THE ROLE OF GAMMAHERPESVIRUSES IN THE DEVELOPMENT OF AUTOIMMUNITY

by

Costanza Casiraghi

B.Sc., Vita-Salute San Raffaele University, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

October 2012

© Costanza Casiraghi, 2012

Abstract

The development of autoimmune diseases is thought to involve both genetic and environmental factors. Epstein-Barr virus (EBV) has been associated with the development of both multiple sclerosis (MS) and systemic lupus erythematosus (SLE). My research projects aimed at identifying the mechanism that this virus is exploiting to cause autoimmunity.

In the first project, the role of EBV infection of the blood brain barrier (BBB) as a trigger of MS was investigated. EBV was found to be able to infect human primary endothelial cells isolated from the BBB. EBV infected brain endothelial cells upregulated pro-inflammatory mediators and supported increased immune cell adhesion. These results suggest that EBV has the ability of increasing the BBB permeability. EBV latency and reactivation in endothelial cells could lead to initial inflammation and infiltration of the first wave of autoreactive immune cells during MS initiation.

The second and third research project were aimed at developing a mouse model to study the interactions between latent gammaherpesvirus infection and the host's immune system that may lead to autoimmunity. The role of murine gamma herpesvirus 68 (γHV-68), the murine equivalent to EBV, was analyzed in the experimental autoimmune encephalomyelitis (EAE) model, an experimentally induced model to study MS, and in the New Zealand Black and White (NZBW) model, a spontaneous SLE mouse model. Mice latently infected with γHV-68 developed more severe EAE that mirrored human MS more closely than EAE in uninfected mice. γHV-68 EAE mice developed lesions composed of CD4 and CD8 T cells, loss of myelin in the brain parenchyma and spinal cord. Further, T cells from the CNS of γHV-68 EAE mice were primarily Th1, producing heightened levels of IFN-γ and T-bet accompanied by IL-17 suppression and decreased regulatory T cells frequencies. γHV-68 NZBW mice exhibited a similar Th1 skewed response and they produced different types of autoantibodies, if compared to uninfected NZBW mice. Clearly, gammaherpesvirus latency polarizes the adaptive immune response in both mouse models, directs a heightened brain pathology following EAE induction reminiscent of human MS and portrays a novel mechanism by which EBV likely influences MS and other autoimmune diseases.

Preface

A version of chapter 3 has been published: Costanza Casiraghi, Katerina Dorovini-Zis and Marc S. Horwitz. Epstein-Barr virus infection of human brain microvessel endothelial cells: A novel role in Multiple Sclerosis. J. Neuroimmunol. 230(1-2):173-7 (2011). I conducted all the experiments, analyzed the data and wrote the manuscript under the guidance of Dr. Marc S. Horwitz and Dr. Katerina Dorovini-Zis.

A version of chapter 4 has been published: Costanza Casiraghi, Iryna Shanina, Sehyun Cho, Michael L. Freeman, Marcia A. Blackman and Marc. S. Horwitz. Gammaherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein-Barr virus and multiple sclerosis. PLoS Pathog 8(5): e1002715 (2012). I conducted all the experiments, analyzed the data and wrote the manuscript under the guidance of Dr. Marc S. Horwitz, with the following exceptions: the ELISA data presented in Figure 4.22 and Figure 4.23 were collected by Sehyun Cho. Iryna Shanina helped me with mouse work, Marcia A. Blackman and Michael L. Freeman provided a reagent.

A version of chapter 5 will be submitted for publication: Costanza Casiraghi, Iryna Shanina and Marc S. Horwitz. Gammaherpesvirus latency affects plasma cells and anti-nuclear autoantibodies in a spontaneous mouse model of systemic lupus erythematosus. I conducted all the experiments, analyzed the data and wrote the manuscript under the guidance of Dr. Marc S. Horwitz. Iryna Shanina helped me with mouse work.

Ethics approval:

Biohazard Approval Certificate

Protocol number: B06-0199

Animal Care Certificate (Animal Care Committee)

Application number: A08-0622

Application number: A08-0415

Table of contents

Abstract ii
Preface iii
Table of contents iv
List of figures viii
List of abbreviations xi
Acknowledgements xv
Dedication xvi
1 Introduction 1
1.1 Herpesviruses 2
1.2 Gammaherpesviruses 3
1.3 Epstein-Barr virus 4
1.4 Host response to Epstein-Barr virus 6
1.4.1 Immune response during acute infection, mononucleosis and latency 6
1.4.2 Viral strategies of immune escape during latency and lytic infection 7
1.5 Modeling Epstein-Barr virus in rodents with murine gamma herpesvirus-68 9
1.5.1 Murine gamma herpesvirus-68 genome: differences and similarities to human gammaherpesviruses 9
1.5.2 Viral replication kinetics and cell targets 10
1.5.3 Innate immune responses to murine gamma herpesvirus-68 infection 11
1.5.4 Adaptive immune responses to murine gamma herpesvirus-68 infection 12
1.5.5 Immune modulation during murine gamma herpesvirus-68 latency 13
1.6 Epstein-Barr virus and autoimmunity 14
1.7 Epstein-Barr virus and multiple sclerosis 15
1.7.1 The development of a hypothesis from 1970 to 2000 15
1.7.2 Association between EBV and MS in epidemiological studies 16
1.7.3 EBV strains in MS patients 17
1.7.4 EBV replication control and reactivation in MS patients 18
1.7.5 EBV humoral responses in MS patients 18
1.7.6 EBV T cell responses in MS patients 19
1.7.7 EBV infection in the MS brain 22
1.7.8 EBV infection of cell types other than B cells 23

1.8 A mouse model of multiple sclerosis: experimental autoimmune encephalomyelitis 25
1.9 Epstein-Barr virus and systemic lupus erythematosus 27
1.9.1 Association between SLE and EBV in epidemiological studies 27
1.9.2 EBV replication control and reactivation in SLE patients 28
1.9.3 EBV humoral responses and molecular mimicry in SLE patients 28
1.9.4 EBV T cell responses in SLE patients 29
1.10 A mouse model of lupus: New Zealand black and white hybrid mouse 31
1.11 Objectives, hypothesis and aims 33
2 Materials and methods 35
2.1 Primary cultures of human brain microvascular endothelial cells (HBMECs) and other cell lines 36
2.2 EBV viral stock 36
2.3 EBV infection of HBMECs, DNA extraction and PCR 37
2.4 HBMECs viability assay 37
2.5 RNA extraction from HBMECs and RT-PCR 37
2.6 Cytokines and chemokine analysis of HBMECs and B95.8 supernatants 38
2.7 HBMECs surface ELISA 38
2.8 HBMECs adhesion assay 38
2.9 Animals 39
2.10 Infections, cidofovir administration and EAE induction 39
2.11 Limiting dilution assay for γHV-68 40
2.12 Nested PCR for γHV-68 40
2.13 Immune cells isolation, staining and flow cytometry 41
2.14 Tetramer staining 42
2.15 Histology and immunohistochemistry 42
2.16 Cytokines and chemokines analysis 43
2.17 Auto-antibodies ELISA 44
2.18 Direct intracellular staining, mixed assays and cell transfers 44
3 Epstein-Barr virus infection of human brain microvascular endothelial cells . 46
3.1 Introduction 47
3.2 Results 49
3.2.1 EBV infects HBMECs 49

3.2.2 HBMECs infected by EBV upregulate pro-inflammatory molecules and support increased immune cell adhesion 53
3.3 Discussion 57
4 Gammaherpesvirus latency accentuates experimental autoimmune encephalomyelitis pathogenesis 59
4.1 Introduction 60
4.2 Results 62
4.2.1 Latent infection with γHV-68 enhances EAE symptoms without CNS infection or increased viral reactivation 62
4.2.2 γHV-68 EAE mice have increased CD8 and CD4 T cells infiltrations, increased inflammation and MS-like lesions in the brain 70
4.2.3 CD8 T cells infiltrating in the CNS of γHV-68 EAE mice express granzyme B and are specific for viral proteins 78
4.2.4 T cells infiltrating in the CNS of γHV-68 EAE mice produce higher amounts of IFN-γ and T-bet along with IL-17 downregulation 81
4.2.5 γHV-68 EAE mice have increased levels of pro-inflammatory cytokines in the serum and a decreased anti-MOG IgG1/IgG2a ratio 88
4.2.6 γHV-68 EAE mice are protected from EAE during acute γHV-68 infection and develop paralysis and CD8 T cell infiltrations in the brain parenchyma only upon establishment of γHV-68 latency 96
4.2.7 CD11b+ CD11c+ cells from γHV-68 EAE mice are able to prime an enhanced Th1 response both in vitro and in vivo 99
4.2.8 Regulatory T cells are decreased in both the CNS and the periphery of γHV-68 EAE mice even before EAE induction 105
4.3 Discussion 108
5 Gammaherpesvirus latency affects plasma cells and anti-nuclear autoantibodies production in a spontaneous mouse model of systemic lupus erythematosus 111
5.1 Introduction 112
5.2 Results 114
5.2.1 γHV-68 NZBW mice have a decreased number of plasma cells and produce different types of anti-nuclear autoantibodies 114

5.2.2 The extent of lupus nephritis and IgGs deposition in the kidneys are comparable between γHV-68 latently infected and uninfected NZBW mice 119
5.2.3 T cells isolated from γHV-68 NZBW mice have an effector phenotype and secrete higher levels of IFN-γ and TNF-α 123
5.3 Discussion 131
6 General discussion, future directions and conclusions 134
6.1 Discussion 135
6.1.1 EBV infection of the blood brain barrier 135
6.1.2 Latent gammaherpesvirus infection affects antigen presentation and skews the T cell response leading to enhanced disease in two different mouse models of autoimmunity 136
6.2 Future directions 139
6.2.1 Establishment of latency and reactivation of EBV infection in HBMECs 139
6.2.2 Modulation of CD40 signaling during EAE in γHV-68 latently infected mice 139
6.2.3 γHV-68 EAE as a model to study CD8 T cell infiltrations inside the CNS 140
6.2.4 B cell activation in γHV-68 NZBW mice as a trigger to accelerate lupus 141
6.2.5 Is latency required? 141
6.3 Concluding remarks 142
References 143

List of figures

Figure 3.1. EBV is able to infect HBMECs 50
Figure 3.2. HBMECs remain viable after EBV infection..... 51
Figure 3.3. Infected HBMECs express both latent and lytic EBV genes..... 52
Figure 3.4. EBV infected HBMECs secrete increased amounts of CCL-5 (RANTES) 24-48 hours post infection 54
Figure 3.5. EBV infected HBMECs express increased levels of ICAM-1 48 hours post infection..... 55
Figure 3.6. Increased PBMCs adhesion on an EBV infected HBMECs monolayer 48 hours post infection 56
Figure 4.1. γHV-68 enhances EAE symptoms..... 63
Figure 4.2. γHV-68 EAE mice show increased mortality 63
Figure 4.3. Infections with other viruses do not alter EAE clinical course..... 64
Figure 4.4. γHV-68 enhances EAE symptoms also when EAE is induced without pertussis toxin 65
Figure 4.5. γHV-68 does not reactivate more upon EAE induction..... 67
Figure 4.6. γHV-68 IL6-KO mice are resistant to EAE induction..... 68
Figure 4.7. γHV-68 does not infect the CNS..... 69
Figure 4.8. γHV-68 EAE mice have increased numbers of T cells inside the CNS..... 72
Figure 4.9. γHV-68 EAE mice show increased percentages of CD8 T cells infiltrating into the CNS..... 73
Figure 4.10. Immunohistochemistry after EAE induction shows enhanced inflammation and immune cell infiltrations inside the brain parenchyma only after γHV-68 infection..... 74
Figure 4.11. γHV-68 EAE mice show increased amount of immune cell infiltrations in the spinal cords 76
Figure 4.12. γHV-68 EAE mice show MS-like lesions in the white matter of the cerebellum and in the corpus callosum 77
Figure 4.13. Granzyme B is upregulated in CD8 T cells isolated from the CNS of γHV-68 EAE mice 79
Figure 4.14. A percentage of CD8 T cells isolated from the CNS of γHV-68 EAE mice is specific for viral proteins..... 80

Figure 4.15. T cells infiltrating in the CNS of γHV-68 EAE mice produce higher amounts of IFN-γ 83
Figure 4.16. Th17 responses are suppressed in γHV-68 EAE mice 84
Figure 4.17. γHV-68 EAE mice upregulate T-bet in CD4 T cells 85
Figure 4.18. T cells in the periphery of γHV-68 EAE mice show increased activation and enhanced production of IFN-γ 87
Figure 4.19. γHV-68 EAE mice have increased amounts of IFN-γ and TNF-α in the serum and in the CNS at different time points post-EAE induction 90
Figure 4.20. γHV-68 EAE mice show upregulation of RANTES and MIG in the serum at day 10 post EAE induction 91
Figure 4.21. CXCR3 is upregulated on splenic T cells at day 10 post EAE induction in γHV-68 EAE mice 92
Figure 4.22. γHV-68 EAE mice secrete more brain extract specific autoantibodies but not anti-MOG autoantibodies 94
Figure 4.23. γHV-68 EAE mice have a decreased Ig1/IgG2a anti-MOG antibodies ratio 95
Figure 4.24. γHV-68 EAE mice are protected from EAE development during acute infection and start to develop EAE symptoms during the establishment of γHV-68 latency 97
Figure 4.25. Only γHV-68 EAE mice show CD8 T cell infiltrations in the brain parenchyma at the onset of EAE symptoms, after γHV-68 acute infection clearance and establishment of γHV-68 latency 98
Figure 4.26. CD11b+CD11c+ cells in the periphery of γHV-68 EAE mice produce more IFN-γ at day 4 post EAE induction 101
Figure 4.27. CD11b+ CD11c+ cells isolated from γHV-68 EAE mice are able to prime an enhanced IFN-γ response in 2D2 CD4+ T cells in vitro 102
Figure 4.28. CD11b+CD11c+ cells isolated from γHV-68 EAE mice express higher levels of CD40 and MHC II 103
Figure 4.29. CD11b+ CD11c+ cells isolated from γHV-68 EAE mice are able to prime an enhanced Th1 response in vivo. 104
Figure 4.30. Regulatory T cells are decreased in the spleens but not in the cervical lymph nodes of γHV-68 mice both during EAE and before EAE induction 106

Figure 4.31. Regulatory T cells are decreased in brains and spinal cords of γHV-68 mice during EAE 107
Figure 5.1. γHV-68 NZBW mice secrete less IgM anti-nuclear antibodies but similar amounts of IgG anti-nuclear antibodies at 8 months of age 115
Figure 5.2. γHV-68 NZBW mice have an increased IgG2a/IgG1 anti-DNA antibodies ratio at 8 months of age 116
Figure 5.3. γHV-68 NZBW mice show decreased numbers of plasma cells and activated B cells at 8 months of age 118
Figure 5.4. Naïve and γHV-68 NZBW mice have similar levels of proteins in the urine at 8 months of age 120
Figure 5.5. Lupus nephritis is similar between γHV-68 NZBW mice and naïve NZBW mice 121
Figure 5.6. The levels of IgGs deposition are similar between γHV-68 NZBW mice and naïve NZBW mice 122
Figure 5.7. γHV-68 NZBW mice produce more IL-6 and IL-12 two weeks after γHV-68 infection 124
Figure 5.8. γHV-68 NZBW mice are still producing more IL-12 at 8 months of age, 200 days post γHV-68 infection 125
Figure 5.9. γHV-68 NZBW mice have increased percentages of T cells capable of secreting IFN–γ and TNF-α at 8 months of age 127
Figure 5.10. γHV-68 NZBW mice have increased numbers of CD8 T cells with an effector/memory phenotype at 8 months of age 128
Figure 5.11. Naïve and γHV-68 NZBW mice show similar numbers of regulatory T cells 130

List of abbreviations

2D2 mice	transgenic mice bearing a MOG 35-55 specific T cell receptor
APCs	antigen presenting cells
ATCC	American Type Culture Collection
BBB	blood brain barrier
Bcl-2	B-cell lymphoma/leukemia-2
BCLF-1	Epstein-Barr virus major capsid protein
BCRF-1	Epstein-Barr virus IL-10 homolog
BHK	baby hamster kidney cells
BHRF-1	Epstein-Barr virus bcl-2 homolog
bp	base pairs
BRLF-1	Epstein-Barr virus immediate early protein
BSA	bovine serum albumin
BZLF-1	Epstein-Barr virus immediate early protein
CBA	Cytometric Bead Array
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund’s adjuvant
CIS	clinically isolated syndrome
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CTL	cytotoxic T cells
CXCR3	C-X-C chemokine receptor type 3
DAB	3,3-diaminobenzidine
DC	dendritic cell
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
dNTPs	deoxynucleotide triphosphate
dsDNA	double strand deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis

EBER	Epstein-Barr virus encoded small RNAs
EBI3	Epstein-Barr induced gene 3
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
Foxp3	forkhead box P3
γHV-68	murine gamma herpesvirus-68
GAPDH	glyceraldehyde-3- phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GrB	granzyme B
H&E	hematoxylin and eosin
HBMECs	human brain microvascular endothelial cells
HHV	human herpesvirus
HRP	horseradish peroxidase
HSV-1	herpes simplex virus 1
HUVECs	human umbilical vein endothelial cells
KSHV	Kaposi's sarcoma virus
IFN-α	interferon alpha
IFN-β	interferon beta
IFN-γ	interferon gamma
ICAM-1	intercellular adhesion molecule 1
Ig	immunoglobulin
IL	interleukin
IL-6 KO	knockout mice without the IL-6 gene
IMDM	Iscove's modified Dulbecco's media
i.p.	intraperitoneally
IRFs	interferon regulatory factors
LCMV	lymphocytic choriomeningitis virus
LCL	lymphoblastoid cell line (human B cells transformed by EBV)
LFB	luxol fast blue

LMP	Epstein-Barr virus latent membrane protein
LNs	lymph nodes
LPS	lipopolysaccharide
M199	medium 199
MCMV	mouse cytomegalovirus
MCP-1	monocyte chemoattractant protein-1 (aka CCL-2)
MEF	mouse embryonic fibroblasts
MEM	minimum essential medium
MHC	major histocompatibility complex
MIG	monokine induced by gamma interferon (aka CXCL-9)
MIP-1α	macrophage inflammatory protein-1 alpha (aka CCL-3)
MIP-1β	macrophage inflammatory protein-1 beta (aka CCL-4)
mLANA	murine latency-associated nuclear antigen
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NGS	normal goat serum
NK	natural killer cell
NZB	New Zealand black mice
$NZBWF_1$	New Zealand black and white F_1 hybrid mouse (also abbreviated as NZBW in the text)
NZW	New Zealand white mice
OCB	oligoclonal bands
OCT	optimal cutting temperature compound
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFU	plaque forming units
PI or p.i.	post infection
PLP	myelin proteolipid protein

PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin
RA	rheumatoid arthritis
RANTES	regulated upon activation, normal T-cell expressed, and secreted protein (aka CCL-5)
RBC	red blood cells
RNA	ribonucleic acid
ROR-γt	retinoic-acid-receptor-related orphan receptor gamma t
RPMI-1640	Roswell Park Memorial Institute culture medium
RT	room temperature
RTA	lytic transactivator protein
RT-PCR	reverse transcription polymerase chain reaction
SLE	systemic lupus erythemathosus
Sm	Smith antigen
SSC	side scatter
ssDNA	single strand deoxyribonucleic acid
T-bet	T-box expressed in T cells
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TCR	T cell receptor
Th	helper T cell
TNF-α	tumor necrosis factor alpha
TLR	toll like receptor
Tregs	regulatory T cells
UPO	urea hydrogen peroxide
VCAM-1	vascular cell adhesion molecule 1
Vero	kidney epithelial cell line from an African green monkey
VZV	varicella zoster virus

Acknowledgements

I would like to first thank my supervisor, Dr. Marc Horwitz, for giving me the opportunity to join his lab and for great interactions and support during these years. It has been a great experience and privilege to work with you, Marc. I would also like to thank my committee, Dr. Ninan Abraham, Dr. Michael Gold and Dr. Katerina Dorovini-Zis, for their feedback, support and suggestions about my research. My gratitude extends to Dr. Jami Bennett and Dr. Jacqueline Quandt for providing initial help for the development of the EAE model.

I am grateful to the University of British Columbia for funding me during my PhD studies and to the endMS Research and Training Network for the funding and opportunity to attend MS summer schools that proved to be valuable for my career.

Thanks for great friendships and all the support from the past and present members of the Horwitz lab and the whole Department of Microbiology and Immunology. Especially, I would like to thank Iryna Shanina, Martin Richer, Maya Poffenberger, Pamela Lincez, Christina Farr, Marine Walic, Sehyun Cho, Lisa Osborne, Adam Plumb, Dan Patton, Munreet Chehal, Alex Sio, Morgan Roberts and Amy MacDonald. It has been an honor to share these years with you all.

I also would like to thank Claudio Erratico who shared with me the first three years of this Canadian adventure. I shall not forget all your help and support.

Thanks to all my Italian friends that kept in touch and always encouraged me during these years I was away from home: Mauro Tiozzo, Maria Rosaria Carbone, Valentina Cavalieri, Francesca Villa, Miriam Magni, Jan de Kruif and Maria Pia Purello.

One special mention goes to Prof. Maria Rosa Montrasio Alfieri and Prof. Fernanda di Miele, two great high school teachers that made me love science from the beginning. Many thanks to Prof. Roberto Burioni and Dr. Nicasio Mancini that transmitted me the passion for the study of viral infections during my BSc studies.

The biggest thanks go to my entire family, my dad Danilo Casiraghi, my mum Marina Merlo, my aunt Patrizia Casiraghi, my grandmothers Ida Sala and Angioletta Perego, my grandfathers Adriano Merlo and Federico Casiraghi. I share this success with all of you: you educated me well, guided me, trusted me and encouraged me during these 27 years. I had a wonderful life for which I am grateful everyday. With all my heart, thank you.

To my parents and my aunt

1 Introduction[1]

[1] A part of this chapter has been published: Costanza Casiraghi and Marc S. Horwitz. Epstein-Barr virus and Multiple Sclerosis. At Press in Multiple Sclerosis: Causes, Diagnosis and Management, Nova publisher, ISBN: 978-1-61324-848-5

1.1 Herpesviruses

Herpesviruses are large enveloped DNA viruses that carry up to 100 different genes. Eight herpesviruses have been characterized as able to cause disease in humans, especially in children and in immunocompromised individuals [1, 2]. All herpesviruses are able to infect a permissive cell type, leading to lytic infection. These viruses enter inside the target cell via pH dependent endocytosis using three glycoproteins, present on the surface of the virus. The viral DNA is then released inside the cell's nucleus where the viral replication process is initiated by the expression of immediate-early viral genes that act both as transactivators of early viral genes and have a role in evading the innate immune response. Expression of early viral proteins leads to viral DNA replication and expression of late proteins (capsid and envelope proteins) that compose the scaffold of the nascent virion. The new viral progeny is then released from the infected cells through lysis or exocytosis [1]. Lytic infection is usually controlled by the adaptive immune response in immunocompetent individuals. Upon acute infection clearance, these viruses are able to establish latency in specific cell types and they are carried through the entire life of the host. Under certain conditions or exogenous stimuli (i.e. stress, UV light, another infection, immunodeficiency), herpesviruses can reactivate the lytic cycle and be transmitted to a new host [2].

