RECOGNITION OF EPSTEIN-BARR VIRUS IN MULTIPLE SCLEROSIS

Gijsbert P. van Nierop

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HERKENNING VAN HET EPSTEIN-BARR VIRUS IN MULTIPLE SCLEROSE

Gijsbert P. van Nierop

The research in this thesis was performed at the Academic Center of Excellence for multiple sclerosis research ErasMS of the departments of Viroscience, Neurology and Immunology at the Erasmus MC, Rotterdam, the Netherlands within the framework of the Erasmus Postgraduate School Molecular Medicine. The research presented in this thesis was financially supported by the Dutch MS research foundation (grant number 09-670MS).

Printing of this thesis was financially supported by the Dutch MS Research foundation (stichting MS research) and GR Instruments BV. All contributors are gratefully acknowledged.

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This thesis was printed by ProefschriftMaken (www.proefschriftmaken.nl)

ISBN/EAN: 9789462958050

Recognition of Epstein-Barr Virus in Multiple Sclerosis
Herkenning van het Epstein-Barr virus in multiple sclerose

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van
het College voor Promoties.

De openbare verdediging
zal plaatsvinden op

Woensdag 7 Februari 2018 om 9:30 uur

door

Gijsbert Paul van Nierop
geboren te Eindhoven

Erasmus University Rotterdam

Promotiecommissie

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Voor mijn meisjes

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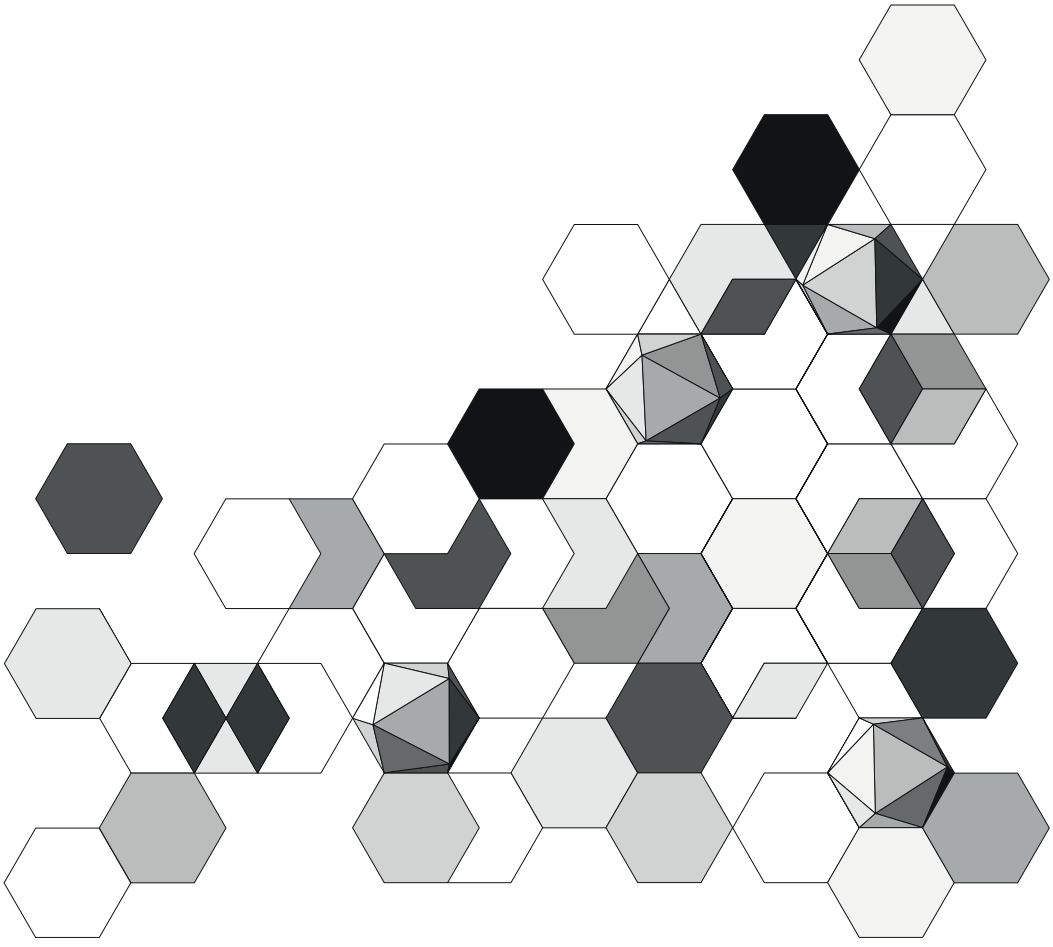
General introduction



Multiple sclerosis: symptoms, disease progression, diagnosis and disease management

Multiple sclerosis (MS) is a very heterogeneous, often devastating neurological disease.[1] In the Netherlands, over 20.000 patients suffer from MS and the incidence is increasing, mainly among woman.[2,3] Typical symptoms are fatigue, sensory and motoric disturbances, visual impairment and paralysis caused by inflammation of the central nervous system (CNS).[1] Clinical presentation and disease progression varies greatly between MS patients and is highly unpredictable. Most patients initially present with a clinically isolated syndrome (CIS) between the age of 20 and 40 years, which is characterized by neurological complaints that last over 24 hours. Over 60% of CIS patients develop MS within one year.[4] A CIS patient that experiences a second clinical attack is classified as having a relapsing remitting (RRMS) disease course.[5] Symptoms typically reside, yet part of the RRMS patients suffer from residual neurological deficit after a relapse.[6] In most cases, RRMS is followed by a secondary progressive (SPMS) phase, characterized by a gradual increase in neurological deficit with a reduction or absence of relapses. 10-15% of MS patients experience a gradual progression from disease onset, termed primary progressive MS (PPMS) (Figure 1A).[1]

Clinically definite MS is diagnosed when a patient presents with neurological complaints for a second time, or with the aid of magnetic resonance imaging (MRI) and occasionally, lumbar puncture to collect cerebrospinal fluid (CSF).[5] MRI is of great added value for diagnosis and to some extend, can visualize MS pathology. Compromised integrity of the blood-brain barrier (BBB) that surrounds the vasculature of the brain characterizes active lesions. This is visualized by contrast enhancement using intravenous administration of gadolinium. The revised McDonalds diagnostic criteria for MS are met when more than one lesion in the brain or spinal cord are detected on MRI, that are disseminated in space and time.[5] Active lesions surround blood vessels and expand radially over time. Early in disease lesions are typically located in periventricular, juxta-cortical, infratentorial or spinal cord white matter.[5] MS lesions are observed in white (WM) and grey matter (GM), e.g. the cortex. Longitudinal imaging studies show GM damage is secondary to WM damage independent of clinical disease course.[7,8] WM inflammation correlates with BBB leakage and is continuous throughout disease development, but is most prominent in the early phase disease.[1,5] GM lesions are associated with meningeal inflammation which is most prominent in progressive forms and late stage MS.[9,10] Gradually, irreversibly damaged areas increase in number and size as a result of the characteristic white matter scars formation, i.e. sclerosis. The resulting neurodegeneration and loss of brain volume, termed brain atrophy, is pronounced in advanced MS (Figure 1B and C).[7]

CSF is analyzed for intrathecal antibody production which is marked by the presence of oligoclonal Ig gamma (IgG) populations.[11] Clonality of IgG populations is shown by iso-electric focusing of CSF IgG. The presence of oligoclonal IgG bands in CIS patients are associated with a near ten-fold increased risk of developing MS.[12]

Currently MS is treated with a variety of immune modulatory drugs that damped the overall immune system, deplete or inhibit specific immune cell subsets or block migration of immune cells to the CNS, with a variable level of success. Recent advances in drug development show great efficacy in the relapsing remitting disease course with lowered clinical relapse rates and reduced MRI activity.[13] However, due to their broad effect they are associated with a wide variety of side effects including secondary autoimmune diseases and opportunistic infections.[13,14] Early treatment initiation with immune-modulatory drugs delays the onset of disease progression. Unfortunately, disease-modifying drugs that specifically target the gradual progressive phase of MS have limited efficacy.[1,13] Several criteria for safe and effective treatment are therefore not met, which underlines the need for more specific targets. Novel insight into the pathogenic mechanism at play are therefore of fundamental importance.

Biomarkers and clinical specimen

Although MS can be diagnosed using clinical parameters alone, or aided by MRI and intrathecal IgG, additional biomarkers for early and reliable diagnosis of MS or the prognosis and monitoring of disease progression are called for. Primarily because early detection and treatment of MS ameliorates disease.[15] Additionally, because biomarkers may help to identify possible therapeutic targets, monitor therapy response or identify factors that are involved in MS pathology.[16,17]

Due to the limited accessibility to the site of inflammation, identifying etiological factors for MS lesions is challenging. Likely, initiating factors are most reliably detected in newly forming lesions, early in disease development. However, collecting brain biopsies of MS lesions during early MS is highly invasive and is therefore only considered in fulminant or atypical MS cases. To gain insight in etiological factors and pathological processes early in MS, most studies rely on more readily accessible clinical specimen including CSF, peripheral blood (PB) and urine. CSF is produced by the choroid plexus in the brain ventricles and flows throughout the subarachnoid space that surrounds the brain and spinal cord (Figure 1B).[18] CSF drains soluble proteins in the interstitial fluid via the perivascular space to cervical draining lymph nodes.[19] Furthermore, intrathecal lymphocytes including their effector molecules are located in these CSF-drained areas. CSF therefore is a good alternative for brain biopsies to study inflammatory mediators and damage-associated proteins, albeit with compromised anatomical localization.[11]

More readily accessible clinical specimen for the identification of biomarkers are peripheral blood and urine. Blood serum or plasma and urine are used to discover CNS- or immune-related markers that associate with CNS pathology.[16,20] PB mononuclear cells (PBMC) are frequently used to identify systemic immune aberrancies.

Neuropathology of MS

The hallmarks of MS pathogenesis are brain and spinal cord inflammation, demyelination, partial remyelination and neurodegeneration.[1,21] In MS lesions oligodendrocytes supplying the protective myelin sheath surrounding the neuronal axons die, leading to demyelinated exposed axons. Bear axons are vulnerable to injury and are functionally impaired without this structural and nutritional support.[22,23] Limitations in remyelination potential of oligodendrocyte progenitor cells result in chronically demyelinated axons and may lead to neurodegeneration.[21]

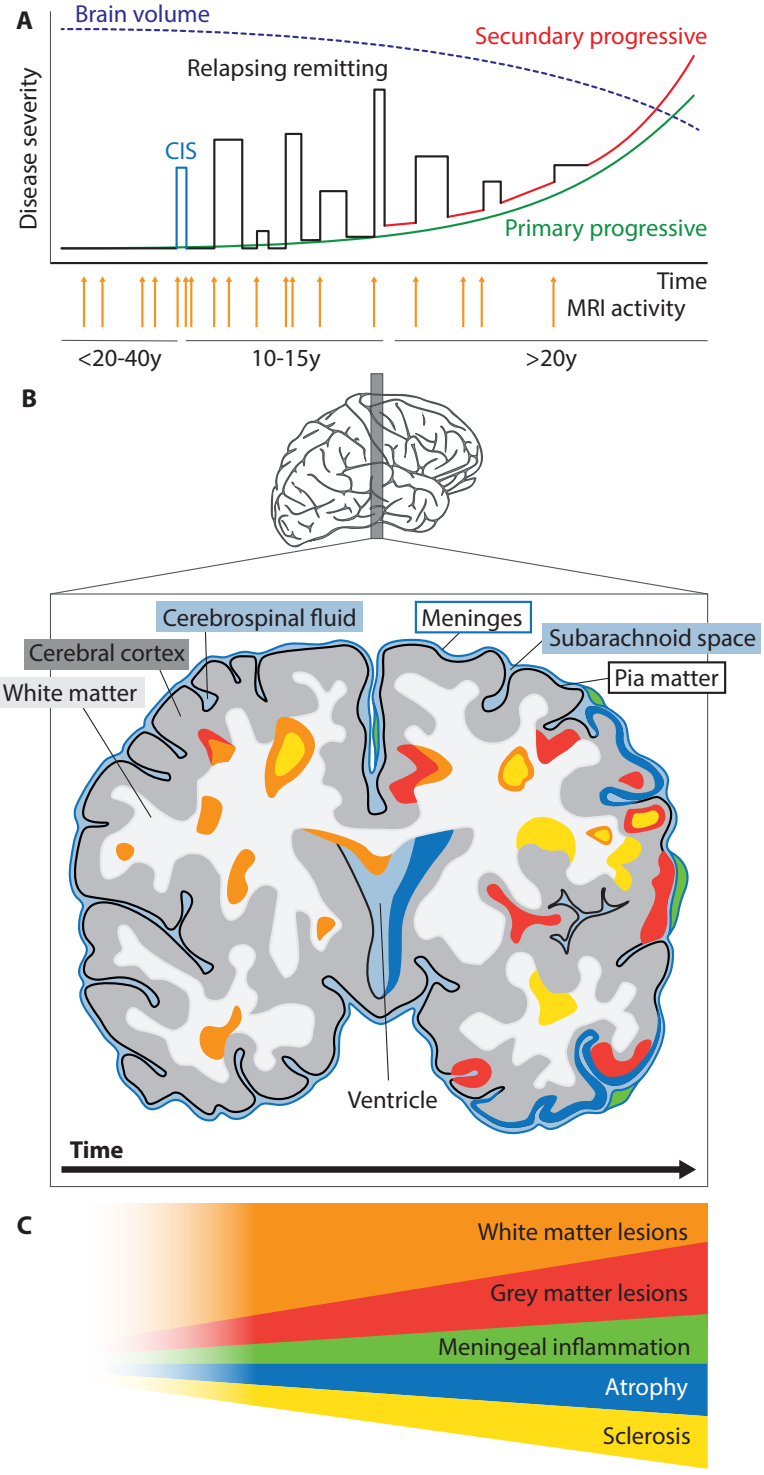
What instigates the inflammation, demyelination and neurodegeneration of MS, and whether the same process is shared between MS patients is unknown. Some regard MS primarily as a neurodegenerative disease with secondary inflammation and demyelination. Others assume oligodendrocytes or neurons are targeted by the adaptive immune system, which leads to demyelination and neurodegeneration, respectively.[1,24,25]

Histopathology of MS

The anatomy of neuropathology is meticulously studied by immunohistochemistry (IHC) on post-mortem collected brain and spinal cord tissues from MS patients and controls. These analyses are therefore mostly limited to progressed or end stage MS patients.

In order to comprehensively compare different studies, several methods have been proposed to classify MS lesions using IHC. Classifiers can be the anatomical location, i.e. WM/GM involvement, or markers of the involved pathological mechanisms, i.e. immune activation, complement deposition, infiltrating leukocytes, demyelinating activity, sclerosis and other factors.[26–28] Furthermore, lesion can be classified based on the progressive developmental stages as they are observed in animal models for MS and MRI.[29] The staging of MS lesions offers an histological timeline of lesion development and has been used to characterize early lesions in progressed MS patients.[29–32] Recently, a unifying comprehensive classification method has been proposed based on the inflammatory and demyelinating activity of lesions.[27] Although many aspects of earlier systems are incorporated in this new classification method, diffuse WM changes in absence of demyelination have not been recognized, as there is still much debate on their origin.[27,29,31] These changes include an

►**Figure 1. Timeline of disease course and neuropathology of multiple sclerosis.** (**A**) Disease progression of the clinically distinct forms of multiple sclerosis (MS) are presented in a schematic graph. Gadolinium enhancing active white matter lesions are shown during the pre-clinical phase of MS using magnetic resonance imaging (MRI activity, orange arrows). Clinically isolated syndrome (CIS, light blue), potentially the first attack of relapsing remitting MS, typically occurs between 20-40 years of age. 30-70% of CIS patients subsequently develop relapsing remitting MS (black line). After a period of 10-15 years secondary progressive MS follows (red line). A minority of patients present with progressive disease from onset, termed primary progressive MS (green line). The brain volume diminished during disease course due to atrophy (dark blue dashed line). (**B**) Disease progression in time (from left to right) is schematically depicted in a cross-sectional view of a MS brain and (**C**) diagram. Early and relapsing remitting MS is associated with perivascular white matter lesions (orange color), which may progress to sclerotic plaques (yellow color). Grey matter lesions (red color) are formed secondary to white matter pathology and are associated with the presence of meningeal inflammation (green color). Grey and white matter pathology leads to atrophy, which is most pronounced surrounding the brain ventricles and in the cerebral cortex (dark blue color).


A
Brain volume
Secundary progressive
Relapsing remitting
Disease severity
CIS
Primary progressive
Time
MRI activity
<20-40y
10-15y
>20y
B
Cerebrospinal fluid
Meninges
Subarachnoid space
Cerebral cortex
Pia matter
White matter
Ventricle
Time
C
White matter lesions
Grey matter lesions
Meningeal inflammation
Atrophy
Sclerosis

increased frequency and immune activation of microglia and macrophages and were previously classified as active non-demyelinating or pre-active lesions. Potentially these changes relate to the cytokine milieu of distant inflammatory processes, neuronal stress by distant demyelination, Wallerian degeneration or pre-lesional changes.[25,27,29,33,34]

MS lesions are characterized by focal (partial) demyelination shown by reduced/absence of myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and luxol fast blue staining. Active lesions are hypercellular and contain immune activated phagocytozing macrophages and microglia. Activated microglia have a ramified appearance, short processes and enlarged cytoplasm.[27,29,32] Immune activated microglia and macrophages have increased expression of CD68 and HLA-DR. Demyelinating microglia and macrophages contain intracellular myelin proteins, stained by myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) or Oil-red-O.[27,29] Active lesions also contain reactive astrocytes, termed hypertrophic gemistocytes, characterized by enlarged cytoplasm and increased expression of glial fibrillary acidic protein (GFAP).[27] Lesions where immune activated and phagocytosing microglia and macrophages cells are distributed throughout the lesion are classified as active and early demyelinating lesions.[27,29] Lesions where HLA-DR+CD68+Myelin+ macrophages and microglia are only present at the border of the lesions, surrounding a demyelinated core are classified as mixed active/inactive lesions[27] or chronically active lesions.[29] Demyelinated areas with very little signs of immune activation or infiltrating macrophages and microglia are regarded as inactive post-demyelination lesions[27] or chronic inactive lesions.[29] These demyelinated areas show increased signs of scarification, as shown by the transition of astrocytes from hypertrophic gemistocytes to fibrous gliosis, characterized by elongated processes.

Infiltrating immune cells forming perivascular cuffs are a prominent feature of active WM lesions. Also GM lesions show perivascular infiltrates, but these are less pronounced than in WM lesions.[21,35] These aggregates primarily consist of T-cells and macrophages, with lower frequencies of B-cells and plasmacells.[14,21,36] These leukocytes are thought to instigate the inflammatory process and activate CNS resident immune cells that subsequently drive inflammation in progressed lesions.[21,37]

In conjunction with inflammatory lesions, neurodegeration is shown by IHC in MS lesions. Transected partially demyelinated axons, stained by Tau or neurofillement, form terminal ovoids (a characteristic union shaped bulb) in MS lesions.[38] Moreover, axonal swelling is observed with aggregated proteins and organelles. These accumulations are marked with amyloid precursor protein (APP) stainings[39] are suggested to aggravate neurodegeneration.[25] These aggregates may result from axonal transport deficits, and have also been shown in absence of demyelination in NAWM.[40] Different views exist on the cause of neuron death. Chronic inflammation may leads to neurodegeneration due to the production of reactive oxygen and nitrogen species (ROS and RNS). ROS an RNS induce cumulative DNA damage in mitochondria, which is detrimental for their energy production function in the form of adenosine triphosphate.[41,42] Combined with the increased energy consumption of neurons due to lowered transduction efficiency of bear axons this may result in neurodegeneration.[14,42] Conversely, axonal damage facilitates chronic inflammation and demyelination.[25]

Immunopathology of MS: innate immune cells

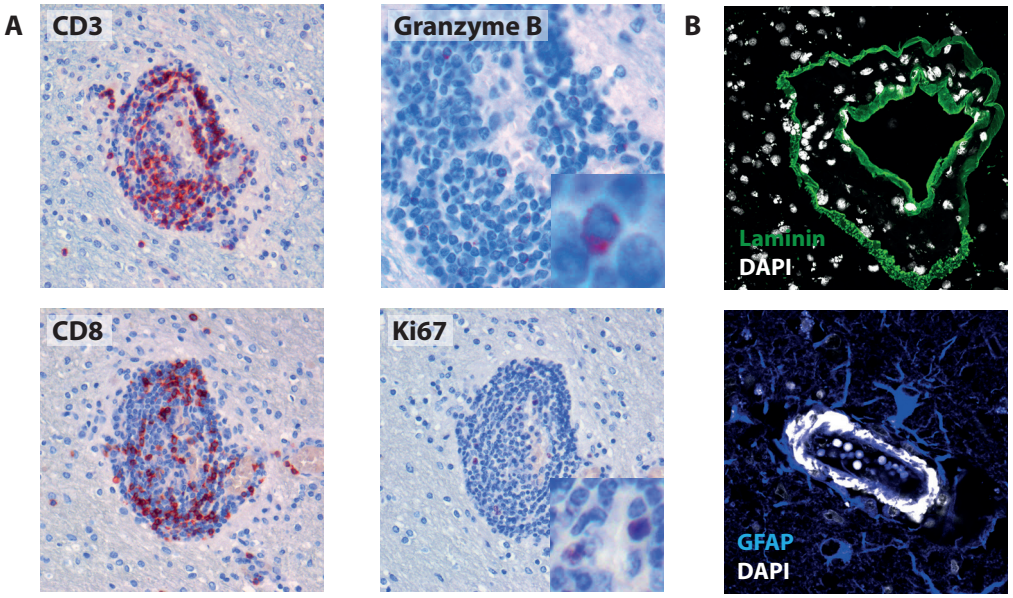
Innate immunity, also known as non-specific or in-born immunity, is an important arm of the immune system that comprises specialized cells and mechanisms that provide an immediate host defense against pathogens. However, unlike the adaptive immune system, innate immunity neither provides a tailored, nor a long-lasting pathogens-specific memory response.

The continuous activation of CNS immune cells and periphery-derived innate immune cells are thought to drive the chronic decline of PP- and SPMS patients.[30,37,43] Although many innate immune cell types may contribute to pathological changes in MS, including astrocytes, dendritic cells, mast cells, and natural killer cells, the most prominent are monocyte-derived macrophages and microglia.[37,43,44] Activated macrophages and microglia phagocytose myelin and their frequency associates with axonal damage.[40] This process is likely a scavenging response to oligodendrocyte death and myelin debris.[30,45] Alternatively, innate immune cells are activated due to neuropathology.[25,42] Although demyelination is detrimental for neuronal function, this process is required for remyelination of axons by oligodendrocyte progenitor cells.[37,44] Pro-inflammatory cytokines that are well known to skew macrophages and microglia into a pro-inflammatory M1 phenotype, including interferon-γ (IFNγ), interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) and monocyte colony stimulating factor (GM-CSF), are highly prevalent in PB of MS patients.[46–48] Furthermore, in untreated MS patients, peripheral monocytes show increased expression of the pro-inflammatory mediators IL-6 and IL-12 and co-stimulatory molecules CD80 and CD86.[49] Contrastingly, in progressing MS lesions, myelin laden foamy macrophages display an anti-inflammatory M2-like phenotype with the expression of IL-10, IL-4, transforming growth factor-β (TGFβ) and C-C motif Chemokine ligand 18 (CCL18).[47,50] Together these data suggests that systemic innate immune aberrancies result in an exaggerated damage response in the CNS of MS patients. This may hamper the local suppressive function of phagocytes in progressing lesions.[50,51]

Immunopathology of MS: adaptive immune cells

Adaptive immunity, also known as the acquired or specific immunity, is composed of highly specialized, systemic lymphocytes that eliminate pathogens in a tailored manner. Although upon primary exposure to a pathogen the onset of the adaptive is delayed compared to innate immune responses, it does provide pathogen-specific immunological memory. This memory response is highly enhanced upon recurrent exposure to the specific pathogen. The adaptive arm of the immune system includes both cell-mediated immunity (T-cells) and humoral immunity (B-cells and antibodies)

Adaptive immune cells are considered pivotal in the initiation or perpetuation of MS lesions.[21,52] Active lesions, particularly in WM but less pronounced in GM, contain characteristic perivascular infiltrates. These infiltrates consist of mostly T-cells, macrophages and lower frequencies of B-cells and plasma cells. Contrary to experimental autoimmune encephalomyelitis (EAE) animal models for MS and earlier reports on


A
CD3
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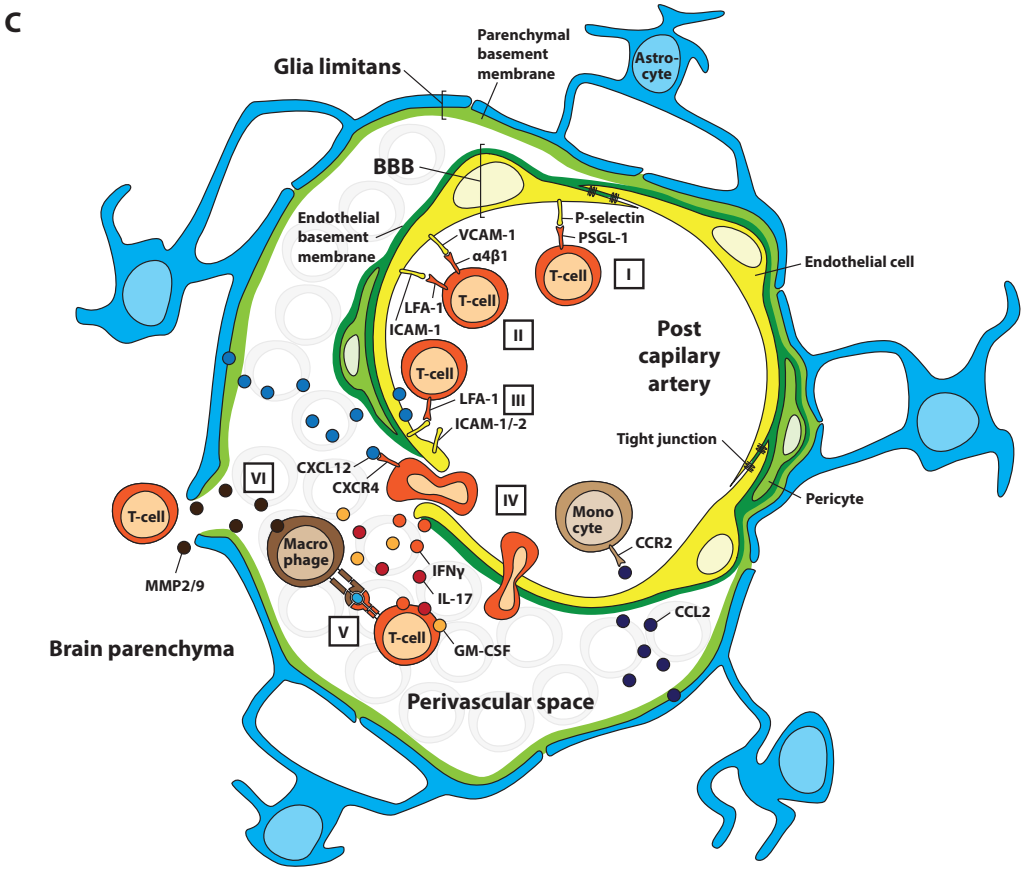
C
Glia limitans
Parenchymal basement membrane
Astro-cyte
BBB
Endothelial basement membrane
VCAM-1
α4β1
P-selectin
PSGL-1
T-cell
I
Endothelial cell
LFA-1
ICAM-1
II
Post capilary artery
III
ICAM-1/-2
Tight junction
CXCL12
CXCR4
VI
IV
Pericyte
Mono cyte
CCR2
Macro phage
IFNγ
IL-17
MMP2/9
V
CCL2
GM-CSF
Brain parenchyma
Perivascular space

◄Figure 2. T-cells infiltrate the brain parenchyma in multiple sclerosis lesions. (**A**) Consecutive 6µm formalin fixed paraffin embedded tissue sections of an active white matter lesions are stained using monoclonal antibodies for specific CD3 (top left), CD8 (bottom left), granzyme B (top right) and Ki67 (bottom right), visualized by 3-amino-9-ethylcarbazole deposition (red color), and nuclear counterstaining with haematoxylin (blue color). Perivascular infiltrates contain mostly CD3+ T-cells, of which the majority are CD8+ T-cells that express granzyme B indicating their cytotoxic potential and in part express Ki67 demonstrating proliferation. CD8+ T-cells partially infiltrate the brain parenchyma in MS lesions. (**B**) 8µm sections of snap-frozen MS brain lesions are stained for nuclei with 4',6-diamidino-2-phenylindole (DAPI, white color) and monoclonal antibodies specific for pan-laminin (green color, top panel), demonstrating the basement membranes of the glia limitans (outer layer) and blood-brain barrier (BBB, inner layer), and glial fibrillary acidic protein staining (GFAP, bleu color, bottom panel), demonstrating the astrocyte end-feet that form the glia limitans surrounding the perivascular space. (**C**) CXCR12 expressed by activated endothelial cells (yellow color) and astrocytes (blue color) recruits CXCR4+ memory T-cells (orange color) to area of inflammation. The specified interactions facilitate T-cell [**I**] capture from the blood stream, [**II**] rolling on the vessel wall, [**III**] crawling to the site of inflammation, [**IV**] extravasation by passing the BBB formed by endothelial cells and the basement membrane (dark green color) by openings in the endothelial cell junctions, or via a trans-cellular pathway, leaving the integrity of tight junctions intact. [**V**] Perivascular T-cells release IFNγ, GM-CSF and IL-17. These cytokines activate astrocytes (blue color) that then release CCL2 that recruits additional CCR2+ myeloid cells (brown color). [**VI**] Activated perivascular macrophages secrete matrix metalloproteinases (MMP)-2 and -9 that cleave the extracellular basement membrane (light green color) and disrupt the glia limitans, enabling T-cells to enter the brain parenchyma.

IHC analysis of human tissues,[53,54] CD8+ T-cells outnumber CD4+ T-cells in MS lesions.[36,55] Perivascular lymphocytes partly express granzyme B and Ki67, indicating their cytotoxic potential and activation-induced proliferation, respectively (Figure 2A).[56]

Although classically regarded as an immune privileged site that is enclosed by the BBB, CNS immune surveillance by adaptive immune cells is now widely recognized.[57,58] With the recent discovery of CNS lymphatics in the meninges that drains fluid and CSF-derived lymphocytes to the cervical lymph nodes, this classic view of immune privilege is further mitigated.[19] Although perivascular lymphocytes were initially thought to traverse the BBB and enter the CNS locally in response to inflammation, they may in part be CNS immune surveilling lymphocytes. Therefore, lymphocytes are able to initiate inflammation beyond the BBB.

Immunopathology of MS: T-cells

The sequential steps needed for T-cells to enter the CNS for immune surveillance and in response to local inflammation are studied in great detail, as these are attractive targets for therapeutic intervention.[59] Blocking entry of lymphocytes to the CNS with specific monoclonal antibodies directed to the α4ß1-integrin (VLA-4) is clinically highly efficacious.[14] The access of lymphocytes is tightly regulated by the barriers that surround the CNS vasculature.[57,58] Endothelial cells form BBB and the blood-spinal cord-barriers. Epithelial cells form the blood-CSF-barrier (BCSFB) in the choroid plexus and blood-leptomeningeal barrier (BLMB).[18] A common feature of these barriers is the formation of tight junctions and a α1- and α2-laminin containing basement membranes that allow regulated permeability for immune cells and soluble molecules.[18,57,59,60] Integrity of the BBB is maintained in continuous cross talk with astrocytes, microglia, pericytes and neurons, but also with circulating immune cells.[18,57,58,61,62] Memory T-cells traverse the BSCFB to patrol the CNS in the choroid plexus, located in the brain ventricles.[57,58] Here, BCSFB epithelial cells

constitutionally express CCL20. The ligand of CCL20, C-C chemokine receptor type 6 (CCR6) is mainly expressed by IL-17 and IL-22 producing T helper 17 cells (Th17), IFNγ, GM-CSF and IL-17 expressing alternative Th17 cells (Th17.1) and IFNγ, IL-17, IL-21 and IL-22 producing cytotoxic CD8+ Tc17 cells.[61–63] All three cell types, including their effector molecules have been described to be pivotal in the induction and effector phase of EAE and MS.[64,65]

Upon secondary activation within the CNS perivascular T-cells release IFNγ, GM-CSF and IL-17.[65,66] These pro-inflammatory cytokines activate astrocytes and endothelial cells that thereby express the chemokine C-X-C chemokine receptor type 12 (CXCR12).[65,67] This recruits CXCR4+ memory T-cells to the site of inflammation.[61,62,68] Activated epithelial cells express P-selectin that captures P-selectin glycoprotein ligand (PSGL-1) positive T-cells from the blood stream. Upon this interaction, T-cells slow down and roll across the vessel wall. T-cell rolling is halted by binding of vascular cell adhesion molecule 1 (VCAM-1) with VLA-4 and intercellular adhesion molecule 1 (ICAM-1) with leukocyte function antigen 1 (LFA-1) on endothelial cells and T-cells, respectively. Next, T-cells crawl across the vessel wall, often opposite to blood flow by interaction of ICAM-1 and ICAM-2 with LFA-1 to the site of inflammation. T-cells may either extravasate by passing the BBB formed by endothelial cells and the basement membrane by openings in the endothelial cell junctions, or via a trans-cellular pathway. Here, T-cells move through BBB endothelial cell leaving the integrity of tight junctions intact. Reactive astrocytes express CCL2, which results in the influx of CCR2+ myeloid cells, like monocytes or dendritic cells, and additional lymphocytes (Figure 2C).

A second barrier, the glia limitans, formed by astrocytes end-feet and supported by a second α4- and α5-laminin containing basement membrane, confounds entry of perivascular lymphocytes to the brain parenchyma during homeostatic conditions.[18,61] However, in MS lesions, parenchymal T-cells are detected (Figure 2A). Astrocyte end-feet and the basement membrane of the glia limitans and BBB can be visualized by IHC using glial fibrillary acidic protein (GFAP) and pan-laminin staining, respectively (Figure 2B). Secondary activation of perivascular T-cells activate perivascular macrophages that secrete matrix metalloproteinases 2 (MMP2) and -9. MMP2 and -9 cleave the extracellular basement membrane and disrupt the glia limitans, enabling T-cells to enter the brain parenchyma (Figure 2C).[18,61] Parenchymal T-cells are likely not part of CNS immune surveilling lymphocytes, but are involved in the inflammatory process in MS lesions. This is supported by studies showing clinical symptoms only manifest in EAE if T-cells breach the glia limitans to enter the brain parenchyma.[69,70]

Clonal enhancement of T-cells, particularly CD8+ T-cells, in lesions and CSF of MS patients implies T-cell proliferation is receptor (TCR)-mediated, i.e. antigen (Ag)-specific activation.[55,71–74] Within the same patients, TCR sequences are shared between lesions, which strongly suggests a common Ag drives T-cell proliferation in distinct lesions.[55,71] Furthermore, increased sharing of TCR is detected in WM lesions, CSF and PB of MS patients.[55] These shared clones are mostly differentiated effector memory T-cells in PB.[55] These data endorse CSF and PB as relevant clinical specimen for the analysis of MS-related

lymphocytes and show potentially pathogenic memory T-cells, partially reside in and may originate from the periphery. It is widely believed that T-cells, that are initially activated in the periphery by pathogens or bystander activation,[75] reactivate within the CNS by cross-reactive self Ag due to molecular mimicry,[76,77] epitope spreading[78] or co-expression of TCRs with different specificities.[79] Upon secondary activation in the CNS, T-cells initiate an inflammatory response that is subsequently aggravated by the activation of local innate immune cells and recruitment of additional leukocytes. Perivascular antigen presenting cells (APC) in WM/GM lesions, meninges and draining lymph nodes are exposed to CNS proteins.[80–83] Phagocytosed CNS proteins are processed and peptides may either be presented on human leukocyte antigen class II (HLA class II) to auto-reactive CD4+ T-cells or cross-presented via HLA class I to auto-reactive CD8+ T-cells. This model concurs with the induction of EAE, where peripheral pathogenic T-cells are induced by immunization with CNS-specific Ag that subsequently evoke CNS inflammation.[54,64]

An alternative hypothesis is that an event intrinsic to the CNS triggers inflammation of local innate immune cells, with the subsequent influx of (autoreactive) lymphocytes as a secondary event. The trigger may be CNS infection with an undefined pathogen or be the result of neurodegeneration.[14,25,33,42] In this view, infiltrating lymphocytes merely amplify the local inflammatory process.

Immunopathology of MS: humoral immunity

Apart from T-cells, clonally expanded B-cells are also observed in the CNS of MS patients.[21,84,85] Intrathecal oligoclonal IgG production is strongly associated with an increased risk of developing MS and is associated with increased cortical lesion load.[5,12,86] Autoreactive antibodies potentially initiate tissue damage.[87] B-cell clones migrate between the meninges, CSF, cervical draining lymph nodes and the periphery.[84,88] Although limited B-cells numbers populate the CSF and parenchyma, large numbers are detected in perivascular infiltrates in inflamed leptomeninges.[1,9,21] Meningeal inflammation is most frequent in rapidly progressing MS patients, with high lesion load and pronounced GM pathology.[9,10]

Perivascular infiltrates in the meninges, containing T-cells, CD20+ B-cells, CD68+ macrophages, CD138+ plasma cells and CD35+ follicular dendritic cells may form tertiary lymphoid structures.[9,10,89] Here, follicular dendritic cells express the B-cell chemo attractant CXCL13. Proliferating B-cells and the presence of Ig+CD138+ plasma cells and plasma blasts indicate that part of these structures form functional ectopic germinal centers.[9,90] This indicates B-cells potentially undergo antigen driven maturation within the CNS environment. Abundant extracellular myelin-derived proteins are selectively detected in the meninges of MS patients.[81] This supports the model that autoreactive B-cell responses are shaped in the meninges of MS patients. However, intracellular myelin in local APC is not increased compared to controls,[81] arguing against increased activation of myelin-specific T-cells in the meninges. Alternatively, B-cells mature in the deep cervical lymph nodes (CLN).[84] Soluble CNS proteins are drained through the cribriform plate to

CLN. Here autoaggressive B- and T-cells are potentially stimulated by phagocytosed CNS antigens presented by macrophages.[83] Neuronal proteins are detected mainly in pro-inflammatory IL-12 and TNFα producing APC and myelin proteins are mainly detected in anti-inflammatory TGFβ and IL-1 receptor antagonist (IL-1ra) producing APC.[82] This may reflect induction of different types of immune responses or be a consequence of the inflammatory state during drainage of CNS antigens. Surgical removal of superficial CLN prior to induction of EAE in mice ameliorates disease severity and illustrates the role of CLN in initiating autoaggressive immune responses.[91]

Apart from their potential role in producing pathogenic IgG,[87] B-cells are considered important APC for pathogenic T-cells. Several lines of research support that B- and T-cell interactions are key in MS including: *i)* a recent metagenomic study showing strong overlap between MS-associated genetic risk factors and expression profiles in B- and T-cells,[92] *ii)* the ability of B-cells to take-up myelin proteins independent of B-cell receptor specificity and present peptides to CD4+ T-cells via human leukocyte allele class II (HLA-II) *in vitro*,[93] *iii)* the success of B-cell depletion therapies.[94] Several anti-CD20 mAb rapidly lower annual relapse rates and MRI lesion load after treatment onset without affecting IgG levels and IgG producing plasma cells.[95] The efficacy of B-cell depletion is likely unrelated to IgG production, but rather to the APC-function and cytokine production of B-cells.[75,94,96] This view is supported by the observed reduced intrathecal T-cell numbers and activation state due to B-cell depletion.[97]

Target antigens of autoreactive B- and T-cells

The striking similarity in pathology between MS and EAE animal models strongly support a role of autoreactive B- and T-cell responses in MS.[54,98] In EAE, MS-like immunopathology is induced by immunization with various CNS antigens in adjuvant. Alternatively, EAE is induced in an antibody independent manner by adoptive transfer of $CD4^+$ T-cells. These studies reinforced the idea that MS is an autoimmune disease mediated by CNS-specific $CD4^+$ Th1, Th17 and Th17.1 cells.[54] However, the presence of clonally enhanced meningeal B-cell and intralesional CD8+ and, to a lesser extent, CD4+ T-cell populations in MS patients is widely considered to prove that Ag-specific B- and T-cell responses drive inflammation in the CNS.[55,71,73,74,84] Also the intrathecally synthesized oligoclonal IgG populations in the CSF of MS patients[12] are suggested to contribute to MS pathology. However, the majority of these IgG clones are directed towards ubiquitous intracellular self-antigens that are released due to tissue damage.[99] Likely, these antigens represent secondary immune targets.

In order to gain insight into the pathogenic mechanism at play and to develop personalized treatment, identification of the enigmatic antigenic targets of auto-aggressive B- and T-cells is of fundamental importance.[100,101] Numerous CNS-restricted autoantigens have been proposed, yet none are exclusively or broadly recognized by MS patients.[37,100–102]

The identification of putative autoantigens involved in MS immune pathogenesis is technically challenging, especially considering that benign autoreactive B- and T-cells are an integral part of a normal immune system.[100,101] These autoreactive regulatory T- and B-cells are though to modulate the immune system by dampening the inflammatory response and restore homeostasis to retain self-tolerance. Strategies to identify candidate target antigens of pathogenic B- and T-cells have mainly relied on their encephalitogenicity in EAE.[37,54,100,102] Oligodendrocyte-specific proteins have been widely studied as candidate auto-antigens in MS, because of the overt axonal demyelination and protein abundance in CSF and meninges of MS patients and because their potent encephalitogenicity in EAE.[37,102,103] More recently, also encyphalogenic glia-, astrocyte-, and neuron-specific proteins were considered as putative autoantigens for B- and T-cells in MS.[104–108] Inducing EAE with oligodendrocyte-specific proteins induces focal white matter lesions and lead to strong clinical relapses. Immunization with neuron-specific proteins predominantly leads to grey matter pathology and progressive disease progression. Immunization with glia-specific proteins induces more diffuse pathology, termed experimental autoimmune panencephalitis, with mild or no clinical symptoms.[104,106] These data suggest different antigens drive the inflammatory process at distinct anatomical sites in the CNS of MS patients or during the relapsing remitting and progressive phase of MS.

Alternative approaches to identify potential target Ag include studying tissue- or CNS protein-binding capacity of serum- and CSF-derived IgG[108,109] and determining the T-cell responses towards lesion-specific proteins.[110] Indeed, $CD4^+$ T-cell and IgG responses to the majority of EAE-inducing CNS proteins and other candidate MS-associated antigens (cMSAg) have been identified in MS patients, but T-cell frequency or IgG levels were indifferent compared to controls, questioning their pathogenic potential.[37,100,102,105,107,109,111] The conflicting outcomes of these studies are likely due to caveats in the experimental design, lack of standardization of applied assays and commonly small sample sizes. An extensive list specifying the tested MS-associated autoantigens is available in an immune epitope database (www.iedb.org), and their relevance is discussed by Vaughan et al.[102]

Etiology of MS

The amount of studies implicating specific factors in the etiology of MS pathogenesis since Jean-Martin Charcot first described it in 1868[112] is vast. Currently, MS is considered to be a result of nature ánd nurture, as both hereditary and environmental factors are implicated in MS development. Although both factors may contribute independently, it is more likely that the interactions between genes and environment dictate MS susceptibility.

Genetics

Concordance studies in monozygotic twins support a genetic component of approximately 30% in the risk of developing MS.[1] In contrast to classically inherited diseases, neither a necessary, nor a sufficient genetic factor to develop MS has been identified.[1]

The dominant genetic factor in MS is the HLA haplotype. In the seventies already, HLA-II alleles were strongly associated with MS. Initially identified by their DR2 and DQ6 serotype, these associations were later refined to the corresponding genotypes, HLA-DRB1*15:01, -DRB5*01:01, -DQA1*01:02, and -DQB2*06:02, respectively.[113] Additional HLA-I and HLA-II alleles have been identified that associate with either elevated or decreased risk of developing MS. The odds risk for developing MS of the HLA haplotype on ranges from an approximate 8-fold increase for homozygous HLA-DRB1*15:01 carriers to a 2-fold decrease for HLA-B*38:01 or homozygous HLA-A*02:01 carriers.[114,115] The role of HLA haplotype in MS is not fully known. Detailed analysis of disease-associated variants in HLA-DRB1 genotype revealed the alterations predominantly influence the conformation of the peptide-binding pocket and consequently, the range of presented peptides.[116] Likely, the MS HLA haplotype shapes the repertoire T-cell specificities and thus the likelihood of initiating pathogenic T-cells. Conversely, MS-associated variants in HLA that do not influence antigen presentation are also detected.[116]

Apart from HLA, in genome wide association studies (GWAS) over 230 disease-associated single nucleotide polymorphisms (SNP) were identified with moderate odds risk for developing MS, ranging from 1.07 to 1.32.[117–119] Including the HLA associations, an estimated 28% of the recurrence rate in siblings is explained by the identified genetic factors. Due to the marginal increase in MS risk, there is limited diagnostic or prognostic potential for these genetic associations.[120] Nevertheless, GWAS offers a valuable tool to reveal genes that are functionally involved in this complex disease and hint upon the involved mechanisms in a hypothesis free manner. However, due to the pervasive interconnection of gene regulatory networks in disease relevant cells, part of the heritable associations that are defined in MS may be explained by effects outside core pathways.[121] The functional contribution of individual genes and networks should therefore be carefully interpreted in the context of disease relevant cell types.

The majority of MS-associated genetic variants are located in non-coding enhancer regions near adaptive immune related genes. Especially in binding sites near key regulators of immune differentiation and activation.[92] There is a striking genetic communality between related autoimmune and chronic inflammatory diseases like rheumatoid arthritis,

systemic lupus erythematosus, celiac disease, Crohn's disease, psoriasis, type 1 diabetes and MS.[122,123] In contrast to neurological and neurodegenerative diseases like migraine, Alzheimer's and progressive supranuclear palsy, epigenetic fine mapping showed the MS-associated risk SNP poorly overlap with transcriptionally active genes of cells in the central nervous system. Contrarily, there is a strong overlap with active genetic regions in T- and B-cells, supporting the hypothesis that adaptive immune responses are key in MS pathogenesis with a minor contribution of neurodegenerative processes.[92,124] Still, three kinesin family member proteins (KIF) that are related to retrograde axonal transport along microtubules contribute to MS susceptibility.[34,117,125] The KIF associations may relate to neuronal transport of active mitochondria to the site where ATP is required. Aged mitochondria, that produce ROS and RNS, are normally removed from the active sites. In MS, stationary organelles and proteins accumulate in axons and form aggregates. These aggregates correlate with disease duration and are not only detected in active lesions but also in normal appearing white matter, which some interpret as an initiating event.[25,34,42]

Environmental factors

The intricate interplay of many environmental factors contribute to the risk of developing MS and influence disease severity. These factors include behavioral, ecological and physiological factors like hormone balance,[126] comorbid diseases,[123] traumatic events or surgery,[127] diet,[128,129] gut microbiome,[130] smoking,[131] vitamin D levels[132,133] and infections.[134] Similar to the genetic traits of MS, these factors are intricately interlinked and therefore may partly reflect indirect associations that are not related to the core pathways involved.

A recent umbrella review of systematic reviews and primary and meta-analysis studies that examined associations between the majority of the above-mentioned environmental factors and multiple sclerosis revealed several caveats.[135] The majority of studies showed large between-study heterogeneity (discordant results), small-study effects (large population studies showing more conservative results compared to smaller population studies) and excess significance bias (more statistically significant results than expected due to reporting bias), casting doubt on their validity.[135] However, using stringent inclusion criteria, two environmental factors stand out from the rest, namely; smoking and infections. Smoking is hypothesized to increase the risk of MS by chronic activation of Th1 and Th17 in the lung,[136] and by altering post-translational modification of autoantigens making them more immunogenic or more prone to cross-reactivity.[137]

The strong correlations of MS with infections is shown by seropositivity for the Epstein-Barr virus as measured by IgG specific for EBV nuclear antigen-1 (EBNA-1) and infectious mononucleosis (IM). Because smoking does not correlate with EBNA-1 IgG levels or IM, it can be regarded as an independent risk factor.[131,138] IM, also known as glandular fever and Pfeiffer's or kissing disease, is a self-limiting lymphoproliferative disease that typically occurs when primary infection with EBV is delayed until adolescence. Increased EBNA-1 IgG levels and IM are therefore closely interlinked and likely associate with MS due to a single core pathway.

The infectious origin of MS is a long-standing hypothesis. The observed sterile CNS inflammation together with epidemiological migration[2,139] and twin studies[140] support an etiopathological role of viruses in MS.[141–143] Also the observation that viruses can cause demyelinating disease in various rodents has fueled this long-held hypothesis.[78,144] Viruses may induce MS immunopathology in different ways. CNS cell infection may induce tissue damage. The innate and adaptive immune responses towards infections may cause by-stander damage to CNS cells.[145–147] Virus-specific immune cells may cross-react with CNS-specific auto-antigens by molecular mimicry.[76,148] Systemic infection and immune activation may create a pro-inflammatory milieu that activates auto-aggressive T-cells in an antigen independent manner.[147,149] Infections may activate self-/pathogen-specific dual TCR T-cells and evoke an auto-immune response.[79]

The various infectious agents considered as potential cause for MS include endogenous retroviruses, measles virus, rubella, mumps, chlamydophyla pneumoniae and human herpesvirusses (HHV).[150–153] Especially HHV are considered attractive candidate viruses for MS. They are highly prevalent worldwide and are able to establish life-long latency in the host. Intermittently, HHV reactivate from this dormant state and may cause recrudescent disease. All HHV can infect lymphocytes during primary infection, and EBV, cytomegalovirus (CMV, also known as HHV5) and HHV6A/B establish latency in specific subsets. During inflammatory conditions, but also during immune surveillance, lymphocytes can traverse the BBB and BCSFB to the CNS. Their high prevalence, potential to reactivate and tropism for migratory lymphocytes argue for their putative role in MS. Most of these pathogens have not withstood the scrutiny of research over time and many of these associations were contradicted in subsequent studies.[147,154–157] As is concluded in the meta-analysis by Belbasis and collegues, the only universal microorganism that is identified is, arguably, the endemic EBV, as is discussed in detail below.[158]

Biology of the Epstein-Barr virus

EBV, also known as HHV4 or human lymphocrypto virus, is one of eight known HHV. Together with Kaposi's sarcoma herpes herpesvirus, also known as HHV8, EBV is classified as a γ-herpesvirus. This class undergoes lytic replication in epithelial cells or fibroblasts and latently persists in lymphoid cells from which the virus intermittently reactivates.

Michael Anthony Epstein, Bert Achong and Yvonne Barr first discovered EBV using electron microscopy in 1964 in Burkitt's lymphoma cell lines.[159] EBV particles are approximately 122-180 nm in diameter and are composed an envelope containing lipids and projecting glycoproteins that mediate infection of host cells. The envelope contains tegument, which consists of specific viral and host proteins and viral RNAs. In the tegument, an icosahedron shaped nucleocapsid composed of core proteins contains the about 172,000 base pair linear double stranded DNA EBV genome (Figure 3A).

The EBV genome was the first herpesvirus that was completely cloned and sequenced. The complex and counterintuitive nomenclature of EBV proteins is still based on the original cloned BamHI restriction fragments. Lytic cycle-associated open reading frames (ORF) were designated according to the size of BamHI fragments (alphabetically from large to small; A, B, C, ..., Z, a, b, c, etc.), orientation (leftward or rightward) and order of appearance of the ORF (from left to right ORF1, ORF2, etc.). For example, BZLF1 is located on BamHI number 26 (Z), Leftwards oriented ORF number 1. Contrastingly, latency-associated ORFs are designated according to cellular location of proteins (nuclear or membrane), regulation of expression (latency expression programme I, II or III) and order of appearance on the genome. For example, EBNA-3B, also known as BERF2, is Epstein-Barr virus Nuclear Antigen expressed during latency 3 located downstream of EBNA-3A (B).

Epstein-Barr virus infection and tropism

EBV is transmitted via saliva. Primary infection occurs in epithelial cells of the naso- and oropharynx (Figure 3B). Here, EBV either traverses the epithelial barrier by transcytosis,[160] or by lytic infection. The EBV glycoproteins gH/gL may bind to $\alpha_V\beta_5$-, $\alpha_V\beta_6$- or $\alpha_V\beta_8$-integrins[161,162] or EBV BMRF2 binds to $\alpha_3\beta_1$- or $\alpha_5\beta_1$-integrins expressed by polarized epithelial.[163] Interaction of gH/gL with its receptors induces a conformational change that enables interaction with gB (gB/gH/gL), which in turn mediates fusion with the cell membrane followed by lytic infection (Figure 3C).[164] Subsequently, submucosal B-cells are infected in the tonsilar crypt or cervical lymph nodes. The EBV 350 and 220 kD glycoproteins (gp350/220) bind with complement receptor 2 (CR2 or CD21)[165] or complement receptor 1 (CR1 or CD35) expressed by B-cells.[166] EBV binding of B-cells improves infection efficiency but is not required. Interaction of gp42 with HLA-II on B-cells is essential for fusion of EBV with B-cells.[167] HLA-II-bound gp42 interacts with gH/gL (gp42/gH/gL). Next, the gp42/gH/gL complex induces a conformational change of the pre-fusion gB trimer that triggers fusion with the cell membrane surface or in endocytic vesicles (Figure 3D).[168,169] The nucleocapsid is transported via microtubules to the cell nucleus, where the viral genome is released together with several tegument proteins to establish latency (see below).

Upon viral reactivation in B-cells, gp42 interacts with immature HLA-II in the endoplasmatic reticulum and is thereby targeted for degradation. Lytic infection in B-cells thereby leads the production of virions with low gp42 content. In HLA-II negative epithelial cells this does not occur, thus epithelial cell-derived virions contain large amounts of gp42. The gp42/gH/gL complex impairs infection of epithelial cells. Consequently, EBV switches cell tropism between epithelial and B-cells.[170]

Alternative pathways of infection are gp350-specific IgA-mediated infection,[171] in-cell infection (phagocytosis of whole EBV infected B-cells),[172] and cell-to-cell contact-mediated infection.[173] These pathways do not rely on glycoprotein-mediated entry of EBV. Consequently, EBV tropism is not limited to epithelial cells and B-cells, which is an important consideration when studying viral presence of putatively EBV-infected clinical specimen.

Epstein-Barr virus latency

EBV selectively establishes life-long latency in B-cells.[174,175] In healthy EBV carriers, 1-50 per 1,000,000 peripheral blood B-cells are latently infected with EBV. After infection of a B-cell, the unmethylated EBV genome first undergoes an abortive lytic replication cycle. Second, viral gene expression is reduced to a growth program (Latency III) with controlled expression by Wp and Cp promoters of EBNA-2, -LP, -3A, -3B, -3C and latent membrane protein 1 (LMP1). Distinct promoters regulate expression of the non-coding RNAs EBER-1 and -2, and approximately 44 miRNAs in the BART and BHRF1 regions.[176,177] EBV induces B-cells to undergo an antigen-independent germinal center reaction that induces differentiation to memory B-cells. Subsequently, EBNA-1 expression by the Qp promoter induces the host DNA repair machinery to circularize the viral genome at the terminal repeats, forming the viral episome. This enables expression of LMP2A/B, the exon region of which are located at either end of the terminal repeat.[178] After the germinal center reaction the mature B-cells enter circulation. The Cp promoter is methylated, limiting gene expression to EBNA-1, LMP1, 2A and 2B, non-coding RNAs and controlled expression of BHRF1 and BARF1 (Latency II). LMP1 functions as a constitutively activated CD40 receptor, and LMP2A mimics an activated B-cell receptor (BCR), enabling proliferation of infected B-cells. BFRH1 is a BCL-2 homologue, protecting cells from apoptosis.[175,179–181]

Full latency is established by down regulation of all viral protein expression by Cp and Wp promoter methylation, and selective expression of non-coding RNA (Latency 0). Expression of viral proteins is fully down regulated to reduce immune-exposure. EBNA-1 is essential for the maintenance of the EBV episome in dividing cells and is selectively induced by the Qp promoter upon B-cell proliferation. EBNA-1 binds the origin of plasmid replication (oriP) on the circular EBV genome and facilitates its replication by the cellular DNA replication machinery (Figure 4).[182,183]

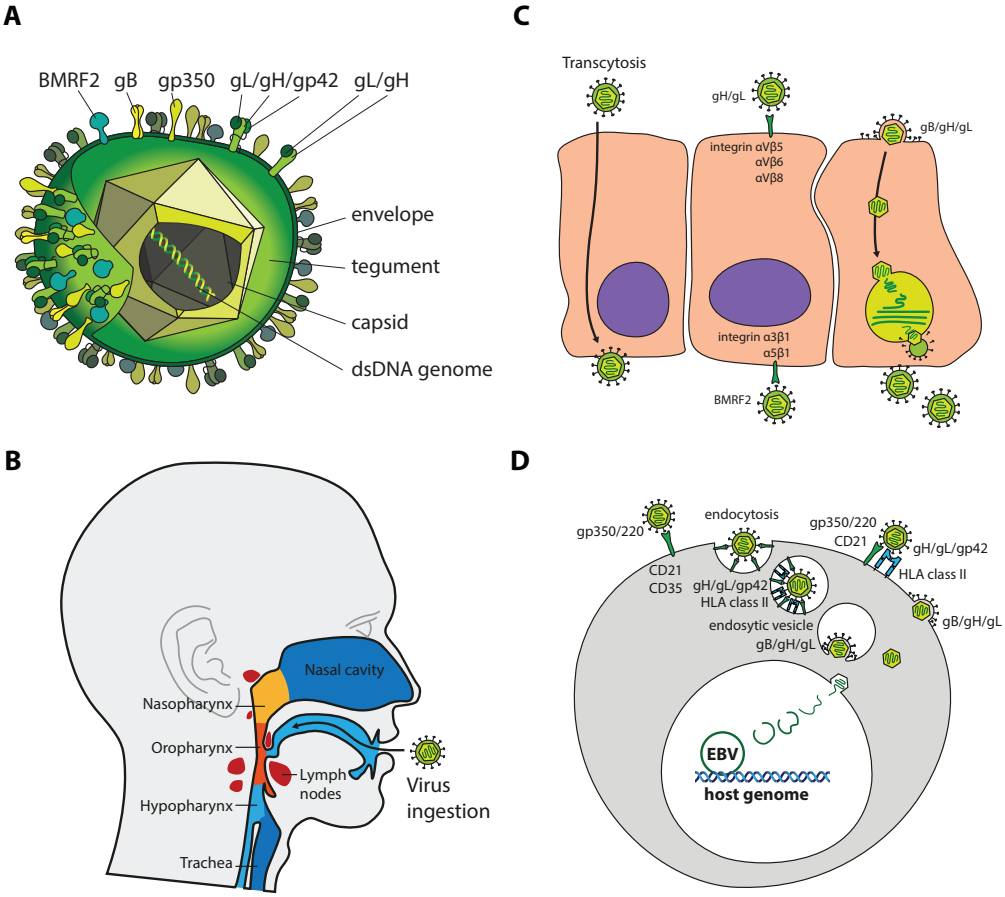

Figure 3. Epstein-Barr virus infects epithelial cells and B-cells in the oro- and nasopharynx. (**A**) The Epstein-Barr virus (EBV) particle is composed of a 170.000 bp dsDNA genome packaged in a nucleocapsid surrounded by tegument and a lipid bilayer envelope with projecting glycoproteins (gp). Epithelial cell-derived EBV particles are rich in gp42/gH/gL trimmers, while B-cell derived particles are rich in gH/gL dimers, which determine the B-cell and epithelial cell tropism, respectively. (**B**) EBV is transmitted via saliva. Primary infection occurs in the oropharynx or nasopharynx. Here, epithelial cells are (**C**) traversed by transcytosis (left), infected via interaction of the gH/gL dimer with $\alpha_V\beta_5$, $\alpha_V\beta_6$ or $\alpha_V\beta_8$ integrins or interaction of BMRF2 with $\alpha_3\beta_1$ or $\alpha_5\beta_1$ integrins (middle). This leads to fusion with the cell membrane and lytic infection of the epithelial cell (right). (**D**) EBV binds to B-cells via interaction of the gp350/220 with CD21 or CD35. Bound EBV may be internalized by endocytosis (left). Interaction of the gp42/gH/gL trimer with HLA class II in endocytic vesicles (middle) or on the cell surface (right) induces fusion with the membrane via gB/gH/gL. The viral capsid is then transported to the nucleus where the viral genome is released. Here, the EBV genome is circularized and establishes latency as a viral episome.

