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Characterization of the B cell response in multiple sclerosis and clinically isolated syndrome using B cell immortalization

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"If you haven't found something strange during the day,
it hasn't been much of a day."
J.A. Wheeler, 1911-2008

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7-AAD	7- aminoactinomycin D
AA	amino acid
Ab	antibodies
AChR	acetylcholine receptor
ADCC	antibody-dependent cell-mediated cytotoxicity
ANA	anti-nuclear antibodies
APC	antigen-presenting cell
APL	altered peptide ligand
APRIL	A proliferation inducing ligand
ASPP2	apoptosis stimulating protein of p53-2
BAFF	B cell activating factor of the TNF family
BAFF-R	B cell activating factor of the TNF family receptor
BBB	blood-brain barrier
BCMA	B cell maturation antigen
BCR	B cell receptor
CCL-19	chemokine C-C motif ligand 19
CD	cluster of differentiation
CD40L	cluster of differentiation 40 ligand
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CIS	clinically isolated syndrome
CLE	cutaneous lupus erythematosus
CLEC16A	C-type lectin-domain family 16 member A
CMV	cytomegalovirus
CNPase	2’,3’-cyclic nucleotide 3’-phosphodiesterase
CNS	central nervous system
CXCL-12	chemokine C-X-C motif ligand 12
CXCL-13	chemokine C-X-C motif ligand 13
CsA	cyclosporine A
CSF	cerebrospinal fluid
D	diversity region
DAB	3,3’ deaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DSS	disuccinimidyl suberate
EAE	experimental autoimmune encephalomyelitits
EAMG	experimental autoimmune myasthenia gravis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3

FR	framework region
GA	glatiramer acetate
GalC	galactocerebroside
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	germinal center
gDNA	genomic deoxyribonucleic acid
GFAP	glial fibrillary acidic protein
Gy	Gray
H	heavy chain
HAMA	human anti-murine antibody response
HC	healthy control(s)
HERV	human endogenous retroviral elements
HHV	human herpes virus
HLA	human leukocyte antigen
HOG	human oligodendroglioma
HRP	horse radish peroxidase
HSCT	hematopoietic stem cell transplantation
HTLV-1	human T-lymphotropic virus 1
ICAM-1	intercellular adhesion molecule 1
IEF	isoelectric focusing
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IL-2	interleukin-2
IL-10	interleukin-10
IL-17	interleukin-17
IL-23	interleukin-23
IL-2Rα	interleukin-2 receptor alpha
IL-7Rα	interleukin-7 receptor alpha
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IP	immunoprecipitation
IRF8	interferon regulatory factor 8
J	joining region
L	light chain
LCL	lymphoblastoid cell line
LFA-1	lymphocyte function-associated antigen-1
LMP	latent membrane protein
mAb	monoclonal antibody
MAC	membrane attack complex
MACS	magnetic activated cell sorting
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MFI	mean fluorescence intensity
MG	myasthenia gravis
MHC	major histocompatibility complex
MMP-9	matrix metalloproteinase 9
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis

NAWM	normal appearing white matter
NIND	non-inflammatory neurological disease
NK	natural killer
OCB	oligoclonal bands
OIND	other inflammatory neurological disease
OSP	oligodendrocyte-specific protein
P	proline
PAD	peptidyl arginine deiminase
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanin 5
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
PLP	proteolipid protein
PML	progressive multifocal leukoencephalopathy
PP-MS	primary progressive MS
R	replacement mutation
RA	rheumatoid arthritis
RR-MS	relapsing-remitting MS
RT	room temperature
S	silent mutation
sd	standard deviation
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SP-MS	secondary progressive MS
SPAG	sperm associated antigen 16
TACI	transmembrane activator and calcium modulator and cytophilin ligand interactor
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFRSF1A	tumor necrosis factor receptor superfamily member 1A
TPI	triosephosphate isomerase
U	Unit
V	variable region
VCAM-1	vascular cell adhesion molecule 1
V_H	variable region heavy chain locus
V_L	variable region light chain locus
VLA-4	very late antigen-4
VZV	Varicella-Zoster virus

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Introduction and aims

Parts of this chapter are based on:

B cell characterization and reactivity analysis in multiple sclerosis.

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B cells and autoantibodies in multiple sclerosis.

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1.1 Basic concepts of multiple sclerosis

1.1.1 Clinical aspects

In 1868, multiple sclerosis (MS) was first described by Jean Martin Charcot as an accumulation of inflammatory cells in a perivascular distribution within the brain and spinal cord of patients with intermittent episodes of neurologic dysfunction[1]. Further evidence of an inflammatory nature was obtained in 1948 by Elvin Kabat who noticed elevated oligoclonal immunoglobulin (oligoclonal bands, OCB) in MS cerebrospinal fluid (CSF)[2]. MS is known today as a chronic inflammatory disease of the central nervous system (CNS) that is characterized by multiple lesions of demyelination, axonal degeneration and gliosis. The myelin sheet surrounding axons is destructed, leading to impaired signalling between neurons. This can give rise to a variety of symptoms such as muscle weakness, unilateral optic neuritis, sensory disturbances, visual loss, cognitive impairment and fatigue[3]. In Europe and Northern America, the prevalence of the disease is 0.1%[4,5]. Most patients develop the disease during young adulthood, women being more frequently affected than men[6,7]. MS has a variable prognosis, although 50% of patients need help walking within 15 years after the disease onset[3].

The majority of patients (85-90%) develop relapsing-remitting MS (RR-MS), in which relapses of clinical symptoms that persist for several days or weeks alternate with periods of remission[6-8]. Relapse frequency and duration increase over time, while recovery from attacks is decreased. This subtype of MS has a female predominance of approximately 2:1[3]. Eventually, about 40% of RR-MS patients develop secondary progressive MS (SP-MS). This disease form is characterized by progressive neurological disease and a decrease in the number of brain lesions. In 10-20% of patients, MS manifests as primary progressive MS (PP-MS) with a progressive course and gradual clinical decline from the onset[9]. Pathological differences have been observed between the different disease forms. In RR-MS, features of an inflammatory process are frequently detected, such as acute CNS lesions with spontaneous resolution. On the other hand, brain degeneration and atrophy that coincide with increased disability are hallmarks of progressive MS. These divergent features are indications for distinct processes in the different phases of disease[7].

The diagnosis of MS is based on clinical and neurological examinations, such as magnetic resonance imaging (MRI) and evoked potentials, that can reveal MS while excluding other autoimmune diseases or infections[4]. MRI is used to visualize multifocal brain lesions, although such lesions are also present in other clinical pictures and are thus not specific to MS[6]. The finding of gadolinium-enhancing lesions on MRI demonstrates the presence of active inflammatory lesions. Furthermore, disease activity and evolution over time can be monitored easily using MRI. CSF analysis is performed to detect OCB, which consist of high concentrations of immunoglobulin (Ig), usually immunoglobulin G (IgG), that are generated by plasma blasts and plasma cells in the CSF or CNS[10]. Detection occurs generally by isoelectric focusing (IEF) followed by immunoblotting. OCB are however not specific for MS, and can merely be used as a very sensitive screening tool. The IgG index, the ratio of IgG to albumin concentration in the CSF compared to the serum, is another CSF parameter that is elevated in about 70-90% of MS patients[10]. Clinically definite MS can only be identified after showing spread of the lesions in time and space, as defined by an international panel in the McDonald diagnostic criteria for MS[11].

In 85% of MS patients, disease starts with a clinically isolated syndrome (CIS)[12]. Patients with a CIS have experienced a first acute or subacute neurological event with demyelination involving the optic nerve, cerebrum, cerebellum, brainstem or spinal cord[12]. Further, CIS patients often present with optic neuritis, brainstem syndrome or partial myelitis[13]. Pathologic abnormalities in white and grey matter are similar to MS although to a lesser degree[12]. More events are generated in 63% of cases over a period of 20 years[14], confirming the diagnosis of clinically definite MS. The occurrence of optic neuritis, partial myelitis and the presence of OCB in the CSF increase the risk of conversion to MS[15], as well as serum antibodies to myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP)[16]. Some reports have indicated that treatment of high risk CIS patients with interferon-beta (IFN-β) or glatiramer acetate could delay onset of MS[17,18]. However, a proportion of CIS patients does not experience a second episode and will never be diagnosed with clinically definite MS[19].

1.1.2 Immunopathogenesis

MS is thought to be caused by a putative autoimmune response to self antigens in a genetically susceptible host after breaking of the existing tolerance mechanisms. The most generally accepted hypothesis claims that MS is triggered by autoreactive T cells that are activated in the periphery by microbial antigens that are crossreactive with myelin, a process that is termed molecular mimicry[6,7]. Alternatively, bystander activation following viral infection could lead to an autoimmune response in the CNS[20]. Autoreactive T cells may otherwise be activated by bacterial or viral superantigens. The degeneration hypothesis, on the other hand, claims that the disease starts when CNS proteins are released into the periphery after primary degeneration, leading to the activation of autoreactive memory T cells that were generated in response to a lifelong asymptomatic infection with herpes viruses[21].

In all hypotheses, peripherally activated autoreactive T cells migrate through the blood-brain barrier (BBB) into the CNS. This migration is promoted by the release of proinflammatory cytokines by the activated T cells and by an upregulation in the expression of endothelial adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), vascular-cell adhesion molecule 1 (VCAM-1) and E-selectin[3]. Proteases, including matrix metalloproteinases, can further enhance the migration of autoreactive T cells by degrading extracellular matrix macromolecules. In the CNS, the autoreactive T cells become reactivated when recognizing myelin antigens that are presented by resident antigen presenting cells (APCs), including glial cells and activated microglia (Fig. 1.1)[7]. The reactivated T cells start producing proinflammatory cytokines such as tumor necrosis factor (TNF) and interferon-γ (IFN-γ)[4]. This eventually results in the upregulation of major histocompatibility complex (MHC) class II molecules on astrocytes and microglia and adhesion molecules on the BBB endothelium, causing further influx of T cells, B cells and macrophages (Fig. 1.1). Demyelination is the conclusive result of this amplified immune response. Some of the proposed myelin target antigens are MBP, MOG, myelin-associated glycoprotein (MAG), proteolipid protein (PLP), glial fibrillary acidic protein (GFAP) and αB-crystallin. Several mechanisms of demyelination have been described, including cytokine-mediated injury, digestion of surface myelin antigens by macrophages, antibody-dependent cytotoxicity, complement-mediated injury and direct injury by cluster of differentiation 4 (CD4) and CD8 T cells. Although

myelin sheats are the primary target of tissue destruction, axons, neurons and astrocytes are also affected[22]. Metabolic impairment of oligodendrocytes through ischemia, toxins or virus infection is also possible[22]. Axonal destruction is triggered by alterations in ion channel permeability that disturb calcium homeostasis within the axons[22]. This leads to activation of calcium-dependent proteases, local degradation of cytoskeletal elements, blockade of axonal transport and finally axonal disruption[23].

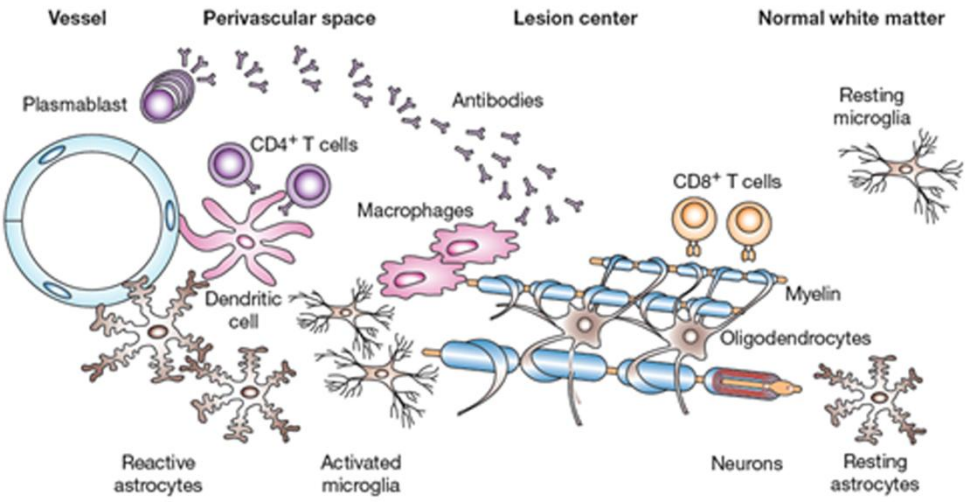

Figure 1.1 Immunopathology of MS. T cells, B cells and macrophages infiltrate the MS lesion through the BBB. Here, autoreactive T cells become reactivated by antigens presented on dendritic and microglial cells, leading to the release of proinflammatory cytokines and other inflammatory mediators. In this way macrophages and B cells are attracted to the lesions. All these cell types cause demyelination and tissue damage by secretion of proinflammatory cytokines (T cells), direct attack of cells expressing MHC class I molecules ($CD8^+$ T cells), production of antibodies (B cells) that activate the complement cascade, phagocytosis and the release of inflammatory and toxic molecules (macrophages). Oligodendrocytes and neurons are the primary targets of these actions. Reactive astrocytes further induce gliosis at the lesion border. Following the inflammatory damage, oligodendrocytes proliferate and remyelinate the demyelinated axons.
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When the target antigen is cleared, the immune cells will undergo activation-induced cell death or redistribute to other tissues. At that time, repair

mechanisms are activated to remyelinate the damaged axons, which is accompanied by clinical stabilization in RR-MS[24]. The myelin membrane can be rebuild by spontaneous remyelination after the inflammatory response. Further, sodium channels can spread from the nodes of Ranvier to axon segments and restore signal conduction. Antibody- and oligodendrocyte precursor cell-mediated remyelination are also possible. Immunoregulatory cells also contribute to the (temporary) resolution of inflammation[24].

T and B cells can become activated against different epitopes of the same protein, termed epitope spreading[6,7]. The whole cascade of events can eventually lead to chronic disease with progressive loss of neurologic function because of irreversible axonal injury, gliotic scarring and exhaustion of the oligodendrocyte progenitor pool. In the progressive phase of MS, the inflammation occurs in a closed CNS environment by repair or closure of the BBB, termed compartmentalization[8]. Inflammation can persist by the development of lymphatic follicle-like structures in the meninges, connective tissue compartments of the CNS and large perivascular spaces[25,26]. Current immunotherapies are often ineffective during progressive disease, partially because the CNS is no longer accessible to the immune system[26].

Lesion pathology

The pathological hallmark of MS is the demyelinated plaque, which consists of a well-demarcated hypocellular area characterized by the loss of myelin, relative preservation of axons and the formation of astrocytic scars[3]. They can appear throughout the brain but more preferentially in the optic nerves, periventricular and spinal cord white matter, brain stem and cerebellum. MS lesions often surround a vessel with extensions into the parenchyma. The inflammatory infiltrate is most often found in the perivascular region and consists of lymphocytes, mainly T cells, and macrophages.

Two types of lesions exist in the CNS of MS patients, namely acute demyelinating lesions and chronic lesions. The composition of acute lesions varies greatly among patients, although heterogeneity between lesions of a particular patient is limited[7]. Cell infiltrates, demyelination, extensive BBB leakage, reactive astrocytes, macrophages containing myelin degradation products and proliferating oligodendroglial cells can be found at the lesion border[27-29]. During the resolution phase, oligodendrocyte progenitor cells enter

the demyelinated region and develop into myelinating oligodendrocytes that are responsible for remyelination of the axons (shadow plaques)[3,7]. However, their remyelinating capacity decreases over time until it fully disappears. In chronic lesions, almost no remyelination is observed although oligodendrocytes are still present. The number of inflammatory cells decreases, first in the center and later also at the edge of the lesion[27]. Macrophages containing cellular degradation products are rare or absent[30]. Adversely, activated microglia are present near the lesion edge, only some containing myelin degradation products. T cells are also present although sparse. Such lesions are mostly accompanied by cortical pathology and diffuse injury of the normal appearing white matter (NAWM)[29].

Lassmann and colleagues described four demyelination patterns: macrophage-associated demyelination (I), antibody-mediated demyelination (II), oligodendrogliopathy-associated demyelination (III) and primary oligodendrocyte degeneration (IV)[22]. These demyelination patterns were described to be heterogeneous between different patients but homogeneous between active lesions of the same patient[31], although these observations have been refuted by others[32,33].

Players

$CD4^+$ helper T cells have long been thought to be the major inflammatory regulators in MS as they are key players in experimental autoimmune encephalomyelitis (EAE), the animal model for MS[7,8]. Moreover, the association of major histocompatibility complex class II (MHC II) genes with MS supports the role of T helper (Th) cells. Their number is increased in the blood of MS patients, while the spectrum of chemokines and cytokines in MS mainly reflect a T helper 1 (Th1)-driven inflammatory response[22,34]. Th cells recruit macrophages by cytokine secretion and help in B cell activation. However, extensive T cell clonal expansion in the CSF of MS patients locates mainly in the cytotoxic $CD8^+$ T cell population[7,35]. Further, CNS cells are able to express major histocompatibility complex class I (MHC I) molecules which are recognized by $CD8^+$ T cells[7]. MS lesions are dominated by $CD8^+$ T cells, regardless of the stage of activity or disease[8]. $CD8^+$ T cells are able to directly attack MHC I-expressing cells, such as neurons and oligodendrocytes. Regulatory T cells, mostly the $CD4^+CD25^+$ T cells with expression of forkhead box P3 (FOXP3), control the

activity of autoreactive T cells. In MS, the function of regulatory T cells appeared to be defective despite normal numbers in the peripheral blood (PB)[36].

More recently, T helper 17 (Th17) cells have been identified as the most essential inflammatory mediators in MS, as well as in other autoimmune diseases[37]. These interleukin-17 (IL-17) producing Th cells are triggered mainly by interleukin-23 (IL-23)[38]. IL-17 concentrations were found to be increased in brain lesions, peripheral blood mononuclear cells (PBMC) and CSF of MS patients[39,40]. Some effects of IL-17 are the induction of proinflammatory cytokines and chemokines, attraction of neutrophils and augmentation of dendritic cell maturation[41]. IL-17 was described as one of the effector cytokines in development and disease course of EAE since neutralization of IL-17 and IL-17 gene knock out led to reduced severity of disease[42,43]. Interestingly, both $CD4^+$ and $CD8^+$ T cells, as well as astrocytes and oligodendrocytes, express this cytokine in active MS lesions[41]. However, the primary role of Th17 cells in MS development has already been refuted, as this T cell subset was shown to play a minor role in the development of autoimmune CNS disease[44].

Macrophages and activated microglia are involved in the MS pathogenesis as well. They dominate the inflammatory reaction in all MS lesions, where they become activated by cytokine-secreting T cells or by Toll-like receptor (TLR) ligands[29]. Moreover, macrophages have the potential to induce tissue injury through a variety of cytotoxic molecules, including proteolytic and lipolytic enzymes, cytotoxic cytokines, reactive oxygen and nitrogen intermediates and excitotoxins. Macrophages also play an important role in the disturbance of mitochondrial function, leading to energy failure and neurodegeneration.

B cells and antibodies have long time been underestimated as players in MS pathogenesis. During the last years, more and more evidence has been obtained for their involvement in the disease process. B cells can become activated by recognition of antigens displayed on dendritic cells in the presence of T cell help but also by direct binding of highly repetitive epitopes in a T cell independent manner. Activated B cells can enter the CNS through the BBB and infiltrate the perivascular cuffs and meninges, where they exert various functions[45]. The role of B cells in MS will be discussed in more detail in section 1.2.

1.1.3 Genetic and environmental factors

MS develops in a genetically susceptible host following certain environmental factors. Thus, both genes and environmental exposures are important in the development of MS.

About 15-20% of MS patients have a family history of MS[46]. The risk of getting the disease is higher in family members of MS patients, while the prevalence in spouses is not different from the general population[7]. Monozygotic twins display a concordance rate of 31%, while this is only 5% for dizygotic twins and 3% in siblings of MS patients[47]. MS is a complex genetic disease with a high number of genes being involved and a considerable heterogeneity in disease alleles. The exact genes that are responsible for MS are not known although the already identified susceptibility genes are related to the immune response. MHC genes on chromosome 6p21 confer the highest risk for development of the disease. More particularly, the human leukocyte antigen (HLA) class II alleles DR15/DQw6 (HLA-DRB1*1501, HLA-DQB1*0602 and HLA-DQA1*0102) have been associated with a 2-4-fold increased risk of getting MS[7,27,48,49]. These alleles code for molecules involved in T cell antigen recognition. Genome wide association studies also identified interleukin-7 receptor alpha chain (IL-7Rα), interleukin-2 receptor alpha (IL-2Rα), C-type lectin-domain family 16 member A (CLEC16A), CD58, tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), interferon regulatory factor 8 (IRF8) and CD6 as potential risk genes for the development of MS[48,50,51]. Further, the VAV2 and ZNF433 gene were found to be possibly associated with MS[52]. IL12A, MPHOSPH9/CDK2AP1 and RGS1 were most recently identified as new MS susceptibility loci[53]. Further research on the exact role of these novel susceptibility genes is required.

The discordance in most monozygotic twins highlights the importance of environmental factors in the disease process. The prevalence of MS greatly varies among different areas of the world, with the highest prevalence in Northern Europe, Southern Australia and the middle part of North America[3,54]. Migration from high- to low-risk areas before adolescence reduces the risk of developing MS[54,55]. The prevalence tends to be higher in areas with moderate climate and in socioeconomical regions with a higher sanitary level and better nutrition[56]. Higher exposure to sunlight at 6-15 years of age has been associated with a decreased risk of MS[57]. This protective effect is probably due to vitamin D

which is produced in the skin after exposure to ultraviolet radiation. The intake of vitamin D in food (supplements) could have a protective effect on MS development. Calcitriol, the active form of vitamin D, was reported to delay induction and decrease progression of EAE[58]. Vitamin D can also promote regulatory T cell function, lead to inhibition of inflammatory cytokines by activated macrophages, to enhanced production of anti-inflammatory cytokines and to reduction of interleukin-2 (IL-2) messenger RNA (mRNA) in the PB[57,58].

Epigenetics could be involved in MS development as well. Hypomethylation of the promoter region in the peptidyl arginine deiminase (PAD) 2 gene has been described in MS, leading to increased amounts of PAD2 in MS brain. PAD enzymes are involved in the process of citrullination, the conversion of arginine into citrulline. PAD2 has been described to cause citrullination of MBP in MS[59]. In addition, epigenetic mechanisms could contribute to the female preponderance through skewed inactivation of the second X chromosome[60]. Other epigenetic factors that have an influence on MS are the increased expression of several microRNAs, histone modifications and polymorphisms of human endogenous retroviral elements (HERV)[61].

Various studies have suggested an association between viral infections and MS. It is known that MS relapses are often accompanied by common viral infection and share similarities with infectious demyelinating diseases[62]. There is however not one disease-causing viral pathogen. Epstein-Barr virus (EBV) has been very strongly associated with MS[63,64]. Virtually all MS patients are EBV seropositive, suggesting that EBV infection is a prerequisite for the development of MS. However, EBV infection alone is insufficient since the majority of EBV seropositive individuals do not develop MS[65,66]. Antibodies to Epstein-Barr nuclear antigen (EBNA) were recovered in 85% of MS patients, compared with 13% of EBV-seropositive controls[67]. Moreover, serum titers of anti-EBV antibodies raised before MS onset, revealing an association between anti-EBV antibodies and the risk of developing MS[68]. An increased humoral and cellular immune response to EBNA was also reported in CIS patients, preceding conversion to MS[69]. Reactivity of CSF IgG OCB towards EBV has been reported[70,71], as well as T cell responses towards EBV in MS patients[72]. In addition, EBV-infected B cells have been discovered in the brain of progressive MS patients[73,74], although these findings could not be confirmed by others[75-77]. Several hypotheses for MS development by EBV have been described. A

pentapeptide sequence of EBNA is homologous with a MBP epitope, underlining the possibility of molecular mimicry[57]. EBV was claimed to induce expression of αB-crystallin on the B cell surface, an MS autoantigen that is normally only expressed in the brain[65]. Another possibility is that an EBV-directed immune response leads to CNS damage as a bystander reaction. Finally, EBV infected autoreactive B cells could seed the CNS in genetically susceptible individuals due to ineffective clearing of the virus[73,78], produce pathogenic autoantibodies and provide costimulation to autoreactive T cells. However, more research is necessary to collect evidence for these hypotheses.

Chlamydia pneumoniae (C. pneumoniae) is another virus often described in relation to MS. 97% of MS patients were shown to be positive for C. pneumoniae in CSF, although this could not be confirmed by others[57]. DNA of C. pneumoniae has been demonstrated in the CSF of both MS patients and neurological controls[79], while IgG titers were higher in the CSF of MS versus control patients[67]. Increased IgG titers were however not correlated with disease duration, disease course, disease activity, disability or the presence of oligoclonal IgG.

Varicella-Zoster virus (VZV), the causative agent of chickenpox, was linked to MS in several epidemiological studies. Findings that contributed to this association were the presence of VZV DNA in CSF and PBMC of MS patients[80,81], although a large evaluation report claimed that there was insufficient evidence to support this association[82]. Contributing to this statement, no VZV DNA was detected in MS CSF or acute MS plaques[83]. In the same study, recombinant antibodies produced from clonally expanded plasma cells of MS CSF did not show reactivity towards VZV. All these findings indicate that VZV is probably not a disease-relevant antigen in MS.

Human herpes virus-6 (HHV-6) has been indicated in MS plaques, blood cells, serum and CSF of MS patients[57]. Constitutive HHV-6 infection of glial cells in inflamed CNS tissue could lead to virus-triggered immunopathologies in MS[84]. Finding of HHV-6 was however not associated with clinical disease[85]. Moreover, DNA of HHV-6 has been identified in normal brains[67], while HHV-6 DNA was not detected in the CSF of MS patients in another study[86].

Cytomegalovirus (CMV) has been associated with MS as well. CMV-reactivity was demonstrated for immunosenescent $CD4^{+}CD28^{null}$ T cells in the PB of 3/4 MS patients[87]. Moreover, studies in the EAE model for rhesus monkey have reported

that CMV shares a mimicry motif with MOG peptide, leading to the reactivation of anti-CMV T cells and their CNS infiltration upon exposure to APC presenting MOG peptide[88]. However, CMV DNA could not be detected in the CSF of MS patients[89].

Other viruses that have been described with reference to MS are retroviruses such as human T-lymphotropic virus 1 (HTLV-1), coronaviruses and the polyoma JC virus[67]. Indications for a link of these viruses and MS are more scarce.

So far, none of the above mentioned viral pathogens could be definitely identified as a causative agent for MS. Infectious agents might shape the immune response against self-antigens and might induce disease under special circumstances. Contrarily, infectious pathogens have also been described to be beneficial in the prevention of autoimmunity in the so-called hygiene hypothesis. According to this hypothesis, allergy and autoimmunity are predominantly diseases of modern industrialized societies as the consequence of advances in sanitation and public health[90]. Immunomodulatory molecules produced by infectious agents can be beneficial and may prevent or ameliorate autoimmune disease[91]. Parasite infections in humans induce regulatory T cells that produce interleukin-10 (IL-10) and transforming growth factor β (TGF-β), inhibit T cell proliferation and suppress IFN-γ production, thus altering the course of MS[92]. Helminth infection or immunization can reduce disease severity in EAE[90].

To summarize, MS is a complex and heterogeneous disease in which both genetic and environmental factors contribute synergistically to development and progression.

1.1.4 Therapy

After several decades of research, MS still cannot be cured. Nevertheless, a lot of new therapies have been developed during the last years that are able to delay disease progression or decrease clinical severity of the relapses.

Immune modulating therapies

As a first line of defence against inflammation, immune modulating drugs are most often used. Several of such therapeutic strategies have been approved by the Food and Drug Administration (FDA)[93]. *Glatiramer acetate* (GA, Copaxone™) binds to MHC II DR molecules, thereby inhibiting antigen-specific T cell

activation and inducing a shift from Th1 to Th2 cytokines[24]. GA was shown to decrease exacerbation rate and prevent the development of new lesions in patients with RR-MS[94,95].

IFN-β also is a FDA-approved therapy for MS. It exists in 2 variants, IFN-β1a (Rebif™, Avonex™) and IFN-β1b (Betaferon™), that were shown to have positive effects in both RR-MS and SP-MS patients[3]. IFN-β can reduce T cell proliferation, TNF-α production and antigen presentation, cause a Th1 to Th2 switch, increase IL-10 secretion, modulate the expression of adhesion molecules and matrix metalloproteinases and can reduce immune cell migration into the CNS[3,24,96]. A side-effect of therapy using these interferons is the development of neutralizing antibodies that sometimes occurs[3].

Fingolimod (FTY720), a fungal metabolite with sphingosine-1-phosphate-receptor agonist activity, causes homing of the lymphocytes to the lymph nodes. The immune cells are trapped in these lymph nodes, preventing their migration into the CNS. FTY720 was recently approved by the FDA for the treatment of MS. Positive effects of FTY720 have been shown on the relapse rate and MRI outcome, although adverse events such as viral infections, hypertension and skin cancer have been observed[97].

Fumarate is a new promising immunomodulating treatment for MS that is still under investigation. The mechanism of action is not yet understood. In RR-MS it reduced gadolinium-enhancing lesions and relapse rate[98]. In some patients, dose-related side effects, such as increase in transaminase levels, were elicited.

Altered peptide ligands (APLs) bind with low affinity to the T cell receptor (TCR), thereby weakening the activation signal[6,24,27]. This causes a switch in the T cell cytokine program from Th1 to T helper 2 (Th2). Instead of using a single APL, peptide mixtures that contain several antigen specificities could be used to treat MS. A disadvantage of APLs is the possibility that they can be recognized as self antigens by disrupted or damaged TCR.

Immunosuppressive therapies

Relapses are often treated by intravenous c*orticosteroids*, such as methylprednisolone. These corticosteroids destroy immune cells and achieve a fast remission state[3,96].

Mitoxantrone is a FDA-approved chemotherapeutic drug that intercalates in the DNA helix, disabling cell division. In MS it is used to suppress the immune

system by eliminating the white blood cells. It causes a reduction in the number of relapses and also has an effect on the secondary progressive phase[99]. However, an increased risk for the development of cancer and cardiac toxicity have been evidenced after prolonged treatment with mitoxantrone.

Further, new therapeutic drugs are currently being investigated in clinical trials for their efficacy and side effects. *Laquinimod* is quinolone-3-carboxamide with proven efficiency in EAE[100]. A reduction in the number of gadolinium-enhanced lesions was shown in a phase II trial[101] and 2 extension phase IIb trials[102,103]. *Teriflunomide* belongs to the group of malononitrilamide agents that block the mitochondrial enzyme dihydro-orotate dehydrogenase, capable of inhibiting T and B cell proliferation[100]. It was also successfully tested in EAE and appeared to reduce MRI disease activity and delay disease progression in a phase II study[104]. Currently, its effects are further investigated in 2 phase III trials[105]. The purine nucleoside analogue *cladribine* specifically induces apoptotic death in resting and dividing lymphoyctes. In a recent phase III trial, cladribine was reported to decrease relapse rate, disability progression and brain lesion count while the relapse-free rate was increased[106].

Other immunosuppressive treatments that were shown to have a positive effect in (subgroups of) MS patients are plasma exchange and haematopoietic stem cell transplantation (HSCT). HSCT is however limited to autologous stem cells and is currently an experimental therapy exclusively assessed in advanced SP-MS or aggressive RR-MS[100].

Monoclonal antibody therapy

Natalizumab (Tysabri) is a FDA-approved recombinant monoclonal antibody (mAb) directed against α4β1 integrin. By blocking this vascular adhesion molecule, entrance of inflammatory cells into the CNS is inhibited[100]. A reduction in relapse rate, MRI activity and disease progression were seen following treatment[107]. The development of progressive multifocal leukoencephalopathy (PML) by the polyomavirus JC has been described in some patients that were treated with Natalizumab and can lead to unilateral paralysis[108]. This emphasizes the need to re-evaluate this therapy in clinical trials.

Other monoclonal antibody therapies are still under clinical investigation. Depletion of T cells, B cells, natural killer (NK) cells and macrophages using *Alemtuzumab,* an *anti-CD52 mAb* (Campath), diminished the relapse rate and

inflammatory MRI activity[109]. A side effect of treatment with Alemtuzumab is the development of autoimmune thyroid disease[110]. B cell depletion by an *anti-CD20 mAb* (Rituximab) was also reported to be successful in RR-MS. Following Rituximab treatment, the number of lesions and relapses was decreased together with inflammation[111-113]. It targets pre-B cells and mature B cells but not plasma cells that do not express CD20. Targeting of the IL-2 receptor using *Daclizumab* also led to encouraging results. A reduction in the number of lesions and a stabilization or even improvement of clinical disability have been demonstrated[100].

Neuroprotective therapy

Neuroprotective strategies could be more effective in progressive MS, as neurodegeneration is described by many studies as the main cause of neurological disability in MS patients[114]. Glutamate antagonists, sodium channel blockers, calcium and potassium channel blockers, cannabinoid receptor antagonists and erythropoietin have all resulted in positive effects in the EAE model. Testing these neuroprotective agents in MS is limited by the lack of robust clinical and surrogate markers of the neurodegenerative process in MS[114]. Further, therapies promoting endogeneous repair mechanisms[115] could be employed, as well as stem cell transplantation[116] or replacement therapies[117]. These are all new strategies that are on their way to entering clinical trials.

1.2 B cells and antibodies in MS

MS has been regarded as a T cell mediated disorder for a long time, while B cells have been neglected due to their minor role in EAE. However, more and more research is now performed concerning the involvement of both B cells and antibodies in the pathogenesis of MS.

1.2.1 Evidence for the involvement of B cells in MS pathogenesis

A substantial amount of evidence has been collected to demonstrate the involvement of the humoral immune response in MS. OCB were first described by Elvin Kabat in 1948 and by EC Laterre in 1964 as being composed of increased Ig generated by antibody-secreting cells in the CNS, thus indicating an intrathecal Ig production[2,118]. They have been found in the CSF but not in the serum of more than 90% of patients with MS[2,119]. These OCB are one of the diagnostic markers of MS[120], although they can be found in other inflammatory diseases of the CNS as well[67].

The presence of B cells, plasma cells, complement and myelin-specific antibodies in chronic MS lesions[119,121-125] and the restricted Ig gene usage pattern in the variable region of the heavy chain (V_H) locus in CSF B cells[126-128] suggested an antigen-driven humoral immune response in the CNS. The antibody response in these patients appeared to be stable over long periods. Oligoclonal anti-MBP IgG has furthermore been described in the CSF of MS patients[129]. More recently, positive results have been achieved using the B cell depleting antibody Rituximab in clinical trials[111,112,130,131]. Pattern II demyelinating lesions, characterized by Ig deposits and involvement of the complement system, are furthermore the most frequent (approximately 58%) lesion type in MS patients[31]. B cell cytokines, such as BAFF (B cell activating factor of the TNF family, tumor necrosis factor ligand superfamily member 13B), were also detected in MS lesions[132].