Herpesviruses are divided in three distinct subfamilies. Alphaherpesviruses (α-herpesviruses) are herpes simplex virus (HSV) and varicella zoster virus (VZV). This subfamily infects epithelial cells and is then able to establish latency in neurons. Betaherpesviruses (β-herpesviruses), like cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6), infect lymphocytes and establish latency in lymphocytes (T cells), monocytes and macrophages. Similarly, gammaherpesviruses (γ-herpesviruses) lead to a lytic infection in lymphocytes (B cells and T cells) and establish latency in lymphocytes, epithelial cells and other cell types of the immune system. Examples of this last subfamily are Epstein-Barr virus (EBV) and Kaposi's Sarcoma virus (KSHV) [1].

1.2 Gammaherpesviruses

The most extensively studied γ-herpesviruses, due to their association with human cancers, are Epstein-Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV), respectively known also as human herpesvirus-4 (HHV-4) and human herpesvirus-8 (HHV-8) [3-5]. Malignancies caused by these viruses are usually associated with immunodeficiency and the majority of latently infected individuals, that represent a large proportion of the human population (close to 90%), carry the infection without any consequences [5]. EBV genome is found in different types of lymphomas (African Burkitt's lymphoma, Hodgkin and non-Hodgkin lymphomas, some T cell and NK cells lymphomas), nasopharyngeal carcinoma and gastric adenocarcinomas [5]. KSHV causes all forms of Kaposi's sarcoma and some lymphoproliferative disorders such as multicentric Castleman disease and primary effusion lymphomas, found especially in HIV positive patients [3].

EBV infects mainly epithelial cells and B cells [6]. Additionally, there is evidence that it also has the ability to infect monocytes [7] and endothelial cells [8, 9]. In contrast, KSHV has the ability to infect more types of cells, including lymphocytes, macrophages, dendritic cells, endothelial cells, fibroblasts and keratinocytes [3].

These two viruses belong to the γ-herpesvirus sub-family and they both carry a similar protein that is a master regulator of the latent-lytic switch [3]. This protein is called the RTA immediate-early protein (encoded by BRLF-1 in the EBV genome and ORF-50 in the KSHV genome). RTA acts as a transcription factor that activates the expression of viral lytic genes and thus controls reactivation from latency. Moreover, EBV also requires another immediate early protein, called BZLF-1 to fully activate the lytic cycle [10], whereas ORF-50 is enough for KSHV.

1.3 Epstein-Barr virus

Epstein-Barr virus (EBV) was first described in 1964 through an electron micrograph of a Burkitt's lymphoma biopsy [11].

EBV is transmitted through saliva and infects B cells and epithelial cells present in the oropharynx [12]. If the infection is acquired early in life, it is usually asymptomatic or is accompanied by a minor febrile illness. Conversely, if the encounter with EBV is delayed to adolescence or early adulthood, it causes infectious mononucleosis, which is a benign lymphoproliferative disease that is usually controlled by the immune system in immunocompetent individuals [5].

EBV initially infects epithelial cells and any kind of B cells in the tonsils and oropharynx and drives their differentiation into memory B cells. EBV infection is present only in memory B cells in the periphery and about 1-50/1 million B cells carry EBV genome in the blood [12]. During latency, the host cell expresses a limited number of EBV gene products: six nuclear protein (EBNA-1/2/3A/3B/3C/LP), three membrane proteins (LMP-1/2A/2B) and EBV-encoded small RNAs (EBER-1 and EBER-2). These viral proteins and transcripts control the cell cycle of the host cell and prevent its apoptosis; they can be upregulated all at the same time or only some of them can be expressed at a given time in a B cell. According to the types of EBV protein that are expressed in an infected cell, five different types of latency programs have been identified in EBV infected individuals (for a review [5]). These latency programs are associated with the stage of development of the infected B cell, with the different types of EBV-associated lymphomas and with the host immune status (immunocompromised or immunocompetent).

EBNAs and LMPs all have a critical role in the immortalization of B cells. EBNA-1 is responsible for the replication of the viral genome during B cell replication and its correct partitioning into daughter B cells. EBNA-2 and EBNA-LP act in concert to transactivate several viral and cellular genes and EBNA-2 is absolutely required for cell immortalization. EBNA-3 family proteins act as EBNA-2 regulators. LMP-1 localizes at the plasma membrane and controls the upregulation of several activation markers, adhesion molecules and growth factor receptors acting as a constitutively active CD40 homolog, physiologically required for B cell survival and proliferation. LMP-2A acts as a constitutively active B cell receptor, thus providing additional survival signals. LMP-2B

promotes motility and spreading of epithelial cells but its function in B cells is poorly characterized [12, 13]. Finally, EBERs, the most abundant transcripts detected in EBV infected cells, bind to several proteins of the type I interferon signaling cascade.

It is not entirely clear what signals reactivate the EBV lytic cycle. Stress or another infection have been shown to be potentially involved. During reactivation, immediate early (IE) lytic genes are expressed (BZLF-1 and BRLF-1). These are transactivators that promote the expression of both early (E) and late (L) lytic genes that encode for structural viral proteins. During the lytic phase, EBV genome is amplified up to 1000-fold and viral proteins stimulate the progression of the cell cycle of the host cell from phase G1 to phase S. This transition is essential for the expression of both IE and E genes [14].

EBV is intermittently shed in the saliva of the majority of EBV seropositive individuals. This allows transmission to another host but also continuous re-infection of more B cells within the host. Usually the start of the lytic cycle and production of new virions is associated with the transition from memory B cell to plasma cell [5, 12].

1.4 Host response to Epstein-Barr virus

EBV infection persists for the entire life of the host in the vast majority of the human population. The virus has evolved several mechanisms to evade the immune response (allowing the establishment of latency) and to permit intermittent reactivation (permitting spread to other hosts).

1.4.1 Immune response during acute infection, mononucleosis and latency

Early during acute infection, natural killer cells (NK cells) have a role in limiting the infection [15, 16]. Subsequently, this role is played mainly by CD8+ cytotoxic T cells (CTLs). During primary lytic infection, CTLs are specific for both latent and lytic EBV antigens [17]. During mononucleosis, up to 40% of the total CD8 response in the blood is directed against one lytic viral antigen, whereas around 2% is specific for latent antigens. The phenotype of these CTLs is mainly effector-memory [18]. Upon resolution of clinical mononucleosis, the CD8 response towards lytic antigens drops between 1-18%, conversely, the frequency of CTLs specific for latent viral epitopes remains stable around 2% [18]. Studies conducted during EBV latency in EBV seropositive donors that never suffered from mononucleosis indicate that the frequency of EBV lytic antigens specific CD8 T cells is much lower (between 0.1 up to 5%), and the response to latent antigens is similar to mononucleosis patients (between 1 up to 4%) [19] [20]. EBV specific CD8 T cells have a major role in controlling EBV reactivation in B cells during latency [21, 22].

EBV specific CD4 T cells have been isolated as well [23, 24] but they have been less characterized than CD8s. Nonetheless, CD4 T cells are thought to play an important role in helping CD8 T cell responses during acute EBV infection. During latency, EBV specific CD4 T cells produce soluble mediators that act on both EBV specific CD8 T cells and EBV infected B cells [25].

Finally, EBV seropositive individuals produce large amounts of antibodies against different viral epitopes. However these antibodies are not protective, since an individual can be super-infected by multiple EBV strains during life [26] and immunocompromised individuals develop lymphoproliferative disorders caused by

uncontrolled EBV replication, despite an increase in titer of EBV specific antibodies [27]. This is likely due to the fact that EBV can spread through cell-cell contact. Increases in antibody titers are often used as a diagnostic marker for EBV associated disease [28-32].

1.4.2 Viral strategies of immune escape during latency and lytic infection

The first immune avoidance mechanism is the establishment of latency itself. Upon clearance of acute infection, only two-three viral proteins, responsible for the maintenance of the viral genome in the infected cells, are expressed at a given time. This limits the numbers of epitopes that can be seen by the immune system [21, 22]. Additionally, the glycine-alanine repeats present in the sequence of EBNA-1 play an important role in inhibiting the proteasomal pathway and thus antigen presentation, allowing infected B cells to remain invisible to the immune system [33]. Antigenic variation that leads to CTL mutant escapes has also been shown to play a role in EBV immune evasion [34, 35]. Finally, during latency, EBV transformed lymphocytes produce high levels of EBV induced gene 3 (EBI3) that is a homolog of the p40 subunit of IL-12. EBI3 has an inhibitory effect on IL-12 that is critical to sustain a T helper type 1 (Th1) response and NK cell activity that are responsible of identifying and killing EBV infected lymphocytes [36, 37].

EBV exploits several mechanisms to dampen both the innate and the adaptive immune response during lytic infection. Several reports have demonstrated that EBV abortive infection of neutrophils triggers apoptosis [38] and secretion of different cytokines [39-41]. EBV has also been shown to infect monocytes in vivo [7] and block their differentiation towards DCs [42]. Additionally, the EBV gene called BCRF-1 encodes for a viral homolog of human IL-10. Viral IL-10 suppresses the development of effector functions of CTLs, promotes the proliferation of infected B cells [43] and interferes with the functions of DCs, inhibiting their migration to regional lymph nodes [44]. Another viral product, BHRF-1, is a homolog of bcl-2, a cellular protein that inhibits apoptosis. The expression of BHRF-1 during lytic infection can confer resistance to EBV infected B cells to CD8 CTLs mediated killing [45]. Finally, EBV specific CD8 T cells show a lower expression of homing receptors that allow entrance in the lymph nodes

(CCR7 and CD62L) [46, 47]. This observation explains why the frequency of EBV specific T cells is much lower in the lymph nodes [46] than in the periphery and why EBV is strictly latent in the peripheral blood and replicates only in the epithelium and in secondary lymphoid tissues as described in paragraph 1.3.

1.5 Modeling Epstein-Barr virus in rodents with murine gamma herpesvirus-68

Human γ-herpesviruses do not infect rodents, thus the study of the immune response towards these viruses as well as viral pathogenicity have been hampered by the lack of a robust small animal model. Some pathogenicity studies have been performed using non-human primates and γ-herpesviruses that are able to infect these animals and are closely related to the human γ-herpesviruses. However these studies are limited by high costs and lack of host genetic systems. Additionally, even if considerable progress has been made in the generation of humanized mice where human γ-herpesviruses infection could be investigated using a small animal model, these mice still have significant limitations since they are not able to mount very strong antigen-specific responses [48]. For these reasons, the isolation of γ-herpesviruses from rodents made feasible a deeper and more comprehensive study of γ-herpesviruses pathogenesis. Murine gamma herpesvirus-68 (γHV-68) has been originally isolated from bank voles and yellow-necked field mice captured in Slovakia [49] and it has been extensively used to study Epstein-Barr virus in mice [50].

1.5.1 Murine gamma herpesvirus-68 genome: differences and similarities to human gammaherpesviruses

The genome of γHV-68 has been sequenced and extensively compared to the genome of human gammaherpesviruses [51]. As expected, γHV-68 carries some genes that are unique to the murine gammaherpesviruses and some that are also conserved in the human gammaherpesvirus family and have the same function.

Examples of homolog genes are: mLANA encoded in the γHV-68 genome is critical for the establishment of latency and the maintenance of the viral genome, thus performing the same function as EBNA-1 in EBV [52, 53]. V-cyclins that control the cellular cycle, thus functioning as cellular cyclins, are encoded by both murine and human gammaherpesviruses [54]. Another γHV-68 protein, called M11, is a viral homolog of Bcl-2 (also designated as vBcl-2) and can inhibit apoptosis [55, 56]; all human gammaherpesviruses encode at least one vBcl-2. Finally γHV-68 K3 protein inhibits antigen presentation by MHC class I [57] and M1 is a superantigen that is

responsible for the expansion of Vβ4+CD8+ T cells and thus for the modulation of host T cell functions during gammaherpesvirus infection [58]. These activities are two other features common to all human gammaherpesviruses [59-61].

However γHV-68 has some unique genes that are not carried by human gammaherpesviruses. For example, M3 is a chemokine binding protein [62, 63] that can inhibit chemokine function and thus modulate inflammatory responses. M3 can bind a wide range of chemokines, including CC, CXC, C and CX(3)C chemokines. Considering the importance that chemokines have in directing immune cells to the site of infection, it is surprising that there is no evidence that M3 is critical for the establishment of latency or for γHV-68 reactivation. Additionally, little is known about the chemokines that are important during γHV-68 infection and how the virus is modulating them.

Another unique γHV-68 protein, called M2, is necessary for both establishment of latency and reactivation from latency [64, 65]. M2 is able to induce IL-10 expression by infected cells [66] and M2 expression in B cells leads to the upregulation of germinal center markers [66] to promote differentiation towards plasma cells that is linked to γHV-68 reactivation from latency [67]. Notably, a viral homolog of IL-10 is encoded by EBV and EBV reactivation is linked as well to plasma cell differentiation.

In summary, even if γHV-68 is encoding for some unique proteins, some of them are playing a similar role and have the same final effect in the host as other proteins encoded by human gammaherpesviruses. These observations, along with the conserved nature of the immune system between mice and humans, make γHV-68 a valuable model to study human gammaherperviruses in a mouse model [68].

1.5.2 Viral replication kinetics and cell targets

γHV-68 establishes latency in B cells, macrophages and splenic DCs [69-71]. The vast majority of studies employ either intranasal or intraperitoneal inoculation. Regardless of the route of infection, γHV-68 establishes latency mainly in the spleen. Acute replication is controlled by day 14-16 post infection. Spontaneous viral reactivation is still high between day 16-18 post infection. This is defined as “peak of viral latency” during which the expression of both lytic and latent viral proteins is still detectable inside infected cells but new viral particles are not produced. However, five

to six weeks post infection, only very few infected splenocytes can reactivate γHV-68 and express viral proteins, suggesting that “true” latency is established few weeks after the clearance of acute infection [72]. During the peak of viral latency, γHV-68 infection is found mainly in naïve and germinal center B cells [73, 74]. Later on, the viral genome is found only in isotype-switched B cells that appear to be memory B cells that have undergone a germinal center reaction [74]. This cell tropism is very similar to the one observed during EBV infection.

Another site of γHV-68 latency, besides the spleen, is the peritoneal cavity. In fact, γHV-68 is found also in peritoneal macrophages during latency [71]. Finally, it is worth noting that B cells are not required for establishment of γHV-68 infection. However, intraperitoneal infection of B cell deficient mice, results in higher levels of viral reactivation that eventually leads to death by day 100 post infection, due to impaired control of viral latency [72]. The cause of death of these mice is unknown but exudative process in the lungs and arteritic lesions are detected, both likely due to chronic viral reactivation.

1.5.3 Innate immune responses to murine gamma herpesvirus-68 infection

Early responses to pathogens are mediated by the recognition of pathogen’s structures by toll like receptors (TLRs) and other intracellular sensors (like RIG-I) that are expressed by innate immune cells. TLR-9 recognizes unmethylated CpG DNA that is found in the genome of DNA viruses like γHV-68. Despite this, it has been shown that TLR-9 has only a modest role in the development of γHV-68 specific immunity [75]. Additionally MyD88, a key molecule used by almost all TLRs to signal, is also dispensable for the control of acute infection [76], but it is required for the establishment of latency. These findings indicate that the primary role of TLRs during γHV-68 infection is not the activation of an antiviral immune response but, instead, TLRs are exploited by the virus to promote latency. Additionally, TLRs triggering during latency, paradoxically, promotes viral reactivation [77]. Interestingly, KSHV reactivation is also induced by TLRs ligands, indicating that this is a common feature shared by all gammaherpesviruses [78].

Type I interferons (IFN-αβ) are the first line of innate cytokines that control viral infection. As such, mice lacking IFN-αβ receptor are not able to efficiently control acute γHV-68 infection and mice infected with high doses (more than 100 pfu) of γHV-68 succumb to acute infection. Mice infected with lower viral doses (100 pfu) can survive the acute infection phase but infected splenocytes reactivate γHV-68 with more efficiency in these mice [79]. Another observation that underscores the importance of IFN- αβ for the control of γHV-68 infection is that the virus has developed strategies to block interferon regulatory factors (IRFs) that are critical to activate the IFN- αβ cascade [80]. This is another evasion strategy that γHV-68 shares with EBV [81, 82].

1.5.4 Adaptive immune responses to murine gamma herpesvirus-68 infection

As has been shown for EBV, the control of lytic acute γHV-68 infection is mainly mediated by CD8 T cells. CD8 T cells specific for γHV-68 lytic antigens start to appear around day 6 post infection, peak around day 10 and contract upon resolution of acute infection [83, 84]. Later, a second wave of late lytic antigen specific CD8 T cells is generated at the peak of latency due to differential expression of viral antigens during acute infection and the peak of latency. This CD8 response is maintained for two months after viral clearance [85, 86]. γHV-68 specific CD8 T cells retain high levels of function, showing an effector memory and central memory phenotype, with production of IFN-γ and TNF-α [87]. CD8 T cells control γHV-68 infection through perforin-granzyme and Fas-dependent cyotoxicity [88, 89]. Oligoclonal CD8 T cells responses are found in γHV-68 mice, as it has been shown in EBV infected individuals (see paragraph 1.4.1). This oligoclonal response preferentially uses a Vβ4 TCR and it arises in response to the viral protein M1 as described in paragraph 1.5.1. In the C57Bl/6 mouse, all the class I restricted γHV-68 epitopes belong to lytic proteins, indicating that latently infected cells are not presenting to CD8 T cells any epitope belonging to viral proteins expressed during latency [68]. This is likely due to γHV-68 immune escape mechanisms that target the MHC I pathway during lytic infection (see paragraph 1.5.1).

CD8 T cell responses are necessary and sufficient to control acute γHV-68 infection, even in the absence of CD4 T cell help or B cells [90]. Thus, these two last components of the adaptive immune response are dispensable for the control of lytic

infection. On the other hand, CD4 T cells play a crucial, non-redundant role in the control of viral reactivation along with CD8 T cells, B cells and IFN-γ signaling (for a review [91]). All these components are required to avoid a chronic viral reactivation that leads to tissue damage, culminating in the death of the infected animals. CD4 T cells specific for γHV-68 can produce IFN-γ for several months following infection [92] and they also display cytolytic function [92]. Finally, the antibody response against γHV-68 is slow to develop [93, 94] but has a role in controlling viral reactivation [95, 96].

1.5.5 Immune modulation during murine gamma herpesvirus-68 latency

Different studies have shown that some inflammatory diseases can be modulated by γHV-68 infection. Experimental autoimmune encephalomyelitis is exacerbated by γHV-68 infection [97]. Worse disease is also observed in γHV-68 infected mice in the context of pulmonary fibrosis [98], atherosclerosis [99, 100], inflammatory bowel disease [101] and Crohn's disease [102]. In all these studies, the mechanisms that the virus is exploiting to lead to this enhanced autoimmune phenotype were not addressed.

Additional work has demonstrated that γHV-68 infection can protect from a lethal challenge with other intracellular pathogens such as Listeria monocytogenes [103], Yersinia pestis [103], Plasmodium [104] and adenovirus [105]. This cross-protection is not antigen specific and it is mediated by innate immunity activation that displays enhanced activation in γHV-68 infected mice and it is thus more efficient in controlling infections with other intracellular pathogens [103].

These findings point towards a complex and long-lasting interaction between the immune system of the host and latent infections, indicating that gammaherpesviruses can have both harmful (autoimmunity, chronic inflammation and tumors) and beneficial (cross-protection) consequences for human immunology.

1.6 Epstein-Barr virus and autoimmunity

A wide array of autoimmune diseases have been linked to EBV infection: systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA), Sjogrens syndrome, autoimmune thyroiditis, autoimmune hepatitis, cryptogenic fibrosing alveolitis and pure red cell aplasia [106-108].

Different studies analyzing EBV specific immunity in patients affected by these autoimmune diseases have shown that molecular mimicry could be implicated. As such, T cells and antibodies specific for both self-antigens and EBV antigens (but also other pathogens, both viruses and bacteria) have been isolated from MS, SLE and RA patients. These data show that many different pathogens have the potential to generate molecular mimicry. So, why is EBV more tightly associated with these autoimmune diseases than other pathogens? Other mechanisms, besides molecular mimicry, can be exploited by this virus to cause autoimmunity. In fact, EBV also has the unique ability to immortalize B cells [107] and affect different arms of the immune system both at the level of antigen presentation and T cell memory responses (see paragraph 1.4.2). Additionally, EBV continuously stimulates the immune system that has to remain vigilant to suppress reactivation from latency. In this regard, it has been proposed that chronic EBV infection at the site of autoimmunity development (i.e. brain for MS and synovium for RA) can lead to initial inflammation that causes the first insult leading to the onset of autoimmunity [107]. Intriguingly, EBV infection has been detected in both the central nervous system and joints of MS and RA patients respectively, even if these observations are still a matter of controversy.

Despite all these findings and the unique ability that EBV has to immortalize B cells and manipulate the immune system of the host, the mechanism that EBV would exploit to trigger autoimmunity is currently not clear.

1.7 Epstein-Barr virus and multiple sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that leads to myelin disruption and progressive disability. MS is believed to be triggered by environmental factors in genetically susceptible individuals but the etiology is still unknown. Myelin damage is thought to be mediated by autoreactive T cells. These T cells are able to cross the blood-brain barrier (BBB), recognize myelin epitopes and produce pro-inflammatory mediators that recruit a second wave of immune cells into the CNS. It is still not clear what is the first event that triggers myelin recognition, BBB breach and T cell entry into the CNS [109].

A number of different viruses have been implicated as etiologic agents of MS since its first description. Jean-Martin Charcot, a French neurologist that first described and named the disease in 1868, related infections with disease onset in some patients [110]. In the past 20 years, increasing evidence has indicated EBV as a potential trigger of MS.

1.7.1 The development of a hypothesis from 1970 to 2000

The first observations of a link between EBV and MS date back to the 1970's and 1980's. In 1979, Fraser and colleagues noticed that lymphoid-cell lines (i.e. B cells transformed by EBV) are established more easily with lymphocytes isolated from patients with clinically active MS than with those from healthy donors. The authors recognized that: "even if there is no primary role for EBV in the immunology of MS, a secondary role of reactivated EBV in its pathogenesis should be considered" [111]. Later, Craig and colleagues published a series of articles in which they demonstrated that T cells from MS patients fail to suppress EBV transformed cells whereas all lymphocytes cultures from healthy controls (except those from EBV seronegative and cord blood donors) are able to suppress EBV immortalized cells. They also found that MS patients have reduced numbers of CD8+ T cells and higher ratio of CD4+/CD8+ T cells [112-114].

During the same years, other papers were published showing increased EBV seropositivity and increased anti-EBV antibody titers among MS cohorts [115-117].

Interestingly, the prevalence of herpes simplex virus (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV), rubella and mumps infections in these cohorts did not differ between MS patients and controls. Moreover, according to a case-control study, MS patients that had infectious mononucleosis before the age of 18 years, have an increased relative risk of multiple sclerosis [118]. The authors of this article pointed out that: “the pathogenesis of multiple sclerosis may involve an age-dependent host response to Epstein-Barr virus infection”. To further corroborate this theory, immigrant studies suggested that the risk of acquiring MS is determined by the area of residence during the first 15 years of life: people moving from a high risk to a low risk area after childhood retain the MS risk of their former area of residence. So, people living in an area where EBV is acquired during childhood and then moving to an area where EBV is more often acquired during/after adolescence would have a reduced risk of developing MS if compared to people that spent the first 15 years of their lives in areas in which EBV infection occurs later in life [119, 120]. There are also striking similarities between MS and mononucleosis prevalence: they both follow the same latitude gradient and affect people with high socio-economic status [119].