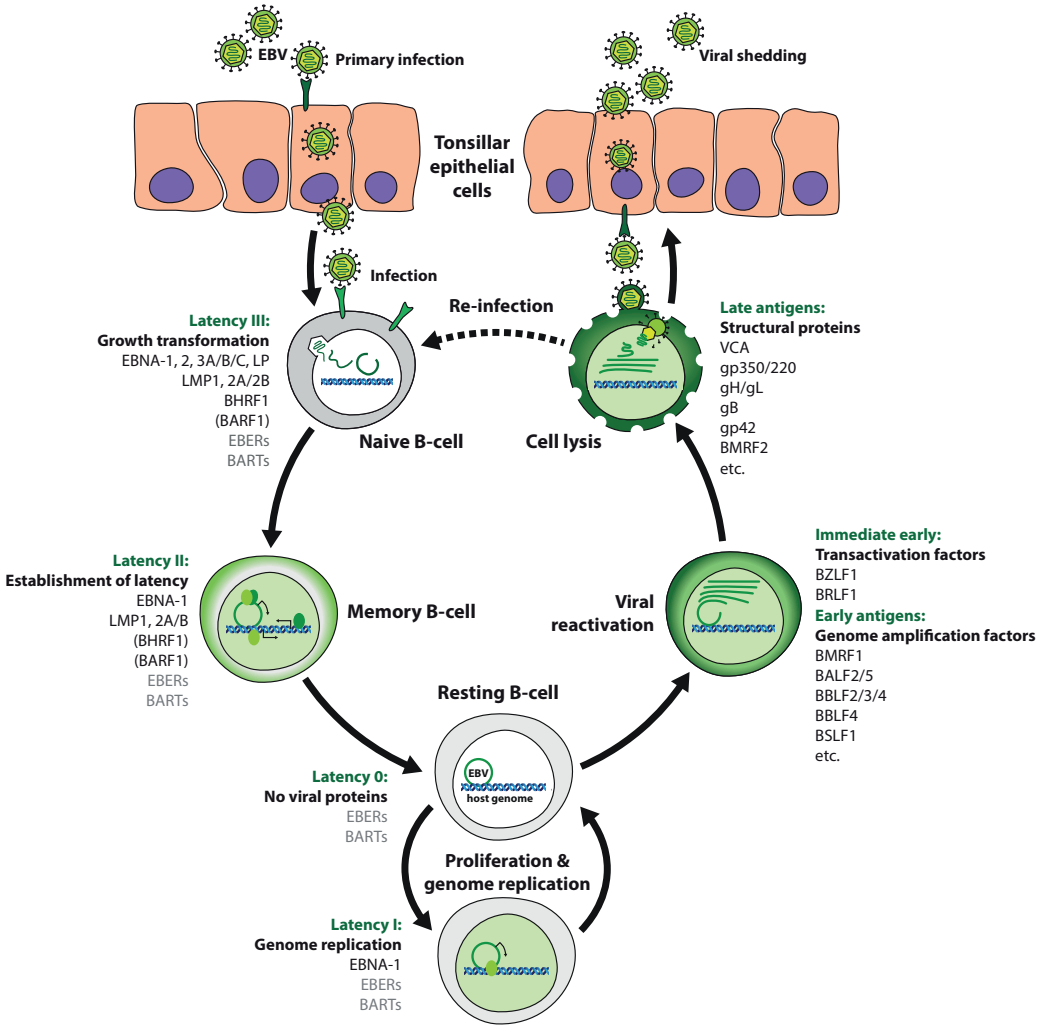

Figure 4. Regulation of the Epstein-Barr virus transcriptome during the virus lifecycle. After initial infection of tonsillar epithelial cells, the Epstein-Barr virus (EBV) infects naïve B-cells in the marginal zone of lymph nodes in the oropharynx. After release of the viral genome in the nucleus the genome is circularized and virus expresses the latency III program, inducing a germinal center reaction in B-cells. The infected B-cell is antigen-independently differentiated to a mature B-cell blast. Latency III includes expression of BHRF1, all EBNA and LMP proteins, the noncoding EBERs and BARTs and optionally BARF1. After growth transformation, EBV expression of EBNAs is limited to EBNA-1 (latency II). When full latency is established in resting B-cells, viral protein expression is shut down by methylation of the viral polymerases and only non-coding EBER and BART transcripts are expressed. Upon cell division the latency I program is expressed, including EBNA-1 and non-coding transcripts, that enables EBV genome replication. Upon differentiation to plasma cell or in response to cellular stress or damage response EBV can reactivate from its dormant state. This is initiated by expression of the immediate early transactivators BZLF1 and BRLF1, which induce expression of early antigens, mainly needed for EBV genome amplification. Subsequently late proteins encoding mainly structural proteins are expressed; viral genomes are encapsulated by budding from the nuclear membrane and enveloped in cellular membranes of the Golgi apparatus. Viral particles are released by exocytosis. Released progeny either infects naïve B-cells or tonsillar epithelial cells. Lytic infection of tonsillar epithelial cells results in virus shedding in the saliva and EBV may be transmitted to a new host.

Epstein-Barr virus reactivation

Activation of memory B-cells via the B-cell receptor may result in differentiation to plasma cells. This process has been shown to switch on the EBV lytic cycle.[184] Stress hormone levels, cellular stress responses and DNA damage have also been linked to EBV reactivation.[185–187]

Reactivation is initiated by regulated expression of three kinetic classes of lytic viral antigens termed immediate early (IE), early (E) and late (L) antigens. Immediate early antigens are the transcriptional activators BZLF1 (also known as Zebra or Zta) and BRLF1. Multiple BZLF1 and BRLF1 responsive elements in early gene promoters induce the expression of 38 early proteins, which in turn induce the expression of 40 late proteins and ultimately result in global bidirectional expression of the viral episome.[188,189] Early proteins are mainly involved in viral genome amplification, immune modulation and host-gene shutdown. Contrary to late genes, early genes are persistently transcribed in the presence of inhibitors of viral DNA synthesis. Late proteins are mainly structural proteins (Figure 4).[183]

The induction of lytic EBV results in several cytopathic effects that are characteristic for herpesvirusses. These include amplification of the viral genome in the center of the nucleus, host-gene shutdown, formation of nucleocapsid at the nuclear border, encapsulation of the viral genome by budding of the nuclear membrane and final envelopment in cytoplasmic membranes.

Epstein-Barr virus-specific immune responses in healthy carriers

Infection of EBV induces a strong innate and adaptive immune response in the host.[190] Most prominent for the innate immune response is the highly increased CD56+ natural killer (NK) cells numbers in peripheral blood and lymph nodes within two days after infection that may persist for several months.[191] NK-cells limit lytic replication of EBV and the pathogenic expansion of CD8+ T-cells that is associated with infectious mononucleosis (IM), which is discussed in detail below.[192] The gradual decrease of NK-cells upon aging is thought to increase risk of IM after primary infection.[190,192]

Antibodies are initially mainly directed towards a wide range of structural lytic viral antigens.[191] Primary EBV infection is diagnosed by the presence of IgM and absence of IgG, specific for the viral capsid antigen (VCA) encoded by the late gene BcLF1.[193] The subsequent VCA-specific IgG response persists for life. Humoral responses towards non-structural lytic cycle early antigens (EA) are delayed for several weeks and decline over time, yet are rapidly increased upon viral reactivation. The EA-D (also known as BMRF1) specific IgG titer is therefore used as a serological marker for EBV reactivation. Antibody responses towards the latent antigens like EBNA-1 are delayed for 3-6 months and gradually increase over time.[191]

Primary infection leads to a dramatic increase in general CD8+ T-cell numbers. Subsequently, a strong EBV-specific CD8+ T-cells response is mounted that limits the initial NK-cell and general CD8+ T-cell numbers. Up to 50% of PB CD8+ T-cells specific for individual EBV antigens have been shown after primary infection using HLA class I

tetramers.[194] The target antigens of EBV-specific CD8+ T-cells are initially mostly lytic immediate early (BZLF1 and BRLF1) and early viral antigens (usually BMRF1, BMLF1, BARF1 and BALF2). Reactivity towards late antigens is rare due to a strong HLA class I down-modulation that is associated with late gene expression.[195,196] The magnitude of the lytic viral antigen response declines and is followed by a relative increase in reactivity towards latent antigens (usually EBNA-3A/B/C, EBNA-1 and LMP2).[191,197] Approximately 1-2% EBV-specific CD8+ T-cells persist in PB in healthy EBV carriers.[191,197]

CD4+ T-cell numbers are not, or only mildly increased upon primary EBV infection.[191,197,198] Using HLA class II tetramers, up to 1% CD4+ T-cells are shown to be EBV-specific.[198] All kinetic classes of lytic viral antigens are targeted by CD4+ T-cells but latent EBV antigens are marginally immunodominant and are mainly CD27+CD28+ throughout the course of infection.[198]

Epstein-Barr virus-associated lymphoproliferative diseases and carcinomas

EBV infection is associated with a wide variety of diseases. Most individuals are infected with EBV during childhood and present with no, or minor symptoms. However, if primary infection is delayed until adolescence or later, IM, also known as glandular fever, Pfeiffer's or kissing disease, frequently occurs. IM patients generally present with fever, headache, sore throat, enlarged tonsils and cervical lymph nodes and fatigue. These symptoms mostly resolve within 2 to 4 weeks, yet fatigue may persist for months. During IM, the immune system responds strongly to EBV infected cells leading to a self-limiting lymphoproliferative disease. Initially, the frequency of infected lymphocytes and lymphocytes specific for EBV infected cells is low. Also the effect of antiviral therapy to limit viral replication in IM is limited. This suggests that neither EBV replication, nor EBV-specific responses drive the proliferation of lymphocytes, but rather a strong pro-inflammatory milieu drives cytokine mediated bystander activation of lymphocytes. Most patients recover by mounting strong EBV-specific immune responses, but immune compromised patients such as transplant recipients, HIV patients and the elderly may suffer from severe lympholiferative disease.[197,199]

EBV infection is associated with an estimated 200,000 cases of B-cell, epithelial cell, T- and NK-cell carcinomas including Burkitt's, Hodgkin's and non-Hodgkin's lymphoma, gastric cancer, nasopharyngeal carcinoma, oral hairy leukoplakia and central nervous system lymphomas.[159,183,200–202] These malignancies are associated with distinct EBV latency expression patterns or chronic active EBV infection and are related to ineffective immunity against EBV.[186,203] Interestingly, possible associations between MS and EBV-related carcinomas are suggested based on epidemiological associations and co-morbidity.[204,205]

Epstein-Barr virus-associated autoimmune diseases

The etiology of autoimmune diseases is generally complex and multifactorial, but there are some striking similarities between different autoimmune diseases. There is a high level of overlap in the involved genes and pathways as is shown by shared genetic risk alleles.[92,123] Also EBV infection is associated with multiple autoimmune diseases. This suggests partially communal pathogenic mechanisms are involved. Systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and MS are most prominently associated with EBV infection.[206]

Virtually all SLE and MS patients are show to be EBV seropositive, which despite the ubiquitous nature of EBV (±95% seropositive among healthy adults) is highly significant.[207] More specifically, alterations in systemic B- and T-cell responses against particular EBV antigens are associated with SLE, RA and MS. Possibly the shared genetic background leads defective regulation of EBV latency or EBV-specific immune aberrancies. We anticipate valuable lessons to elucidate the pathological mechanisms in MS can be learned from other immune diseases.

SLE is a chronic inflammatory autoimmune disease affecting several organs; mostly skin, joints, kidneys and CNS. Severe SLE may lead to multi-organ failure. The prevalence of SLE is dependent on gender and race, with highest incidence among women of African descent.[207] SLE is characterized by non-tissue specific mature B-cell responses directed towards nuclear antigens like chromatin, dsDNA, and various spliceosomal and ribonucleoproteins components. Structural mimicry between several of these nuclear antibodies, as well as epitope spreading, has been shown for EBNA-1 and EBNA-2 specific IgG in SLE patients and various animal models.[208] In SLE, EBV viral load, EA-D specific IgG seroprevalence and titers are increased.[208,209] Furthermore, the frequency of EBV-specific $CD8^+$ T-cells is increased, yet the cytotoxic potential of these cells is reduced.[179,207] Together, these findings indicate EBV infection is poorly controlled in SLE patients.

RA is a widespread chronic inflammatory autoimmune disease, selectively affecting joints. Patients have painfull, stiff and swollen joints, due to inflammation of the synovial membrane. In contrast to MS and SLE, EBV seroprevalence in RA patients is indifferent from healthy controls indicating EBV infection is not a prerequisite of developing RA. Nevertheless, EBV-specific IgG and viral load are increased in blood and synovial fluid.[210,211]

Citrullinated fibrinogen, filament aggregating protein and keratin proteins are detected in the RA synovia. Auto-antibodies specific for these citrullinated proteins arise years before disease onset. Cross-reactive IgG against the citrullinated glycine–arginine rich region in EBNA-1 and collagen and keratin filaments are shown in affected joints.[212] Furthermore, cross-reactive T- and B-cell responses against EBV glycoprotein 110 (BALF1) and peptides of the dominant RA-associated genetic risk allele HLA-DRB1-04:01 have been shown.[213] Molecular mimicry may therefore result in autoreactive B- and T-cell responses in RA.

Systemic EBNA-1-specific CD8+ T-cell responses are increased in RA patients compared to controls.[214] Synovial fluid of RA patients is enriched for EBV-specific CD8+ T-cells targeting various immunodominant latent (EBNA-3A/B/C and LMP2) and lytic target antigens (BZLF1, BMLF1, BRLF1, BCRF1, BMRF1 and BALF4).[215,216] However, EBV-specific CD8+ T-cells expressed reduced amounts of IFNγ,[217,218] which suggests impaired T-cell control of EBV. These results suggest chronic antigenic challenge of EBV-specific CD8+ T-cells in the joints of RA patients. A study by Scotet and colleagues showed similar enrichment of EBV-specific and, to a far lesser extent, CMV-specific CD8+ T-cells in lesions of psoriatic arthritis, ankylosing spondylitis, arthrosis, encephalitis, uveitis and MS patients.[216] Although, they conclude EBV-specific CD8+ T-cells are trapped in chronic lesions by an undefined non-disease-related mechanism, one may also argue these results hint upon a common pathogenic EBV-related pathway in these chronic inflammatory diseases, like is suggested based on the shared genetic risk factors.[92,123]

Epstein-Barr virus and multiple sclerosis

Epstein-Barr virus and multiple sclerosis: sero-epidemiology

Besides smoking, the strongest associated environmental risk factor for developing MS is EBV infection.[135] The initial link between EBV and MS was the striking epidemiological similarity between IM and MS.[219] Areas with high EBV seroconversion rates before adolescence and consequently a low incidence of IM (e.g. Africa, Orient and Polynesia), coincide with low incidence of MS. Conversely, areas with low, or delayed EBV seroconversion and consequently, a high incidence of IM (e.g. Northern Europe), coincide with high MS incidence. These data are supported by migration data that suggest that the place of residence before the age of 16 years, and therefore the risk of contracting EBV infection, determines the risk of developing MS.[219] A high incidence of IM and MS coincide with regard to age, geographical distribution, socio-economical status, ethnicity and hygiene status.[134,219] Individuals with a history of IM have a 2- to 3-fold higher risk of developing MS.[220–223]

Several hypotheses exist on how a history of IM contributes to MS risk. The widespread EBV infection of B-cells during IM may include infection and differentiation of naive autoreactive B-cells populations without negative selection.[224] The strong Ag-independent bystander activation and proliferation of CD8+ T-cells in the initial phase of IM may include auto-reactive CD8+ T-cells, or skews regulatory auto-reactive regulatory T-cells to a pro-inflammatory phenotype. Primary EBV infection in a mature immune system with reduced plasticity may result in suboptimal immune control of EBV, making EBV more prone to reactivate and chronically stimulate the immune system. During IM, cross-reactive B- and T-cell responses may be initiated. Notably, IM patients temporarily have antibodies that recognize unique cross-reactive epitopes in EBNA-1 and several host proteins[225,226] that have been implicated in systemic lupus erythematosus.[208,209]

MS patients are almost universally infected with EBV, compared to 90-95% of age and gender matched controls.[149,158] The odds ratio of developing MS was found to be 0.06 in EBV seronegative individuals.[134] Moreover, recently the validity of the negative serostatus in these rare MS patients is questioned.[158,227] Longitudinal studies among over 3 million military personnel showed EBV infection precedes the development of MS.[228] The elevated EBNA-complex and EBNA-1 IgG levels occurred years before clinical manifestation of MS.[229] EBNA IgG is therefore a robust prospective biomarker for MS risk. Together these data imply EBV infection is a prerequisite for developing MS.

In MS patients increased serum IgG responses towards EBNA-1 are consistently shown, whereas responses towards other viral antigens such as EA-D and VCA are more variable.[227] Whereas some show EBNA-1 IgG levels, but not VCA IgG, correlate with lesion load on MRI and disease severity,[145,230] others show VCA IgG correlate with brain atrophy and lesion load.[231,232]

The sero-epidemiological evidence of the association between EBNA-1 IgG levels and MS is compelling, but the biological relevance of these antibodies is uncertain. Potentially, the increased EBNA-1 IgG levels are a consequence of the MS-associated genetic factors. Serum EBNA-1 IgG titers are shown to contribute to MS-risk independent of the dominant MS-risk allele HLA-DRB1*1501.[233] Still, HLA-DRB1*1501 carriership correlates with increased EBNA-1 titers in the general population,[234,235] which points towards an independent additive effect. Potentially, other MS-associated genetic factors associate with EBNA-1 IgG levels. Alternatively, IgG specific for epitopes in EBNA-1 cross-react with yet undefined CNS autoantigens like described for SLE and RA.[208,209] If cross-reactive EBNA-1 IgG are involved in MS pathogenesis, their intrathecal synthesis is anticipated. Oligoclonal bands of MS patients have been reported to bind EBV antigens.[236] However these are low affinity poly-specific antibodies that are a consequence of the chronic inflammatory response in MS patients.[237,238] What portion of these are functionally relevant high-affinity antibodies and if they are produced intrathecally remains to be determined.

Epstein-Barr virus and multiple sclerosis: intrathecal prevalence

Aberrancies in the humoral response against EBV suggest the control of EBV in MS patients is compromised which suggests EBV is more prone to reactivate which may be reflected in the viral load. Although in some MS patients an increased EBV load is detected in PBMC, these results were not significant compared to healthy EBV carriers.[239]

Alternatively, EBV may induce MS pathology by infection or CNS resident cells or by reactivation of infected intrathecal B-cells. In some studies EBV genomes are detected by PCR in the CSF of a minority of MS patients,[157,240–242] this suggests ongoing lytic EBV infection is not a common CNS feature of MS. Furthermore, some report these findings were indifferent from other neurological diseases which refutes disease association.[241] Even though, the highly cell-associated nature of latent EBV may lead to misdiagnosis in cell-poor CSF, these findings argue against fulminant lytic intrathecal infection in MS. In sharp contrast with these findings, several studies by the group Aloisi and collaborators

showed EBER expression by EBV infected B-cells in meningeal tertiary lymphoid structures and perivascular cuffs of active WM lesions in the majority of MS patients.[85,243–246] They showed most EBV infection in B-cells expressed the latency III growth program with the expression of EBNA-2 and LMP1.[243] Part of EBV-infected B-cells expressed the IE and E lytic viral antigens BZLF1 and BFRF1 which indicates of intrathecal reactivation of EBV.[243,245] These data implicate a key role for active EBV in WM and GM pathology of MS patients. However, these results could not be confirmed by others.[247,248] In these studies EBER expressing B-cells were detected in a single MS but not in 105 other MS cases including 12 that were documented as EBV-positive by Aloisi and colleagues. They conclude EBV infection is not a characteristic feature of the MS brain. This discrepancy may either be methodological or relate to selective presence of EBV in ectopic follicles near early MS lesions, which are rarely sampled.[223,246,249] Conclusive evidence of intrathecal EBV infection in MS patients is of fundamental importance to decipher the role of EBV in the etiology and pathology of MS. Despite this ongoing debate, even in absence of virus within the CNS, EBV may also evoke pathogenic immune responses elsewhere.

Epstein-Barr virus and multiple sclerosis: T-cell responses

Systemic elevated EBV-specific humoral responses suggest also the cellular immunity against EBV is altered. The overall peripheral T-cell responses, mainly of CD8+ T-cells, to EBV infected B-cells are decreased in MS patients, as determined by IFNγ secretion of PB-derived CD4+ and CD8+ T-cells stimulated by EBV-infected autologous B-cells (BLCL).[250] Notably, these results may reflect reduced numbers of BLCL-specific CD8+ T-cells, or reduced functionality analogous to findings in SLE and RA patients.[179,207,217,218] Other studies that document increased frequencies of CD8+ T-cell frequencies specific for immunodominant latent peptides (EBNA-3A and LMP2A)[146] and antigen-HLA pentamers (EBNA3C and LM2A)[224] or lytic viral antigens-HLA pentamers (BZLF1 and BMLF1)[224,245] in MS patients support the latter. Peripheral CD8+ T-cell frequencies specific for lytic antigens correlate with disease activity,[224,245] but show decreased polyfunctionality[224] possibly due to exhaustion. These data support the hypothesis that viral reactivation due to an impaired CD8+ T-cell control induces MS pathology. It is also hypothesized that a defective peripheral CD8+ T-cell control of EBV reactivation leads to an expanded population of EBV-infected B-cells. Because EBV is able to differentiate B-cells in an Ag-independent manner without negative selection, these may include autoreactive B-cells.[224]

To a lesser extent peripheral CD4+ T-cell responses are altered in MS patients. EBNA-1 specific CD4+ Th1 (but not Th17) effector memory frequencies are selectively increased and show broadened specificity against EBNA-1 peptides.[148,239] Part of EBNA-1 specific CD4+ T-cells cross-react with myelin-derived peptides.[239] Also BALF5-specific CD4+ T-cells cross-react with myelin basic protein (MBP).[76] This suggests EBV can induce virus/host cross-reactive CD4+ T-cell responses due to molecular mimicry.

EBV infection also induces expression of αB-crystallin in B-cells, an antigen that is normally restricted to the CNS and is strongly upregulated in MS lesions.[46,251] αB-crystallin peptides are selectively presented via HLA-DR to pro-inflammatory IFNγ producing CD4+ T-cells.[251,252] EBV may therefore induce autoreactive T-cells.

Another possible implication of EBV in inducing auto-aggressive T-cells is suggested by the altered autoantigen processing and presentation as is shown in a rhesus monkey EAE model. Here, B-cells infected with an EBV-related lymphocryptovirus are selectively capable of presenting a MOG core epitope that is normally degraded. Lymphocryptovirus infected B-cells may therefore selectively be capable of inducing autoaggressive T-cell responses.[253] It is currently unknown if this also holds true for human B-cells and EBV.

If EBV-specific or EBV-induced autoaggressive T-cells are involved in the immunopathogenesis of MS, intrathecal enrichment of specific clones is anticipated. Several studies have consistently shown intrathecal EBV-specific T-cell responses using various methods. Increased CD4+ T-cell responses towards EBV-infected B-cell lines (BLCL) were reported in MS patients but in this study the cognate Ag was not defined.[254] Early in disease increased CD8+ T-cells frequencies specific for latent (EBNA-3A) and lytic viral antigens (BMLF1) were shown using HLA-peptide tetramers in CSF.[255] Intrathecal CD4+ and CD8+ T-cells responses towards autologous dendritic cells laden with total EBV and EBNA-1 lysates (but not CMV or myelin lysates) were increased in MS patients.[256] In one patients a cross-reactive BALF5 and myelin basic protein specific CD8+ T-cell clone was identified using synthetic peptides.[257] T-cell receptor (TCR) sequencing of intrathecal T-cells showed sequences that overlap with previously described EBV-specific CD8+ TCRs, including the immunodominant lytic antigen BMLF-1.[258] In one study by the group of Aloisi and colleagues, CD8+ T-cells were shown to closely interact with BFRF-1 expressing B-cell in a GM lesion and express granzyme B and membrane bound CD107a, indicative of recent degranulation and cytotoxicity.[244] These data strongly suggest a pro-inflammatory T-cell response in the CNS that is fueled by reactivation of EBV. However, in light of the current debate on EBV infected B-cells in the CNS of MS patients, these data need independent confirmation. Detailed characterization of T-cells specific for EBV or candidate autoantigens is therefore warranted.

Scope of this thesis

T-cells are widely considered pivotal in the initiation or perpetuation of MS lesions and are thought to recognize CNS-specific antigens. Reactivity towards candidate MS-associated autoantigens (cMSAg) is mostly assessed in PB of MS patients and controls. While some show elevated cMSAg-specific T-cell responses in MS patients, others find similar frequencies in healthy controls and contradict disease association. These conflicting outcomes that may be due to caveats in the experimental design, lack of standardization of applied assays, HLA mismatched patients and controls and the commonly small sample sizes. Likely, disease-initiating auto-aggressive T-cells are enriched in the CNS of MS patients, early in disease development. In **Chapter 2**, we setup a platform to simultaneously determine CD4+ and CD8+ T-cell responses towards endogenously synthesized candidate antigens and determined intrathecal T-cell responses towards 7 putative MS-associated autoantigens in CIS and early stage MS patients.

Human herpesvirus (HHV) infections are associated with several non-infectious neurological diseases, including MS. However, due to the highly cell-associated nature of HHVs, intrathecal presence of herpesvirusses may often be misdiagnosed in cell-poor CSF samples. The prevalence of EBV in the CNS of MS patients is debated. Evidence of intrathecal HHV infection is of fundamental importance to decipher the etiological role in MS pathogenesis and other neurological diseases. In **Chapter 3** we determined the prevalence of all known HHV infections in CSF cell pellets of a large cohort of MS patients and other neurological disease patients lacking typical clinical signs of infection, sampled early after onset of disease symptoms.

Systemic EBV-specific humoral immune responses, particularly EBNA-1, are elevated in MS patients. Serum EBNA-1 IgG levels are elevated before onset of MS and subsequently correlate with disease activity and prognosis. However, the biological relevance of these antibodies is uncertain. Intrathecal synthesis of high affinity EBV-specific IgG is anticipated if they are involved in the immune pathogenesis of MS. In **Chapter 4** we determined the spectrum of EBV antigens recognized in serum and CSF, and defined highly specific B-cell epitopes in the immunodominant EBNA-1. Next, we determined whether the highly EBNA-1-specific IgG are produced intrathecally in MS and non-infectious neurological disease patients.

Alternatively, the increased peripheral EBNA-1 specific IgG in MS patients is a consequence of a shared genetic background. MS-associated genetic factors may either influence EBV latency in B-cells, or affect EBV-specific humoral response. The contribution of MS-associated genetic risk variants in EBNA-1 and EA-D specific IgG responses was determined in **Chapter 5**.

EBV-specific T-cell responses may be involved in MS immunopathology. Intrathecal virus-specific T-cells may recognize EBV-infected cells in the brain or cross-react with CNS antigens. Auto-aggressive T-cells may recognize autoantigens induced in, or selectively presented by EBV infected B-cells. In **Chapter 6** we determined reactivity of intrathecal T-cells towards autologous EBV-infected B-cells in early MS patients and

controls. Subsequently, we assayed the specific EBV-antigens recognized by these CSF-derived CD8+ T-cells, including EBV-induced αB-crystallin. In **Chapter 7**, we determined the phenotype and function of T-cells at the site of immunopathology; the MS lesion. In paired post-mortem blood, CSF, NAWM and white matter lesion tissue biopsies of a large cohort of end-stage MS patients we characterized the phenotype, clonal repertoire and reactivity towards a panel of candidate MS-associated autoantigens and autologous EBV-infected B-cells antigen specificity of T-cells.

Key findings are summarized, their merits discussed in context of current literature and future directions for further research are proposed in **Chapter 8**.

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Chapter 2

Intrathecal CD4+ and CD8+ T-cell responses to endogenously synthesized candidate disease-associated human autoantigens in multiple sclerosis patients

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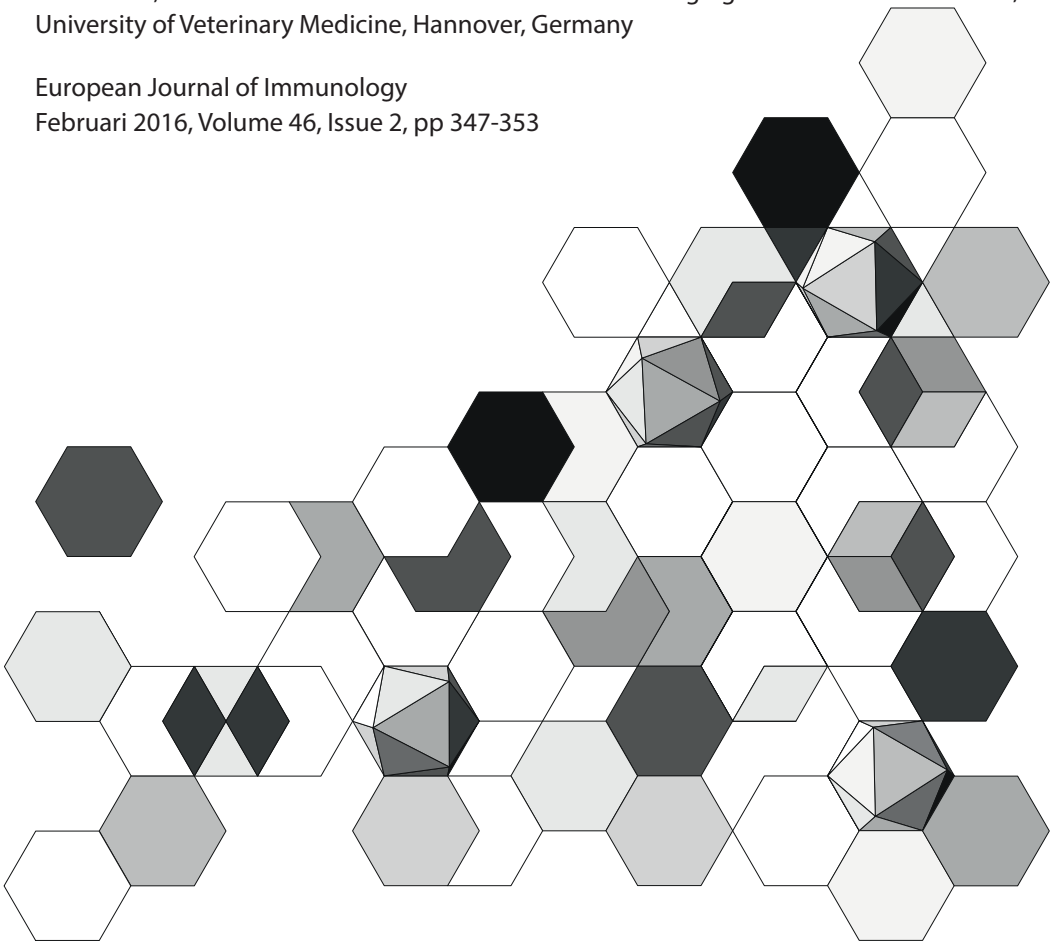
European Journal of Immunology
Februari 2016, Volume 46, Issue 2, pp 347-353



Abstract

Multiple sclerosis (MS) pathology is potentially orchestrated by autoreactive T-cells, but the antigens recognized remain unknown. A novel antigen presenting cells (APC)/T-cell platform was developed to determine intrathecal CD4+ and CD8+ T-cell responses to candidate MS-associated autoantigens (cMSAg) in clinically isolated syndrome (CIS, n=7) and MS (n=6) patients. Human cMSAg encoding open reading frames (n=8) were cloned into an Epstein–Barr virus (EBV)-based vector to express cMSAg at high levels in EBV-transformed B-cells (BLCLs). Human cMSAg cloned were myelin-associated MAG) and -oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein (PLP), ATP-dependent potassium channel ATP-dependent inwards rectifying potassium channel 4.1 (KIR4.1), S100 calcium-binding protein B (S100B), contactin-2 (CNTN2), and neurofascin (NFASC). Transduced BLCLs were used as autologous APC in functional T-cell assays to determine cMSAg-specific T-cell frequencies in cerebrospinal fluid derived T-cell lines (CSF-TCLs) by intracellular IFNγ flow cytometry. Whereas all CSF-TCL responded strongly to mitogenic stimulation, no substantial T-cell reactivity to cMSAg was observed. Contrastingly, measles virus fusion protein-specific CD4+ and CD8+ T-cell clones, used as control of the APC/T-cell platform, efficiently recognized transduced BLCL expressing their cognate antigen. The inability to detect substantial T-cell reactivity to eight human endogenously synthesized cMSAg in autologous APC do not support their role as prominent intrathecal T-cell target antigens in CIS and MS patients early after onset of disease.

Introduction

MS is a chronic neurological disorder characterized by inflammation, demyelination, and axonal loss leading to accumulative disability.[1] MS immunopathology has been widely studied in EAE models.[2] EAE is induced by immunization of animals using various, mainly myelin-derived CNS proteins, or by adoptive transfer of autoreactive T-cells. Autoreactive CD4+ and CD8+ T-cells are analogously assumed to orchestrate MS pathology, but candidate MS autoantigen (cMSAg) targets remain enigmatic.[1,2]

Several cMSAgs have been proposed including oligodendrocyte-specific proteins like myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), glia-specific proteins like inwards rectifying potassium channel (KIR4.1, ATP-dependent inwards rectifying potassium channel 4.1) and S100 calcium-binding protein (S100B) and neuron-specific proteins like contactin-2 (CNTN2) and neurofascin 155kD isoform (NFASC).[1,2] The majority of studies detailing cMSAg-specific T-cells in MS patients assayed peripheral blood with conflicting outcome.[1,2] Some observed increased auto-reactive T-cell frequencies in MS, while others described equivalent numbers in patients and controls questioning their role in MS.[3] The poor correlation between blood and intrathecal T-cell phenotypes, and TCR repertoires[4] dispute extrapolation of systemic T-cell data to MS immunopathology.

Most studies assayed intrathecal cMSAg-specific T-cell responses using autologous PBMC, pulsed with synthetic peptides or recombinant (animal) cMSAg, as APC.[2] This strategy has several limitations.[2,3] First, cMSAg of different species and bacterial-produced antigens have a different amino acid composition and conformation compared with native human cMSAg, respectively, which potentially affect processing and presentation of the cognate epitope.[5] Second, peptide binding is highly HLA allele-specific. Distinct HLA alleles are dominant MS risk or even protective factors.[1] Thus, differences in cMSAg T-cell responses in MS patients and controls may be due to intercohort HLA genotype disparity[1] ensuing mandatory HLA matching of patients and controls. Third, CD8+ T-cells preferentially recognize endogenously synthesized proteins. This is only partially overcome using cMSAg-spanning synthetic peptides as corresponding epitopes may not be presented when processed intracellularly and might need length optimization.[5] Finally, previous studies commonly used superphysiological peptide concentrations, typically in the 10–250 µM range, which may lead to activation of disease-irrelevant T-cells expressing low-avidity TCR.[6]

To overcome the aforementioned limitations we developed a novel APC/T-cell platform that facilitates high and stable expression of individual cMSAgs in Epstein–Barr virus (EBV)-transformed B-cells (BLCL), an easy to generate and efficient APC system to assay CD4+ and CD8+ T-cell responses simultaneously by flow cytometry. This APC system was used to determine the frequency of CD4 and CD8 T-cells directed to eight different human cMSAgs in short-term cerebrospinal fluid (CSF)-derived T-cell lines (CSF-TCLs) obtained from MS (n=6) and clinically isolated syndrome (CIS; n=7) patients.

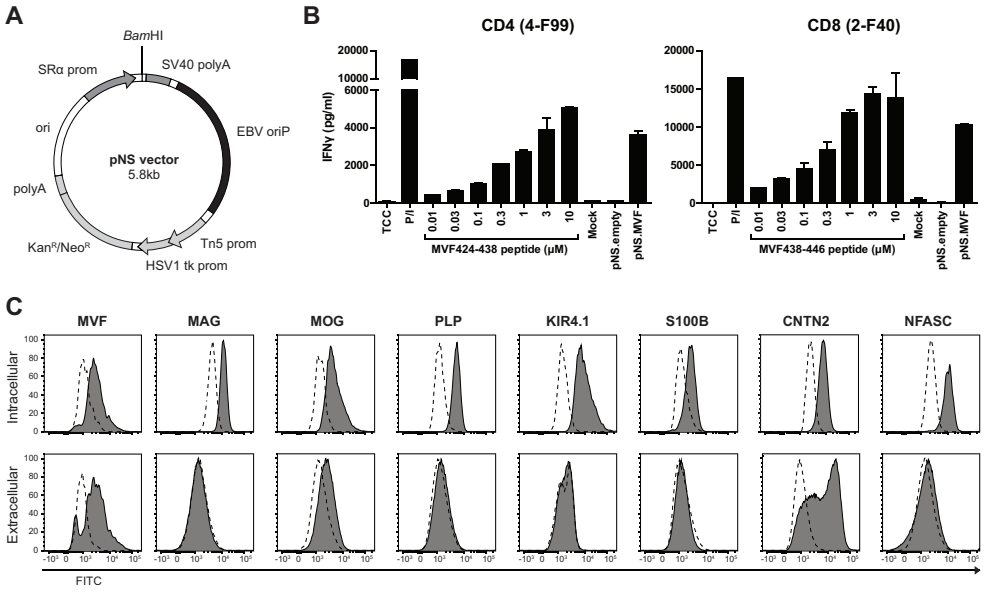


Figure 1. EBV-based episomal pNS vector enables stable expression and efficient presentation of a cloned gene in BLCL to CD4+ and CD8+ T-cells. (**A**) Schematic representation of the pNS expression vector containing EBV origin of latent replication P (EBV oriP) for episomal replication in EBV-transformed B-cell lines (BLCLs). Combined kanamycin/neomycin transferase under control of the prokaryotic transposon Tn5 and eukaryotic herpes simplex type 1 (HSV-1) promoter enabled positive geneticin selection of transduced BLCL. Antigens of interest were cloned in BamHI site and expressed under control of SRα promoter and SV40 polyadenylation signal (poly A). (**B**) Histograms of flow cytometry analyses of cells gated for lymphocytes, based on forward and side scatter and viability using fluorescent viability staining of representative BLCL transduced with pNS encoding measles virus fusion protein (MVF) and seven individual candidate human MS-associated autoantigens (cMSAg) cultured under gentamycin selection for >2 weeks. Dark area represents antigen-specific staining and the dotted line represents the respective isotype control staining. Vertical axis shows cell counts normalized to mode. Histograms are representative of two independent experiments. (**C**) HLA-matched BLCL pulsed with increasing synthetic peptide concentrations encompassing epitopes of MVF-specific CD4+ (4-F99) and CD8+ (2-F40) T-cell clones (TCCs) and stably pNS.MVF-transduced BLCL expressingMVFprotein were cocultured for 24 h with respective TCC. IFNγ levelswere determined by ELISA. As controls, TCC were nonstimulated (TCC), stimulated with phorbolmyristate-acetate and ionomycin (P/I) or cocultured with untransfected BLCL (mock) and BLCL transducedwith empty pNS vector (pNS.empty). Data are shown as mean±SD (n=4) and are pooled from two independent in-duplo experiments.

Results and discussion

BLCL express and present recombinant proteins to CD4 and CD8 T-cells efficiently

The advantage of using immortalized BLCL as APC is their high expression of HLA class I and II (HLA-I/II) and costimulatory molecules crucial for T-cell activation. Ectopic expression of EBV nuclear antigen 1 facilitates episomal replication of the origin of latent replication (oriP) containing EBV genomes. We optimized an eukaryotic EBV-based vector, containing the EBV OriP and a geneticin transferase expression cassette, facilitating stable plasmid maintenance in BLCL.[7] The original pNS vector was modified to clone and express human cMSAg proteins more efficiently (Figure 1A).

To validate that stable transduction of BLCL leads to efficient processing and presentation of endogenously expressed antigen by both HLA-I and II, we cloned measles virus fusion protein (MVF) into the pNS vector (pNS.MVF). The pNS.MVF-transduced BLCL grown for several weeks under geneticin selection showed uniform MVF surface expression (Figure 1B; left panel) (Supplementary Figure 1A). Next, we assayed recognition of transduced HLA-matched BLCL (GR-BLCL) by well-defined MVF-specific CD4+ (4-F99) and CD8+ T-cell clones (2-F40; TCCs).[8,9] Both TCC showed a dose-dependent response to BLCL pulsed with their cognate peptides (Figure 1C). TCCs cultured alone or cocultured with mock-transduced GR-BLCL secreted negligible IFNγ amounts, whereas both TCCs stimulated with phorbolmyristate-acetate and ionomycin (P/I) secreted high IFNγ levels. Notably, both TCCs efficiently recognized pNS.MVF-tranduced GR-BLCL, but not empty vector (pNS.empty)-transduced BLCL (Figure 1C). Thus, the pNS/BLCL-system facilitates high expression and efficient presentation of CD4+ and CD8+ T-cell epitopes of an endogenously expressed antigen.

Table 1. Patient demographics and diagnosis

Patient ID	Age; Sex[a]	Disease entity[b]	Disease duration[c]	CSF WBC ($x10^3$/mL)[d]	CSF OCB[e]	IgG index[f]	Follow-up (months)	Progression to MS[g]
1	37; F	CIS	10	5	no	0.5	50	no
2	34; F	CIS	22	5	no	0.5	3	no
3	21; F	CIS	8	5	multiple	0.7	46	yes, to RRMS
4	25; M	CIS	1	12	multiple	0.9	42	yes, to RRMS
5	37; M	CIS	4	3	no	0.5	25	no
6	37; M	CIS	2	2	no	0.5	24	no
7	42; M	CIS	4	3	multiple	0.7	30	no
8	51; F	PPMS	54	11	multiple	0.9	2	n.a.
9	36; F	RRMS	102	4	multiple	1.4	29	n.a.
10	34; F	RRMS	9	14	multiple	1.0	26	n.a.
11	29; F	RRMS	30	25	multiple	1.0	32	n.a.
12	41; F	RRMS	36	7	multiple	2.1	51	n.a.
13	38; F	RRMS	18	5	n.d.	0.5	51	n.a.

[a]Age in years; F, female; M, male.
[b]CIS, clinical isolated syndrome and PP, primary progressive MS; RRMS, relapsing remitting MS.
[c]Disease duration in months at time of lumbar puncture.
[d]White blood cell count (WBC) per mL cerebrospinal fluid (CSF).
[e]Presence and number of oligoclonal bands (OCB) in CSF only; n.d. not determined
[f]IgG index was determined by the following formula: (CSF [IgG]/CSF [albumin]) /(serum [IgG]/serum [albumin]).
[g]No, no progression to MS during follow-up; n.a. not applicable

No evidence for intrathecal T-cell responses to cMSAg in CIS and MS patients

No consensus exists on autoantigens specifically recognized by systemic and intrathecal T-cells in MS. Limited studies have addressed cMSAg-specificity of CSF-derived T-cells in MS.[10–12] Ideally, antigen specificity of CSF-derived T-cells is determined *ex vivo*. However, limited cell numbers in surplus CSF samples, particularly in CIS cases, prohibited functional T-cell assays for multiple cMSAgs directly (Table 1). Therefore, we generated short-term CSF-TCLs by stimulating CSF-derived cells from CIS and MS patients in a single round of mitogenic stimulation to characterize intrathecal human cMSAg-specific CD4+ and CD8+ T-cell responses simultaneously and to perform repetitive analysis to determine interassay variability.

The coding sequences of human cMSAg for oligodendrocyte (i.e. MAG, MBP1, MOG, and PLP), glia (i.e. KIR4.1 and S100B) and neuronal origin (i.e. CNTN2 and NFASC) were cloned in the pNS vector and subsequently used to nucleofect autologous BLCL (autoBLCL). Flow cytometry showed that all viable transduced BLCLs uniformly expressed the respective cMSAg intracellularly after 2–3 weeks of geneticin selection (Figure 1B). Whereas all assayed cMSAg, except MBP1 and S100B, are transmembrane proteins only MOG- and CNTN2-specific mAb target extracellular domains of the protein. Transmembrane localization of MOG and CNTN2 was confirmed by extracellular staining. Unfortunately, no MBP1-specific mAb was available.

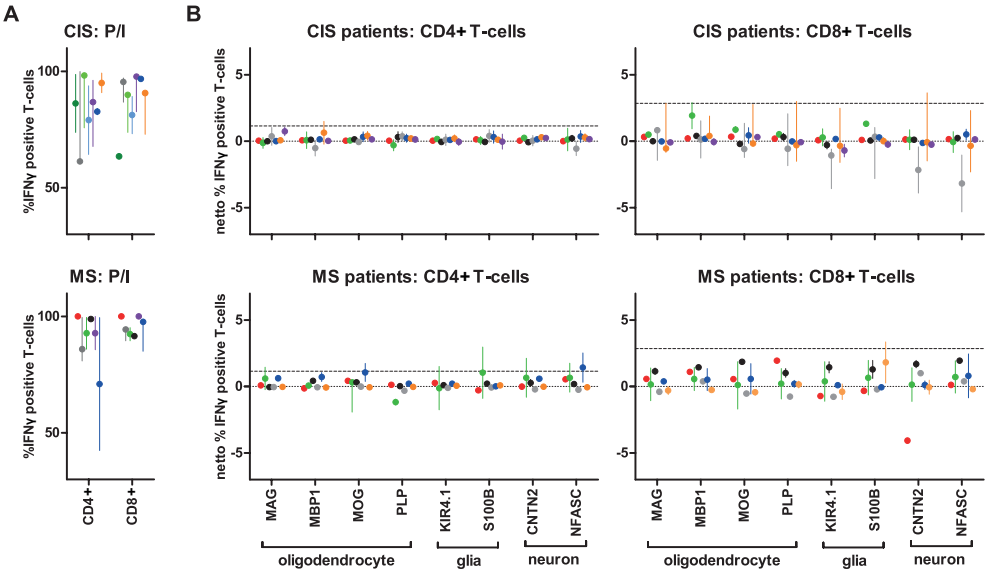


Figure 2. CD4+ and CD8+ T-cell reactivity to candidate MS-associated human autoantigens in CSF-derived T-cell lines from CIS and MS patients. (**A**) CSF-derived T-cell lines (CSF-TCLs) generated from patients diagnosed for clinically isolated syndrome (CIS; n=7) and MS (n=6) were stimulated with phorbolmyristate-acetate and ionomycin (P/I). Phenotype and frequency of responding T-cells was determined by flow cytometry. Cells were gated on viable lymphocytes using fluorescent viability staining, expression of CD3, subdivided on CD4 and CD8 and subsequently intracellular IFNγ expression. Colored dots and vertical bars represent median reactivity and range of individual CSF-TCL. (**B**) Autologous BLCL (autoBLCL) were nucleofected with pNS vector encoding candidate MS-associated antigens (cMSAgs; n=8) and positively selected in culture for >2 weeks with geneticin to generate stable cMSAg expressing BLCLs. CSF-TCLs were cocultured with cMSAg-transduced autologous BLCLs. The netto frequency of cMSAg-specific T-cells, corrected for reactivity toward pNS.empty-transduced BLCL, is shown as percentage of IFNγ+ CD4 (left panels) or CD8 T-cells (right panels). Threshold for positive cMSAg T-cell reactivity, indicated by a horizontal dashed line, was calculated as 3.09-times SD of all mean netto cMSAg-specific CD4+ and CD8+ T-cell responses, allowing a 0.1% one-tailed false discovery rate. Significance of variation in T-cell reactivity per cMSAg was determined by one-way analysis of variance for CD4+ and CD8+ T-cells independently. CSF-TCL T-cell reactivity to cMSAg-transduced autoBLCLs was determined at least twice, except for RRMS patient #9 (red color) and #12 (black color), which were measured only once.

To determine the lower level of detection of antigen-specific CD4 and CD8 T-cells in T-cell lines using the APC/T-cell platform developed, we spiked a T-cell mixture consisting of two irrelevant varicella zoster virus-specific CD4+ (TCC 146) and CD8+ (TCC 38) with increasing numbers of the MVF-specific CD4+ and CD8+ TCCs and measured the response to pNS.MVF-transduced GR-BLCL by intracellular IFNγ flow cytometry.[8,9] The estimated detection limit of CD4+ and CD8+ T-cells directed to endogenously expressed protein, here represented by MVF, in transduced GR-BLCL was 1 and 3%, respectively (Supplementary Figures 1B and 2). Next, the frequency of cMSAg-specific CSF-derived CD4+ and CD8+ T-cells was determined by coculturing CSF-TCL with human cMSAg-transduced autoBLCL. P/I stimulation of all CSF-TCL showed that the majority of CD4 and CD8 T-cells produced high levels of IFNγ indicating that the CSF-derived T-cells cultured were immunecompetent and not exhausted (Figure 2A). To correct for background T-cell reactivity (e.g. intrathecal EBV-specific T-cells recognizing BLCL),[13] cMSAg-specific CD4+ and CD8+ T-cell responses were normalized for reactivity toward pNS.empty-transduced autoBLCL (Supplementary Figure 3). Interassay variation for CD4+ and CD8+ T-cell responses was determined by calculating the SD of all mean netto cMSAg-specific responses. The variation was lower for CD4+ T-cells (0.33%) as compared with CD8+ T-cells (0.86%). Consequently, the threshold for positive calls was set at 1.2% for CD4+ T-cells and 2.9% for CD8+ T-cells allowing a one-tailed 0.1% false discovery rate, respectively.[14] This closely resembles the detection limit determined using titrated MVF-specific TCCs and MVF-transduced GR-BLCL as APC (Supplementary Figure 2). Notably, the only positive call for cMSAg reactivity was the CSF-TCL of patient #13 containing a median 1.4% (range 0.3–2.5%) frequency of NFASC-specific CD4 T-cells. However, no significant variation was observed between pMSAg for CD4+ (p=0.74) and CD8+ T-cells (p=0.30, one-way analysis of variance) (Figure 2B).

Concluding remarks

Intrathecal autoreactive T-cells are anticipated to be involved in MS pathology, but we were unable to detect substantial CD4+ and CD8+ T-cell reactivity to a broad panel of cMSAgs (n=8) in short-term cultures of CSF-derived T-cells of seven MS and six CIS patients. Few studies enumerated intrathecal antigen-specific CD4+ and CD8+ T-cells responses simultaneously using endogenously processed antigen.[2,3] Whereas antigen processing and presentation may differ between BLCL and the currently undefined local APC in MS lesions, the use of cMSAg-transduced autoBLCL as artificial APC represents a reasonable compromise to detect intrathecal cMSAg-specific T-cells. Congruent to our findings, Wuest and colleagues also did not observe significant reactivity of CSF-derived T-cells toward self-antigens using cell-, myelin-, and brain-derived lysate pulsed autologous dendritic cells used as APC.[12]

Still, a cautious interpretation of our data is warranted, as there are a few limitations. First, the assay format is not well equipped to detect low frequencies of cMSAg-specific T-cells, as the overall background was 1.1±0.3% and 2.6±0.9% (mean±SD) reactive CD4+

and CD8+ T-cells, respectively. Nevertheless, local frequencies of Ag-specific T-cells observed by others typically exceeded our detection limit in compartmentalized T-cell mediated diseases like Chronic Chagas' Cardiomyopathy[15] and autoimmune thyroiditis.[16,17] Moreover, using BLCL as APC we have previously detected profound frequencies (>5%) of intralesional T-cells. T-cells directed to the triggering herpes virus in patients with herpetic ocular diseases.[18,19] Second, a limited number of 13 patients were included in this study, which warrants further studies on larger cohorts of patients and disease controls. However, we included patients early in disease development, particularly seven CIS patients. Notably, two of them progressed to RRMS during clinical follow-up, suggesting that intrathecal reactivity against the explored antigens did not contribute to their disease evolution.

In conclusion, the inability to detect substantial intrathecal T-cell reactivity to eight human cMSAgs in CIS and MS patients do not support their role as prominent target antigen for intrathecal T-cell responses in CIS and MS patients.

Materials and methods

Patients and clinical specimens

Paired blood and CSF samples from CIS (n=7) and MS (n=6) patients were obtained as part of diagnostic workup at the Erasmus MC (Rotterdam, the Netherlands) (Table 1). No patient received immunomodulatory therapy at time of lumbar puncture, except one CIS patient (patient #2) who received Avonex treatment. CSF was always obtained >1 month after start of clinical symptoms. The study was performed according to the tenets of the Helsinki declaration, approved by the local medical ethical committee and written informed consent was obtained from all participants.

Generation of BLCL and CSF-TCL

Isolation of PBMC and generation of autoBLCL were performed as described previously.[8,13] CSF-TCL were generated from surplus CSF cells by stimulation with 1 µg/mL phytohemagglutinin-L (Roche, Branfort, CT) in the presence of 30 Gray γ-irradiated allogeneic PBMC used as feeder cells.[13] Cells were grown in RPMI-1640 supplemented with antibiotics and 10% heat-inactivated bovine (B-cells) or pooled human (T-cells) serum. T-cells received recombinant human IL-2 (25 U/mL) and IL-15 (25 ng/mL; Myltenyi biotec, Bergisch Gladbach, Germany) every 3–4 days and were harvested around day 14, aliquoted and stored frozen.[13] Generation, characterization, and maintenance of MVF-specific CD4+ and CD8+ TCC was described.[8]

Cloning of human cMSAg in the EBV-based episomal pNS vector

The eukaryotic expression vector pNS.CD8α described by Mazda *et al*.[7] was modified to the current pNS vector by removing murine CD8α by PvuII/HindIII digestion, generation of blunt ends using T4 DNA polymerase and subsequent self-ligation (all from New England Biolabs, Ipswich, MA) (Figure 1A). ORFs encoding human MAG, MBP1, MOG, PLP1, S100B, CNTN2, and NFASC were PCR amplified from cDNA clones obtained from Darmacon (Lafayette, CO) and cloned in pCR4.1-TOPO (Invitrogen). Restriction endonuclease target sequences engineered into amplification primers were used to shuttle ORFs to pNS vector's BamHI site (Supplementary Table 1). ORFs encoding MVF were cloned from pEC12.MVF[20] and human KIR4.1 (Damacon) from a cDNA clone by SacI or SacII/SmaI digestion into pNS vectors's BamHI site after generating blunt ends using T4 DNA polymerase, respectively. DNA sequences of all cloned vectors were verified by Sanger sequencing using an ABI prism 3130XL genetic Analyzer (Applied Biosystems, Foster City, CA) with pNS vector specific 5´- and 3´-end cMSAg flanking primers and if no overlapping sequences were obtained internal cMSAg-specific primers (data not shown).

Generation of BLCL expressing MVF and candidate MS-associated neuroantigens

BLCL were nucleofected using Amaxa Cell line nucleofection kit V (Lonza; Bazel, Switzerland) per manufacturer's instructions and transduced cells positively selected using 500 µg/mL geneticin (Life Technologies; Carlsbad, CA) for >2 weeks. Expression of cMSAgs and MVF were determined by extracellular and intracellular flow cytometry on a FACSCanto II (BD Biosciences; San Jose, CA) using specific antibodies. Intracellular staining was performed using Cytofix/Cytoperm Kit (BD Biosciences) per manufacturer's instructions. The following mAbs were used at predefined concentrations: Mouse-anti-MVF (clone F3-5), mouse-anti-MAG (clone 513; Millipore; Darmstadt, Germany), mouse-anti-MOG (clone 8–18C5; Millipore), mouse-anti-PLP1 (clone sc-73336; Santa Cruz; Dallas, TX), mouse-anti-KIR4.1 (clone 1C11; Sigma-Aldrich; St. Louis, MO), mouse-anti-S100B (clone 19/S100B; BD Biosciences), mouse-anti-CNTN2 (clone 372913; R&D systems; Minneapolis, MN), and rabbit-anti-NFASC (clone ab31457; AbCAM; Cambridge, UK). Secondary fluorescein-conjugated goat-anti-mouse IgG (BD Pharmingen; San Diego, CA) or swine-anti-rabbit IgG (Dako; Glostrup, Denmark) were used. Respective mouse IgG1, IgG2a, IgG2b, or Rabbit IgG isotypes were used as negative controls. Data analysis on viable stained cells was performed using FlowJo software version 10 (Ashland, OR). Gating strategy for BLCL is shown in Supplementary Figure 1A.

T-cell reactivity to endogenously expressed MVF in BLCL

CD4+ and CD8+ MVF-specific TCCs were incubated with pNS.MVF-transduced HLA-matched BLCL (GR-BLCL). Negative controls included TCC alone, TCC cocultured with BLCL or BLCL nucleofected with the pNS vector without insert. Positive control for T-cell stimulation included stimulation with phorbolmyristate-acetate (50 ng/mL) and ionomycin (500 ng/mL) (Sigma-Aldrich). Additionally, TCC were incubated with GR-BLCL

pulsed overnight with increasing concentrations of synthetic peptide, range 0.01–10 μM, representing minimal T-cell epitopes.[8] In all assays, 5×10^4 BLCL were cocultured with either TCC at a 1:1 ratio for 24 h. Secreted IFNγ was quantified by ELISA in conditioned culture medium (eBioscience; San Diego, CA). Experiments were performed twice.

The detection limit of T-cell responses toward endogenously expressed antigen was determined by titrating the MVF-specific CD4+ (4-F99) and CD8+ TCC (2-F40) into a T-cell mixture, consisting of VZV-specific CD4+ (TCC 146; patient 4) and CD8+ TCC (TCC 38; patient 3),[9] ranging from 0.1 to 10% of either MVF-specific TCC in the T-cell mixture. T-cell mixtures (total 10^5 cells) were cocultured with pNS.MVF -transduced GR-BLCL in a 1:1 ratio for 6–8 h in the presence of Golgistop per manufacturer's instructions (BD Biosciences). GR-BLCL transduced with pNS.empty and GR-BLCL pulsed overnight with 3 μM of aforementioned synthetic peptides were used as negative and positive control APC, respectively. T-cells were phenotyped with fluorochrome-conjugated mAbs directed to CD3 (clone SP34-2) (BD Pharmingen; San Diego, CA), CD4 (clone SK3; BD Biosciences), and CD8α (clone RPA-T8; eBioscience) stained for viability (violet live/dead stain; Invitrogen), fixed, permeabilized, and subsequently stained for intracellular IFNγ (clone B27; BD Pharmingen) followed by flow cytometry. Lymphocytes were first gated based on forward and side scatter characteristics and viability using violet live/dead staining and subsequently on differential expression of surface markers CD3, CD4, and CD8 (Supplementary Figure 1B). Finally, intracellular IFNγ expression was defined for both gated T-cell subsets (Supplementary Figure 2). Experiment was performed twice.

Intrathecal T-cell reactivity to endogenously expressed human cMSAg expressed in autologous BLCL

CSF-TCL (10^5 cells) were cocultured with autoBLCL stably transduced with aforementioned cMSAg (n=8) in a 1:1 ratio for 6–8 h in the presence of Golgistop per manufacturer's instructions (BD Biosciences). Negative and positive controls included CSF-TCL incubated with pNS.empty-transduced autoBLCL and CSF-TCL solely stimulated with P/I, respectively. T-cells were stained and analyzed using flow cytometry as described above (Supplementary n Figure 3). Experiments were performed at least twice, except for RRMS patient #9 and #12 where limited numbers of CSF-TCL cells were available.

Statistical analysis

Netto T-cell reactivity toward cMSAg-transduced BLCL was calculated by subtracting reactivity toward pNS.empty-transduced BLCL. Threshold for positive cMSAg T-cell reactivity was calculated as 3.09-times SD of all mean netto cMSAg-specific CD4+ and CD8+T-cell responses, allowing a 0.1% one-tailed false discovery rate.[14] Significance of variation in T-cell reactivity per cMSAg was determined by one-way analysis of variance for CD4+ and CD8+ T-cells independently.

Acknowledgements

This work was supported in part by grants from Dutch MS Research Foundation (#MS 09-670) and the Niedersachsen-Research Network on Neuroinfectiology (N-RENNT) of the Ministry of Science and Culture of Lower Saxony (Germany). We kindly acknowledge Dr. Osam Mazda (Kyoto Prefectural University of Medicine, Kyoto, Japan) for sharing the pNS. CD8α vector, Dr. Werner J Ouwendijk, Sarah Getu, and Samira Michels (Dept. Viroscience, Erasmus MC) for technical assistance.

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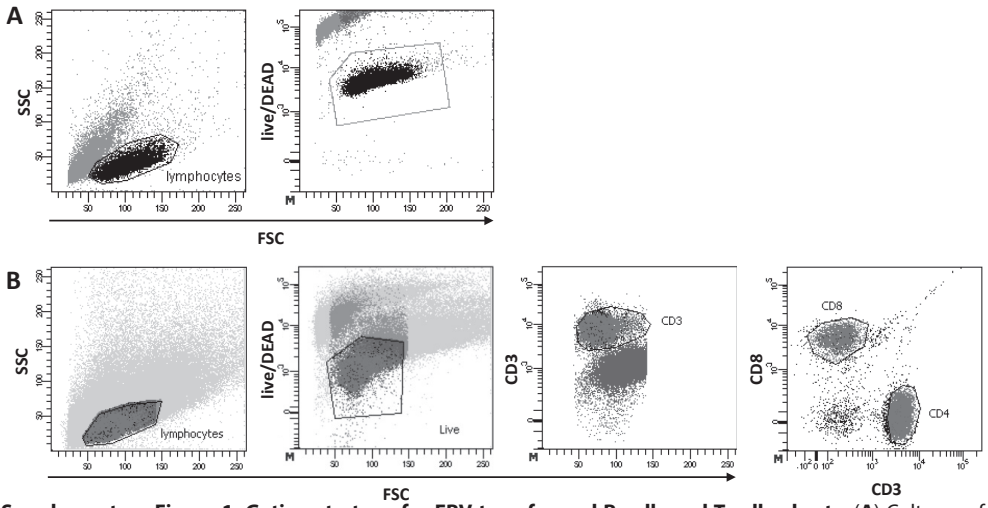
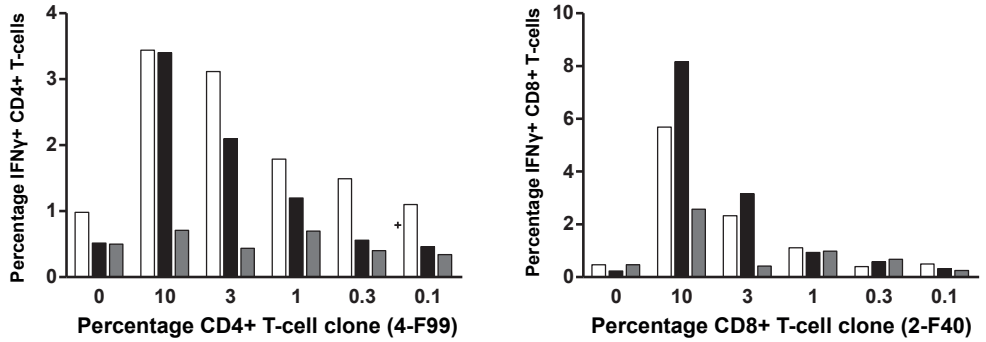
Supplementary Table 1. Cloning of MS-associated antigens in pNS vector

Gene[a]	GenBank Accession No	Length (aa)[b]	Primer sequence[c] 5'-end forward (5'-3')	Primer sequence[c] 3'-end reverse (5'-3')	Restriction site ORF[d] 5'-end	Restriction site ORF[d] 3'-end
MAG	BC053347	626	CT*CTTAAG*TACAGA**ATG**ATATTCCTCAC	CT*ATGCAT*ACTTGACCCGGATTTCAG	*Afl*II (T4)	*Nsi*I (T4)
MBP1	BC065248	197	CT*CTTAAG*ATTCAGG**ATG**GGAAACCAC	CT*ATGCAT*GCTACGTGCCAGTTCTTCC	*Afl*II (T4)	*Nsi*I (T4)
MOG	BC035938	295	CT*CTTAAG*TAGAG**ATG**GCAAGCTTATC	CT*ATGCAT*TCTAACAGCTGGCTTCTTTGC	*Afl*II (T4)	*Nsi*I (T4)
PLP	NM_000533	277	CT*GGATCC*CAAAGAC**ATG**GGCTTGTTAG	CT*GGATCC*GAACTTGGTGCCTCGGC	*Bam*HI	*Bam*HI
KIR4.1	BC131627	379	n.a.	n.a.	*Sac*II (T4)	*Sma*I
S100B	BC001766	92	CT*GGATCCG*AGG**ATG**TCTGAGCTGGAGAAGGCC	CT*GGATCC*TCAAAGAACTCGTGGCAGGCAG	*Bam*HI	*Bam*HI
CNTN2	BC129986	1040	CT*GGATCC*ATCCACC**ATG**GGGACAGCCAC	CT*GGATCC*GAAGGGGCTGGCTGTGTCCG	*Bam*HI	*Bam*HI
NFASC	BC137013	1169	GA*GGATCC*GGGCCAGGTGCCGAGG**ATG**	GA*GGATCC*GTGGTTTTGTCTCCCCTTCTC	*Bam*HI	*Bam*HI
MVF	AM237414	553	n.a.	n.a.	*Sac*I (T4)	*Sac*I (T4)

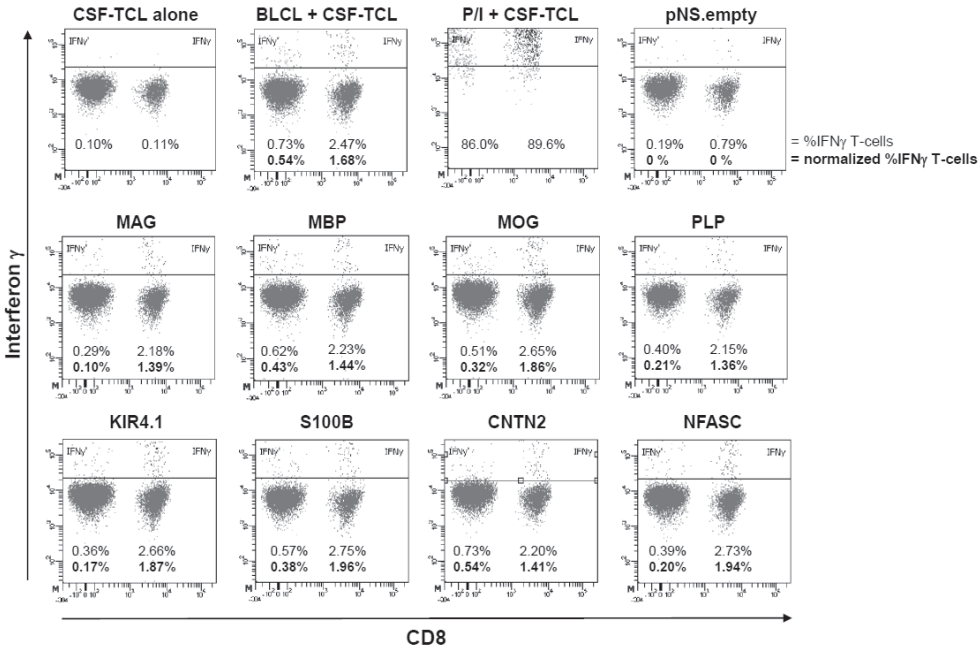
[a]MAG; myelin associated glycoprotein, MBP1; myelin basic protein isoform 1, MOG; myelin oligodendrocyte protein, PLP; proteolipid protein, KIR4.1; inward rectifying potassium channel 4.1, S100B; S100 calcium binding protein B, CNTN2; contactin-2, NFASC; neurofascin, and MVF; measles virus fusion protein.
[b]aa, amino acid.
[c]Primer sequence annotation: *restriction site*, **start codon**; n.a. not applicable.
[d]ORF, open reading frame; T4, restriction site blunted using T4 DNA polymerase.