In addition, B cell follicle-like structures were characterized in the meninges of MS patients[25]. These are large B cell aggregates consisting primarily of memory B cells that are localized in the sub-arachnoid space, mainly inside the cerebral sulci. They are called B cell follicle-like structures as they display several GC-like features including the presence of dendritic cells expressing CXCL13 (chemokine

C-X-C motif ligand 13), B cell proliferation, expression of activation-induced cytidine deaminase and plasma cell differentiation[133]. However, the typical structure of lymphoid follicles with a GC and a mantle zone could not be recovered. Ectopic lymphoid follicles have also been denoted in EAE[134].
Taken together, these findings all contribute to the compelling evidence that the humoral immune response is implicated in the pathogenesis of MS.

1.2.2 B cell maturation and B cell subsets

In order to examine the role of B cells in the pathogenesis of MS, it is important to understand the B cell maturation process. The distribution of the different B cell subpopulations that make up the B cell repertoire in healthy individuals and MS patients has already been thoroughly analyzed.

B cell maturation

Mature naïve B cells, which are characterized by expression of the general B cell marker CD19 and the absence of CD27 and CD38 on their surface[135], are released in the PB after development in the bone marrow and secondary lymphoid organs[136,137]. Recognition of an antigen in lymphoid tissue triggers B cell activation, which is accompanied by an upregulation of CD80 and CD86 surface expression. After activation, B cells proliferate into plasma blasts that produce low-affinity antibody for a few days[138] or further differentiate into different effector B cell subtypes in germinal centers (GC) via the centrocyte and centroblast stages[139], which is accompanied by an upregulation of CD38 surface expression[140-142]. In this case, memory B cells, plasma blasts or plasma cells are developed after extensive mutation and maturation (Fig. 1.3)[143,144]. Memory B cells, expressing both CD19 and the memory surface marker CD27[135], are able to reactivate and differentiate into plasma cells very fast upon a new challenge with their antigen. Plasma blasts are short-lived antibody-secreting cells expressing CD19, CD27, CD38 but also the plasma cell marker CD138. Plasma cells have lost proliferating capacity and are mostly long-lived antibody-producing cells that no longer display CD19 but do express CD38, CD138 and CD27. Another B cell subset consists out of regulatory B cells that are involved in the regulation of the immune response. The 2 major regulatory B cell subpopulations in mice are $CD19^{+}CD24^{high}CD28^{high}CD1d^{high}$ transitional 2

marginal zone precursor (T2-MZP) and $CD19^{+}CD5^{+}CD1d^{high}$ B10 cells[165,166]. In humans, regulatory B cells have not been characterized in detail, although $CD19^{+}CD24^{high}CD28^{high}CD1d^{high}$ regulatory B cells have been described[167]. As this regulatory B cell subset also expresses high levels of CD5, it seems that there is more concordance between transitional and B10 regulatory B cells in humans[163].

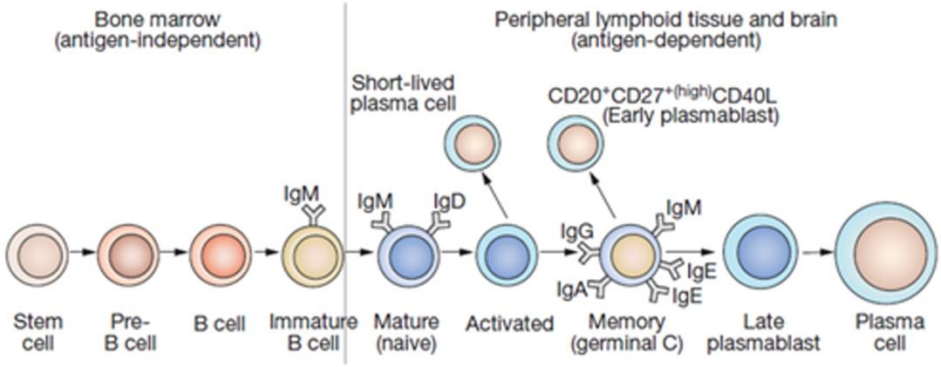

Figure 1.2 B cell maturation process. B cells develop in the bone marrow from stem cells into immature B cells that are released into the periphery and travel to lymphoid tissue. Here, they become mature antigen-inexperienced (naïve) B cells that upon activation by their target antigen differentiate into short-lived antibody-secreting cells or further mature in GC into memory B cells. These can further develop into plasma blasts and plasma cells.
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B cell repertoire in healthy individuals and MS patients

In healthy individuals, most B cells that populate the PB belong to the naïve B cell population, although some circulating memory B cells can be denoted. The antibody-secreting effector cells move into the target tissues to eliminate their target cells and consequently cannot be found in the blood. B cells are rarely found in the CSF of healthy persons and patients with a non-inflammatory neurological disease (NIND)[144].

The PB of MS patients displays a similar B cell subtype distribution as that of healthy individuals, mostly harbouring B cells with a $CD19^{+}CD27^{-}$ naïve phenotype[145,146]. The CSF of MS patients and patients with other inflammatory neurological disorders (OIND) contains on average 4-5% of $CD19^{+}$ B cells,

opposite to the situation in healthy persons. Variability in CSF B cell numbers was seen between patients[144,145,147-149], but the majority were mature $CD19^+CD27^+CD38^-CD138^-$ memory B cells[145,146,150]. A population of IgG^+/IgA^+CD27^- memory B cells exists as well[151], but numbers of this B cell subset have not yet been studied in MS. $CD19^+CD38^{high}CD77^+$ $Ki67^+Bcl\text{-}2^-$ centroblasts, that normally only occur in GC in lymphatic tissues[152], were demonstrated in the CSF[144,146], either as the result of circulating memory B cell recruitment to the CNS or intrathecal differentiation of naïve B cells. Both processes can be promoted by ectopic GC that were identified in the brain meninges of MS patients and suggest that antibody-secreting cells can be generated within the CNS[25].

Plasma blasts ($CD19^+CD38^+CD138^+$) have also been described as an important CSF B cell population. Numbers of plasma blasts were significantly increased in CSF of MS and CIS patients when compared with OIND patients[148]. Furthermore, the number of mature memory B cells and plasma blasts in the CSF of MS patients was correlated with intrathecal IgG and immunoglobulin M (IgM) production, higher disease activity and lesions on MRI, total CSF leukocyte numbers and intrathecal production of matrix metalloproteinase 9 (MMP-9), and the homing chemokine CXCL-13[145,148]. These findings all indicate that CSF B cells play a role in acute inflammation. Being one of the most prevalent antibody-secreting cells in the CSF, plasma blasts are held responsible for intrathecal IgG production and are consequently mentioned as main effector B cell population in acute inflammation in MS[145]. Normally, plasma blasts are short-lived antibody-secreting cells that rapidly differentiate into fully mature $CD19^-CD138^+$ plasma cells. In MS, however, plasma blast numbers remained virtually constant over time[145], which might demonstrate an antigen-driven chronic B cell stimulation in the CNS.

Disagreement exists concerning the contribution of $CD19^-CD38^+CD138^+$ plasma cells to the B cell repertoire in the CSF compartment. While some studies pointed out very low numbers of plasma cells[145,148,153], another study demonstrated a high number of plasma cells in the CSF of MS patients[146]. Thus, the distribution of plasma blasts and plasma cells in the CSF remains vague, although $CD19^+CD138^+$ and $CD19^-CD138^+$ cells have recently been shown to be derived from the same progenitor pool and thus represent convergent responses[153]. The absence of plasma cells in the CSF of MS patients could be

explained by the fact that plasma cells migrate to the brain meninges and parenchyma so they are hard to detect in CSF. Moreover, surface marker expression profiles have not been entirely clarified for plasma cells and plasma blasts. Previously, it has even been shown that CD138 was not expressed on the surface of plasma blasts and consequently was the only surface marker allowing to differentiate between plasma cells and plasma blasts[154]. Thus the phenotypic differences between these two cell types still have to be determined in detail. Table 1.1 gives an overview of the different B cell subsets, their surface marker expression profiles and distribution among PB and CSF.

Table 1.1 Overview B cell subsets in healthy individuals and MS: expression of surface markers and distribution in PB and CSF.

	naïve B cell	memory B cell	plasma blast	centroblast	plasma cell	references
surface markers	$CD19^+$ $CD27^-$ $CD38^-$	$CD19^+$ $CD27^+$ $CD138^-$ $CD38^-$	$CD19^+$ $CD27^{high}$ $CD138^+$ $CD38^+$	$CD19^+$ $CD77^+$ $Ki67^+$ $Bcl\text{-}2^-$ $CD38^{high}$	$CD19^-$ $CD27^{high}$ $CD138^+$ $CD38^+$	121,144-147,153
healthy persons						
periphery	+	(+)	-	-	-	146,154
CSF	-	-	-	-	-	145,148,155
multiple sclerosis						
periphery	+	(+)	-	-	-	145,146,148,155
CSF	-	+	+	+	+/(+)	144-149,153,155

+ present; - not present; (+) present in low amounts

1.2.3 Functional roles of B cells and autoantibodies in MS

One of the most important functions of B cells in MS is the production of autoantibodies. Plasma cells and plasma blasts in the CNS generate antibodies that recognize self antigens. These pathogenic antibodies can clear target cells, such as oligodendrocytes or neurons, via antibody-dependent cell-mediated cytotoxicity (ADCC) or complement activation (Fig. 1.2A)[119,128,156]. In ADCC, autoantibodies bind Fc receptors on macrophages, neutrophils and NK cells that

consequently attack myelin by phagocytosis. This further leads to activation of phagocyte oxidase and secretion of hydrolytic enzymes that induce tissue damage and inflammation[128]. Alternatively, autoantibodies bind directly to their target antigen and activate the complement cascade through complement fixation. The Fc region of the antibody binds to complement component C1q, resulting in activation of the complement cascade. This ultimately leads to the generation of a membrane attack complex (MAC) that is inserted into the cell membrane of the target cell to cause lysis and cell death[128,157].

B cells exert antibody independent functions in MS pathogenesis as well. Although their antigen presenting capacity is low when capturing antigen in a nonspecific manner, B cells are potent APC for the activation of T cells of the same specificity[158,159]. In this way, B cells participate in the generation and maintenance of T cell clonal expansion and cytokine production (Fig. 1.2B).

Further, B cells can secrete a variety of cytokines. Proinflammatory cytokines, such as IL-6 and TNF-α, enhance tissue damage in the CNS by activation of macrophages and T cells (Fig. 1.2C)[160]. The resulting T cell response is dependent on the profile of secreted cytokines. B effector (Be) 1 cells produce IFN-γ and promote Th1 responses, while Be2 cells release IL-4 and induce Th2 cells[143]. Proinflammatory cytokines can further modulate the migration of dendritic cells and provide feedback stimulatory signals for B cell activation[156]. Duddy *et al.* described the decreased capacity of B cells in MS to produce IL-10[161], an immunosuppressive cytokine. IL-10 can be produced by naïve and memory B cells, dependent on the activation signals[162], but is typically produced by regulatory B cells[125,144]. The secretion of IL-10 and/or TGF-β by regulatory B cells can inhibit proliferation and cytokine production by effector $CD4^+$ T cells and can induce regulatory T cells[163]. In EAE, B cell deficiënt mice were unable to recover from acute EAE, suggesting a role for B cells in regulation of the disease[164].

Furthermore, B cells are involved in the formation and maintenance of ectopic lymphoid follicles (Fig. 1.2D). The formation of such follicular structures is driven by dendritic cells and memory B cells through the actions of cytokines and chemokines including CXCL-12 (chemokine C-X-C motif ligand 12), CXCL-13 and lymphotoxin[146]. In addition, B cells in MS produce neurotrophic factors such as nerve growth factor[168], brain-derived neurotrophic factor[169] and neurturin[170]. These might help in restoring CNS integrity. Finally, B cells provide co-

stimulation in the T cell activation process[119,125]. During MS exacerbations, the number of circulating B cells that express the costimulatory receptors CD80 and CD86 (B7-1) is significantly increased[171,172]. This again contributes to T cell clonal expansion and cytokine production.

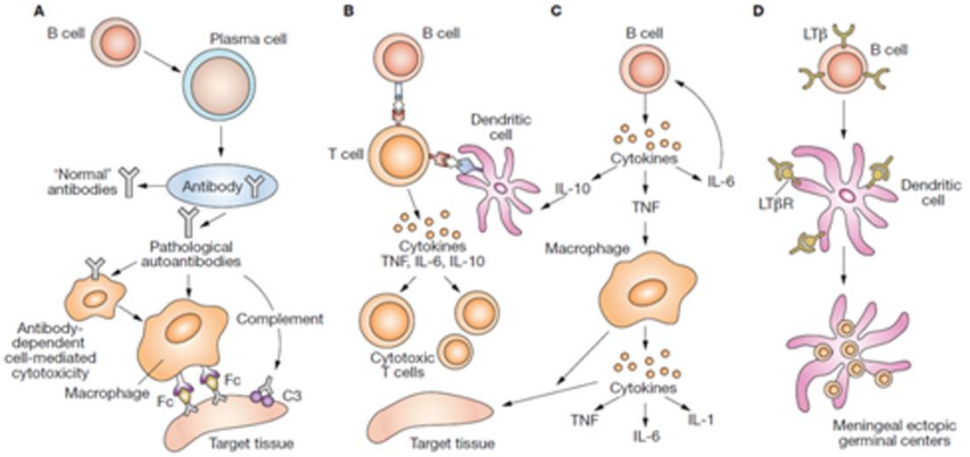

Figure 1.3 Main B cell functions in MS. B cells in MS exert 4 important functions that contribute to disease pathogenesis. (A) Production of autoantibodies that cause tissue damage through complement activation or ADCC. (B) B cells function as APC in the activation of cytotoxic T cells, leading to T cell clonal expansion and increased cytokine production. (C) Proinflammatory cytokines are also produced by activated B cells, causing activation of macrophages and T cells and enhancement of tissue damage. (D) Clonally expanded B cells form ectopic GC, maintaining a pathogenic humoral immune response in the CNS.

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1.2.4 B cell migration and survival in the CNS

Migration of B cells to the brain is directed by CXCL-13, CXCL-10 and CXCL-12 that are secreted by endothelial cells and are upregulated in the brain of MS patients[45,144,173]. In addition, migration is facilitated by very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1)[45]. These adhesion molecules bind to VCAM-1 and ICAM-1 on endothelial cells to direct B cells from the circulation into the CNS.

BAFF and A proliferation inducing ligand (APRIL) are two important regulators of B cell survival and activation in the CNS, but also of differentiation, GC formation and Ig production[174,175]. Both members of the TNF family are produced by monocytes, macrophages and dendritic cells[156]. Their receptors on the B cell surface are BAFF-receptor (BAFF-R), B cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cytophilin ligand interactor (TACI)[175]. BAFF may synergize with CXCL-12, CXCL-13 and CCL-19 (chemokine C-C motif ligand 19) to attract and maintain B cells in the inflamed CNS of MS patients[176]. Levels of BAFF, BAFF-R and APRIL mRNA are increased in patients with MS, where they are produced by astrocytes[177-179]. BAFF could also be produced by autoreactive B cells, as the group of Serafini recently reported strong BAFF immunoreactivity in infiltrating EBV-infected B cells that were organized in ectopic B cell follicles in the meninges of MS brains[74]. The increase in BAFF could in turn favour the production of autoreactive antibodies[180]. BAFF and APRIL have furthermore been described to be relevant targets for therapy of various autoimmune disorders and could be used in that context since intravenous Ig contains anti-BAFF and anti-APRIL antibodies[181,182].

Whether B cells are implicated in the initiation of the disease or whether their involvement is secondary to the causative immune response is still not clear. However, all together, these findings point toward an important role of B cells in MS. The B cell repertoire in the CSF of MS patients has already been intensively studied. However, the relative contribution of the different B cell subsets to intrathecal IgG production and OCB formation still has to be defined. In addition, the site of CSF B cell activation (periphery, CNS of both) and the phenotypic differences between plasma cells and plasma blasts have to be determined in detail.

1.3 B cell reactivity analysis in MS

Numerous approaches are currently used to investigate reactivity of the humoral immune response with different success rates, and despite a lot of effort the targets of the autoreactive B cells and antibodies are not yet known. Since MS is characterized by a heterogeneous disease course and genetic background, novel markers for the diagnosis of patients and a multiplex treatment strategy are needed for better control of the disease. Studying the humoral immune response thus remains a hot topic in MS research where identification of the target antigens is one of the main goals.

1.3.1 Focus on antibodies or B cells in serum or CSF

Most studies investigating the specificity of the humoral immune response have focused on autoantibodies directly by analyzing reactivity of the antibodies in serum or CSF of MS patients versus healthy controls or other neurological control patients. A substantial amount of the already identified candidate autoantigens have been denoted in this way. Several techniques can be used in this setting, such as protein microarrays[183], phage display[184] or proteomics-based approaches[185].

CSF can be a useful surrogate for the brain microenvironment and is therefore suitable to study antibody responses in MS. Moreover, oligoclonal Ig have mainly been described in the CSF of MS patients. However, CSF may also comprise antibodies originating from the PB that are not specific to the disease. Disease-relevant autoreactive antibodies may even bind to their targets or to Fc receptors in the CNS tissue, rendering them undetectable in CSF. Serum can be used as an alternative to study B cell reactivity in MS. Intrathecally produced antibodies are able to diffuse from the CSF into the circulation, leaving them detectable in the serum. In addition, several studies have described the increased presence of specific antibody reactivity in serum of MS patients when compared to neurological controls, demonstrating that serum autoantibody profiles can be used to distinguish MS patients from control subjects[70,186-188]. An approach that does not suffer from antibody binding to target antigens or from antibody dilution and breakdown would be to investigate the reactivity of the

(autoreactive) B cells in CSF and PB of MS patients. Several target antigens that have been identified by both approaches are described below (Table 1.2).

1.3.2 Target antigens identified by autoantibody profiling in MS

Multiple candidate antigens of the humoral immune response in MS have already been identified but evidence for their role in the disease process is still lacking or their involvement is not specific to MS. MOG[121,189,190], MBP[129,191], PLP[192], MAG[193], oligodendrocyte-specific protein (OSP)[194] phosphatidylcholine[195] and galactocerebroside (GalC)[196] are all myelin antigens that have been mentioned as possible autoantibody targets. Humoral responses to axonal and neuronal antigens, such as cytoskeletal neurofilament proteins, gangliosides, tubulin, neurofascin and Nogo-A, have been reported in MS and were sometimes correlated with disease progression[185,197-201]. Anti-lipid antibodies directed against sulfatide, sphingomyelin, oxidized phosphatidylcholine and others were detected in MS CSF using lipid microarrays[202]. In addition, antibodies specific for DNA have been demonstrated in brain and CSF of MS patients[203]. A panel of 8 novel autoantibody targets for MS was recently identified in our research group[184]. Further, B cell responses towards viruses, including EBV, HHV-6, CMV, measles and C. pneumonia, were reported[128,197]. CSF OCB of MS patients even showed binding to EBV proteins[70]. In addition, some protective antibodies that cause remyelination and myelin repair have been found, such as antibodies directed against oligodendrocyte surface antigens or heath shock proteins[197].

1.3.3 Target antigens identified by B cell reactivity analysis in MS

The group of Link *et al.* performed different studies on B cell reactivity in MS using an immunospot assay. Nitrocellulose microtiter plates were coated with myelin antigens to detect binding of PBMC or CSF cells. When studying CSF cells and PBMC of 19 MS patients, MBP-reactive CSF B cells were found in 57% of MS patients while none were present in PB. MAG-reactive B cells were indicated both in CSF and PBMC of MS patients (55% and 11% of patients, respectively)[204]. Furthermore, anti-MOG IgG secreting cells were demonstrated in PBMC from 8 of 16 MS patients and in CSF from 8 of 10 MS patients[189].

Other studies made use of recombinant antibodies that were produced from patients' B cells. $CD19^+$ or $CD138^+$ cells from CSF were single-cell sorted into PCR plates, the variable regions of the heavy and light Ig chains (V_H and V_L) were amplified and cloned into a plasmid vector which was then expressed in a bacterial expression system. The corresponding recombinant antibodies were used for reactivity screenings with immunoprecipitation, Western blot, ELISA (enzyme-linked immunosorbent assay) or immunohistochemistry. Lambracht-Washington and coworkers discovered reactivity of their recombinantly produced antibodies towards MBP, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and GFAP[205]. On the other hand, von Büdingen *et al.* produced 9 recombinant MS antibodies following the same procedure, and in this case 8 displayed myelin reactivity and 1 was directed against astroglia[206]. Apart from myelin binding in MS brain tissue, their targets could not be identified using several techniques such as costaining with known myelin antigens on brain tissue in immunohistochemistry, Western blot with human myelin or ELISA using recombinant and native MOG and MBP. In yet another study from Zhang *et al.*, recombinant antibodies from MS CSF demonstrated reactivity to axons with pathologic changes in MS brain tissue[207]. The targets were later identified as TPI and GAPDH[208]. Very recently, Yu *et al*. employed 19 recombinant antibodies from clonally expanded plasma cells in the CSF of MS patients in combination with phage displayed random peptide libraries to demonstrate peptide reactivity[209]. Several antigenic candidates were identified, including stress proteins, cell surface proteins and neuronal proteins.

Another possibility for determination of the B cell target antigens and characterization of the B cell repertoire is the immortalization of B cells from MS patients. Using this technique immortalized B cell clones, that show continuous antibody production, are generated. The target antigens of the produced antibodies can be determined and the B cell response that is going on in vivo can be studied. B cell immortalization will be discussed in more detail in the next section.

Table 1.2 Targets of antibodies and B cells in MS

Targets of antibodies in MS	Reference
Myelin antigens	
Myelin oligodendrocyte glycoprotein (MOG)	121,190
Myelin basic protein (MBP)	129,191
Proteolipid protein (PLP)	192
Myelin associated glycoprotein (MAG)	193
Galactocerebroside (GalC)	196
Oligodendrocyte antigens	
Oligodendrocyte-specific protein (OSP)	194
Transaldolase	210
2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)	211
Lipid antigens	
Sulfatide	202
Sphingomyelin	202
Phosphatidylcholine	195,202
Axonal and neuronal antigens	
Neurofilament	212
Gangliosides	198
Tubulin	200
Neurofascin	185
Nogo-A	201
Viral antigens	128,197
Epstein-Barr virus (EBV)	70
Human Herpes virus 6 (HHV-6)	
Cytomegalovirus (CMV)	
Measles	
Chlamydia pneumoniae (C. pneumoniae)	
Other antigens	
DNA	203
Heath shock proteins	213
Targets of B cells in MS	
Myelin antigens	
Myelin basic protein (MBP)	204,205
Myelin associated glycoprotein (MAG)	204
Myelin oligodendrocyte glycoprotein (MOG)	189
Oligodendrocyte antigens	
2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)	205
Other antigens	
Glial fibrillary acidic protein	205
Glyceraldehyde-3-phosphate dehydrogenase	208
Triosephosphate isomerise	208

1.4 Methods for monoclonal antibody production

Several approaches for the production of mAb exist[214], including the hybridoma technology, phage display technology and recombinant antibody production after single-cell sorting or laser-capture microdissection. An alternative for these methods is the immortalization of human B cells, leading to the generation of continually dividing and antibody-producing immortalized B cells.

1.4.1 Hybridoma technology

The hybridoma technology, first described in 1975 by Köhler and Milstein[215], is the longest existing and most widely used method for the generation of mAb. Antibodies are selected in murine species by the use of mouse hybridomas generated from the stable fusion of immortalized myeloma cells with B cells from immunized mice. The successful use of such mouse mAb appeared to be low in drug development[216]. There are several causes for this low success rate. Mouse mAb display high immunogenicity in humans by a human anti-murine antibody immune response (HAMA) and sometimes a low efficiency due to weak interactions between mouse mAb and complement or Fc receptors. Further, mouse antibodies do not bind the human receptor FcRn that regulates the serum half-life of antibodies[217], leading to a terminal half-life that is typically less than 20 hours[218,219].

Humanization or chimerization is necessary to avoid side effects when administered to humans. In chimerization, the variable domains of a mouse mAb are joined to the constant domains of a human antibody[220,221]. Humanization of mouse mAb can be achieved using several methods, one of the easiest being the transfer of the complementarity determining regions (CDR) from a mouse antibody into a human antibody[222-224]. However, the generation of humanized antibodies with a high binding affinity for antigen generally requires the transfer of additional framework region (FR) residues from the mouse mAb[225]. These are time-consuming procedures after which some murine protein sequences still remain present in the antibody molecule[215,225]. Changes in the functional and immunogenic properties of the resulting antibodies can occur, limiting their efficacy.

Human/human homohybridomas[226] and human/murine heterohybridomas[227] have sometimes been used as an alternative but these turned out to be unstable and were therefore not commonly used. Mice transgenic for human Ig loci (XenoMouse strains) can alternatively be used for hybridoma production but again the antibodies are selected in a murine environment[214,228].

1.4.2 Antibody phage display

Phage display of antigens was first described in 1985 by Smith *et al*[229] and is based on the ability of phage virions, virus particles that infect and amplify in bacteria, to incorporate foreign DNA into their genome, coupled to a gene encoding a phage coat protein[229]. The peptide/protein product, encoded by a DNA insert, is thus displayed at the phage particle surface where it is available for experimental strategies. A very successful application of phage display is the isolation of mAb by the use of large phage antibody libraries[230]. Such antibody libraries can be made from IgG genes of an immunized animal or immune donor, non-immunized donors or synthetic antibody libraries can be constructed by in vitro assembly of Ig genes[231]. Antibody libraries made from B cells of immune donors are especially useful in analyzing natural humoral responses in patients with autoimmune disease[232,233].

Phage display allows the selection of antibodies against human antigens, although this selection occurs in an experimental in vitro situation. Some advantages of this technology are the possibility to select for specific binding properties such as species crossreactivity[234] and the generation of very large collections of antibodies[235]. Nevertheless, in vitro affinity maturation is often necessary to obtain high-affinity antibodies.

1.4.3 Recombinant antibody production following single-cell sorting or laser-capture microdissection

B cells at different stages of development can be isolated by fluorescence-activated cell sorting (FACS), based on different surface marker expression profiles[236]. Alternatively, fluorescently labelled B cells can be isolated from tissue, for example post-mortem brain tissue from MS patients, using laser-capture microdissection[237]. The corresponding heavy and light chain genes are

then amplified and cloned into eukaryotic expression vectors. Recombinant monoclonal antibodies can thus be produced in vitro with the same specificity of their in vivo counterparts, favouring the production of relevant antibodies[238]. However, antibody-secreting plasma cells and plasma blasts are difficult to isolate using flow cytometry because they move into the target tissues to eliminate their target cells, limiting their presence in the periphery. Using laser-capture microdissection only limited cell numbers can be isolated, which possibly hampers a reliable representation of the in vivo B cell repertoire. Moreover, fixation and staining of the tissue could disrupt the target molecules and could possibly interfere with the downstream PCR amplification.

1.4.4 B cell immortalization

B cell immortalization by EBV has been widely used in immunological, molecular and genetic research as it provides high amounts of DNA and large numbers of B cells and antibody molecules. EBV infects B cells via their CD21 receptor[239] and subsequently transforms them into continually dividing, lymphoblastoid cell lines. Its advantage over the other mAb producing technologies is the generation of antibodies that represent both the specificity and diversity of the humoral immune response in vivo. Consequently, antibodies produced by B cell immortalization can be useful in several settings in which humoral immunity is involved. Antibodies can even be generated against an infectious agent or tumour cells, rendering these antibodies attractive for therapy.

In vivo EBV infection

EBV is a gamma herpes virus that infects about 90% of the world population[240]. Primary infection during childhood is usually asymptomatic[241] while primary infection of adolescents can cause infectious mononucleosis[242]. Associations of EBV with Burkitt's lymphoma and nasopharyngeal carcinoma have been found[243,244]. In vivo, naïve B cells in the lymphoid tissue are preferentially infected by EBV through attachment of the gp350/220 viral membrane glycoprotein to CD21 on the B cell[239,245-247]. A second interaction between gp42 and HLA II molecules is also required[248]. This combined signalling results in activation of lck and Ca^{2+} mobilization followed by elevated messenger RNA (mRNA) synthesis, surface CD23 expression and IL-6 production[247,249,250]. Next,

the viral genome is uncoated and delivered to the nucleus, leading to genome circularization and Wp promoter expression. A cascade of events is activated that leads to activation of the growth program or latency III program in order to obtain proliferating blast cells[251]. At this stage, EBNA 1, 2, 3A, 3B, 3C and LP (or EBNA1-6) as well as the latent membrane proteins (LMP) 1, 2A and 2B are expressed[65]. An overview of the most important functions of these EBV viral proteins is given in Fig. 1.4. The latency II (default) program subsequently drives infected cells into the memory B cell state in a GC reaction[252]. Expression of EBNA 2, 3A, 3B, 3C and LP is downregulated, while EBNA1, LMP1 and LMP2 expression is continued. Following entrance into the circulation, expression of the viral genes is shutdown (latency I program) which renders these latently infected memory B cells undetectable for the host's immune system[253]. Consequently, the virus can stay present in the circulation for long periods in terms of a latent infection. Reactivation of the virus is possible when infected memory B cells become accidentally reactivated. EBNA1 expression is upregulated during cell division and activates the host cell DNA polymerase to elicit transmission of the viral genome into the daughter cells[65]. Under normal conditions this is prevented by EBV-specific cytotoxic memory T cells[254,255]. In cases of immune suppression, however, the virus can become reactivated and give rise to lymphoproliferative disease, although this can also occur in immunocompetent patients[253].

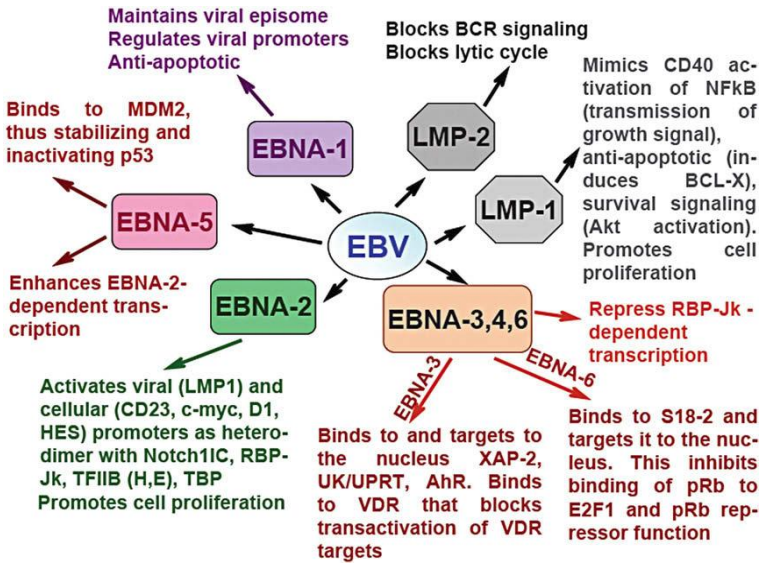

Figure 1.4 Functions of EBV viral proteins. EBV infection of the B cell leads to the expression of viral proteins that exert various functions in B cell transformation.
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In vitro EBV infection

In vitro infection of B cells from human PB by EBV causes a transformation into actively proliferating lymphoblastoid cell lines (LCL)[256]. The continuous proliferation of these LCL may be the consequence of the absence of a GC environment where EBNA2, 3A, 3B, 3C and LP are normally downregulated[257]. Shortening of the telomeres occurs because of fast cell division, until the transformed cells go into a proliferative crisis[258]. At this point little or no telomeric DNA remains, leading to apoptosis of a large proportion of the LCL[259]. A small proportion of the transformed cells survive the proliferative crisis through activation of telomerase, the enzyme that elongates telomeres de novo (Fig. 1.5)[260-262]. They become truly immortalized B cell lines that are characterized by downregulation of p16/Rb[256], mutation of the p53 gene[263], modulation of apoptosis[259], upregulation of WRN helicase[263,264], chromosomal instability[263] and a change in cytotoxicity to various chemical compounds[265]. All these features contribute to the evasion of cell death and continuous cell growth.

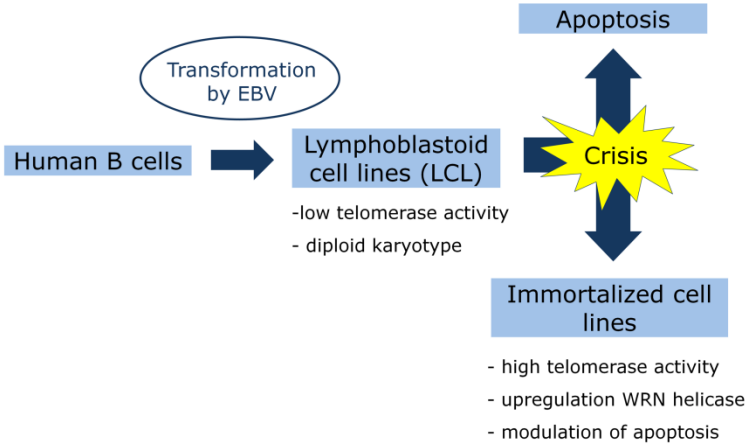

Figure 1.5 In vitro B cell immortalization process. Human B cells can become infected by EBV and will be transformed into lymphoblastoid cell lines (LCL), characterized by low telomerase activity and normal diploid karyotypes. When telomeres are very short, the transformed B cells go into a proliferative crisis in which most of the cells die because of apoptosis. A small portion of the B cells survive and become immortalized through activation of telomerase. Some other features of immortalized B cells are the upregulation of WRN helicase and modulation of apoptosis.

History B cell immortalization technology

The B cell immortalization technology was introduced in 1972 when B cells were transformed by culturing them in the presence of EBV, obtained from a human lymphoblastoid cell line (883L) derived from a patient with transfusion-induced infectious mononucleosis, and a feeder layer consisting of human placental fibroblasts[266]. The method was further adjusted in subsequent years to make use of EBV that was produced by the marmoset lymphocyte cell line B95-8 and has been used in this way for several decades[267-269]. The resulting LCL were mainly unstable IgM producing clones with low affinity. Since then, many efforts have been put in increasing the immortalization efficiency and B cell growth rate. Various adjustments have been tried, such as fusion of the immortalized B cell lines with myelomas[270], transfection of the immortalized B cells with activated c-myc DNA[271], B cell pre-activation by CD19 and/or B cell receptor (BCR) ligation[272], the use of fresh or frozen whole blood for immortalization[273,274] or the addition of growth factors and cytokines[275,276], but all without considerable improvements.