Finally, evidence of molecular mimicry between EBV and myelin antigen was demonstrated through a series of articles. Wucherpfennig and colleagues showed that three T cells clones isolated from MS patients and specific for myelin basic protein (MBP) can be also activated by both bacterial and viral peptides, including one EBV DNA polymerase peptide [121]. Additionally, anti-EBV antibodies were found to be cross-reactive for neuroglial cells epitopes [122] and transaldolase, a protein expressed in olygodendrocytes [123].

1.7.2 Association between EBV and MS in epidemiological studies

Early studies identified increased prevalence of EBV seropositivity among MS patients. Ascherio and colleagues reviewed eight studies [124], including 1,005 cases and 1,060 controls, demonstrating that EBV seroprevalence among MS patients is virtually 100%, whereas it is 95-85% among the general population. More compelling results came subsequently from MS pediatric cohorts. In fact, EBV seropositivity is lower in children than in the adult population and differences in EBV seroprevalence in

MS pediatric cases against controls are even more striking than in adults: 80-90% of children with MS have also been infected by EBV. In contrast, only 40-50% of children in the control groups were EBV seropositive [125-127]. Importantly seropositivity for other herpesviruses did not differ between the groups.

Additionally, EBV seropositivity and history of mononucleosis further increases the risk of developing MS [128-131]. A large Canadian study analyzed over 14,000 MS cases and 7,600 spouse controls asking about history of measles, mumps, rubella, varicella and infectious mononucleosis. They found no differences between the groups for all the other infectious diseases except for mononucleosis: 699 cases and 165 controls indicated a history of infectious mononucleosis [130]. Moreover, a review of previously published articles about the link between MS and mononucleosis, including over 19,000 cases and 16,000 controls, showed that MS risk is strongly associated with mononucleosis with a relative risk of 2.17 ($p=10^{-54}$) [128]. Another recent interesting study showed that MS risk increases if EBV seroconversion occurs. The authors found that all EBV negative cases converted into EBV positive before MS onset. On the other hand only 35.7% of controls seroconverted in the same period of time [131].

1.7.3 EBV strains in MS patients

The prevalence of EBV infection is very high in adults that never developed MS. So it is conceivable that MS patients are infected by a particular EBV strain that is able to cause MS. Only few article in the last years have addressed this question. In 2008, Lindsey and colleagues sequenced EBV using blood collected from MS patients and healthy controls. They sequenced EBV latent membrane protein-1 (LMP-1) because variations in this gene are associated with lymphoproliferative diseases. Different LMP-1 sequences were isolated from both cases and controls but there was no difference between the groups [132]. Similar results were obtained in a subsequent study that sequenced the N- and C-terminus regions of EBNA-1 and, again, LMP-1 [133]. Another group sequenced EBNA-1 and BRRF-2 because of their ability to induce dysregulated immune responses in MS patients. Different polymorphisms were identified in both genes, in both cases and controls. Two SNPs were found to occur slightly more frequently in MS patients, but the study was not conclusive [134]. More studies with larger numbers of cases and controls are likely needed to better address this question.

1.7.4 EBV replication control and reactivation in MS patients

Another mechanism that EBV could exploit to trigger MS is the ability to replicate better or reactivate more often in MS patients. This frequent reactivation could constantly stimulate the immune system leading to impairments in the maintenance of peripheral tolerance. Serological and genetic markers of EBV infection and reactivation were analyzed monthly for one year on MS patients. Patients with clinical symptoms due to progression or relapses have frequent EBV reactivation and EBV genome is detected in 100% of these cases but in none of the MS patients with stable diseases. [135]. These data were partially confirmed by another study that measured EBV RNA and DNA levels in peripheral blood leukocytes (PBMCs) from cases and controls using quantitative PCR. There was no significant difference between the two groups, but paired samples showed EBV increase before and during relapses [136]. EBV plasma viral loads were also measured in a small group of MS patients and controls in another study. In this case, the authors did not find any difference between MS patients and control [137]. Discrepancies in these studies maybe due to the small numbers of cases-controls analyzed, to the different type of starting material (plasma, blood or PBMCs) and different methodology (PCR or serology) employed to detect EBV reactivation.

1.7.5 EBV humoral responses in MS patients

Oligoclonal bands (OCB), are found in the cerebrospinal fluid (CSF) of MS patients. Their presence is used as a diagnostic criteria for MS [109]. The specificity of these antibodies is still largely unknown but some groups have investigated the possibility that they are specific for EBV proteins. In one of the early articles, OCB were found to be enriched in antibodies specific for EBV proteins, especially EBNA-1, in a subset of MS patients [138, 139]. Following investigations detected EBV reactivity along with reactivity against other herpesviruses; or did not find any reactivity; or found the same EBV reactivity in MS and other neurological inflammatory diseases [140-142]. This indicates that EBV humoral response in the CSF may be only part of the multispecific antibody production in MS that targets different viruses.

On the other hand, studies on sera samples have produced more consistent results. In fact, patients affected by MS show a dysregulated humoral immune response against EBV in the serum both before the onset and during the course of the disease. This observation was first made in 2001, when Ascherio and colleagues analyzed blood samples collected before MS onset and they showed that MS patients have higher serum titers of anti-EBV antibodies but not anti-CMV antibodies [143]. Following studies confirmed and further strengthened these results. Prospectively collected serum samples were analyzed for EBV EBNA-1, CMV, HHV-6, HSV, VZV and measles reactivity. Only high activity to EBNA-1 significantly increases the risk of MS and it is more pronounced five years before onset of clinical symptoms [28, 144]. Another study determined that EBNA-1 antibodies start to be elevated in comparison with matched controls even up to fifteen to twenty years before disease initiation. The antibody titer then remains elevated until clinical manifestations occur [145]. Interestingly, these findings have been shown to be specific for MS. EBNA-1 antibodies levels are not increased in the CSF or in the serum of patients suffering from other inflammatory and non-inflammatory neurological diseases [146]. Overall, these results indicate that elevated IgG responses against EBNA-1 are strongly linked to MS onset and they can predict conversion to MS. This has been observed in a study that analyzed humoral immune responses against EBV and other viruses (HHV-6, CMV and measles) in patients with clinically isolated syndrome (CIS) to assess if viral antibody titers can predict conversion to MS. EBNA-1 IgG titers were the only ones that correlate with number of brain lesions, disability severity and predict conversion to MS [147].

Finally higher anti EBNA-1 antibody titers are associated with increased presence of new MS lesions in the brain [148], with increased disability and increased loss of brain volume and grey matter atrophy [149].

1.7.6 EBV T cell responses in MS patients

Molecular mimicry between EBV and myelin antigens was demonstrated for the first time in 1995 with the isolation of a cross-reactive T cell clone from a MS patient [121]. The authors showed that the TCR of this T cell clone can bind and recognize with high degree of structural equivalence two different MHC alleles/peptide complexes:

DRB1*1501 presenting a myelin antigen and DRB5*0101 presenting an EBV antigen. Intriguingly both MHC alleles are associated with MS risk [150]. Cross-reactive T cells can be also detected in the CSF fluid of MS patients, indicating that these lymphocytes can gain access to the CNS: CD4+ T cells specific for both an EBV polymerase peptide and a myelin basic protein peptide were in fact isolated from two MS patients [151].

Another mechanism that EBV could exploit to trigger MS is through an increased or decreased EBV specific CD8 or CD4 response. Increased EBV specific immunity with more cytotoxic activity or more IFN-γ production can lead to a bystander activation of auto-reactive T cells. On the other hand, decreased T cell immunity against EBV could lead to frequent viral reactivation and chronic stimulation of the immune system with loss of peripheral tolerance. Different studies have shown that both scenarios could be possible. CD8 T cells responses against two EBV antigens HLA-B7 restricted have been found increased in MS patients if compared to healthy controls. CD8 responses to these epitopes were measured by an ELISPOT assay detecting IFN-γ production [152]. Different results were obtained in another article in which the authors tested the frequency of CD8 T cells specific for other HLA-B7 restricted EBV peptides. The authors did not find any significant difference between MS patients and healthy controls [153]. This might be due to the fact that different peptides were tested. On the other hand, increased frequency of EBV specific CD8 T cells in MS patients was again confirmed by another group with a different experimental approach: T cells from cases and controls were incubated with autologous EBV-transformed B cells and then stained for IFN-γ production [154]. All the studies mentioned above examined patients with established disease. Additional studies looking at earlier time points, closer to MS onset, were performed: for example, it has been shown that EBV specific CD8 responses are more elevated at MS onset and then tend to decrease over time [155]. Moreover, higher numbers of EBV specific CD8 T cell in the CSF of MS patients at early disease stage were detected using a CFSE-based cytotoxic assay [156] and EBV specific CD8 T cells isolated from MS patients expressed higher levels of both IFN-γ and IL-4 [157]. This might indicate that EBV has an important role as initial disease trigger. The results of these studies are specific for MS and EBV infection only, since dysregulation of EBV immunity is not detected in other inflammatory and non-inflammatory neurological diseases. Additionally CMV specific responses are equivalent between MS patients and controls.

All these studies point in favor of the hypothesis that MS patients have an increased and dysregulated immune response against EBV that could lead to bystander activation of an autoimmune response. On the other hand, as mentioned at the beginning of this paragraph, there is also evidence that MS patients may have a decreased EBV specific T cells response, leading to impairment in EBV infection control. Pender and colleagues analyzed the frequency of PBMCs that produce IFN-γ when exposed to autologous EBV infected B cells isolated from MS patients and EBV positive controls. They found decreased CD8 T cell responses against transformed B cells in MS patients. They also showed that this impairment was not due to decreased HLA expression on B cells or a primary defect in the function of the CD8 compartment [158]. A similar result was found by another group that detected decreased EBV specific CD8 responses in MS patients using the same experimental approach of Pender and colleagues [159]. All the studies on EBV specific CD8 T cell responses mentioned in this paragraph examined a heterogeneous population of MS patients, at different stages of disease (relapsing-remitting MS and/or secondary progressive MS and/or primary progressive MS). Additionally, the authors of these articles do not always specify if the blood samples were collected during active or inactive disease. This is likely the reason why different results were obtained from different groups. It is conceivable that EBV specific CD8 immunity changes over time in MS patients; i.e. increased responses closer to disease onset, followed by a decrease at later time points or during remission.

Finally other published work analyzed EBV specific CD4 T cells responses, especially EBNA-1 specific response, since MS patients have dysregulated antibody response against EBNA-1 and EBNA-1 specific CD4 T cells have been shown to be important for EBV control in healthy subjects. A first study showed increased frequency of EBNA-1 specific CD4+ memory T cells in MS patients. These cells are also able to proliferate more and produce more IFN-γ. T cell responses against other EBV latent and lytic immunodominant peptides and CMV epitopes did not differ between cases and controls [160]. A second article further characterized EBNA-1 specific CD4 T cells as central memory Th1 helper precursors and Th1 polarized effector memory T cells. Intriguingly these T cells are able to cross-recognize myelin epitopes more frequently. Additionally myelin cross-reactive CD4 cells are able to produce both IFN-γ and IL-2; whereas EBNA-1 specific, non cross-reactive CD4 cells are producing IFN-γ only [161]. This IL-2 and IFN-γ polyfunctional phenotype is typical of persistent infections and gives

credit to the theory that both molecular mimicry and bystander activation, through dysregulated cytokine production, can have a role in MS onset.

1.7.7 EBV infection in the MS brain

Since MS was first described in 1868, different investigators postulated that myelin damage was caused by a virus infecting the CNS. As soon as EBV was implicated in MS pathogenesis, the quest to find EBV in MS plaques started. Few negative articles were initially published [162, 163]. The main technique used to detect EBV infection in these articles was in situ-hybridization using probes against EBER, an EBV RNA transcript expressed during latency. The authors recognized some caveats of this approach: RNA degradation was present in some of the samples analyzed and the majority of lesions tested were chronic lesions, not representative of the initial stages of the disease [163]. Another group employed a highly sensitive PCR approach, to detect EBV infection in paraffin embedded active demyelinating MS lesion collected at autopsy. All the lesions analyzed with this approach tested negative for EBV infection [164].

In 2007, Aloisi's group published for the first time evidence that EBV is able indeed to infect the MS brain [165]. Eight cases of MS patients with ectopic follicles in the meninges and B cells/plasma cells infiltration in the white matter were analyzed. Additionally, brain specimens from other twelve MS cases and two cases of acute MS were stained. Ectopic follicles are lymphoid structures in the meninges and have germinal centre features: they are composed of CD20+ cells surrounded by stromal/dendritic cells. Moreover CXCL13 expression, proliferation and differentiation of B cells into plasma cells are detected in the follicles. Both intrameningeal ectopic follicles and perivascular cuffs of acute and chronic active white matter lesions stained positive for EBER through in situ-hybridization. The author termed the brain specimen from these MS patients as "EBV high". Lower levels of EBV infection are also detected in all the other brain samples analyzed (termed as "EBV low") except one. The negative brain sample was also negative for B cells infiltrations, while in the EBV low cases, EBV infection was present in B cells and plasma cells scattered in the parenchyma or close to the perivascular area. Brain section of other inflammatory and non-inflammatory CNS

diseases tested negative for EBV infection. Immunofluorescence and immunohistochemistry for different EBV proteins showed that only latency proteins are generally expressed. But, in some EBV high cases and in acute MS cases lytic EBV protein were also detected, mainly in plasma cells, indicating EBV reactivation. EBV replication is likely abortive since viral glycoprotein expression was not detected. EBV high cases presented with CD8+ T cells that are producing perforin and IFN-γ and they co-localize with infected B cells. This indicates that the presence of a lytic EBV infection in the CNS is recruiting pro-inflammatory immune cells, both T cells and antigen presenting cells (APCs) to counteract the infection. This response could start a pro-inflammatory loop with initial myelin damage, recruitment and bystander activation of myelin autoreactive T cells. A second article by Aloisi's group, that further confirms EBV infection, employed laser–capture microdissection to isolate and analyze a fraction of the tissue of interest [166]. The presence of other viruses, apart from EBV, in the CNS of MS patients was not investigated by Aloisi's group. Other groups were not able to reproduce these findings and they did not find evidence of EBV infection in the MS brain [167-169].

How can these different results be reconciled? It is unlikely that these results are false negative or false positive: Aloisi's tissue sections were selected from patients with prominent B cells infiltration and high levels of inflammation (in the case of the two acute MS cases). On the other hand, the articles with disparities are looking at less well-selected tissues that are not enriched in B cells infiltrations or ectopic follicles structures. Future studies should focus on the prevalence of B cells infiltration and ectopic follicles by screening plaques from different patients at different disease stages to confirm if EBV infection is a common feature of a certain subset of MS patients.

1.7.8 EBV infection of cell types other than B cells

Inconsistent EBV detection in the CNS of MS patients indicates that EBV might be triggering disease by infecting a small subset of cells in the CNS or by another mechanism. The presence of autoreactive myelin-specific lymphocytes is not sufficient to cause MS and such cells have been isolated from healthy individuals [170, 171]. In mouse models, MS-like disease can be triggered only after a leakage of

the blood brain barrier (BBB) is induced [172, 173]. The BBB is critical for maintenance of brain homeostasis. Brain vessels are composed of a specialized type of endothelial cell that protects the brain by preventing the passage of molecules and immune cells. In MS patients, the formation of new demyelination plaques is associated with a disruption of the BBB [174]. Intriguingly, EBV has been shown to infect other types of cells like epithelial cells [6], endothelial cells [8, 9] and monocytes [7]. EBV infection and reactivation in these cells may work in concert with EBV influence on B cells.

In conclusion, there is convincing epidemiological evidence that EBV is involved in MS but the pathogenic mechanisms remain elusive. It is conceivable that genetically susceptible individuals infected by this virus could respond differently to other infections leading to the development of an autoimmune response. Previous studies focused mainly on the humoral and cellular immune response against EBV in MS patients and on the presence of EBV infection in the CNS. EBV infects and transforms B cells, so future studies should investigate the effect that EBV infection has on antigen presentation and its ability to influence polarization and cytokine expression by B cells, within the CNS or the periphery. Most notably, this should be followed in individuals that have had mononucleosis and carry genes linked to MS susceptibility.

1.8 A mouse model of multiple sclerosis: experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was first described as a model to study MS in vivo over 50 years ago [175]. To induce active disease, mice are immunized with a myelin peptide emulsified in complete Freund's adjuvant (CFA). The type of peptide used depends on the mouse strain that it is chosen. CFA is composed of mineral oil and desiccated Mycobacterium tuberculosis. Two injections of pertussis toxin (PTX) may also be required depending, again, on the mouse strain. PTX has been hypothesized to be important to both facilitate the entry of T cells in the CNS [172] and to activate a strong T cell response to break tolerance [176, 177]. Two mouse strains have been most commonly used: SJL mice immunized with a peptide derived from myelin proteolipid protein (PLP) [178] and C57Bl/6 mice immunized with a peptide derived from myelin oligodendrocyte glycoprotein (MOG) [179]. Mice usually develop ascending paralysis starting at day 10-12 post EAE induction. SJL mice develop a relapsing-remitting disease course, conversely, C57Bl/6 mice develop a chronic progressive disease [179]. Besides active EAE, passive EAE can also be induced by injecting mice with myelin-reactive T cells that have been isolated from an active EAE host and expanded in vitro [180].

In this thesis, only active EAE in C57Bl/6 mice has been employed. The C57Bl/6 model has been preferred over SJL mice, because the vast majority of knockouts and transgenic mice are available only on the C57Bl/6 background.

EAE pathogenesis in C57Bl/6 mice is mainly driven by MHC-II-restricted CD4+ T cells [181-183]. A percentage of MOG specific CD4+ T cell has a T helper 1 (Th1) phenotype and secretes IFN-γ and TNF-α. Additionally another subset of T helper cells that produces IL-17, known as Th17, has been shown to play an equally important role in EAE pathology [184, 185]. During the induction phase of EAE, MOG autoreactive CD4+ T cells, that escaped central tolerance and are naturally present in the T cell repertoire of C57Bl/6 mice [186], are primed and expanded in the periphery. During the effector phase, activated autoreactive T cells migrate into the CNS, where they are locally reactivated by APCs [187, 188], thus starting a pro-inflammatory loop that recruits additional immune cells. C57Bl/6 mice immunized with MOG present with CD4 T cell and macrophage infiltrations in the white matter of the spinal cord, with minimal

brain inflammation. In MS patients, however, the vast majority of myelin lesions are found within the brain parenchyma and equivalent numbers of CD8 T cells are found alongside with CD4 T cells, both playing critical roles in the disease pathology [109, 189].

EAE has proven to be a valuable tool in the development of therapies that are now being successfully used to treat MS [190] but, since EAE does not mirror all the features found in MS patients, many successful EAE treatments are not effective in MS patients. This indicates that a better mouse model that recapitulates all MS feature is needed to improve our ability to develop effective drugs.

1.9 Epstein-Barr virus and systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a heterogeneous multi-organ autoimmune disease. It affects between 2 and 4 million people worldwide [191]. It has been calculated that between 400 and 800 SLE patients die every day world-wide [191]. SLE patients, 90% of whom are women, develop autoantibodies against different components of the cell's nucleus such as DNA, histones and chromatin. Deposition of immune complexes (antibody and cognate antigens complexes) starts an immune response in the target organ leading to tissue inflammation and damage [192]. Skin, kidneys, lungs, brain and heart are the most affected organs. Anti-DNA autoantibodies bound to nuclear material preferentially deposit in the kidneys leading to glomerulonephritis and kidney failure in SLE patients [193].

Genetic predisposition and environmental factors have both been shown to be important contributors to SLE. However, the trigger that leads to the production of autoantibodies and immune dysregulation is currently unknown [192, 194]. The concordance rate of SLE between monozygotic twins is 24% [195, 196] indicating that genetic susceptibility alone is not enough to start the disease process but it is clearly important since the concordance rate of SLE between dizygotic twins is only 2%. Different viruses have been indicated as environmental triggers of SLE [197, 198] and Epstein-Barr virus is the most closely associated with SLE development.

1.9.1 Association between SLE and EBV in epidemiological studies

EBV seropositivity has been measured in different studies to investigate if an association is present between EBV exposure and development of SLE. Since EBV seropositivity in the adult population is around 90-95%, pediatrics cohorts, which have a lower EBV prevalence, can be more informative, as it has been proven for multiple sclerosis. One of the first studies published showed that 99% of pediatric SLE patients have been exposed to EBV. In contrast only 70% of children in the control cohort have been infected by EBV [199]. Interestingly there was no difference in prevalence between the two cohorts when other herpesviruses were tested. Subsequent serologic results on both children and adults confirmed these early results [196, 200].

1.9.2 EBV replication control and reactivation in SLE patients

Another mechanism by which EBV could trigger SLE is the ability to replicate better or reactivate more frequently in SLE patients. To address this, different studies have been performed in order to assess the EBV viral load in the blood of SLE patients and healthy controls. EBV positive B cells rate normally declines with age but not in SLE patients [201]. Intriguingly SLE flares correlates with increases in the frequency of EBV positive PBMCs [202]. Two additional studies have found that SLE patients have from 15 fold [203] to 40 fold [204] higher EBV viral loads than healthy individuals. In another study, the levels of EBV infected cells in SLE patients were similar to the ones normally found only in immunosuppressed patients, even if the SLE patients included in the study were not taking any immunosuppressive drugs [202]. Additionally, in the same study, an abnormal expression of BZLF-1, LMP-1 and LMP-2a was found in the blood of SLE patients [202]. These findings have been also reconfirmed by more recent work [205]. In this study, Larsen et al also show that increases in EBV viral load always occur one week or more after the onset of a SLE flare. Higher percentages of EBV infected B cells in the blood of SLE patients could contribute to the enhanced autoantibody production.

1.9.3 EBV humoral responses and molecular mimicry in SLE patients

Anti-Sm autoantibodies (where Sm stands for Smith antigen, a set of nuclear proteins) are found in SLE patients and used as diagnostic criteria. Up to 20% of total IgGs is specific for Sm in the sera of some lupus patients [206]. Analysis of serum samples collected before and after SLE diagnosis discovered that SLE patients are initially producing anti-Sm antibodies against the same epitope (PPPGMRPP) [207, 208]. This epitope was found to have homology with EBNA-1 and anti- PPPGMRPP antibodies isolated from SLE patients were able to bind EBNA-1 [208]. Additionally, immunization of rabbits with the EBNA-1 peptide was enough to initiate an anti-Sm antibody response in the immunized animals, thus suggesting that an autoimmune response could start in response to a viral protein structure. This discovery made EBV and its protein EBNA-1 good candidates as SLE environmental triggers. Further work

showed similar results when the Ro antigen, another nuclear antigen, was analyzed [209-211]. Anti-Ro antibodies are produced by SLE patients at an early stage of the disease. The initial epitope recognized by the anti-Ro antibody response cross reacts again with an EBNA-1 peptide, even if there is no sequence homology in this case. Intriguingly, mice immunized with the viral peptide progressively develop antibodies specific for multiple Ro epitopes and other autoantigens, developing symptoms of SLE such as renal dysfunction [209]. Interestingly, it has been shown that the antibody response against EBNA-1 is directed to the large alanine-glycine repeat in healthy individuals infected by EBV. On the other hand, EBNA-1 specific antibodies in SLE patients are directed against other EBNA-1 epitopes that are cross-reactive with Ro and Sm [210]. These cross-reactive antibodies have also been found in mononucleosis patients [211]. However anti-EBNA-1/autoantigen cross-reactive antibodies disappear after mononucleosis resolution in healthy individuals but they persist in SLE patients [211].

