School of Medicine

Division of Clinical Neuroscience

Clinical Neurology Research Group

THE VIRAL HYPOTHESIS IN MULTIPLE SCLEROSIS: Role of Epstein-Barr virus and Human Endogenous Retroviruses

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Doctor of Philosophy

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Declaration of authenticity

I hereby declare that this thesis is my original work and has not been submitted for a degree or examination to any other university.

The experimental material presented herewith is my own work, except where otherwise stated.

The use of StatsDirect (Version 2.8.0) for the meta-analysis in Chapter 5 was supervised by Prof Zhang at the Division of Rheumatology, Orthopaedics and Dermatology, in the University of Nottingham.

Experiments in chapter 6 section 3.2 were performed with the contribution of the MSc student Deema Alghabban. She isolated the cells and performed Flow Cytometry staining under my instructions. I did the data acquisition and analysis.

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I confirm that where other sources of information have been used or quoted, this has been indicated and accordingly acknowledged by complete references.

Elena Morandi

Supervisor: Dr Bruno Gran

Co-supervisor: Prof Cris Constantinescu

Abstract

Epstein Barr Virus (EBV) is a major risk factor in Multiple Sclerosis (MS), via as yet unclear mechanisms. Several hypotheses have been proposed to explain how EBV infection could cause MS and the aim of this thesis was to better understand the mechanisms of action of EBV in the context of MS studying a) the role of EBV in myelin antigen presentation by B cells and b) the association of HERVs with MS.

In a non-human primate experimental autoimmune encephalomyelitis (EAE) model, an EBV-related lymphocryptovirus enables B cells to protect a proteolysis-sensitive immunodominant myelin oligodendrocyte glycoprotein (MOG) peptide (residues 35-55) against destructive processing. This facilitates its cross-presentation to autoaggressive cytotoxic MHC-E-restricted cytotoxic T cells. The present study extends these observations to human B cells and identifies a key role of autophagy. EBV infection upregulated antigen presentation-related markers on B cells and activated the cross-presentation machinery. Although human MOG protein was degraded less in EBV-immortalized B-lymphoblastoid cell lines (LCL) than in uninfected B cells, induction of cathepsin G activity by EBV led to total degradation of the immunodominant peptides. Inhibition of cathepsin G or citrullination of the arginine residue within a LC3-interacting regions (LIR) motif of immunodominant MOG peptides abrogated their degradation. Internalized MOG co-localized with autophagosomes, which may protect it from destructive processing. Thus, EBV infection switched MOG processing in B cells from destructive to productive possibly facilitating cross-presentation of disease-relevant epitopes to CD8+ T cells. This mechanism could facilitate presentation of myelin autoantigens that may be involved in MS induction and progression.

The first part of this thesis shows a possible EBV-mediated mechanism involved in MS pathogenesis, but it is likely that different mechanisms act alternatively or cumulatively in different individuals based on environmental and genetic differences. A further mode of action of EBV is through the activation of Human Endogenous Retroviruses (HERVs). In normal conditions HERVs are silenced or expressed at low levels, but in some pathological cases, like MS, their expression is higher than in the healthy population. We performed a systematic review and meta-analysis of the literature on the association between HERVs and MS. The systematic review suggested a strong association between HERV expression and MS, in particular with the HERV-W family. The meta-analysis showed odds ratios of 22, 44, and 6 for the expression of MSRV*pol* in serum/plasma, MSRV*env* in PBMC and MSRV*pol* in CSF respectively. Furthermore, we confirmed the association experimentally. An increased expression of MSRV/HERV-W*env* and TLR4 RNA was detected in blood of MS patients compared with control groups and the viral protein Env was expressed mainly by B cells and monocytes, but not by T cells. Our finding that EBV infection can induce the expression of MSRV/HERV-W*env* is consistent with previous reports in the literature. We also established that such increased expression was not due to a repression of retroviral restriction factors in LCL.

A further connection between HERVs and MS is supported by the observation that people infected by HIV may have a lower risk of developing MS than the HIV non-infected, healthy population. We found that the expression of MSRV/HERV-W*env* RNA in HIV-infected people was lower than in MS patients and similar to healthy controls. Nevertheless, there was no difference in MSRV/HERV-W*env* expression between antiretroviral drug -treated and -untreated HIV patients. The expression of MSRV/HERV-W*env* was also detected in vitro in LCL treated with different classes

of antiretroviral treatments (ART) and only Efavirenz (NNRI) reduced MSRV/HERV-W*env* expression.

In conclusion, taking in consideration the multifactorial aetiology of MS, it is likely that EBV infection and increased expression of MSRV/HERV-W are significant contributing factors in genetically predisposed individuals. This thesis helps to better understand the mechanisms of action of EBV and HERVs in the context of MS.

List of Publications

EBV infection empowers human B cells for autoimmunity – Role of autophagy and relevance to multiple sclerosis.

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Human Endogenous Retroviruses and Multiple Sclerosis: Causation, Association or After-Effect?

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Morandi E*, Tanasescu R, Tarlinton RE, Constantinescu CS, Zhang W, Tench C, Gran B. PLoS One.* 2017 Feb 16;12(2):e0172415.

EBV Infection and Multiple Sclerosis: Lessons from a Marmoset Model.

't Hart BA, Kap YS, ***Morandi E,*** *Laman JD, Gran B. Trends Mol Med*. 2016 Dec; 22(12):1012-1024. Review.

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TLR2 stimulation regulates the balance between regulatory T cell and Th17 function: a novel mechanism of reduced regulatory T cell function in multiple sclerosis.

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Awards

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14-17 September 2016 (London)

ICI 2016. International Congress of Immunology. Poster presentation.
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21-26 August 2016 (Melbourne)

Division of Clinical Neuroscience research day. Poster presentation.
Prize for the best poster
11 January 2016 (Nottingham)

ECTRIMS 2015. Congress of the European Committee for Treatment and Research in Multiple Sclerosis. Poster presentation.
Travel Grant award.
7-10 October 2015 (Barcelona)

MS Frontiers 2015. Conference MS Society. Oral presentation.
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To my origins, my mum and dad, my sister, my lake

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List of Abbreviations

Ab	Antibody
AEP	Asparagine endopeptidases
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cells
APRIL	A proliferation-inducing ligand
Arg	Arginine
ART	Antiretroviral therapy
Atg	Autophagy-related (Atg) proteins
BAF	Bafilomycin
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCL	B-cell Lymphoma
BCR	B-cell Receptor
BD	Bipolar disorder
BEC	Brain endothelial cell
BL	Burkitt lymphoma
BLyS	B lymphocyte stimulator
BM-DC	Bone marrow derived Dendritic cells
BMI	Body mass index
BPRC	Biomedical Primate Research Centre
BSA	Bovine serum albumin
CalHV3	Callithrichine herpesvirus-3
Cat	Cathepsin
CCL	Chemokine ligand
CD	Cluster of differentiation
CFA	Complete Freund`s adjuvant
CI	Confidence intervals
CIS	Clinically isolated syndrome
Cit	Citrulline
Cj	Common marmoset
CLIP	Class II-associated invariant chain
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T cells
CTRL	Control
CXCL	Chemokine ligand
DC	Dendritic cells
DMF	Dimethyl Fumarate
DMT	Disease modifying treatment
EA	Early antigen
EAE	Experimental autoimmune encephalomyelitis
EBER	Epstein-Barr virus encoded small RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus

EDSS scale	Expanded disability status
EDTA	Ethylenediaminetetraacetic acid
ENV	Envelope
ER	Endoplasmic reticulum
FC	Flow cytometry
FCS	Foetal calf serum
fDC	Follicular dendritic cells
FMO	Fluorescence minus one
GABA	Gamma-aminobutyric acid
GAG	Group-specific antigen
GC	Germinal centre
GCT	Germ cells tumour
GM-CSF	Granulocytes-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HC	Healthy controls
HD	Hodgkin’s disease,
HERV	Human endogenous retrovirus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leucocytes antigen
HMBS	Hydroxymethylbilane synthetase
HML	Human mouse mammary tumour virus like
HSV	Herpes simplex viruses
HTLV-1	Human T cell leukemia virus 1
IFA	Incomplete Freund`s adjuvant
IFN	Interferon
II	Integrase inhibitor
IL	Interleukin
IL-R	Interleukin-receptor
IM	Infectious mononucleosis
JCV	John Cunningham virus
KSHV	Kaposi's Sarcoma-associated herpesvirus
LC3	Light chain 3
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LIR	LC3-interacting region
LMP	Latent membrane protein
LTR	Long terminal repeats
LTα	Lymphotoxin-α
MACS	Magnetic activated cell sorter
MAIT	Mucosa-associated invariant T cells
MBP	Myelin basic protein
mDC	Myeloid dendritic cells
MDM	Monocyte-derived macrophages
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility complex

MLV	Murine leukaemia virus
MMTV	Mouse mammary tumour virus
MOG	Myelin oligodendrocyte glycoprotein
MR1	MHC class I-related molecule
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSRV	MS-associated retrovirus
NHP	Non-human primate
NK	Natural Killer cells
NKT	Natural Killer T cells
NLR	NOD-like receptors
NMSS	National Multiple Sclerosis Society
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOS	Newcastle-Ottawa assessment scale
NOS	Nitric acid synthase
NPC	Nasopharyngeal carcinoma
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
O.N.	Over-night
OCB	Oligoclonal IgG
ODN	Oligodeoxynucleotide
OND	Other neurological disease
OR	Odds ratio
PAD	Peptidyl arginine deïminase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
PLP	Proteolipid protein
PML	Progressive multifocal leukoencephalopathy
POL	Polymerase
PP-MS	Primary progressive MS
PRO	Protease
PRR	Pattern recognition receptor
PVDF	Polvinylidene fluoride
PZLF	Promyelocytic leukaemia zinc finger protein
RA	Rheumatoid arthritis
RAP	Rapamycin
rhMOG	recombinant human MOG
RIPA	Radioimmunoprecipitation assay
RLR	RIG-I-like receptors
rMFI	Relative mean fluorescence intensity
ROI	Regions of interest
RPMI	Roswell Park Memorial Institute medium

RR-MS	Relapsing-remitting MS
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
Sag	Superantigen
SE	Standard error
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
SP-MS	Secondary progressive MS
Syn-1	Syncytin-1
TCR	T-cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
TJ	Tight Junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
VCA	Viral capsid antigen
VCAM	Vascular cell adhesion molecule
VitD	Vitamin D
VLA	Very latent antigen
VSV	Varicella-zoster virus
WT	Wild type

CHAPTER 1: Introduction

1.1. Multiple Sclerosis

1.1.1. Multiple Sclerosis disease

Multiple Sclerosis (MS) is one of the most common causes of neurological disability in young adults. It is a chronic demyelinating disease of the central nervous system (CNS) characterized by inflammatory and degenerative changes in the brain and spinal cord. These features are the cause of the clinical symptoms of MS: disturbances in visual acuity that may result in blindness and double vision, motor disturbances affecting walking and use of hands, incoordination, bowel and bladder incontinence, spasticity and sensory disturbances in touch, pain, vibration and temperature sensory modalities (Sospedra and Martin, 2005). Pathological examinations of brains of patients with MS show characteristic perivascular inflammatory infiltrates, in particular T cells and macrophages, together with myelin breakdown and degeneration of axons (Stys et al., 2012). There are two main theories about the aetiology of MS (Stys et al., 2012): the traditional "outside-in" model considers MS an autoimmune disease in which T cells enter the CNS and together with macrophages and B cells destroy various elements in the CNS. The result is an inflammatory reaction that leads to demyelination and tissue injury. The second theory is the "inside-out" model. This argues that the initial event is a cytodegeneration in the CNS followed by releasing of antigenic constituents and the activation of autoimmunity and inflammation.

1.1.2. Clinical course

In 1996, the US National Multiple Sclerosis Society Advisory Committee on Clinical Trials in Multiple Sclerosis defined the clinical subtypes of MS based mainly on the

clinical context of the patient (Lublin and Reingold, 1996). In 2013, MS phenotypes were re-examined and categorized as relapsing or progressive (Lublin et al., 2014) (Fig. 1.1). Clinically isolated syndrome (CIS) is a single clinical episode of demyelination that may or may not progress to MS.

The most frequent MS course is relapsing-remitting MS (RR-MS), which accounts for 85-90 % of cases (Sospedra and Martin, 2005). It is characterised by a series of attacks that result in varying degrees of disability followed by recovery in a remission period of variable duration before the next exacerbation. RR-MS can be considered active or non-active based on disease activity detected by clinical relapses or magnetic resonance imaging (MRI) (Fig. 1.1A).

Progressive MS can be divided into primary progressive (PP)-MS (10-15% of patients) if there is progressive accumulation of disability from onset or secondary progressive (SP)-MS if the progression is after an initial relapsing course. Progressive disease can be described as active/non active and/or with/without progression (Fig. 1.1B). The factors that are responsible for different clinical courses or reliable biomarkers that can predict the clinical course remain unknown.

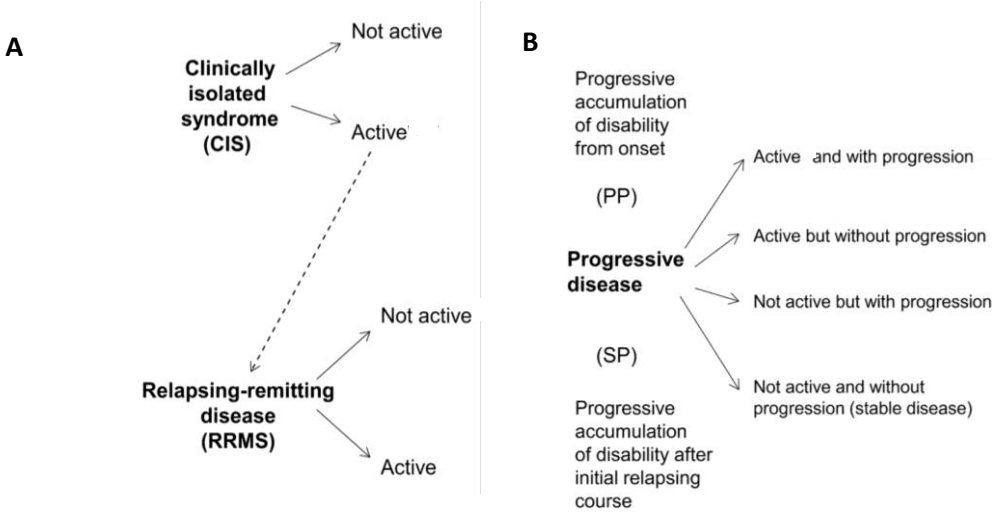


Fig. 1.1 MS Clinical course. The 2013 multiple sclerosis phenotype descriptions for **(A)** relapsing and **(B)** progressive disease. Activity is determined by clinical relapses assessed at least annually and/or MRI activity. Progression is measured by clinical evaluation, assessed at least annually. Adapted from (Lublin et al., 2014).

1.1.3. Epidemiology

The incidence and prevalence of MS increase with latitude: MS is frequent in Europe, United States, Canada, New Zealand and part of Australia, while it is rare in Asia, tropics and subtropics (Fig. 1.2). The estimated number of people with MS has increased from 2.1 million in 2008 to 2.3 million in 2013 (33 per 100,000) (ATLAS, https://www.msif.org/wp-content/uploads/2014/09/Atlas-of-MS.pdf). The UK MS Society estimates that the number of people living with MS in the UK is approximately 107,000 (166 per 100,000 people), and every year in this country there are 5,000 new diagnoses (Mackenzie et al., 2014). The risk is higher in women with an approximate female-to-male ratio of 2-3 in most populations; the incidence usually increases after 18 years of age and the peak age at onset is between 25 and 35 years (Ascherio and Munger, 2007).

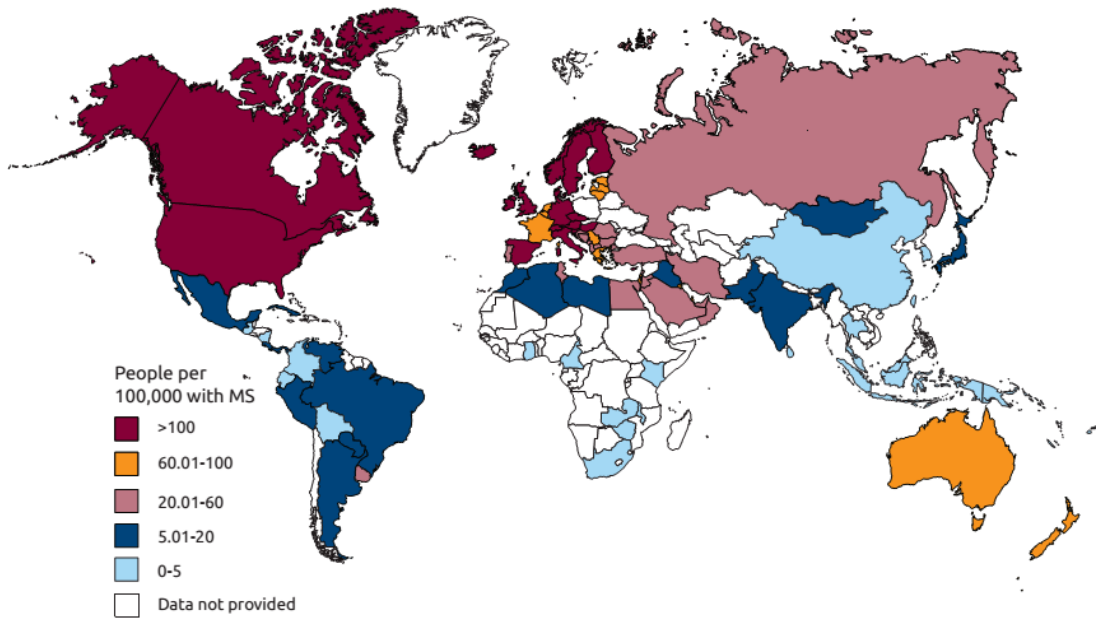


Fig. 1.2 MS Prevalence by country. Multiple Sclerosis International Federation 2013 (ATLAS, https://www.msif.org/wp-content/uploads/2014/09/Atlas-of-MS.pdf)

1.1.4. Pathology

The main pathological hallmark of MS is the formation of sclerotic plaques displaying demyelination of white and grey matter in brain and spinal cord. These plaques represent the end stage of accumulation of inflammation, demyelination and remyelination, oligodendrocytes depletion and astrocytosis, and neuronal and axon degeneration (Compston and Coles, 2008) (Fig. 1.3). Under physiological conditions, the blood brain barrier (BBB) represents a tight barrier between the circulating blood and the CNS and is formed by dense tight junctions, which seal the space between adjacent brain endothelial cells. Disruption of the BBB is a crucial event that is involved in the entry of inflammatory molecules into the brain, a prerequisite for the subsequent entry of inflammatory cells (Prat et al., 2002). Autoreactive lymphocytes mount aberrant responses against CNS autoantigens, the precise nature of which is still unresolved and the failure of local regulatory mechanisms within the brain permits the formation of MS lesions. Ongoing disease results in gradual neuroaxonal loss that correlates with patient disability and in brain atrophy accompanied by ventricular enlargement. In white matter lesions there is the presence of sclerotic glial scars formed by astrocytes. Demyelinated areas in the white matter can be partially repaired by remyelination. Inflammation is present at all stages of multiple sclerosis, but it is more pronounced in the acute as compared to the chronic phases. In early lesions macrophages dominate the infiltrate, followed by CD8+ T cells, whereas lower numbers of CD4+ T cells, B cells and plasma cells can also be found. As the disease continues, inflammatory T cell and B cell infiltrates, microglia and astrocyte activation, and diffuse myelin reduction and axonal injury are evident. This results in a more pronounced atrophy of the grey and white matter (Fig. 1.3) (Compston and Coles, 2008; Dendrou et al., 2015).

In SP-MS tertiary lymphoid structures have been found to form in the meninges, and these inflammatory aggregates may contribute to cortical demyelination and tissue injury at a later stage. In PP-MS, reduced plaque load, less evidence for inflammation, and absence of lymphoid follicles are the pathogenic features that differentiate from other MS clinical courses (Compston and Coles, 2008; Dendrou et al., 2015).

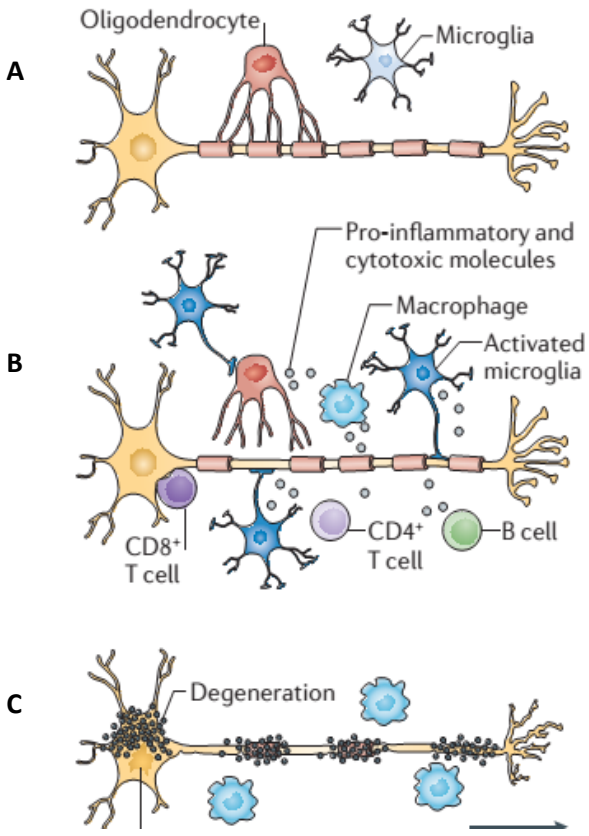


Fig. 1.3 Inflammatory neurodegeneration mechanisms. (A) Microglia typically exist in a resting state and continually survey the microenvironment. **(B)** Pro-inflammatory and cytotoxic molecules released by inflammatory cells, as well as cell contact-dependent mechanisms of T cell-mediated damage, may lead to microglia and/or macrophage activation and oligodendrocyte injury. **(C)** This process can directly or indirectly lead to axonal injury and death of the neuronal cell. Figure adapted from (Calabrese et al., 2015).

1.1.5. Diagnosis

Diagnostic criteria for MS include clinical and paraclinical laboratory assessments emphasizing the need to demonstrate dissemination of lesions in space and time and to exclude alternative diagnoses. Although the diagnosis can be made on clinical grounds alone, MRI of the CNS can support, supplement, or even replace some clinical criteria, emphasized by the McDonald Criteria of the International Panel on Diagnosis of MS (McDonald et al., 2001), that have been revised in 2010 (Polman et al., 2011). The McDonald Criteria have resulted in earlier diagnosis of MS with a high degree of both specificity and sensitivity, allowing for improved counselling of patients and earlier treatment.

1.1.6. Genetic factors

The genetic component in MS is suggested by familial aggregation of cases and high prevalence in certain ethnic populations. Several studies indicate that the risk of developing MS is higher for people with MS in the family and it is related to the number of genes that are shared with family members diagnosed with MS. The degree of familial aggregation can be determined by estimating the ratio between the prevalence of MS in siblings versus the prevalence in the population. This value is between 20 and 40 that means risk heritability (Oksenberg and Hauser, 2005). Moreover, the pairwise concordance of 20-40% in identical twin pairs compared to 2-5% in like-sex fraternal twin pairs provides additional evidence for a genetic aetiology (although concordance of 2% in siblings vs 5% of fraternal twins suggests that the maternal intrauterine environment plays a role as well). Genes within the human leucocytes antigen (HLA) complex provide the strongest genetic risk factors for MS.

Significant correlation has been found between HLA class II region on chromosome 6p21 and susceptibility to MS. This region has been refined to the DR15 and DQ6 subtypes of DR2 and DQw6 respectively, which correspond to phenotypic expression of the DRB1*1501-DRB5*0101 (DR15) and DQA1*0102-DQB1*0602 (DQ6) genotypes (Barcellos et al., 2002). In contrast, some alleles are thought to have protective effects, such as HLADRB1*01, HLA-A*02 and A2Cw7B58DR2DQ1 (Pastorino et al., 2009).

Of note, the relationship between familial recurrence risk and genetic relatedness is non-linear, suggesting that multiple risk alleles determine the susceptibility to MS, each allele with modest individual effects. Genome-wide association studies (GWAS) have proved to be a powerful tool for identifying particular genetic variants associated with complex diseases and traits. MS GWAS have detected almost 110 non-HLA single nucleotide polymorphism (SNP) related to immune function, providing further evidence that MS is an immune-mediated disease (Olsson et al., 2017). Examples are the CD24 molecule on locus 6q21, the Neuropeptide Preprotachykinin-1 (TAC1) on 7q21-22, Interleukin 4-receptor (IL-4R) on 16p11-12, Interleukin-2 receptor (IL-2R) on 10p15, Nitric Acid Synthase (NOS2a) on 17q11, β-chemokines ligands (CCLs) on 17q11, Protein kinase Cα (PRKCA) on 17q24 and Intereukin-7 receptor (IL-7R) on 5p13 (Oksenberg and Hauser, 2005).

1.1.7. Environmental factors

The rationale for an environmental contribution to the aetiology of MS is provided by a combination of genetic and epidemiologic studies.

Firstly, as reported before, genetic studies show an association with the disease and certain genes, but studies of monozygotic twins show a concordance rate of 30%, which drops to 5% in dizygotic twins (Willer et al., 2003). Moreover, genetic predisposition only partially explains the geographic variation in MS incidence, while migration and epidemiological studies show an increased disease incidence with latitude both in Northern and Southern hemispheres and differences in risk between people of common ancestry who migrate to areas of high or low MS prevalence (Ascherio and Munger, 2007). Indeed, MS risk tends to decline among individuals migrating from high to low-risk areas, and increase for those who migrate from a low-risk to a high-risk area before adolescence (McLeod et al., 2011). This evidence indicates that MS is a complex multifactorial disease in which there is an interaction between genetic and environmental factors.

In table 1.1 Odds Ratios (OR) of environmental risk factors are presented alone or in combination with HLA gene interaction.

Table 1.1 Established and possible lifestyle and environmental risk factors for MS (Olsson et al., 2017)

Factor	OR	HLA gene interaction	Combined OR (nongenetic factor + HLA allele)	Effect during adolescence	Immune system implied	Level of evidence
Smoking	~1.6	Yes	14	No	Yes	+++
EBV infection (seropositivity)	~3.6	Yes	~15	Yes	Yes	+++
Vitamin D level <50 nM	~1.4	No	NA	Probably	Yes	+++
Adolescent obesity (BMI >27 at age 20 years)	~2	Yes	~15	Yes	Yes	+++
CMV infection (seropositivity)	0.7	No	NA	Unknown	Yes	++
Night work	~1.7	No	NA	Yes	Yes	++
Low sun exposure	~2	No	NA	Probably	Yes	++
Infectious mononucleosis	~2	Yes	7	Yes	Yes	++
Passive smoking	~1.3	Yes	6	No	Yes	+
Organic solvent exposure	~1.5	Unknown	Unknown	Unknown	Unknown	+
Oral tobacco/nicotine	0.5	No	NA	Unknown	Yes	+
Alcohol	~0.6	No	NA	Unknown	Yes	+
Coffee	~0.7	No	NA	Unknown	Yes	+

Abbreviations: EBV, Epstein-Barr virus; BMI: Body mass index; CMV, Cytomegalovirus; OR, Odds Ratio.

The OR represents the odds that an outcome (MS) will occur in the presence of a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure.

1.1.8. Non-infectious factors

The non-infectious environmental factors that have had the most extensive investigation are smoking and Vitamin D (VitD).

Smoking is associated with an increased MS risk (Hedström et al., 2009) and passive exposure to smoking has also been associated with the disease (Hedström et al., 2011). On the other hand, oral tobacco (snuff) has an association with decreased risk (Hedström et al., 2009). This observation suggests that smoking may drive the risk to MS by activating autoantigen-specific cells present in the lung through lung inflammation itself and toxins contained in smoke but not in chewed tobacco.

Human populations situated at relative lower latitudes, and therefore with a greater duration and intensity of sunlight, or those populations situated at relatively higher latitudes but with a VitD rich diet have a lower MS prevalence (Kakalacheva and Lunemann, 2011). The molecular mechanism of the effect of VitD in MS remains elusive, but VitD may act as an immunomodulator: it suppresses the maturation and activity of antigen presenting cells (APC), reduces the production of pro-inflammatory T cells and increases the activity of regulatory T cells (Koch et al., 2013).

Another factor that can increase the susceptibility to MS is obesity during adolescence. Lastly, available data to support a negative association between MS and alcohol or coffee are inconclusive (Olsson et al., 2017).

1.1.9. Infectious factors

Since the early period following the characterisation of MS, infections have been proposed as a possible cause of the disease, resulting in two main hypotheses (Ascherio and Munger, 2007):

- The "*Prevalence hypothesis*" suggests that MS is caused by a pathogen that is more frequent in areas with high incidence of MS. This agent causes in most individuals an asymptomatic persistent infection, while only rarely neurological symptoms (years after the primary infection) (Kurtzke, 1993);
- The "*Hygiene hypothesis*" (poliomyelitis hypothesis), postulates that a pathogen can increase the risk of developing MS if acquired in late childhood or adulthood. Furthermore, exposure to infectious agents early in life is protective against the disease and the risk increases with the age of infection (Poskanzer et al., 1976).

In addition to epidemiological observations, immunological evidence and experiments in animal models also may provide a proof for an infectious cause in MS. For instance the presence of oligoclonal IgG (OCBs) in the CSF of at least 90% of MS patients is noteworthy (Owens et al., 2011). Usually, human CNS diseases associated with this feature are inflammatory and/or infectious, and OCBs are directed against the agent that causes the disease, while there are no genetic diseases characterised by these bands. Therefore it is possible that in the case of MS, IgG or IgM OCBs (Villar et al., 2014) target an as yet unidentified antigen, with some evidence for Epstein-Barr virus as a possible target (Cepok et al., 2005; Owens et al., 2011).

Among infectious agents Chlamydia pneumoniae (a gram-negative bacterium) has been included as candidate. Chlamydia DNA and antibodies have been detected in CSF of some patients with MS (Sriram et al., 1999), but a real association between the bacterium and the disease has not been established. Furthermore, MS brain cells have been analysed for infectious agents with different techniques, but no antigen has been detected yet. It is important to note that is unlikely that a microorganism larger than a

virus will have been missed in MS plaques given the optical resolution of electron microscopy.

Herpesviruses form a large family of enveloped double-strand DNA viruses. In total, there are more than 130 herpesviruses of which eight types can infect humans: Herpes Simplex Viruses 1 and 2 (HSV-1, HSV-2), Varicella-zoster virus (VSV), Epstein-Barr virus (EBV), Human Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV-6), Human Herpesvirus 7 (HHV-7), and Kaposi's Sarcoma-associated Herpesvirus (KSHV). These different members are similar in terms of viral structure and organization and can cause lytic or latent infection. Four main herpesviruses have been studied as possible risk factors in MS: HHV-6, VSV, CMV and especially EBV that is discussed in Section 1.3.

1.1.9.1. HHV-6

HHV-6 is a β-herpesvirus with seroprevalence of more than 95% in children by the age of 2 years (Prober, 2011). It is a neurotropic virus, reported to infect T cells, oligodendrocytes and microglia cells and can be found in a lytic or latency phase in the CNS tissue. The primary infection of the virus can lead to severe neurological complications, such as encephalitis and epilepsy. A significantly higher prevalence of HHV-6 DNA was found in brains, CSF, serum and blood of MS patients as compared with healthy control (HC) subjects (Ascherio and Munger, 2007). An association with MS was further supported by bystander activation and molecular mimicry. In fact the HHV-6 protein U24 shares sequence homology with the myelin basic protein (MBP), a suspected target of autoreactivity in MS (Kakalacheva and Lunemann, 2011). Despite that, so far there is no data that would unequivocally support an etiologic role of HHV-6 in the pathogenesis of MS (Leibovitch and Jacobson, 2014).

1.1.9.2. Varicella Zoster Virus (VSV)

VZV is a ubiquitous pathogen that establishes latency in the dorsal root ganglia in about 95% of adults (Barnes and Whitley, 1986). It is the causative agent of varicella (chickenpox), and can occasionally lead to symptomatic reactivation known as zoster. Although there are several epidemiological studies that investigated a link between VZV and MS, and in some of these the presence of VZV DNA was reported in blood cells and CSF from RR-MS patients, a meta-analysis including 40 reports revealed insufficient evidence to support such association (Marrie and Wolfson, 2001).

1.1.9.3. Cytomegalovirus (CMV)

CMV has a seroprevalence ranging from 45% to 100% worldwide (Cannon et al., 2010), but usually the infection remains asymptomatic in the vast majority of infected individuals. This virus infects epithelial and endothelial cells, monocytes and lymphocytes. Several studies have been performed on the association between CMV and MS, but many had non-significant results. A recent study showed a negative association of CMV seropositivity with paediatric MS (Waubant et al., 2011) and the same result has been obtained in a large population of adult-onset MS pathology (Sundqvist et al., 2014). Moreover a meta-analysis of 13 previous studies confirmed this negative association (Sundqvist et al., 2014). One hypothesis is that EBV and CMV are involved in an immune response competition and in double-seropositive individuals there could be a balance in immune response between these viruses, while in CMV seronegative subjects only EBV could trigger the immune system towards an MS phenotype (Sundqvist et al., 2014). Another possible mechanism is molecular mimicry. Indeed, in experimental autoimmune encephalomyelitis (EAE) in rhesus monkeys a cross-reaction between CMV and the myelin oligodendrocyte glycoprotein (MOG) has been shown, without inducing EAE (Brok et al., 2007).

1.2. The immune system in MS and its animal models

1.2.1. Introduction to the immune system

Immunity is a state of resistance to infection and the immune system is constituted by a wide variety of cells and secreted molecules. It can be classified in innate and adaptive immunity (Owen et al., 2013; Todd et al., 2015). Innate immunity is rapidly activated in the early stage of an infection and its role is to limit the spread of a pathogen within the body. This type of immunity is moderately efficient and its capabilities remain the same on repeated exposure to the same microbe. Therefore, the resolution of an infection usually also requires adaptive immunity. Adaptive immunity takes longer to activate but generates a defence that is more effective (specificity) and improves upon repeated exposure to the same microbe (memory). Innate and adaptive immunity are complementary and cooperate in order to give the best defence. The main actors in adaptive immunity are T and B lymphocytes. They possess different surface receptors that enable them to specifically recognize individual antigens (Owen et al., 2013; Todd et al., 2015).

MS was thought to be mediated principally by CD4+ T cells that react against myelin antigens (Frohman et al., 2006), but recently also other cell types, such as B cells (Michel et al., 2015), have been reconsidered. These immune cells are activated in the periphery and express adhesion molecules which facilitate interactions with ligands present on vascular endothelial cells, resulting in extravasation across the BBB (Michel et al., 2015; Compston and Coles, 2008) (Fig.1.4). Once in the CNS, these myelin-reactive cells contribute to the demyelination and progressive axonal pathology characteristic of MS (Frohman et al., 2006).

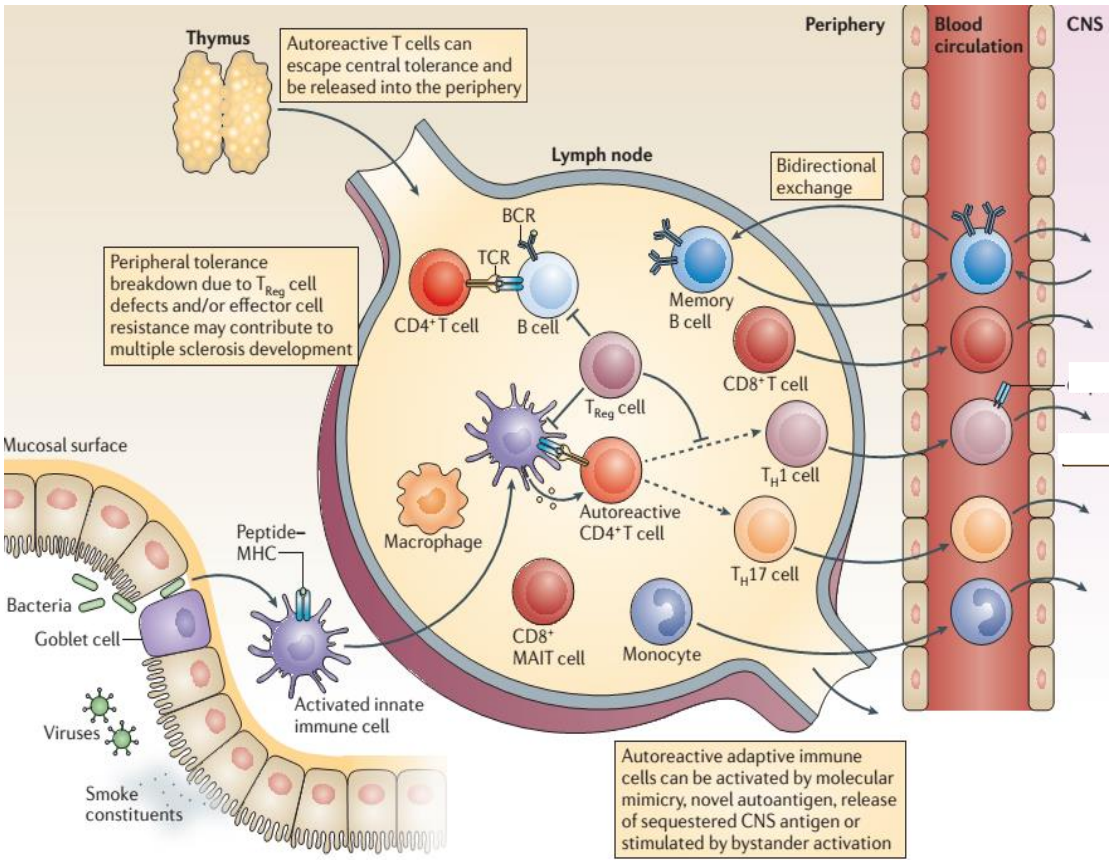


Fig. 1.4 Immune system dysregulation in MS. During the establishment of central tolerance in the thymus, most autoreactive T cells are deleted; however, this process is imperfect, and some autoreactive T cells are released into the periphery. In health, peripheral tolerance mechanisms keep these cells in check. If this tolerance is broken CNS-directed autoreactive B cells and T cells can be activated in the periphery to become aggressive effector cells. CD8 + T cells, differentiated CD4+ T helper 1 (Th1) and Th17 cells, B cells and innate immune cells can infiltrate the CNS, leading to inflammation and tissue damage. B cells trafficking out of the CNS can undergo affinity maturation in the lymph nodes before re-entering the target organ and promoting further damage. Dashed arrows indicate differentiation. BCR, B cell receptor; CD8+ MAIT cell, CD8+ mucosa-associated invariant T cell; TCR, T cell receptor (Dendrou et al., 2015).

Such immunological mechanisms are explained in the next sections.

1.2.2. Antigen processing and presentation

B and T lymphocytes can recognize antigens in different forms. B cells can recognize an antigen in its native unmodified state through its B cell receptor (BCR) or through antibodies, while T cells can only recognize a processed antigen bound to HLA (or major histocompatibility complex, MHC) expressed by APC.

Antigen processing is the process that involves partial degradation of protein antigens by proteases to yield antigenic peptides. In this way peptides can be of the appropriate length and amino acid sequence to bind HLA and be presented to T cells (Owen et al., 2013; Todd et al., 2015).

Classically endogenous and exogenous antigens follow two different pathways. Peptides derived from the nucleus and the cytosol are generated from proteins synthesized in the cells that are marked for disposal (e.g. ubiquitination) or that signal a viral infection. These proteins are degraded trough the proteasome, a protease complex designed to carry out selective and efficient hydrolysis of proteins, and transported to the endoplasmic reticulum where they will be associated with the HLA class I. If the cell is infected by a virus, this will produce interferons. Interferons induce the expression of a different set of proteosomal enzymes that form the immunoproteasome. Peptides (8-10 amino acids) presented on the cell surface associated with the HLA-I have the ability to activated cytotoxic CD8+ T cells (Fig. 1.5A).

Exogenous antigens are internalized by different ways depending on the cell. Dendritic cells (DC) and macrophages perform endocytosis that permits the entry of material bound to specialized cell surface receptors (e.g. pattern recognition receptors PRRs, Fc receptor, complement receptors). DC can also engulf large quantities of

surrounding fluid through micropinocytosis. B cells use their BCR to bind with high affinity conformational protein and after BCR clustering they internalize the antigen. After the uptake, antigens enter endosomes, cytoplasmic vesicles that contain proteases called cathepsins with the ability to specifically degrade antigens. In the meantime, in the endoplasmic reticulum the newly synthesized HLA class II associates with the invariant chain and migrates to the endosomes. Once the HLA-II is in the endosome, this will bind the antigen through the release of a specific part of the invariant chain (Class II-associated invariant chain, CLIP) that was occupying the peptide-binding groove. Only professional APC, such as DC, macrophages and B cells, express HLA-II (Owen et al., 2013; Todd et al., 2015) (Fig. 1.5B) In contrast to HLA-I, the HLA-II binding groove is open and peptides presented can be 14-18 amino acids long. Under certain conditions, however, shorter or longer peptides can be presented. The HLA-II transports the antigen on the cell surface ready to be presented to the CD4 T cells. Optimal presentation requires (Stoeckle and Tolosa, 2010):

- Limited proteolytic cleavage of the peptide;
- Competition between different peptides on the base of binding affinity;
- Binding of long peptides fragments to HLA heterodimers and further trimming by exopeptidases;
- Final editing of bound peptides.

In some situations, cross-presentation of antigens between the HLA-I and -II pathways can occur. In particular, it has been reported that DC have the ability to divert exogenous antigens into the class I processing pathway for presentation to cytotoxic T cells (Dudziak et al., 2007). Moreover, this mechanism has also been observed in

primary B cells stimulated with a CpG DNA motif (Heit et al., 2004; Jiang et al., 2011). (Fig. 1.5C)

Regarding autoimmune diseases, the sequence of events that lead the processing and presentation of autoantigens is still incompletely understood. The presence of self-reactive cells, which escape the central and peripheral tolerance, is thought to be required for the emergence of autoimmunity, but it should be noted that these cells are also present in healthy people.

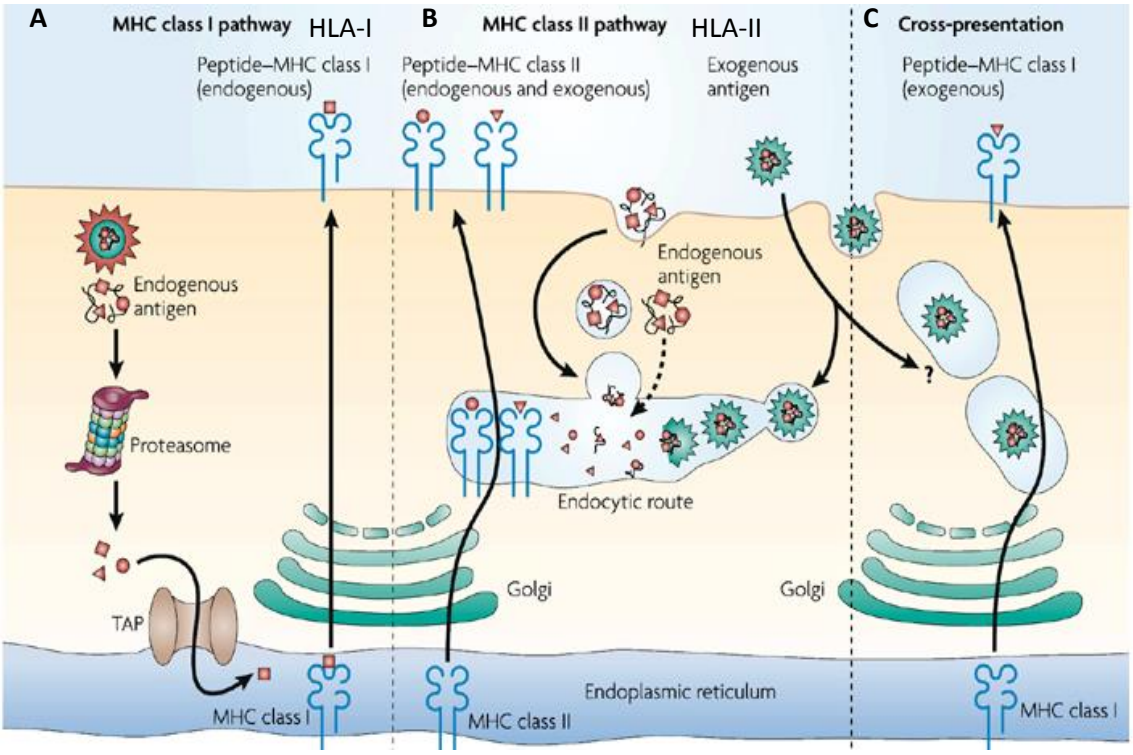


Fig. 1.5 The antigen-presentation pathways. (A) MHC-I (or HLA-I) molecules present peptides that are derived from endogenous proteins degraded mainly in the cytosol. **(B)** MHC-II (or HLA-II) molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous components, such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that access the endosomes by autophagy. **(C)** Some cells have the ability to deliver exogenous antigens to the MHC-I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood. Adapted from (Villadangos and Schnorrer, 2007).

1.2.3. T cells

1.2.3.1. T-cell development and thymic selection

The precursors of T lymphocytes leave the bone marrow at very early stage and migrate to the thymus (becoming thymocytes). At this stage each thymocyte expresses both T cell surface glycoproteins cluster of differentiation (CD)4 and CD8 that are members of the immunoglobulin superfamily. Each thymocyte is subjected to two contrasting selective processes (Education): positive selection and negative selection. This selection depends on the interaction between the T cell receptor (TCR) and HLA protein on the surface of cortical epithelial cells. Thymocytes can fail to bind self-HLA and undergo programmed cell death by apoptosis. Those thymocytes whose TCR interacts with low avidity with HLA-I maintain the expression of CD8 and lose CD4, while those thymocytes whose TCR interacts with HLA-II maintain the expression of CD4 and lose CD8. Negative selection involves thymocytes that strongly react with high avidity with autoantigens associated with HLA proteins on thymic stromal cells. These autoreactive T cells undergo apoptotic death and this deletion is called central tolerance. Central tolerance occurs in the thymus and bone morrow, while peripheral tolerance is in lymph nodes and other tissues and their role is to prevent over-reactivity of the immune system to various self-antigens. When self-reactive cells escape central and peripheral tolerance, autoimmunity can be induced (Fig.1.4) (Owen et al., 2013; Todd et al., 2015).

1.2.3.2. CD4+ T helper cells (Th)

CD4+ T cells recognise antigen complexed with HLA-II, which is expressed by professional APC. Based on their cytokine secretion and transcription factor expression, CD4+ T cells were divided into four major subsets: T helper (h)1, Th2, Th17 and Tregs (Legroux and Arbour, 2015).

Th1 cells express mainly interferon-γ (IFN-γ) and tumour necrosis factor (TNF) and are the key cellular immune response to intracellular pathogens. Th1 have been considered causative agents in MS since MS animal models are driven by this T cell subset. Some groups documented that myelin specific CD4+ T cells secreted INF-γ are sufficient to transfer the disease into naïve mice (Fletcher et al., 2010). In humans Th1 are increased in MS patients in relapse compared to MS remission and HC (Nakajima et al., 2004). A correlation between INF-γ secretion and contrast-enhancing lesions in MS was also found (Calabresi et al., 1998; Tejada-Simon et al., 2001) and intravenously injection of IFN-γ caused increase of relapses in a small pilot trial (Johnson and Panitch, 1989). Despite this, injection of antibodies blocking IFN-γ augmented the disease severity in the mouse (Lublin et al., 1993).

In 2005 a new subset of CD4+ T cells was identified (Harrington et al., 2005). Th17 cells express IL-17, IL-21 and IL-22 and their main role is to provide protection against certain bacterial and fungi infection (Harrington et al., 2005). Proinflammatory Th17 cytokines are present in higher amount in MS patients compared to HC (Matusevicius et al., 1999) and the frequency of Th17 cells is significantly higher in the CSF of patients with RR-MS during relapse in comparison to remission or to patients with other neurological diseases (OND) (Brucklacher-Waldert et al., 2009). Moreover, cells that product both IFN-γ and IL-17 had a greater capacity to cross the human BBB and were detected in post-mortem brain tissues (Kebir et al., 2007; Murphy et al., 2010).

Transfer of activated myelin-specific Th17 cells can induce the disease in naïve mice (Langrish et al., 2005), but IL-17 is not indispensable for EAE induction (Haak et al., 2009).

More recent some studies pointed out the crucial role of granulocyte-macrophage colony-stimulating factors (GM-CSF) (Codarri et al., 2011). This cytokine can be secreted by both Th1 and Th17 cells and is upregulated in MS patients (Rasouli et al., 2015). GM-CSF deficient mice were resistant to the induction of EAE (McQualter et al., 2001; Codarri et al., 2011).

Th2 cells express mainly IL-4, IL-5, and IL-13 and play an essential role in the defence against extracellular parasites and in allergy. In EAE, initially it was reported that Th2 cells specific for myelin antigens could induce EAE upon adoptive transfer (Lafaille et al., 1997), but more recently another study failed to show the same result (Jager et al., 2009). Moreover, in some instances, induction of a Th2 response during ongoing autoimmune inflammation can have therapeutic effect probably due to IL-4 that is known to inhibit Th1 differentiation and to suppress EAE (Constantinescu et al., 2001).

1.2.3.3. Regulatory T cells (Tregs)

Tregs were initially identified as a subtype of CD4+ T cells expressing the α-chain of IL-2 receptor (IL-2Rα) CD25 that had the function to protect against the development of autoimmunity (Sakaguchi et al., 1995). It was discovered that Tregs were involved in tolerance to self and non-self in mice and humans (Asano et al., 1996). Many groups investigated Tregs taken from peripheral blood of MS patients and they found that these cells have impaired regulatory functions and migratory properties, but not necessarily altered frequency compared to cells from HC (Viglietta et al., 2004). In mouse EAE, the depletion of Tregs using an anti-CD25 monoclonal antibody resulted in increased susceptibility to EAE (McGeachy et al., 2005). Myelin specific Treg cells are able to migrate and accumulate in the CNS in animals with EAE. Interestingly,

Treg cell accumulation and frequency in the CNS have been shown to correlate with recovery from EAE (McGeachy et al., 2005).

1.2.3.4. CD8+ T cells

CD8+ T cells recognise antigens complexed with HLA-I and can exert cytotoxic functions. The implication of CD8+ T cells in the pathophysiology of MS is suggested by the presence of these cells, in a greater number than CD4+ T cells, in the brain lesions of MS patients (Lucchinetti et al., 2011). These lesion-infiltrating CD8+ T cells express granzyme B and IFNγ, and these cells were shown to be able to cause axonal damage (Sauer et al., 2013). Indeed, up-regulation of HLA-I molecules has been observed in MS lesions early during the course of disease before demyelination develops (Gobin et al., 2001). Overall, MS patients have been reported to display elevated responses of autoreactive CD8+ T cells specific for a variety of neuroantigens in comparison to HCs (Crawford et al., 2004).

1.2.4. B cells

1.2.4.1. B-cell development

B cells originate in the bone marrow. BCR and secreted antibodies derived from five immunoglobulin classes which differ in the heavy chain: IgM, IgD, IgG, IgA and IgE. B cells precursors are pre-B cells that produce only IgM. Bone marrow stromal cells provide a variety of signal to guide B cells along the developmental pathway. If B cells generate receptors that recognise self-antigen, they will undergo either apoptosis (anergy) or rearrangements of the light chain (receptor editing). Naïve B cells that leave the bone marrow express both IgM and IgD on their surface. They will move to

lymphoid follicles of the spleen and lymph nodes, where they encounter and respond to thymus-independent antigens that directly stimulate B cell proliferation or thymus-dependent antigens that require help from T cells and have to be presented on HLA-II on follicular (f)DC. Lymphoblastoid cells will then proliferate, and either differentiate into plasma cells and start to synthesize large quantities of immunoglobulin molecules or enter Germinal Centre (GC) reactions. The GC reactions are characterized by clonal expansion, class switch recombination, somatic hypermutation, and selection for increased affinity of a BCR for its unique antigenic epitope through affinity maturation. Lymphoblasts can also revert their state in a resting state and form a memory population specific for the antigen that induced the primary response (Owen et al., 2013; Todd et al., 2015).

In MS, B cell GC-like structures were discovered in brains of patients with SP-MS (Serafini et al., 2004). These aggregates were always localized adjacent to subpial lesions, within structures reminiscent of GC containing B cells, plasma cells, T cells, macrophages and a network of fDC comparable to classical peripheral lymphoid follicles (Serafini et al., 2004; Aloisi and Pujol-Borrell, 2006). Brains containing B cell aggregates exhibited more severe grey matter lesions with increased demyelination and neuronal damage while white matter lesions did not differ from other MS patients (Magliozzi et al., 2007).

1.2.4.2. B cells as precursors to antibody-producing cells

Most MS patients show IgG oligoclonal bands in the CSF, indicating an intrathecal antibody (Ab) synthesis. These Abs are produced by plasma cells, but their specificity is not clear yet. Myelin-reactive Abs have been found in MS patients, but their role is still controversial. Autoreactive Abs in the CNS can act through opsonisation,

facilitating Fc receptor mediated phagocytosis and cytotoxicity, or activating the complement system. Indeed, antibodies and complement deposition have been noticed in active demyelinating lesions (Lucchinetti et al., 2000)

1.2.4.3. B cells as antigen-presenting cells

The importance of the key role of B cells in MS is reflected in new results from clinical trials. Indeed, B-cell depletion with anti-CD20 monoclonal Ab (rituximab or the humanized ocrelizumab) is effective in reducing disease activity in both RR-MS and PP-MS (Hauser et al., 2008; Hauser et al., 2017; Montalban et al., 2017). CD20 is a glycosylated phosphoprotein expressed on the surface of all stages of B cell development except on either early pro-B cells or plasma blasts and plasma cells. In the clinical study immunoglobulin levels were not significantly altered in number, suggesting that other B cells functions, rather than autoantibodies production, are important in MS (Cross et al., 2006).

In the context of EAE, it was reported that EAE induced with recombinant MOG protein, was independent of myelin-specific antibodies and required B cells with APC function (Molnarfi et al., 2013; Parker Harp et al., 2015).

1.2.4.4. B cells as proinflammatory cytokine-producing cells

B cells of MS patients produced abnormally higher levels of TNFα, Lymphotoxin-α (LTα), IL-6 and IL-15 upon stimulation (Li et al., 2015a). In particular, GM-CSF-producing B cells were abnormally increased in MS patients and B cell depletion in patients with MS resulted in a B cell GM-CSF-dependent decrease of pro-inflammatory myeloid-cell responses (Li et al., 2015b).

1.2.4.5. Regulatory B cells (Bregs)

B cells expressing IL-10 and IL-35 possess anti-inflammatory roles. In humans, both

naïve and memory B cells are capable of producing IL-10 in a context-dependent manner. Upon CD40-ligand stimulation naïve B cells were able to produce IL-10, but this response was found to be abnormally deficient in B cells of MS patients (Duddy et al., 2007). On the other hand, another study reported the opposite result (Iwata et al., 2011).

In mice deficient for IL-10 selectively from B cells, EAE was more severe (Fillatreau et al., 2002), and adoptive transfer of in vitro-induced IL-10-producing B cells suppressed EAE in an IL-10-dependent manner (Fillatreau et al., 2002; Yoshizaki et al., 2012). Similarly, B cells producing IL-35, an anti-inflammatory cytokine, were found to play important roles in recovery from EAE (Shen et al., 2014).

1.2.5. Innate immunity

1.2.5.1. Monocytes/macrophages

Monocytes are blood mononuclear cells and are renewed continually from bone marrow hematopoietic stem cells throughout their life. During inflammation, monocytes migrate from the bloodstream into affected tissues, including the CNS, where they differentiate into "infiltrating" macrophages (Ransohoff and Cardona, 2010). Based on different environmental signals, macrophages can direct their phenotype into a number of functional phenotypes (at least nine distinct programs (Xue et al., 2014)), including M1, proinflammatory macrophages and M2, quiescent/anti-inflammatory macrophages (Michell-Robinson et al., 2015). Active MS lesions are characterized by the presence of macrophages that predominantly display M1 characteristics, but a major subset of these cells also co-express M2 markers (Vogel et al., 2013). Indeed, phenotypically similar macrophages in the CNS can not only contribute to the generation of inflammatory lesions and perform a pathogenic role in the demyelination process, but can also contribute to regenerative repair mechanisms to resolve inflammation (Greter et al., 2015). Monocytes/macrophages have been implicated in inducing neural pathology in MS by secretion of toxic molecules, antigen presentation to cytotoxic T lymphocytes, and degradation of synapses (Miron and Franklin, 2014).

In EAE, the destruction of myelin and axons as well as oligodendrocyte cell death were directly related to inflammation and to the presence of monocytes/macrophages (Miron and Franklin, 2014).

Microglia have often been referred to as resident macrophages in the CNS; and only in the past few years, it has been possible to differentiate between monocyte-derived

macrophages (MDM) and microglia. True microglia are resident mononuclear phagocytes of the brain parenchyma that originate during embryogenesis from the yolk sac and are maintained independently of hematopoietic stem cells (Katsumoto et al., 2014). When the CNS is inflamed, microglia can differentiate into macrophages, whose functions are distinct from those of infiltrating monocytes. In the healthy brain, microglia physically contribute to brain development and homeostasis, including regulation of cell death, synapse pruning and elimination, neurogenesis, and neuronal surveillance (Katsumoto et al., 2014).

1.2.5.2. Natural Killer (NK) cells

NK cells contribute to both effector and regulatory innate immune functions through cytotoxic activity mainly against viral infected cells or tumour cells and through cytokines secretion (Moretta et al., 2008). Based on their cytokine pattern, NK cells can be subdivided into subtypes that have a proinflammatory or regulatory function. Studies in human MS suggest a beneficial effect of various subsets of NK cells. During relapses a decreased cytotoxic activity of circulating NK cells has been described (Gandhi et al., 2010) and subset of NK cells was reported to negatively regulate the activation of antigen-specific autoreactive T cells (Takahashi et al., 2004).

In EAE, NK cells were shown to be mainly protective in the course of disease and depletion of NK cells led to EAE exacerbation (Xu et al., 2005). However, other studies suggested that NK cells may also be pathogenic when they found that depletion of NK cells exacerbated EAE and decreased IFN-γ and TNF-α production (Winkler-Pickett et al., 2008).

