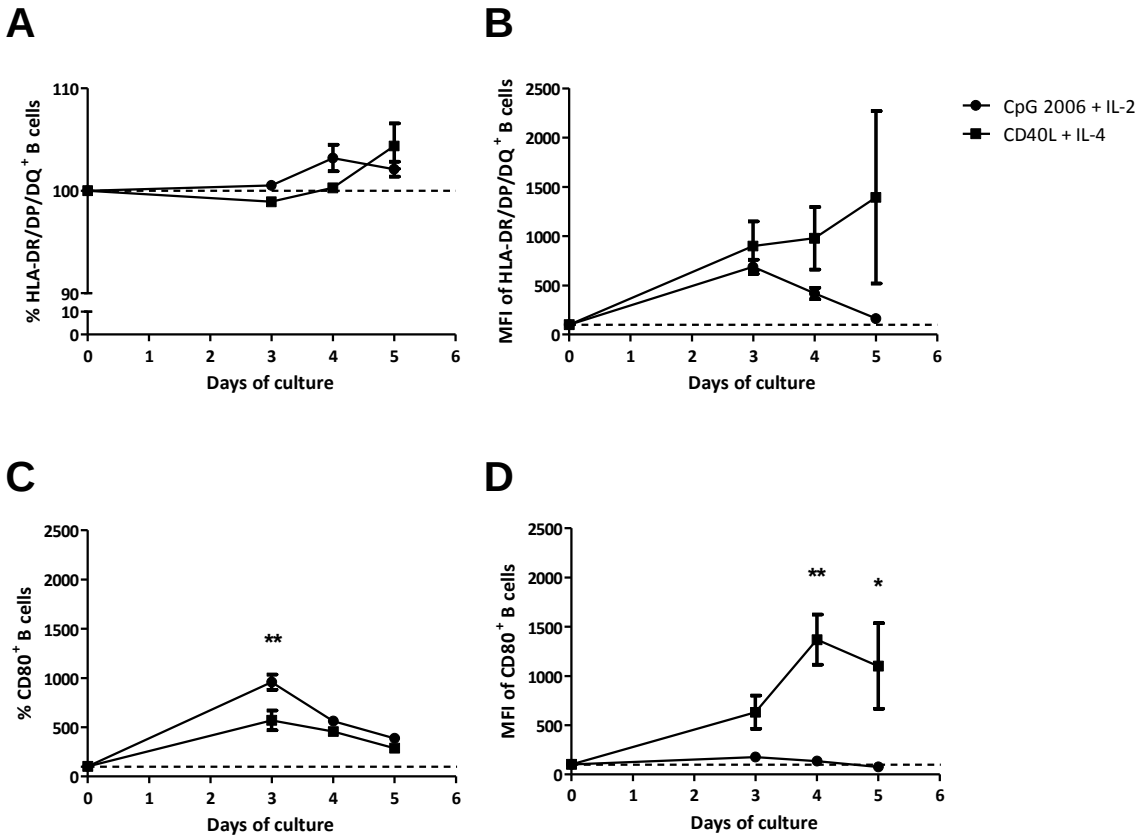

Figure 10. Expression of APC and (co)stimulatory molecules on B cells following TLR-dependent and T cell-dependent B cell activation. The percentage and MFI of HLA-DR/DP/DQ$^+$ (A-B) and CD80$^+$ (C-D) B cells was determined by flow cytometry at day 0 and after 3, 4 and 5 days of activation in 2 HCs. All measurements were performed in duplicate. The relative expression levels are shown in which the culture medium condition is set at 100%. *=0,05 and **=0,01

3.4 Optimization of CFSE assay

In order to determine the B cell proliferative response and expression of APC and (co)stimulatory molecules following antigen-specific B cell stimulation, a CFSE assay was used. B cells were labeled with CFSE and cultured with self (i.e. MBP, MOG and PLP) and nonself (i.e. CMV and TT) antigens. TT is a superantigen to which most recently vaccinated individuals have developed an immune response, while CMV is a human herpesvirus that has been implicated in the development of MS. In parallel, CFSE labeled B cells were cultured with culture medium as a negative control. The proliferating (i.e. reactive) capacity of B cells was examined through flow cytometry by measuring the dilution of CFSE. In addition, the expression of HLA-DR, HLA-A/B/C and CD80 on B cells was determined after 10-14 days of culture. A number of parameters, such as the CFSE concentration, method of B cell isolation, B cell-feeder cell ratio and duration of cell culture, were first determined.

3.4.1 Concentration of CFSE

The CFSE concentration was optimized to achieve an optimal labeling of the B cells. An efficient labeling is necessary to detect multiple cell divisions before the fluorescent CFSE signal fades to the

background and to avoid spill-over of the CFSE signal into other channels. PBMCs labeled with 0,5, 1 and 2 µM CFSE were stimulated with anti-CD3 during 7 days. Proliferation was measured by analyzing the decrease in the CFSE signal, while the viability was evaluated using 7AAD. After a 7 day culture with anti-CD3, the proliferation and viability of CFSE labeled PBMCs were determined using flow cytometry. The proliferative fraction and viability of PBMC was comparable when using 0,5 and 1 µM CFSE (Figure 11B and E). However, cell divisions could be evaluated more efficiently when using 1 µM CFSE, because the peak of the labeled cells was located more to the right. The viability of 2 µM CFSE labeled PBMC showed an increase in the 7AAD signal, probably due to spill-over of the CFSE signal (Figure 11I). Therefore, the optimal CFSE concentration was determined to be 1 µM .