Intriguingly, there are some reports that show that mononucleosis can induce the onset of SLE symptoms [212-214]. One can speculate that, after EBV infection, SLE patients are not able to control the production of autoantibodies that continue on increasing in number and on expanding their specificities to other antigens over time, until overt clinical disease manifests.

1.9.4 EBV T cell responses in SLE patients

As discussed above, SLE patients have an impaired control of EBV replication. This could be due to an impaired ability of the EBV specific T cell response to control viral reactivation in SLE patients. In fact, an early study found that T cells from SLE patients are not as efficient as T cells from healthy controls in controlling EBV infection of autologous B cells [215]. Kang et al. detected increased frequencies of EBV-specific CD4+ IFN-γ+ T cells in SLE patients but decreased frequencies of EBV-specific CD8+positive IFN-γ+ T cells suggesting that impaired control of EBV replication could be due to impaired EBV specific CD8 immunity [204]. Similar results were found in a subsequent study [216] where the absolute frequency of EBV-specific CD8 T cells were increased in SLE patients but their function was impaired, especially in IFN-γ

production, as detected by Kang et al.

More recent work have shown that the frequency of polyfunctional EBV specific CD8 T cells capable of producing IFN-γ, TNF-α, IL-2 and MIP-1β upon antigen stimulation is decreased in SLE patients. Moreover EBV specific CD8 T cells from SLE patients are less efficient in the exocytosis of cytotoxic granules than CD8s isolated from healthy control. This functional impairment was shown to be due to increased expression of PD-1 on EBV specific CD8 T cells and could be reversed through PD-1 blockade [205].

Overall, these data show that EBV is closely linked to SLE development. Additionally several EBV proteins make this virus a good candidate to be the environmental trigger of SLE. EBV produces a homologue of IL-10 than can stimulate antibody production and also it carries a homologue of bcl-2 that is a survival factor and it can thus render B cells that are secreting autoantibodies resistant to apoptosis. Finally, both LMP-1 and LMP-2a inhibit apoptosis and promote B cell survival. In fact LMP-1 is a homologue of CD40 (provides a co-stimulatory signal when is activated) and LMP-2a interacts with the BCR receptor providing enhanced survival signal to the B cell that is expressing it [217, 218].

1.10 A mouse model of lupus: New Zealand black and white hybrid mouse

New Zealand black/white hybrid (NZBWF1 or NZBW) mice are a F1 hybrid obtained from the crossing of a New Zealand black (NZB) with a New Zealand white (NZW) mouse. The hybrid was first described in 1961 by Heyler and Howie [219] as the first mouse model that resembled human SLE. While both NZB and NZW display limited autoimmunity, NZBW hybrids develop an autoimmune disease that includes: lymphadenopathy, splenomegaly, elevated serum antinuclear (DNA, histones and chromatin) autoantibodies and immune complex-mediated glomerulonephritis [220, 221]. As in SLE patients, disease in NZBW mice is biased in favor of females. In fact, the mean survival time for female NZBW mice is 245 days, conversely it is 406 days for male NZBW mice [221]. For the first three months of life, NZBW mice are clinically normal [220], but starting from 4-5 months of age the titer of anti-nuclear antibodies increases and switches from IgM to IgG and subsequently from IgG1 to IgG2 subclasses. The onset of glomerulonephritis is associated with the appearance of anti-nuclear antibodies IgG2a and IgG3, which are the most pathogenic [222-224]. Glomerulonephritis appears in females around 6 months of age, with 50% mortality at 8.5 months of age [225]. Moreover both the production of autoantibodies and the development of glomerulonephritis are dependent on IFN-γ [226-228] and CD4 T cells. In fact, depletion of CD4 T cells, either through depleting antibody administration or development of CD4 KO in NZBW mice, leads to downregulation of autoantibody production and protection from glomerulonephritis development [229-234]. On the other hand the role of CD8 T cells in NZBW mice is still controversial. In fact CD8s have been shown to have both a pathogenic and a regulatory function [229, 235]. A number of studies have investigated the cytokine profiles of cloned T cells from NZBW mice to identify if a skewing towards a certain type of T helper response was present [236-238]. From these studies, it emerged that a distinct skewing of cytokines towards either a Th1 or a Th2 phenotype was not evident before SLE development. A number of B cell defects have been also characterized in the NZBW mouse [221, 239]. Increase in B cell numbers has been detected early in life. This is accompanied by premature maturation at an accelerated rate that results in increased percentages of mature B cells earlier in life and an accelerated loss of B cell precursors later in life [240, 241]. Interestingly, if a nuclear autoantigen, such as DNA, binds simultaneously a self reactive DNA specific

BCR and a TLR (like TLR-9) on the same B cell, it can trigger both BCR and TLR signaling pathways, reinforcing activation of autoreactive B cells [242]. As such, TLR-9 that synergistically activates B cells along with BCR engagement has been shown to be important for the production of autoantibodies. Activation of TLR-9 leads to enhanced disease [243], conversely TLR-9 KO have decreased autoantibody levels [244].

1.11 Objectives, hypothesis and aims

The overall objective of this thesis is to determine the link between latent gammaherpesvirus infection and the development of autoimmunity.

Two different mechanisms could be exploited by EBV to trigger autoimmune diseases: a direct mechanism or an indirect mechanism. The direct mechanism relies on the infection of the organ or a subset of cells in the organ where autoimmunity develops. As such, I hypothesized that EBV could trigger MS by infecting endothelial cells of the BBB. Reactivation from latency could upregulate an inflammatory response in endothelial cells and permeabilize the barrier leading to the recruitment of the first wave of inflammatory cells that will start to damage myelin, resulting in the first MS insult. The indirect mechanism relies on EBV influence on antigen presentation in the periphery. As such, I further hypothesized that EBV could affect antigen presentation. In individuals that already have a critical number of autoreactive T cells or other immune genes that predispose them to the development of autoimmunity, an alteration of antigen presentation, especially autoantigens presentation, could affect the skewing of the T helper response and the frequencies of regulatory T cells, thus tipping the balance towards autoimmunity.

Experiments were designed to determine if EBV was able to infect human primary endothelial cells isolated from the blood brain barrier of healthy individuals and whether EBV infection was able to cause a pro-inflammatory response in these cells. To address this, after having confirmed the ability of EBV to infect these cells, I measured the levels of different pro-inflammatory markers on EBV infected brain endothelial cells and I tested the degree of immune cell adhesion on an EBV infected endothelial monolayer.

In order to fully address the global interaction between latent gammaherpesviruses, the host's immune system and development of autoimmunity, I developed two in-vivo models to study the role of EBV infection in the development of MS and SLE. I infected with the murine homolog of EBV two previously described mouse models of MS and SLE, EAE and NZBW mice respectively. I then studied the development of autoimmunity in these latently infected mice assessing disease progression, organ damage and different aspects of the immune response. Additionally, in the EAE model, experiments were designed to address the role of specific APCs in the modulation of the T helper response in a latently infected host. Finally, I also

performed the same experiments using different herpesviruses to demonstrate that acceleration of autoimmunity was a phenomenon specific for latent gammaherpesvirus infection only.

2 Materials and methods

2.1 Primary cultures of human brain microvascular endothelial cells (HBMECs) and other cell lines

Primary cultures of HBMECs were established from normal brains at autopsy as previously described [245]. The endothelial origin and purity of these cells were confirmed by the strong positive staining for Factor VIII-related antigen and binding of Ulex europeaus agglutinin. Primary HBMECs cultures retained important morphological and functional characteristic of their in vivo counterparts, mainly, interendothelial tight junctions that are impermeable to proteins, high electrical resistance and paucity of cytoplasmic vesicles [245, 246]. All experiments were performed using primary HBMECs cultures at passage 0-1 from six different donors. HBMECs were plated on fibronectin coated tissue culture plates and cultured in medium 199 (M199) (Invitrogen) supplemented with 10% horse serum (Cocalico Biologicals), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (all from Invitrogen), 20 µg/ml endothelial cell growth supplement and 100 µg/ml heparin (both from Sigma). B95.8, a marmoset EBV positive B cell line, and an EBV positive human lymphoblastoid cell line (LCL) were obtained from Dr. R. Tan and were maintained in RPMI 1640 with L-glutamine (Invitrogen) supplemented with 10% FBS (Invitrogen). PBMCs were isolated from human blood using Histopaque-1077 (Sigma) according to manufacturer's instruction and stored in liquid nitrogen.

2.2 EBV viral stock

EBV was harvested from the supernatant of B95.8 cells by centrifugation at 1200 rpm for 10 min and stored at -80°C. SYBR green real-time PCR was performed to quantify the number of EBV genome copies in the viral stock. Briefly, DNA was extracted from the viral stock using a QIAamp DNA Mini Kit (Qiagen) following manufacturer's instructions. Quantitated EBV DNA for the standard curve was purchased from Advanced Biotechnologies. Primers specific for the EBER gene were used [247]. Ten fold serial dilutions of the standard and the viral stock DNA were run in triplicate with 0.4 μM of each primer and 12.5 μl of IQ SYBR green mix (Biorad) in a final volume of 25 μl. The reaction was incubated at 95°C for 3 min followed by 35

cycles at 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. Fluorescence was measured after the extension step. Melting curve analysis (65-95°C at a rate of 0.5°C/sec) and visualization of the PCR products on a 2% agarose gel were performed to ensure specific amplification.

2.3 EBV infection of HBMECs, DNA extraction and PCR

HBMECs were grown to 40-50% confluence on a 24-well plate and infected with 5 copies of EBV genome per each plated cell for 1 hour at 37°C or mock-infected with M199 alone for 1 hour at 37°C. After incubation, cells were provided with fresh media to ensure removal of viral nucleic acid contamination. Subsequently media was changed every other day. At 5 days PI, after several PBS washes, DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) following manufacturer's instructions. A PCR was performed using primers specific for EBV genome [247]. 5 μl of DNA were added to a PCR mix composed of 0.2 mM dNTPs, 0.4 μM of each primer and 2.5 U of Pwo SuperYield DNA polymerase (Roche). The reaction was incubated at 95°C for 2 min followed by 45 cycles at 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min.

2.4 HBMECs viability assay

The LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen) that measures esterase activity was used according to the manufacturer's instruction following the fluorescence microscopy protocol.

2.5 RNA extraction from HBMECs and RT-PCR

HBMECs were infected as described above. At day 9 PI, RNA was extracted using an RNeasy Mini kit (Qiagen) following manufacturer's instructions. On-column DNA digestion was performed using an RNAse-free DNase set (Qiagen). 2 μg RNA was reverse-transcribed into cDNA with a reverse transcription system using random hexamers (Promega) following manufacturer's instructions. 4 μl of cDNA were added to

a PCR mix composed of 0.2 mM dNTPs, 0.4 μM of each primer [7, 248, 249] and 2.5 U of Pwo SuperYield DNA polymerase (Roche). The reaction was incubated at 94°C for 2 min followed by 45 cycles at 94°C for 1 min, a gradient of 60-67°C or 55-62°C (see below for single annealing temperatures) for 1 min, 72°C for 1 min: EBNA-1 (60°C); EBNA-2 (61.3°C); LMP-1 (60.5°C); LMP-2A (55°C); LMP-2B (55°C); EBER-1 (62°C); BZLF-1 (62.5°C); BCLF-1 (65.6°C); GAPDH (61.3°C).

2.6 Cytokines and chemokine analysis of HBMECs and B95.8 supernatants

Protein levels of cytokines and chemokines were measured in HBMECs supernatants and B95.8 supernatants using a human inflammation CBA kit (BD Bioscience) or a human flex set (BD Bioscience), both cross-reactive with non-human primates. Samples were prepared according to the manufacturer's instructions and analyzed on a BDFacsArray with FCAP software (BD Bioscience).

2.7 HBMECs surface ELISA

Confluent HBMECs cultures were infected with EBV as described above. Cells were fixed with 0.025% glutaraldehyde for 10 min and incubated for 1 hr with a mouse anti-human ICAM-1 or VCAM-1 monoclonal antibody (Beckam-Coulter) diluted 1:40 in PBS containing 5% BSA and 4% NGS. Cells were washed and incubated with a peroxidase-conjugated goat anti-mouse IgG (1:2,000, Jackson ImmunoResearch) for 1 hr. After three PBS washes, OPD dissolved in citrate phosphate buffer pH 5 was added for 45 minutes. The reaction was stopped with 2M H_2SO_4 and the absorbance was read at 490 nm.

2.8 HBMECs adhesion assay

Confluent HBMECs cultures were infected with EBV as described in paragraph 2.3. 48 hours post infection and 24 hours post TNF-α treatment (100U/ml, used as

positive control to maximize the expression of adhesion molecules), HBMECs cultures were washed with 10% horse serum in M199 twice. The media was then replaced with 50 μl of PBMCs suspension (10^6 cells/ml) in RPMI 1640 with 10% AB serum. HBMECs were incubated with PBMCs for 1hr at 37°C. Cell monolayers were washed three times with M199, once with PBS to remove non-adherent PBMCs and then fixed with 3% paraformaldehyde for 20 min at RT. Adherent PBMCs were stained as previously described [250]. Briefly, the endogenous peroxidase was blocked for 30 minutes at RT with 3% H_2O_2 in methanol. HBMECs were incubated with a mouse anti-human CD45 (1:70, Dako) for 1 hr and then with a peroxidase-conjugated goat anti-mouse IgG for 1 hr (1:200, Jackson ImmunoResearch). Finally DAB was added for 20 min at 4°C and the monolayers were counterstained with hematoxylin (Vector Laboratories). The number of lymphocytes bound per mm^2 was determined by counting the number of adherent lymphocytes in five randomly selected fields.

2.9 Animals

Chapter 4: IL-6 deficient C57BL/6 (IL-6KO); C57BL/6 wild type and 2D2 mice were purchased from Jackson Laboratory (Bar Harbor, ME). These mice were maintained in the Wesbrook Animal Facility or in the Centre for Disease Modeling at the University of British Columbia (Vancouver, Canada).

Chapter 5: $NZBWF_1$ (called NZBW for brevity in the rest of the text) mice were purchased from Jackson Laboratory (Bar Harbor, ME). These mice were maintained in the Centre for Disease Modeling at the University of British Columbia (Vancouver, Canada). For kidney failure assessment, the levels of proteins in the urine were measured using Chemstrip GP (Roche).

2.10 Infections, cidofovir administration and EAE induction

Mice were infected intraperitoneally (i.p.) between 7-10 weeks of age with 10^4 pfu of γHV-68 WUMS strain (purchased from ATCC, propagated on BHK cells); or 10^4 pfu of LCMV Armstrong strain 53b (originally acquired from Dr. M.B. Oldstone, propagated on BHK cells); or 2,500 pfu of MCMV (from ATCC, generous gift of Dr. S.M. Vidal); or

10^4 pfu of HSV-1 McIntyre strain (generous gift of Dr. E. Pryzdial, propagated on Vero cells); or 200 μl of MEM as a control.

Cidofovir (Vistide; Gilead Sciences) was diluted in PBS and filter sterilized. It was administered subcutaneously in the scruff of the neck at a dose of 25 mg/kg or 15 mg/kg as previous reports [251, 252]. Mice were given a two days loading dose four weeks post γHV-68 infection and 10 days before EAE induction. After, mice were injected every 3rd day until day 15 post EAE induction when they were euthanized.

EAE was induced 35-40 days post infection by injecting subcutaneously each mouse with 100μl of emulsified complete Freund's adjuvant (DIFCO) with 200μg of MOG 35-55 (GenWay biotech, purity > 95%) and 400 μg of desiccated Mycobacterium tuberculosis H37ra (DIFCO). Mice also received two i.p. injections with 200 ng of pertussis toxin (List Biologicals) at the time of immunization and 48 hours later. Mice were scored on a scale of 0 to 5: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 2 loss of coordinated movements; 2.5; one hind limb paralyzed; 3, both hinds limbs paralyzed; 3.5, hind limbs paralyzed, weakness in the forelimbs; 4, forelimbs paralyzed; 5, moribund or dead.

2.11 Limiting dilution assay for γHV-68

At indicated time points post EAE, mice were euthanized and spleen harvested. A single cell suspension was generated after RBC lysis. A limiting dilution assay was performed, as previously described [253], to analyze the amount of ex-vivo reactivation (using live splenocytes) and the amount of pre-formed virus (using lysed splenocytes through one cycle of freeze-thaw). Splenocytes were plated on a 96 well plate on a monolayer of mouse embryonic fibroblasts (MEF). Twelve 2-fold serial dilutions were prepared starting at 10^5 cells/well. Twelve wells were plated for each dilution. Plates were incubated at 37°C for 15-20 days and then scored for cytopathic effect.

2.12 Nested PCR for γHV-68

DNA was extracted from brains, spinal cords and spleens of perfused mice at indicated time points before and post EAE induction using QIAamp DNA mini kit

(QIAGEN) following manufacturer's instruction. A nested PCR to detect γHV-68 was performed as previously described [97]. Briefly, 2 μl of DNA were added to a PCR mix composed of 0.2 mM dNTPs, 0.4 μM primers and 2.5 U of Taq polymerase. PCR cycles were the following: 95°C for 2 min, followed by 20 cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min followed by 7 minutes at 72°C. The primers used were 5' CCA TCT AGC GGT GCA ACA TTT TCA TTA C 3' (forward) and 5' TTT ACT GGG TCA TCC TCT TGT TTG GG 3' (reverse). Then, 2.5 μl from the previous PCR reaction were used for the second PCR reaction using the following internal primers with the same cycles: 5' CGA ACA ACA ATC CCA CTA CAA TTA TGC G 3'(forward) and 5' GTA TCT GAT GTG TCA GCA GGA GCG TC 3' (reverse). The samples were run on a 2% agarose gels (Invitrogen) and the 462 bp expected band was visualized using SYBR safe (Invitrogen).

2.13 Immune cell isolation, staining and flow cytometry

Chapter 4: mice were perfused with 30cc of PBS and spinal cords, brains, spleens, inguinal and cervical lymph nodes were isolated at indicated time points post EAE induction. A single cell suspension was generated from all the organs. Immune cells were further isolated from the CNS using a 30% Percoll gradient.

Chapter 5: at the onset of kidney failure (8 months of age), mice were euthanized, spleens were harvested and a single cell suspension was generated.

For intracellular staining, CNS mononuclear cells, splenocytes and lymph node cells were stimulated for 4 hours in IMDM (Gibco) containing 10% FBS (Gibco), GolgiPlug (BD Biosciences), 10 ng/ml PMA and 500 ng/ml ionomycin; or for 24 hours with MOG peptide (100 μM) with addition of GolgiPlug during the last 5 hours of incubation.

Cells were incubated with Fc block (BD Biosciences) on ice for 15 minutes for both surface and intracellular staining. Antibodies for the cell surface markers were added to the cells in PBS with 2% FBS for 30 min on ice. After washing, if intracellular staining needed to be performed, cells were resuspended in Fix/Perm buffer (eBiosciences) for 30-45 min on ice, washed twice and incubated with Abs for intracellular antigens (cytokines and transcription factors) in Perm buffer (30 min, on

ice). Fluorescently conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone N418), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD19 (clone eBio1D3), CD3 (clone eBio500A2), CD45 (clone 30-F11), CD44 (clone IM7), CD62L (clone MEL-14), CD69 (clone H1. 2F3), CD21 (clone eBio809), CD23 (clone B3B4), IgM (clone eB121-15F9), CD80 (clone 16-10A1), CD86 (clone GL1), CD40 (clone 1C10), MHC I (clone 28-14-8), MHC II (clone M5), IL-6 (clone MP5-20F3), IL-10 (clone JES5-16E3), IL-17 (eBio17B7), IL-4 (clone 11B11), IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), T-bet (clone eBio4B10), ROR-γt (clone AFKJS-9), Foxp3 (clone FJK-16s), granzyme B (clone 16G6) were all purchased from eBiosciences. Anti IL-12 antibody (clone C15.6) was purchased from BD Biosciences. Anti-CCR5 (clone HM-CCR5), CXCR3 (clone CXCR3-173) and CD138 (clone 281-2) antibodies were purchased from Biolegend. Samples were acquired using a FACS LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc).

2.14 Tetramer staining

Immune cells were isolated as detailed above. One million cells were incubated with a mixture of two class I tetramers, both conjugated with APC (provided by the Trudeau Institute Molecular Biology Core Facility). Tetramer p56 (D^b/ORF6$_{487-495}$ AGPHNDMEI) was diluted 1:300 and tetramer p79 (K^b/ORF61$_{524-531}$ TSINFVKI) was diluted 1:400. Cells were incubated for 1 hour at RT and then washed. Surface staining was then performed and cells were fixed in 1% PFA for 20 min on ice before acquisition.

2.15 Histology and immunohistochemistry

Spinal cords and brains harvested from perfused EAE mice and kidneys harvested from NZBW mice were formalin fixed and paraffin embedded. Six-micron thick sections were stained with eosin and hematoxylin (kidneys and spinal cords) or luxol fast blue (spinal cords and brains) (all from Sigma) following standard protocols. Brains from perfused mice and kidneys from NZBW mice were frozen in OCT (Fisher Scientific) and ten-micron thick sections were processed for immunohistochemistry.

Briefly, sections were fixed in ice cold 95% ethanol for 15 min and washed in PBS several times. This was followed by washes in TBS with 0.1% Tween (TBST) and incubation for 10 min with 3% H_2O_2 to block endogenous peroxidase. After washing, blocking buffer was added for 1 h (10% normal goat serum in PBS). Primary antibody was added overnight at 4°C: purified rat anti-mouse CD4, anti-mouse CD8, anti-mouse F4/80 (all from eBiosciences) or biotinylated-anti-mouse IgG (Vector), diluted 1:100 in PBS 2% normal goat serum. After washes in TBST, biotinylated secondary antibody (anti-rat IgG, mouse absorbed, Vector) was added for 1 h, if needed, diluted 1:200 in PBS 2% normal goat serum. After washes in TBST, Vectastain ABC reagent was used (Vector) following manufacturer's instruction. Then, DAB (Sigma) was added as a substrate and, after incubation for 8 min in the dark and several washes in distilled water, sections were counterstained with Harris hematoxylin for 20 seconds, in lithium carbonate for 30 sec, washed in several changes of distilled water and mounted with VectaMount AQ (Vector).

2.16 Cytokines and chemokines analysis

Serum cytokine and chemokine levels were measured at the indicated time points using a mouse inflammation CBA kit (BD Bioscience) for detection of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 or a cytokine flex set (BD Bioscience) allow for detection of CXCL-9 (MIG), CCL-3 (MIP-1α), CCL-4 (MIP-1β), CCL-5 (RANTES), IL-17A, GM-CSF and G-CSF. Samples were prepared according to manufacturer's instructions and analyzed on a BDFacsArray equipped with FCAP software (BD Biosciences). Serum levels of IFN-β were measured using a VeriKine Mouse interferon beta kit (PBL interferon source) according to the manufacturer's instructions. For CNS supernatant analysis, brains and spinal cords were homogenized in 10 mls of FACS buffer to obtain a single cell suspension. The suspension was then centrifuged for 10 min at 1200rpm. A total of 10 mls of CNS supernatant was obtained for each sample, 1 ml was frozen at -80°C and then 50 μl were analyzed using the mouse inflammation CBA kit as detailed above.