Supplementary Figure 1. Gating strategy for EBV-transformed B-cells and T-cell subsets. (**A**) Cultures of EBV-transformed B-cells (BLCL) were stained for viability with the violet live/dead buffer and subsequently for intra-cellular or surface expression of candidate multiple sclerosis-associated antigens as depicted in Figure 1B. Viable BLCL were identified based on lymphocyte forward and side scatter characteristics and cell viability. (**B**) Cell cultures, consisting of T-cells and BLCL, were stained for surface markers CD3, CD4 and CD8 and viability using violet live/dead buffer. Viable T-cells were identified based on lymphocyte forward and side scatter characteristics, live cells and subsequently CD3 expression. Finally, viable CD3+ cells were separated into CD4+ and CD8+ viable T-cells.



Supplementary Figure 2. Estimation of CD4+ and CD8+ T-cell detection limit recognizing recombinant protein expressing EBV- transformed B-cells used as antigen presenting cells. EBV-transformed B-cells (BLCL) were nucleofected with a eukaryotic expression vector (pNS) encoding measles virus F (MVF) protein to generate stable high MVF protein expressing BLCL. Indicated percentages of two different MVF- specific T-cell clones (TCC), one CD4+ (clone 4-F99) and one CD8+ (clone 2-F40) TCC, were spiked into a T-cell population consisting of two irrelevant varicella zoster virus-specific CD4+ and CD8+ TCC. T-cell mixtures were co-cultured with MVF-expressing BLCL in a 1:1 ratio for 6 to 8 hrs at 37˚C in the presence of Golgistop and subsequently stained for intracellular IFNγ and surface CD4 and CD8 followed by flow cytometry analysis (black bars). Cells were gated for viable lymphocytes, based on forward and side scatter and cell viability using violet live/dead buffer, expressing CD3 and subsequently subgated for viable CD4+ and CD8+ T-cells expressing IFNγ. Frequencies of reactive T-cells are shown as percentage of IFNγ+ CD4+ or CD8+ T-cells. T-cell reactivity towards non-transduced BLCL pulsed overnight with 3 μM synthetic peptide encompassing the MVF-specific TCC cognate MVF epitopes were included as positive control (white bars). BLCL transduced with the empty pNS vector were used as negative control (grey bars). Data of an experiment, performed in monoplo, are shown. The experiment was performed twice.



Supplementary Figure 3. T-cell reactivity towards endogenously synthesised cMSAg of a cerebrospinal fluid-derived T-cell line (CSF-TCL) of multiple sclerosis (MS) patient #11. Autologous EBV-transformed B-cells (BLCL) were nucleofected with the eukaryotic expression vector pNS encoding candidate human MS-associated antigens (cMSAg; n=8) and positively selected *in vitro* with geneticin for >2 weeks at 37˚C to generate stable cMSAg expressing autologous BLCL. CSF-TCL generated from surplus CSF of patient #11 were co-cultured with cMSAg-transduced autologous BLCL in a 1:1 ratio for 6 to 8 hrs at at 37˚C in the presence of Golgistop and subsequently stained for intracellular IFNγ and surface CD3, CD4 and CD8 followed by flow cytometry analysis. Cells were gated for lymphocytes forward and side scatter characteristics, viability using violet live/dead buffer and subsequently CD3 expression. Finally, viable CD3+ cells were subgated for CD4+ (pink) and CD8+ (blue) T-cells and subsequently intracellular IFNγ expression. Frequency of reactive T-cells are shown as percentage of IFNγ+ CD4+ (pink) or CD8+ (blue) T-cells. The normalized frequency of cMSAg-specific T-cells, corrected for reactivity towards autologous BLCL transduced with the empty pNS vector (pNS.empty, is shown as netto percentage of IFNγ+ CD4+ or CD8+ T-cells (bold). CSF-TCL alone and co-cultured with non- transduced autologous BLCL served as negative controls. CSF-TCL stimulated solely with phorbolmyristate-acetate and ionomycin (P/I) served as positive control for T-cell activation. Data of an experiment, performed in monoplo, are shown. The experiment was performed twice.

Chapter 3

Prevalence of human *Herpesviridae* in cerebrospinal fluid of patients with multiple sclerosis and noninfectious neurological disease in the Netherlands

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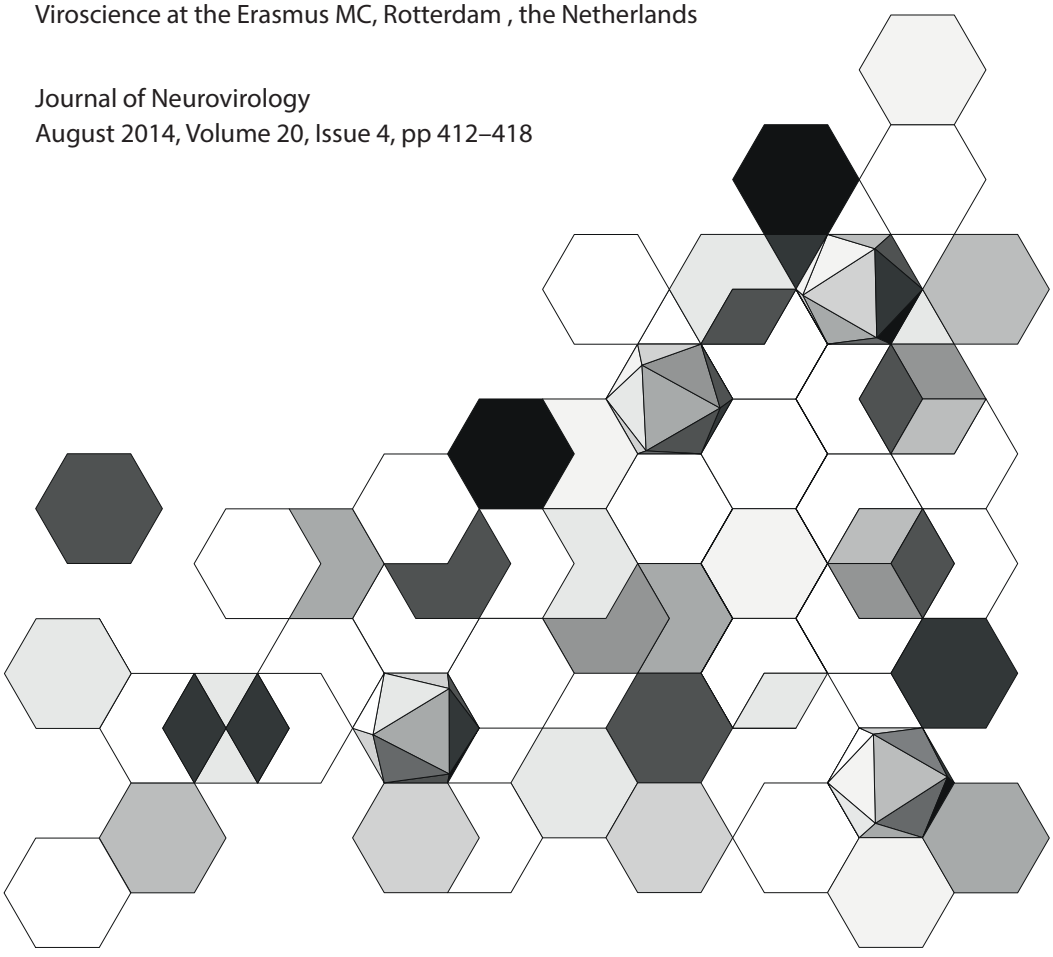
Journal of Neurovirology
August 2014, Volume 20, Issue 4, pp 412–418



Abstract

Prevalence of eight human herpesviruses (HHV1–8) was determined by real-time PCR in cell-rich cerebrospinal fluid (CSF) samples, obtained early after disease symptoms, of Dutch patients with multiple sclerosis (MS) and other noninfectious central nervous system diseases (NIND). Whereas HHV1–8 DNA was undetectable in CSF samples of MS patients, HHV6 DNA was detected in a plexus neuritis case and HHV7 DNA in an ependymoma and a Behçets' disease patient. However, intrathecal HHV infection was not detected. Data indicate that HHV1–8 are rarely detected in CSF of Dutch NIND patients and do not support the role of intrathecal HHV infection early after onset of disease symptoms in MS.

Introduction

Human herpesviruses (HHV) are endemic worldwide. The HHV family includes eight members: herpes simplex types 1 (HSV-1; HHV1) and 2 (HSV-2; HHV2), varicella zoster virus (VZV; HHV3), Epstein-Barr virus (EBV; HHV4), cytomegalovirus (CMV; HHV5), human herpesviruses 6 (HHV6) and 7 (HHV7), and Kaposi's sarcoma-associated herpesvirus (KSHV; HHV8). A hallmark of HHV is their ability to establish a lifelong latency of the host and reactivate intermittently to shed virus or cause recrudescent disease.[1]

All HHV can infect lymphocytes during primary infection but also establish latency in specific lymphocyte subsets (e.g., HHV4, HHV6, and HHV7). Predominantly in central nervous system (CNS) disease, but also during normal immune surveillance, lymphocyte can pass the blood–cerebrospinal fluid barrier or the blood–brain barrier to enter the cerebrospinal fluid (CSF).[2] The high prevalence of HHV infections worldwide and their ability to infect migratory lymphocytes argue for their potential etiopathogenic role in CNS diseases.[3,4]

Multiple sclerosis (MS) is an inflammatory CNS disease leading to demyelination and neurodegeneration. Environmental risk factors, including infectious agents like viruses and bacteria, determine whether MS develops in genetically predisposed individuals.[5–7] Various herpesviruses including HHV3, HHV6, and, more recently, HHV4 have been suggested to be involved in MS pathology.[6] In addition to MS, herpesvirus infections have also been advocated to trigger other neurological diseases without clinical evidence of an infectious origin.[3] Current data on the potential role of HHVs in MS and other noninfectious neurological diseases are inconclusive.[3,4,6] Contradictory studies have been reported on the association with MS and HHV3,[8–13] HHV4,[4,9–17] and HHV6 (Supplementary Tables 1 and 2).[9–12,14,17–23] Discordant data on the etiologic role of HHV and CNS diseases may in part be due to the sampling interval after disease onset, content of the CSF samples analyzed, and methodology used to detect HHV DNA. The time window in which cell-free HHV is detectable in clinical samples like CSF after infection is usually short due to enzymatic degradation of the virus. Furthermore, HHVs are commonly highly cell-associated and may often be misdiagnosed in cell-poor CSF samples.[24,25]

The aim of the current study was to determine the prevalence of HHV infection in the Netherlands in a large cohort of patients with MS (*n*=53) and patients lacking typical clinical signs of infectious neurological disease (*n*=135) by analyzing cell-rich CSF samples obtained early after onset of disease symptoms.

Materials and Methods

Patients and clinical specimens

At the Department of Neurology (Erasmus University Medical Center, Rotterdam, the Netherlands), between years 2004 and 2008, CSF samples from MS patients (*n*=53) and patients with clinical noninfectious neurological disease (NIND; *n*=135) were obtained by lumbar puncture. The CSF studied were surplus samples collected as part of the diagnostic workup within 2 weeks after onset of disease symptoms. The patient characteristics are summarized in Table 1. The MS patient cohort consisted of 28 cases with relapsing–remitting (RRMS), 12 primary progressive (PPMS), and 13 patients with clinically isolated syndrome (CIS).[26,27] The NIND patient cohort consisted of 135 cases suffering from a variety of clinical symptoms including cancer (e.g., leukemia and lymphoma; *n*=25), headache (*n*=19), subarachnoid hemorrhage (*n*=8), Behçet's disease (*n*=4), and epilepsy (*n*=4) (data not shown). A patient with clinically diagnosed herpetic encephalitis was included as positive control for the combined detection of HHV-specific DNA and RNA. Each enrolled patient had consented in future testing of archived clinical samples. The study was approved by the local ethical committee and performed according to the tenets of the Helsinki Declaration.

Isolation of DNA and RNA from CSF samples

CSF aliquots (2–5 mL) were centrifuged at 3,000×*g* for 10 min, and CSF cell pellets (CSFpel) and supernatant (CSFsup) were stored at −80˚C. Total nucleic acid was isolated using MagNA Pure LC total nucleic acid isolation kit combined with the MagNA Pure LC isolation station (Roche Diagnostics, Mannheim, Germany) from the CSFpel or 200 µL CSFsup per manufacturer's instructions.

Table 1. General characteristics of the study population

Neurological disease entity	Number	Gender male: female	Mean age (years ± SD)
Multiple sclerosis	53	21 : 32	39 ± 12
Relapsing remitting	29	10 : 19	39 ± 11
Primary progressive	11	5 : 6	47 ± 9
Clinically isolated syndrome	13	6 : 7	32 ± 10
Non-infectious neurological diseases	135	63 : 72	50 ±18

Quantification of cells and HHV1–8 DNA in CSF samples

Real-time quantitative PCR (qPCR) was used to determine both the total amount of cells and HHV1–8 genome equivalent copies (gec) in CSF-derived nucleic acid preparations using an ABI Prism 7500 and Taqman Universal Master Mix (both from Applied Biosystems, Nieuwerkerk, the Netherlands) as described previously.[28,29] The qPCR assay used to determine the number of cells was based on the human single-copy housekeeping gene *Homo sapiens hydroxymethylbilane synthase* (*HMBS*).[29] The HHV-specific sequences and target genes of the primers/probe pairs used are described elsewhere: HHV1 gene *US4* (glycoprotein G),[28] HHV2 gene *US6* (glycoprotein D),[28] HHV3 gene *ORF38* (thymidine kinase),[28] HHV4 gene *BNRF1* (tegument protein p143),[30] HHV5 gene *UL75* (DNA polymerase),[28] HHV6 gene *U38* (DNA polymerase),[31] HHV7 gene *U57* (DNA polymerase),[31] and HHV8 gene *ORF25* (major capsid protein).[32] For standardization of the HHV-specific qPCR assays, commercially available quantified DNA control panels (Advanced Biotechnologies, Columbia, MD) and high-titer virus preparations derived from culture supernatants were used.[28] The sensitivity of the HHV-specific qPCR assays, as defined by the 95 % hit rate on the electron microscopy-counted virus stocks, was ~100 HHV gec/mL. For HMBS standardization, an HHV4-transformed B-cell line was used as reference.[29]

Quantification of HHV transcripts in CSF samples

Nucleic acid preparations of HHV DNA-positive CSF samples were treated with recombinant DNase (Roche Diagnostics) followed by complementary DNA (cDNA) synthesis using random hexamer primers and subsequently subjected to qPCR analysis with the same HHV-specific primer/probe pairs used to detect the respective late HHV transcripts.[29–31] Reverse transcribed qPCR (RT-qPCR) analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Applied Biosystems) was always run in parallel to calculate the relative HHV transcript levels, calculated by the following formula: $\Delta Ct(GAPDH) = Ct(viral\ transcript) - Ct(GAPDH)$. In case of HHV6 U38 transcript detection, cDNA was synthesized using primers that cross-react between HHV6A and HHV6B in a well-defined splice junction in the HHV6 *U100* gene region.[33] HHV6 transcripts were amplified by a nested PCR and detected using ethidium bromide staining and agarose gel electrophoresis. Omission of reverse transcriptase in cDNA synthesis resulted in no detectable HMBS, GAPDH, and HHV qPCR signals demonstrating that the RT-qPCR signals obtained represent transcript levels (data not shown).

Statistical analysis

Statistical differences between CSF cell numbers were determined by the Mann–Whitney *U* test. $p<0.05$ was considered significant.

Results

Estimation of cell numbers in CSF samples

Discordant data on the role of HHV in the etiology of noninfectious neurological diseases, particularly the association of MS and HHV3,[8–13] HHV4,[4,9–17] and HHV6,[9–12,14,17–23] may be due to the CSF format assayed.[24,25] Because most HHVs are lymphotropic and cell-free viral DNA is more vulnerable to enzymatic destruction, we postulated that qPCR analysis on DNA isolated from CSF-derived cells is more appropriate to assay the role of intrathecal HHV1–8 infections in relation to MS and other noninfectious neurological diseases.

To estimate the cell content in the pelleted CSF samples of our study cohort, consisting of 53 MS and 135 NIND patients, we performed qPCR analysis specific for the single-copy human gene *HMBS* on nucleic acids extracted from the CSF cell pellets of all patients.[29] The median estimated number of cells ± interquartile range in all CSF samples was 551±2,248 cells/CSF (range, 0–1.18×10^6). HMBS qPCR signal was undetectable in the CSF of six (4%) NIND, two (15%) CIS, six (55%) PPMS, and eight (28%) RRMS patients. The median estimated cell numbers in NIND patients (618±2,159; range 0–1.18×10^6 cells/CSF) was significantly higher compared to those in MS patients (166±3,019; range 0–217,432 cells/CSF; p=0.04) (Figure 1A). Within the MS patient group, the median CSF cell numbers in PPMS patients (0±122; range 0–7,598) was significantly lower compared to both RRMS patients (654±2,172; range 0–27,196; p=0.037) and CIS patients (1,394±217,431; range 0–217,431; p=0.013) (Figure 1B). In neither patient group, CSF cell numbers correlated with the patients' age and gender (data not shown).

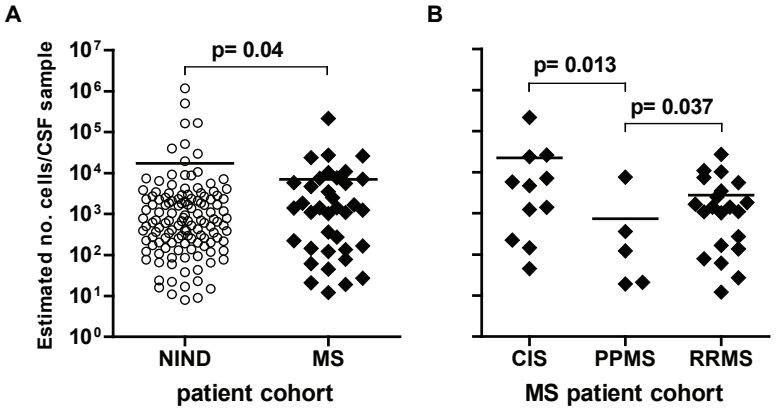


Figure 1. Comparison of estimated cell numbers in cerebrospinal fluid (CSF) samples between patients with multiple sclerosis (MS) and noninfectious central nervous system disease (NIND). (**A**) Comparison betweenMS and NIND patients. (**B**) Comparison betweenMS patients with clinically isolated syndrome (CIS), primary progressive MS (PPMS), and relapsing–remitting MS (RRMS). Statistical differences between CSF cell numbers were determined by the Mann–Whitney U test.

Prevalence and state of intrathecal HHV1–8 infection in MS and NIND patients

We determined the presence of HHV1–8 DNA in CSF samples of all MS (*n*=53) and NIND patients (*n*=56) with suspected mononuclear pleocytosis, arbitrarily set at >1,500 HMBS gec/CSF (*n*=49) (Figure 1A). Whereas HHV1–8 DNA was undetectable in CSF samples of MS patients, HHV6 DNA was detected in one plexus neuritis case (patient #2) and HHV7 DNA in both an ependymoma (patient #3) and a Behçet's disease patient (patient #4, Table 2). In comparison, CSF cell pellet of the plexus neuritis case contained a relatively high HHV6 DNA load (4.3 HHV6 gec/CSF cell).

Detection of HHV-specific DNA implicates a disease-relevant lytic HHV infection or the presence of latently infected cells that may have entered the subarachnoid space nonspecifically upon inflammation-induced disruption of the blood–brain barrier.[24,25] To differentiate between both options, HHV DNA-positive CSFpel samples were analyzed for lytic transcripts of the respective HHVs. To validate our assays, we included a CSF sample of a herpetic encephalitis case (patient #1). We confirmed the clinical diagnosis by identifying HHV2 as causative agent but also detected HHV4 DNA in the same CSFpel sample. Consistent with the pathology of herpetic encephalitis, which is predominantly mediated by the cytopathic effect of the virus,[34] we detected the HHV2 US6 transcript encoding the glycoprotein D and the presence of HHV2 in the patient's CSFsup sample. Moreover, the HHV2 DNA load in CSFsup was lower compared to the paired CSFpel sample (Table 2). Notably, no viral transcripts and DNA of the respective HHVs were detected in both the CSFpel and paired CSFsup samples of the other HHV DNA-positive patients, respectively (Table 2; data not shown).

Table 2. Characteristics of patients with human herpesvirus DNA positive cerebrospinal fluid (CSF) samples[a]

			Estimated cell number and quantity of HHV-specific DNA and RNA in CSF				
Patient No.	**Gender; Age (yrs)**	**Clinical diagnosis**	**Cell number/ CSF pellet**	**HHV type**	**CSF pellet HHV gec/cell**	**CSF supernatant HHV gec/mL**	**HHV RNA level ΔCt (HHV-GAPDH)**
1	Male; 60	HHV2 encephalitis	$1.8X10^6$	HHV2	$4.6x10^{-2}$	$8.5x10^4$	-3.95
				HHV4	$2.8x10^{-2}$	UND	UND
2	Female; 59	Plexus neuritis	$3.2X10^3$	HHV6	4.3	UND	UND
3	Male; 32	Ependymoma	$1.17X10^6$	HHV7	$4.4x10^{-4}$	UND	UND
4	Female; 27	Behcet's disease	$1.9X10^3$	HHV7	$2.2x10^{-2}$	UND	UND

[a]HHV, human herpesvirus; gec/cell, genome equivalent copies per cell; ΔCt (HHV-GAPDH), delta Ct value of respective HHV and the hous-keeping gene GAPDH; UND, undetectable and ND, not determined.

Discussion

Central nervous system infections are predominantly caused by viruses, commonly HHVs and enteroviruses.[25,35] However, the etiology of the vast majority of neurological diseases has not been defined.[3] Epidemiological data and clinical presentation of specific sterile CNS diseases, particularly MS, hint at the etiopathological role of viruses.[4,6] Human herpesviruses are potential candidates as they are endemic worldwide and establish a lifelong persistent infection with intermittent reactivation.[1] The aim of the current retrospective study was to determine the prevalence and state of HHV1–8 infection in surplus CSF samples of MS patients (*n*=53) and individuals with neurological disease lacking typical clinical signs of CNS infection (*n*=135), who underwent a diagnostic lumbar puncture at early onset of disease symptoms in the Netherlands.

Real-time PCR is considered the gold standard to detect and quantify viruses in clinical specimens. However, viral loads in CSF samples are commonly presented as genome equivalent copies per milliliter, potentially leading to misinterpretation or even false-negative results as most viruses, including HHVs, are cell-associated, and cell-free virus is more vulnerable to degradation.[24,25] We postulate that diagnostic analysis aimed to unravel both the viral etiology and the burden of virus infection in CSF of patients with CNS diseases should preferably be performed on CSF cell pellets. The estimated cell content of the CSFpel samples, determined by HMBS-specific qPCR, varied extensively within and between CSF samples of MS and NIND patients (Figure 1A). Sterile CSF pleocytosis, arbitrarily set at >1,500 HMBS copies/sample, was found in 16 of 53 (30%) MS patients and correlated with the clinical MS subtype. Pleocytosis was detected in CSF samples of 6 of 13 (46%) CIS and 9 of 28 (32%) RRMS patients compared to only 1 of 12 (8%) PPMS patients who had no signs of clinical relapse. The HMBS copy numbers were significantly higher in both CIS and RRMS patients compared to those in PPMS patients (Figure 1B). The data are in line with the reported association between CSF pleocytosis and clinical activity in MS.[27] Moreover, 49 of 135 (36%) NIND patients showed signs of pleocytosis, which was not restricted to a specific CNS disease, and significantly more cells were detected in the CSF of NIND compared to MS patients (Figure 1A).

The presence of HHV1–8 DNA in CSFpel samples was assayed by qPCR in all MS patients and 49 NIND cases with suspected pleocytosis. We did not detect HHV1–8 DNA in CSF samples of the MS patients, which concur with previous reports that did not found an association with HHV1, HHV2, HHV5, HHV7, and HHV8 and MS (Supplementary Tables 1 and 2).[9–12,14,36,37] However, our data contrast previous reports that documented a strong disease association between HHV3,[10,12,13] HHV4,[12,16,17] and HHV6 DNA[10,11,14,17–22] in CSF. For all three HHVs, however, analogous studies on CSF samples of MS patients by multiple labs in different countries were also unable to confirm these findings.[4,8,9,11,12,18,23,36] These discordant data are puzzling and may be related to various methodological variables that influence the sensitivity of detecting HHV DNA in CSF, including patient selection (i.e., country and MS entity) and sampling date of CSF in relation to disease symptoms, the CSF format analyzed (i.e., whole CSF, CSFsup, or CSFpel), and the PCR assays (i.e., qPCR, conventional and nested conventional PCR) used.[24,25,38] However, detailed analyses of the

design and data of analogous studies did not identify a specific variable that explains the conflicting PCR data on the presence of HHV3, HHV4, and HHV6 DNA in the CSF of MS patients (Supplementary Tables 1 and 2).

HHV-specific DNA was detected in 3 of 49 (6%) CSFpel samples of NIND cases with suspected pleocytosis. A similar low prevalence of HHV DNA-positive CSF samples of NIND patients have been reported in Sweden.[39] We detected HHV7 DNA in an ependymoma and a Behçet's disease patient and HHV6 DNA in a plexus neuritis case (Table 2). Because the HHV7 DNA load/CSF cell was very low and no viral DNA was detected in the paired CSFsup sample, an intrathecal HHV7 infection at time of sampling was excluded.[24,25] Moreover, as two and three additional ependymoma patients and a Behçet's disease patient tested were HHV7 negative, the etiopathogenic role of HHV7 infection in both diseases is unlikely. Based on the same arguments, we conclude that the plexus neuritis case did not have an intrathecal HHV6 infection and that HHV6 was not associated with the disease. Moreover, the relatively high HHV6 DNA load (4.3 HHV6 gec/CSF cell) does not imply an active infection but most likely represents chromosomal integrated HHV6 (ciHHV6).[1,40] HHV6 is the only HHV that is integrated into the host germ line genome and can be detected in lymphocytes and hair follicle cells, at ~1–4 HHV6 gec/cell, in about 1% of the US and UK population.[41] Recent studies have shown that detection of HHV6 DNA in CSF is commonly due to infiltrating ciHHV6 lymphocytes and does not indicate an active HHV6 CNS infection.[41] Surplus blood sample of the plexus neuritis case was not available to determine the patient's ciHHV6 status. Consequently, we postulate that both lymphotropic HHVs did not originate from the CNS itself but have most likely entered the subarachnoid space nonspecifically within migratory lymphocytes upon inflammation-induced disruption of the blood–brain barrier.[2]

In conclusion, our data do not support the role of intrathecal HHV infection in MS early after disease onset and demonstrate that incidental detection of HHV1–8 DNA in CSF samples of NIND patients does not indicate viral infection. Paired analysis of the CSFpel and CSFsup sample, supplemented with RT-qPCR to detect viral transcripts as shown for the HHV2 encephalitis case, can differentiate between virus-induced disease and nonspecific presence of HHVs in CSF samples. Future collaborative studies between MS centers are recommended to share clinical specimens and use standardized molecular assays to validate the postulated etiopathogenic role of HHVs in MS.[15,38]

Acknowledgements

We thank the clinical staff from the Department of Neurology (Erasmus MC), especially Mrs. Kim van Gasteren and Kim Zuidwijk, for the collection and management of clinical samples. The study was in part funded by the Dutch MS Research Foundation (project #MS 09-670 MS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supporting information

Supplementary Table 1. MS patient characteristics of previous PCR studies on prevalence of human herpesviruses in cerebrospinal fluid

				No. patients with disease entity[c]					
Reference	Country	Gender (F/M)[a]	Age (years)[b]	CIS	RRMS	PPMS	SPMS	Duration disease (moths)[d]	Time after onset disease or exacerbation
Alvarez-Lafuente et al. 2008	Spain	34 / 14	Mean: 33	48	na	na	na	40 [1-94]	<72 hours after onset disease (n=48)
Franciotta et al. 2009	Italy	34 / 20	32 [20-52]	na	54	na	na	108 ± 60	<14 days (n=34) and <6 months (n=20)
Sotelo et al. 2008	Mexico	21 / 6	32 ± 10	na	27	na	na	64 ± 78	<7 days post-exacerbation (n=15)
Ordonez et al. 2010	Mexico	13 / 7	33 ± 7	na	na	4	16	128 ± 71	Not clearly defined
Mancuso et al. 2007	Italy	24 / 14	38 ± 12	na	23	15	na	84 [12-168]	Not clearly defined
Mancuso et al. 2010	Italy	36 / 15	41 [16-77]	12	24	15	na	Not clearly defined	Not clearly defined
Virtanen et al. 2014	USA	18 / 19	42 ± 11	na	28	7	2	66 ± 79	Not clearly defined
Mirandola et al. 1999	Italy	NCD	37 [19-57]	na	32	na	na	Not clearly defined	<7 days post-exacerbation (n=32)
Taus et al. 2000	Italy	18 / 7	39 ± 10	na	25	na	na	28 ± 56	<14 days post-exacerbation (n=25)
Burgoon et al. 2009	USA	7 / 1	29 ± 3	3	5	na	na	Not clearly defined	8 days post-exacerbation (n=4 RRMS)
Tejada-Simon et al. 2002	USA	21 / 9	Mean: 38	na	26	na	4	Mean: 6	Not defined clearly
Rotola et al. 2004	Italy	NCD	NCD	32	na	na	na	Not clearly defined	Not clearly defined
Yao et al. 2009	USA	11 / 7	37 [27-54]	na	18	na	na	59 [2-336]	Median 5 months post-exacerbation
Ahram et al. 2009	Jordan	25 / 11	30 ± 8	na	30	1	5	Mean: >36	Not clearly defined
Ramroodi et al. 2013	Iran	56 / 22	29 [16-52]	na	22	10	6	Mean: >24	Obtained at exacerbation (RRMS/SPMS)
Gustafsson et al. 2013	Sweden	25 / 2	Mean: 49	27	na	na	na	Not clearly defined	Not clearly defined

[a]F, female; M, male.
[b]Age is provided as median age [range], mean age ± SD or overall mean age. Data are based on records provided in the corresponding papers.
[c]CIS, clinically isolated syndrome; RRMS, relapsing remitting MS; PPMS, primary progressive MS; SPMS, secondary progressive MS; na, not applicable.
[d]Duration disease at time of sampling is provided as median [range], mean ± SD or overall mean disease duration. Data are based on records provided in the corresponding papers, which on occasion are “not clearly defined” (NCD).

Supplementary Table 2. Summary results of previous PCR studies on prevalence human herpesviruses in cerebrospinal fluid in multiple sclerosis

			Number CSF samples positive (%)[c]							
Reference	**Format CSF sample[a]**	**PCR assay[b]**	**HHV1**	**HHV2**	**HHV3**	**HHV4**	**HHV5**	**HHV6**	**HHV7**	**HHV8**
Alvarez-Lafuente et al. 2008	CSFsup	qPCR	1 (2)	0	0	1 (2)	0	5 (10)	nd	nd
Franciotta et al. 2009	CSFpel	qPCR	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	nd	nd
Sotelo et al. 2008	CSFsup	qPCR	nd	nd	25 (95)	nd	nd	nd	nd	nd
Ordonez et al. 2010	CSFsup	qPCR	0 (0)	0 (0)	13 (65)	2 (10)	nd	0 (0)	nd	nd
Mancuso et al. 2007	CSFsup	nesPR	0 (0)	0 (0)	12 (43)	1 (4)	1 (4)	3 (11)	0 (0)	0 (0)
Mancuso et al. 2010	CSFpel	qPCR	nd	nd	0 (0)	0 (0)	nd	0 (0)	nd	nd
	CSFsup	qPCR	nd	nd	0 (0)	1 (2)	nd	1 (2)	nd	nd
Virtanen et al. 2014	CSFtot	nesPCR	nd	nd	nd	3 (8)	nd	5 (14)	nd	nd
Mirandola et al. 1999	CSFsup	nesPCR	nd	nd	nd	nd	nd	0 (0)	0 (0)	nd
Taus et al. 2000	CSFpel	conPCR	nd	nd	nd	nd	nd	0 (0)	0 (0)	nd
Burgoon et al. 2009	CSFpel	qPCR	nd	nd	0 (0)	nd	nd	nd	nd	nd
Tejada-Simon et al. 2002	CSFsup	nesPCR	nd	nd	nd	nd	nd	14 (46)	nd	nd
Rotola et al. 2004	CSFpel	nesPCR	nd	nd	nd	nd	nd	3 (9)	nd	nd
	CSFsup	nesPCR	nd	nd	nd	nd	nd	7 (22)	nd	nd
Yao et al. 2009	CSFsup	nesPCR	nd	nd	nd	nd	nd	0 (0)	nd	nd
Ahram et al. 2009	CSFtot	nesPCR	nd	nd	nd	nd	nd	8 (27)	nd	nd
Ramroodi et al. 2013	CSFtot	qPCR	nd	nd	nd	nd	nd	11 (29)	nd	nd
Gustafsson et al. 2013	CSFtot	nesPCR	nd	nd	nd	nd	nd	1 (4)	nd	nd

[a]CSFsup, cerebrospinal fluid (CSF) supernatant; CSFpel, CSF cell pellet; CSFtot, unseparated total CSF.
[b]qPCR, quantitative real-time PCR; nesPCR, nested conventional PCR; conPCR, conventional PCR.
quantitivge is provided as median age [range], mean age ± SD or overall mean age. Data are based on records provided in the corresponding papers.
[c]HHV, human herpesvirus type 1 through 8; nd, not determined. “0”, DNA of respective HHV type not detectable.

Chapter 4

No evidence for intrathecal IgG synthesis to Epstein–Barr virus nuclear antigen–1 in multiple sclerosis

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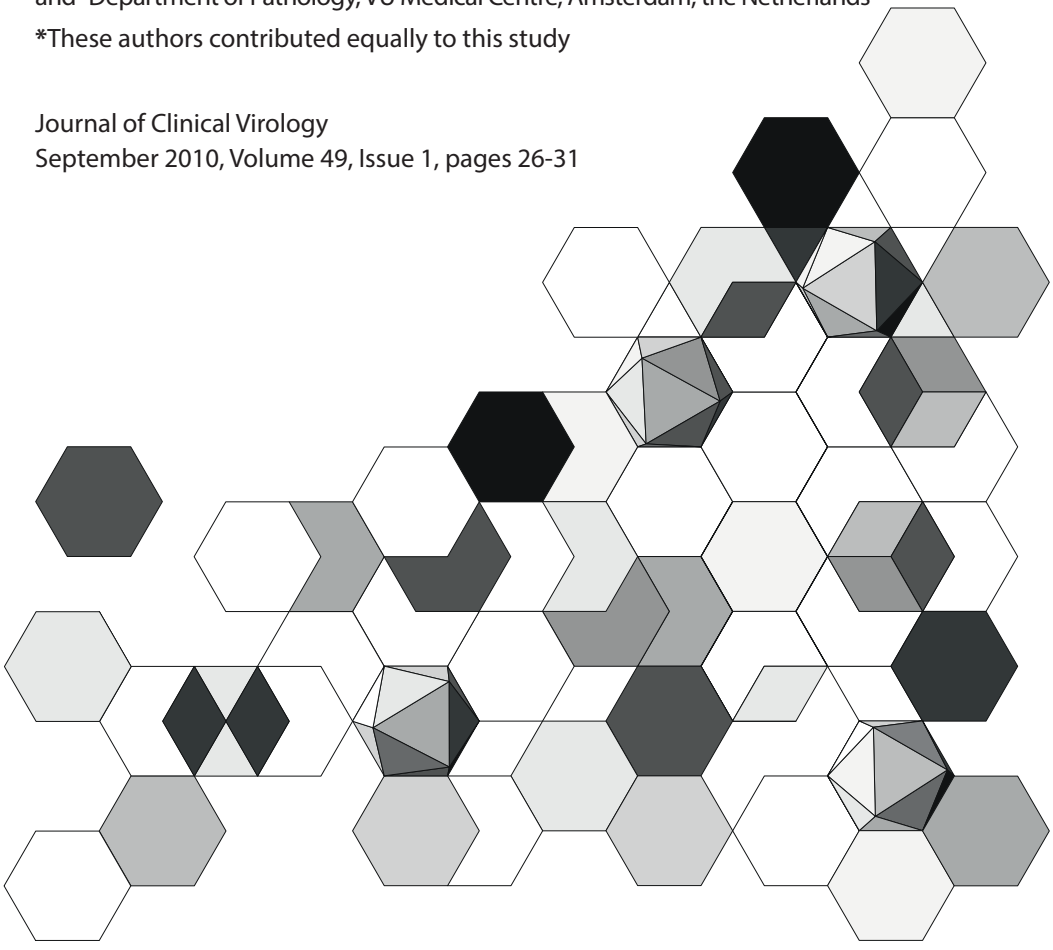
Journal of Clinical Virology
September 2010, Volume 49, Issue 1, pages 26-31



Abstract

Background: Recent studies suggest an intrathecal IgG response against Epstein-Barr virus (EBV) in multiple sclerosis (MS), implicating a pathogenic role for the virus in MS.

Objectives: To determine the spectrum of anti-EBV antibodies and B-cell epitopes within EBV nuclear antigen-1 (EBNA-1). Furthermore, to determine whether EBNA-1-specific IgG is produced intrathecally.

Study design: Immunoblot analysis was used to study the anti-EBV IgG response in serum and cerebral spinal fluid (CSF) in MS and controls. EBNA-1 B-cell epitopes were identified by immunoscreening of 12 residue long peptides, with 11 residue overlap, spanning EBNA-1. Thirteen peptides containing all immunoreactive regions were constructed and used in paired serum and CSF of MS patients (n=17) and controls (n=18). Subsequently, reactivity to the identified immunodominant peptide was analyzed in a large cohort of serum and CSF of MS patients (n=114) and disease controls (n=62).

Results: No difference was observed in the overall anti-EBV antibody diversity, but EBNA-1 reactivity was increased in MS patients versus controls for immunoblot and ELISA ($p<0.0001$). Epitope analysis on EBNA-1 revealed one immunodominant region covering residues 394–451: EBNA-$1^{394–451}$. Anti-EBNA-$1^{394–451}$ IgG levels in serum and CSF were significantly higher in MS patients compared to controls. However, normalization for total IgG content of paired serum and CSF samples abrogated this disease association.

Conclusions: MS patients have normal overall anti-EBV antibody responses with increased reactivity to EBNA-$1^{394-451}$. No evidence was found for intrathecal EBNA-1-specific IgG synthesis in MS.

Background

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) resulting in demyelination and neurodegeneration. The disease develops in genetically predisposed individuals in response to environmental factors, most likely viral infections.[1] Although many viruses have been postulated to be implicated in MS pathology, including varicella zoster virus,[2] human herpesvirus 6[3] and measles virus,[4] none of these were irrefutably linked. However, recent studies advocate the role of Epstein-Barr virus (EBV) in MS.[5] EBV seroprevalence is higher in MS patients compared to controls (99% versus 90–95%) and MS is shown to have a clear and reproducible clinical relation with infectious mononucleosis.[6] Serum and intrathecal IgG levels to the latency-associated EBV nuclear antigen-1 (EBNA-1) are elevated before onset of MS and correlates with disease activity and prognosis.[5–10] Contrastingly, IgG to lytic EBV proteins including the viral capsid antigen (VCA) are not changed or only marginally increased, suggesting that EBV abnormalities in MS are associated with B-cell responses to latent EBV antigens.[5,8] Recently, Serafini et al. reported the presence of EBV-infected B-cells in meninges and perivascular regions of MS lesions.[11] However, this observation as well as the involvement of a local EBV-specific B- and T-cell response is still under debate.[5,12–14]

Objectives

The aim of our study was twofold. To substantiate the postulated EBV-MS association, we first identified the overall anti-EBV antibody reactivity and defined EBNA-1 B-cell epitopes recognized by serum and CSF IgG of MS patients and controls. Second, we determined whether EBNA-1-specific IgG is produced intrathecally.

Study design

Study population

The study group included 114 MS patients and 62 patients with non-inflammatory neurological diseases (NIND) recruited at the Erasmus Medical Centre (Rotterdam, Netherlands) and the diagnosis were controlled by an experienced neurologist (RQH) based on diagnostic McDonald criteria for MS.[15] As for the controls, the patient files and follow-up have been thoroughly checked to exclude possible MS in the NIND cohort. Serum and CSF samples were obtained for diagnostic purposes. For detailed EBNA-1 epitope analyses we obtained sera from 18 healthy EBV seropositive individuals from the USA (n=7), The Netherlands (n=6) and Hong Kong (n=5). Sera from 4 EBV seronegative individuals were used for background measurements. Informed consent was obtained from all patients and the study was approved by the local ethical committee.

Routine serology

Overall IgG antibody responses to EBV proteins and defined EBNA and VCA markers were determined by standardized immunoblot and ELISA assays as described before.[16–18] Before analysis, all serum samples were diluted one-hundred times and all CSF ten times. Antibody responses to human cytomegalo virus (HCMV) antigens were determined by ELISA using a purified glycine-extracted antigen preparation.[19,20]

Epitope mapping of EBNA-1

To specify EBNA-1 B-cell epitopes from healthy EBV seropositive individuals, the IgG reactivity was determined to 12-mer synthetic peptides (n=630), with 11 residue overlap, spanning the entire 641 residue long EBNA-1 sequence of the B95-8 EBV strain.[21] Peptide synthesis and immunoscreening were performed as described elsewhere from 18 EBV seropositive healthy individuals from globally distinct regions.[21,22] Mean OD_{450} values of four healthy EBV seronegative individuals was used to determine individual background value for each peptide.

EBNA-1 combipeptide reactivity

EBNA-1 peptide-specific IgG for the thirteen high affinity epitopes (Table 1) were determined by ELISA and validated by Western blotting using recombinant full-length EBNA-1 as described previously.[16–18] Sera from peptide-immunized and pre-immune rabbits were used as positive and negative controls, respectively. Monoclonal antibodies OT1x and 2B4, reacting with an alpha-helical epitope located at residues 430–442 and a linear epitope at 446–451, respectively were used as positive controls.[23,24] Additionally, sera from healthy EBV seropositive and seronegative donors were included in each ELISA assay for further standardization. Background levels of the serum ELISAs were determined using four EBV seronegative controls. Serum cut-off values (COV) were defined as the mean OD450 values plus 2-times standard deviation (SD) of these EBV seronegative controls. CSF ELISA COV was defined as the mean of all NIND CSF plus 2-times the SD. CSF ELISA OD_{450} values were standardized by dividing the mean of duplicate measurements for the clinical sample by the COV. Total IgG was determined with the PeliClass human IgG kit (Sanquin Reagents, Amsterdam, The Netherlands). EBNA-1-specific IgG was normalized for total IgG levels according to the following formula:

$$\frac{OD_{450}\ \text{EBNA-1 IgG CSF}/OD_{450}\ \text{EBNA-1 IgG serum}}{IgG_{total}\ \text{CSF}/IgG_{total}\ \text{serum}}$$

Results were statistically analyzed with Mann–Whitney U and Spearman correlation test. P values <0.05 were considered statistically significant.

Table 1. Epstein-Barr Nuclear Antigen 1 (EBNA-1) synthetic peptides used in ELISA

Antigen	aa Position	aa sequence
EBNA-1	1-44	MSDEGPGTGPGNGLGEKGDTSGPEGSGGSGPQRRGGDNH GRGRG
Gly-Ala	147-168 174-195 268-289	AGAGGGAGGAGAGGGAGGAGG
Gly-Arg	348-369	GGSGGRRGRGRERARGGSRERA
EBNA-1	368-387	RERARGGSRERARGRGRGRG
EBNA-1	394-420	PPRRPPPGRRPFFHPVGEADYFEYHQE
EBNA-1	424-451	DGEPDVPPGAIEQGPADDPGEGPSTGPR
EBNA-1	436-461	QGPADDPGEGPSTGPRGQGDGGRRKK
EBNA-1	450-477	PRGQGDGGRRKKGGWFGKHRGQGGSNPK
EBNA-1	460-486	KKGGWFGKHRGQGGSNPKFENIAEGLR
EBNA-1	477-502	KFENIAEGLRALLARSHVERTTDEGT
EBNA-1	506-531	GVFVYGGSKTSLYNLRRGTALAIPQC
EBNA-1	459-607	RKKGGWFGKHRGQGGSNPKFENIAEGLRALLARSHVERTTD EGTWVAGVFVYGGSKTSLYNLRRGTALAIPQCRLTPLSRLPF GMAPGPGPQPGPLRESIVCYFMVFLQTHIFAEVLKDAIKDLVM TKPAPTCNIRVTVCSFDDGVDLP
EBNA-1	614-641	VEGAAAEGDDGDDGDEGGDGDEGEEGQE

EBNA-1 Epstein Barr Nuclear Antigen 1; ***aa*** amino acid; ***Gly-Ala*** Glycine-Alanine repeat; ***Gly-Arg*** Glycine-Arginine rich domain

Results

Clinical characteristics

Patients analysed included 114 MS and 62 NIND cases. The mean (±SD) age of MS and NIND patients was 38 (±11.5) and 44 (±18.6) years, respectively. Among MS patients, clinical definite MS was confirmed in 61 of 114 (54%) and clinically isolated syndrome (CIS) in 53 of 114 patients (46%). The mean (±SD) age at MS onset was 34.2 (±10.1) years. Within definitive MS group, 35 and 26 patients had a relapsing remitting (RR-MS) and primary progressive (PP-MS) course, respectively. The control group consisted of 62 NIND patients.

Similar repertoire of anti-EBV IgG response in serum and CSF of MS and NIND patients

Immunoblot analysis revealed a normal diversity pattern for anti-EBV IgG response in serum and CSF from both MS and NIND patients. Serum and CSF showed the characteristic limited diversity of anti-EBV IgG, involving antibodies directed to the recombinant antigens EBNA-1 (BKRF1), VCA-p18 (BFRF3), VCA-p40 (BDRF1) and the EBV transactivator protein Zebra (BZLF1), as observed for healthy EBV carriers (data not shown).[16,18,22] MS cases frequently showed more intense EBNA-1 IgG reactivity compared to NIND (data not shown),[25,26] which led us to investigate the anti-EBNA-1 response in more detail.

Relevance of EBNA-1 for intrathecal anti-EBV response in MS patients was substantiated by testing all samples for VCA-p18 IgG using ELISA. Only serum, not CSF, showed significantly elevated VCA reactivity for MS compared to NIND. Parallel analysis of anti-HCMV IgG responses, revealed no differences between any of the study groups (data not shown).

Identifying EBNA-1 B-cell epitopes

Epitope mapping by immunoscreening of 630 twelve residue long peptides spanning the entire EBNA-1 antigen showed that the B-cell epitopes are largely confined to the N-terminal part of the protein (Figure 1). Here, mostly the glycine–alanine repeat consisting of residue 90–325 (Gly–Ala, EBNA-1$^{90–325}$) and to a lesser extent, the glycine–arginine rich domain (Gly–Arg, EBNA-1$^{348–369}$) contained many B-cell epitopes. However, numerous proteins contain similar repeat sequences gainsaying that the IgG responses measured for these Gly–Ala and Gly–Arg repeats are EBNA-1-specific.[27,28] Thus, EBNA-1-specific IgG responses in healthy EBV seropositive individuals are predominantly directed to EBNA-1$^{394–451}$.

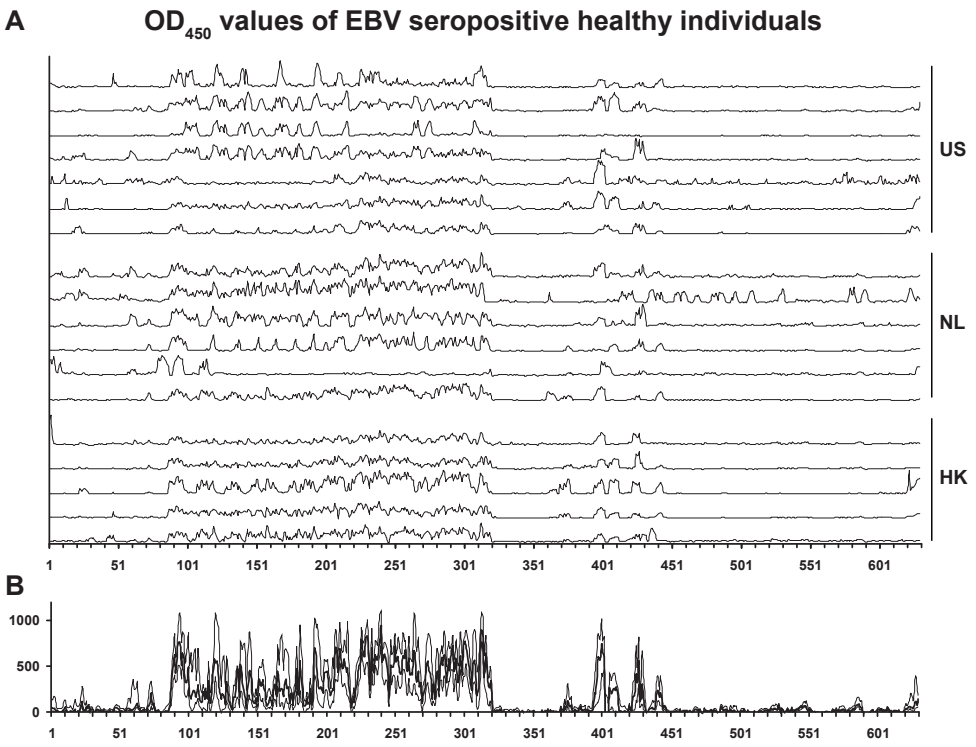


Figure 1. Epstein-Barr virus nuclear antigen 1 peptidescan of serum IgG of heatlhy EBV carriers. Plot of enzyme-linked immunosorbent assay OD_{450} values of 18 Epstein Barr virus (EBV) seropositive healthy individuals from United States (US), The Netherlands (NL) and Hong Kong (HK) for 12-mer peptides with an 11 residue overlap spanning the entire EBV nuclear antigen-1 (EBNA-1) protein (**A**). Horizontal axis shows peptide number: peptide 1–630. Average of all sera (**B**) shows that high reactive B-cell epitopes are confined to the glycine–alanine repeat (EBNA-1$^{90–325}$) and EBNA-1$^{395–451}$.

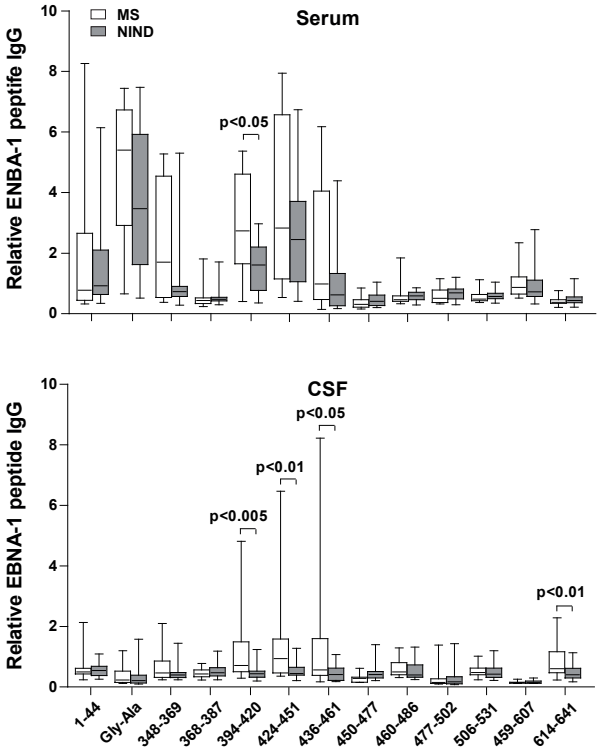


Figure 2. Epstein-Barr nuclear antigen-1 (EBNA-1)-specific IgG responses in serum and cerebrospinal fluid (CSF) samples towards EBNA-1 peptides. Enzyme linked immunosorbent assay of serum and CSF samples towards synthetic peptides covering the indicated residues of EBNA-1 of multiple sclerosis patients (MS; n=17; white bars) and non-inflammatory neurological disease controls (NIND; n=22; grey bars). EBNA-1 peptide responses are presented as the relative IgG levels, calculated as described in the study design section. Median values (centre line), interquartile ranges (boxes) and minimal and maximum values (whiskers) are indicated. Results were statistically analysed with the Mann–Whitney U test.

Increased IgG levels to EBNA-1$^{394–451}$ in serum and CSF of MS patients

Elevated levels of EBNA-1 IgG have been reported in serum and CSF of MS patients.[7–10] To confirm this finding and to delineate recognized EBNA-1 B-cell epitopes, 13 partly overlapping immunoreactive EBNA-1-specific peptides (Table 1) were synthesized based on the preceding EBNA-1 B-cell epitope mapping (Figure 1). By computer aided minimal energy calculations, these longer peptides are predicted to acquire their normal structural conformation, as proven for EBNA-1$^{394–451}$ by OT1x and 2B4 monoclonal antibody reactivity, and thereby include epitopes having secondary structures (data not shown).[24]

IgG reactivity to these larger EBNA-1 peptides was determined in paired serum and CSF samples of 17 MS and 22 NIND patients. The overall pattern in serum and CSF was comparable between both patient groups (Figure 2). In contrast to CSF, serum IgG responses were mainly directed to Gly–Ala and Gly–Arg repeat domains of EBNA-1 (Figure 2). Interestingly, in serum and particularly CSF of MS patients significantly increased IgG responses to EBNA-1$^{394–461}$ and EBNA-1$^{614–641}$ were detected (Figure 2). Low reactivity to EBNA-1$^{450–477}$ advocates EBNA-1$^{394–451}$ as the immunodominant region.

Subsequent analyses of EBNA-1$^{394–451}$ in a larger set of MS patients (n=114) and controls (n=62) substantiated the significantly elevated IgG levels to EBNA-1$^{394–451}$ in MS patients (Figure 3A). Western blotting using recombinant EBNA-1$^{394–451}$ confirmed ELISA data, showing that 95% of the MS sera and only 82% of NIND were EBNA-1$^{394–451}$ positive.

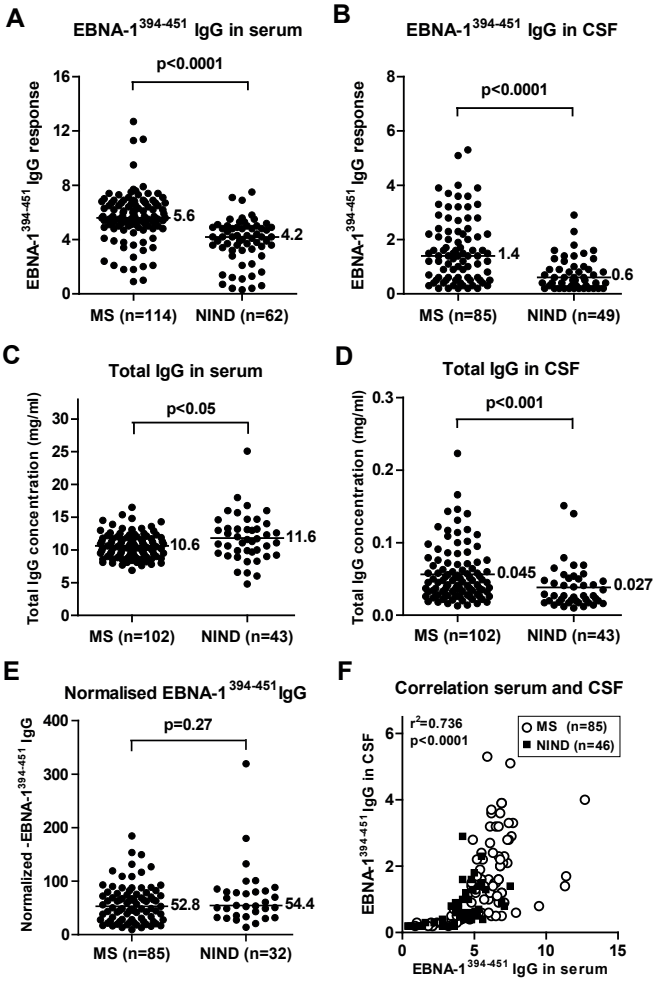


Figure 3. Elevated IgG levels towards Epstein Barr nuclear antigen-1 protein residues 394–451 (EBNA-1$^{394–451}$) in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients is not attributable to intrathecal IgG synthesis. EBNA-1$^{394–451}$-specific IgG levels in serum(n=114) (**A**) and CSF samples (n=85) (**B**) of MS patients are significantly higher compared to patients with non-inflammatory neurological diseases (NIND; serum (n=62) and CSF (n=49)). Total IgG levels in serum (**C**) and CSF samples (**D**) of MS (n=102) and NIND patients (n=43) differ significantly. Normalized EBNA-1$^{394–451}$ IgG levels (**E**), calculated as described in the study design section and the correlation of EBNA-1$^{394–451}$ IgG levels (**F**) in paired serum and CSF samples of MS (n=85) and NIND (n=32inE; n=49 in **F**) argues against intrathecal synthesis. (**A**–**E**) Horizontal lines represent median IgG levels. Results were statistically analysed with the Mann–Whitney U (**A**–**E**) and Spearman correlation test (**F**).

Blood-brain barrier dysfunction attributes to increased anti-EBNA-1$^{394–451}$ IgG levels in CSF of MS patients

The elevated CSF EBNA-1$^{394–451}$ IgG levels may be due to intrathecal synthesis or leakage of serum IgG into the CSF compartment. To differentiate between both options, anti-EBNA-1$^{394–451}$ IgG levels in paired serum and CSF of 85 MS and 46 NIND patients were normalized for total IgG of the respective samples. Total IgG in serum of MS patients were significantly lower compared to controls (Figure 3C) and were significantly elevated in CSF of MS patients (Figure 3D). Contradictory, normalized anti-EBNA-1$^{394–451}$ responses were similar between MS and NIND (Figure 3E) and anti-EBNA-1$^{394–451}$ IgG levels in paired serum and CSF samples correlated significantly (Figure 3F). Thus, the data suggest that elevated anti-EBNA-1$^{394–451}$ IgG responses in CSF of MS patients is not due to intrathecal IgG

synthesis, but more likely associated with blood-brain barrier dysfunction. This conclusion is also supported by correlation of the Q albumin (albumin CSF/albumin serum) with Q EBNA-1 (EBNA-1$^{394–451}$ CSF/EBNA-1$^{394–451}$ serum). Albumin was tested by routine diagnostic assays and the data were available for 77 MS cases and 31 NIND patients. Spearman correlation was statistical significant for the Q albumin and Q EBNA-1$^{394–451}$ with r^2=0.35 (95% CI 0.17–0.51; p=0.0002).

Discussion

During the past three decades, several viruses including measles virus and human herpesviruses have been suggested to play a role in initiation and perpetuation of MS pathology.[1,8] Whereas most MS-associated viruses have not withstood scrutiny in time, the long-held assumption of EBV as causative agent in MS has recently been reinforced by the demonstration of latently EBV-infected B-cells in MS lesions and increased IgG responses to latency-associated EBV protein EBNA-1 in both serum and CSF of MS patients.[5,9–11] Confirming previous findings, we observed that all MS patients were EBV seropositive, either by IgG immunoblot or ELISA. Immunoblot analysis revealed that the recognized spectrum of EBV proteins was similar in serum and CSF from MS and NIND patients. Moreover, both patient groups showed similar patterns of limited EBV antigen diversity as observed in healthy EBV carriers. This indicates that aberrant lytic replication is unlikely to play a role in MS and is in agreement with the non-elevated EBV–DNA levels in both CSF and circulation of MS patients.[11,12]

The EBNA-1 immunoblot analyses confirmed increased aberrant EBNA-1 IgG reactivity in MS serum and CSF, suggestive of a role for latent EBV antigens in MS. Therefore, the main aim of our study was to delineate the EBNA-1 B-cell epitopes and to determine whether anti-EBNA-1 IgG is produced intrathecally in MS patients. We first identified EBNA-1 B-cell epitopes recognized by serum IgG in healthy EBV carriers and then compared it to the response in serum and CSF of MS and NIND patients. We identified one immunodominant EBNA-1 protein region (EBNA-1$^{394–451}$) in serum and CSF samples of MS (Figure 2). The data are in line with a recent study by Sundström et al.,[25] describing significantly elevated serum IgG titers to EBNA-1$^{385–420}$ in MS patients. Notably, our data extend this association by also demonstrating significantly increased anti-EBNA-1$^{394–451}$ IgG levels in CSF next to serum (Figure 3). Sundström et al. also suggests that antibodies to EBNA-1-specific domains and HLA DRB1*1501 interact as risk factors.[25] Although this has not been tested in our study, we do not expect this would influence the message of our study since the higher prevalence of HLA DRB found in MS would only contribute to a positive result.