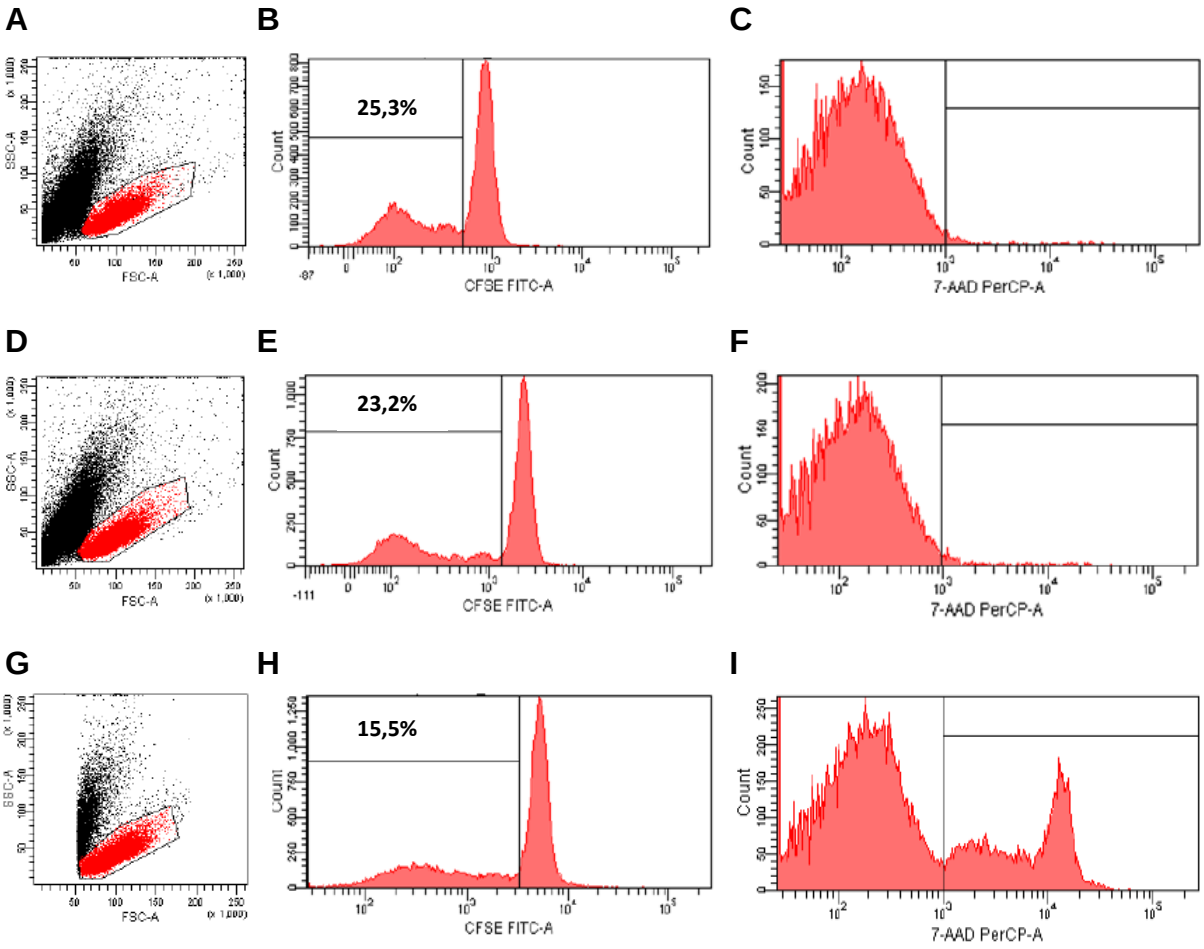

Figure 11. Optimization of the CFSE concentration. After a 7 day culture with anti-CD3, the proliferation (B, E and H) and viability (C, F and I) of 0,5, 1 and 2 µM CFSE labeled PBMC was determined by flow cytometry. The CFSE labeled PBMC were gated by a lymphocyte gate (A, D and G). The PBMC viability was similar in all CFSE concentrations used, namely between 99% and 99,6%.

3.4.2 Method of B cell isolation

Two procedures of B cell isolation were compared in order to select the optimal (i.e. high purity and efficiency; and low B cell activation status) isolation method. B cells of one donor were isolated using

the positive magnetic selection and negative magnetic B cell enrichment kit (EasySep). The purity of B cells after negative B cell selection was 99,81% (Figure 12B), whereas the B cell purity was 97,56% after positive selection (Figure 12E). In addition, the negative B cell selection yielded the lowest percentage of $CD25^+$ activated B cells (Figure 12C) in comparison to the positive B cell selection (Figure 12F). Prior to the selection procedure, the percentage of $CD25^+$ B cells was 24,52%. In previous experiments, B cells were isolated by cell sorting, but this isolation method was not efficient due to long duration and low yield.

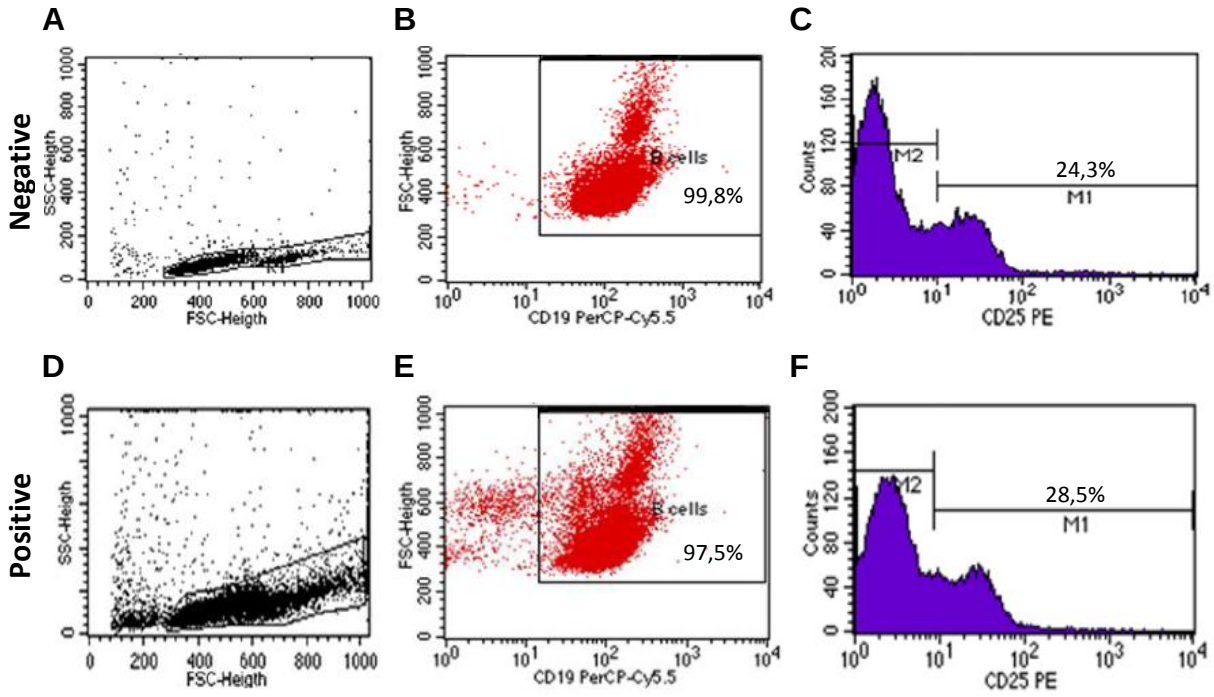

Figure 12. Positive and negative magnetic selection of B cells. After B cell enrichment (negative selection), the purity of B cells (B) isolated from PBMCs (A) was 99,75%. The percentage of $CD25^+$ (i.e. activation marker) B cells was 24,3% (C). Positive magnetic B cell selection yielded a B cell purity of 97,5% (E) isolated from PBMCs (D). The percentage of $CD25^+$ B cells was 28,52% (F). Prior to the isolation method the percentage of $CD25^+$ B cells was 24,51%. Both selection procedures were repeated multiple times by our research group.

In brief, the B cell enrichment kit was used in further experiments due to the high purity, short duration and low percentage of $CD25^+$ B cells.

3.4.3 Duration of antigen stimulation

The duration of antigen stimulation and B cell-feeder cell ratio were evaluated in order to achieve an optimal antigen reactivity (i.e. maximal proliferation, viability and expression of (co)stimulatory molecules). Therefore, CFSE labeled B cells and autologous feeder cells were cultured in different conditions (i.e. culture medium, TT, MBP and CMV) and two different ratios, namely 1:1, and 1:2, respectively. After 10-14 days of culture, the proliferation and viability of the B cells was determined by flow cytometric analysis in addition to the B cell expression of APC and (co)stimulatory molecules.

The proliferating fraction (ΔPF) was determined by deducting the percentage of proliferated B cells in the absence of antigen from the percentage of proliferative B cells in response to a certain antigen. The proliferative response was considered significant with ΔPF exceeding 2, similar to thresholds set for positive proliferation in CFSE based assays documented previously.[52] The viability of B cells and their expression of APC and (co)stimulatory molecules in the culture medium condition were set at 100%, accordingly the other conditions were normalized.