2.17 Auto-antibodies ELISA

Nunc immunoplates were coated with 10 μg/ml of MOG peptide, 10 μg/ml of brain extract (purchased from Santa Cruz Biotechnology), 8 μg/ml of histones (purchased from Worthington Biochemical Corporation), 40 μg/ml of dsDNA (purchased from Worthington Biochemical Corporation) or 20 μg/ml of ssDNA (obtained by boiling for 10 min dsDNA) in carbonate buffer and incubated overnight at 4°C. Plates were washed three times with wash buffer (PBS, 0.05% Tween). Blocking buffer (PBS with 1% BSA; 0.05% Tween and 10% FBS) was added in each well for 1 h. After removal of the blocking buffer, day 28 post EAE induction sera samples diluted 1:20 and 1:40 in blocking buffer or NZBW sera samples diluted 1:50 in blocking buffer were added for 1.5 h at RT. After washing, anti-mouse IgG-HRP or IgM-HRP (Sigma), diluted 1:1000 in blocking buffer, were added for 1 h. After washing, OPD (0.2 mg/ml, Sigma) and UPO (0.2 mg/ml) in citrate buffer were added for 30 min and the reaction was either stopped or not with 25% H_2SO_4. Absorbance was read at 490 nm or 450nm respectively. For IgG1/IgG2a ELISA the following antibodies were used: biotin anti-mouse IgG1 (diluted 1:10000 in blocking buffer) and biotin anti-mouse IgG2a (diluted 1:6250 in blocking buffer), both from Jackson Immunoresearch (generous gift of Dr. J. Quandt). In this case, after washing and before adding the substrate, streptavidin-HRP was added (diluted in blocking buffer 1:1000, Jackson Immunoresearch). No secondary antibody controls, sera from healthy mice (for lupus) or sera from non-immunized mice (for EAE) were added as negative controls in each ELISA plate.

2.18 Direct intracellular staining, mixed assays and cell transfers

Sterile Brefeldin A was purchased from Sigma and 250 μg were injected intravenously in mice at day 4 post EAE induction [254]. Six hours later mice were euthanized and spleens and inguinal lymph nodes were harvested. Cells were prepared and stained to detect IL-6, IL-12, TNF-α, IFN-γ and IL-10 as described above. Intracellular staining was performed without any further ex-vivo restimulation.

For mixed assays, spleens and inguinal lymph nodes were harvested at day 4 post EAE induction. A single cell suspension was prepared and stained with anti-CD11c

and anti-CD11b antibodies (see above for details). CD11c+ CD11b+ cells were sorted with a FACSAria cell sorter (BD Biosciences). CD4 T cells from 2D2 mice were isolated from spleens with a CD4 T cells negative selection kit following manufacturer's instructions (STEMCELL technologies). Isolated CD11b+CD11c+ ($2x10^4$/well) and 2D2 CD4 T cells ($5x10^5$/well) were seeded on a 24 well plate in RPMI, 10% FBS and Pen/Strep (all from GIBCO) with or without 10 or 100 μM MOG peptide. After 72 hours, T cells were restimulated with PMA, ionomycin and GolgiPlug and stained for CD4 and IFN-γ as described above.

For cell transfers, CD11b+CD11c+ cells were sorted as described above. Each mouse received 100,000 cells intra peritoneally. EAE was induced 24 hours post-transfer.

3 Epstein-Barr virus infection of human brain microvascular endothelial cells[2]

[2]A version of chapter 3 has been published: Costanza Casiraghi, Katerina Dorovini-Zis and Marc S. Horwitz. Epstein-Barr virus infection of human brain microvessel endothelial cells: A novel role in Multiple Sclerosis. J. Neuroimmunol. 230(1-2):173-7 (2011).

3.1 Introduction

Despite decades of focused biomedical research, the etiology of multiple sclerosis (MS) is currently not well understood. Epidemiological data and geographic patterns imply that MS is caused by an environmental insult in genetically predisposed individuals [109]. Epstein-Barr virus (EBV) has been identified as a putative environmental trigger of MS. The risk of MS is 20 times greater among people who have contracted infectious mononucleosis, a disease caused by EBV, as compared with seronegative individuals [255]. A steep increase in the titer of antibodies specific for EBV has been detected in the serum of some MS patients: it begins to elevate 10-20 years prior to the onset of symptoms and correlates with the risk of developing the disease [28]. CD4+ T cells that cross-react with both EBV and myelin basic protein and co-produce IFN-γ and IL-2 have been isolated from MS patients [161]. Interestingly, a recent study detected a decreased T cell reactivity against EBV infected B cells in MS patients indicating a dysregulated EBV immune response [158]. Despite this evidence, the mechanism by which EBV would trigger MS remains elusive [256]. Intriguingly, the infection of endothelial cells by other herpesviruses such as cytomegalovirus or Kaposi's sarcoma virus upregulates the expression of cytokines, chemokines and adhesion molecules [257]. Taking a fresh approach, we reasoned that EBV infection of atypical target cells such as endothelial cells may be an essential component to pathogenesis in MS.

In transgenic TCR mouse models, MS is triggered only after the blood-brain barrier (BBB) is compromised [172]. The presence of autoreactive myelin-specific lymphocytes is not sufficient to cause MS since such cells have been isolated from healthy individuals [170]. In MS patients, lesion and plaque formation are associated with a disruption of the BBB. Thus, an increase in BBB permeability, along with autoreactive T cells activation, is a requirement for the development of MS. However, it is unclear how changes in the BBB are initiated prior to the initial immune cell entry into the brain [258].

We hypothesized that EBV infection of a subset of brain endothelial cells would increase the potential for an inflammatory breach of the BBB, particularly following reactivation of latent virus. Prior work has established that EBV can infect macrovascular endothelial cells both in human tissues [259, 260] and in culture with

human umbilical vein endothelial cells (HUVECs) [8, 9]. However, the BBB is composed of microvascular endothelial cells that differ significantly from macrovascular endothelial cells, such as HUVECs, in a number of characteristics, most importantly in terms of susceptibility to viral infection by *Herpesviridae* [261]. To investigate our premise, we asked whether EBV could infect primary human brain microvascular endothelial cells (HBMECs) leading to increased endothelial cell activation that would be relevant for MS pathogenesis.

3.2 Results

3.2.1 EBV infects HBMECs

HBMECs isolated from three different donors were successfully infected with EBV (Figure 3.1) under standard laboratory culture conditions. The viral genome was detected by standard PCR in infected HBMECs and was absent from mock-infected HBMECs, indicating that the donors' endothelial cells were EBV negative (Figure 3.1).

EBV infection did not diminish the viability of HBMECs and this was confirmed using esterase activity assays (Figure 3.2).

Further, EBV gene expression was detected by RT-PCR 9 days post infection (PI). To ensure removal of viral nucleic acid contamination, media was changed every other day between infection and RNA harvest; a non-HBMEC control (virus added on fibronectin coated wells) was performed and primers were designed on different exons [7]. Moreover infection with heat-inactivated EBV [262] did not lead to any detectable viral gene expression (data not shown). Latency and immediate early gene expression were detected in separate experiments with cells derived from two different donors (Figure 3.3), thereby demonstrating viral replication and EBV gene splicing. Expression of BZLF-1 and EBNA-1, both involved in the transactivation of the transcription of other viral genes, were detected PI in HBMECs from one donor; whereas LMP-2B and EBNA-1 were found to be expressed PI in HBMECs from a second donor (Figure 3.3).

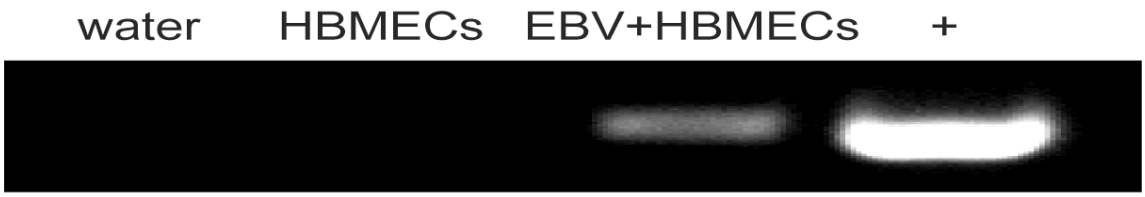


Figure 3.1. EBV is able to infect HBMECs

PCR to detect the presence of viral genome using DNA extracted from mock-infected HBMECs (HBMECs, incubated for 1h with M199), EBV infected HBMECs (EBV+HBMECs) 5 days post infection (PI) and EBV viral stock (+).

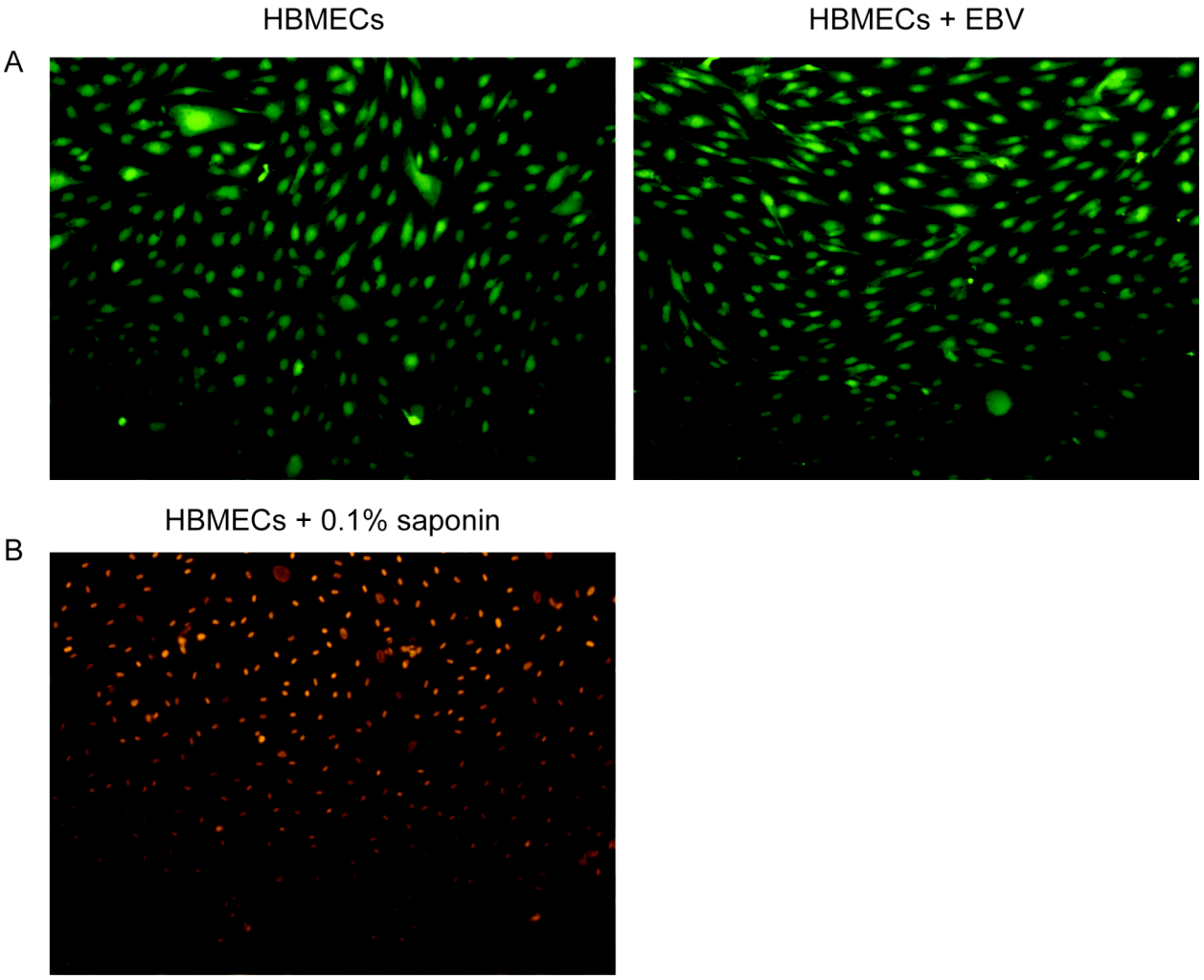


Figure 3.2. HBMECs remain viable after EBV infection

Live-dead cell assay performed 7 days post EBV infection in **(A)** mock-infected HBMECs (left panel); EBV infected HBMECs (right panel) and **(B)** HBMECs treated with 0.1% saponin for 10 minutes, as a positive control for cytotoxicity. The assay is composed of two dyes: calcein AM and EthD-1. Live cells will appear green thanks to the presence of intracellular esterase activity which converts the non-fluorescent calcein AM into the green fluorescent calcein. Conversely, dead cells will appear orange due to the binding of EthD-1 to the nucleic acids. EthD-1 is excluded by the intact plasma membrane of live cells.

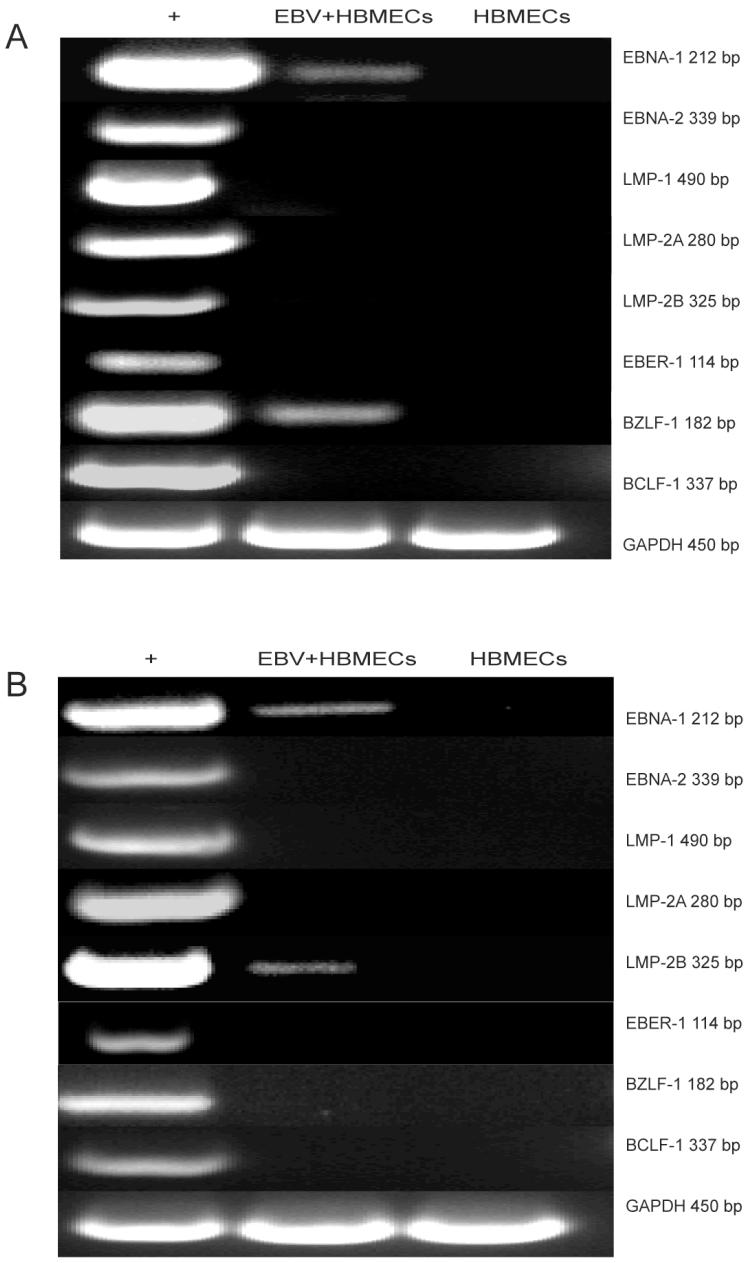


Figure 3.3. Infected HBMECs express both latent and lytic EBV genes

RT-PCR for EBV genes expression (EBNA-1, EBNA-2, LMP-1, LMP-2A, LMP-2B, EBER-1, BZLF-1 and BCLF-1) using RNA extracted from a human lymphoblastoid cell line transformed with EBV (+), EBV infected HBMECs (EBV+HBMECs) 9 days PI and mock-infected HBMECs (HBMECs). **A-B** are two different donors, RNA were pooled from triplicate wells. Results representative of four separate experiments.

3.2.2 HBMECs infected by EBV upregulate pro-inflammatory molecules and support increased immune cell adhesion

We next investigated whether EBV infection of HBMECs could lead to activation and increased production of pro-inflammatory molecules. Uninfected HBMECs expressed basal levels of CCL-2 (MCP-1) and IL-8 (data not shown) consistent with previous reports [263, 264]. Increased CCL-5 (RANTES) production was observed at 24 and 48 hours PI in the culture supernatants (Figure 3.4). Further, the surface expression of the adhesion molecule, ICAM-1, was significantly increased 48 hours PI, whereas VCAM-1 expression was very low in either naive or EBV infected HBMECs (Figure 3.5). Importantly, both ICAM-1 and CCL-5 are involved in firm adhesion of leukocytes to the endothelium [246, 250, 265].

To determine whether the observed EBV-mediated upregulation of ICAM-1 and CCL-5 was sufficient to increase the adhesion of peripheral blood mononuclear cells (PBMCs), we performed PBMC adhesion assays. While only a few PBMCs adhered to naive HBMECs (Figure 3.6A and 3.6D), significantly greater numbers of PBMCs adhered to EBV infected HBMECs (Figure 3.6B and 3.6D, $p<0.0001$). As a positive control, HBMECs treated with TNF-α for 24 hours showed significant levels of PBMC adhesion (Figure 3.6C and 3.6D).

The supernatant used for the infection was obtained from a marmoset B cell line (B95.8) transformed by EBV. To rule out the possibility that cytokines produced by B95.8 cells are contributing to the observed changes in HBMECs, B95.8 supernatant was tested for the presence of proinflammatory mediators using a human kit with cross-reactivity to non-human primates (see Materials and Methods). B95.8 supernatant was positive for IL-10 but negative for IL-6, IL-1β, TNF-α and CCL-5. In summary, EBV infection upregulates inflammatory molecules in HBMECs.

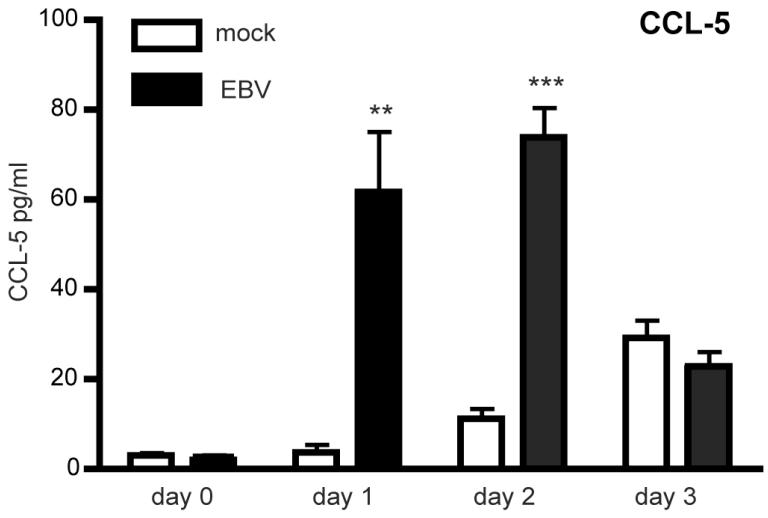


Figure 3.4. EBV infected HBMECs secrete increased amounts of CCL-5 (RANTES) 24-48 hours post infection

CCL-5 expression detected in the supernatant of mock-infected HBMECs (mock, HBMECs incubated for 1h with M199) and EBV infected HBMECs (EBV) before infection (day 0) and every subsequent 24 hours PI (day 1, 2, 3; ** p=0.0018; *** p<0.0001) (triplicate wells from four separate experiments using four different donors). Data analyzed with t-test.

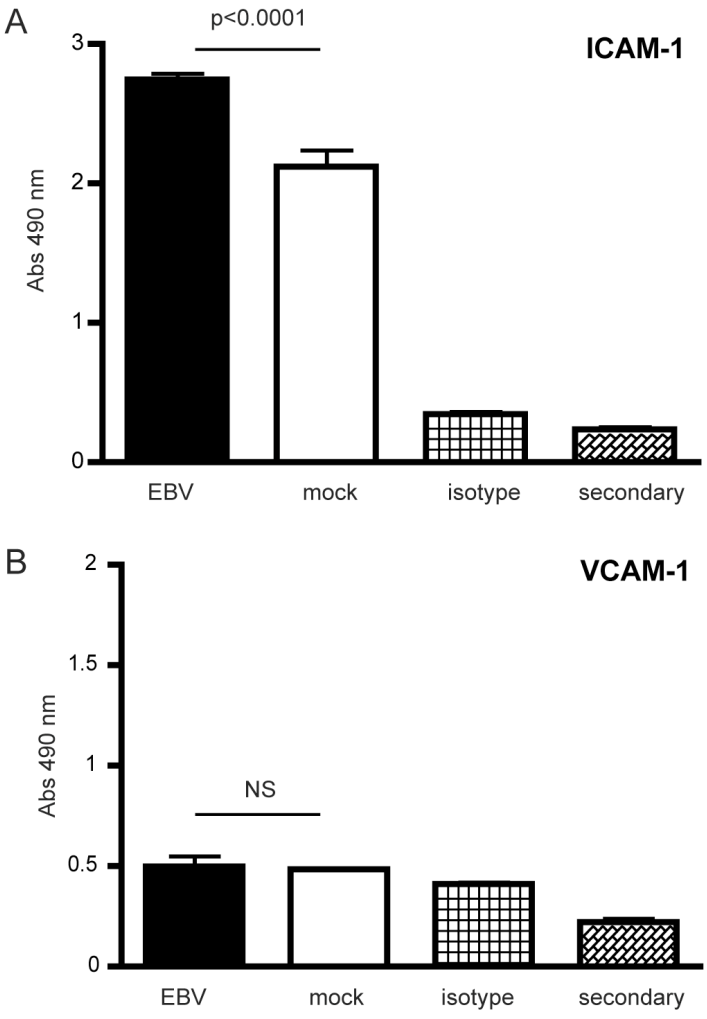


Figure 3.5. EBV infected HBMECs express increased levels of ICAM-1 48 hours post infection

ICAM-1 **(A)** and VCAM-1 **(B)** expression detected with a surface ELISA in EBV infected HBMECs (EBV) and mock-infected HBMECs (mock) 48 hours PI (triplicate wells from three separate experiments using three different donors). An irrelevant antibody control with the same isotype of the ICAM/VCAM primary antibody (isotype) and only secondary antibody control (secondary) were added. Data analyzed with t-test.