1.2.5.3. Natural Killer T cells (NKT)

Natural killer T (NKT) cells are a particular subset of T cells that share characteristics of NK cells and T cells. Classical NKT cells (iNKT) recognise glycolipid antigens presented by CD1d, a lipid monomorphic glycoprotein. These cells are capable of producing large amounts of cytokines, such as IFN-γ, IL-10, IL-4, IL-13 and transforming growth factor (TGF)-β, mainly associated with protection from autoimmunity. In MS patients a decreased number of iNKT cells was reported (Gandhi et al., 2010). In EAE, it was reported that activation of iNKT cells reduced IFN-γ but increased IL-10 and IL-4 production leading to EAE suppression (Singh et al., 2001).

Besides iNKT cells, diverse subsets of T cells that co-express a TCR and NK cell antigens have been identified, termed NKT-like cells, among which mucosal-associated invariant T (MAIT) cells. In human peripheral blood, MAIT cells usually represent 1–10% of T lymphocytes. They are rare in lymphoid tissues, and preferentially home to peripheral tissues, such as the gut, the lung, and the liver. MAIT cells are restricted by a monomorphic MHC class I-related molecule (MR1) and exert an important antimicrobial function (Bianchini et al., 2017). The contribution of these cell subsets to the pathogenesis of MS has been investigated both in patients with different forms of MS and in the EAE mouse model. Contradictory observations have been reported, and it is yet not clear whether they exert a protective or a pro-inflammatory and harmful role (Miyazaki et al., 2011; Annibali et al., 2011).

1.2.5.4. Dendritic cells (DC)

DC are professional APC that transport antigens from tissues to local lymph nodes and can prime naïve T cells by presenting processed antigen complexed with HLA-II. DC are classified into different categories based on their surface markers and a widely accepted classification distinguishes human DC into myeloid (m)DC and lymphoid/plasmacytoid (p)DC (Gandhi et al., 2010). In MS patients, DC have been reported to express increased levels of activation markers and secretion of proinflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-23 (Vaknin-Dembinsky et al., 2008), suggesting a role in MS pathogenesis. In addition, altered DC phenotype and dysfunctional interaction of DC with T cells has been reported in MS patients (Navarro et al., 2006).

EAE could be induced by the transfer of bone marrow derived DC (BM-DC) presenting MOG_{35-55} into naïve mice (Weir et al., 2002). Furthermore, mDC have been demonstrated to accumulate in the CNS during EAE, where they could present myelin auto-antigens to CD4+ T cells inducing their differentiation in Th17 cells (Bailey et al., 2007).

1.2.5.5. Innate immune receptors (TLR, CLR, etc)

The innate immune system can initiate rapid and potent responses to microbes recognised on the basis of the expression of pathogen-associated molecular patterns (PAMPs). DC, tissue macrophages, microglia, and other innate immune cells express pattern recognition receptors (PRRs) that recognize PAMPs. Several types of PRRs cooperate in responses against pathogens, including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors (ALRs) (Akira et al., 2006). TLRs are type I transmembrane proteins consisting of three

domains, which include the extracellular leucine-rich repeat (LRR) receptor with avidity for PAMPs, a transmembrane and the intracellular toll-interleukin (IL)-1 receptor (TIR) domain, which interacts with downstream adapter proteins. TLRs could possibly play a dual role as both a proinflammatory and anti-inflammatory molecules in MS (Podda et al., 2013).

TLR2 is ubiquitously expressed by immune (both innate and adaptive) cells, endothelial cells, epithelial cells and nervous system cells and it has the unique ability to form heterodimers with either TLR6 or TLR1 and thus recognizes a large variety of PAMPs from bacterial, viral or also endogenous ligands (Hossain et al., 2016). The effect of TLR2 activation is tissue and ligand-specific. It can induce inflammation in MS and EAE inducing Th17 and reducing Treg response (Nyirenda et al., 2015), but it could also promote Th2 response and exert anti-inflammatory effects (Nyirenda et al., 2009; Hossain et al., 2016).

1.2.5.6. TLR4

TLR4 is a cellular transmembrane receptor that detects LPS, a component of the outer membrane of Gram-negative bacteria, and it is expressed by macrophages, mDC, mast cells, and B cells.

In cultures of human microglia, LPS-stimulation of TLR4 or TLR3 by double strand RNA induced the secretion of chemokine ligand (CXCL)10, which in human brain can attract T cells towards MS lesions (Jack et al., 2005). Moreover, in human MS lesions TLR4 and TLR2 were reported to induce the expression of the proinflammatory cytokine IL-23 in macrophages and microglia (Li et al., 2007).

In EAE TLR4 is involved in the induction of the disease due to its recognition of the adjuvant substances during the immunisation process. In mice TLR4 showed both

regulatory and proinflammatory function. Small doses of LPS administrated prior to the induction of EAE was reported to delay disease onset, without affecting severity. This effect was associated with an early production of TNF-α, TGF-β, and IFN-β (Buenafe and Bourdette, 2007). In addition, TLR4 deficient mice developed more severe EAE than wild-type (WT) mice (Marta et al., 2008) and the loss of TLR4 solely in CD4 + T cells was reported to abrogate disease symptoms almost completely, mainly through blunted Th17 and, to a lesser degree, Th1 responses (Reynolds et al., 2012).

Moreover TLR4 stimulation was also shown to break immune tolerance in a PLP-specific TCR transgenic mouse strain resistant to EAE, activating APCs and rendering it susceptible to disease (Waldner et al., 2004).

1.2.5.7. TLR9

TLR9 is expressed on B cells and DC and it is essential for responses to bacterial and viral DNA. It also recognises synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides (CpG DNA). CpG dinucleotide frequency and methylation status differs between vertebrates and bacteria (Krieg et al., 1995). CpG dinucleotides are generally present at the expected frequency of 1 per 16 dinucleotides in microbial DNA, but they are only one-quarter as prevalent in vertebrates. In addition, they are highly methylated in vertebrates, but not in bacteria. Methylation of CpG abolished mitogenicity, and this is the main reason why bacterial CpG dinucleotide can be recognized by the vertebrate immune system and enhance innate and adaptive immunity. Activation of TLR9 on B lymphocytes with CpG leads to proliferation, production of antibodies and IL-6, increased expression of CD86, CD40, CD54 and MHC-I and II (Hartmann and Krieg, 2000). Naïve B cells are poor APC, but, interesting, when they are activated with CpG they become potent APC for class

I HLA-restricted T cell responses (Jiang et al., 2011). The mechanism at the base of this phenomenon is still not clear, but this study showed that also B cells, and not only DC, have the ability of cross-presentation.

In T cells stimulation with the TLR9 ligands CpG ODN reduced the suppressive functions of Tregs, but on the other hand TLR9 may also exert a protective effect in MS through the induction of IL-10 in B cells (Podda et al., 2013). TLR9 expression and TLR9-induced IL-10 production was reduced in B cells of MS patients compared to controls (Hirotani et al., 2010). TLR9 has also been reported to contribute to the recognition of EBV by human primary monocytes and pDC (Fiola et al., 2010) suggesting that such TLR may mediate some of the putative effects of EBV in human MS.

In EAE, TLR9 deficient mice exhibited more severe EAE than WT mice (Marta et al., 2008).

1.2.6. Candidate myelin self-antigens in MS

1.2.6.1. Proteolipid Protein

Proteolipid protein (PLP) is the most abundant myelin protein. There are two isoforms: the full-length PLP is exclusively expressed in the CNS, while DM20, a splice variant of PLP, is expressed in peripheral organs such as the thymus and lymph nodes and only DM20 plays a role in negative selection in the thymus (Klein et al., 2000). The epitope $PLP_{139-151}$ seems to be a target of autoreactive T cells which can escape central tolerance in the thymus, but enhanced reactivity to this epitope has not been confirmed and T-cell responses to PLP has been reported to be quite heterogeneous (Riedhammer and Weissert, 2015).

1.2.6.2. Myelin Basic Protein

Myelin basic protein (MBP) is an important candidate autoantigen in MS. This is the second most abundant myelin protein after PLP and it can be found in both central and peripheral myelin. MBP reactive T cells have been shown in MS patients and HC, with significantly more IFN-γ-secreting T cells in response to different MBP peptides in MS patients than in controls (Moldovan et al., 2003).

The central region of MBP_{84-102}, MBP_{83-99}, can bind to several HLA-DRB molecules and was identified as immunodominant in several studies (Martin et al., 1991; Valli et al., 1993; Martin et al., 2001; Mycko et al., 2004). However, results varied with respect to differences in reactivity to MBP_{84-102} between MS patients and HC.

Other epitopes have been described as immunodominant are MBP_{13-32}, $MBP_{144-163}$, $MBP_{143-168}$ and $MBP_{151-170}$ and higher reactivity to these peptides was seen in MS patients (Riedhammer and Weissert, 2015). It was also shown that T-cell reactivity against myelin components can correlate with disease activity (Bielekova et al., 2004).

1.2.6.3. Myelin Oligodendrocyte glycoprotein

Myelin oligodendrocyte glycoprotein (MOG) is a minor component of the myelin sheath. It was not detected in the thymus and therefore it might evade presentation in central tolerance induction. On the other hand, MOG and other myelin antigens have been observed in cervical lymph nodes and perivascular spaces of MS patients (Kooi et al., 2009).

Higher numbers of autoreactive T cells to DRB1*0401/MOG_{97-109}-tetramer or to MOG_{1-20} and MOG_{35-55} epitopes have been detected in MS patients compared to HC (Wallstrom et al., 1998; Varrin-Doyer et al., 2014).

Interestingly, some data suggest that MOG could play a role in humoral autoimmunity in MS. MOG-specific autoantibodies have been discovered in acute lesions of MS patients (O'Connor et al., 2005). Some studies also found higher levels of MOG-specific Abs in the serum of MS patients than in control sera (Khalil et al., 2006). However, other studies did not observe any differences between the sera or CSF of MS patients and HC or patients with OND (Karni et al., 1999; O'Connor et al., 2005). Anti-MOG Abs were shown to be demyelinating in an EAE experiment (Zhou et al., 2006).

Data about a possible association of anti-MOG-antibodies (and anti-MBP-antibodies) with a progression from CIS to definite MS remain controversial, rendering these antibodies difficult the use as biomarkers (Ramanathan et al., 2016).

1.2.7. Animal models of MS

1.2.7.1. Rodent models of MS

Experimental autoimmune encephalomyelitis (EAE) is the most used MS animal model. EAE is a spectrum of neurological disorders that is induced in laboratory animals following the induction of autoimmunity to CNS elicited either by immunization with CNS antigen such as CNS homogenate, myelin proteins, fusion proteins and single myelin peptides or by passive transfer of myelin protein-specific CD4+ T cells (Constantinescu et al., 2011).

Inbred and specific pathogen free (SPF) laboratory strains of mice and rats are used for studying MS disease pathogenesis and drug screening. Immunization with antigens requires the administration of complete Freund`s adjuvant (CFA). The bacterial components of the adjuvant provide danger signals which help to overcome tolerance mechanisms. Injection of autoantigen formulated with adjuvants lacking microbial antigens, such as incomplete Freund's adjuvant (IFA) mineral oil, usually fails to evoke EAE in SPF rodents; immune tolerance mechanisms are enforced instead.

Relapsing remitting EAE can be induced with $PLP_{139-151}$ in SJL mice (McRae et al., 1992) and chronic EAE induced with MOG_{35-55} in C57BL/6 mice (Iacomini et al., 1995). In these models Th1 and Th17 cells are induced. CNS lesions in EAE are characterized by inflammation and demyelination caused by myelin-specific CD4+ T cells, which translocate into the CNS followed by permeabilization of the BBB. In the CNS, these T cells are reactivated by local and infiltrating APCs, which present MHC-II-associated peptides, leading to inflammatory processes and the eventual demyelination and axonal damage (‘t Hart et al., 2011).

Although the rat has become less frequently used, various active rat EAE models have been developed (Adelmann et al., 1995). Dark Agouti (DA) rats with EAE present mainly focal spinal cord, cerebellar lesions and optic neuritis, Brown Norway (BN) rats develop clinical features similar to neuromyelitis optica, while Lewis (LEW.1AR1) rats can also present cortical lesions(‘t Hart et al., 2011).

Important discrepancies exist between EAE mice or rats and MS patients (Steinman and Zamvil, 2006; ‘t Hart et al., 2011; Lassmann and Bradl, 2017). First MS develops spontaneously without an obvious external trigger, while EAE requires the injection of autoantigen formulated in potent adjuvants. Second, EAE lesions in rodent models are predominantly observed in the spinal cord whereas in MS lesions are commonly found in the brain as well. Third, immunological differences, such as the absence of CD8+ T cells in brain lesions, reflect the large evolutionary distance between rodents and man. Finally, these models comprise a limited number of genetically homogeneous mice that are not exposed to environmental factors.

1.2.7.2. Non-human primate models of MS

Although EAE mouse model was sometimes useful in the translation of some immunomodulatory drugs from the animal model to MS (e.g. glatiramer acetate, natalizumab), this was not always the case, indicating the requirement of a link between the mouse model and the human disease. In non-human primate (NHP) models these differences are less evident. The common marmoset (Cj) is a small-bodied neotropical primate that breeds well in captivity. Marmosets share the outbred nature and a high degree of genetic, immunological, and microbiological similarity with humans. Indeed the MHC class II region of the marmoset, termed Caja, comprises equivalents of HLA-DR-DQA and -DQB and marmosets, unlike mice, are naturally

infected with a lymphocryptovirus (LCV) related to EBV, or callithrichine herpesvirus-3 (CalHV3), which infects B cells (‘t Hart et al., 2015).

In the marmoset model two different pathways of the EAE disease can be recognized. In the classical rhesus macaque or marmoset EAE model, after the administration of recombinant human MOG_{1-125} (rhMOG) in CFA, the initiation pathway starts and this comprises the classical synergy of pro-inflammatory MHC-II-restricted Th1 and Th17 cells, the production of anti-MOG antibodies and inflammation in the white matter of brain and spinal cord. This mechanism is comparable between mice and primates. When marmosets are immunized with $MOG_{34–56}$ peptide in IFA, the progression pathway is predominant. Selective activation of autoaggressive CD8+ T cells and prominent demyelination in the white and cortical grey matter of marmoset brains is observed, presenting more similarity with human MS (‘t Hart et al., 2015).

1.2.8. Current immunotherapies for MS

In the last 20 years the range of treatments for MS has incredibly expanded, although MS patients can currently benefit only from disease modifying treatments (DMT) that can improve the course of the disease, but without curing it. So far, more than 10 DMTs have been approved for RR-MS (Comi et al., 2016).

Interferon β (IFN-β) is an injectable drug and was the first drug approved for RR-MS. It is a cytokine with anti-inflammatory properties, reducing the presentation of myelin antigens, preventing lymphocytes crossing of the BBB, inducing Treg and IL-10, and reducing Th1 and Th17 cells (Kasper and Reder, 2014).

Glatiramer acetate is another injectable drug composed of a mixture of peptides of four amino acids from MBP that induces an anti-inflammatory immune response similar to IFN-β. This action includes driving DC, monocytes, T cells and B cells

towards anti-inflammatory phenotype, competition for the binding of antigen presenting cells, and downregulating of both Th1 and Th17 cells promoting a Th2 immune response (Aharoni, 2014).

IFN-β and glatiramer acetate have similar therapeutic efficacy in terms of 30% of relapse rate reduction and MRI activity decrease. For these drugs side-effect are rare and often present as reactions at the injection site due at the frequent injections (Comi et al., 2016).

Teriflunomide, Dimethyl fumarate and Fingolimod are oral drugs. Teriflunomide is an active metabolite of leflunomide that inhibits the proliferation of autoreactive B and T cells preserving the replication of haematopoietic and memory cells. Moreover, it can induce a shift to anti-inflammatory response. Similarly to IFN-β, Teriflunomide reduces the relapse rate and MRI lesions volume.

The exact mechanism of action of Dimethyl fumarate (DMF) remains to be elucidated. It acts on the immune system reducing the lymphocyte count, promoting a Th2 shift and an anti-inflammatory DC differentiation. In addition, DMF can exert neuroprotective function reducing toxic oxidative stress (Kretzschmar et al., 2016).

Fingolimod is a sphingosine 1-phosphate analogue that acts as antagonist of sphingosine 1-phosphate receptor, essential to lymphocytes to leave the lymphoid tissue. The main effect of Fingolimod is a decreased number of circulating lymphocytes with a reduced entry in the CNS. In clinical trials Fingolimod reduced the relapse-rate by 48-54% compared with placebo (Doggrell, 2010), and 38-52% compared with IFN-β (Cohen et al., 2010). Common adverse events include lymphopenia, liver enzyme level abnormalities, bradycardia and opportunistic infections.

Natalizumab is a humanised monoclonal antibody directed against the α-4 integrin, component of the very late antigen (VLA)-4 expressed by lymphocytes, that binds the vascular cell adhesion molecule 1 (VCAM-1) on the BBB and mediates the entry of lymphocytes in the CNS. Natalizumab in one clinical trial reduced the relapse rate by 68% and the worsening of disability by 42% (Polman et al., 2006). Cases of progressive multifocal leukoencephalopathy (PML) with a mortality rate of more than 20% were reported during the treatment. PML is caused by the reactivation of John Cunningham (JC) virus, and because 50% of the population is JC seropositive, the risk can be stratified (Comi et al., 2016).

Alemtuzumab is a humanised monoclonal antibody directed against CD52 present on the surface of lymphocytes and monocytes, causing B and T cell depletion. In a comparative trial with IFN-β, Alemtuzumab reduced the relapse rate by 74% and the worsening of disability by 71% although it was associated with autoimmunity (Coles et al., 2008),

All these DMTs are effective for RR-MS, but not for the progressive forms of MS.

Ocrelizumab is a humanised monoclonal antibody directed against CD20 expressed by B cells. This was the only drug effective in RR-MS and also in PP-MS. In two identical phase 3 trials (OPERA I and OPERA II), ocrelizumab was compared with IFN-β and the annual relapse rate was 46% lower with ocrelizumab than with IFN-β (Hauser et al., 2017). In patients with PPMS, ocrelizumab was compared with placebo for a treatment period of at least 120 weeks and until a pre-specified number of confirmed disability progression events had occurred. Ocrelizumab was associated with significantly lower rates of clinical and MRI progression than placebo (Montalban et al., 2017).

1.3. Epstein Barr Virus

1.3.1. The biology of the virus

1.3.1.1. EBV infection

EBV is the Human Herpesvirus 4 and it belongs to lymphocryptovirus or gamma subfamily of herpesviruses (Ascherio and Munger, 2010). EBV can infect both naïve and memory B cells, and epithelial cells with two different mechanisms. To enter B cells, the viral glycoprotein gp350 binds to cellular receptor CD21 and then, viral glycoprotein gp42 interacts with cellular MHC-II molecules. On the other hand, to enter epithelial cells, viral protein BMRF-2 interacts with cellular β1 integrin. These interactions trigger the fusion of the viral envelope with the cell membrane, allowing EBV to enter the target cell (Thorley-Lawson, 2015).

This virus is very common and infects over 90% of individuals within the first decades of life (Ascherio and Munger, 2010). Primary infection usually occurs through contact with infected saliva and is asymptomatic during the acute phase, whereas in adolescents and adults is symptomatic in 75% of cases and presents as infectious mononucleosis (IM).

1.3.1.2. Replication cycle

The EBV virus is a particle of about 120 nm and it has a double strand linear DNA containing about 85 genes. The DNA is enclosed by a protein capsid that is surrounded by a tegument and then by an envelope.

When EBV infects a cell, the genome converts to an episome typically present as a single copy at a frequency of 1 to 50 per million B lymphocytes and the virus enters the latency phase. During the latency there is no production of virions and EBV

typically persists for the life of the host. There are three latency programmes that seem to parallel the physiological process of B cell maturation (Fig1.6) (Thorley-Lawson, 2015). EBV is spread through saliva and crosses the epithelial barrier of Waldeyer's ring to interact with naïve B cells. Upon infection of the naïve B cells, it drives the infected cells to mature into proliferating blasts using the growth transcription program in which nine latent proteins are expressed (Latency III, EBNA1 to 6, LMP1, 2A and 2B, Table 1.2). Then EBV-infected blasts migrate into the follicle where they switch their transcription program to the default program which provides surrogate antigen and T cell help signals (Latency II, EBNA1, LMP1, LMP2A, Table 1.2). Later the latently infected B cells leave the follicle as memory B cells which are quiescent with respect to viral latent protein expression and they can be dividing (Latency I, EBNA1 only program) or resting (Latency 0) cells. If an infected, resting, memory B cell latently infected with EBV returns to Waldeyer's ring and receives signals that initiate plasma cell differentiation, it will trigger the release of infectious virus entering the lytic phase. Lytic proteins are produced in three consecutive stages: immediate-early (BZLF1, BRLF1, Table 1.2), early (BNLF2, Table 1.2), and late (gp350, gp220, VCA, Table 1.2).

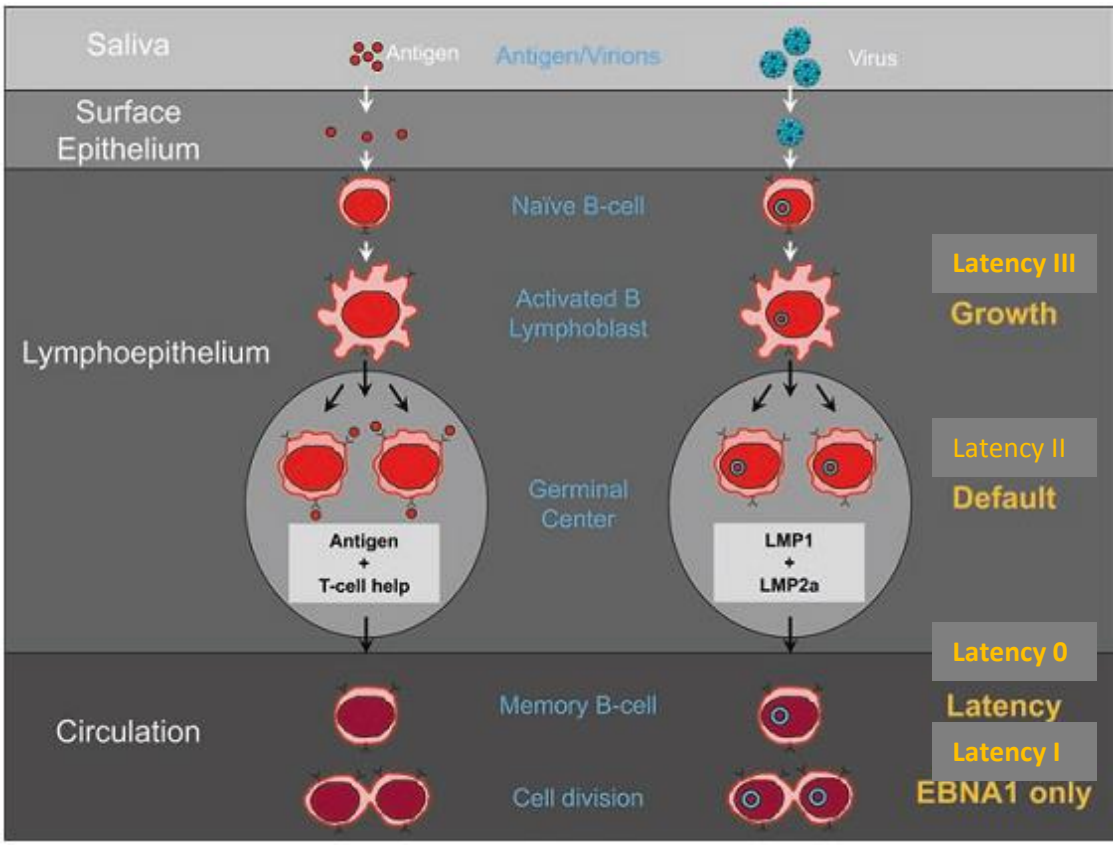


Fig. 1.6 EBV biology mirrors B cell biology. The left diagram shows a typical mucosal humoral immune response. Antigen in saliva is recognized by naïve B cells that become activated blasts and migrate to the follicle to undergo a germinal centre (GC) reaction. If they receive signals from antigen and antigen-specific Th cells, they can leave to become resting memory B cells that occasionally undergo division as part of memory B cell homeostasis. The right diagram shows how EBV uses the same pathways. EBV is spread through saliva, crosses the epithelial barrier, and infects naïve B cells. These become B cell blasts that enter the GC. Here, the viral latent proteins LMP1 and LMP2 have the capacity to provide surrogate antigen and Th survival signals that allow the latently infected B cells to leave the GC as resting memory cells that also divide through homeostasis. (Adapted from Thorley-Lawson, 2015a).

In addition to genes for these proteins, some noncoding RNAs are also expressed. These include Epstein-Barr virus encoded small RNAs (EBERs), which can be secreted from latent cells.

EBV-transformed B cells in vitro are called lymphoblastoid cell lines (LCL). They are immortalized B cells that express all the proteins of the latent phase (Latency III).

Table 1.2 EBV proteins and their functions (Farrell, 2015)

EBV proteins	Phase	Function
EBNA LP (EBV Nuclear Antigen Leader Protein)	*Latent*	Regulator of latent viral gene transcription
EBNA1 (EBV Nuclear Antigen 1)	*Latent*	It maintains the viral episome, enhances EBER transcription and inhibits its own proteasome processing
EBNA2 (EBV Nuclear Antigen 2)	*Latent*	Major regulator of viral gene transcription. Upregulates EBV specific and cell promoters
LMP1 (Latent Membrane Protein 1)	*Latent*	Constitutive CD40 mimic oncoprotein, it induces cytotoxic T lymphocyte apoptosis, upregulates IL-10
LMP2A (Latent membrane protein 2A)	*Latent*	Constitutive B cell receptor mimic, it increases degradation of IFN receptors and induces cytotoxic T lymphocyte apoptosis
LMP2B (Latent Membrane Protein 2B)	*Latent*	Regulator of LMP2A and LMP1 function
BZLF1	*IE-Lytic*	Transactivators that bind viral and cellular promoters to activate viral gene expression
BRLF1	*IE-Lytic*	
BNLF2	*E-Lytic*	Involved in DNA replication and metabolism
VCA (Viral Capsid Antigen)	*L-Lytic*	Structural protein of the capsid virion
Gp350	*L-Lytic*	Virion binding to CD21, B cell infection
Gp220	*L-Lytic*	Virion binding to CD21, B cell infection

Abbreviations: *IE*, immediate-early; *E*, early; *L*, late.

1.3.1.3. Subtypes of EBV

There are two main strains of viruses: type 1 and type 2. They differ in the sequences of the latent genes (primarily in EBNA-2) and in the number of repeats within the genome. In US, Europe and Southeast Asia EBV-1 is more common, while in Africa and New Guinea the frequency of EBV-1 and EBV-2 are similar (Abdel-Hamid et al., 1992). It is unclear whether type 1 and type 2 viruses differ in the spectrum of diseases they cause or in the frequency with which they cause them. The current view is that both viruses can cause disease with the same efficiency (Farrell, 2015).

1.3.2. Immune system and EBV

1.3.2.1. Immune response to EBV

The initiation of EBV-specific immune control is probably mediated by APC, in particular by DC which are cross presenting EBV antigens detected by TLRs from infected B cells to CD4+ and CD8 T+ cells and to NK cells (Chijioke et al., 2013).

TLR3 and TLR9 seem to be the TLRs involved in this mechanism and their activation due to the stimulation of DC and NK cells, causes IFN-γ production, delaying latent EBV antigen expression (Chijioke et al., 2013). After the innate immunity, EBV infection causes B cell activation with increased IgG, IgM and IgA levels. At onset, during the infection, IgM antibodies to VCA and to early antigens (EA) are detected (although non-EBV specific antibodies are also produced), while IgG to VCA and to EBNA-1 are produced in a later stage.

An expansion of CD8+ T cells is found mostly against EBV lytic antigens although as the infection progresses, also against latent antigens. A small CD4+ T cell response

has also been detected, but, in healthy EBV carriers the CD8+ T cell count is considerably higher than the CD4+ T cell (Taylor et al., 2015).

1.3.2.2. EBV immune evasion strategies

EBV utilizes different strategies to evade detection and elimination by the host immune system. It can modulate either the immune cell function, or the antigen presentation pathways, or apoptotic pathways (Hatton et al., 2014).

First, EBV encodes some lytic cycle proteins such as BCRF1and BARF1 that have the ability to suppress the production of anti-viral molecule including IFN-γ, IFN-α, IL-2, and IL-6 by CD4+ T cells (Cohen and Lekstrom, 1999; Jochum et al., 2012).

Second, EBNA-1 contains a glycine–alanine repeat that inhibits its processing and presentation by HLA-I (Tellam et al., 2004). Moreover some early lytic proteins (BNLF2a, BGLF5 and BILF1) prevents the expression and the peptide loading of HLA-I molecules (Zuo et al., 2009).

Finally, EBV has evolved several tactics to prevent apoptosis of the infected cell in order to augment viral persistence. A functional B-cell lymphoma (bcl)-2 homolog encoded by BHRF1 can inhibit apoptosis (Desbien et al., 2009). Moreover LMP signalling can modulate cell death pathways. Indeed LMP1 is a functional homolog of CD40 and can provide survival signals in concert with LMP2A which mimics BCR signalling (Snow et al., 2006).

1.3.3. EBV role in disease

1.3.3.1. Infectious Mononucleosis

IM is usually a self-limited disease of a few weeks and it is the result of a marked increase in circulating EBV–specific cytotoxic T lymphocytes and release of inflammatory cytokines. Clinical symptoms are pharyngitis, fever, fatigue, cervical lymphadenopathy and hepatosplenomegaly. The reason why some individuals develop IM and not others, remains to be determined. Researchers have presented evidence for a genetic influence, showing increased concordance of IM within monozygotic versus dizygotic twins and identifying polymorphisms in immune response genes that correlate with EBV infection or disease severity (Taylor et al., 2015). In addition, the strong correlation between IM and adolescence age could be explained by the more intense viral transmission or by the age-related differences in CD8 T lymphocytes response (Ascherio and Munger, 2010), but until now it is still unknown.

1.3.3.2. EBV-associated cancers

It is well established that EBV latent proteins can drive the proliferation of B lymphocytes and their deregulated expression could play a causative role in tumour development. Convincing epidemiological, serological, and molecular biological evidence associate EBV infection to lymphoma in the immunosuppressed, Hodgkin's disease (HD), and Burkitt lymphoma (BL) (Thorley-Lawson, 2015). Patients who are immunosuppressed, such as transplant patients (particularly EBV seronegative graft recipients) and late stage acquired immune deficiency syndrome (AIDS) patients, have a suppressed immune response that allows uninhibited growth of EBV-infected cells.

These are Latency III malignancies in which B cells express a full spectrum of latent EBV proteins.

HD is a tumour of germinal centre cells and tumour cells are characterized by the expression of EBNA1 and LMP1 and 2 (Latency II).

BL is characterized by the expression of a single EBV protein (EBNA1 whose phenotype suggests a germinal centre origin (latency I)). BL cells are well adapted to escape cytotoxic T cells (CTL) recognition since they have down-regulated expression of MHC-I and of the transporters associated with antigen processing (TAP-1 and/or TAP-2).

EBV can also establish malignancies in a non-B cell environment. Examples of non-B-cell malignancies include some epithelial tumours, such as the nasopharyngeal carcinoma (NPC) that has a latency II phenotype (Thorley-Lawson, 2015; Taylor et al., 2015).

1.3.3.3. EBV-associated autoimmune-mediated diseases

Although EBV has been suspected as a causing agent in many autoimmune diseases, only for MS and systemic lupus erythematosus (SLE) there is evidence of an association. There is little to no epidemiological evidence in favour of a role of EBV in juvenile rheumatoid arthritis and myasthenia gravis (Ascherio and Munger, 2015). Virtually all SLE patients, whether adult or paediatric, are EBV-seropositive (Hanlon P, 2014). Nevertheless, there is no mechanistic explanation as to how EBV infection might predispose to SLE. The two main hypothesis are molecular mimicry in humoral response to EBNA1 and at least three targeted antigens in SLE (e.g. Sm/Ro ribonucleoproteins) and the induction of IFN- α through TLR activation in DC by EBV DNA and RNA (EBERs) (Kang et al., 2004).

1.3.4. EBV role in MS

1.3.4.1. Association between EBV and MS

The link between EBV and MS is proposed first of all to explain the similarity between the epidemiology of IM and of MS in terms of age, geographical distribution, socioeconomic status and ethnicity. Both diseases are rare in developing countries where hygiene is poor and most children are infected with EBV very early without developing IM. On the contrary, in western countries, where just 50% of individuals are infected with EBV during adolescence and young adulthood, MS risk is two-to threefold higher among individuals with history of IM (Ascherio and Munger, 2010). This would suggest that the late EBV infection has a principal role in MS development (Handel et al., 2010). In a case-control study among military personnel including 305 individuals who developed MS and 610 matched controls, time of EBV infection was determined by measuring antibody titers in serial serum samples collected before MS onset. 3.3% cases and 5.2% controls were initially EBV negative. All of the 10 EBV-negative cases became EBV positive before MS onset; comparatively, only 35.7% of normal control samples seroconverted (Levin et al., 2010). This suggests that EBV is a requirement for MS prior to development. Interesting, the risk for people seronegative is about 15 times lower than that of EBV-positive individuals (Ascherio and Munger, 2007). This observation strongly supports that EBV infection is an important risk factor, or, an alternative explanation is that individual EBV negative are genetically resistant to both virus infection and MS.

In addition, there are further observations that confirm the link EBV-MS. A systematic review and meta-analysis of the epidemiological association between EBV and MS, taking in consideration 39 studies from 1960 to 2012, found, as previous reviews, a significant difference for seropositivity to anti EBNA IgG and anti-VCA IgG in MS

cases, but not to anti-EA IgG, indicative of recent infection (Almohmeed et al., 2013). In a further study, not only IgG, but also EBNA-1 specific memory CD4 T cells were found elevated in the blood of MS patients (Lunemann et al., 2006). Moreover a recent study (Kvistad et al., 2014) confirms the positive correlation between anti EBNA1 IgG antibodies and MRI disease activity already reported in other independent papers (Ascherio and Munger, 2010). In addition to anti-EBV antibodies being increased in the serum of MS, they are also elevated in the CSF, which could indicate specific synthesis of antibodies against EBV or simply reflect a polyspecific antibody synthesis (Pender, 2011). Further indirect evidence of the presence of EBV in the CNS, is provided by the finding that EBV-specific CD8+ T cells are enriched in the CNS in early MS but not in patients with other neurological disease. Again, effects on relapse rate in MS due to the depletion of CD20+ B cells by rituximab (Hauser et al., 2008), could be another proof of the association between EBV and MS.

1.3.4.2. Viral pathogenesis of EBV in MS

Several hypotheses have been proposed to explain how EBV infection could cause MS: 1) the EBV bystander damage hypothesis 2) the EBV cross-reactivity hypothesis due to molecular mimicry 3) the αB-crystallin hypothesis 4) the EBV-infected autoreactive hypothesis 5) the EBV-infected APC hypothesis and 6) the Human Endogenous Retrovirus hypothesis.

1) The EBV bystander damage hypothesis: In meninges of patients with progressive disease and very severe inflammatory and neurodegenerative pathology, large B cell aggregates with features of lymphoid B cells follicles similar to germinal centres have been found (Serafini et al., 2007). Recent studies using different techniques such as immunohistochemistry for EBV latent and lytic proteins, in situ hybridization for EBER and reverse

transcriptase-polymerase chain reaction (RT-PCR) for EBV nucleic acids, show a higher presence of EBV-infected B cells in MS post-mortem brain tissue compared with other CNS inflammatory diseases (Serafini et al., 2010; Serafini et al., 2013). Infiltration of EBV-infected B cells could drive the immunopathology of MS eliciting cytotoxic T lymphocyte response and activating TLR3 by EBER. This immune response could induce type I IFN and proinflammatory cytokines with damage to surrounding tissue (Iwakiri et al., 2009). However, other research groups were unable to detect EBV in brain tissues of the same and other cohorts of patients with MS using similar techniques (Lassmann et al., 2011).

2) The EBV cross-reactivity hypothesis due to molecular mimicry: The molecular mimicry between EBV and myelin antigens proposes that T cells primed by exposure to EBV antigens cross-react with CNS antigens. Consistent with this hypothesis is the presence of specific EBNA-1 CD4+ T cells that can cross react with myelin antigens (Lunemann and Munz, 2009), but this mechanism does not explain the unique role of EBV since all infectious agents have the potential to induce cross-reactivity with CNS (Pender, 2011).
3) The αB-crystallin hypothesis: The exposure of lymphoid cells to EBV induces the expression of αB-crystallin (heat shock protein) and there is the possibility that the immune system recognizes the endogen protein as a microbial antigen generating a CD4+ T cells response (Van Noort et al., 2000).
4) The EBV-infected autoreactive hypothesis: This hypothesis proposes that in genetically susceptible individuals it is present a deficiency of the cytotoxic CD8+ T cells that normally keep EBV infection under tight control (Pender et al., 2009). EBV-infected autoreactive cells could produce pathogenic

oligoclonal antibodies and costimulatory survival signals to autoreactive T cells that inhibit their apoptosis. In this way the autoreactive T cells could attack the CNS and lead to spreading of the immune response to CNS antigens (Pender, 2011).

5) The EBV-infected APC hypothesis: Latent EBV infection confers survival advantages to B cells by mimicking signals of T cell help and B cell receptor, but also it induces modification in other biological mechanisms of B cells. It could be that these EBV-infected B cells have a different expression of enzymes that process antigens and present them to T cells in a different way compared to uninfected cells. The first part of this thesis will test this hypothesis (Part 1, Chapters 2, 3 and 4).

6) The Human Endogenous Retrovirus hypothesis: EBV can activate the expression of human endogenous retrovirus that in turn can activate innate immunity. The second part of this thesis will test this hypothesis (Part 2, Chapters 5, 6, 7, and 8).

1.4. HERVs

1.4.1. The biology of HERVs

Retroviruses are enveloped viruses with single-strand positive RNA genome. After the infection of the target cell, they can reverse transcribe their RNA and integrate the DNA product into the cellular chromosomes, forming a provirus. Occasionally some types of retrovirus can infect germ line cells and colonize the host`s germ line by forming endogenous retroviruses. From 70 to 30 million years ago, exogenous retroviruses integrated themselves into human`s cells of the germ line, becoming part of the human DNA and being transmitted through a Mendelian pattern by generations. Indeed, almost 8% of the human genome is constituted of human endogenous retroviruses (HERVs) ranging in copy number from one to many thousands. They are part of our history and evolution and not always retain only a passive role (Gifford and Tristem, 2003). The genomic persistence of HERV sequences during evolution led to the accumulation of several mutations, insertions and deletions that have generally compromised their coding capacity. Even though mutations and control mechanisms prevent their expression, some HERVs maintain anyway the ability of reverse transcription, protein expression and viral particles production in many normal tissues and cultured cells. In fact, probably between 7 and 30% of all HERV sequences in the genome is transcriptionally active and the extent of expression varies from tissue to tissue and also between individuals. Nevertheless their activity seems to be modulated in pathological conditions such as in cancer and autoimmune diseases (Voisset et al., 2008).

1.4.2. Structure and genome

HERVs have the same gene structure of exogenous retroviruses. The *gag* gene encodes the structural components of matrix, capsid and nucleocapsid; the *pro-pol* genes determine the production of the three viral enzymes protease, reverse transcriptase (RT) and integrase; and the *env* gene is responsible for encoding the envelope surface and transmembrane elements (Fig. 1.7). The 5′- and 3′ long terminal repeats (LTR) delimitate the genome and the promoter and enhancer regions in the LTR regulate HERV expression. Probably HERVs had initially the capacity of retrotrasposition or reinfection, but during the time, in the passage from a generation to the next, endogenous retroviruses have accumulated a series of mutations or recombination events and the evolution has selected only integrations more harmless to humans (Gifford and Tristem, 2003). HERVs that have recently invaded the germ line are more likely found as whole virus with all the full-length sequence, while older HERVs are usually more truncated. The majority of HERVs (90%) are found as a single LTR. (Kassiotis, 2014) Although infectious ERVs have been identified in some organisms such as cats, mice and koala, human ERVs cannot replicate due to mutations in *pol* and *gag* genes. In fact no HERVs have been shown to date to produce infectious virions (Antony et al., 2011).

Fig. 1.7 HERV genome. LTR (Long Terminal Repeat) regions bound the genome with four major viral genes: *gag* (encoding matrix and retroviral core), *pol* (reverse transcriptase and integrase), *pro* (protease), and *env* (envelope).

1.4.3. HERV families

There is no standard nomenclature for HERVs. They are classified into three classes on the base of their sequence identity with known exogenous retroviruses: class I, II and III respectively similar to gammaretroviruses, betaretroviruses or spumaviruses. These classes are divided into several families in which the letter added to HERV (HERV-W, HERV-K, HERV-H..) corresponds to the tRNA specificity of the primer binding site. To date, according to this classification, 31 HERV families have been identified, while, considering the sequence homology, HERVs can be grouped in 80 distinct families (Gifford and Tristem, 2003). They can include some exogenous and horizontally transmissible strains, so their transmission can be Mendelian or non-Mendelian (Perron et al., 2009).

The W family is the most studied family of HERVs and it is found very often associated with MS. In the DNA this is a multi-copy gene family integrated 40 million years ago. HERV-W elements occurred on all chromosomes showing no recognizable cluster distribution, except chromosome 16 that apparently do not contain HERV-W sequences. A recent study using complex bioinformatics tools reported the presence of 213 HERV-W sequences located mainly in intergenic regions, with the exception of 55 elements inserted into human coding genes, almost exclusively into intronic regions (Grandi et al., 2016).

Another well-studied HERV family associated with MS is HERV-Fc, integrated about 10-15 million years ago. This is part of the HERV-H/F family and has almost complete *gag, pol, pro* and *env* genes. Transcripts from HERV-Fc genes have been detected in different human tissues (testis, skin and trachea), suggesting that their promoters are active.

HERV-K, called also HML (Human mouse mammary tumour virus like), is the most young family of human proviruses, integrated recently (30 million years ago) in the human genome and these retroviruses are phylogenetically similar to exogenous betaretroviruses. In our genome probably about 550 HERV-K proviruses and 6400 solitary LTRs exist. More than 90 proviruses of this group maintain an intact open reading frame with the ability of encode viral proteins.

1.4.4. HERVs role in disease

1.4.4.1. HERV-associated cancers

The first tumorigenic infectious human retrovirus, the human T cell leukaemia virus 1 (HTLV-1), was described for the first time in 1980 in T cell lymphoma/leukaemia (ATL) (Robert-Guroff et al., 1982). Although HERVs have been suspected to be involved in cancer, real evidence for a causal role of ERVs for tumorigenesis has been shown only in the murine model (Kassiotis, 2014). Nevertheless, HERV-encoded RNA and proteins have been detected in germ cells tumour (GCT), melanoma, breast cancer, ovarian cancer, endometrial carcinoma, astrocytoma and neuroblastoma (Ruprecht et al., 2008).

Potential mechanisms that link HERVs with cancer are described below. Transformation by insertional mutagenesis involves insertional activation of oncogenes or disruption of tumour-suppressor genes. These mechanisms have been described in the mouse studying the murine leukaemia virus (MLV) and the mouse mammary tumour virus (MMTV) (Fan and Johnson, 2011). Nevertheless, in humans no replication-competent HERV has been discovered.

Some retroviral proteins have been shown to act as tumour-promoting proteins. In particular, two accessory proteins, Rec and Np9, produced as alternative splicing products of the HERV-K env gene are frequently found in transformed cells from GCT tumours but not in healthy cells (Ruprecht et al., 2008). These two accessory proteins interact with the promyelocytic leukaemia zinc finger protein (PZLF) and co-expression of Rec and Np9 with PLZF abrogated the transcriptional repression of the c-Myc gene promotor by PLZF and resulted in c-Myc overproduction, leading to increased cell proliferation and reduced apoptosis (Denne et al., 2007).

In addition to the effect of HERV proteins, noncoding RNAs of certain HERVs could have more direct effects on genome function. For instance, HERV-K11 provirus can transcribe noncoding RNAs that binding the premRNA splicing factor PSF inhibits its function of proto-oncogenes repressor (Li et al., 2009).

Moreover, the repetitive nature of HERVs provides an ideal substrate for nonallelic homologous DNA recombination, which results in chromosomal rearrangements. Examples include recurrent chromosomal translocations in human prostate cancer, which create fusions between a HERV-K provirus and an otherwise dormant oncogene of the ETS (E26 transformation–specific) family (Tomlins et al., 2007).

1.4.4.2. HERV-associated autoimmune diseases

Association of HERVs with MS is discussed in the second part of this thesis (Chapter 5). Besides MS, other autoimmune diseases have been associated with HERVs, including type 1 diabetes mellitus, rheumatoid arthritis (RA), and SLE.

Potential mechanisms that link HERVs with autoimmune disease are described below. Molecular mimicry could be one possible autoimmune mechanism. For instance, molecular mimicry due to sequence similarity between HERV-K10 gag and IgG1Fc (a key target for rheumatoid factor) could mediate the autoimmune response (Nelson et al., 2014). In addition, molecular mimicry between ERV-3 env protein and a number of lupus autoantigens plus collagen type IV has been observed also in SLE (Trela et al., 2016).

HERV-K18 is implicated in type 1 diabetes through the encoding of a superantigen (SAg) stimulating Vbeta7CD4 T cells (Conrad et al., 1997). SAgs have the ability to selectively bind Vβ chains on T cells in a variety of different modes indiscriminately and without regard for their antigen specificity.

Although evidence that suggests that human autoimmunity is caused or influenced by HERVs is weak, another hypothesis is that HERV expression induce chronic stimulatory signals, which targeting both innate immune cells and lymphocytes, result in autoimmunity.

1.4.4.3. HERV-associated neurological diseases

Both HERV-W and HERV-K families have been linked with schizophrenia and bipolar disorder (BD), mostly in PCR-based studies in CSF or post-mortem brains (Christensen et al., 2010).

Pro-inflammatory properties of HERVs may be at the base of the immune aberrancies observed in individuals with schizophrenia. In addition, HERVs can also act in the transcriptional regulatory networks affecting the transcription levels of genes they are inserted in or near. Indeed, a whole-genome analysis of enhancer activity of HERVs, identified a HERV-K-related insert acting as an enhancer for the schizophrenia-linked gene proline dehydrogenase 1 (PRODH) (Suntsova et al., 2013). PRODH is one of the candidate genes for susceptibility to schizophrenia and other neurological disorders. Moreover, one HERV-W LTR element was identified within the regulatory region of the gamma-aminobutyric acid (GABA) receptor B1 gene which is down-regulated in schizophrenia (Hegyi, 2013).

PART 1:

EBV infection empowers human B cells for autoimmunity. Role of autophagy and relevance to multiple sclerosis

Strong evidence suggests that B cells have a central pathogenic role in MS pathogenesis and progression (Section 1.2.4). First, B-cell depletion with anti-CD20 monoclonal antibodies is effective in reducing disease activity in both relapsing and progressive forms of MS (Hauser et al., 2008). Second, the presence of OCB in the CSF is a sensitive biomarker of MS and has been associated with MS activity, progression, and prognosis (Joseph et al., 2009). Third, the presence of B cell aggregates within tertiary lymphoid follicles has been detected in the meninges of patients with advanced MS (Serafini et al., 2004).

MS epidemiological studies reveal that various environmental risk factors, such as infection with EBV, smoking and vitamin D have a strong contribution in the disease (Koch et al., 2013). The mechanisms underlying this association, however, have not been clearly elucidated.

Although the association of remote EBV infection with increased susceptibility to MS is well established (Ascherio and Munger, 2015), the contrast between the high prevalence of EBV infection (60-90%) and low prevalence of MS (±0.1%) in the human population remains a strong paradox. The hypothesis reported and investigated in this thesis supports the novel concept that the high MS risk in EBV infected individuals might be explained by pathogenic properties acquired by B cells upon infection with EBV under specific circumstances (‘t Hart et al., 2016). Indeed, presentation of antigen by resting B cells usually does not result in immune activation, but rather in tolerance of the corresponding T cell (Raimondi et al., 2006). However, when B cells are infected by EBV a different scenario can occur. In particular, only a small fraction of all B cells ($0.5 – 300 / 10^6$ memory B cells) in the blood carries the virus (Thorley-Lawson, 2015). Consequently, the difference between the prevalence

of MS and the prevalence of EBV infection, might depend on the fraction of B cells that are both specific for myelin autoantigen and infected by EBV. Indeed, evidence from a NHP EAE model shows that the EBV-related LCV-infected B cells have a central pathogenic role in the disease (‘t Hart et al., 2016). When the naturally LCV-infected marmoset B cells are depleted in vivo using anti-CD20 monoclonal antibodies, B-cell therapy is effective. This is in contrast with ineffective treatments directed at B-cell survival factors such as B lymphocyte stimulator (BLyS) and A proliferation-inducing ligand (APRIL), which did not affect LCV viral load (‘t Hart et al., 2013).

EBV could induce modification in the autoantigen processing and presentation, activating autoreactive pathogenic T cells. Interestingly, in the literature a case report described a patient who developed a severe encephalopathy following IM. Clinicians detected no EBV DNA in the blood or in the CSF and no EBV-specific antibodies in the CSF, but a potent MOG-specific cellular and humoral immune response (Jilek et al., 2007). This report suggests that EBV infection can trigger an immune response against MOG in humans.

The processing of MOG by intact human EBV- infected cells has been studied here in Nottingham, reported in this thesis and in press in the Journal of Immunology *(“EBV infection empowers human B cells for autoimmunity; Role of autophagy and relevance to multiple sclerosis”, E. Morandi 2017*). In parallel, the same mechanisms have been investigated also in cell lysates of NHP models in collaboration with the Immunobiology department in the Biomedical Primate Research Centre (BPRC) in Rijswijk, Netherlands. Observations found in the marmoset model have been reported in a study led by our collaborators and published in the Journal of Immunology in 2016 *(“Lymphocryptovirus infection of non-human primate B cells induces*

conversion of destructive into productive processing of the pathogenic CD8 T cell epitope in myelin oligodendrocyte glycoprotein"; (Jagessar et al., 2016). Indeed, evidence from the NHP EAE model shows that the LCV CalHV3 can influence the ability of B cells to process and present autoantigens to T cells. The development of this EAE model requires the administration of the human MOG in presence of CFA in mice and rhesus macaques or IFA in marmoset (Chapter 1.2.7.2). Interestingly, the infusion of autologous LCV-infected B cells pre-pulsed in vitro with MOG_{34-56} induced autoreactive T-cell activation and meningeal inflammation in marmosets (Haanstra et al., 2013a). Specifically, LCV-infected B cells had a central role in the activation of highly pathogenic MHC-E restricted effector memory cytotoxic T cells presenting the critical epitope MOG_{40-48}. To present MOG to CD8+ T cells, LCV-infection confers cross-presentation capacity to NHP B cells through the activation of the cross-presentation machinery and the prolongation of the CTL epitope half-life. The fast proteolytic degradation of the antigen in the endolysosomal compartment is suppressed to enable translocation to the MHC-I loading pathway. In this processing, cathepsin G (CatG) has a central role in the degradation of MOG_{35-55}, but substitution of the Arginine 46 (Arg46) residues for Citrulline (Cit) makes the peptide completely resistant against proteolytic degradation in the NHP cell lysates. The Arg to Cit substitution is a physiologically relevant modification of antigenic peptides, mediated by the enzyme peptidyl arginine deïminase (PAD), which can occur in autophagosomes in stressed B cells.

In MOG processing by intact human EBV-infected B cells similar observations have been made. EBV infection renders B cells potent APC with the ability to cross-present autoantigens. Unlike uninfected B cells, EBV infected ones can partially degrade rhMOG, but completely degrade MOG peptides (destructive processing) due to an

increased activity of CatG. If peptides are citrullinated in particular position they can be rescued and not degraded (productive processing). In the model proposed here, EBV infection induces autophagy in B cells facilitating the activation of PAD, which mediates citrullination of Arg residues in motifs that mediate association of rhMOG peptides with autophagosomes. Citrullinated MOG peptides are protected from degradation and can be presented to autoreactive T cells on MHC-I. This mechanism could facilitate presentation of a disease-relevant myelin autoantigen that may be involved in MS induction and progression.

CHAPTER 2: EBV-infected cells are strong APC able to internalize and process MOG

2.1. Background

In order for an APC to be competent for the initiation of primary immune response, the APC must be able to bind, take up, process, and express the antigen on the HLA class I/II complexes in association with costimulatory molecules.

To study the effect of EBV latent infection in B cells processing and antigen presentation, EBV-immortalized B-lymphoblastoid cell lines (LCL) were generated in vitro. LCL represent a tissue culture model for human B cell transformation and virus latency and they express the genes of the latency III program. They have been already shown to be effective APC to activate specific T cells (Thorley-Lawson and Mann, 1985; Livingston et al., 1997).

LCL were studied in comparison with other cell groups derived from the same healthy subjects: primary uninfected isolated CD20+ B cells, CD20+ B cells activated over-night with CpG and CD20- peripheral blood mononuclear cell (PBMC) subfraction (the remaining PBMC after CD20+ isolation). CpG is a synthetic oligodeoxynucleotide (ODN) containing a motif present in unmethylated bacterial DNA that through TLR9 can activate B cells and pDC (Chapter 1.2.5.6). These cells were included as positive control cells because previous reports indicate that CpG-activated B cells are capable of antigen cross-presentation (Jiang et al., 2011).

In this Chapter phenotypical expression of extracellular B cells APC-related markers such as HLA-I (A, B, C), HLA-I (E), HLA-II, CD80, CD86, CD70 and CD40 was detected in the different cell groups. HLA-I and HLA-II are the molecules directly related to antigen presentation and EBV can increase or decrease their expression

depending on the viral phase of infection and cell type (Masucci et al., 1987; Gregory et al., 1990; Griffin et al., 2013). HLA-E is a non-classical human MHC (class Ib) and increased frequency of EBV specific HLA-E restricted CD8+ T cells has been found to be associated with MS (Jorgensen et al., 2012). CD80 and CD86 are costimulatory molecules that bind to the T-cell CD28 and their expression prepare B cells for the interaction with T cells (Fig. 2.1). As well, CD40 binds to CD40ligand product by T cells and it is a costimulatory signal for B-cell activation, proliferation and differentiation. CD70 is expressed by LCL and binding CD27 on T cells activates their cytotoxic activity (Yamada et al., 2002). (Fig. 2.1)

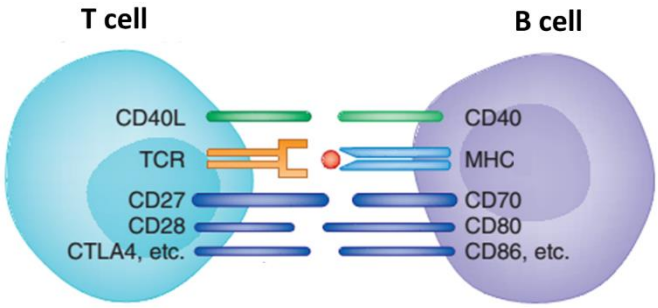


Fig. 2.1 Cross-talk between T – B cells. Principal markers involved in antigen presentation by B cells. CD40L, CD40 ligand; TCR, T-cell antigen receptor; CTLA4, cytotoxic T lymphocyte–associated protein 4; MHC, major histocompatibility complex. Adapted from (Haanen and Schumacher, 2007).

In addition, the intracellular expression of IFN-γ, IL-10 and TNF-α was studied to better understand if LCL have also a role in the production of soluble cytokines in the immune activation.

Once cells are activated to express APC markers, the antigen presentation process includes three main stages: Antigen binding, internalization and breakdown. B cells usually recognize a conformational protein through the BCR that mediates the internalization inside the endosomes. MOG (described in section 1.2.6.3) was the

antigen utilised in this study due to its use in the NHP animal model and its role in MS autoreactivity. The binding of recombinant human (rh)MOG (Fig 2.2) on the surface of LCL and its internalization was analysed by Flow Cytometry (FC). At the same time, the expression of co-stimulatory molecules was detected after incubation with rhMOG to measure any variation in their expression. After the internalization in the endosome/lysosome compartments, rhMOG is processed and peptides of the correct length are generated and loaded on the HLA. Primary uninfected CD20+ B cells, CpG activated CD20+, LCL and CD20- cells were incubated with rhMOG and the presence of the antigen was tested through protein electrophoresis. As well, the processing assays were repeated using the peptide MOG_{35-55} (Fig 2.2) to test if the immunogenic epitope MOG_{40-48} was degraded or not.

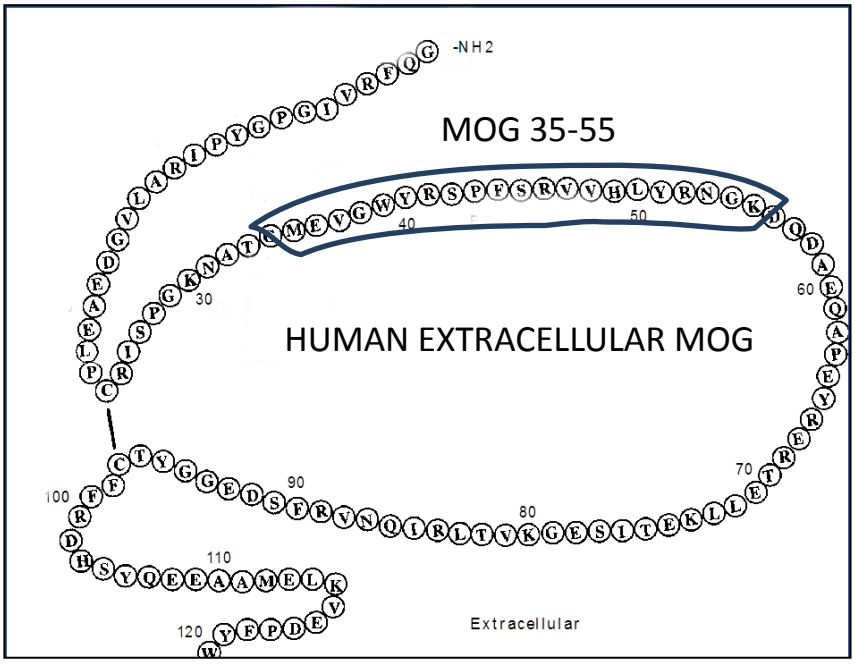


Fig. 2.2 Human extracellular rhMOG and peptide MOG_{35-55} sequence.