To compare distinct disease courses of MS (RRMS, PPMS and CIS), we included a relatively high number of PPMS cases (43% of MS samples; normal 10–15% of MS population). However, no significant differences between these subgroups were observed (data not shown).

We demonstrated that EBNA-1-specific IgG responses are elevated both in serum and CSF of MS patients compared to controls. Confirming earlier studies,[8–10,12] this was not shown for other EBV proteins, including the VCA-p18 marker (data not shown). To determine whether increased EBNA-1-specific IgG levels in MS were due to intrathecal synthesis, we corrected for the possible dysfunction of the blood-brain barrier by normalizing for total IgG (Figure 3C and D). This revealed comparable normalized anti-EBNA-1$^{394–451}$ IgG levels in MS and NIND patients, arguing against intrathecal IgG synthesis (Figure 3E). Significant correlation between EBNA-1$^{394–451}$ IgG levels in paired serum and CSF samples in both MS patients and controls strengthens this conclusion (Figure 3F).

In conclusion, our data showed no evidence for intrathecal anti-EBV IgG synthesis, as also supported by others.[29] Whether peripheral infection or immune responses play a pathogenic role remains to be determined. Notably, the MS-associated EBNA-1$^{394–451}$ region identified encompasses several immunodominant HLA DR-, including potential HLA DRB1*1501-restricted CD4+ T-cell epitopes.[30,31] Moreover, MS patients have elevated frequencies and broader epitope reactivity of EBNA-1-specific CD4+ T-cells,[31] including specific T-cells that cross-reacted with MS-associated myelin proteins.[24,26–32] We hypothesize that in genetically predisposed individuals,[1,33] EBNA-1 expression evokes a neuroantigen cross-reactive anti-EBNA-1 T-cell response, that upon entry into the CNS recognizes and targets cells expressing the cognate neuroantigen.

Acknowledgments

The ErasMS centre located at the Erasmus MC is supported by MS Research Foundation Netherlands. This study was approved by the local ethical committee and was performed according to the tenets of the Helsinki declaration. We thank Mrs. Hedy Juwana for technical support.

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Chapter 5

Elevated EBNA-1 IgG in MS is associated with genetic MS risk variants

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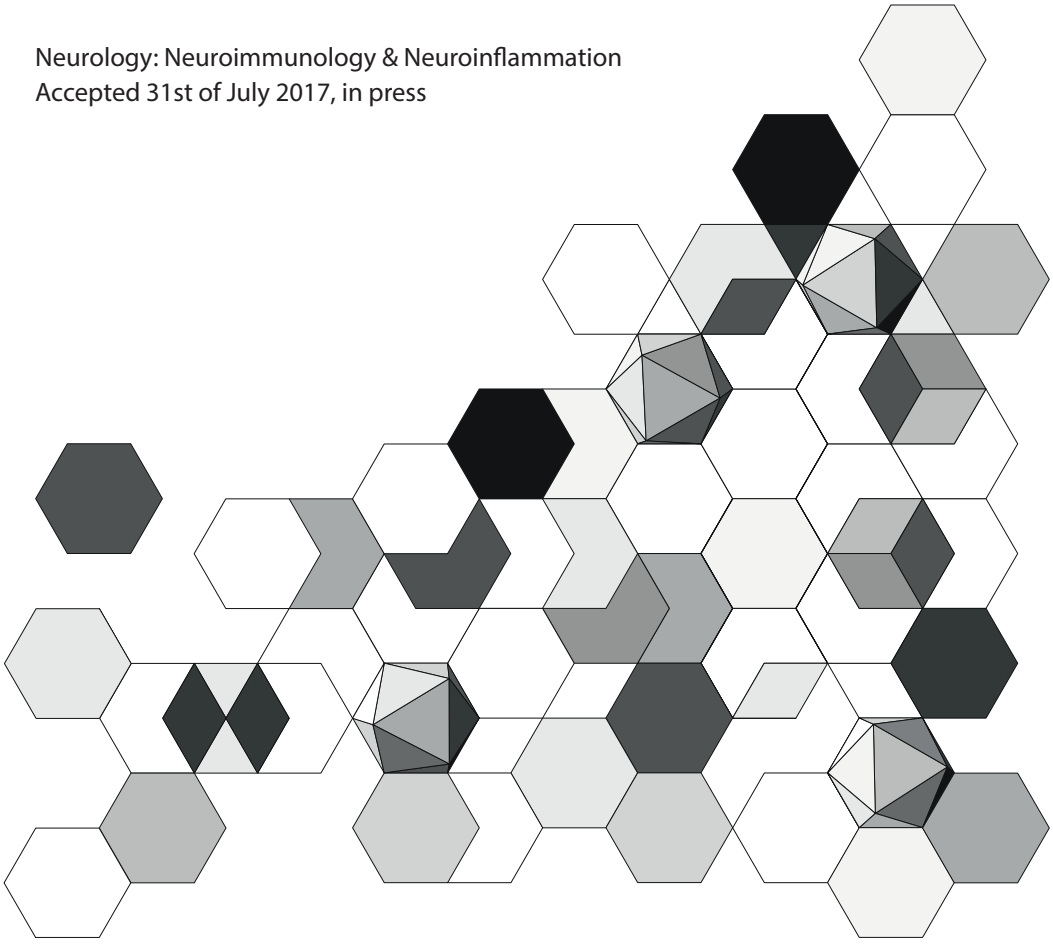
Neurology: Neuroimmunology & Neuroinflammation
Accepted 31st of July 2017, in press



Abstract

Objective: To assess whether multiple sclerosis (MS) genetic risk polymorphisms (SNP) contribute to the enhanced humoral immune response against Epstein Barr virus infection (EBV) in MS patients.

Methods: Serum anti-EBV nuclear antigen-1 (EBNA-1) and early antigen-D (EA-D) IgG levels were quantitatively determined in 668 genotyped MS patients and 147 healthy controls. Anti-varicella zoster virus (VZV) IgG levels were used as a highly prevalent, non-MS associated control herpesvirus. Associations between virus-specific IgG levels and MS risk SNP were analyzed.

Results: IgG levels of EBNA-1, but not EA-D and VZV, were increased in MS patients compared to HC. Increased EBNA-1 IgG levels were significantly associated with risk alleles of SNP rs2744148 (SOX8), rs11154801 (MYB), rs1843938 (CARD11) and rs7200786 (CLEC16A/CIITA) in an interaction model and a trend towards significance for rs3135388 (HLA-DRB1*1501). Additionally, risk alleles of rs694739 (PRDX5/BAD) and rs11581062 (VCAM1) were independently associated and interacted with normal EBNA-1 IgG levels. None of these interactions were associated with EA-D and VZV IgG titers.

Conclusions: Several MS-associated SNPs significantly correlated with differential IgG levels directed to a latent, but not a lytic EBV protein. The data suggest that the aforementioned immune related genes orchestrate the aberrant EBNA-1 IgG levels.

Introduction

The etiology of multiple sclerosis (MS) involves genetic and exposure to environmental factors, including Epstein-Barr virus (EBV) infection.[1–3] Genetic risk factors for MS include specific human leucocyte antigen (HLA) alleles and currently approximately 100 mainly adaptive immune related single nucleotide polymorphisms (SNP) with modest odds ratios (OR) compared to the major HLA-DRB1*1501 association[4–6] and EBV exposure.[7]

Almost all MS patients are infected with EBV, compared to 90-95% in healthy controls.[7,8] A history of EBV-related infectious mononucleosis (IM) and elevated EBV nuclear antigen-1 IgG levels increases the risk to develop MS later in life.[9–11] Recently, a meta-analysis showed that antibodies against the latency associated EBNA-1 are consistently increased in MS patients compared to healthy EBV carriers, whereas results for the lytic viral capsid antigen (VCA) and early antigen D (EA-D), are more heterogeneous.[7]

Epidemiological and genetic studies support interactions between EBV and HLA-DRB1*15 on an additive scale.[12–14] Whether the MS risk SNP are associated with enhanced IgG levels against EBV is currently unclear.[13,14]

We hypothesized that certain MS risk SNP are involved in the increased humoral immune response against EBV. Serum IgG levels against the EBV antigen EBNA-1 were measured and associations with MS risk SNPs were assessed.

Table 1. Clinical and demographic characteristics of patients and controls

	HC (n=147)	MS (n=668)	p-value
Age at sampling (SD) in years	41 (12)	42 (13)	0.46
Age at onset (SD) in years	n.a.	34 (11)	n.a.
Number of female: male	78:60	477:190	$5x10^{-4}$
Disease duration in months (IQR)	n.a.	98 (13-152)	n.a.
CSF abnormal		82%	
IgG index raised		72%	
Oligoclonal bands:	n.a.		n.a.
Multiple		61%	
1 additional		12%	
Treated patients (%)	n.a.	17	n.a.

HC; healthy controls, MS; multiple sclerosis, SD; standard deviation, IQR; interquartile range, CSF; cerebrospinal fluid, n.a.; not applicable, CSF abnormal defined as: either oligoclonal bands specific in CSF or a raised IgG-index >0.67 or both.

Materials and methods

Patients and controls

Consecutive MS patients (n=668) seen at the MS center ErasMS between 2003 and 2013 were included. A diagnosis of MS was made based on the 2005 McDonald criteria for MS[15] and sub classified as clinically isolated syndrome (CIS), primary progressive (PP), relapsing remitting (RR) or secondary progressive (SP) MS based on the clinical course of disease. Unrelated healthy controls (HC, n=147) were persons accompanying MS patients to our outpatient clinic. HC were age matched to the MS patients. Exclusion criteria for HC to participate in this study were prior neurological symptoms suggestive for MS or the use of immunomodulatory drugs for other autoimmune diseases.

Standard Protocol Approvals, Registrations, and Patient Consents

This study was performed according to the guidelines specified in the declaration of Helsinki, approved by the medical ethical committee of the Erasmus MC and all participants gave written informed consent.

Determination of IgG levels against viral proteins

Plasma from blood collection tubes containing EDTA (BD) was used to measure IgG levels against EBNA-1, EA-D and VZV as a control. Subsequently in samples negative for EBNA-1 and EA-D, we measured anti-VCA IgG to ascertain anti-EBV serostatus. All samples were determined using well validated chemoluminescent assays and IgG levels measured on a Liaison XL (all Diasorin) according to the manufacturers' guidelines at the national referral center for virus diagnostics at the Erasmus MC. All samples were automatically diluted by a factor 20 by the Liaison XL. If antibody levels were below or above the threshold, these samples reanalyzed undiluted or manually pre-diluted by factor 10 and subsequently diluted by the Liaison XL, respectively. Using this protocol, all samples were in the linear range of the assays. Patients negative for EBNA-1, EA-D and VCA IgG were omitted from further study to prevent bias between MS patients and HC due to differences in EBV seroprevalence.

DNA isolation and genotyping.

Genomic DNA was isolated from peripheral blood cell pellets using standard laboratory techniques. Isolated DNA was resuspended in TE-buffer and stored at -20˚C. Genotyping was performed using Illumina 610K Quad array (Illumina) or Sequenom platform (on both platforms, genotyping was performed in 1 run according to good laboratory practice following the manufacturers' protocol) for 78 MS-associated risk SNP at the Erasmus MC (Supplementary Table 1).[4,5] All SNP were in Hardy-Weinberg equilibrium (all p>0.30).

Statistical analysis

IgG levels for EBNA-1, EA-D and VZV were ^{10}log transformed to normalize titers. T-tests were used to compare groups and ANOVA was used to compare more than two groups (clinical parameters: use of immunomodulatory drugs, age, gender, disease form). For logistic regression, IgG levels were dichotomized as above or below the 75th percentile and the odds ratio (OR) for MS risk SNP were determined. In the first phase of this study, 330 MS patients were included and all MS risk SNP included in this study (Table e-1) were assessed. All SNP (in interaction) that associated with EBNA-1 IgG with a p-value <0.10 (i.e. VCAM-1, PRDX5, SOX8, MYB, CARD11, CLEC16A and HLA-DRB1*1501), EBNA-1, EA-D, VZV, and optionally VCA IgG levels were subsequently determined in a validation cohort of 336 MS patients and 147 healthy controls. All associations were confirmed in the validation cohort. Subsequently, both datasets were pooled for further analysis. Lastly, interaction models between MS risk SNP associated with IgG levels were constructed using forward conditional logistic regression. Initially, in a univariate model SNP associated with increased EBV IgG were identified. Subsequently, the most significant SNP was assessed in an interaction term with all remaining SNP. Significant interactions were subsequently taken into the next model with an additional SNP in the interaction term, until no more significant interactions were found. Lastly, using this interaction term, remaining interactions between the other SNP were assessed using the same method. Logistic regression models were determined both uncorrected and corrected for aforementioned clinical parameters. If the number of homozygous SNP carriers was smaller than 40 persons, this group was pooled with heterozygous allele carriers.

95% confidence interval (CI) were calculated using bootstrapping analysis with 10.000 random samples from the assessed population with a Bias corrected accelerated method (BCa, unless indicated otherwise). All analyses were performed using SPSS version 20 (IBM).

P-values <0.05 were considered statistically different and significance is indicated in the figures as * p<0.05, ** p<0.01, *** p<0.001. Graphs were constructed using GraphPad Prism version 5.04 (GraphPad Software Inc.).

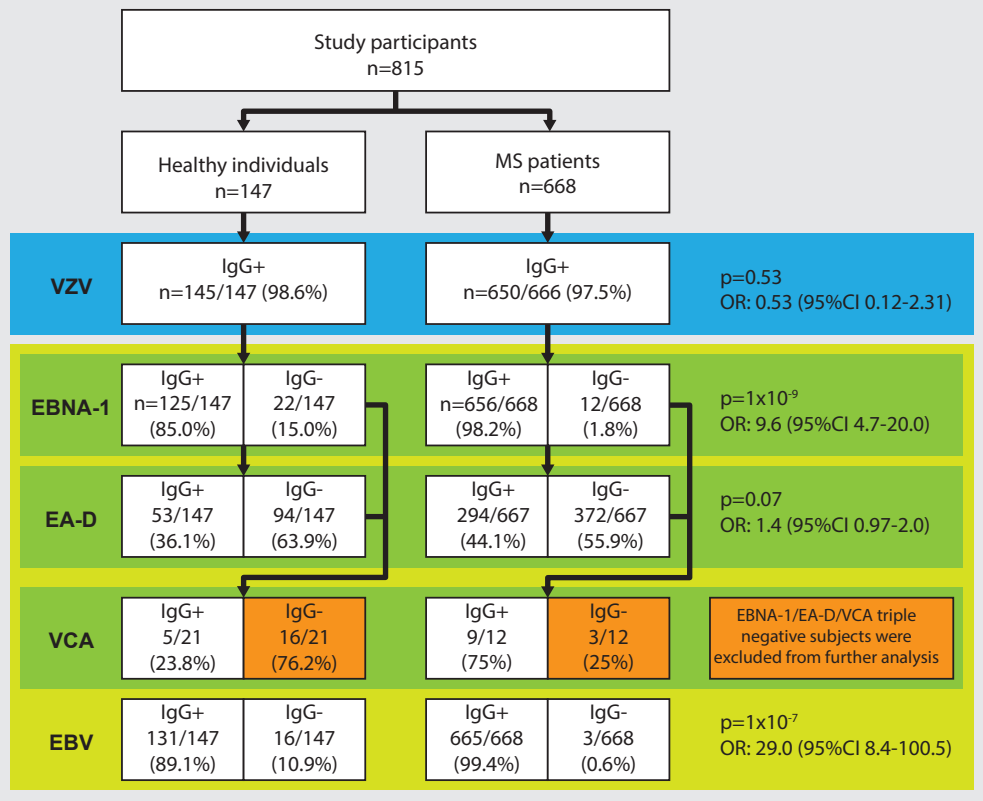


Figure 1 Flowchart of the study and virus seroprevalence. Immunoglobulin G (IgG) levels against varicella-zoster virus (VZV) and the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) and early antigen D (EA-D) peptides were determined. For EBNA-1 and EA-D IgG double-negative patients, viral capsid antigen (VCA) IgG was assessed to ensure EBV serostatus. IgG levels against VZV was used as a highly prevalent herpes control virus to assure the specificity of the outcomes. EBNA-1, EA-D, and VCA triple negative patients were excluded from further study. CI=confidence interval; OR=odds ratio.

Results

Patients and controls

MS patients and HC were age matched, however the gender distribution was significant different. Demographic characteristics and clinical information are shown in Table 1.

EBV seroprevalence and EBNA-1 IgG levels are increased in MS patients compared to controls.

More MS patients (665 of 668; 99.6%) compared to HC (131 of 147; 89.1%) were EBV seropositive based on EBNA-1, EA-D and VCA IgG levels (OR 29.0, 95% CI 8.4-100.5; $p=1x10^{-7}$, Figure 1). This difference in EBV seroprevalence was irrespective of HLA-DRB1*1501 carriership (HLA-DRB1*1501 adjusted OR 43.6, 95% CI 9.7-196.7; $p=9x10^{-7}$).

No difference in VZV seropositivity between MS and HC was observed (p=0.40, Figure 1). Additionally, EBNA-1 IgG levels were higher in MS patients compared with healthy EBV carriers (HEC) ($p=9x10^{-7}$, Figure 2A). Differences in EBNA-1 IgG levels were not related to gender differences (p=0.36, Figure 2D), clinical disease course (p=0.16, Figure 2E) or immunomodulatory drug treatment (p=0.62, Figure 2F). Contrastingly, no differences were observed for EA-D (p=0.55, Figure 2B) and VZV IgG levels between MS and HC (p=0.08, Figure 2C).

*HLA-DRB1*1501 is associated with enhanced serum EA-D IgG levels*

EBNA-1, EA-D and VZV IgG levels were dichotomized as above or below the 75th percentile of MS patients. Subsequently, OR of HLA-DRB1*1501 carriers for high IgG levels was determined. Previous associations of HLA-DRB1*1501 with elevated EBNA-1 IgG titers[12] showed a trend for significance (OR: 1.43; 95%CI 0.96-2.13; p=0.08) using a multivariate logistic regression model (Figure 3A). Moreover, we observed an association of HLA-DRB1*1501 with EA-D IgG levels (OR: 2.08, 95% 1.19-3.64; p=0.01), but not VZV (data not shown).

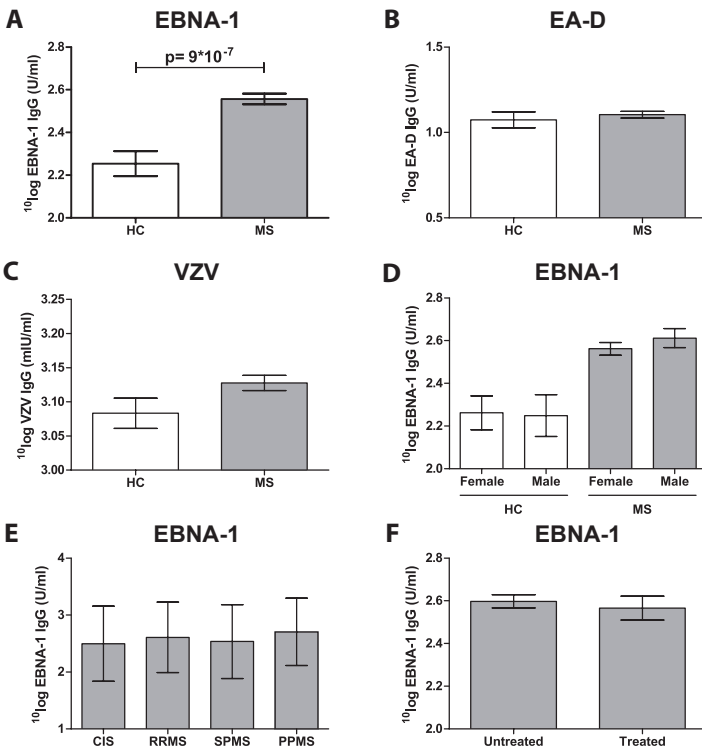


Figure 2. EBNA-1, but not EA-D nor VZV IgG, are increased in patients with MS. (**A**) Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) immunoglobulin g (IgG) titers are significantly increased in patients with MS (grey bars) compared with healthy EBV carriers (HC; white bars). No significant differences in IgG levels between MS and healthy controls (HCs) were observed for (**B**) early antigen D (EA-D) and (**C**) varicella-zoster virus (VZV). (**D**) IgG levels against EBV are similar in (**D**) women and men, (**E**) relapsing-remitting (RR) MS, secondary progressive (SP) MS, primary progressive (PP) MS, or clinically isolated syndrome (CIS) patients, and (**F**) patients treated with immunomodulatory drugs or untreated.

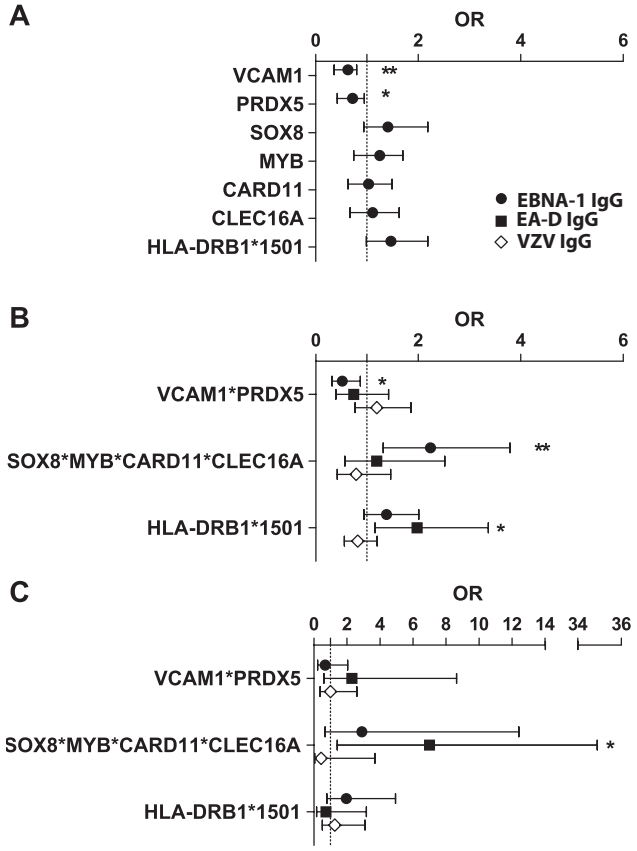


Figure 3. VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A interactions are associated with EBNA-1 and EA-D IgG levels. Epstein-Barr nuclear antigen 1 (EBNA-1; filled circle), early antigen D (EA-D; filled square), and varicella-zoster virus (VZV) (open diamond) immunoglobulin G (IgG) levels were dichotomized, and logistic regression was performed to assess which MS risk SNPs are associated with EBNA-1, EA-D, and VZV IgG levels. (**A**) Univariate logistic regression in patients with MS showed significance for EBNA-1 IgG above the 75th percentile for VCAM1 (odds ratio [OR] 0.54, 95% confidence interval [CI] 0.36–0.81; p=0.003) and PRDX5 (OR 0.63, 95% CI 0.42–0.95; p=0.028). Multivariate logistic regression with interaction terms between MS risk SNPs showed significant interactions of VCAM1*PRDX5 (OR 0.54, 95% CI 0.32–0.89; p=0.01) and SOX8*MYB*CARD11*CLEC16A (OR 2.1, 95% CI 1.23–3.66; p=0.004) for EBNA-1 IgG, but not HLA-DRB1*1501 (p=0.1), in patients with MS (**B**) and in healthy Epstein-Barr virus carriers (**C**). HLA 5 human leukocyte antigen.

PRDX5 and VCAM1 protective alleles are associated with elevated EBNA-1 IgG levels

Next, we assessed the OR of 78 MS-associated risk SNP (Table e-1) for high serum EBNA-1, EA-D and VZV levels. The non-MS-risk alleles of PRDX5 (OR: 0.63; 95%CI 0.42-0.95; p=0.028) and VCAM1 (OR: 0.54; 95%CI 0.36-0.81; p=0.003) were correlated with high EBNA-1 IgG levels. Conversely, MS-risk alleles of MYB, SOX8, CARD11, CLEC16A and HLA-DR showed a trend towards significance for high EBNA-1 IgG levels (p<0.10, Figure 3A). No significant differences were found for the remaining 71 MS risk SNP. No association between these MS risk SNP and EA-D IgG and VZV IgG was found (all p>0.52).

Complex interaction between VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A associated with EBNA-1 IgG

Next, we assessed whether interactions between MS risk SNP are associated with the enhanced anti-EBNA-1 humoral response observed in MS patients. All associated SNP with p<0.10 and HLA-DRB1*1501 (Figure 3A) were included in logistic regression models with interaction terms. Interaction was observed between the non-MS-risk polymorphisms in VCAM1 and PRDX5 (p=0.01, Figure 3B) in association with high EBNA-1 IgG levels. A

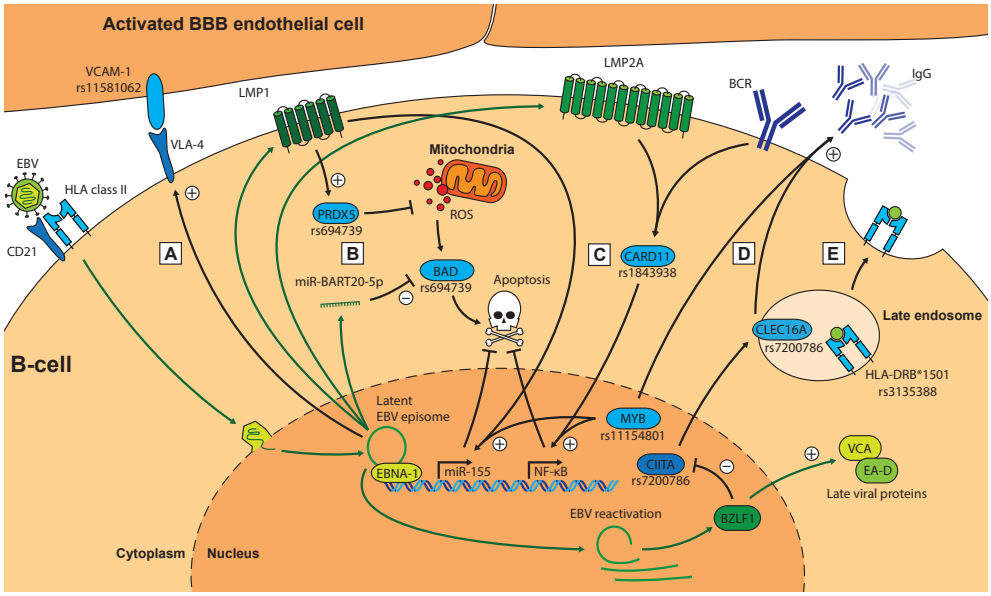


Figure 4. Model proposing interaction between EBV latency proteins with here described MS risk genes. (**A**) Epstein-Barr virus (EBV) infection of B-cells induces very late antigen 4 (VLA-4) expression, the ligand of VCAM-1 expressed by activated brain epithelial cells. (**B**) Latency-associated membrane protein 1 (LMP1) of EBV induces expression of the peroxiredoxin family of antioxidant enzymes (PRDX5), thereby reducing reactive oxygen species (ROS) and preventing caspase-mediated apoptosis. EBV-expressed micro RNA (miR)-BART20-5p targets B-cell chronic lymphocytic leukemia/BAD transcripts, reduces BAD protein levels, and inhibits apoptosis. (**C**) Analogous to B-cell receptor signaling, EBV LMP2A activates miR-155 and NF-kB expression via CARD11 and thereby inhibits apoptosis and promotes proliferation. (**D**) Immediate early lytic EBV protein BZLF1 blocks loading of peptides to HLA class II in late endosomes by inhibiting CIITA and induced expression of early antigen D (EA-D) and viral capsid antigen (VCA). (**E**) CLEC16A is involved in HLA-II antigen presentation via regulation of the endosomal compartment and is important for the production of Ig. Plus and minus indicate increased and reduced expression, respectively. EBNA-1=Epstein-Barr nuclear antigen 1; HLA=human leukocyte antigen; IgG=immunoglobulin G; NF-kB=nuclear factor kappa B.

second interaction term between the risk alleles of SOX8-MYB-CARD11-CLEC16A was associated with high EBNA-1 IgG (p=0.004). In a combined model, HLA-DRB1*1501 was not associated with increased EBNA-1 IgG titers (p=0.1, Figure 3B). Adjustment for gender, clinical disease course and immunomodulatory treatment did not affect the results (p>0.20, data not shown). Age at sampling was significantly associated with EBNA-1 IgG, adjusting our model did not alter the conclusions (data not shown). No associations between these interacting genes and EA-D or VZV IgG were found (Figure 3B). Similar, though less robust trends in HEC were observed (Figure 3C). Lastly, the 95% CI of these interactions were assessed using a bootstrapping approach with 10.000 random samples to non-parametrically fit the model. Only very minor differences after bootstrapping were observed (approximate bias between the actual data and the bootstrapped dataset was 0.019 or less), indicating that the OR on EBNA-1 IgG levels are accurate. In conclusion, gene-gene interaction between VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A is specifically associated with EBNA-1 IgG and not with EA-D and VZV.

Gene-gene interaction associated with EBNA-1 IgG is not secondary to MS

Next, we assessed whether the different gene-gene interactions are related to the MS risk SNP or secondary to MS disease activity. Therefore, we used logistic regression with abundant EBNA-1 IgG as the dependent variable and MS patients as a covariate. Correcting for MS as a covariate did not alter the previously described interactions, indicating that the gene-gene interactions itself are important for the increased EBNA-1 IgG and that the associations are not secondary to MS alone. Additionally, correcting for HLA-DR did not affect our results (data not shown).

Discussion

Increased EBV seroprevalence and more specifically, enhanced immunoglobulin γ (IgG) specific for EBNA-1 immune response in MS patients have been widely reported[2,8,10,11] and confirmed in this study. Longitudinal studies demonstrating EBV infection in the years preceding onset of MS[2,8,10,11] are often used as (circumstantial) evidence for a causal role of EBV in MS. Alternatively, this might be an epiphenomena as a result of a dysregulated immune system observed in MS patients, leading to an altered handling of the virus. Additionally, the virus might be more active in MS patients, although the antibody titers against EA-D, a marker for active viral replication, are not increased in MS patients in the majority of published studies[7] as well as in our study. Lastly, it might be explained that MS patients develop infectious mononucleosis at a later age than healthy controls, especially because EBV infection at later age is more likely to cause infectious mononucleosis.[16] The enhanced EBNA-1 IgG titers in MS might at least partially be explained by both HLA and non-HLA MS-associated risk genes.[13,14] Recently, a study with 1367 Mexican Americans assessed association between genome wide over 900.000 SNP and EBNA-1 seropositivity, irrespective of antibody titers. They found that only HLA-DRB1 and HLA-DQB1 were associated with EBNA-1 positivity. This is slightly in contrast with the current study, which might be explained by the fact that we took the EBNA-1 IgG titers into account in our models. Additionally, their study might have suffered from a power problem with multiple testing issues, whereas we only assessed MS-associated SNP. Lastly, differences in population (patients with cardiovascular disease and diabetes mellitus) might explain the different results.[13]

MS-associated risk SNP are shown to affect the Th- and B-cell transcriptome.[17,18] This may on the one hand, influence humoral immune responses against EBV and on the other, influence regulation of EBV latency in B-cells. To assess whether MS risk genes are associated with EBNA-1 IgG, we stratified quantitative EBNA-1, EA-D, and VZV IgG titers as above the 75th percentile to MS associated SNP in healthy EBV carriers and EBV positive MS patients and determined the OR of these polymorphisms.

In this study, we observed a similar trend between EBNA-1 IgG and of HLA-DRB1*1501 in line with previously reported associations.[12] Moreover, here HLA-DRB1*1501 was also associated with EA-D IgG levels, but not VZV IgG. The HLA-DRB1*1501 influences

the repertoire of T-cells recognizing peptide antigens.[19] It can therefore be suggested that this HLA-DRB1*1501 association with EBNA-1 and EA-D IgG levels reflects Th-/B-cell interactions, having an effect on the EBV-specific humoral immune response of MS patients.

In contrast, we found that the HLA-independent MS-risk SNPs in VCAM-1 and PRDX5 inversely correlated with EBNA-1 IgG levels. Interestingly, in a multivariate logistic regression model, interactions between VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A were associated with EBNA-1 IgG levels in a contrasting manner. Strikingly, the HLA-DRB1*1501 effect on EBNA-1 IgG titer is overshadowed by these two genetic interactions associated with altered EBNA-1 humoral responses. The increased EBNA-1 IgG observed in MS patients is partially explained by these genetic interactions.

A similar genetic association was found in healthy controls previously exposed to EBV, although not statistically significant presumably due to lack of statistical power. The in part common genetic background of healthy controls and MS patients (e.g. the quite high proportion of healthy controls carrying MS risk SNP) might explain the common effects observed in the immune response against EBV observed in this study. The identified MS risk genes associated with EBNA-1 IgG are likely candidates to be functionally involved in the enhanced humoral immune response, as discussed here below.

EBNA-1 is expressed in all phases of the EBV lifecycle except latency 0, where only non-coding EBV transcripts are expressed.[20] EA-D, an EBV polymerase processivity protein encoded by BamHI fragment M rightward open reading frame 1 (BMRF1), is restricted to lytic phase EBV.[20] The observed association with EBNA-1 IgG levels therefore suggests that the genes associated with MS risk SNP found in this study interact with latency associated transcripts of EBV in infected B-cells. Strikingly, the majority of genes associated with the SNP that correlate with EBNA-1 IgG levels identified in this study have been shown to interact with EBV associated proteins and transcripts.

VCAM1 is expressed by cytokine-activated blood-brain barrier endothelial cells in MS lesions.[21] Memory B-cells constitutionally express the ligand of VCAM1, integrin α4β1 (Very Late Antigen-4; VLA-4).[17] EBV infection considerably increases VLA-4 expression in B-cell,[22] suggesting increased migration of EBV B-cells to the central nervous system (Figure 4A).

PRDX5 is a member of the peroxiredoxin family of antioxidant enzymes. PRDX5 expression prevents reactive oxygen species (ROS) mediated damage in mitochondria, thereby inhibiting caspase induced apoptosis. The EBV latent membrane protein 2A (LMP2A) induces PRDX5 expression in B-cells.[23] Alternatively, the PRDX5 associated SNP, rs694739 is related to B-cell CLL/lymphoma 2-associated antagonist of cell death (BAD) in a type I diabetes study.[24] Expression of BAD is induced during caspase-mediated apoptosis. The constitutionally EBV expressed miR-BART20-5p targets the 3'-UTR of BAD resulting in decreased BAD levels and reducing apoptosis in infected B-cells.[25] EBV inhibits caspase mediated apoptosis via PRDX5 and BAD during latent and lytic phase infection (Figure 4B) resulting in reduced immune exposure of EBNA-1, which may partly explain the PRDX5 association with lowered EBNA-1 IgG levels in MS patients (Figure 4B).

The rs11154801 SNP is linked to Abelson helper integration site 1(AHI1) or MYB.[26] MYB is a transcription factor required for B-cell development and expression is reduced during the process. Aberrant expression of MYB leads to lymphoproliferative disorders by inducing NF-κB and miR-155 expression.[27] Direct interaction of EBV transcripts or proteins with MYB are not shown. However, LMP1 induces miR-155 and LMP2A/B potently induces NF-κB expression during latency I and II, preventing apoptosis and promoting proliferation.[27–29] CARD11 is a scaffolding protein required for NF-κB activation. Interestingly, gain of function mutations in CARD11 lead to B-cell malignancies and anergic T-cells by continuous NF-κB activation, which is severely exacerbated by EBV infection (Figure 4C).[30]

The rs7200786 SNP is in close proximity of CLEC16A, DEXI and CIITA and alters expression of DEXI.[31] CIITA and CLEC16A are involved in the regulation of HLA class II via the endosomal system.[32] In addition, CLEC16A knock-out in mice leads to reduced numbers of B-cells and an altered immunoglobulin spectrum.[33] The immediate early lytic cycle associated EBV transcription factor BZLF1 downregulates MHC class II antigen presentation through inhibition of CIITA during EBV reactivation (Figure 4D,E).

rs2744148 is located 36.6kb downstream of the nearest gene SOX8, sex determining region Y-box 8. To date, no interactions of rs2744148 with SOX8 or EBV transcripts are known.

As we here followed a candidate gene approach by using a set of predefined recently discovered MS risk genes, we did not correct for multiple testing. This can be seen as a limitation of this study, with a risk of being underpowered. To attenuate this issue we used a first and second cohort to validate the findings. Moreover, higher numbers obtained by pooled analysis of patients and controls gave results in the same direction with lower p-values. The results are plausible as the SNP associated with elevated EBNA-1 IgG are all, except SOX8, highly expressed in B-lymphocytes (data not shown, based on www.biogps.org) and already shown to be involved in EBV latency machinery. In the current study, we were able to assess seventy-eight of the one hundred MS risk SNP currently published, thereby possibly missing some genetic associations with EBNA-1 IgG. It is unlikely that this has affected the underlying results. It might be an underestimation of the real genetic contribution to EBNA-1 IgG levels in MS, as there are even more MS risk genes to be identified. Lastly, although we assessed whether or not our results are secondary to MS disease itself, and we found that correcting for MS in our regression models did not alter the results, we can not fully rule out that MS disease itself is at least partially associated with increased EBNA-1 IgG. To fully assess this, we would need a larger cohort of healthy controls to draw reliable conclusions for this association.

Taken together, the genes associated with the identified genomic MS-risk SNP have previously been shown to be involved in migration (VCAM1), anti-apoptosis (PRDX5/BAD, MYB/NHI1, CARD11) and MHC class II antigen presentation (HLA-DRB1*1501, CLEC16A/CIITA) in latently EBV infected B-cells presumably resulting in altered EBNA-1 IgG levels. Functional differences in these pathways may lead to distinct viral inhibition and appear at the same time in MS related autoimmunity. This deserves to be further explored at a functional level.

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Supporting information

Supplementary Table 1. Genes associated with the MS-SNP included in this study

SNP from: International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium et al,

rs11154801 MYB/NHI1	rs11581062 VCAM1	rs12368653 CYP27B1	rs228614 NF-κB1	rs2293370 TMEM39A	rs2303759 DKKL1
rs2744148 SOX8	rs4648356 MMEL1	rs630923 CXCR5	rs7200786 CLEC16A/CIITA	rs1800693 TNFRSF1A	rs2243123 IL12A
rs9282641 CD86	rs9891119 STAT3	rs2300603 BATF	rs1870071 EVI5	rs1335532 CD58	rs1323292 RGS1
rs7522462 C1ORF106	rs12466022 no gene	rs7595037 PLEK	rs17174870 MERTK	rs11129295 EOMES	rs669607 no gene
rs2028597 CBLB	rs6897932 IL7R	rs4613763 PTGER4	rs2546890 IL12B	rs12212193 BACH2	rs13192841 no gene
rs1738074 TAGAP	rs354033 ZNF746	rs1520333 IL7	rs4410871 MYC	rs2019960 PVT1	rs3118470 IL2RA
rs1250550 ZMIZ1	rs7923837 HHEX	rs650258 CD6	rs10466829 CLECL1	rs949143 ARL6IP4	rs4902647 ZFP36L1
rs2119704 GALC	rs13333054 IRF8	rs180515 RPS6KB1	rs7238078 MALT1	rs1077667 TNFSF14	rs8112449 TYK2
rs874628 MPV17L2	rs2425752 CD40	rs6062314 TNFRF6B	rs2283792 MAPK1	rs140522 SCO2	

HLA related SNP

rs6457110 HLA-A2	rs3135388 HLA-DRB1*15:01

SNP from International Multiple Sclerosis Genetics Consortium (IMSGC) et al, Nat Genet. 2013 Nov;45(11):1353-60.

rs1843938 CARD11	rs2050568 FCLR1	rs2288904 SLC44A2	rs694739 PRDX5/BAD	rs12148050 TRAF3	rs3007421 PLEKHG5
rs11587876 DDAH1	rs666930 PHGDH	rs842639 FLJ16341	rs9828629 FOXP1	rs756699 TCF7	rs941816 PXT1
rs2456449 no gene	rs793108 no gene	rs2688608 C10ORF55	rs7120737 AGBL2	rs4772201 MIR548AN	rs8042861 IQGAP1
rs7204270 MAPK3	rs11865086 MAPK3	rs1886700 CDH3	rs7196953 MAF	rs1870071 EPS15L1	

Chapter 6

Intrathecal CD8+ T-cells of multiple sclerosis patients recognize lytic Epstein-Barr virus proteins

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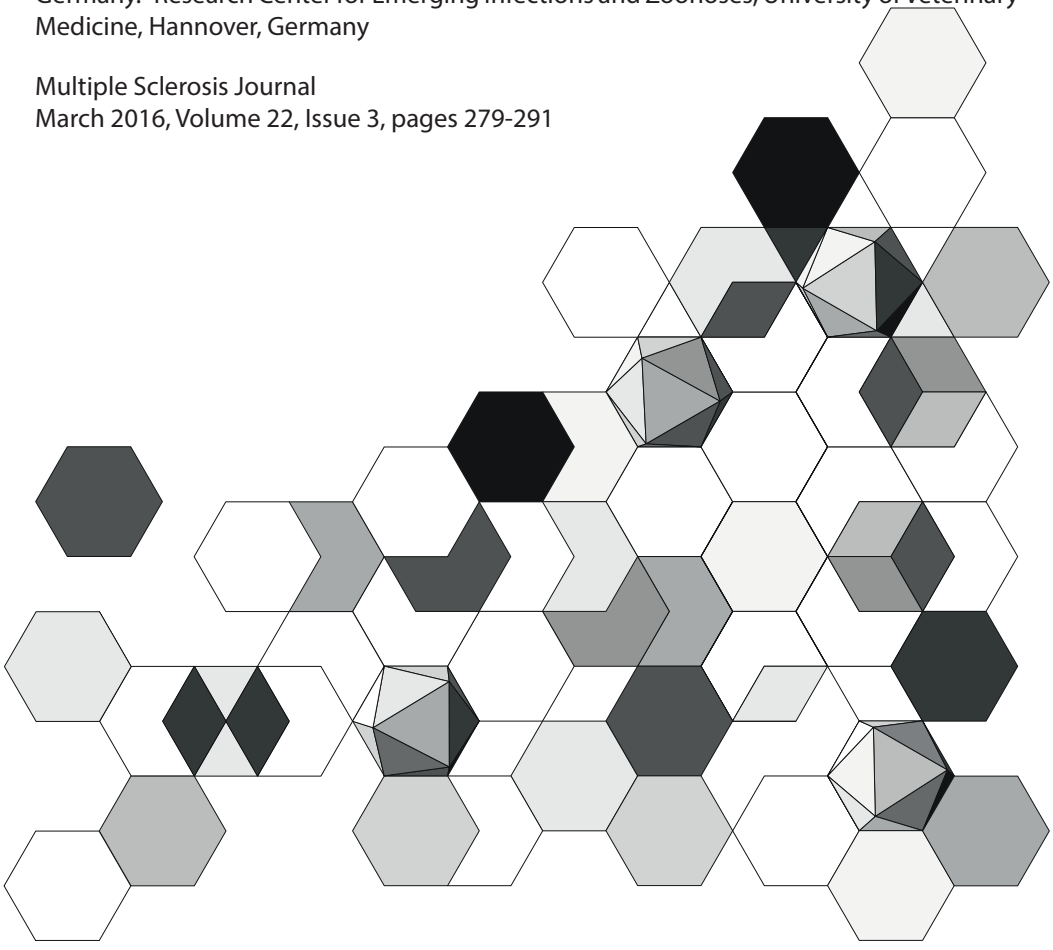
Multiple Sclerosis Journal
March 2016, Volume 22, Issue 3, pages 279-291



Abstract

Background: The association between Epstein-Barr virus (EBV) and multiple sclerosis (MS) may involve intrathecal EBV-specific T-cell responses targeting the virus or indirectly, autoantigens.

Objective: Compare the prevalence and fine-specificity of EBV-specific T-cells in the cerebrospinal fluid (CSF) of patients with MS (n=12), clinically isolated syndrome (CIS) (n=17) and other neurological diseases (OND) (n=13).

Methods: Intrathecal EBV-specific T-cell reactivity was assayed using CSF-derived T-cell lines (CSF-TCL) and autologous EBV-transformed B-cells (autoBLCL) as antigen-presenting cells (APC). EBV proteins recognized by autoBLCL-specific CD8+ T-cells were identified using human leukocyte antigen class I (HLA-I)-negative monkey cells as artificial APC, co-transfected with 59 different EBV genes and the corresponding patient's HLA-I alleles that were involved in autoBLCL T-cell reactivity. Reactivity towards the MS-associated autoantigen αB-crystallin (CRYAB) was determined analogously.

Results: CSF-TCL from CIS and MS patients had significantly higher frequencies of autoBLCL-reactive CD4+ T-cells, compared to the OND patients. CIS patients also had significantly higher autoBLCL-reactive CD8+ T-cells, which correlated with reactive CD4+ T-cell frequencies. AutoBLCL-specific CD8+ T-cell responses of four CSF-TCL analyzed in detail were oligoclonal and directed to lytic EBV proteins, but not CRYAB endogenously expressed by autoBLCL.

Conclusions: Enhanced intrathecal autoBLCL-specific T-cell reactivity, selectively directed towards lytic EBV proteins in two CSF-TCL, suggested a localized T-cell response to EBV in patients with MS. Our data warrant further characterization of the magnitude and breadth of intrathecal EBV-specific T-cell responses in larger patient cohorts.

Introduction

Multiple sclerosis (MS) is characterized by the development of foci of demyelinating and axon-damaging inflammation that spreads throughout the central nervous system (CNS). Clinically-isolated syndrome (CIS) may precede definitive MS. The clinical course of MS may follow a variable pattern over time and MS is classified into three categories: relapsing–remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS).[1] Although the clinical course of MS is highly variable, most patients eventually develop severe neurological disability.

The cause and pathogenic mechanisms of MS remain enigmatic. MS is considered the result of a local inflammatory response, involving both resident cells (e.g. microglia) and infiltrating T-cells in the CNS, in genetically susceptible individuals; initiated by environmental factors, most likely viruses.[1,2] Recent studies advocate the role of Epstein-Barr virus (EBV) during the initiation and potentially the perpetuation of MS pathogenesis.[2] Indicative of this are *i)* both higher EBV seroprevalence and systemic virus-specific B- and T-cell responses in MS patients, compared to controls,[3–5] *ii)* the epidemiological relationship between infectious mononucleosis and MS[6], *iii)* the presence of EBV-infected B-cells in MS lesions and viral deoxyribonucleic acid (DNA) in cerebrospinal fluid (CSF).[7–9] However, evidence for the presence of EBV in the lesions and CSF of MS patients remain controversial.[10–12]

EBV is a human B-cell tropic γ-herpesvirus that is endemic worldwide.[13] The virus' hallmark is to establish a lifelong latent infection in B-cells. The T-cells are pivotal in controlling viral latency.[13,14] EBV may evoke T-cell-mediated MS pathology in different ways. Virus-specific T-cells may recognize EBV-infected cells in the brain,[7,15] cross-react with myelin antigens,[16,17] or recognize autoantigens (e.g. αB-crystallin (CRYAB)) induced in B-cells upon EBV infection.[18] Given the limited access to brain tissue, studies on local antigen-specific T-cell responses in MS patients are restricted to CSF samples. CD4+ and CD8+ T-cells that are reactive to autologous EBV-transformed B-cell lines (BLCL), in part directed to EBV nuclear antigen 1 (EBNA-1), can be cultured from CSF from MS patients.[17,19,20] Recently, two analogous studies on larger patient cohorts, including MS patients and disease controls, compared EBV- and cytomegalovirus (CMV)-specific T-cell responses in paired blood and CSF.[21,22] Whereas both groups describe a selective increase of intrathecal EBV-specific, but not CMV-specific T-cells, the EBV T-cell responses were either restricted to CD8+ T-cells mainly in patients with early MS[21] or limited to CD4+ T-cells in RRMS patients.[22] Furthermore, deep sequencing of T-cell receptor β (TCRBV) gene usage in paired blood and CSF samples confirm intrathecal enrichment of EBV-specific CD8+ T-cells in MS.[23] However, the latter studies did not completely determine the viral proteins recognized or were limited to an analysis of a selected set of known immunodominant EBV CD8+ T-cell epitopes.[19–22]

The aim of the current study was to determine the prevalence and fine specificity of EBV-specific T-cells cultured from the CSF of CIS and MS cases, and patients with other neurological disease (OND). A schematic overview of the subsequent experimental procedures performed to address these important issues in MS pathology is presented in Figure 1.

Materials and methods

Patients and clinical specimens

Between December 2008 and June 2012, paired blood and CSF samples from CIS (n=17), MS (n=12) and OND patients (n=5) were obtained as part of a diagnostic workup at the Erasmus Medical Center (Rotterdam, the Netherlands). Informed consent was obtained from each patient. The study was approved by the local ethical committee and performed according to the tenets of the Helsinki declaration. The MS patient cohort consisted of RRMS (n=10) and PPMS (n=2) and CIS (n=17) patients, according to the 2010 Revised McDonald Criteria. The OND cohort consisted mainly of paraneoplastic neurological syndrome patients (n=9) with high serum Human neuroantigen D (HuD)-specific antibody titers; and OND patients (n=4), whom suffered from a variety of clinical symptoms, including headache and subarachnoid hemorrhage, all sampled between February 2005 and September 2007.[24] Patients received neither corticosteroids, nor immunomodulatory therapy at the time of their lumbar puncture, which was always performed >1 month after the clinical symptoms began. We typed the HLA Class I (HLA-I) genotype of specific patients, using standard diagnostic polymerase chain reaction (PCR) at a 4-digit resolution level.

EBV serology and real-time PCR

The total IgG levels, and the IgG levels directed to EBV virus capsid antigen (VCA) and EBNA-1, were determined from the serum, using chemoluminescent assays on a Liaison XL analyzer (Diasorin, Torino, Italy), as per the manufacturer's instructions. The EBV-specific intrathecal IgG production was determined for EBNA-1 by calculating the Golmann-Witmer coefficient (GWC), as described previously.[25] A GWC >3 was considered indicative for intrathecal EBV-specific IgG production. We determined the presence of EBV DNA in the CSF, and human CRYAB and β-actin transcript levels in BLCL, by real-time PCR (qPCR) and reverse-transcribed qPCR on an ABI Prism 7700 with Taqman Universal Master Mix and the custom-made (EBV gene BNRF1)[26] or commercial intron-spanning primer/probe-pairs specific for human CRYAB and β-actin (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's instructions, respectively. We determined the relative transcript levels by the formula: $10{,}000 \times 2^{-(Ct\ CRYAB\ -\ Ct\ \beta-actin)}$

Generation of BLCL and CSF-TCL

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation of heparinized peripheral blood and the B-cells were infected with EBV strain B95.8 to generate BLCL, as described previously.[27] The CSF-derived T-cell lines (CSF-TCL) were obtained by nonspecific stimulation of cells from CSF with 1 µg/ml phytohemagglutinin-L (PHA-L) (Roche, Branford, CT, USA) in the presence of γ-irradiated allogeneic PBMC (3000 rad; 10^5/well) in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated pooled human serum (Sanquin, Amsterdam, the Netherlands) and 50 IU/ml recombinant interleukin-2 (IL-2) (Miltenyi, Bergisch Gladbach, Germany); and then cultured for

about 2 weeks at 37°C.[24,27] The CSF-TCL with insufficient cell numbers (i.e. < $5x10^6$) were restimulated with an anti-CD3, from clone OKT3 (Janssen-Cilag, Tilburg, the Netherlands).[28] At the time of inclusion, the co-authors who generated the CSF-TCL and autologous BLCL (autoBLCL) were unaware of the patients' clinical diagnosis. Identical protocols were used previously, to generate paired CSF-TCL and autoBLCL from HuD-PNS patients at the same lab (Department of Viroscience, Erasmus Medical Center, the Netherlands), under supervision of the senior author.[27] For the latter patient group, the researchers were aware of their clinical diagnosis beforehand. Notably, of the 47 paired peripheral blood and surplus CSF samples obtained between December 2008 and June 2012, the generation of BLCL and CSF-TCL with sufficiently high T-cell numbers was unsuccessful for five and 13 patients, respectively. We obtained successful CSF-TCL to high numbers from all the MS patients, but only in 17 of 28 (61%) CIS, and 3 of 5 (60%) of the OND patients. Overall, the paired BLCL and CSF-TCL of 29 MS patients and 13 OND patients were available to study intrathecal EBV-specific B- and T-cell reactivity (Table 1).

T-cell reactivity to autologous BLCL

EBV reactivity of CSF-TCL was determined by intracellular staining (ICS) for interferon γ (IFNγ) using autologous BLCL (autoBLCL) as antigen presenting cells (APC).[28] As a positive control for T-cell activation, CSF-TCL were stimulated for 6 hours with a combination referred to as P/I, of phorbolmyristate-acetate (PMA) at 50 ng/ml and ionomycin at 500 ng/ml (Sigma, St Louis, MO, USA).[27,28] T-cells were phenotyped with fluorochrome-conjugated monoclonal antibody (mAb) directed to human CD3 (clone SP34-2; BD Pharmingen, San Diego, CA, USA), CD4 (clone SK3; BD Biosciences, San Jose, CA, USA), CD8α (clone RPA-T8; eBiosciences, San Diego, CA, USA) and IFNγ (clone B27, BD Pharmingen, San Diego, CA, USA) and stained for viability (violet live/dead stain; Invitrogen); and then analyzed using a FACSCanto flow cytometer and CellQuest Pro software (BD Biosciences), as described.[27,28]

Experiments were performed at least two times and as negative controls, the appropriate isotype- and fluorochrome-matched unrelated mAbs were included. Except for the HuD-PNS patient cohort, the team was blinded for the patients' clinical diagnosis during autoBLCL T-cell reactivity analyses. All subsequent in-depth T-cell analyses were performed on CSF-TCL of selected high autoBLCL responding CIS and MS patients. Enumeration of activated T-cells, based on their intracellular IFNγ expression, was defined by gating on the viable cells that expressed surface CD3, CD4 or CD8. For all assays, at least $5x10^3$ gated viable CD4+ and CD8+ T-cells were obtained; and the threshold gate for autoBLCL-reactive T-cells was set at 0.1% IFNγ+ CD4+ and CD8+ T-cells, based on unstimulated CSF-TCL for each donor. All T-cell assays were performed and the data interpreted by the team, according to longstanding in-house standard operating procedures (SOPs) in the Department of Viroscience (Erasmus MC).

Identification of the HLA-I allele involved in CD8+ T-cell reactivity to BLCL

Identification of the patient's HLA-I allele involved in autoBLCL CD8+ T-cell reactivity was determined using partially HLA-I matched allogenic BLCL as the APC. Furthermore, CSF-TCL clonality was determined using a multiparametric analysis tool (IOTest Beta Mark mAb Kit; Beckman Coulter, Marseille, France), which contains multiple fluorochrome-conjugated mAbs directed to approximately 70% of all known TCRBV.[29] Combined extracellular TCRBV and intracellular IFNγ staining facilitated the differentiation between BLCL and allo-HLA reactivity of the corresponding CSF-TCL by flow cytometry. Experiments were performed at least two times.

T-cell reactivity to the EBV proteome and human αB-crystallin

The generation and validation of individual expression vectors (cloned in the pCMV-EHis vector) containing the EBV proteome, covering a total of 59 of 85 (69%) of all known EBV proteins that were used as target antigens for CSF-TCL, have been detailed elsewhere.[30] Human CRYAB cDNA (Darmacon, Lafayette, CO, USA) was cloned in the custom-made eukaryotic pNS.CD8α vector.[31]

We determined the EBV proteome and CRYAB T-cell reactivity on artificial APC, consisting of Cos-7 monkey fibroblasts (American Type Culture Collection, Manassas, VA, USA) that were co-transfected with the patient-matched HLA-I allele-specific cDNA (cloned in vector pcDNA3) and expression vectors encoding 59 individual EBV protein-encoding genes or human CRYAB, as described.[28] All co-transfections were performed in duplicate; and the appropriate negative (untransfected Cos-7 cells) and positive controls (autologous BLCL and P/I) were included. CSF-TCL were added to co-transfected Cos-7 cells; and then after 24 hrs, the culture supernatants were collected for IFNγ ELISA.[28] Expression of specific EBV proteins (i.e. EBNA-1 [clone OT1x], LMP1 [clone OT21c], BZLF1 [clone BZ1], BALF2 [clone OT9-2] and BMRF1 [clone OT14e-2]) by BLCL, and expression of human CRYAB (clone 2E8) (AbD Serotec, Oxford, UK) by co-transfected Cos-7 cells, was confirmed by conventional immunocytology on fixed cells, using fluorescein-conjugated goat-anti-mouse IgG (BD Biosciences). Experiments were performed at least two times.

Statistical analyses

Statistical differences were determined by the Mann-Whitney U test, or in the case of paired specimens, by Spearman's rank correlation test using Prism (GraphPad 5.0 Software, La Jolla, CA, USA).

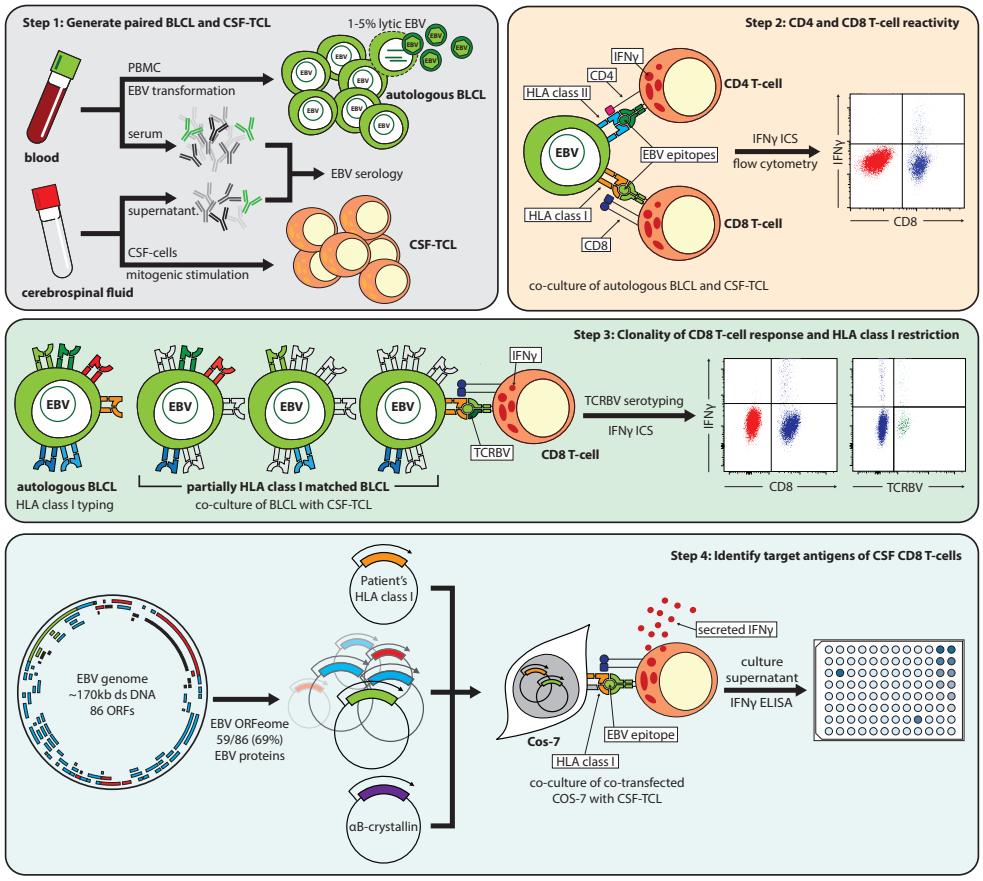


Figure 1. Schematic overview of experimental procedure to identify intrathecal EBV-response. We generated paired EBV-transformed BLCL and CSF-TCL. Co-culture of CSF-TCL with the autologous BLCL as APC; followed by CD3, CD4, CD8, ICS IFNγ and flow cytometry allowed simultaneous analysis of CD4 and CD8 T-cell reactivity. Subsequently, the clonality and HLA-I restriction of BLCL-specific CD8+ T-cells was assayed by co-culture of CSF-TCL with partially HLA-I-matched allogeneic BLCL, followed by combined IFNγ ICS and T-cell receptor Vβ analysis. Next, EBV targets of BLCL-reactive CD8+ T-cells were identified. Therefore, an EBV ORF library encoding 59 of the 86 EBV proteins and EBV-induced CRYAB was cloned in expression vectors. HLA-I-deficient Cos-7 cells were co-transfected with expression vectors encoding the EBV ORFeome or CRYAB and the previously-identified patients' HLA-I and used as the artificial APC in a co-culture with CSF-TCL. The conditioned culture supernatants were screened for secreted IFNγ using ELISA, for a qualitative identification of the CD8+ T-cell targets. APC: antigen-presenting cells; BLCL: blood-derived EBV-transformed B-cell lines; CRYAB: alpha-B-crystallin; CSF-TCL: CSF-derived T-cell lines; EBV: Epstein-Barr virus; ELISA: enzyme-linked immunosorbent assay; ICS: intracellular staining; IFN: interferon; HLA-I: human leukocyte antigen Class I; ORF: open reading frame.

Table 1. General characteristics of the study population

Neurological disease entity	Number	Gender male: female	Median age (years and IQR)[a]	Median disease duration (months and IQR)[a]
Multiple sclerosis patients	29	10 : 19	38 (34-41)	7 (2-22)
Relapsing remitting	10	2 : 8	37 (33-39)	16 (3-69)
Primary progressive	2	1 : 1	51 & 55[b]	10 & 54[b]
Clinically isolated syndrome	17	7 : 10	38 (34-41)	4 (1.5-10)
Disease controls[c]	13	7 : 6	66 (54-70)	3 (2-8)
HuD-PNS	9	3 : 6	66 (54-70)	3 (3-8)
miscellaneous	4	4 : 0	64 (57-72)	1 (0.08-8)

[a]All values are medians (interquartile ranges; IQR).
[b]The age and disease duration of 2 patients with primary progressive MS are presented.
[c]HuD-PNS: paraneoplastic neurological syndrome patients with high serum HuD-specific antibody titers; miscellaneous: patients with variety of clinical symptoms including headache and subarachnoid hemorrhage.