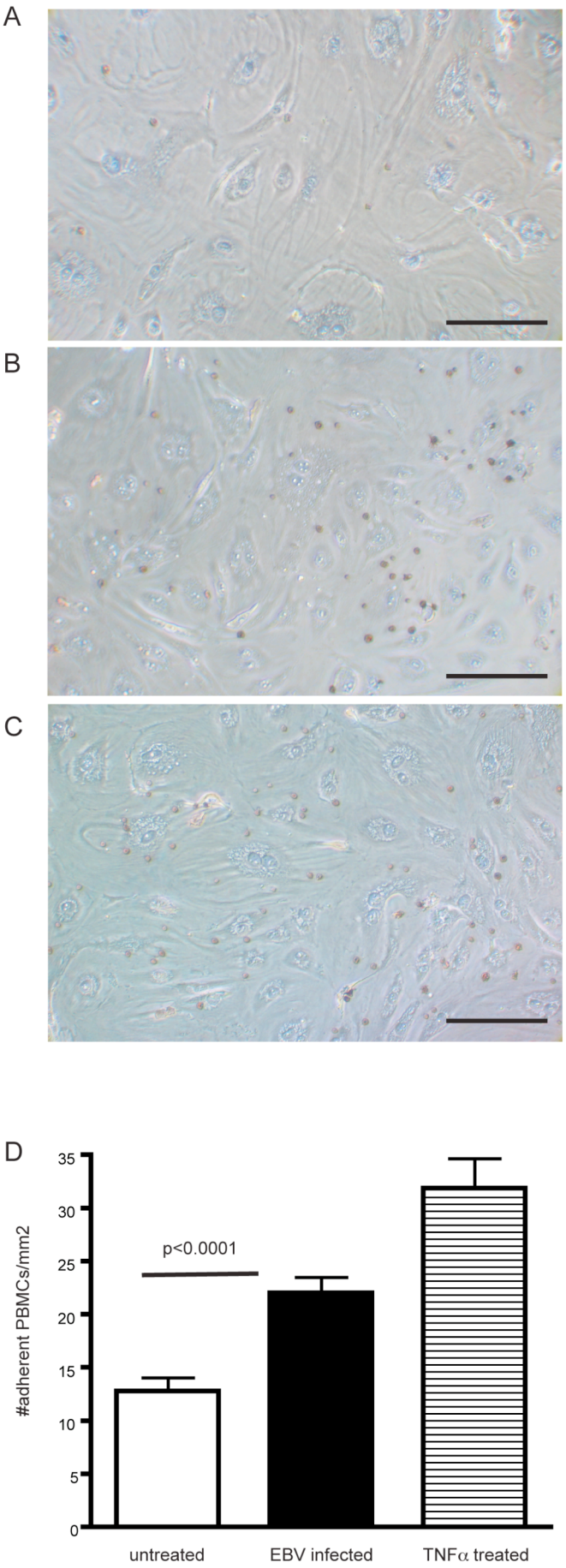


Figure 3.6. Increased PBMCs adhesion on an EBV infected HBMECs monolayer 48 hours post infection

A-C: Adherent PBMCs (brown spots) on 1 mm^2 fields acquired with a 20X objective and a digital camera with a 4X zoom: mock-infected HBMECs monolayer **(A)**, EBV infected HBMECs monolayer 48 hours PI **(B)** and TNF-α (100U/ml for 24 hours) treated HBMECs monolayer (C) as positive control. Scale bar 200 μm. **D**: Quantification of the amount of adherent PBMCs/mm^2 from 5 randomly selected fields (with 1 mm^2 grid) from triplicate wells (three separate experiments using three different donors). Data analyzed with t-test.

3.3 Discussion

This is the first report demonstrating successful EBV infection and gene expression in HBMECs. We found that distinct EBV genes are expressed in HBMECs from different donors (BZLF-1 and EBNA-1 in HBMECs from the first donor and LMP-2B and EBNA-1 from the second donor). BZLF-1 is an immediate early gene that is responsible for the transition from latency to lytic cycle reactivation [12]. EBNA-1 is a latency gene responsible for maintenance of the viral genome during host cell replication [12]. LMP-2B is a latency gene that promotes motility and spreading of epithelial cells [13], while its function in EBV infected B cells is poorly understood [12]. Although these patterns are different from those observed in infected B cells, they are similar to what is found in EBV infected epithelial cells. In fact, primary epithelial cells in vitro have been shown to express EBNA-1, LMPs, and BZLF-1 at day 5 PI with similar levels of variability at the single cell level and with primary cultures from different donors [249, 266].

We also found increased production of pro-inflammatory molecules, such as CCL-5 and ICAM-1, in EBV infected HBMECs that support increased adhesion of PBMCs on their surface. Intriguingly, both CCL-5 and ICAM-1 have been associated with MS. Polymorphisms in CCL-5 and its receptor, CCR5, modify the course and the outcome of MS [267]. The low-producer CCL-5 allele is associated with reduced risk of axonal loss; whereas the high-producer CCL-5 allele is associated with more severe clinical disease. CCL5 expression is upregulated in BBB cells before clinical signs appear [268]. MS derived HBMECs express higher levels of ICAM-1 and circulating leukocytes of MS patients express higher levels of LFA-1, the ligand of ICAM-1 [269]. Therefore, the ability of EBV to upregulate CCL-5 and ICAM-1 in HBMECs in a similar manner to what is observed in the BBB of MS patients, could in part describe the mechanism of MS pathogenesis.

Herein, we demonstrate that EBV infection of HBMECs leads to endothelial cell activation and PBMC adhesion. Based on our observations and the work of others [8, 9, 266], it is our expectation that only a minority of cells is infected by EBV and expresses viral genes. We propose that reactivation of latent EBV infection in brain endothelial cells could upregulate cytokines, chemokines and adhesion molecules that would induce a local breach in the BBB and attract autoreactive lymphocytes into the brain. In

an individual with an increased level of peripheral autoreactive T cells, this could lead to an initial localized entry of immune cells and the development of CNS lesions. In this manner, EBV would only need to infect a small population of HBMECs and reactivation would be required to occur within a minority of these cells. This proposed model would serve to explain the inconsistent detection of EBV infection in MS brains [165-169]. This mechanism further accounts for important features of MS including: lack of detectable virus in the MS plaques; infiltration by macrophages, and both viral and myelin specific lymphocytes; the presence of oligoclonal antibodies in the cerebrospinal fluid and the success of anti-viral treatments like interferon-β on preventing MS relapses.

4 Gammaherpesvirus latency accentuates experimental autoimmune encephalomyelitis pathogenesis[3]

[3] A version of chapter 4 has been published: Costanza Casiraghi, Iryna Shanina, Sehyun Cho, Michael L. Freeman, Marcia A. Blackman and Marc. S. Horwitz. Gammaherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein-Barr virus and multiple sclerosis. PLoS Pathog 8(5): e1002715 (2012).

4.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). In MS, the myelin sheath, which insulates and protects neurons, is attacked and destroyed by the host's immune system leading to progressive disability [109]. MS is considered to be the result of an environmental influence in genetically susceptible individuals. Different environmental triggers have been associated with MS development and viral infections are the most common suspects [109, 255].

To date, Epstein-Barr virus (EBV) has the strongest correlation with MS development [255]. EBV is a DNA virus of the γ-herpesvirus family. It establishes a life-long latent infection mainly in B cells [12], but it has been shown to have the ability to infect other cell types such as epithelial cells, endothelial cells and monocytes [7, 8]. It infects 90% of the human population and it is usually acquired during childhood. When primary infection takes place during adolescence or in adulthood, it leads to infectious mononucleosis [5]. All MS patients are EBV seropositive [124] and individuals with a history of mononucleosis have a 20-fold higher risk of developing MS later in life [255]. Moreover the EBV-specific humoral and cellular immunity seems dysregulated in MS patients: there is an increase in anti-EBV antibodies titer years before MS onset [147], a decreased CD8+ EBV response [158] and the presence of polyfunctional myelin/EBV cross-reactive CD4+ T cells in MS patients [161]. Finally, although still controversial, EBV infected B cells have been found in ectopic follicles in the brain of MS patients [165, 167-169]. Despite all this evidence, it is still not clear how EBV would trigger CNS autoimmunity. Although EBV does not infect rodents, murine gamma herpesvirus-68 (γHV-68) has been a useful tool in studying the relationship between the immune system and latent γ-herpesvirus infection in mice [50].

Experimental autoimmune encephalomyelitis (EAE) is a well-studied and accepted model for the study of MS in rodents [179]. After immunization with myelin peptides emulsified with adjuvants, mice develop ascending paralysis and present with CD4 T cell and macrophage infiltrations in the white matter of the spinal cord, with minimal brain inflammation. In MS, however, the vast majority of myelin lesions are found within the brain parenchyma and equivalent numbers of CD8 T cells are found alongside CD4 T cells, both playing critical roles in the disease pathology [109, 189].

Despite these differences and others, EAE has proven to be a valuable tool in the development of therapies that are now being successfully used to treat MS [190].

Although γHV-68 genome differs from that of EBV, it elicits an immune response in mice that shares many features with EBV. In fact both viruses establish a life long infection in B cells, modulating the immune response of the host, leading to the expansion of a potent CD8 response similar to that detected in humans during mononucleosis [270]. Because of these similarities, we decided to test the impact of a γHV-68 latent infection during the development of EAE. When EAE is induced, mice latently infected with γHV-68 showed a significantly modified disease phenotype that recapitulated aspects of human MS not typically observed in EAE. γHV-68 EAE mice presented with greater ascending paralysis, more neurological symptoms, brain inflammation with myelin lesions driven by a potent Th1 response and CD8 T cell infiltration.

4.2 Results

4.2.1 Latent infection with γHV-68 enhances EAE symptoms without CNS infection or increased viral reactivation

To determine the impact of γHV-68 latency on the development of EAE in mice, C57Bl/6 mice were infected with γHV-68 and allowed to recover for five weeks to enable complete clearance of the acute infection and establishment of latency prior to EAE induction. Viral clearance was demonstrated by absence of plaques on viral plaque assays on spleens harvested on day 35 post infection (p.i.). When tested, mice previously infected with γHV-68 presented with earlier onset of EAE (around day 7-8 post induction), compared with naïve mice induced for EAE that developed paralysis around day 10-12 post EAE induction (Figure 4.1). Moreover γHV-68 EAE mice presented with worse clinical EAE scores (Figure 4.1) and this includes a heightened mortality rate (15%) between day 12-18 post EAE induction (Figure 4.2). To demonstrate the specificity of the disease phenotype to γHV-68 infection, EAE was induced in mice previously infected with lymphocytic choriomeningitis virus (LCMV). LCMV induces a strong well-characterized Th1 response as well as a strong memory CD8 T cell response similar to γHV-68. Mice previously infected with LCMV (LCMV EAE), showed a clinical disease course similar to uninfected EAE mice (Figure 4.3A). At the dose given, LCMV does not establish a latent or persistent infection, so to further test the γHV-68 specificity of our phenotype, mice were infected with a β-herpesvirus (murine cytomegalovirus, MCMV), capable of establishing a latent infection similar to γHV-68. MCMV EAE mice developed EAE with the same clinical disease course as uninfected EAE mice and showed 100% survival after immunization (Figure 4.3B). Additionally, EAE was induced in γHV-68 mice without administering pertussis toxin (γHV-68 MOG CFA). Interestingly, γHV-68 MOG CFA mice (Figure 4.4, blue line) developed milder paralysis than γHV-68 EAE mice, yet still displayed a disease course similar to EAE alone (Figure 4.4, black line). These data clearly demonstrate that this phenotype is a feature specific for γHV-68 latent infection as only latent γHV-68 infection confers susceptibility to a more severe form of EAE that includes a heightened level of mortality.

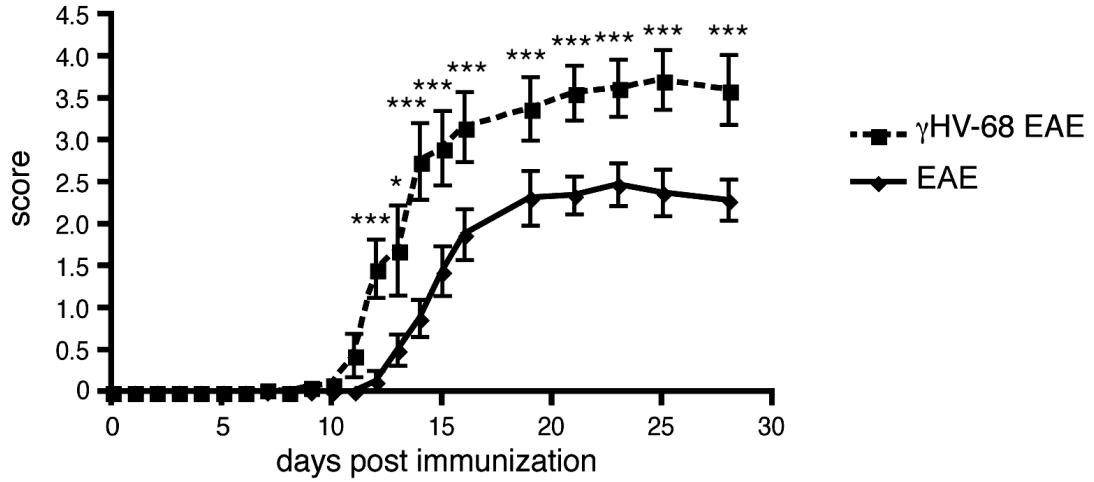


Figure 4.1. γHV-68 enhances EAE symptoms

Mice were infected with γHV-68 (dashed line) or MEM only (solid line). Five weeks p.i. EAE was induced (day 0 on the graph). The graph shows EAE scores up to day 28 post EAE induction (three separate experiment, n=16/group). Data analyzed with two-way ANOVA followed by Bonferroni's post test; *** p<0.001; ** p<0.01 * p<0.05.

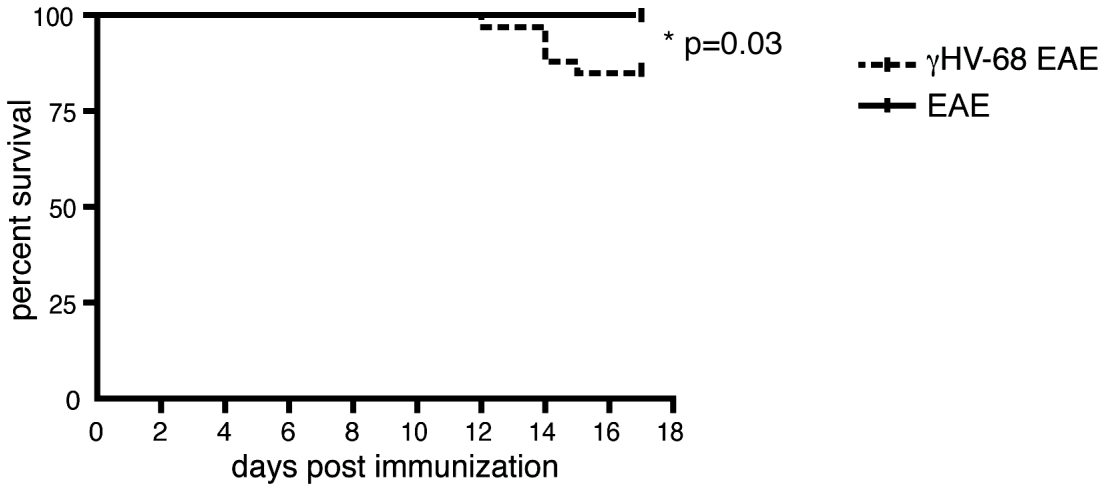


Figure 4.2. γHV-68 EAE mice show increased mortality

Mice were infected with γHV-68 (dashed line) or MEM only (solid line). Five weeks p.i. EAE was induced (day 0 on the graph). The graph shows survival up to day 18 post EAE induction (four-five separate experiments, n=28/group). Data analyzed with Kaplan-Meier analysis.

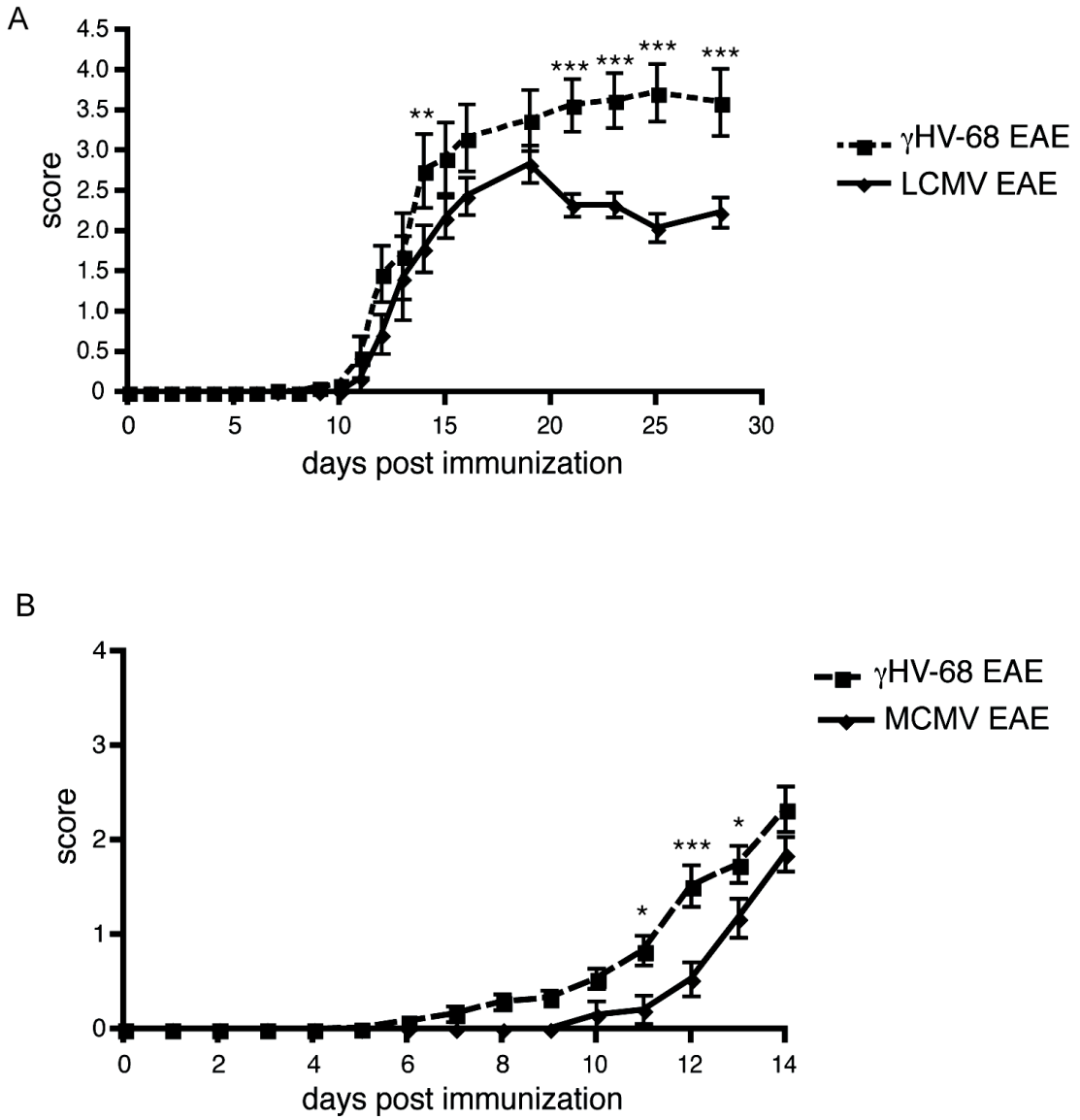


Figure 4.3. Infections with other viruses do not alter EAE clinical course

Mice were infected with γHV-68 (dashed line) or LCMV **(A)** or MCMV **(B)** (solid lines). Five weeks p.i. EAE was induced (day 0 on the graph). The graphs show EAE scores up to day 28 or 14 post EAE induction (three separate experiment, at least n=16/group). Data analyzed with two-way ANOVA followed by Bonferroni's post test; *** $p<0.001$; ** $p<0.01$ * $p<0.05$.

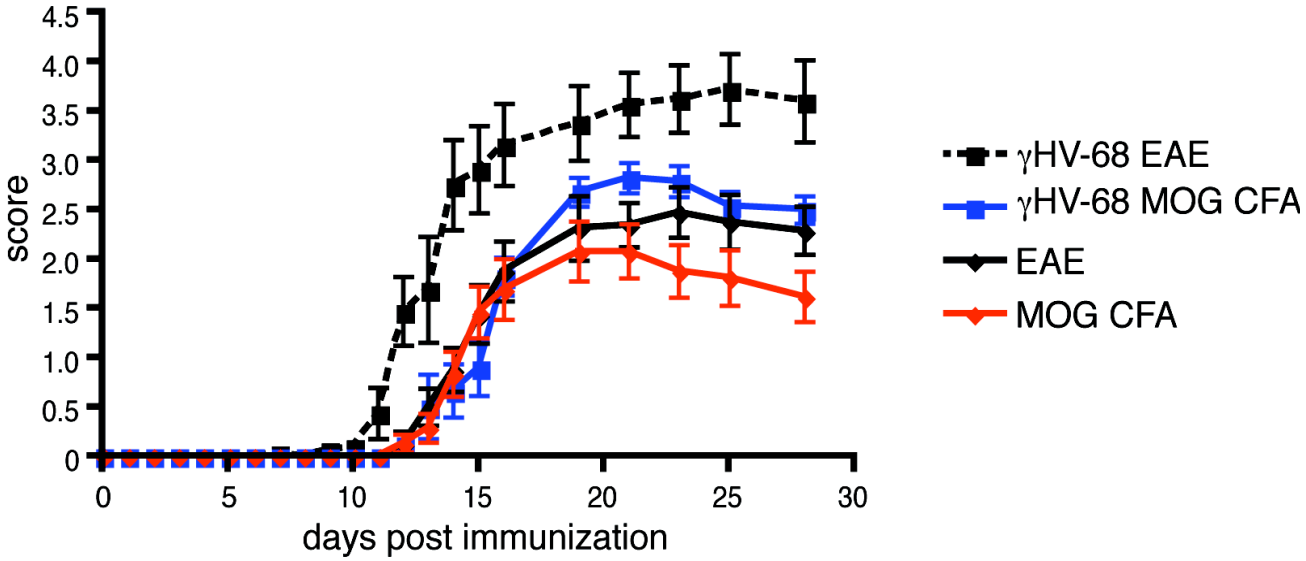


Figure 4.4. γHV-68 enhances EAE symptoms also when EAE is induced without pertussis toxin

Mice were infected with γHV-68 (dashed black line and blue line) or MEM only (solid black line and red line). Five weeks p.i. EAE was induced (day 0 on the graph) with (EAE) or without (MOG CFA) pertussis toxin. The graph shows EAE scores up to day 28 post EAE induction (three separate experiment, n=16/group).

Previous work has demonstrated that reactivation of γHV-68 in latently infected mice occurs following treatment with toll like receptors (TLR) ligands such as poly I:C or LPS [77]. For EAE induction, mice are injected with complete Freund's adjuvant (CFA) and pertussis toxin (PTX), which contain TLR ligands. To determine whether the increased EAE signs observed were due to increased viral reactivation and replication, limiting dilution assays were performed to allow for the simultaneous quantification of ex-vivo γHV-68 reactivation (Figure 4.5A) and pre-formed virus (Figure 4.5B). γHV-68 EAE mice showed a similar extent of ex-vivo reactivation and similar amount of pre-formed virus in the spleen both at day 7 and day 14 post EAE induction when compared to γHV-68 infected mice (5-7 weeks post infection) without EAE induction. TLR ligands present in the CFA are not reactivating more γHV-68 and enhanced EAE scores are not due to increased viral replication.

To further confirm the lack of dependence on viral replication/reactivation, EAE was also induced in uninfected IL-6KO mice and in γHV-68 IL-6KO mice. IL-6KO mice are resistant to EAE induction and both latently infected and uninfected mice retained this resistance (Figure 4.6). IL-6 is not required to control γHV-68 infection but it is critical for EAE induction. γHV-68 IL-6KO mice retain EAE resistance indicating that the heightened clinical disease observed in γHV-68 mice is not solely due to an increased viral replication upon EAE induction. Moreover, γHV-68 mice were treated with cidofovir, a drug known to suppress γHV-68 replication [251], before and after EAE induction. γHV-68 mice treated with cidofovir showed no differences in phenotype and clinical disease as compared to untreated γHV-68 mice (data not shown).

Finally, to ask whether virus replication was detectable in the CNS, DNA was extracted from brains and spinal cords and the presence of viral DNA was assessed using the most sensitive PCR approach (nested PCR; Figure 4.7). All brains and spinal cords tested negative for γHV-68 DNA following EAE induction. Overall these results indicate that the observed phenotype is not the result of a reactivated replicating virus and that latent γHV-68 infection is inducing greater disease through an indirect mechanism.