In this chapter the following studies will be presented:

- Antigen presentation-related markers and cytokines expressed by primary CD20+, CpG activated CD20+, LCL and CD20- cells;
- Binding and internalization of rhMOG by LCL;
- Variation in the expression of APC co-stimulatory molecules after rhMOG internalization;
- Degradation of rhMOG by primary CD20+, CpG activated CD20+, LCL and CD20- cells;
- Degradation of MOG_{35-55} by primary CD20+, CpG activated CD20+, LCL and CD20- cells.

2.2.Materials and Methods

2.2.1. Human Samples:

All subjects provided informed consent as approved by the ethics review board (Appendix I pag. 258). Informed consents were signed and obtained from all donors. Healthy subjects (N=8, mean age= 37.88 Std deviation= 9.58, Appendix II pag. 261) were recruited among volunteers in the Division of Clinical Neuroscience of the University of Nottingham. 50ml of heparinised venous blood were collected from each study participant.

2.2.2. Isolation of PBMC

PBMC were isolated from blood using Ficoll density gradient centrifugation. Blood was diluted with an equal volume of Phosphate buffered saline (PBS; Sigma-Aldrich). 30ml of diluted blood samples were layered into 15ml of Histopaque-1077 (Sigma-Aldrich) density medium and centrifuged at 600g for 30min without break. PBMC layer was removed and washed twice with PBS. After the final wash the cell pellet was re-suspended in 10ml of PBS. Cell count was performed by pipetting 10µl of sample into 90µl of trypan blue (Sigma-Aldrich). 10µl of the mixture was pipetted into a Neubauer haemocytometer and cell count was reported as cells/ml.

2.2.3. Isolation of CD20+ cells

CD20+ B cells were purified from PBMC by positive selection using CD20+ cell isolation kit (Miltenyi Biotec). Cell suspension was centrifuged at 300g for 10min. Supernatant was pipetted off completely and the cell pellet was re-suspended in magnetic activated cell sorter (MACS) buffer [PBS ph7.2, 0.5% bovine serum albumin (BSA), 2mM Ethylenediaminetetraacetic acid (EDTA)] per 10^7 cells. The CD20 Microbeads cocktail was added at 20µl per 10^7 cells. Cell suspension was mixed and

incubated for 15min at 4˚C. The cells were then washed by adding 5ml of buffer and centrifuged at 300g for 10min then re-suspended in 500µl of MACS buffer. MS column was placed in the magnetic field and rinsed with 500µl of MACS buffer. The cell suspension was applied into the column and the CD20+ cells were bind to the column. The column was washed 3 times with 500µl of MACS buffer and the unlabelled CD20- cells were collected. Then the column was removed from the separator and 1ml of buffer was pipetted in the column and the fraction with the magnetically labelled cells was flushed out by applying a plunger. Collected CD20+ and CD20- cells were finally counted with haemocytometer. Purity of CD20+ cells was assessed using an anti-CD20 Ab by FC as described below.

2.2.4. Activation of CD20+ cells with CpG

10^6 CD20+ isolated B cells were cultured in 400µl of complete medium [Roswell Park Memorial Institute medium (RPMI) with 10% foetal calf serum (FCS), 100 units penicillin - 1mg/ml streptomycin (pen/strep), 20mM L-glutamine all from Sigma-Aldrich] with 30µg/ml of CpG OND 2006 (Invivogen, sequence "TCG TCG TTT TGT CGT TTT GTC GT") in 48-wells plate for 24h. Activation of CD20+ cells was assessed using an anti-CD86 Ab by FC as described below.

2.2.5. Generation of LCL

5-10 x 10^6 of isolated PBMC were centrifuged at 300g for 10min. 3-4ml of supernatant from B95.8 EBV infected marmoset cell line (kindly donated by Jill Brooks, Birmingham) which had not been fed for at least 5 days was collected and centrifuged 200g for 5min. The B95.8 supernatant was filtered on the PBMC pellet using a 0.45µm syringe filter and a 10ml syringe and 3-4 drops of FCS were added. The PBMC mixed with the virus were incubated over-night (O.N.) at 37˚C. After centrifugation at 300g

for 5 min supernatant was discard and infected PBMC were re-suspended in 2ml of CSA medium (complete RPMI + 1ug/ml Cyclosporine A from Sigma) in 24-wells plate. After 1 week they were re-feed and cultured with CSA. After have achieved the correct concentration, they were expanded in 24cm^2 and 75cm^2 flasks. Once new B-LCL were established from each different donor, these were expanded in culture and used for further experiments. Infection of the cells with EBV and the handling of EBV-infected cells were performed following the University of Nottingham risk assessment protocols for Biological agents.

2.2.6. Extracellular Flow cytometer staining APC-related markers

Surface staining for Pe-Cy5 anti-CD20 (2H7), Pe anti-CD86 (2331), Pe-Cy7 anti-CD80 (L307.4), Alexa Fluor 488 anti-CD40 (5C3), Alexa Fluor 488 anti-CD70 (Ki-24), APC anti-HLA- DR, DP, DQ (Tu39), Pe anti-HLA-ABC (G46-2.6) all from BD and Pe-Cy7 anti-HLA-E (3D12, Biolegend) was performed on the different cell groups (CD20+, CpG activated CD20+, LCL, and CD20- cells). Isotype controls were included and Fluorescence minus one (FMO) samples were used to set the gating (one example in Appendix III pag. 262). Cells were divided in 10^6 cells per FACS tube (Sarstedt, Germany) and washed with 2ml of FACS buffer (PBS+2% FCS) and centrifuged at 300g for 6min. Supernatants were discarded and pellets were re-suspended and stained with 5-20µl of Abs as suggested by datasheet. Cells were incubated in the dark for 30min at 4˚C. After incubation, cells were washed 2 times with 2ml of FACS buffer and centrifuged at 300g for 6min. Cells were then fixed with 500ul Fixation buffer (2% paraformaldehyde, BD). Cells were analysed by FC using LSRII flow cytometer (BD Biosciences, USA) and FlowJo software (version V10, FlowJo, LLC, USA). Mean fluorescence intensity (MFI) raw values were divided by the MFI of isotype control in each experiment to give a relative MFI (rMFI) value. To

ensure the comparability of measurements on different days, FC performance was standardized each day by the use of rainbow calibration particles (8 peaks, BD Biosciences) to demonstrate sensitivity, linearity and accuracy of the machines in accordance with the manufacturer's instructions.

2.2.7. Intracellular Flow cytometer staining cytokines

Intracellular staining for APC anti-IFN-γ (XMG1.2), Pe anti-IL-10 (JES3-19F1) and PeCy-5 anti-TNF-α (Mab11) was performed on the different cell groups (CD20+, CpG activated CD20+, LCL, and CD20- cells). Isotype controls were included and FMO samples were used to set the gating. After extracellular staining, 250µl of Fix/Perm (BD Bioscience) was added in each tube and incubate for 20min at 4˚C. Cells were washed two times with Perm/Wash buffer (containing Saponin; BD Bioscience) and centrifuged at 300g for 6min. Supernatants were discard and pellets were re-suspended and stained with 5-20µl of Abs as suggested by datasheet. Cells were incubated in the dark for 30min at 4˚C. Cells were washed two times with Perm/Wash buffer (BD Bioscience) and centrifuged at 300g for 6min. Cells were analysed by FC using LSRII flow cytometer (BD Biosciences, USA) and FlowJo software (version V10, FlowJo, LLC, USA).

2.2.8. Incubation of cells with rhMOG

After trying different concentrations of cells with different concentrations of protein, the best ratio chosen was 10^5 cells for 1µg of rhMOG (Cambridge Bioscience) or 2µg of MOG_{35-55} peptide (Peptide 2.0). Cells were incubated with rhMOG or MOG_{35-55} at 37°C in 96-wells plate (Costar) in complete medium for different time points as indicated in the legend. When Fc blocker was used, cells were incubated with rhMOG in presence of 2.5µg per 10^6 cells of human BD Fc Block (BD Bioscience).

2.2.9. Flow cytometer staining for rhMOG detection

LCL previously incubated with rhMOG for different time points (1h, 3h, 6h, O.N.) and only LCL with no rhMOG as control were divided in 10^6 cells per FACS tube (Sarstedt, Germany), washed with 2ml of FACS buffer (PBS+2% FCS) and centrifuged at 300g for 6min. Supernatants were discard and pellets were re-suspended and stained with 1µl, 5µl or 10µl of primary mouse IgG anti human MOG (8-18C5, kindly donated by prof. Linington, University of Glasgow) incubated in the dark for 1h at 4°C. 10µl was chosen as best volume. After incubation cells were washed 2 times with 2ml of FACS buffer and centrifuged at 300g for 6min. Cells were then stained with 10µl of secondary FITC goat F(ab`)2 anti-mouse IgG (R&D Systems) and washed twice as previously described. When rhMOG was assessed intracellularly cells were fix/perm as described above and stained with the same primary and secondary at the same concentrations. Cells were analysed by FC using LSRII flow cytometer (BD Biosciences, USA) and FlowJo software (version V10, FlowJo, LLC, USA).

2.2.10. Protein electrophoresis-based assay for processing experiments

10^5 LCL, CD20+, CpG activated CD20+, CD20- cells previously incubated with rhMOG for different time points (4h, O.N.) were washed and frozen at -20˚C. Samples with 4x loading buffer and 10x reducing agent (both Life Technologies) were loaded in NuPage Novex Bis-Tris precast protein gels, 4-12% (Life Technologies) and run at 100V. Controls (CTRL) were added loading only the rhMOG protein or peptides incubated without cells and only-cells without the protein. The gels were stained with SimplyBlue Safestain (Life Technologies) for 2-3h and decolorized over-night. The imaging was at Odyssey scanner (LI-COR Biosciences) and the protein bands were

quantified with Image Studio Lite software (version 4.0, LI-COR Biosciences). Percentage of degradation was calculated with the formula:

*(MOG incubated without cells – MOG incubated with cells) / MOG incubated without cells * 100.*

2.2.11. MOG Western blotting

LCL previously incubated with rhMOG were washed and mixed with 4x loading buffer and 10x reducing agent (both Life Technologies). Then they were loaded in NuPage Novex Bis-Tris precast protein gels, 4-12% (Life Technologies) and run at 100V. Proteins were transferred to polvinylidene fluoride (PVDF) transfer membrane (GE Healthcare Life Sciences) (30 V, 90 min) and blocked in PBS-tween 2% BSA (Sigma-Aldrich) for 1h. Blots were incubated over-night with 1:100 primary mouse IgG anti human MOG (8-18C5, kindly donated by prof. Linington, University of Glasgow) antibody at 4°C. Then incubated with 0.06µg/ml secondary 680RD Donkey anti-mouse IgG (H + L) (LICOR Biosciences) for 1h at room temperature. The membrane was scanned with an Odyssey scanner (LI-COR Biosciences) at 800nm.

2.2.12. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Paired T test or repeated One-way ANOVA was used for statistical comparisons in FC experiments. Paired non-parametric Friedman test was used for statistical comparisons in quantification of gels. The graphs show mean with standard error of the mean (SEM) if a parametric test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤ 0.05 were considered significant and only significant p values were reported in the graphs.

2.3.Results

2.3.1. EBV infection upregulates antigen-presentation – related markers

After blood collection and PBMC isolation, different groups of cells were obtained from 8 different healthy subjects (Appendix II pag. 261). LCL were generated infecting PBMC with B95.8 cells supernatant and CD20+/- cells were purified using magnetic bead isolation. After isolation, the phenotype was assessed by FC to determine the purity of cells positive for CD20+. The percentage of CD20-positive cells was always >95% for primary CD20+, >85% for LCL and < 5% for CD20- cells (Fig 2.3). A fraction of CD20+ isolated cells was activated with CpG for 24h and the activation of the CpG-stimulated cells was assessed by FC detecting the activation marker CD86. The percentage of CD86 positive cells was always >40% for CpG activated CD20+, >70% for LC and < 5% for CD20+ and CD20- cells (Fig 2.3).

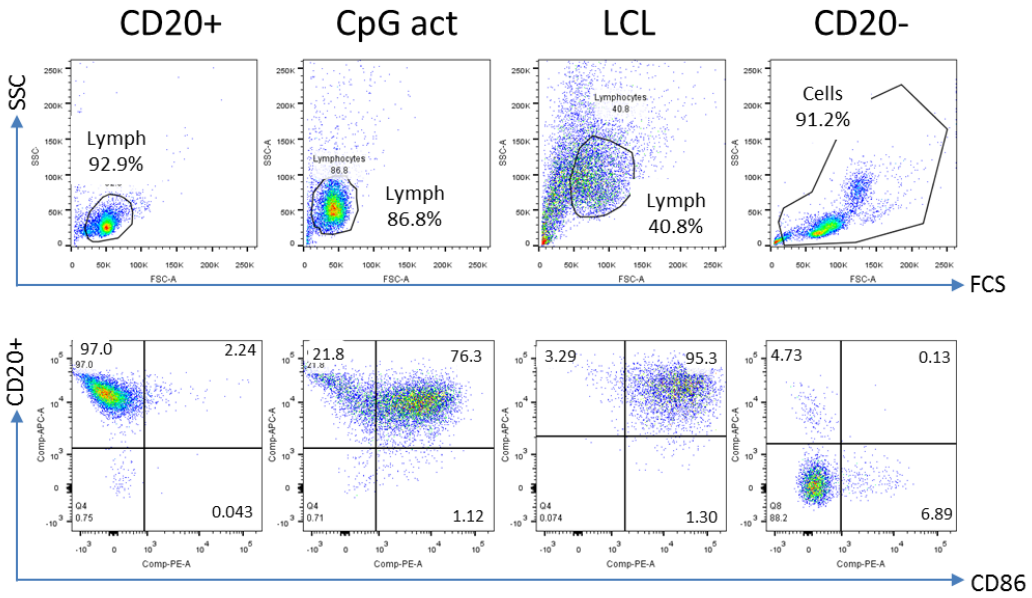
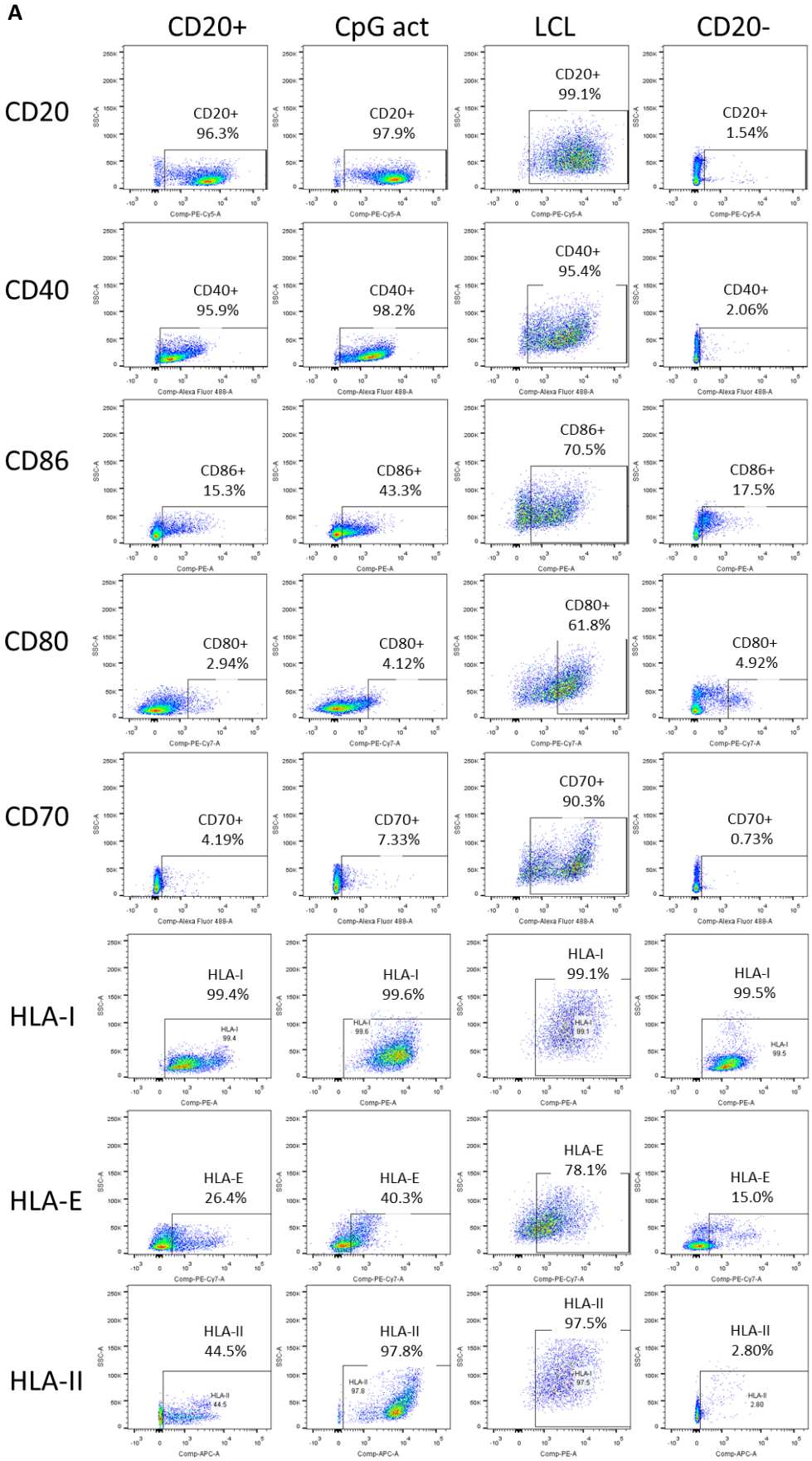


Fig. 2.3 Cell groups. Isolated CD20+ cells, CpG activated CD20+ cells, LCL and CD20- cells were obtained from the same subjects. One representative dot plot out of 8 independent experiments is shown. Lymphocytes (Lymph) are gated based on physical parameters side scatter (SSC) and forward scatter (FSC). Purity of CD20+ cells and activation of the cells were evaluated through detection of CD20 and CD86 by FC.

The principal APC-related markers were checked in the different cell groups to investigate if EBV could phenotypically induce their expression. CD40, CD86, CD80, CD70, HLA-I (A, B, C), HLA-I (E), and HLA-II (DR, DP, DQ) were detected through extracellular staining. EBV increased the percentage of the number of cells positive (Fig 2.4) and the rMFI (relative mean fluorescence intensity) (Fig 2.5) for CD40, CD80, CD86, CD70, HLA-E, and HLA-II compared with all the other groups. All groups were 100% positive for HLA-I, but EBV infection increased its expression on the cell surface (Fig 2.5). In the CpG activated cells there was an increased number of cells positive for CD40, CD86, CD80 and HLA-E and increased rMFI of CD40, CD86, CD80 and HLA-II compared to the primary uninfected CD20+ cells (Fig 2.4, Fig. 2.5).

The expression of cytokines, such as IFN-γ, TNF-α and IL-10, was assessed through intracellular staining. In LCL there was an increased production of IFN-γ, but not TNFα and IL-10, compared with CD20+ cells (Fig 2.6).

These results confirm the previous indication (Thorley-Lawson and Mann, 1985; Livingston et al., 1997) that EBV infection leads to a potent activation of B cells, with increased expression of some of their antigen presentation markers.


A
CD20+
CpG act
LCL
CD20-
CD20
CD20+ 96.3%
CD20+ 97.9%
CD20+ 99.1%
CD20+ 1.54%
CD40
CD40+ 95.9%
CD40+ 98.2%
CD40+ 95.4%
CD40+ 2.06%
CD86
CD86+ 15.3%
CD86+ 43.3%
CD86+ 70.5%
CD86+ 17.5%
CD80
CD80+ 2.94%
CD80+ 4.12%
CD80+ 61.8%
CD80+ 4.92%
CD70
CD70+ 4.19%
CD70+ 7.33%
CD70+ 90.3%
CD70+ 0.73%
HLA-I
HLA-I 99.4%
HLA-I 99.6%
HLA-I 99.1%
HLA-I 99.5%
HLA-E
HLA-E 26.4%
HLA-E 40.3%
HLA-E 78.1%
HLA-E 15.0%
HLA-II
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HLA-II 97.8%
HLA-II 97.5%
HLA-II 2.80%

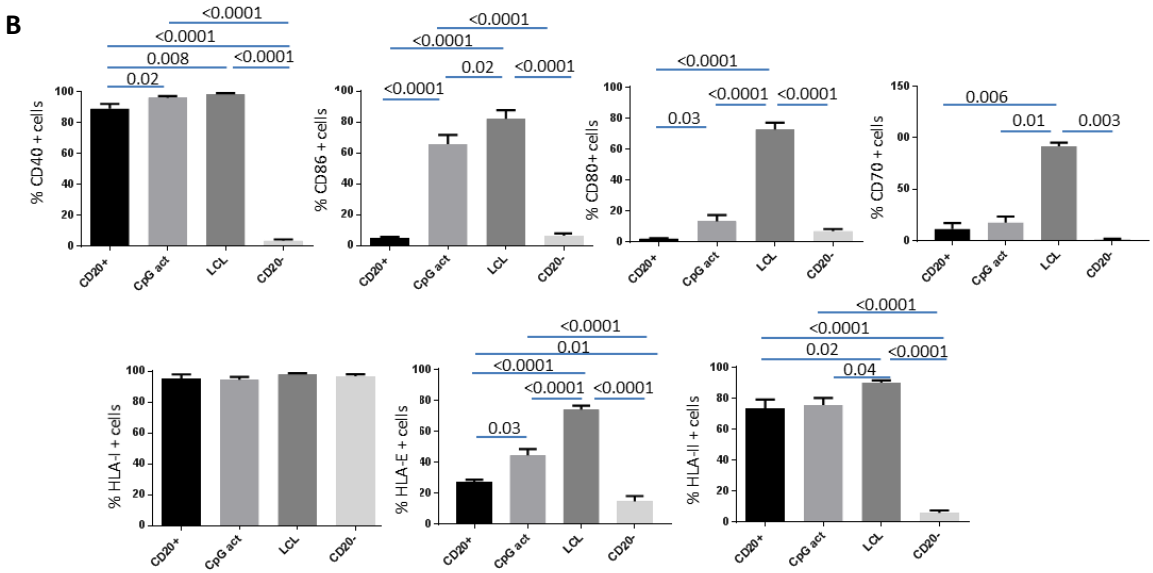


Fig. 2.4 EBV increases the % expression of antigen presentation - related markers by B cells. (A) One representative dot plot is shown for each marker CD20, CD40, CD80, CD86, CD70, HLA-I (A, B, C), HLA-E, and HLA-II (DR, DP, DQ) for each cell group (CD20+, CpG activated CD20+, LCL and CD20- cells). **(B)** Bar chart data are representative of 8 independent experiments from different donors showing the mean and SEM of extracellular expression of cells positive for each marker (n=8, peired repeated one-way ANOVA test).

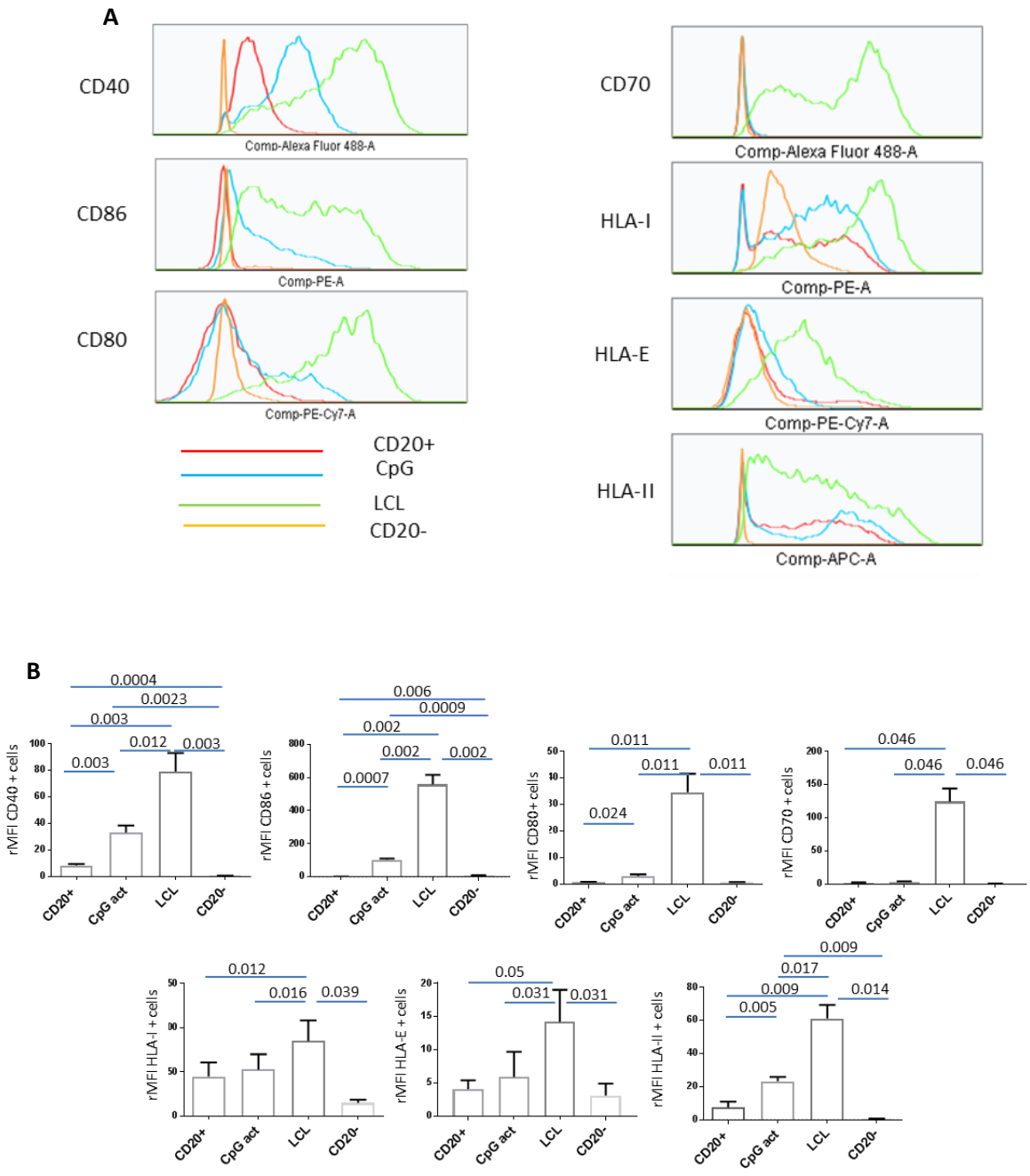


Fig. 2.5 EBV increases the rMFI expression of antigen presentation - related markers by B cells. (A) One representative histogram overlap of different cell groups is shown for each marker CD40, CD86, CD80, CD70, HLA-I (A, B, C), HLA-E, and HLA-II (DR, DP, DQ). **(B)** Bar chart data are representative of 8 independent experiments from different donors showing the mean with SEM of extracellular expression of mean fluorescence intensity (MFI) relative to the isotype control (rMFI) (n=8, paired repeated one-way ANOVA test).

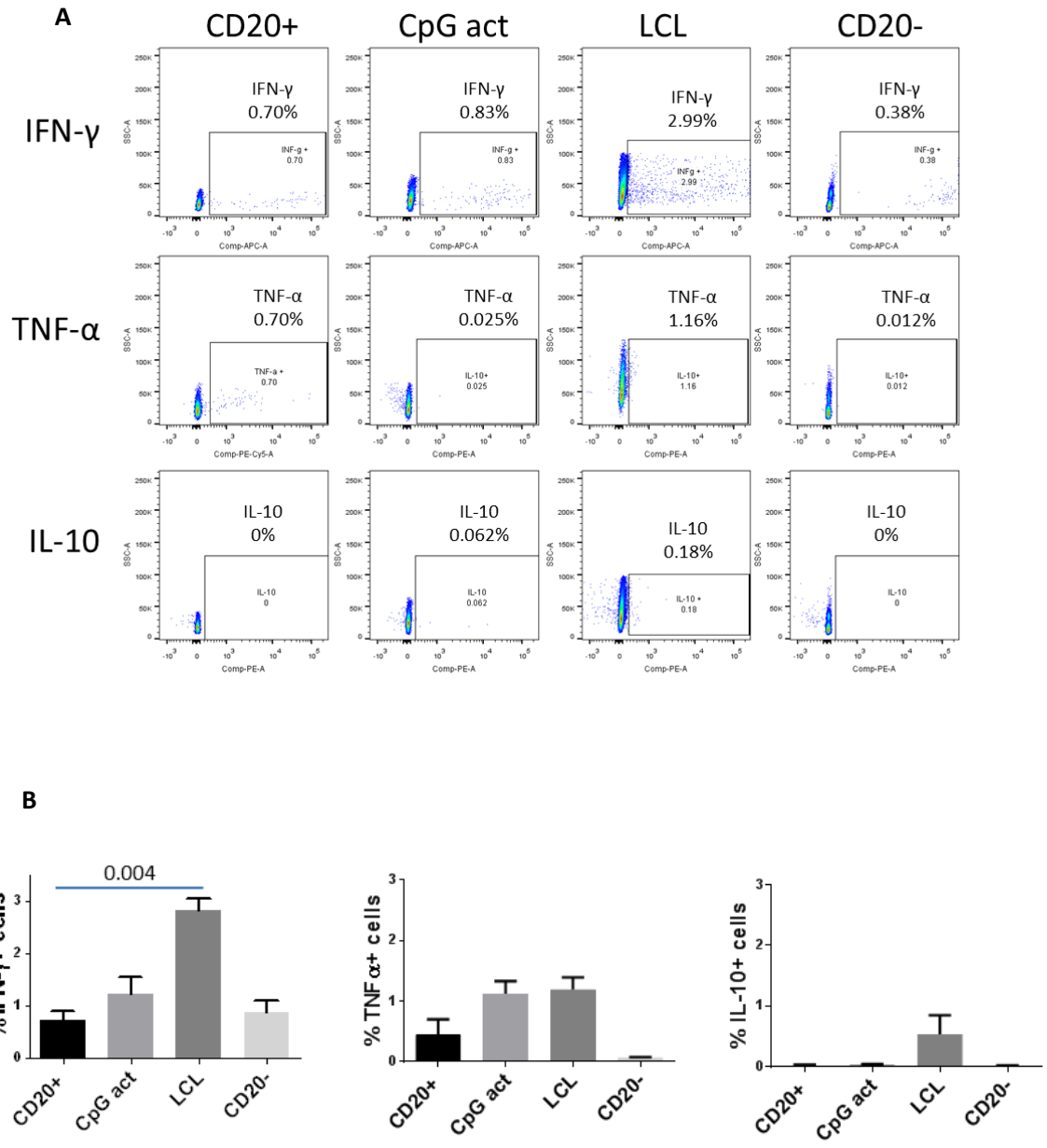


Fig. 2.6 EBV increases IFN-γ production. (A) One representative dot plot is shown for each cytokine IFN-γ, TNF-α, IL-10 for each cell group (CD20+, CpG activated CD20+, LCL and CD20- cells). **(B)** Data are representative of 6 independent experiments from different donors showing the mean and SEM of intracellular expression of cells positive for each marker (n=6, paired repeated one-way ANOVA test).

2.3.2. LCL internalize and process rhMOG and MOG$_{35-55}$

The binding and internalization of rhMOG by LCL was analyzed. rhMOG was detected through FC using the 8-18 C5 anti-MOG Ab. This Ab recognizes only the conformational epitope, which is formed by the apical B-C, F-G, C'C" loops (Adelmann et al., 1995). After the incubation of rhMOG with LCL for 1h, LCL samples were split in 2 tubes: in one tube, cells had been fixed and permeabilized, for intracellular staining only (Fig 2.7 lower panels); in the other, they were not fixed and permeabilized, but surface-stained only (Fig 2.7 upper panels). On the surface the percentage of cells binding the protein was high (mean 41.8%) while was lower inside the cells (mean 9.66%) (Fig 2.7B). To test if the cells were binding rhMOG through the FC receptor, a FC receptor blocker was added in the culture (Fig 2.7A). The result was similar to the experiment without the blocker.

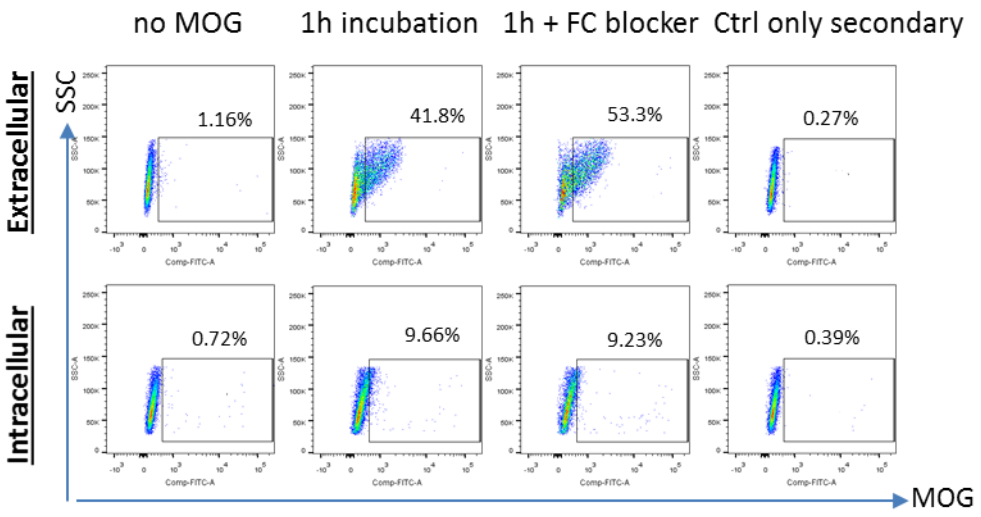


Fig. 2.7 LCL bind and internalize rhMOG after 1h incubation. After incubation of LCL with rhMOG for 1h, rhMOG can be detected extra- and intracellularly by FC with primary 8-18C5 anti-MOG Ab and secondary FITC-anti mouse IgG. The expression of rhMOG after 1h incubation has been detecting also in presence of Fc blocker. The last panel represents the control of the staining with no primary anti-MOG Ab. One representative dot plot is shown out of 3 experiments.

LCL were incubated with rhMOG for different time points and a reduction in rhMOG detection during the time was observed (about 50% every 2-3 hours of incubation) reaching 0% after O.N. incubation (Fig 2.8). The decrease of rhMOG could suggest an internalization and degradation of the protein, since the loss of rhMOG extracellular confirmation is enough to lose the conformational epitope that bind 8-18C5 Ab.

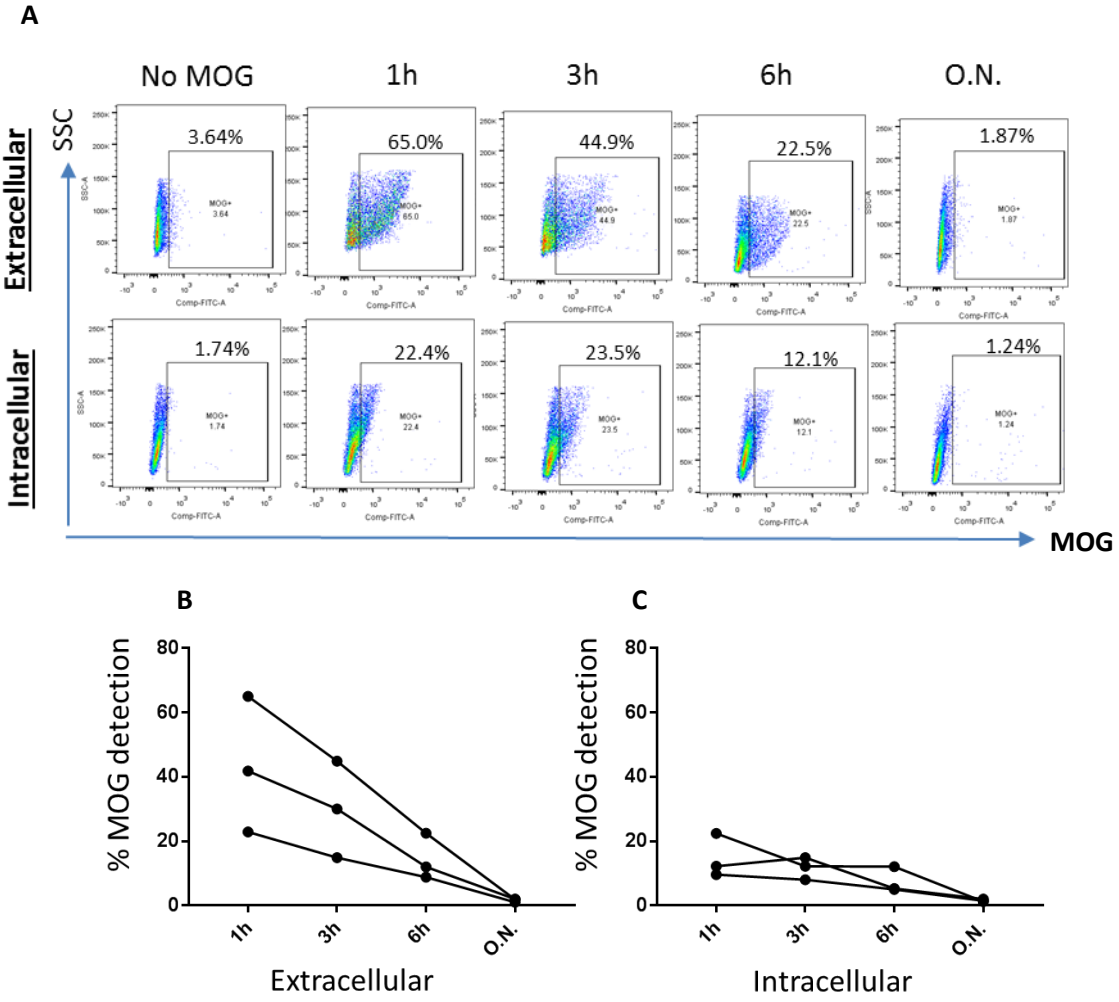


Fig. 2.8 Binding and internalization of rhMOG by LCL in the time. (A) After incubation of LCL with rhMOG for 1h, 3h, 6h and O.N., rhMOG can be detected extra- and intracellularly by FC with primary 8-18C5 anti-MOG Ab and secondary FITC-anti mouse IgG. One representative dot plot is shown. **(B)** Extracellular and **(C)** Intracellular percentage MOG detaction in the time in 3 different LCL is illustrated in dots.

Other experiments were performed to test if the antibody used after permeabilization detected only the intracellular or both intra- and extracellular rhMOG. A sequence of extracellular staining (with primary mouse anti-MOG 8-18C5 followed by anti-mouse IgG-FITC), cell fixation and permeabilization, and intracellular staining (with primary mouse anti-MOG 8-18C5 followed by anti-mouse IgG coupled to a different fluorochrome, CF 568) was used in the same tube. Surface staining for membrane-bound rhMOG was unequivocally abolished by fixation and permeabilization of LCL (Fig. 2.9). By contrast, intracellular staining for rhMOG using the CF568 fluorochrome was clearly positive (37.9%), in spite of a degree of non-specific staining with the secondary Ab only (11.7%) (Fig. 2.9). The percentage of "true positive" intracellular staining for rhMOG (26.2% in this example) was therefore approximately the same as that shown in Fig. 2.7 (22.4%). Permeabilization using Cytofix/Cytoperm kit (BD Bioscience, containing saponin) or 0.5% Tween 20 yielded similar results. The loss of rhMOG surface staining with the use of permeabilization reagents was probably a consequence of a relatively weak binding of soluble rhMOG to the cell membrane.

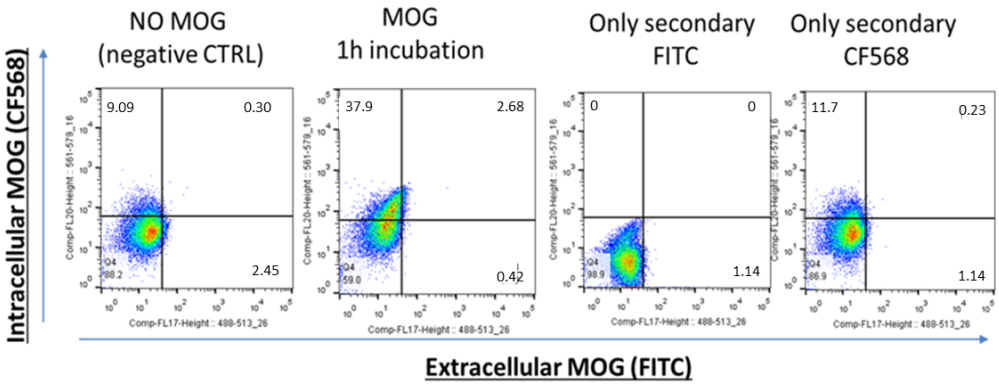


Fig. 2.9 Surface staining for membrane-bound MOG is abolished by permeabilization of LCL. After incubation of LCL with rhMOG for 1h, LCL were stained on the surface with primary 8-18C5 anti-MOG Ab and secondary FITC-anti mouse IgG, fixed and permeabilized, and stained intracellularly with primary 8-18C5 anti-MOG Ab and secondary CF586-anti mouse IgG. One representative dot plot is shown out of 2 experiments.

To test if the internalization of rhMOG would further activate LCL, the expression of APC – related markers was analyzed after the incubation of the cells with rhMOG. An upregulation of CD80, but not CD40 and CD86, was observed (Fig 2.10).

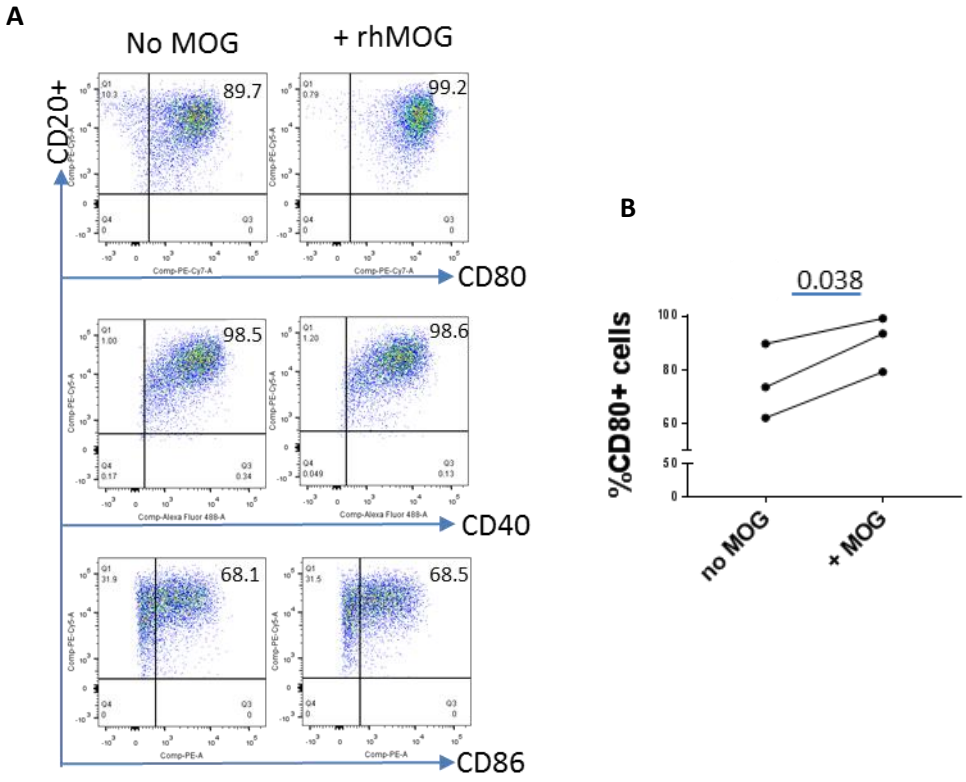


Fig. 2.10 Increased expression of CD80 after rhMOG internalization. (A) After incubation of LCL with rhMOG for 1h, the expression of CD80, CD40 and CD86 was detected by FC. **(B)** The graph shows the number of cells positive for CD80 before and after incubation with rhMOG for 1h (n=3; paired T test).

2.3.3. EBV infection rescues rhMOG from degradation

rhMOG degradation by the different cell groups was then analyzed to study whether EBV infection has a specific effect on processing of the protein by B cells. After optimizing the test using different protein concentrations and different incubation time, rhMOG was incubated for 4h and O.N. with the different cell groups (LCL, CD20+, CpG activated CD20+, CD20- cells) and then analyzed by SDS gel and quantified. Intact rhMOG was detected as control as a single band of ± 14 kDa in the gel (Fig 2.11 A, "CTRL" line). One representative gel is shown for 4h and O.N. incubation. Graphs show dots with medians and interquartile range of % of degradation in 4 experiments calculated only after O.N. incubation because after 4h no major differences were detected. High degradation of rhMOG was detected after O.N. incubation with CD20+ and CD20- cells while less degradation was observed with LCL and CpG activated B cells (Fig 2.11 A, B). With LCL an intermediate degradation product was detected at 6 kDa. Controls are presented showing a gel loaded with only-cells (Fig. 2.11 C) and only LCL with or without rhMOG (Fig. 2.11 D). Moreover, the correct detection of rhMOG was verified in a Western Blot (WB) incubating LCL and CD20+ cells with rhMOG and detecting the protein with the 8-18C5 anti-MOG Ab (Fig. 2.11 E).

These data indicate that while B cells are capable of high proteolytic degradation of rhMOG, this activity is partially abolished by EBV infection.

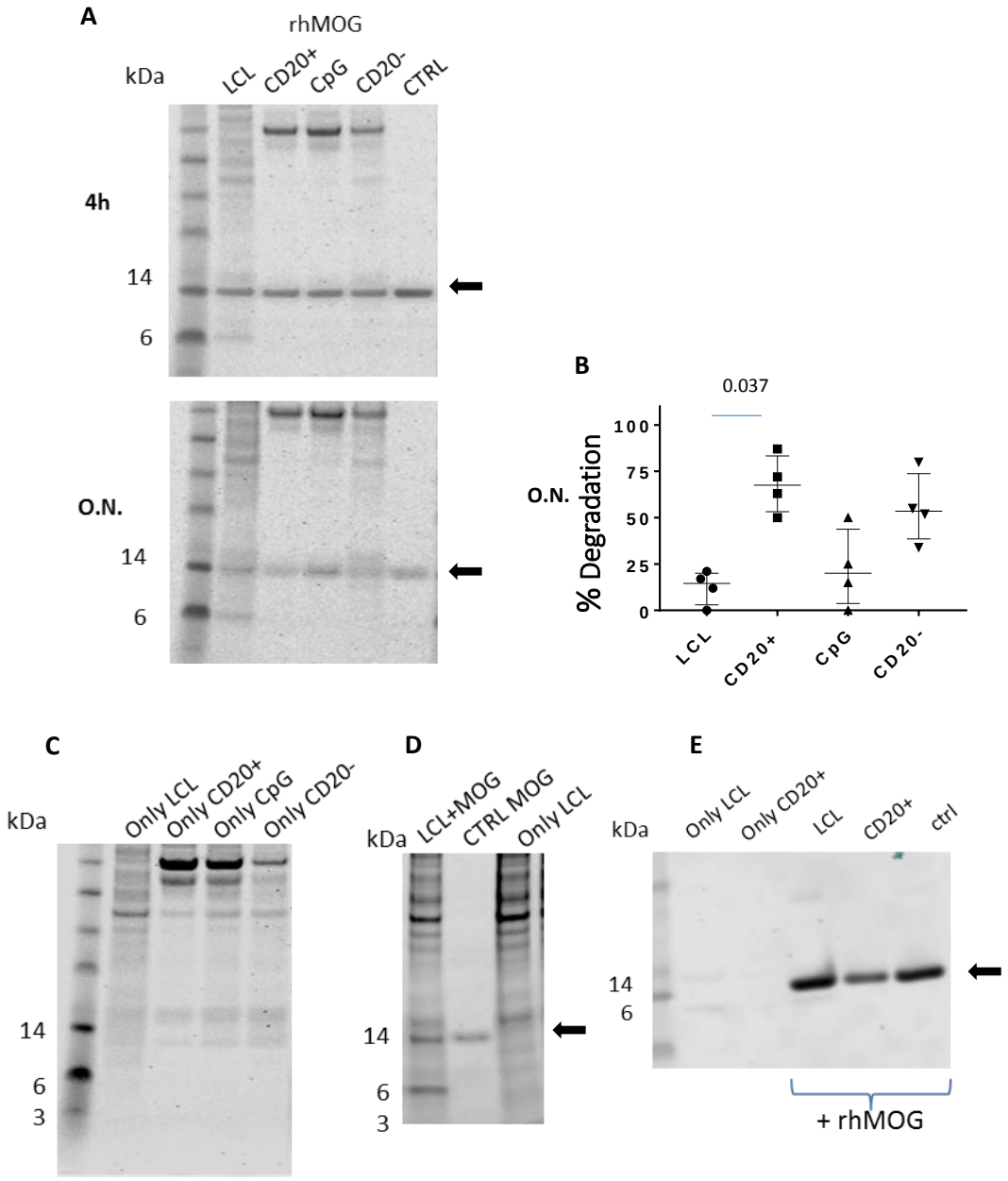


Fig. 2.11 rhMOG degradation by cell groups. The presence of MOG can be detected with SDS gel (⬅). **(A)** Same number of LCL, CD20+, CpG activated CD20+, CD20- cells were incubated with 1µg of rhMOG for 4h or O.N. CTRL (control) shows the protein incubated alone (14kDa), while all the other lines have been loaded with the mix cells-protein after the incubation. Gels from one representative experiment are shown. **(B)** Percentage of degradation of rhMOG after O.N. incubation in 4 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=4; paired non-parametric Friedman test). Control gels show **(C)** LCL, CD20+, CpG activated CD20+, CD20- cells incubated with no protein/peptide, **(D)** LCL incubated with/without rhMOG and **(E)** detection of MOG trough WB with primary 8-18C5 anti-MOG Ab.

2.3.4. EBV infection increases MOG_{35-55} degradation

The same assay was used for testing the proteolytic degradation of MOG_{35-55}. MOG_{35-55} is an immunodominant peptide used to induce EAE in the mouse and NHP model and it is also a human T cell epitope. The peptide was presented in the gel as control as a band of 3 kDa (Fig 2.12A last line "CTRL" line). This was completely degraded by LCL and CD20-, while unstimulated and CpG activated CD20+ cells did not process completely the peptide (Fig 2.12). These results suggest that EBV leads to modifications in MOG processing, rescuing rhMOG from major degradation but allowing B cell to degrade MOG_{35-55}.

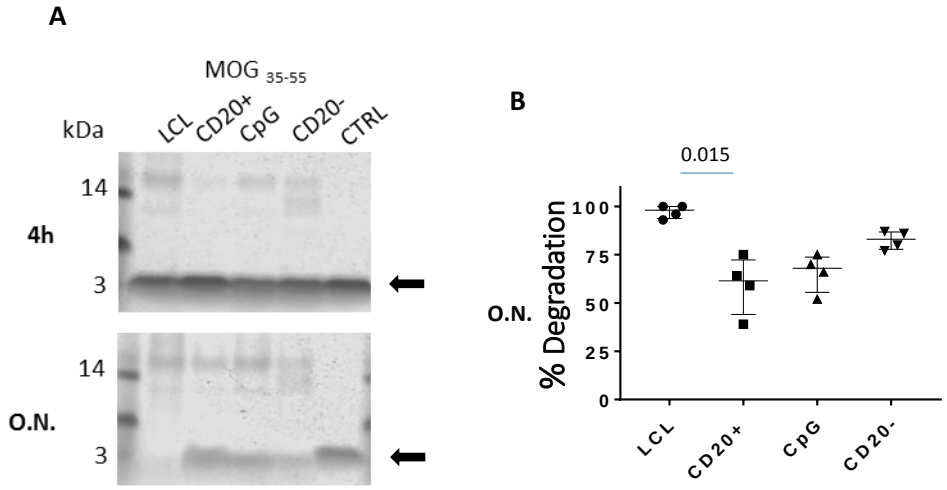


Fig. 2.12 MOG_{35-55} degradation by cell groups. The presence of MOG_{35-55} can be detected with SDS gel (⬅). **(A)** LCL, CD20+, CpG activated CD20+, CD20- cells were incubated with 2μg MOG_{35-55} for 4h or O.N. CTRL (control) shows the peptide incubated alone (3kDa), while all the other lines have been loaded with the mix cells-peptide after the incubation. Gels from one representative experiment are shown. **(B)** Percentage of degradation of MOG_{35-55} after O.N. incubation in 4 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=4; paired non-parametric Friedman test).

2.4. Conclusions

Primary uninfected B cells are efficient APC that can capture exogenous Ag via their clonal BCR and then process it in their endolysosomal compartment. All these mechanisms seem to be more potent if the B cells are infected by EBV.

LCL had much stronger APC phenotype upregulating the expression of HLA-I and II and costimulatory molecules compared to the other groups of cells. They also produced more IFN-γ suggesting that they can activate the inflammatory immune response. As expected (Decker et al., 2000), the stimulation of activated CD20+ B cells with CpG also induces increased expression of costimulatory molecules CD40, CD80, and CD86, confirming the ability of these cells to prime the immune response. Together, these data emphasize that both EBV infection and CpG activation (Jiang et al., 2011) can render B cells more potent APC and specifically, able to cross-present exogenous antigens in an MHC-I-restricted manner. However, some differences between the two types of activated B cells were found. While the % of cells with upregulation of costimulatory molecules CD40 and CD86 was comparable, the expression level per cell differed markedly. Moreover, only on LCL strong induction of CD70, CD80 and HLA-E, was observed, both in cell frequency as well as the expression level per cell. These data extend the observation of T cell activation mechanisms induced by LCV infection of NHP B cells (Jagessar et al., 2016). In the marmoset EAE model CD80 and CD70 mediate the cross-talk of LCV-infected B cells and autoaggressive MOG_{40-48} specific MHC-E restricted CTLs (Dunham et al., 2017). These similar findings in human B cells may reflect close mechanisms of relevance to the pathogenesis of MS.

These data confirm previous indication (Thorley-Lawson and Mann, 1985; Livingston et al., 1997) that EBV infection leads to a potent activation of B cells transforming

them in strong APCs. In particular, the ability of LCL to present EBV antigens to T cells is well known, and for example autologous LCL have been used to determine EBV-specific T cells cultured from the CSF of CIS, MS and OND cases, finding an enhanced intrathecal auto LCL-specific T cell reactivity in MS patients (van Nierop et al., 2016). Opposite, processing and presentation of myelin antigens in LCL has been poorly explored in humans.

Interestingly, B cells of MS patients are thought to express higher levels of costimulatory molecules than HC (Huang et al., 2000; Mathias at al., 2016; Ireland et al., 2016) suggesting an enhanced APC function of B cell in MS. Moreover, recently it has been demonstrated that B cells from MS patients, but not HC, could incite proliferation and IL-17 cytokine production by Th17 cells in response to neuroantigens (Ireland et al., 2016).

In this Chapter the binding, internalization and processing of MOG by human LCL was studied. LCL could bind MOG on the surface and internalize it. After incubation of LCL with MOG, the presence of the protein extracellularly and intracellularly peaked at 1 and 3 h, respectively, followed by a rapid reduction in detectable protein. During this process the conformational B cell epitope remained initially intact, as could be deduced from binding of the 8-18C5 anti-MOG Ab. It is likely that reduced surface staining reflects internalization, whereas reduced intracellular staining reflects degradation of the conformational epitope (Breithaupt et al., 2003).

Blockade of the Fcγ receptor did not significantly reduce either surface or intracellular staining indicating that the observed effects were not due to MOG captured on LCL surface in immune complexes.

The increased expression of CD80 after 1 h suggests simultaneous activation of the antigen presentation machinery during uptake of rhMOG, while probably the regulation of CD40 and CD86 required further co-stimulations.

rhMOG was detected also by gel electrophoresis and was less degraded by LCL compared to uninfected CD20+ and CD20- cells. These observations of reduced degradation of rhMOG upon EBV infection of B cells suggest that EBV confers protection from processing of the whole protein. However, the generation of smaller peptides in the 6-14 kDa range was observed in LCL, but not in CD20+ uninfected B cells, suggesting the breakdown of MOG protein into 10-20 amino acid long peptides. For the peptide MOG_{35-55} the opposite result was observed: MOG_{35-55} was completely degraded by LCL, but not by uninfected and CpG-activated B cells.

Unfortunately it was not possible to detect the binding and internalization of the peptide MOG_{35-55} by FC and by WB due to the lack of an Ab that can recognize this linear sequence.

Mass spectrometry could have been a more precise technique for the study of protein degradation, but for the purpose of the study (not focussing on proteomics) and for the high number of experiments planned we elected to use SDS gel electrophoresis as an adequate technique.

The next Chapter will investigate why LCL have an increased capacity of process the peptide and which is the main protease involved.

CHAPTER 3: MOG processing is led by CatG but can be blocked by citrullination

3.1. Background

Professional APCs express a wide range of proteases involved in MHC-II antigen processing, but different APCs express a distinct protease profile. The endocytic proteases comprise cathepsins (Cat) and asparagine endopeptidases (AEP). Cathepsins have different substrate specificity and tissue distribution, and they are grouped in cysteine (CatB, C, F, H, L, S, V, and X), serine (CatG and A) and aspartic (CatD and E) cathepsins. Despite this variability, there is strong evidence for a "protease-determined" model of antigen processing, in which a dominant proteolytic activity "unlocks" the antigen delivering the initial cleavage, opening up the antigen for further degradation by other endoproteases and controlling the efficacy of T cell activation (Stoeckle and Tolosa, 2010). Action of the unlocking protease can both create and destroy epitopes, and its nature depends on the cell type. For example, it has been shown that AEP and CatS dominate MBP processing in monocytes and monocyte-derived DC, while CatG is the MBP unlocking protease in primary peripheral blood DC and microglia (Burster et al. 2004). Indeed, several observations have suggested that cysteine cathepsins may play a role in MS. Increased CatB activity was observed in MS lesions, CSF and PBMC and an increased expression of CatS has been documented in PBMC, hematopoietic stem cell, serum and CSF of MS patients (Staun-Ram and Miller, 2011; Martino et al., 2013), as well as in the EAE model (Clark and Malcangio, 2012). Therapeutic targeting of cysteine cathepsins has received strong interest in MS, because inhibiting CatS in mice resulted in protection from the development of EAE (Baugh et al., 2011). A recent study (Allan and Yates,

2015) shows that bone marrow-derived macrophages deficient in CatB, S, or L are equally efficient at presenting MOG to CD4+ T cells in mice. Consistently, mice deficient in either CatB or S are not clinically or immunologically protected from EAE, and cysteine cathepsin inhibitor prevents EAE in a CatS-independent manner. On the other hand, mice deficient in CatL are protected from EAE, presumably owing to thymic immunodeficiency (Allan and Yates, 2015).