In 2004, an improved B cell immortalization method based on a combination of EBV and the polyclonal B cell activator CpG oligonucleotide CpG2006, a ligand of TLR9, was published[277]. Using this method, antibodies with the capacity to neutralize SARS coronavirus were produced. An alternative procedure, characterized by B cell activation prior to EBV infection, was described in 2008 to generate antibodies reactive against cytomegalovirus (CMV)[278]. These adapted immortalization procedures displayed higher immortalization efficiencies, although they had only been used for the generation of virus neutralizing antibodies from infected individuals and still presented low reproducibility. Subcloning of the resulting immortalized B cells is necessary in all described B cell immortalization methods to obtain monoclonal antibody-producing cells.

There is still need for a B cell immortalization method with high efficiency and reproducibility that could be adapted for the production of (autoreactive) antibodies from patients with autoimmune disease. A high efficiency method for low B cell numbers could be advantageous when B cells are not readily available, such as autoreactive B cells in the PB and CSF of MS patients or in the synovial fluid of rheumatoid arthritis (RA) patients.

1.5 Aims of the study

MS is an inflammatory disorder of the CNS that is marked by a heterogeneous disease course and genetic background. Despite a lot of progress in MS research, the disease process and the triggering event are still not entirely understood. The role of B cells in this disease has been studied extensively during the past years, but the biology of the aberrant B cell response and the B cell target antigens remain largely unknown. Numerous autoantibody profiling studies have made use of antibodies that were recovered from the CSF or serum of MS patients. This approach is limited by the dilution and breakdown of antibodies on the one hand and the masking of interesting antibodies because of binding to their target or Fc receptors in the CNS on the other hand. Alternatively, the repertoire of clonally expanded B cells could be studied instead of the antibody spectrum, thereby restricting attention to the B cell subsets that are most relevant to the disease process.

The aim of this study was to use B cell immortalization in order to characterize the B cell population that is present in the PB and CSF of MS patients, CIS patients and control individuals. B cell immortalization can be a powerful tool to study B cell responses in patients with autoimmune disease as it allows the production of fully human antibodies that truly reflect both the specificity and diversity of the human immune response in vivo. A further characterization of B cell diversity, phenotypes and antibody specificity could give more insight into the disease process of MS and could eventually lead to novel markers for disease.

1.5.1 Aim 1: Development of a B cell immortalization method with high efficiency and reproducibility for low B cell numbers without the need for cloning

B cell immortalization technologies have always suffered from low efficiency and reproducibility. Although promising results were achieved using the polyclonal B cell stimulus CpG2006, low reproducibility remained in our hands. Moreover, cloning of the resulting immortalized B cell lines appeared to be time consuming and suffered from low efficiencies.

In chapter 2, an improved B cell immortalization method is described. Simultaneous and sequential B cell stimulation and infection are tested in combination with different culture conditions to search for a B cell immortalization method with high efficiency for 50 B cells per well. Both methods are tested on total PBMC but also following enrichment of antigen-experienced IgG^+ B cells. Different techniques for the isolation of this B cell subset are analyzed to select the one that provides the highest purity, yield and immortalization efficiency. We further search for high monoclonality rates in the resulting immortalized cultures using B cell spectratyping to eliminate the need for subcloning.

The development of a B cell immortalization procedure with high efficiency and reproducibility for low B cell numbers reduces bias towards the preferential outgrowth of fast growing immortalized B cells. Moreover, it can be especially advantageous for the study of rare B cells, such as memory B cells in the PB or CSF of MS patients.

1.5.2 Aim 2: Characterization of the B cell repertoire in MS and CIS in terms of B cell clonal expansion and diversity

B cell diversity has been extensively studied in the CSF and brain of MS patients by examination of the BCR genes, and more specifically the V_H region genes. A restricted intrathecal B cell response has been evidenced by high mutation frequencies and the preferential usage of certain Ig VH genes. Further, B cells expressing identical sequences that code for antibody specificity have been reported in the CSF of patients with MS and CIS. These findings all point towards an intrathecal clonally expanded B cell response with ongoing affinity maturation that is triggered by repeated antigen exposure. However, the role of peripheral B cell responses in MS and CIS has remained unclear up to now.

In chapter 3, we aim at a further characterization of B cell diversity and clonal expansion in the PB of MS and CIS patients and a confirmation of the restricted B cell response in the CSF. For this purpose, we use a detailed Ig V_H region sequencing analysis using a panel of immortalized B cell lines that are produced from CSF but mostly from PB of MS patients, CIS patients and healthy controls. Further study of B cell diversity and evaluation of the extent of clonal expansion

can give more insight into the involvement of peripheral B cells in MS pathogenesis.

1.5.3 Aim 3: Phenotypic B cell characterization in MS and antibody specificity analysis

The involvement of B cells in the pathogenesis of MS is indisputable owing to the substantial amount of evidence that has been collected in recent years. Memory B cells and plasma cells were reported to be clonally expanded in the CSF of MS and CIS patients. One of the most important functions of B cells in the pathogenesis of MS is the production of (pathogenic) autoantibodies. Despite a lot of effort using many different approaches, the target antigens of the expanded B cells in MS have not been fully identified. Reactivity of B cells in the CSF of MS patients towards known myelin autoantigens but also towards viral epitopes and axonal antigens has already been discovered. However, these antibodies are often not specific to MS and evidence for their role in the disease process is scarce.

In chapter 4, we describe a detailed phenotypic and reactivity analysis of peripheral and CSF B cells. The distribution of B cell subsets in PB and CSF is studied in MS patients and healthy subjects. Reactivity of a panel of immortalized B cell lines that was obtained from the PB of MS patients, CIS patients and NIND/OIND patients but also from the CSF of 1 MS and 1 OIND patient is examined using several technologies. Binding of antibodies from the immortalized B cells to several cell types, brain tissue, viruses that were previously associated with MS and the myelin antigen MOG is examined using flow cytometry, immunocytochemistry, immunohistochemistry and immunoprecipitation. Candidate antigenic targets are identified using proteomic approaches.

Characterization of B cell repertoires in disease and health can lead to more information on aberrant B cell responses and the involved B cell subsets. Reactivity analysis of the peripheral and intrathecal B cell repertoire of MS and CIS patients and identification of antigenic targets can result in novel disease markers for diagnosis and therapy.

2

Optimization and validation of the B cell immortalization procedure

Based on:

A novel method for making human monoclonal antibodies.

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ABSTRACT

B cell immortalization by Epstein-Barr virus (EBV) is an established method for antibody production. Different B cell immortalization procedures with insufficient efficiency and/or reproducibility have been described, emphasizing the need for the development of an improved B cell immortalization method when seeding low B cell numbers per well.

The immortalization efficiency of i) sequential versus ii) simultaneous B cell stimulation and infection was tested on total peripheral blood mononuclear cells (PBMC) and isolated immunoglobulin G+ (IgG^+) B cells. Various experimental conditions were tested, such as duration and concentration of EBV infection, different methods for the isolation of IgG^+ B cells and the use of CpG2006 and/or interleukin-2 (IL-2) for B cell stimulation.

We have developed a B cell immortalization method for low B cell numbers per well using simultaneous B cell stimulation by CpG2006 and B cell infection by EBV, followed by an additional CpG2006 and IL-2 stimulus. Using this method, IgG-producing immortalized B cell lines were generated from peripheral blood IgG^+CD22^+ B cells with an efficiency of up to 83%. Antibody could already be obtained from the culture supernatant after 3-4 weeks. Clonality analysis demonstrated monoclonality in 87% of the resulting immortalized B cell lines. Given the high immortalization efficiency and monoclonality rate, subcloning of the immortalized cultures was no longer necessary. Moreover, the resulting immortalized B cell lines represented the ongoing humoral immune response in vivo.

This B cell immortalization method can be applied for the characterization of (autoreactive) B cells of patients with autoimmune disease. This could eventually lead to the identification of new autoantigens, disease markers or targets for therapy.

2.1 Introduction

B cell immortalization by EBV has been widely used in immunological, molecular and genetic research as it provides high amounts of DNA and large numbers of B cells and antibody molecules. EBV infects B cells via their CD21 receptor[239] and subsequently transforms them into continually dividing, lymphoblastoid cell lines that produce antibodies representing the humoral immune response in vivo. Antibodies can be generated against an infectious agent or tumour cells, rendering the resulting antibodies attractive for therapy. In addition, B cell immortalization can be a valuable tool for the production and characterization of autoreactive antibodies from patients with autoimmune diseases. This can provide more insight into the underlying mechanisms of humoral immune responses in autoimmunity and can lead to the identification of new autoantigens, disease markers or targets for therapy[184,186,199]. The major advantage of B cell immortalization, when compared to other antibody producing techniques[214,215,226,227,238,279], is the generation of fully human antibodies that truly reflect both the specificity and diversity of the human immune response, generated from the human B cell repertoire, without the need for specific immunization.

The use of EBV particles of the marmoset lymphocyte B95-8 cell line for B cell immortalization was first described in 1977[267,269]. B cell immortalization has suffered from low efficiency and reproducibility since then, despite various procedure adjustments. Introduction of the polyclonal B cell activator CpG2006 in the B cell immortalization process and B cell activation prior to EBV infection recently led to the successful production of antibodies neutralizing SARS coronavirus and CMV, respectively[277,278]. However, these methods resulted in low reproducibility in our hands. The current study was aimed at developing a B cell immortalization procedure with high efficiency and reproducibility when seeding low B cell numbers per well, that could easily be adopted for the production of (autoreactive) antibodies from patients with autoimmune disease. Such a method could be especially advantageous when autoreactive B cells are not easily available, for example in the CSF and PB of MS patients. Moreover, seeding low B cell numbers per well limits bias towards the preferential outgrowth of fast growing immortalized B cells.

In this chapter, we describe the optimization experiments that were performed to develop a B cell immortalization method that was applicable for the seeding of low B cell numbers per well. Two different approaches were tested in this setting, namely simultaneous and sequential B cell stimulation by CpG2006 in the presence or absence of IL-2 and B cell infection by EBV.

2.2 Materials and methods

2.2.1 Cell isolation

PB of healthy donors was obtained with informed consent. PBMC were isolated by Ficoll Hypaque density gradient centrifugation (Sigma-Aldrich, Bornem, Belgium).

Three methods for isolation of memory B cells were tested on 1 x 10^7 PBMC of 1 healthy donor. Negative B cell selection followed by positive selection of IgG^+ cells was performed using magnetic cell separation (MACS) according to the manufacturer's instructions ("MACS", IgG^+ memory B cell isolation kit, Miltenyi Biotec B.V., Utrecht, The Netherlands). Alternatively, PBMC were stained with PE-Cy5-labelled (phycoerythrin-cyanin 5) anti-CD22 antibodies (Ab) and PE-labelled (phycoerythrin) anti-IgG Ab for 30 min at 4°C and subsequently enriched by means of FACS sorting ("sort") using a FACSAria II cell sorter (all from BD Biosciences, Erembodegem, Belgium). As a third approach, B cells were first enriched using negative MACS selection (B cell isolation kit II, Miltenyi Biotec B.V.) according to the manufacturer's instructions, after which FACS sorting for IgG was performed as described above ("MACS + sort").

2.2.2 Analysis of CpG2006 and IL-2 effects on B cell activation

PBMC from 3 healthy donors were activated during 7 days using CpG2006 (ODN2006, 5'-tcgtcgttttgtcgttttgtcgtt-3', InvivoGen, Toulouse, France) and IL-2 (Roche Diagnostics, Vilvoorde, Belgium). Cyclosporine A (CsA, Sigma-Aldrich) was added at 250 ng/ml to suppress T cell responses. To obtain sufficient cell numbers for flow cytometric analysis, 1 x 10^4 PBMC (about 1,000 B cells) per well were cultured with 20 µg/ml CpG2006 and 0, 1,000, 2,000 or 5,000 U/ml IL-2. Since PBMC comprise about 10% B cells, these concentrations correspond with 1 µg/ml CpG2006 and 0, 50, 100 or 250 U/ml IL-2 for 50 B cells. The percentage of live $CD25^+$ activated B cells was measured at 1-7 days after stimulation by flow cytometry. PBMC were stained with PE-labelled anti-CD19 Ab, 7-aminoactinomycin D-negative (7-AAD) and fluorescein isothiocyanate (FITC)-labelled anti-CD25 Ab (all from BD Biosciences) for 30 min at 4°C. Cells

were washed twice and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

2.2.3 B cell immortalization procedure

Fresh total PBMC or IgG^+CD22^+ B cells were cultured in U-bottom 96-well plates (Nunc, Roskilde, Denmark) at respectively 500 (assuming a mean B cell percentage of 10% in the PB) or 50 cells per well in RPMI 1640 medium supplemented with L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin (all obtained from Invitrogen Life Technologies, Merelbeke, Belgium) and 10% heat-inactivated fetal bovine serum (FBS, HyClone Europe, Erembodegem, Belgium). Immortalization experiments were performed on cells of 2 healthy donors in 30 wells for each tested condition. As feeder cells, 1×10^5 autologous or allogenic irradiated (83 Gy) PBMC were used. CsA was added at 250 ng/ml when starting with total PBMC to suppress growth of (cytotoxic) T cells that would otherwise target EBV-infected B cells, causing immortalization failure.

In case of sequential B cell stimulation and infection (method 1), cells were stimulated during 3 days with 1 µg/ml CpG2006 with or without 50 U/ml IL-2 (Fig. 2.1A). B cells were subsequently infected with 50% v/v or 30% v/v EBV-containing supernatant (3.36×10^8 viral copies/ml) from the B95-8 cell line (ATCC, Manassas, USA) during 18 hours or 7 days, respectively. After this B cell immortalization phase, cells were restimulated during 7 days using 1 µg/ml CpG2006 in the presence or absence of 50 U/ml IL-2. Culture medium was replaced and cells were continuously cultured without extra stimuli. Immortalization was verified 28 days after seeding by screening the culture supernatant for antibody production using dot blot analysis and by light microscopic examination of cell growth. Both parameters were included since positive antibody measurements were sometimes observed in the absence of B cell growth, due to B cell activation at the start of the culture.

Simultaneous B cell stimulation and infection (method 2) was performed by culturing the cells during 2 weeks in the presence of 30% v/v EBV-containing supernatant and 1 µg/ml CpG2006 with or without 50 U/ml IL-2 (Fig. 2.1B). After immortalization, cells were restimulated during 7 days by 1 µg/ml CpG2006 in the presence or absence of 50 U/ml IL-2. Cells were subsequently

cultured for another 7 days in bare culture medium. Again, cultures were tested for immortalization after 28 days by performing dot blot analysis for antibody production and light microscopic cell growth examination.

2.2.4 Dot blot analysis for antibody production

Culture supernatant (5µl) of potentially immortalized B cells was spotted on a nitrocellulose blotting membrane (Protran BA-85, VWR International, Leuven, Belgium). After drying, blots were blocked with 5% (w/v) milk powder in PBS (MPBS) during 30 min on a shaker. Membranes were then incubated during 1 hour with HRP-labelled rabbit anti-human IgG or rabbit anti-human IgM (both from Dako, Heverlee, Belgium) 1:100 (13 µg/ml) in MPBS and extensively washed in PBS supplemented with 0.05% (v/v) Triton X-100 (PBS-T). Antibody production was detected using 3,3' deaminobenzidine (DAB) substrate (Sigma-Aldrich). Serial dilutions of human IgG (Invitrogen) or human IgM (Jackson ImmunoResearch, Suffolk, UK) ranging from 500 µg/ml to 0.5 µg/ml were included as a positive control. Culture medium was used as a negative control.

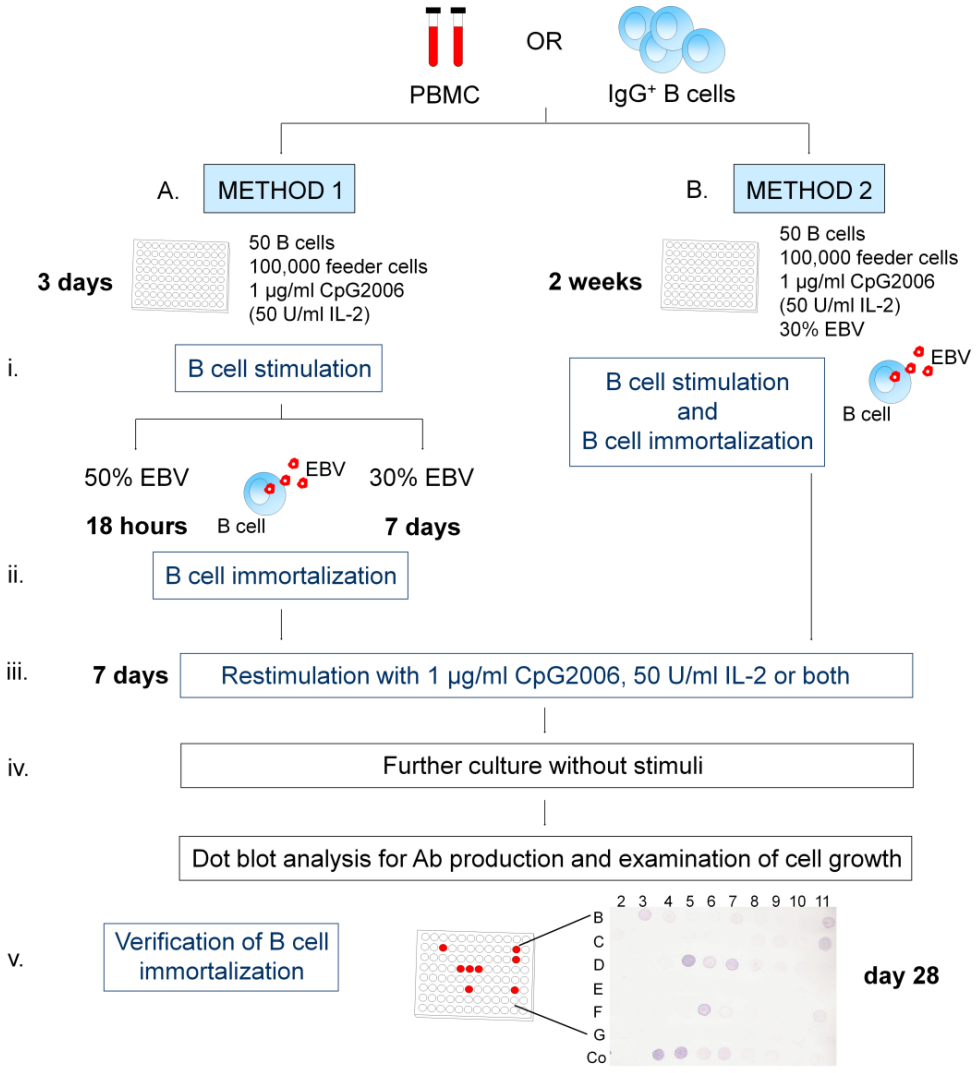


Figure 2.1 Overview B cell immortalization procedures. PBMC or IgG$^+$CD22$^+$ B cells were immortalized using the sequential (method 1) or simultaneous (method 2) procedure. In method 1, B cell stimulation with CpG2006 in the presence or absence of IL-2 (i) was performed during 3 days before infection with 30% v/v or 50% v/v EBV-containing supernatant during 7 days or 18 hours, respectively (ii). Method 2 was performed by B cell stimulation using CpG2006 with or without IL-2 together with infection using 30% v/v EBV during 2 weeks (i and ii). Following the B cell immortalization phase, cells from both methods were stimulated with CpG2006, IL-2 or both during 7 days (iii). After this restimulation, culture medium was replaced (iv) and cells were cultured further. Immortalization status was verified after 28 days of culture by performing dot blot analysis for antibody production and by light microscopic examination of cell growth (v).

2.2.5 B cell spectratyping

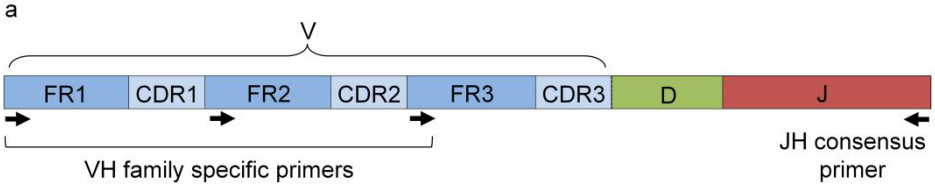
B cell spectratyping was performed on randomly selected immortalized B cell lines to determine monoclonality rates. Genomic DNA (gDNA) was first isolated from immortalized B cells present in single wells. Cells were lysed overnight at 37°C in lysisbuffer (10 mM Tis, 0.4 M NaCl, 2.4 mM EDTA), 20% sodium dodecyl sulphate (SDS) and 0.2% proteinase K solution in 35 mM SDS, 2.4 mM EDTA. The gDNA was then purified using a chloroform based extraction process, precipitated by ethanol and resuspended in sterilized water. gDNA concentrations were determined using a SmartLadder (Eurogentec, Seraing, Belgium) on a 1% agarose gel.

The variable region of the BCR heavy chain (HC) locus (V_H) was subsequently amplified from 100 ng gDNA using the IdentiClone™ IGH Gene Clonality Assay (Invivoscribe Technologies, La Ciotat, France) according to the manufacturer's recommendations. This standardized assay was extensively validated using Revised European/American Lymphoma (REAL) Classification[280]. Primers that target the conserved FR were used in combination with a FAM-labelled consensus primer targeting the conserved joining region (J) in order to amplify the variable region in which genetic rearrangements occur during B cell development (Fig. 2.2). The B cell receptor genes are highly polymorphic and subject to diversity and mutations. To maximize identification of the clonal rearrangements, a polymerase chain reaction (PCR) was performed for each FR using VH family-specific forward primers. Analysis of the resulting fragments was accomplished using automatic capillary electrophoresis (ABI3730, Applied Biosystems, Halle, Belgium). Polyclonal populations resulted in a Gaussian distribution of amplification products with different lengths, while a monoclonal population yielded a single amplification product. This spectratyping procedure can detect clonal populations with a sensitivity of 0.5-5%.

2.2.6 Statistical analysis

All statistical analyses were performed using Prism software version 4.00 (Graphpad). We analyzed the data using unpaired Student's *t*-test. A p value of <0.05 was considered statistically significant.

a



b

Target region	Sequence
VH1-FR1	5′ GGCCTCAGTGAAGGTCTCCTGCAAG 3′
VH2-FR1	5′ GTCTGGTCCTACGCTGGTGAAACCC 3′
VH3-FR1	5′ CTGGGGGGTCCCTGAGACTCTCCTG 3′
VH4-FR1	5′ CTTCGGAGACCCTGTCCCTCACCTG 3′
VH5-FR1	5′ CGGGGAGTCTCTGAAGATCTCCTGT 3′
VH6-FR1	5′ TCGCAGACCCTCTCACTCACCTGTG 3′
VH1-FR2	5′ CTGGGTGCGACAGGCCCCTGGACAA 3′
VH2-FR2	5′ TGGATCCGTCAGCCCCCAGGGAAGG 3′
VH3-FR2	5′ GGTCCGCCAGGCTCCAGGAA 3′
VH4-FR2	5′ TGGATCCGCCAGCCCCCAGGGAAGG 3′
VH5-FR2	5′ GGGTGCGCCAGATGCCCGGGAAAGG 3′
VH6-FR2	5′ TGGATCAGGCAGTCCCCATCGAGAG 3′
VH7-FR2	5′ TTGGGTGCGACAGGCCCCTGGACAA 3′
VH1-FR3	5′ TGGAGCTGAGCAGCCTGAGATCTGA 3′
VH2-FR3	5′ CAATGACCAACATGGACCCTGTGGA 3′
VH3-FR3	5′ TCTGCAAATGAACAGCCTGAGAGCC 3′
VH4-FR3	5′ GAGCTCTGTGACCGCCGCGGACACG 3′
VH5-FR3	5′ CAGCACCGCCTACCTGCAGTGGAGC 3′
VH6-FR3	5′ GTTCTCCCTGCAGCTGAACTCTGTG 3′
VH7-FR3	5′ CAGCACGGCATATCTGCAGATCAG 3′
J	5′ CTTACCTGAGGAGACGGTGACC 3′

c

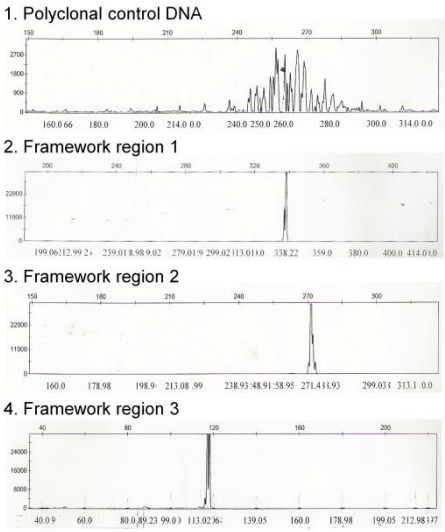


Figure 2.2 Spectratyping method. (a) The variable region of the BCR heavy chain locus consists of a variable (V), diversity (D) and joining region (J). 3 framework regions (FR) determine antibody structure, while 3 complementarity-determining regions (CDR) code for antigen specificity. The VDJ region is amplified by the use of VH family specific primers that bind to the conserved FR in combination with a FAM-labelled consensus J primer. (b) Overview of all primer sequences for the different VH families in the 3 FR and the consensus J-targeted primer. (c) Polyclonal populations resulted in a Gaussian distribution of amplification products with different lengths (1), while a monoclonal population yielded a single amplification product (2-4).

2.3 Results

An improved B cell immortalization method for the seeding of low B cell numbers per well was developed by testing sequential (method 1, Fig. 2.1A) and simultaneous (method 2, Fig. 2.1B) B cell stimulation and infection.

2.3.1 Addition of CpG2006 and IL-2 for B cell stimulation

CpG2006 was previously reported to increase immortalization efficiency when administered during EBV infection[277]. First, the effect of CpG2006 on B cell activation status was tested throughout time in the presence and absence of IL-2. A CpG2006 concentration of 1 µg/ml was optimal for stimulation of 50 B cells per well, resulting in maximal B cell activation after 3 days (Fig. 2.3). The addition of 50 U/ml IL-2 together with 1 µg/ml CpG2006 resulted in similar percentages of activated B cells and did not increase the CpG2006 induced B cell activation. Furthermore, IL-2 concentrations higher than 50 U/ml resulted in lower B cell activation, indicating that addition of IL-2 was not necessary for B cell activation and could even decrease CpG2006 induced activation. However, IL-2 might still exert an enhancing effect on growth of immortalized B cells. B cell activation using 0.5 µg/ml CpG2006 was tested as well, but appeared to be insufficient for B cell immortalization (data not shown). Activated B cells could not be detected when culture medium or CsA alone were used (data not shown).

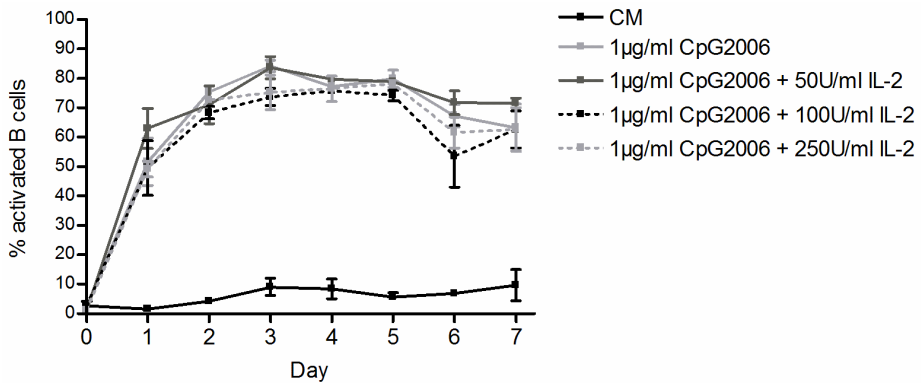


Figure 2.3 Optimization of CpG2006 and IL-2 conditions. The effect of CpG2006 and IL-2 on B cell activation was assessed at 1-7 days using flow cytometry. Averaged results from experiments on 3 healthy donors are shown. CM = culture medium

2.3.2 Immortalization of total PBMC using sequential or simultaneous B cell stimulation and infection

Sequential (method 1) and simultaneous (method 2) B cell stimulation and infection were tested on total PBMC using optimized CpG2006 and IL-2 conditions. An immortalization efficiency of 95-100% was measured using simultaneous B cell stimulation and infection (Fig. 2.4a). This was significantly higher than the sequential protocol with 30% EBV (IgG and IgM: $p<0.0001$) or 50% EBV (IgG: $p=0.0002$; IgM: $p=0.0019$). However, the largest proportion of resulting B cell lines appeared to be IgM^+. Following simultaneous B cell stimulation and infection (method 2) with restimulation using CpG2006 and IL-2, 76% of immortalized B cell lines were IgM^+, while 23% was IgG^+. The use of IL-2 during B cell stimulation (method 1) or infection (method 2) was tested as well, showing no increase in immortalization efficiency (data not shown).

Clonality analysis of the immortalized B cells was performed to evaluate the need for cloning of the B cell lines. B cell spectratyping resulted in 70% monoclonal and 30% biclonal immortalized B cell lines (Fig. 2.4b), emphasizing a high monoclonality rate when seeding low B cell numbers per well.

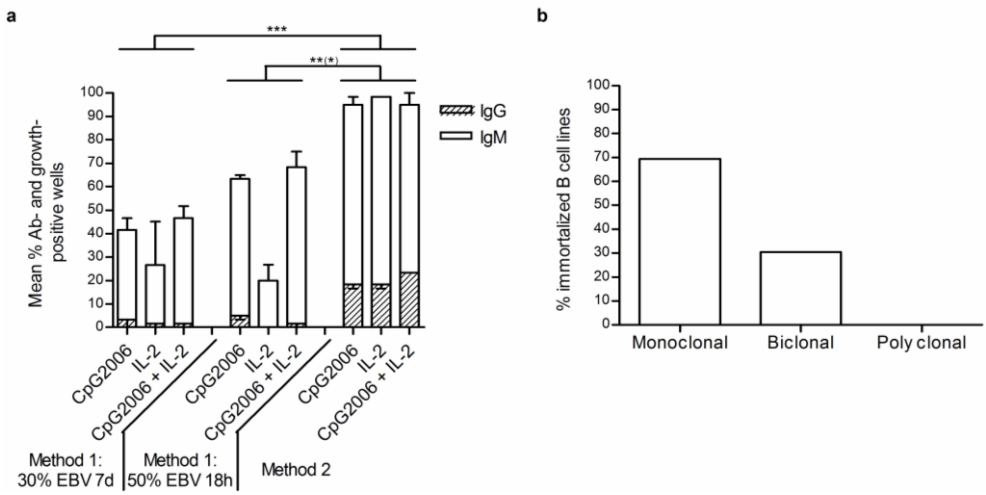


Figure 2.4 Immortalization efficiency and clonality using total PBMC. (a) Immortalization efficiency of the sequential (method 1) or simultaneous (method 2) procedure is depicted as mean percentage of antibody- and growth-positive wells. IgG^+ and IgM^+ immortalized B cell lines are shown with restimulation using CpG2006, IL-2 or both. Results are the average of experiments on 2 healthy donors. (b) Clonality of 34 randomly selected B cell lines from both donors is shown. *** $p<0.001$; ** $0.001<p<0.01$

2.3.3 Immortalization of IgG$^+$ B cells isolated by different methods

As memory B cell responses and IgG antibodies are most often implicated in autoimmune diseases, IgG$^+$ B cells were isolated prior to immortalization to select for IgG-producing B cell lines. Isolation of memory B cells was tested by 3 different methods prior to immortalization using simultaneous B cell stimulation and infection followed by restimulation using CpG2006 and IL-2. IgG$^+$CD22$^+$ FACS sorting resulted in the highest yield, purity and immortalization efficiency when compared to magnetic selection alone or in combination with FACS sorting (Table 2.1). No IgM$^+$ immortalized B cell lines were recovered after FACS sorting, although several IgM$^+$ wells could be found after magnetic selection (data not shown). Consequently, IgG$^+$CD22$^+$ FACS sorting was proven to be the best choice for IgG$^+$ B cell isolation prior to B cell immortalization.

Table 2.1 Comparison methods for isolation of IgG$^+$ B cells

Method	Amount of cells	Yield	Purity	Immortalization efficiency[d]
sort[a]	1 x 10^7	14 x 10^3	>99%	83%
MACS[b]	1 x 10^7	10.3 x 10^3	89.4%	62.5%
MACS + sort[c]	1 x 10^7	8.6 x 10^3	97%	73%

[a] sort: IgG$^+$CD22$^+$ FACS sorting; [b] MACS: magnetic selection IgG$^+$ B cells; [c] MACS + sort: magnetic negative B cell selection followed by FACS sorting for IgG; [d] immortalization efficiency was calculated by the percentage of IgG- and growth-positive wells

2.3.4 Immortalization of IgG$^+$ B cells using sequential or simultaneous B cell stimulation and infection

Finally, method 2 was confirmed as the most optimal B cell immortalization procedure by testing the sequential and simultaneous protocol on peripheral IgG$^+$CD22$^+$ B cells. To exclude differences in immortalization efficiency due to donor or experimental variability, the same healthy donors and experimental conditions were used as in previous experiments with total PBMC.

Simultaneous B cell stimulation and infection (method 2) followed by restimulation using CpG2006 and IL-2 resulted in the highest mean immortalization efficiency (55%) (Fig. 2.5a). The addition of CpG2006 or IL-2 alone after EBV infection generated lower immortalization efficiencies of 35-

45%, although this was not significant. Moreover, method 2 was characterized by small variability between donors which emphasizes its high reproducibility. A very low immortalization efficiency was obtained using the sequential procedure on 1 of the donors, leading to high donor variation and the absence of significant differences when comparing immortalization procedures. Again, IL-2 addition during the immortalization phase showed no additional benefit on the B cell immortalization success rate for both tested methods (data not shown).

Clonality analysis of immortalized B cell lines generated by sequential and simultaneous B cell stimulation and infection pointed to a very high monoclonality rate of 87% (Fig. 2.5b). The remaining 13% of the investigated B cell lines appeared to be biclonal.