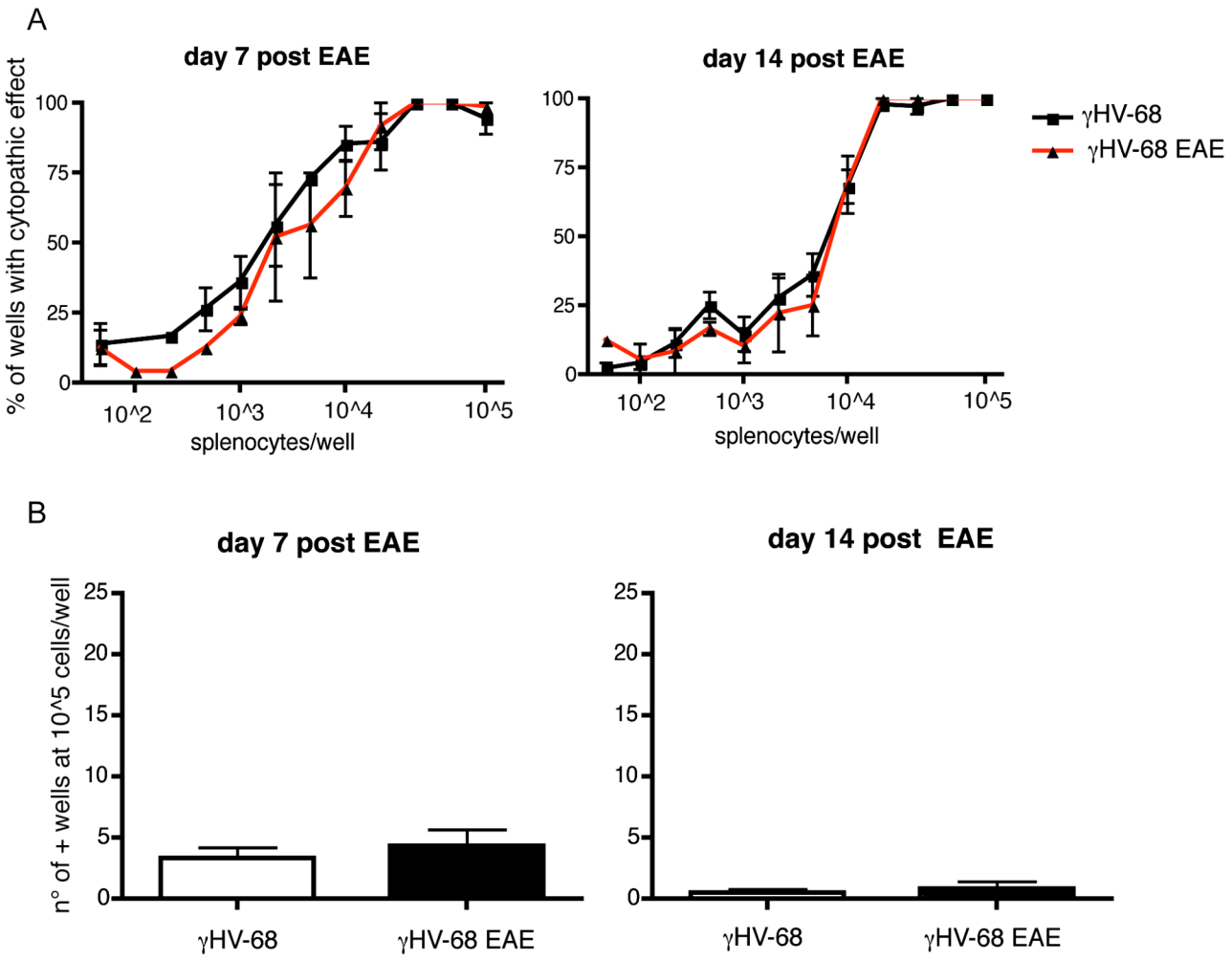


Figure 4.5. γHV-68 does not reactivate more upon EAE induction

Mice were infected with γHV-68. Five weeks p.i., EAE was induced (red line with triangles, black bar) or not induced (black line with squares, open bar). At day 7 (left panel) and day 14 (right panel) post EAE induction, spleens were harvested and a limiting dilution assay was performed using **(A)** live splenocytes to assess the amount of ex-vivo viral reactivation and **(B)** lysed splenocytes to assess the amount of pre-formed virus (three separate experiments using 3-4 mice/group).

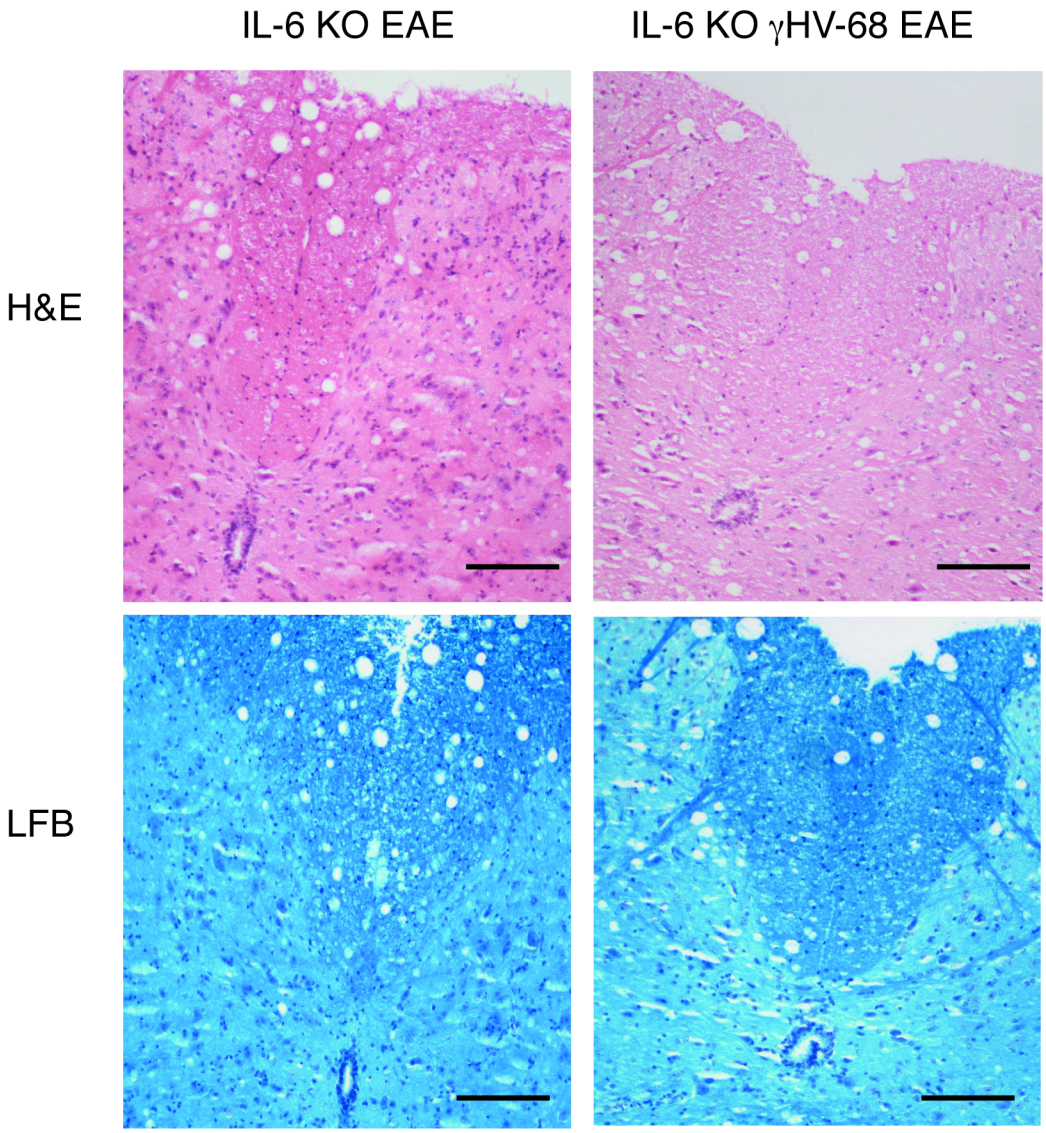


Figure 4.6. γHV-68 IL6-KO mice are resistant to EAE induction

C57Bl/6 mice IL6-KO were infected with γHV-68 (right panels) or MEM only (left panels). Five weeks p.i., EAE was induced. Both groups did not develop any signs up to day 40 post EAE induction. At this timepoint, mice were perfused and spinal cords were harvested and fixed in formalin. Upper panels show H&E staining, lower panels show luxol fast blue staining to assess myelin damage. Two separate experiments with 6 mice/group. Scale bar: 100 μm.

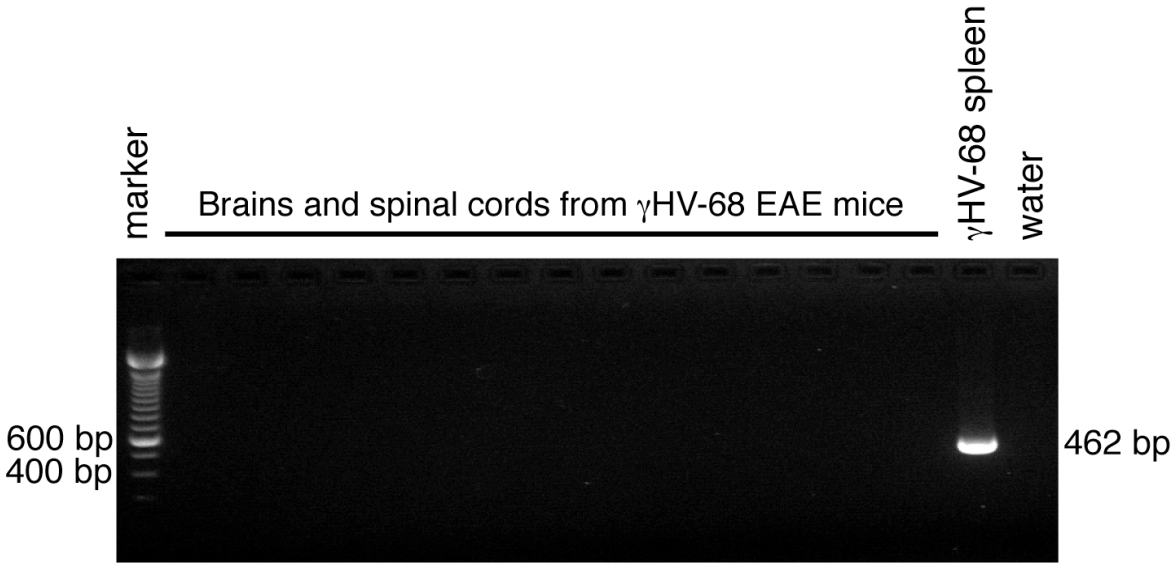


Figure 4.7. γHV-68 does not infect the CNS

Brain and spinal cords were harvested from perfused γHV-68 EAE mice at different timepoints pre and post EAE (day 0 before EAE induction; day 3; day 7 and day 14 post EAE). DNA was extracted and a nested PCR was performed to detect γHV-68 DNA. A spleen was used as a positive control. The figure shows a representative gel of three separate experiments (15 brains/spinal cords harvested at day 14 post EAE induction have been loaded on the gel displayed here; 37 brains and 39 spinal cords were analyzed in total).

4.2.2 γHV-68 EAE mice have increased CD8 and CD4 T cells infiltrations, increased inflammation and MS-like lesions in the brain

Since in addition to severe ascending paralysis, γHV-68 EAE mice also displayed unusual symptoms such as loss of balance, ataxia and hunched posture; we investigated both spinal cord and brain histopathology. During the course of EAE, mice develop ascending paralysis due to spinal cord inflammation. Immune infiltrations in the brain cortex are atypical and, if present, are restricted to the meninges. To assess the composition and to quantify infiltrating immune cells in γHV-68 EAE mice and in EAE mice, CNS infiltrates were isolated and stained at day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time). γHV-68 EAE mice presented with an increased number of T cells in the CNS, both in the brains and in the spinal cords when compared to EAE mice (Figure 4.8A). On the other hand, the number of infiltrating macrophages was similar in both groups (Figure 4.8B). CD4 T cells are the primary T cells type infiltrating the CNS during EAE (Figure 4.9B). Surprisingly, increased percentages of CD8 T cells were detected in both the spinal cords and the brains of γHV-68 EAE mice (Figure 4.9A).

To confirm the FACS data and demonstrate that T cells in the brain were infiltrating into the parenchyma and were not confined to the meninges, immunohistochemistry on brain sections was performed both at day 15 and day 28 post EAE induction. Figure 4.10 shows data from day 28, equivalent results were obtained at day 15 post EAE. γHV-68 EAE mice (upper left panels) showed heightened CD4 (Figure 4.10A) and CD8 (Figure 4.10B) T cell infiltrations inside the brain parenchyma. Brain sections were also stained for F4/80 that is expressed both on infiltrating macrophages and on activated microglia, indicating areas of inflammation in the CNS (Figure 4.10C). γHV-68 EAE mice showed heightened staining in multiple areas of the brain. Conversely, the brains from EAE mice (lower left panels) showed fewer infiltrating CD4 T cells, mostly lining the blood vessels and the meninges, no CD8 infiltrations and weaker F4/80 staining when compared to brains from γHV-68 EAE mice. Additionally, EAE was induced in γHV-68 mice without administering pertussis toxin (γHV-68 MOG CFA). Pertussis toxin has been shown to be important both as an adjuvant to prime the MOG specific response and to permeabilize the blood-brain barrier [172]. γHV-68 MOG

CFA mice developed milder paralysis than γHV-68 EAE mice but still showed T cells infiltrations into the brain parenchyma and inflammation (Figure 4.10A-B-C, upper right panels). Further, LCMV EAE mice did not display any observable lymphocyte infiltrations in the brain and had an EAE course equal to that observed in uninfected mice (Figure 4.10D-E). MCMV EAE mice showed localized CD4 T cell infiltrations, fewer in number and size than the ones observed in γHV-68 EAE (Figure 4.10D). However, no CD8 T cell infiltrations were detectable in MCMV EAE mice (Figure 4.10E).

Spinal cords were analyzed at the same time points as brains. Hematoxylin and eosin staining showed increased amounts of immune cells infiltrating in the spinal cords of γHV-68 EAE mice, thus confirming the FACS data (Figure 4.11).

Finally and most importantly, the development of lesions in the brain parenchyma similar to MS was observed in γHV-68 EAE mice with localization of infiltrating CD4/CD8 T cells and increased inflammation (F4/80 staining) on consecutive sections (Figure 4.12). Similar to MS, multiple pronounced yet small lesions of mononuclear cells with areas of myelin loss were observed within the white matter of both the cerebellum (Figure 4.12) and corpus callosum. These lesions were found only in γHV-68 EAE mice at day 28 post EAE induction (two separate experiments with 8 mice/group/experiment). This demonstrates that γHV-68 latent infection leads to strong T cell activation post EAE induction with infiltrations inside the white matter of the brain leading to myelin damage.

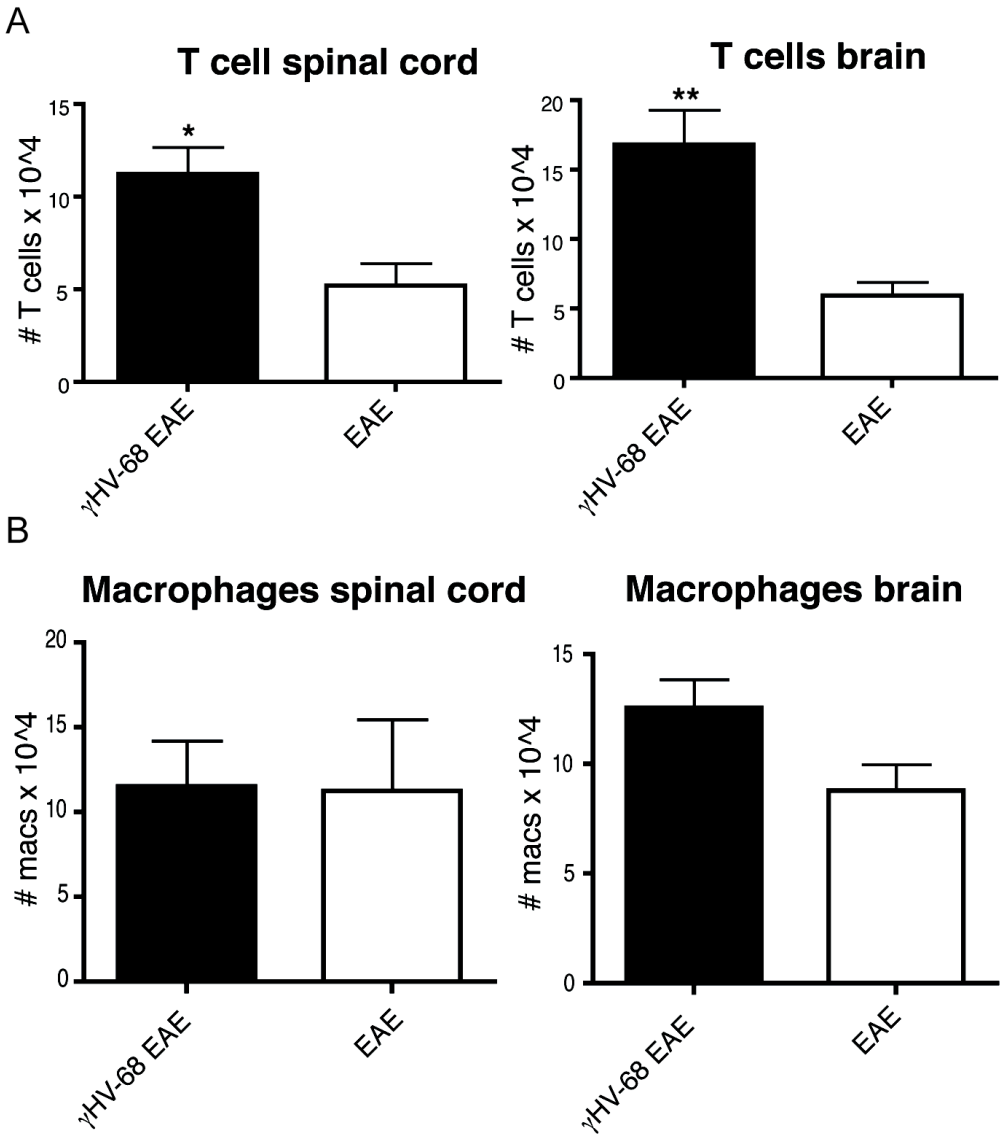


Figure 4.8. γHV-68 EAE mice have increased numbers of T cells inside the CNS
Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains (right panels) and spinal cords (left panels) were harvested and processed to isolate immune infiltrates. **(A)** Total number of infiltrating T cells (CD45+ CD11b- CD3+). **(B)** Total numbers of infiltrating macrophages (CD45+ CD11b high). Three separate experiments with 8-6 mice/group; data were analyzed with t-test: ** $p<0.01$, * $p<0.05$.

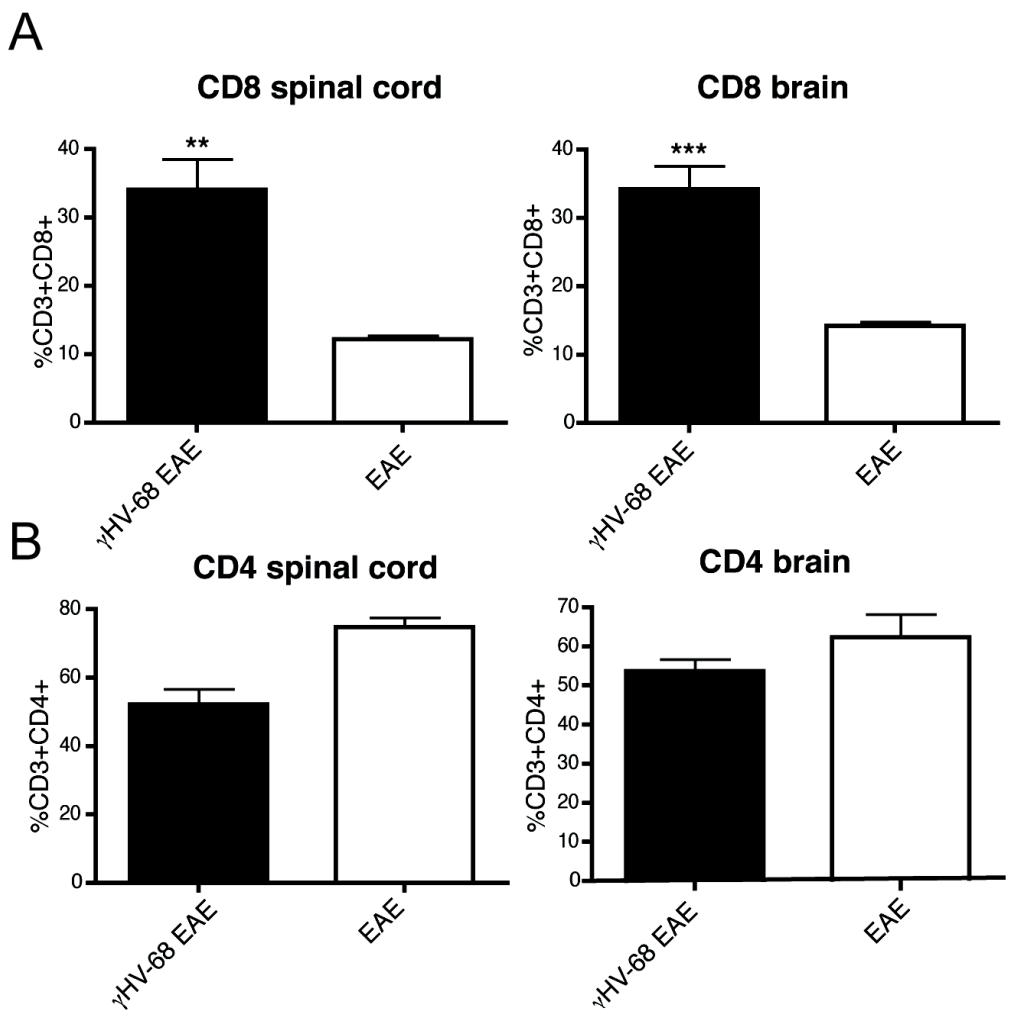


Figure 4.9. γHV-68 EAE mice show increased percentages of CD8 T cells infiltrating into the CNS

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains (right panels) and spinal cords (left panels) were harvested and processed to isolate immune infiltrates. **(A)** Percentage of infiltrating CD3+ CD8+ lymphocytes and **(B)** CD3+ CD4+ lymphocytes. Three separate experiments with 8-6 mice/group; data were analyzed with t-test: *** $p<0.001$; ** $p<0.01$.