Other classes of cathepsins have not been study in detail in MS. In the marmoset EAE model CatG had the ability to destroy the pathogenically critical MOG_{40-48} epitope during processing in uninfected and LCV-infected B cell lysate. CatG has chymotrypsin- and trypsin-like activity across a broad pH spectrum. The CatG active site is composed by aspartate, histidine and serine residues and can hydrolyse a peptide bond after aromatic and strongly positively charged residue (F, K, R or L) in the P1 position (Burster et al., 2010). In this chapter the ability of CatG and cysteine proteases to process MOG and CatG enzyme activity were studied in LCL, primary uninfected isolated CD20+ B cells, CpG-activated CD20+, and CD20- cells.

Several factors can affect cathepsin activation and antigen processing. These include inflammatory stimuli, cellular stress and post-translational modifications. Regarding inflammation, LPS treatment of monocyte-derived DC and macrophages results in an increase in CatS (Burster et al., 2005), CatB and CatL activity without changing the Cat RNA expression (Creasy and McCoy, 2011). Processing can also be affected by post-translational modifications present in the antigen. Citrullination is an enzymatic post-translational modification of proteins where the imino group of arginine is replaced by a keto group by the enzyme peptidylarginine deiminase (PAD) (Wang and Wang, 2013) (Figure 3.1). Citrullination usually occurs with high concentration of Ca^{2+} in autophagosomes (Ireland and Unanue, 2012)

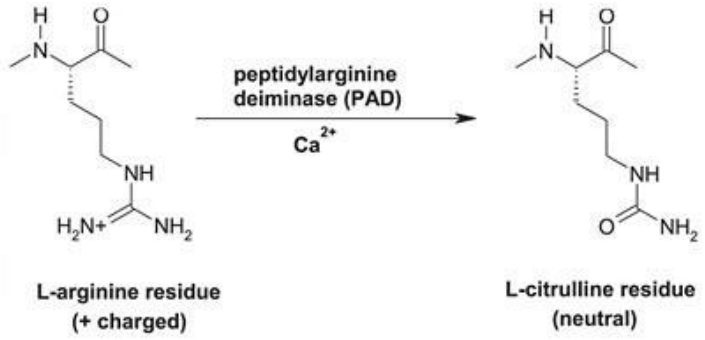


Fig. 3.1 Citrullination. Conversion of the amino acid arginine into the amino acid citrulline. Enzymes called peptidylarginine deiminases (PADs) replace the primary ketimine group (=NH) by a ketone group (=O) in presence of Ca^{2+}.

The substitution of the positively charged Arg residue for neutral Cit modifies the biological function and immunogenicity of proteins. Citrullination may affect processing by affecting the cleavage site and masking it, but also altering the three-dimensional structure and, consequently, the cleavage site accessibility. For example increased citrullination of MBP in MS brain was associated with increased susceptibility to proteolysis by CatD (Cao et al., 1999). In this chapter the function of citrullination in MOG processing was investigated. The pathogenic MOG_{40-48} peptide contains two arginine (position 41 and 46) that could function as potential CatG cleavage sites (Figure 3.2) (Burster et al., 2010). Peptides citrullinated in these motives were incubated with CatG and different cell groups and the MOG degradation was analyzed by gel electrophoresis.

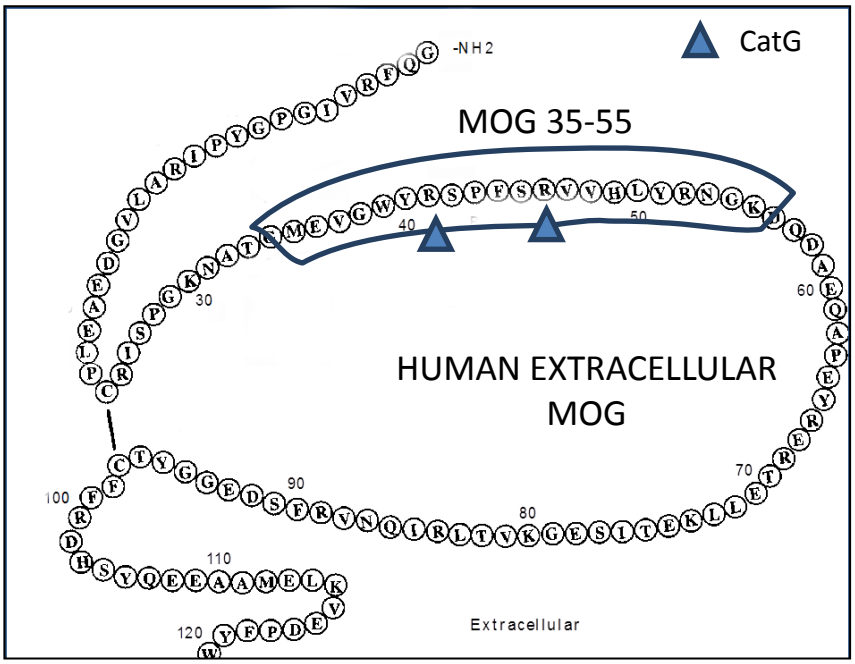


Fig. 3.2 human MOG. MOG_{35-55} contains the pathogenic peptide MOG_{40-48} in which there are 2 arginine (41 and 46) that are 2 possible CatG cleavage sites.

In this chapter the following studies will be presented:

- Degradation of rhMOG and MOG_{35-55} by primary CD20+, LCL and CD20- cells with or without cathepsin inhibitors;
- Degradation of rhMOG and MOG_{35-55} by human purified CatG;
- CatG activity in primary CD20+, CpG activated CD20+, LCL and CD20- cells;
- Degradation of citrullinated MOG_{35-55} by primary CD20+, LCL and CD20- cells.

3.2.Materials and Methods

3.2.1. Generation of different cell groups

CD20+ cells, CpG activated CD20+ cells, CD20- cells, and LCL were obtained as described previously in Section 2.2.

3.2.2. Incubation of cells with MOG

10^5 cells were incubated with 1μg of rhMOG (Cambridge Bioscience) or 2μg of MOG peptides (Peptide 2.0). CD20+, LCL, and CD20- cells were incubated for 4h and O.N. at 37°C in 96-wells plate (Costar) in complete medium with the protein and with/without the Z-Gly-Leu-Phe-CMK CatG inhibitor (0.1mM, Sigma) or a different CatG inhibitor (0.1mM, Calbiochem) or the E-64 cysteine inhibitor (0,01mM, sigma). Citrullinated peptides were purchase from Peptide2. Sequences were downloaded from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) (Appendix IV pag. 263). Modifications included substitution of the positively charged arginine residues on positions 41 and 46 for neutrally charged citrulline. In CatG purified incubation, 5mU/ml of human CatG (Sigma-Aldrich) was used.

3.2.3. Protein electrophoresis-based assay for processing experiments

Cells previously incubated with MOG and inhibitors for different time points (4h, O.N.) were washed and frozen at -20˚C. Samples with 4x loading buffer and 10x reducing agent (both Life Technologies) were loaded in NuPage Novex Bis-Tris precast protein gels, 4-12% (Life Technologies) and run at 100V. Controls (CTRL) were added loading only the MOG protein or peptides incubated without cells and only-cells without the protein. The gels were stained with SimplyBlue Safestain (Life Technologies) for 2-3h and decolorized over-night. The imaging was at Odyssey

scanner (LI-COR Biosciences) and quantified with Image Studio Lite software (version 4.0, LI-COR Biosciences). Percentage of degradation was calculated with the formula:

*(MOG incubated without cells – MOG incubated with cells) / MOG incubated without cells * 100.*

3.2.4. Cell lysing and protein quantification

Cell pellets were washed twice with PBS and re-suspended with radioimmunoprecipitation assay (RIPA) buffer (Sigma). The cell lysate was kept on ice for at least 30min and centrifuged for 10min at 8,000g at 5°C. Supernatant was transferred to a new Eppendorf ready for quantification. Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) was used for estimation of total protein content in the cell lysates. A standard curve of seven different albumin concentrations (1mg/ml – 0.015mg/ml) was employed and RIPA buffer was used as blank. Colour was measured at 562nm with a Benchmark Plus spectrophotometer (Bio-Rad) and results analysed with the Microplate Manager software.

3.2.5. Cathepsin G activity assay

CatG activity was measured in 200µg/ml cell lysates in a total volume of 40µl 160mM Tris-HCL +1.6M NaCl + 5mM dithiothreitol (DTT), pH 7.4, and with/without 0.01mM Z-Gly-Leu-Phe-CMK CatG inhibitor (Sigma). After a pre-incubation of 30min at 37°C, 10µL of 200µM Z-Gly-Gly-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) was added. The total mixture was incubated for different time points (1h, 3h, O.N.) at 37 °C and fluorescence was measured with the Fluorostar OMEGA plate reader at excitation wavelength 355nm and emission 460nm. As positive control

5mU/mL human CatG was used (Sigma-Aldrich). Values were normalized with the background measured with the cell lysates with no substrate.

3.2.6. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Paired non-parametric Friedman test was used for statistical comparisons in quantification of gels. When more than one variable was considered, paired repeated Two-way ANOVA was used. The graphs show mean with standard error of the mean (SEM) if a parametric test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤ 0.05 were considered significant and only significant p values were reported in the graphs.

3.3. Results:

3.3.1. Cathepsin G is the main protease involved in the degradation of MOG peptides

In the previous chapter, it was shown that different cell types have different ability to process rhMOG and MOG_{35-55}. To understand which are the main cathepsins involved, rhMOG and MOG_{35-55} were incubated with different cell groups (CD20+ cells, LCL and CD20- cells) in presence or absence of cathepsin inhibitors. CMK is a specific CatG inhibitor and E-64 is a broad cysteine cathepsin inhibitor. These inhibitors were chosen because CatG is the main protease involved in MOG processing in marmoset LCV-infected cells (Jagessar et al., 2016) and because cysteine proteases have been largely investigated in MS patients (Staun-Ram and Miller, 2011; Martino et al., 2013).

One representative gel is shown for 4h and O.N. incubation. Graphs show dots with medians and interquartile range of % of degradation in 4 experiments calculated only O.N. incubation because after 4h no major differences were detected. After O.N. incubation, CMK inhibited slightly the degradation of rhMOG in all the cell groups, while E-64 did not have any major effect (Fig 3.3). More strongly CMK inhibited the degradation of MOG_{35-55} in all the cell groups, while again E-64 did not have any major effect (Fig.3.4). In particular in LCL the inhibition of CMK was statistically different. Controls are presented showing a gel loaded with only-cells with or without CMK (Fig. 3.4C).

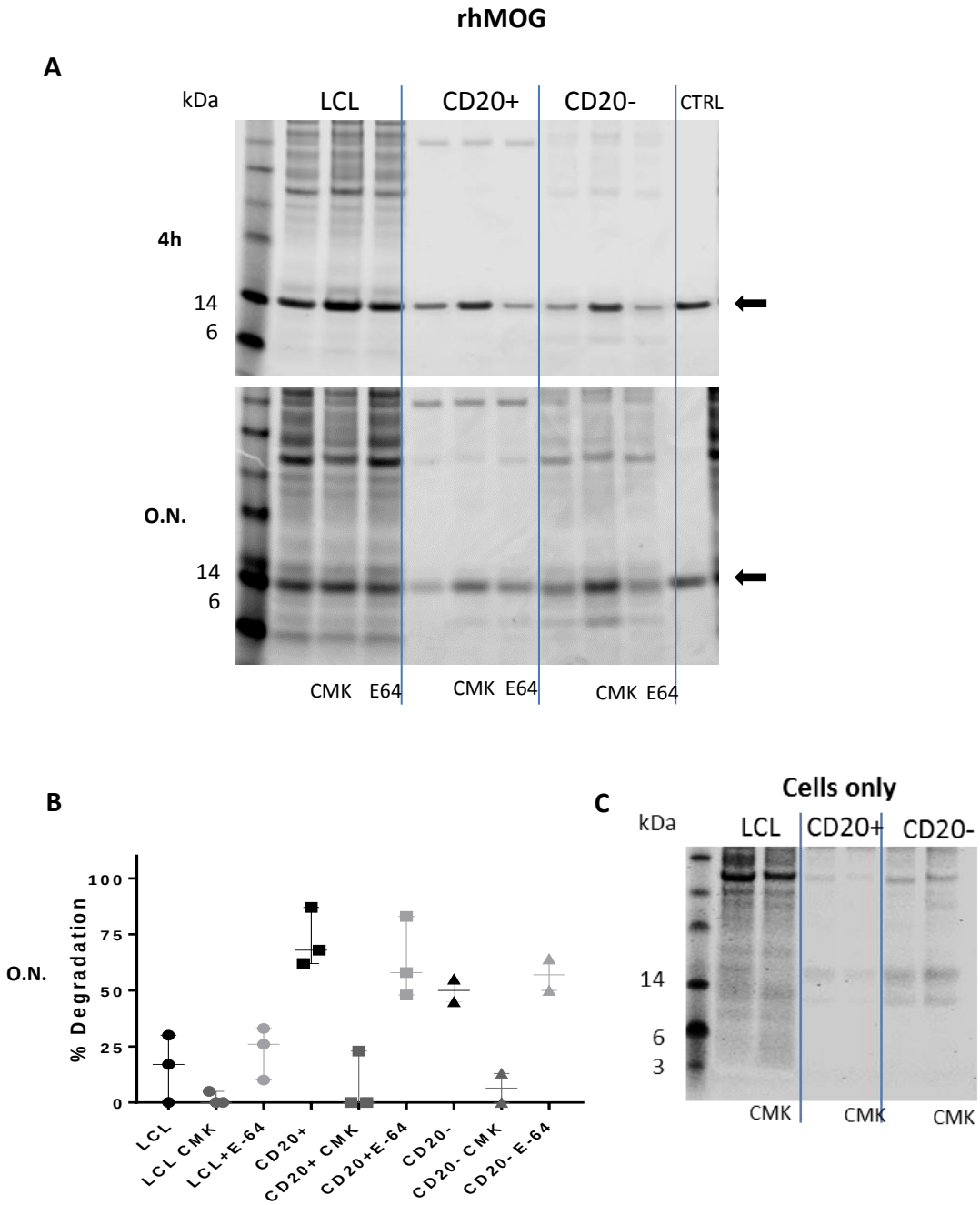


Fig. 3.3 Inhibition of rhMOG degradation by CatG and cysteine protease inhibitor in LCL, CD20+, and CD20- cells. (A) LCL, CD20+, and CD20- cells from the same subject were incubating 4h and O.N. with 1µg of rhMOG with or without CMK as CatG inhibitor and E-64 as cysteine protease inhibitor. The last line (CTRL) shows rhMOG alone as control Gels from one representative experiment are shown. **(B)** Percentage of degradation of rhMOG after O.N. incubation in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3; paired non-parametric Friedman test). **(C)** Control gel shows LCL, CD20+, CD20- cells with or without CMK incubated with no protein/peptide.

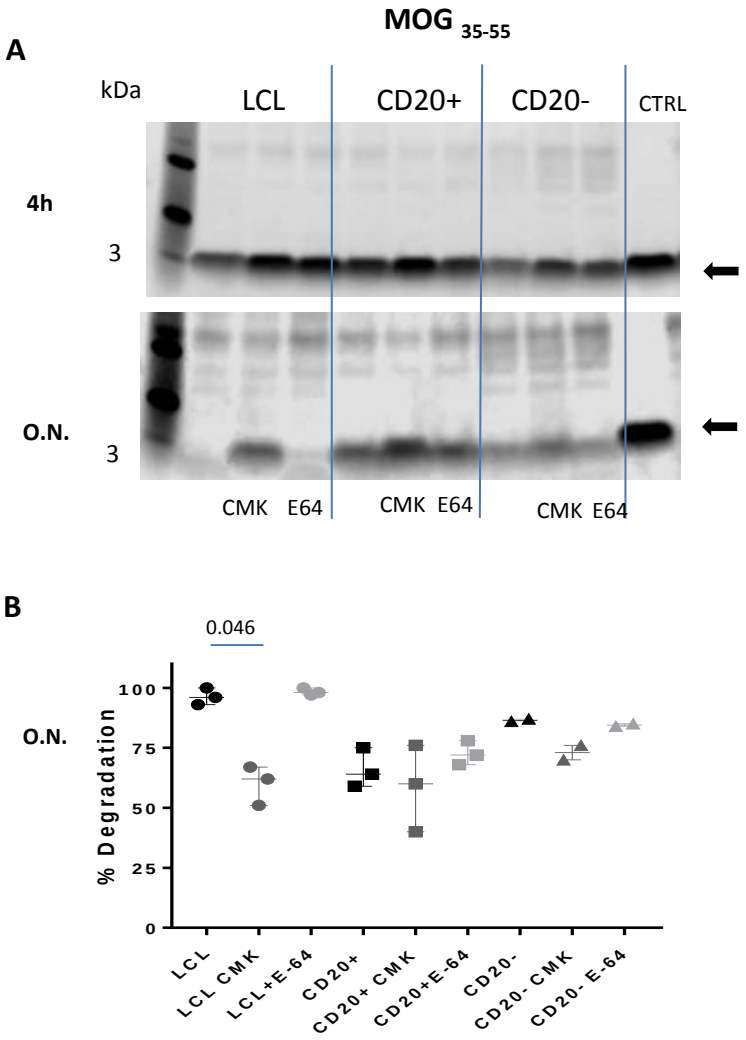


Fig. 3.4 Inhibition of MOG_{35-55} degradation by CatG and cysteine protease inhibitor in LCL, CD20+, and CD20- cells. (A) LCL, CD20+, and CD20- cells from the same subject were incubating 4h and O.N. with 2µg of MOG_{35-55} with or without CMK as CatG inhibitor and E-64 as cysteine protease inhibitor. The last line (CTRL) shows MOG_{35-55} alone as control. Gels from one representative experiment are shown. **(B)** Percentage of degradation of MOG_{35-55} after O.N. incubation in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3, paired non-parametric Friedman test).

These results indicate that in all cell types analyzed the serine protease CatG is the most important protease for the degradation of MOG and MOG_{35-55}. To confirm this role of CatG, processing experiments were repeated incubating LCL with MOG_{35-55} in presence or absence of a second CatG inhibitor (In2, $C_6H_{33}N_2O_6P$) different from CMK. Again, also the second CatG inhibitor could protect MOG_{35-55} from degradation (Fig 3.5).

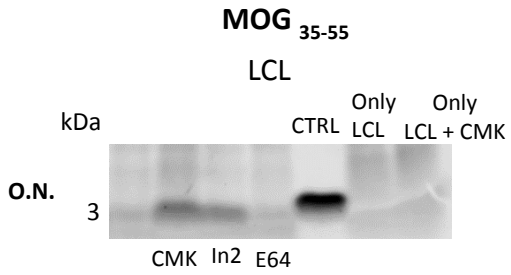


Fig. 3.5 Inhibition of MOG_{35-55} degradation by a second CatG inhibitor in LCL. LCL were incubated O.N. with MOG_{35-55} with CMK, a second CatG inhibitor (In2), and E-64. "CTRL" (control) line represents the peptide alone as positive control, while negative controls are "only LCL" line showing the cells alone and "only LCL + CMK" line showing the cells alone with CMK. One representative gel is shown out of 3 experiments.

To further test the key role of CatG in MOG processing, rhMOG and MOG_{35-55} were incubated with purified human CatG. A strong rhMOG degradation with the formation of a band at 6kDa was observed after 4h and O.N. (Fig 3.6 A), and a complete degradation of MOG_{35-55} was detected after O.N. incubation (Fig 3.6 B). The presence of CMK or In2 rescued the protein from degradation.

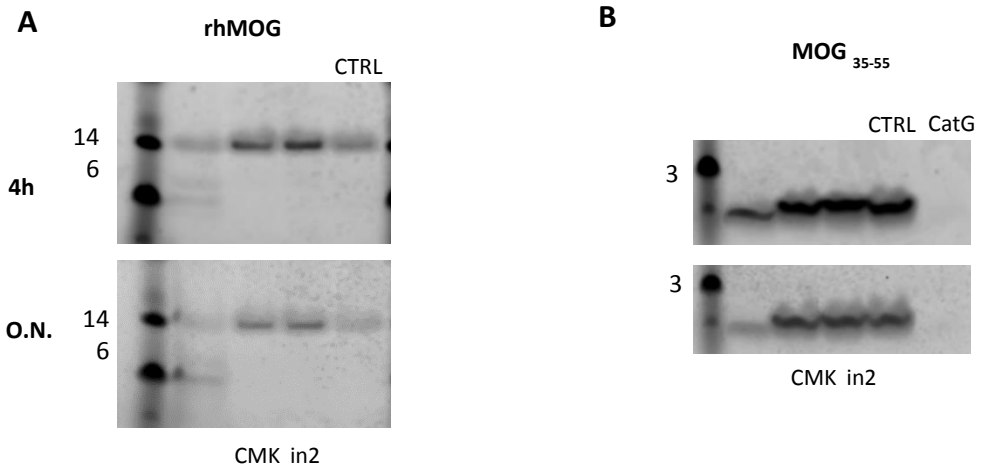


Fig. 3.6 CatG degrades rhMOG and MOG_{35-55}. Purified human CatG was incubated 4h and O.N. with **(A)** rhMOG and **(B)** MOG_{35-55} with or without CMK and In2 as CatG inhibitors. "CTRL" (control) line represents the protein/peptide alone as positive control, while "CatG" line shows the purified CatG without MOG_{35-55}. Gels from one representative experiment out of 3 are shown.

3.3.2. EBV infection upregulates Cathepsin G activity

CD20+ cells do not express CatG mRNA (Jagessar et al., 2016; Burster et al. 2004). CatG is an enzyme produced mainly by neutrophils, but cells that do not express it endogenously can internalize CatG from the serum by a thrombin-like receptor present on the cell surface (Yamazaki et al., 1997). Indeed CatG protein has been found in primary monocytes, B cells, dendritic and murine microglia cells (Burster et al., 2010). Given the difference in MOG_{35-55} degradation between primary CD20+ cells and LCL, it is possible that EBV infection increases the activity of CatG in B cells. To test this hypothesis the level of CatG activity in the different cell groups was measured after 1h (Fig 3.7 A), 3h (Fig 3.7 B) and O.N. (Fig 3.7 C) of incubation with a protease-specific fluorescent substrate. Human purified CatG was used as positive control. CatG activity was higher in LCL compared to CD20+, CpG activ CD20+ and CD20- after all time points CMK could inhibit the activity of CatG (Fig. 3.7).

Different concentrations of LCL lysate were used to confirm the specificity of the test. 0.2, 0.6 and 1.8 mg/ml of protein lysate were incubated with protease-specific fluorescent substrate and measured after 1h, 3h and O.N. (Fig 3.8). The activity increased with the concentration and in the time. CMK completely inhibited 0.2 and 0.6 mg/ml of lysate, and reduced by 50% the activity of 1.8 mg/ml. These experiments show that CatG has a clear role in the degradation of MOG_{35-55} and its activity is induced by EBV infection.

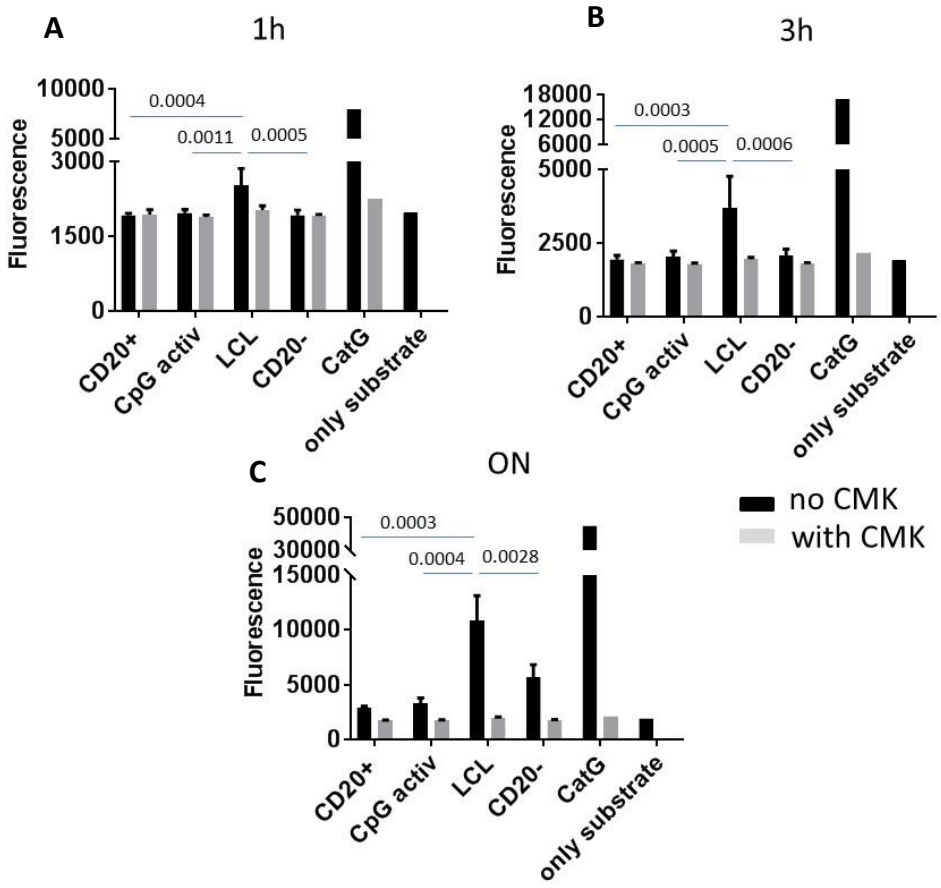


Fig. 3.7 CatG activity increases in LCL. CatG activity was detected in the indicated cell lysates (0.6 mg/ml) after **(A)** 1h, **(B)** 3h, and **(C)** O.N. incubation with a protease-specific fluorescent substrate. Graphs show means and SEM of 3 different experiments. Purified CatG was used as positive control and substrate alone as negative. Measurements were performed in the absence or presence of the inhibitor CMK. (n=3; paired repeated 2-way ANOVA comparing different group cells).

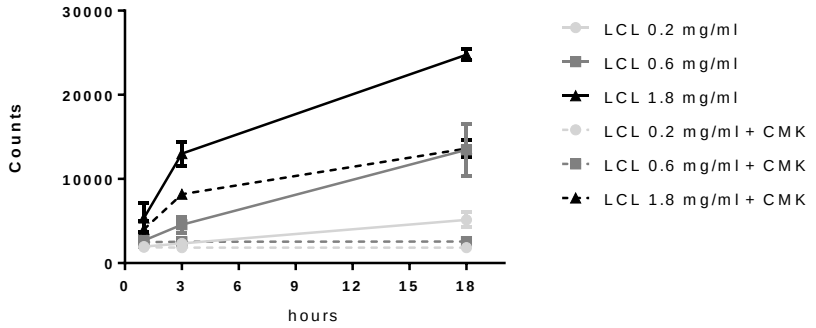


Fig. 3.8 CatG activity is concentration-dependent and increases over time. The kinetic (1h, 3h, O.N.) of different concentration of LCL lysates (0.2, 0.6, 1.8 mg/ml) with or without CMK was measured using a CatG-specific fluorescent assay. Symbols show mean ± SEM values of 3 different experiments.

3.3.3. Citrullination of Arg46 rescues MOG_{35-55} from degradation in EBV-infected B cells

The CatG active site is composed by aspartate, histidine and serine residues and can hydrolyze a peptide bond after aromatic and strongly positively charged residue (F, K, R or L) in the P1 position (Burster et al., 2010). The MOG_{40-48} epitope (YRPPFSRVV) contains four potential CatG cleavage sites: Arg (R41), Arg (R46) Tyr (Y40) and Phe (F44) (Fig 3.2). Substitution of the Arg41 or Arg46 residues by neutrally charged citrulline might affect the degradation of the peptide by CatG, therefore MOG_{35-55}cit41, MOG_{35-55}cit46 and MOG_{35-55}cit41,46 (Fig. 3.9 A,B,C respectively) were incubated with the human purified CatG for 4h and ON. All the different peptides were protected from the degradation by CatG.

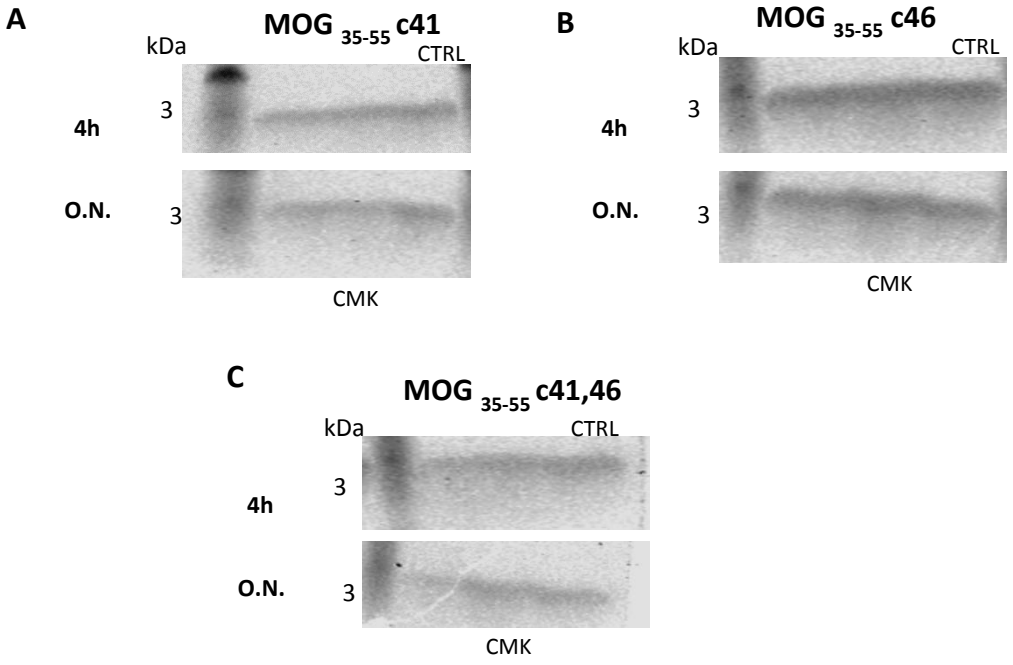


Fig. 3.9 Citrullinated peptides are protected from degradation by CatG. Purified human CatG was incubated 4h and O.N. with **(A)** MOG_{35-55}cit41, **(B)** MOG_{35-55}cit46, and **(C)** MOG_{35-55}cit41,46 with or without CMK as CatG inhibitor. "CTRL" (control) line represents the peptides alone as positive control. Gels from one representative experiment out of 3 are shown.

Next, processing experiments were performed incubating MOG_{35-55}cit41, MOG_{35-55}cit46 and MOG_{35-55}cit41,46 with the different cell groups. The replacement of the Arg41 did not alter the sensitivity of the peptide for O.N. degradation by all the cells (Fig. 3.10 A,B,G), while the Arg46Cit substitution protected from the degradation by LCL and primary and CpG activated CD20+ cells (Fig. 3.10 C,D,G). Regarding the peptide citrullinated in position 41 and 46, more variability was observed between cells from different subjects in different experiments (Fig. 3.10 E,F).

Next, the degradation of citrullinated MOG_{35-55} peptides with or without inhibition of CatG (CMK) or cysteine proteases (E-64) was tested. After ON incubation, CMK decreased the degradation of MOG_{35-55}cit41 in LCL and in CD20- cells (Fig.3.11 A,B), while in CD20+ cells and in incubation with MOG_{35-55}cit46 and MOG_{35-55}cit41,46 there were no major differences (Fig.3.11 C,D,E,F). In conclusion, the substitution of the Arg46 to Cit made the MOG_{35-55} peptide resistant against proteolytic degradation by CatG in LCL.

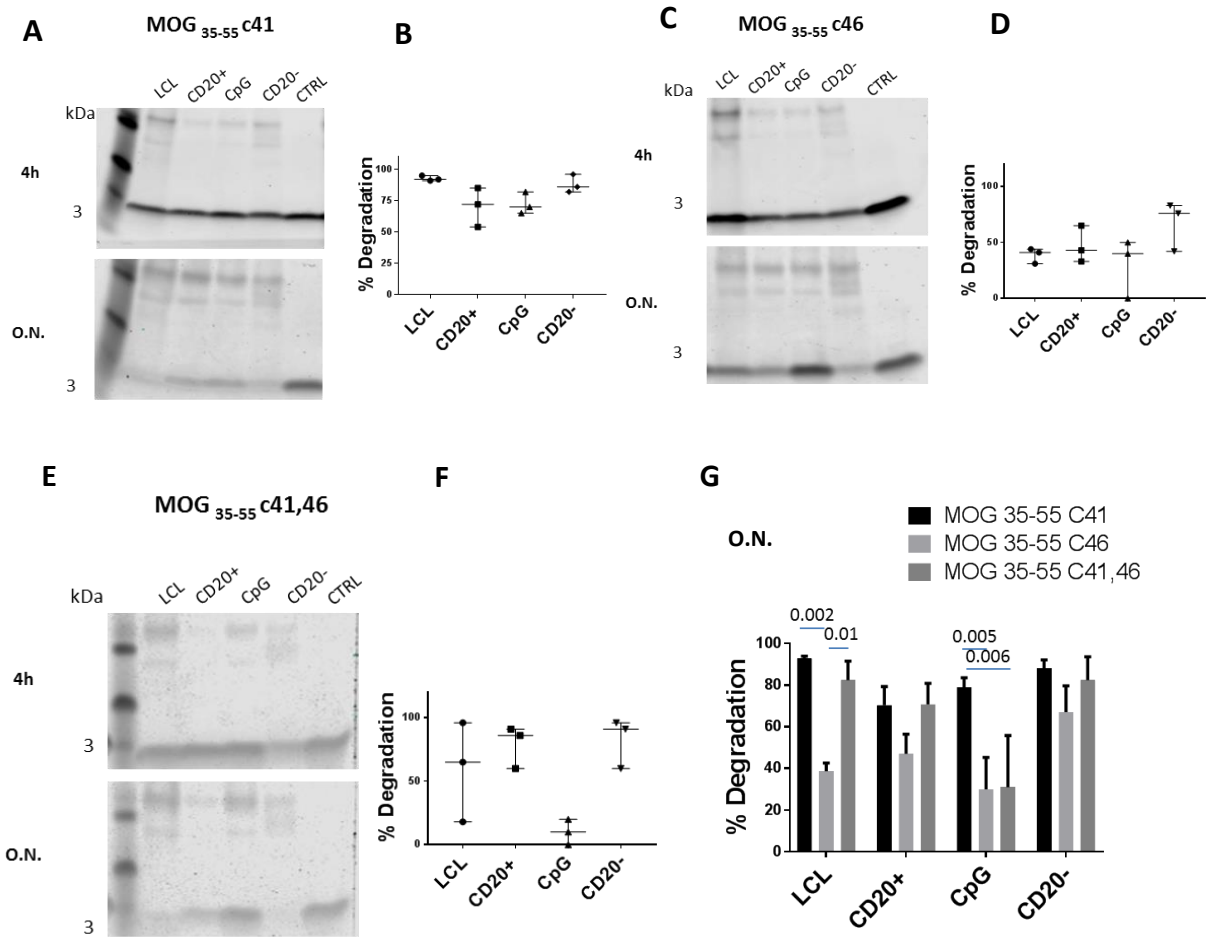


Fig. 3.10 Citrullination of MOG$_{35-55}$ in position 46 rescues the peptide from degradation. LCL, CD20+, CpG activated CD20+, and CD20- cells from the same subject were incubating 4h and O.N. with 2µg of **(A)** MOG$_{35-55}$cit41, **(C)** MOG$_{35-55}$cit46 and **(E)** MOG$_{35-55}$cit41,46. Gels from one representative experiment are shown. Percentage of degradation of **(B)** MOG$_{35-55}$cit41, **(D)** MOG$_{35-55}$cit46 and **(F)** MOG$_{35-55}$cit41,46 after O.N. incubation in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3; paired non-parametric Friedman test). **(G)** Means with SEM of the percentage of degradation of MOG$_{35-55}$cit41, MOG$_{35-55}$cit46 and MOG$_{35-55}$cit41,46 by different cell types are compared in the bar graph (n=3; paired repeated 2-way ANOVA).

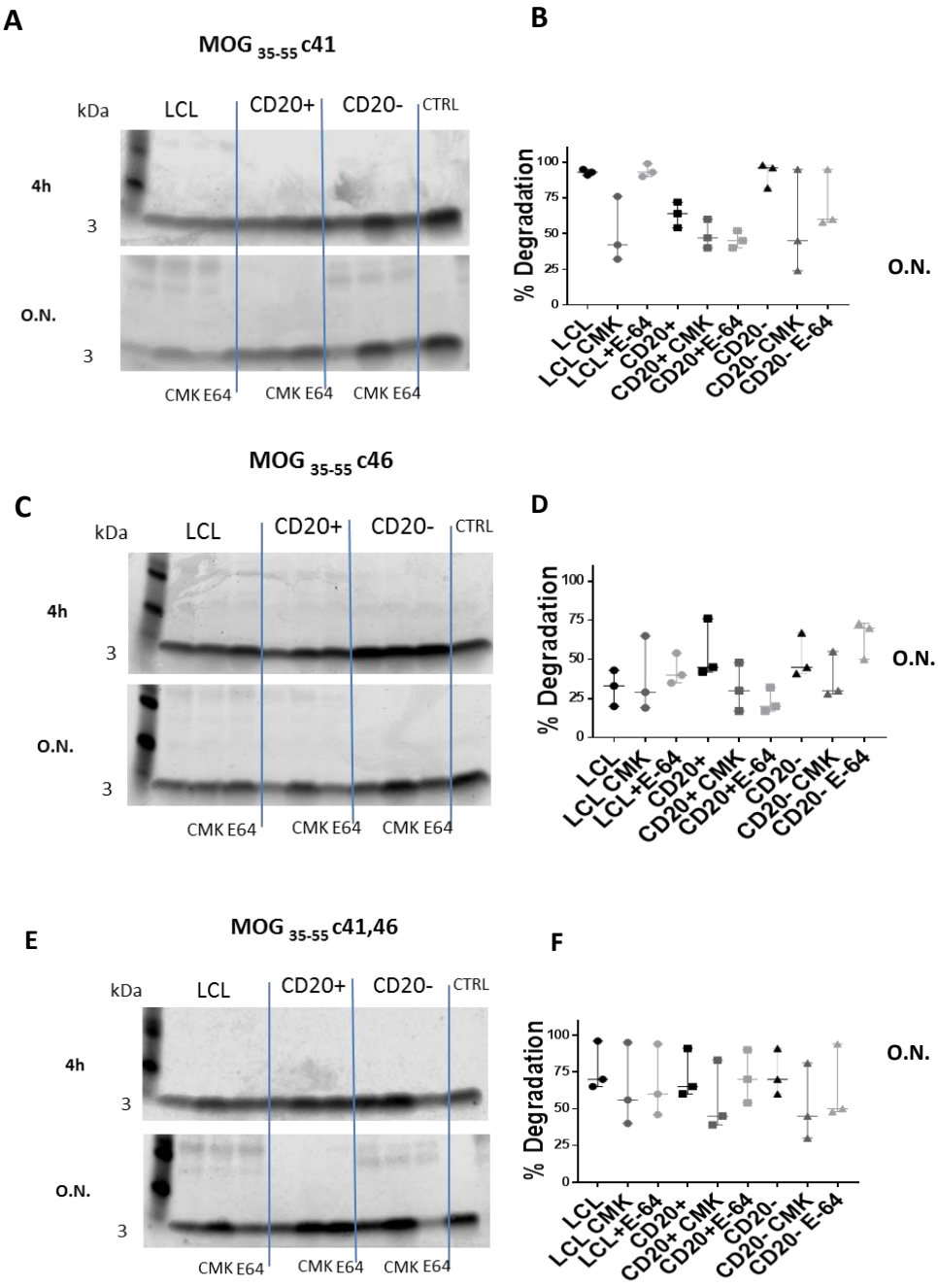


Fig. 3.11 Inhibition of MOG$_{35-55}$cit41, MOG$_{35-55}$cit46 and MOG$_{35-55}$cit41,46 degradation by CatG and cysteine protease inhibitors. LCL, CD20+, and CD20- cells from the same subject were incubating 4h and O.N. with 2µg of **(A)** MOG$_{35-55}$cit41, **(C)** MOG$_{35-55}$cit46 and **(E)** MOG$_{35-55}$cit41,46 with or without CMK as CatG inhibitor and E-64 as cysteine protease inhibitor. Gels from one representative experiment are shown. The last lines show the peptide alone as control. Percentage of degradation of **(B)** MOG35-55cit41, **(D)** MOG35-55cit46 and **(F)** MOG35-55cit41,46 after O.N. incubation in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3; paired non-parametric Friedman test).

3.4. Conclusions

These data show that CatG is the main protease involved in MOG processing and that CatG activity is increased by EBV infection. This could explain the differences in MOG_{35-55} degradation by primary uninfected B cells and LCL.

CMK inhibited rhMOG and MOG_{35-55} degradation by all cell types and especially by LCL. On the contrary E-64, the covalent cysteine cathepsin inhibitor, did not have any major effect, confirming the key role of CatG.

The role of CatG in B cells and EBV infection is controversial. In primary human B lymphocytes no RNA expression of CatG was detected, although CatG protein was present in lysosomal extracts of this cell type (Burster et al. 2004). As an explanation, it has been proposed that B cells may take up exogenous CatG present in human serum following the binding to the surface of human B cells in a receptor-mediated fashion (Yamazaki et al., 1997). Data in this thesis showed no CatG activity in primary B cells, as well as CpG-activated B cells. On the other hand, the enzymatic activity increased after EBV infection. The inactive form of CatG may be activated by the serine protease activator CatC, the production of which is increased by EBV infection of B cells (Jagessar et al., 2016). The current observation that CatG activity in LCL increased upon prolonged incubation supports this explanation. Some CatG activity was also present in CD20- cells, probably related to monocytes and DC (Burster et al., 2010).

CatG was already shown to be the key cathepsin for the processing of MBP in B cells (Burster et al. 2004). Moreover, in lysosomes derived from primary murine microglia, CatD, CatS, AEP and CatG were involved in the processing of MBP. But when microglia were treated with INF-γ to mimic the inflammatory environment of MS, CatG, and no other cathepsins, was drastically down-regulated. This resulted in less

MBP processing (Burster et al., 2007). CatG activity was strongly upregulated in LCL and this led to an increased processing of the peptide $MOG_{35-55,}$ but not of the whole protein rhMOG. This observation is in direct contrast to the MOG degradation by purified CatG, suggesting that in the LCL cell compartments rhMOG protein is in some way protected from CatG.

Citrullination is a post-translational modification that can affect protein degradation and it is dependent upon autophagy and PAD activity. All the citrullinated MOG_{35-55} peptides were protected from degradation by purified CatG, indicating that Arg41 and Arg46 are two CatG cleavage sites. Interestingly, when the peptides were incubated with different cell groups only citrullination in position 46 protected from degradation by all cell types. Most likely, MOG_{35-55}cit41 after O.N. incubation in the cells was degraded by either CatG that targeted other cleavage sites or by other cathepsins. This did not happen with MOG_{35-55}cit46, suggesting that the position of the CatG cleavage site in the peptide is important for its degradation inside the cells. Surprisingly, citrullination in both positions 41 and 46 did not always protect from degradation, but cells from different subjects behaved differently. This could be due to the physical influence of the double citrulline in the uptake of peptide by the cells, in the accessibility of the cleavage site to CatG or other enzymes, and in the stability of the peptide (Stoeckle and Tolosa, 2010).

The de novo recognition of self-antigens that have been citrullinated is an appealing basis for breakdown in immunologic self-tolerance and the immune system would target the modified self-antigen as if it were pathogen derived.

In animal models of MS, it has been reported that citrullinated forms of myelin autoantigen can induce or exacerbate autoimmunity. Citrullinated MBP was highly encephalitogenic in Lewis rats upon active immunization, and adoptive transfer of

citMBP-specific T cells led to severe clinical EAE in these animals (Cao et al., 1998). In the mouse EAE, T cells specific for citrullinated MOG peptide could provoke exacerbation of pathology if transferred into mice with ongoing EAE (Carrillo-Vico et al., 2010). Of note, mice lack an orthologue of EBV-infected B cells. The study in the NHP showed that infusion of rhesus monkeys with autologous B cells infected with an EBV-related lymphocryptovirus and pre-pulsed with citrullinated MOG_{34-56} peptide induced autoreactive T cell activation and early signs of encephalitis (Haanstra et al., 2013b).

There is increasing evidence that citrullination may also play an important role in human MS pathogenesis. About 18% of MBP protein is citrullinated in healthy individuals, while 45% or more of MBP is citrullinated in MS patients (Wood et al., 1996). Increased citrullinated MBP was found in areas of both ongoing and past demyelination in active and chronic active MS lesions, while lower levels of citrullinated proteins were observed in control white matter (Bradford et al., 2014). As well, increased citrullination was noticed in brains of EAE mice (Nicholas et al., 2005). In the inflamed brain, the myelin sheath becomes compromised when myelin is hyper-citrullinated. It becomes partially unfolded and its interaction with phospholipids is weakened, leading to sheath instability. In addition to these biophysical effects of citrullination, published evidence also suggests an immunological role for protein citrullination in MS. It was shown that stimulation with citrullinated MBP generated a higher number of T-cell lines from MS patients and that these cell lines generally responded with greater sensitivity to citrullinated MBP than to the unmodified protein (Tranquill et al., 2000). Moreover, DRB1*15:01 and DRB5*01:01, which comprise the DR2 haplotype that confers the greatest genetic risk for MS, preferentially present peptides that have been citrullinated at key HLA-

binding residues. Therefore, although definitive evidence remains lacking, it is highly probable that citrullinated myelin antigens are preferentially presented by DRB1*15:01 and DRB5*01:01 and recognized by T cells and antibodies in MS patients (Nguyen and James, 2016).

Little is known about the cause of MS brain citrullination and it has been proposed that environmental factors associated with MS could cause this post-translational modification. For instance, smoking can induce cell necrosis, with cell membrane damage and perturbation of intracellular Ca^{2+} homeostasis. The high cytoplasmic concentration of Ca^{2+} in autophagosomes could initiate the citrullination process (Klareskog et al., 2011). As well, viruses could be the cause of both foreign and host citrullinated proteins, leading to autoimmune cross-reactivity against host peptides. Nevertheless, how those environmental factors act on citrullination in MS remains to be investigated. The next Chapter will evaluate the importance of the position 46 in MOG_{35-55}, its connection with autophagosomes, and the possibility that EBV could induce citrullination through autophagy.

CHAPTER 4: Role of Autophagy in antigen processing in LCL

4.1.Background

Citrullination has been shown to occur in autophagosomes. Autophagy is a cell biological process that was first discovered as survival mechanism of cells under conditions of nutritional restriction. It participates in multiple cellular processes, including clearance of intracellular pathogens, misfolded proteins and dysfunctional organelles, and antigen (cross-) presentation (Munz, 2009; Mintern et al., 2015; Shibutani et al., 2015). In starvation-induced autophagy essential nutrients are produced by recycling of cell components. Autophagy induction involves the assembly of membrane pieces to a concave double membrane structure (isolation membrane) with the help of multiple autophagy-related (Atg) proteins. By fusion of several phagophores closed double membrane vacuoles are formed, called autophagosomes (Fig 4.1). Prior to the assembly with lysosomes, autophagosomes may fuse with endosomes, containing proteins sorted for degradation and endocytosed receptor-ligand complexes; these fusion products are indicated as amphisomes. Calcium ions seem to play a role as important co-factor in autophagosome maturation and accumulate in autophagosomes and amphisomes (Fader and Colombo, 2009). Amphisomes travel to the perinuclear region of the cell, to fuse with lysosomes and form autophagolysosomes (Fig 4.1). Inside the autophagolysosomes the cargo is degraded by lysosomal proteases, including cathepsins. The microtubule-associated light chain 3 (LC3), in particular the phosphatidylethanolamine-linked isoform LC3-II, is expressed on the double membrane of the autophagosome and serves as a docking molecule for proteins targeted for degradation (Fig 4.1).

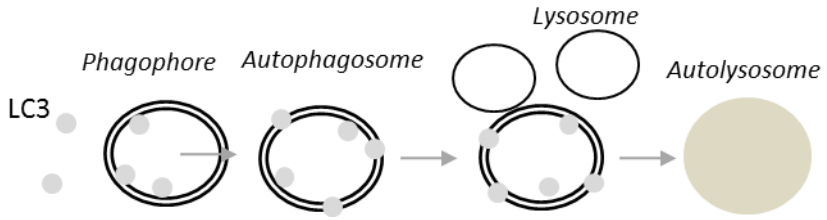


Fig. 4.1 Autophagy flux. The assembly of autophagosomes includes the cooperation of Atg8/LC3, the docking molecule to which autophagy receptors bind. Fusion of autophagosomes with lysosomes induces degradation of enclosed cargo.

Selective targeting of cargo to autophagosomes is mediated by autophagy receptors, which contain LC3-interacting region (LIR) motifs (Birgisdottir et al., 2013). In the previous Chapter it was found that the Arg to Cit46 substitution made MOG_{35-55} resistant to proteolytic degradation by LCL, while citrullination of the Arg41 residue seemed to have no effect. The Arg46 residue is located in the ^{44}FSRV^{47} LIR motif of MOG_{35-55} (Fig. 4.2). It is possible that protection of a LIR motif against cleavage by CatG via citrullination permits binding to LC3-II and association with the autophagosome. This hypothesis was tested in further processing experiments together with another immunodominant MOG peptide, i.e., MOG_{1-20}, which contains a putative F-LIR motif in residues ^{3}FRVI^{6} (Fig. 4.2).

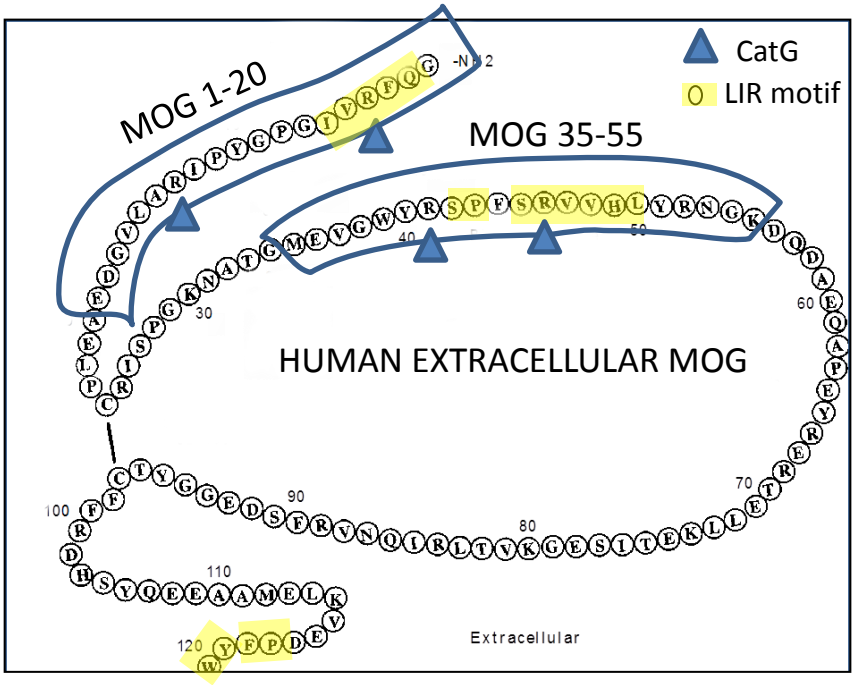


Fig. 4.2 Human MOG. rhMOG contains 3 LIR motifs, one in MOG_{35-55} (45-49), one in MOG_{1-20} (3-6) and another in $MOG_{118-122}$.

To further explore the role of autophagy in MOG processing, LCL were treated with drugs inducing or inhibiting autophagy and incubated with different MOG peptides. Moreover, to verify if EBV could induce autophagy, LC3 protein expression was assessed in different cell types by Western blotting and immunofluorescence.

Citrullination is the key peptide modification in the context of autophagy and it is mediated by the catalysing peptidylarginine deïminase (PAD) (Fig. 3.1) (Ireland and Unanue, 2012). Five isoforms of PAD exist (PAD1, 2, 3, 4 and 6), which differ in tissue distribution: PAD1 is mainly expressed in epidermis and uterus; PAD2 is widely expressed in multiple tissues, including blood, secretory glands, brain, uterus, spleen, pancreas, skeletal muscle; PAD3 is localized to epidermis and hair follicles; PAD4 is detected mainly in white blood cells including granulocytes and monocytes and in

tumours; PAD6 was originally identified in eggs and embryos (Wang and Wang, 2013).

PAD2 and 4 are relevant in EAE model and their overexpression in transgenic mice increases the amount of citrullinated MBP and accelerates the development of demyelination (Carrillo-Vico et al., 2010). Indeed, PADs are up-regulated under pathological conditions, such as in tissue inflammation (Klareskog and Catrina, 2015), and in particular higher expression of PAD2 has been found in PBMC of MS patients compared to HC (Calabrese et al., 2012).

EBV infection could cause citrullination inducing also PAD expression other than autophagosomes. The RNA relative expression of PAD2 was analysed in different cell groups.

In this chapter the following studies will be presented:

- Degradation of native and citrullinated MOG_{1-20} by primary CD20+, CpG activated Cd20+, LCL and CD20- cells;
- Co-localization between autophagosomes and rhMOG;
- Degradation of MOG peptides by LCL following autophagy modulation;
- Autophagy marker LC3 expression in primary CD20+, CpG activated Cd20+, LCL and CD20- cells;
- PAD2 RNA expression in primary CD20+, CpG activated Cd20+, LCL and CD20- cells;

4.2.Methods

4.2.1. Generation of different cell groups, incubation of cells with MOG and protein electrophoresis-based assay for processing experiments

As described previously in Section 2.2 and 3.2. MOG_{1-20} and citrullinated peptides were purchased from Peptide2. Sequences were downloaded from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) (Appendix IV pag. 263). Modifications included substitution of the positively charged arginine residues on positions 4, 13, 41, and 46 with neutrally charged citrulline.

4.2.2. Immunofluorescence

Cells were washed with PBS with 2mM EDTA and 0.5% BSA, and 50,000 cells in 200µl were cytospun on SuperFrost microscope slides (Menzel-Glazer, Thermo Fisher Scientific) with the Cytospin 4 Cytocentrifuge (ThermoFisher Scientific) for 12min at 17.88g. Cells were fixed and permeabilized for 10min with cold 100% methanol, air dried, blocked for 10min with PBS + 5% FCS and incubated with 1:100 rabbit monoclonal anti human LC3A/B (D3U4C) antibody (Cell Signaling), 1:100 mouse monoclonal anti human ß actin antibody (Sigma-Aldrich) and 1:100 mouse IgG anti human MOG (8-18C5, kindly donated by prof. Linington) in PBS+ 5% BSA over-night at 5˚C. Cells were washed three times with PBS and incubated with 1:1000 secondary CF™ 488A goat anti-rabbit IgG (H+L) antibody and CF™ 568 goat anti-mouse IgG (H+L) antibody (both from Sigma-Andrich) for 1h at room temperature in PBS + 1% BSA. After four final wash steps with PBS, coverslips were mounted with Vectashield mounting medium for fluorescence with DAPI (VECTOR). Control slides with single colour and no secondary antibodies were included as controls. Imaging was performed with LSM880 confocal laser scanning microscope (Zeiss). Measure of

LC3 relative expression was calculated with ImageJ (ImageJ-win64, NIH). Thresholds were set to define Regions of Interest (ROIs) using the actin images. These ROIs were then used to analyze the expression of actin and LC3 within each "particle" (individual cell, at least 10 cells per image). Three images were acquired for each different condition. The mean pixel intensity of CD20+ cells was used as denominator to normalize the expression of LC3 in the other cell types. Different experiments were performed maintaining identical image acquisition settings and exposure times.

4.2.3. Autophagy modulation

To modulate autophagy, LCL were cultured either with 800nM Rapamycin (RAP, Calbiochem) for 4h or 500nM Bafilomycin (BAF, Calbiochem) for 3h or 10mM 3-MA (Sigma-Aldrich) for 1h. Cells were the either incubated with MOG peptides as described above or lysed for LC3 Western blotting.

4.2.4. Cell lysing and protein quantification

Cell pellets were washed twice with PBS and re-suspended with RIPA buffer (Sigma). The cell lysate was kept on ice for at least 30min and centrifuged for 10min at 8,000g at 5°C. Supernatant was transferred to a new Eppendorf ready for quantification. BCA Protein Assay Kit (Thermo Scientific) was used for estimation of total protein content in the cell lysates. A standard curve of 7 different albumin concentrations (1mg/ml – 0.015 mg/ml) was employed and RIPA buffer was used as blank. Colour was measured at 562nm with a Benchmark Plus spectrophotometer (Bio-Rad) and results analysed with the Microplate Manager software.

4.2.5. LC3 Western blotting

Equal amounts of cell lysates (5-15µg) were resolved by a 12% denaturating SDS–polyacrylamide gel electrophoresis (180 V, for 60min). Proteins were transferred to

polvinylidene fluoride (PVDF) transfer membrane (GE Healthcare Life Sciences) (30 V, 90 min) and blocked in PBS-tween 2% BSA (Sigma-Aldrich) for 1h. Blots were incubated over-night with 1:1000 rabbit monoclonal anti human LC3A/B (D3U4C) antibody (Cell Signalling) and 1:10000 mouse monoclonal anti human β-actin antibody (Sigma-Aldrich) at 4°C. Then incubated with 0.06µg/ml secondary 800CW Donkey anti-Rabbit IgG (H + L) and 680RD Donkey anti-mouse IgG (H + L) (both from LICOR Biosciences) for 1h at room temperature. The membrane was scanned with an Odyssey scanner (LI-COR Biosciences) at 700nm (LC3) and 800nm (actin), and band intensities were quantified with Image Studio Lite version 4.0 software (LI-COR Biosciences). To calculate LC3 relative expression, in each Western Blot the LC3 band intensity for each cell group was divided by the corresponded band intensity of the actin and of CD20+ cells (considered as 1).