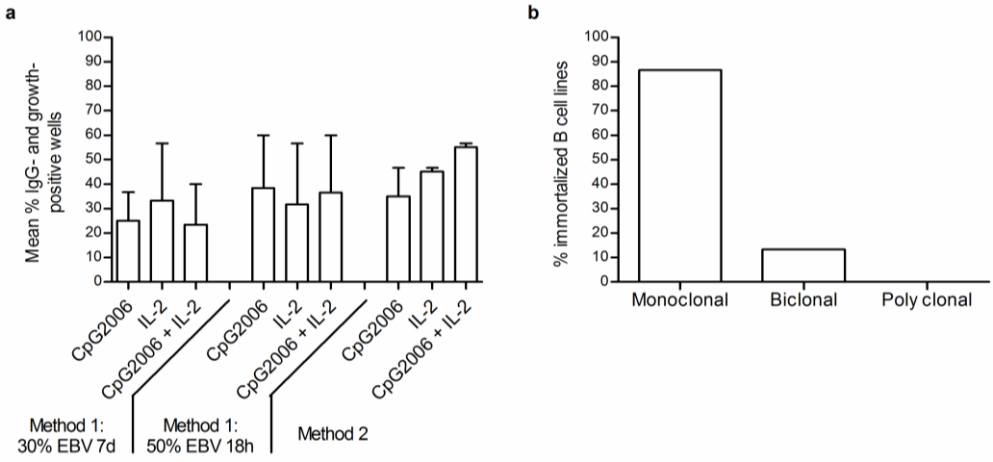


Figure 2.5 Immortalization efficiency and clonality using IgG$^+$CD22$^+$ cells. (a) Immortalization efficiency following the sequential (method 1) or simultaneous (method 2) procedure on IgG$^+$CD22$^+$ cells is shown as the percentage of IgG- and growth-positive wells with the addition of CpG2006, IL-2 or both during restimulation. Results are the average of experiments in 2 healthy donors. (b) Clonality of 36 immortalized B cell lines from both donors is depicted.

2.3.5 Improved B cell immortalization procedure

In conclusion, we developed an improved B cell immortalization procedure that was characterized by simultaneous B cell stimulation by CpG2006 and infection by EBV followed by a restimulation using CpG2006 and IL-2 (Fig. 2.7). Using

this improved immortalization procedure, immortalization efficiencies ranging from 53% to 83% could be achieved when using IgG^+ B cells of 3 healthy donors (Fig. 2.6a). On average, an immortalization efficiency of 55% was obtained using IgG^+ B cells (Fig. 2.6b). The improved procedure could also be applied on total PBMC with an efficiency of 72% for the generation of IgM^+ B cell lines and 23% for production of IgG^+ immortalized B cell lines. B cell spectratyping indicated high monoclonality rates following immortalization. About 87% of the immortalized B cells appeared to be monoclonal when starting with IgG^+CD22^+ cells, while this was about 70% for total PBMC (Fig. 2.6c).

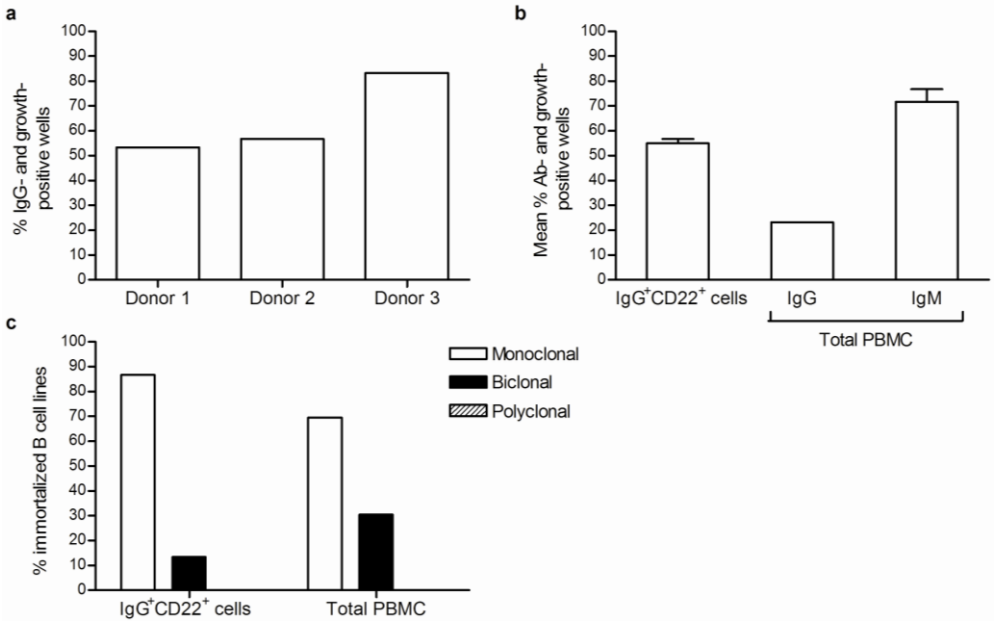


Figure 2.6 Features of the improved B cell immortalization method. (a) Immortalization efficiencies of 53-83% were obtained on IgG^+ B cells of 3 donors. (b) An average immortalization efficiency of 55% was achieved when starting with IgG^+ B cells. When using PBMC, immortalization efficiencies of 23% and 72% were obtained for the generation of IgG- and IgM-producing B cell lines, respectively (c) Monoclonality rates of 87% for IgG^+CD22^+ cells and 70% for total PBMC were measured.

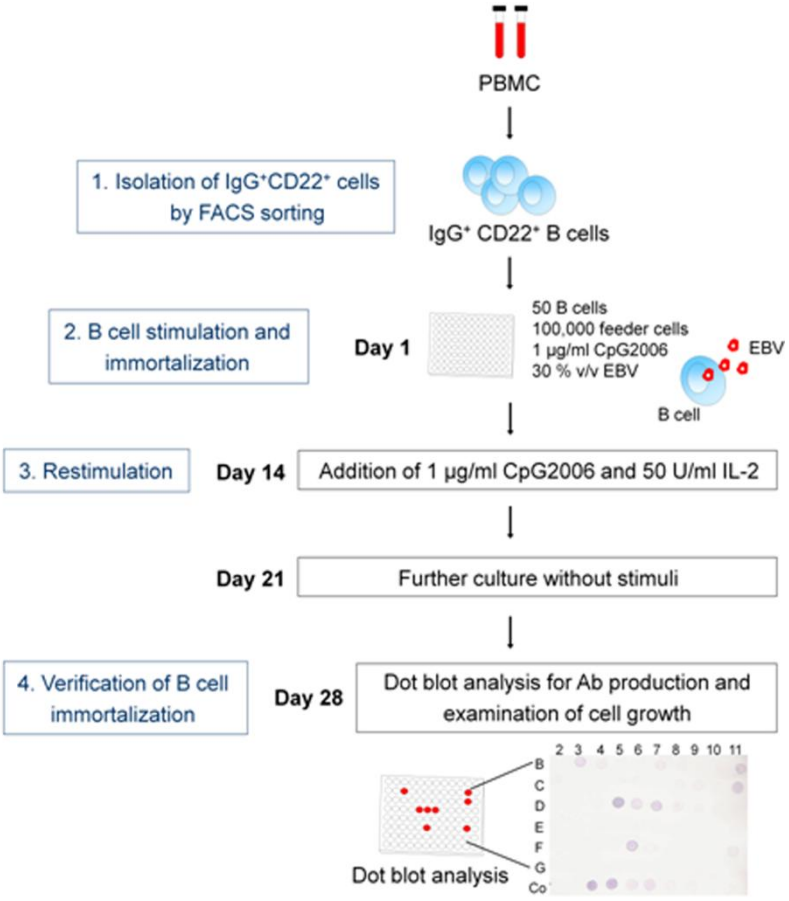


Figure 2.7 Improved B cell immortalization procedure. IgG^+CD22^+ B cells are isolated from PBMC by FACS sorting (1) and thereafter immortalized using simultaneous B cell stimulation and infection. B cells are cultured during 2 weeks in microtiter plates at 50 cells per well in the presence of 1 x 10^5 autologous irradiated feeder cells, 1 µg/ml CpG2006 and 30% v/v EBV-containing supernatant of the B95-8 cell line (2). After this immortalization phase, cells are restimulated during 7 days with 1 µg/ml CpG2006 in combination with 50 U/ml IL-2 (3). Culture medium is then replaced and cultures are continued without the addition of stimuli for 7 days. Immortalization status is verified after 28 days of culture by performing dot blot analysis for antibody production and by light microscopic examination of cell growth (4).

2.3.6 Validation of the B cell immortalization technology

B cell immortalization was validated in order to determine whether the immortalized B cells represented the in vivo B cell response. For this purpose, immortalized B cell lines were generated from peripheral B cells of 3 MS and 2 CIS patients. B cell spectratyping of the V_H region was performed using FR1-specific primers on the resulting immortalized B cell lines but also on total PBMC of these patients to compare the B cell repertoire before and after B cell immortalization.

Fragment sizes of the immortalized B cell lines are shown as red arrows in the total PBMC spectratyping profiles of each patient separately in Fig. 2.8. As expected, B cell fragment analysis of total PBMC resulted in a Gaussian-shaped curve consisting of fragments of varying size. From the 81 immortalized B cell lines of MS-1, a proportion carried identical V_H fragment sizes which suggested the use of similar V_H genes and a possibility for the presence of identical clones. When a large panel of immortalized B cell lines was generated, all fragment sizes of the PBMC repertoire were represented in the immortalized B cells (MS-1). Not all PBMC clones were recovered in the immortalized B cell lines when lower numbers of B cell lines were available. In this case, the immortalized B cell lines mainly corresponded to the most frequent PBMC fragment sizes (MS-2, MS-3, CIS-1 and CIS-2). This was reasonable since B cell clones that are highly prevalent in PBMC have a higher chance to be immortalized than less frequent clones, emphasizing the need of high immortalization efficiencies.

In conclusion, immortalized B cell lines represented the in vivo B cell response as fragment sizes of immortalized B cell lines covered most fragment sizes of the total PBMC pool. However, B cell immortalization leads to the outgrowth of a panel of in vivo occurring B cell clones that is taken at random from the whole population. Not all PBMC B cell clones can be immortalized due to restrictions in immortalization efficiency.

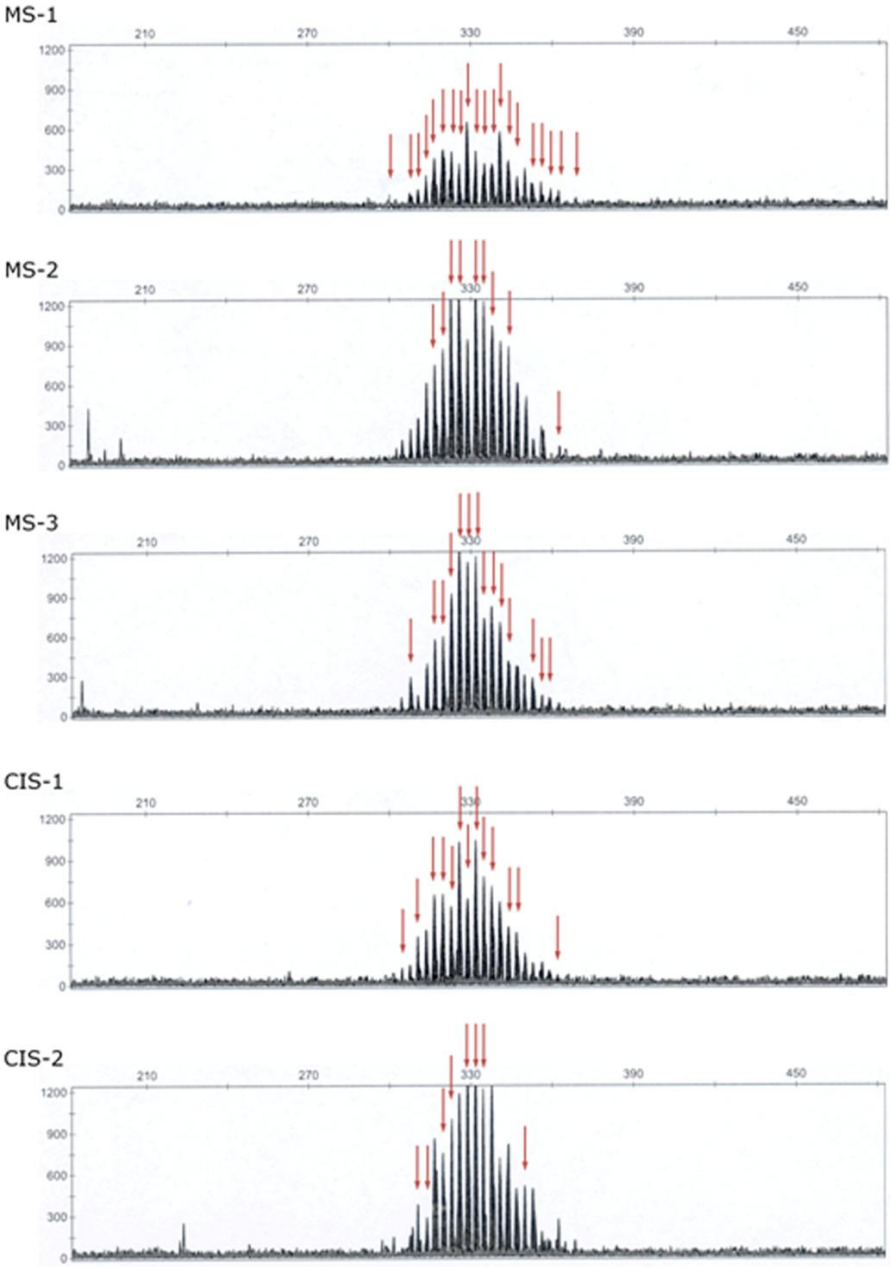


Figure 2.8 Representation of immortalized B cell lines in the total PBMC repertoire. B cell spectratyping analysis was performed on immortalized B cell lines and total PBMC from 3 MS patients and 2 CIS patients. The spectrum of PBMC fragment sizes is depicted for all 5 patients. Peaks corresponding to fragment sizes that were identified in the immortalized B cell lines are marked by red arrows.

2.4 Discussion

Numerous B cell immortalization procedures have been proposed to increase antibody production and growth rate of the transformed B cell lines. Antibodies with the capacity to neutralize SARS coronavirus, H5N1 influenza and CMV were recently generated by adapted B cell immortalization procedures[277,278,281]. Despite these successes, reproducibility remained low in our hands due to incomplete method descriptions and faint statements concerning the use of CpG2006 and IL-2. Most studies made use of large B cell numbers, which led to the generation of polyclonal immortalized B cell lines that had to be cloned out. In this study, we developed a high efficiency B cell immortalization procedure when seeding low B cell numbers.

The highest immortalization efficiency and reproducibility were obtained using simultaneous B cell stimulation and infection (method 2) both on total PBMC and isolated IgG^+ B cells, emphasizing the need of CpG2006 stimulation during B cell infection. CpG2006 was used for B cell stimulation as it was previously shown that this ligand of Toll-like receptor 9 (TLR9)[282] increases immortalization efficiency when administered during EBV infection[277] and causes polyclonal B cell activation and proliferation[283-285]. Some studies revealed that CpG2006 suppresses viral infection as it protects against several viruses[286,287] and induces the production of anti-viral cytokines and other proteins[240,288]. Concerning EBV, CpG2006 has only been shown to suppress the initiation of lytic EBV infection via inhibition of lytic gene expression[240]. Such connection, however, has never been demonstrated for latent EBV infection during B cell immortalization in vitro. Moreover, we showed a positive effect of CpG2006 on B cell activation and immortalization efficiency when CpG2006 was administered when immortalization was in progress (data not shown).

When testing the sequential and simultaneous protocol on total PBMC, no difference could be detected between the addition of CpG2006, IL-2 or both after immortalization since efficiencies reached approximately 100%. The larger proportion of IgM^+ immortalized cells was reasonable, as most B cells in the PB belong to the naïve B cell population[289]. Naïve B cells also express the EBV receptor CD21 (chapter 4) and can be activated by CpG2006 to produce large amounts of IgM[284]. Moreover, the percentages of IgM^+ and IgG^+ immortalized B

cell lines that were produced from total PBMC, namely 72% and 23%, resemble the proportion of naïve and memory B cells present in the PB[290].

Autoantibodies of the IgG isotype are mostly implicated in the pathogenesis of autoimmune diseases. For example, IgG OCB have been described in the CSF of patients with MS and are used as a diagnostic indicator[2,119]. In several autoimmune diseases, including MS, myasthenia gravis (MG)[233,291], systemic lupus erythematosus (SLE) and RA[292], IgG responses against autoantigenic targets are present. Therefore, it is interesting to focus on IgG responses when examining humoral immunity in autoimmune diseases. The enrichment of IgG^+ B cells prior to B cell immortalization creates the opportunity to select for the production of IgG^+ immortalized B cell lines. IgG^+CD22^+ FACS sorting was proven to be the best choice for IgG^+ B cell selection because of the resulting high yield, purity and immortalization efficiency.

The sequential protocol (method 1) was more subject to variation in immortalization efficiency than the simultaneous protocol (method 2) when tested on IgG^+ B cells. The reason for this lower reproducibility is not known, although isolated IgG^+ B cells are likely to react differently in culture than total PBMC due to stress factors induced during FACS sorting and the presence of fluorescently labelled antibodies on their surface, at least in the beginning of the culture.

In contrast to the increase that has been described in CpG2006 elicited B cell activation[293] or immortalization[294] by IL-2, we could not detect an amplification of B cell immortalization when IL-2 was administered during the immortalization phase (data not shown). Since the IL-2 receptor is mainly present on activated B cells[295], it is probable that IL-2 exerts a stimulating effect on B cell proliferation and growth rather than on B cell activation. This is also reflected in the higher immortalization efficiency when CpG2006 and IL-2 were combined for restimulation in comparison with the addition of CpG2006 or IL-2 alone, although the difference was not statistically significant. Addition of CpG2006 increases the effect of IL-2 given that TLR9 expression is elevated after EBV infection and CpG2006 stimulation[296], rendering the immortalized cells even more responsive to CpG2006.

A monoclonality rate of 87% was measured for the immortalized B cell lines that were generated from isolated IgG^+ B cells. The preferential generation of monoclonal immortalized populations can be explained by the immortalization

process itself. EBV infected B cells undergo a proliferative crisis in which most of the cells die because of apoptosis. A small proportion of surviving B cells become activated and eventually transform into lymphoblastoid immortalized cell lines[256]. Consequently, the outgrowth of multiple immortalized B cell lines is rare when starting with low B cell numbers per well. The high monoclonality rate provides a major advantage for our B cell immortalization method in combination with the high immortalization efficiency. Subcloning using limiting dilution is no longer necessary, which considerably reduces the time span in which monoclonal antibodies can be produced.
B cell immortalization has proved to be a valuable method to study humoral immune responses as the immortalized B cell lines represented the repertoire in vivo, based on B cell spectratyping. The most frequent B cells were most likely to become immortalized so that in vivo clonal expansions should readily be recovered in the immortalized B cell lines. However, PBMC fragment sizes usually represent multiple B cell clones with V_H fragment sizes in the same range. Representation of specific PBMC B cell clones in the immortalized B cell repertoire could only be studied by sequencing approaches and single-cell sorting.

In summary, a reproducible B cell immortalization method was developed with an efficiency of up to 83% for fast IgG production from isolated $CD22^+IgG^+$ PB B cells, by simultaneous B cell stimulation using CpG2006 and B cell infection by EBV followed by an additional stimulus of CpG2006 and IL-2. B cell spectratyping demonstrated monoclonality in almost 90% of the immortalized B cell lines originating from isolated IgG^+ B cells. This unique combination of a high immortalization efficiency and monoclonality rate eliminates the need for cloning of the immortalized cells. For the first time, a B cell immortalization procedure is introduced with high efficiency and reproducibility for low B cell numbers that makes immediate generation of monoclonal antibodies, representing the in vivo humoral immune response, possible.

3

Molecular analysis of B cell diversity and clonal expansion in MS and CIS patients

Based on:

Clonally expanded B cells in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis and clinically isolated syndrome.

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Submitted

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ABSTRACT

Molecular analysis of the immunoglobulin (Ig) heavy chain variable region (V_H) of B cells from clinically isolated syndrome (CIS) and multiple sclerosis (MS) patients has previously demonstrated restricted clonally expanded B cells in cerebrospinal fluid (CSF) but not in peripheral blood (PB). We aimed at further characterizing diversity and clonal expansion of B cells from MS patients, CIS patients and healthy controls (HC) following B cell immortalization.

Sequencing analysis of the Ig V_H region was performed on genomic DNA of 284 immortalized B cell lines from 11 MS patients, 4 CIS patients and 2 HC. More specifically, 206 B cell lines were studied from PB of 10 MS patients, 6 from CSF of 1 MS patient, 32 from PB of 4 CIS patients and 40 from PB of 2 HC. Analyzed parameters included V_H family gene distribution, mutation frequency, clonal expansion and features of complementarity-determining region 3 (CDR3).

Clonally expanded B cell populations were demonstrated in CSF and PB of MS patients and in PB of CIS patients. Average Ig V_H mutation frequency was significantly higher for peripheral B cells from MS patients (12.5%) when compared to CIS patients (8.7%) and HC (5.6%). CIS patients also displayed significantly higher mutation frequencies in their PB B cells than HC. These findings indicate a restricted antigen-stimulated clonally expanded B cell response with possible affinity maturation not only in CSF but also in PB of MS and CIS patients. Moreover, MS and CIS PB B cells showed expression of long and negatively charged CDR3, which could point towards autoreactivity and an association with the disease process.

Together, these data indicate a role for possible autoreactive peripheral B cell responses in MS and CIS patients. Identification of the target antigens of these clonally expanded B cells could provide more insight into their relevance in the disease process but could also lead to the identification of novel antigenic targets in MS.

3.1 Introduction

An antibody molecule consists of 2 heavy (H) and 2 light (L) chains, each comprising a variable region for antigen recognition and a constant region encoding effector functions. The variable region of the H chain is shaped by the combination of a variable (V), diversity (D) and J segment. Each V region is further subdivided into 3 hypervariable CDR and 4 conserved FR. The centre of the antigen-binding site is made up of CDR3, the most diverse of all domains.

Functional antibodies are formed during B cell development by the aggregation of several germline VDJ gene segments[297,298]. Substantial diversity is generated in the antibody repertoire by the combination of different VDJ segments but also by means of imprecise joining, addition of P and N nucleotides, somatic mutation and exonuclease activity[299,300]. Upon antigenic stimulation in the presence of T cell help, relevant B cell clones are positively selected and undergo hypermutation of the BCR V genes in order to select B cells expressing a receptor with increased affinity for the stimulating antigen. This affinity maturation occurs in GC of the lymphoid organs[300], although this location is not a prerequisite[301-303]. B cells that have evolved from a single precursor cell are characterized by identical V gene rearrangements, although the number and distribution of point mutations can vary.

Analysis of BCR genes allows the study of B cell responses and the detection of abnormalities in the generation of antibodies. Production of high-affinity autoreactive antibodies in autoimmune diseases could be elicited by a skewed usage of certain genes or gene families, the preferential usage of particular H and L chain combinations, defective receptor editing or aberrations in types and frequencies of point mutations[299]. Antigen-stimulated clonal expansion of B cells can be evidenced by a high prevalence of identical CDR3 sequences in combination with high numbers of point mutations[127]. Moreover, the effects of positive and negative antigen selection on B cell responses and the association between the occurrence of certain mutations and antibody pathogenicity can be evaluated[304,305].

In the last decade, a lot of studies have been performed to analyze the Ig V_H locus of B cells from MS patients, usually following single-cell sorting. B cells from the CSF and brain of MS patients were characterized by a chronic antigen-driven B cell response, as evidenced by the restricted usage of VDJ gene

segments[126,127,306-310]. These clonally expanded B cells sometimes experienced intraclonal diversification[127,149,306,311]. Furthermore, skewed V_H family gene usage with an overrepresentation of VH1, VH3 and especially VH4 family members has been pointed out[126,127,306-308,310,311]. Several studies demonstrated increased mutation numbers in Ig V_H genes of B cells from CSF and plaques of MS patients, suggesting ongoing affinity maturation[308,311-313]. Similar features were reported for CSF B cells from CIS patients[310,314] but also for B cells or antibodies in other autoimmune diseases, such as antibodies directed against the acetylcholine receptor (AChR) in experimental autoimmune myasthenia gravis (EAMG)[315]. Indications for intact receptor editing were found in MS CSF B cells[308,311,312]. Some studies showed GC B cell selection through mutation targeting to RGYW/WRCY motifs in the CDR[310,312], although this could not be confirmed by others[311]. The role of peripheral B cell responses in MS and CIS has remained unclear up to now. Some studies that examined Ig V_H sequences of peripheral B cells could not detect clonal B cell populations in PB and were unable to discriminate between peripheral B cell responses in healthy controls (HC) and MS or CIS patients[127,314,316].

The aim of this study was to use sequencing analysis of the BCR V_H region in order to examine the diversity of immortalized B cell lines that were generated from the PB of MS patients, CIS patients and HC, as well as from the CSF of 1 MS patient. We intended to characterize the B cell repertoire in the PB of MS and CIS patients in more detail to evaluate the extent of clonal expansion. Molecular characterization of B cells in CIS patients could further contribute to the gathering of knowledge on B cell responses in the beginning of disease.

3.2 Materials and methods

3.2.1 Study population

PB and CSF were obtained with informed consent from MS and CIS patients, that were diagnosed based on the McDonald criteria[11]. A total of 206 Ig V_H sequences were analyzed from PB of 10 MS patients with an average age of 42.8 ± 9.9 years, a mean disease duration of 9.4 ± 8.5 years and a median EDSS of 2.5 ranging from 1.5 to 6 (Table 3.1). Further, 6 Ig V_H sequences were examined from CSF of 1 female RR-MS patient (Table 3.2). Another 32 sequences from PB B cells of 4 CIS patients were included (Table 3.3). This CIS group had an average age of 36.3 ± 14.5 years with 0.5-12 year disease duration and a median EDSS of 1.3. As a control, 40 immortalized B cell lines from PB of 2 HC were included, 1 27 year-old male and 1 62 year-old female.

Table 3.1 Characteristics of MS patients

Subject	Sex	Age	MS type[a]	Disease duration[b]	Treatment[c]	EDSS[d]	OCB[e]	Analyzed sequences
MS-1	F	27	RR	2	untreated	6.0	10(2)	7
MS-2	M	27	RR	3	untreated	2.0	18(2)	2
MS-3	F	42	RR	17	untreated	3.0	8(0)	7
MS-4	F	49	PP	2	untreated	2.0	0(NA)	9
MS-5	F	47	RR	0.5	untreated	1.5	8(0)	11
MS-6	F	60	SP	23	untreated	3.0	NA	4
MS-7	F	43	RR	18	untreated	3.0	NA	18
MS-8	F	46	RR	4	IFN-β	3.5	NA	18
MS-9	F	40	RR	6	IFN-β	1.5	NA	65
MS-10	F	47	RR	0.5	untreated	2.0	15(0)	65

[a] RR: relapsing-remitting, PP: primary progressive, SP: secondary progressive; [b] in years; [c] at sampling, IFN-β: interferon-beta; [d] expanded disability status scale; [d] oligoclonal bands in CSF, (): oligoclonal bands in serum, NA: not available

Table 3.2 Characteristics of MS CSF

Subject	Sex	Age	MS type[a]	Disease duration[b]	Treatment[c]	EDSS[d]	OCB[e]	Analyzed sequences
MS-CSF	F	43	RR	4	untreated	1.5	>10	6

[a] RR: relapsing-remitting MS; [b] in years; [c] at sampling; [d] expanded disability status scale; [e] oligoclonal bands in CSF

Table 3.3 Characteristics of CIS patients

Subject	Sex	Age	Disease duration[a]	Treatment[b]	EDSS[c]	OCB[d]	Analyzed sequences
CIS-1	F	40	1	untreated	2.0	0(NA)	3
CIS-2	F	36	12	IFN-β	0.0	0(NA)	3
CIS-3	F	52	NA	untreated	NA	0(NA)	6
CIS-4	M	17	0.5	untreated	2.0	0(NA)	20

[a] in years; [b] at sampling, IFN-β: interferon-beta; [c] expanded disability status scale; [d] oligoclonal bands in CSF, (): oligoclonal bands in serum; NA: not available

3.2.2 Sequencing analysis Ig V_H region

gDNA was first isolated from 0.5 – 1 x 10^6 immortalized B cells as described before[317]. Concentrations of gDNA were determined using a SmartLadder (Eurogentec, Seraing, Belgium) on a 1% agarose gel and were then adjusted to 100 ng/µl.

The Ig V_H region was amplified from 100 ng gDNA using a reverse consensus primer directed against the conserved J region in combination with a mixture of forward primers targeting the different V_H families of FR1. Reaction mixtures consisted of 1 x PCR buffer, 0.25 mM dNTP, 1 U Taq polymerase (Roche Diagnostics, Brussels, Belgium) and 10 pmol of all primers (Eurogentec, Seraing, Belgium). PCR conditions were 7 min at 95°C, 35 cycles of 95°C for 45s, 60°C for 45s and 72°C for 90s and a final elongation step of 10 min at 72°C. Primer sequences were designed and tested during the BIOMED-2 Concerted Action as described by Van Dongen *et al.*[280] and are shown in Table 3.4. PCR products were separated on 2% agarose gels to verify successful amplification.

The reverse sequence of the purified PCR products (ExoSAP-IT, Affymetrix, Ohio, USA) was determined with the reverse J primer and Big Dye TMT Terminator Cycle Sequence Ready Reaction Kit II (Applied Biosystems, Warrington, United

Kingdom). The PCR program contained 25 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4 min. PCR products were subsequently purified using sephadex, followed by analysis on ABI Prism 310 Genetic Analyser (Applied Biosystems). V_H family usage was determined by comparing the nucleotide sequences to GenBank databases with the Ig basic local alignment search tool of NCBI (IgBLAST, http://www.ncbi.nlm.nih.gov/igblast/)[318] and the DNAPLOT tool of the MRC Centre for Protein Engineering (VBase, http://vbase.mrc-cpe.cam.ac.uk)[319]. The Ig V_H region was subsequently sequenced in the forward direction by means of a V_H family specific forward primer using the same procedure. Forward and reverse sequences were aligned using the multiple sequence alignment program of EBI (ClustalW2, http://www.ebi.ac.uk/Tools/clustalw2/index.html) in order to establish the complete VDJ region sequence.

3.2.3 Ig VDJ recombination analysis

Analysis of the VDJ region of the immortalized B cell lines was accomplished using JOINSOLVER® software (http://joinsolver.niaid.nih.gov)[320]. Using this online program, alignment of the Ig V_H sequences to germline V gene segments, mutation analysis and CDR3 analysis were performed.
Amino acid (AA) charge and composition of the CDR3 region were determined using DNAMAN software Version 4.15. Phylogenetic trees were made of AA CDR3 sequences from the different patient groups and individual patients with the help of Bosque software[321].

3.2.4 Statistical methods

All statistical analyses were performed using Prism software version 4.00 (Graphpad). Usage of particular V, J and D families, as well as CDR3 length and charge, was compared between patient groups using Fisher's exact test. For all other analyses, Mann-Whitney *t*-test or Student's *t*-test were performed. A *p* value of < 0.05 was considered statistically significant.

Table 3.4 Primer sequences for Ig V_H region sequencing analysis

Target region[a]	Gene family[b]	Primer sequence
FR1	VH1	5′ GGCCTCAGTGAAGGTCTCCTGCAAG 3′
	VH2	5′ GTCTGGTCCTACGCTGGTGAAACCC 3′
	VH3	5′ CTGGGGGGTCCCTGAGACTCTCCTG 3′
	VH4	5′ CTTCGGAGACCCTGTCCCTCACCTG 3′
	VH5	5′ CGGGGAGTCTCTGAAGATCTCCTGT 3′
	VH6	5′ TCGCAGACCCTCTCACTCACCTGTG 3′
FR2	VH1	5′ CTGGGTGCGACAGGCCCCTGGACAA 3′
	VH2	5′ TGGATCCGTCAGCCCCCAGGGAAGG 3′
	VH3	5′ GGTCCGCCAGGCTCCAGGAA 3′
	VH4	5′ TGGATCCGCCAGCCCCCAGGGAAGG 3′
	VH5	5′ GGGTGCGCCAGATGCCCGGGAAAGG 3′
	VH6	5′ TGGATCAGGCAGTCCCCATCGAGAG 3′
	VH7	5′ TTGGGTGCGACAGGCCCCTGGACAA 3′
FR3	VH1	5′ TGGAGCTGAGCAGCCTGAGATCTGA 3′
	VH2	5′ CAATGACCAACATGGACCCTGTGGA 3′
	VH3	5′ TCTGCAAATGAACAGCCTGAGAGCC 3′
	VH4	5′ GAGCTCTGTGACCGCCGCGGACACG 3′
	VH5	5′ CAGCACCGCCTACCTGCAGTGGAGC 3′
	VH6	5′ GTTCTCCCTGCAGCTGAACTCTGTG 3′
	VH7	5′ CAGCACGGCATATCTGCAGATCAG 3′
J	/	5′ CTTACCTGAGGAGACGGTGACC 3′

[a] FR: framework region, J: joining region; [b] VH: variable region Ig heavy chain

3.3 Results

The Ig V_H region was studied on immortalized B cell lines from 11 MS patients, 4 CIS patients and 2 HC. Molecular analysis of these BCR genes could give more insight into B cell biology and diversity in health and disease. B cell immortalization was performed using simultaneous or sequential B cell stimulation and infection. Low numbers of sequences were obtained for some patients by the use of a B cell immortalization procedure that was not fully optimized yet (Tables 3.1 – 3.3). Sequencing of the Ig V_H region was successfully performed on 328/431 immortalized B cell lines (76.1%). This success rate is in line with the monoclonality rate of the immortalized B cells (79.6%, data not shown). Furthermore, V_H sequences containing errors were excluded from further analysis. Unproductive rearrangements were manifested for some Ig V_H sequences by an out-of-frame J region or occasionally a stop codon in the V_H region and could be due to amplification of the unproductively rearranged allele. In total, 284 immortalized B cell lines expressed productive rearrangements, including 206 B cell lines from the PB of 10 MS patients, 6 from the CSF of 1 MS patient, 32 from the PB of 4 CIS patients and 40 from the PB of 2 HC. We confined further VDJ region analysis to sequences with productive rearrangements since these give rise to functional antibodies.