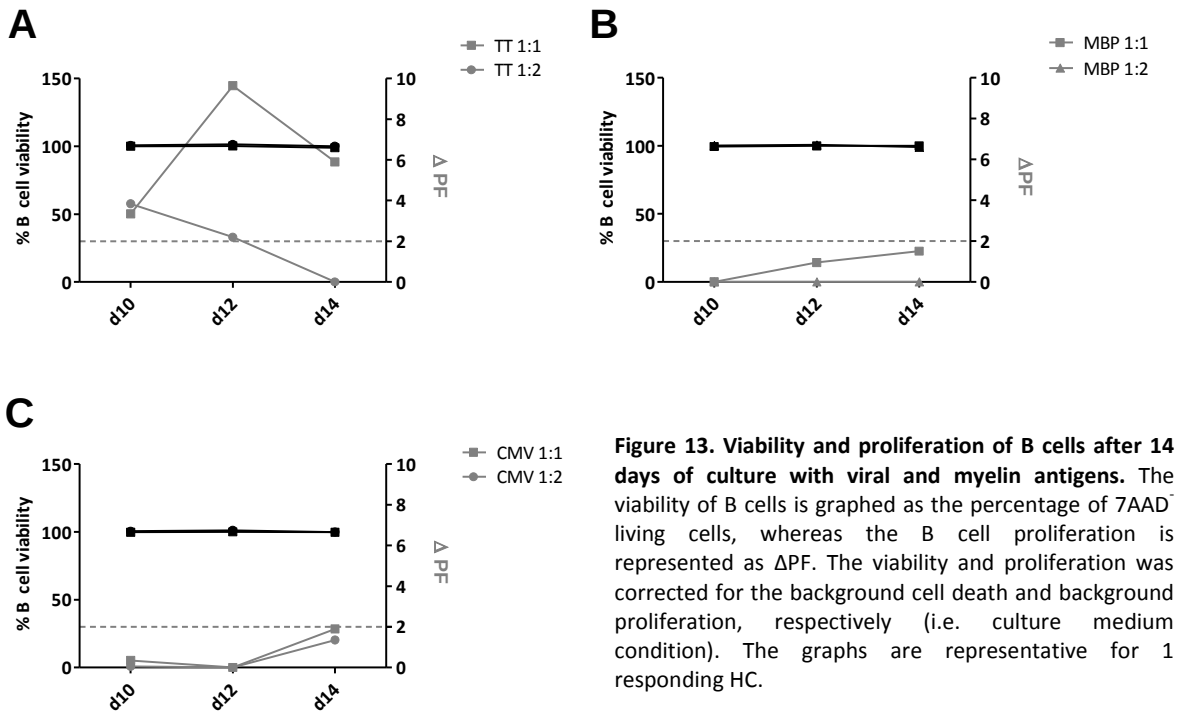

Figure 13. Viability and proliferation of B cells after 14 days of culture with viral and myelin antigens. The viability of B cells is graphed as the percentage of 7AAD$^-$ living cells, whereas the B cell proliferation is represented as ΔPF. The viability and proliferation was corrected for the background cell death and background proliferation, respectively (i.e. culture medium condition). The graphs are representative for 1 responding HC.

The B cell viability and proliferation of responding HCs were used to determine the optimal culture duration and B cell-feeder cell ratio. In Figure 13, 1 representative responding HC is shown. B cell viability was maintained at $\pm$ 100% after 10-14 days of culture in all tested ratios (Figure 13 A-C; left Y axis). However, B cell proliferation was higher when using a B cell-feeder cell ratio of 1:1 when compared to 1:2 (Figure 13A-C; right axis). The proliferative response of B cells against TT was considered significant at each time point (i.e. 10, 12 and 14 days of culture) as ΔPF exceeded 2 (Figure 13A). When considering duration of the culture, the highest B cell proliferation against TT was observed after 12 days of culture.

The percentage and MFI of B cells expressing HLA-DR and HLA-A/B/C (Figure 14A-F) did not differ between the 2 ratios after 10-14 days of culture with TT, MBP or CMV. However, the percentage and MFI of CD80$^+$ B cells increased after 12-14 days of culture with TT (Figure 14A-B) and CMV (Figure 14E-F). This elevation was more prominent in the 1:1 ratio.

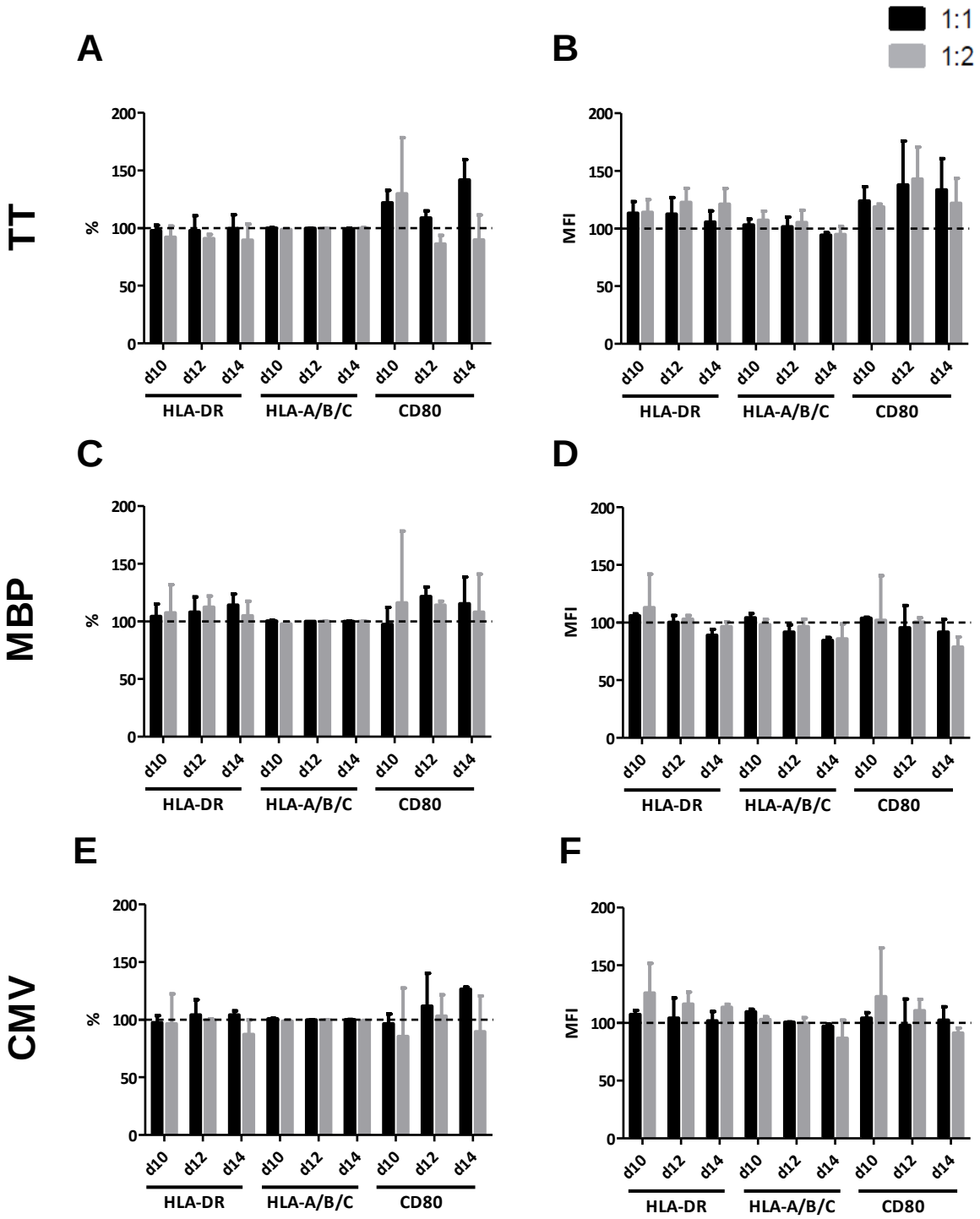

Figure 14. The percentage and MFI of APC and (co)stimulatory molecules on peripheral B cells following TT, MBP and CMV stimulation using different B cell-feeder cell ratios. The percentage and MFI of HLA-DR$^+$, HLA-A/B/C$^+$ and CD80$^+$ B cells was determined following stimulation with TT (A-B), MBP (C-D) and CMV (E-F) after 10, 12 and 14 days of culture. Results are the average of experiments in 3 HCs. The relative expression levels are shown in which the culture medium condition is set at 100%.