4.2.6. RNA extraction

Total RNA was isolated from 1-5.10^6 cells from different cell groups using High pure RNA isolation kit (Roche) following the manufacturer's instructions. Cells were lysed with the Lysis/Binding buffer and lysate was transferred to the High Pure filter tube. RNA was selectively bound to the High Pure filter tubes Remaining contaminants were removed in several wash steps and the membrane was treated with DNase I to remove trace amounts of bound DNA. After the wash steps, RNA was extracted in the elution buffer provided by the kit and stored at -80ºC. RNA concentration was determined by measuring the absorbance at 260nm using Nanodrop ND-100 (Thermo Scientific).

4.2.7. cDNA synthesis

For cDNA acquisition, 10µl RNA samples (0.5µg), 2µl Random hexamers and 1µl dNTPs mix (10mM) (all Promega) were mixed, followed by a 5min incubation at 65^{o}C for first strand cDNA synthesis. A master mix containing 1µl RN-ase inhibitor (Promega), 1µl DTT (0.1M) and 4µl 5x First-Strand Buffer was added, along with 1µl Superscript III reverse transcriptase (RT) (220 units/µl) – an engineered Moloney murine leukemia virus RT (all Invitrogen). Negative control reactions replacing the RNA template or the RT with DNase/RNase free H_20 were included. Samples were incubated as followed: 5 min at 95^{o}C, 60 min at 50^{o}C and 25 min at 70^{o}C. cDNA was stored at -80^{o}C.

4.2.8. PAD2 Real-time RT-PCR

After a titration of different cDNA dilutions, 1:2 DNase/RNase free H_20 dilution was selected. Relative quantification was performed using Hydroxymethylbilane Synthetase (HMBS) as housekeeping gene. Each RT-PCR reaction included 2µl of diluted cDNA and 12.5µl of Faststart Universal probe master (Rox) (Roche). PAD2 Taqman (TaqMan®/Invitrogen) probe/forward and reverse primer mix at final concentration 900nM were used. DNase/RNase free H20 was added up to 25µl. All samples run in duplicates. RT- PCR reactions were held using the 7900HT Fast Real-Time PCR system (Applied Biosystems) in 96-well plates. The following incubation protocol was imposed: 10 min at 95^{o}C and 40 cycles of 10 sec at 95^{o}C followed by 30 sec at 60^{o}C. All the samples run in duplicates during RT-PCR, thus the average cycle threshold (Ct) value of each sample was obtained. The Ct values of PAD2 was normalised compared to the Ct value of HMBS. Comparison of the relative amounts of PAD2 in CpG activated CD20+, LCL and CD20- cells compared to primary CD20+

was performed using the $2^{-\Delta\Delta Ct}$ method. Any change in gene expression between cell groups was expressed as fold change using the formula below: Fold increase for each cell group compared to the CD20+cell group = $2^{-\Delta\Delta Ct}$. Where:

For each sample: $\Delta\Delta Ct = [(Ct\ PAD2 - Ct\ HMBS) - \Delta CtCD20+]$

$\Delta CtCD20+ = [(Ct\ PAD2\ CD20+ - Ct\ HMBSCD20+) + ...] / n$

n=number of samples

4.2.9. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Paired non-parametric Friedman test was used for statistical comparisons in quantification of gels and RT-PCR due to the small population analysed (n<6). For immunofluorescence experiments One-way ANOVA was used. When more than one variable was considered, paired repeated Two-way ANOVA was used. The graphs show mean with standard error of the mean (SEM) if a parametric test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤0.05 were considered significant and only significant p values were reported in the graphs.

4.3. Results

4.3.1. Citrullination of the residue in the LIR motif rescues the peptide from processing

The Arg46 residue is located in the ^{44}FSRV^{47} LIR motif and its citrullination, that prevents the cleavage by CatG, may permit the binding of the peptide to LC3-II enclosing MOG_{35-55} in the autophagosomes. The consensus sequence of LIR motifs is [W/Y/F]xx[L/I/V], where x can be any amino acid (Birgisdottir et al., 2013). In addition to this core motif, the presence of an acidic residue (E, D, S, T) close to the N-terminal site of the LIR motif is important. Using this algorithm the rhMOG sequence was scanned and 3 other potential LIR motifs were found: residues 3 to 6 (FRVI), 44 to 47 (FSRV) and 118 to 122 (FYWV) (Fig 4.2). To test this concept, processing experiments were performed using the immunodominant peptide MOG_{1-20} (Bielekova et al., 2004). After incubating MOG_{1-20} with different cell types, it was observed that the peptide was degraded by LCL and CD20- after O.N. incubation, while primary and CpG activated CD20+ cells did not process it (Fig. 4.3).

LCL were incubated with MOG_{1-20} with or without CatG and cysteine protease inhibitors. CMK and the second CatG inhibitor (In2) could protect MOG_{1-20} from degradation by LCL, thus further confirming the predominant role of CatG in this process (Fig 4.4).

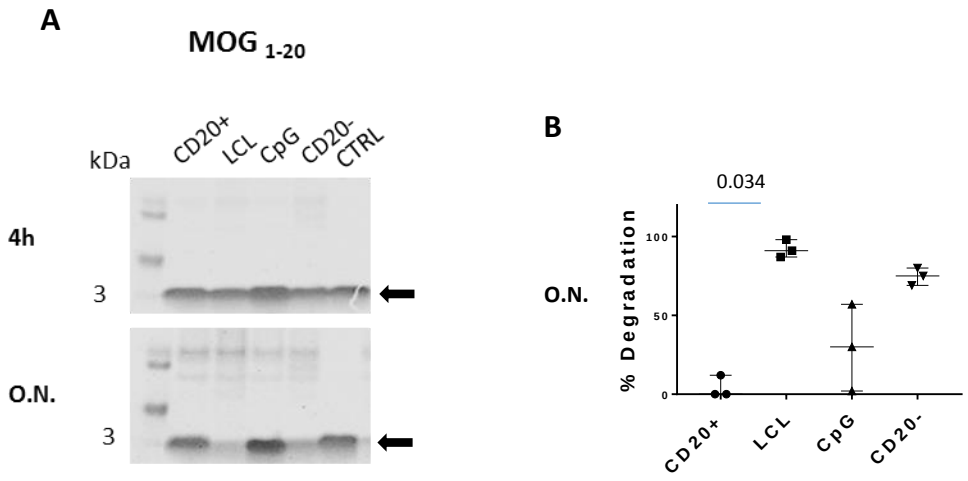


Fig. 4.3 MOG_{1-20} is totally degraded by LCL and CD20- cells. The presence of MOG_{1-20} can be detected with SDS gel (⬅). (A) CD20+, LCL, CpG activated CD20+, and CD20- cells from the same subjects were incubating 4h and O.N. with 2µg of MOG_{1-20}. The last line shows the peptide alone as control. Gels from one representative experiment are shown **(B)** Percentage of degradation of MOG_{1-20} after O.N. in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3; paired non-parametric Friedman test).

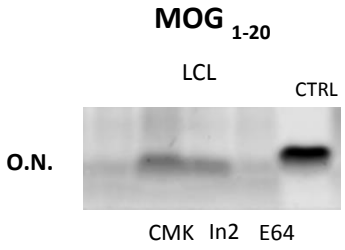


Fig. 4.4 Inhibition of MOG_{1-20} degradation by CatG inhibitor. LCL were incubating O.N. in presence or absence of CMK and In2 as CatG inhibitors and E-64 as cysteine inhibitor with 2µg of MOG_{1-20}.The last line shows the peptide alone as control. Gel from one representative experiment out of 3 is shown.

In MOG_{1-20} sequence there are 3 Arg in position 4,9,13 (Fig.4.2). Substitution of the Arg4 or Arg13 residues by neutrally charged Cit might affect the degradation of the peptides, therefore processing experiments were performed incubating MOG_{1-20}cit4, MOG_{1-20}cit13 and MOG_{1-20}cit4,13 with the different cell groups. The replacement of the Arg4 protected the peptide from the degradation by all cell types (Fig. 4.5 A, B), while citrullination in position 13 did not alter the sensitivity of the peptide for degradation by LCL after O.N. incubation (Fig. 4.5 C, D). The peptide citrullinated in both position 4 and 13 appeared to be highly degraded by all the groups except CD20+ cells (Fig 4.5 E, F). Of note, the citrullination in position 4 is within the LIR motif (Fig 4.2). As in MOG_{35-55}, also in $MOG_{1-20,}$ the citrullination of the residue in the LIR motif (4) rescues the peptide from the processing, suggesting the association between this MOG peptide and autophagosomes.

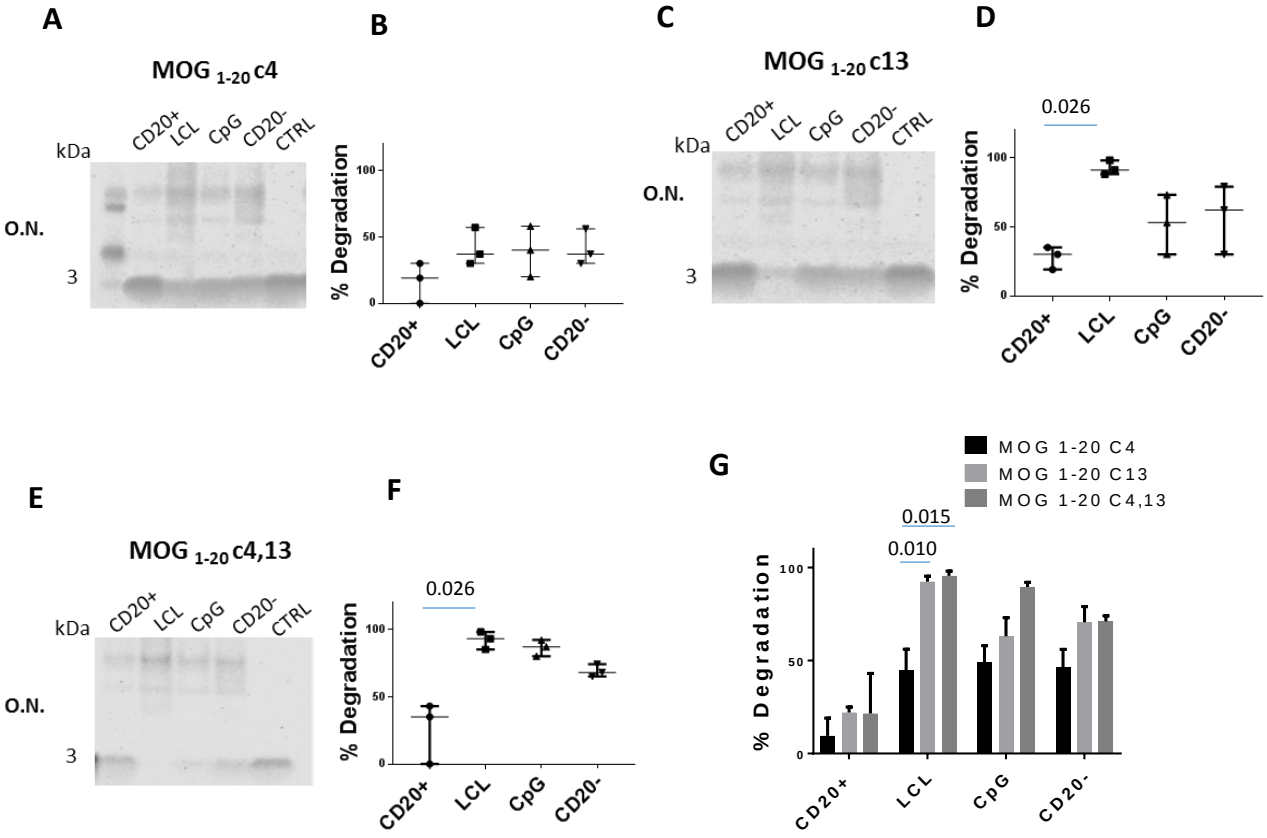


Fig. 4.5 Citrullination of MOG$_{1-20}$ in position 4 rescues the peptide from degradation by LCL. CD20+, LCL, CpG activated CD20+, and CD20- cells from the same subject were incubating O.N. with 2µg of **(A)** MOG$_{1-20}$cit4, **(C)** MOG$_{1-20}$cit13 and **(E)** MOG$_{1-20}$cit4,13. The last line (CTRL) shows the peptide alone as control. Gels from one representative experiment are shown. Percentage of degradation of **(B)** MOG$_{1-20}$cit4, **(D)** MOG$_{1-20}$cit13 and **(F)** MOG$_{1-20}$cit4,13 $_{20}$ after O.N. in 3 experiments is illustrated in dots. Median and interquartile range are indicated by bars. (n=3; paired non-parametric Friedman test). **(G)** Means with SEM of the percentage of degradation of MOG$_{1-20}$cit4, MOG$_{1-20}$cit13 and MOG$_{1-20}$cit4,13 by different cell types are compared in the bar graph (n=3; paired repeated 2-way ANOVA).

4.3.2. Internalized MOG co-localizes with autophagosomes

Citrullination of the Arg residue within a LIR motif rescues the peptides from degradation, suggesting a role for the autophagosome in their processing.

LC3, and in particular the phosphatidylethanolamine-linked isoform LC3-II, is the most used phagophore/autophagosome marker. After having showed in the second Chapter (section 2.3.2) internalization of rhMOG by LCL, here its localization and LC3 expression were detected by immunofluorescence simultaneously. MOG co-localized with LC3, suggesting the internalization of the protein in the autophagosomes (Fig. 4.6).

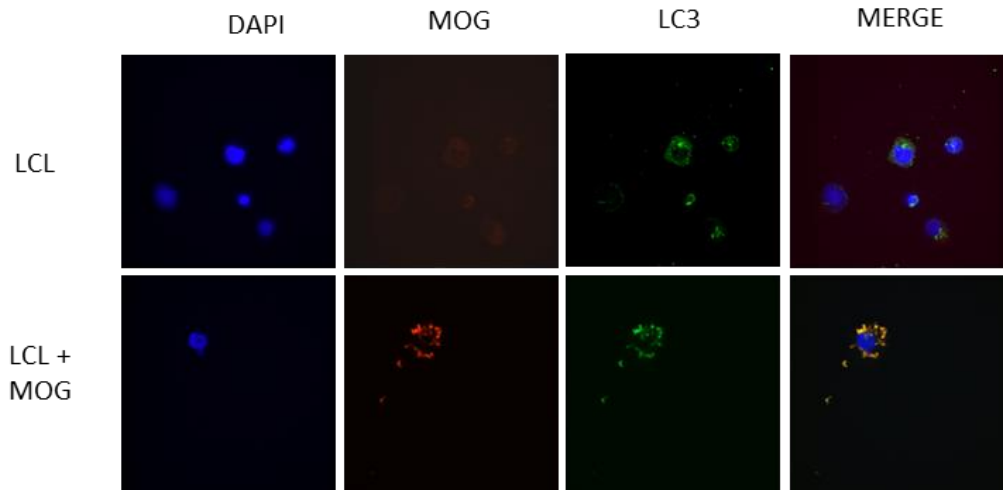


Fig. 4.6 Co-localization between MOG and LC3. LCL were incubated 1h with or without MOG and stained for immunofluorescence with primary 8-I8C5 anti-MOG (red) and anti-LC3 Abs (green). One representative experiment is shown out of 3 experiments. Original magnification X400.

4.3.3. Inducing autophagy in LCL further protects MOG peptides citrullinated in LIR motif from degradation

To further explore the role of autophagy in MOG processing, the effect of autophagy modulation was studied. LCL were treated with Rapamycin (RAP), Bafilomycin (BAF) or 3-MA (Fig 4.7). Rap, inhibiting mTOR signaling, induces the activation of autophagy; Baf is an inhibitor of the fusion between autophagosomes and lysosomes; 3-MA is an inhibitor of the formation of phagophores.

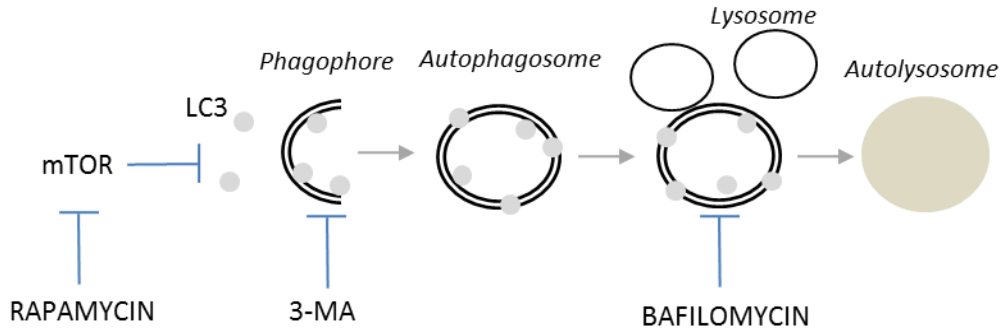


Fig. 4.7 Autophagy flux modulation. LCL were treated with the autophagy inducer Rapamycin (RAP), the autolysosome inhibitor Bafilomycin (BAF) and the autophagy inhibitor 3-MA.

Treated LCL were split; some were used to detect LC3-II expression by WB, and some were incubated with MOG peptides. As expected, treatment of LCL with Rap and with Baf led to increased levels of LC3-II, due to its increased expression and accumulation, respectively. 3-MA had modest effects (Fig. 4.8). The same LCL treated with the above-mentioned stimuli were incubated with MOG_{35-55}, MOG_{35-55}cit41, MOG_{35-55}cit46 (Fig 4.9), MOG_{1-20}, MOG_{1-20}cit4, MOG_{1-20}cit13 (Fig. 4.10). The key finding was that Rap treatment further reduced degradation of the citrullinated peptides MOG_{35-55}cit46 (Fig. 4.9 C, D) and MOG_{1-20}cit4 (Fig. 4.10 B, D), while 3-MA increased their degradation. This suggests that stimulation of autophagy enhances the protection of peptides with a CatG-resistant LIR motif from degradation in LCL.

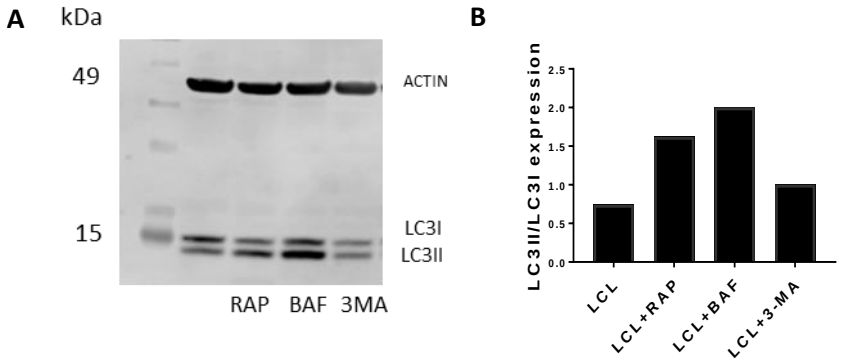


Fig. 4.8 Autophagy flux in treated LCL. Autophagy activity was detected through WB in cells treated with Rapamycin (RAP), Bafilomycin (BAF) or 3-MA. **(A)** Treated LCL were immune-blotted for LC3 and actin. **(B)** The density of the LC3-I and LC3II bands were quantified, corrected for the actin, and the ratio LC3-II/LC3-I was plotted in the graph. One representative experiment is shown out of 3 experiments. These treated cells were used for experiments showed in Fig. 9 and Fig.10.

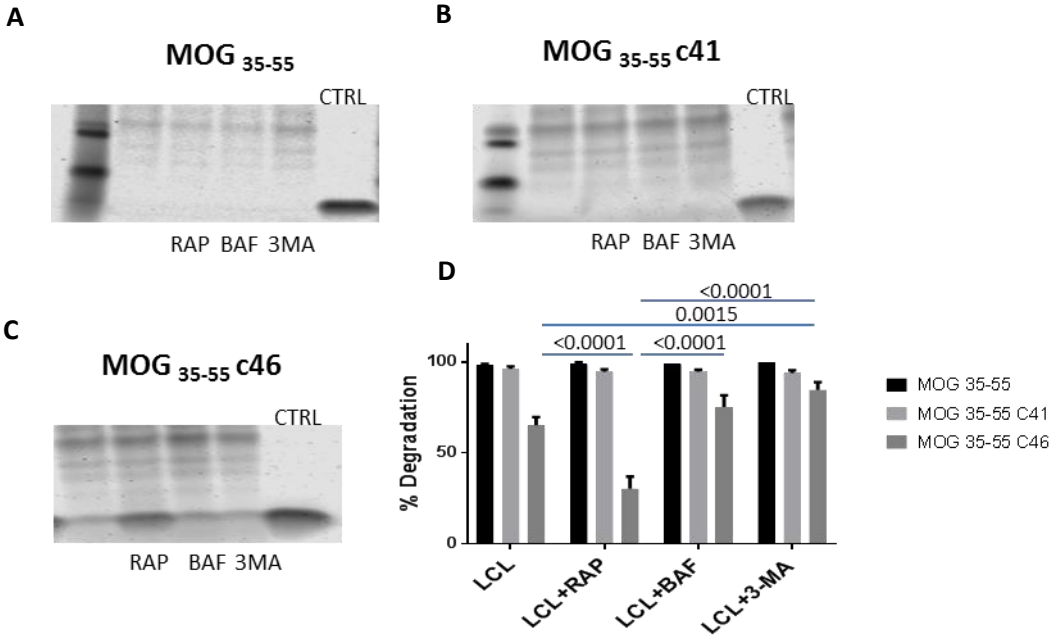


Fig. 4.9 Autophagy is directly involved in MOG_{35-55} processing. LCL treated with Rapamycin (RAP), Bafilomycin (BAF) or 3-MA were incubated O.N. with the peptides **(A)** MOG_{35-55}, **(B)** MOG_{35-55}cit41, and **(C)** MOG_{35-55}cit46. **(D)** Means with SEM of the percentage of degradation of MOG_{35-55}, MOG_{35-55}cit41 and MOG_{35-55}cit46 by treated LCL are compared in the bar graph (n=3; paired repeated 2-way ANOVA).

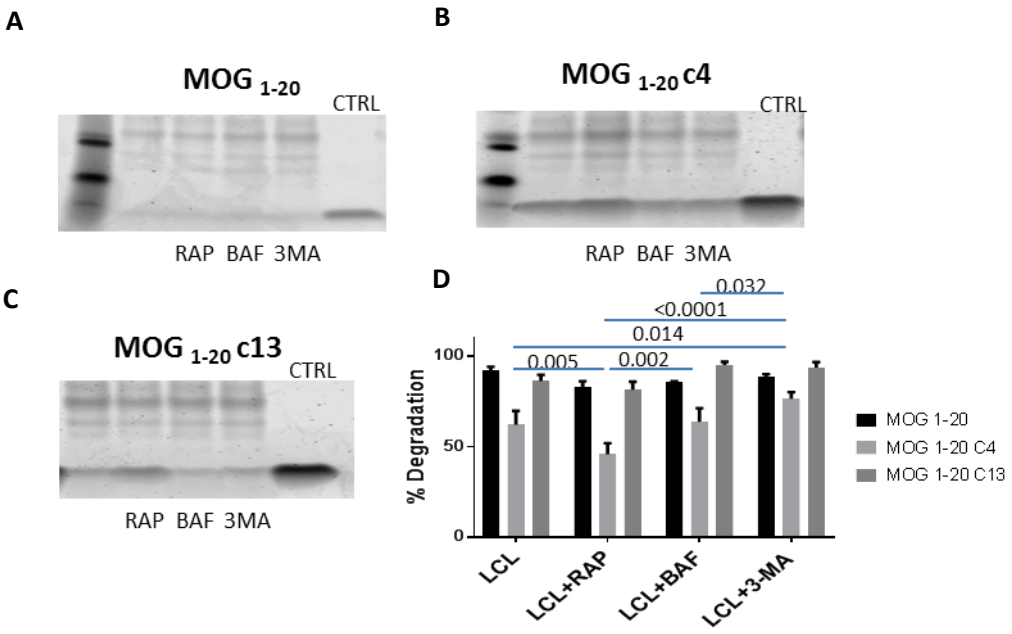


Fig. 4.10 Autophagy is directly involved in MOG$_{1-20}$ processing. LCL treated with Rapamycin (RAP), Bafilomycin (BAF) or 3-MA were incubated O.N. with the peptides **(A)** MOG$_{1-20}$, **(B)** MOG$_{1-20}$cit4 and **(C)** MOG$_{1-20}$cit13**. (D)** Means with SEM of the percentage of degradation of MOG$_{1-20}$, MOG$_{1-20}$cit4 and MOG$_{1-20}$cit13 by treated LCL are compared in the bar graph (n=3; paired repeated 2-way ANOVA).

4.3.4. EBV infection induces autophagosomes

Next, the effect of EBV infection on LC3 expression as a marker of autophagy was studied. CD20+, LCL and CpG activated CD20+ cells from the same donor were lysed and equal concentrations of protein lysate were immunoblotted to detect LC3 proteins. Higher expression of LC3-I and LC3-II were detected in LCL, as well as in CpG activated cells, compared to primary CD20+ cells (Fig 4.11). Baf is a drug that blocks the fusion between autophagosomes and lysosomes (Fig. 4.7) and in presence of Baf, if there is LC3-II expression, this will accumulate in the cytoplasm. Treating the cells with Baf O.N. permitted to further assess the increased production of LC3-II by EBV infected and CpG activated CD20+ cells compared to uninfected CD20+ and CD20- cells (Fig 4.12).

Similar results were obtained staining the cells for immunofluorescence (Fig. 4.13). Relative LC3 expression in LCL treated with Baf was higher compared to LCL and to Baf-treated CD20+ and CD20- cells.

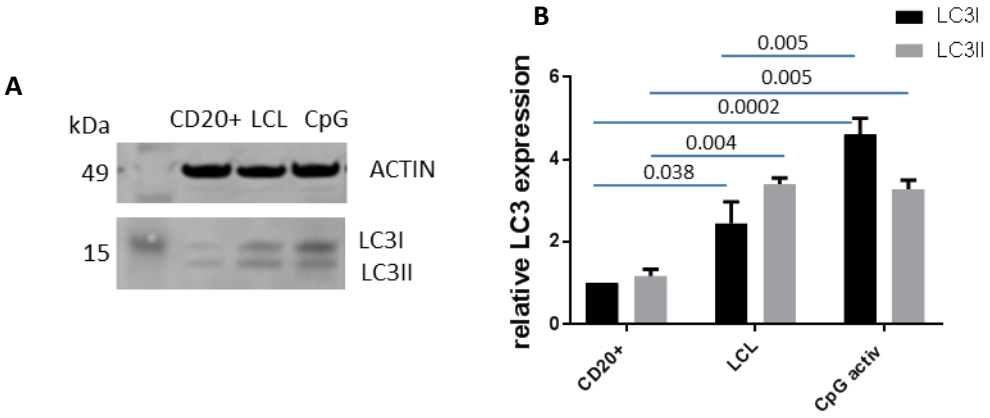


Fig. 4.11 Increased expression of LC3-II in LCL and CpG activated CD20+ cells. Autophagy activity was detected in cell groups by WB. (A) Indicated cell lysates were immuno-blotted for LC3 and actin as house-keeping gene and **(B)** the density of LC3-I and LC3II bands were quantified, corrected for the background and normalized for actin expression and for the expression in CD20+ cells. Means with SEM of relative expression in 3 experiments were plotted in the graph (n=3; paired repeated 2-way ANOVA).

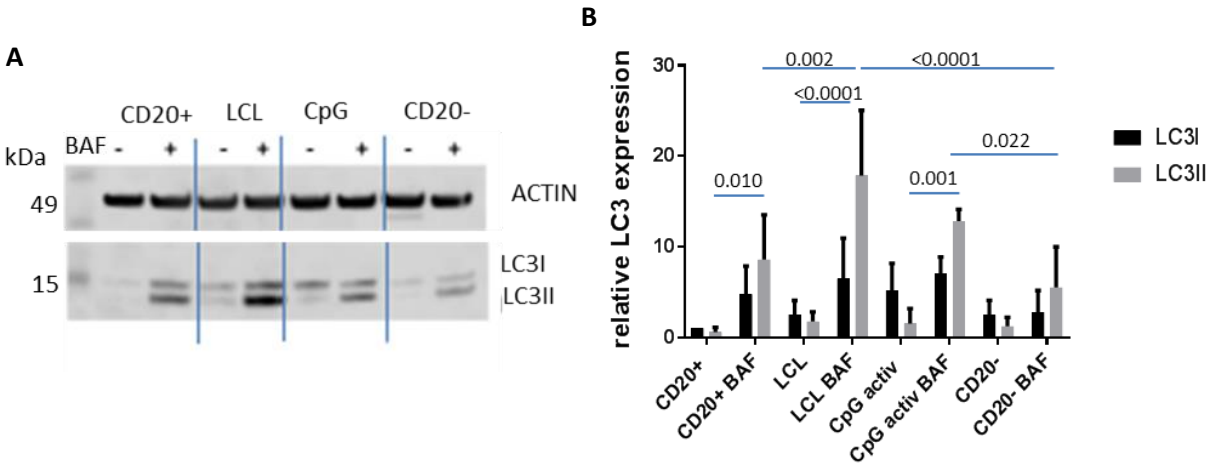


Fig. 4.12 Increased expression of LC3-II in LCL treated with Baf. Autophagy activity was detected in cells cultured with or without Bafilomycin (BAF) by WB**. (A)** Indicated cell lysates were immuno-blotted for LC3 and actin as house-keeping gene, and **(B)** the density of LC3-I and LC3II bands were quantified, corrected for the background and normalized for actin expression and for the expression in CD20+ cells. Means with SEM of relative expression in 3 experiments were plotted in the graph (n=3; paired repeated 2-way ANOVA).

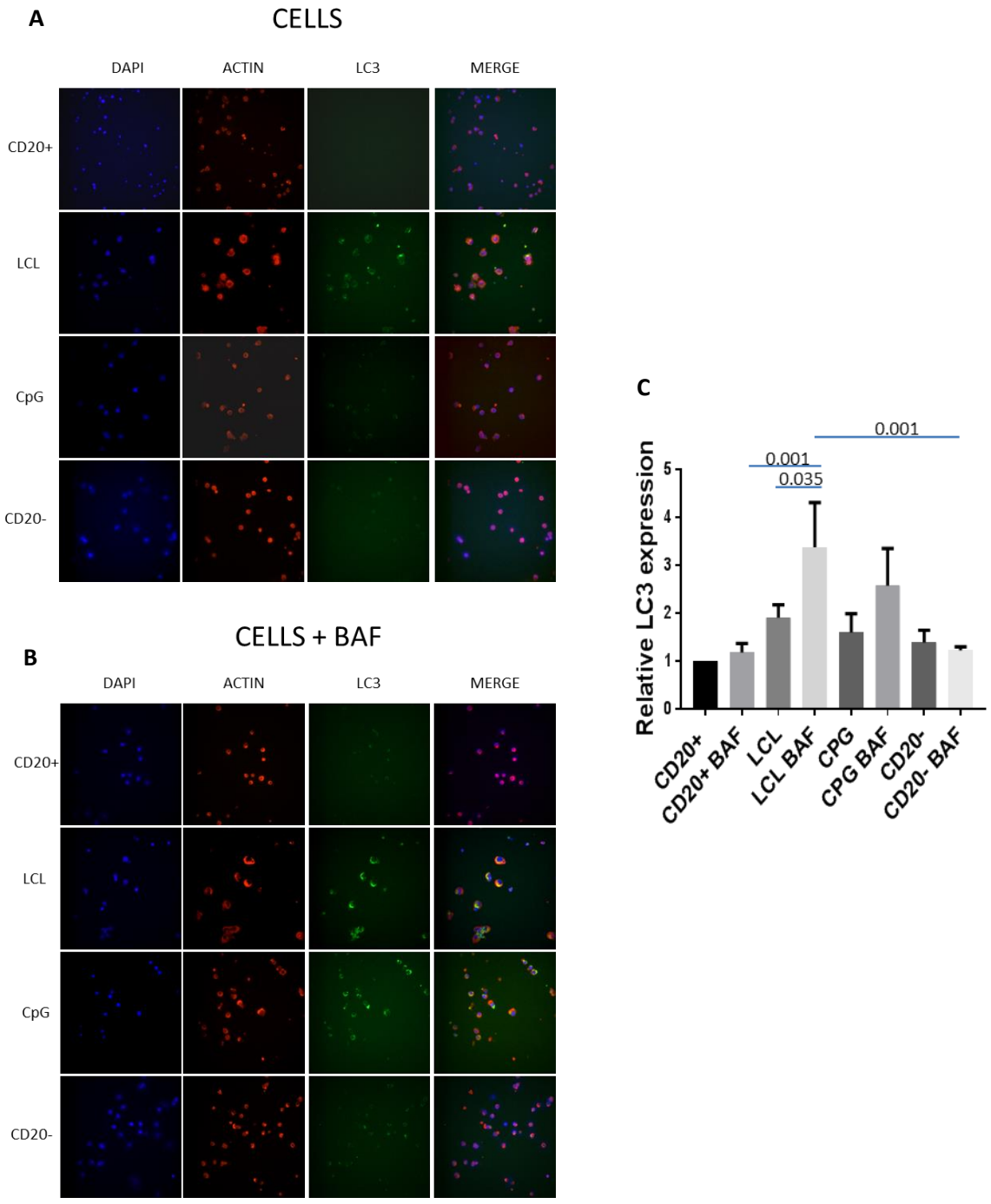


Fig. 4.13 EBV infection induces autophagosomes. Autophagy activity was detected trough immunofluorescence in cells cultured **(A)** without or **(B)** with Bafilomycin (BAF). Cells were stained for immunofluorescence with anti-actin (red) and anti-LC3 (green) Abs and representative images of 3 images acquired for slide in 3 independent experiments are shown. Original magnification X400. **(C)** Bar chart represents the mean with SEM of the quantification of LC3 relative to CD20+ cells in 3 independent experiments (n=3; repeated 1-way ANOVA).

4.3.5. LCL does not increase PAD2 expression

Autophagosomes are induced by EBV infection and are clearly involved in MOG processing. The high concentration of Ca^{2+} in autophagosomes can activate PAD, but there is also the possibility that PAD expression itself is modulated by EBV infection. The RNA expression of PAD2 was analyzed in LCL, CD20+, CD20- and CpG activated cells using real-time RT-PCR. Relative expression of PAD2 RNA was not found increased in LCL compared to uninfected or CpG activated CD20+ cells (Fig 4.14). By contrast, CD20- cells expressed higher PAD2 level compared with CD20+ cells, probably due to high enzyme expression in monocytes (Wang and Wang, 2013).

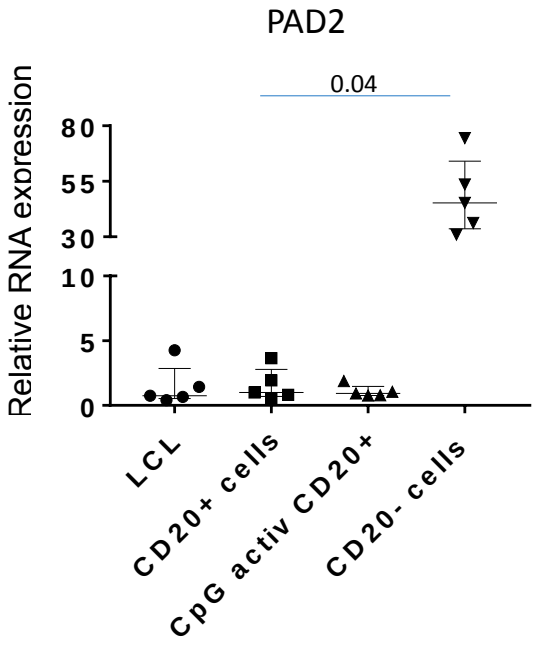


Fig. 4.14 PAD2 RNA expression does not increase in LCL. Total RNA was extracted from CD20+, CpG activated CD20+, LCL and CD20- cells from the same donors to determine expression of mRNA encoding PAD2 with real-time RT-PCR. Transcript levels were normalized against the reference gene HMBS and then against the mean of the expression in CD20+ cells to calculate relative expression. Relative PAD2 RNA expression in 5 HC is illustrated in dots. Median and interquartile range are indicated by bars. (n=5, paired non-parametric Friedman test)

4.4. Conclusion

The data presented in this Chapter show that autophagosomes are engaged in the protection of MOG against destructive processing. In the model proposed MOG is internalized by EBV-infected B cells, it is incorporated inside endosomes/ autophagosomes, citrullinated and protected from CatG processing.

The critical role of the citrullinated residues appears to be their participation in a LIR motif, through which peptides can associate with LC3-II in autophagosomes (Birgisdottir et al., 2013). To better study this mechanism, a further immunogenic MOG peptide (MOG_{1-20}) was tested. In LCL MOG_{1-20} was completely processed by CatG and only CatG inhibitor or citrullination in position 4 inhibited its degradation. Interestingly, when both MOG_{35-55} and MOG_{1-20} were citrullinated within the LIR motif, they were protected from CatG processing. The intact LIR motif allows peptides to associate with autophagosomes, thus prolonging their survival. Although localization of the citMOG peptides inside LCL through IF was difficult to visualize, it was possible to detect the whole MOG protein with the anti-MOG Ab. MOG protein co-localized with LC3, confirming the internalization of MOG in autophagosomes. Of note, degradation of MOG protein was low in LCL (Chapter 2.3). In line with this concept, modulation of the autophagy flux affected the processing of MOG peptides citrullinated in the LIR motif. The increasing formation of autophagosomes induced with rapamycin increased protection of MOG_{35-55}cit46 and MOG_{1-20}cit4. By contrast, inhibition of autophagy with 3-MA enhanced their degradation. Of note, the lack of effect of Baf on peptides processing may be due to its effects being distal to autophagosome formation.

Established the important function of autophagosomes in MOG processing, the next observation was the increased autophagy activity in LCL compared to uninfected CD20+ cells.

Cells use autophagy to clear viral infections, but EBV has learned how to manipulate the autophagic pathway for its own benefit (Granato et al., 2014). Depending on the stage of the infection EBV can induce or block autophagy. Indeed EBV-positive latency II and latency III primary B cell from diffuse large B cell lymphoma and post-transplant lymphoproliferative disorder, but neither EBV-negative nor EBV-positive latency I Burkitt lymphoma, were found to have autophagy constitutively activated (Pujals et al., 2015). On the other hand, the autophagic flux is blocked at the final steps during the reactivation of EBV from latency. Lytic EBV exploits the autophagic machinery for its transportation in order to enhance viral production (Granato et al., 2014) using the autophagosome membrane for envelope acquisition (Nowag et al., 2014).

LCL are B cells infected with EBV in phase latency III and the protein LMP1 constitutively activates autophagy (Lee and Sugden, 2008). After MOG internalization in LCL, the antigen is located in autophagosomes induced by EBV. Here it is protected from total degradation. The peptide that survives the processing in autophagosomes may then be loaded on HLA-E and presented to autoreactive cytotoxic T cells facilitating autoimmunity as it was observed in the marmoset EAE model (Jagessar et al., 2012). Indeed in Chapter 2 it was shown that EBV increases the expression of HLA-E (Fig. 2.3) that has been shown in macrophages to be retained in the vesicles of the autophagy-lysosome network (Camilli et al., 2016).

EBV infection induces autophagosomes and activates the cross-presentation machinery. On the other hand, EBV did not increase PAD2 RNA expression.

Nevertheless, PAD2 activity better correlates with Ca2+ concentration and therefore with autophagy activity. In fact, comparable RNA expression of the enzyme can result in different levels of activation and citrullination (Ireland and Unanue, 2011). Moreover, this study focused on PAD2 because higher expression of PAD2 was found in PBMC of MS patients compared to HC (Calabrese et al., 2012), but it is also possible that EBV modulates the expression of other PAD isoforms, such as PAD4, not investigated here.

In conclusion, to our knowledge these are the first data to show direct implication of autophagy induced by a virus in the processing of autoantigens by human immune cells.

In particular this thesis focuses on the autoantigen processing. To test cross-presentation of citrullinated protein to human cytotoxic CD8+ T cells, further observations are required. First, the direct detection of MOG citrullination in autophagosomes of EBV-infected cells was not shown. Different WB experiments were performed using different anti-citrulline Abs: F95 Ab, used to stain human brain samples to show anatomical localization of citrullinated proteins (Bradford et al., 2014) and a commercial (Merck-Millipore) polyclonal anti-citrulline used to detect citrullinated vimentin in synoviocytes (Sorice et al., 2016). In both the cases no result was obtained due to the difficulties in detecting citrullination (Clancy et al., 2016). Also detection of citrulline by mass spectrometry (MS) would have been challenging due to the small change in mass that occurs when an arginine residue is deiminated upon citrullination (the observed parent mass of a peptide is increased by 0.98 Da) (Clancy et al., 2016). Nevertheless, other studies report increased citrullination directly linked to increased autophagy activity (Ireland and Unanue, 2011; Ireland and

Unanue, 2012; Durrant et al., 2016; Sorice et al., 2016). Second, the presentation of MOG peptides to CD4+ or CD8+ cells was not demonstrated here. However, the cross-presentation of MOG peptides by LCV-infected cells has been demonstrated in vivo in the EAE marmoset model (Haanstra et al., 2013b).

PART 2:

HERVs and MS

Viral involvement in MS pathogenesis is a concept that has gained ever increasing appreciation by the scientific community. In addition to herpesviruses, such as HHV-6, VZV, and especially EBV (Ascherio and Munger, 2007), the expression of HERVs has been considered as a risk factor for developing MS and for disease progression (Perron et al., 2009).

In 1989, Perron et al. discovered the presence of extracellular virions harbouring retrotrascription activity in leptomeningeal cells (LM7) of CSF of MS patients (Perron et al., 1997). MS-associated retrovirus (MSRV) was the name assigned to these virions due to their reproducible detection in MS patients, but not healthy individuals (Perron et al., 1997). MSRV retroelement belongs to the W family of HERV and its RNA harbours *gag*, *pol* and *env* regions within LTRs (Mameli et al., 2009).

Both in vitro and in vivo experiments have illustrated that the immunopathogenicity induced by these proteins may be mediated by their acting as superantigens, inducing polyclonal T-cell activation and secretion of proinflammatory cytokines and redox reactants. The latter can have neurotoxic effects in oligodendrocytes (Perron et al., 2001; Firouzi et al., 2003; Antony et al., 2004). Indeed, the MSRV/HERV-W protein has been shown to possess pro-inflammatory properties through direct interaction with TLR4 (Rolland et al., 2006). TLR4 is a pattern recognition receptor (PRR) located on the plasma membrane of cells that primarily senses Gram-negative bacteria lipopolysaccharide (LPS), but also viral proteins like Respiratory Syncytial Virus (RSV) fusion protein. Upon TLR4 engagement, signalling pathways are activated leading to secretion of proinflammatory cytokines like IL-1β, IL-6 and TNF-α. MSRV/HERV-W can activate TLR4 in monocytes in such a manner but also stimulate dendritic cell activation and drive naïve T cells towards Th1-like responses (Rolland et al., 2006).

In addition to intrinsic factors (epigenetics and retroviral restriction factors), external events, and in particular viral infections, can act as activators of MSRV/HERV-W expression, or de-regulators of the mechanisms that normally prevent HERV expression, either at the time of infection or via longer term epigenetic modification. One possible mechanism of MS pathogenesis is that EBV infection triggers MSRV/HERV-W expression (Perron et al., 2009).

Moreover, there has been a recent report outlining the fact that co-morbidity with HIV and MS is vanishingly rare. One possibility is that most HIV-positive people in developed countries are on anti-retroviral drug therapy. These inhibitors of retroviruses may also protect from MS development.

The literature on MSRV/HERV-W and MS has been quite confusing and created an unresolved debate in the scientific community. This project aims to clarify the association between HERVs and MS through a systematic review and meta-analysis of the literature, as well as by producing original data. Moreover, the hypotheses that a) EBV can activate MSRV and b) antiretroviral therapy could repress MSRV protecting from MS development will be tested.

CHAPTER 5: The association between HERVs and MS: a systematic review and meta-analysis

5.1. Background

HERV families are classified via a naming system on the basis of the tRNA specificity of the primer binding site, corresponding to the amino acid that would be added to the HERV were it translated into viral proteins (HERV-W,-K,-H etc.) (Section 1.4.3) (Gifford and tristem, 2003). The first HERV reported to be associated with MS in the late 1980s MSRV, a member of the HERV-W family (Perron et al., 1997). In addition to HERV-W, an increased expression of HERV-K and HERV-H families in the blood, brain or CSF from people with MS has also been reported by some groups (Christensen et al., 2005), but not others (Antony et al., 2007).

Indeed the literature on this topic has been confused by a number of issues. The original studies on MSRV (Perron et al., 1997; Ménard et al., 1997) assumed that functional viral particles were involved and focussed on detection of cell-free (presumably virion associated) RNA. The later realisation that none of the 213 HERV-W loci in the human genome are fully replication competent (Grandi et al., 2016) cooled enthusiasm for the hypothesis of retroviral involvement in MS. Reports of an association between MSRV/HERV-W sequences and MS however continued, some affirming the association, some refuting it. Further confusions arose from these reports due to the plethora of detection methods (PCR and protein based), patient cohorts and sample types (blood, CNS, cell free and cell based) analysed and the variety of names given to the sequences detected. Recent detailed analysis of reported HERV-W/MSRV/Syncytin-1 sequences has demonstrated that they originate from a mosaic

of loci and it is unlikely that the methods used to date are able to distinguish those from a single locus (Grandi et al., 2016).

In this thesis the available data on the expression of different HERV families in MS patients were systematically reviewed and a meta-analysis of the expression of HERV-W *env* and *pol* RNA in MS patients and controls was performed. This study entitled "*The association between human endogenous retroviruses and multiple sclerosis: a systematic review and meta-analysis*" was published by Plos One in 2017 (Morandi et al., 2017).

5.2.Methods

In performing this study, the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) protocol (Moher et al., 2009) was followed. Details of the protocol for this systematic review were registered on PROSPERO (registration number CRD42016047290) and can be accessed at http://www.crd.york.ac.uk/PROSPERO/#index.php

5.2.1. Data source and search

Medline, Embase and the Cochrane Library up to 9th September 2016 were searched using the keywords "MULTIPLE SCLEROSIS" AND ("HERV" OR "HERVS" OR "HERV-W" OR "HERV-H" OR "HERV-K" OR "MSRV") with no time restriction. Only papers published in English were included. Two independent investigators (EM and RTan) extracted the data from the literature databases and the references cited in the identified papers.

5.2.2. Inclusion Criteria

Full text articles were included in the systematic review if they were case-control studies containing data on the expression of viral proteins or RNA or DNA of any HERV family, in any type of tissue, from patients diagnosed with MS and from control groups (either healthy or pathological). Studies were further sub-grouped based on the retroviral family, the techniques employed, the protein/nucleic acid identified, and the type of tissue. A subset of these articles was used for a quantitative meta-analysis if they studied the same protein or nucleic acid in the same type of tissue, using the same techniques. An additional inclusion criterion for the meta-analysis was the use of the same type of control group (healthy subjects or other controls). A meta-analysis was

performed only if a minimum of 4 comparable studies met these criteria. Reviews and conference abstracts were excluded.

5.2.3. Data Extraction

EM and RTan independently screened the papers yielded by the search to eliminate duplicates and to verify that inclusion criteria were met. Any disagreement was resolved through discussion with a third investigator (BG). The articles extracted were first organised in five different groups on the basis of the HERV families studied. From each eligible study the first author, the year of publication, the protein/nucleic acid investigated, the type of tissue analysed, the techniques used, the populations studied (number of cases and controls, number of women, mean age, source and type of disease), the country of origin, and a summary of the results (percentage or number of cases and controls positive for the HERV expression) were extracted independently by EM and RTan.

5.2.4. Meta-analysis

The association of different viral protein/nucleic acid with MS was analysed separately. The odds ratio (OR) were estimated by the Mantel-Haenszel test and the standard error (SE) and the 95% confidence intervals (CI) were calculated using SPSS (IBM, version 22). The total OR, the p-value of fixed effects (inverse of variance) and of random effects (DerSimonian-Laird test) were calculated using StatsDirect (Version 2.8.0). The heterogeneity was evaluated calculating the Inconsistency (I^2) and the Cochrane Q p-value. Egger's regression test was used to examine bias.

5.2.5. Quality assessment

The quality assessment of included studies was based on the Newcastle-Ottawa assessment scale (NOS) (Wells et al., 2012).

5.3. Results

5.3.1. Study selection

The online search identified 324 articles (Fig. 5.1). After removing the duplicates, 244 articles were retained. A further 203 articles (58 reviews, 4 methodological papers, 136 articles that did not study the association between HERVs and MS, and 5 studies that were not case-control studies) were excluded. Forty-one papers were deemed eligible and 2 additional articles were identified from the references of the selected articles. The 43 papers included were categorised into 5 groups based on the HERV family studied and are presented in a world map in Fig 5.2. Six papers studied more than one HERV family and were included in more than one table. Twenty-five articles focused on HERV-W; 13 on HERV-H; 9 on HERV-K, whilst HRES-1 was studied in 5 and HERV-15 in 1 paper.

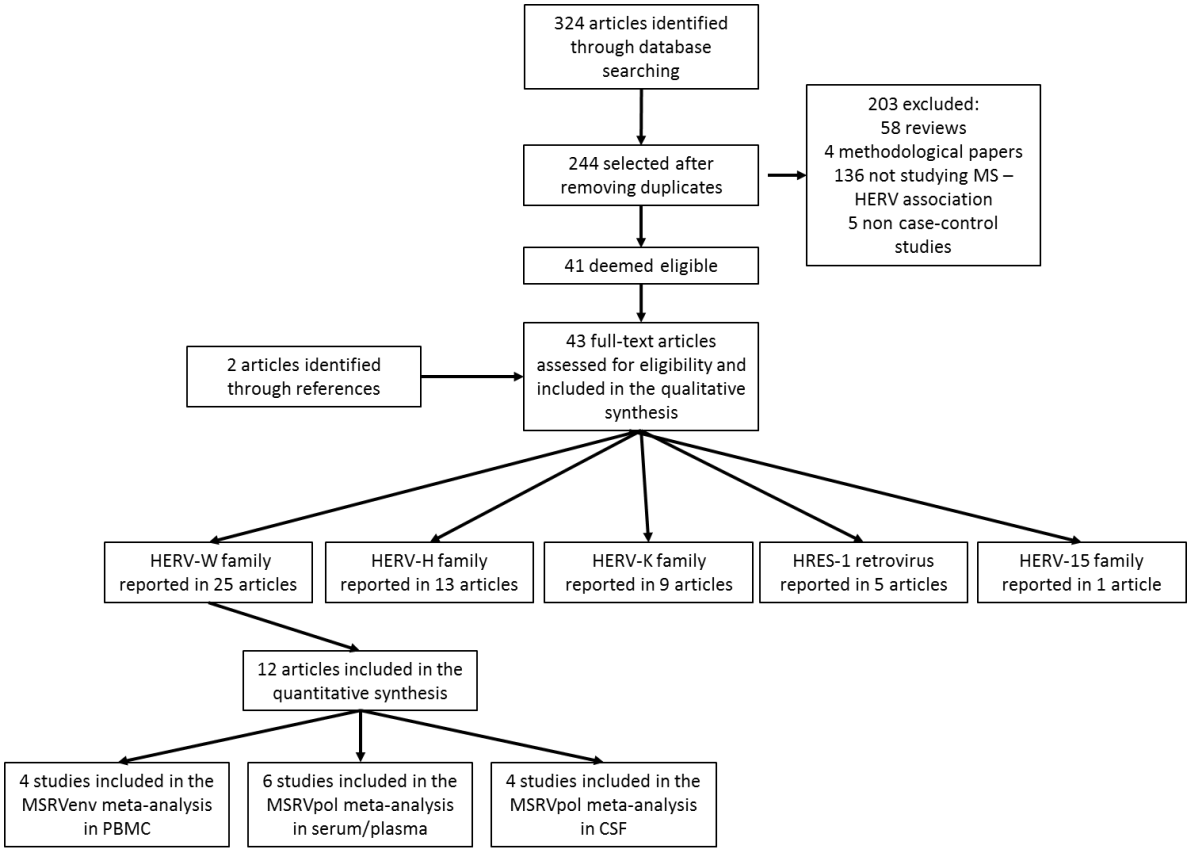


Fig. 5.1 Flow chart of the study selection and procedure

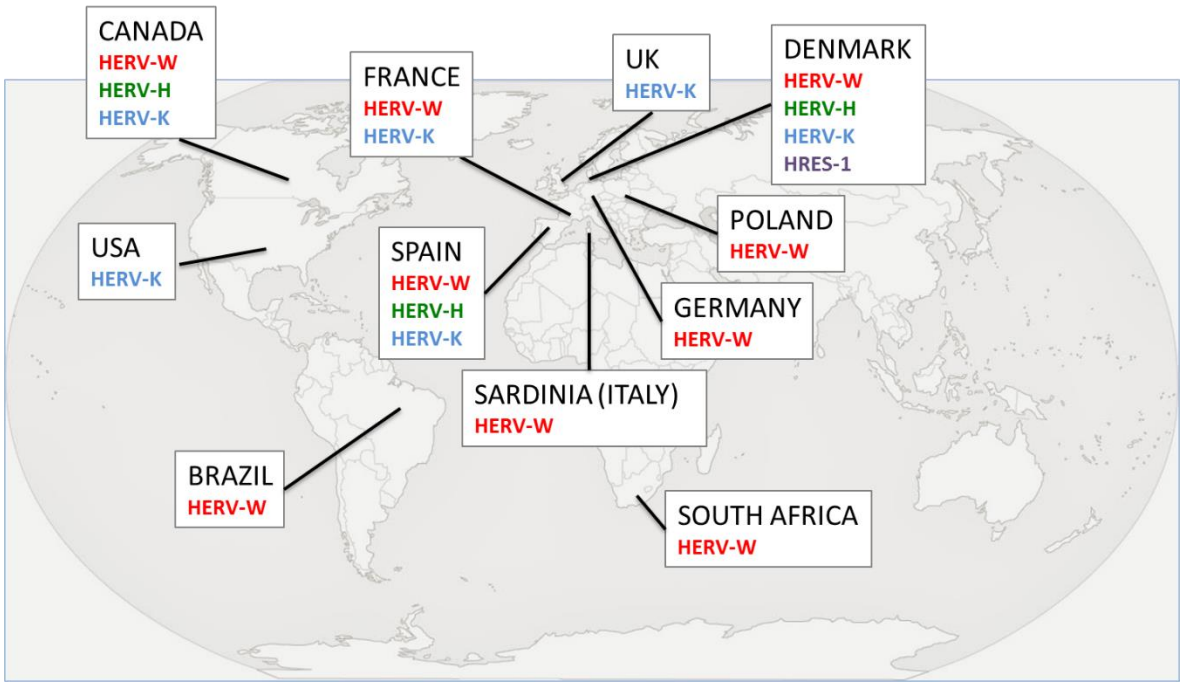


Fig. 5.2 Overview of the countries in which different research groups investigated specific HERV families referenced in the study.

5.3.2. HERV-W

The literature search found 25 articles reporting on an association between HERV-W and MS by 9 different research groups (Fig.5.2).

5.3.2.1. HERV-W in peripheral blood

Expression of the MSRV/HERV-W *env* and *pol* in the blood (PBMC or serum/plasma) was reported in 20 publications by Sardinian, French, Polish, Spanish, Brazilian, South African, Danish, German, and Canadian groups. In 15 studies MSRV/HERV-W*env* (Arru et al., 2007; Mameli et al., 2007; Mameli et al., 2009; Perron et al., 2012; Garcia-Montojo et al., 2013; do Olival et al., 2013; Garcia-Montojo et al., 2014) or MSRV/HERV-W*pol* (Ménard et al., 1997; Perron et al., 1997; Garson et al., 1998; Serra et al., 2001; Dolei et al., 2002; Zawada et al., 2003; Nowak et al., 2003; de Villiers et al., 2006; Arru et a., 2007) RNA and protein were found to be increased in serum/plasma or PBMC of MS patients compared to control groups (HC or OND) by different techniques (RT-PCR, FC and ELISA).

Two studies demonstrated different levels of expression of the MSRV/HERV-W*env* in different types of blood cells (Brudek et al., 2009; Mameli et al., 2012). In these studies MSRV/HERV-W*env* RNA and protein expression detected by RT-PCR and FC were increased in monocytes, NK and B cells, but not in $CD4^+$ and $CD8^+$ T cells of MS patients compared to controls (Brudek et al., 2009; Mameli et al., 2012). Three studies (from Canada and Germany) did not detect increased levels of HERV-W*env* RNA (either MSRV or syncytin-1 that share 94% sequence identity at RNA level) in plasma, total PBMC and cell subtypes from MS patients compared to control groups (Antony et al., 2006; Antony et al., 2007; Laufer et al., 2009).

5.3.2.2. HERV-W in the CSF

Six papers studied the expression of HERV-W in the CSF from MS patients and controls. MSRV/HERV-W *pol* and *env* RNA detected by RT-PCR were found to be over-expressed in people with MS compared with HC and OND by the French (Perron et al., 1997) and Italian groups (Dolei et al., 2002; Arru et al., 2007). The Spanish (Alvarez-Lafuente et al., 2008) and Canadian groups (Antony et al., 2006; Antony et al., 2007) did not find an increased expression of HERV-W*env* RNA by RT-PCR in MS patients.

5.3.2.3. HERV-W in the brain

Nine publications studied the expression of MSRV/HERV-W in the brain tissue. A French and an Italian group showed the presence of MSRV/HERV-W Pol, Gag and Env proteins and RNA in infiltrating macrophages clustered around endothelial cells in MS lesions (Perron et al., 2005; Mameli et al., 2007; Perron et al., 2012; van Horssen et al., 2016), but not in the brain of HC or OND using immunohistochemistry and RT-PCR. By contrast, using the same techniques, a Canadian group found an increased expression of HERVWE1*env* RNA (syncytin-1) but not MSRV*env*, in the brains of MS patients compared to OND (Johnston et al., 2001; Antony et al., 2004; Antony et al., 2006; Antony et al., 2007). A German group used Next Generation Sequencing (NGS) to detect the expression of HERV-W loci in the brain, without significant differences between HC and MS (Schmitt et al., 2013).

5.3.2.4. HERV-W in different types of MS

Three studies in MS patients and HC looked at MSRV/HERV-W expression in different forms of MS. The French group found differences between RR-, PP- and SP-MS with an increase of MSRV/HERV-W*env* DNA copy number, but not of RNA and protein expression, in the progressive forms of MS (Perron et al., 2012). The Spanish

group detected a higher expression of MSRV/HERV-W*env* RNA in SPMS than RRMS (Garcia-Montojo et al., 2014). Patients with an elevated MSRV/HERV-W*env* DNA copy number had a higher degree of disability, according to their Expanded Disability Status Scale (EDSS) score (Garcia-Montojo et al., 2013).