Results

Intrathecal T-cell reactivity to autologous BLCL increased in CIS and MS patients

To study the role of EBV infection in MS, we analyzed virus-specific B- and T-cell responses in surplus CSF from CIS (n=17), MS (n=12) and OND patients (n=13) (Table 1). Laboratory diagnostics on paired serum and CSF of the CIS and MS cases showed that all patients were EBNA-1 seropositive, except one CIS patient, whom was EBNA-1 seronegative but VCA IgG seropositive. Local antibody response to EBNA-1 and EBV DNA were undetectable in the CSF of all CIS and MS patients (data not shown). Paired surplus CSF and serum of OND patients were not available for retrospective EBV diagnostics.

To compare intrathecal EBV-specific T-cell responses between patient cohorts, we expanded the CSF T-cells non-specifically, with PHA-L and subsequent anti-CD3, to obtain high T-cell numbers (Figure 1). In contrast to CD4+ T-cells, CD8+ T-cell frequencies were significantly higher in the CSF-TCL of CIS patients (mean ± standard error=31±4; p=0.009), compared to the MS (28±6) and OND patients (16±4). AutoBLCL were generated by EBV infection of blood-derived B-cells. Concordant with earlier studies, the majority of BLCL cells expressed the viral proteins EBNA-1 and LMP1, which represent latently-infected B-cells.[13] Notably, about 2–5% of the BLCL cells had entered the lytic cycle, and most likely expressed the whole EBV proteome (Supplementary Figure 1). Accordingly, BLCL were considered appropriate APC to analyze intrathecal EBV-specific T-cell reactivity.[14,30]

To determine if our protocol to generate CSF-TCL from different patient cohorts, and in particular long-term storage of HuD-PNS cell lines in liquid nitrogen, influenced the functionality of CSF-derived T-cells, all CSF-TCL were assayed for IFNγ expression upon P/I stimulation. The average frequency of IFNγ+ CD4+ and CD8+ T-cells in P/I-stimulated CSF-TCL was >80% in all patient groups, indicating that the methodology used and long-term storage of CSF-TCL did not negatively affect T-cell immune competence of CSF-derived T-cells (Supplementary Figure 2). Subsequently, the CSF-TCL were assayed for autoBLCL reactivity.

Frequencies of autoBLCL-reactive CD4+ T-cells were significantly higher in CSF-TCL of CIS (mean ± SE: 1.5±0.3; p=0.003) and MS patients (2.0±0.5; p=0.001), compared to disease controls (0.3±0.1), as seen in Figure 2A. For CD8+ T-cells, CIS patients (5.4±1.6; p=0.02), but not MS patients (3.5±1.2; p=0.05) had significantly higher autoBLCL-reactive

T-cell frequencies, compared to disease controls (1.2±0.6). No significant differences were detected between MS and CIS patients (Figure 2A). Overall, frequencies of autoBLCL-reactive CD8+ T-cells (3.7±0.8) were significantly higher than in CD4+ T-cells (1.2±0.2; p=0.01). Notably, intra-patient frequencies of CD4+ and CD8+ autoBLCL-reactive T-cells correlated significantly; which upon analysis of the three patient groups separately, was only significant for the CIS patients (Figure 2B).

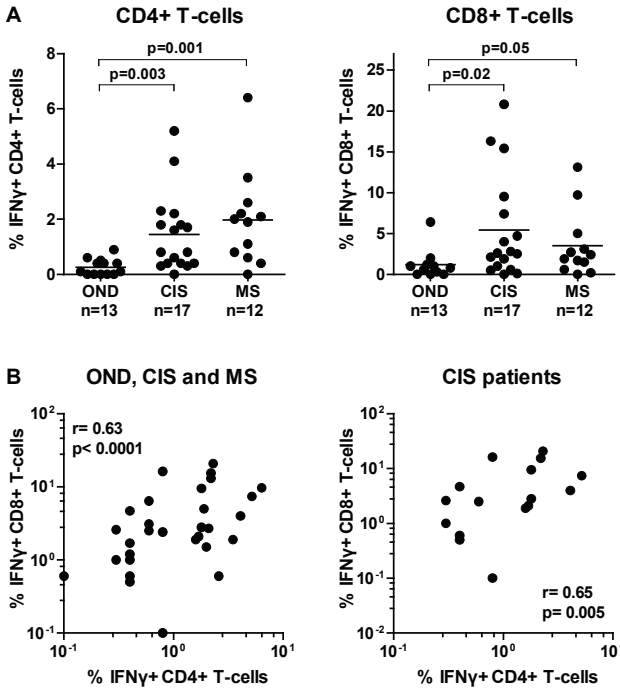
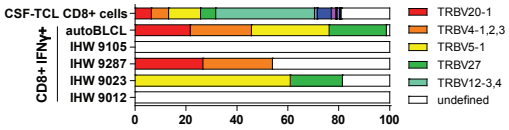


Figure 2. Increased intrathecal CD4+ and CD8+ T-cell reactivity to autologous BLCL, in CIS and MS patients. (**A**) T-cell lines generated from CSF of MS and CIS patients, and as a control, patients with OND; were incubated with autologous EBV-transformed B-cell lines. Phenotype and frequency of the reactive T-cells was determined by flow cytometry, using specific monoclonal antibodies directed to CD3, CD4, CD8 and intracellular IFNγ. (B) The correlation of autoBLCL-reactive CD4+ and CD8+ T-cell frequencies of individual CSF-TCL of all three patient groups (left panel) or solely CIS patients: Dots represent data of individual CSF-TCL, presented as the percentage of IFNγ+ cells of all CD4+ or CD8+ T-cells in the corresponding CSF-TCL, and the bars show the average values of each patient group. The statistical analyses used were the Mann-Whitney U test (**A**) and the Spearman's rank correlation test (**B**). autoBLCL: autologous EBV-transformed B-cell lines; CSF: cerebrospinal fluid; CSF-TCL: T-cell lines generated from CSF; IFN: interferon; OND: other neurologic diseases

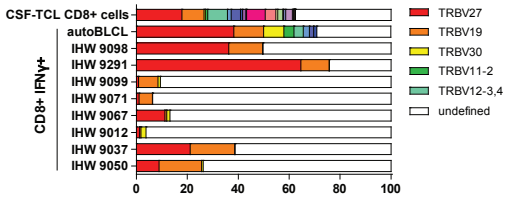
Intrathecal CD8+ T-cell reactivity to autologous BLCL is directed to lytic EBV proteins

Recent data argue for the potential role of CD8+ T-cells in MS pathology.[1,2] Increased frequency of autoBLCL-reactive CD8+ T-cells, predominantly in CIS patients (Figure 2A), prompted us to determine if these CSF-derived CD8+ T-cells recognized EBV proteins (Figure 1). EBV protein specificity was determined using a recently-developed viral proteome approach, using artificial APC, consisting of Cos-7 cells co-transfected with 59 individual EBV protein-encoding genes and the patient HLA-I alleles identified as the restriction element of the autoBLCL-specific CD8+ T-cell response.[28] Because both high numbers of T-cells and relatively high frequencies of reactive CD8+ T-cells (>4%) are mandatory for definitive viral proteome screening hits, the EBV proteome approach was only possible for CSF-TCL of three CIS patients (LP33, LP52 and LP54) and one RRMS patient (LP51).[28]

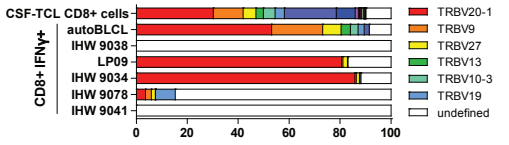
LP33	HLA-A		HLA-B		HLA-C		%CD8+IFNγ+
autoBLCL	01:01	01:01	07:02	08:01	07:01	07:02	8.5
IHW 9105	01:01	01:01	—	—	—	—	0.2
IHW 9287	—	—	07:02	—	—	07:02	12.1
IHW 9023	01:01	01:01	—	08:01	07:01	—	9.8
IHW 9012	—	—	—	—	07:01	—	7.4



LP51	HLA-A		HLA-B		HLA-C		%CD8+ IFNγ+
autoBLCL	02:01	29:02	40:01	45:01	03:04	06:02	15.4
IHW 9098	—	—	40:01	40:01	03:04	03:04	8.5
IHW 9291	—	—	40:01	40:01	—	—	2.1
IHW 9099	02:17	02:17	—	—	03:03	03:03	5.6
IHW 9071	—	—	—	—	—	03:04	16.6
IHW 9067	02:01	02:01	—	—	—	—	6.8
IHW 9012	02:01	02:01	—	—	—	—	4.1
IHW 9037	—	29:02	40:01	—	—	—	1.9
IHW 9050	—	29:02	—	—	—	—	2.1



LP52	HLA-A		HLA-B		HLA-C		%CD8+ IFNγ+
autoBLCL	02:01	03:01	07:02	35:01	04:01	07:02	44.8
IHW 9038	02:01	02:01	—	—	07:01	07:01	2.4
LP09	—	—	07:02	—	07:02	—	26.0
IHW 9034	03:01	03:01	07:02	07:02	07:02	07:02	24.0
IHW 9078	03:01	—	—	—	—	—	6.5
IHW 9041	—	—	35:02	35:02	04:01	04:01	2.4



LP54	HLA-A		HLA-B		HLA-C		%CD8+ IFNγ+
autoBLCL	02:01	26:08	27:05	51:01	04:01	16:02	70.1
IHW 9032	02:01	02:01	—	—	—	—	36.7
IHW 9026	26:01	26:01	—	—	—	—	0
IHW 9005	—	—	27:05	27:05	—	—	2.7
IHW 9041	—	—	—	—	04:01	04:01	0
IHW 9105	—	—	—	—	04:01	04:01	6.5

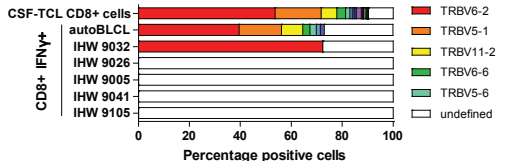
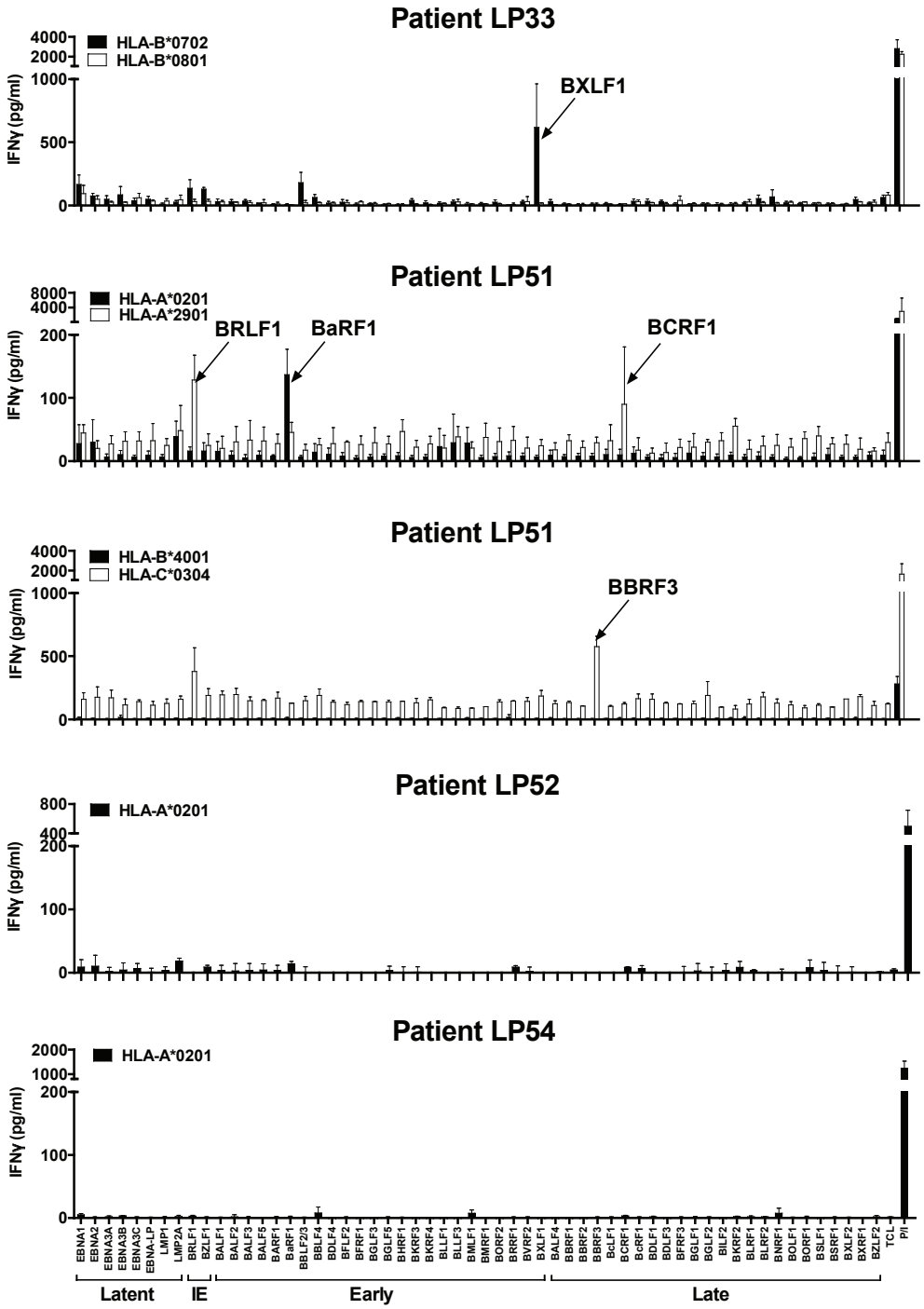


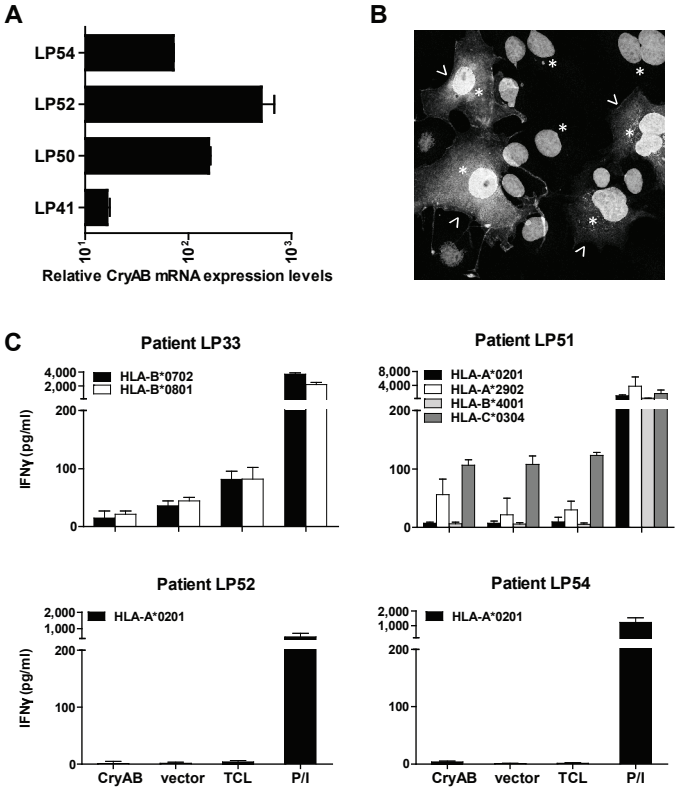
Figure 3. Identification of the patient's HLA-I allele involved in intrathecal CD8+ T-cell reactivity to autologous BLCL. TCL generated from CSF (CSF-TCL) of three CIS patients (numbers LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated with autoBLCL and various partially-matched HLA-I allogeneic BLCL. The TCRBV gene usage of BLCL-reactive CD8+ T-cells was determined by flow cytometry, using specific mAbs directed to different TCRBV (covering about 70% of all known TCRBV), CD8 and intracellular IFNγ. The left panel shows the HLA-A, HLA-B and HLA-C alleles, expressed by the allogeneic BLCL, that match the patients' HLA-I genotype and the corresponding frequency of BLCL-reactive CD8+ T-cells, presented as the percentage of IFNγ+ cells of all the CD8+ T-cells in the corresponding CSF-TCL (last column). The right panel shows the TCRBV gene usage of all CD8+ T-cells in the CSF-TCL (upper row) and the autoBLCL-reactive CD8+ T-cells (subsequent rows). For the latter analysis, the percentage of autoBLCL-reactive CD8+ T-cells was set to 100%, to facilitate inter-BLCL comparison of the TCRBV gene usage of the BLCL-specific CD8+ T-cells. aHerein, undefined indicates that the corresponding TCRBV could not be determined with the TCRBV-specific mAbs used. autoBLCL: autologous EBV-transformed B-cell lines; BLCL: blood-derived EBV-transformed B-cell lines; CIS: clinically-isolated syndrome; CSF: cerebrospinal fluid; EBV: Epstein-Barr virus; HLA-I: human leukocyte antigen Class One; mAbs: monoclonal antibodies; MS: multiple sclerosis; RRMS: relapsing–remitting MS; TCL: T-cell lines; TCRBV: T-cell receptor V beta

First, we determined the patients' HLA-I alleles involved in the autoBLCL CD8+ T-cell responses, by using a combined flow cytometry assay with partially HLA-I-matched BLCL as APC and TCRBV phenotyping, to differentiate between BLCL and allo-HLA-reactive T-cells (Figure 1 and 3). Whereas all four CSF-TCL contained a polyclonal CD8+ T-cell population, selective IFNγ expression combined with restricted TCRBV usage indicated that CD8+ T-cell reactivity was restricted by one (LP52: HLA-B*0702 and LP54: HLA-A*0201), two (LP33: HLA-B*0702 and HLA-B*0801) and four (LP51: HLA-A*0201, HLA-A*2902, HLA-B*4001 and HLA-C*0304) HLA-I alleles. Our data implicate that the autoBLCL-reactive CD8+ T-cell repertoire in all four CSF-TCL is oligoclonal (Figure 3). Finally, the CSF-TCL were subjected to the EBV proteome assay, using the identified HLA-I alleles (Figure 1). The EBV proteome collection contained 59 of 85 (69%) of all known EBV genes and covered viral proteins that are selectively expressed during latency and those co-expressed during lytic EBV infection; including immediate early, early and late viral proteins.[13,30] In total, four out of eight (50%) autoBLCL/HLA-I allele combinations tested revealed reproducible specific CD8+ T-cell EBV protein hits (Figure 4).

CD8+ T-cell recognition of autologous BLCL is not directed to αB-crystallin

The inability to identify the cognate EBV protein of the remaining four out of eight autoBLCL/HLA-I allele combinations may be because the EBV proteome collection was not complete: 26 of the 85 (31%) known EBV proteins are not included.30 Alternatively, CD8+ T-cells are directed to host cell antigens expressed by BLCL. The heat shock protein CRYAB is a potential candidate host protein, as this protein is identified as a T-cell autoantigen in MS patients, and EBV infection of B-cells induces CRYAB expression.[1,18] Indeed, readily-detectable levels of CRYAB transcript were detected in the BLCL of representative patients (Figure 5A). Next, we cloned and expressed human CRYAB in Cos-7 cells, along with the respective HLA-I alleles identified as the restriction element of the autoBLCL CD8+ T-cell response (Figure 3). The CRYAB protein expression was detectable in transfected Cos-7 cells (Figure 5B). However, no CSF-TCL recognized Cos-7 cells transfected with the respective CRYAB/HLA-I combinations, indicating that the intrathecal autoBLCL CD8+ T-cell response was not directed to CRYAB (Figure 5C).


Patient LP33
HLA-B*0702
HLA-B*0801
BXLF1
IFNγ (pg/ml)
Patient LP51
HLA-A*0201
HLA-A*2901
BRLF1
BaRF1
BCRF1
Patient LP51
HLA-B*4001
HLA-C*0304
BBRF3
Patient LP52
HLA-A*0201
Patient LP54
HLA-A*0201
EBNA1 EBNA2 EBNA3A EBNA3B EBNA3C EBNA-LP LMP1 LMP2A BRLF1 BZLF1 BALF1 BALF2 BALF3 BALF5 BARF1 BaRF1 BBLF2/3 BBLF4 BDLF4 BFLF2 BFRF1 BGLF3 BGLF5 BHRF1 BKRF3 BKRF4 BLLF1 BLLF3 BMLF1 BMRF1 BORF2 BRRF1 BVRF2 BXLF1 BALF4 BBRF1 BBRF2 BBRF3 BcLF1 BCRF1 BcRF1 BDLF1 BDLF3 BFRF3 BGLF1 BGLF2 BILF2 BKRF2 BLRF1 BLRF2 BNRF1 BOLF1 BORF1 BSLF1 BSRF1 BXLF2 BXRF1 BZLF2 TCL PI
Latent
IE
Early
Late



◄Figure 4. Intrathecal autoBLCL-reactive CD8+ T-cells recognize lytic EBV proteins. T-cell lines generated from CSF, of three CIS patients (numbers LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated, in duplicate, with Cos-7 cells that co-expressed the respective donor-specific HLA-I allele and individual EBV proteins (n=59); and T-cell reactivity was measured by quantifying IFNγ secretion in the cultures' supernatants. The EBV proteins are arrayed on their kinetic class level in a nominal genomic order on the x-axis and the IFNγ secretion levels (mean ± SD) of 2–3 independent experiments are shown. The names of EBV proteins that are specifically recognized are indicated by an arrow. The results of the EBV proteome analyses on the CSF-TCL for individual HLA-I alleles of patients LP33 (n=2) and LP51 (n=4) are shown in black and grey values. The negative and positive controls were CSF-TCL incubated with untransfected Cos-7 cells or CSF-TCL stimulated with PMA and ionomycin, respectively.

autoBLCL: autologous EBV-transformed B-cell lines; CIS: clinically-isolated syndrome; CSF: cerebrospinal fluid; EBV: Epstein-Barr virus; IFN: interferon; MS: multiple sclerosis; P/I: PMA and ionomycin; PMA: phorbol-myristate acetate; RRMS: relapsing–remitting MS; TCL: T-cell lines.

▲Figure 5. CD8+ T-cell recognition of autologous BLCL is not directed to αB-crystallin. (**A**) Relative CRYAB transcript levels were determined by reverse transcriptase human CRYAB-specific real-time PCR on RNA isolated from BLCL from the indicated patients. Relative transcript levels were determined by the formula 10,000 x $2^{(-delCt)}$, where delCt equals Ct ((CRYAB) - Ct (β-actin)). (**B**) The Cos-7 cells transfected with human CRYAB were assayed for CRYAB protein expression by immunocytology, using a specific mAb, and subsequently visualized using a fluorescein-conjugated secondary antibody (arrowheads). The nuclei were stained with DAPI (asterisks). Original magnification was 400x. (**C**), T-cell lines generated from CSF of three CIS patients (patients LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated, in duplicate, with Cos-7 cells that co-expressed the respective donor-specific HLA-I allele and human CRYAB. T-cell reactivity was measured by quantifying IFNγ secretion in conditioned medium, presented as (mean ± SD) of 2–3 independent experiments. The results of the CRYAB analyses on the CSF-TCL for individual HLA-I alleles, of patients LP33 (n=2) and LP51 (n=4), are shown in multiple colors. The negative and positive controls were CSF- TCL incubated with Cos-7 cells, transfected with a control vector (vector) or untransfected Cos-7 cells (TCL), and the CSF-TCL were stimulated with P/I.

BLCL: EBV-transformed B-cell lines; CRYAB: alpha B crystalline; DAPI: 4′, 6-diaminidino-2- phenylindole; EBV: Epstein-Barr virus; mAb: monoclonal antibody; P/I: PMA and ionomycin; TCL: T-cell lines.

Discussion

Despite strong epidemiological evidence, the potential pathological mechanisms initiated by EBV in MS remain unclear.[1,2,11] If EBV is involved then the virus' signature, either directly as viral remnants (e.g DNA) or indirectly as target of a local host immune response, should be selectively detectable or increased in brain tissue or alternatively CSF of MS patients. Most likely at early onset of disease.[1,2] Data presented here supported the involvement of intrathecal EBV-specific T-cell responses in MS, particularly in CIS patients. We detected significantly higher frequencies of autoBLCL-reactive CD4+ T-cells in the CSF-TCL from both CIS and MS patients, compared to OND patients (Figure 2A). Moreover, CIS patients also had significantly higher autoBLCL CD8+ T-cell reactivity, which only in this patient group was correlated with paired reactive CD4+ T-cell frequencies. This suggested a coordinated intrathecal CD4+ and CD8+ T-cell response directed toward either EBV or host antigens, expressed by autoBLCL (Figure 2B).

Data are consistent with earlier reports on T-cell reactivity to autoBLCL in the CSF of MS patients.[17,19,20] In contrast, neither EBV DNA nor intrathecal virus-specific IgG production was detectable in the CSF of the CIS and MS patients (data not shown). Whereas the latter data concur with reports that refute the role of EBV in MS,[10–12,25] other groups did find EBV remnants and increased virus-specific IgG in the CSF of MS patients.[7–9,32,33] The relatively long time interval at which the CSF samples of our patient cohort were obtained (>1 month after onset of disease) may have impeded detection of EBV DNA, but most likely not intrathecal EBV-specific IgG production.[12,14] This B-cell/T-cell discrepancy may be methodological.[2,3] Whereas intrathecal T-cell reactivity was performed on BLCL that in part expressed the whole EBV proteome, local antibody production was assayed only on one EBV protein (EBNA-1). Alternatively, the autoBLCL T-cell reactivity was not directed to EBV proteins, but to host proteins like the MS-associated autoantigen CRYAB, endogenously expressed by BLCL.[2,18] Previous studies had not completely deciphered this autoBLCL phenomenon.[17,19,20] They were either restricted to the analysis of a selective set of known immunodominant EBV peptides or used autologous dendritic cells pulsed with EBV or BLCL protein lysates as APC.[19–22] Besides the limited number of peptides tested, which discounts issues like inter-HLA allele peptide affinity and post-translational peptide modifications, the caveat of the latter strategy is restriction to structural viral proteins and potential contamination of host proteins in case of EBV and BLCL protein lysates, respectively.[13,14,30]

The artificial APC system applied here overcomes these potential drawbacks, by assaying CD8+ T-cell reactivity on cells that both endogenously express individual EBV proteins or human CRYAB, along with the HLA-I alleles that are involved in autoBLCL CD8+ T-cell recognition.[28] Concurrent with a previous report no autoBLCL reactive T-cell recognized CRYAB.[18] Although BLCL predominantly contained B-cells expressing latent viral proteins (Supplementary Figure 1), the intrathecal CD8+ T-cell targets were restricted to the lytic EBV proteins, classified as immediate early (BRLF1), early (BaRF1 and BXLF1), and late (BCRF1 and BBRF3) proteins (Figure 3).

The antigen diversity and kinetic class of the EBV CD8+ T-cell targets identified do not support CD8+ T-cell cross-reactivity between EBV and neuroantigens, but agreed with the current concept that early lytic EBV proteins are prominent CD8+ T-cell targets, respectively.[13,14] Herein, we provide the first evidence for CD8+ T-cell recognition of the lytic EBV proteins BXLF1, BCRF1 and BBRF3. However, we did not confirm specific EBV proteins, especially EBNA-1 and DNA polymerase (BALF5), as targets of the intrathecal EBV-specific T-cells in MS patients.[17,20,23] This discrepancy, likewise increased intrathecal EBV CD4+ T-cell reactivity though indifferent between MS and OND patients as reported by two independent groups[19,22] is puzzling that needs follow-up. Despite differences on patient cohorts and experimental set-up, including autoBLCL versus peptides/lysates as antigens in functional assays on T-cells *ex vivo* versus CSF-TCL, the current data support the involvement of EBV-specific T-cells in the intrathecal inflammatory response in MS patients in the absence of a detectable intrathecal EBV infection.[17,19–23] Additional support for this notion is provided by recent studies on the experimental autoimmune encephalomyelitis (EAE) model in common marmosets, suggesting the pivotal role of CD20 B-cells, infected with the EBV-like simian γ-herpesvirus CalHV3, as the APC for the pathogenic T-cell response.[34]

Potential limitations of our study are *i)* a relatively large part of CSF-TCL from OND patients were generated several years ago, from a HuD-PNS study group (9/13 OND patients), *ii)* the potential bias in data interpretation due to prior knowledge of the patients' diagnosis.[27] First, all CSF-TCL were generated and subsequently analyzed in functional T-cell assays in the same lab (Department of Viroscience, Erasmus Medical Center) according to the same in-house SOPs. Moreover, we showed that even long-term storage of CSF-TCL, particularly those generated from HuD-PNS patients, does not impair the immunocompetence of T-cells upon stimulation with P/I (Supplemental Figure 2). Second, the co-authors involved in generating and assaying the CSF-TCL for autoBLCL reactivity were blinded from the patients' diagnosis, except for the HuD-PNS patient cohort. Subsequent in-depth analyses (i.e. TCRBV, EBV target antigen and CRYAB T-cell analyses) of selected high autoBLCL responders were performed on CSF-TCL of unmasked CIS and MS patients. In our opinion, the use and interpretation of T-cell assays performed according to in-house SOPs by the same operators, using appropriate controls and settings, have contained this potential drawback of our study.

In conclusion, the current study provided novel insights into the association between EBV and MS. First, intrathecal T-cell reactivity towards autoBLCL was significantly increased in both CIS and MS patients, compared to other diseased controls. Second, in-depth analysis of four high autoBLCL T-cell responders demonstrated that the intrathecal autoBLCL-specific CD8+ T-cell responses were oligoclonal and in two CSF-TCL directed toward the lytic EBV proteins, but not to CRYAB that was endogenously expressed by BLCL. The data suggested a local T-cell response to EBV in MS. This warrants further characterization of the magnitude and breadth of intrathecal EBV-specific T-cell responses using the whole EBV proteome in larger cohorts of patients and disease controls.

Acknowledgements

We thank Prof. JM Middeldorp (VUMC, Vrije Universiteit Medical Center; Amsterdam, Netherlands) for providing the EBV-specific mAbs, M Janssen for technical assistance and the clinical staff from the Department of Neurology, Erasmus Medical College (Rotterdam, Netherlands); we particularly thank JWK De Beukelaar for the collection and management of HuD clinical samples. This work was partially funded by the Dutch MS Research Foundation (project #09-670 MS, to GPVN); and the Niedersachsen-Research Network on Neuroinfectiology, of the Ministry of Science and Culture of Lower Saxony, Germany (to JGM and GMGMV).

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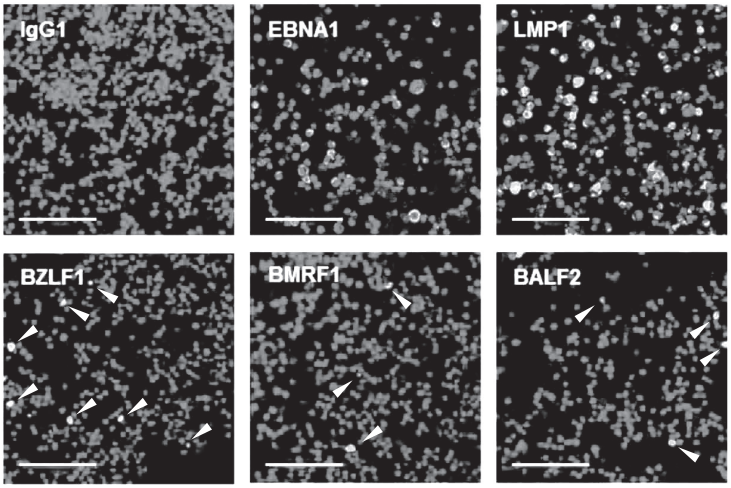
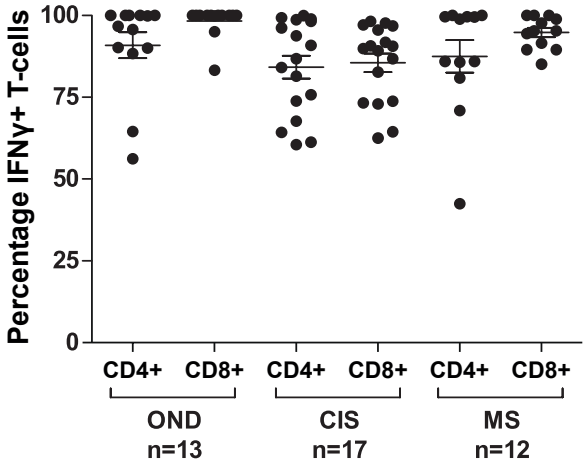
Supporting information



Supplementary Figure 1. Human Epstein-Barr virus transformed B-cell lines express latent and lytic cycle viral proteins. Immunocytology of a representative Epstein-Barr virus (EBV) transformed B-cell line that shows widespread expression of latent (EBNA-1 and LMP1) and lower frequency expression of lytic cycle EBV proteins (BZLF1, BALF1 and BMRF1, indicated with white arrowheads). Cells were spotted on glass slides and stained with monoclonal antibodies directed to indicated EBV proteins and subsequently incubated with a fluorescein-conjugated secondary antibody (white color). Nuclei were stained with DAPI (grey color). Bars indicate 100 μM and original magnification was 100-times.



Supplementary Figure 2. T-cells in cerebral spinal fluid-derived T-cell lines are immunologically competent. Cerebrospinal fluid-derived T-cell lines (CSF-TCL) obtained from patients with other neurological disease (OND), clinically isolated syndrome (CIS) and multiple sclerosis (MS) were stimulated with a combination of phorbolmyristate-acetate (PMA; 50 ng/ml) and ionomycin (500 ng/ml) to determine the immunecompetence of CD4+ and CD8+ T-cells. Stimulated cells were stained for viability, surface CD3, CD4 and CD8, and intracellular interferon-γ and subsequently analyzed using flow cytometry. Dots represent data of individual CSF-TCL, presented as percentage IFNγ+ cells of all CD4+ or CD8+ T-cells in the corresponding CSF-TCL, and bars and whiskers indicate the mean and standard deviation.

Chapter 7

Phenotypic and functional characterization of T–cells in white matter lesions of multiple sclerosis patients

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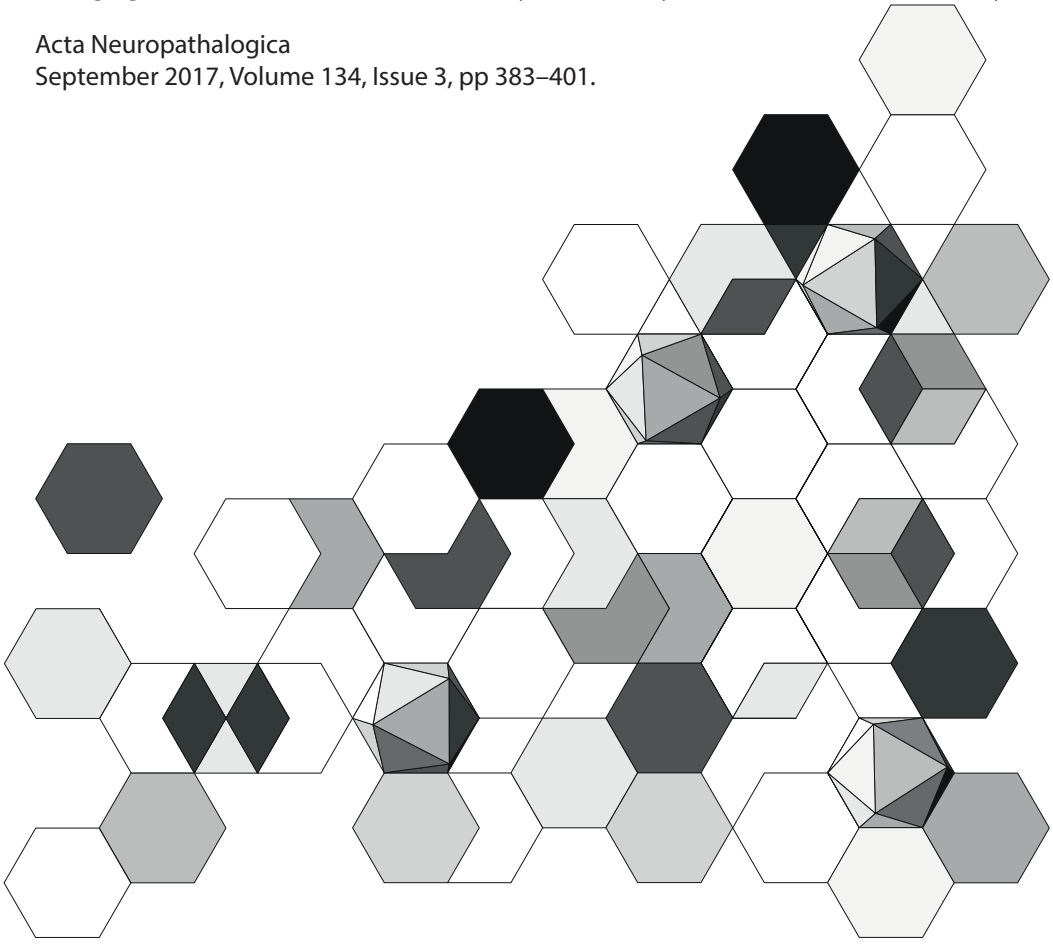
Acta Neuropathalogica
September 2017, Volume 134, Issue 3, pp 383–401.



Abstract

T-cells are considered pivotal in the pathology of multiple sclerosis (MS), but their function and antigen-specificity are unknown. To unravel the role of T-cells in MS pathology, we performed a comprehensive analysis on T-cells recovered from paired blood, cerebrospinal fluid (CSF), normal-appearing white matter (NAWM) and white matter lesions (WML) from 27 MS patients with advanced disease shortly after death. The differentiation status of T-cells in these compartments was determined by *ex vivo* flow cytometry and immunohistochemistry. T-cell reactivity in short-term T-cell lines (TCL), generated by non-specific stimulation of T-cells recovered from the same compartments, was determined by intra-cellular cytokine flow cytometry. Central memory T-cells predominated in CSF and effector memory T-cells were enriched in NAWM and WML. WML-derived CD8+ T-cells represent chronically activated T-cells expressing a cytotoxic effector phenotype (CD95L and granzyme B) indicative for local antigenic stimulation (CD137). The same lesions also contained higher CD8+ T-cell frequencies expressing co-inhibitory (TIM3 and PD1) and co-stimulatory (ICOS) T-cell receptors, yet no evidence for T-cell senescence (CD57) was observed. The oligoclonal T-cell receptor (TCR) repertoire, particularly among CD8+ T-cells, correlated between TCL generated from anatomically separated WML of the same MS patient, but not between paired NAWM and WML. Whereas no substantial T-cell reactivity was detected towards seven candidate human MS-associated autoantigens (cMSAg), brisk CD8+ T-cell reactivity was detected in multiple WML-derived TCL towards autologous Epstein-Barr virus (EBV) infected B-cells (autoBLCL). In one MS patient, the T-cell response towards autoBLCL in paired intra-lesional TCL was dominated by TCRVβ2+CD8+ T-cells, which were localized in the parenchyma of the respective tissues expressing a polarized TCR and CD8 expression suggesting immunological synapse formation *in situ*. Collectively, the data suggest the involvement of effector memory cytotoxic T-cells recognizing antigens expressed by autoBLCL, but not the assayed human cMSAg, in WML of MS patients.

Introduction

Multiple sclerosis (MS) is a chronic debilitating disease characterized by inflammation of the central nervous system (CNS), leading to demyelination and axon damage. The cause and pathogenic pathways involved remain enigmatic. MS is considered the result of a local inflammatory response in genetically susceptible individuals initiated by environmental factors.[1,2]

MS immunopathology has been widely studied in experimental autoimmune encephalomyelitis (EAE) animal models. EAE is induced by immunization with candidate MS-associated CNS autoantigens (cMSAg) in adjuvant and by adoptive transfer of cMSAg-specific CD4+ T-cells. These studies reinforced the idea that MS is an autoimmune disease mediated by cMSAg-specific CD4+ Th1/Th17-cells.[3] Indeed, CD4+ T-cell responses to the majority of EAE-inducing cMSAg have been identified in peripheral blood (PB) and occasionally CSF of MS patients.[2–4] However, their frequency in PB of MS patients was indifferent compared to controls, questioning their pathogenic potential.[2,5] The additional role of CD8+ T-cells in MS and EAE immunopathology has only recently emerged.[3,5] In MS patients, CD8+ T-cells outnumber CD4+ T-cells in active lesions and closely interact with oligodendrocytes and demyelinating axons.[1,5,6] CD8+ T-cells infiltrates in active white matter lesions (WML) are oligoclonal and persist in CSF and peripheral blood.[7–9] Human cMSAg-specific CD8+ T-cells lyse human leukocyte antigen (HLA) class I-matched oligodendrocytes *in vitro*.[10–12] The cytotoxic effector mechanisms involve both the Fas/FasL and perforin/granzyme B (grB) pathways.[12–15]

Current data advocate Epstein-Barr virus (EBV) infection as risk factor in MS.[16,17] The epidemiological similarity of MS and EBV-induced infectious mononucleosis (IM), enhanced systemic B- and T-cell responses to EBV in MS patients compared to (disease) controls, and the putative presence of EBV-infected B-cells in MS lesions are indicative.[18–20] Even though evidence for the presence of EBV in MS lesions remains incredulous,[21] EBV may evoke T-cell-mediated MS immunopathology in different ways.[22,23] Virus-specific T-cells may recognize EBV-infected cells in the brain or alternatively cross-react with cMSAg.[24] Alternatively, IM-associated systemic immune activation may result in a cytokine environment that activates pathogenic T-cells, including cMSAg-specific T-cells, in an antigen independent manner.[16,17]

The aims of this study were to characterize the differentiation status and antigen-specificity of T-cells recovered from paired white matter tissues containing WML, normal-appearing white matter (NAWM), CSF and PB from 27 patients with advanced MS.

Materials and methods

Clinical specimens

Heparinized PB (3-6 ml; n=19), CSF (8-20 ml; n=17), and macroscopically-defined NAWM (n=23) and WML (n=29), were obtained from 27 MS patients (median age 59 yr, range 35-95 yr) at autopsy with a median post-mortem interval of 9.6 hr (range 4.8-14 hr) (Supplementary Table 1). All patients had advanced disease (median disease duration 25 yr, range 10-54 yr), expanded disability status scale >6 and the majority had primary or secondary progressive MS (22 of 25, 88%). Leading cause of death was legally granted euthanasia for 11 of 27 (44%) patients. Additionally, 12 formalin-fixed and paraffin-embedded (FFPE) tissues of 10 diseased MS patients were collected (Supplementary Table 2). Clinical specimens of deceased MS patients were collected by the Netherlands Brain Bank (NBB; Amsterdam, The Netherlands) and genital skin biopsies of six patients with herpes simplex virus type 2 (HSV-2) genital herpes (GH) were obtained by the department of Dermatology and Venereology (Erasmus MC, Rotterdam, The Netherlands). Written informed consent for brain autopsy and genital skin biopsies, use of clinical specimens and clinical information for research purposes have been obtained in advance from all study participants. Study procedures were performed in compliance with Dutch legislation and institutional guidelines, approved by the respective local ethical committees for use of MS patient material (project number 2009/148; VU University Medical Center, Amsterdam) and skin biopsies of GH patients (project number MEC 167.153/1998/15; Erasmus MC) performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. During autopsy, WML was distinguished macroscopically from NAWM based on grey appearance and by being firm to touch. PB was used to generate EBV-transformed B-cell lines (BLCL) and for 2-digit HLA typing as described.[25] Part of white matter tissues (1-4 gr) were snap-frozen and stored at -80°C for subsequent *in situ* analysis. Remaining tissue was dispersed to single cell suspensions as described.[25] About one-tenth of the single cell suspension was snap-frozen for subsequent EBV-specific EBER1 transcript expression analysis and remaining cells were used for *ex vivo* flow cytometric analysis and to generate short-term T-cell lines (TCL) as described.[25] In brief, CSF and lymphocyte-enriched brain tissue cell suspensions were stimulated with phytohemaglutinin-L (1 µg/ml; Roche, Branford, CT) for 10-14 days and subsequently with an anti-CD3 monoclonal antibody (mAb) (clone OKT-3; Janssen-Cilag, Tilburg, the Netherlands) for 10-14 days in the presence of γ-irradiated (3,000 rad) allogeneic PBMC and recombinant human interleukin 2 (rIL-2 IU/ml) and rIL-15 (25 ng/ml; both Miltenyi biotec, Bergisch Gladbach, Germany).[20]

In situ analyses of brain tissues

To classify the WM tissues of MS patients, brain tissues were characterized by immunohistochemistry (IHC) as NAWM, diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL) and inactive lesions (IL) as described in the recently updated classification system for MS brain lesions[26] (representative stainings of defined WM tissues of MS patients are shown in Supplementary Figure 1). In brief, consecutive 7-9 µm cryostat brain sections were air-dried, fixed with acetone and subsequently assayed for: *i)* (de-) myelination by anti-myelin oligodendrocyte protein (MOG) (clone Z12; kindly provided by prof. Sandra Amor, VU medical center, Amsterdam), *ii)* immune activation using anti-HLA-DP/DQ/DR mAb (CR3/43; Dako, Glostrup, Denmark), *iii)* presence of macrophages and microglia using anti-CD68 (clone EMB11; Dako). Granzyme B expressing T-cells were detected in consecutive 6 µm sections of FFPE mAIL tissue of four representative MS patients stained for CD3 (clone F7.2.38), CD8 (C8/144B), grB (GrB-7, all Dako). Stainings were visualized by 3-amino-9-ethylcarbazole as described.[25] Intracellular localization of grB and cytotoxic potential of CD8+ T-cells was assessed in 8 µm FFPE sections by triple immunofluorescent stainings using mAb directed to CD8 (clone YTC182.20, AbD Serotec), grB and cleaved caspase 3 (cCASP3; clone G7481, Promega, Madison, USA). The presence of tissue-resident T-cells (T_{RM}) was assessed analogously by detecting CD8, CD69 (FN50, Biolegend) and CD103 (2G5.1, Thermo Fisher) expression in WML of MS patients and as control skin biopsies of GH patients. CD8+ T-cells expressing grB in the parenchyma and perivascular space of mAIL sections were counted in multiple z-stack scans acquired at 400x magnification. To determine the CNS cell types encountered by intra-lesional CD8+ T-cells, double immunofluorescence stainings were performed on 8 µm FFPE sections of WML biopsies. CNS cell types were visualized using mAbs directed to neuron-specific neurofilament heavy chain (NF-H, clone SMI-31, Biolegend), microglia-specific ionized calcium-binding adapter molecule 1 (Iba1; clone 019-19741, Wako, Osaka, Japan), oligodendrocyte-specific proteolipid protein (PLP; clone plpc1, AbD serotec) and astrocyte-specific glial fibrillary acidic protein (GFAP; clone Z0334, Dako) combined with an anti-CD8 mAb (C8/144B). Antibody binding was visualized by AF488-conjugated Rat IgG (A-11006), AF568- conjugated IgG1 (A-21124), AF488- or AF647-conjugated IgG2a (A-21131 or A-21241) or AF647- conjugated rabbit IgG-specific (A-21246, all Invitrogen) antibodies. To detect T-cell receptor beta chain 2 (TCRVβ2) expressing T-cells *in situ*, 8 µm cryostat brain sections were stained with APC-conjugated CD8 (RPA-T8, BD), FITC-conjugated TCRVβ2 (MPB2D5; Beckman Coulter) and laminin (L9393; Sigma) mAb combined with AF594-conjugated anti-rabbit IgG (R37119; Invitrogen). TCRVβ2 staining was amplified using the FASER kit (Miltenyi biotec). For all fluorescent stainings, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei and sections scanned using LSM700 confocal microscope and Zen 2010 software (Zeiss; Oberkochen, Germany). Appropriate (fluorochrome-) matched Ig isotype antibodies were used as negative controls for all stainings and stainings scored by two independent observers.

Ex vivo T-cell phenotyping

Paired PB-, CSF-, NAWM- and WML-derived cells were incubated with combinations of fluorochrome-conjugated mAb directed to following markers: CD3 (clone UCHT1), CD4 (SK3 or RPA-T4), CD8 (SK1 or RPA-T8), CD27 (M-T271), CD45 (TU116), CD56 (B159), CD137 (4-1BB; all Becton Dickinson (BD), Franklin Lakes, NJ), CD57 (HCD57), CD95L (NOK-1), ICOS (C398.4A), PD1 (EH12.2H7), TIM3 (F38-2E2; all Biolegends, San Diego, CA) and CD45RA (HI100; Pharmingen, San Diego, CA). Cells were measured on a Canto II flow cytometer (BD). Negative controls consisted of stainings with appropriate Ig isotype control antibodies. If a parent-population contained <100 events, the gated cell population was omitted from further analysis.

Quantitative EBV transcript expression analysis

Snap-frozen brain tissue-derived single cell pellets were used for RNA isolation and cDNA synthesis as described.[25] EBV EBER1 transcript expression was quantified by real-time quantitative PCR (qPCR) using forward primer 5'-TCATAGGGAGGAGACGTGTGT-3', reverse primer 5'-TGACCGAAGACGGCAGAAAG-3' and probe 5'FAM-AGACAACCACAGACACCGTGGTGACCA-3'MGB (all Eurogentec; Liege, Belgium). RNA isolated from water and BLCL served as negative and positive controls in each run, respectively. For each sample β-actin transcripts (Hs01060665_g1, Applied Biosystems) were used as positive control for RNA isolation and cDNA synthesis. Sensitivity of the EBER1-specific qPCR was determined as 1-10 BLCL per 10^6 PBMC (data not shown). An ABI prism 7500 and Taqman Universal Master Mix (both Applied Biosystems; Nieuwerkerk, the Netherlands) were used for all qPCR reactions as described.[25]

Functional T-cell assays

T-cell reactivity towards EBV-infected B-cells in CSF-, NAWM- and WML-TCL was determined by incubation with autologous EBV-transformed B-cell lines (autoBLCL) as antigen presenting cell (APC) as described previously.[20] The frequency of B-cells in BLCL lines (n=5) undergoing spontaneous lytic cycle infection was determined by flow cytometric analysis of EBV glycoprotein 350 expression (clone OT.1C-2, kindly provided by Prof.dr. J.M. Middeldorp, VU medical Centre, Amsterdam, the Netherlands). An allogeneic BLCL (BLCL-GR; HLA-A*01;03, HLA-B*07;27, HLA-C*02;07, HLA-DRB1*13;15, HLA-DQB1*06;06), carrying the major MS-associated HLA class I and II risk alleles (HLA-A*03, HLA-DRB1*15 and HLA-DRB1*13),[27] was transduced to express the 7 following human cMSAg constitutively: *i)* oligodendrocyte-specific proteins [myelin-associated glycoprotein (MAG), myelin basic protein isoform 1 (MBP1) and myelin oligodendrocyte glycoprotein (MOG)], *ii)* neuron-specific proteins [contactin-2 (CNTN2) and 155kD isoform of neurofascin (NFASC)] and *iii)* glia-specific proteins [inwards rectifying potassium channel (KIR4.1) and S100 calcium binding protein B (S100B)].[28] Stable cMSAg-expressing BLCL-GR lines, validated by flow cytometry using cMSAg-specific mAb, were used as allogeneic APC to detect cMSAg-specific CD4+ and CD8+ T-cells simultaneously in TCL of HLA-matched MS patients (Supplementary Table 1).[28] To validate this BLCL platform,

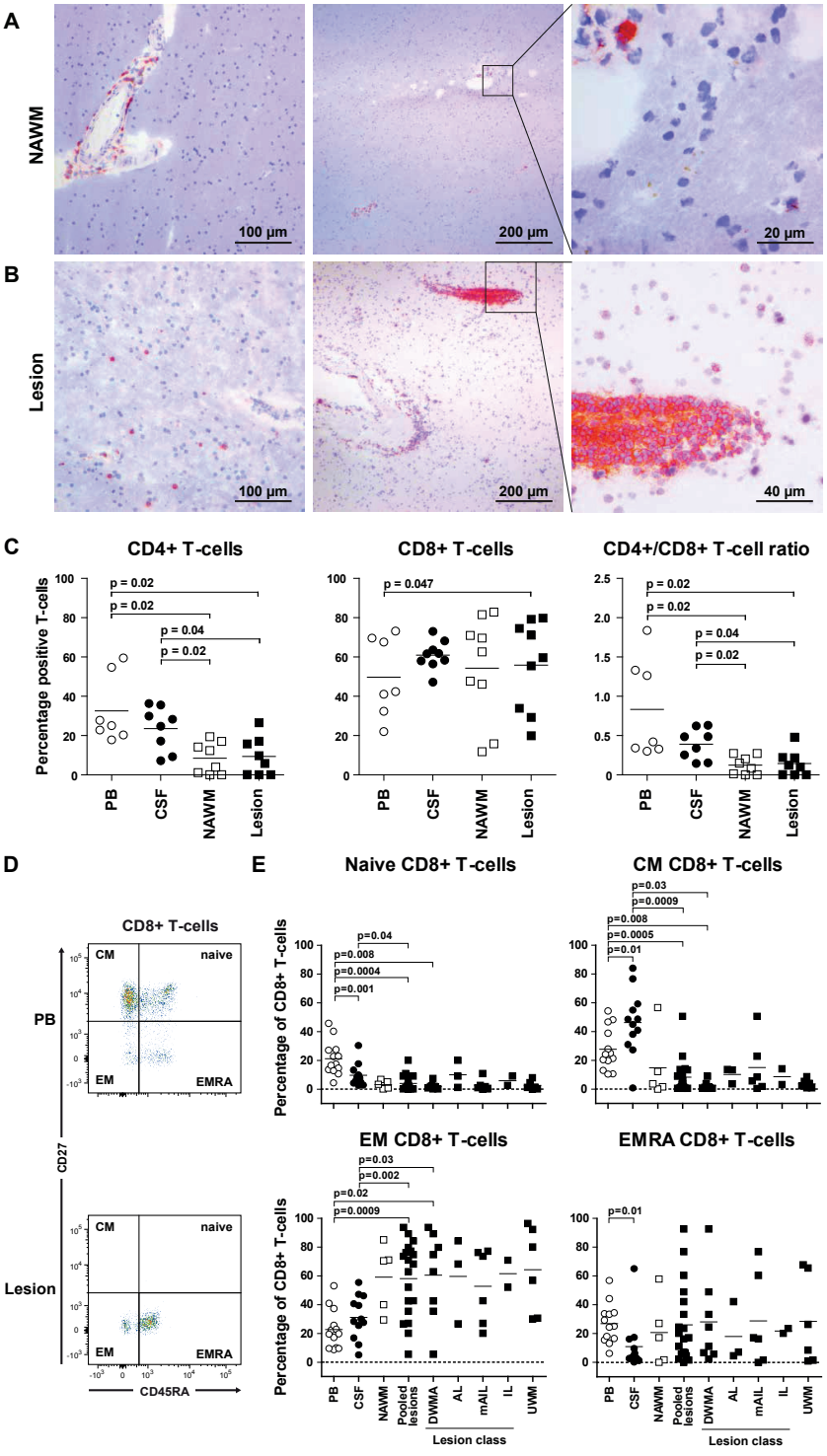
we also transduced BLCL-GR to express measles virus fusion protein (MVF) and assayed their APC function by incubation with MVF-specific CD4+ (4-F99) and CD8+ (2-F40) T-cell clones (TCC) as described previously.[28] Stimulated T-cells were stained with fluorochrome-conjugated mAb to human CD3 (SP34-2), CD4 (SK3), CD8α (RPA-T8) and for viability (violet live/dead stain; Invitrogen). Subsequently, cells were fixed, permeabilized and stained for intracellular IFNγ (B27) and CD137 (4-1BB, all BD) and finally measured on a Canto II flow cytometer. Combined intracellular IFNγ and CD137, two independent T-cell activation markers induced upon antigenic stimulation, was set as criterion to identify antigen-specific T-cells.[20,29] TCL stimulated with phorbolmyristate-acetate (PMA; 50 ng/ml) and ionomycin (Iono; 500 ng/ml; both Sigma), or mock-stimulated TCL, were used as positive and negative control, respectively. Netto reactivity of CD4+ and CD8+ T-cells towards cMSAg-transduced BLCL-GR was calculated by subtracting T-cell reactivity to cMSAg-transduced BLCL-GR with mock-transduced BLCL-GR. Threshold for positive cMSAg T-cell reactivity was calculated as previously reported.[28] Experiments were performed in at least two independent occasions.

T-cell receptor repertoire analysis

The clonality of NAWM- and WML-derived TCL was determined using a set of fluorochrome-conjugated mAb to 24 different human TCRVβ chains covering approximately 70% of the known human TCRVβ repertoire (IOTest Beta Mark mAb kit; Beckman Coulter, Marseille, France) combined with mAb to CD3, CD4 and CD8. TCRVβ usage of autoBLCL-specific T-cells in WML-TCL was identified by CD3, CD4, CD8 and TCRVβ combined with intracellular IFNγ staining and flow cytometry. T-cell clonality was also determined by the TCR gamma (TCRγ) gene rearrangement Assay 2.0 (Invivoscribe, San Diega, CA) performed in duplo on DNA isolated from FACSsorted viable CD8+ T-cells of paired brain tissue-derived TCL of 8 MS patients, and as control PBMC and two monoclonal human T-cell leukemic cell lines (MOL3 and KL 1985-001), according to manufacturers' instructions (Invivoscribe).

Data analysis and statistics

Flow cytometry data was analyzed with FlowJo (Tree Star Inc.; Ashland, OR) software. Paired data was assessed using Wilcoxon-signed rank test. Significance of variation in cMSAg- and autoBLCL-specific T-cell reactivity was determined by ANOVA for CD4+ and CD8+ T-cells separately. Unpaired t-test was used to determine significance of autoBLCL T-cell reactivity. All statistical analyses were performed using Graphpad Prism 5 software (Graph Pad Inc.; La Jolla, CA).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Percentage positive T-cells
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CSF
NAWM
Pooled lesions
DWMA
AL
mAIL
IL
UWM
Lesion class

◄ **Figure 1. CD8+ T-cells in normal-appearing and diseased white matter tissues of MS patients preferentially express an effector memory phenotype.** (**A** and **B**) Parenchymal T-cells are selectively detected in active MS lesions. 10-µm cryostat sections of paired (**A**) normal-appearing white matter (NAWM) and (**B**) white matter lesion (WML) of two representative patients of five MS patients analyzed. CD3 expressing cells (T-cells) were stained with 3-amino-9-ethylcarbazole (red color) and counterstained with hematoxylin (blue color). Whereas perivascular T-cells were detected in both NAWM and WML, parenchymal T-cells were exclusively detected in active WML of MS patients. (**C**) Percentages of CD4+ and CD8+ T-cells, and CD4+/CD8+ T-cell ratio, are shown for paired PB (open circles), CSF (closed cir- cles) and histologically defined as NAWM (open squares) and WML (filled squares). Lymphocytes were isolated from paired peripheral blood (PB), cerebrospinal fluid (CSF), NAWM and WML (lesion) from patients with advanced MS (n=17) and subjected to multi- plex flow cytometry. Gating procedure of CD4+ and CD8+ T-cells is shown in Online Resource 3. (**D**) CD8+ T-cells were subdivided in naïve (CD27+CD45RA+), central memory (CM; CD27+CD45RA–), effector memory (EM; CD27–CD45RA–) and terminally differenti- ated effector memory (EMRA; CD27–CD45RA+) T-cells. Gating procedure is shown for representative paired PB and WML-derived CD8+ T-cells. (**E**) The frequency of naïve, CM, EM and EMRA CD8+ T-cells is shown for paired PB, CSF and white matter brain tissues that were immunohistologically classified as NAWM, diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mIAL), inactive lesion (IL) or unconfirmed white matter tissues (UWM) (see Online Resource 3 for criteria applied for MS WM classification). Horizontal lines represent the mean frequencies. Wilcoxon matched pairs test was used to calculate significance Sensitivity

Results

Macroscopically-defined NAWM of MS patients with advanced disease contain diffuse white matter abnormalities

To characterize the role of T-cells in MS, we collected paired PB, CSF, NAWM and WML specimens from 27 deceased patients with advanced MS (Supplementary Table 1). The presence and classification of the WM tissues sampled was determined by IHC on representative snap-frozen sections of surplus NAWM and WML sections (Supplementary Figure 1);[26] provided adequate size of brain tissues was available. All macroscopically-defined lesions were confirmed as WML and all contained demyelinated areas classified as AL, mAIL or IL.[26] Notably, 9 of 20 (45%) macroscopically-defined NAWM tissues presented as DWMA consisting of increased numbers of macrophage/microglia and high HLA class II expression (Supplementary Table 1). These DWMA represent periplaque abnormalities, Wallerian degeneration or pre-lesional changes.[26] The spatial distribution of T-cells was determined on paired NAWM and WML tissues from 5 MS patients. In both NAWM and active WML, T-cells were mainly detected in the Virchow-Robin space (Figure 1A). However, parenchyma-infiltrating T-cells were selectively observed in WML suggesting that these T-cells are not part of immune surveillance, but rather involved in the local inflammatory response.[6,30]

Effector memory CD8+ T-cells are the main T-cell subset in NAWM and WML of MS patients

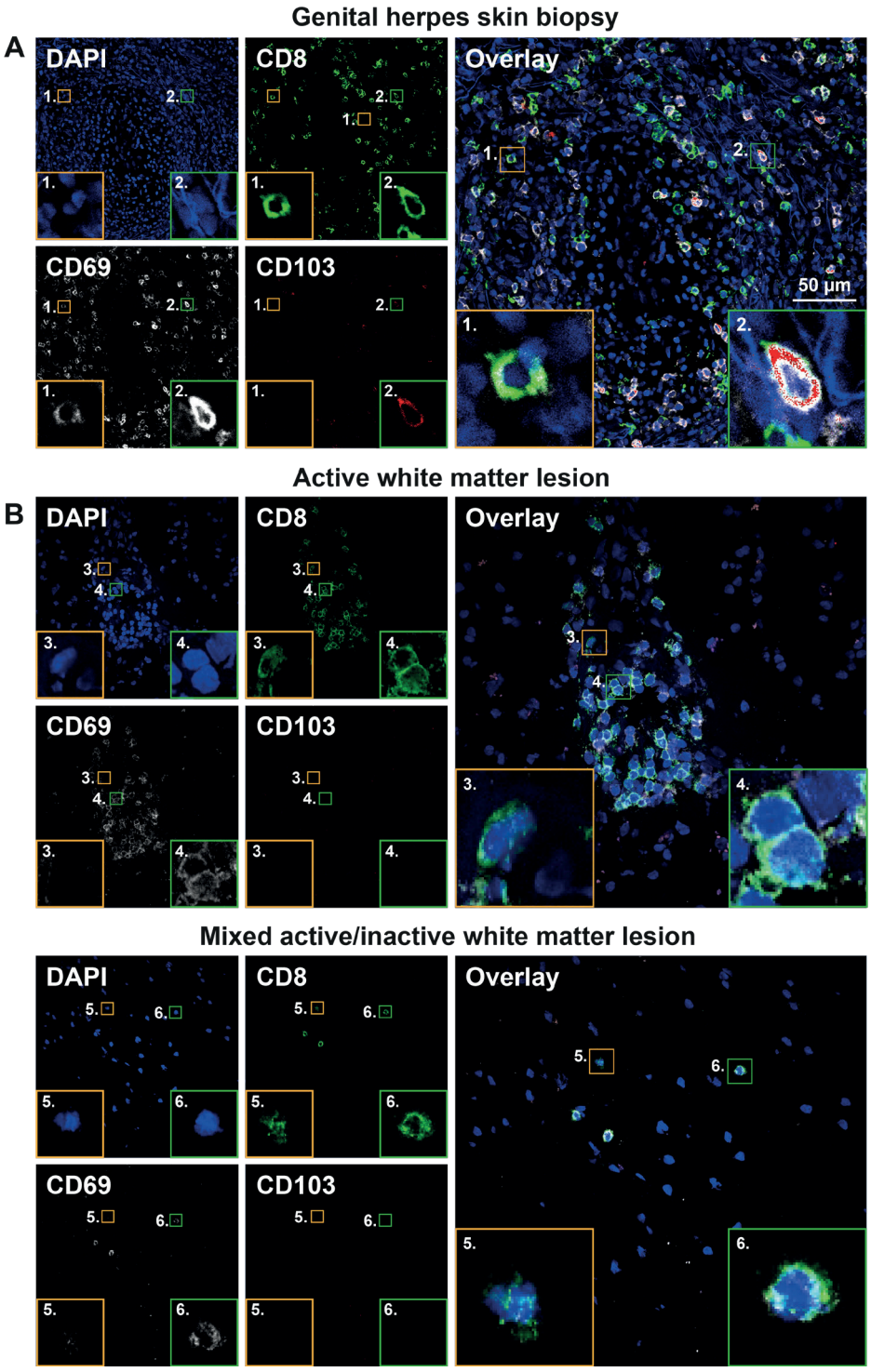
To determine the phenotype and differentiation status of T-cells in MS patients *ex vivo*, paired PB, CSF, and lymphocyte-enriched NAWM- and WML-derived single cell suspensions of 17 MS patients were subjected to multiplex flow cytometric analysis. T-cells were selected by lymphocyte, $CD45^{high}$ and CD3 gating, and finally sub-classified as $CD3+CD4^{high}$ and $CD3+CD8^{high}$ T-cells based on PB-derived T-cells. In contrast to PB and CSF, brain tissue derived CD3+ cells frequently expressed low levels of CD4 or CD8, and on occasion were devoid

of CD4 and CD8 expression (Supplementary Figure 2). As these different T-cell subtypes are difficult to differentiate by flow cytometry and no consensus exists in literature on their origin, we omitted them from further analysis and only focused on T-cells expressing high levels of CD4 or CD8. The latter uniform gating strategy was chosen to compare the activation and differentiation status of the same CD4+ and CD8+ T-cells between multiple anatomic locations of the same individual. In both NAWM and WML, CD8+ T-cells dominated as shown by the significantly lower CD4+/CD8+ T-cell ratio in NAWM and WML compared to PB and CSF (Figure 1C).