Taking into account that an optimal proliferative response of B cells was obtained after 12 days of culture and the expression of APC and (co)stimulatory molecules, more specifically CD80, on the B cells was maximal after 14 days of culture, the optimal duration of the CFSE assay was determined to be 13 days of culture and the optimal B cell-feeder cell ratio was 1:1.

3.5 B cell antigen reactivity and expression of APC and (co)stimulatory molecules after antigen-specific activation

The proliferative response of CFSE labeled B cells cultured with TT, MOG, PLP, MBP or CMV was determined after 13 days of stimulation. In addition, the expression of APC and (co)stimulatory molecules on B cells was assessed.

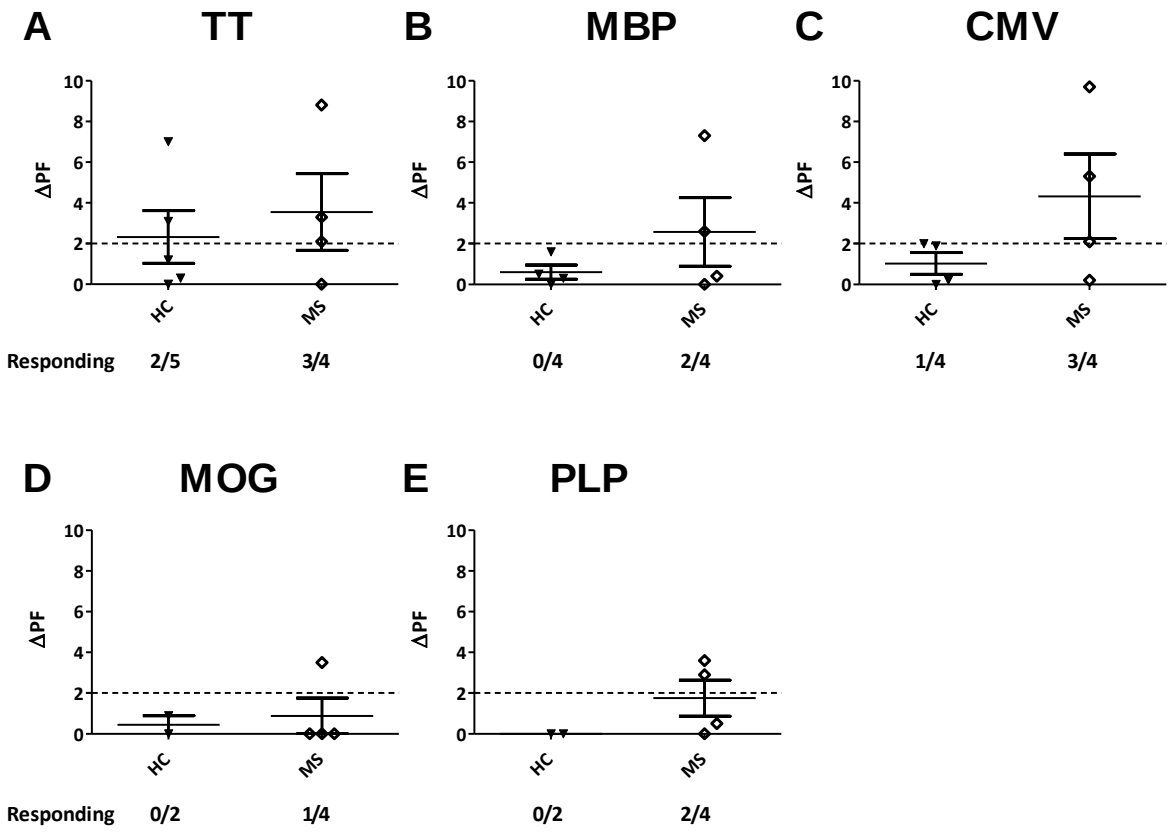

Figure 15. The proliferative response of peripheral B cells cultured with TT, MBP, CMV, MOG or PLP. The antigen reactivity of B cells against TT, MBP, CMV, MOG and PLP is shown for 2-5 HC and 4 MS patients. ΔPF is determined by deducting the background proliferation from the proliferation against a specific antigen. A threshold value for positive proliferation was set at 2%.

As expected, the proliferative response of B cells against TT was comparable between MS patients and HC (Figure 15A). The self antigens MBP (Figure 15B) and PLP (Figure 15E) did not cause B cell reactivity in HC, whereas half of the MS patients did show a proliferative response against these antigens. In addition, MOG reactivity was observed in 1 MS patients versus 0 HC (Figure 15D). Interestingly, 3 out of 4 MS patients versus 1 HC demonstrated B cell reactivity against CMV (p=0,14; Figure 15C). However, no statistically significant differences were found in the proliferative B cell response between HC and MS patients.