5.3.2.5. MSRV/HERV-W meta-analysis

Examining all the publications found, 3 meta-analyses of the association of MSRV/HERV-W with MS were performed, in which at least 4 studies of association between HERV and MS reported the same viral target, tissue sample and technique compared to the same type of control group (either healthy or neurological controls). Twelve articles were suitable for inclusion according to the criteria described in the Methods. Together, these studies included 478 MS patients, 330 HC and 145 OND. The characteristics of participants in the included studies are summarised in Table 5.1. A table with the PCR primers used for each study is reported in Appendix V pag. 264. A separate meta-analysis was performed for each viral protein (*env* and *pol*) and for each different tissue. Four studies investigated MSRV/HERV-W*env* RNA in PBMC (111 MS patient and 58 HC, Table 5.1A), 6 studies investigated MSRV/HERV-W*pol* RNA in serum/plasma (309 MS patients and 272 HC, Table 5.1B), and 4 studies investigated MSRV/HERV-W*pol* RNA in CSF (187 MS patients and 145 HC, Table 5.1C).

Table 5.1. Characteristics of participants in the included studies. Participants in meta-analysis A) MSRV/HERV-W*env* expression in PBMC B) MSRV/HERV-W*pol* expression in serum/plasma and C) MSRV/HERV-W*pol* expression in CSF.

A ***Env expression in PBMC***

Study ID	HERV	SAMPLE	METHOD	MS CASES							CONTROLS				COUNTRY
				TOT	SEX	MEAN AGE	SOURCE	RR MS	SP MS	PP MS	TOT	SEX	MEAN AGE	SOURCE	
S. do Olival 2013	*MSRV env*	PBMC	RT-PCR	10			Clinically definite MS				10			HC with no familiar history of MS	Brazil
Perron 2012	*MSRV env*	PBMC	RT-PCR	58			Hospital Neurologic. Depart.				26	Sex-matched	Age-matched	HC from transfusion centre	European
Mameli 2007	*MSRV env*	PBMC	RT-PCR	35		37.1	Active MS				14		32.3	HC from transfusion centre	Sardinia
Mameli 2009	*MSRV env*	PBMC	RT-PCR	8	F 4	43	Depart. of Neurosc. University Sassari	5	1	2	8			6 Blood donors and 2 Health Care Operators	Sardinia

B

Pol expression in serum/plasma

Study ID	HERV	SAMPLE	METHOD	MS CASES							CONTROLS				COUNTRY
				TOT	SEX	MEAN AGE	SOURCE	RR MS	SP MS	PP MS	TOT	SEX	MEAN AGE	SOURCE	
Arru 2007	*MSRV pol*	PLASMA	nested PCR	147	F 102	33.8	Sardinia, Ferrara Pamplona Stockholm				98	Sex-matched	Age-matched	HC from Sardinia and Spain	European (Sweden, Spain, Italy, Sardinia)
Dolei 2002	*MSRV pol*	PLASMA	RT-PCR	39	F 25	36.9	Sardinian origin, free of IT	24	4		39		37	HC from transfusion centre	Sardinia
Serra 2001	*MSRV pol*	PLASMA	RT-PCR	25		36.6	Sardinian origin, free of IT for at least 3 months	15	2		25		37.6	HC without known MS risk	Sardinia
Garson 1998	*MSRV pol*	SERUM	RT-PCR	17			French clinically active MS				36			Healthy UK adults	France
de Villiers 2006	*MSRV pol*	SERUM	RT-PCR	49			South African of European descent	37	7	5	39		Age-matched	HC and laboratory personnel from same ethnic group	South Africa (European descendent)
Nowak 2003	*MSRV pol*	SERUM	RT-PCR	32		Median 37.7	Clinically definite untreated				27		Median 34.5	Healthy adults	Poland

C

Pol expression in CSF

Study ID	HERV	SAMPLE	METHOD	MS CASES							CONTROLS				COUNTRY
				TOT	SEX	MEAN AGE	SOURCE	RR MS	SP MS	PP MS	TOT	SEX	MEAN AGE	SOURCE	
Alvarez-Lafuente 2008	*HERV-W*	CSF	RT-PCR	48	F 34	32.6	First clinically evident demyelinating event	48			44	F 29	OIND 43.7 ONIND 39.4	23 OIND 21 ONIND	Spain
Arru 2007	*MSRV pol*	CSF	nested PCR	98			Sardinia, Ferrara Pamplona Stockholm				81	Sex-matched	Age-matched	OND from Sardinia, Ferrara Stockholm	European (Sweden, Spain, Italy, Sardinia)
Dolei 2002	*MSRV pol*	CSF	RT-PCR	31		36.7	Sardinian origin, free of IT for at least 3 month				10		31.9	4 CNS OIND, 6 CSF ONIND	Sardinia
Perron 1997	*MSRV pol*	CSF	RT-PCR	10	F 6	34.7	Grenoble Paris Milan	2	3	5	10	F 7	36.7	Grenoble Paris Milan	France

Abbreviations: pol, Polymerase; env, Envelope; PBMC, Peripheral Blood Mononuclear Cells; CSF, Cerebrospinal Fluid; RT-PCR, Reverse Transcription Polymerase Chain Reaction; MS, Multiple Sclerosis; HC, Healthy Control; OND, Other Neurological Disease; RRMS, Relapsing-Remitting MS; SP, Secondary-Progressive MS; PP, Primary-Progressive MS; F, Female; OIND, Other Inflammatory Neurological Disease; ONIND, Other Non-Inflammatory Neurological Disease; IT, Immunomodulatory Treatments

Seventy of 111 MS patients (63%) and 10 of 58 HC (17.2%) expressed MSRV/HERV-W*env* in PBMC with an OR of 22.66 (95%CI 6.32-81.20; p<0.0001 for fixed and random effects, 0% inconsistency and Egger test for publication bias p=0.22) (Fig. 5.3). Two-hundred forty-three of 309 MS patients (78.6%) and 41 of 272 HC (15%) expressed MSRV/HERV-W*pol* in plasma/serum with an OR of 18.12 (95%CI 10.61-30.92; p<0.0001 for fixed effects) and OR of 44.11 (95%CI 12.95-150.30; p<0.0001 for random effect, inconsistency 61.2% and Egger test for publication bias p=0.0088) (Fig. 5.4). One-hundred nine of 187 MS patients (58%) and 37 of 145 OND (25%) expressed MSRV/HERV-W*pol* in CSF with an OR of 6.00 (95%CI 3.35-10.74; p<0.0001 for fixed and random effects, 0% inconsistency and Egger test for publication bias p=0.55) (Fig. 5.5).

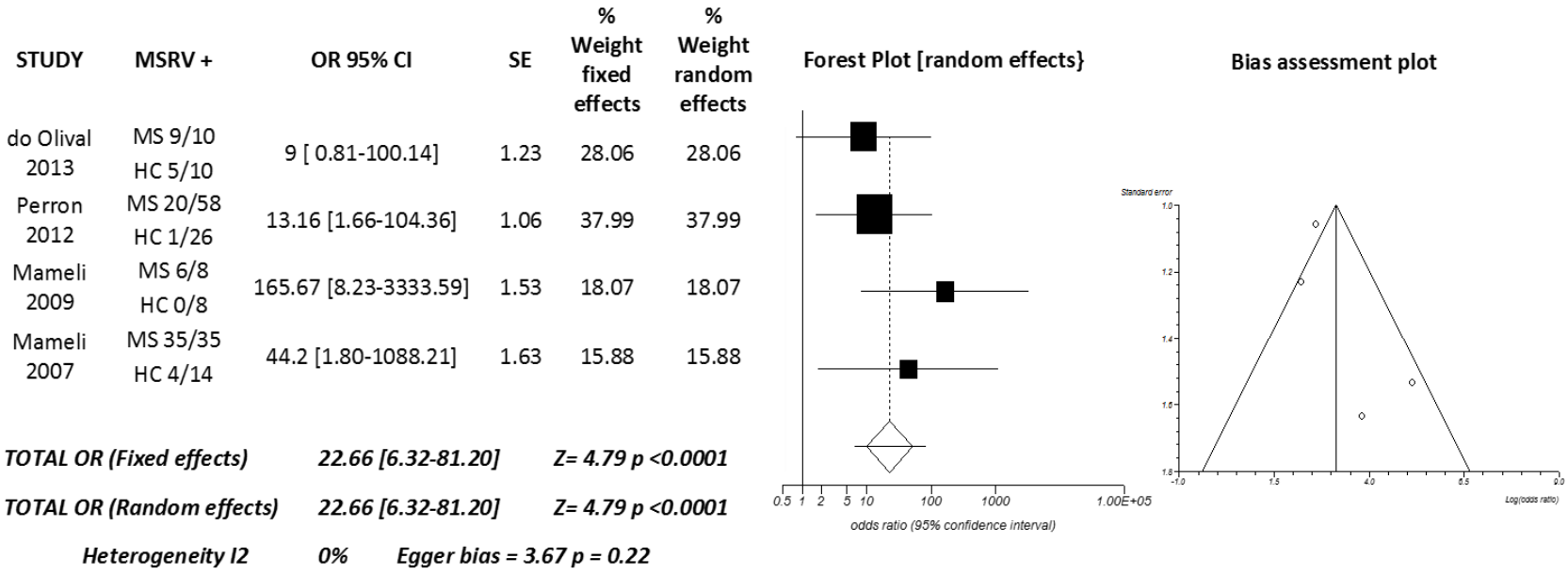


Fig. 5.3 Summary meta-analysis of MSRV*env* expression in PBMC in MS patients and HC. Statistical analysis, forest plot and bias assessment funnel plot of comparison of the expression of MSRV*env* in PBMC in MS and HC. Odds Ratio (OR), 95% confidence interval (CI), standard error (SE), Z test, p-value (p) of fixed effects (inverse of variance) and of random effects (DerSimonian-Laird test) were calculated. The heterogeneity was evalueted calculating the Inconsistency (I2) and publication bias through Egger's regression test.

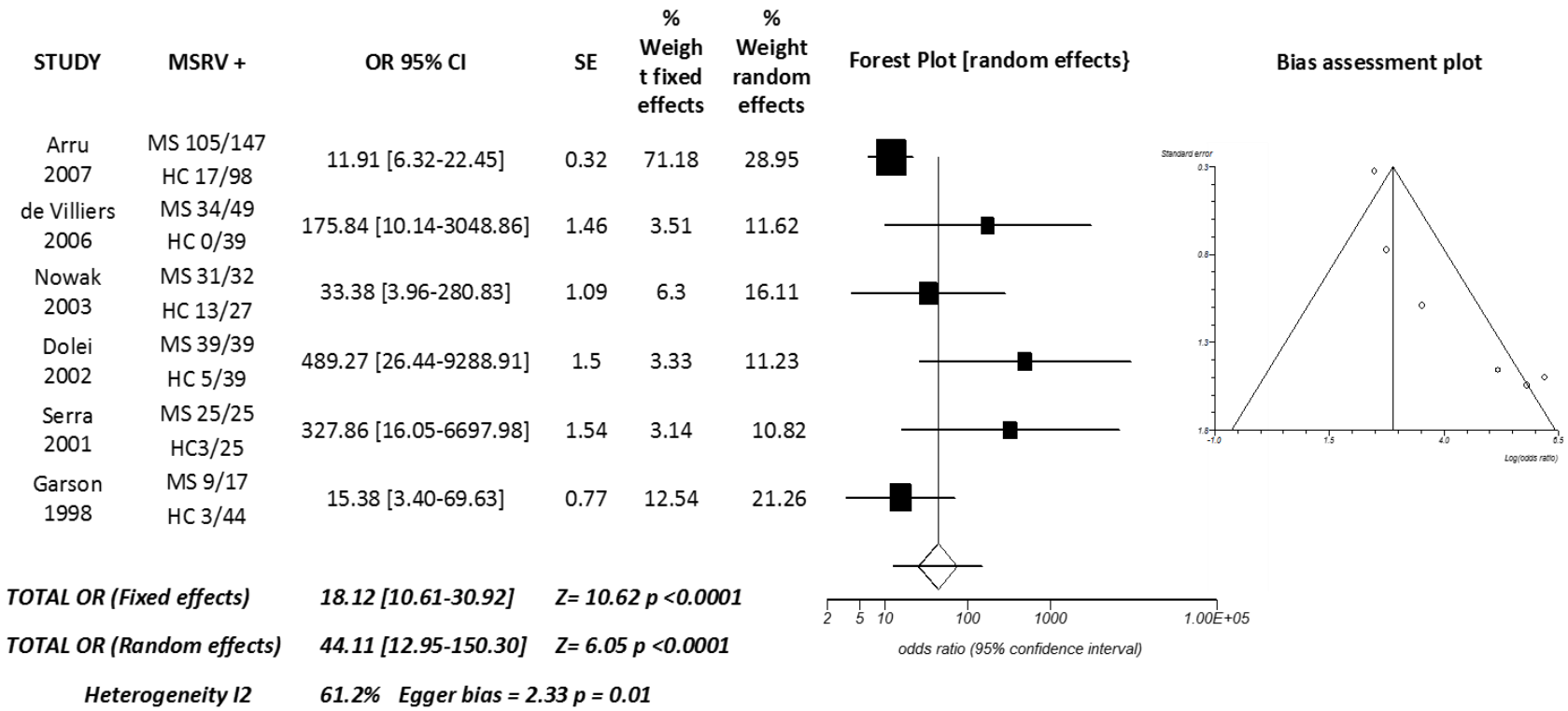


Fig. 5.4 Summary meta-analysis of MSRV*pol* expression in serum/plasma in MS patients and HC. Statistical analysis, forest plot and bias assessment funnel plot of comparison of the expression of MSRV*pol* in serum/plasma in MS and HC. Odds Ratio (OR), 95% confidence interval (CI), standard error (SE), Z test, p-value (p) of fixed effects (inverse of variance) and of random effects (DerSimonian-Laird test) were calculated. The heterogeneity was evalueted calculating the Inconsistency (I2) and publication bias through Egger's regression test.

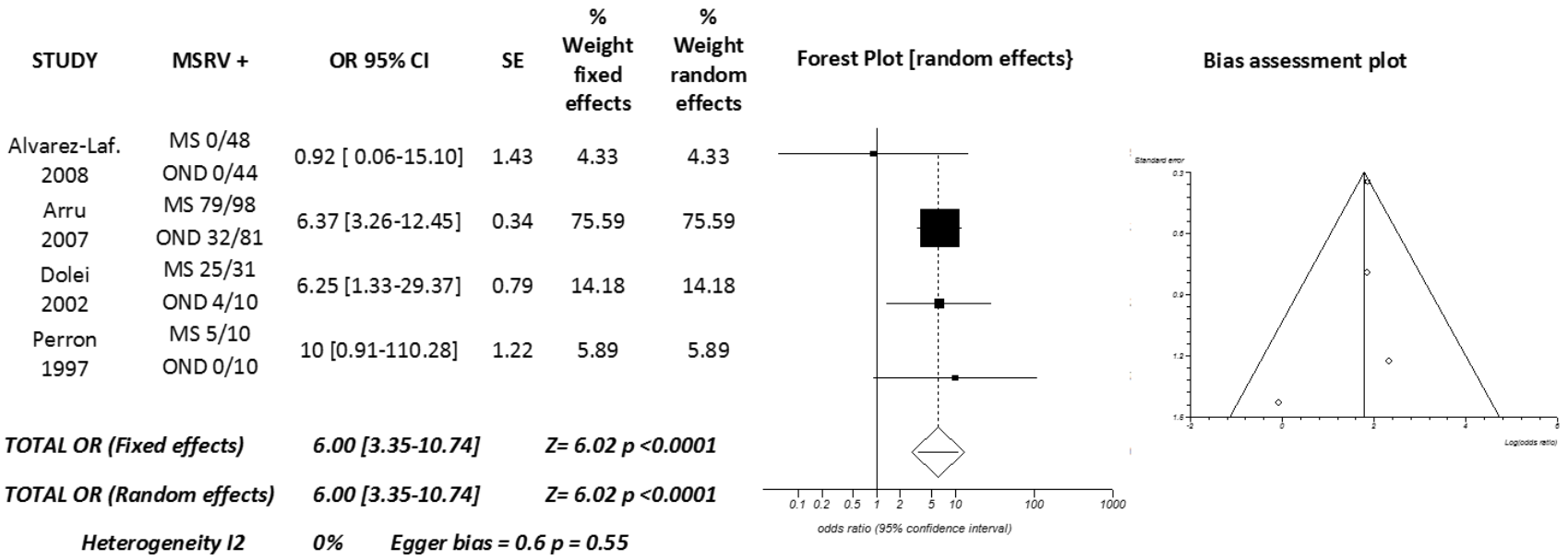


Fig. 5.5 Summary meta-analysis of MSRVpol expression in CSF in MS patients and OND. Statistical analysis, forest plot and bias assessment funnel plot of comparison of the expression of MSRVpol expression in CSF in MS and OND. Odds Ratio (OR), 95% confidence interval (CI), standard error (SE), Z test, p-value (p) of fixed effects (inverse of variance) and of random effects (DerSimonian-Laird test) were calculated. The heterogeneity was evalueted calculating the Inconsistency (I2) and publication bias through Egger's regression test.

5.3.2.6. Quality assessment

The NOS scale is a quality scale to assess primary studies included in the meta-analysis. The maximum score that could be achieved by a study was 10 stars. The majority of the studies scored less than half of the maximum score, with a mean of 4.5 (Table 5.2). The highest scoring study was Perron et al. 2012 with score of 8 stars. With regards to selection criteria, most studies had adequate definitions of cases and controls. The comparability of cases and controls was good for 6 studies that matched at least the age (Table 5.2). For the exposure criteria, all the studies used appropriate molecular techniques; 3 studies reported blinding sample analysts. No studies reported a non-response rate (Table 5.2).

Table 5.2. Quality assessment of included studies

	Selection				Comparability		Exposure			**TOT**
STUDY ID	**S1**	**S2**	**S3**	**S4**	**C1**	**C2**	**E1**	**E2**	**E3**	
S. do Olival 2013	x			x			x			3
Perron 2012	x	x	x		x	x	xx	x		8
Mameli 2009	x	x	x				xx	x		6
Mameli 2007	x		x		x		x			4
Arru 2007	x	x			x	x	xx	x		7
Dolei 2002	x		x		x		x			4
Serra 2001	x	x	x	x	x		x			6
Garson 1998			x				x			2
de Villiers 2006			x		x	x	x			4
Nowak 2003			x				x			2
Alvarez-Lafunte 2008		x	x				x			3
Perron 1997		x	x		x	x	x			5

Abbreviations: S1 case definition; S2 representativeness cases: defined hospital, over period of time, defined area; S3 selection controls: community controls; S4 definition controls: no history of MS; C1 matched for age; C2 matched for other factor; E1 ascertainment of exposure (x, appropriate detection technique; xx, appropriate detection technique and blinding); E2 same method for case and controls; E3 non-response rate

5.3.3. HERV-H

Thirteen articles from 3 different research groups from Spain, Denmark and Canada focused on the association between HERV-H and MS (Fig. 5.2).

The presence of HERV-H in MS samples was detected for the first time by Christensen in 1998 (Christensen et al., 2000). The expression of HERV-H*env* and *gag* RNA by PCR was increased in the serum and PBMC of Danish MS (Christensen et al., 2000; Christensen et al., 2003). B cells and monocytes, but not T cells, from patients with active MS expressed higher levels of HERV-H Env protein detected by FC when compared to patients with stable MS and to controls (Brudek et al., 2009).

In contrast, no difference in RNA expression was detected by CSF PCR between Spanish MS patients and neurological controls (Alvarez-Lafuente et al., 2008). Three studies did not find differences in the expression of HERV-H*env* RNA in the brain (Antony et al., 2004) and PBMC (Antony et al., 2006) and HERV-H*gag* RNA in the brain (Johnston et al., 2001) between Canadian MS and OND patients.

The Danish group (Nexø et al., 2001; Nexø, et al., 2016) identified a SNP (rs391745) on HERV-Fc1 (a HERV-H retrovirus on chromosome X) that was associated with RRMS and SPMS in the Danish and Norwegian populations (Hansen at al., 2011). They showed an increased expression of HERV-Fc1*gag* RNA in plasma and of HERV-Fc1*Gag* protein in T cells and monocytes in active MS patients compared to non-active MS and HC (Laska et al., 2012). The same group did not find differences in the HERV-Fc1*gag* DNA copy number in PBMC between the MS and HC groups (Nissen et al., 2012).

A systematic review summarized the presence of HERV-Fc1 in the Spanish, Danish and Norwegian MS cohorts (de la Hera et al., 2014). The authors found an initial OR

of 1.17 (p=0.004; 95%CI: 1.05-1.30) in favour of the association which increased to 1.27 (95%CI: 1.11-1.45) after the Spanish cohort was excluded, bringing the heterogeneity from 82% to 0% (Hansen et al., 2011).

5.3.4. HERV-K

Nine studies published by 6 different research groups in Denmark, France, Canada, UK, USA and Spain describe an association between MS and the HERV-K family (Fig. 5.2). More specifically, the studies focussed on the HERV-K10,-K115,-K113 and –K18 loci of this family.

The first report in 1997 by a French group found no difference in the expression of HERV-K10*env* RNA in the PBMC and brain samples in MS patients compared to HC (Rasmussen et al., 1997). The Canadian group reported an increased expression of HERV-K*pol* (Johnston et al., 2001), but not of HERV-K*env* (Antony et al., 2004; Antony et al., 2006) in the brain of MS patients compared to control groups, using RT-qPCR. HERV-K113 and HERV-K115 are HERV-K copies that are only found in certain percentage of the population. A British group used DNA PCR and showed an increased frequency of HERV-K113, but not HERV-K115, in MS and Sjogren`s syndrome compared to HC in a British population (Moyes et al., 2005). The same authors could not reproduce the result in a larger study using unaffected parents of MS patients as control group (Moyes et al., 2008), but a Danish group found an association between the SNP rs2435031 near HERV-K113 and MS (Nexø, et al., 2016).

A meta-analysis studying the link between HERV-K18 polymorphisms on chromosome 1 and autoimmune diseases found an association between the haplotype HERV-K18.3 (97Y-154W) and the American and Spanish MS population with an OR of 1.22 (95%CI:1.09-1.38) (de la Hera et al., 2013, Tai et al., 2008). A stronger

association was detected in the subgroup of MS patients carrying the HLADRB1* 15:01 risk allele, known as a genetic risk factor for MS (de la Hera et al., 2013).

5.3.5. HRES and HERV-15

Rasmussen et al. in Denmark produced the 5 publications dealing with the relationship of the human T cell leukemia virus-related endogenous sequence (HRES) with MS (Fig.5.2). These authors reported an association between MS and different haplotypes of HRES-1 defined on the basis of a SNP. They detected an increased frequency of haplotype 1 in British (Rasmussen et al., 2000) and haplotypes 2 and 3 in Danish MS patients (Rasmussen and al., 1996; Rasmussen and Clausen, 1999), but no haplotype association in Chinese MS patients (Rasmussen and el., 1998) compared to HC. There was no difference in the level of RNA expression of HRES-1 between Danish MS patients and HC (Rasmussen et al., 1997).

A GWAS in progressive MS identified a locus on chromosome 7q35 (rs996343(G)) that resides in a retroviral element of the HERV-15 family (Martinelli-Boneschi et al., 2012).

5.4. Discussion

With this systematic review and meta-analysis a strong association between MS and HERVs was demonstrated.

The HERV-W family has been studied in most detail since the discovery of its member MSRV in biological samples obtained from MS patients (Perron et al., 1997). A qualitative and quantitative analysis were performed. Overall, 20 of 25 articles that studied MSRV/HERV-W in MS report an association between MS and increased expression of MSRV/HERV-W in blood, CSF, and brain tissue. The aim of the meta-analysis was to identify retroviral family-specific articles that studied the presence or absence of the same protein or nucleic acid in the same type of tissue, using the same techniques. An additional inclusion criterion for the meta-analysis was the presence of the same control group. The meta-analysis was performed only if a minimum of 4 studies met these criteria. On this basis, a quantitative meta-analysis could only be performed for a) HERV-W family MSRV*env* detected in PBMC of MS and HC by RT-PCR b) MSRVpol detected in serum or plasma of MS and HC by RT-PCR and c) MSRVpol detected in CSF of MS and OND by RT-PCR.

All the performed meta-analyses showed a strong association between MSRV/HERV-W*pol* and MSRV/HERV-W*env* and MS.

Using healthy blood donors as control group, the results of the *env* meta-analysis showed a high OR (22.66) with no inconsistency or publication bias. High OR were obtained from the *pol* meta-analysis in serum/plasma as well, but a high heterogeneity between studies (61.2%) and evidence of publication bias were noted. The study with the largest population in the *pol* meta-analysis was a multicentre study including patients from Sweden (32%), Spain (41%) and Sardinia (27%) (Arru et al., 2007),

while the studies with very high OR, but a relatively small population, were only from Sardinia (Serra et al., 2001; Dolei et al., 2002), an Italian island with peculiar genetics and a high incidence of MS (Sotgiu et al, 2002). This can suggest either a publication bias due to observation that all the publications with high OR related to the Sardinian population come from a single research group, or a strong connection between MSRV/HERV-W and genetics. Indeed genetic differences between the studied populations could underlie the high heterogeneity. Other important confounders could be sex, age and source of case and controls. Unfortunately due to limitations in the information provided by these articles (age, sex, type of MS, and source), it was not possible to stratify the population based on these parameters.

In line with the results found in the blood comparing MS and HC, the *pol* meta-analysis in CSF of MS and OND showed strong OR (6.00) as well, with no inconsistency or publication bias. This suggests that MSRV/HERV-W*pol* expression is specifically associated with MS rather than general neurological diseases.

The major limitations of these meta-analyses are the relatively small population samples included in the analysis, which might not be representative of the whole population of patients with MS or controls, and the small number of studies included. Of all the articles studying HERV-W, only 4 were included in the meta-analysis for MSRV*env* in PBMC, 6 for MSRV*pol* in serum/plasma and 4 for MSRV*pol* in CSF. Only one of the publications with negative results was eligible for the quantitative analysis (Alvarez-Lafuente et al., 2008), while all the others did not meet the inclusion criteria, creating a potential bias. Specifically, Antony et al 2006 and 2007, Schmitt et al 2013 and Laufer et al 2009 did not report data that could be interpreted as number of MSRV-positive and –negative MS and control subjects, but presented them as relative expression and often reporting only the mean or the p value for the differences.

Arbitrary cut-off values to identify "positive" and "negative" samples were not established because it was not assumed that every author would be able and willing to provide with original data. On the other hand, positive/negative cut-off values are not explicitly presented in the publications included in the meta-analysis. For these reasons, the data were used as they are actually presented in the peer-reviewed articles selected. Such papers were included in the qualitative, rather than quantitative analysis.

Among negative studies, Antony et al reported an association between MS and syncytin-1 rather than MSRV*env*. The MSRV*env* sequence is thought to originate from distinct HERV-W loci in different parts of the genome as well as recombination among them (Antony et al., 2011). By contrast, syncytin-1 only describes the Env protein encoded by ERVWE-1, a replication-incompetent sequence on chromosome 7q21-22. At the RNA level MSRV*env* and syncytin-1 share 94% sequence identity, only differing for a 12-nucleotide insertion, making their discrimination difficult (Mameli et al., 2009). Differences in real-time PCR methods (Garson et al., 2009) may also contribute to such complexity, which will require further clarification. PCR is the most commonly used method for HERV detection, but it does have limitations, including the inclusion or exclusion of different loci from the same group of viruses depending on the design of the primers used, which vary between studies (Appendix V pag. 264). Although it was not found any correlation between primers used in studies included in the serum and PBMC meta-analysis and OR found, for the MSRV*pol* meta-analysis in CSF the unique study that used different set of primers was the one with the lowest OR (Alvarez Lafuente, 2008). This underlies the difficulty in state which HERV locus/loci is/are responsible for observed differences. Moreover,

creation of recombinants between different sequences in vitro may also lead to detection of RNAs that do not originate from genomic DNA.

The majority of the studies scored poorly (<5) in the NOS scale for quality assessment. This could be related to poor reporting rather than poor conduct (for example only 3 of 12 studies specified to use the same technique for cases and controls). Difficulties in the enrolment of community controls matched with at least two factors (age and sex) with MS patients could be a real bias to take in consideration in the analysis.

Overall, the association between MSRV/HERV-W and MS is strong. Moreover, the expression of MSRV/HERV-W has also been associated with the occurrence of disease progression (Sotgiu et al., 2010).

In addition to the HERV-W family, studies on HERV-H, HERV-K, HRES and HERV-15 also showed an association with MS in the qualitative analysis.

For these HERV families, 17 of 28 studies reported a significant association between HERV expression and MS, whereas the other 11 studies did not. More limited agreement between these studies may be explained by genetic differences among populations (Rasmussen et al., 1996; Rasmussen et al., 1998), differences in the patterns of expression of different HERVs in CNS or peripheral tissues at different stages of MS (Antony et al., 2006), and by methodological issues. Technical issues were raised with regards to the adequate storage conditions of the samples (Alvarez-Lafuente et al., 2008) or the RT-PCR methodology used (Garson et al., 2009). Unfortunately, for HERV families different from HERV-W, there were fewer than 4 studies meeting the mentioned inclusion criteria, therefore a quantitative analysis was not possible. With regards to HERV-Fc1 (H family, included in our qualitative analysis), a meta-analysis was already published by De la Hera et al 2014 (de la Hera

et al., 2014), only focussing on the strength of association between the presence of the rs391745 SNP and MS.

In conclusion, these findings strongly support the evidence for an association between HERVs, and in particular MSRV/HERV-W, and MS. In the next Chapter, this association will be tested experimentally.

CHAPTER 6: Experimental data on the association of MSRV/ HERV-W and MS

6.1. Background

There are three main HERV families found associated with MS in literature: HERV-H/F, HERV-K and in particular HERV-W (Chapter 5). In HERV-W family there are two main elements that are thought to be related with MS: MSRV/HERV-W and syncytin-1 (syn-1). The first is a complete retrovirus with RT activity, a polyA RNA formed by LTRs, *env*, *gag* and *pol* sequences. The second indicates only the protein Env encoded by ERVWE-1, a replication-incompetent sequence on chromosome 7q21-22. This is an intracellular protein with a physiological role in pregnancy. In fact Syn-1 has been reported to mediate placental cytotrophoblast fusion in vivo, and thus may be important in human placental morphogenesis (Mi et al., 2000). At the RNA level MSRV/HERV-W*env* and *syn-1* share the 94% of identities and differ only for an insertion of 14 nucleotides (Fig. 6.1). Until now, because the antibodies identified are not specific only for a unique HERV-W, there is no method to discriminate between MSRV and syncytin-1 at the protein level. On the other hand, at the RNA level, a real-time RT-PCR has been optimized to discriminate the two sequences on the basis of the 12-nucleotide insertion (Fig.6.1) (Mameli et al., 2009).

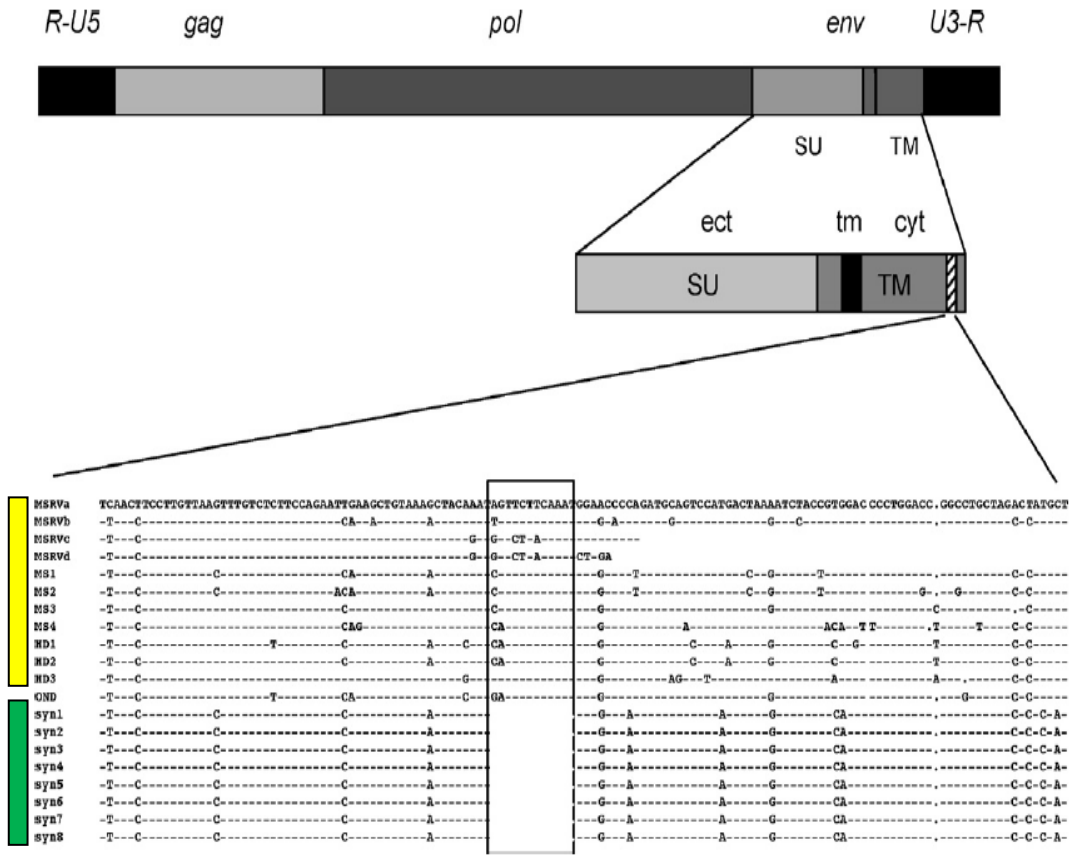


Figure 6.1 Organization of MSRV genome, and comparison of the corresponding env sequence from MSRV and from syncytin-1 (Mameli et al., 2009).

Little is known about the effect of MSRV/HERV-W on the immune system, but some studies have shown that it can activate innate immunity through CD14/TLR4 and induce the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (Rolland et al., 2006).

UK is a high-prevalence country for MS, but to our knowledge there has been no study performed to establish possible association between MSRV/HERV-W presence in the British population and MS. In this Chapter, the RNA expression of MSRV/HERV-W *env* and TLR4 was evaluated in MS patients, HC and OND patients using the optimized RT-PCR described above (Mameli et al., 2009).

Basal expression of MSRV/HERV-W is present in different immune cell types, including astrocytes, monocytes, NK and B cells. On the contrary, in T cells there is no expression of this endogenous retrovirus (Brudek et al., 2009; Mameli et al., 2012). The presence of the virus was studied in PBMC and different immune cell types of HC and MS patients through Flow Cytometry with help of the MSc student Deema Alghabban.

In this chapter the following studies will be presented:

- RNA expression of MSRV/HERV-W*env* and TLR4 in total blood of MS patients, HC and OND patients;
- Protein expression of MSRV/HERV-WEnv in PBMC, B cell, monocytes and T cells of HC and MS patients.

6.2. Materials and Methods

6.2.1. Human Samples:

Blood samples for gene expression analysis were collected in PAXgene Blood RNA tubes (Qiagen) and directly stored at -80^{o}C from patients attending Queen's Medical Centre in Nottingham (part of Nottingham University Hospitals NHS Trust) and University Hospital of North Staffordshire, another participating centre. MS diagnosis was established according to McDonald's criteria (Polman et al., 2011). All patients and HC signed informed consent (Appendix I pag. 258). Patients' and HC' age, gender and clinical status are illustrated in Appendix VI pag. 265 (n HC =22, mean age= 42.2 sdt=11.7; n MS= 22, mean age=42.8 sdt= 8.4; n OND= 14, mean age 62.0 sdt=14).

Blood samples for FC analysis were collected in heparin tubes from patients attending Queen's Medical Centre in Nottingham and processed the same day. All patients and HC signed informed consent. Patients' and HC' age, gender and clinical status are illustrated in Appendix VII pag.268 (n HC =6, mean age= 39.7 sdt=13.1; n MS= 12, mean age=44.3 sdt= 11.9)

6.2.2. RNA isolation:

Intracellular RNA was purified using the PAXgene Blood RNA kit (Qiagen) following the manufacturer's instructions. After centrifugation at 3000g, nucleic acids pellet was washed, resuspended, and incubated in optimized buffers together with proteinase K for protein digestion. An additional centrifugation through the PAXgene Shredder spin column was carried out to homogenize the cell lysate and remove residual cell debris, and the supernatant of the flowthrough fraction was transferred to a fresh microcentrifuge tube. Ethanol was added to adjust binding conditions, and the lysate was applied to a PAXgene RNA spin column. During a brief centrifugation, RNA was

selectively bound to the PAXgene silica membrane. Remaining contaminants were removed in several wash steps and the membrane was treated with DNase I to remove trace amounts of bound DNA. After the wash steps, RNA was extracted in the elution buffer provided by the kit and stored at -80ºC. RNA concentration was determined by measuring the absorbance at 260nm using NanoDrop ND-100 (Thermo Scientific).

6.2.3. cDNA synthesis:

For cDNA acquisition, 10µl RNA samples (between 0.2-0.5ug), 2µl Random hexamers and 1µl dNTPs mix (10mM) (all Promega) were mixed, followed by a 5 min incubation at 65ºC for first strand cDNA synthesis. A master mix containing 1µl RN-ase inhibitor (Promega), 1µl DTT (0.1M) and 4µl 5X First-Strand Buffer was added, along with 1µl Superscript III RT (220 units/µl) (all from Invitrogen). Negative controls replacing the RNA template or the RT with DNase/RNase free H_20 were included. Samples were incubated as followed: 5 min at 95ºC, 60 min at 50ºC and 25 min at 70ºC. cDNA was stored at -80ºC.

6.2.4. Real Time RT-PCR:

After a titration of different cDNA dilutions, 1:2 DNase/RNase free H_20 dilution was selected. Relative quantification was performed using HMBS as a housekeeping gene. Each RT-PCR reaction included 2µl of diluted cDNA and 12.5µl of Faststart Universal probe master (Rox, Roche). For MSRV//HERV-W*env* RT-PCR primers were designed as reported previously in literature (Mameli et al., 2009) and 1µl of probe at the final concentration of 250nM and 1µl of each forward and reverse primer at the final concentration of 900nM (TaqMan, Invitrogen) were used, whereas for TLR4 and HMBS RT-PCR, 1.25µl of final concentration 900nM of probe/forward and reverse primer mix were used (TaqMan, Invitrogen). DNase/RNase free H_20 was added up to

25µl. All samples run in duplicates. RT- PCR reactions were held using the 7900HT Fast Real-Time PCR system (Applied Biosystems) in 96-well plates. The following incubation protocol was imposed: 10 min at 95ºC and 40 cycles of 10 sec at 95ºC followed by 30 sec at 60ºC. All the samples run in duplicates during RT-PCR, thus the average Ct value of each sample was obtained. The Ct values of MSRV//HERV-W*env* or TLR4 were normalised compared to the Ct value of HMBS. Comparison of the relative amounts of MSRV//HERV-W*env* and TLR4 was performed using the $2^{-\Delta\Delta Ct}$ method considering an HC reference gene (HC_{15}, selected for the high amount of RNA concentration). The reference gene HC_{15} was analysed in all the different plates run as inter-run calibrator. Any change in gene expression between HC, MS and OND patients towards the HC_{15} was expressed as fold change using the formula below:

Fold increase for each individual patient compared to the $HC_{15} = 2^{-\Delta\Delta Ct}$

Where for each patient:

$\Delta\Delta Ct = [(Ct\ GI - Ct\ HMBS) - \Delta CtHC_{15}]$ GI = MSRV*env* or TLR4

$\Delta CtHC_{15} = \Delta Ct\ reference\ gene\ (HC_{15}) = (Ct\ GIHC_{15} - Ct\ HMBSHC_{15})$

6.2.5. DNA gel electrophoresis:

10µl of cDNA mixed with 5µl of 10x loading buffer run on a 2% agarose gel in 1x TBE after staining with Ethidium Bromide. A 50bp DNA ladder (Invitrogen) was used.

6.2.6. Isolation of PBMC

PBMC were isolated form blood donors using Ficoll density gradient centrifugation. Blood was diluted with an equal volume of PBS (Sigma-Aldrich). 30ml of diluted blood samples were layered into 15ml of Histopaque-1077 (Sigma-Aldrich) density medium and centrifuged at 600g for 30min without break. PBMC layer was removed

and washed twice with PBS. After the final wash the cell pellet was re-suspended in 10ml of PBS. Cell count was performed by pipetting 10µl of sample into 90µl of trypan blue (Sigma-Aldrich). 10µl of the mixture was pipetted into neubauer hemocytometer and cell count was reported as cells/ml.

6.2.7. Extracellular Flow cytometer staining HERV-W

Surface staining for Pe-Cy7 anti-CD3 (UCHT1), PE anti-CD14 (M5E2) and Pe-Cy5 anti-CD20 (2H7) all from BD was performed on PBMC. HERV-W was detected using 3µl primary anti-HERV-WEnv rabbit polyclonal antibody (Allele Biotech) with 1.5µl secondary CF 488A goat anti-rabbit antibody (Sigma-Aldrich). Control with only secondary CF 488A goat anti-rabbit antibody was included and FMO samples were used to set the gating. Cells were divided in 10^6 cells per FACS tube and washed with 2 ml of FACS buffer (PBS+2% FCS) and centrifuged at 300g for 6min. Supernatants were discard and pellets were re-suspended and 10µl of human serum were added to all samples to avoid non-specific antibody binding. Then they were stained with 5-20µl of Abs as suggested by datasheet. Cells were incubated in the dark for 30min at 4˚C. After incubation cells were washed 2 times with 2ml of FACS buffer and centrifuged at 300g for 6 min. Cells were then fixed with 500µl Fixation buffer (2% paraformaldehyde, BD). Cells were analysed by FC using LSRII flow cytometer (BD Biosciences, USA) and FlowJo software (version V10, FlowJo, LLC, USA).

6.2.8. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Unpaired T test or One-way ANOVA were used for RT-PCR and FC analysis ($n > 6$). All statistical tests have been indicated in the figure legends. The graphs show mean with standard error of the mean (SEM) if a parametric

test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤ 0.05 were considered significant and only significant p values were reported in the graphs.

6.3.Results

6.3.1. Expression of MSRV/HERV-W*env* RNA in MS, HC and OND

Total RNA was extracted from PAXGENE tubes of 22 MS patients, 22 HC and 14 OND. MS patients and HC were age-matched, while OND were older (average age MS=47.54, HC=42.18, OND=62.09) (Appendix VI pag. 2265). Relative quantification of MSRV/HERV-W*env* and TLR4 gene expression was assessed with real-time RT-PCR considering the HC reference gene (HC 15, Appendix VI pag. 265) as 1. Means of MSRV/HERV-W*env* fold change were 3.41 for MS, 1.72 for HC and 0.74 for OND (Table 6.1). The expression of MSRV/HERV-W*env* in the MS group was significantly higher compared with HC ($p=0.002$) and OND ($p<0.001$) (Fig. 6.2 A). Means of TLR4 fold change were 2.65 for MS, 1.47 for HC and 2.07 for OND (Table 6.1). TLR4 expression was significantly higher in MS compared to HC ($p=0.016$) (Fig. 6.2 B). The fold change was not similar across samples. MS patients had MSRV/HERV-W*env* fold change ranging from 1.12 to 10.76 and TLR4 from 0.95 to 6.28. Interestingly, the majority of patients that exhibited more than ~2.5-fold MSRV//HERV-W*env* increase, had also elevated TLR4 expression (Fig. 6.2 C).

Table 6.1 Fold change of MSRV//HERV-W*env* and TLR4 expression in A) HC, B) MS patients and C) OND patients.

A

HC	MSRV	TLR4
HC 1	1.31	0.38
HC 2	1.52	0.86
HC 3	1.64	1.66
HC 4	2.26	2.29
HC 5	2.21	0.73
HC 6	1.15	1.88
HC 7	2.11	4.23
HC 8	1.49	1.13
HC 9	2.74	1.65
HC 10	1.19	0.83
HC 11	0.58	1.32
HC 12	1.55	1.88
HC 13	1.48	3.32
HC 14	1.51	1.84
HC 15	**1.00**	**1.00**
HC 16	1.88	0.62
HC 17	2.93	1.25
HC 18	2.00	1.43
HC 19	2.08	1.51
HC 20	2.16	0.71
HC 21	1.45	0.71
HC 22	1.66	1.10
Aver.	1.72	1.47

B

MS	MSRV	TLR4
MS 1	3.19	1.26
MS 2	3.27	2.55
MS 3	2.91	2.26
MS 4	1.30	2.46
MS 5	2.10	1.28
MS 6	1.63	1.38
MS 7	2.47	1.74
MS 8	2.99	5.01
MS 9	3.90	2.75
MS 10	4.66	3.43
MS 11	7.32	5.29
MS 12	10.76	6.28
MS 13	1.44	1.90
MS 14	2.95	2.81
MS 15	3.11	2.91
MS 16	1.21	1.20
MS 17	2.56	1.96
MS 18	2.01	2.65
MS 19	1.12	0.95
MS 20	2.39	0.85
MS 21	2.37	3.46
MS 22	9.42	3.98
Aver.	3.41	2.65

C

OND	MSRV	TLR4
OND 1	0.13	0.29
OND 2	0.68	0.87
OND 3	0.42	1.55
OND 4	0.87	7.64
OND 5	0.71	1.82
OND 6	0.33	1.68
OND 7	0.84	2.58
OND 8	0.83	0.87
OND 9	1.26	2.46
OND 10	0.77	1.72
OND 11	0.88	1.03
OND 12	1.09	1.15
OND 13	1.54	3.59
OND 14	0.00	1.80
Aver.	0.74	2.08

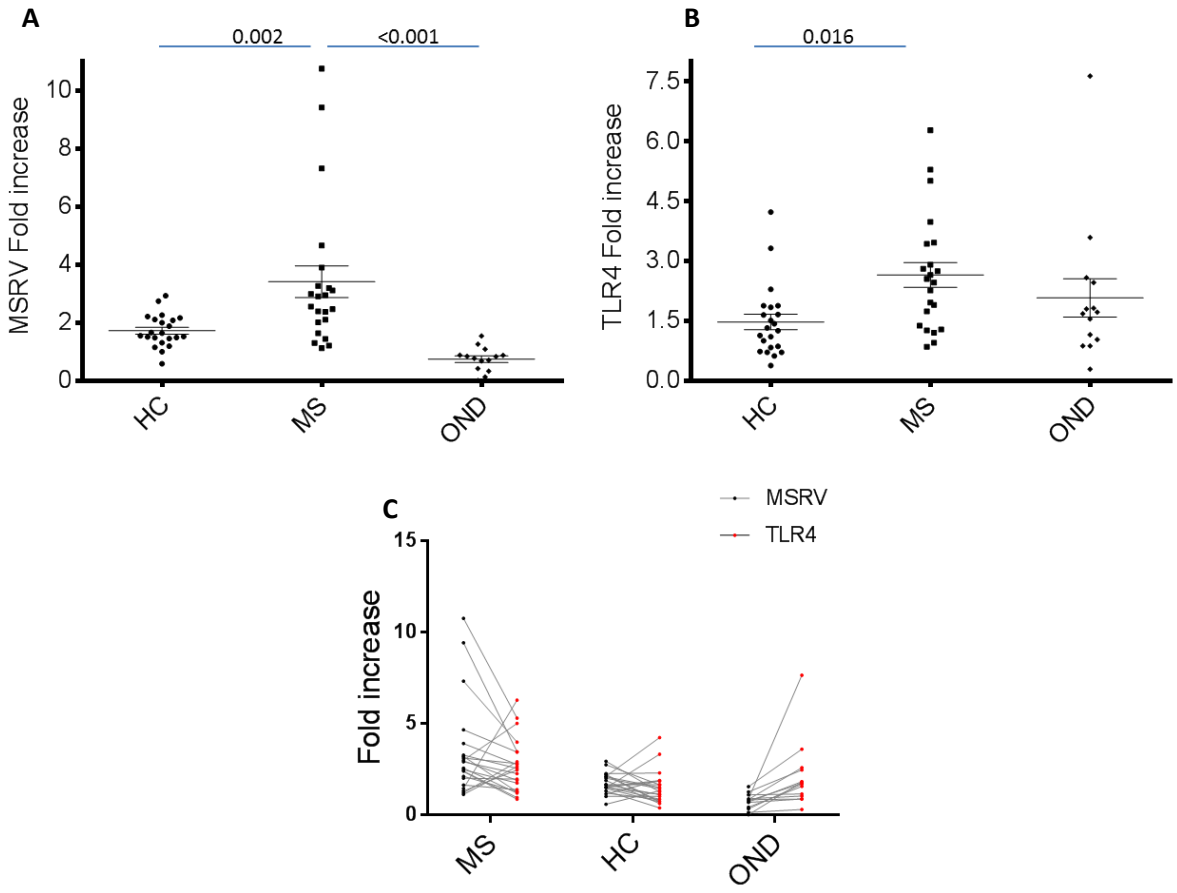


Fig. 6.2 MSRV//HERV-W*env* and TLR4 gene expression are higher in MS patients, compared to control groups. MSRV/HERV-W*env* and TLR4 expression were evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Fold changes of **(A)** MSRV/HERV-W*env* and **(B)** TLR4 expression of each individual sample in each group are illustrated in dots. MS patients were compared to HC and OND using HC_{15} as reference gene. Mean and SEM are indicated by bars (n MS=22, n HC=22, n OND=14; 1-way ANOVA test). **(C)** The expression of MSRV/HERV-W*env* and TLR4 is connected for each individual in each group.

Different groups were not sex-matched since in the MS group only 4 out of 22 patients were male. Categorizing the subjects based on gender, the average fold increase for MSRV/HERV-W*env* expression in females and males was 3.25 and 4.12 in MS patients, 1.79 and 1.62 in HC and 0.37 and 0.44 in OND respectively (Fig. 6.3 A). Average fold increase for TLR4 expression in the same groups was 2.64 and 2.71 in MS patients, 1.93 and 1.04 in HC and 1.88 and 1.13 in OND respectively (Fig. 6.3 B). Only the expression of TLR4 between HC male and female was significantly different.

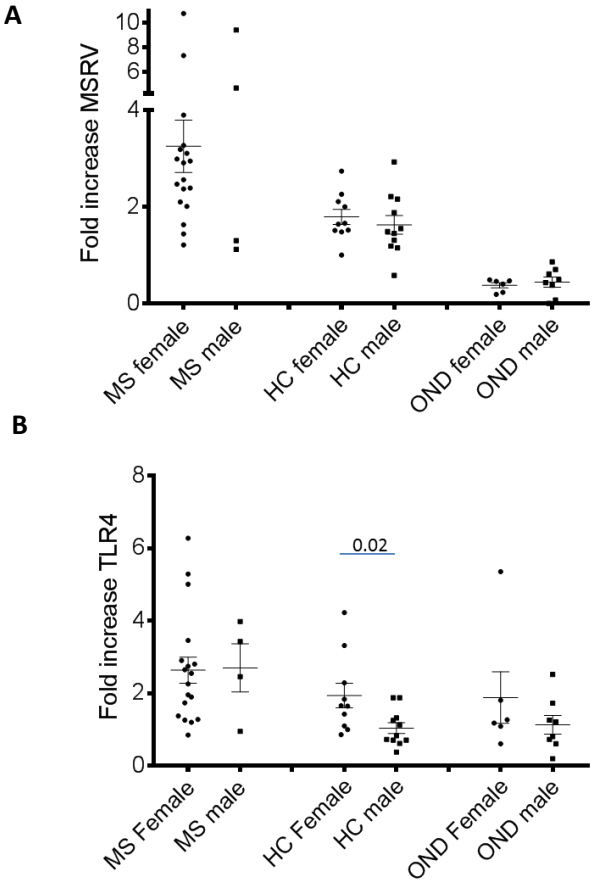


Fig. 6.3 MSRV/HERV-W*env* and TLR4 gene expression in MS patients, HC and OND patients categorized on gender. MSRV/HERV-W*env* and TLR4 expression were evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Fold changes of **(A)** MSRV/HERV-W*env* and **(B)** TLR4 expression of each female and male subject in MS, HC and OND are illustrated in dots. Mean and SEM are indicated by bars (n female MS=18, n male MS=4, n female HC=11, n male HC=11, n female OND=6, n male OND=8; unpaired T test).

Furthermore, the average expression of MSRV/HERV-W*env* and TLR4 for the 10 MS patients in RR-MS in remission stage was compared to the 6 patients that were in the SP-MS (Appendix VI pag. 265). MSRV/HERV-W*env* expression was 3.19 fold increased in the patients that were in RR-MM and 4.49 fold increased for the patients that were in the SP stage (Fig. 6.4). TLR4 expression was 2.28 fold increased for RR patients and 3.03 fold change for SP patients (Fig. 6.4). None of these differences was significant.

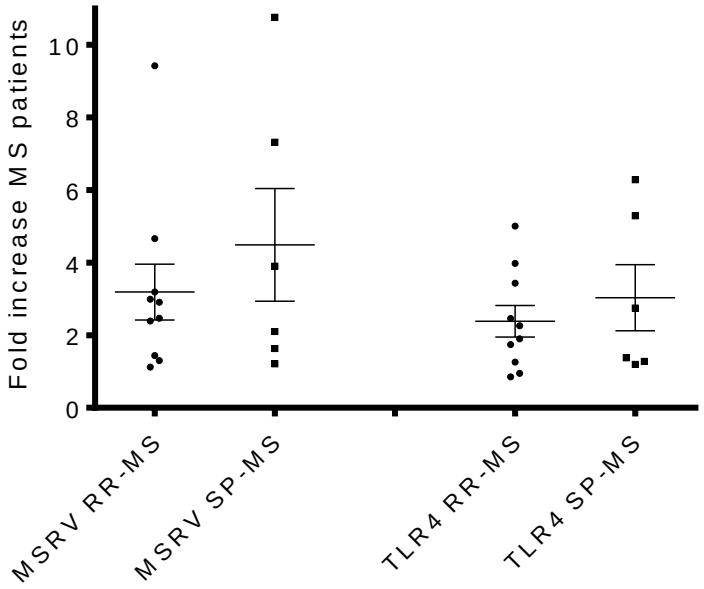


Fig. 6.4 MSRV/HERV-W*env* and TLR4 gene expression in MS patients categorized on the disease course. MSRV/HERV-W*env* and TLR4 expression were evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Fold changes of MSRV/HERV-W*env* and TLR4 expression of RR-MS and SP-MS are illustrated in dots. Mean and SEM are indicated by bars (n RR-MS=10, n SP-MS=6; unpaired T test).

In order to validate that the primers used in RT-PCR recognised the correct gene, a few samples of cDNA amplified in each RT-PCR run were selected randomly and analysed by gel electrophoresis (Fig. 6.5). The bands size acquired were the expected for all three genes (MSRV/HERV-W*env*, TLR4 and HMBS) under examination.

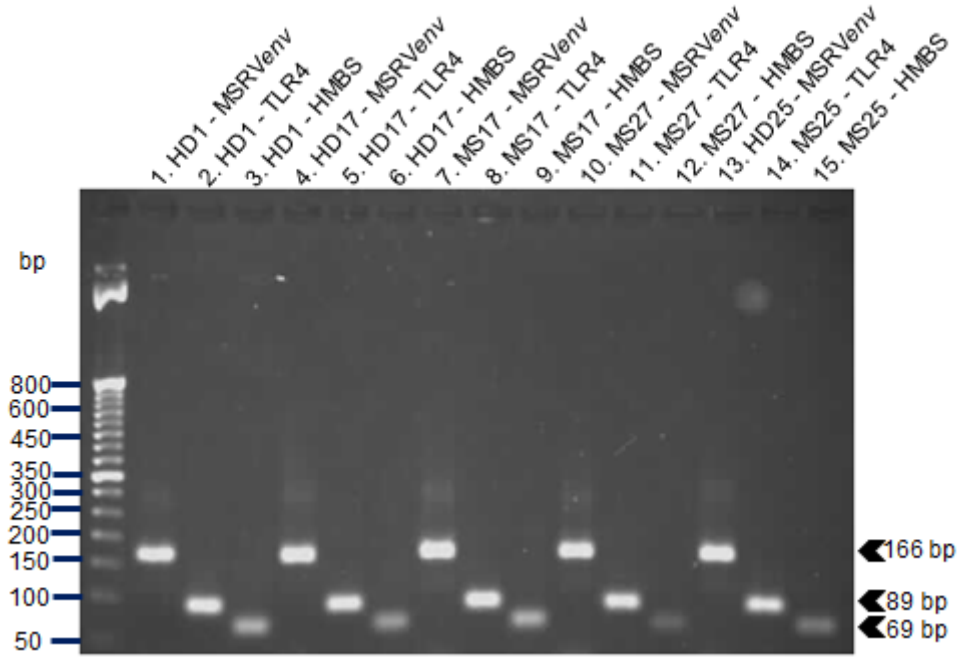


Fig. 6.5 DNA gel electrophoresis for validation of proper gene amplification in RT-PCR. Samples run on a 2% agarose gel stained with Ethidium Bromide. The first line accommodates a 50 base pairs (bp) primer. Each of the 3 lines that follow represents one of a randomly selected subject analysed for MSRV/HERV-W*env*, TLR4 and HMBS gene amplification. MSRV*env* amplicon size = 166bp, TLR4 amplicon size = 89bp, HMBS amplicon size = 69bp.

6.3.2. Expression of HERV-WEnv protein in different cell types of MS and HC

HERV-W*env* can be translated into protein and detected on the surface of immune cells. HERV-WEnv protein expression was detected on the cell surface of PBMC from 10 MS (6 RR-MS, 1 PP-MS, 3 SP-MS) patients and 6 age-matched HC (Appendix VII pag. 268) using a primary anti-HERV-WEnv antibody and a CF488A-labelled secondary antibody by FC (Mameli et al., 2012). HERV-WEnv was expressed by the 20% of PBMC of which mainly by CD20+ B cells and CD14+ monocytes. CD3+ T cells were negative (Fig. 6.6).

Comparing the mean of MS and HC samples, there was no significant difference in HERV-WEnv expression in total PBMC, T cells, B cells, or monocytes between the two groups (Fig.6.7).

Categorizing the samples based on gender (Appendix VII pag. 268), HERV-WEnv expression was similar between male and female MS patients (Fig. 6.8). Due to the small number of HC samples (6), gender comparison was not possible in this group.