Next, the differentiation status of T-cells was compared between compartments based on differential surface expression of CD45RA and CD27 (Figure 1C).[29] Naive (T_{NA}; CD27+CD45RA+) CD8+ T-cells were readily identified in PB, less frequently in CSF and rarely in NAWM and WML (Figure 1D). Central memory (T_{CM}; CD27+CD45RA-) CD8+ T-cells were the dominant phenotype in CSF. Effector memory (T_{EM}; CD27-CD45RA-) CD8+ T-cells predominated in both NAWM and WML, with frequencies 2-fold higher compared to PB and CSF. Finally, terminally differentiated memory (T_{EMRA}; CD27-CD45RA+) T-cell frequencies were equivalent in PB, NAWM and WML, but lower in CSF. No significant differences in CD4+/CD8+ T-cell ratio and CD8+ T-cell differentiation status were observed between different WM types. Low numbers of CD4+ T-cells in most WML and NAWM specimens precluded conclusive definition of their differentiation status (data not shown). In conclusion, CD8+ T_{EM} cells are enriched in both NAWM and WML of the MS patients analyzed.

►Figure 2. CD8+T-cells in white matter lesions of MS patients preferentially express CD69, but not CD103. Representative triple immunofluorescent stainings on 8 µm cryosections of (**A**) skin biopsies from six genital herpes patients and (**B**) immunohistochemically classified white matter lesions (WML) of six MS patients. Tissues were stained for CD8 (green color), CD69 (white color) and CD103 (red color) using specific monoclonal antibodies (mAbs) and isotype specific fluochrome-conjugated secondary antibodies. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue color) and analyzed using a Zeiss LSM-700 confocal laser microscopy and ZEN software. Skin biopsies of genital herpes patients were stained as positive control to validate staining strategy by confirming localization tissue-resident CD8+ T-cells based on differential CD69 and CD103 staining: CD8+CD69+CD103- (**A**; inset 1) and CD8+CD69+CD103+ T-cells (**A** inset 2). In WML of MS patients (**B**), perivascular (top panels) and parenchymal CD8+ T-cells (bottom panels) were incidentally CD69-CD103- T-cells (**B**; insets 3 and 5) and predominantly CD69+CD103- T-cells (**B**; inset 4 and 6). Size bar is indicated in top right image.

The expression of CD69 and CD103, two proteins described to enhance long-term retention of T-cells within peripheral tissues also referred as T_{RM} cells,[31] was determined on CD8+ T-cells in WML cryosections of 6 MS patients by triple immunofluorescence staining. We first validated our staining protocol by analyzing skin biopsies of 6 HSV-2 GH patients. In agreement with a previous study, the majority of human genital skin CD8+ T_{RM} expressed CD69 and about 20% of these cells co-expressed CD103 (Figure 2A).[31,32] In contrast, both perivascular and parenchymal CD8+ T-cells in WML of MS patients exclusively expressed the CD8+CD69+CD103- phenotype (Figure 2B). These data contrast previous studies on mouse CNS- and human glioma-derived CD8+ T-cells showing that about half of CD8+CD69+ T_{RM} cells in CNS tissue co-express CD103.[33] Because CD69 is also expressed by T-cells early after TCR and cytokine activation,[29] and since CD103 is debated as reliable T_{RM} marker,[34] the CD8+CD69+CD103- T-cells detected in WML of the MS patients analyzed may denote genuine CD8+ T_{RM} cells, represent activated T-cells or a mixture thereof.[29,31,33,34]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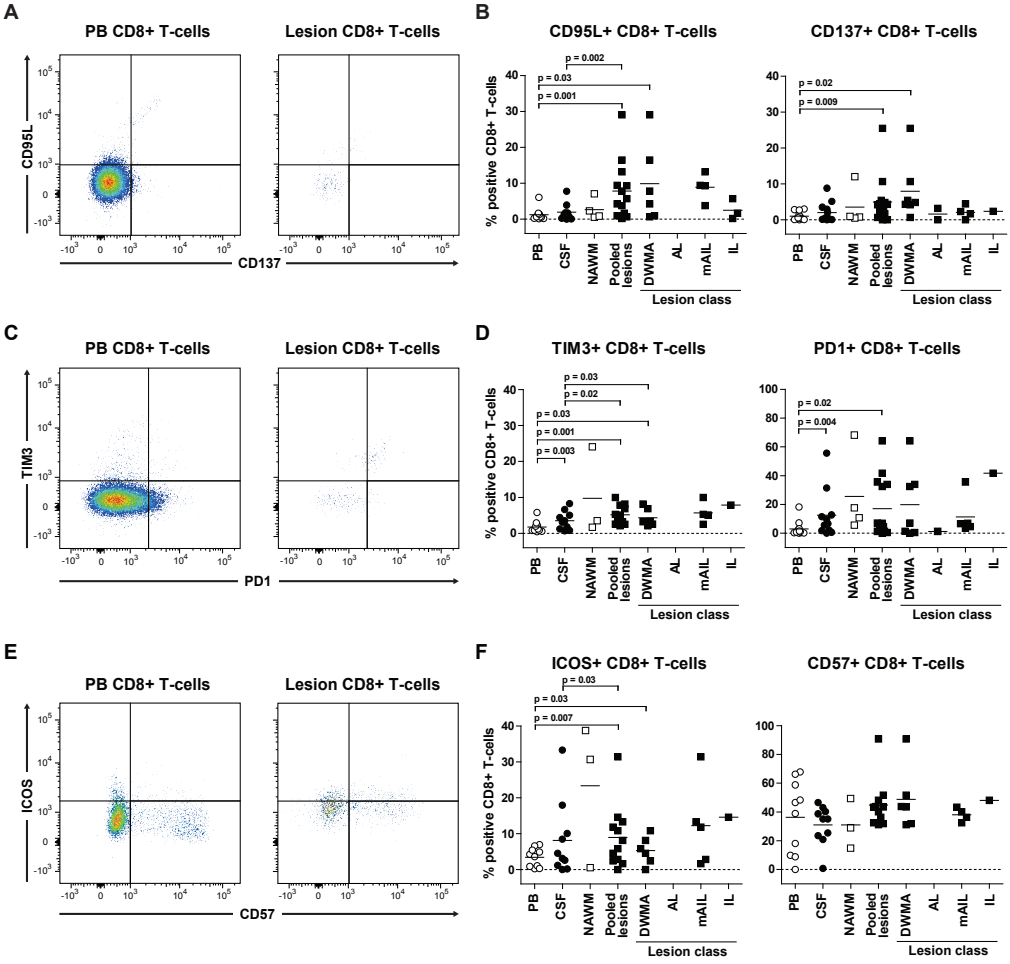


Figure 3. CD8+ T-cells in white matter lesions of MS patients are chronically activated T-cells expressing a cytotoxic effector T-cell phenotype. Flow cytometric analysis of cytotoxic molecule CD95L and co-stimulatory receptor CD137 (**A** and **B**), co-inhibitory receptors TIM3 and PD1 (**C** and **D**) and co-stimulatory molecule ICOS and senescence marker CD57 (**E** and **F**) expression on CD8+ T-cells in paired peripheral blood (PB, open circles), cerebrospinal fluid (CSF, closed circles) and brain tissues histologically defined as normal-appearing white matter (NAWM, open squares) and white matter lesions (WML, filled squares) classified as diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mIAL) and inactive lesions (IL) of 17 MS patients (see Supplementary Figure 1for criteria applied for MS WM classification). Lymphocyte gating was performed as described in the legend of Supplementary Figure 2. Gating strategies and percentages of marker positive CD8+ T-cells in paired anatomic compartments are shown. If a parent population contained <100 events, daughter populations were omitted in further analysis. Horizontal lines indicate the mean. Wilcoxon matched pairs test was used to calculate significance.

Phenotype of CD8+ T-cells in white matter lesions of MS patients implicate a local antigen-specific cytotoxic T-cell function

To determine the effector function and activation status of CD8+ T-cells *ex vivo* between different compartments of MS patients, expression of the death signal and co-stimulatory receptor FasL (CD95L) and the early activation marker CD137 were determined by multiplex flow cytometry on CD8+ T-cells (Figure 3A). Expression of both CD95L and CD137 is induced on antigen stimulated CD8+ T-cells and interaction of FasL with its receptor Fas may lead to target cell lysis.[29] Compared to PB, significantly more CD8+ T-cells in WML were positive for CD95L and CD137, which was predominantly observed in DWMA (Figure 3B).

In addition to the Fas/FasL-pathway, T-cell cytotoxicity involves exocytosis of granules containing perforin and granzymes.[35] Previous studies showed lowered[30] or absent[36] intrathecal expression of perforin and grB by CD8+ T-cells in non-inflammatory CNS conditions, suggesting a functionally repressed phenotype of CNS surveilling T-cells. Contrastingly, grB+CD8+ T_{EM} cells appeared enriched in CSF of MS patients.[37] To address this issue, consecutive sections of mAIL WML from four MS patients were stained for CD3, CD8 and grB (Figure 4A). Whereas the majority of CD8+ T-cells showed abundant whilst punctated grB expression, a fraction of CD8+ T-cells showed a more polarized grB expression pattern in both perivascular and parenchymal CD8+ T-cells (Figure 4A). GrB distribution and cytotoxic potential of CD8+ T-cells was confirmed using Z-stack confocal laser microscopy on 12 WML biopsies of 10 additional MS patients assayed by triple immunefluorescence staining for CD8, grB and the early apoptotic marker "cleaved caspase 3" (cCASP3). Occasionally adjacent cells expressed cCASP3 suggesting CD8+ T-cell mediated cytotoxity *in situ* (Figure 4B). Parenchymal CD8+ T-cells were most frequently identified in mAIL (data not shown). The frequency of grB-expressing CD8+ T-cells in the parenchyma tended to be higher compared to the paired perivascular regions in mAIL (Figure 4C). Collectively, the data indicate that CD8+ T-cells in WML of MS patients analyzed express a cytotoxic phenotype indicative for T-cells recognizing their cognate antigen *in situ*.[35]

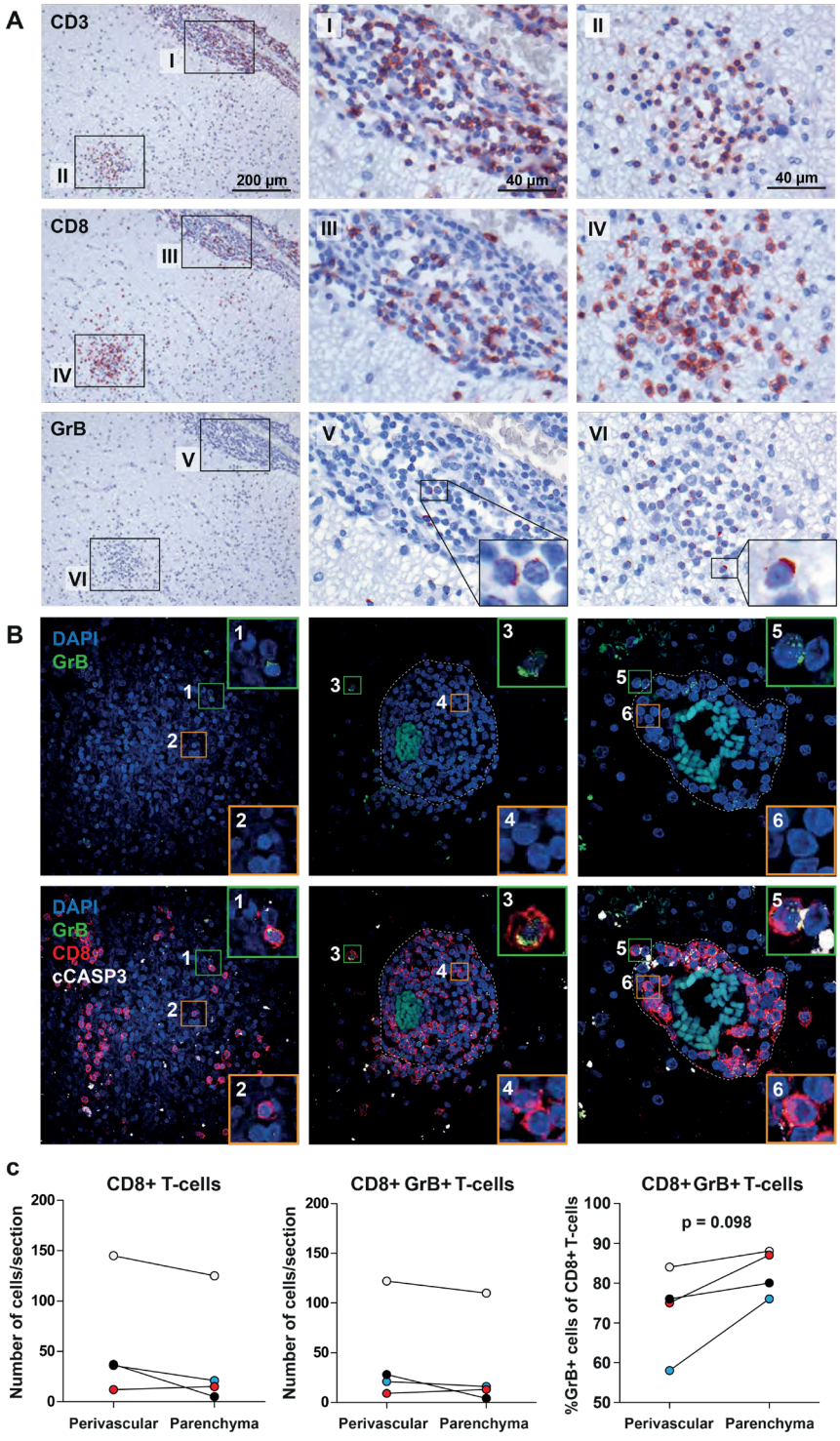
CD8+ T-cells in white matter lesions of MS patients are chronically activated T-cells expressing a cytotoxic effector T-cell phenotype

The repertoire of co-stimulatory and co-§inhibitory receptors determines the fate of T-cells after antigenic challenge.[29,38] Chronic antigenic stimulation leads to upregulation of co-inhibitory receptors that negatively affect their proliferative potential, effector function and survival.[29] In CNS, co-signaling via transmembrane immunoglobulin mucin 3 (TIM3) and programmed cell death type 1 (PD1) are important immunoregulatory mechanisms.[39] Hence, the frequency of CD8+ T-cells expressing TIM3 and PD1 *ex vivo* was compared between different compartments by multiplex flow cytometry (Figure 3C and 3D). Compared to PB, significantly more CD8+ T-cells in both CSF and MS lesions expressed TIM3 and PD1. In WML, this was largely attributed to CD8+ T-cells in DWMA. Chronic T-cell

stimulation may ultimately result in an apoptosis-resistant and senescent phenotype, characterized by human natural killer-1 receptor expression (CD57). This phenotype is potentially reverted by specific cytokines (e.g., IL-2 and IL-15) resulting in upregulated expression of the inducible co-stimulator (ICOS) molecule on T-cells.[38] No significant difference was observed in *ex vivo* CD57 expression by CD8+ T-cells between different compartments, arguing against senescent CD8+ T-cells in MS (Figure 3E and 3F). Frequencies of ICOS+CD8+ T-cells were significantly elevated in WML compared to PB and CSF, but were similar between NAWM and the different types of WML. Collectively, the data demonstrate that CD8+ T-cells in WML of MS patients exhibit characteristics of chronically activated T-cells.

To identify the brain cell types that interact with CD8+ T-cells, double immunofluorescence stainings on WML tissue sections of 12 FFPE tissues of 10 MS patients were performed (Supplementary Table 2). Hereto, anti-CD8 mAb was combined with antibodies directed to specific proteins expressed by human astrocytes (GFAP), microglia (Iba1), oligodendrocytes (PLP) and neurons (NF-H) (Figure 5). Perivascular CD8+ T-cells interacted preferentially with astrocytes and microglia. Parenchymal CD8+ T-cells interacted with astrocytes, microglia, oligodendrocytes in fully myelinated and partially demyelinated areas. Moreover, they interacted with neurons in areas with and without prominent axonal swelling, indicative of neuronal stress or axonal damage due to demyelination.[40] Hence, CD8+ T-cells in WML of the MS patients analyzed showed no preferential interaction with a specific brain cell type (Figure 5).

►**Figure 4. CD8+ T-cells in white matter lesions of MS patients express granzyme B.** (**A**) Representative stainings on 6 µm sections of a formalin-fixed and paraffin-embedded (FFPE) mixed active/inactive white matter lesion (mAIL) of 1 of 4 MS patients analyzed. CD3 (top panel), CD8 (middle panel) and granzyme B (grB) expressing cells (bottom panel) were stained with 3-amino-9-ethylcarbazole (red color) and counterstained with hematoxylin (blue color). Abundant punctate expression of grB was detected in perivascular (insets I, III and V) and parenchymal (insets II, IV and VI) CD8+ T-cells. Granzyme B polarization was observed in both perivascular and parenchymal CD8+ T-cells (insets V and VI). (**A**) Representative maximum intensity projections of z-stack laser confocal microscopy images of immunofluorescent triple stainings for grB (green color), CD8 (red color) and the early apoptotic cell marker "cleaved caspase-3" (cCASP3; white color). Stained sections were counterstained with DAPI (blue color). Representative stainings of three mAIL are shown of 12 immunohistochemically classified WML tissues of 10 MS patients analyzed. Punctated (inset 1) and polarized grB expression by CD8+ T-cells (insets 3 and 5), as well as grB-negative CD8+ T-cells are shown (insets 2, 4 and 6). Co-localization of grB and cCASP3 is observed in a cell adjacent to a CD8+ T-cell with polarized grB suggesting grB-mediated killing of the respective target cell (inset 5). Dotted line represents the glia limitans separating the perivascular space and parenchyma. (**C**) The grB-expressing CD8+ T-cells were counted in the perivascular space and parenchyma of mAIL of four MS patients analyzed. Wilcoxon matched pairs test was used to calculate significance.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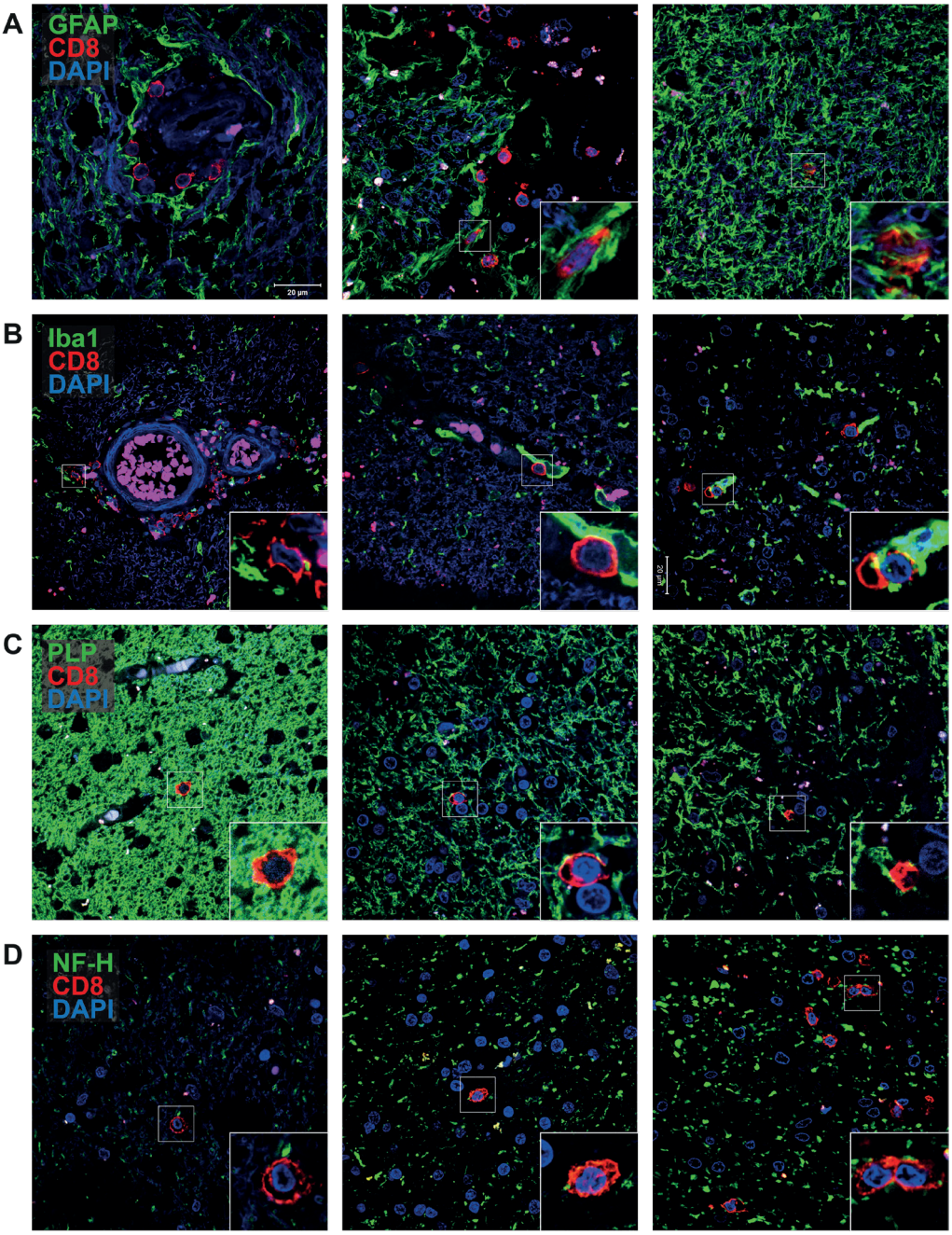
Perivascular
Parenchyma
CD8+ GrB+ T-cells
Number of cells/section
200
150
100
50
0
Perivascular
Parenchyma
CD8+GrB+ T-cells
p = 0.098
%GrB+ cells of CD8+ T-cells
100
90
80
70
60
50
Perivascular
Parenchyma


A
GFAP
CD8
DAPI
20 µm
B
Iba1
CD8
DAPI
C
PLP
CD8
DAPI
D
NF-H
CD8
DAPI

◄ **Figure 5. CD8+ T-cells interact with all major brain-resident cell types in white matter lesions of MS patients.** Representative double immunofluorescence stainings on 8 µm sections of 12 formalin-fixed paraffin-embedded white matter lesion (WML) tissues from 10 MS patients are shown for (**A**) glial fibrillary acidic protein (GFAP: marker for astrocytes), (**B**) ionized calcium-binding adapter molecule 1 (Iba1: marker for microglia), (**C**) proteolipid protein (PLP: marker for oligodendrocytes) and (**D**); neurofilament heavy chain (NF-H: marker for neurons; all green color) combined with CD8 (red color), counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue color) and finally analyzed using a Zeiss LSM-700 confocal laser microscopy and ZEN software. Perivascular CD8+ T-cells interact with astrocytes (**A**; left and middle panel) and microglia (**B**; left and middle panel). Parenchymal CD8+ T-cells also interact with astrocytes (**A**; right panel), microglia (**A**; right panel) and oligodendrocytes (**C**) in fully myelinated (left panel) and partially demyelinated areas (middle and right panels). Parenchymal CD8+ T-cells also interact with neurons (**D**) in areas without (left panel), with moderate (middle panel) and with prominent axonal swelling (right panel). The latter is indicative of axonal damage. Insets show specific interactions between CD8+ T-cells and the major brain-resident cells analyzed for. Scale bar is indicated (**A**, top left panel).

Correlation of TCR repertoire between TCL generated from distinct lesions of the same MS patient

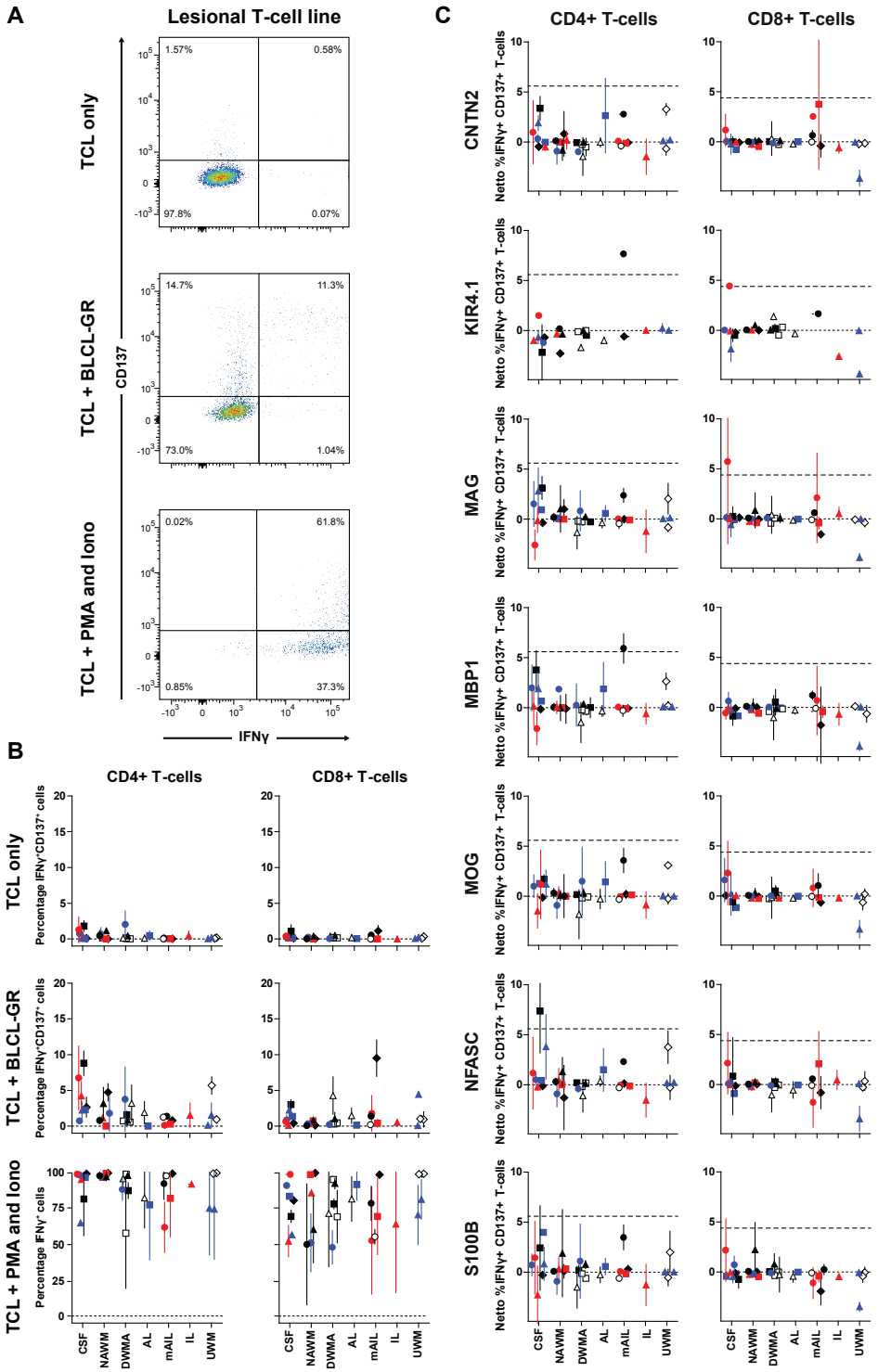
The markers expressed by CD8+ T-cells in WML suggest antigen-driven activation and potentially retention of specific TCC in affected tissue of MS patients (Figure 3 and 4). Given the low T-cell numbers in the clinical specimens obtained, T-cell clonality was assayed on short-term TCL generated by non-specific stimulation of T-cells recovered from paired CSF, NAWM and WML specimens. TCRVβ chain usage of CD4+ and CD8+ T-cells was determined by flow cytometry on paired NAWM- and WML-TCL (n=4 patients) and paired WML-TCL (n=6 patients) (Supplementary Figure 3). Most TCL showed oligoclonal TCRVβ repertoires, but no specific TCRVβ predominated among patients. Whereas the TCRVβ repertoire of paired NAWM- and WML-TCL did not match, a significant correlation was observed for CD4+ and particularly CD8+ T-cells in paired WML-TCL of 2/6 and 4/6 MS patients, respectively (Supplementary Figure 3). The shared TCRVβ chain usage implicates enrichment of specific TCC in distinct WML of the same MS patient. To address this issue in more detail, we assayed the composition of the TCR repertoire of CD8+ T-cells in paired NAWM/WML- (n=3) and WML/WML-TCL (n=5) by performing TCRγ-rearrangement spectratyping on sorted CD8+ T-cells (Supplementary Figure 4).[41] The TCRG locus is rearranged early during T-cell development in both TCR-αβ and -γδ lineage precursors and is considered the prototypic TCR locus for the detection of T-cell clonality in clinical specimens.[41] Typical polyclonal Gaussian curves signifying polyclonality, or one or two peaks illustrative for the rearrangement of the TCRG locus on one or both chromosomes, were detected when human PBMC or monoclonal T-cell leukemic cell lines (MOLT3 and KL 1985-001) were assayed, respectively (Supplementary Figure 4).[41] TCRG GeneScan analyses demonstrated a reproducible, markedly restricted pattern of TCRG rearrangements by CD8+ T-cells in the brain-derived TCL analyzed. These data confirm that the oligoclonal CD8+ T-cell populations in TCL generated from paired NAWM and WML of the same MS patient were clonally distinct (patients #2, #4 and #8; Supplementary Figure 4), whereas CD8+ T-cells in paired WML-TCL of multiple MS patients showed a strong clonal overlap (patients #5, #6, #7 and #9; Supplementary Figure 4). These data suggest that the paired WML sampled contained identical CD8+ TCC potentially involved in the disease process.

No substantial T-cell responses towards human candidate MS-associated autoantigens in T-cell lines generated from cerebrospinal fluid and white matter of MS patients

Previous studies, both on EAE and MS patients, support a key role of pathogenic T-cells directed to various cMSAg including glia- (KIR4.1, S100B), oligodendrocyte- (MAG, MBP, MOG) and neuron-specific proteins (CNTN2 and NFASC).[2,4,5] However, autoreactive T-cells are also a physiological component of the healthy immune system making disease association difficult.[2,4,5] Among the various genetic predictors of MS development, 3 specific HLA-I and -II alleles have been identified: HLA-A*0301, -DRB1*1501 and -DRB*1301.[27] Their association with MS may involve presentation of specific cMSAg-derived peptides that trigger pathogenic T-cell responses.[42]

To test this hypothesis, CD4+ and CD8+ T-cell reactivity towards these 7 human cMSAg was determined for TCL of MS patients (n=14) carrying the three MS-associated HLA risk alleles (Supplementary Table 1). Hereto, an allogeneic BLCL (BLCL-GR), matched for these three HLA alleles, was transduced and selected *in vitro* to express the individual cMSAg, and measles virus fusion protein as control T-cell antigen, constitutively at high level (data not shown).[28] Because no human cMSAg-specific T-cell clone or lines were available, we used MVF-transduced BLCL-GR as APC to validate the applicability of our expression system to process and present endogenously expressed antigens by both HLA-I and II. As shown previously, both the MVF-specific CD4+ (4-F99) and CD8+ TCC (2-F40) recognized MVF-transduced GR-BLCL efficiently (data not shown).[28] Next, human

►Figure 6. T-cell lines generated from paired cerebrospinal fluid and white matter brain tissue of MS patients show no substantial T-cell reactivity towards candidate human MS-associated autoantigens. Short-term T-cell lines (TCL) were generated by non-specific stimulation of T-cells recovered from paired cerebrospinal fluid (CSF) and white matter brain tissues from 14 MS patients, which were immunohistologically classified as normal-appearing white matter (NAWM), diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL), inactive lesions (IL) or undefined white matter tissue (UWM) (see Supplementary Figure 1 for criteria applied for MS WM classification). An HLA-matched Epstein-Barr virus transformed B-cell line (i.e., BLCL-GR) was used to assay T-cell reactivity towards candidate human MS-associated autoantigens (cMSAg). (**A**) Antigen-specific T-cells were enumerated by determining co-expression of intracellular interferon gamma (IFNγ) and CD137 using multiplex flow cytometry. Gated CD8+ T-cells from mAIL-derived TCL of MS patient #27 (see Supplementary Table 1) is representatively shown. CD8+ T-cells alone (top panel), stimulated with un-transduced BLCL-GR (middle panel) or with phorbolmyristate-acetate (PMA) and ionomycin (Iono) are shown. (**B**) The frequency of IFNγ and CD137 co-expressing CD4+ (left panel) and CD8+ T-cells (right panels) that were cultured alone (top panels) or co-cultured with untransduced BLCL-GR (middle panels) are shown. The bottom panel shows the frequency of IFNγ-expressing CD4+ (left panel) and CD8+ T-cells (right panel) after stimulation with a cocktail of T-cell mitogens (i.e., PMA and Iono). (**c**) BLCL-GR were nucleofected with human candidate MS autoantigens (cMSAg) expression vectors encoding human contactin-2 (CNTN2), inwards rectifying potassium channel (KIR4.1), myelin associated glycoprotein (MAG), myelin basic protein isoform 1 (MBP1), myelin oligodendrocyte glycoprotein (MOG), neurofascin (NFASC) or S100 calcium binding protein B (S100B). TCL were co-cultured with the respective cMSAg-expressing BLCL-GR and the phenotype and frequency of cMSAg-specific T-cells determined by flow cytometry. The netto frequency of cMSAg-specific T-cells, corrected for reactivity towards un-transduced BLCL-GR, is shown as the percentage IFNγ+CD137+ CD4+ (left panel) and CD8+ T-cells (right panel). Symbols represent the individual MS patients analyzed (n=14; specified at the bottom of the figure). The majority of TCL were assayed at least 2-times, of which vertical lines represent the mean and standard deviation. Horizontal dashed lines depict the cut-off for positive calls for CD4+ and CD8+ T-cells, allowing a 0.1% false discovery. Significance of variation in cMSAg-specifc T-cell reactivity was determined by ANOVA for CD4+ and CD8+ T-cells separately.


A
Lesional T-cell line
TCL only
TCL + BLCL-GR
TCL + PMA and Iono
CD137
IFNγ
1.57%
0.58%
97.8%
0.07%
14.7%
11.3%
73.0%
1.04%
0.02%
61.8%
0.85%
37.3%
B
CD4+ T-cells
CD8+ T-cells
Percentage IFNγ+CD137+ cells
C
CD4+ T-cells
CD8+ T-cells
CNTN2
KIR4.1
MAG
MBP1
MOG
NFASC
S100B
Netto %IFNγ+ CD137+ T-cells
CSF
NAWM
DWMA
AL
mAIL
IL
UWM
Patient #1○ ,#2● ,#3● ,#4● ,#5△ ,#7▲ ,#8▲ ,#17▲ ,#19□ ,#20■ ,#21■ ,#22■ ,#24◇ ,#27◆

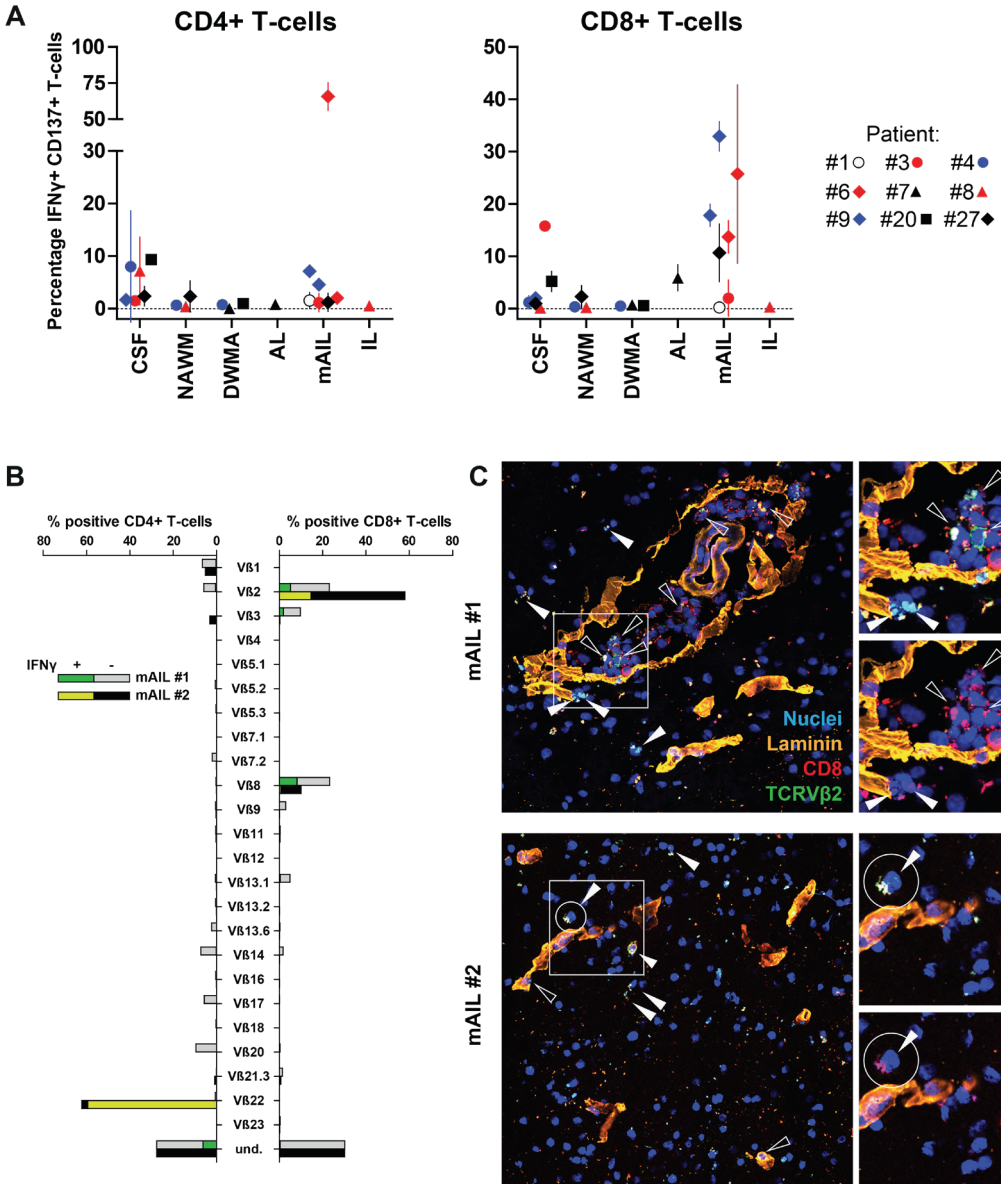
cMSAg-specific T-cell reactivity was determined by multiplex flow cytometry on CSF-, NAWM- and WML-TCL of HLA-matched MS patients using the cMSAg-transduced BLCL-GR as antigen presenting cells (Figure 6). Whereas TCL only and TCL co-cultured with untransduced BLCL-GR showed limited T-cell responses, TCL stimulated with a cocktail of the T-cell mitogens PMA and Iono showed a uniformly high expression of the T-cell activation marker IFNγ demonstrating that the T-cells in the TCL were immunecompetent and not exhausted (Figure 6A and B).

The BLCL-GR reactive CD4+ (mean ± SD: 2.1% ± 2.4) and CD8+ (1.3% ± 2.0) T-cell responses of the CSF- and brain tissue-derived TCL were low and the inter-assay variation was 1.69% for CD4+ T-cells and 1.44% for CD8+ T-cells in independent replicate experiments. Consequently, the threshold for positive calls was 5.6% for CD4+ T-cells and 4.4% for CD8+ T-cells allowing a one-tailed 0.1% false discovery rate.[28] Marginal cMSAg-specific T-cell responses were detected in only 4 of 207 (1.9%) cMSAg/TCL-combinations, including two CSF-TCL (Figure 6C). Overall, the limited cMSAg-specific T-cell reactivity in paired CSF-, NAWM- and WML-TCL argues against their involvement in the perpetuation of disease in the 14 MS patients analyzed.

T-cells recovered from white matter lesions of MS patients recognize autologous Epstein-Bar virus infected B-cells

Epstein-Bar virus infection is a major environmental risk factor to develop MS in genetically predisposed individuals.[16,27] Recent data argue for a role of EBV-specific T-cells in MS pathology,[20,43,44] but proof of their presence in brain tissues of MS patients is lacking. This prompted us to investigate T-cell responses towards autoBLCL in paired CSF-, NAWM- and

►Figure 7. White matter lesion-derived CD8+ T-cells recognize autologous Epstein-Barr virus transformed B-cells and localize in the parenchyma to form immune synapses. (**A**) short-term T-cell lines (TCL) were generated by non-specific stimulation of T-cell recovered from paired cerebrospinal fluid (CSF) and white matter brain tissues from 9 MS patients, which were immunohistologically classified defined as normal-appearing white matter (NAWM), diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL) and inactive lesions (IL) (see Supplementary Figure 1 for criteria applied for MS WM classification). The TCL were incubated with autologous Epstein-Barr virus transformed B-cell lines (autoBLCL). Next, the phenotype and frequency of autoBLCL-specific T-cells was determined by co-expression of intracellular interferon gamma (IFNγ) and CD137 using multiplex flow cytometry. The frequency of autoBLCL reactive T-cells is shown as the percentage of IFNγ+CD137+ CD4+ (left panel) and CD8+ T-cells (right panel). Symbols represent individual donors (n=9; specified in the legend) and vertical lines represent the mean and standard deviation of at least two independent experiments per TCL. Significance of variation in autoBLCL T-cell reactivity was determined by ANOVA for CD4+ and CD8+ T-cells separately. (**B**) TCL generated from two anatomically distinct mAIL of MS patient #6 (see Supplementary Table 1) were cultured with autoBLCL to assay the T-cell receptor variable β chain (TCRVβ) usage of the reactive T-cells, determined by intracellular IFNγ expression, using multiplex flow cytometry. The frequency of CD4+ (left x-axis) and CD8+ T-cells (right x-axis) of specific TCRVβ families (y-axis) are depicted (gray bars = lesion #1, black bars = lesion #2). The frequency of autoBLCL reactive CD4+ T-cells and CD8+ T-cells of each TCRVβ family is shown (stacked green bars = IFNγ+ T-cells). Results shown are representative for two independent experiments. "Und." refers to T-cells expressing a TCRVβ chain not covered by the TCRVβ-family specific monoclonal antibody panel used. (**C**) Triple immunofluorescence staining for TCRVβ2 (green color), laminin (orange color) and CD8 (red color) in surplus WML tissue sections (8 µm) containing WML #1 and #2, from which the corresponding TCLs shown in panel "**A**" were generated. Nuclei were stained with DAPI (blue color). TCRVβ2+ CD8+ T-cells reside in the perivascular cuff (open arrowhead) and the parenchyma (closed arrowhead) of both distinct WML of the same patient. The majority of parenchymal T-cells show polarization of both CD8 and TCRVβ2 (encircled cells in top-right insets). Images of representative stainings are shown.


A
CD4+ T-cells
CD8+ T-cells
Percentage IFNγ+ CD137+ T-cells
CSF
NAWM
DWMA
AL
mAIL
IL
Patient:
#1 #3 #4
#6 #7 #8
#9 #20 #27
B
% positive CD4+ T-cells
% positive CD8+ T-cells
IFNγ + -
mAIL #1
mAIL #2
Vß1
Vß2
Vß3
Vß4
Vß5.1
Vß5.2
Vß5.3
Vß7.1
Vß7.2
Vß8
Vß9
Vß11
Vß12
Vß13.1
Vß13.2
Vß13.6
Vß14
Vß16
Vß17
Vß18
Vß20
Vß21.3
Vß22
Vß23
und.
C
mAIL #1
mAIL #2
Nuclei
Laminin
CD8
TCRVβ2

WML-TCL. Concordant with earlier studies, about 5% of autoBLCL cells expressed the late lytic viral antigen glycoprotein 350, demonstrating that these cells have spontaneously entered the lytic cycle and most likely expressed the whole EBV proteome (Supplementary Figure 5). Accordingly, autoBLCL were considered appropriate APC to analyze EBV-specific T-cell reactivity.[20,45] Due to limited cell numbers and viability, autoBLCL could only be generated for 9 MS patients. In contrast to NAWM- and DWMA-TCL, substantial T-cell responses towards autoBLCL, predominantly CD8+ T-cells, were detected in multiple mAIL- and the sole AL-TCL analyzed. This response was most pronounced among mAIL-TCL: 5 of 7 mAIL-TCL assayed had brisk T-cell responses towards autoBLCL-specific (Figure 7A). Notably, these strong CD8+ T-cell responses correlated between paired TCL generated from distinct mAIL of the same patient: MS patients #6 and #9 (Figure 7A). The data implicate enrichment of autoBLCL reactive CD8+ T-cells in AL and mAIL of MS patients that are potentially involved in the disease process.[12]

These autoBLCL reactive T-cells are potentially enriched in brain tissue due to local stimulation by EBV proteins.[18] Hence, we determined the presence of the EBV transcript EBER1, which is highly expressed during both latent and lytic EBV infection,[46] in surplus brain tissue from which the respective NAWM- and WML-TCL were generated. No EBER1 RNA was detected implicating that productive EBV infection or the presence of latently EBV-infected B-cells in the brain tissues sampled is highly unlikely (data not shown).

Clonally expanded autologous BLCL reactive CD8+ T-cells are located in brain parenchyma and form immune synapses

The combined data on congruent TCR clonality, selective TCRVβ expression by CD8+ T-cell showing reactivity towards autoBLCL in paired intra-lesional TCL suggest local enrichment of the same CD8+ TCC. Among the high autoBLCL T-cell responders, sufficient T-cell quantities were only available for the mAIL-TCLs of MS patient #6 for follow-up studies. TCRVβ analysis revealed that TCRVβ2+ T-cells dominated the oligoclonal CD8+ T-cell response towards autoBLCL in the paired intra-lesional TCL of MS patient #6: mAIL-TCL#1 (32%) and #2 (89%) (Figure 7B). Furthermore, the autoBLCL reactive CD4+ T-cell response in mAIL-TCL#2 was restricted to TCRVβ22 (99%) expressing T-cells. Next, we localized TCRVβ2+CD8+ T-cells, as proxy for the dominant autoBLCL reactive CD8+ TCC, by IHC in the respective WML tissues. The TCRVβ2+CD8+ T-cells were detected in the Virchow-Robin space and parenchyma in both mAIL of patient #6 (Figure 7C). Notably, the majority of parenchymal TCRVβ2+CD8+ T-cells showed polarization of both TCR and CD8, indicative for immune synapse formation and confirmed by Z-stack analysis (Data not shown). Collectively, the data suggest the involvement of CD8+ T-cells recognizing antigens expressed by autoBLCL, and potentially the same TCC, in the inflammatory process in two distinct mAIL of the same MS patient.

Discussion

The current study provides novel insights into the phenotypic and functional characteristics of the T-cell response in CSF and (non-)affected brain tissue of MS patients. Three main findings are reported. First, T-cells in MS lesions are predominantly CD8+ T_{EM} cells expressing a cytotoxic effector phenotype indicative for local antigenic stimulation. Second, T-cells cultured from WML in 4 of 9 MS patients recognize autoBLCL. This reactivity was profound in TCL generated from AL and mAIL. Third, no substantial T-cell reactivity was observed towards 7 human cMSAg in CSF- and brain tissue-derived TCL of MS patients expressing the major MS-associated HLA risk alleles HLA-A*03, -DRB1*15 and -DRB1*13.

During the past decade, the focus on the role of CD4+ T-cells in MS pathology has shifted towards CD8+ T-cells; the most abundant T-cell subset identified in active WML of MS patients.[1,5] The antigen-specificity (e.g., autoantigens and/or EBV proteins) and the potential role of intralesional CD8+ T-cells (cytotoxic or regulatory) in MS pathology is still a matter of debate.[6,37,47,48] The infrastructure of the Netherlands Brain Bank and the commitment of MS patients to donate their tissues after death for research purposes, offered us the unique opportunity to compare the phenotype, function and reactivity of T-cells between paired PB, CSF, NAWM and WML of 27 MS patients. Notably, the study design enabled comparison of T-cells between compartments intra-individually, hereby limiting influence of inter-patient immune differences that are potentially unrelated to MS. A limitation of our study was, however, that all MS patients included had a long progressive disease course. Nevertheless, *in situ* characterization of the macroscopically-defined NAWM and WML tissues obtained clearly demonstrated the presence of lesions covering the complete spectrum of MS disease activity ranging from DWMA to IL.[26] Markedly, about half of the macroscopically defined NAWM contained DWMA that represent periplaque abnormalities, Wallerian degeneration or pre-lesional abnormalities.[26] The relative high frequency of these type of diffuse abnormalities outside of focal MS lesions in end-stage MS patients raises doubt if all DWMA will progress to active lesions, which necessitates identification of specific markers that identifies the origin and fate of DWMA in time.

Concordant with previous studies, T-cells were located in perivascular spaces of both NAWM and WML, whereas parenchymal T-cells were restricted to WML of MS patients.[6,49] The *ex vivo* distribution of CD8+ T_{NA}, T_{CM}, T_{EM} and T_{EMRA} cells in NAWM, WML and CSF of MS patients resembled data on WM and CSF under normal CNS conditions.[30,50] However, compared to paired PB, CD8+ T-cells in WML of the MS patients analyzed showed significantly increased expression of markers indicative for antigen-induced cytotoxicity (CD59L and grB) and activation (CD69, grB and CD137). The increased expression of the co-inhibitory (TIM3 and PD1) and co-stimulatory receptors (ICOS), whilst not accompanied with increased CD57 expression, suggests that the CD8+ T-cell underwent chronic activation *in situ* while retaining their proliferative potential. In comparison, CSF-derived CD8+ T-cells had increased expression of both co-inhibitory markers, which refutes active involvement in CNS pathology.[49] Contrary to normal CNS conditions,[30] CD8+ T-cells in MS lesions expressed grB, suggesting that they encountered their cognate antigen locally and are potentially cytotoxic T-cells.[35,36] Indeed, occasionally CD8+ T-cells with

polarized grB expression were localized adjacent to cells showing signs of early apoptosis. However, detailed *in situ* analysis could not demonstrate a specific brain-resident cell type preferentially targeted by CD8+ T-cells in the WML analyzed. Furthermore, the majority of WML-derived CD8+ T-cells expressed CD69, which in the absence of CD103 co-expression may reflect their activated state or indicate a specific subset of T_{RM} cells.[29,33] Overall, the activated and cytotoxic effector memory phenotype of CD8+ T-cells in WML, along with overlapping TCRVβ repertoires between paired WML-TCL of the same patient, implicate clonally expanded T-cells recognizing their cognate antigen locally. These CD8+ T-cells are potentially involvement in the deleterious CNS inflammation that contributes to MS pathogenesis.[35]

To decipher the cognate antigen driving T-cell activation in MS lesions, we analyzed T-cell reactivity in short-term TCL generated from different compartments of the same patient using autologous or alternatively an allogeneic HLA-matched BLCL stably transduced with 7 human cMSAg (i.e., CNTN2, KIR4.1, MAG, MBP1, MOG, NFASC and S100B).[20,28] The expression of 3 well-described MS-associated HLA-I and -II risk alleles (i.e., HLA-A*0301, -DRB1*1501 and -DRB*1301)[27] by the allogeneic BLCL-GR facilitated simultaneous detection of CD4+ and CD8+ T-cell responses in TCL of HLA-matched MS patients towards endogenously synthesized and processed human cMSAg.[28] Notably, no substantial cMSAg-specific T-cell reactivity was detected in TCL generated from CSF, NAWM and WML of 14 MS patients. The data concur with recent studies on CSF-TCL of patients with early MS describing negligible intrathecal cMSAg-specific T-cell reactivity,[28,51] but are in contrast to other studies showing brisk systemic and occasional local T-cell reactivity towards distinct cMSAg-specific synthetic peptides in MS patients.[2,4,52–54] These discrepancies may be methodological or related to the timing of tissue sampled during the course of disease. Most studies assayed cMSAg-specific T-cell reactivity using autologous PBMC as APC pulsed with high concentrations synthetic peptides (10-250 μM) or recombinant (animal) cMSAg. However, disease-irrelevant T-cells expressing low-avidity TCR are potentially activated by these super-physiological peptide concentrations.[55] Also, due to species differences, amino acid composition and conformation of the synthetic peptides used may differ from natural T-cell epitopes.[56] The BLCL/cMSAg platform used in this study offered a more physiological approach to detect cMSAg-specific CD4+ and CD8+ T-cell responses as compared to more conventional APC platforms used previously.[28] Our inability to detect cMSAg-specific T-cell reactivity may be due to the inclusion of specimens from patients with advanced MS disease in which the disease-initiating cMSAg-specific T-cell response that potentially sparked of disease is not actively involved in perpetuating MS pathology. Alternatively, cMSAg-derived peptides were presented by other HLA alleles not shared with the allogenic BLCL-GR. Nevertheless, the inability to detect intra-lesional cMSAg-specific T-cell responses in end-stage MS patients, as well as in CSF of patients with clinical isolated syndrome and early MS,[28] does not support the long-held hypothesis that the cMSAg assayed here are prominent targets of local pathogenic T-cell responses in MS patients.

Contrastingly, brisk T-cell reactivity (mainly CD8+ T-cells) was detected towards autoBLCL, preferentially among TCL generated from AL and mAIL, but not DWMA and IL, suggesting their involvement in lesion activity. On occasion, these strong CD8+ T-cell responses were identified in TCL generated from anatomically separated WML of the same MS patient, suggesting involvement of the same TCC in both lesions. Indeed, even though observed in a single MS patient and not confirmed at the TCR level, the autoBLCL reactive CD8+ T-cell response in the paired mAIL-TCL of this particular MS patient was dominated by TCRVβ2+ CD8+ T-cells. These T-cells were detected in the parenchyma of the respective WML tissues of the same patient with polarized TCR and CD8 expression suggesting immunological synapse formation *in situ*. Recently, we and others have shown intrathecal CD8+ T-cell reactivity to EBV antigens in patients with early MS.[20,57,58] Because no EBV transcripts could be detected in the corresponding WML assayed here, the autoBLCL-specific CD8+ T-cell responses identified are most likely not involved to combat intracerebral EBV infection as suggested by others.[18,44] The cognate antigen could be an EBV protein, an autoantigen induced or selectively processed in EBV-infected B-cells or an EBV/cMSAg-cross-reactive autoantigen.[24,43,59–62] A limitation of our APC platform, using BLCL as APC, is that it withholds clear distinction between these options. Unfortunately, autologous activated B-cells were not available to determine if the T-cell reactivity towards autoBLCL truly targeted EBV antigens. Furthermore, due to limited numbers of T-cells available from the autoBLCL reactive TCL, in-depth cognate EBV antigen discovery as described previously was also not possible.[20]

In conclusion, the data presented suggest that cytotoxic CD8+ T_{EM} cells directed to autoBLCL, but not to the seven human cMSAg assayed in combination with three major MS-associated HLA risk alleles, are potentially involved in the immunopathology of white matter lesions of the MS patients analyzed. Follow-up studies on WML-derived T-cells are warranted to identify the cognate virus and/or host antigen recognized by this potential deleterious autoBLCL reactive T-cell response in MS patients.

Acknowledgements

We kindly acknowledge Tamara Wabeke, Karim L. Kreft, Sarah Getu and Monique van Velzen (all Erasmus MC) for their technical assistance and Jaap M. Middeldorp (VU medical center, Amsterdam, the Netherlands) for providing the gp350-specific mAb. This research was in part financially supported by the Dutch MS Research Foundation (grant ID 09-670 MS) and the Niedersachsen-Research Network on Neuroinfectiology (N-RENNT) of the Ministry of Science and Culture of Lower Saxony (Germany).

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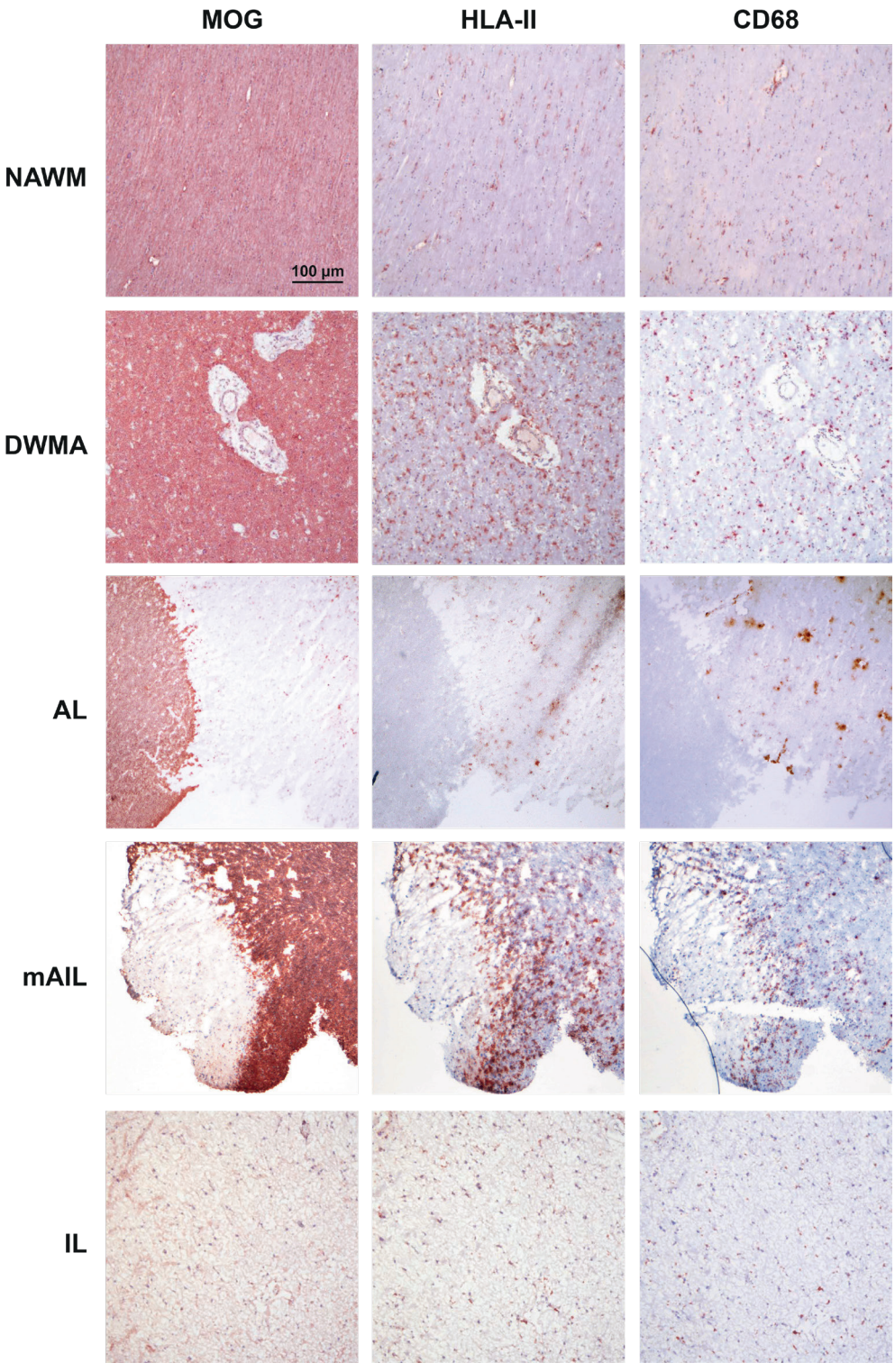
Supporting information

► **Supplementary Figure 1. Examples of distinct white matter phenotypes of MS patients.** Representative examples of MS white matter stainings according to Kuhlmann et al., Acta Neuropathol (2017).[26] We used myelin oligodendrocyte glycoprotein (MOG), HLA class II (HLA-II) and CD68, expressed by macrophages and microglia, as markers to discriminate normal appearing white matter (NAWM: HLA-II+/–Myelin+CD68+/-), diffuse white matter abnormalities (DWMA: HLA-II++Myelin+CD68+), active lesions (AL: HLA-II++Myelin+/–CD68+), mixed active/inactive lesions (mAIL; hypercellular rim: HLA-II++Myelin+/–CD68++ and hypocellular center: HLA-II+/–Myelin–CD68+/–) and inactive lesions (IL: hypocellular: HLA-II+/– Myelin–CD68+/-) in consecutive 8 µm sections of snap-frozen MS brain tissues. Scale bar is indicated in top left image.