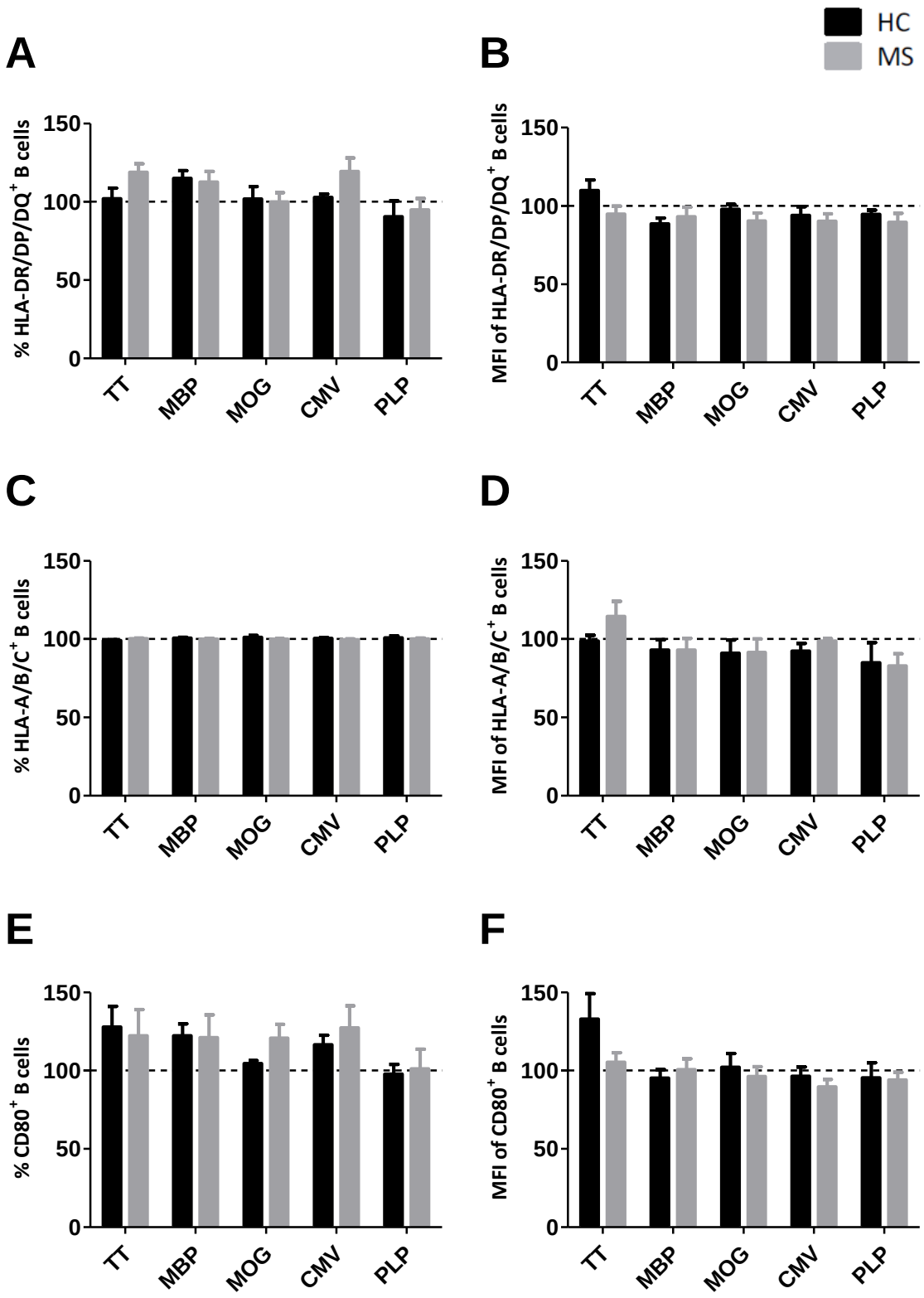

Figure 16. Expression of APC and (co)stimulatory molecules on peripheral B cells after 13 days of culture with TT, MBP, MOG, CMV or PLP. The percentage and MFI of B cells expressing HLA-DR (A-B), HLA-A/B/C (C-D) and CD80 (E-F) is shown for 2-5 HC and 4 MS patients after 13 days of stimulation with TT, MBP, MOG, CMV and PLP. The relative expression levels are shown in which the culture medium condition is set at 100%.

The percentage of B cells expressing HLA-DR and CD80 (Figure 16A and E) increased after antigen-specific stimulation. More specifically, the CMV (p=0,2; Figure 16A) and TT (p=0,11; Figure 16A)

antigen seem to elevate the percentage of HLA-DR$^+$ B cells more in MS patients than HCs. In addition, the percentage of B cells expressing CD80 seems to increase after MOG stimulation in MS patients (p=0,2; Figure 16E). However, the percentage and MFI of B cells expressing HLA-DR (Figure 16A-B), HLA-A/B/C (Figure 16C-D) and CD80 (Figure 16E-F) did not significantly differ between HC and MS patients after 13 days of stimulation with TT, MBP, MOG, CMV and PLP, which is probably due to the low number of HC and MS patients currently tested.

4 Discussion

MS is generally considered a $CD4^+$ T cell mediated CNS inflammatory disease. The type of APCs causing the activation of these autoreactive T cells has not been completely elucidated. In the periphery, antigen presentation is commonly performed by dendritic cells, B cells and macrophages, while astrocytes and microglia are the main APCs within the CNS. We evaluated the capacity of peripheral and intrathecal B cells as APCs in MS, since anti-CD20 mediated B cell depletion significantly reduced the number of gadolinium-enhancing lesions as well as the T cell number in RRMS patients.[42] These observations indicated that B:T cell interactions are important for the expansion of autoreactive T cells in MS.

Efficient T cell activation requires the engagement of TCR with the Ag-MHC complex. Secondly, the (co)stimulatory molecules CD80/CD86, expressed on APCs, are essential for T cell proliferation and cytokine secretion. In the absence of CD80/CD86 costimulation, T cell anergy occurs.[31] Aberrant (co)stimulatory molecule expression is considered to play a role in a number of autoimmune disorders, including RA, systemic lupus erythematosus and MS.[55] In the EAE model, the upregulation of CD80 is necessary for the onset of monophasic EAE and the recurrent exacerbations in chronic relapsing EAE, which are elicited by a Th1 immune response. In addition, CD86 is more likely to play a role in the immune response, because it is induced much faster than CD80.[50]

Reports of Boylan et al[55] and Svenningsson et al[56] did not find significant differences between peripheral B cells expressing CD80 in stable RRMS patients and HC, which is in accordance with the study of Genç and coworkers.[50] However, the latter research group showed that active RRMS patients had a significant increase in B cells expressing CD80 in comparison to HC and stable RRMS patients, which was not observed in the aforementioned studies.[55, 56] In accordance to the previous reports, we did not find significant differences in the percentage of $CD80^+$ B cells between RRMS patients and HC. Interestingly, we did show an increased percentage and MFI of peripheral B cells expressing CD80 in the total MS patient group (Figure 5E and 6D; $p<0,05$) without distinguishing between active and stable MS patients. Opposed to CD80, the percentage of B cells expressing CD86 tended to be elevated in RRMS ($p=0,09$; Figure 5D) and MS patients ($p=0,065$; Figure 5D) in comparison to HC, but no differences were found in the MFI of $CD86^+$ B cells, which was in the line with the report of Boylan et al[55]. However, Genç and colleagues did observe an elevated number of $CD86^+$ lymphocytes in active RRMS patients in comparison to the control group.[50] This contradictory finding can be explained by the fact Genç et al[50] used PBMCs, while Boylan et al[55] worked with whole blood samples.

The elevated presence of $CD80^+$ B cells in the PB of MS patients may be involved in the progression of autoimmune diseases. B cell-deficient mice cannot elicit autoreactive T cell responses after immunization with self cytochrome c peptides. However, reconstituting the $CD80/CD86^+$ B cell population caused T cell autoreactivity in the B cell deficient mice.[57] This type of B cells may play a role in antigen presentation when only low antigen levels are present. Even though dendritic cells are the main APCs in T cell activation, antigen-specific B cells can be up to 10^4 fold more efficient in antigen presentation to T cells due to their BCR-mediated antigen uptake.[50]

Besides costimulatory molecules, APCs also express MHC class II molecules that induce T cell activation. Genç and colleagues found an increased percentage of B cells expressing HLA-DR/DP/DQ in active RRMS patients.[50] However, we observed a significant decrease in the percentage of HLA-$DR/DP/DQ^+$ B cells ($p<0,05$-$0,001$; Figure 5B) in the MS groups, which can be attributed to the fact that we did not distinguish between active and stable MS. The percentage of $CD40^+$ B cells was also lower in all MS patient groups in comparison to HC (Figure 5F; $p<0,05$-$0,001$). In addition, the percentage of B cells expressing CD27 (i.e. memory B cell) was determined in the PB of MS patients as well. In our cohort, the MS patients had a decreased percentage of peripheral $CD27^+$ B cells when compared to the control group (Figure 5G; $p<0,01$). These findings are not in agreement with a similar report which found comparable percentages of memory B cells in the periphery of MS patients.[52] The observed decline in peripheral HLA-$DR/DP/DQ^+$, $CD40^+$ and $CD27^+$ B cells may be explained by their migration to the inflamed CNS. In addition, the percentage and MFI of B cells expressing HLA-DR/DP/DQ, CD40 and CD27 can be affected by MS patients undergoing treatment. At present, we are evaluating the effect of treatment on the expression of APC and (co)stimulatory molecules on B cells, but the number of patients currently included is too low to make definite conclusions.

The PB may not be the ideal specimen to evaluate the antigen presenting capacity of B cells, because the CNS white matter is the target of the immune response.[30] Therefore, we also assessed the expression of APC and (co)stimulatory molecules in CSF, which can be an appropriate surrogate for the CNS environment.