3.3.1 Skewed VH family gene usage in a subgroup of MS patients

VH family gene usage patterns approximated the germline distribution (expected frequency, VBase Centre for Protein Engineering) in all study groups (Fig. 3.1a). Peripheral B cells from HC showed a VH family distribution of VH3 > VH4 > VH1 > VH5 > VH2. VH usage patterns were VH3 > VH1 > VH4 > VH5 = VH2 for PB B cells of MS patients and VH3 > VH1 > VH4 = VH2 > VH5 of CIS patients. The largest VH gene family, VH3, was also the most frequently expressed in PB B cells of all patient cohorts. An overrepresentation of VH1 family members was evidenced in peripheral B cells of MS patients when compared to HC ($p = 0.03$) that could be attributed to skewed usage towards VH1 in 2 out of 10 MS patients (Fig. 3.1b, MS-7 and MS-9). VH family distributions similar to HC PB were evident from the other MS patients, as demonstrated for 2 representative MS

patients (Fig. 3.1b, MS-8 and MS-10). The longer disease duration of MS-7 and MS-9 (18 and 6 years, respectively) in comparison to MS-8 and MS-10 (4 and 0.5 years, respectively) could contribute to more extensive B cell expansion with restricted VH gene usage following chronic antigen stimulation (Table 3.1). The majority of MS CSF Ig sequences (4/6) expressed VH4 family genes, which confirmed previous data on CNS B cells in MS[307,316].

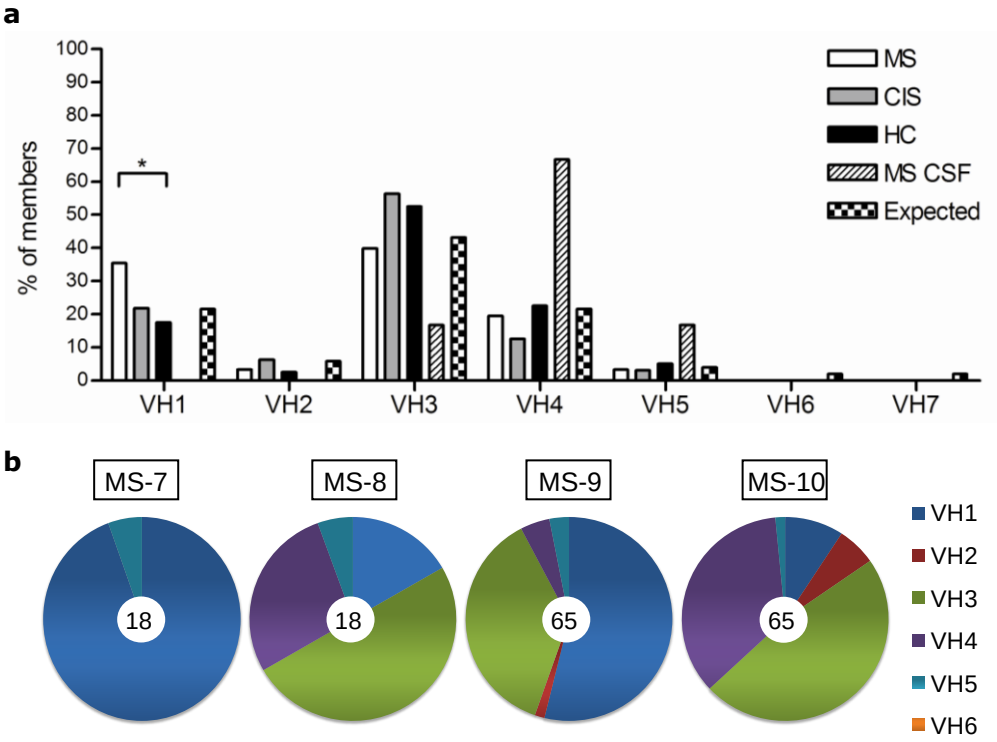

Figure 3.1 Heavy chain V gene usage in MS, CIS and HC. (a) Distribution of VH families in immortalized B cells from PB of MS patients, CIS patients and HC, as well as from CSF of 1 MS patient. Expected values indicate the number of functional germline genes. Comparison between patient groups was done using Fisher's Exact test. * $p<0.05$ (b) VH family gene usage is depicted for PB B cells of 4 representative MS patients (MS-7, MS-8, MS-9 and MS-10). Central oval "n" indicates the number of productive sequences examined.

Segment VH4-39 was previously reported to be the most frequently expressed VH4 gene in MS brain[126,307]. Therefore, we examined the presence of VH4-39 gene segments in our study population (Fig. 3.2). VH4-39 expression was

significantly higher in MS CSF when compared to MS PB (p = 0.0002), CIS (p = 0.0003) and HC (p = 0.0012), although only 6 MS CSF sequences were included. Moreover, VH4-39 expression was found in peripheral B cells from MS and CIS patients but not from HC, indicating a possible association of VH4-39 gene usage with disease or autoimmunity.

Additionally, we analyzed DH and JH family gene usage. In all patient groups, JH4, DH3 and DH5 were the most frequently used DH and JH gene families (data not shown), which was similar to germline expression and previous reports[126,322,323].

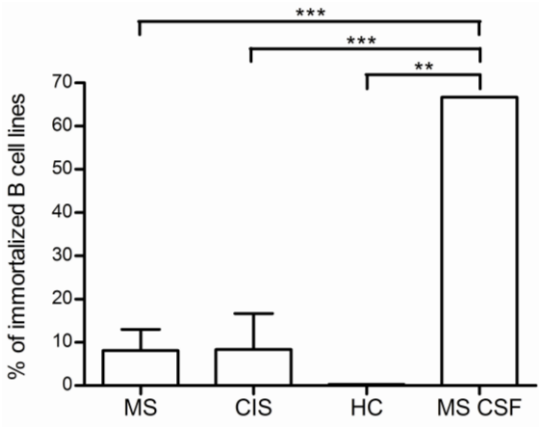

Figure 3.2 Expression of VH4-39 germline gene segments. The percentage of immortalized B cell lines expressing the VH4-39 germline segment is depicted for MS patients, CIS patients and HC. Statistical analysis was performed using Mann-Whitney *t*-test. ** 0.001<p<0.01; *** p<0.001

3.3.2 Peripheral and intrathecal B cells from MS patients are clonally expanded

Ig V_H CDR3 amino acid (AA) sequences were compared in and between patient populations to unravel clonal expansion of B cells. The identification of several immortalized B cell lines expressing identical CDR3 AA sequences indicated clonally related B cells that probably recognize the same antigenic target (Table 3.5). A total of 10 B cell clonal expansions was retrieved, including 1 from MS CSF and 9 from PB of MS (Fig. 3.5) and CIS (Fig. 3.4) patients. B cells from PB of HC showed a very diverse repertoire that lacked identical clones (Fig. 3.3).

Clonally restricted B cells have previously been reported once in PB of MS patients by expression of homogeneous levels of surface Ig belonging to one light chain class[324]. However, B cell clonal expansions could not be identified in MS PB in recent reports using molecular analysis of the Ig V_H region[127,311].

All identified clonally expanded B cell populations showed expression of VH1, VH3 or VH4 family genes, as described before in MS CSF and brain[126,127,307]. The largest clonally expanded PB B cell population comprised 49 related B cells, 1 of 1 CIS patient and 48 of 2 MS patients (MS-7 and MS-9, clone 1). All clone members expressed VH1-69, clarifying the overrepresentation of VH1 family genes in MS-7 and MS-9 (Fig. 3.1b). VH1-69 has furthermore been described to be increased in B cells from MS white matter lesions[126]. The clonal expansion that was detected in MS CSF consisted of 2 clonally related B cell lines. VH4-39 gene usage was an extra indication for their autoreactivity and involvement in the disease process. Several B cell clones (No. 1, 2, 3 and 5) were found in PB of multiple MS and CIS patients, emphasizing their possible relevance in the disease process. Furthermore, B cell clones 5 and 7 were retrieved exclusively from CIS patients and could therefore be implicated very early in pathogenesis. All expanded B cell clones from MS patients were derived from patients with the RR-MS type. However, only 1 PP-MS an 1 SP-MS patient were included in the study. Based on these results, peripheral B cell clonal expansion could not be associated with disease subtype.

Members of expanded B cell clones were characterized by high deviation from germline sequences, indicating high mutation frequencies (supplemental Table S1.1). Moreover, differences in homology to the closest germline V, D and J segment pointed towards differences in frequency and location of Ig V_H mutations. High mutation frequencies that differ between members of the same clonal population are indications for intraclonal diversification and affinity maturation.

In conclusion, we evidenced antigen-stimulated clonal expansion of B cells with indications for ongoing affinity maturation in PB of CIS patients and in CSF and PB of MS patients.

Table 3.5 Overview clonally expanded B cell populations

Clone number	Frequency	Patients	CDR3 AA sequence	VH gene	DH gene	JH gene
Peripheral blood						
Clone 1	49 x	2 MS (33x;16x); 1 CIS (1x)	AGLFAYSYGPLDY	V1-69*01	D5-5*01	J4*02
Clone 2	8 x	1 MS (7x); 1 CIS (1x)	ARDFFGSGSYHHPGGMDV	V3-13*01	D3-10*01	J6*02
Clone 3	5 x	2 MS (4x;1x)	GRAQLSLGAIDY	V3-33*01	D3-10*02	J4*02
Clone 4	4 x	1 MS	AREFPSGSYYGLGY	V3-7*01	D1-26*01	J4*02
Clone 5	3 x	2 CIS (2x;1x)	TSDHILDTAK	V3-15*01	D5-5*01	J4*02
Clone 6	2 x	1 MS	AKDIGPYYYDSSGYPDS	V3-9*01	D3-22*01	J4*02
Clone 7	2 x	1 CIS	ARVILDGYNNDDGFDV	V1-69*02	D5-24*01	J3*01
Clone 8	2 x	2 MS (1x;1x)	ARLRGYAETRWFDI	V4-39*04	D3-16*01	J5*02
Clone 9	2 x	1 MS	AKDIGVTSLYFFYGLDV	V3-30*18	NA	J6*02
Cerebrospinal fluid						
Clone 10	2 x	1 MS CSF	VSSYSSGWFG	V4-39*01	D6-19*01	J4*02

NA: not available; V, D and J genes that are expressed by the Ig heavy chain are shown for each clonal B cell population

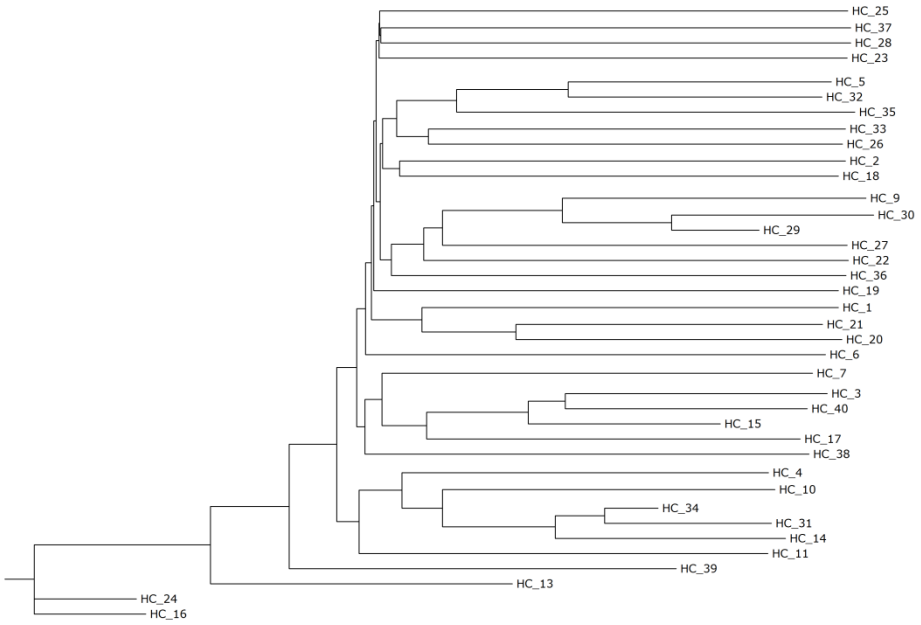

Figure 3.3 Phylogenetic tree CDR3 AA sequences HC. B cells from the PB of HC make up a very diverse polyclonal population, showing no identical CDR3 sequences.

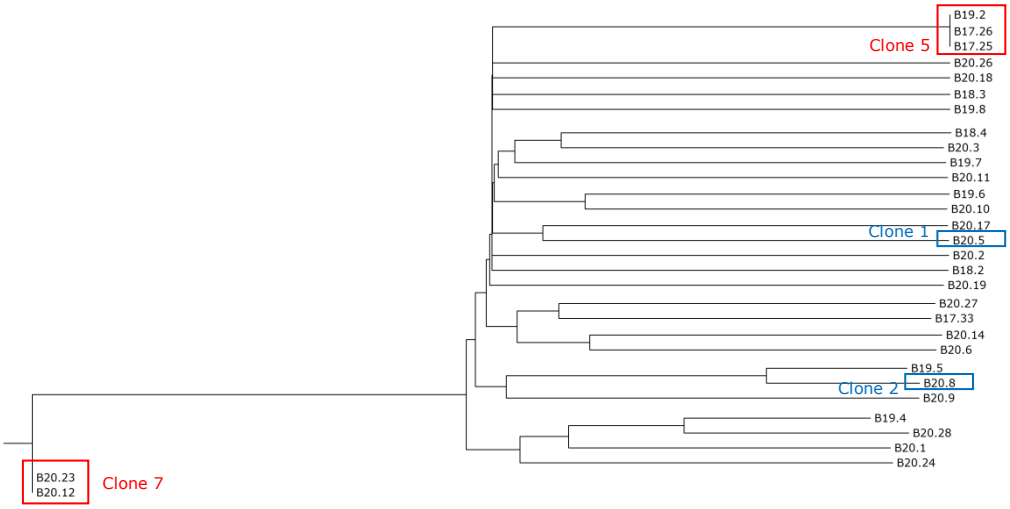

Figure 3.4 Phylogenetic tree CDR3 AA sequences CIS. Some clonal populations were identified in the PB of 4 CIS patients, of which 2 appeared to be specific to CIS (clone 5 and 7). Some other B cell clones were part of B cell clonal expansions that could be found in MS PB (clone 1 and 2).

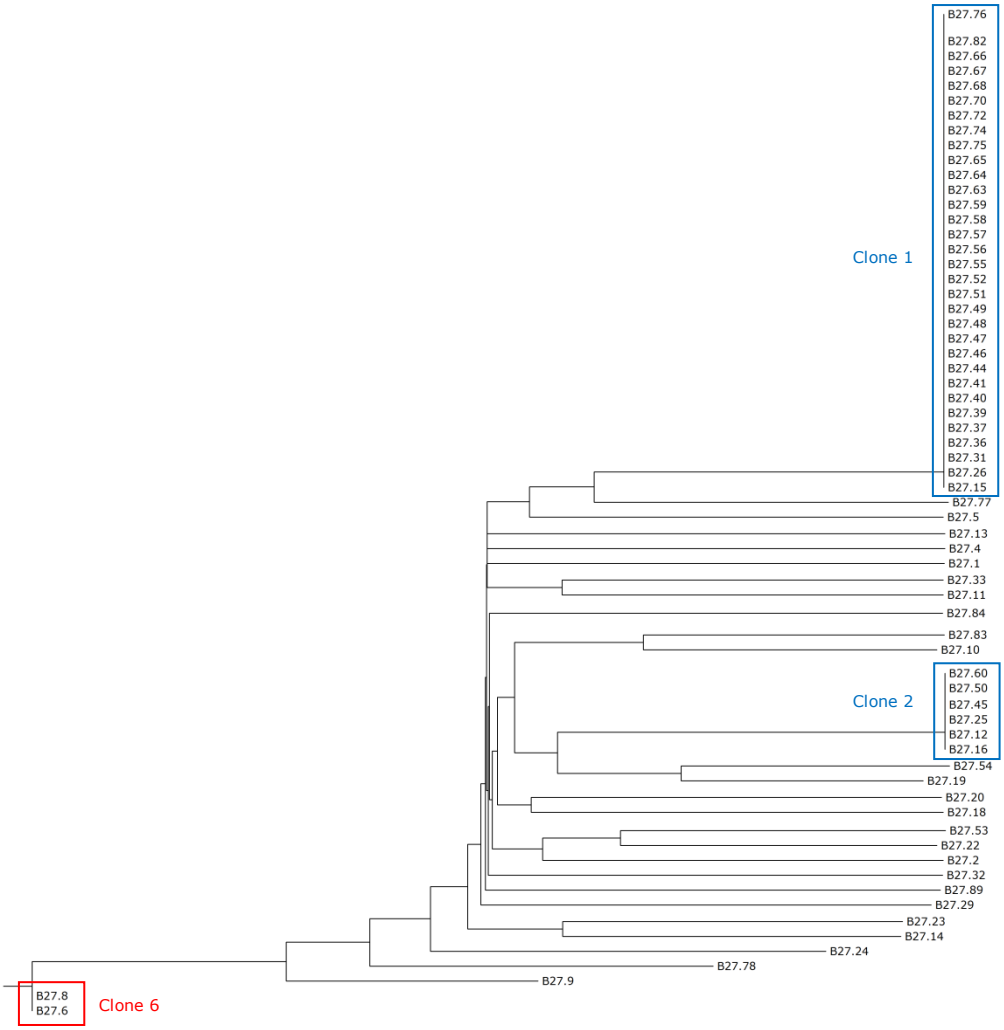

Figure 3.5 Phylogenetic tree CDR3 AA sequences MS-9. The peripheral B cell repertoire of MS patients is more restricted when compared to HC. Several clonal expansions in a representative MS patient are demonstrated. Clone 6 was only found in this MS patient, while clone 1 and 2 were also found in other patients.

3.3.3 Peripheral B cells from MS and CIS patients display high mutation frequencies in their Ig V_H genes

After having obtained indications for intraclonal diversification in peripheral clonally expanded B cell populations of MS and CIS patients, we decided to look further into the Ig V_H mutation frequencies.

Here we found that immortalized B cell lines from PB of MS patients displayed significantly higher mutation numbers (Fig. 3.6a) and mutation frequencies (Fig. 3.6b) when compared to CIS patients ($p = 0.02$ and $p = 0.04$, respectively) and HC ($p < 0.0001$ for both parameters). Further, Ig V_H sequences from CIS patients displayed significantly higher mutation frequencies than those from HC PB ($p = 0.03$). Average Ig V_H mutation frequency of peripheral B cells was 12.5 ± 9.3 for MS patients, 8.7 ± 7.1 for CIS patients and 5.6 ± 3.3 for HC. The percentage of Ig V_H mutations in MS CSF B cells was on average 11.4 ± 9.5 (data not shown), which approximates MS PB mutations. Increased Ig V_H mutations in peripheral B cells were also observed at patient level in MS and CIS patients (data not shown). Similar observations were done when analyzing the number of transitions (interchanges of purines or pyrimidines) and transversions (interchanges of purine for pyrimidine bases) in MS, CIS and HC (data not shown). These results again demonstrate an antigen-stimulated B cell response, possibly with affinity maturation, in the PB of MS and CIS patients. The discrepancy between MS and CIS patients could be explained by the earlier disease phase in CIS, limiting time that was available for the accumulation of mutations.

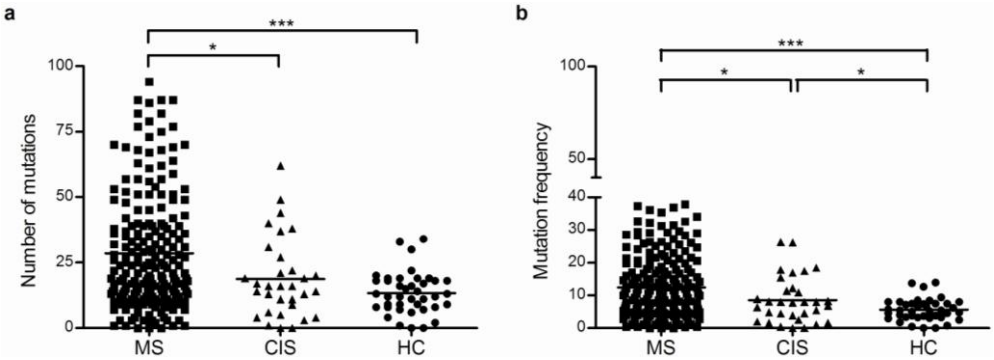

Figure 3.6 Mutation analysis Ig V_H region peripheral B cells from MS, CIS and HC. The number (a) and percentage (b) of Ig V_H mutations was determined for all immortalized B cell lines from the PB of MS patients, CIS patients and HC. Mann-Whitney or Student's *t*-test was used for statistics. * $p<0.05$; *** $p<0.001$

To our knowledge, it is the first time that increased mutation frequencies are described in peripheral B cells from MS and CIS patients following V_H region sequencing. Increased accumulation of mutations in the BCR genes is unlikely to

be caused by the B cell immortalization method, given the low mutation percentages in immortalized B cells from HC. Moreover, when comparing IgG^+ and IgM^+ immortalized B cell lines, higher percentages of mutations and Ig V_H regions containing >2% of mutations were observed the former (data not shown), indicating that the in vivo mutation mechanisms are correctly represented in the immortalized B cells.

3.3.4 Peripheral B cells from MS and CIS patients express long and negatively charged CDR3 regions

Long CDR3 regions have been detected in MS CSF B cells[312], as well as in B cells reactive to self antigens (anti-nuclear (ANA) polyreactive B cells) in SLE[325]. Therefore, it became of interest to determine if the V_H CDR3 length of peripheral B cells from MS and CIS patients was also increased. Indeed, some indications for longer CDR3 sequences in peripheral B cells of MS patients were obtained although overall, this was not significant. Decreased expression of small CDR3 sequences (<9 AA) was observed by B cells of MS patients when compared to HC ($p = 0.015$) (Fig. 3.7a). CDR3 sequences of 10-14 AA were more frequent in the MS population, but this was not significant. Further, average AA CDR3 length was increased in MS patients, although not significant (Fig. 3.7b). Average CDR3 length of HC peripheral B cells was similar to measurements in previous studies[322,326], refuting the possibility of bias introduction by B cell immortalization.

ANA polyreactive B cells from SLE patients were shown to have a high propensity towards positive CDR3, presumably to enhance binding of negatively charged DNA[325]. Moreover, B cells from MS CSF were characterized by net negative CDR3, possibly conferring an advantage to enter and reside in the CNS[312]. In our study, B cells of MS patients showed increased expression of negatively charged CDR3 sequences as well. A trend towards increased negative CDR3 was seen for MS patients in comparison to HC ($p = 0.05$) although this was not significant for CIS patients ($p = 0.34$) (Fig. 3.8). Moreover, expression of positive CDR3 was significantly decreased in MS patients versus HC ($p < 0.01$). CIS patients also displayed less positive CDR3 sequences than HC but again not significant. Neutral CDR3 sequences were equally present in all 3 patient groups.

Taken together, these data point towards a trend of B cells expressing longer and negatively charged CDR3 sequences in the PB of MS patients and in the PB of MS and CIS patients, respectively.

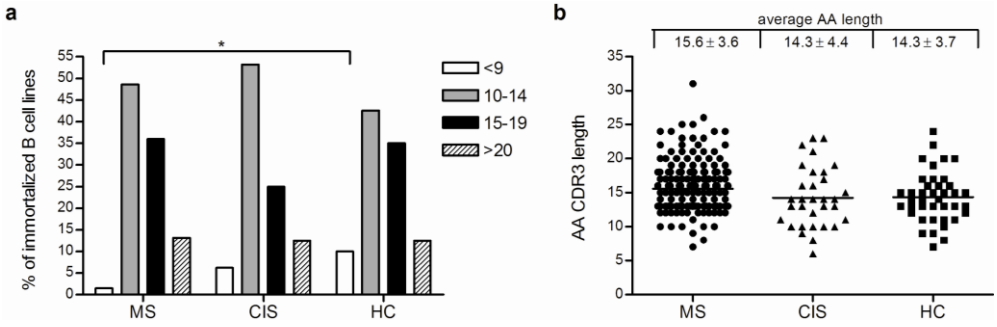

Figure 3.7 CDR3 AA length of Ig V_H sequences from MS, CIS and HC. (a) Ig V_H CDR3 lengths of B cells were separated into 4 groups of varying length for MS, CIS and HC. (b) Ig V_H CDR3 length is shown in amino acids (AA) for peripheral B cells from all groups. Average CDR3 lengths are listed above (± standard deviation). Mann-Whitney *t*-test was used for statistical analysis. * $p<0.05$

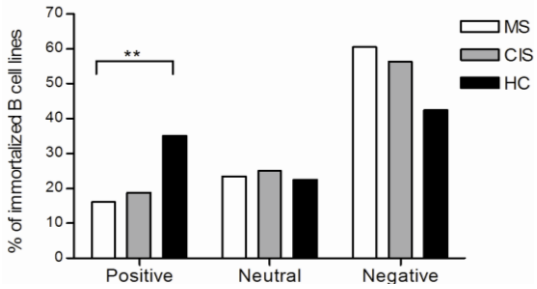

Figure 3.8 CDR3 AA charge of Ig V_H sequences from MS, CIS and HC. The percentage of immortalized B cells that expressed a positive, neutral or negative CDR3 are shown for all groups. Statistics was performed using Mann-Whitney *t*-test. ** $0.001<p<0.01$

3.4 Discussion

In this study, we demonstrated clonally expanded B cells in CSF and PB of MS patients and in PB of CIS patients. A large population of clonally related B cells even led to skewed VH1 usage in PB of 2 out of 10 MS patients. Increased Ig V_H mutation frequencies in PB of MS and CIS demonstrated diversification and affinity maturation. Moreover, peripheral B cells from MS and CIS patients showed a tendency towards somewhat longer and negatively charged CDR3 when compared to HC, suggesting an association with the disease process.

Two clonally related B cells were detected in the CSF of an MS patient, while 9 populations of clonally expanded B cells were identified in the PB of MS and CIS patients. The finding of clonally expanded B cells in the PB did not coincide with serum OCB. However, serum OCB were not routinely measured for all patients. High prevalence of irrelevant polyclonal B cells in the PB could further lead to sensitivity issues when detecting serum OCB. An increased Ig V_H mutation frequency was observed in PB of MS and CIS patients, indicating affinity maturation following chronic antigen stimulation. MS patients showed a significantly higher Ig V_H mutation frequency than CIS patients, probably owing to their more advanced disease course. All together, these data point towards a role for PB B cells in MS pathogenesis.

Whether PB clonally expanded B cells represent a primary or secondary pathological response remains unclear. The absence of shared clones between CSF and PB of MS patients could point towards diverging immune responses in these compartments. However, paired CSF and PB B cells should be analyzed in order to examine this in more detail. Several findings suggest a pathologic causative role of clonally expanded B cells in the PB. First, clonally related B cells were identified early in the disease phase (CIS). Second, peripheral B cells of MS and CIS patients but also CSF B cells of MS patients showed expression of the VH4-39 gene, that was previously associated with MS[126]. Finally, the majority of PB B cell clonal expansions (5/9) were found in multiple patients, emphasizing their relevance in disease.

Expanded PB B cells could reflect a pathologic intrathecal B cell response due to several mechanisms. It is not yet known whether initial antigen recognition in MS occurs in the CNS or circulation. Clonally expanded peripheral B cells could

represent a population of B cells that were initially triggered in the PB. These autoreactive B cells could then travel towards the CNS and become reactivated. Serafini *et al.* suggested that a dysregulated EBV infection is involved in B cell pathology in MS[73], which could also cause peripheral expansion of EBV-infected autoreactive B cells that later travel towards the CNS. On the other hand, BBB opening could lead to leakage of autoreactive B cells from the CNS into the circulation. Autoreactive memory B cells could possibly recirculate in the PB to recruit more inflammatory cells into the CNS. Autoreactive B cells could be reactivated in the periphery by brain antigens that have leaked into the circulation following tissue damage. Moreover, they could expand due to the decreased presence of regulatory cells. Another possibility is clonal expansion of peripheral B cells as the result of a secondary immune response that could be initiated by epitope spreading or leakage of brain antigens into the PB. Reactivity screening of the clonally expanded B cell populations could provide more insight into the underlying mechanisms.

The identification of clonally related B cells in MS CSF confirmed previous reports on a restricted intrathecal B cell response in MS[127,308,310]. However, to our knowledge, we are the first to demonstrate clonally expanded B cells in the PB of MS and CIS patients using molecular Ig V_H analysis. Clonally restricted B cell populations were reported once in MS PB using a cytofluorometric technique[324]. However, the absence of clonally related B cells in the PB of MS or CIS patients was described by multiple studies using molecular Ig V_H analysis[127,314,316]. Colombo *et al.* could not identify shared clones between CSF and PB[127], while Monson *et al.* could only detect a single CSF B cell clone in the PB of 1 MS patient[311]. Naïve B cells were mostly studied in these studies, since total PBMC or PB $CD19^+$ cells were analyzed[289]. However, clonally related B cells are unlikely to be found in the heterogeneous naïve B cell pool. In our study, peripheral IgG^+ memory B cells were analyzed for all patient groups, as IgG^+ B cells were selected prior to B cell immortalization. We thereby excluded the observation of differences that are inherent to different B cell subsets, while focusing on B cells with high relevance in pathogenesis.

Evidence for the involvement of peripheral B cells in MS pathogenesis comes from other studies as well. The group of Link detected MAG- and MOG-reactive B cells in MS PB[189,204], demonstrating autoreactive peripheral B cells. Moreover, some MS patients show OCB in serum as well as CSF[327], as was evidenced for

two MS patients included in this study (MS-1 and MS-2). A pathogenic role of PB antibodies was further supported by the beneficial effects of plasma exchange in a subgroup of MS patients with severe relapses and antibody deposition within inflammatory CNS lesions[328-330].

When analyzing Ig CDR3, PB B cells from MS patients showed a tendency towards somewhat longer and negatively charged CDR3, features that were previously linked to autoimmunity and/or MS[312,325]. B cells with negative CDR3 charges could have a higher propensity to bind proteins with net positive charge, such as MOG and MBP. However, the position of particular negative residues also plays a role in antigen-antibody interactions. CDR3 length and charge were not statistically different in CIS when compared to HC. The same was seen when comparing average CDR3 length between MS and HC. Non disease-related PB B cell clones could decrease significance. B cells that are irrelevant to the disease process probably contribute more to the peripheral B cell repertoire and thus exert a stronger effect on statistical significance early in disease (CIS).

Analysis of the number of replacement (R) and silent (S) mutations can give more information on B cell selection mechanisms. A higher proportion of R mutations in the CDR is a typical feature of antibodies that were affinity matured in GC following antigenic stimulation[331]. GC selection has sometimes been reported for B cells isolated from MS CSF[312], while alternative mechanisms such as ectopic GC were suggested by others[311]. In this study, different methods for analysis of R and S mutations were applied but were omitted due to inconclusive results (data not shown). Previous reports have also questioned the relevance of R/S analysis due to ambiguity of current R/S methods[332-334].

In conclusion, we have identified an antigen-stimulated clonally expanded B cell response in the PB and CSF of MS and in the PB of CIS patients. High Ig V_H mutation frequencies indicate hypermutation with a possibility for affinity maturation. A lower Ig V_H mutation frequency in B cells from CIS patients reflects the earlier phase in the disease process. Peripheral B cells from MS and CIS patients were further characterized by long and acidic CDR3 regions, which are probably associated with an autoreactive immune response.