MOG
HLA-II
CD68
NAWM
DWMA
AL
mAIL
IL
100 µm

Supplementary Table 1. General characteristics of multiple sclerosis patients and white matter brain tissues obtained for *ex vivo* and *in vitro* T-cell analyses.

Patient ID	Clinical course[a]	Gender[b]	Age (yrs)	Disease duration (yrs)[c]	EDSS[d]	Cause of death	CSF (pH)	PMI (hrs)[e]	IHC of viable tissues[f]		HLA class I[g]			HLA class II[g]	
									NAWM	Lesion(s)	A	B	C	DRB1	DQB1
1	SPMS	F	64	31	≥8	Urosepsis	6.20	10:10	NAWM	mAIL	*02, *03	*07, *51	*02, *07	*11, *15	*03, *06
2	PPMS	F	88	25	6-7	Exhaustion by chronic colitis ulcerosis	6.85	7:55	NAWM	mAIL	*01, *02	*07, *38	*07, *12	*15, *15	*06, *06
3	SPMS	M	45	10	≥8	Pulmonary embolisms and cardiac arrest	6.22	7:45	n.a.	mAIL	*01, *01	*07, *08	*07, *07	*03, *15	*02, *06
4	SPMS	M	76	44	≥8	Cerebrovascular incident	6.42	7:35	NAWM	DWMA	*01, *26	*27, *37	*01, *06	*10, *16	*05, *05
5	RRMS	F	59	24	7-8	Euthanasia	7.08	4:45	DWMA	AL	*11, *24	*35, *52	*03, *12	*01, *13	*05, *06
6	SPMS	F	68	37	≥8	Euthanasia	6.40	10:20	mAIL	mAIL	*02, *68	*14, *57	*06, *08	*13, *15	*03, *06
7	PPMS	M	83	21	n.d.	Pneumonia and lung cancer	6.40	7:50	DWMA	mAIL	*02, *29	*40, *45	*03, *06	*12, *13	*03, *06
8	SPMS	F	74	25	≥8	Cardio-respiratory insufficiency	6:43	10:15	NAWM	IL	*03, *03	*07, *44	*07, *16	*07, *15	*02, *06
9	PPMS	M	54	12	7-8	Euthanasia	6.39	8:15	n.a.	2xmAIL	*11, *29	*35, *44	*04, *16	*01, *15	*02, *06
10	PPMS	F	66	23	≥8	Euthanasia	6.45	9:35	NAWM	mAIL	n.d.	n.d.	n.d.	n.d.	n.d.
11	SPMS	F	56	32	≥8	Pneumonia	6.16	8:25	n.a.	AL	n.d.	n.d.	n.d.	n.d.	n.d.
12	RRMS	M	56	13	7-8	Suicide	7.10	10:10	DWMA	DWMA	n.d.	n.d.	n.d.	n.d.	n.d.
13	SPMS	M	73	43	≥8	Urosepsis	6.40	8:45	NAWM	IL	n.d.	n.d.	n.d.	n.d.	n.d.
14	PPMS	F	54	31	≥8	Heart Failure	6.27	9:20	DWMA	IL	n.d.	n.d.	n.d.	n.d.	n.d.
15	SPMS	M	54	25	≥8	Euthanasia	6.26	10:50	DWMA	mAIL	n.d.	n.d.	n.d.	n.d.	n.d.
16	SPMS	F	95	54	≥8	Infection, cachexia and dehydration	6.40	10:00	NAWM	AL	n.d.	n.d.	n.d.	n.d.	n.d.
17	n.d.	M	71	15	6.5	Metastasized pulmonary carcinoma	6.38	8:45	n.a.	n.d	*01, *02	*07, *07	*07, *07	n.d.	n.d.
18	SPMS	M	59	21	7	Euthanasia	6.50	10:45	DWMA	mAIL	n.d.	n.d.	n.d.	n.d.	n.d.
19	SPMS	F	53	16	7-8	Euthanasia	6.81	7:15	DWMA	DWMA	*02, *03	*07, *35	*04, *07	*01, *15	*05, *06
20	RRMS	F	87	18	≥8	Dehydration and renal insufficiency	5.92	9:30	NAWM	DWMA	*02, *02	*15, *44	*03, *07	*15, *15	*06, *06
21	SPMS	F	48	22	≥8	Respiratory failure	6.10	11:50	NAWM	mAIL	*01, *11	*15, *52	*03, *12	*04, *11	*03, *03
22	PPMS	M	66	25	≥8	Euthanasia	7.28	10:55	mAIL	AL	*02, *03	*07, *15	*03, *07	*15, *15	*06, *06
23	PPMS	M	57	25	6.5	Sepsis	6.80	10:15	NAWM	mAIL	n.d.	n.d.	n.d.	n.d.	n.d.
24	SPMS	F	56	21	6.5	Suicide	6.42	14:00	n.d.	n.d.	*24, *30	*18, *40	*03, *05	n.d.	n.d.
25	PPMS	F	51	15	≥8	Euthanasia	6.60	9:45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	n.d.	F	35	10	≥8	Euthanasia	6.37	10:20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	SPMS	F	74	50	>7.5	Euthanasia	6.40	7:50	NAWM	mAIL	n.d.	n.d.	n.d.	n.d.	n.d.

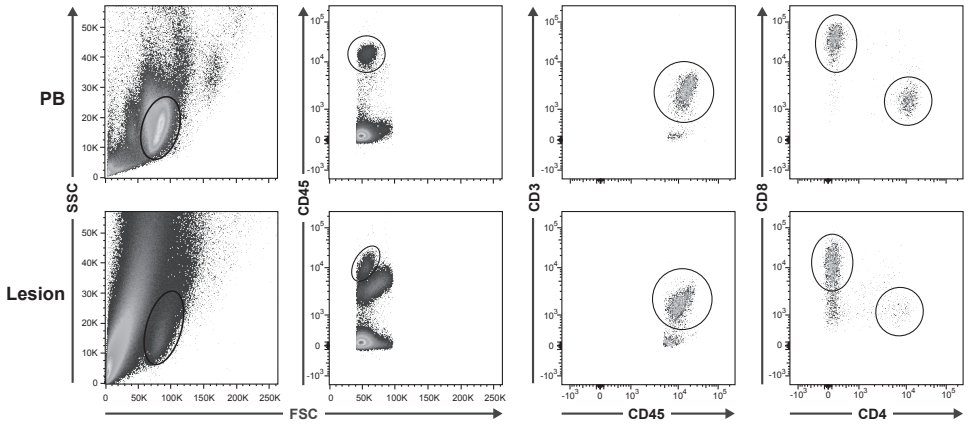
▲

[a]RR, relapsing remitting MS; PP, primary progressive MS; SP, secondary progressive MS; Und., undefined disease course. [b]Gender: Male or Female, [c]Duration disease since clinical diagnosis of MS. [d]Expanded disability status scale. [e]Post-mortem interval: time between death and the end of autopsy. [f]Macroscopically characterized normal appearing white matter (NAWM) and MS lesion tissues obtained were subjected to immunohistochemistry (IHC) to classify WM as: NAWM, diffuse white matter abnormalities (DWMA), active lesion (AL), mixed active/inactive lesion (mAIL) and inactive lesion (IL) (see reference 29). n.a., not available; n.d., not determined. [g]HLA genotypes overlapping with BLCL-GR (HLA-A*01,*03, -B*07,*27, -C*02,*07, -DRB1*13,*15, -DQB1*06,*06) are underlined.

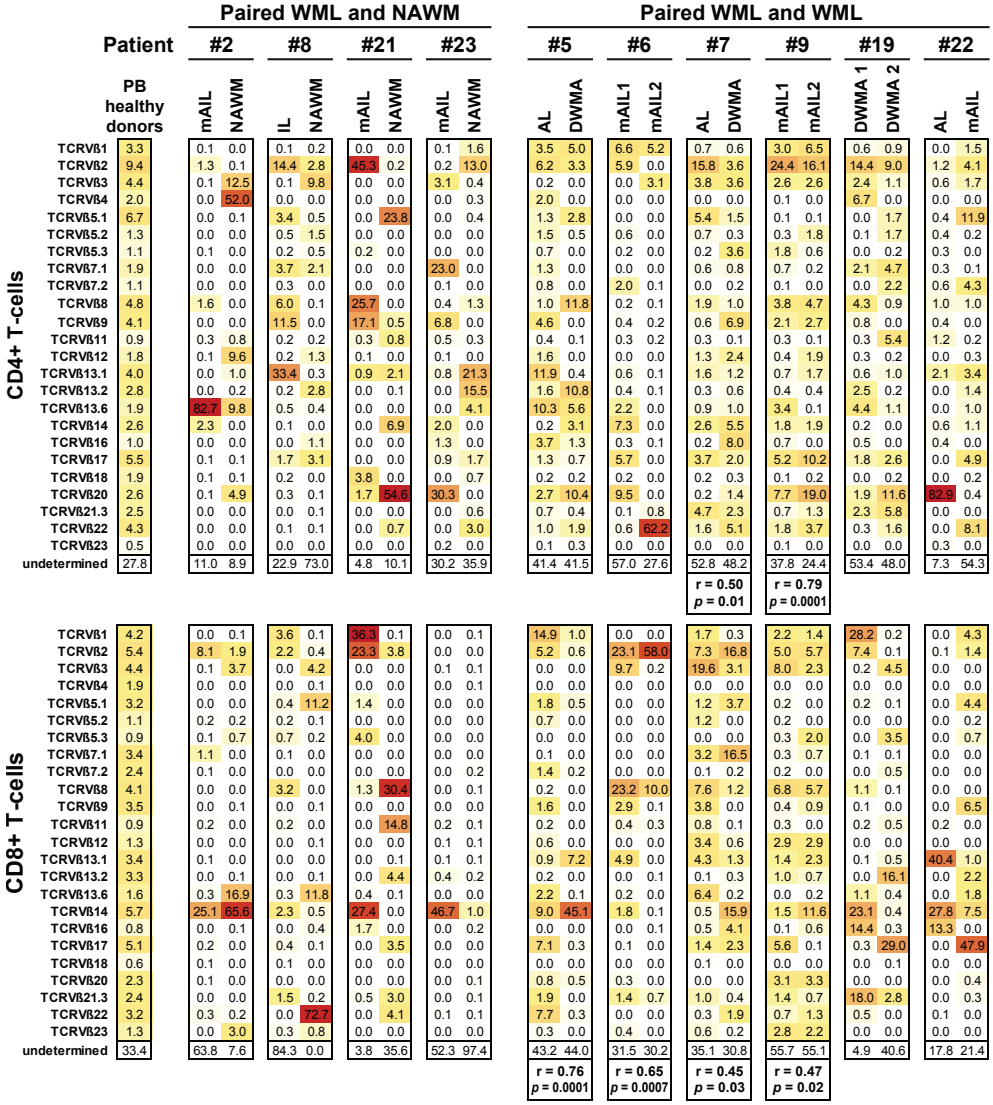
Supplementary Table 2. General characteristics of multiple sclerosis patients and white matter brain tissues obtained for *in situ* T-cell analyses.

Patient ID[a]	Clinical course[b]	Gender[c]	Age (yrs)	Disease duration (yrs)[d]	EDSS[e]	Cause of death	CSF (pH)[f]	PMI (hrs)[g]	IHC of FFPE tissue(s)[h]
S99/128	PPMS	M	74	40	≥8	Resiratory insufficiency	6.91	8:30	IL
S00/274	SPMS	M	64	34	≥8	End stage MS	6.84	7:30	AL
S02/052	SPMS	F	75	34	7-8	Pneumonia	6.40	8:00	IL
S06/054	PPMS	F	44	6	≥8	Decompensation	6.34	10:15	IL
S06/139	SPMS	M	56	21	≥8	Pneumonia	6.65	8:00	mAIL
S07/216	SPMS	M	71	26	≥8	Pneumonia	6.60	7:00	mAIL
S09/251	SPMS	M	75	39	6.5	Euthanasia	6.53	7:45	mAIL
S10/020 (#6)	SPMS	F	68	37	≥8	Euthanasia	6.40	10:20	DWMA, AL & mAIL
S11/080 (#11)	SPMS	F	56	32	≥8	Pneumonia	6.16	8:25	mAIL
S12/008	SPMS	F	66	17	≥8	Pulmonary hypertension	6.73	10:45	AL

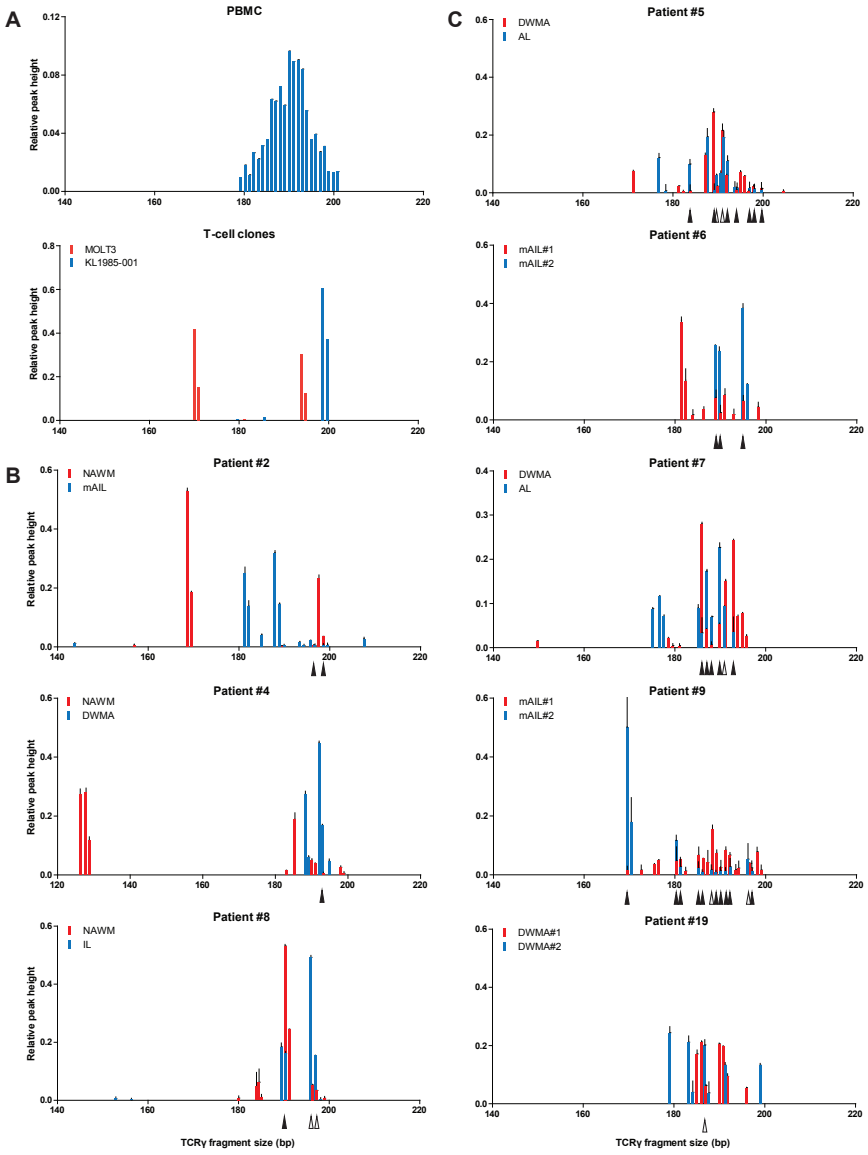
[a]Patient number as assigned by the Netherlands Brain Bank (#6 and #11 refer to patient ID in Online Resource 1). [b]RR, relapsing remitting MS; PP, primary progressive MS; SP, secondary progressive MS; Und., undefined disease course. [c]Gender: Male or Female. [d]Duration disease since clinical diagnosis of MS. [e]Expanded disability status scale. [f]CSF, cerebrospinal fluid. [g]Post-mortem interval: time between death and the end of autopsy. [h]Formallin-fixed and paraffin-embedded (FFPE) white matter lesion tissues were characterized by immunohistochemistry (IHC) to classify the sampled WML as: diffuse white matter abnormality (DWMA), active lesion (AL), mixed active/inactive lesion (IAL) and inactive lesion (IL) (see reference 29).



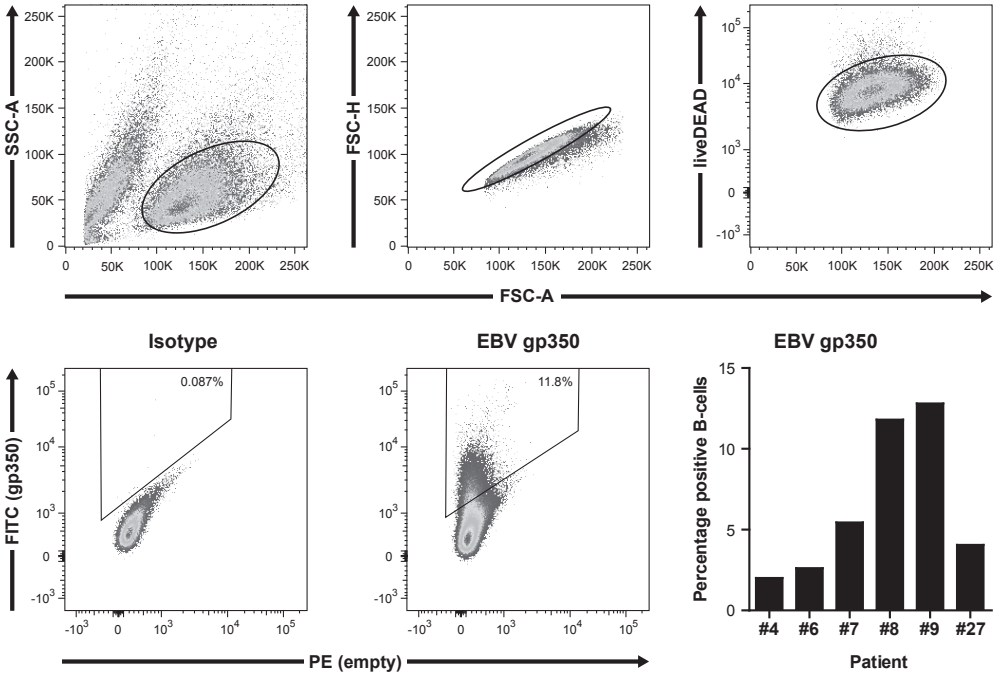
Supplementry Figure 2. Gating procedure of paired PB- and white matter lesion-derived lymphocytes from MS patients. Lymphocytes were isolated from paired peripheral blood (PB), cerebrospinal fluid (CSF), normal-appearing white matter (NAWM) and white matter lesions (lesion) from patients with advanced MS (n=17) and subjected to multiplex flow cytometry. Pseudocolored density plots of paired PB (top panels) and white matter lesion (bottom panels) samples from one representative MS patient are shown. Lymphocytes were identified based on low forward, intermediate sideward scatter (FSC and SSC, respectively) and high CD45 expression, which was followed by subgating for CD3 and either high CD4 or high CD8 expression.



Supplementary Figure 3. Correlation in T-cell receptor variable beta chain protein expression of CD4+ and CD8+ T-cells expanded from anatomically distinct white matter lesions of the same MS patient. Short-term T-cell lines (TCL) were generated by non-specific stimulation of T-cells recovered from paired white matter brain tissues that were histologically classified as normal-appearing white matter (NAWM) and white matter lesios (WML) classes; diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL) and inactive lesions (IL) (see Online Resource 2 for criteria applied for MS WM classification). The T-cell receptor variable beta chain (TCRVβ) protein expression profile was determined for 10 MS patients by flow cytometry using monoclonal antibodies (mAb) directed to 24 distinct human TCRVβ chains for CD4+ (upper rows) and CD8+ T-cells (lower rows) of the indicated TCL for each MS patient (column pairs). Light to dark color grading indicate the lowest to highest frequencies of specific TCRVβ expressed by CD4+ and CD8+ T-cells in each TCL. The mean TCRVβ chain expression of PB-derived CD4+ and CD8+ T-cells from 85 unrelated healthy individuals are provided as reference (data provided by Beckman Coulter). "Undetermined" refers to T-cells expressing a TCRVβ chain not covered by the TCRVβ-specific monoclonal antibody panel used. Spearman's rank correlation test were used for statistical analysis.



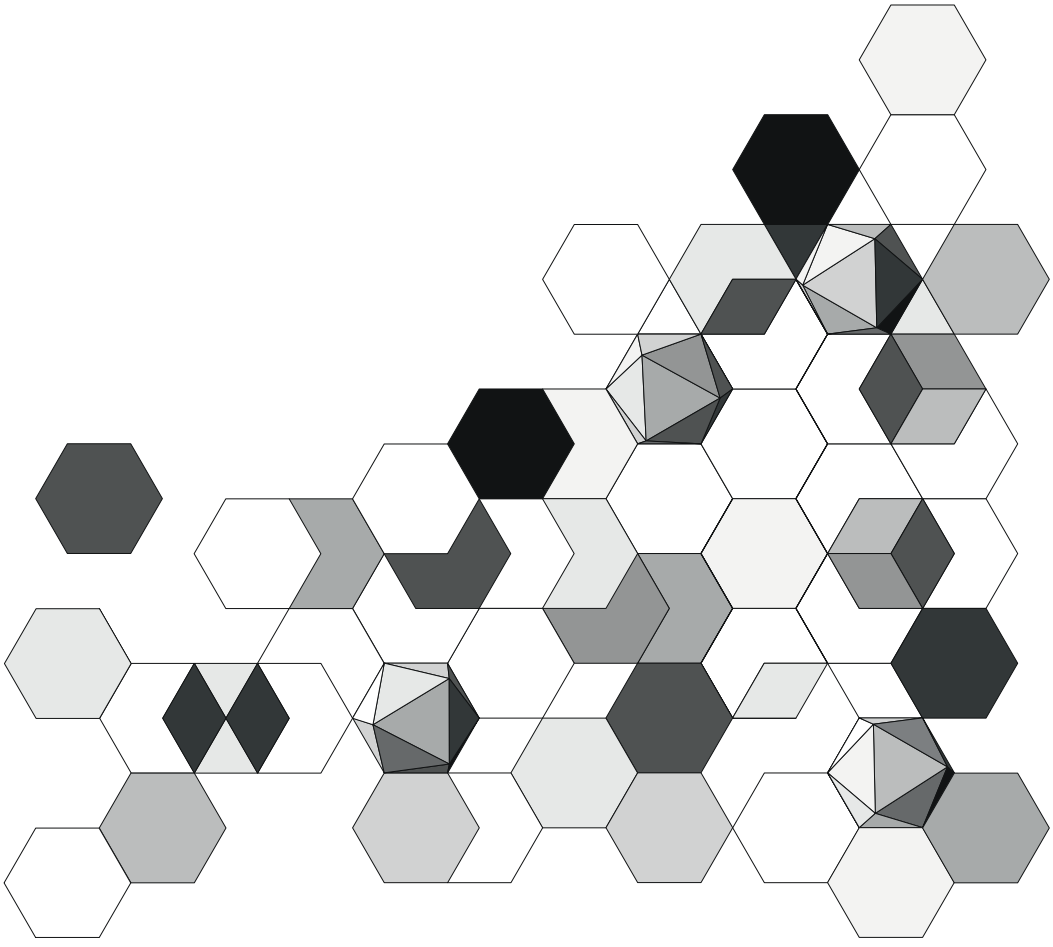
Supplementary Figure 4. Increased clonal overlap in paired white matter lesions compaired to normal appearing white matter derived CD8+ T-cells in MS patients. The T-cell receptor gamma chain (TCRγ) rearrangement spectra was determined by multiplex PCR and fragment length analysis on DNA isolated from (**A**) peripheral blood mononuclear cells (PBMC, top panel) and two T-cell clones (bottom panel) with one (blue bars; KL1985-001) or two rearranged alleles (red bars; MOLT-3). Both TCC display additional +1 base pair (bp) peaks due to the addition of a 3'-adenosine residue by the non-proof reading polymerase used. TCRγ rearrangement spectra of DNA isolated from 10.000-200.000 sorted viable CD8+ T-cells of short-term T-cell lines generated from (**B**) histologically classified paired normal appearing white matter (NAWM; red bars), diffuse white matter abnormalities (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL) and inactive lesions (IL) (blue bars) of 3 MS patients and (**C**) paired white matter lesions (blue and red bars) of 5 MS patients are shown. The avarage size and peak height (bars) and peak height range (vertical line) of two independent PCRs are shown. Overlapping fragments are indicated by black (0-0.25 bp length difference) and white arrow heads (0.25-0.5 bp length difference).



Supplementary Figure 5. Low frequency of cultured latently Epstein-Barr virus infected human B-cell lines express the viral glycoprotein 350. Long-term cultured EBV-infected B-cell lines (BLCL) were stained using fluorescent viability marker and a monoclonal antibody (moAb) specific for EBV glycoprotein 350 (gp350), or as control an IgG1 isotype moAb, and staining visualized using a FITC-conjugated rabbit-anti- mouse IgG secondary antibody. EBV-specific glycoprotein 350 is expressed on the cell surface of EBV-infected B-cells undergoing productive virus infection, but not on latently EBV-infected B-cells. Single viable B-cells were gated based on sideward scatter (SSC-A) and forward scatter area (FSC-A) and height (FSC-H), intermediate liveDEAD staining. Gating of donor #8 is shown representative for 6 donors analysed.

Chapter 8

Summary and general discussion



Summary

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system leading to motoric, cognitive, sensory deficits and fatigue. The etiology of MS is unknown. Both a genetic predisposition and infection with the herpesvirus family member Epstein-Barr virus (EBV) are risk factors for the development of MS.

Innate and adaptive immune cells, mainly T-cells, are involved in the inflammatory process in white matter lesions (WML) of MS patients yet the phenotype and function are poorly defined. In Chapter 7 we determined the phenotype and function of T-cells in WML, normal appearing white matter (NAWM), cerebrospinal fluid (CSF) and blood of deceased end-stage MS patients. In NAWM and WML T-cells were located in the perivascular space. However, additional T-cells in the brain parenchyma were selectively detected in WML, suggesting these are involved in lesion development. NAWM- and WML-derived T-cells were mainly effector memory CD8+ T-cells. T-cells in WML expressed markers of chronic antigenic stimulation and cytotoxicity. Furthermore, CD8+ T-cells cultured from WML and NAMW by mitogenic stimulation were clonally enriched. The repertoire of CD8+ T-cells corresponded between anatomically distinct WML of the same patient but not between paired NAWM and WML. This suggests T-cells from distinct WML recognize a shared antigen, and are likely enriched due to antigen-specific activation and proliferation. These results indicate an important role of CD8+ T-cells in WML of MS patients.

The cognate Ag driving activation and proliferation of T-cells in WML is unknown. Several oligodendrocyte-, glia- and neuron-specific antigens are suggested to drive WML T-cells. In **Chapter 2** we developed and validated a T-cell/antigen presenting cell (APC) platform to readout T-cell reactivity towards a panel of endogenously expressed candidate MS-associated autoantigens (cMSAg). In **Chapter 2** we used this platform to readout cMSAg-reactivity of T-cells cultured from CSF of early MS patients and in **Chapter 7** of T-cells cultured from CSF, NAWM and WML of deceased end-stage MS patients. No substantial reactivity towards cMSAg was detected in MS patients. These data do not support the role of T-cells specific for these cMSAg in MS pathogenesis.

MS is thought to result from intrathecal virus infection with a yet unidentified causal infectious agent. Human herpes viruses (HHV), are likely candidates to cause refractory disease episodes or induce chronic intrathecal inflammation in MS patients because of their endemic nature, their ability to establish lifelong latency with intermittent reactivation and their ability to modulate immune responses. Because HHV are highly cell-associated, we determined the prevalence of all known HHV in CSF-derived cells of early MS patients and controls with other neurological diseases (OND) in **Chapter 3**. No HHV DNA was detected in CSF-derived cells of MS patients. These data do not support intrathecal HHV infection early in disease development.

The presence of intrathecal antibodies may indicate local infection in absence of detectable viral DNA. In **Chapter 4** we determined if the intrathecal EBV-specific humoral response in MS patients is indicative of local infection. The repertoire of CSF-derived EBV-specific IgG targets was comparable between MS patients and controls. EBV IgG responses were strongest towards the latency-associated Epstein-Barr nuclear antigen 1 (EBNA-1). Amino acids 394 to 451 of EBNA-1 (EBNA-1394-451) contained the immunodominant EBNA-1-specific epitopes. EBNA-1394-451 IgG levels were increased in CSF of MS patients

compared to controls. However, correcting EBNA-1394-451 IgG for total CSF IgG or albumin levels abolished this difference. These data indicate increased EBNA-1394-451 levels in MS patients are not the result of intrathecal synthesis, but rather of leakage from serum due to blood-brain barrier dysfunction.

The risk of developing MS is partly genetic. Currently 230 different, mostly immune related single nucleotide polymorphisms are associated with MS risk (MS-SNP). Potentially, MS-SNP are associated with EBV-specific humoral immune responses. In **Chapter 5** we determined the association of 78 MS-SNP with the EBV-specific latency-associated EBNA-1 IgG and the lytic cycle-associated EA-D IgG levels. Additionally, VZV IgG levels were determined as a non-MS-associated highly prevalent control herpesvirus. The dominant MS-associated genetic risk factor HLA-DRB1*1501 associated with EBNA-1 and EA-D IgG levels, but not VZV IgG levels. Furthermore, MS-SNP and combinations of MS-SNP associated with B-cell migration (VCAM-1), inhibition of apoptosis (PRDX5/BAD, MYB/NHI1 and CARD11) and HLA class II antigen presentation (CLEC16A and CIITA) correlated with EBNA-1 IgG levels. Notably, regulation of these genes or their ligands is highly affected by EBV infection, which strengthens these associations. These data indicate the genetic background for MS in part determines EBV-specific humoral responses.

In absence of detectable EBV DNA and intrathecal EBV-specific IgG synthesis, intrathecal EBV-specific T-cells may indicate local EBV infection. Alternatively, T-cells specific for EBV-infected B-cells (BLCL) may be involved in MS pathogenesis. BLCL-specific T-cells may recognize EBV antigens, EBV-/host-cross-reactive antigens or autoantigens that are expressed by EBV-infected B-cells (BLCL) like αB-crystallin. In **Chapter 6** we determined the frequency of T-cells specific for BLCL and their fine-specificity in short-term T-cell lines (TCL) cultured from the CSF of early MS patients and OND. The frequency of BLCL-specific T-cells, especially CD8+ T-cells, was increased in MS patients. Furthermore, the frequencies of BLCL-specific CD4+ and CD8+ T-cells correlated, suggesting a coordinated response. CD8+ T-cells in CSF of MS patients selectively recognized lytic EBV antigens but not latent EBV antigens or αB-crystallin.

To determine if BLCL-specific T-cells are involved in MS pathogenesis, we determined their frequency in TCL cultured from post mortem collected paired CSF, NAWM and WML of end-stage MS patients in **Chapter 7**. Contrary to NAWM and inactive WML, TCL from active and mixed active/inactive WML showed brisk CD8+ T-cell reactivity towards autologous BLCL. In two anatomically separated mixed active/inactive WML of one MS patient the BLCL-specific CD8+ T-cell response was oligoclonal. In both lesions, T-cell receptor variable β-chain 2 (TCRVβ2) expressing CD8+ T-cells dominated the response. TCRV2+CD8+ T-cells were localized in the perivascular space and brain parenchyma of both WML. Furthermore, these TCRVβ2+CD8+ T-cells showed polarized expression of the T-cell receptor and CD8α co-receptor indicating the formation of an immunological synapse. However, no EBV transcripts were detected in the corresponding tissues. Although not confirmed at the clonal level, these results indicate BLCL-specific CD8+ T-cells recognize an autoantigen in WML. This may be an autoantigen induced by EBV in BLCL, an EBV/self cross-reactive antigen due to molecular mimicry or an autoantigen that is targeted due to epitope spreading. Together, these results show a prominent role for chronically activated BLCL-specific memory CD8+ T-cells in active WML of MS patients.

General discussion

Multiple sclerosis (MS) is widely classified as an autoimmune disease, but there is currently no conclusive evidence for this.[1] A unifying cognate autoantigen that causes immunopathology is unknown and therefore neither the original,[2] nor the revised Witebsky's criteria to classify MS as an autoimmune disease are met.[3] Moreover, it is currently unknown if MS is a neurodegenerative disease with a secondary (auto-) immune effect or and (auto-) immune disease with secondary neurodegeneration.[1,4] Defining whether MS has an autoimmune component and dissecting the temporal relation between immunopathology and neurodegeneration is key to our understanding of MS. This would ultimately pave the way for improved and more specific therapeutic intervention, like immune tolerization. Potentially, MS is the result of a spectrum of different (autoimmune) diseases, where different neurological deficits or immune targets drive clinically distinct disease forms. The large variation in the clinical presentation and disease progression between different patients,[1] as well as in the pathological features of MS lesions[5,6] supports this view.

Currently the only known common feature of MS patients is infection with Epstein-Barr virus (EBV), which is considered a prerequisite for developing MS.[7] All MS patients are latently EBV-infected and virus-specific immune responses are altered in MS patients compared to healthy EBV carriers, in advance of clinical disease.[8,9] Potentially, EBV drives immunopathogenesis in MS by activating autoreactive or EBV/autoantigen cross-reactive lymphocytes. Depending on the mechanism, therapies that enhance EBV immune responses might therefore be beneficial or detrimental for MS patients.

In this thesis, we aimed to clarify the putative role of T-cells in MS pathogenesis by analyzing their phenotype and function in cerebrospinal fluid (CSF) of early MS patients and in white matter lesions (WML) of end-stage MS patients and controls. We further set out to clarify the putative role of EBV in MS pathogenesis by analyzing the breadth and magnitude of humoral and cellular EBV-specific immune responses in MS patients and (disease) controls.

Phenotype and function of T-cells in white matter lesions of MS patients

Based on genetic associations, histopathology and the efficacy of current treatments targeting specific lymphocyte subsets a key role of B- and T-cells in the immunopathology of MS is anticipated.[6,10–13] Infiltrating lymphocytes in white matter lesions (WML) are mostly T-cells, with lower frequencies of B-cells.[13,14] Supported by early pathology studies of MS lesions[15] and experimental autoimmune encephalomyelitis (EAE) animal models for MS, specific CD4+ T-helper subsets are considered key regulators of CNS inflammation.[16] In EAE, activation of Th17 and Th17.1 subsets in the central nervous system results in expression of interferon gamma (IFNγ), interleukin 17 (IL-17), IL-22 and granulocyte monocyte colony stimulating factor (GM-CSF). This leads to activation of endothelial cells of the vessel wall, increased permeability of the blood-brain barrier (BBB) and bystander activation of astrocytes. Subsequently, activated astrocytes express CCL2 and CXCL12 whereby

additional lymphocytes are recruited.[17,18] However, contrary to earlier reports on MS[15] and EAE,[19] the frequency of CD4+ T-cells in human WML is low and CD8+ T-cells predominate.[20] Clonal expansion of T-cells in WML is most prominent among CD8+ T-cells,[21–23] which hints at antigen-specific proliferation of CD8+ T-cells in WML of MS patients. However, the phenotype and functions of these intralesional CD8+ T-cells are poorly defined.

In **Chapter 7** we analyzed the spatial orientation and phenotype of T-cells in *post mortem* collected paired normal appearing white matter (NAWM) and WML biopsies and compared these T-cells to peripheral blood (PB), cerebral spinal fluid (CSF) T-cells of the same patient.[24] A limitation of this study was that all included patients had a long progressive disease course, hereafter classified as end-stage MS. Nevertheless, by characterizing the NAWM and WML tissues by immunohistochemistry (IHC), we demonstrated WML covered the complete spectrum of MS disease activity, including diffuse white matter abnormalities in absence of demyelination (DWMA), active lesions (AL), mixed active/inactive lesions (mAIL) and inactive lesions (IL).[6] By IHC we showed that T-cells in NAWM are located in the perivascular Virchow-Robin space, confined by the glia limitans. Notably, WML contain parenchymal T-cells suggesting these T-cells are not part of surveilling lymphocytes, but are rather involved in the local inflammatory process.[25,26]

Brain tissues were dispersed to single cell suspensions and T-cells were phenotypically characterized by *ex vivo* flow cytometry. We confirmed the predominance of CD8+ T-cells in NAWM and WML. The proportion of CD27+CD45RA+ naïve (T_{NA}), CD27+CD45RA- central memory (T_{CM}), CD27-CD45RA- effector memory (T_{EM}) and CD27-CD45RA+ terminally differentiated (T_{EMRA}) CD8+ T-cells was approximately evenly distributed in PB of end-stage MS patients. In all CNS samples, however, negligible frequencies of CD8+ T_{NA} cells were detected. While CSF-derived CD8+ T-cells were mostly T_{CM}, NAWM- and WML-derived CD8+ T-cells preferentially expressed a T_{EM} or T_{EMRA} phenotype.[26,27] To determine if these differentiated intralesional CD8+ T-cells are long-lived tissue-resident memory T-cells (T_{RM}), we performed IHC on WML tissues of MS patients to assay expression of the T_{RM}-associated cell surface markers CD69 and CD103 on CD8+ T-cells.[28] Perivascular and parenchymal CD8 T-cells were mostly CD69+CD103- T-cells. Because CD69 is also expressed within a few hours upon T-cell receptor (TCR) mediated stimulation, or cytokine and chemokine activation[27], and CD103 is debated as T_{RM}-specific marker,[29] these CD69+CD103-CD8+ T-cells may be denote genuine T_{RM}, activated T-cells or a mixture thereof.[27–30]

Compared to PB, increased frequencies of CD8+ T-cells in MS lesions expressed the co-stimulatory molecules ICOS, CD137 and CD95L, indicative of co-stimulation, Ag-specific stimulation and cytotoxic effector function respectively.[27] Also the frequency of the co-inhibitory molecules TIM3 and PD1 was increased on CD8+ T-cells in WML compared to PB, suggesting both functional inhibition and chronic stimulation.[27,31] Expression of co-stimulatory and –inhibitory molecules on CD8+ T-cells was most prominent in DWMA tissues, which may represent periplaque abnormalities, Wallerian degeneration or pre-lesional changes.[6] Contrastingly, CD57 expression was not increased arguing against senescence of intralesional CD8+ T-cells.[31] The majority of CD8+ T-cells in WML expressed the cytolytic molecule granzyme B (grB).[32] The frequency of grB-expressing cells tended to

be higher among parenchymal compared to perivascular CD8+ T-cells. While the majority of these T-cells showed a punctated and distributed expression of grB, a minority showed polarized grB expression. Occasionally the adjacent cells expressed the early apoptotic marker cleaved caspase-3, suggesting active CD8+ T-cell mediated cytolysis.[32]

To define the brain cell type targeted by CNS-infiltrating CD8+ T-cells in MS patients, we performed double-IHC stains using monoclonal antibodies (mAbs) directed to CD8 and the neuron-specific neurofilament heavy chain (NF-H), microglia-specific ionized calcium-binding adaptor molecule (Iba1), oligodendrocyte-specific proteolipid protein (PLP) and astrocyte-specific glial fibrillary acidic protein (GFAP). Close interactions of perivascular CD8+ T-cells with astrocytes and microglia were observed. Parenchymal CD8+ T-cells interacted with astrocytes and oligodendrocytes in fully myelinated and partially demyelinated areas and with neurons in areas with and without prominent axonal swelling, which indicates neuronal stress or damage.[4] However, no preferential interaction between CD8+ T-cells and a specific brain cell type was observed.

Using a panel of T-cell receptor variable ß-chain (TCRVß)-specific mAbs, combined with multiplex PCR-based TCR gamma-chain spectratyping, we confirmed that brain-derived T-cells are clonally enriched and showed there is increased sharing of TCRVß usage in paired WML compared to NAWM, especially among CD8+ T-cells. This supports the model that T-cells specific for an antigen that is shared between different lesions contribute to immune pathology.[21–23] Collectively these data demonstrate that CD8+ T-cells in WML of end-stage MS patients are activated memory cytotoxic T-cells that recognize their target antigen locally and are potentially actively involved in MS pathogenesis.

Putative targets of auto-reactive T-cells in MS patients

The notion that lesion-infiltrating pathogenic T-cells are autoreactive is mainly based on the striking similarity in pathology between MS and the various EAE mouse, rat and monkey models.[19,33–35] EAE is induced using various CNS-specific proteins or peptides and results in an encephalogenic T-cell response that induces brain and spinal cord inflammation and demyelination. Moreover, adoptive transfer of autoreactive T-cells is sufficient to induce MS-like disease in naïve animals.[19,36] Depending on the antigen used to immunize animals, distinct forms of neuropathology can be induced with variable clinical effect. The different forms of clinical disease and variation in disease progression suggest that intra-cerebral immune responses to various antigens may cause different MS entities.[37–41]

Studies on EAE models have provided novel insight into intra-cerebral inflammatory processes, lymphocyte migration and testing of potential therapeutic agents, best exemplified by the therapeutically highly efficacious VLA-4 mAb.[12,42,43] However, despite the pathological similarity between EAE and MS, care should be taken in extrapolating findings to human disease. Especially, in relation to MS-associated etiological factors because *i)* the non-physiological nature of inducing autoimmune responses with peptides in adjuvant, *ii)* interspecies immune differences, *iii)* the mostly monophasic disease in mouse and rat EAE compared to the often relapsing remitting nature of human disease and *iv)* interspecies differences in the virome and microbiome.

EAE-inducing autoantigens are nevertheless considered candidate MS-associated antigens (cMSAg). These cMSAg are mostly oligodendrocyte myelin proteins including myelin-associated glycoprotein (MAG), myelin basic protein isoform 1 (MBP1), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP).[33–35,44] Also various glia-specific proteins, including inwards rectifying potassium channel 4.1 (KIR4.1) and S100 calcium binding protein B (S100B) and neuron-specific proteins including contactin-2 (CNTN2) and the 155kDa isoform of neurofascin (NFASC) are considered cMSAg.[33–35,44] Many studies have focused on analyzing T-cell responses against these targets in MS patients and controls, but with conflicting outcome.[33–35] Some observed increased frequencies of peripheral cMSAg-specific T-cells, but others described similar frequencies in HLA-matched controls, questioning their relevance.[44,45] As the HLA haplotype endows dominant MS-associated risk, and even protective alleles,[10,46] a strict HLA-matching of patients and controls is mandatory when assaying autoreactive T-cells.

T-cells specific for cMSAg are mostly analyzed using synthetic peptides or recombinant (animal) cMSAg using autologous peripheral blood mononuclear cells (PBMC) as antigen presenting cells (APC).[33,45] This type of study design poses several limitations. The poor correlation in T-cell phenotype between blood and CSF refutes extrapolation of systemic T-cell data to the CNS.[47] These recombinant, synthetic or even xenotypic antigens may have different amino acid compositions, post-translational modifications and structural conformations compared to naturally expressed human antigens. For instance, the immunogenicity of MBP largely depends on its citrullination profile, which may not be physiological.[48–51] These differences may affect the antigen processing and presentation of cMSAg-derived peptides.[52] Also, CD8+ T-cells preferentially recognize intra-cellular synthesized and processed antigens or alternatively exogenous antigens cross-presented by professional APC like dendritic cells (DC). This is only partially overcome by using protein-spanning synthetic peptides, as the derived epitopes may not be presented in similar form when endogenously expressed.[52] Furthermore, typically super-physiological peptide or protein concentrations (10-250 μM) are used to pulse APC. This may lead to activation of disease-irrelevant T-cells expressing low-avidity T-cell receptors.[53]

In **Chapter 2**, we developed a novel *in vitro* APC/T-cell platform to simultaneously assay antigen-specific CD4+ and CD8+ T-cell responses against intracellular synthesized human antigens.[54] This platform enables stable expression of individual antigens in EBV-transformed B-cells (BLCL) to be used as APC in functional T-cell assays. A limitation of this APC/T-cell platform is that the background EBV-specific T-cell response hampers detection of low frequency T-cell responses. However, the magnitude of specific T-cell responses usually exceeds these frequencies in compartmentalized autoimmune diseases like chronic Chagas' cardiomyopathy[55] and auto immune thyroiditis.[56,57] Another limitation is that the use of BLCL refutes analysis of potential EBV/cMSAg-cross-reactive peptides. Even though antigen processing and presentation of antigens may differ between BLCL and the yet unidentified local APC in MS lesions, we consider this a reasonable compromise to detect cMSAg-specific T-cells.

In **Chapter 2 and 7** we used the APC/T-cell platform to determine the frequency of T-cells specific for a panel of cMSAg in (1) CSF from patients with clinical isolated syndrome (CIS) and early MS, and (2) paired CSF, NAWM and WML of end-stage MS patients.[24,54] The cMSAg analyzed were: oligodendrocyte-specific antigens (MAG, MBP isoform 1 (MBP1), MOG and PLP), glia-specific proteins (KIR4.1 and S100B), and neuron-specific proteins (CNTN2 and NFASC). Ideally, the specificity of T-cells is determined *ex vivo*. However, limited T-cell numbers in CSF samples and white matter tissues precluded direct analysis of isolated T-cells. Therefore, short-term T-cell lines (TCL) were generated by a single round of non-specific stimulation of CSF-derived cells of CIS and early MS patients, and two rounds of non-specific stimulation for paired CSF-, NAWM- and WML-derived cells of end-stage MS patients. No substantial T-cell reactivity was detected against any of the assayed cMSAg in CSF-TCL of early MS patients and in paired CSF-, NAMW- and WML-TCL of end-stage MS patients. Few studies have quantified cMSAg-specific T-cell responses in CSF or brain tissues using endogenously processed antigens.[44,45,58] In line with our study, Wuest and colleagues also did not observe significant reactivity of CSF-derived T-cells towards cMSAg using human cell-, myelin and brain-derived lysates and autologous DCs as APC.[58] However, these data are in contrast to the only other study that determined cMSAg-specific T-cell reactivity in human WML.[59] Here, post-mortem tissue samples were collected from 16-year-old female with aggressive RRMS. Contrary to our study cohort, CD4+ T-cells were predominant in 2 of 3 WML assayed, but not the paired NAWM tissue. T-cell reactivity in the WML- and NAWM-TCLs was determined using HLA class II matched allogeneic PBMC pulsed with synthetic peptides spanning parts of the proteins cyclic nucleotide phosphodiesterase, myelin-associated oligodendrocyte basic protein, MOG, MBP and PLP.[59] CD4+ T-cells from WML showed reactivity towards MOG aa11-30 (MOG^{11-30}), MOG^{21-40}, $PLP^{190-209}$ and MBP^{13-32}. Notably, T-cell reactivity towards the same peptides and additionally towards PLP^{89-106} was observed in the paired NAWM-TCL, indicating that this T-cell reactivity is not specific for WML. These discrepancies may be methodological or related to the timing of tissue sampling during the disease course. In conclusion, our data do not support that T-cells specific for the assayed cMSAg are involved in MS pathology, and refute these cMSAg as prominent target antigen in both early and end-stage MS patients.

Prevalence of intrathecal human herpesviruses in MS patients

Besides a genetic predisposition, the exposure to environmental factors comprises crucial risk factors for the development of MS. The CNS inflammation in absence of detectable microbes (bacteria, fungi, protozoans, etcetera) in MS hints at an initiating role of virusses.[60–62] Bearing in mind the endemic nature of human herpes viruses (HHVs), their ability to establish lifelong latency, intermittent reactivation of latent virus and their ability to modulate immune responses, these viruses are considered likely candidates to cause refractory disease episodes or induce chronic intrathecal inflammation in MS patients. The role of the prime MS-associated HHV, EBV (HHV4), is widely debated.[63–66] Ideally, the presence of HHV is assayed at the site of disease, during onset of inflammation,

early in disease development. As the access to brain tissue is very limited early in disease development, studies on the prevalence of HHV in MS patient are restricted to CSF samples collected early in disease or brain biopsies collected post-mortem after prolonged disease. In **Chapter 3** we report on a comprehensive study of the prevalence of all eight known HHVs in CSF of CIS patients,[1,67] and patients with relapsing remitting (RR-) and primary progressive (PP-)MS and non-inflammatory neurological disease (NIND) patients as disease controls.[68]

HHV are typically highly cell-associated and may be misdiagnosed in cell-poor CSF samples.[69,70] Also the interval between disease onset or disease exacerbation and time of sampling may affect HHV detection.[69] In our study, we focused on cell-rich CSF samples that were collected within 2 weeks after onset of clinical symptoms. No genomic DNA of HHV1-8 was detected; implicating that intrathecal HHV infection is not involved in the early phase of MS disease. However, considerable discrepancies on the prevalence of intrathecal HHV in MS patients are reported. While disease association with VZV,[71–73] EBV,[72,74–79] and HHV6 were reported,[73,75,80–86] others could not verify these findings,[61,72,80,83,87–90] including our group (**Chapters 3, 6 and 7**)[24,68,91] and collaborators.[92] These discordances may relate to: *i)* the types of clinical specimen analyzed. HHV prevalence in CSF was either analyzed in unfractioned CSF samples,[75,80,82,84] or CSF samples that were split into cell-free supernatant[71–73,83,85,86,89–91] and cell pellets.[83,85,87,88,93] Presence of EBV-specific nucleic acids and protein were analyzed on either preselected WML tissues, which included the juxtaposition meninges that contained abundant B-cell infiltrates,[74,76,79] or extensive collections of randomly selected WML tissue specimens that partly included the associated inflamed meninges.[24,92,94] *ii)* the clinical characteristics of the included MS patients. Most studies included patients with various MS entities, some studies focused on CIS[81,82,85], while others selectively included RRMS patients.[71,88–90,93] For studies on CSF, the mean disease duration varied greatly between studies (between 0 and 128 months, where specified), as did the timeframe between disease onset or exacerbation and sampling (within 72 hours to 14 days, if specified at all). The presence of EBV in MS brain tissues was selectively analyzed on *post mortem* tissues from MS patients with end-stage disease. *iii)* the sensitivity and specificity of the applied methods. While most studies aimed to detect HHV genomic DNA by conventional PCR,[93] quantitative PCR[71,72,81,83,84,87,88] or nested PCR[73,75,80,82,85,86,89,90], others selectively screened for EBV transcripts by reversed-transcriptase PCR (RT-PCR)[24,79], *in situ* RNA hybridization (ISH)[76,77,92,94,95], or EBV protein-specific IHC.[76,77,79,92,94,95]

Despite detailed assessment of all aforementioned variations in clinical specimens, patient characteristics and detection methods, no consensus was obtained that explains these discrepancies in CSF and brain tissue samples.[68] To discuss the discordancy on EBV prevalence CNS tissue of MS patients, two focused expert meetings were organized: March 2010 (at the VUMC; Amsterdam, the Netherlands) and second one in June 2010 (at the center for brain research; medical university of Vienna, Austria).[63] Reagents, techniques and clinical specimens were exchanged between participating groups to identify potential technical caveats. Consecutive sections of suspect EBV-positive brain

tissues were stained by different labs, again with conflicting results.[63] These experiments excluded that differences in the selected patients, post-mortem autolysis times, fixation and handling of tissue blocks were culprit for the conflicting results. Instead the sensitivity and specificity of the applied methods to detect EBV RNA and protein are the likely cause of the conflicting results.[63,95] Using well-validated EBV-specific ISH, RT-PCR and quantitative RT-PCR protocols, EBV could be readily detected with high specificity and sensitivity in EBV-induced glioma and oral hairy leukoplakia tissues,[92] EBV-spiked PBMC[24] and in a CSF cell pellet of an herpes simplex type 2 encephalitis patient[68]. Thus, our data do not support a role of intrathecal and -cerebral HHV infection in patients with early and end-stage MS.[24,68,91]

Intrathecal EBV-specific humoral responses in MS patients

A hallmark for MS is the presence of intrathecally synthesized oligoclonal IgG bands, which correlate with high risk of MS and WML load.[1,67,96] These are clonally related to peripheral B-cell clones and are generally directed against ubiquitous intracellular autoantigens that are not CNS-specific.[97,98] Intrathecal IgG in MS patients are therefore considered to be mainly produced as a secondary response to chronic CNS inflammation and tissue damage.[98] Nonetheless, fractions of intrathecally produced IgG may be involved in MS pathogenesis. The peripheral and intrathecal humoral response against the latency-associated Epstein-Barr virus nuclear antigen 1 (EBNA-1) is elevated in MS patients compared to healthy EBV carriers and correlates with disease prognosis and activity.[7,9,65,99–101] Contrastingly, the humoral response towards lytic EBV proteins, particularly the viral capsid antigen (VCA), is not or only marginally increased in MS patients.[7,99] This suggests that increased EBV-specific humoral responses in MS patients are mainly directed to latent EBV proteins, although one may argue that viral proteins that are located in the nucleus of EBV-infected cells like EBNA-1 are only accessible to antibodies after cell death. EBNA-1 may therefore represent a secondary immune target. The presence of EBV in CSF, perivascular regions and meninges of MS patients is unclear.[63] Chronic local EBV infection may result in intrathecal IgG production specific for viral antigens. Intrathecal EBV-specific IgG production may therefore indicate intrathecal EBV infection. Alternatively, if EBNA-1 IgG are involved in immune pathogenesis, local synthesis of EBNA-1 IgG is anticipated. Detection of EBNA-1 IgG cross-reactivity with citrullinated collagen and keratin in rheumatoid arthritis patients[102] and several nuclear host antigens in systemic lupus erythematosus patients,[103] has fueled this hypothesis.

In **Chapter 3** we determined the breadth of EBV antigens recognized by intrathecal IgG by immunoblot using cell lysates of lytically EBV-infected B-cells.[104] A normal diversity pattern of IgG reactivity was observed towards proteins that (co-) migrated with the EBV proteins EBNA-1 (BKRF1), VCA-p18 (BFRF3), VCA-p40 (BDRF1) and Zebra (BZLF1) and several undefined proteins, with immunodominance of EBNA-1 in both MS and NIND patients. Subsequently, the EBNA-1 B-cell epitopes were defined in serum of healthy EBV carriers using 12-meric synthetic peptides spanning the entire EBNA-1 protein. Immunodominant regions in EBNA-1 ranged from amino acid 395 to 451 (EBNA-1^{395-}

451) and the glycine-alanine repeat EBNA-1$^{90-325}$. However, numerous proteins contain a glycine-alanine repeat,[105,106] implicating that EBNA-1$^{90-325}$ IgG reactivity may not be EBNA-1 specific. Based on the predefined immunoreactive regions in EBNA-1, longer synthetic peptides - predicted to have secondary structures - were designed using *in silico* minimal energy calculations. Using these longer peptides, EBNA-1 IgG reactivity was determined in paired serum and CSF samples of both MS and NIND patients. Whereas the overall IgG reactivity profile was similar in both patient groups, reactivity towards EBNA-1$^{394-420}$, EBNA-1$^{424-451}$ and to a lesser extent EBNA-1$^{436-461}$ were selectively increased in both serum and CSF of MS patients. Based on the 12-mer and longer synthetic peptides we showed that EBNA-1$^{394-451}$ contains the immunodominant epitopes of EBNA-1 specific IgG.

The increased intrathecal EBNA-1$^{394-451}$ IgG levels in MS patients may have been synthesized by local plasma cells or merely reflect leakage of serum IgG into the CSF due to BBB dysfunction. Normalization of EBNA-1$^{394-451}$ IgG for the total IgG levels in the respective CSF samples annulled the difference between MS and NIND patients. Furthermore, the strong correlations between intrathecal EBNA-1$^{394-451}$ IgG and total IgG and albumin levels in CSF suggests the increased local EBV response is not due to intrathecal synthesis, but rather due to blood-brain barrier (BBB) dysfunction. While others detected intrathecally synthesized EBV-specific IgG in a subset of MS patients, these antibodies represent low-affinity IgG. These are likely not involved in MS pathogenesis because affinity maturation of disease relevant antibodies expressed by chronically stimulated B-cells would result in high-affinity IgG.[107] Whether high-affinity EBNA-1 IgG are involved in MS pathogenesis remains to be determined. Potentially, the increased intrathecal EBNA-1 IgG levels are because they affect BBB integrity directly. Recent insights in fluid dynamics of the CSF that include paraventricular flow (glymphatics)[108] and drainage to cervical lymph nodes (CNS lymphatics)[109,110] imply that soluble inflammatory mediators like IgG which are produced distant from the brain ventricles, like in WML, cannot be sampled by lumbar puncture.[111,112] This suggests IgG measured in CSF, which is collected via lumbar puncture, mainly reflects disease processes near ventricles such as the meninges. The lack of intrathecal EBV-specific IgG synthesis therefore negates the role of a widespread intrathecal or meningeal EBV infection in MS patients and is congruent with our studies being unable to demonstrate EBV nucleic acids in CSF samples of patients with early and end-stage disease.[24,68,91,92]

Involvement of MS-associated genetic factors in EBV-specific humoral immune responses

The risk of developing MS is partially dictated by genetic predisposition.[1,113] The majority of MS-associated genetic variants are single nucleotide polymorphisms (MS-SNP), which are located in enhancer regions of multiple immune related genes.[10,11,114,115] There is a striking overlap in the open chromatin profile of activated B-cells and MS-SNP, indicating their functional relevance in B-cells.[116] EBV establishes a latent infection in B-cells, which is regulated by host and viral proteins. The latency-associated EBV proteins LMP1 and LMP2A chronically activate B-cells. Therefore, the MS-SNP linked genes are potentially functionally affected by EBV infection and *vice versa*. As virtually all MS patients are infected with

EBV,[7,117] MS-SNP may be functionally involved in the regulation of EBV latency in B-cells or in EBV-specific immunity. HLA-DRB1*1501 is the strongest MS-associated genetic factor, which also poses increased risk to infectious mononucleosis and MS on an additive scale.[118,119] Recently, HLA-DRB1*1501 carriership has also been correlated with increased EBNA-1 IgG titers in otherwise healthy EBV carriers.[120,121] Furthermore, the prevalence of MS-SNP was considerably increased in healthy EBV carriers with high EBNA-1 titers, but which variants contribute to this association was only partially defined.[120]

In **Chapter 4** we determined the association of MS-SNP[10,114] with the EBV-specific IgG responses towards EBNA-1 and the early lytic cycle associated EBV early-antigen-D (EA-D, encoded by BMRF1) in MS patients and healthy controls. Total varicella zoster virus (VZV)-specific IgG were determined as a highly prevalent, non-MS-associated control HHV.[87] We confirmed the increased serum EBNA-1 IgG responses in MS patients[7], associated with unchanged serum IgG levels towards the EBV protein EA-D and VZV. Furthermore, we confirmed the EBNA-1 IgG association with HLA-DRB1*1501 carriership.[120] We observed an additional HLA-DRB1*1501 association with EA-D IgG levels, but not VZV IgG. Of the 78 MS-SNP analyzed, one SNP related to VCAM-1 and one related to PRDX5 and BAD (PRDX5/BAD)[10,122] were associated with low EBNA-1 IgG levels. In a multivariate analysis, interactions between MS-SNP related to VCAM-1 and PRDX5/BAD were associated with low EBNA-1 IgG. These associations are counterintuitive given the positive correlations between EBNA-1 IgG titers and MS-SNPs with MS risk.[8] VCAM-1 is associated with migration of B-cells. The increased expression of the ligand for VCAM-1, VLA-4 on B-cells by EBV infection[123] suggests increased migration to the CNS. PRDX5/BAD are both associated with caspase-induced apoptosis. EBV inhibits both genes during latent and lytic infection, respectively.[124,125] The resulting reduced immune exposure of ENBA-1 may partly explain the lowered EBNA-1 IgG levels in MS patients that carry this SNP. Interactions between MS-SNP related to SOX8, MYB/NHI1, CARD11 and CLEC16A/CIITA were selectively associated with high EBNA-1 IgG levels. The respective MS-SNP host genes are involved in lymphocyte migration (VCAM-1),[42] anti-apoptosis (PRDX5/BAD, MYB/NHI1 and CARD11)[124–127] and HLA class II presentation (CLEC16A, CIITA and HLA-DRB1*1501).[128,129] Because not all known MS-SNPs were assessed, our study potentially underestimates genetic association with EBNA-1 IgG. The majority of genes that are associated with the identified EBNA-1 IgG correlated MS-SNP have been shown to be functionally affected by EBV proteins and transcripts.[123–127,130,131] Functional differences in the affected pathways (migration, apoptosis and HLA class II presentation) may therefore lead to distinct viral inhibition by EBNA-1 IgG that are more likely to occur in MS-related autoimmunity. Taken together, the aberrant EBV-specific humoral immune responses are partly due to the genetic background of MS patients.

EBV-specific intrathecal T-cell responses in early MS patients

Even in absence of detectable EBV DNA, transcripts and proteins,[24,68,91] and intrathecal EBV-specific IgG synthesis,[104] the virus' signature may be reflected by the presence of intrathecal EBV-specific T-cells in MS patients. Studies on peripheral blood of MS patients indicated that EBV-specific T-cell responses are most prominently increased early in disease development.[132] Apart from indicating a cleared infection,[74,76] EBV-specific T-cells may be involved in MS pathology by recognizing viral peptides cross-reacting with CNS-specific antigens, referred to as molecular mimicry.[133–135] Alternatively, autoreactive T-cells are activated by B-cells that upon EBV infection express autoantigens, like αB-crystallin.[136,137] EBV-specific CD4+ and CD8+ T-cells have been demonstrated in CSF of MS patients using peptide[138,139] or autologous EBV transformed B-cells (autoBLCL) stimulation,[138] tetramer staining using immunodominant EBV epitopes[140] and by T-cell receptor sequencing.[141] However, these studies have not, or only partially scrutinized the EBV proteins recognized by intrathecal EBV-specific T-cells.