The expression of $CD80^+$ B cells tended to be higher in the CSF of MS patients in comparison to their peripheral counterparts (Figure 6D: $p=0,0921$), while Sellebjerg et al[58] and Svenningsson et al[56] did observe a significant increase of $CD80^+$ B cells in the CSF compartment. This observation can be due to the low number of MS patients currently included in our study and the lack of CSF control groups. Monteyne and coworkers also detected CD80 messenger ribonucleic acid (mRNA) in half of the CSF cells from active MS patients, but not in stable or progressive patients.[59] As previously discussed, these findings can be related to the increased peripheral percentage of $CD80^+$ B cells in active disease

and not in stable RRMS.[50] However, the presence of CD80 mRNA is not confined to MS.[59] The number of $CD86^+$ B cells was highly elevated in the CSF opposed to the PB of MS patients (Figure 7D; p<0,001), which was in line with the observations of Sellebjerg et al[58]. In addition, the MFI of B cells expressing CD86 was increased in the CSF (Figure 8C; p=0,0069). Even though Monteyne and colleagues[59] did not find differences in the CD86 mRNA expression between the CSF of the control group and, stable and active MS cases, post-translational modifications can elevate the protein levels while the gene expression remains constant.

Besides the CSF compartment, postmortem studies showed an elevated expression of $CD80^+$ lymphocytes in acute MS lesions, but not control brains and inflammatory infarcts. CD80 staining was primarily located in perivascular cuffs.[56, 60] In contrast, the predominant expression of CD86 was found on macrophages present in MS plaques and cerebral infarcts, indicating that MS lesions and not cerebral infarcts are associated with CD80 expression.[60, 61] Since Windhagen et al. also observed a low expression of CD86 in noninflammatory brains, CD86 may be constitutively expressed on microglia.[60] As previously discussed, Monteyne and coworkers found a similar expression of $CD86^+$ CSF cells between control and MS groups.[59] Our preliminary results showed the presence of $CD80^+$ B cells in the MS brain (Figure 9F), whereas no B cells and other inflammatory infiltrates were detected in healthy brain tissue. In summary, the elevated percentage and MFI of peripheral $CD80^+$ B cells and CSF $CD86^+$ B cells in MS patients and the presence of $CD80^+$ B cells in MS brain indicate that costimulatory molecules increase the antigen presention capacity of B cells in MS.

In the second objective, we evaluated the expression of APC and (co)stimulatory molecules after TLR-dependent (i.e. CpG 2006 and IL-2) and T cell-dependent (i.e. CD40L and IL-4) B cell activation. Therefore, B cells were activated *in vitro* using two systems, namely the CpG+IL-2 and CD40L+IL-4 system. Thereby, a comparison could be drawn between bacterial B cell stimulation through TLR9 and T cell-dependent B cell activation. Jiang and co-workers showed that CpG activated B cells had an increased expression of MHC class II, CD40 and CD80, which enabled them to activate allogeneic T cells.[62] However, similar to the study of Harp and colleagues we observed that the CD40L+IL-4 system upregulated the MHC class II and the co-stimulatory molecule CD80 more efficiently in comparison to the CpG+IL-2 system (p<0,05; Figure 10A-D).

In order to determine the proliferative response of B cells against antigen-specific stimuli, a CFSE assay was performed in which CFSE labeled B cells were cultured with nonself antigens (i.e. TT and CMV) and self antigens (i.e. MOG, MBP and PLP) in the presence of irradiated feeder cells. The proliferative response, viability and HLA and (co)stimulatory molecule expression of B cells was determined by flow cytometric analysis. To study the B cell reactivity against common nonself

antigens, the B cell specificity to TT was compared to the myelin reactivity of B cells. The nonself antigen TT was selected since most individuals have been vaccinated with inactivated TT (i.e. tetanus), giving them an elevated immune response. In parallel, the myelin self antigens MBP, MOG and PLP were included to determine the role of B cells in MS pathogenesis. MBP and PLP are components of the myelin sheath, which have been extensively studied due to their abundance and easy isolation. Multiple research groups have successfully isolated MBP- and PLP-reactive T cells from MS patients as well as healthy donors, demonstrating that self reactive T cells can be part of the normal T cell repertoire.[4] However, the frequency of activated MBP-reactive T cells is elevated in MS patients and are less dependent on CD28 stimulation for their activation.[4, 63] Furthermore, MBP-specific Abs are increased in the serum and CSF of MS patients and are also detected in MS lesions.[28] Aside from MBP and PLP, MOG is another candidate autoantigen as it is highly immunogenic. MOG is the only myelin antigen that causes T cell reactivity and a demyelinating Ab response in EAE models.[4] In addition, the presence of MOG-specific Abs in serum, CSF and MS lesions have been reported in many studies over the years.[4, 28, 33, 64] The presence of myelin-specific Abs and myelin-reactive T cells suggests that B and T cells have escaped negative selection during their development. This fraction of autoreactive cells may have the potential to induce a detrimental immune response, leading to inflammatory autoimmune CNS disease. Finally, CMV was included since this virus has been implicated as a causative agent in the development of MS through molecular mimicry. 't Hart and colleagues, namely, demonstrated that CMV is able to reactivate MOG-specific T cells in EAE rhesus monkeys.[11]

To our knowledge, this study is the first to evaluate the proliferative response of B cells towards various myelin and viral antigens. By using the CFSE assay, cell division of proliferating cells was tracked relatively easily. However, the frequency of myelin-specific B cells in the PB has to be taken into account. The number of cultured B cells has to be sufficiently high in order to distinguish the small amount of myelin-reactive B cells from the background proliferation.

Because HC and MS patients are apparently vaccinated with tetanus at a similar frequency, both groups should have a comparable number of residual memory B cells. As expected, we observed a similar proliferative response of B cells against TT in HC and MS patients ($p>0,05$; Figure 16A). In extent, Harp and colleagues found a comparable $CD4^+$ T cell proliferation in response to TT when cultured with naïve or memory B cells.[52]

The antigen reactivity of B cells was also determined in response to the self antigens MBP, MOG and PLP. Half of the MS patients had a proliferative response against MBP (Figure 15B) and PLP (Figure 15E), while no HC showed a response to these myelin antigens. Furthermore, 1 MS patient was MOG-reactive versus 0 HC (Figure 15D). However, no significant differences were found between HC

and MS patients, which can be due to the low patient and HC number currently included. In a way, the presence of myelin-reactive B cells is apparent, since Abs directed against MBP, MOG and PLP have been isolated from the PB, CSF and lesions in MS patients.[28] Harp and colleagues also identified memory B cells from HC and MS patients that bound to MOG and MBP. Still, only memory B cells of MS patients elicited $CD4^+$ T cell proliferation, which may be attributed to certain features on memory B cells.[52] Their role in the immunopathogenesis of MS, however, needs to be further elucidated.

A proliferative response of B cells against CMV was found in 3 out of 4 MS patients, whereas 1 of the HC were responsive (p=0,14; Figure 15C). Besides the antigen reactivity of B cells, the expression of APC and (co)stimulatory molecules on B cells was evaluated. The percentage of B cells expressing HLA-DR was elevated in MS patients after TT and CMV stimulation (Figure 16A), whereas the percentage of $CD80^+$ B cells was increased in MS patients after MOG stimulation (Figure 16E). However, no significant differences were found between HC and MS patients. Additional subjects need to be included to make accurate conclusions about the expression of APC and (co)stimulatory molecules on B cells and the proliferative responses of B cells after stimulation with self and nonself antigens.

5 Conclusion

Longtime, the main function of B cells in MS was considered to be antibody production. Recently, clinical trials using Rituximab demonstrated that B cells can also play a role as APC, since Rituximab reduced the total number of T cells in the blood of RRMS patients as well as the number of the lesions, while Ab titers remained constant. To our knowledge, little research has investigated the capability of B cells to participate in MS pathogenesis by (auto)antigen presentation to autoreactive T cells.