Table S3.1 Features of Ig V_H sequences in MS and CIS clonal populations

Clone	Patient	CDR3 AA sequence	VH segment	Homology (%)	DH segment	Homology (%)	JH segment	Homology (%)
Clone 1								
B20.5	CIS-3	AGLFAYSYGPLDY	V1-69*01	93	D5-05*01	100	J4*02	97.6
B24.2	MS-7	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	100	J4*02	97.5
B24.3	MS-7	AGLFAYSYGPLDY	V1-69*01	91.7	D5-05*01	93.8	J4*02	97.6
B24.4	MS-7	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	100	J4*02	97.6
B24.6	MS-7	AGLFAYSYGPLDY	V1-69*01	91.4	D5-05*01	92.9	J4*02	97.4
B24.7	MS-7	AGLFAYSYGPLDY	V1-69*01	94.1	D5-05*01	100	J4*02	97.5
B24.8	MS-7	AGLFAYSYGPLDY	V1-69*01	92.7	D5-05*01	100	J4*02	97.6
B24.9	MS-7	AGLFAYSYGPLDY	V1-69*01	94.2	D5-05*01	93.8	J4*02	97.6
B24.10	MS-7	AGLFAYSYGPLDY	V1-69*01	92.3	D5-05*01	93.8	J4*02	97.6
B24.11	MS-7	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	100	J4*02	97.6
B24.12	MS-7	AGLFAYSYGPLDY	V1-69*01	91.9	D5-05*01	93.8	J4*02	97.6
B24.13	MS-7	AGLFAYSYGPLDY	V1-69*01	93.2	D5-05*01	100	J4*02	97.6
B24.14	MS-7	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	100	J4*02	97.6
B24.15	MS-7	AGLFAYSYGPLDY	V1-69*01	92	D5-05*01	100	J4*02	97.6
B24.16	MS-7	AGLFAYSYGPLDY	V1-69*01	92.6	D5-05*01	93.8	J4*02	97.5
B24.17	MS-7	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	100	J4*02	97.6
B24.19	MS-7	AGLFAYSYGPLDY	V1-69*01	93.5	D5-05*01	93.8	J4*02	97.3
B27.15	MS-9	AGLFAYSYGPLDY	V1-69*01	93.1	D5-05*01	93.8	J4*02	97.5
B27.26	MS-9	AGLFAYSYGPLDY	V1-69*01	89.9	D5-05*01	93.8	J4*02	97.5
B27.31	MS-9	AGLFAYSYGPLDY	V1-69*01	92	D5-05*01	94	J4*02	98
B27.36	MS-9	AGLFAYSYGPLDY	V1-69*01	87.7	D5-05*01	93.8	J4*02	97.6
B27.37	MS-9	AGLFAYSYGPLDY	V1-69*01	92.8	D5-05*01	93.8	J4*02	97.6
B27.39	MS-9	AGLFAYSYGPLDY	V1-69*01	91.8	D5-05*01	93.8	J4*02	97.6
B27.40	MS-9	AGLFAYSYGPLDY	V1-69*01	90.9	D5-05*01	93.8	J4*02	97.5
B27.41	MS-9	AGLFAYSYGPLDY	V1-69*01	93.2	D5-05*01	93.8	J4*02	97.6
B27.44	MS-9	AGLFAYSYGPLDY	V1-69*01	93.5	D5-05*01	93.8	J4*02	97.6
B27.46	MS-9	AGLFAYSYGPLDY	V1-69*01	93.3	D5-05*01	93.8	J4*02	97.6
B27.47	MS-9	AGLFAYSYGPLDY	V1-69*01	94	D5-05*01	93.8	J4*02	97.6
B27.48	MS-9	AGLFAYSYGPLDY	V1-69*01	92.6	D5-05*01	93.8	J4*02	97.6
B27.49	MS-9	AGLFAYSYGPLDY	V1-69*01	93.5	D5-05*01	93.8	J4*02	97.6
B27.51	MS-9	AGLFAYSYGPLDY	V1-69*01	92.8	D5-05*01	93.8	J4*02	97.6
B27.52	MS-9	AGLFAYSYGPLDY	V1-69*01	91.1	D5-05*01	93.8	J4*02	97.6

Table S3.1 (continued) Features of Ig V_H sequences in MS and CIS clonal populations

Clone	Patient	CDR3 AA sequence	VH segment	Homology (%)	DH segment	Homology (%)	JH segment	Homology (%)
B27.55	MS-9	AGLFAYSYGPLDY	V1-69*01	94.2	D5-05*01	93.8	J4*02	97.6
B27.56	MS-9	AGLFAYSYGPLDY	V1-69*01	92.3	D5-05*01	93.8	J4*02	97.6
B27.57	MS-9	AGLFAYSYGPLDY	V1-69*01	92	D5-05*01	93.8	J4*02	97.6
B27.58	MS-9	AGLFAYSYGPLDY	V1-69*01	93.1	D5-05*01	93.8	J4*02	97.6
B27.59	MS-9	AGLFAYSYGPLDY	V1-69*01	91.8	D5-05*01	93.8	J4*02	97.2
B27.63	MS-9	AGLFAYSYGPLDY	V1-69*01	92.7	D5-05*01	91.7	J4*02	97.6
B27.64	MS-9	AGLFAYSYGPLDY	V1-69*01	94.2	D5-05*01	93.8	J4*02	97.6
B27.65	MS-9	AGLFAYSYGPLDY	V1-69*01	94.2	D5-05*01	93.8	J4*02	97.6
B27.66	MS-9	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	93.8	J4*02	97.6
B27.67	MS-9	AGLFAYSYGPLDY	V1-69*01	93.3	D5-05*01	93.8	J4*02	97.6
B27.68	MS-9	AGLFAYSYGPLDY	V1-69*01	92.7	D5-05*01	93.8	J4*02	97.6
B27.70	MS-9	AGLFAYSYGPLDY	V1-69*01	94.2	D5-05*01	93.8	J4*02	97.6
B27.72	MS-9	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	93.8	J4*02	97.6
B27.74	MS-9	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	93.8	J4*02	97.5
B27.75	MS-9	AGLFAYSYGPLDY	V1-69*01	88.2	D5-05*01	94	J4*02	97.6
B27.76	MS-9	AGLFAYSYGPLDY	V1-69*01	93.2	D5-05*01	93.8	J4*02	97.6
B27.82	MS-9	AGLFAYSYGPLDY	V1-69*01	93.8	D5-05*01	94	J4*02	97.6
Clone 2								
B20.8	CIS-3	ARDFFGSGSYHHPGGMDV	V3-13*01	96	D3-10*01	94	J6*02	100
B27.12	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	94	D3-10*01	94	J6*02	95.6
B27.16	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	96	D3-10*01	87	J6*02	100
B27.25	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	95.3	D3-10*01	100	J6*02	100
B27.45	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	93	D3-10*01	87	J6*02	100
B27.50	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	96.4	D3-10*01	87	J6*02	100
B27.60	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	94.5	D3-10*01	87	J6*02	100
B27.62	MS-9	ARDFFGSGSYHHPGGMDV	V3-13*01	94.8	D3-10*01	82.6	J6*02	97.7
Clone 3								
B26.2	MS-8	GRAQLSLGAIDY	V3-33*01	96.1	D3-10*02	100	J4*02	97.6
B29.11	MS-10	GRAQLSLGAIDY	V3-33*01	96.1	D3-10*02	100	J4*02	97.6
B29.20	MS-10	GRAQLSLGAIDY	V3-33*01	95.3	D3-10*02	100	J4*02	97.6
B29.27	MS-10	GRAQLSLGAIDY	V3-33*01	94.3	D3-10*02	100	J4*02	97.6
B29.51	MS-10	GRAQLSLGAIDY	V3-33*01	94.5	D3-10*02	100	J4*02	97.6

Table S3.1 (continued) Features of Ig V_H sequences in MS and CIS clonal populations

Clone	Patient	CDR3 AA sequence	VH segment	Homology (%)	DH segment	Homology (%)	JH segment	Homology (%)
Clone 4								
B6.3	MS-1	AREFPSGSYYGLGY	V3-07*01	95.9	D1-26*01	100	J4*02	100
B6.6	MS-1	AREFPSGSYYGLGY	V3-07*01	96.3	D1-26*01	100	J4*02	100
B6.7	MS-1	AREFPSGSYYGLGY	V3-07*01	95.9	D1-26*01	100	J4*02	100
B6.8	MS-1	AREFPSGSYYGLGY	V3-07*01	95.1	D1-26*01	100	J4*02	100
Clone 5								
B17.25	CIS-1	TSDHILDTAK	V3-15*01	97.6	D5-05*01	100	J4*02	100
B17.26	CIS-1	TSDHILDTAK	V3-15*01	98.4	D5-05*01	100	J4*02	100
B19.2	CIS-2	TSDHILDTAK	V3-15*01	98	D5-05*01	100	J4*02	100
Clone 6								
B27.6	MS-9	AKDIGPYYYDSSGYPDS	V3-09*01	97.7	D3-22*01	100	J4*02	97.5
B27.8	MS-9	AKDIGPYYYDSSGYPDS	V3-09*01	94.3	D3-22*01	100	J4*02	97.6
Clone 7								
B5.2	MS-CSF	VSSYSSGWFG	V4-39*01	96.1	D6-19*01	93.8	J4*02	100
B5.4	MS-CSF	VSSYSSGWFG	V4-39*01	96.9	D6-19*01	93.8	J4*02	100
Clone 8								
B20.12	CIS-3	ARVILDGYNNDDGFDV	V1-69*02	93	D5-24*01	100	J3*01	95.6
B20.23	CIS-3	ARVILDGYNNDDGFDV	V1-69*02	92.2	D5-24*01	93.8	J3*01	93.8
Clone 9								
B26.1	MS-8	ARLRGYAETRWFDI	V4-39*04	92	D3-16*01	81.2	J5*02	93.6
B29.35	MS-10	ARLRGYAETRWFDI	V4-39*04	90	D3-16*01	100	J5*02	100
Clone 10								
B26.9	MS-8	AKDIGVTSLYFFYGLDV	V3-30*18	93.1	NA	NA	J6*02	91.2
B26.23	MS-8	AKDIGVTSLYFFYGLDV	V3-30*18	93	NA	NA	J6*02	97.6

Each line identifies the name of the clone member, patient number, CDR3 AA sequence, most homologous V, D and J germline segments and the degree of homology to the closest germline segment for the H chain V region amplified from that single B cell clone. NA: not available

4

Analysis B cell distribution and antibody specificity in MS and CIS

Based on:

Reactivity profile of memory B cells from peripheral blood and cerebrospinal fluid in multiple sclerosis and clinically isolated syndrome.

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ABSTRACT

B cells are key players in the pathogenesis of multiple sclerosis (MS) and clinically isolated syndrome (CIS). However, composition of the B cell repertoire in peripheral blood (PB) and cerebrospinal fluid (CSF) is not entirely clear. Further, the specificity of autoreactive B cells remains largely unknown. In this study, we analyzed B cell subset distribution in PB and CSF of MS patients. B cell specificity in CIS and MS was studied following B cell immortalization.

Phenotypic analysis showed that MS CSF consisted of plasma cells (4.4%), memory B cells (3.4%) and plasma blasts (2.3%), while naïve (6.1%) and memory (4.1%) B cells made up MS PB. Plasma cells were the main population of intrathecal antibody secreting cells, but they are not subject to immortalization because they lack the Epstein-Barr virus (EBV) receptor CD21. Memory B cells were the only B cell subset present in PB and CSF of MS patients and showed high CD21 expression.

Antibody specificity of 353 immortalized B cell lines from MS, CIS and non-inflammatory neurological disease (NIND) patients was analyzed by flow cytometry using different target cell lines. Binding to intracellular antigens was demonstrated for 24% and 22% of PB B cells from MS and CIS patients, respectively. Different antibody staining patterns suggested specificity for different antigens. Immortalized B cells from NIND PB and MS CSF were negative for antibody binding. However, cystatin-A and apoptosis-stimulating protein of p53-2 (ASPP2) were suggested as candidate antigens of 2 MS CSF B cell clones.

In conclusion, memory B cells were identified as main target population of B cell immortalization that harbours a large proportion of disease-relevant B cell clones. Immortalized memory B cells from the PB of MS and CIS patients showed specificity for intracellular autoantigens. Moreover, candidate target antigens of 2 CSF memory B cell clones were identified, although repetition experiments and confirmation are necessary.

4.1 Introduction

The underlying cause of MS is still unknown, although it is widely accepted that the acquired immune response plays a key role in development of disease[7,335]. B cells and antibodies have been clearly implicated in MS pathogenesis. The majority of disease-related B cells in the CSF and PB of MS patients have a memory $CD19^+CD27^+$ phenotype[145,146,150]. Plasma blasts ($CD19^+CD138^+$) and plasma cells ($CD19^-CD138^+$) can only be retrieved in the CSF of MS patients, since these effector B cells migrate to their target tissue for antibody production. However, it is not clear whether plasma blasts or plasma cells make up the largest intrathecal antibody-secreting B cell population[146,148,153]. A restricted, clonally expanded response of memory B cells and plasma cells has been reported in the CSF of MS patients[126,149,308]. This intrathecal B cell clonal expansion results in the production of OCB[145,146], one of the immunological hallmarks of MS[336]. Previous results demonstrated the presence of B cell clonal expansions, not only in the CSF but also in the PB of MS and CIS patients (Chapter 3).

The oligoclonal intrathecal B cell response is not specific for MS but can be found in other inflammatory CNS disorders as well, where they are always directed against the underlying infectious agent[337-339]. It is thus conceivable that the persistent IgG response in MS patients also targets disease-relevant antigens. The antigenic targets of OCB in MS remain unclear as they usually lack reactivity with commonly studied myelin antigens such as MOG and MBP[206,340]. Binding of oligoclonal IgG to EBV proteins has recently been reported[70]. However, many autoantibody reactivities that do not correspond to the major OCB have been recovered from CSF and serum of MS patients. Myelin-specific B cells and antibodies are often found in the CSF or serum of MS patients[189-193], although they can also be found in other neurological diseases and even in healthy controls[121,341-343]. Other antigenic specificities could be relevant as well, including neural protein targets, carbohydrate and lipid moieties or even protective antibodies[195,196,202,344,345]. A panel of 8 novel MS antigenic targets was identified in a recent study, of which a proportion was directed to alternative isoforms of proteins[184]. In addition, antibodies directed against a variety of pathogens have been demonstrated in the CSF of MS patients. These could be part of a polyspecific anti-viral antibody response as a bystander reaction in MS patients,

although the underlying mechanism is unclear[346-348]. The prevalence of possible disease-relevant antibodies, their association with particular MS phenotypes and their utility as biomarkers have remained difficult to establish[349]. Moreover, the use of different assays to measure autoantibodies in MS using solid-phase, soluble-phase, denaturing and non-denaturing conditions have made interpretation of results difficult[350].

Instead of focusing on autoantibody profiling, some studies have analyzed the reactivity of the clonally expanded B cells and plasma cells in the CSF and PB of MS patients. This has led to the identification of the axonal autoantigens TPI and GAPDH[207,208] and to the detection of MOG-reactive B cells in PB and CSF of MS patients[189]. In addition, myelin and intracellular filament reactivity were shown for intrathecal plasma cells from MS patients although the exact target antigens could not be identified[206].

In this study, we first characterized the B cell repertoire in PB and CSF of MS patients using phenotypic analysis. The portion of plasma cells and plasma blasts that make up the CSF repertoire of antigen-secreting cells in MS patients was analyzed. Next, we aimed at determining antibody specificity of peripheral and intrathecal B cells from the PB and CSF of MS patients and from the PB of CIS patients following B cell immortalization. We previously observed clonally expanded B cells in the PB of MS and CIS patients that were elicited by repeated antigen exposure (Chapter 3). We hypothesized that peripheral B cells in MS also displayed autoreactivity and were thus involved in the autoreactive immune response. Identification of their targets could lead to new biomarkers for diagnosis, monitoring of disease activity and/or prognosis. In addition, specific antigen-directed therapy could lead to better treatment of (subgroups of) MS patients.

4.2 Materials and methods

4.2.1 Patient population

PB and/or CSF was obtained from all patients and HC with informed consent. MS and CIS patients were diagnosed according to the McDonald criteria[11].
Distribution of B cell subsets and their CD21 expression was examined in 17 MS patients and 5 HC. The average age of the MS group was 43.8 ± 11.1 years, mean disease duration 4.8 ± 5.2 years and median EDSS 2.5 (Table 4.1). Parallel CSF and PB samples were available from 11 MS patients (No. 1-8, 12-13, 16) while PB was obtained from 6 other MS patients (No. 9-11, 14-15, 17). PB was also obtained from 5 HC that had an average age of 28 ± 6.4 years.

Table 4.1 Characteristics of MS patients used for phenotypic B cell characterization

Subject	Sex	Age	Disease duration[a]	Treatment[b]	EDSS[c]	Active disease	OCB[d]
RR-MS							
1	F	40	<0.5	corticoids	NA	yes	>1
2	F	33	3	Natalizumab	1.0	NA	5
3	M	33	<0.5	untreated	1.5	NA	NA
4	F	27	2	untreated	6.0	yes	10
5	F	51	5	untreated	2.0	no	25
6	F	43	7	untreated	2.5	no	7
7	F	34	1	untreated	2.0	NA	0
8	F	38	0.5	untreated	2.0	yes	5
9	F	39	0.5	IFN-β	2.5	yes	19
10	F	66	>5	Natalizumab	2.0	no	1
11	F	43	2	Natalizumab	2.5	NA	6
SP-MS							
12	F	35	9	GA	6.0	yes	14
13	F	58	8	IFN-β	6.0	yes	6
14	F	42	16	untreated	7.0	no	5
RP-MS							
15	M	46	1	untreated	5.0	yes	11
PP-MS							
16	M	54	7	untreated	2.5	NA	9
17	F	63	15	untreated	5.5	NA	NA

RR-MS: relapsing-remitting MS, SP-MS: secondary-progressive MS, RP-MS: relapsing-progressive MS; PP-MS: primary-progressive MS; [a] in years; [b] at sampling, IFN-β: interferon-beta; GA: glatiramer acetate; [c] expanded disability status scale; [d] oligoclonal bands in CSF; NA: not available

Immortalized B cell lines were obtained from 18 other MS patients, 4 CIS patients and 4 NIND patients. This MS population was characterized by an average age of 41.9 ± 10.3 years, a mean disease duration of 6.6 ± 7 years and a median EDSS of 2 (Table 4.2). The CIS group had an average age of 36.3 ± 14.5 years, 0.5-12 years disease duration and EDSS from 0-2 (Table 4.3), while the control group had an average age of 40 ± 5.9 years (Table 4.4). Diagnosis was not yet known for one of the included NIND patients.

Table 4.2 Characteristics of MS patients used for B cell immortalization

Subject	Sex	Age	Disease duration[a]	Treatment[b]	EDSS[c]	Active disease	OCB[d]
RR-MS							
MS-1	F	43	4	untreated	1.5	yes	>10(NA)
MS-2	F	27	2	untreated	6.0	yes	10(2)
MS-3	M	27	3	untreated	2.0	yes	18(2)
MS-4	F	42	17	untreated	3.0	no	8(0)
MS-5	F	35	10	untreated	5.0	no	0(NA)
MS-6	F	32	1	corticoids	2.0	yes	18(NA)
MS-7	F	23	1	untreated	1.0	NA	2(NA)
MS-8	F	57	3	untreated	0.0	no	0(0)
MS-9	F	39	<0.5	untreated	NA	NA	NA
MS-12	F	46	4	IFN-β	3.5	NA	NA
MS-13	F	47	0.5	untreated	1.5	no	8(0)
MS-14	F	43	18	untreated	3.0	NA	NA
MS-15	F	40	6	IFN-β	1.5	NA	NA
MS-17	F	47	0.5	untreated	2.0	no	15(0)
SP-MS							
MS-11	F	60	23	untreated	3.0	no	NA
MS-16	F	44	13	cyclophosph.	4.0	NA	NA
PP-MS							
MS-10	F	54	10	untreated	5.0	no	8(NA)
MS-18	F	49	2	untreated	2.0	no	0(NA)

RR-MS: relapsing-remitting MS, SP-MS: secondary progressive MS, PP-MS: primary progressive MS; [a] in years; [b] at sampling, IFN-β: interferon-beta, cyclophosph.: cyclophosphamide; [c] EDSS: expanded disability status scale; [d] oligoclonal bands in CSF, (): oligoclonal bands in serum; NA: not available

Table 4.3 Characteristics of CIS patients used for B cell immortalization

Subject	Sex	Age	Disease duration[a]	Treatment[b]	EDSS[c]	Active disease	OCB[d]
CIS-1	F	40	1	untreated	2.0	yes	0(NA)
CIS-2	F	36	12	IFN-β	0.0	no	0(NA)
CIS-3	F	52	NA	untreated	NA	no	0(NA)
CIS-4	M	17	0.5	untreated	2.0	yes	0(NA)

[a] in years; [b] at sampling, IFN-β: interferon-beta; [c] EDSS: expanded disability status scale; [d] oligoclonal bands in CSF; NA: not available

Table 4.4 Characteristics of NIND patients used for B cell immortalization

Subject	Sex	Age	Disease specification
NIND-1	F	35	B cell non-Hodgkin lymphoma
NIND-2	F	36	headache
NIND-3	F	48	ischemic attack
NIND-4	F	41	NA

NA: not available

4.2.2 Cell culture

PBMC were isolated by Ficoll Hypaque density gradient centrifugation (Sigma-Aldrich, Bornem, Belgium). CSF cells were obtained by centrifugation of the CSF at 4°C at 1500 rpm and kept on ice. Immortalized B cell lines were kept in RPMI 1640 medium supplemented with L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen) and 10% heat-inactivated FBS (HyClone Europe).

The human oligodendroglioma (HOG) cell line was kindly provided by Dr. G. Dawson (University of Chicago, Chicago, IL, USA). A549 cells were obtained from ATCC (Manassas, USA). Both cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS (Hyclone Europe, Erembodegem, Belgium), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen).

4.2.3 Phenotypic B cell characterization

$CD19^+CD27^-CD138^-$ naïve B cells, $CD19^+CD27^+CD138^-$ memory B cells, $CD19^+CD27^+CD138^+$ plasma blasts and $CD19^-CD27^+CD138^+$ plasma cells were measured in PB and CSF of MS patients and PB of HC using flow cytometry. PBMC (1×10^5) or CSF cells (2,500-4,000) were incubated in the presence of FITC, PE and peridinin chlorophyll protein (PerCP) conjugated antibodies against CD19, CD27, CD138 and CD21 (all from BD Biosciences) during 30 min at 4°C. Cells were washed in FACS buffer (PBS/2% FBS/0.09% Na_3) and analyzed on a FACSCalibur flow cytometer using CellQuest software (both from BD Biosciences, Erembodegem, Belgium).

4.2.4 B cell immortalization

B cell immortalization of PBMC and CSF cells was performed using sequential or simultaneous B cell stimulation and infection as described in section 2.2.2 and figure 2.1. Sequential B cell stimulation and infection was performed when starting with 50 IgG^+CD22^+ cells per well (method 1). Simultaneous B cell stimulation and infection (method 2) was applied using 50 total PBMC (5 B cells) per well (method 2A) or 500 total PBMC (50 B cells) per well (method 2B). The improved B cell immortalization procedure was performed on 50 isolated IgG^+CD22^+ B cells per well (method 2C).

4.2.5 Isotyping of the immortalized B cell lines

Isotype determination (IgG, IgM) of the immortalized B cell lines was performed using dot blot analysis, as described in section 2.2.4. IgG1, IgG2, IgG3 and IgG4 isotypes were defined by the use of HRP-labelled mouse anti-human IgG1, IgG2, IgG3 (all from Invitrogen) or IgG4 (AbD Serotec, Düsseldorf, Germany) 1:50 in MPBS. Antibody concentration in the culture supernatant was estimated using serial dilutions of known concentrations of human IgG (Invitrogen) or human IgM (Jackson ImmunoResearch, Suffolk, UK).

4.2.6 Clonality analysis of the immortalized B cell lines

Clonality analysis was performed for most of the immortalized B cell lines using B cell spectratyping of the Ig V_H region, as described in section 2.2.5 and figure 2.2. Monoclonal populations were defined as yielding a single amplification product, while 2 or more amplification products were generated for biclonal or polyclonal immortalized B cell lines, respectively.

4.2.7 Flow cytometry of antibody binding to different cell types

Flow cytometry was used to measure binding of antibodies produced by immortalized B cell lines to the HOG cell line, A549 cell line and PBMC. Incubations were done at 4°C for PBMC and on ice for HOG and A549. Extracellular antibody binding was measured by incubation of 1 x 10^5 cells with 100 µl culture supernatant of the immortalized B cell lines (IgG concentrations ranging from 0.5 to 50 µg/ml) and 7-AAD (BD Biosciences) during 1 hour. Cells were washed using FACS buffer and incubated in the presence of a FITC-conjugated goat anti-human IgG (1:100, 10 µg/ml, AbD Serotec) during 30 min. After 3 washing steps, cells were analyzed on a FACSCalibur flow cytometer.
To measure intracellular antibody binding, 1 x 10^5 cells were first incubated with 7-AAD during 30 min. Cells were washed in FACS buffer, subsequently fixed and permeabilized using the BD Cytofix/Cytoperm^{TM} Kit (BD Biosciences). Next, cells were incubated with 100 µl culture supernatant of the immortalized B cell lines during 1 hour. Following 3 washing steps in Perm/Wash buffer (BD Biosciences), a FITC-labelled goat anti-human IgG (1:100) was added during 30 min. Cells were washed again in Perm/Wash buffer and suspended in FACS buffer for analysis on a FACSCalibur flow cytometer.
Data acquisition and analysis of mean fluorescence intensity (MFI) were performed using CellQuest software. MFI analysis was done by gating on living 7-AAD negative cells, followed by correction for binding of an isotype control, a human antibody specific for the AChR (Prof. De Baets, Maastricht, The Netherlands) (Fig. 4.1). Mean + 3x standard deviation (sd) of the negative samples was used as cut-off for a positive signal. Samples that gave positive signals were tested again to confirm specific binding. GalC was stained in HOG

cells using a rabbit anti-GalC Ab and a FITC-labelled sheep anti-rabbit IgG (1:100, both from Sigma-Aldrich).

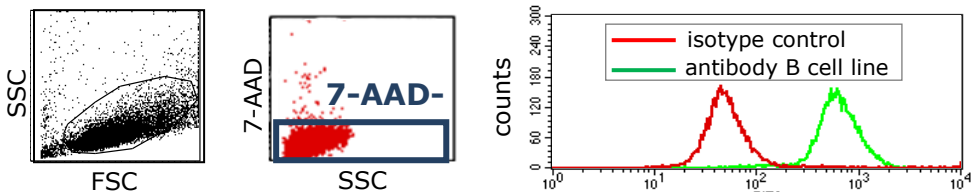

Figure 4.1 MFI analysis of antibody binding to HOG, A549 and PBMC. Analysis is focused on living cells by gating on 7-AAD negative cells. MFI of antibody produced by the immortalized B cell line was corrected for binding of an isotype control.

4.2.8 Immunocytochemistry of antibody binding to HOG cell line

HOG cells were cultured on glass slices and fixed in 4% paraformaldehyde (PFA) during 15 min at room temperature (RT). After 3 washing steps in PBS, HOG cells were permeabilised using 0.2% (v/v) PBS/Triton X-100 during 30 min. Culture supernatant of the immortalized B cell lines or an anti-AChR isotype control were incubated with the cells during 1 hour at 4°C. A secondary FITC-labelled goat anti-human IgG (1:100, AbD Serotec) was added during 30 min at 4°C. Nuclear staining was performed during 10 min at RT with 4',6-diamidino-2-phenylindole (DAPI). Finally, glass slices were coverslipped with fluorescence mounting medium (Dako). In addition, HOG cells were stained with a rabbit anti-human GalC Ab and a FITC-labelled sheep anti-rabbit IgG.

4.2.9 Antibody reactivity to MOG and viruses

Antibody reactivity against recombinant human MOG was tested by the group of Dr. B. 't Hart (BPRC, Rijswijk, The Netherlands), as previously described[351]. Culture supernatant of the immortalized cultures was used for this ELISA.
Immortalized B cell lines were screened for viral reactivity using routine clinical diagnostic tests (Prof. Dr. J-L. Rummens, Jessa Ziekenhuis, Hasselt, Belgium). Binding to viruses that were previously associated with MS, including EBV, CMV,

measles virus, VZV and Rubella, was examined on culture supernatant of immortalized B cell lines.

4.2.10 Purification of antibodies from culture supernatant

To increase IgG concentrations and limit background issues due to FBS in the culture supernatant, antibodies were purified and concentrated. Monoclonal antibodies were purified from 1 l of cell-free culture supernatant using a protein A/G column (Thermo Fisher Scientific, Erembodegem, Belgium) in combination with affinity-chromatography (BioCAD). Eluted antibodies were further purified and concentrated using Slide-A-Lyzer G2 dialysis cassettes (Thermo Fisher Scientific). Purity of the produced mAb was analyzed by 12% SDS-PAGE, while concentrations were determined with the BCA Protein Assay Kit (Thermo Fisher Scientific) following instructions of the manufacturer.
We managed to purify antibodies from 7 clones: 2 HOG-specific B cell lines (B29.73 and B29.9), 3 MS CSF B cell lines (B5.1, B5.4 and B5.5), 1 B cell line that showed binding to HOG, A549 and PBMC (B26.2) and 1 that was part of the largest clonal expansion in MS and CIS patients (B27.44: V1-69*01, D5-05*01, J4*02).

4.2.11 Immunoprecipitation

Normal human brain tissue was homogenized with a rotor-stator in lysis buffer (150 mM sodium chloride, 1% (v/v) NP-40 (Nonidet P-40, Sigma-Aldrich), 50 mM Tris, pH 8.0) containing EDTA-free nuclease inhibitors (Roche Diagnostics, Vilvoorde, Belgium), followed by clearing of the lysate by centrifugation. Lysates were produced from HOG and A549 cells using the Pierce® Crosslink Immunoprecipitation Kit (Thermo Fisher Scientific) following manufacturer's instructions. The protein concentration in the homogenate was determined using the BCA Protein Assay Kit, divided in aliquots and stored at -80°C.
Immunoprecipitation (IP) of the purified mAb with HOG, A549 or brain tissue lysate was performed using the Pierce® Crosslink IP Kit. First, 50 µg of monoclonal antibody was coupled to protein A/G agarose and covalently attached by a disuccinimidyl suberate (DSS) crosslinker. Lysates were pre-

cleared with control agarose to reduce non-specific binding. Next, antibody-coupled columns were incubated overnight at 4°C with 1 mg of lysate proteins. Bound proteins were eluted in 4 fractions (pH 2.8) and collected in 1M Tris buffer (pH 9.5). To control for non-specific binding, IP was also performed using a human IgG1 or IgG2 isotype control that was raised against a hapten that is non-existent in mammalian cells (Eureka Therapeutics, Emeryville, CA, USA).

4.2.12 Mass spectrometry

To identify the purified antibody targets, 30 µl of IP eluates were evaporated and separated on 12% SDS-PAGE followed by silver staining using the SilverQuest™ Silver Staining Kit (Invitrogen). Protein bands and total protein eluates were analyzed by electrospray ionization-tandem mass spectrometry on a LCQ Classic (ThermoFinnigan, San Jose, CA) equipped with a nano-liquid chromatography column switching system. All mass spectrometric data was searched using SEQUEST version 1.0.43.0 (ThermoFinnigan) and MASCOT version 2.2 (Matrix Sciences, London, UK) against ipi.HUMAN database (86379 entries). Scaffold version 3_00_03 (Proteome Software Inc.) was used for validation of search engine output.

4.2.13 Statistical analysis

Statistical analysis of phenotypic B cell characterization data was performed using Prism software version 4.00 (Graphpad). Mann-Whitney *t*-test was performed. A p value of < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Phenotypic B cell characterization in PB and CSF

The distribution of 4 major B cell subpopulations was examined in PBMC of 17 MS patients and 5 HC, as well as in CSF of 11 MS patients. $CD19^+CD27^+CD138^-$ memory B cells made up 4.1% and 3.9% of total lymphocytes in the PB of MS patients and HC, respectively (Fig. 4.2a). Similar numbers of memory B cells were found in the CSF of MS patients (3.4%), emphasizing their relevance in MS pathogenesis. $CD19^+CD27^+CD138^+$ plasma blasts and $CD19^-CD27^+CD138^+$ plasma cells were absent in the PB but were significantly increased in the CSF of MS patients, comprising 2.3% plasma blasts and 4.4% plasma cells ($p < 0.0001$ and $p = 0.0002$, respectively). $CD19^+CD27^-CD138^-$ naïve B cells were the predominant B cell population in the PB of both MS patients and HC (6.1% and 4.7% respectively). The percentage of naïve B cells was significantly higher in PB than in CSF of MS patients ($p < 0.0001$), where almost no naïve B cells were retrieved.

Next, susceptibility to B cell immortalization of the different populations in PB and CSF was examined by measuring expression of CD21, the EBV receptor. The majority of memory B cells showed CD21 expression, both in PB of MS patients (93%) and HC (93.5%) and in CSF of MS patients (73%) (Fig. 4.2b). About half of plasma blasts (49.4%) in the CSF of MS patients evidenced CD21 expression, while only 11.6% of plasma cells displayed CD21 on their surface. CD21 was also expressed on 88% and 94% of naïve B cells from MS patients and HC, respectively. In MS CSF, no naïve B cells were present to measure EBV receptor expression.

These findings indicate that the memory B cell subset, containing many disease-relevant B cells, is the most important study population in PB and CSF when applying B cell immortalization with a preselection of IgG^+ B cells. Plasma blasts and plasma cells migrate to the CNS, together with memory B cells, where they are responsible for oligoclonal IgG production. Half of the plasma blasts can also be immortalized but this is in contrast to plasma cells that lack CD21 expression. No differences in B cell distribution and CD21 expression were observed between different MS types or between MS and HC. A high percentage of plasma cells was retrieved in the PB of 1 MS patient receiving Natalizumab (RR-MS 2), while

no plasma blasts or plasma cells were found in the paired CSF. However, 2 other MS patients (RR-MS 10 and 11) under Natalizumab treatment did not show this deviating B cell distribution.

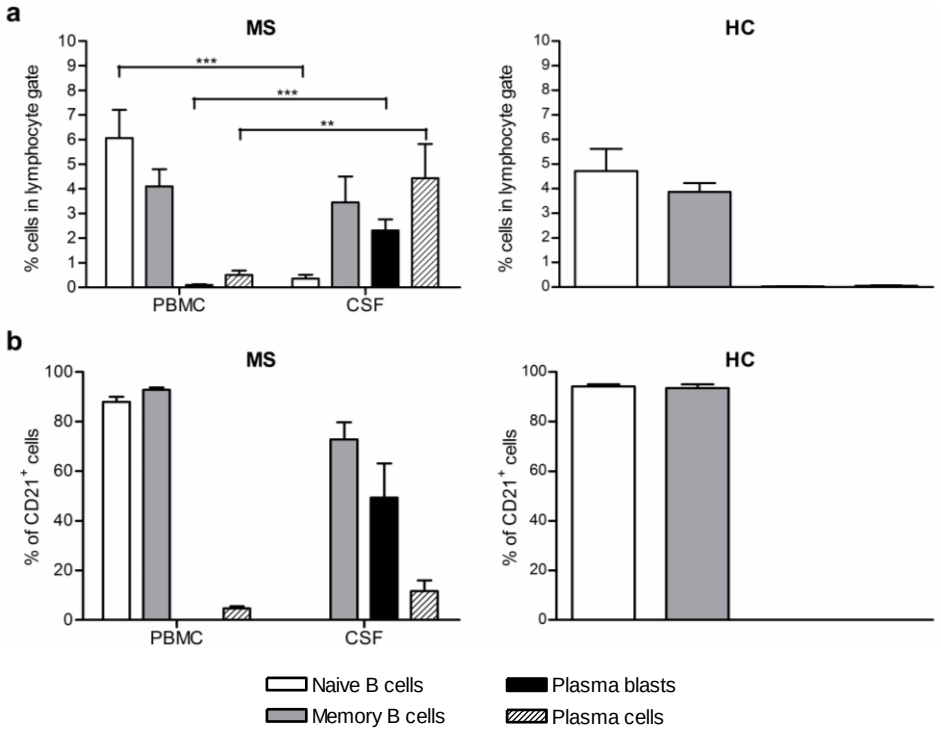

Figure 4.2 Phenotypic B cell characterization in the PB and CSF. The distribution (a) and CD21 expression (b) of naïve B cells, memory B cells, plasma blasts and plasma cells are shown in the PBMC and CSF B cell repertoire of MS patients and in the PB of HC.

4.3.2 Analysis of the antibody specificity of immortalized B cell lines from MS, CIS and NIND patients

We analyzed the antibody specificity of 301 B cell lines from 18 MS patients, 45 B cell lines from 4 CIS patients and 7 B cell lines from 4 NIND patients (353 in total). Characteristics of all immortalized B cell lines that were produced for each patient are shown in Table 4.5. Only 9 immortalized B cell lines were generated from the CSF although B cell immortalizations were started with CSF cells of almost all patients included in the study. This indicates low efficiency for the immortalization of CSF B cells.