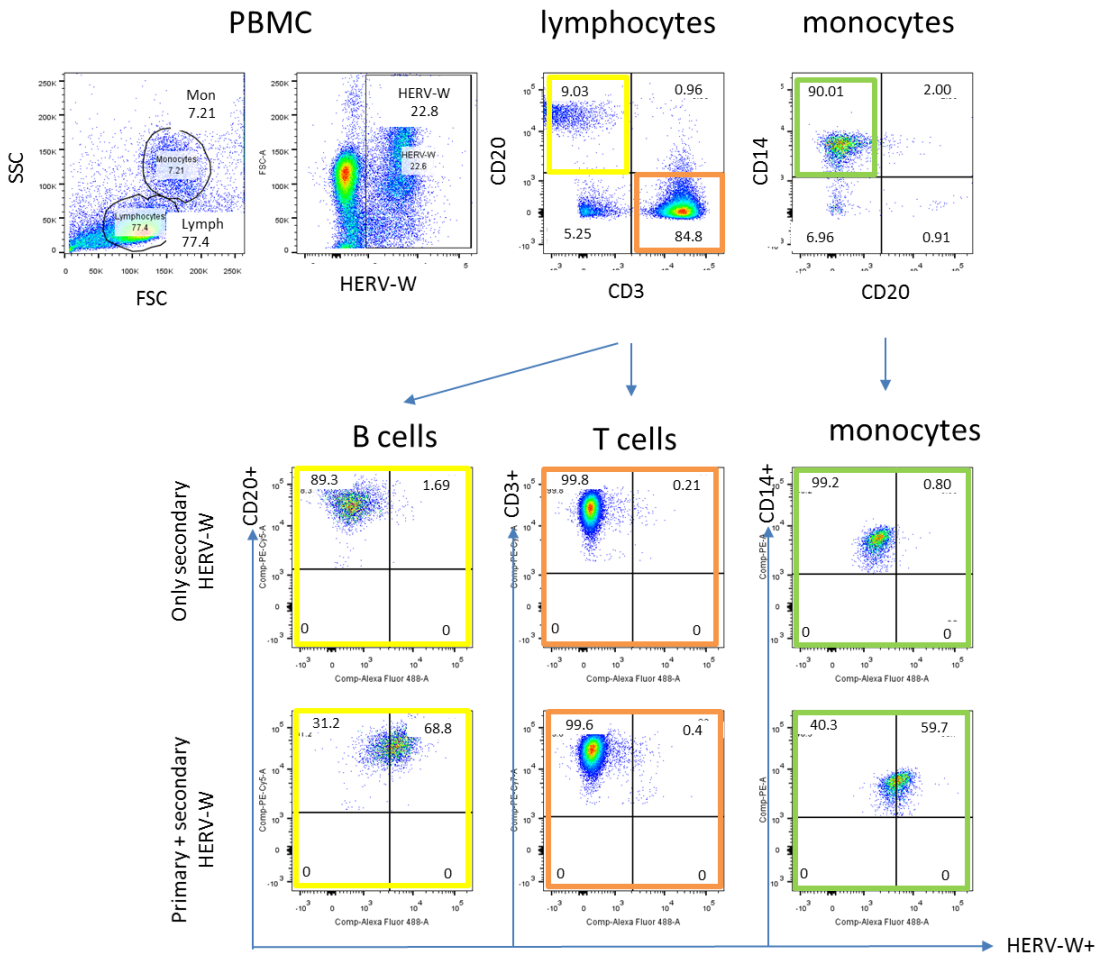


Fig. 6.6 Gating strategy and FC analysis of HERV-WEnv expression in PBMC. Expression of HERV-WEnv was detected in total PBMC, as well as CD20+ and CD3+ lymphocytes and CD14+ monocytes. The staining with only secondary CF 488A anti-rabbit antibody (without anti-HERV-WEnv primary Ab) was used as negative control. Cells from one HC are shown as example.

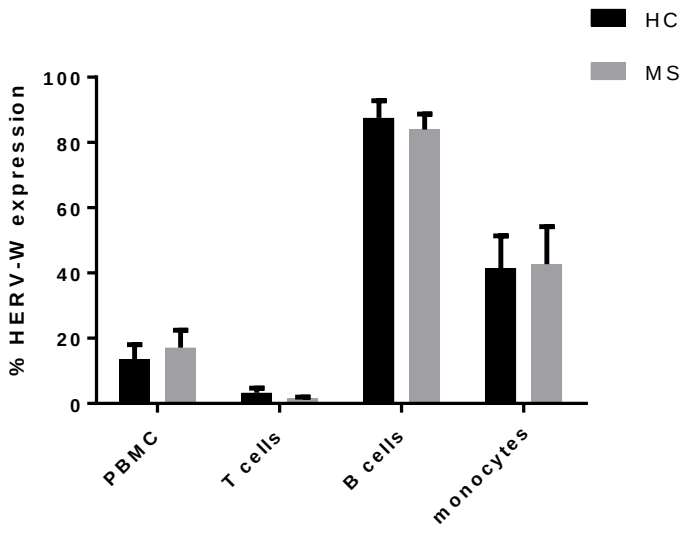


Fig. 6.7 No difference in the expression of HERV-WEnv in different immune cells between HC and MS. Expression of HERV-WEnv was detected in total PBMC, CD3+ T cells, CD20+ B cells and CD14+ monocytes from 10 MS patients and 6 HC. The bars represent the mean of percentage of expression of HERV-WEnv in different cell groups and error bars represent SEM (n MS=10, n HC=6; unpaired T test).

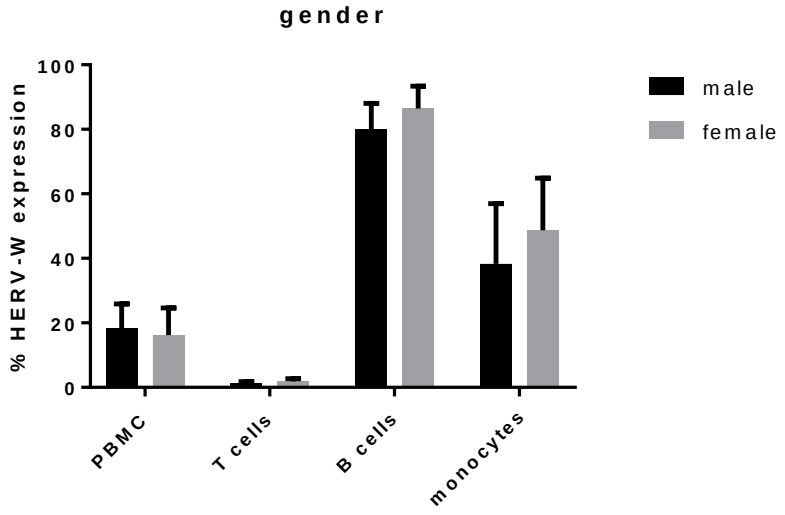


Fig. 6.8 No difference in the expression of HERV-WEnv in different immune cells of MS patients between male and female. FC analysis of surface expression of HERV-WEnv in PBMC, CD3+ T cells, CD20+ B cells and CD14+ monocytes from 10 MS grouped on gender base. The bars represent the mean of percentage of expression of HERV-WEnv in different cell groups and error bars represent SEM (n male MS=5, n female MS=7; unpaired T test).

6.4. Discussion

Several studies in MS patients and control populations show MSRV/HERV-W correlation with MS disease and prognosis (Sotgiu et al., 2010; Garcia-Montojo et al., 2014) (Chapter 5). The mechanism of action of MSRV/HERV-W seems to involve TLR4 in activating a pro-inflammatory immune response. In this chapter the expression of MSRV/HERV-W*env* and TLR4 were studied in total blood of MS patients, HC and OND patients. Amplification signals were obtained in all samples analysed, but with a difference degree of expression. Both MSRV/HERV-W*env* and TLR4 gene expressions were higher in the MS group compared to HC, with a statistical power of 85%. It is noteworthy that the majority of MS patients that exhibited more that ~1.5-fold increase in the MSRV/HERV-W*env* gene expression, had also an increased TLR4 gene expression. This would be consistent with the observation that MSRV/HERV-W can activate TLR4 (Rolland et al., 2006).

Sequence alignment of the immunopathogenic MSRV*env* sequence revealed that chromosome X has three homologous MSRV*env* DNA copies inserted (Antony et al., 2011). If these copies are more expressed in females, this might contribute to explain why MS is more present in females. Nevertheless, in the present study, MSRV/HERV-W*env* did not differ among genders. This could be due to the small population analysed and to the fact that the primers used do not specifically recognise MSRV*env* sequence on chromosome X (Mameli et al., 2009). In line with this concept, TLR4 was higher in female HC compared with males. Although the link between innate immunity and gender is not completely understood, a study investigated the mechanism of gender-related differences in innate response measuring the level of inflammatory cytokine production in whole blood and PBMC culture after LPS stimulation (Imahara et al.,

2005). They found that women produced significantly less LPS-induced inflammatory cytokines, suggesting a different TLR4 activation between males and females.

Furthermore, the expression of MSRV/HERV-W*env* was assessed in RRMS patients that were in the remission phase of the disease and in SPMS patients. No significant difference was noticed between the two different groups. It would have been interesting to compare longitudinally the same RRMS patients in relapse and remission, but blood from patients in relapse was more difficult to collect.

Limitations of this study were the relatively small number of subjects included and the gender/age bias between different groups. Moreover, as already discussed in Section 5.4, the RT-PCR protocol used here is based on what is currently consider the best way to detect MSRV in literature, but it has been clarified that PCR-based methods detect a mosaic of sequences rather than specific loci, making PCR results difficult to interpret (Grandi et al., 2016).

Despite the detection of an increased expression of MSRV/HERV-W*env* RNA in MS patients by RT-PCR, HERV-WEnv protein was detected in all samples of MS and HC using FC with no difference in the number of HERV-W positive cells between the two groups. At the protein level, no antibody has been developed yet to discriminate MSRVEnv from Syn-1 (Mameli et al., 2009). Moreover, the issue of HERV-W complexity extends to studies of HERV protein expression: it often remains unclear as to which genomic locus the observed HERV proteins are derived from and therefore, whether the precise identity of the protein recognized by the HERV-WEnv antibody can be established. This could explain why HERV-W is expressed in similar percentage in both groups: the antibody could detect either MSRV*env* or Syn-1, or both together. Another new technique was used to try to detect specifically MSRV*env*.

The Prime Flow RNA Assay (Thermo Fisher Scientific) permits to combine RNA hybridization and antibody staining with the advantage to detect RNA and protein expression using FC. This technique is not only able to target RNA itself, but also to amplify the signal of RNA transcript. Unfortunately, it was not possible to detect MSRV*env* RNA signal, probably because the probe was not sensitive enough to detect the expression of the retrovirus.

HERV-WEnv was expressed in whole PBMC, in high level in B cells and in monocytes. No expression of HERV-WEnv was detected in T cells. These results are in line with previous publications. It has been reported that in peripheral blood, MSRV/HERV-W RNA and proteins are expressed mainly in monocytes/macrophages and B cells and their expression is higher in MS patients, while in T cells the retrovirus is undetectable at the RNA and protein level in both HC and MS (Brudek et al., 2009; Mameli et al., 2012). These observations underline the key role of B cells and monocytes in HERV-W expression. In particular, monocytes isolated from the peripheral blood of patients with active MS and stimulated in vitro with either PMA or LPS produced significant levels of gliotoxic activity, which correlated with increased HERV RNA and retrotranscriptase activity (Ménard et al., 1997). This observation suggested a role for HERV expression in mediating gliotoxicity in MS monocytes/macrophages. Moreover, high amounts of TNFα, IL-1β and IL-6 were secreted by monocytes from HC treated for 24 h with MSRV/HERV-Wenv (Rolland et al, 2006). Interestingly, the secretion of TNFα was blocked by anti-TLR4 and anti-CD14, but not by anti-TLR2 Ab, suggesting that TLR4 is specifically involved in the proinflammatory effects of HERV-WEnv (Rolland et al., 2006). Consistent with these observations, in chronic active MS brain lesions MSRV/HERV-W Env was detected in microglia/macrophages in proximity to TLR4-positive oligodendroglial precursor

cells (OPC). The recombinant HERV-WEnv induced the production of iNOS and proinflammatory cytokines such as TNFα, IL-1β and IL-6 in cultured rat OPC, with associated reduction in myelin protein production and differentiation capacity (Kremer et al., 2013). Moreover, MSRV/HERV-WEnv induced phenotypic and functional maturation of DC and enabled them to support the development of Th1-like effector lymphocytes (Rolland et al., 2006). This pathogenic role of MSRV/HERV-W has also been demonstrated in MOG_{35-55}-induced EAE in C57-BL/6 mice, where MSRV/HERV-W Env could substitute for mycobacterial lysate as a component of CFA, thereby inducing CNS disease. In the same model MSRV/HERV-WEnv also activated cells of the innate immune system, leading to a proinflammatory cytokine production through TLR4 in association with CD14 (Perron et al., 2013).

The trigger factor(s) for MSRV/HERV-W reactivation is still not clear, but there is evidence to suggest that other viral infections can act as co-factors (Dual viral hypothesis). The next chapter will investigate the role of EBV in MSRV/HERV-W activation.

CHAPTER 7: MSRV/HERV-W and EBV

7.1.Background

EBV is strongly associated with MS (Chapter 1.3.4). Interestingly, MS risk is 2-3fold higher among individuals with a history of IM (Ascherio and Munger, 2010). In vitro the expression of MSRV/HERV-W genes/proteins is activated by some viruses such as EBV, herpes simplex virus type 1 or by influenza virus (Dolei and Perron, 2009). One study showed that binding of the EBV surface glycoprotein gp350 can activate the expression of MSRV/HERV-W in cells from blood and brain (U-87MG astrocytes) (Mameli et al., 2012). In PBMC exposed to EBV gp350, HERV-W*env* is also expressed at higher levels in B cells and particularly in the monocyte/macrophage. The latter cells, particularly after differentiation to macrophages, are the most responsive to EBVgp350, expressing higher levels of HERV-W*env* than B cells (Mameli et al., 2012). In another study the same research group measured the expression of HERV-W*env* in EBV-uninfected healthy individuals, patients with IM, and individuals with high anti-EBNA-1 IgG titres, suggesting a past infection (Mameli et al., 2013). Compared with uninfected individuals, IM patients had 2-fold higher frequency of HERV-W-positive B cells, and 4-fold and 5.5-fold increase in NK cells and monocytes, respectively. In patients with past EBV infection, B cells showed similar percentages of HERV-W-positive B cells as those with IM (2-fold higher than seronegative, uninfected individuals), intermediate percentages HERV-W-positive monocytes (2.6-fold higher than uninfected individuals), whereas NK cells were mostly HERV-W-negative (Mameli et al., 2013). In this Chapter, the RNA and protein expression of MSRV/ HERV-Wenv will be analysed in B cells infected or not with EBV and in B cells stimulated with CpG, a TLR9 agonist.

The mechanism through which EBV can activate HERV-W is not clear. This could be related to epigenetics or retroviral restriction factors. Retroviral Restriction factors are a set of proteins and biological pathways that play an integral role in suppression and control of replication of retroviruses in normal tissues. They were originally identified in work looking at cellular restriction of HIV replication, though subsequent work has identified them also as active in suppression of endogenous retroviruses and in inhibition of replication of several other classes of viruses including herpesviruses (Suspene et al., 2011). At least 5 such restriction factors have been identified robustly. They are a diverse set of proteins and pathways, targeting viral uncoating and reverse transcription (the APOBEC family of genes, TRIM 5 alpha and SAMHD1), translation (Schlafen11) and release (Tetherin) (Harris et al., 2012). Lentiviruses are known to encode proteins (such as Vif, Vpx and Vpr) that specifically inhibit retroviral retroviral restriction factors, and the expression of versions of restriction factors capable of inhibiting particular lentiviruses is a major determinant of the ability of these viruses to replicate in a particular host species or cell line (Simon et al., 2015). Specifically TRIM family proteins interact with the viral capsid and target the core to the ubiquitin-proteasome pathway to be degraded (Ozato et al., 2008). One member of interest is TRIM5α, as it was found in rhesus monkeys as a component of the innate immune response able to inhibit HIV-1 replication in Old World monkey cells (Sakuma et al., 2007b). Another big family of restriction factors are the APOBEC3 genes, which encode for 7 different cytosine deaminases. APOBEC3 proteins are packed into the viral particles during their formation and during reverse transcription they produce mutations in the sequence of the genome of the virus, hence making the virus particle unable to infect other cells (Chiu and Greene, 2008). There is recent evidence that some markers in TRIM5, TRIM22 and APOBEC3 genes have been statistically

associated with a genetic risk for MS (Nexø, et al., 2013). One hypothesis is that the infection by EBV modulates the expression of retroviral restriction factors, downregulating their expression, and resulting in aberrant expression of the immunogenic MSRV/HERV-WEnv protein. To test this hypothesis, protein expression of APOBEC3G and TRIM5 were analysed through WB in uninfected and EBV-infected CD20+ B cells with help of the MSc student Gemma Vidal Pedrola. CpG-activated B cells and the CD20-negative PBMC sub fraction were also included as control groups.

In this chapter the following studies will be presented:

- RNA expression of MSRV/HERV-W*env* in LCL, CD20+, CpG active CD20+ and CD20- cells;
- RNA expression of MSRV/HERV-W*env* in LCL from HC and from MS;
- Protein expression of APOBEC3G and TRIM5 in LCL, CD20+, CpG active CD20+ and CD20- cells in MS and HC.

7.2.Materials and methods

7.2.1. Human Samples

Blood samples were collected in heparin tubes from patients attending Queen's Medical Centre in Nottingham (part of Nottingham University Hospitals NHS Trust). MS diagnosis was established according to McDonald's criteria (Polman et al., 2011). All patients and HC signed informed consent (Appendix I). Patients' and HC' age, gender and clinical status are illustrated in Appendix VIII pag. 269 (n HC =5, mean age= 39.6 sdt=10.9; n MS= 4, mean age=54 sdt= 7.4).

7.2.2. Generation of different cell groups, cDNA synthesis, real-time RT-PCR and extracellular FC staining for HERV-W

CD20+ cells, CpG activated CD20+ cells, CD20- cells, and LCL were obtained as described previously in Section 2.2.

cDNA synthesis and real-time RT-PCR were performed as described in Section 6.2. The Ct values of MSRV/W*env* were normalised compared to the Ct value of HMBS. Comparison of the relative amounts of MSRV/W*env* in CpG activated CD20+, LCL and CD20- cells compared to primary CD20+ was performed using the $2^{-\Delta\Delta Ct}$ method. Any change in gene expression between cell groups was expressed as fold change using the formula below:

Fold increase for each cell group compared to the CD20+cell group = $2^{-\Delta\Delta Ct}$. Where:

For each sample: $\Delta\Delta Ct = [(Ct\ MSRV/Wenv - Ct\ HMBS) - \Delta CtCD20+\ ]$

$\Delta CtCD20+ = [(Ct\ MSRV/Wenv\ CD20+ - Ct\ HMBSCD20+) +...] /n$

n=number of samples Extracellular FC staining for HERV-W was performed as described previously in Section 6.2

7.2.3. RNA extraction

Total RNA was isolated from 1-5.10^6 cells from different cell groups using High pure RNA isolation kit (Roche) following the manufacturer's instructions. Cells were lysed with the Lysis/Binding buffer and lysate was transferred to the High Pure filter tube. RNA was selectively bound to the High Pure filter tubes Remaining contaminants were removed in several wash steps and the membrane was treated with DNase I to remove trace amounts of bound DNA. After the wash steps, RNA was extracted in the elution buffer provided by the kit and stored at -80^{o}C. RNA concentration was determined by measuring the absorbance at 260nm using Nanodrop ND-100 (Thermo Scientific).

7.2.4. Cell lysing and protein quantification

Cell pellets were washed twice with PBS and re-suspended with RIPA buffer (Sigma). The cell lysate was kept on ice for at least 30 min and centrifuged for 10 min at 8,000g at 5°C. Supernatant was transferred to a new Eppendorf ready for quantification. BCA Protein Assay Kit (Thermo Scientific) was used for estimation of total protein content in the cell lysates. A standard curve of seven different albumin concentrations (1mg/ml – 0.015mg/ml) was employed and RIPA buffer was used as blank. Colour was measured at 562nm with a Benchmark Plus spectrophotometer (Bio-Rad) and results analysed with the Microplate Manager software.

7.2.5. Restriction factors Western blotting

Equal amounts of cell lysates were resolved by a 12% denaturating SDS–polyacrylamide gel electrophoresis (180 V, for 60 min). Proteins were transferred to

PVDF transfer membrane (GE Healthcare Life Sciences) (30 V, 90 min) and blocked in PBS-tween 2% BSA (Sigma-Aldrich) for 1h. Blots were incubated over-night with 1:200 rabbit anti-TRIM5α antibody (ab4389; Abcam Ltd.), 1:1000 rabbit anti-APOBEC3G antibody (ab54257 Abcam Ltd.) and 1:10000 mouse monoclonal anti human β- actin antibody (Sigma-Aldrich) at 4°C. Then incubated with 0.06μg/ml secondary 800CW Donkey anti-Rabbit IgG (H + L) and 680RD Donkey anti-mouse IgG (H + L) (both from LICOR Biosciences) for 1h at room temperature. The membrane was scanned with an Odyssey scanner (LI-COR Biosciences) at 700nm (APOBEC3G and TRIM5α) and 800nm (actin), and band intensities were quantified with Image Studio Lite version 4.0 software (LI-COR Biosciences). To calculate restriction factors relative expression, in each Western Blot the band intensity for each cell group was divided by the band intensity of CD20+ cells (considered as 1).

7.2.6. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Nonparametric Mann-Whitney or paired Wilcoxon test (2 groups) or Friedman test (>2 groups) were used for WB and RT-PCR ($n < 6$) analysis. The graphs show mean with standard error of the mean (SEM) if a parametric test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤ 0.05 were considered significant and only significant p values were reported in the graphs.

7.3. Results

7.3.1. Expression of MSRV/HERV-W*env* increases EBV-infected cells

To investigate if EBV could activate MSRV/HERV-W, MSRV/W*env* RNA expression was measured in isolated CD20+ cells, CD20+ cells activated with CpG (TLR9 agonist), LCL and CD20- cells from 5 different HC donors (Appendix VIII pag. 269). Quantification of MSRV/HERV-W*env* gene expression relative to the mean of the expression in CD20+ cells (considered as 1), was assessed with real time RT-PCR. LCL showed 12-fold increase in MSRV/HERV-W expression compared to the uninfected CD20+ cells (Fig. 7.1A). Similarly, CpG activation induced the expression of MSRV/HERV-W*env*. HERV-WEnv protein has been detected in LCL also by Flow Cytometry (Fig. 7.1B).

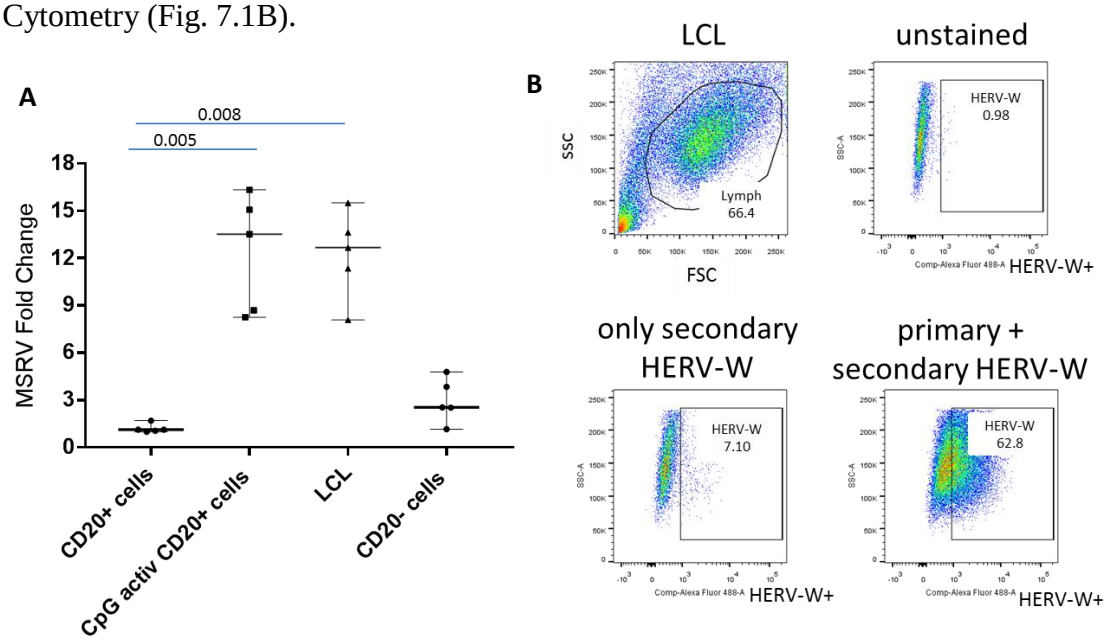


Fig. 7.1 MSRV/HERV-Wenv expression increases in LCL and CpG activated cells. (A) MSRV/HERV-Wenv expression was evaluated by relative quantification RT-PCR using the 2-ΔΔCt method. Fold changes of MSRV/HERV-Wenv in CD20+, CpG activated, LCL and CD20- cells are illustrated in dots. Medians and interquirtile range are indicated by bars (n=5, paired nonparametric Friedman). **(B)** Expression of HERV-WEnv was detected in LCL. The staining with only secondary CF 488A anti-rabbit Ab (without anti-HERV-W primary Ab) was used as control. Data for one representative LCL are shown.

Relative MSRV/HERV-W*env* RNA expression was measured in LCL from 3 HC and from 3 MS to test if MS patients express more MSRV/HERV-W*env* RNA after EBV infection compared to HC. Quantification of MSRV/HERV-W*env* gene expression relative to the mean of the expression in HC LCL (considered as 1), was assessed with real time RT-PCR. No difference was detected between LCL from HC and from MS (Fig. 7.2).

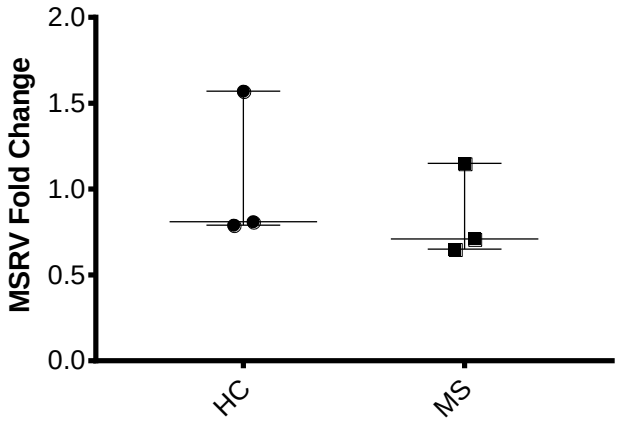


Fig. 7.2 MSRV/HERV-Wenv expression is similar in LCL from HC and MS. MSRV/HERV-Wenv expression was evaluated by relative quantification RT-PCR using the 2-ΔΔCt method. Fold changes of MSRV/HERV-Wenv in LCL from HC and MS patients are illustrated in dots. Medians and interquirtile range are indicated by bars (n HC=3, n MS= 3; Mann-Whitney nonparametric test).

7.3.2. EBV and retroviral restriction factors

To determine the effect of EBV infection on the expression of the restriction factors, LCL and other cell types from the same subjects were tested for the protein expression of APOBEC3G and TRIM5α. Cells were lysed and protein quantification was performed to analyse the same amount of total protein. Restriction factors APOBEC3G and TRIM5α were detected by WB. Using two different concentrations of the anti APOBEG3G antibody (1/500 and 1/1000) two bands were noticed, one at 42 kDa, probably representing APOBEC, and another at approximately 20kDa (Fig.7.3 A). Expression of TRIM5α was not detected at the suggested size of 56 kDa, but another band was visible around 27 kDa using 1/200 antibody dilution (Fig.7.3 B). This band could represent a different isoform for TRIM5, called TRIM5ι, as it is the smaller known isoform that have a molecular weight of 29 kDa. It is possible that the total amount of protein loaded (1µg to 13µg) was not enough to detect TRIM5α. In order to determine if this was the reason, different concentrations of protein ranging from 5 to 40µg from LCL were loaded in the gel. A band around 56 kDa that could correspond to TRIM5α was detected when 40 and 20µg of protein were loaded (Fig. 7.3 C). At these concentrations of protein, a wide range of unspecific band were also detected in addition to the highly intense 27 kDa band already detected before and thought to be TRIM5 isoform.

All the bands analysed in the WB were bigger in LCL compared to CD20+ cells (Fig. 7.3 A,B).

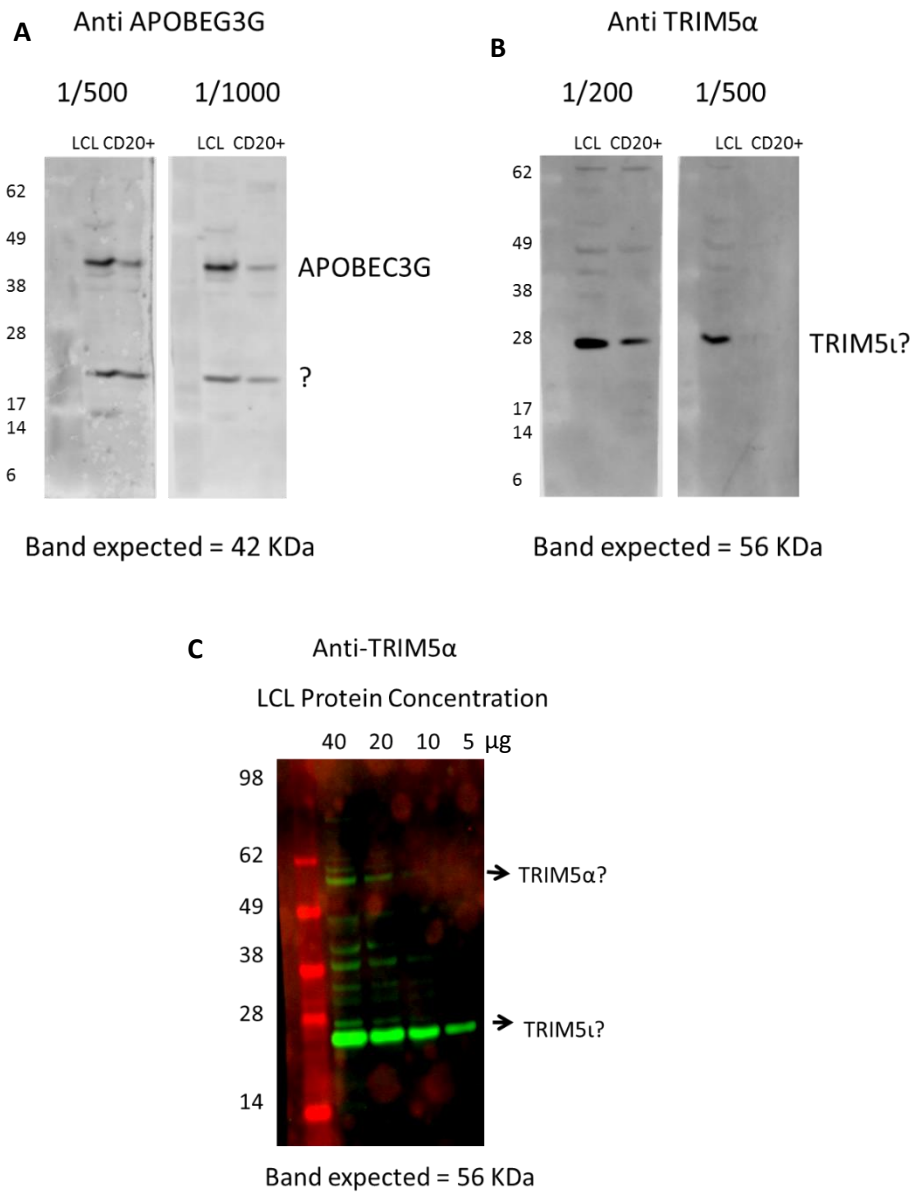


Fig. 7.3 Detection of APOBEC3G and TRIM5 by WB. LCL and CD20+ cells were lysed and 12µg of protein was loaded in the SDS gel. (A) Anti APOBEC3G Ab was used with 1/500 and 1/1000 dilution and APOBEC3G was detected at 42kDa. **(B)** Anti TRIM5α Ab was used with 1/200 and 1/500 dilution and the putative TRIM5ι was detected at 27kDa. **(C)** 40, 20, 10, and 5µg of LCL protein lysate were loaded and TRIM5 was detected with the anti TRIM5α Ab diluted 1/200.

CD20- cells and LCL showed higher expression of APOBEC3G compared to B cells (Fig. 7.4 A,B). CpG-activated B cells had also higher expression of APOBEC3G although this expression was slightly lower compared to LCL. Expression of the putative TRIM5ι isoform was higher in LCL and CpG-activated cells, while it was low in B cells and almost undetectable in CD20-negative cells (Fig. 7.4 A,C).

Anyway, none of these differences were statistically significant when the medians of 4 experiments were compared in a paired non-parametric test (Fig. 7.4 B,C).

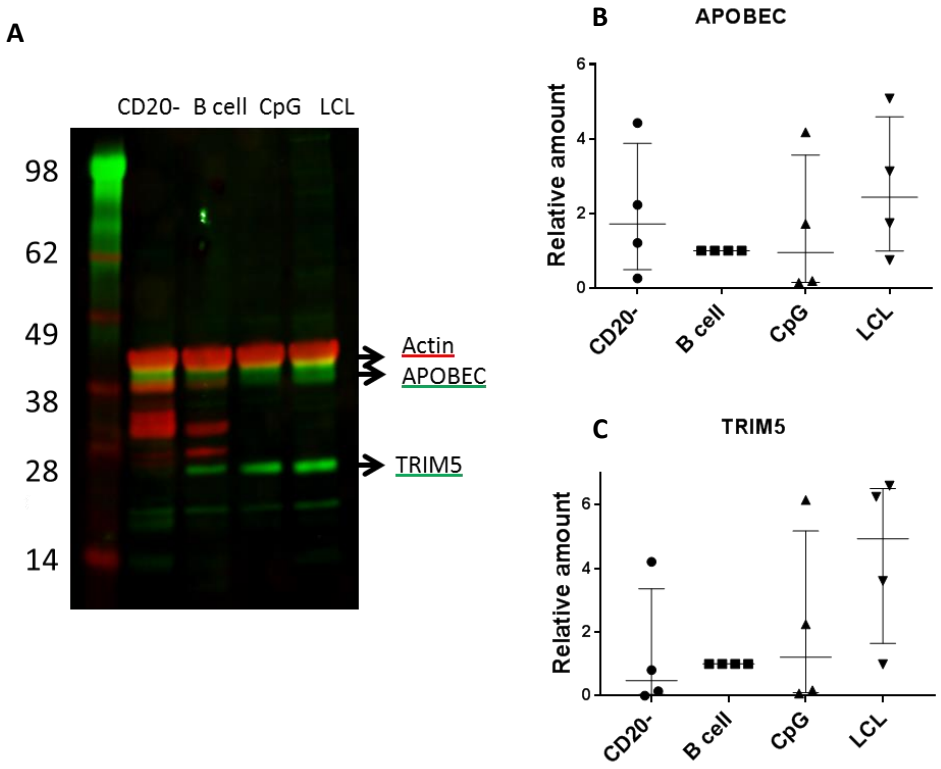


Fig. 7.4 The expression of APOBEC3G and TRIM5 is higher in LCL. (A) CD20-, CD20+, CpG activated CD20+ cells and LCL were lysed and 12µg of protein was loaded in the SDS gel. APOBEC3G (42kDa) and TRIM5ι (27kDa) are detected in green, while the housekeeping gene ACTIN (42kD) is in red. One representative WB is shown. Quantification of **(B)** APOBEC3G and **(C)** TRIM5ι relative to the expression in CD20+ cells in cells from 4 different HC is represented in dot. Median and interquartile range are indicated by bars. (n=4; paired nonparametric Friedman test).

To investigate if there were differences between the expression of the two restriction factors in HC and in MS patients, the same procedure was performed with cells isolated from blood of age- and sex-matched MS patients. Similar results were found, with high expression of APOBEC3G in CD20-negative cells and in LCL (Fig. 7.5 A, B). TRIM5α was again undetectable, but its probable isoform TRIM5ι was more highly expressed in LCL compared to the other cells types (Fig. 7.5 A, C). For both restriction factors, APOBEC3G and TRIM5ι, cells from MS patients had higher expression than cells from HC (Fig. 7.5 D, E).

Again, none of these differences were statistically significant (Fig. 7.5 B, C, D, E)

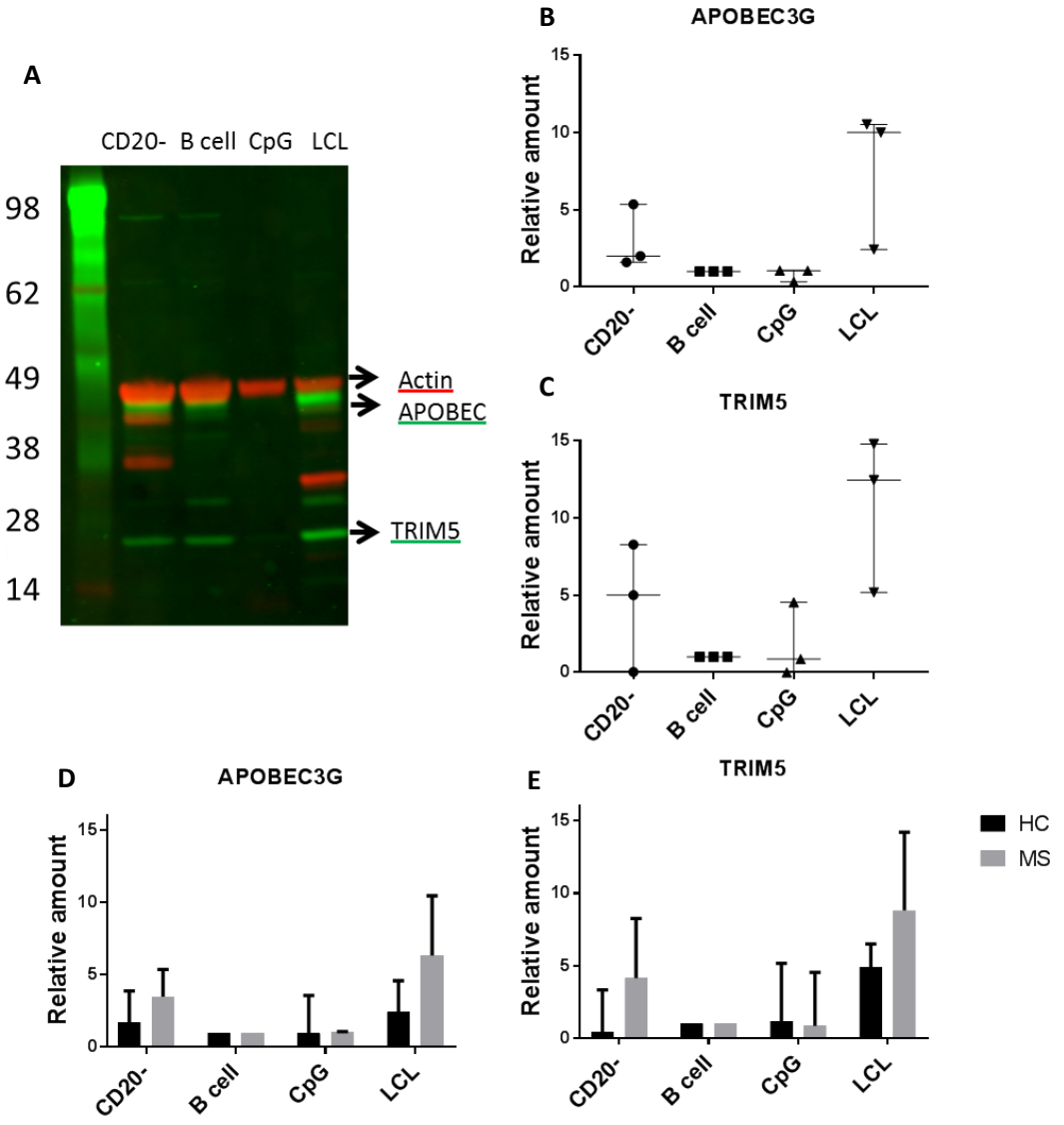


Fig. 7.5 The expression of APOBEC3G and TRIM5 is higher in LCL in MS patients. (A) CD20-, CD20+, CpG activated CD20+ cells and LCL from one MS patient were lysed and 12µg of protein was loaded in the SDS gel. APOBEC3G (42kDa) and TRIM5ι (27kDa) are detected in green, while the housekeeping gene ACTIN (42kD) is in red. One representative WB is shown. Quantification of **(B)** APOBEC3G and **(C)** TRIM5ι relative to the expression in CD20+ cells in cells from 4 different HC is represented in dot. Median and interquartile range are indicated by bars. (n=4; paired nonparametric Friedman test). Medians with interquirtile range of the relative expression of **(D)** APOBEC3G and **(E)** TRIM5 in each cells type is compared between HC and MS (n HC=4, n MS=4; Mann-Whitney test between HC and MS).

7.4. Conclusions

As reported in the literature, EBV infection induces the expression of MSRV/HERV-W*env*. On the other hand, the activation of the retrovirus is not specific only for EBV, but it increases also when B cells are activated with CpG. Bacterial or synthetic unmethylated CpG ODNs mediate their effects by interacting with TLR9 that is expressed in pDC and B cells. Activation of B cells with CpG leads to proliferation, production of antibodies and cytokines. It is possible therefore that MSRV/HERV-W activation is induced either directly by TLR9 activation as in presence of a bacterial infection or by pro-inflammatory cytokines expressed by CpG-activated B cells. Indeed it has been reported that pro-inflammatory stimuli, such as IFN-γ and TNF-α can activate MSRV/HERV-W*env* expression in B cells (Mameli et al., 2012).

MSRV/HERV-W*env* expression was not different in LCL from HC and people with MS, suggesting that EBV infection may potently induce MSRV/HERV-W*env* and mask the original difference in MSRV/HERV-W*env* expression between B cells of HC and MS patients.

The increased HERV-W transcriptional activity in EBV infected cells could be explained with epigenetic regulation, escape from viral restriction factors, or other yet unknown mechanisms. One hypothesis is that EBV could downregulate the expression of retroviral restriction factors, releasing the expression of MSRV/HERV-WEnv proteins in MS patients. Data presented in this Chapter disprove this theory and shows the opposite results.

Higher expression of APOBEC3G was observed in LCL compared to uninfected B cells. It is possible that in EBV-infected B cells, the virus activates expression of these HERVs and the expression of these endogenous retroviruses activates the expression of the restriction factors as a protective mechanism. That would explain the higher

levels of APOBEC3G found in LCL cells. Another study has proven that the viral nuclear protein EBNA3C directly targets and induces the activation-induced cytidine deaminase (AID) activity (Kalchschmidt et al., 2016). AID is an APOBEC-related enzyme which is essential for the affinity maturation of Ig heavy and light chains during B cell differentiation. Because AID has a similar function of APOBEC3, there is the possibility that EBNA3C is also able to induce specifically APOBEC3G, contributing to this higher expression in LCL.

Expression of TRIM5α could not be determined as the antibody could not detect a band at the expected molecular weight. The increase of protein loaded in the gel for the WB enabled to detect TRIM5α only when more than 20µg of protein were used. The limitation was that the higher the amount of protein loaded in the WB, the more antibody specificity was lost. Moreover, it was difficult to extract high protein concentration from B cells that represent a low percentage of the PBMC isolated.

On the other hand, a band around 27 kDa was detected. There are six isoforms of TRIM5 described and they are shorter than TRIM5α. They are characterised to be truncated isoforms, which lack the domain responsible for the interaction of the restriction factor with the viral capsid (Battivelli et al., 2011). TRIM5ι, with a molecular weight of 29 kDa, is the isoform that has the most similar molecular weight to 27 kDa. This TRIM5 isoform is the second most abundant isoform and it inhibits the anti-viral activity of TRIM5α presumably by formation of inactive heterodimers (Battivelli et al., 2011). It would be interesting to further asses first, if the band detected really represents TRIM5i and second, if the activity of TRIM5α in these cells is inhibited. Various factors can also influence the activity of TRIM5α in human cells, like for example the amount of exposure of cells to IFN-α, known to activate the antiviral activity of TRIM5α in human and rhesus monkey cells (Sakuma et al.,

2007a). The CpG used to stimulate de B cells is CpG class B. This class strongly stimulates B cells and NK cells but it produces a weak activation of the secretion of IFN-α (Gursel et al., 2002). Because this class of CpG does not produce a robust release of IFN-α, B cells are not properly stimulated to increase expression of the restrictions factors as TRIM5α, but further studies are required to evaluate this possibility.

Therefore, other mechanisms might be playing a role in the higher expression of HERVs seen in EBV-infected cells as some EBV proteins activating cellular transcription factors or epigenetics. Epigenetic mechanisms can methylate DNA and bring histone modification to silence or repress viral RNA transcription. A direct link between MS and altered epigenetic regulation has already been reported in studies on the immunopathology of MS in particular on the differentiation of Th1, Th2 and Th17 cells and on cytokine production (Kucukali et al., 2015). Similar epigenetic alterations could influence the expression of MSRV/HERV-W.

When the expression of the restriction factors in HC and MS patients was compared, the expression was generally higher in MS patients than in HC. This is an interesting finding as MS patients have a higher expression of HERVs and it is possible that to counteract this increased expression cells would overexpress the restriction factors. In conclusion, these experiments suggest that the expression of viral restriction factor proteins is not suppressed by EBV-infection and therefore another mechanism may be at the base of MSRV/HERV-W activation in LCL.

CHAPTER 8: HERVs and HIV

8.1.Background

The Human Immunodeficiency Virus (HIV) is an exogenous retrovirus that causes HIV infection and over time Acquired Immunodeficiency Syndrome (AIDS) (Broder and Gallo, 1984). AIDS is a condition of immune depression in which progressive failure of the immune system allows the development of life-threatening opportunistic infections and cancers. HIV-1 infects primarily CD4 T cells, monocytes/macrophages and, to a lesser extent, dendritic cells (Maartens et al., 2014). HIV-1 immune activation is associated with the production of several cytokines and the activation of cells of the innate immune system via various pathways. By its nucleic acids, HIV-1 activates innate signalling in infected cells through TLR7/8 and TLR3, thus inducing TNF-α and type I IFN, which contribute to immune activation and viral replication (Maartens et al., 2014).

Despite the strong immune activation during HIV infection, a recent report outlines the fact that comorbidity with HIV and MS is vanishingly rare. A recent comparative cohort study (Gold et al., 2015) examined the association between HIV and MS using an English medical database with a cohort of 21,207 HIV-positive patients and 5,298,496 controls stratified by age, sex, year of first hospital admission, region of residence, and socioeconomic status. They calculated that the rate ratio of developing MS was statistically significant lower in people infected than in those not infected by HIV (0.38 (95% CI 0.15 to 0.79). The authors discussed two different hypotheses that can explain this correlation. The first is related to the HIV viral infection itself. Because HIV targets CD4+ lymphocytes, the reduction of CD4+ T cells in infected people could decrease the possibility of autoimmunity and therefore reduce the risk to

develop MS. But this is not the case for many autoimmune diseases: the list of reported autoimmune diseases associated with HIV/AIDS includes rheumatologic syndromes, systemic lupus erythematosus, anti-phospholipid syndrome, vasculitis, primary biliary cirrhosis, polymyositis, Graves' disease, and idiopathic thrombocytopenic purpura (Zandman-Goddard and Shoenfeld, 2001). Molecular mimicry, upregulation of autoreactive CD8+ T cells, polyclonal B cell activation with synthesis of autoantibodies and dysregulation of the B/T lymphocytes interaction could be at the basis of autoimmunity in HIV-infected patients. In the literature there are different clinical cases that report the diagnosis of MS or CNS demyelinating disorders after HIV infection (Delgado et al., 2014, Chin, 2015, Gonzalez-Duarte et al., 2011), suggesting that HIV itself cannot protect from MS. On the other hand, some HIV-infected MS patients that started ART (Antiretroviral Therapy) had a better MS clinical course (Delgado et al., 2014; Maruszak et al., 2011; Chalkley and Berger, 2014). For this observation, the second hypothesis that explains the inverse correlation between HIV infection and MS refers to the effect of ART. Unfortunately, in the comparative cohort study published by Gold, the authors did not have available data on retroviral drugs, but they assumed that most patients were under ART (Gold et al., 2015).

Different classes of antiretroviral drugs exist based on the phase of the retrovirus life-cycle that the drug targets (Fig. 8.1) and usually drugs from different classes are used in combination to treat HIV infection (termed ART, or combination anti-retroviral therapy (cART) or highly active anti-retroviral therapy (HAART).

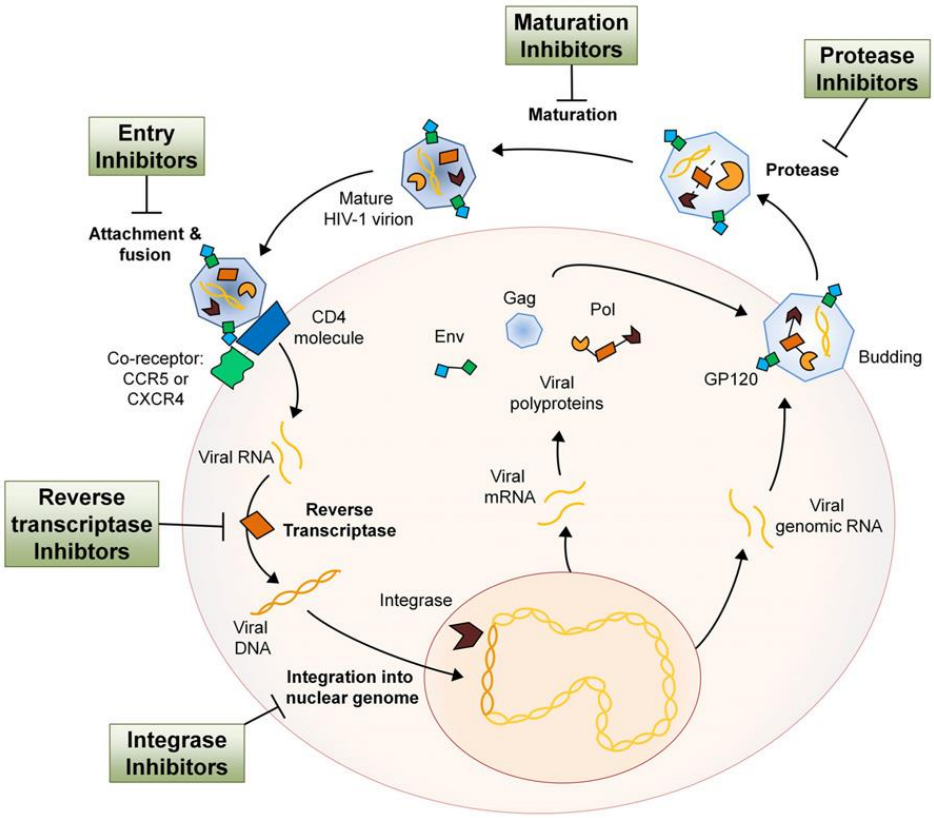


Fig. 8.1 The HIV-1 life cycle and antiretroviral drugs. Antiretroviral drugs are divided into entry inhibitors, reverse transcriptase inhibitors (RTI), integrase inhibitors (II), protease inhibitors (PI) and maturation inhibitors (Smith et al., 2012).

Entry inhibitors interfere with binding, fusion and entry of HIV to the host cell (e.g. Maraviros and Efuvirtide). Reverse transcriptase inhibitors inhibit the HIV reverse transcription and are divided in Nucleoside Reverse Transcriptase Inhibitors (NRTI), Nucleotide Reverse Transcriptase Inhibitors (NtRTI) and Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI). NRTI and NtRTI are nucleoside and nucleotide analogues which act as competitive substrate inhibitors. NRTIs are chain terminators without a 3' OH group, that once incorporated prevent other nucleosides from being incorporated into the DNA chain (e.g. currently used NRTIs include Zidovudine, Abacavir, Lamivudine, Emtricitabine, and Tenofovir). NNRTIs inhibit reverse transcriptase by binding to an allosteric site of the enzyme (e.g. Nevirapine and

Efavirenz). Integrase inhibitors (II) inhibit the viral enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell (e.g. Raltegravir). Protease inhibitors (PI) block the viral protease enzyme necessary to produce mature virions upon budding from the host membrane, and prevent the cleavage of gag and gag/pol precursor proteins (e.g. Lopinavir, Indinavir, Nelfinavir, Amprenavir and Ritonavir).

These antiretroviral therapies are specific not only against HIV, but could probably also inhibit endogenous retroviruses preventing the development of MS. A phase II clinical trial (INSPIRE) studying the effect of the integrase inhibitor Raltegravir on RR-MS patients has recently been completed. Unfortunately, this trial was negative regarding the impact of the drug on MS inflammatory activity detected by MRI (Marta et al., 2016). However, as HERVs are already integrated in the genome, it is possible that classes of antiretroviral drugs that target other stages of the retroviral lifecycle, or their combinations, could still be effective.

In the current thesis, a small cohort of HIV+ patients that were either treated or not with ART was recruited to study the effect of antiretroviral drugs on the expression of human MSRV/HERV-W in a pilot study in vivo.

In parallel, the same classes of drug were used to treat LCL to test their efficacy in MSRV/HERV-W inhibition in vitro.

In this chapter the following studies will be presented:

- RNA expression of MSRV/HERV-W*env* and TLR4 in HIV patients compared to HC and MS;
- RNA expression of MSRV/HERV-W*env* in LCL treated with ART;
- Protein expression of MSRV/HERV-W*env* in LCL treated with ART.

8.2.Materials and Methods

8.2.1. Human Samples:

Blood samples for analysis were collected in PAXgene Blood RNA tubes (Qiagen) in collaboration with Dr Prith Venkatesan, Consultant in Infectious Diseases at Nottingham University Hospital. Blood samples obtained from HIV+ patients at the Nottingham City Hospital Campus were safely sent to the Microbiology Department at the Queen`s medical centre campus to the attention of Dr Fouzia Jabeen, Consultant Virologist, and retrieved by researchers in Dr Gran's team for processing at the Division of Clinical Neuroscience laboratories. All patients signed informed consent. Patients' age, gender and clinical status are illustrated in Appendix IX pag. 270 (n HIV =9, mean age= 44.2 sdt=7.8). Handling of HIV-infected blood was performed following the University of Nottingham risk assessment protocols for Biological agents.

8.2.2. RNA extraction, cDNA synthesis, real-time RT-PCR, generation of LCL and extracellular FC staining for HERV-W

PAXgene RNA extraction, cDNA synthesis and Real-time RT-PCR were performed as described in Section 6.2. All the samples run in duplicates during RT-PCR, thus the average Ct value of each sample was obtained. The Ct values of MSRV//HERV-W*env* or TLR4 were normalised compared to the Ct value of HMBS. Comparison of the relative amounts of MSRV//HERV-W*env* and TLR4 was performed using the $2^{-\Delta\Delta Ct}$ method considering an HC reference gene (HC_{15}, Section 6.3). The reference gene HC_{15} was analysed in all the different plates run as inter-run calibrator. Any change in gene expression towards the HC_{15} was expressed as fold change using the formula below:

Fold increase for each individual patient compared to the $HC_{15} = 2^{-\Delta\Delta Ct}$

Where for each patient:

$\Delta\Delta Ct = [(Ct\ GI - Ct\ HMBS) - \Delta CtHC_{15}]$ GI = MSRV*env* or TLR4

$\Delta CtHC_{15} = \Delta Ct\ reference\ gene\ (HC_{15}) = (Ct\ GIHC_{15} - Ct\ HMBSHC_{15})$

LCL were obtained as described previously in Section 2.2.

Extracellular FC staining for HERV-W was performed as described previously in Section 6.2. Blue Live/dead marker (ThermoFisher Scientific) was included in the staining to assess the viability.

8.2.3. Drug treatments

10^6 LCL in complete medium in 48-wells plate were treated with Lamivudine, Tenofovir, Daranuvir, Efavirenz and Raltegravir at concentrations of 10µM, 1µM, 0,1µM for 5 days. At day 3 the medium was changed and fresh drugs were added.

8.2.4. Statistics

Statistics was performed consulting the division`s statistician. GraphPad Prism 7 was used for all statistical analysis. Unpaired T test and One-way ANOVA test were used for RT-PCR analysis (n >6). Nonparametric paired Friedman test was used for RT-PCR analysis (n <6). The graphs show mean with standard error of the mean (SEM) if a parametric test was used or median and interquartile range for non-parametric tests. All statistical tests have been indicated in the figure legends. P values of ≤ 0.05 were considered significant and only significant p values were reported in the graphs.

8.3. Results

8.3.1. Expression of MSRV/HERV-W in HIV patients with or without ART treatment

MSRV/HERV-W*env* and TLR4 RNA expression were analysed by RT-PCR in 9 HIV-infected patients and compared with 22 age-matched HC and 22 age-matched MS patients previously analysed (Section 6.3.1). To compare PCR data obtained at different time, one reference sample (HC_{15}) has been run in all different PCR plates and used as calibrator. All HIV patients had a detectable MSRV/HERV-W*env* and TLR4 expression (Table 8.1). Compared with MS patients, MSRV/HERV-W*env* expression was lower in HIV patients (Fig. 8.2 A), while TRL4 expression increased (Fig. 8.2 B). MSRV/HERV-W*env* expression was similar in HIV patients and HC (Fig. 8.2 A).

Table 8.1 MSRV/HERV-W*env* and TLR4 fold increase, HIV viral load and ART information of in HIV patients are reported.