We hypothesized that B cells of MS patients have an increased antigen presentation capacity. Indeed, the percentage and MFI of peripheral $CD80^+$ B cells and CSF $CD86^+$ B cells was elevated in MS patients in comparison to HC. Additionally, the presence of $CD80^+$ B cells was detected in MS brain, while no inflammatory infiltrates were found in healthy brain tissue. These findings suggests that the elevated expression of costimulatory molecules on B cells of MS patients increase their antigen presentation capacity.

Furthermore, we evaluated the B cell proliferative response and expression of APC and (co)stimulatory molecules in response to TT, MBP, MOG, CMV and PLP. B cell reactivity was observed in MS patients in response to myelin antigens, while no HC had a proliferative response against these antigens In addition, 3 out of 4 MS patients versus 1 HC showed B cell reactivity towards CMV. The expression of HLA-DR and $CD80^+$ B cells was also elevated in MS patients after antigen-specific stimulation. However, no significant differences in the B cell reactivity and expression were observed due to the low number of persons currently included.

In future experiments, the expression of APC and (co)stimulatory molecules on peripheral and CSF B cells of MS patients will be determined in more RRMS and SPMS patients and a distinction will be made between active and stable disease. Additional CSF controls (i.e. noninflammatory neurological disorders [NIND], other inflammatory neurological diseases [OIND] and HC) are included to make appropriate comparisons between patient and control groups. Furthermore, we will evaluate whether treatment affects the expression of APC and (co)stimulatory molecules on peripheral and CFS B cells by performing a follow-up of MS patients undergoing treatment and comparing them to untreated patients. Additionally, the presence of $CD80^+$ and HLA-DR^+ B cells is determined in more post-mortem MS brains.

The proliferative response of B cells and the expression of APC and (co)stimulatory molecules on peripheral B cells in response to TT, MBP, MOG, CMV and PLP is assessed further by including more MS patients and HC (i.e. at least 10 individuals per group). In addition, we will determine whether B cells of MS patients can elicit a higher T cell proliferation in response to TT, MBP, MOG, CMV and PLP

than HC. Harp and colleagues[47] demonstrated that CD40L+IL-4 activated B cell elicited $CD4^+$ T cell proliferation in response to MBP in both MS patients and HC. In a more recent study, Harp et coworkers[52] also observed that memory B cells from responding MS patients caused an increased autologous $CD4^+$ T cell proliferation in response to MOG and MBP. Besides evaluating the T cell proliferation in response to self antigens, we will also examine whether MOG-reactive T cells proliferate in response to CMV-specific antigen presenting B cells. 't Hart and coworkers, namely, stated that MOG-reactive T cells can be reactivated by the encountering of CMV-APC in the lymph nodes.[11] Besides studying T cell proliferation, the subsequent T cell response will be characterized on gene, protein and cellular level in order to investigate whether B cells of MS patients induce a more proinflammatory/anti-inflammatory T cell response than HCs. Briefly, the inflammatory Th1 and Th17 cytokines IFN-γ and IL-17 in addition to the anti-inflammatory Th2 cytokine IL-4 will be determined by qPCR and ELISA. In addition, the T cell differentiation will be assessed by flow cytometry.

In conclusion, MS is a chronic and debilitating disorder of the CNS, which affects 2,5 million persons worldwide. The most frequent symptoms include sensory, cognitive and visual impairment and paralysis.[4] Depending on the disease progression and severity, job performance and employment are affected. The chronic and disabling nature of MS is associated with a considerable socioeconomical burden for the individual and the health care system. The annual health care costs exceed 7 billion dollar in the United States.[65, 67] To date, the true etiology of MS remains unknown. Current therapies can only partially reduce the clinical disease activity and formation of new MS lesions. At present, there is no treatment available that intervenes in the underlying disease mechanism, thereby halting the progression of the disease.[66]

A more in-depth knowledge of the B-T cell interplay will lead to further insights into the underlying disease mechanism of MS. This may enable researchers to develop therapies, which are directed against certain B-T cell interactions. This study could provide appropriate markers in order to specifically target pathological B-T cell interactions and give insights into possible effects of such targeted therapies.

6 References

1. Hemmer B, Archelos JJ, Hartung HP. New concepts in the immunopathogenesis of multiple sclerosis. Nature reviews Neuroscience. 2002;3(4):291-301.
2. Zuvich RL, McCauley JL, Pericak-Vance MA, Haines JL. Genetics and pathogenesis of multiple sclerosis. Seminars in immunology. 2009;21(6):328-33.
3. Sospedra M, Martin R. Immunology of multiple sclerosis. Annual review of immunology. 2005;23:683-747.
4. Hellings N, Raus J, Stinissen P. Insights into the immunopathogenesis of multiple sclerosis. Immunologic research. 2002;25(1):27-51.
5. Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmunity reviews. 2010;9(5):A387-94.
6. Hemmer B, Nessler S, Zhou D, Kieseier B, Hartung HP. Immunopathogenesis and immunotherapy of multiple sclerosis. Nature clinical practice Neurology. 2006;2(4):201-11.
7. Hoffjan S, Akkad DA. The genetics of multiple sclerosis: an update 2010. Molecular and cellular probes. 2010;24(5):237-43.
8. Baranzini SE. Revealing the genetic basis of multiple sclerosis: are we there yet? Current opinion in genetics & development. 2011;21(3):317-24.
9. Kalman B, Lublin FD. The genetics of multiple sclerosis. A review. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 1999;53(8):358-70.
10. Hafler DA. Multiple sclerosis. The Journal of clinical investigation. 2004;113(6):788-94.
11. t Hart BA, Hintzen RQ, Laman JD. Multiple sclerosis - a response-to-damage model. Trends in molecular medicine. 2009;15(6):235-44.
12. Hoppenbrouwers IA, Hintzen RQ. Genetics of multiple sclerosis. Biochimica et biophysica acta. 2011;1812(2):194-201.
13. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. Lancet neurology. 2010;9(6):599-612.
14. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Annals of neurology. 2007;61(4):288-99.
15. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell. 1995;80(5):695-705.
16. Pohl D. Epstein-Barr virus and multiple sclerosis. Journal of the neurological sciences. 2009;286(1-2):62-4.
17. Kakalacheva K, Lunemann JD. Environmental triggers of multiple sclerosis. FEBS letters. 2011;585(23):3724-9.
18. Ebers GC. Environmental factors and multiple sclerosis. Lancet neurology. 2008;7(3):268-77.
19. Ascherio A, Munger KL. Epstein-barr virus infection and multiple sclerosis: a review. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology. 2010;5(3):271-7.
20. Swanborg RH, Whittum-Hudson JA, Hudson AP. Infectious agents and multiple sclerosis--are Chlamydia pneumoniae and human herpes virus 6 involved? Journal of neuroimmunology. 2003;136(1-2):1-8.
21. 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. Journal of neuroimmunology. 2007;182(1-2):135-52.
22. Tsai JC, Gilden DH. Chlamydia pneumoniae and multiple sclerosis: no significant association. Trends in microbiology. 2001;9(4):152-4.