In **chapter 6** we determined the frequency and phenotype of intrathecal EBV-specific T-cells in patients with CIS and early MS, and as control cohort patients with other neurological diseases (OND). Short-term CSF-derived T-cell lines (CSF-TCL) were generated by non-specific stimulation and autoBLCL were generated.[91] We demonstrated that about 1-5% of autoBLCL spontaneously entered the lytic cycle *in vitro*, representing B-cells expressing the whole EBV proteome. Thus, autoBLCL are the ideal autologous APC to determine EBV-specific T-cell responses. Intrathecal CD4+ and particularly CD8+ T-cell responses towards autoBLCL were increased in early MS patients compared to OND patients. Interestingly, autoBLCL-specific CD4+ and CD8+ T-cell responses correlated, suggesting a coordinated T-cell response. However, congruent with our previous studies, neither EBV DNA[68,104] nor intrathecally synthesized EBNA-1 IgG[104] was detected in the respective CSF samples. The inability to detect EBV DNA and EBNA-1 IgG is potentially due to the relatively long interval (> 1 month) between disease onset and collection of the respective CSF samples because soluble IgG and cell-free virus particles are continuously drained to the cervical lymph nodes and are sensitive to enzymatic degradation compared to CSF cells.[110,111]

The EBV antigens recognized by intrathecal autoBLCL-specific T-cells was determined using an artificial APC system consisting of non-human primate Cos7 cells expressing the majority of the EBV proteome (69% of all annotated EBV proteins) together with the responsive HLA-I allele of the MS patient analyzed. Alternatively, T-cells may be directed towards host antigens which expression is induced by EBV: e.g. heat shock protein αB-crystallin[136] that is highly upregulated in MS lesions.[142] αB-crystallin has been identified as a possible T-cell autoantigen in MS patients.[136,143] Interestingly, oligoclonal autoBLCL-specific CD8+ T-cell responses selectively recognized lytic viral antigens of distinct kinetic classes, presented on several specific HLA-I alleles. These responses included immediate early (BRLF1), early (BaRF1 and BXLF1) and late EBV proteins (BBRF3 and BCRF1). Four out of eight identified autoBLCL/HLA-I combinations did not show reactivity to EBV proteins.

This may be explained by the incomplete EBV antigen collection (31% EBV proteins were not assayed), or by reactivity towards host antigens. It is plausible that the intrathecal autoBLCL-specific T-cell response in early MS patients comprises EBV-reactive and autoreactive T-cells.

These data concur with previous studies describing T-cell reactivity towards structural EBV proteins, particularly the lytic viral proteins,[58,140,144,145]. However, other studies showing selective T-cell reactivity towards the latency-associated antigens EBNA-1 and EBNA-3A.[133,139,141,145] These differences may relate to T-cell culture conditions applied, because some groups used EBV protein-spanning synthetic peptide pools to expand Ag-specific CSF-derived T-cells,[133,139,145] which may lead to an overrepresentation of specific EBV reactive T-cell clones. Furthermore, latency-associated EBV protein reactive intrathecal T-cells were detected using PBMC pulsed with synthetic peptides as APC.[133,145] This may result in activation of disease-irrelevant T-cells, as discussed previously.[50,52–54] Nevertheless, the MS patient cohorts studied are too small to draw firm conclusions on whether intrathecal EBV-specific T-cells are preferentially directed towards the latent or lytic EBV proteins.

Several lines of evidence support a key role in antigen presentation, co-stimulation and cytokine production functions of B-cells.[146–149] The coordinated oligoclonal autoBLCL-specific CD4+ and CD8+ T-cell response in CSF of early MS patients suggest BLCL are the culprit APC.[150] Potentially, EBV-infected B-cells may erroneously stimulate autoreactive T-cells. In support of this hypothesis, B-cells infected with the common marmoset lymphocryptovirus (CalHV3), a simian γ-herpesvirus resembling EBV, are considered pivotal in the development of MS-like pathology in the marmoset EAE model.[151] This non-human primate EAE model, that closely resembles MS pathology and progressive disease progression, is mediated by HLA-E-restricted CD8+ T-cells induced by CalHV3-infected B-cells. Due to alterations in the antigen presentation pathways, these CalHV3-infected B-cells selectively present erroneous MOG peptides to pathogenic T-cells and induce a pathogenic autoreactive T-cell respons.[152]

Our study provides insights in the potential role of EBV in MS pathogenesis. The intrathecal T-cell response towards autoBLCL is oligoclonal and is increased in MS patients compared to disease controls. AutoBLCL-specific CD8+ T-cells selectively reacted towards lytic EBV antigens which supports a role for EBV reactivation in MS. Studies in larger cohorts to determine the immunodominant kinetic class of EBV antigens recognized by intrathecal T-cells are warranted.

Intralesional T-cell responses towards autologous EBV-transformed B-cells

It is not known if the increased autoBLCL-specific CSF-derived T-cells responses observed in early MS patients are involved at the site of disease: the MS lesion. This prompted us to investigate the frequency of autoBLCL-specific T-cells in paired CSF-, NAWM- and WML-derived short-term TCL of end-stage MS patients (**Chapter 7**). In contrast to NAWM- and DWMA-TCL, substantial autoBLCL-specific T-cell responses were detected in AL- and mAIL-TCL. These T-cell responses were most prominent among CD8+ T-cells in mAIL, where 5 of 7 TCL analyzed from 5 MS patients showed brisk T-cell reactivity towards autoBLCL. Anatomically distinct paired WML-derived TCL of two patients showed a strong correlated CD8+ T-cell response in both lesions. The presence of autoBLCL-specific CD8+ T-cells in WML argues for their role in disease progression. These T-cells are potentially enriched due to local stimulation by EBV antigens. Hence, we determined the presence of the most abundant EBV transcript (EBER1) in surplus dispersed brain tissue originally used to generate the respective TCL. Because no EBER1 RNA was detected lytic EBV infection or the presence of latent EBV in the sample CNS tissues are unlikely.

By TCRVβ analysis combined with functional T-cell analysis, we determined the TCRVβ usage of autoBLCL-specific CD8+ T-cells in TCL generated form two distinct mAIL of one MS patient. The oligoclonal autoBLCL-specific CD8+ T-cell response in WML of this specific MS patient was dominated by TCRVβ2+CD8+ T-cells, which were readily detected in both WML by IHC. Notably, these particular CD8+ T-cells were detected in both lesions in the Virchow-Robin space and the parenchyma. The majority of the parenchymal TCRVβ2+CD8+ T-cells showed strong polarization of both TCR and CD8, indicative of the formation of an immunological synapse. Unfortunately, insufficient T-cells were available to define if the cognate antigen is EBV or host-specific. Although not confirmed at the clonal level, these data suggest that the same CD8+ T-cell clone, specific for an antigen expressed by autoBLCL, is involved in the inflammatory process in two distinct mAIL of the same MS patient. The autoBLCL-specific CD8+ T-cells in active WML, intriguingly devoid of detectable EBV DNA and RNA, suggest that these T-cells are not targeting a local EBV infection like suggested by others.[74,76,140] The cognate antigen may be an EBV antigen, and autoantigen expressed or selectively processed in EBV-infected B-cells or and EBV/cMSAg cross-reactive autoantigen.[133–136,152,153] These data suggest autoBLCL-specific cytotoxic T-cells are involved in MS immunopathology and warrant the follow-up studies to identify the cognate antigen of WML T-cells.

Future perspectives

In **Chapter 2 and 7** we reported no substantial T-cell reactivity to a panel of well-known cMSAg. In **Chapter 7** we showed that part of the WML-derived T-cells are chronically stimulated and express markers suggesting their status of exhausted T-cells.[27,154] Potentially, these cells are less responsive when stimulated *in vitro* and as a consequence were underrepresented in our T-cell cultures. Currently, antagonists of these co-inhibitory pathways are available including PD-1, TIM3 and LAG-3 blocking mAbs and p38MAPK inhibitors.[154–156] Improved T-cell culture methods that include these compounds may rescue responsiveness and proliferation of these T-cells *in vitro*.

Using BLCL as APC for cMSAg, the analysis of potential EBV/cMSAg cross-reactive T-cells is hampered because we normalized for reactivity towards BLCL that may intrinsically present the cross-reactive epitope. Currently, alternative methods to immortalize autologous B-cells independent of EBV are available using expression of Bcl-6 and Bcl-xL.[157] Using these EBV negative B-cell lines as APC for expression individual EBV and cMSAg would enable the analysis of potential EBV/cMSAg cross-reactive T-cells.[158]

The prevalence of EBV nucleic acids and protein in CSF and brain tissues of MS patients remains a matter of debate. In **Chapter 3, 6 and 7** we were unable to detect EBV DNA and RNA remnants in CSF samples of early MS patients and WML of end-stage MS patients. Together with the absence of intrathecal EBV-specific IgG as shown in **Chapter 4**, these findings do not support lytic EBV infection and presence of latently EBV-infected B-cells in the CNS of MS patients. Besides the difficulty in collecting relevant clinical specimen, the sensitivity and specificity of detection may be culprit to the conflicting results.

Strikingly, all studies aimed to detect EBV by ISH used the same EBER-specific probe sequences. Although these probes robustly detect EBV in EBV-related tumors, the conflicting reports[63,74,76,92,94,95] show the necessity of improved *in situ* staining methods. Current available ISH methodologies facilitate detection of (fragmented) RNA and DNA molecules with high specificity and sensitivity. Lead companies in ISH technology, including ACD (https://acdbio.com), indicate that their ISH platforms enable detection of only a few RNA molecules. To our knowledge, no studies are reported on the presence of EBV in WML tissues using these novel ISH platforms. Recent studies on the EBV transcriptome revealed several additional transcripts that are abundantly expressed during different latency programs of EBV, besides EBER.[159–161] Re-designing EBV-specific probes for EBER, and several other putatively expressed EBV transcripts like EBNA-1, BHRF1 and the BHRF1 and BART miRNAs[162], in comparison with the classic EBER probe sequences may aid in increasing the sensitivity and specificity of detection of EBV transcripts.

The sensitivity of current qPCR based methods to detection of EBV DNA is limited due to the relatively low copy number of EBV genomes compared to EBV transcripts.[161] Analysis using RT-qPCR specific high abundant EBV transcripts may increase the sensitivity of detection for EBV compared to qPCR-specific for viral DNA sequences.[161] To broaden the scope of PCR-based analysis also next-generation sequencing (NGS) may be beneficial

to detect EBV transcripts. Despite the recent advances in NGS, only a few studies used these methods to analyze CSF or WML of MS patients.[163,164] Enrichment of the B-cells from WML by *ex vivo* fluorescent assisted cell sorting (FACS) or laser-capture microscopy, combined with advanced ultra-deep NGS methods and enrichment of EBV targets[165] may considerably increase sensitivity of detection.

Intrathecal synthesis of EBV-specific IgG was not observed in **Chapter 4**. Nevertheless, the clonally enhanced repertoire of intrathecal B-cells indicate that antigen-specific B-cell responses are triggered, particularly in the ectopic germinal centers in the leptomeninges of MS patients.[13,79,166] Currently, these B-cells are thought to mainly have an important role in GM pathology during the progressive phase of MS, possibly as a source of autoantibodies.[167–171] After EBV infection, naïve B-cells differentiate into memory B-cells independent of antigenic stimulation and without negative selection. The EBV-transformed memory B-cell pool may therefore include autoreactive B-cells. Even though the presence of EBV in these cells is debated,[63] recent insights implicate EBV infection as a potential source of intrathecal autoreactive B-cells.[172] Because EBV replication efficiency in B-cells is recently shown to be limited, part of infected B-cells may become EBV negative after multiple replication cycles.[172] Identifying the antigenic targets of these potentially autoreactive B-cells may shed light on inflammatory mediators in progressed disease. This is of particular interest because the efficacy of current therapies that target the progressive phase of MS is limited.[1,12]

In **Chapter 5** we showed the increased EBNA-1 IgG response is associated with specific MS-associated genetic factors. However, further studies are needed to functionally dissect these interactions. Recent advances in CRISP/Cas9-mediated genome editing enables the analysis of the functional contribution of individual MS-SNP on the regulation of host genes and pathways and the regulation of EBV latency *in vitro*. Additionally, including MS-SNP and HLA-I/II genotyping of patients and controls that are analyzed using functional T-cell analysis may provide novel insights on the causal pathways involved in the aberrant T-cell response towards EBV or cMSAg in MS patients.

The increased frequency of BLCL-specific T-cells in CSF of early MS patients in **Chapter 6** and active WML of end-stage MS patients in **Chapter 7** suggests a key role of autoBLCL-specific T-cells in MS pathology. Of note, because no other virus than EBV has withstood the scrutiny of scientific research, we did not include reactivity towards other viruses in our T-cell assays. Potentially, this leads to an excess significance bias due to the limited number of parameters. Including chronic and acute control viruses like VZV or cytomegalovirus and influenza and measles would strengthen our associations with EBV.

The selective reactivity towards lytic viral antigens in the CSF of early MS patients is in sharp contrast to healthy EBV carriers, where mostly latent EBV antigens are immunodominant CD8+ T-cell targets.[173–175] These data suggest that the intrathecal T-cell control of latent EBV in MS patients is impaired. Potentially, this leads to more frequent or a further progressed EBV reactivation due to a delayed T-cell response in MS patients. Contrastingly, the increased humoral response towards EBNA-1, and not EA-D and VCA,

suggests the control of latent virus is affected. However, one may argue that nuclear antigens are only accessible for IgG after cell death, and therefore EBNA-1 may represent a secondary immune target.

Recently, Pender and colleagues studied the function and frequency of peripheral EBV-specific CD8+ T-cells in a large cohort of age, gender and HLA-I matched MS patients and healthy EBV carriers.[176] They showed the BLCL-specific T-cell function decreases as disease progresses. Interestingly, similar compromised EBV-specific T-cell responses have been shown in rheumatoid arthritis[177,178] and systemic lupus erythematosus which strengthens the hypothesis of a shared pathogenic mechanism in these autoimmune diseases.[179,180] Contrary to our findings, they showed EBV-specific CD8+ T-cells in MS patients mainly recognize latent antigens and mainly lytic EBV antigens in healthy EBV carriers. However, this was shown with very limited HLA-I-restricted peptide pools, including 13 peptides from EBV latent antigens and 5 peptides from EBV lytic antigens, which is neither proportional to nor representative for the complete EBV ORFeome. To draw firm conclusions on a potential altered immunodominance of EBV antigens, these data should be confirmed by screening intrathecal T-cell reactivity towards the entire EBV proteome. The high BLCL-reactivity and large number of T-cells needed for EBV ORFeome screens as detailed in **Chapter 6**, hampers the analysis patients with lower frequencies of autoBLCL-specific CD8+ T-cells and limits the number of patients that can be included in these type of analysis. Alternatively, CRISPR/Cas9-mediated genome editing of EBV is used to target latent or lytic cycle EBV in autoBLCL.[181] Full EBV knock-out autoBLCL or autoBLCL with BZLF1 and BMRF1 knock-out EBV variants can be generated and used as APC to determine if latent or lytic viral antigens are immunodominant for intrathecal EBV-specific T-cells.

The potential discrepancy in immune dominance of EBV antigens[91] and T-cell functionality[176,182] between MS patients and healthy EBV carriers is of particular interest because it would provide a rational for boosting T-cell immunity against specific EBV antigens by subunit vaccines or adoptive immunotherapy to restore T-cell control of latent EBV in MS patients. The adoptive transfer of *in vitro* stimulated and expanded autologous CD8+ T-cells specific for several immunodominant epitopes of the latent antigens EBNA-1, LMP1 and LMP2A was used to treat one patient with severe SPMS.[183] This patient showed substantial clinical improvement without serious adverse events. Also, intrathecal IgG production and disease activity on MRI was decreased.[183] This type of therapy is not without risk as CNS inflammation may be aggravated due to the potential cross-reactivity of the re-infused CD8+ T-cells with autoantigens but may offer a opportunity to treat MS.[184]

The cognate antigens recognized by WML-derived autoBLCL-specific T-cells in the absence of detectable EBV DNA or transcripts are puzzling (**Chapter 7**). More detailed knowledge of what host antigens are induced by EBV in B-cells would be highly interesting to identify novel cMSAg for MS T-cells. Host transcriptomics and proteomics of B-cells prior and after EBV infection, after establishment of latency and by reactivation of EBV can be used to identify cMSAg. Alternatively, characterizing the HLA-associated peptidome using

mass spectrometry of BLCL during latency and reactivation may aid to identify disease relevant epitopes for MS-relevant T-cells.[185] Of the identified cMSAg, surface proteins are attractive candidates as primary B-cell targets of lesion- or meninges-derived B-cells of MS patients. Genetic programming using Bcl-6 and Bcl-xL may enable the culture of the respective B-cells of MS patients and controls. These B-cell lines may be used to generate human IgG, which can be used to screen reactivity towards identified cMSAg. Preferentially these IgG are screened using cell-based assays that express the relevant conformational B-cell epitopes.[186,187]

To study the role of EBV infected B-cells in MS *in vivo*, the marmoset EAE animal model is particularly interesting.[188] In this model CalHV infected B-cells are shown to be capable of presenting erroneous MOG peptides towards autoreactive CD8+ T-cells *in vitro*.[152] Notably, it is unknown if EBV has a similar potential and this deserves follow-up. It is not known if CalHV infected B-cells are required to induce EAE as currently no CalHV seronegative marmoset are available for comparative analysis. The previously discussed CRISPR/Cas9-mediated genome editing may aid in the adoptive transfer of CalHV knockout B-cells after B-cell depletion.

The T-cell culture methods used in **Chapter 2, 6 and 7** are inadequate to obtain sufficient T-cell numbers for in-depth characterization of the target antigen repertoire and the respective epitopes. The above suggested improved culture methods with the addition of antagonists of inhibitory pathways may result in larger T-cell numbers, but the *in vitro* proliferative capacity of T-cells may nevertheless be limited. Furthermore, prolonged expansion of TCL may lead to a skewed T-cell repertoire, which impedes the accurate quantification of Ag-specific T-cell responses. Alternatively, *ex vivo* isolated CNS-derived CD4+ and CD8+ T-cells are analyzed at the single cell level using fluorescence activated cell sorting. Sorted single cells can be used for α- and β-chain TCR sequencing and quantifying expression of phenotypic or functional transcripts including co-inhibitor (TIM3, PD-1, CD57 and CTLA-4) and stimulatory receptors (CD137, FasL, CD107a), cytokines (TNFα, IL-2, IL-17 and GM-CSF) and effector molecules (granzymes, perforin). Thereby the phenotype and function of the dominant T-cell clones can be determined.[189,190] Furthermore, by ISH using probes complementary to the variable CDR3 region of the TCR, Ag-specific T-cells can be localized in surplus tissue and interactions with CNS resident (microglia, oligodendrocytes and neurons) or infiltrating cells (B-cells, T-cells, macrophages) can be determined. The TCR cDNA sequence of dominant WML-derived T-cell clones can be cloned and using synthetic DNA constructs. These TCR cDNA can be cloned and expressed in recombinant T-cell lymphoma reporter cells.[191] High-throughput screening of these TCR-recombinant reporter cells using peptide libraries or endogenously synthesized proteins of newly identified cMSAg can be used to identify the culprit target antigen(s).

Concluding remarks

Autoreactive T-cells likely contribute to MS immunopathology yet their target antigen remains enigmatic. Detailed knowledge of the culprit MS antigen(s) would enable the development of new therapies to tolerize MS patients in an autoantigen-specific manner. Despite conflicting reports on cMSAg, these therapies are currently being implemented in clinical trials for several myelin peptides in MS patients.[192,193] Host antigens expressed by BLCL may be valuable candidates for this type of immune therapy. On the other side of the spectrum, sub-unit vaccines to boost immunity towards particular EBV antigens may aid to restore the immune control of EBV in MS patients. Besides the potential benefits for MS patients, given the wide arrange of EBV-related diseases and a lack of effective antiviral therapies, safe and preferably therapeutic EBV subunit vaccines are called for.[183,184,194,195] However, defining if specific EBV target antigens are involved in MS pathology is imperative to assure that EBV vaccine targets do not induce a cross-reactive autoimmune response.

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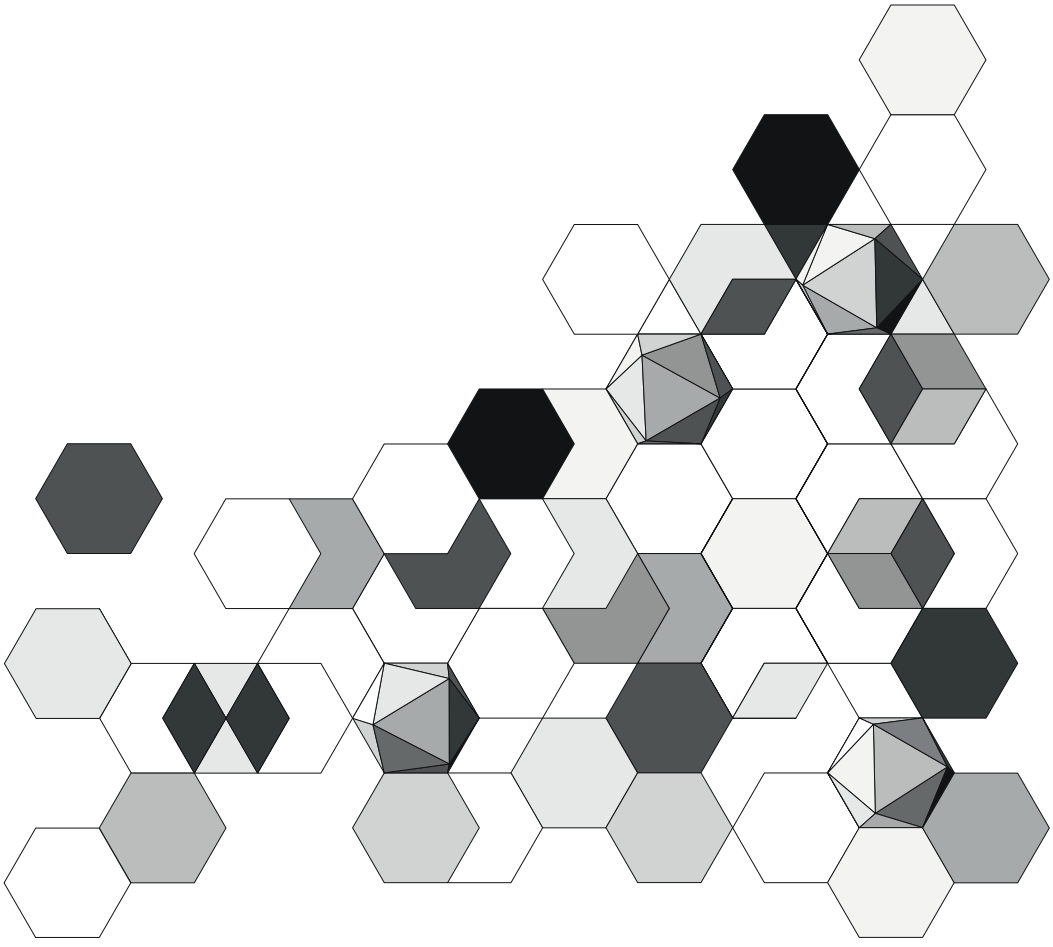
Chapter 9

Nederlandse samenvatting



Multiple sclerose (MS) is een chronische ontstekingsziekte van de hersenen en ruggenmerg, oftewel het centraal zenuwstelsel (CZS). Deze ontstekingen leiden tot vermoeidheid en problemen met cognitie, bewegen, tast en zien. MS komt bij ongeveer 1 op de 1000 individuen voor en is daardoor, buiten trauma, de meest voorkomende oorzaak van invaliditeit. Genezing van MS is op het moment niet mogelijk maar er zijn diverse therapieën die het verloop van de ziekte kunnen vertragen of de plotselinge aanvallen verzachten. Helaas hebben de meest effectieve therapieën ook de ernstigste bijwerkingen.

Vroeg in de ontwikkeling van MS bevinden de meeste ontstekingen zich in de witte stof van de hersenen en het ruggenmerg en later ook in de grijze stof. In de witte stof bevinden zich de uitlopers van zenuwcellen (axonen) die de verschillende delen van de hersenen met elkaar verbinden en oligodendrocyten. In de grijze stof bevinden zich de zenuwcellichamen. De oligodendrocyten beschermen en voeden de axonen door hier een eiwitmantel omheen te vormen (myelineschede). Kenmerkend voor MS is dat deze myelineschede beschadigd raakt en verloren gaat (demyelinisatie), waardoor zenuwcellen kwetsbaar worden en niet goed meer kunnen functioneren. De aangeboren afweercellen in de hersenen (glia) en afkomstig uit bloed (macrofagen) zijn betrokken bij het afbreken van myeline. Daarnaast zijn verworven afweercellen (lymfocyten) betrokken bij de ontstekingsreactie en de beschadiging van oligodendrocyten. De verworven afweer bestaat uit antistof producerende cellen (B-cellen) en celgemedieerde afweer (T-cellen).

In de witte stof zitten twee unieke barrières rond de bloedvaten die de toegang van afweercellen reguleren om mogelijke beschadiging als gevolg van een lokale ontstekingsreactie te beperken. Vanuit de bloedbaan zijn dit eerst de bloed-brein-barrière en als tweede de glia limitans. In de hersenontstekingen in de witte stof van MS patiënten (verder laesies genoemd) bevinden zich karakteristieke infiltraten van uit bloed afkomstige afweercellen; met name T-cellen, en in mindere mate B-cellen en macrofagen. De mate van infiltratie is gerelateerd aan de hoeveelheid schade. Het is echter onbekend of deze afweercellen in het hersenweefsel de oorzaak of een gevolg zijn van laesies.

De oorzaak van MS is nog niet bekend. Wel is aangetoond dat zowel een genetische aanleg als blootstelling aan omgevingsfactoren zoals virus infecties, in het bijzonder Epstein-Barr virus (EBV), ten grondslag liggen aan het ontwikkelen van MS. EBV is één van de acht humane herpesvirussen (HHV). Herpesvirussen komen vaak voor en blijven levenslang in een rustende, latente staat aanwezig in de gastheer. Sporadisch reactiveren deze virussen waardoor zij herhaaldelijk voor ziekteverschijnselen kunnen zorgen. Herpesvirussen zijn in staat de immuniteit van de gastheer sterk te onderdrukken. Bekende voorbeelden van herpesvirussen zijn herpes simplex virus (HSV), de oorzaak van de koortslip, en varicella zoster virus (VZV), de oorzaak van waterpokken en gordelroos. Infectie met EBV op jonge leeftijd is meestal asymptomatisch. Bij infectie op latere leeftijd, zoals tijdens de pubertijd leid dit echter vaak tot infectieuze mononucleose, ook bekend als de ziekte van Pfeiffer. Personen met een voorgeschiedenis van Pfeiffer hebben een verhoogde kans op het ontwikkelen van MS op latere leeftijd.

Er wordt verondersteld dat met name T-cellen een belangrijke rol spelen in het ontstaan en/of voortduren van laesies, maar de eigenschappen van deze cellen zijn onduidelijk. In **hoofdstuk 7** van dit proefschrift hebben we een vergelijking gemaakt van T-cellen in bloed, hersenvocht, laesies en niet-aangedane witte stof ('normal appearing white matter': NAWM) van overleden MS patiënten met een relatief lang ziektebeloop van gemiddeld 25 jaar. In laesies en NAWM vonden we hoofdzakelijk T-cellen rondom bloedvaten tussen de bloed-brein-barrière en de glia limitans, ook perivasculaire ruimte genoemd. Uitsluitend in laesies vonden we sporadisch T-cellen voorbij de glia limitans in het zogenoemde parenchym van het hersenweefsel. Deze T-cellen zijn hoogstwaarschijnlijk direct betrokken bij de ontstekingsreactie en het voortduren van laesies.

In zowel laesies als NAWM vonden we overwegend CD8+ T-cellen, ook wel bekend als cytotoxische T-cellen, en slechts lage frequenties van CD4+ T-cellen, ook wel helper T-cellen genoemd. In laesies, NAWM en hersenvocht waren deze CD8+ T-cellen vrijwel uitsluitend geheugen T-cellen, terwijl we in bloed ook naïeve T-cellen detecteerden. Naïeve T-cellen ontwikkelen zich tot geheugen T-cellen wanneer ze het specifieke eiwit waardoor ze geactiveerd worden (antigeen) hebben herkend met hun T-cel receptor. De CD8+ T-cellen uit laesies vertoonde kenmerken van chronisch antigeen-specifieke stimulatie en cytotoxiciteit. Daarnaast vonden we een verrijking van een beperkte set van T-cel receptoren op CD8+ T-cellen (klonale verrijking) in laesies. Het repertoire aan T-cel receptoren vertoonde een sterke gelijkenis bij verschillende laesies van dezelfde patiënten. Deze gelijkenis vonden we niet bij laesies en NAWM. Klonale verrijking kan ontstaan wanneer antigeen-specifiek geactiveerde T-cellen gaan delen. Mogelijk zorgt een gemeenschappelijk antigeen daarom voor de klonale verrijking van CD8+ T-cellen in verschillende laesies. Deze resultaten suggereren dat cytotoxische geheugen T-cellen een belangrijke rol spelen in de pathogenese van MS.

Voor onderzoek naar het ontstaan van MS laesies wordt met name gebruik gemaakt van experimentele auto-immuun encefalomyelitis (EAE) proefdiermodellen. De laesies van MS en EAE diermodellen lijken veel op elkaar. Bij EAE wordt door middel van immunisatie met myeline-, glia- of neuroneiwitten een auto-immuun T-cel reactie geïnduceerd die leidt tot hersenontstekingen en -schade. Hierdoor wordt verondersteld dat vergelijkbare auto-immuun T-cellen ook in MS een oorzakelijke rol spelen.

In **hoofdstuk 2** hebben we een nieuwe methode ontwikkeld en gevalideerd om herkenning van potentiële MS geassocieerde kandidaat antigenen (MSAg) door T-cellen uit te kunnen lezen. Hierbij hebben we gebruik gemaakt van EBV-geïnfecteerde B-cel lijnen (BLCL) die MSAg produceren en presenteren aan T-cellen. Al kan antigeen stimulatie verschillen tussen BLCL en het nog niet gedefinieerde verantwoordelijke celtype in het CZS, biedt dit systeem een meer natuurgetrouwe stimulatie dan de eerder gebruikte T-cel stimulatie methodes. In **hoofdstuk 2** hebben we dit systeem toegepast op uit hersenvocht gekweekte T-cellen van MS patiënten met een kort ziektebeloop en in **hoofdstuk 7** op T-cellen gekweekt uit hersenvocht, laesie en NAWM monsters van overleden MS patiënten met een lang ziektebeloop. We hebben een panel aan myeline-,

glia- en neuron-specifieke MSAg getest maar vonden geen noemenswaardige herkenning van MSAg. De veronderstelde rol van MSAg-specifieke T-cellen in de pathogenese van MS wordt door onze resultaten niet ondersteund.

Door de specifieke eigenschappen van HHV wordt verondersteld dat MS het gevolg is van een CZS infectie met deze virussen. In **hoofdstuk 3** hebben we de aanwezigheid van alle tot nu toe bekende herpesvirussen onderzocht in hersenvocht van MS patiënten met een zeer kort ziekte beloop (minder dan 2 weken) en als controle patiënten met andere neurologische aandoeningen met een onbekende oorzaak (ANA). Doordat latente herpesvirussen zich in cellen bevinden is het lastig om hun aanwezigheid in hersenvocht, waarin zich slechts weinig cellen bevinden, vast te stellen. Om de pakkans te vergroten hebben we uit hersenvocht de cellen geïsoleerd voor analyse. Ondanks de verrijking voor latent virus en het gebruik van zeer gevoelige en specifieke polymerase ketting reactie-gebaseerde meetmethode hebben we geen HHV-specifiek DNA gedetecteerd in cellen geïsoleerd uit hersenvocht van MS patiënten. De veronderstelde betrokkenheid van intrathecale herpesvirus infectie in een vroeg stadium van MS wordt door onze resultaten niet ondersteund.

Ondanks dat we geen herpesvirus infectie van het CSZ van MS patiënten hebben aangetoond is er overtuigend bewijs voor een rol van EBV in MS pathogenese. Op basis van epidemiologisch onderzoek wordt verondersteld dat infectie met EBV voorafgaand aan het ontwikkelen van MS vereist is. EBV is latent in B-cellen. Er zijn EBV positieve B-cellen aangetoond in laesies en de naburige hersenvliezen van MS patiënten, maar andere studies hebben dit niet kunnen bevestigen. In afwezigheid van aantoonbaar EBV-specifiek DNA kan een EBV infectie van het CZS mogelijk worden aangetoond aan de hand van lokale productie van EBV-specifieke antistoffen IgG. De antistof-gemedieerde afweer, met name immuunglobuline G (IgG), tegen EBV-specifieke eiwitten is zowel in bloed als hersenvocht verhoogd bij MS patiënten ten opzichte van gezonde controles. Als een bepaald EBV-specifieke IgG betrokken is bij MS pathogenese worden deze ook mogelijk intrathecaal geproduceerd.

In **hoofdstuk 4** hebben we aangetoond dat er geen noemenswaardige verschillen zijn in het repertoire van EBV-specifieke antigenen welke herkend worden door IgG in hersenvocht tussen MS patiënten en patiënten met ANA. De IgG waarden gericht tegen Epstein-Barr nucleair antigeen 1 (EBNA-1) waren het meest sterk verhoogd bij MS patiënten. Binnen het EBNA-1 eiwit bleek een klein stukje van aminozuur 394 tot 451 (EBNA-1$^{394\text{-}451}$) het sterkst herkend te worden door IgG en in zowel serum als hersenvocht van MS patiënten. Na correctie voor de totale hoeveelheid IgG of albumine eiwit in het hersenvocht was het EBNA-1$^{394\text{-}451}$ IgG niveau echter gelijk bij MS en ANA patiënten. Hieruit concluderen wij dat er geen lokale productie is van EBNA-1$^{394\text{-}451}$ IgG, maar dat het verhoogde niveau in het hersenvocht van MS patiënten het gevolg is van lekkage uit serum door de bloed-brein-barrière van MS patiënten. Een mogelijke intrathecale infectie

met EBV wordt niet ondersteund door onze resultaten. Of EBNA-1$^{394-451}$ IgG betrokken is bij de immuun pathogenese voor MS is echter niet duidelijk. Mogelijk zijn EBNA-1$^{394-451}$ IgG betrokken bij beschadiging van de bloed-brein-barrière in MS patiënten.

Het risico op het ontwikkelen van MS is deels genetisch bepaald. In tegenstelling tot klassieke overerfbare genetische aandoeningen is er niet één enkele mutatie bekend die afdoende of vereist is voor het ontwikkelen van MS. Wel is momenteel van ongeveer 230 natuurlijk voorkomende genetische varianten ('single nucleotide polymorphisms': SNPs) aangetoond dat deze vaker voorkomen bij MS patiënten dan bij gezonde controles. Deze MS-geassocieerde varianten (MS-SNPs) zijn hoofdzakelijk gerelateerd aan genen die betrokken zijn bij immuniteit, wat de rol van het immuunsysteem bij MS pathogenese onderschrijft. Een groot deel van deze genen zijn actief in geactiveerde B-cellen. Van het dominante MS risico gen HLA-DRB1*1501 is aangetoond dat dit voorspellend is voor een verhoogd EBNA-1 IgG niveau in bloed van gezonde EBV dragers.

Mogelijk zijn andere MS-SNPs ook voorspellend voor EBV-specifieke IgG niveaus in MS patiënten. In **hoofdstuk 5** hebben we van 78 SNP bekeken of deze associëren met specifieke IgG niveaus voor het latent EBNA-1 en reactiverend EBV 'early antigen-D' (EA-D) in bloed van MS patiënten en gezonde EBV-geïnfecteerde individuen. Ter controle hebben we IgG specifiek voor het niet-MS-geassocieerd VZV, geanalyseerd. In onze studie hebben we de HLA-DRB1*1501 associatie bevestigd in MS patiënten. Verder vonden wij dat HLA-DRB1*1501 ook geassocieerd is met verhoogd EA-D IgG, maar niet met VZV IgG. Tevens vonden we een aantal SNP, of combinaties van SNP, die geassocieerd waren met EBNA-1 IgG, en niet met EA-D of VZV IgG waarden in serum. Het merendeel van deze MS-SNPs zijn geassocieerd met genen die betrokken zijn bij migratie van B-cellen (VCAM-1), bij remming van gecontroleerde celdood (PRDX5/BAD, MYB/NHI1 en CARD11) en bij antigeen presentatie aan CD4+ T-cellen (CLEC16A, CIITA en HLA-DRB1*1501). Ook de SOX8-geassocieerde MS-SNP was met EBNA-1 IgG waarden geassocieerd maar de functie hiervan is onbekend. De regulatie van de EBNA-1 en EA-D IgG-geassocieerde genen wordt sterk beïnvloedt door EBV-specifieke eiwitten in B-cellen wat deze associaties onderschrijft. De genetische achtergrond voor MS is dus deels bepalend voor de antistof-gemedieerde afweer tegen EBV.

In afwezigheid van een detecteerbare EBV infectie en specifieke intrathecale antistof productie kan een lokale rol voor EBV mogelijk gedetecteerd worden door de aanwezigheid van lokale EBV-specifieke T-cellen. EBV-specifieke T-cellen zijn potentieel betrokken bij de ziekteontwikkeling van MS. EBV-specifieke T-cellen herkennen mogelijk ook lichaamseigen antigenen doordat de eiwitten op elkaar lijken, dat wil zeggen door middel van kruisreactie. Anderzijds kunnen BLCL-specifieke T-cellen lichaamseigen eiwitten herkennen die door EBV infectie geproduceerd worden door BLCL, zoals het door EBV geïnduceerde lichaamseigen eiwit α B-crystalline. De frequentie van EBV-specifieke T-cellen in bloed is verhoogd ten opzichte van gezonde virus dragers, met name in een vroeg stadium van de ziekteontwikkeling. In **hoofdstuk 6** hebben we daarom de frequentie van T-cellen die patiënt-eigen BLCL (autoBLCL) herkennen in het hersenvocht bepaald bij

MS patiënten in een vroeg stadium van ziekteontwikkeling en ANA. De frequentie van CD4+ T-cellen, en met name van CD8+ T-cellen, gericht tegen autoBLCL was verhoogd bij MS patiënten. De frequenties van deze CD4+ en CD8+ T-cellen waren gecorreleerd, hetgeen duidt op een gecoördineerde T-cel reactie. Deze CD8+ T-cellen herkende selectief EBV antigenen gerelateerd aan reactiverend EBV, en niet met latent EBV of α B-crystalline. Omdat CD8+ T-cellen in gezonde EBV-geïnfecteerde individuen juist hoofdzakelijk latente eiwitten herkennen, suggereert dit dat de T-cellen in MS patiënten hoofdzakelijk reageren met eiwitten die later tot expressie komen na EBV-reactivatie.

Om te bepalen of BLCL-specifieke T-cellen betrokken zijn bij de vorming of continuering van laesies hebben we in **hoofdstuk 7** gekeken of er een verrijking is van deze T-cellen in laesies ten opzichte van NAWM van dezelfde MS patiënt. In tegenstelling tot inactieve laesies en NAWM vonden we in actieve laesies veel autoBLCL-specifieke CD8+ T-cellen. In twee actieve laesies van één MS patiënt zagen we dat de autoBLCL-specifieke CD8+ T-cel respons slechts bestond uit enkele klonen: de dominante kloon werd in beide laesies gevonden. Om te bepalen of deze CD8+ T-cel kloon betrokken was bij de ontstekingsreactie hebben we deze cellen gelokaliseerd in een ingevroren deel van het laesieweefsel waaruit de desbetreffende T-cellen geïsoleerd waren. Al hebben we niet kunnen aantonen of het exact dezelfde kloon betrof, bevonden deze T-cellen zich zowel in de perivasculaire ruimte als in het parenchym van beide laesies. Het merendeel van deze T-cellen in het parenchym hadden gepolariseerde expressie van CD8 en de T-cel receptor wat suggereert dat ze hun antigeen herkende. Waarschijnlijk was dezelfde autoBLCL-specifieke T-cel kloon betrokken bij het ontstekingsproces in beide laesies.

Om te bepalen of autoBLCL-specifieke CD8+ T-cellen lokaal EBV herkennen hebben we in een restant van de cel-pellets van alle laesies waar deze T-cellen uit gekweekt zijn de aanwezigheid van EBV-specifieke transcripten onderzocht. In geen enkele laesie detecteerden we EBV transcripten, wat impliceert dat deze T-cellen mogelijk lichaamseigen antigeen herkennen die geproduceerd worden door autoBLCL. Dit kan bijvoorbeeld onstaan door moleculaire gelijkenis van EBV en lichaamseigen antigenen.

Deze resultaten tonen een prominente rol voor autoBLCL-specifieke CD8+ T-cellen in laesies van MS patiënten aan, waarvan het antigeen nog niet bekend is. Identificatie van het oorzakelijke antigeen maakt het mogelijk MS patiënten hier specifiek tolerant voor te maken. Een dergelijke hoogst specifieke behandeling heeft mogelijk veel minder bijwerkingen dan de huidige breedspectrum immuun regulerende therapieën. Anderzijds, kan het induceren of versterken van een immuunrespons tegen latente EBV antigenen mogelijk voordelig zijn voor MS patiënten om de immuun controle van latent EBV te versterken, bijvoorbeeld door vaccinatie. Hiervoor moet uitgesloten worden dat deze EBV latente antigenen een rol spelen in de immunopathogenese van MS om een kruis-reactieve auto-immuun respons te voorkomen.

In dit proefschrift hebben we specifieke kenmerken van de T-cellen die betrokken zijn bij de ontwikkeling van MS laesies aangetoond (**Hoofdstuk 7**). We hebben geen bewijs gevonden voor betrokkenheid van T-cellen gericht tegen een panel aan MSAg in het hersenvocht vroeg in de ziekteontwikkeling (**Hoofdstuk 2**) en in laesies laat in de ziekteontwikkeling (**Hoofdstuk 7**). Ook hebben we geen bewijs voor een EBV infectie (**Hoofdstuk 3**) of EBV-specifieke antistof productie in het hersenvocht van MS patiënten gevonden (**Hoofdstuk 4**). De verhoogde EBV-specifieke antistofproductie bleek deels te verklaren aan de hand van de genetische aanleg voor het ontwikkelen van MS (**Hoofdstuk 5**). De T-cel gemedieerde afweer tegen EBV in het hersenvocht van MS patiënten was verhoogd en gericht tegen lytische EBV Wen (**Hoofdstuk 6**). Eveneens hebben we een belangrijke rol voor CD8+ T-cellen gericht tegen EBV-geïnfecteerde B-cellen in actieve laesies aangetoond (**Hoofdstuk 7**).

Epilogue

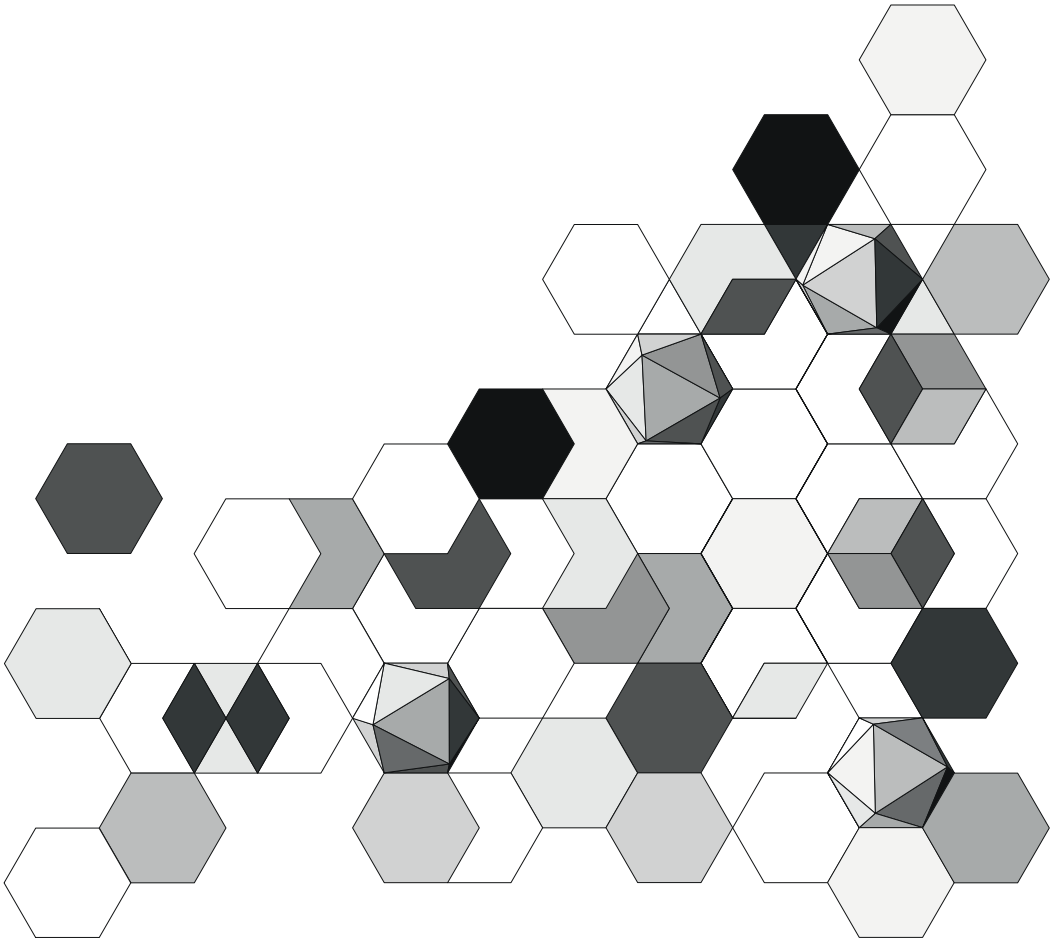
About the author

Curriculum Vitae

PhD portfolio

List of publications

Dankwoord



Curriculum Vitae

Gijsbert P. van Nierop was born on 8th of August 1977 in Eindhoven, the Netherlands. He graduated from the Athenaeum (VWO) on 1997 at the Emmaus College in Rotterdam, the Netherlands. Thereafter he commenced by studying Chemistry at the University of Utrecht, and from 1999-2002 at the Hogeschool Utrecht where he received his Bachelor of Applied Science degree, well ahead of schedule. During his studies he worked at Det Norske Veritas Petroleum Services in Rotterdam performing fuel quality testing for the shipping industry and followed additional courses in Organic Chemistry and Biochemistry to pursue a career in Life Sciences. His interest in molecular biology and translational research was instigated during his internships at the department of Virus and Stem Cell biology at the Leiden University Medical Center under supervision of Dr. Antoine A.F. de Vries, Dr. Manuel A.F.V. Gonçalves and Prof.dr. Rob C. Hoeben. Here he continued his research on development of therapeutic strategies for Duchene's disease by genetic complementation and homology-directed gene editing using chimeric viral vectors as a research technician. In 2008 he started as a senior research technician on a collaborative project of the departments of Viroscience and Neurology at the Erasmus Medical Center in Rotterdam supervised by Dr. Georges M.G.M. Verjans and Prof.dr. Rogier Q. Hintzen. Here, he mainly studied role of local T-cell responses in multiple sclerosis pathology. In 2013 he was allowed to continue this research as a PhD candidate at the Molecular Medicine research school.

PhD portfolio

Name:	Gijsbert Paul van Nierop
Research groups:	Erasmus MC, department of Neurology and Viroscience
Research school:	Post-graduate Molecular Medicine
PhD period:	2013-2018
Promotors:	Prof.dr. Rogier Q. Hintzen
	Prof.dr. Georges M.G.M. Verjans

In-depth courses

2010	Course in Virology
2013	Adobe Photoshop and Illustrator
2015	Adobe InDesign
	Research Integrity
2016	Basic course in "R"
	Gene Expression Data Analysis using "R"
	Writing Successful Grant Proposals

Attended congresses, symposia and meetings

2010	MS Research days (Alphen a/d Rijn, the Netherlands)	
	14th Molecular Medicine Day (Rotterdam, the Netherlands)	Poster
	Workshop "EBV and B-cells in MS brain" (Amsterdam, the Netherlands)	Oral
2011	MS Research days (Leiden, the Netherlands)	Oral
	5th joint ECTRIMS/ACTRIMS (Amsterdam, the Netherlands)	
2012	35th International Herpesvirus Workshop (Calgary, Canada)	Poster
	MS research days (Arnhem, the Netherlands)	Poster
	16th Molecular Medicine Day (Rotterdam)	Oral
	Symposium "MS, Herpesviruses and Aging" (Rotterdam)	Invited speaker
2013	17th Molecular Medicine Day (Rotterdam)	Poster
	MS research days (Hasselt, Belgium)	Two posters
	Audit ErasMS center, department of Neurology (Rotterdam)	Oral
2014	6th joint ECTRIMS/ACTRIMS (Boston, USA)	Poster
	Neuroinfectiology symposium, N-RENNT (Hannover, Germany)	
	18th Molecular Medicine day (Rotterdam)	Poster
2015	NVVI (Noordwijkerhout, The Netherlands)	Oral
	31st ECTRIMS (Barcelona, Spain)	Poster
	MS research days (Oegstgeest, The Netherlands)	Oral and poster
2016	T-cell Consortium (Rotterdam, The Netherlands)	Invited speaker
	ECTRIMS/ACTRIMS (London, United Kingdom)	Oral and poster

Supervision and teaching activities

2010-2017	Lab rotations MSc students Infection and Immunity Master
2014	Co-supervision MSc student (JGM)
2016-2017	Lecture Infections and Immunity Master winter course

Awards

2013	MS research days, 1st and 2nd poster award
2015	31st ECTRIMS, poster was selected as Scientific Highlight
2016	Netherlands Centre for One Health, Research Image Award

List of publications

van Nierop GP, van Luijn MM, Michels SS, Melief M-J, Janssen M, Langerak AW, Ouwendijk WJ, Hintzen RQ, Verjans GM. Phenotypic and functional characterization of T-cells in white matter lesions of multiple sclerosis patients. Acta Neuropathol. 2017, 134: 383-401.

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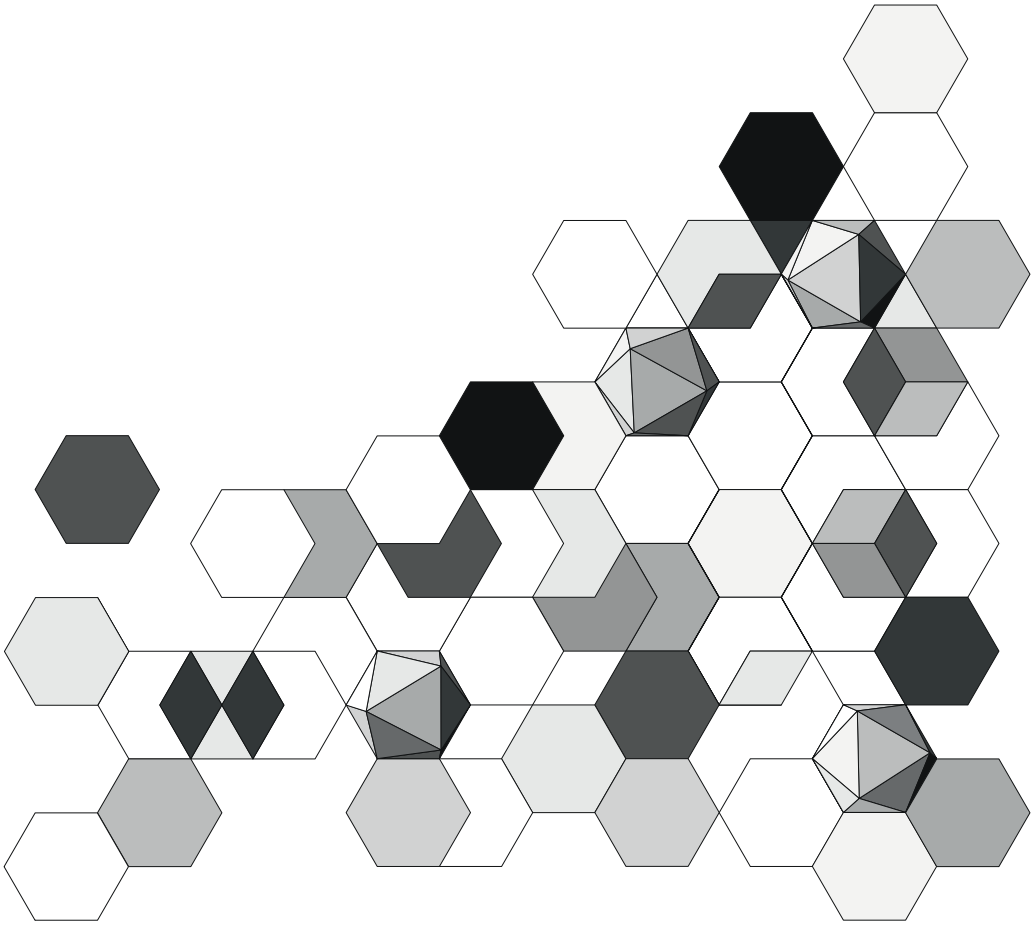
*These authors contributed equally to the preperation of this manuscript

Dankwoord



Lieve mensen,

Het voelt als een klein wonder dat dit proefschrift tot een goed eind is gekomen, aangezien ik aan het begin van het traject geen benul had van afweercellen, laat staan van hun rol bij MS immunopathogenese. De totstandkoming van dit proefschrift was dan ook alleen mogelijk met de begeleiding, ondersteuning en hulp van velen. Bij deze wil ik iedereen die bijgedragen heeft aan dit proefschrift hartelijk bedanken voor hun inzet en de leuke en vruchtvolle samenwerking. Al zijn er uit dit onderzoek genoeg interessante leads voortgekomen die nader onderzoek verdienen, omwille van deze thesis is het tijd om er een punt achter te zetten,

MS is een rotziekte voor zowel de patiënten als hun naasten. Voor het onderzoek beschreven in deze thesis hebben veel patiënten, tijdens de onzekere tijd rond het stellen van de diagnose, bloed en hersenvocht gedoneerd. Anderen hebben toestemming gegeven voor het gebruik van hun hersenen kort na overlijden. Dit getuigt van een, door mij gedeelde, sterke wil om MS de wereld uit te helpen. Het gedoneerde materiaal is van doorslaggevend belang geweest voor mijn onderzoek. De patiënten, hun familie, vrienden en eventuele nabestaanden welke tijdens deze emotionele tijden mee hebben gewerkt aan dit onderzoek wil ik daarvoor hartelijk bedanken.

Mijn promotoren Rogier en Georges, jullie gaven me het vertrouwen dat ik de overstap van virale gentherapie naar MS onderzoek kon maken. Naast inzicht in de complexe materie van herpes virologie en neuro-immunologie was het jullie visie en vastberadenheid die het mogelijk hebben gemaakt om naar dit mooie resultaat toe te werken. Jullie zeer uiteenlopende manieren van overleggen, problemen oplossen en schrijven van manuscripten heeft mij geleerd dat er meerdere wegen zijn die naar Rome leiden. Ik heb ongelooflijk veel van jullie geleerd. Daarnaast heb ik genoten van alle gezelligheid bij congressen, feestjes en "meetings" in huiselijke kring.

Ik zou bijna vergeten dat het een beetje awkward is om trots lid van een herpesclub te zijn, want wat een fantastische club is het om onderzoek bij te doen. Monique, de manier waarop je me bij de eerste MS donor (en enige die tijdens "kantooruren" opgewerkt is) hebt gered is tekenend voor de rest van onze samenwerking. Altijd stond je voor me klaar met persoonlijk en wetenschappelijk advies of gewoon voor de gezelligheid. Bedankt voor je geduld bij mijn eindeloze stroom aan vragen over neuro-immunologie. Werner, je tomeloze energie en gedrevenheid (tot 22:00 uur) is ongekend. Ik heb genoten van de wetenschappelijke discussies over drie onderwerpen tegelijk zonder het geven van enige context. Mede dankzij jouw snelle schakelen, overzicht en inzicht in details is deze thesis

tot een goed einde gekomen. Freek, ouwe lab-reus. Je hebt me geleerd dat megaproeven altijd groter kunnen. Nu nog hoe je deze op tijd af hebt. Sarah, ik heb genoten van hoe je alles tot op het bot analyseert. Ik mis je 'six-o-clock questions'. Eef, kort maar krachtig. Suuz, leuk om even je kamergenoot geweest te zijn. Shirley, zorg jij er voor dat de herpesclub zijn mojo behoud? Je gaat er vast, net als ik, een hoop plezier beleven.

In het bijzonder dank ik mijn paranimfen Johanna en Samira. Johanna, je startte precies op het goede moment om de boel weer op de rit te krijgen. Vanaf de eerste dag ging je meteen vol gas en je was niet te stoppen, op één "stoooopid" vaccinatie na ;o). Meteen had je een klik met iedereen en dat zegt veel over jou; je bent een leuk mens. Samira, in korte tijd ging je van wat terughoudend en verlegen naar eigengereid, standvastig en zelfverzekerd. En terecht. De mooiste plaatjes van dit proefschrift hebben we aan jouw skills te danken. We hebben bergen moeten verzetten om het Acta stuk succesvol af te ronden en zonder jouw tomeloze inzet was dat nooit gelukt. Ik ben vereerd dat jullie beiden mij als paranimf willen ondersteunen. Allen bedankt voor de leuke tijd tijdens lab-stamp-sessies, congressen, feestjes, uitjes, koffietjes, etentjes, borrels, …

Iedereen van het ErasMS bij de afdeling Neurologie, veel van jullie zag ik vrijwel alleen tijdens meetings, congressen, sportieve uitstapjes en feestjes. Toch is het een hechte groep en was iedere bijeenkomst gezellig en memorabel. Naghmeh, het onderzoek wat we samen deden was niet eenvoudig maar dankzij je doorzettingsvermogen en goed getimede koffie 's-avonds, hebben we onze "negatieve" bevindingen tot een mooi positief en goed geciteerd verhaal gemaakt. Dorine, ik weet niet of ik het destijds helemaal over heb kunnen brengen, maar ik ben je zeer dankbaar voor hoe je voor Jacqueline en mij klaar stond in lastige tijden. Tessel, Daniëlle, Julia, Roos, Yuyi, Dorine, Rinze, Kim[2], Immy, jullie enthousiasme is aanstekelijk. Bedankt voor alle hulp, discussies en gezelligheid. Laten we een drank doen.

Iedereen van het ErasMS bij de afdeling Immunologie, fijn dat ik altijd kon komen aanwaaien om te sparren of voor gebruik van apparatuur en leuke labspeeltjes. Jon, al ben je al enige tijd verbonden aan de RUG, bedankt voor je begeleiding, de uitnodigingen om te mogen spreken op symposia en afdelingsmeetings en de maatwerk cursus neuro-immunologie aan het begin van het traject. Marvin, bedankt voor alle discussies, je inzet en kritische blik tijdens het schrijven van ons Acta stuk. Karim, onze ogenschijnlijk eenvoudige studie bleek nogal een rollercoaster. Zonder jouw rotsvaste vertrouwen in eigen data en kritische blik op het werk van derden was het nooit gelukt om deze studie te publiceren. Malou, promoveren kan een (z)ware beproeving zijn. Ik wens je alle succes en vertrouw erop dat je het B-cel project tot een goed einde brengt. Marie-José, Annet, Jamie, Liza, bedankt voor jullie hulp op het lab en input bij alle meetings. Hopelijk kruisen onze paden in de toekomst vaker.

Alle (ex-)collega's van de afdeling Viroscience, de afdeling heeft naast wetenschappelijk ook "quasi-wetenschappelijk" een reputatie hoog te houden. Sinterkerst, de vele borrels en het congres in Dakar, Senegal, zijn slechts enkele van de hoogtepunten waarvan ik blij ben dat ik die met jullie heb meegemaakt. Ik wil iedereen bedanken die heeft bijgedragen aan de unieke sfeer op de afdeling. Een aantal wil ik hier nog extra benoemen, zonder anderen tekort te doen. Ab, bedankt dat ik bij je op de afdeling terecht kon voor dit onderzoek, ook al bleef het woord "virus" in mijn presentaties op de afdeling soms onbenoemd. Rik, als mede vriezerachterwacht ben je de redder van vele onderzoeken, promoties en carrières, inclusief die van mij. Bedankt dat je altijd tijd voor me hebt gemaakt onder het mom van: "Ik heb nooit tijd, dus ik heb altijd tijd". Daarnaast mijn lab- en kamergenoten (My lab- and office-roomies): Rory, Tien, Selma, Joyce, Alwin, Selma, Bri, Petra, Mart, Patrick, Stella[2], Maarten, Marine, Henk-Jan, Fatiha, Selma, David, Arno, Stalin, Claudia, Debby S, Mart, and (provided the availability of calories) Do. Bedankt voor de samenwerking, (on)zinvolle discussies en gezelligheid. Virologieband "the Spikes": Maarten, Carel, Sabine, dr. Martin, Monique, Stefan, Björn, Rob, Marleen, Esther. Zonder vals over te komen; we rocked! Simone, Maria, Loubna, Anouk, Wim, Robert, Dennis, Peter, bedankt voor al het geregel. Marion, bedankt voor de kans en steun om aansluitend aan deze promotie het B-cel platform uit te kunnen rollen op de afdeling. Ik heb er zin an. Barry, Byron, Ron, Petra, Noreen, Stella en Nella, we maken er wat moois van.

Natuurlijk wil ik ook mijn familie en vrienden bedanken voor de broodnodige relativering en het bieden van een uitlaatklep wanneer in het lab de werkelijkheid zich niet naar de theorie wilde voegen. Martijn, Jean, Pjotr, Jelly en Tthijs, bedankt voor de bijeen gesprokkelde Rock & Roll punten. Pa, ma en broers Jasper en Bas, bedankt voor de onvoorwaardelijke steun, interesse en geduld. Mijn schoonouders Simon en Jarka, jullie zijn misschien wel de belangrijkste reden geweest om de switch te maken naar MS onderzoek. Het feit dat jullie ondanks tegenslagen nooit bij de pakken hebben neergezeten is inspirerend.

En dan diegenen waar het mij allemaal om te doen is: mijn drie meisjes. Het is moeilijk in woorden te vatten wat jullie allemaal voor me betekenen. Allereerst mijn grote liefde en rotsvaste pijler, Jacqueline. Door de vele 'Het-wordt-wat-later-vandaag'-appjes is er onbedoeld veel op jouw schouders terecht gekomen. Ik bewonder hoe je desondanks altijd alles onder controle hebt. Ik ben ongelooflijk gelukkig met jou. Mijn kleine lieve meisjes: Mila en Lenka. Jullie maken me onbedoeld haarfijn duidelijk wat echt belangrijk is in het leven. Ik geniet elke dag van jullie open blik, van wat jullie allemaal kunnen en wat jullie ongetwijfeld nog gaan bereiken. Ik hou van jullie!

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. Symptoms of MS include cognitive, motoric, sensory and visual impairment, pain and fatigue. The genetic background of the host and infection with the herpesvirus family member Epstein-Barr virus (EBV) are risk factors for developing MS but the pathogenic mechanisms are unknown. In this thesis we set out to clarify the putative role of EBV in MS by analyzing the intrathecal viral prevalence, breadth and magnitude of humoral and cellular EBV-specific immune responses and autoimmune responses in MS patients.