Table 4.5 Panel of obtained immortalized B cell lines

Subject	No. immortalized B cell lines	Source	Isotype[a]		Clonality		Immortalization method[b]
			IgG	IgM	Monoclonal	Biclonal or polyclonal	
MS patients							
Selection for IgG- and IgM-producing B cell lines							
MS-1	7	CSF	3	3	6	1	2A
MS-2	9	PBMC	1	3	8	1	2A
MS-3	5	PBMC	2	1	5	0	1
MS-4	9	PBMC	5	4	7	2	1
MS-5	1	PBMC	0	1	1	0	1
MS-6	1	PBMC	0	1	ND		2A
MS-7	4	PBMC	0	4	1	2	1
MS-8	1	PBMC	1	0	ND		2A
MS-9	1	PBMC	0	1	1	0	1
Selection for IgG-producing B cell lines							
MS-10	4	PBMC	4	0	2	1	2B
MS-11	8	PBMC	8	0	6	1	2C
MS-12	24	PBMC	24	0	20	3	2C
MS-13	17	PBMC	17	0	9	5	2B
MS-14	19	PBMC	19	0	12	2	2C
MS-15	89	PBMC	89	0	60	22	2C
MS-16	2	PBMC	2	0	2	0	2C
MS-17	88	PBMC	88	0	26	6	2C
MS-18	12	PBMC	12	0	10	2	2B

[a] for some subjects, antibody concentrations of immortalized B cell lines were below detection limits; [b] method 1: sequential method using 50 PBMC per well, method 2A: simultaneous method using 50 PBMC per well, method 2B: simultaneous method using 500 PBMC per well, method 2C: simultaneous method using 50 IgG^+CD22^+ B cells per well (improved procedure);
ND: not determined

Table 4.5 (continued) Panel of obtained immortalized B cell lines

Subject	No. immortalized B cell lines	Source	Isotype[a]		Clonality		Immortalization method[b]
			IgG	IgM	Monoclonal	Biclonal or polyclonal	
CIS patients							
Selection for IgG- and IgM-producing B cell lines							
CIS-1	5	PBMC	2	2	2	1	2B
Selection for IgG-producing B cell lines							
CIS-2	4	PBMC	4	0	1	0	2B
CIS-3	8	PBMC	8	0	7	0	2B
CIS-4	28	PBMC	28	0	18	5	2B
Control patients							
Selection for IgG- and IgM-producing B cell lines							
NIND-1	2	CSF	1	1	2	0	2A
NIND-2	1	PBMC	0	1	1	0	2A
NIND-3	3	PBMC	2	1	2	1	2A
NIND-4	1	PBMC	0	1	1	0	2A

[a] for some subjects, antibody concentrations of immortalized B cell lines were below detection limits; [b] method 1: sequential method using 50 PBMC per well, method 2A: simultaneous method using 50 PBMC per well, method 2B: simultaneous method using 500 PBMC per well, method 2C: simultaneous method using 50 IgG^+CD22^+ B cells per well (improved procedure);
ND: not determined

Analysis of antibody reactivity to HOG, A549 and PBMC

Autoreactivity of several peripheral immortalized B cell lines was indicated by examining binding of their antibodies to HOG cells, PBMC and A549 cells using flow cytometry. The HOG cell line was established from a surgically removed oligodendroglioma and was shown to express some oligodendrocyte-specific proteins such as MBP and CNPase[352]. A549 cells, which are adenocarcinomic human alveolar basal epithelial cells[353], were included as a negative control.

Table 4.6 Overview of antibody binding patterns to PBMC, HOG and A549

Patient	HOG binding (%)	PBMC binding (%)	HOG and A549 binding (%)	HOG, A549 and PBMC binding (%)
MS patients				
MS-1	0/7 (0)	0/7 (0)	0/7 (0)	0/7 (0)
MS-2	0/9 (0)	0/9 (0)	0/9 (0)	1/9 (11.1)
MS-3	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
MS-4	1/9 (11.1)	0/9 (0)	1/9 (11.1)	0/9 (0)
MS-5	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
MS-6	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
MS-7	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)
MS-8	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
MS-9	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
MS-10	0/4 (0)	0/4 (0)	1/4 (25)	1/4 (25)
MS-11	0/8 (0)	0/8 (0)	4/8 (50)	3/8 (37.5)
MS-12	0/24 (0)	0/24 (0)	1/24 (4.2)	8/24 (3.3)
MS-13	0/17 (0)	0/17 (0)	1/17 (5.9)	1/17 (5.9)
MS-14	0/19 (0)	0/19 (0)	0/19 (0)	0/19 (0)
MS-15	1/89 (1.1)	0/89 (0)	4/89 (4.5)	7/89 (7.9)
MS-16	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
MS-17	2/88 (2.3)	0/88 (0)	14/88 (15.9)	17/88 (19.3)
MS-18	0/12	0/12	0/12	1/12 (8.3)
CIS patients				
CIS-1	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
CIS-2	0/4 (0)	0/4 (0)	0/4 (0)	1/4 (25)
CIS-3	0/8 (0)	1/8 (12.5)	1/8 (12.5)	0/8 (0)
CIS-4	0/28 (0)	0/28 (0)	6/28 (21.4)	1/28 (3.6)
Control patients				
NIND-1	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
NIND-2	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
NIND-3	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
NIND-4	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)

Extracellular binding to HOG cells, A549 cells or PBMC was not observed (data not shown). However, intracellular antibody binding to one or more of the tested cell types was detected in 80/353 (22.7%) immortalized B cell lines, indicating specificity for an autoantigen (Table 4.6). This panel of autoreactive immortalized B cell lines included 70/294 (24%) PB B cells from MS patients and 10/45 (22%) PB B cells from CIS patients.

Exclusive binding to the HOG cell line was demonstrated for 4/294 (1.4%) B cell lines from MS patients (B10.8, B27.53, B29.9 and B29.73, Fig. 4.3), indicating their specificity for HOG cells. Antibody binding to PBMC but not to HOG and A549 cells was indicated for 1/45 (2.2%) PB B cell lines from CIS patients. These are probably directed against antigens that are specific to PBMC. A total of 75/339 (22%) immortalized B cell lines from MS and CIS patients showed antibody binding to all tested cell types, suggesting specificity for a more common intracellular target. This panel of B cell lines that produced antibodies against common intracellular antigens included 42 B cell lines that were positive for binding to HOG, A549 and PBMC and 33 that showed staining of HOG and A549 but not PBMC. Two of these immortalized B cell lines were part of clonally expanded B cell populations (clones 2 and 3, Chapter 3).

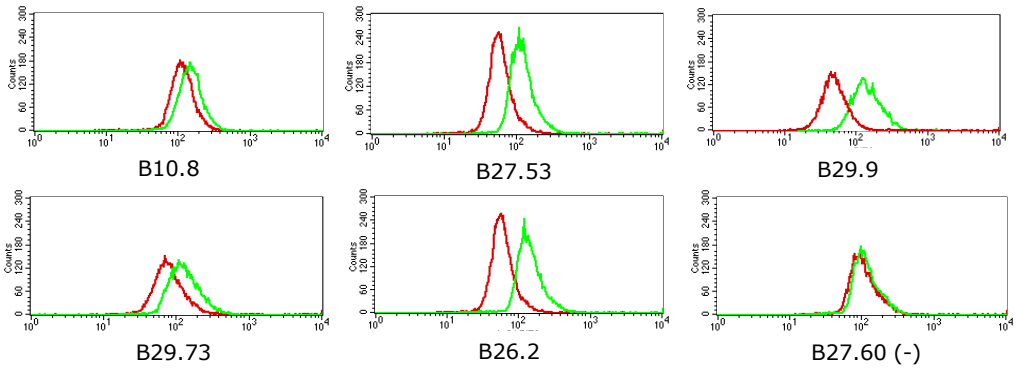

Figure 4.3 MFI analysis of intracellular antibody binding to HOG cells. MFI histograms are shown for 4 HOG-specific B cell lines (B10.8, B27.53, B29.9 and B29.73), 1 B cell line reactive against all 3 cell types (B26.2) and 1 negative immortalized B cell line (B27.60).

The majority of MS (10/17) and CIS (3/4) patients showed autoreactivity in at least 1 peripheral B cell line. Patients MS-9, MS-10 and MS-16 in particular

showed a high proportion of autoreactive peripheral B cells (50, 87.5 and 37.5%, respectively). No difference was observed between MS subtypes, as autoreactive B cells were found in RR-MS, PP-MS and SP-MS patients. The 7 immortalized B cell lines from the CSF of MS-1 were negative for binding to HOG, A549 and PBMC.

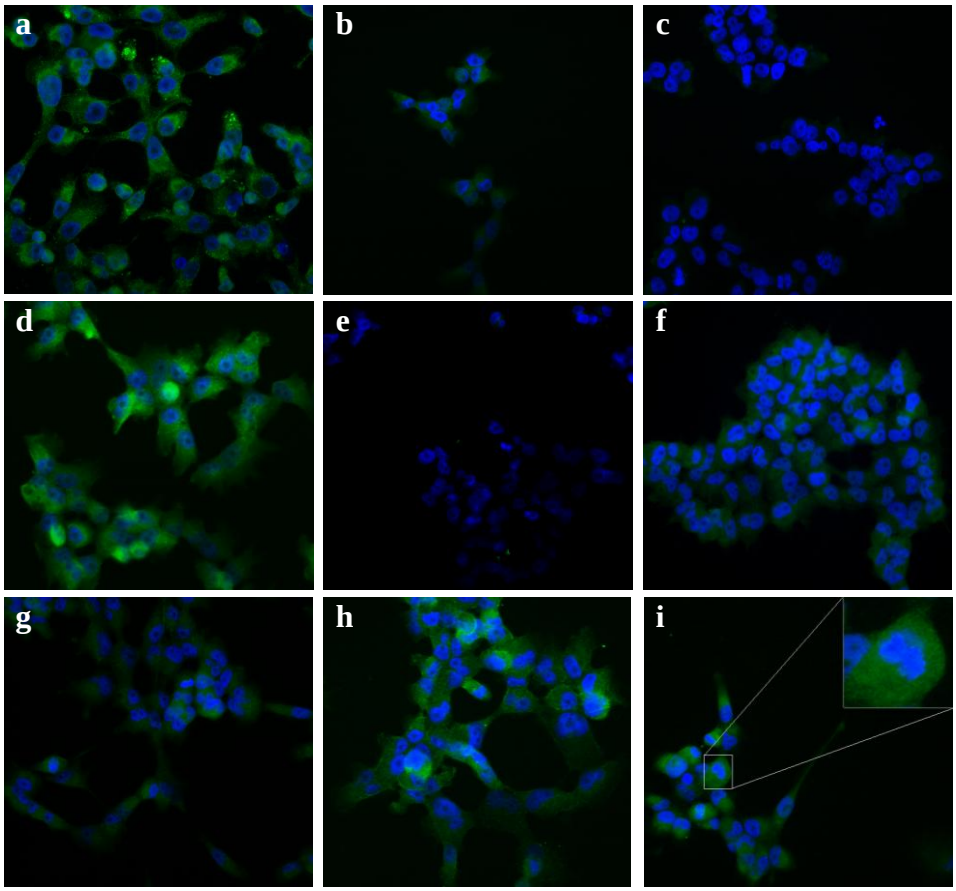

Figure 4.4 Immunocytochemistry of antibody binding to HOG cells. B10.8 (a), B27.53 (b), B29.9 (c) and B29.73 (d) showed HOG-specific binding using flow cytometry. A representative image of an anti-AChR IgG1 isotype control (e) and GalC (f) are depicted. B29.39 (g) was positive for binding to HOG and A549, while B26.2 (h) and B27.33 (i) reacted against HOG, A549 and PBMC in flow cytometric analysis. Images are 400x magnified.

Binding to HOG cells was then confirmed by means of immunocytochemistry. Intracellular HOG staining was demonstrated for all HOG-specific immortalized B cell lines, although 2 B cell lines (B10.8 and B29.73) showed higher reactivity when compared with the other 2 (B27.53 and B29.9, Fig. 4.4 a-d). This could be caused by slightly lower antibody concentrations in the culture supernatant of B27.53 and B29.9, or the lower abundance of their specific antigen. Different staining patterns were distinguished. A speckled pattern was observed using antibodies from B10.8 while the staining pattern of the other B cell lines was more homogeneous. In addition, intracellular HOG staining was confirmed for 1 B cell line that was reactive against HOG and A549 and 2 B cell lines that showed binding to all 3 cell types in flow cytometry (Fig. 4.4 g-i). These results confirmed flow cytometric analysis of HOG cells.

In conclusion, autoreactivity was detected in 80 immortalized B cell lines from the PB of MS and CIS patients. Some HOG-specific (4) and PBMC-specific (1) B cell clones were demonstrated although the majority of autoreactive B cells were directed against a general intracellular target (75). Staining of HOG cells using flow cytometry was confirmed using immunocytochemistry. Moreover, different antibody staining patterns that were observed for some clones suggested different antigenic targets.

Analysis of antibody reactivity to MOG, viruses and brain tissue

Reactivity of antibodies from the immortalized B cells was determined against recombinant human MOG. MOG reactivity could not be detected in 7 CSF immortalized B cell lines from MS-1, 3 HOG-specific clones (B27.53, B29.9 and B29.73), 3 clones positive for HOG and A549, 3 clones positive for HOG, A549 and PBMC and 3 negative clones. Furthermore, none of the 45 immortalized B cell lines from MS-1 to MS-8 and NIND-1 to NIND-4 were positive for reactivity towards EBV, CMV, measles virus, VZV and Rubella. However, only a panel of 19/353 and 45/353 immortalized B cell lines were tested for MOG and viral reactivity, respectively. Detection limits might hamper the detection of reactivity since antibody concentrations in the culture supernatant were often only 0.5-10 µg/ml.

Antibodies were purified from 3 MS CSF B cell lines (B5.1, B5.4 and B5.5), 2 HOG-specific B cell lines (B29.9 and B29.73), 1 with general intracellular reactivity (B26.2) and 1 belonging to the largest clonal expansion (B27.44,

Chapter 3). B5.1, B5.4, B26.2 and B29.73 were IgG1, while B5.5, B27.44 and B29.9 were demonstrated to be IgG2 by dot blotting. Specific binding to brain tissue could not be demonstrated using these purified and concentrated antibodies. Several tissues (healthy and EAE rhesus monkey, EAE mouse and MS brain tissue) and different blocking and fixation conditions were tested on frozen and paraffin-embedded tissue, as well as antigen retrieval.

Target antigen identification using IP on HOG cells

IP of the purified antibodies with lysates of HOG cells resulted in the identification of candidate autoantigens of 2 MS CSF B cell lines. The potential target of clone B5.1 was cystatin-A. It was detected with 1 unique peptide and a probability of 79.5% by both SEQUENCE and MASCOT search engines. The full tryptic peptide spectrum (Fig. 4.5a) showed overlap in the peptide sequence for y and b ions, corresponding to C- and N-terminal related ions, respectively. Moreover, the spectrum was consistent with the occurrence of one proline (P) at sequence position 3 (16 AA away from the C-terminus), as evidenced by the occurrence of y16 (as single as well as double charged ion). The complementary b2 ion (corresponding to the dipeptide ion 'SL') was missing probably due to the inability of the ion trap to detect ions with m/z smaller than "parent ion m/z divided by 4". These findings increased confidence for the identification of cystatin-A as target antigen of B5.1. However, molecular weight of cystatin-A (11kDa) did not correspond to its location in the SDS-PAGE (near 150kDa, data not shown). This could be explained by complex formation of cystatin-A with other proteins.

Apoptosis stimulating protein of p53-2 (ASPP2) was identified as potential target peptide for clone B5.4, that was part of a CSF clonal expansion. It was identified by both search engines with a protein identification probability of 92.5%. The full tryptic peptide spectrum was consistent with one P in sequence position 6, as evidenced by the y11 ion and corresponding b6 ion (Fig. 4.5b). However, minimal sequence overlap was present between b and y ions, the latter even showing a gap in the peptide sequence. The incorrect location of the 125.6kDa ASPP2 between 50-75kDa in the SDS-PAGE could be explained by the detection of its break-down products.

In conclusion, cystatin-A and ASSP2 were identified as candidate target antigens of MS CSF clones B5.1 and B5.4. The latter immortalized B cell line was one of

the two clonally related B cells that were identified in MS CSF (clone 9, chapter 3). However, repeated experiments and further confirmation are necessary to clarify these findings and to identify these proteins with certainty as targets of B cells in MS CSF. Other IP experiments using A549 and human brain lysates were evaluated using Western blotting but results remained elusive.

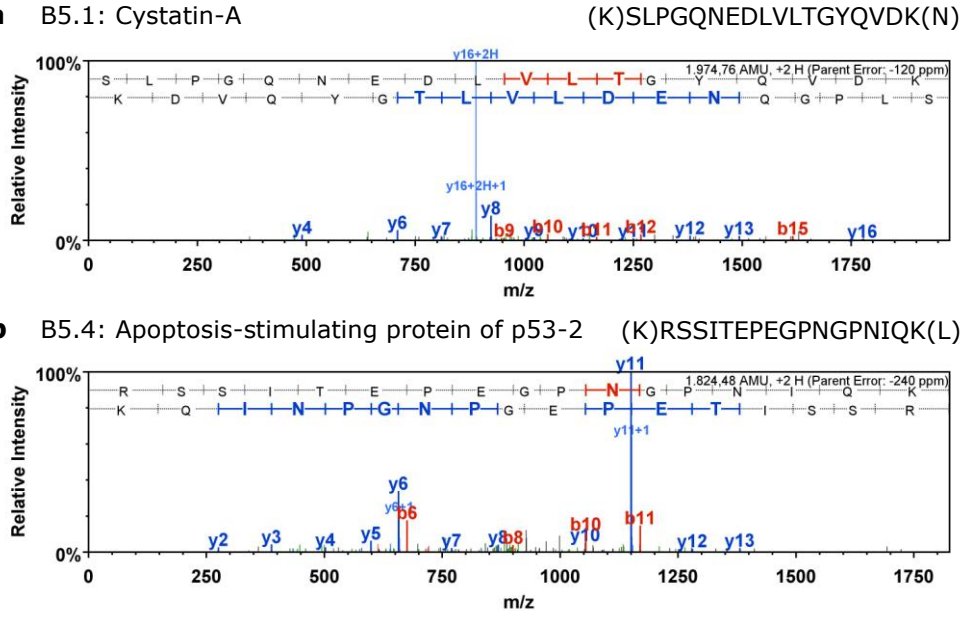

Figure 4.5 Peptide spectra of potential target antigens of B5.1 and B5.4. The mass spectra of the candidate target antigens of 2 MS CSF clones are shown, namely cystatin-A for B5.1 (a) and isoform 1 of apoptosis-stimulating protein of p53-2 for B5.4 (b). Peptide sequences are shown for each candidate.

4.4 Discussion

In this study, phenotypic B cell analysis indicated plasma cells as predominant B cell population in the CSF of MS patients, although they were not susceptible to B cell immortalization. Memory B cells were identified as main target population of B cell immortalization in PB and CSF of MS patients. Moreover, antibodies from 24% of PB B cell lines from MS patients and 22% of PB B cell lines from CIS patients demonstrated specificity for intracellular antigens. Immortalized B cells from NIND PB and MS CSF were negative for binding to HOG, A549 or PBMC. However, cystatin-A and ASPP2 were suggested as target antigens of 2 B cell clones from the CSF of an MS patient.

Memory B cells were shown to be major constituents of the B cell repertoire both in PB and CSF of MS patients and were identified as main target population of B cell immortalization. Naïve B cells made up the largest B cell population in MS PB and showed high susceptibility to B cell immortalization. Nevertheless, peripheral naïve B cells comprise a lot of clones that are irrelevant to the disease process. Plasma cells were the most prevalent B cell subpopulation in the CSF of MS patients, but appeared to be unsusceptible to immortalization. Previous studies pointed out a low number of plasma cells in MS CSF[145,148,153]. Patient cohorts used in these studies showed similar clinical characteristics but were of a larger size. Corcione *et al.*, however, denoted high plasma cell numbers in MS CSF as well[146]. Our findings are in line with these results, indicating that long-lived plasma cells are the major antibody-secreting cells in MS CSF.
It would be very interesting to characterize the antibody specificity of plasma cells in MS and CIS. However, the memory B cell pool contains all precursor B cells that eventually mature into plasma cells. Thus, analysis of memory B cells is most likely to reveal the same antigen specificities. All together, the memory B cell pool represents an ideal repository of antibodies for analysis in MS including all specificities that are generated in the course of an immune response.

In a second part of the study, we confirmed our hypothesis that a proportion of peripheral B cells from MS and CIS patients showed autoreactivity. B cell lines producing antibodies directed against intracellular antigens were observed in PB

and CSF of MS patients and PB of CIS patients following B cell immortalization. HOG-specific B cell lines could be directed against oligodendrocyte (precursor) specific proteins including MBP, MOG and CNPase[352,354,355] or myelin-specific lipids such as GalC and sulfogalactosylceramide[355]. Candidate target antigens of PBMC-specific B cell lines could be various proteins or peptides, such as cytokines, HLA or TCR molecules. These autoreactive B cells could function in the elimination of cytokine-producing lymphocytes or regulatory cells. Antigenic targets of B cells that were positive for binding to HOG and A549 could be involved in cell cycle regulation, DNA replication or other cellular processes that are increased in fast growing cell lines. In that context, the glycolytic enzymes TPI and GAPDH have been identified as targets of autoreactive antibodies in MS CSF[208]. Further, anti-DNA antibodies have been demonstrated in brain and CSF of MS patients[203], while antibodies against oncogenes have recently been evidenced in RA[356]. Immortalized B cell lines that were negative for antibody binding could still be directed against other viral or brain-derived antigens or lipids. No extracellular antibody binding was evident, which could be due to insufficient antibody concentrations and/or specificity for low abundant cell surface proteins. Trypsin could remove or damage surface antigens, although GalC was even detected on the cell surface following trypsinization (data not shown).

Extracellular target antigens are pathogenically very interesting since they are readily available for antibody binding. However, antibodies directed against intracellular autoantigens could play a role in disease pathology as well. Various antibodies against intracellular targets have already been reported in CSF and/or serum of MS patients. Differential mRNA expression patterns were evidenced for intracellular and intranuclear autoantigens in MS lesions[357], which could account partly for the increased occurrence of anti-nuclear antibodies (ANA) in MS sera[358,359]. Autoreactive antibodies to yet unidentified intracellular proteins, both nuclear and cytoplasmic, were further detected in sera and not in CSF of 10 MS patients[360]. Antibodies with specificity for intracellular targets could be induced as by-products of systemic immune activation. Increased apoptosis and inflammation in MS could lead to increased post-translational modifications of intracellular antigens that might subsequently be presented as novel self-proteins, as was seen in patients with SLE[361]. Autoantibodies against intracellular antigens could also function in immune regulation. Natural

antibodies that are directed against intracellular antigens have been implicated in self-protective mechanisms such as blocking epitopes in damaged tissues or facilitating clearance of self-proteins[362,363]. Alternatively, antibodies with specificity for intracellular targets can have pathologic relevance in the primary autoreactive immune response. Antibodies that recognize intracellular enzymes, such as CNPase and transaldolase[211,364], or proteins with distinct sequences, translated Alu repeats[365], have been implicated in myelin and oligodendrocyte loss. Furthermore, effects on EAE pathology were evidenced by antibodies to neurofilament light chain[366] and sperm associated antigen 16 (SPAG 16) isoform 2 (unpublished results from our own research group). However, how these antibodies are generated remains unknown. The finding of antibodies that are specific for intracellular HOG antigens also suggests their involvement in demyelination or loss of oligodendrocytes, although this has to be further investigated. Identification of the target antigens could give more insight in the pathological relevance of these B cells in the disease process.

Cystatin-A and ASPP2 were identified as candidate target antigens of MS CSF B cell clones. Both proteins were retrieved by single-peptide protein identifications, that were previously reported in the CSF of MS patients[367]. The presence of prolines in the spectrum causes a high sensitivity to fragmentation, which leads to the occurrence of smaller peaks. The incorrect location of both proteins in SDS-PAGE needs further research. Cystatin-A could participate in complex formation with other proteins, although no interaction partners such as p53 were identified. ASPP2 could be identified in the lower molecular weight region as a consequence of protein processing and truncation. We also have to take into account the possibility that cystatin-A was introduced accidentally in the sample, as is often the case with keratins.

Cystatins function in the regulation of cysteine proteases such as cathepsin B, H and L that are involved in cellular processes including protein degradation and antigen processing[368]. Cystatin-C has already been described in demyelination and neurodegeneration[369,370]. Cystatin-A levels were increased in serum and sometimes also in CSF of MS patients[367]. Further, cystatin-A was reported in neurons and pericytes of human brain and was upregulated in neuritic plaques in Alzheimer's disease[371]. Increased levels of cystatin-A could reflect increased apoptosis, which probably leads to the production of autoantibodies. ASPP2 is

known to enhance damage-induced apoptosis through binding to p53[372]. Defective p53-mediated apoptosis has been reported in a subset of MS patients, leading to defective elimination of autoreactive cells[373]. It is thus possible that B cells directed against ASPP2 increase the defect in p53 functioning. Experimental repetition and confirmation experiments are necessary to address the reproducibility of these findings. Competition experiments using recombinant antibodies or analysis of antibody binding to the purified protein are necessary to confirm antibody specificity.

The absence of reactivity to MOG, viruses and brain tissue of our panel of immortalized B cell lines can be caused by insufficient antibody concentrations in the culture supernatant. Alternatively, immortalized B cells could be directed against lipids. Autoantibodies specific for lipids have previously been detected in MS CSF and serum by several research groups[187,195,198,202,374,375]. Identification of lipid antigens as targets of autoreactive B cells is hindered by technical difficulties, the large number of potential lipid antigens and their hydrophobicity.

In conclusion, memory B cells were identified as major disease-related B cell subset in PB and CSF that was susceptible to B cell immortalization. Moreover, autoreactive B cells were demonstrated in the PB of MS and CIS patients that were directed against a variety of autoantigens. In addition, cystatin-A and ASPP2 were identified as autoantigenic candidates for 2 B cell clones from the CSF of an MS patient. These identifications have to be confirmed by further analysis.

5

Summary, General discussion & Future perspectives

Substantial evidence for the involvement of an aberrant humoral immune response in the pathogenesis of MS has been gathered. Increased antibody levels in the CSF of MS patients lead to the production of OCB, one of the immunological hallmarks for diagnosis. Many investigations have attempted to identify the target antigens of these antibodies, although no unequivocal evidence of their reactivity has been established to date. B cells in the CSF and brain of MS patients are most certainly associated with pathogenesis, as indicated by clonal expansion of B cells in CSF and MS lesions[309,376], antibody and complement deposition in (a subset of) lesions[31] and follicle-like GC in the meninges of chronic progressive MS patients[25]. No clear link has been established between PB B cells and MS pathogenesis, although autoreactive B cells and antibodies have been identified in the PB or serum of MS patients[70,187-189]. Moreover, plasma exchange is used with success to treat a subset of MS patients[328-330], suggesting a role for peripheral B cells in the disease process.
In this thesis, we developed an improved B cell immortalization method for the fast generation of mAb from human B cells. This B cell immortalization technology was successfully applied on B cells from the PB and CSF of MS and CIS patients in order to further study the humoral immune response in terms of clonal expansion and antibody specificity. In the following paragraphs, the main results are described and discussed.

I. B cell immortalization is a valuable technology for the study of human B cell responses

B cell immortalization has already proved to be a useful technique for the fast generation of large B cell numbers and antibodies. However, there was still need of a B cell immortalization method with high efficiency and reproducibility, that could easily be adopted for the production of (autoreactive) antibodies from patients with autoimmune disease.
In **chapter 2**, we describe the development of an improved B cell immortalization method with an efficiency of up to 83% for the generation of IgG-producing immortalized B cell lines from IgG^+ B cells. This procedure was characterized by simultaneous B cell stimulation by CpG2006 and EBV infection during 2 weeks, followed by restimulation during 7 days using CpG2006 and IL-

2. IgG^+CD22^+ FACS sorting resulted in the highest cell yield, purity and immortalization efficiency when preselecting for IgG^+ B cells. Moreover, an average immortalization efficiency of 95% could be achieved using this improved method on total PBMC, leading to the preferential outgrowth of IgM^+ B cell lines. B cell spectratyping profiles of immortalized B cell lines and total PBMC of the same patients showed considerable overlap, indicating that the immortalized B cells represented in vivo B cell clones. Although we always have to take into consideration that the panel of immortalized B cells represents a random sample from the total population, these results demonstrated that B cell immortalization can be used to study humoral immune responses in settings of autoimmunity.

What is the advantage of our improved B cell immortalization method in comparison to previous immortalization protocols?

Most existing B cell immortalization protocols are based on EBV infection of high B cell numbers, leading to the outgrowth of oligoclonal or polyclonal B cell populations that have to be cloned by limiting dilution[267,269]. This causes substantial bias, hampering representation of the in vivo B cell pool. We demonstrated high monoclonality rates of 70-87% using our improved immortalization method. In this way, the introduction of bias is reduced and the process of mAb generation is accelerated. Monoclonal B cell populations are highly frequent when seeding 50 B cells per well, since EBV infection causes activation of cellular protection mechanisms and induces a proliferative growth crisis, leading to apoptosis of a proportion of the infected B cells. Furthermore, our improved B cell immortalization method is characterized by high reproducibility as many immortalized B cell lines were produced from patients and healthy individuals. It is also successfully applied in the research group of Prof. De Baets (K. Vrolix, Maastricht University, Maastricht, The Netherlands) for characterization of the B cell response in myasthenia gravis (MG).

Some variation in immortalizaton efficiency between donors will always be evident. We hypothesize that decreased expression of TLR9 or IL-2 receptor in some donors could undo the enhancing effect of B cell stimulation. In addition, cytotoxic T cells are able to kill EBV-infected B cells in seropositive donors[377,378]. This effect is inhibited by adding cyclosporine A or by immortalizing isolated IgG^+ B cells. However, the presence of EBV-specific or EBV-infected B cells in the culture could still influence the effectiveness of EBV infection.

What is the advantage of B cell immortalization in comparison to alternative mAb production technologies?

Single-cell PCR and laser-capture microdissection have been used repeatedly for molecular B cell analysis in MS and CIS[308,380]. The main advantage of these techniques is the possibility to study Ig genes of B cells or plasma cells from the brain and CSF, thereby focusing on disease-related B cells. However, the most limiting factor of these methods is the labour intensity of the protocols. Consequently, the number of patients and B cells that are studied is often limited. Laser-capture microdissection makes use of tissue slices that have been frozen or fixed, which could interfere with the structure of the target cells and the downstream PCR. In addition, the low amount of DNA that is isolated from individual cells is sometimes insufficient for successful PCR amplification. These limitations can hamper the representation of the in vivo B cell or antibody repertoire.

Hybridoma technology and antibody phage display are alternative technologies that have been successfully used for the production of mAb. However, humanization of the produced antibodies is necessary when using hybridomas in order to apply the mAb to humans. In antibody phage display, selection of the antibodies occurs in an in vitro setting. Moreover, affinity maturation is often necessary to obtain antibodies with sufficient affinity for their target antigen.

B cell immortalization allows the generation of fully human in vivo selected antibodies that reflect the diversity and specificity of the human immune response. The main strengths of this approach are: i) most of the immortalized B cell lines are monoclonal, ii) it is rapid, iii) human IgG can be selected to a variety of antigens from a small amount of PB, iv) almost unlimited amounts of antibodies, cells and DNA are generated and v) it paves the way for various screening and characterization procedures that allow further characterization of the produced clones. In addition, large patient cohorts and large B cell numbers can be analyzed. A limitation of our improved B cell immortalization method is that it mainly focuses on PB-derived B cells, since CSF-derived B cell lines were generated with low efficiency. However, the finding of a restricted clonally expanded B cell response in the PB of MS and CIS patients (chapter 3) now highlights the importance of peripheral B cell responses in MS. Bias towards the preferential outgrowth of fast growing immortalized B cells is reduced by culturing low B cell numbers per well, although some bias is always present.

In summary, the above described techniques for mAb production and B cell profiling are complementary and should be combined to obtain better insight into the underlying disease processes in MS. However, one always has to take into consideration that the B cells that are analyzed using these technologies represent a random sample that is taken from the whole population of in vivo B cells. B cell immortalization technology is a promising tool to further study B cell responses in autoimmunity. In addition, it can be used for a variety of other applications, such as transcriptomic studies[379]. Unlimited amounts of DNA are readily available for molecular B cell analysis or the establishment of cell line resources. Virus neutralizing antibodies can be isolated for effective therapy. In addition, detailed characterization of in vivo B cell responses, both in health and disease, can result in the development of novel immunotherapeutic strategies.

In conclusion, we have developed an improved B cell immortalization with high efficiency and reproducibility for the production of monoclonal IgG^+ B cell lines that represent the in vivo B cell population. It is the first immortalization procedure that eliminates the need for cloning, which speeds up the production of mAb. Taken together, this B cell immortalization technology can easily be applied for the study of the humoral immune response in autoimmune disease.

II. Antigen-experienced clonally expanded B cells are present both in PB and CSF of MS and CIS patients

In **chapter 3**, we applied B cell immortalization for the characterization of the diversity of the B cell repertoire in MS and CIS patients, primarily in the PB but also in the CSF, to evaluate the extent of clonal expansion. Hereby, clonality analysis of the Ig V_H region of immortalized B cell lines was performed.

Are B cells from the CSF and PB of MS and CIS patients clonally expanded?

A lot of evidence has been obtained for intrathecal antigen-stimulated clonally expanded B cells in MS and CIS[126,307,314]. In this study, only 7 immortalized B cell lines from the CSF of 1 MS patient were analyzed. Nevertheless, 2 clonally related CSF B cells were identified that expressed a VH gene that was previously linked to MS brain, namely VH4-39[126,307]. These findings emphasize an association of the clonally related CSF B cells with disease pathogenesis and confirm previous reports on restricted intrathecal B cell responses in MS.

No evidence for a restricted peripheral B cell response has been found to date. We are the first to demonstrate clonally expanded B cells in the PB of both MS and CIS patients using molecular BCR analysis. Nine populations of clonally related B cells were identified out of 238 Ig V_H sequences from the PB of 10 MS patients and 4 CIS patients. These B cell clonal expansions shared identical CDR3 sequences and used VH gene segments of the VH1, VH3 or VH4 family, which corresponds to previous reports on expanded intrathecal B cells in MS[306,308,310,311]. The presence of a large VH1-69 expressing B cell clonal expansion in 1 CIS and 2 MS patients even skewed VH family usage to an overrepresentation of VH1 family members in these patients. In contrast, B cells from the PB of HC were heterogeneous and did not display clonal expansion.

Next to clonal expansion, indications of affinity matured PB B cells from MS and CIS patients were obtained. B cells from the PB of MS and CIS patients showed increased Ig V_H mutation frequencies in comparison to HC, suggesting antigen-experienced B cells undergoing affinity maturation. In addition, PB B cells from MS patients were more frequently mutated than those from CIS patients. The discrepancy in disease phase between early diseased CIS patients and clinically definite MS patients could account for the difference in mutation frequencies between these patient groups.

Previous reports on Ig V_H region sequencing did not find a restricted B cell response in MS PB and could not discriminate between PB B cells in MS and HC[127,316]. When considering that isolated $CD19^+$ B cells or total PBMC were examined in these studies and that the majority of B cells in the PB have a naïve $CD19^+CD27^-$ phenotype (chapter 4), we can conclude that these studies mainly focused on naïve B cells. It is therefore reasonable that no similarities were detected between naïve B cells from the PB and memory B cells and plasma cells

from the CSF. Our results show that a clonally expanded B cell response with possible affinity maturation can be found in the PB of MS and CIS patients when focusing on memory B cells. Memory B cells that are irrelevant to the disease are present in the PB as well. However, we have demonstrated binding of PB B cell clones to the HOG cell line (chapter 4), emphasizing the importance of peripheral B cells in the autoreactive immune response in MS pathogenesis.