PATIENTS	fold increase MSRV	fold increase TLR4	HIV viral load	ART
HIV 1	1.21	6.45	30 010	-
HIV 2	0.55	5.27	608	-
HIV 3	0.32	2.09	<40	YES
HIV 4	0.58	1.29	<40	YES
HIV 5	0.74	3.09	106 361	-
HIV 6	1.62	9.45	<40	YES
HIV 7	1.22	2.9	<40	YES
HIV 8	1.81	5.59	<40	YES
HIV 9	0.76	2.42	<40	YES
average	0.98	4.28		

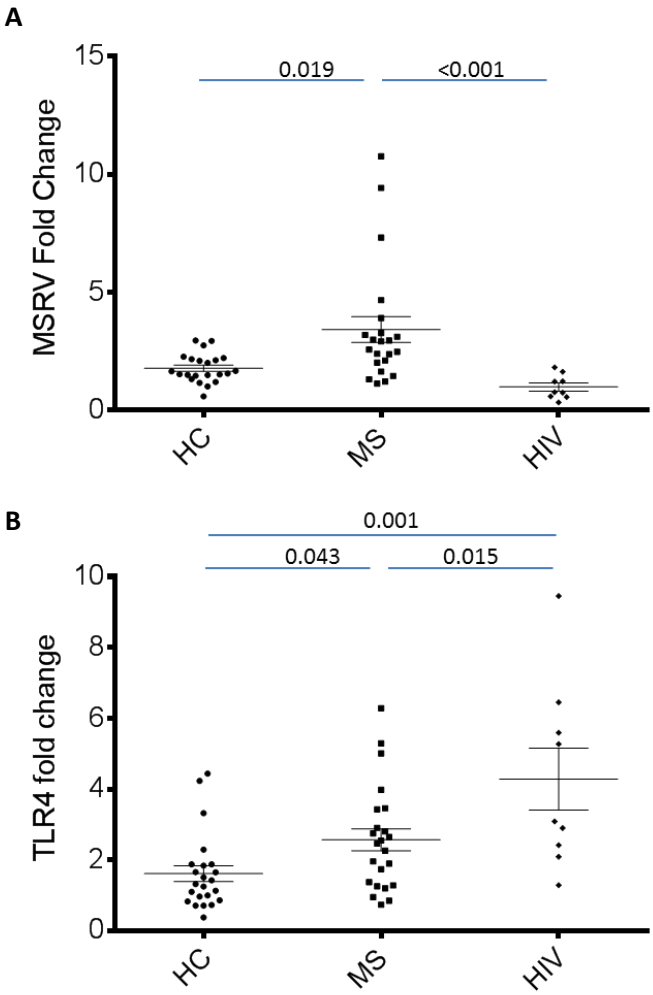


Fig. 8.2 MSRV/HERV-W*env* and TLR4 gene expression in HIV patients compared to MS and HC. MSRV/HERV-W*env* and TLR4 expression were evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Fold changes of **(A)** MSRV/HERV-W*env* and **(B)** TLR4 expression of each individual sample in each group are illustrated in dots. HIV patients were compared to HC and MS using HC_{15} as reference gene. Mean and SEM are indicated by bars (n HC=22, n MS=22, n HIV=9; 1 way-ANOVA Test).

The HIV patients were then grouped in patients that were taking antiretroviral drugs and patients that were not taking them. Only 3 patients were not under treatment (Table 8.1). No difference was found in MSRV/HERV-W*env* (Fig. 8.3 A) and TLR4 (Fig. 8.3 B) expression between the two groups.

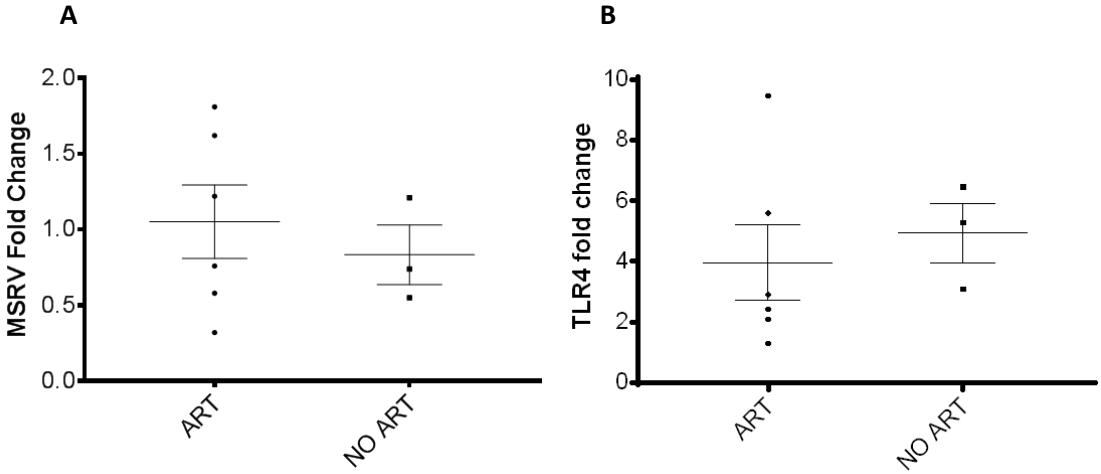


Fig. 8.3 MSRV/HERV-W*env* and TLR4 gene expression in HIV patients categorized in patients that were taking ART and patients that were not. MSRV/HERV-W*env* and TLR4 expression were evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Fold changes of **(A)** MSRV/HERV-W*env* and **(B)** TLR4 expression of 9 HIV patients were categorized in ART-treated and -untreated patients and are illustrated in dots. Mean and SEM are indicated by bars (n ART=6, n noART=3; unpaired T test).

8.3.2. Expression of MSRV/HERV-W in LCL treated with different antiretroviral drugs in vitro

MSRV/HERV-W*env* expression was then analysed in cells treated in vitro with antiretroviral drugs. MSRV/HERV-W*env* RNA expression was analysed by RT-PCR in LCL treated with the same classes of drugs that the patients were taking (Appendix IX pag. 270). In this in-vitro system, LCL were used because they express high level of MSRV/HERV-W*env* (Section 7.3.1). The drugs used were Lamivudine (NRTI), Tenofovir (NtRTI), Daranuvir (PI), Efavirenz (NNRTI) and Raltegravir (PI). LCL from 3 different HC were treated with drugs at the concentrations of 10μM, 1μM, 0,1μM for 5 days (Fig. 8.4). Quantification of MSRV/HERV-Wenv gene expression relative to the mean of the expression in the untreated LCL (CTRL, considered as 1), was assessed with real time RT-PCR.

Only Efavirenz (NNRTI) at the highest concentration decreased the expression of MSRV/HERV-W*env* (Fig. 8.4 D).

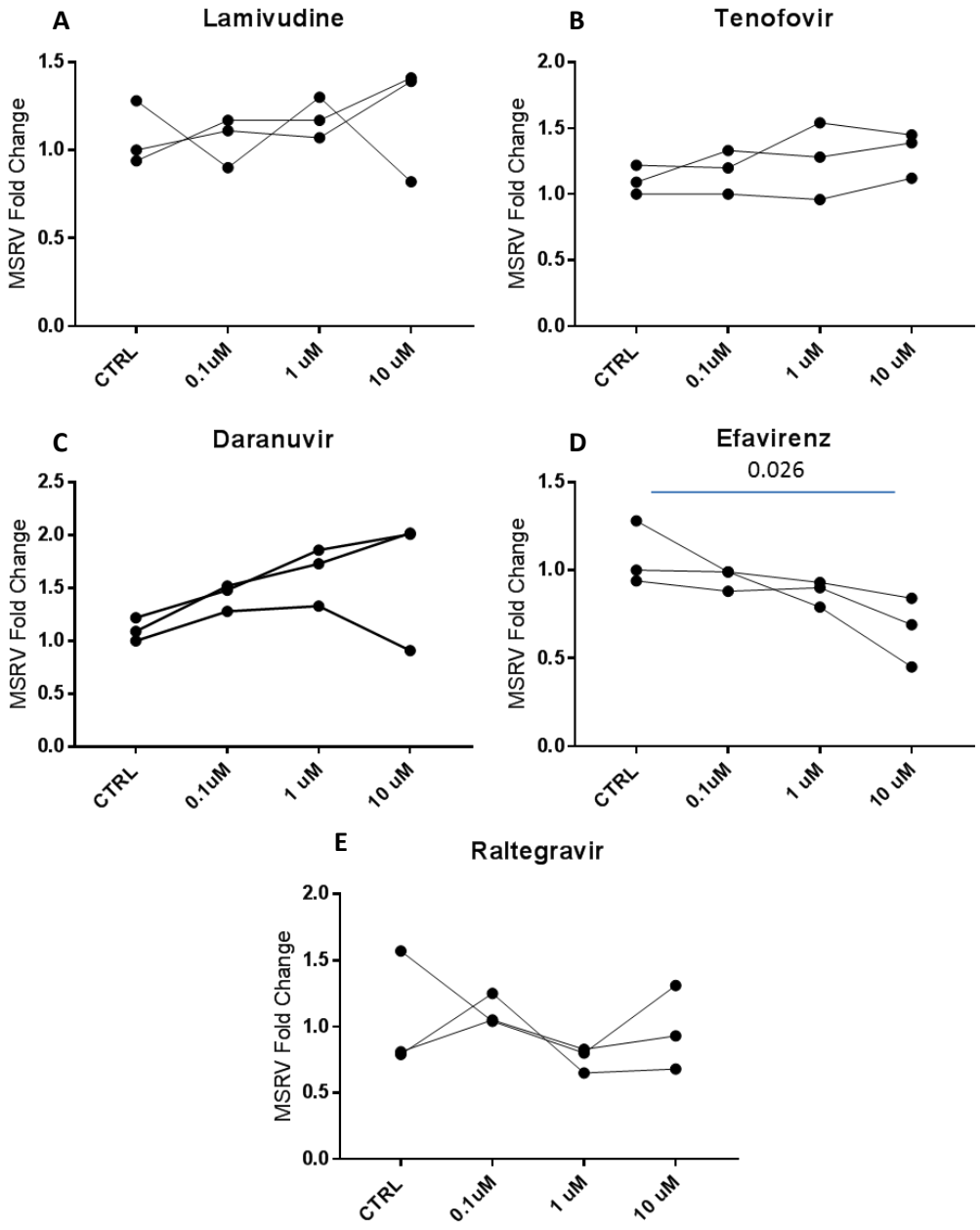


Fig. 8.4 Efavirenz decreases the expression of MSRV/HERV-W*env*. LCL were treated with 0.1μM, 1μM and 10μM of **(A)** Lamivudin, **(B)** Tenofovir, **(C)** Daranuvir, **(D)** Efavirenz and **(E)** Raltegravir for 5 days. CTRL represnts LCL treated only with DMSO. MSRV/HERV-W*env* expression was evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method and is illustrated in dots in 3 LCL (n=3; paired nonparametric Friedman test).

Cells were then treated combined all the drugs together, to mimic ART in vivo. MSRV/HERV-W*env* expression was detected through RT-PCR (Fig. 8.5). Although MSRV/HERV-W*env* expression had a trend to decrease, the difference was not significant. HERV-W/HERV-WEnv protein was detected through FC when the cells were treated with 1μM of combined drugs (Fig. 8.6) and a significant reduction in HERV-WEnv expression was detected.

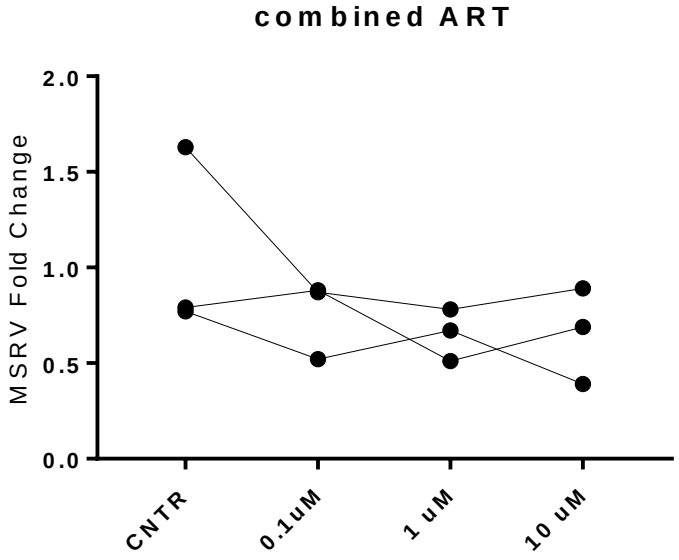


Fig. 8.5 Combined ART does not decrease the expression of MSRV/HERV-W*env* RNA. LCL were treated with combined Lamivudin, Tenofovir, Daranuvir, Efavirenz and Raltegravir at 10μM, 1μM and 0.1μM of for 5 days. CTRL represents LCL treated only with DMSO. MSRV/HERV-W*env* expression was evaluated by relative quantification RT-PCR using the $2^{-\Delta\Delta Ct}$ method and is illustrated in dots in 3 LCL (n=3; paired nonparametric Friedman test).

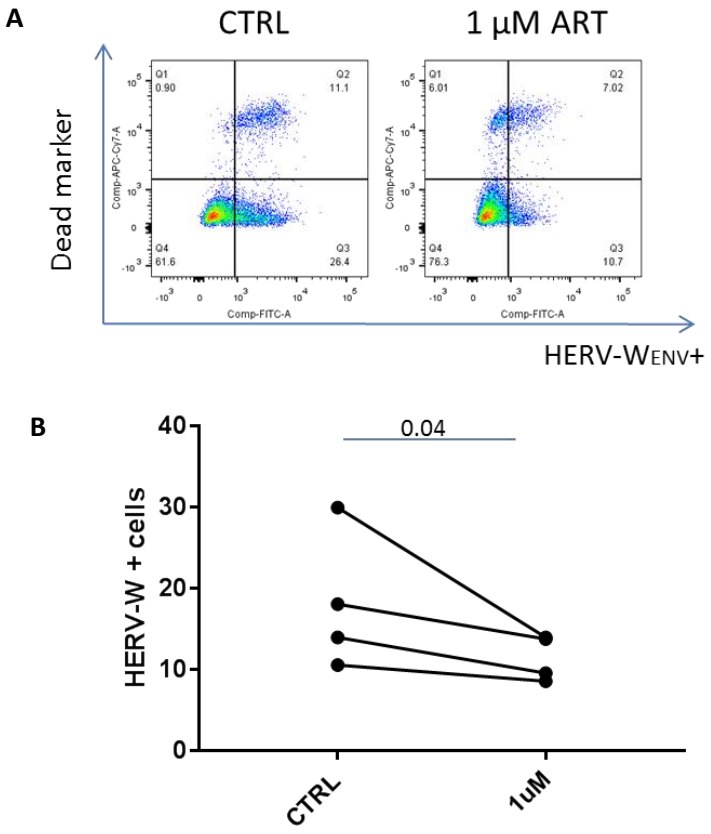


Fig. 8.6 Combined ART decreases the expression of HERV-WEnv protein. LCL were treated with combined Lamivudin, Tenofovir, Daranuvir, Efavirenz and Raltegravir at 1µM for 5 days. **(A)** HERV-WEnv expression was evaluated by extracellular FC and dead cell marker was included. One example is shown. **(B)** HERV-WEnv expression of 4 individual LCL is illustrated in dots before and after the combined ART treatment (n=4; paired Wilcoxon test).

8.4. Conclusions

MSRV/HERV-W*env* and TLR4 expression were evaluated in HIV-infected patients. They showed an expression of MSRV/HERV-W*env* similar to HC and significantly lower than MS patients. The relationship between exogenous and endogenous retroviruses is quite peculiar. All retroviruses have a similar genetic make-up, and homologous proteins encoded by one retrovirus could theoretically perform comparable functions for another member of the family, and could thus complement a defective virus (Van der Kuyl, 2012). Indeed, it has been reported that HIV, through its protein TAT, increases the expression of MSRV and HERV-K, as well as TLR4, in isolated B cells, NK cells and monocytes in vitro (Uleri et al., 2014). In vivo HIV, although it dysregulates all immune cells, preferentially targets T cells. T cells do not express MSRV/HERV-W*env* (chapter 6.3.2) and this could be a possible explanation for the failed detection of increased MSRV/HERV-W*env* expression in the blood of HIV patients. Analysing the patients' HIV viral load, there was no correlation between HIV viral load and MSRV/HERV-W*env* and TLR4 expression (Table 8.1).

When HIV patients were divided into ART treated and untreated, no difference was found between the two groups. However, due to the low number of HIV patients not under ART treatment (3), we cannot conclude that ART in vivo does not affect MSRV/HERV-W expression. In the literature a reduction of HERV-K expression in people that were receiving suppressive ART has been reported (Contreras-Galindo et al., 2006; Laderoutea MP, 2007), but other HERV families have not been investigated. It is unclear whether HERV-K was directly inhibited by the antiretroviral drugs, or whether the loss of activation by HIV was responsible for the reduction of HERV-K viral load.

It is possible that the different ethnicity of HIV patients that were not receiving ART (Jamaican, Greek and Filipino) and those treated (all white British; Appendix IX pag. 270) could influence the expression of MSRV/HERV-W*env* on the basis of genetic differences.

Opposite to MSRV/HERV-W*env*, TLR4 was highly expressed in HIV patients, more than in HC and MS. This suggests that HIV infection induces TLR4. Indeed, it has been reported that mDC from HIV patients had an increased expression of TLR2 and TLR4 compared to HC (Hernandez et al., 2012). Remarkably, the expression was higher in cells from patients who did not use ART and in monocytes there was a positive correlation between both the expression of TLR2/4 and viral load, but no correlation with CD4+ T cell numbers (Hernandez et al., 2012).

LCL were then treated in vitro with different concentrations of the same classes of drugs that the patients were taking (Appendix IX pag. 270). Only treatment with 10μM of Efavirenz (NNRTI) showed a reduction in MSRV/HERV-W*env* expression. NNRTIs are non-competitive inhibitors that bind to an allosteric site of the enzyme affecting the active site responsible for the formation of the DNA double helix, leading to a drastic reduction in enzyme efficiency. It is possible that the inhibition of the RT in some way affects also the transcription of the HERV RNA. And this concept found some evidence in the literature for HERV-K. Indeed, although there is no evidence that HERV-W can function as a retrovirus capable of retro transcribe, some HERVs, and in particular HERV-K (Hohn et al., 2013), can. Protease inhibitors targeted at HIV are not active against HERV-K (Towler et al., 1998), but other classes of antiretroviral drugs, in particular the ones that target the RT enzyme, might be. Interestingly, the NNRTI Nevirapine and Efavirenz have been already showed to efficiently inhibit endogenous RT activity that was detectable in many human cell lines and leukaemic

cells (Sciamanna et al., 2005; Mangiacasale et al., 2003), suggesting a possible efficacy of this drug class in the inhibition of HERV. Nevertheless, considering the HIV patients that were taking NNRTI drugs as part of the ART mix (patients HIV 4,6,7,8 Appendix IX pag. 270), they did not show a decreased expression of MSRV*env*.

When LCL were treated with the combined treatments a suggested, but not significant, reduction of MSRV/HERV-W*env* RNA and a significant reduction of HERV-WEnv protein was detected.

The difference between the in vitro (LCL-treated cells) and in vivo (HIV patients) system could be explained by different hypotheses.

First, RNA extracted from HIV patients was isolated from peripheral total blood and is not representative of a specific cell type. During HIV infection, there is alteration in immune cell populations (e. g. in B cells there is hyper activation, as well as increased apoptosis that results in B cells exhaustion (Moir and Fauci, 2009)). Therefore, it is possible that in HIV patients, even if MSRV/HERV-Wenv expression increases in some cell subpopulations, this change is not detected in whole blood. Moreover, as discussed in Chapter 2 and 3, RT-PCR may not be the best method to detect MSRV expression.

Second, although most of the literature associates MSRV/HERV-W with MS, there is evidence that others HERV families are also involved in the disease (Chapter 5). Therefore, it is possible that ART inhibits other HERV families, such as HERV-K, thus protecting from the development of MS.

Third, the number of HIV patients in this pilot study is too small to draw definite conclusions about the association of HIV with MSRV and the role of ART. It was particularly difficult to enrol untreated HIV patients. Considering a statistical power of 80%, a significance level of 0.05, and standard deviation and effect size measured here, a future study would need to enrol at least 58 treated and 58 untreated HIV patients to detect a difference in the two groups.

Before larger studies are conducted, we can at this stage only speculate about these mechanisms.

CHAPTER 9: General discussion

9.1. Conclusions of the study

The association of remote EBV infection with increased susceptibility to MS is well established. The mechanisms underlying this association, however, have not been clearly elucidated. Several hypotheses have been proposed to explain how EBV infection could cause MS and the aim of this thesis was to better understand the mechanisms of action of EBV in the context of MS studying a) the role of EBV in antigen presentation by B cells and b) the association of HERVs with MS.

9.1.1. PART 1 - EBV infection empowers human B cells for autoimmunity. Role of autophagy and relevance to multiple sclerosis

In the first part of this thesis we reported that EBV alters the ability of B cells to process a pathogenic relevant myelin autoantigen in a way that may lead to autoimmunity.

EBV infection of human B cells led to upregulation of HLA class I (A, B, C, and E) and II (DR, DP, and DQ) molecules, as well as the costimulatory molecules CD86, CD40, CD80, and CD70 (Section 2.3.1). In the marmoset EAE model, CD80 and CD70 mediate the cross-talk of LCV-infected B cells and autoaggressive $MOG_{34–56}$-specific, MHC-E-restricted CTLs (Jagessar et al., 2012; Dunham et al., 2017). The increased expression of CD80, CD70 and HLA-E may therefore indicate the activation of the cross-presentation machinery in human cells as well.

MOG is a crucial myelin component for the establishment of chronic neuroinflammation in NHP (Jagessar et al., 2008; Haanstra et al., 2013a) and, potentially, in humans (Kooi et al., 2009). After incubation of LCL with rhMOG, its binding to the cell surface as well as its internalization were detected by FC (Section 2.3.2). A rapid reduction in detectable protein was reported during the time, probably reflecting internalization (after 1h of incubation, a proportion of rhMOG could be detected in autophagosomes by immunofluorescence; Section 4.3.2) and degradation of the conformational epitope. After 1h of incubation with rhMOG, the increased expression of CD80 suggests simultaneous activation of the antigen presentation machinery.

The presence of rhMOG in LCL was also detected through SDS gel. The observation of reduced degradation of rhMOG upon EBV infection of B cells suggests that EBV confers partial protection from processing of the whole protein (Section 2.3.3), probably due to the internalization in autophagosomes. Despite this, in LCL the generation of smaller peptides in the 3-14 kDa range was observed, suggesting the breakdown of MOG into 10-20 amino acid-long peptides.

Opposite to rhMOG, after direct incubation of the cells with the immunodominant peptide MOG_{35-55}, this was completely degraded by LCL, but not by non-infected and CpG activated B cells (Section 2.3.4).

MOG degradation was mediated by CatG (Section 3.3.1) and CatG activity was increased by EBV infection (Section 3.3.2), explaining the complete degradation of the peptide MOG_{35-55} by LCL. CatG is expressed also by DC and cortical thymic epithelial cells (cTEC) (Stoeckle et al., 2009). The expression of CatG in cTEC could be involved in the escape of thymic selection and the release in periphery of MOG-

autoreactive cells. For instance, autoreactive T cells specific for MBP epitopes that are sensitive to proteolysis by AEP in TEC may escape thymic selection (Manoury et al., 2002). Activation of these T cells in the periphery is likely prevented by destructive processing of the same epitopes by AEP and/or CatG in B cells (Burster et al. 2004). In the case of MOG, B cells in the peripheral immune system may prevent autoaggressive T cell activation by destructive processing of the critical autoantigen epitope MOG_{40-48}. EBV infection could convert this tolerogenic mechanism into productive processing of the epitope enclosing MOG in autophagosomes. Indeed, citrullination of only one of the two CatG targeted Arg residues (position 46, but not 41) protected MOG_{35-55} against degradation (Section 3.3.3).

The critical role of the Arg46 residue appears to be its participation in a LIR motif, through which the peptide can associate with LC3-II in autophagosomes (Birgisdottir et al., 2013). When this motif is intact, the peptide can associate with autophagosomes, thus prolonging its survival. Results obtained in processing experiments with MOG_{1-20} that contains a LIR motif in position 4 are consistent with these concepts (Section 4.3.1). In line with this hypothesis, increasing the formation of autophagosomes with rapamycin increased protection of MOG_{35-55}cit46 and MOG_{1-20}cit4, whereas inhibition of autophagy with 3-MA enhanced its degradation (Section 4.3.3). EBV infection itself induced formation of autophagosomes in B cells (Section 4.3.4) that may confer to them the ability to citrullinate and cross-present MOG peptides. Finally we measured the RNA expression of PAD2 and we did not find it increased in EBV-infected cells (Section 4.3.5). Nevertheless, comparable RNA expression of the enzyme can result in different levels of PAD2 activation and citrullination that correlate with autophagy activity (Ireland and Unanue, 2011).

The results presented in this study suggest one mechanism through which EBV infection could promote the activation of autoreactive myelin specific-T cells with a pathogenic role in MS (Fig. 9.1).

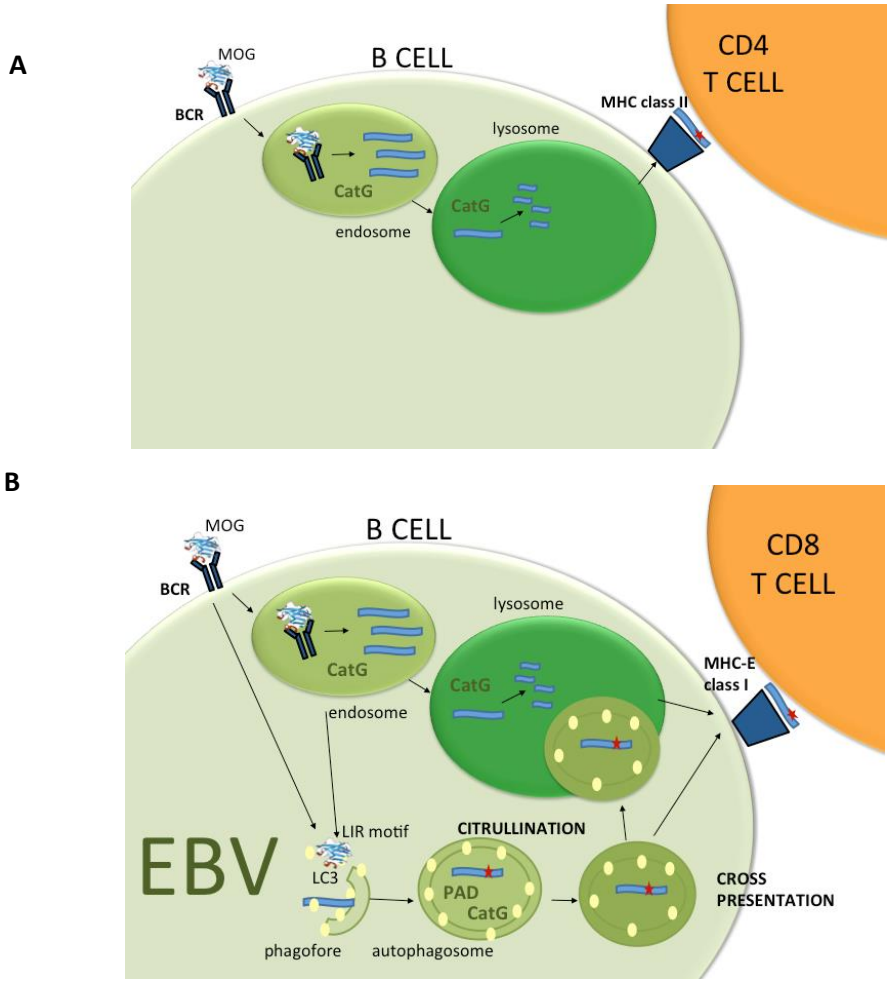


Fig. 9.1 Model of the processing of human MOG in non-infected and EBV-infected B cells. B cells capture MOG via clonal specific antigen receptors. **(A)** In uninfected B cells the captured protein is taken up in endosomes where MOG is processed by CatG and then peptides are presented on MHC-II to activate CD4 T cells. **(B)** When EBV infects B cells, it induces the autophagy flux; peptides with LC3-interacting region (LIR) motif are inserted in phagophore through the microtubule-associated protein light chain 3 (LC3). This process is accompanied by increased Ca2+, a condition in which peptidylarginine deiminase (PAD) enzymes are activated, leading to citrullination of MOG antigens. These citrullinated peptides are protected by CatG cleavage and are cross-presented on MHC-E to activate CD8 T cells.

The project started in collaboration with the Immunobiology department in the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. At the BPRC they studied the comparative RNA sequencing of LCV-infected and non-infected NHP B cells, the transcription profiles and activity of cathepsins in NHP APC subsets and the proteolytic degradation of rhMOG and native and citrullinated MOG_{35-55} in cell lysates from marmosets and rhesus monkeys (Jagessar et al., 2016).

In this thesis, we extended these studies to human intact immune cells and we demonstrated that EBV infection converts destructive processing of immunodominant peptides of MOG into productive processing through molecular and cellular processes involved in autophagy.

9.1.2. PART 1 - Limitations

The main limitation of the study is the lack of direct demonstration of MOG citrullination induced in autophagosomes by EBV infection (Section 4.4). Detection through WB failed to discriminate between citrullinated and non-citrullinated MOG peptides and advances in methods may be needed to demonstrate this post-translational modification.

Another important limitation is the use of LCL as EBV-infected human cells in vitro. Indeed, functional properties of LCL may be different from the small number of latently infected memory B cells in a patient with MS. The in vitro generated LCL used in the current study represent a tissue culture model for human B cell transformation and virus latency (Merchant et al., 2001) and they express the latency III program. In one study immunohistochemical analysis and RT-PCR of post-mortem MS brain samples revealed that most B cells in white matter lesions, meninges, and

ectopic B-cell follicles co-express LMP1, LMP2A, and EBNA-1 (Serafini et al., 2010), suggesting a strong similarity with LCL.

9.1.3. PART 2 - HERVs and MS

In the second part of this thesis we confirmed the association between the presence of MSRV/HERV-W and MS, and we studied the link between EBV and HERVs, and between ART and HERVs.

The published literature on HERVs and MS is confusing and often contradictory. Although different reviews on the role of HERVs in MS have been published, these have tended to focus on smaller groups of studies or specific hypotheses about the potential pathogenesis of HERVs in MS. The volume of published studies at this point in time was large enough to apply the method of systematic review to the available information. The systematic review and meta-analysis of the literature on the association between HERVs and MS suggested a strong association between HERV expression and MS, in particular with regards to the HERV-W family (Chapter 5). The meta-analysis showed odds ratios of 22, 44, and 6 for the expression of MSRV*pol* in serum/plasma, MSRV*env* in PBMC and MSRV*pol* in CSF respectively. Despite some limitations, including the small populations investigated and the low number of studies analysed, this Chapter strongly supports the association of MSRV/HERV-W reported in literature.

Furthermore, we confirmed this association also experimentally. An increased expression of MSRV/HERV-W*env* and TLR4 were detected in MS patients compared with control groups through RT-PCR (Section 6.3.1). Flow cytometry data showed that the viral protein Env was produced mainly by B cells and monocytes, but not by

T cells, although different expression between MS patients and HC was not observed (Section 6.3.2).

As already reported in literature (Mameli et al., 2012: Mameli et al., 2013), EBV infection could induce the expression of MSRV/HERV-W*env* (Section 7.3.1) and this was not due to a repression of retroviral restriction factors in LCL (Section 7.3.2). We suggest that epigenetic and other mechanisms may be involved and will require further study.

Finally, we investigated the involvement of HIV and ART in MS. Indeed, a further connection between HERVs and MS is supported by an observation that people infected by HIV may have a lower risk of developing MS than the HIV non-infected healthy population (Gold et al., 2015). Expression of MSRV/HERV-W*env* in HIV-infected people was lower than in MS patients and similar to HC (section 8.3.1), while expression of TLR4 was higher in HIV-infected people than in HC and MS. Nevertheless, there was no difference between ART-treated and -untreated HIV patients. The expression of MSRV/HERV-W*env* was detected also in LCL treated with different classes of ART and only Efavirenz (NNRI) reduced MSRV/HERV-W*env* expression. Further experiments are needed to clarify the potential role of ART in protecting from MS.

9.1.4. PART 2 - Limitations

The main limitation in the study of MSRV/HERV-W in MS is the lack of a technique that can unequivocally identify MSRV. Flow Cytometry cannot discriminate between MSRV/HERV-W and Syncytin viral proteins and it is now clear that also PCR-based methods detect a mosaic of sequences rather than a sequence derived from a specific loci, making such analysis difficult to interpret (for this reason in this thesis MSRV

has been referred as MSRV/HERV-W) (Grandi et al., 2016). Moreover, creation of recombinants between different sequences in vitro may also lead to detection of RNAs that do not originate from genomic DNA. A method that could overcome these limitations is Next generation Sequencing (NGS). This technique potentially allows comprehensive coverage of all the HERVs present within a sample without pre-selection of which loci will be examined (Brown et al., 2012: Paces et al., 2013). For the time being, however, it is very expensive to perform NGS on a high number of samples. It is likely that future improvements in detection techniques will lead to the discovery of additional HERVs associated with MS, but potentially also to disproving previously reported significant associations.

Other important limitations concern the populations studied. Different groups were not always age and sex-matched and the number of people enrolled was not always appropriate. In Chapter 8, more HIV-infected people should be included in the analysis to draw more definite conclusion.

9.2.Future directions

Future work is required to better understand the role of EBV-infected B cells as APC in MS.

First, this thesis focused on MOG processing, but the key mechanism to understand autoimmune reactions is related with the presentation of the antigen. Co-cultures of LCL and uninfected B cells with T cells are needed to understand the differences in T cell activation by the presentation of rhMOG and citrullinated and non-citrullinated peptides. It is important to clarify if MOG is actually cross-presented by human cells and which type of T cells are activated. Moreover, presentation assays should be performed modulating autophagy flux and testing if increasing autophagy results in increasing presentation and activation of T cells. We performed different experiments pulsing LCL with or without MOG and co-culturing them with autologous T cells. INF-γ production by CD4+ and CD8+ T cells was measured, but no difference was found between T cells activated by LCL alone or LCL pre-pulsed with MOG. A strong INF-γ production was detected in all the cases, probably due to activation of T cells by EBV antigens and the response against MOG might therefore be masked. Different protocols could help to overcome this problem.

Second, the hypothesis that EBV influences antigen presentation by B cells could be tested with myelin antigens other than MOG. MBP could be a good candidate, as MBP is expressed both in the central and peripheral nervous system and citrullinated proteins were detected in the brain of MS patients (Bradford et al., 2014).

Third, if the role of autophagy in MS were definitely established, the identification and development of a drug targeting autophagy could be worthwhile. The regulation of autophagy is complex and involves many signalling pathways. Most chemical

inhibitors of autophagy are not entirely specific and one should be cautious in interpreting the findings obtained with the use of these compounds in vitro, especially regarding their dose and incubation time. For instance, 3-MA has a dual role in autophagic regulation: it suppresses starvation-induced autophagy, but can also promote autophagic flux when administered under nutrient-rich conditions with a prolonged period of treatment (Yang et al., 2013). Further investigations are required to assess the clinical potential of autophagy inhibitors in human diseases.

Future work is also required to clarify the role of MSRV/HERV-W in MS. In this thesis the association of HERVs with MS was confirmed, but further studies are needed to determine whether the presence of these HERVs is a cause or an effect of immune dysregulation in MS. Studying ART treated and untreated HIV-infected people can be a good way to test in vivo if ART is effective also against endogenous retrovirus and if there is a beneficial effect in retroviral suppression. Amplifying the pilot study presented in this thesis could bring a significant progress in the current knowledge on HERVs in MS.

Moreover, studies focusing of epigenetic changes after EBV infection could be useful to better understand the EBV-HERVs link.

9.3.Implication of the study

MS is an important inflammatory and neurodegenerative disease and clinical research in this field is extensive. Nevertheless, there are still many open questions. In 2013 the MS Society identified the 10 research priorities that matter most to people affected by MS and healthcare professionals to include in the MS research strategy 2013-2017 (https://www.mssociety.org.uk/ms-news/2013/09/top-10-ms-research-priorities-identified). Immediately at the second position was the question: *How can MS be prevented?* To prevent MS the research community needs to understand 1) the factors required in MS pathogenesis and 2) the biological mechanism involved by these factors.

The cause of MS is not known, but genetic linkage studies and the beneficial effect of immunosuppressive/immunomodulatory treatments indicate a pathogenic role of the immune system (Dendrou et al., 2015). Activation of the autoimmune process in MS results from the interaction of genetic and environmental factors (which include infections, smoking, and vitD deficiency) (Olsson et al., 2017). Smoking and VitD seem to act as modulators of the immune system, while the role of EBV and HERVs are less clear.

Regarding EBV, MS does not develop in EBV-seronegative adult individuals, and infectious mononucleosis (typical of late EBV infection) substantially enhances the risk of developing MS (Ascherio and Munger, 2010). Form this observation two hypothesis can be formulated: either individuals who are EBV negative are genetically resistant to both EBV infection and MS, or EBV infection increases susceptibility to MS. The first hypothesis cannot be excluded, although it is rather implausible. Some HLA-DR alleles have been linked to risk of IM (Ramagopalan et al., 2011), but a

common genetic basis between IM and MS is not supported. Rather, MS-risk alleles appear to have additive or multiplicative effects with history of IM (Disanto et al., 2013) and anti-EBNA1 titres in promoting disease susceptibility (Xiao et al., 2015).

If the association between EBV and MS is considered as causative, prevention of EBV infection would be expected to substantially reduce MS incidence. Different studies have been performed to discover a vaccine that can prevent EBV infection, but so far they have been unsuccessful. On the other hand, a gp350 EBV vaccine exists that may prevent IM in a small cohort of young adults (Sokal et al., 2007). Hypothetically, reducing IM could reduce also MS incidence, but this may depend on the specific biological efficacy of the vaccine, and mostly, on the mechanism of action of EBV in MS. For instance, three placebo-controlled trials tested the efficacy of the antiviral alacyclovir, or its precursor valacyclovir, in MS, suggesting overall a positive trends by clinical measures, although not statistically significant (Lycke et al., 1996: Bech et al., 2002: Friedman et al., 2005). Of note, these studies were too small to be conclusive, and showed that alacyclovir did not decrease the number of latently infected B cells that are likely to be involved in MS. Therefore, it is essential to understand the biological link between the virus and MS to design to best therapy against EBV.

The study reported in this thesis investigates the novel concept that pathogenic properties conferred to B cells upon EBV infection may explain the high MS risk in EBV-infected individuals. EBV infection (in LCL phase III latency) broadens the APC function of B cells and affects the processing of MOG. Indeed our data show that EBV infection switches MOG processing in B cells from destructive to productive through the induction of autophagy. In this process autophagy may have a double function: to activate citrullination of MOG peptides and to load the peptide into HLA-E class I.

These results together with those observed in the EAE marmoset model (Jagessar et al., 2016), suggest that this mechanism facilitates cross-presentation of disease-relevant epitopes to CD8+ T cells, thus inducing demyelination in the CNS. Indeed, in the marmoset EAE model LCV-infected B cells were capable of activating $MOG_{40–48}$-specific autoaggressive CD8+CD56+ T cells driving EAE progression (Haanstra et al., 2013a).

Only a small fraction of all B cells (0.5 – 300 / 10^6 memory B cells) carries the virus (Thorley-Lawson, 2015). Consequently, the difference between the prevalence of MS (affecting approximately 0.1% of the population) and of EBV infection (60-90% of the population, depending on age and environment), might depend on the fraction of myelin antigen-specific B-cell clones that are infected by EBV.

This hypothesis leads to two main observations. First, although these mechanisms are of particular relevance for MS, they may also underlie other autoimmune diseases in which EBV and citrullination are involved, such as rheumatoid arthritis (Costenbader and Karlson, 2006). Second, as EBV infection is not the only trigger of autophagy and cross-presentation (for instance also CpG activation may induce similar consequences), other stimuli could create a similar immune process in the few MS cases (mainly pediatric) that are EBV negative. These may include other viruses or comorbidities.

The first part of the thesis shows a possible EBV-mediated mechanism involved in MS pathogenesis, but it is likely that different mechanisms act alternatively or cumulatively in different individuals based on environmental and genetic differences (Olsson et al., 2017).

A further mode of action of EBV is through the activation of HERVs. This theory, in which an exogenous factor can induce the expression of genetically transmitted

endogenous retroviruses, potentially provides at least one missing link between environmental triggers, and the immunopathogenic cascades leading to the MS.

Although there remains a possibility that the increased retroviral expression in MS patients is a consequence of inflammation, the association between HERVs and MS is quite strong and the balance of evidence clearly favours a pathogenic role of HERVs. MSRV/HERV-W has the pathogenic potential to activate inflammatory immunity (Rolland et al., 2006), which could be relevant to both disease susceptibility and progression.

The association between MSRV/HERV-W expression and MS susceptibly and progression makes MSRV/HERV-W a good biomarker candidate as a diagnostic and prognostic factor (Mameli et al., 2008). For biomarkers to become clinically applicable they have to go through discovery, verification, clinical validation, and then multicentre validation before being available for implementation (El Ayoubi and Khoury, 2017). The MSRV/HERV-W can be easily detected in serum of patients, but for now the lack of a standard and optimized technique is the major obstacle to its use as a biomarker in clinical practice. Moreover, it is difficult to establish cut-off in retrovirus expression and the potential different levels of expression of MSRV/HERV-W in different genetic populations could create a strong bias (Morandi et al., 2017).

A humanized antibody called GNbAC1 has been developed to specifically target MSRV/HERV-W. In a phase I clinical study, the antibody safety was tested in 33 healthy male subjects with no adverse events reported (Curtin et al., 2012). Subsequently, a phase IIa, single-blind, placebo-controlled, dose-escalation randomized study with an open-label extension up to 12 months of treatment, was performed (Derfuss et al., 2015). The goal of the study was to assess the safety,

immunogenicity, the pharmacodynamics and pharmacokinetics of repeated administrations of GNbAC1 in people with MS. Ten patients were enrolled (3 women and 7 men; 1 RRMS, 3 PPMS, and 6 SPMS) and divided in two dose cohorts of five patients each. Four patients were blindly randomized to receive GNbAC1 and one patient to receive placebo for the first administration, then all patients of the two dose cohorts received 11 open-label infusions of GNbAC1 (either at 2 mg/kg or at 6 mg/kg) at four week intervals. A decrease in MSRV/HERV-W*env* and MSRV/HERV-W*pol* transcripts levels was observed at 3 months and 6 months of treatment. After 12 months of follow-up, the pharmacodynamics and pharmacokinetics were assessed, with no evidence of significant toxicity or immunogenicity. MRI appearances were stable at 6 months in 9 of the 10 MS patients; the tenth patient had a new T2 lesion and dropped out after 6 months (Zimmermann et al., 2015: Derfuss et al., 2015). A longer observation and a phase III larger trial will be required to assess the real efficacy of the drug.

In conclusion, taking in consideration the multifactorial aetiology of MS (Olsson et al., 2017), it is likely that EBV infection and increased expression of MSRV/HERV-W are significant contributing factors in genetically predisposed individuals ("dual viral hypothesis"). If these observations are further substantiated, future personalised treatments of MS may well include antiretroviral drugs and the possibility to prevent MS could be achieved through the formulation of an anti-EBV vaccine.

Appendix

Appendix I. Approval of the study "Role of the innate immune system in Multiple Sclerosis" by the Nottingham Research Ethics Committee.

NHS

National Research Ethics Service

Nottingham Research Ethics Committee 2
1 Standard Court
Park Row
Nottingham
NG1 6GN

Telephone: 0115 8839368
Facsimile: 0115 9123300

05 January 2009

Dr Bruno Gran
Clinical Associate Professor and Honorary Consultant Neurologist
University of Nottingham
Division of Clinical Neurology
Room B31, Medical School
QMC, Derby Road
Nottingham
NG7 2UH

Dear Dr Gran,

Full title of study: **Role of the innate immune system in Multiple Sclerosis**
REC reference number: **08/H0408/167**

Thank you for your letter of 23 December 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). The favourable opinion for the study applies to all sites involved in the research. There is no requirement for other Local Research Ethics Committees to be informed or SSA to be carried out at each site.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Investigator CV		26 September 2008
Application	12208/14135/1/324	10 November 2008
Letter of invitation to participant	1.0	26 September 2008
Evidence of Insurance		05 August 2008
Letter from Sponsor		25 September 2008
Letter from Funder		17 December 2007
Peer Review		
EU Funding		17 October 2006
Peer Review		
Advertisement	1.0	26 September 2008
Response to Request for Further Information		23 December 2008
Participant Consent Form: Patients giving a blood and CSF sample	2.0	23 December 2008
Participant Consent Form: Participants giving a blood sample	2.0	23 December 2008
Participant Information Sheet: Patient controls giving a blood sample	2.0	23 December 2008
Participant Information Sheet: MS patients giving a blood cerebralspinal fluid sample	2.0	23 December 2008
Participant Information Sheet: Healthy Controls giving a blood sample	2.0	23 December 2008
Participant Information Sheet: MS Patients giving a blood sample	2.0	23 December 2008
Participant Information Sheet: Patient Controls giving a blood and cerebrospinal fluid sample	2.0	23 December 2008
Letter of invitation to participant	Patient control - 1.0	23 December 2008
Protocol	2.0	23 December 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review –guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0408/167	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely,

Dr Martin Hewitt / Ms Linda Ellis
Chair / Committee Coordinator

Email: linda.ellis@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Paul Cartledge - University of Nottingham*
R&D office for NHS care organisation at lead site - NUH (via email)

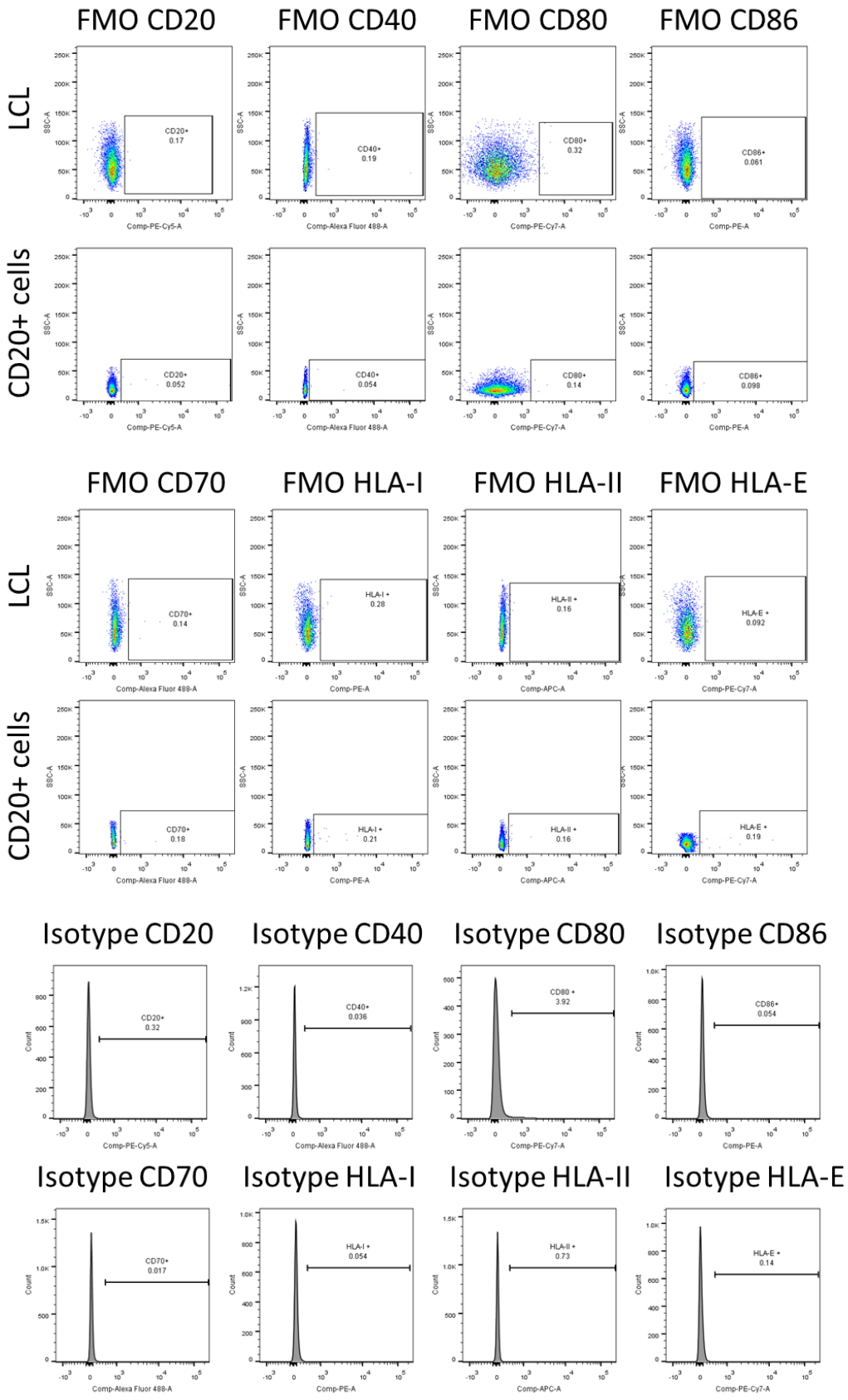
Appendix II – Participants at the study on antigen-presentation – related markers detected by FC in different cell groups (Section 2.3.1)

Information on HC

Samples No	**AGE**	**GENDER**
HC 1	32	M
HC 2	44	F
HC 3	47	F
HC 4	38	F
HC 5	27	M
HC 6	54	M
HC 7	31	F
HC 8	30	M
	mean 37.87	

Appendix III. Example of Flow Cytometer controls (Section 2.3.1)

The example of FC controls in one staining is reported. FMOs were included for both LCL and CD20+ cells due to the physical difference in the two cell groups. Isotype were included only for LCL



Appendix IV. MOG sequences

NAME	SEQUENCE
rhMOG (MOG_{1-125})	>MASLSRPSLPSCLCSFLLLLLLQVSSSYAGQFRVIGPRHP IRALVGDEVELPCRISPGKNATGMEVGWYRPPFSRVVHL YRNGKDQDGDQAPEYRGRTELLKDAIGEGKVTLRIRNV RFSDEGGFTCFFRDHSYQEEAAMELKVEDPFYWVSPGV LVLLAVLPVLLLQITVGLVFLCLQYRLRGKLRAEIENLHR TFDPHFLRVPCWKITLFVIVPVLGPLVALIICYNWLHRRL AGQFLEELRNPF
MOG_{35-55}	>MEVGWYRPPFSRVVHLYRNGK
MOG_{35-55}c41	>MEVGWY-**Cit**-PPFSRVVHLYRNGK
MOG_{35-55}c46	>MEVGWYRPPFS-**Cit**-VVHLYRNGK
MOG_{1-20}	>GQFRVIGPRHPIRALVGDEV
MOG_{1-20}c4	>GQF-**Cit**-VIGPRHPIRALVGDEV
MOG_{1-20}c13	>GQFRVIGPRHPI-**Cit**-ALVGDEV

Peptides were derived from the human MOG sequence downloaded from the National Centre for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/protein). Modifications included substitution of the positively charged Arg residues on positions 41 and 46, and 4 and 13, for uncharged Cit.

Appendix V– RT-PCR primers used in study included in the meta-analysis (Section 5.3.3)

	NAME	FORWARD	REVERSE	PROBE	REF METHOD
MSRVenv in PBMC					
do Olival 2013	HERV-Wenv	5`-CCAATGCATCAGGTGGGTAAC-3`	5`-GAGGTACCACAGACAAAAAATATTCCT-3`		Nellaker 2006
Perron 2012	MSRV/HERV-W env	5`-CTTCCAGAATTGAAGCTGTAAAGC-3`	5`-GGGTTGTGCAGTTGAGATTTCC-3`	FAM-TTCTTCAAATGGAGCCCCAGATGCAG-TAMRA	Mameli 2009
Mameli 2009	MSRV/HERV-W env	5`-CTTCCAGAATTGAAGCTGTAAAGC-3`	5`-GGGTTGTGCAGTTGAGATTTCC-3`	FAM-TTCTTCAAATGGAGCCCCAGATGCAG-TAMRA	Mameli 2009
Mameli 2007	HERV-W env	5′-GCCCCATCGTATAGGAGTCTTTC-3′	5′-AGTGGCAGAGTGATAGCAGTTG-3′	FAM-CCCACCTTCACTGCCCACACCCAT-3′-TAMRA	Mameli 2007
MSRVpol in SERUM					
Arru 2007	HERV-W/MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
de Villiers	MSRVpol st1.1	5`-AGGAGTAAGGAAACCCAACGGAC-3`	5`-TAAGAGTTGCACAAGTGCG-3`		
	MSRVpol st1.2	5`-TCAGGGATAGCCCCCATCTAT-3`	5`-AACCCTTTGCCACTACATCAATTT-3`		Perron 1997
Nowak	MSRVpol st1.1	5`-AGGAGTAAGGAAACCCAACGGAC-3`	5`-TAAGAGTTGCACAAGTGCG-3`		
	MSRVpol st1.2	5`-TCAGGGATAGCCCCCATCTAT-3`	5`-AACCCTTTGCCACTACATCAATTT-3`		Perron 1997
Dolei 2002	MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
Serra 2001	MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
Garson 1998	MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
MSRVpol in CSF					
Alvarez-Laf. 2008	HERV-Wpol	5`-ACMTGGAYYRTBYTRCCYCA-3`	5`-GTAAATCATCCACMTAYYGAAGGAYMA-3`	FAM-TYAGGGATAGCCCYCATCTRTTTGGYCAGGCA-TAMRA	Forsman 2005
Arru 2007	MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
Dolei 2002	MSRVpol	5'-GGCCAGGCATCAGCCCAAGACTTGA-3'	5'-TGCAAGCTCATCCCTSRGACCT-3'		
		5'-GACTTGAGCCAGTCCTCATACCT-3'	5'-CTTTAGGGCCTGGAAAGCCACT-3'		Garson 1998
Perron 1997	MSRVpol st1.1	5`-AGGAGTAAGGAAACCCAACGGAC-3`	5`-TAAGAGTTGCACAAGTGCG-3`		
	MSRVpol st1.2	5`-TCAGGGATAGCCCCCATCTAT-3`	5`-AACCCTTTGCCACTACATCAATTT-3`		Garson 1998

Appendix VI – Participants at the study on MSRV*env* and TLR4 expression (Section 6.3.1)

Information on HC

Samples No	**AGE**	**GENDER**
HC 1	32	M
HC 2	44	F
HC 3	47	F
HC 4	38	F
HC 5	27	M
HC 6	54	M
HC 7	31	F
HC 8	30	M
HC 9	51	F
HC 10	42	M
HC 11	27	M
HC 12	44	M
HC 13	60	F
HC 14	44	F
HC 15	***55***	***F***
HC 16	28	M
HC 17	60	M
HC 18	53	F
HC 19	59	
HC 20	31	M
HC 21	27	M
HC 22	44	F
	mean 42.18	

Information on MS patients

Samples No	AGE	GENDER	Disease course	Current DMT
MS 1	41	F	RR	no
MS 2	56	F		no
MS 3	38	F	RR	no
MS 4	54	M	RR	no
MS 5	55	F	SP	no
MS 6	46	F	SP	no
MS 7	44	F	RR	no
MS 8	46	F	RR	no
MS 9	52	F	SP	no
MS 10	21	M	RR	no
MS 11	61	F	SP	no
MS 12	46	F	SP	no
MS 13	51	F	RR	no
MS 14	56	F	PP	no
MS 15	56	F		no
MS 16	52	F	SP	no
MS 17	46	F		no
MS 18	48	F		no
MS 19	49	M	RR	no
MS 20	45	F	RR	no
MS 21	41	F		no
MS 22	42	M	RR	no
	mean 47.55			

Information on OND

Samples No	AGE	GENDER	Disease course	Current DMT
OND 1	48	M	Epilepsy	no
OND 2	86	M	Cerebrovascular disease/Focal seizures	no
OND 3	69	F	Complex partial seizures	no
OND 4	45	F	Complex partial seizures/left hippocampal sclerosis	no
OND 5				no
OND 6	52	F	Migraine/probable temporal lobe epilepsy	no
OND 7	86	F	Previous stroke/ unilateral limp symptoms	no
OND 8	67	F	Stroke/cerebrovascular disease	no
OND 9	52	F	Neck and facial spasis	no
OND 10	56	M	Non epileptic attacks/lower limp symptoms	no
OND 11	63	M	Parkinson's disease	no
OND 12	59	M	Temporal lobe epilepsy/migraine	no
OND 13				no
OND 14			Parkinson's disease	no
	mean 62.09			

Appendix VII – Participants at the study on HERV-WEnv protein detection on immune cell types (Section 6.3.2)

Information on HC

Samples No	AGE	GENDER
HC1	47	F
HC2	29	F
HC3	28	M
HC4	27	F
HC5	51	M
HC6	56	M
	mean 39.66	

Information on MS patients

Samples No	AGE	GENDER	Disease course	Current DMT
MS1	35	F	RR	no
MS2	60	M	PP	no
MS3	27	F	RR	no
MS4	31	M	RR	no
MS5	48	F	RR	no
MS6	35	F	RR	no
MS7	42	M	RR	no
MS8	39	F	RR	no
MS9	45	F	SP	no
MS10	66	F	SP	no
MS11	57	M	SP	no
MS12	46	M	RR	no
	mean 44.25			

Appendix VIII – Participants at the study on restriction factors (Section 7.3.2)

Information on HC (same HC as Appendix II)

Samples No	AGE	GENDER
HC 1	32	M
HC 2	47	F
HC 3	38	F
HC 4	27	M
HC 5	54	M
	mean 39.6	

Information on MS patients

Samples No	AGE	GENDER	Disease course	Current DMT
MS1	49	F	RR	no
MS2	52	M	RR	no
MS3	65	F	RR	no
MS4	50	M	SP	no
	mean 54			

Appendix IX – HIV patients participating at the study on MSRV*env* and TLR4 expression (Section 8.3.2)

Samples No	AGE	GENDER	ETHNICITY	ART	Duration on ART	Duration of undetectable viral load	viral load
HIV 1	46	M	Greek	No	-	-	30,010
HIV 2	31	M	Jamaican	No	-	-	608
HIV 3	48	M	White British	Truvada Darunavir	4 yrs	4 yrs	<40
HIV 4	49	F	White British	Kivexa Efavirenz	13 yrs	13 yrs	<40
HIV 5	34	F	Filipino	No	-	-	106,361
HIV 6	42	M	White British	Atripla	3 yrs	4 yrs	<40
HIV 7	57	M	White British	Atripla	9 yrs	9 yrs	<40
HIV 8	47	F	White British	Combivir Efavirenz	9 yrs	9 yrs	<40
HIV 9	44	M	White British	Truvada Darunavir	12 yrs	12 yrs	<40

ARTs (antiretroviral treatments):

Trivada: Tenofovir + Emitricitabine (NRTI + NRTI)

Daranuvir (PI)

Combivir: Lamivudine + Zidovudine (NRTI + NRT

Efavirenz (NNRTI)

Atripla: Tenofovir + Emitricitabine + Efavirenz (NRTI + NNRTI)

Kivexa: Abacavir + Lamivudine (NRTI)

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