23. Gran B, Hemmer B, Vergelli M, McFarland HF, Martin R. Molecular mimicry and multiple sclerosis: degenerate T-cell recognition and the induction of autoimmunity. Annals of neurology. 1999;45(5):559-67.
24. Nairn R, Helbert M. Immunology for medical students. 2nd ed. Philadelphia: Mosby; 2007.
25. Burgoon MP, Gilden DH, Owens GP. B cells in multiple sclerosis. Frontiers in bioscience : a journal and virtual library. 2004;9:786-96.
26. Frohman EM, Racke MK, Raine CS. Multiple sclerosis--the plaque and its pathogenesis. The New England journal of medicine. 2006;354(9):942-55.
27. Cross AH, Waubant E. MS and the B cell controversy. Biochimica et biophysica acta. 2011;1812(2):231-8.
28. Cross AH, Trotter JL, Lyons J. B cells and antibodies in CNS demyelinating disease. Journal of neuroimmunology. 2001;112(1-2):1-14.
29. Abbas AK, Lichtman AH. Basic immunology : functions and disorders of the immune system. 3rd ed. Philadelphia, PA: Saunders/Elsevier; 2009.
30. Fraussen J, Vrolix K, Martinez-Martinez P, Losen M, De Baets MH, Stinissen P, et al. B cell characterization and reactivity analysis in multiple sclerosis. Autoimmunity reviews. 2009;8(8):654-8.
31. Duddy M, Bar-Or A. B-cells in multiple sclerosis. International MS journal / MS Forum. 2006;13(3):84-90.
32. Bartok B, Silverman GJ. Development of anti-CD20 therapy for multiple sclerosis. Experimental cell research. 2011;317(9):1312-8.
33. Weber MS, Hemmer B, Cepok S. The role of antibodies in multiple sclerosis. Biochimica et biophysica acta. 2011;1812(2):239-45.
34. Berer K, Wekerle H, Krishnamoorthy G. B cells in spontaneous autoimmune diseases of the central nervous system. Molecular immunology. 2011;48(11):1332-7.
35. 25th forum in immunology. Immunological aspects of multiple sclerosis. Research in immunology. 1989;140(2):173-248.
36. Bar-Or A, Fawaz L, Fan B, Darlington PJ, Rieger A, Ghorayeb C, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? Annals of neurology. 2010;67(4):452-61.
37. Barun B, Bar-Or A. Treatment of multiple sclerosis with Anti-CD20 antibodies. Clin Immunol. 2011.
38. Franciotta D, Salvetti M, Lolli F, Serafini B, Aloisi F. B cells and multiple sclerosis. Lancet neurology. 2008;7(9):852-8.
39. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol. 2004;14(2):164-74.
40. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. The Journal of experimental medicine. 2007;204(12):2899-912.
41. Willis SN, Stadelmann C, Rodig SJ, Caron T, Gattenloehner S, Mallozzi SS, et al. Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. Brain : a journal of neurology. 2009;132(Pt 12):3318-28.
42. Ray A, Mann MK, Basu S, Dittel BN. A case for regulatory B cells in controlling the severity of autoimmune-mediated inflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. Journal of neuroimmunology. 2011;230(1-2):1-9.
43. Townsend MJ, Monroe JG, Chan AC. B-cell targeted therapies in human autoimmune diseases: an updated perspective. Immunological reviews. 2010;237(1):264-83.
44. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. The New England journal of medicine. 2008;358(7):676-88.

45. Bar-Or A, Calabresi PA, Arnold D, Markowitz C, Shafer S, Kasper LH, et al. Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. Annals of neurology. 2008;63(3):395-400.
46. Cross AH, Stark JL, Lauber J, Ramsbottom MJ, Lyons JA. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. Journal of neuroimmunology. 2006;180(1-2):63-70.
47. Harp CT, Lovett-Racke AE, Racke MK, Frohman EM, Monson NL. Impact of myelin-specific antigen presenting B cells on T cell activation in multiple sclerosis. Clin Immunol. 2008;128(3):382-91.
48. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. The Journal of clinical investigation. 2008;118(10):3420-30.
49. Weber MS, Prod'homme T, Patarroyo JC, Molnarfi N, Karnezis T, Lehmann-Horn K, et al. B-cell activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity. Annals of neurology. 2010;68(3):369-83.
50. Genc K, Dona DL, Reder AT. Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. The Journal of clinical investigation. 1997;99(11):2664-71.
51. 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. Annals of neurology. 2011;69(2):292-302.
52. Harp CT, Ireland S, Davis LS, Remington G, Cassidy B, Cravens PD, et al. Memory B cells from a subset of treatment-naive relapsing-remitting multiple sclerosis patients elicit CD4(+) T-cell proliferation and IFN-gamma production in response to myelin basic protein and myelin oligodendrocyte glycoprotein. European journal of immunology. 2010;40(10):2942-56.
53. Mingari MC, Gerosa F, Carra G, Accolla RS, Moretta A, Zubler RH, et al. Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. Nature. 1984;312(5995):641-3.
54. Idzerda RL, March CJ, Mosley B, Lyman SD, Vanden Bos T, Gimpel SD, et al. Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. The Journal of experimental medicine. 1990;171(3):861-73. Epub 1990/03/01.
55. Boylan MT, Crockard AD, McDonnell GV, Armstrong MA, Hawkins SA. CD80 (B7-1) and CD86 (B7-2) expression in multiple sclerosis patients: clinical subtype specific variation in peripheral monocytes and B cells and lack of modulation by high dose methylprednisolone. Journal of the neurological sciences. 1999;167(2):79-89.
56. Svenningsson A, Dotevall L, Stemme S, Andersen O. Increased expression of B7-1 costimulatory molecule on cerebrospinal fluid cells of patients with multiple sclerosis and infectious central nervous system disease. Journal of neuroimmunology. 1997;75(1-2):59-68.
57. Roth R, Nakamura T, Mamula MJ. B7 costimulation and autoantigen specificity enable B cells to activate autoreactive T cells. J Immunol. 1996;157(7):2924-31. Epub 1996/10/01.
58. Sellebjerg F, Jensen J, Ryder LP. Costimulatory CD80 (B7-1) and CD86 (B7-2) on cerebrospinal fluid cells in multiple sclerosis. Journal of neuroimmunology. 1998;84(2):179-87.
59. Monteyne P, Van Laere V, Marichal R, Sindic CJ. Cytokine mRNA expression in CSF and peripheral blood mononuclear cells in multiple sclerosis: detection by RT-PCR without in vitro stimulation. Journal of neuroimmunology. 1997;80(1-2):137-42.
60. Windhagen A, Newcombe J, Dangond F, Strand C, Woodroofe MN, Cuzner ML, et al. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. The Journal of experimental medicine. 1995;182(6):1985-96.
61. De Simone R, Giampaolo A, Giometto B, Gallo P, Levi G, Peschle C, et al. The costimulatory molecule B7 is expressed on human microglia in culture and in multiple sclerosis acute lesions. Journal of neuropathology and experimental neurology. 1995;54(2):175-87.

62. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. European journal of immunology. 2007;37(8):2205-13.
63. de Rosbo NK, Ben-Nun A. T-cell responses to myelin antigens in multiple sclerosis; relevance of the predominant autoimmune reactivity to myelin oligodendrocyte glycoprotein. Journal of autoimmunity. 1998;11(4):287-99.
64. Archelos JJ, Storch MK, Hartung HP. The role of B cells and autoantibodies in multiple sclerosis. Annals of neurology. 2000;47(6):694-706.
65. Prescott JD, Factor S, Pill M, Levi GW. Descriptive analysis of the direct medical costs of multiple sclerosis in 2004 using administrative claims in a large nationwide database. Journal of managed care pharmacy : JMCP. 2007;13(1):44-52.
66. Vanderlocht J, Hellings N, Hendriks JJ, Stinissen P. Current trends in multiple sclerosis research: an update on pathogenic concepts. Acta neurologica Belgica. 2006;106(4):180-90.
67. Bainbridge JL. Economics of multiple sclerosis and its treatment. Ad Stud Phar. 2004;4(11) 330-3.

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