Can the obtained results be caused by interference of the B cell immortalization process?

B cell immortalization could interfere with molecular analysis of B cells due to the introduction of viral DNA and cellular transformation that is accompanied by chromosomal instability and modulation of apoptosis[259,263]. In that case, mutations that were introduced in vitro could interfere with our results. However, several lines of evidence refute this possibility. VH family gene usage was similar to the germline distribution in PB of both patients and controls[322]. IgG^+ B cells expressed more mutations than IgM^+ B cells, mimicking the in vivo situation. Furthermore, immortalized B cells from HC showed a very heterogeneous repertoire with low Ig V_H mutation numbers and frequencies. Thus, the clonal expansion and increased Ig V_H mutation frequency of PB B cells from MS and CIS patients truly represents a restricted in vivo B cell response that is going on in response to repeated antigen exposure.

Are the clonally expanded PB B cells involved in disease pathogenesis?

It is possible that some of the retrieved B cell clonal expansions represent an irrelevant response towards a foreign antigen. However, the presence of large groups of clonally related B cells or their presence in multiple patients point towards their pathologic involvement. This is also suggested by the occurrence of clonally expanded PB B cells already early in disease (CIS). More prove is given by the presence of B cell clones expressing VH4-39, the most frequent VH4 gene in MS brain[126,307], in the PB of MS and CIS patients, but their lack in PB of HC. Moreover, PB B cells from MS and CIS patients displayed several features of autoreactive B cells. Indications were obtained for longer CDR3 regions in MS patients when compared to HC, as was previously demonstrated for B cells from MS CSF[312]. Additionally, PB B cells from MS and CIS patients were characterized by a higher frequency of negatively charged CDR3 sequences

in comparison to HC. This could confer an advantage for the recognition of positively charged antigens, such as the MS-related autoantigens MBP and MOG. Moreover, the finding of negatively charged CDR3 in B cells from MS CSF was previously linked to their entrance in the CNS[312]. Thus, B cells with negatively charged CDR3 in the PB of MS and CIS patients could have the capacity to travel towards the CNS and be implicated in disease pathogenesis.

It has to be noted that paired CSF and PB B cells were not included in this study, due to the lack of immortalized B cell lines from the PB and CSF of the same patient. In order to study the relation between clonal B cells in PB and CSF of MS patients in more detail, molecular B cell analysis of paired CSF and blood samples is warranted.

Together, the above findings support the possibility that peripheral B cells in MS patients carry a large proportion of disease-related and/or autoreactive B cells. This hypothesis is further confirmed by the data presented in chapter 4, showing autoreactivity for 24% and 22% of immortalized B cell lines from the PB of MS and CIS patients, respectively. Interestingly, 2 populations of clonally expanded B cells (clones 2 and 3) displayed reactivity to common intracellular antigens.

Which are the mechanisms by which PB B cells can be involved in disease pathogenesis?

The pathologic relevance of clonally expanded B cells in the PB of MS and CIS patients is not yet clear. Several possible pathways exist for the generation of restricted peripheral B cell responses. The PB clonally expanded B cells could represent the intrathecal immune response by diffusion of B cells and antibodies through the BBB into the circulation[381-383]. Increasing evidence shows that BBB impairment and leakage in MS is not only restricted to acute disease but starts already at an early stage[384], which could explain the finding of clonally related PB B cells in CIS patients. Furthermore, indications for BBB disturbance have now been collected in normal appearing white matter (NAWM) and inflammatory silent inactive lesions[385-387]. Alternatively, autoreactive memory B cells could recirculate to attract other inflammatory cells into the CNS by functioning as APC[158,159]. Another possibility is the development of a secondary immune response in the PB. B cells could be activated by antigens that have leaked in the PB or because of epitope spreading[6,7]. In this case, the observed peripheral

B cell response is an epiphenomenon of tissue damage instead of being primarily involved in disease pathogenesis.

Expansion of autoreactive B cells could occur in the periphery due to decreased suppressive functions of regulatory T cells[36] and could be sustained by repeated antigen exposure as a consequence of BBB leakage. The same mechanism could account for the activation of recirculating memory B cells and their differentiation into antibody-secreting cells that travel towards the CNS.

III. Memory B cells: major players in MS and B cell immortalization

The distribution of different B cell subsets in the PB and CSF of MS patients was further analyzed in **chapter 4**. Memory B cells were identified as major constituents of the B cell repertoire in PB (4.1%) and CSF (3.4%) of MS patients. Moreover, memory B cells were identified as main target population of B cell immortalization when IgG^+ B cells were first isolated, both in PB and CSF. Naïve B cells were the predominant B cell subset in MS PB and were also sensitive to B cell immortalization. However, most naïve B cells are not related to disease and can be excluded from further analysis by the use of isolated IgG^+ B cells. The presence of plasma blasts and plasma cells was limited to MS CSF (2.3% and 4.4%, respectively), where they are responsible for oligoclonal IgG production[146,148,153]. Plasma cells were indicated as the main B cell effector population in the CSF of MS patients but did not express the EBV receptor. It is interesting to note that half of the plasma blasts were sensitive to B cell immortalization. The possibility to further analyze CSF plasma blasts is very valuable, since these cells were previously described as main effector B cell subset in the CNS of MS patients[145,153].

In conclusion, memory B cells are major constituents of the B cell population in the PB and CSF. Next to plasma cells, memory B cells are clonally expanded in MS CSF and PB due to chronic antigen stimulation (Chapter 3)[126,149,308]. The memory B cell pool thus represents an ideal repository of antibody specificities that were generated in the course of an immune response and form a valuable population for analysis in MS.

IV. The B cell pool in PB and CSF of MS patients and in PB of CIS patients contains autoreactive B cell clones

The second part of **chapter 4** comprised an antibody specificity screening of immortalized B cell lines from MS and CIS patients to gain more insight into their role in the disease process. Identification of the specific target antigens could even lead to novel markers for disease.

Does our panel of immortalized B cell lines from MS and CIS patients contain autoreactive B cells?

Several antigenic targets of autoreactive B cells in MS have been suggested, but their specificity and pathological relevance remain largely unknown. Based on the results described in chapter 3, we hypothesized that a proportion of PB B cells in MS and CIS are autoreactive and are involved in the disease process.

Flow cytometric analysis of antibody binding to HOG, A549 and PBMC demonstrated antibody specificity to intracellular autoantigens for PB immortalized B cells from MS and CIS patients. HOG-specific binding was demonstrated for 4 B cell clones from the PB of MS patients, which was also confirmed by immunocytochemistry. In addition, 1 PB B cell clone from the CIS group showed binding to PBMC but not to HOG and A549 cells, suggesting its specificity for antigens that are exclusively present in PBMC. Reactivity to more common intracellular targets was indicated for 22% of immortalized B cells from MS and CIS patients by binding to all tested cell types. This group of B cell clones with reactivity to intracellular targets also included 2 populations of clonally expanded B cells (chapter 3). The other B cell clonal expansions, as well as the CSF B cell lines, did not show reactivity to the tested cell types. These results confirm that autoreactive B cells are present in the PB of MS and CIS patients and emphasize the pathological relevance of these peripheral B cells.

What is the role of autoantibodies specific for intracellular antigens in autoimmune disease?

Using flow cytometric analysis, we demonstrated reactivity of immortalized B cell lines from the PB of MS and CIS patients towards intracellular targets. Extracellular reactivity could not be measured, although this could be due to insufficient antibody concentrations and/or specificity for low abundant cell

surface proteins. Antibodies directed against intracellular targets have already been described in autoimmune diseases including SLE[388], RA[389], MG[291] and MS[357-359]. In MS, these antibodies recognize targets such as CNPase[211], transaldolase[364], translated Alu repeats[365] and DNA[358,359].

Antibodies and B cells directed against intracellular autoantigens could be produced during chronic brain inflammation as a secondary response to tissue damage. During apoptosis, proteins are modified by cleavage through proteases, caspases, endonucleases or post-translational modifications[390-392]. Furthermore, caspases and granzymes can be activated following killing of virally infected cells by cytotoxic T cells and NK cells[393]. Modified proteins can then be presented on apoptotic cells, as in SLE[394], and subsequently be recognized by the immune system[395]. Peripheral tolerance mechanisms are thus circumvented and autoimmunity is triggered. Once this secondary immune response has been established, epitope spreading can lead to the generation of autoantibodies against the whole protein. Antibodies with reactivity towards cleaved or modified autoantigens have already been described in SLE, scleroderma, psoriasis and RA[396]. In MS, the citrullinated form of MBP can induce EAE and is increased in MS brain[397,398].

However, antibodies directed against intracellular autoantigens could be part of the pathologic autoimmune response in MS as well. Previously, several intracellular brain antigens were identified as targets of the humoral immune response in MS. Increased numbers of antibodies binding to heath shock proteins were detected in the CSF of MS patients[213]. Further, autoantibodies directed against intracellular oligodendrocyte antigens, such as CNPase[211] and transaldolase[210], but also against axonal and neuronal antigens, including tubulin[200] and neurofilament[212], have been demonstrated in MS patients. Immunization of mice with neurofilament light chain protein was shown to induce MS-like symptoms resulting in axonal loss and gray matter pathology[366]. This effect was thought to be mediated by anti-neurofilament antibodies that enter the axon and interfere with the axonal skeleton. Results from our own research group indicated sperm associated antigen 16 (SPAG16) isoform 2 as novel target antigen of B cells and antibodies in MS patients. Injection of anti-SPAG16 isoform 2 antibodies at disease onset in EAE animals resulted in an exacerbation of the disease course and increased infiltration of inflammatory cells (unpublished results). These data all demonstrate the ability of antibodies

against intracellular antigens to exert pathologic effects on their target cells and thus contribute to disease pathogenesis.

Antibodies directed against intracellular targets could further be involved in immune regulation since natural antibodies have been implicated in blocking epitopes in damaged tissues or facilitating the clearance of self-proteins[362,363].

The origin of these antibodies is not exactly known. Next to the modification of autoantigens, brain inflammation can also lead to enhanced antigen processing and presentation. Moreover, intracellular antigens are exposed to the immune system during apoptosis. This leads to the formation of immune complexes of intracellular antigens, which amplifies the ongoing immune response.

In conclusion, antibodies specific for intracellular antigens could play a primary role in MS pathology. The finding of HOG-specific B cell clones in this study is in favour of this hypothesis. Alternatively, they could arise as by-products of inflammation and thereby aggravate ongoing chronic inflammation or evoke secondary symptoms. In this case, they could still be relevant as biomarkers for disease. Identification of the target antigens and analysis of the functional significance of these antibodies reactive to intracellular antigens is warranted to further elucidate their role in the disease process of MS.

What are the target antigens of autoreactive B cells in MS?

Preliminary experiments using immunoprecipitation on HOG lysates and identification of the purified proteins indicated cystatin-A and ASPP2 as possible target antigens of 2 MS CSF B cell clones. Immune responses directed against both antigens could be part of a secondary immune response due to tissue damage and apoptosis, as described above. Anti-ASPP2 antibodies could contribute to decreased apoptosis of autoreactive lymphocytes in MS[373]. This could in turn lead to increased damage to brain cells and the increased presentation of intracellular autoantigens, generating antibodies reactive against intracellular targets such as cystatin-A. Both identifications could not be assigned with 100% certainty, as they were only found with 1 unique peptide and were not located at the correct position in SDS-PAGE. Therefore, these experiments have to be repeated and further characterization of the 2 MS CSF clones is necessary to confirm the identifications.

Candidate target antigens of several other B cell lines could not be identified, including 1 clonally expanded and 3 HOG-specific PB B cell clones. In addition,

no reactivity of the immortalized B cells was detected against MOG, several viruses and brain tissue, although not all immortalized B cell lines were tested. Nevertheless, many research groups have experienced difficulties in identifying B cell target antigens in MS. The group of Owens was unable to detect reactivity of recombinant antibodies from MS CSF towards MBP, MOG or PLP[340]. Von Büdingen *et al.* showed binding of recombinant antibodies from MS CSF plasma cells to MS brain tissue but the target antigens remained unknown[206]. Several explanations can account for the difficulty of identifying B cell target antigens. First, poor affinity of the produced antibodies and loss of reactivity due to tissue fixation could hamper antigen identification. Second, it is possible that the antigen is only minimally represented in target tissue which renders its detection almost impossible using existing immunological and proteomic techniques. Alternatively, autoreactive B cells are directed against modified proteins, that are often not represented in brain tissue or cells. Finally, B cells and antibodies in MS might not be directed against myelin or other proteins but against (glyco)lipids. Anti-lipid autoantibodies can exert pathophysiological effects, as was shown for antibodies against gangliosides in Guillain-Barré syndrome, a group of neuropathies with motor and/or sensory peripheral nerve dysfunction[399]. In addition, anti-lipid antibodies have already been identified in MS[187,195,198,202,374,375]. Antibodies directed against lipids are more difficult to isolate and identify owing to inappropriate representation by Western blotting or phage display, their large number and hydrophobicity. To circumvent these problems, antibody reactivity to myelin lipids could be determined using ELISA or lipid microarrays.

General conclusion and future perspectives

The results obtained in this thesis demonstrate that B cell immortalization is a valuable technology for analysis of the humoral immune response in autoimmune diseases such as MS. The finding of clonally expanded, highly mutated and autoreactive B cells in the PB of MS and CIS patients point towards the involvement of peripheral B cells in MS pathogenesis. B cell clones specific to the HOG cell line and PBMC were evidenced in the PB of MS and CIS patients, while the CSF of an MS patient harboured 2 B cell clones directed against

cystatin-A and ASPP2. Further research is necessary to determine whether these peripheral and intrathecal autoreactive B cells are involved in the primary pathological response or a secondary immune response following tissue damage. However, B cells reactive to oligodendrocytes were indicated in the PB of MS patients by binding of their antibodies to HOG cells, suggesting their pathological relevance. This autoantibody response can be evoked by exposure of intracellular antigens following increased apoptosis in the MS brain. Chronic brain inflammation can result in repeated antigen exposure, which can ultimately result in clonal expansion of B cells with reactivity towards intracellular autoantigens in the CSF, but also in the PB following BBB leakage.

Further identification of the target antigens of clonally expanded and autoreactive B cells from MS and CIS patients is necessary to confirm their pathological relevance. In addition, novel disease markers for diagnosis and prognosis or novel targets for therapy could be identified. A wide antibody reactivity screening could aid in identifying the specific antigens. Antibodies from immortalized B cell lines could therefore be screened for reactivity towards known myelin lipids and proteins, viruses and multiple human brain cell lines. Specificity for myelin proteins and lipids could be investigated by the use of microarrays and ELISA. In addition, phage display technology could be applied for target antigen identification when using cDNA libraries from MS or normal human brain. Further, IP of the antibodies with lysates of HOG cells and brain tissue followed by mass spectrometry should be continued to determine the target antigens of all relevant B cell lines. Cystatin-A and ASPP2 also have to be confirmed as target antigens of the 2 MS CSF B cell clones. Hereby, competition for binding with the target antigen can be tested using a recombinant antibody of the same specificity. Alternatively, binding of the antibodies to the purified antigen can be examined to confirm specificity.

Moreover, the pathological relevance of the intracellular autoantibodies should be studied in order to get more insight into the underlying disease processes. The ability of the intracellular autoantibodies to neutralize their target cells, for example the HOG cell line, could be investigated by in vitro killing assays. When the target antigen is identified, a cell line transfected with this antigen could be used to screen for cytotoxicity. Injection of the purified antibodies in EAE could be done to analyze their in vivo relevance, by monitoring the effect on disease course, clinical score, demyelination and inflammatory infiltration. Binding of MS

OCB from the CSF to the target antigen using IEF and immunoblotting could further demonstrate their in vivo relevance, as well as measurement of the antibodies in a large cohort of MS and CIS patients by means of ELISA. In order to obtain large amounts of purified antibodies for all these analyses, recombinant antibodies could be produced without the need of reculturing the immortalized B cell lines.

In order to compare oligoclonal B cell responses between the PB and CSF, immortalized B cells from paired PB and CSF could be used for molecular Ig V_H analysis. However, the current B cell immortalization procedure should then first be optimized for the immortalization of B cells originating from the CSF. Large volumes of CSF should be used to obtain sufficient B cell numbers for efficient transformation. Stronger and faster activating stimuli might be needed to help the CSF cells overcome EBV-induced stress. In this context, polyclonal B cell stimuli that target an extracellular ligand, such as CD40L, could be used instead of CpG2006 that binds the intracellular TLR9.

All together, the results of this thesis indicate that both intrathecal and peripheral autoreactive and/or clonally expanded B cells are implicated in MS pathogenesis. Further research on the targets and pathological relevance of these B cells is necessary to elucidate the underlying disease mechanisms and for the identification of novel disease markers.

6

Samenvatting

Multiple sclerose (MS) is een auto-immuunziekte die voorkomt bij 1 op 1000 personen. De isolerende myelineschede rond de zenuwuitlopers in het centrale zenuwstelsel (CZS) wordt afgebroken (demyelinisatie) door een chronische inflammatoire reactie, waardoor de signaal geleiding tussen neuronen verstoord wordt. Dit leidt tot symptomen zoals spierzwakte, neuritis optica en vermoeidheid. In de hersenen zijn zogenaamde plaques of lesies zichtbaar die infiltraten van immuuncellen bevatten. De oorzaak van MS is niet gekend, alhoewel wetenschappelijke studies aangetoond hebben dat zowel genetische als omgevingsfactoren een rol spelen. Huidige therapieën zijn gericht op het verbeteren van symptomen en het verminderen en uitstellen van opstoten.
In 85% van de gevallen start MS met een *clinically isolated syndrome* (CIS). Dit is een eerste klinische opstoot die gepaard gaat met demyelinisatie, neurologische plaques en symptomen die lijken op MS. Bij het voorkomen van een 2^{e} opstoot kan de definitieve diagnose van MS gesteld worden. Een deel van de CIS-patiënten ondergaat geen verdere opstoten.

T-cellen werden lange tijd beschouwd als de belangrijkste spelers in het ziekteproces. Recent is er echter ook meer aandacht voor de betrokkenheid van de humorale immuunrespons. Het belangrijkste bewijs voor een rol van B-cellen in MS is het voorkomen van oligoklonale banden die gevormd worden door verhoogde productie van immunoglobuline G (IgG) antilichamen in het ruggenmergvocht (CSV). Ondanks intensief onderzoek zijn de specifieke antigenen van de oligoklonale antilichamen nog steeds onbekend. Klonale expansie van B-cellen in het CSV en lesies, antilichaam en complement depositie in lesies en het voorkomen van ectopische germinale centra in de hersenen van progressieve patiënten wijzen tevens op een associatie van B-cellen met MS. De betrokkenheid van B-cellen uit het perifere bloed (PB) is nog onduidelijk. Verscheidene autoreactieve B-cellen en antilichamen werden wel al aangetoond in het PB of serum van MS-patiënten. Bovendien wordt plasmaferese met succes gebruikt om een subgroep van MS-patiënten te behandelen.

In deze studie werd B-cel immortalisatie gebruikt om de humorale immuunrespons in MS en CIS verder te karakteriseren. B-cel immortalisatie wordt gebruikt voor de productie van humane antilichamen waarbij B-cellen na infectie met Epstein-Barr virus (EBV) getransformeerd worden in continu

groeiende B-cellijnen. Aangezien de geproduceerde antilichamen representatief zijn voor de *in vivo* B-cel respons, kan B-cel immortalisatie toegepast worden voor het bestuderen van B-cellen bij patiënten met een auto-immuunziekte. In dit hoofdstuk worden de belangrijkste resultaten van dit doctoraat samengevat aan de hand van de voorop gestelde doelstellingen.

I. Ontwikkeling van een verbeterde B-cel immortalisatie procedure

In een eerste luik van dit doctoraat werd een verbeterde methode voor B-cel immortalisatie ontwikkeld. Bestaande immortalisatie procedures werden gekenmerkt door gebrekkige efficiëntie of reproduceerbaarheid. Door het gebruik van grote aantallen B-cellen voor EBV-infectie kunnen B-cellijnen met een groeivoordeel de andere B-cellen overgroeien, waardoor een juiste representatie van de *in vivo* B-cel respons onmogelijk wordt. Er was dus nood aan een B-cel immortalisatie methode met een hoge efficiëntie voor het immortaliseren van lage B-cel aantallen per well. Met behulp van deze methode zou de productie van (autoreactieve) antilichamen van patiënten met een auto-immune aandoening mogelijk moeten zijn.

In **hoofdstuk 2** werd de ontwikkeling beschreven van een verbeterde B-cel immortalisatie methode voor de snelle aanmaak van IgG-producerende B-cellijnen. Deze werd gekenmerkt door gelijktijdige CpG2006 stimulatie en EBV-infectie van B-cellen gedurende 2 weken, gevolgd door restimulatie gedurende 1 week. IgG^+ B cellen werden op voorhand geselecteerd omdat deze het meest relevant zijn in het ziekteproces. *Fluorescence activated cell sorting* (FACS) voor IgG en CD22 resulteerde hierbij in de hoogste opbrengst, zuiverheid en immortalisatie efficiëntie. Door gebruik te maken van deze procedure konden immortalisatie efficiënties tot 83% en een percentage monoklonale B-cellijnen van 87% behaald worden. De verbeterde immortalisatie methode kon ook toegepast worden op totale perifere bloed mononucleaire cellen (PBMC) met een efficiëntie van 95% en een percentage monoklonale B-cellijnen van 70%. De meerderheid van de resulterende B-cellijnen was in dit geval wel immunoglobuline M+ (IgM^+), naargelang de verdeling van naïeve en geheugen B-cellen in het PB. Door de combinatie van een hoge immortalisatie efficiëntie en

hoge monoklonaliteit bij de resulterende B-cellijnen, is onze verbeterde B-cel immortalisatie methode de eerste waarbij uitkloneren niet langer noodzakelijk is. Bijgevolg kan de productie van monoklonale antilichamen veel sneller en eenvoudiger gebeuren. Bovendien werd aangetoond dat de gevormde geïmmortaliseerde B-cellijnen een goede representatie geven van de B-cellen die *in vivo* voorkomen.

Mogelijke toepassingen van deze technologie zijn onder andere de analyse van B-cel reacties in auto-immune aandoeningen en de productie van monoklonale antilichamen voor immunotherapie of het neutraliseren van een virus. In dit doctoraat werd de B-cel immortalisatie technologie gebruikt om meer inzicht te verkrijgen in de B-cel respons in MS and CIS.

II. Analyse van B-cel klonale expansie en diversiteit in MS en CIS

Een 2^{e} doelstelling was een verdere karakterisatie van B-cel diversiteit en klonale expansie in MS- en CIS-patiënten. Klonaal geëxpandeerde B-cellen werden reeds meermaals aangetoond in het CSV en in de hersenen van MS- en CIS-patiënten, maar niet in het PB. Affiniteitsmaturatie trad hierbij op om een B-cel receptor met hogere affiniteit voor het antigen te creëren. In deze studie werd een sequentie analyse van het variabele gebied van de zware keten van de B-cel receptor (V_H) uitgevoerd op geïmmortaliseerde B-cellen uit het PB en CSV van MS-patiënten en uit het PB van CIS-patiënten.

Zoals beschreven in **hoofdstuk 3**, werden klonaal geëxpandeerde B-cel populaties niet enkel in het CSV maar ook in het PB van MS- en CIS-patiënten aangetoond met indicaties voor affiniteitsmaturatie. Een bevestiging voor de gerestricteerde intrathecale B-cel respons in MS werd verkregen door de identificatie van 2 klonaal gerelateerde B-cellen in het CSV van een MS-patiënt. Deze B-cel kloons maakten bovendien gebruik van het VH4-39 gen dat reeds aangetoond werd in de hersenen van MS-patiënten, wat wijst op een verband met het ziekteproces en autoreactiviteit van deze B-cellen doet vermoeden. Negen populaties van klonaal geëxpandeerde B-cellen werden geïdentificeerd in het PB van 10 MS- en 4 CIS-patiënten. Deze brachten allemaal VH

gensegmenten van de VH1, VH3 of VH4 familie tot expressie, zoals eerder gerapporteerd werd voor intrathecaal geëxpandeerde B-cellen in MS. Verhoogde V_H mutatiefrequenties werden waargenomen in B-cellijnen uit het PB van MS- en CIS-patiënten in vergelijking met gezonde controles (HC). Dit wijst op de aanwezigheid van chronisch geactiveerde B-cellen die affiniteitsmaturatie ondergaan. Bovendien vertoonden PB B-cellen van MS-patiënten een hogere V_H mutatiefrequentie dan die van CIS-patiënten. Het verschil in mutatiefrequentie tussen MS en CIS kan verklaard worden door het verschil in de ziektefase, die verder gevorderd is bij MS-patiënten. B-cellijnen uit het PB van HC waren heterogeen en vertoonden geen klonale expansie of verhoogde immunoglobuline (Ig) V_H mutatiefrequentie, wat bewijst dat onze resultaten niet beïnvloed werden door de B-cel transformatie. Dus, de klonaal geëxpandeerde en sterk gemuteerde B-cellen bij MS- en CIS-patiënten wijzen op een gerestricteerde *in vivo* B-cel respons ten gevolge van chronische antigen stimulatie.
Verder werden aanwijzingen verkregen voor het voorkomen van langere en negatief geladen *complementarity determining region 3* (CDR3)-sequenties in het PB van MS-patiënten. Lange CDR3-gebieden werden reeds geassocieerd met MS CSV B-cellen en autoreactiviteit. Een negatieve CDR3-sequentie zou een voordeel kunnen geven voor binnengaan in het CZS of voor herkenning van positief geladen antigenen, zoals *myelin basic protein* (MBP). Het is dus mogelijk dat het PB van MS-patiënten een aanzienlijke proportie ziekte-gerelateerde en autoreactieve B-cellen bevat.

Al deze resultaten wijzen op een rol voor perifere B-cellen in het ziekteproces bij MS en CIS. Eerdere moleculaire B-cel studies vonden geen verschil tussen PB B-cellen bij MS of CIS en HC, mogelijk door het vergelijken van CSV geheugen B-cellen met PB naïeve B-cellen. Onze studie toont aan dat klonale B-cel expansies in het PB wel aangetoond kunnen worden bij analyse van geheugen B-cellen. Of de perifere B-cel klonale expansies deel uitmaken van de primair pathologische of secundaire immuunrespons als gevolg van weefselschade, moet nog verder onderzocht worden. Hiertoe moeten B-cellen uit gepaard CSV en PB vergeleken worden en de specifieke antigenen van de perifere klonale expansies bepaald worden. Een eerste karakterisatie van de B-celreactiviteit werd uitgevoerd in hoofdstuk 4.

III. Analyse van B-cel distributie en antigen reactiviteit in MS en CIS

In het 3e deel van dit doctoraat werd het B-cel repertoire in het PB en CSV van MS-patiënten gekarakteriseerd met behulp van fenotypische analyse. Vervolgens werd de antigen reactiviteit van geïmmortaliseerde B-cellijnen uit het PB en CSV van MS-patiënten en het PB van CIS-patiënten bepaald. Op basis van de voorgaande experimenten werd als hypothese gesteld dat een proportie van de PB B-cellen in MS en CIS autoreactief is en dus een rol speelt in de pathologische immuunrespons. Identificatie van hun target antigenen kan leiden tot meer inzicht in het onderliggende ziekteproces, maar ook tot de ontwikkeling van nieuwe merkers voor diagnose, prognose en nieuwe targets voor therapie.

In **hoofdstuk 4** werden geheugen B-cellen geïdentificeerd als belangrijkste doelgroep voor B-cel immortalisatie na preselectie van IgG^+ B-cellen. Deze B-cel populatie omvat het volledige spectrum van antilichaam specificiteiten die tijdens een immuunrespons gevormd worden in zowel CSV als PB van MS-patiënten. Ongeveer de helft van de plasmablasten, die in sommige studies beschreven werden als belangrijkste effector B-cel populatie in het CZS bij MS, was ook vatbaar voor EBV-infectie. In onze studie waren plasmacellen de meest voorkomende antilichaam-secreterende cellen in het CSV van MS patiënten, hoewel ze niet vatbaar bleken voor EBV-infectie door het gebrek aan CD21-expressie. CD21-expressie was wel terug te vinden op nagenoeg alle naïeve B-cellen, die de grootste B-celpopulatie vormden in het PB maar minder relevant zijn in het ziekteproces van autoimmune aandoeningen zoals MS.

Intracellulaire autoreactiviteit werd aangetoond voor perifere B-cellen van MS- en CIS-patiënten door FACS-analyse van antilichaambinding aan verschillende celtypes. Specificiteit voor humane oligodendroglioma (HOG) cellen werd aangetoond voor 4 B-cel kloons uit het PB van MS-patiënten, terwijl 1 B-cel kloon uit het PB van de CIS-patiënten specifieke binding vertoonde aan PBMC. Algemene intracellulaire reactiviteit werd dan weer gemeten voor 22% van de geïmmortaliseerde B-cellijnen van MS- en CIS-patiënten door antilichaambinding aan HOG-cellen, PBMC en de controle humane alveolaire epitheel cellijn A549. HOG-specifieke B-cellen kunnen ondermeer gericht zijn tegen oligodendrocyt (precursor) antigenen zoals MBP. Cytokines, *human leukocyte antigen* (HLA)

moleculen of T-cel receptor moleculen kunnen dan weer targets zijn voor PBMC-specifieke B-cellen.

Antilichamen gericht tegen intracellulaire antigenen werden reeds aangetoond in MS en kunnen betrokken zijn bij de primaire pathologische immuunrespons of bij een secondaire immuunrespons ten gevolge van weefselschade. Het terugvinden van HOG-specifieke B-cellen in het PB van MS-patiënten wijst op de eerste hypothese. De manier waarop antilichamen gericht tegen intracellulaire autoantigenen ontstaan, is nog onbekend. Verhoogde apoptose bij chronische ontsteking van de hersenen kan wel aanleiding geven tot verhoogde antigen-presentatie. Verder onderzoek naar de specifieke targets van de autoreactieve B-cellen is nodig meer duidelijkheid te geven over hun betrokkenheid in het ziekteproces.

Tenslotte werden cystatine-A en *apoptosis-stimulating protein of p53-2* (ASPP2) geïdentificeerd als kandidaat antigenen van 2 B-cel kloons uit het CSV van een MS-patiënt. Een B-cel respons tegen deze antigenen kan eveneens het resultaat zijn van een secundaire immuunrespons ten gevolge van apoptose en weefselschade. Antilichamen specifiek voor ASPP2 kunnen bijdragen tot de verminderde apoptose van autoreactieve immuuncellen in MS. Dit kan leiden tot verhoogde schade aan hersencellen en presentatie van intracellulaire hersenantigenen. Hierdoor kunnen dan weer autoantilichamen tegen intracellulaire eiwitten zoals cystatine-A gevormd worden. Herhaling van deze experimenten is echter nodig om reproduceerbaarheid aan te tonen en verdere karakterisatie van deze geïmmortaliseerde B-cellijnen is gewenst om cystatine-A en ASPP2 te bevestigen als kandidaat antigenen van MS CSV B-cellen.

Conclusie

De resultaten die in dit doctoraat behaald werden, bewijzen dat B-cel immortalisatie een waardevolle technologie is voor analyse van de humorale immuunrespons in auto-immuunziekten zoals MS. Het vinden van klonaal geëxpandeerde, sterk gemuteerde en autoreactieve B-cellen in het PB van MS- en CIS-patiënten wijst duidelijk op de betrokkenheid van perifere B-cellen in de

pathogenese van MS. B-cel kloons specifiek voor de HOG-cellijn en PBMC werden aangetoond in het PB van MS- en CIS-patiënten. Het CSF van een MS-patiënt bevatte 2 B-cel kloons die gericht waren tegen cystatine-A en ASPP2. Verder onderzoek is noodzakelijk om te bepalen of deze perifere en intrathecale autoreactieve B-cellen betrokken zijn in de primaire pathologische respons of in een secundaire immuunrespons ten gevolge van weefselschade. De aanwezigheid van B-cellen gericht tegen oligodendrocyten, zoals aangetoond door binding van hun antilichamen aan de HOG-cellijn, wijst echter op hun relevantie in de ziekte pathologie. Intracellulaire antilichamen ontstaan mogelijk door verhoogde blootstelling van autoantigenen als gevolg van de sterke apoptose in de hersenen van MS-patiënten. Chronische ontsteking van de hersenen kan resulteren in herhaalde blootstelling aan de autoantigenen, die uiteindelijk kan leiden tot klonale expansie van B-cellen met intracellulaire autoreactiviteit, niet enkel in het CSV maar ook in het PB na verstoring van de bloed-hersen barrière.

Verder onderzoek naar de target antigenen en pathologische relevantie van de klonaal geëxpandeerde en autoreactieve B-cellen in PB en CSV van MS- en CIS-patiënten is noodzakelijk. Op deze manier kunnen de onderliggende ziektemechanismen verhelderd worden en kunnen nieuwe ziektemerkers voor diagnose en therapie geïdentificeerd worden.

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Curriculum Vitae

Judith Fraussen werd geboren op 21 februari 1984 te Bilzen. In 2002 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Latijn-Wiskunde-Wetenschappen aan het Heilig-Hart College te Lanaken. Vervolgens startte ze haar opleiding Biomedische Wetenschappen aan de Universiteit Hasselt/transnationale Universiteit Limburg (tUL) waar ze in 2005 haar diploma bachelor in de Biomedische Wetenschappen met onderscheiding behaalde. Aansluitend voltooide ze de master in de Biomedische Wetenschappen eveneens aan de Universiteit Hasselt waar ze in 2006 afstudeerde met grote onderscheiding. Haar eindwerk, getiteld 'Rol van de $CD4^{+}CD28^{nul}$ T-cellen in de pathogenese van auto-immune aandoeningen' werd uitgevoerd aan het Biomedisch Onderzoeksinstituut van de Universiteit Hasselt in het labo van Prof. Dr. Piet Stinissen. Deze afstudeerthesis was eveneens goed voor de 3^{e} prijs in de rangschikking voor de beste afstudeerscriptie in de masteropleiding biomedische wetenschappen, afstudeerrichting Klinische Moleculaire Wetenschappen. In september 2006 startte ze haar doctoraat aan het Biomedisch Onderzoeksinstituut van de Universiteit Hasselt rond de humorale immuunrespons in multiple sclerose (MS) met een tUL-beurs. Tijdens deze periode van 4 jaar volgde ze een onderwijsprofessionaliseringstraject en een cursus academisch Engels.

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"Success is not final, failure is not fatal:
it is the courage to continue that counts"
Winston S. Churchill, 1874-1965