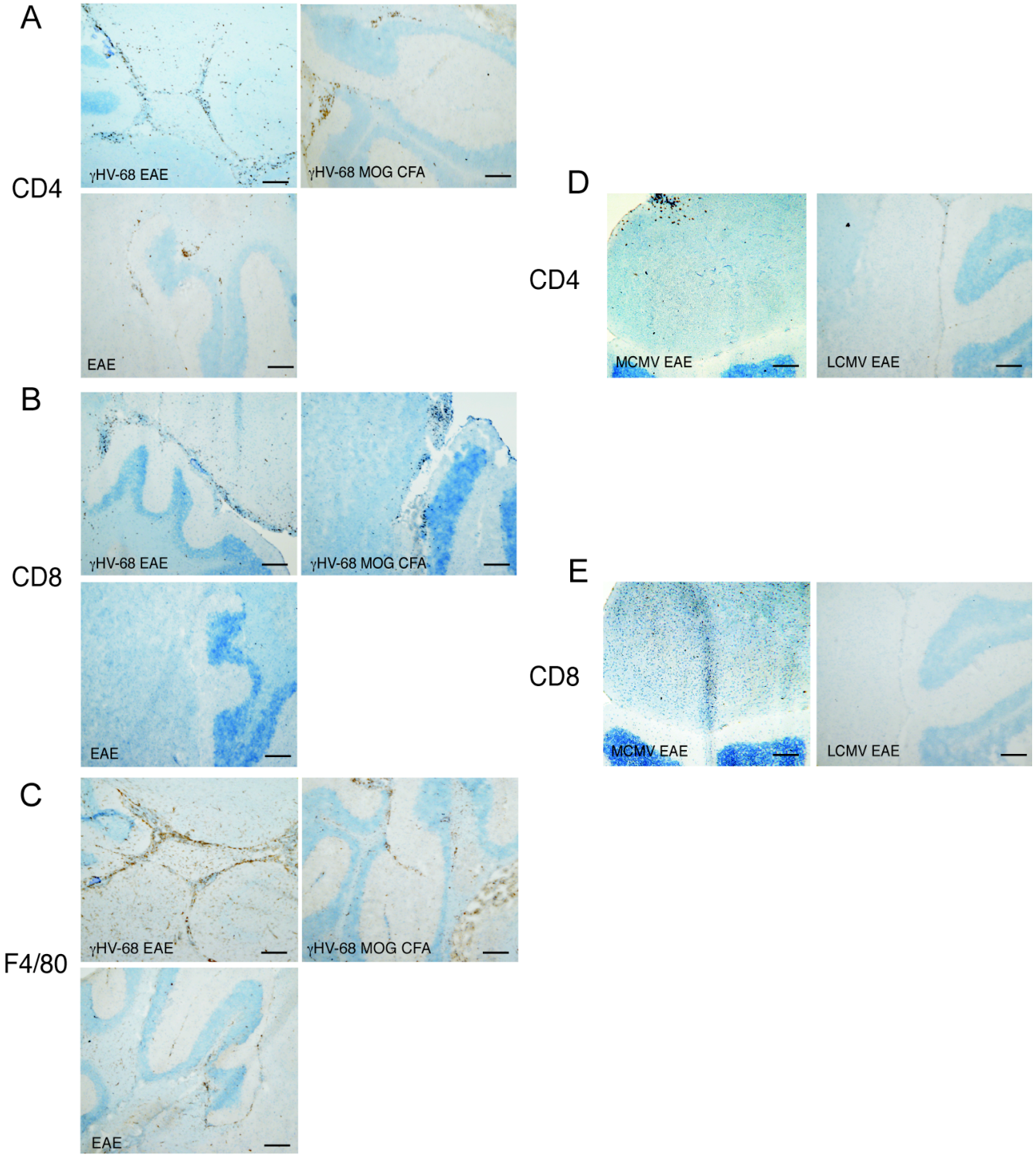

A
CD4
γHV-68 EAE
γHV-68 MOG CFA
EAE
B
CD8
γHV-68 EAE
γHV-68 MOG CFA
EAE
C
F4/80
γHV-68 EAE
γHV-68 MOG CFA
EAE
D
CD4
MCMV EAE
LCMV EAE
E
CD8
MCMV EAE
LCMV EAE

Figure 4.10. Immunohistochemistry after EAE induction shows enhanced inflammation and immune cell infiltrations inside the brain parenchyma only after γHV-68 infection

Mice were infected with γHV-68 (**A-B-C** upper panels: γHV-68 EAE and γHV-68 MOG CFA); or MEM only (**A-B-C** bottom panel: EAE); or MCMV or LCMV (**D-E**: MCMV EAE and LCMV EAE). Five weeks p.i. EAE was induced with (EAE) or without (MOG CFA) pertussis toxin. At day 28 post EAE induction (day 28 shown here, similar results obtained at day 15 post EAE induction), mice were perfused and brains were embedded in OCT, snap frozen, cut and stained with antibodies specific for CD4 **(A-D)**, CD8 **(B-E)** and F4/80 **(C)**. The pictures are representative of three separate experiments (n=16/group). Scale bar: 100 μm.

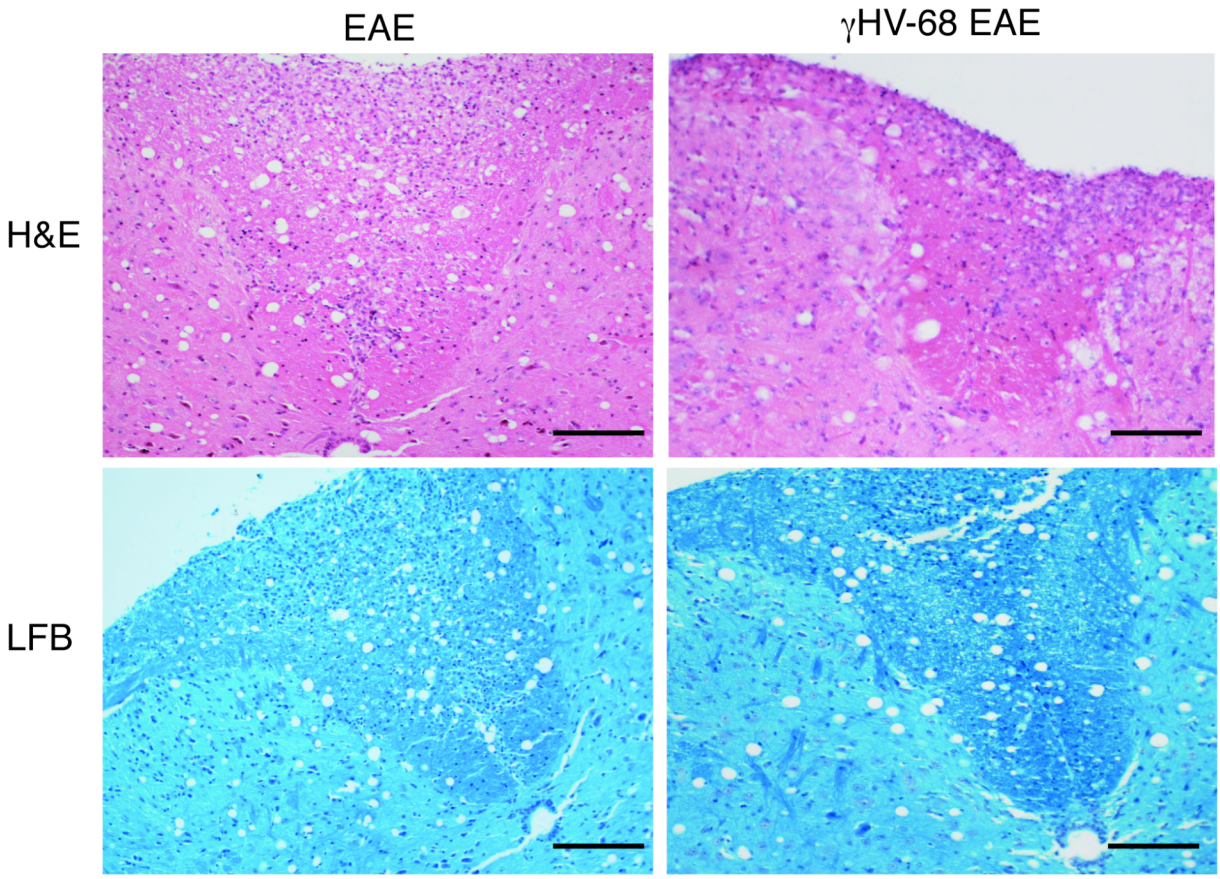


Figure 4.11. γHV-68 EAE mice show increased amount of immune cell infiltrations in the spinal cords

Mice were infected with γHV-68 (right panels) or MEM only (left panels). Five weeks p.i. EAE was induced. At day 28 post EAE induction mice were perfused and spinal cords were harvested, fixed in formalin and paraffin embedded (similar results obtained at day 15 post EAE induction). Cross-sections were stained with H&E (upper panels) and luxol fast blue (lower panels). Representative pictures of three separate experiments (n=16/group). Scale bar = 100 μm.

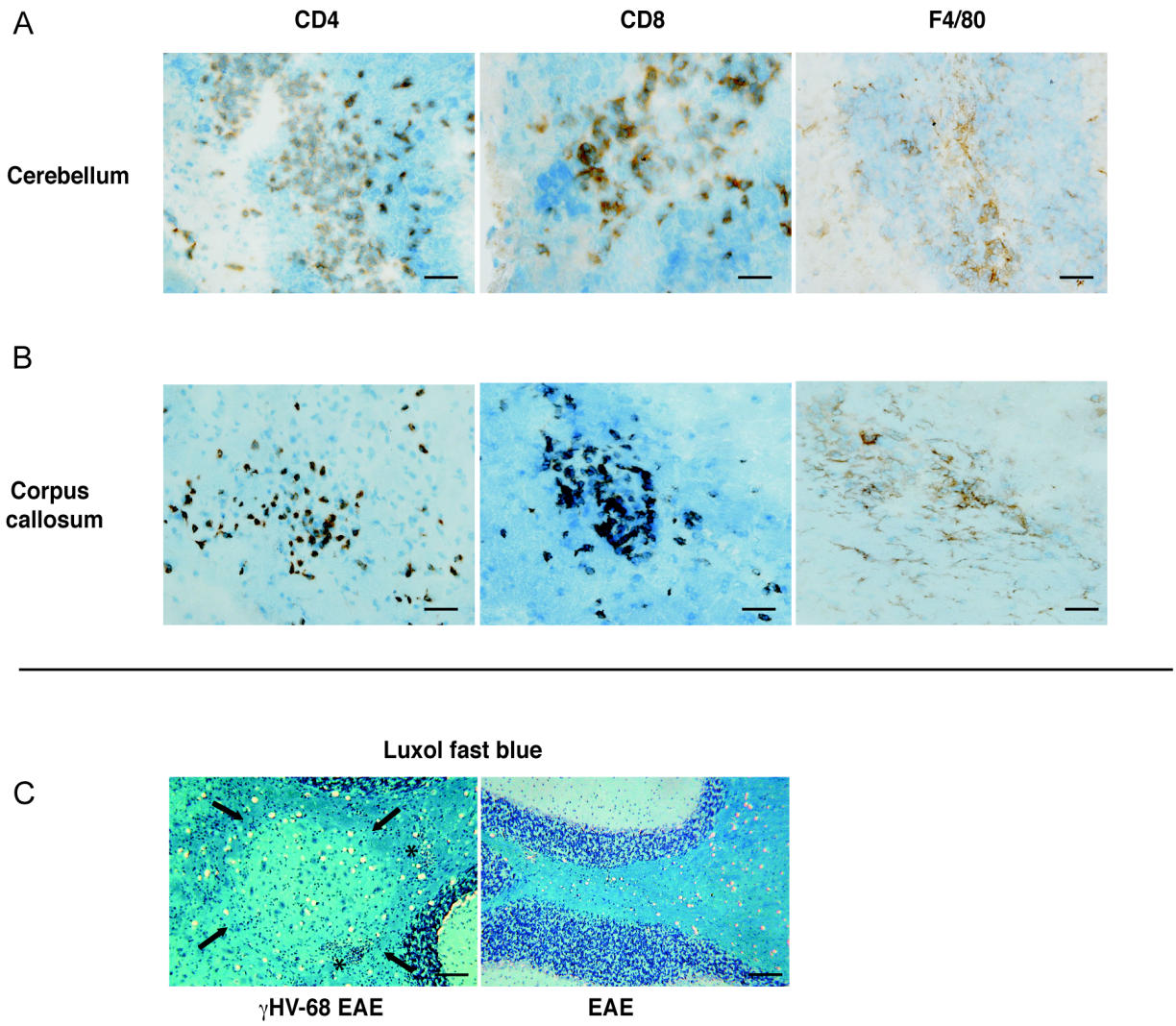


Figure 4.12. γHV-68 EAE mice show MS-like lesions in the white matter of the cerebellum and in the corpus callosum

Mice were infected with γHV-68 or MEM only. Five weeks p.i. EAE was induced. At day 28 post EAE induction mice were perfused and brains were embedded in OCT, snap frozen, cut and stained with antibodies specific for CD4, CD8 and F4/80; or were formalin fixed and embedded in paraffin and stained with luxol fast blue. **(A-B)** The images displayed for CD4-CD8-F4/80 staining are consecutive sections cut from the same cerebral hemisphere of the same mouse (all images from γHV-68 EAE mice). Scale bar: 50 μm. **(C)** The arrows show an area of demyelination in the cerebellum of a γHV-68 EAE mouse and the asterisks highlight immune cell infiltrations. The right panel shows a normal cerebellum from a naïve EAE mouse. Scale bar: 100 μm. All pictures are representative of two separate experiments (n=16/group).

4.2.3 CD8 T cells infiltrating in the CNS of γHV-68 EAE mice express granzyme B and are specific for viral proteins

CD8 T cell infiltrations, usually not present in the CNS of EAE mice, were detected in the brain and spinal cords of γHV-68 EAE mice in significantly higher percentages (Figure 4.9 and Figure 4.13A). CD8 T cells were further characterized for granzyme B production, which has the potential to damage oligodendrocytes [271]. γHV-68 EAE mice presented with higher levels of CD8+ Granzyme B+ T cells in both the brain and the spinal cord (Figure 4.13B).

As it was possible that γHV-68 specific memory CD8 T cells could become activated in a bystander manner upon EAE induction and driven into the site of inflammation in the CNS, the specificity of the CD8 T cells in γHV-68 EAE mice was investigated. A percentage of CD8 T cells infiltrating in both the brains (4.9 ± 0.5%) and the spinal cords (3.4 ± 0.5%) of γHV-68 EAE mice (Figure 4.14) were specific for the two immunodominant viral epitopes (p56 and p79). The first epitope is from ORF 6 (p56) that encodes a single stranded DNA binding protein. The second epitope is from ORF 61 (p79) that encodes the large ribonucleotide reductase subunit [86]. Both are lytic genes and are expressed during γHV-68 acute infection. The percentages of p56 and p79 specific CD8 T cells detected in the CNS post EAE (49 days post infection) were equivalent to their respective percentages in spleens (4.3 ± 3.2%) of γHV-68 infected mice at day 40 post primary infection (Figure 4.14, upper right panel); consistent with previously published results [86].

These results demonstrate that infiltrating CD8 T cells expressing granzyme B in γHV-68 EAE mice provide a considerable potential for CNS pathology. Moreover, the size of the population of virus specific CD8 T cells found in the CNS post EAE is similar to that observed in the spleens during latency and before EAE induction. This indicates that EAE induction is not eliciting an expansion of γHV-68 specific CD8 memory T cells. Their migration into the brain in the absence of γHV-68 infection inside the CNS is likely due to the fact that memory T cells express higher levels of adhesion molecules (like CD44) and they are able to enter any inflammation site (i.e. the CNS if EAE has been induced). It is not clear if γHV-68 specific CD8 T cells are then retained into the brain and contribute to the pro-inflammatory loop or if they enter and then readily re-exit the CNS because they do not encounter the antigen they are specific for.

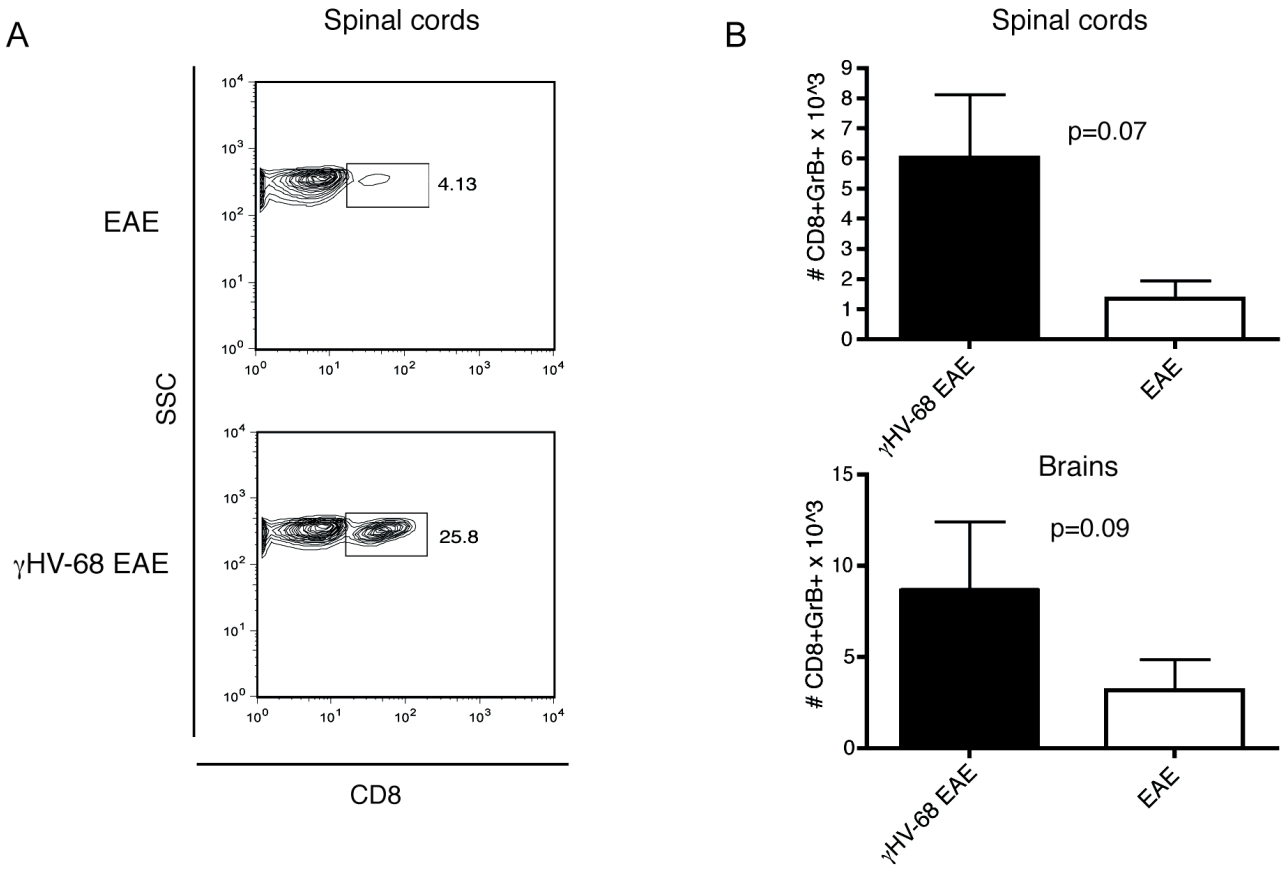


Figure 4.13. Granzyme B is upregulated in CD8 T cells isolated from the CNS of γHV-68 EAE mice

Mice were infected with γHV-68 or MEM only. Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains and spinal cords were harvested and processed to isolate immune infiltrates. **(A)** Representative FACS plots showing the percentages of CD8 T cells infiltrating in the spinal cords (similar results were obtained in the brains) and histogram **(B)** showing the number of CD8+ granzyme B+ T cells infiltrating in the spinal cords and in the brains (immune cells were not restimulated before granzyme B staining). Two separate experiments with 6 mice/group, data analyzed with t-test.

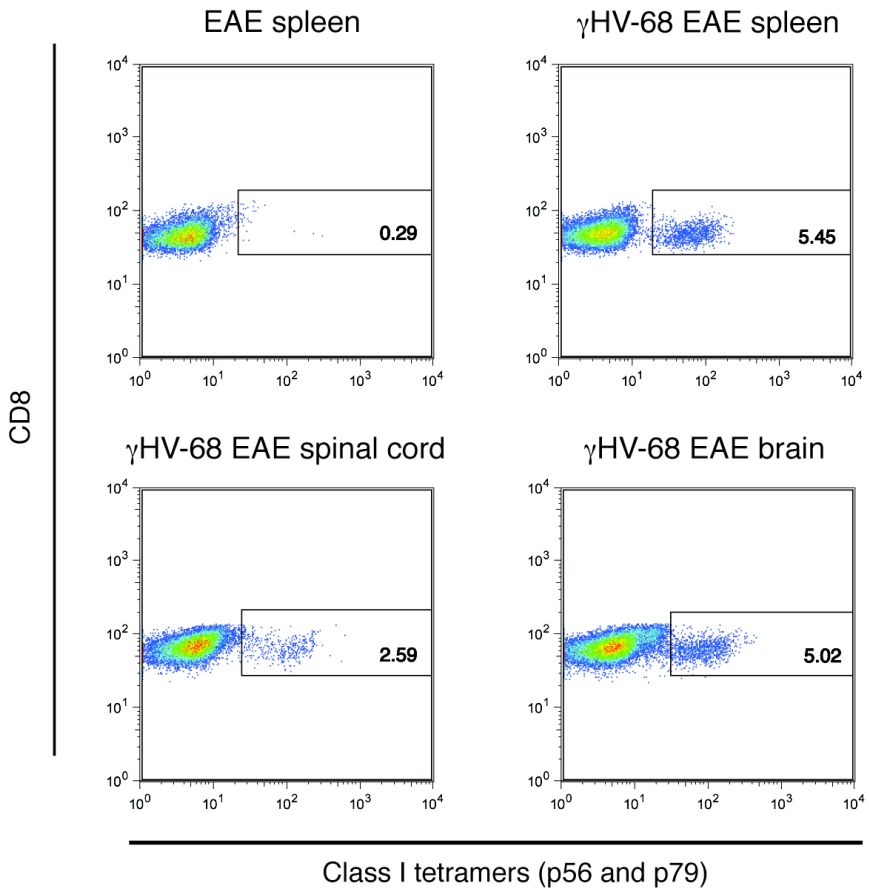


Figure 4.14. A percentage of CD8 T cells isolated from the CNS of γHV-68 EAE mice is specific for viral proteins

Mice were infected with γHV-68 or MEM only. Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains and spinal cords were harvested and processed to isolate immune infiltrates. The figure shows representative FACS plots displaying percentages of CD8+ T cells with TCR specific for γHV-68 epitopes (p56 and p79) in the spleen of EAE mice (negative control); in the spleen of γHV-68 EAE mice (positive control; 3.3 ± 1.6%), in the spinal cord (3.4 ± 0.5%) and in the brain (4.9 ± 0.5%) of γHV-68 EAE mice. Two separate experiments with 6 mice/group. Errors indicated in brackets in this figure legend are s.d.

4.2.4 T cells infiltrating in the CNS of γHV-68 EAE mice produce higher amounts of IFN-γ and T-bet along with IL-17 downregulation

To determine the cytokines produced by the T helper response after EAE immunization, T cells were isolated from the CNS and restimulated ex-vivo (Figure 4.15 and Figure 4.16). The T cell response primed upon EAE induction in naïve mice is a mixed Th1-Th17 CD4 T cell response with production of both IFN-γ and IL-17 primarily in spinal cords [272]. In contrast, in γHV-68 EAE mice, CD4 T cells produced significantly increased amounts of IFN-γ (Figure 4.15A-B) particularly within the brain parenchyma, along with suppressed levels of IL-17 (Figure 4.16). Moreover increased percentages of these CNS CD8 T cells from γHV-68 EAE mice produced IFN-γ (Figure 4.15C-D). As expected, the amount of CD8 T cells infiltrating the CNS of naïve EAE mice was too low to perform the assay. When restimulated with the MOG specific peptide, similar results were observed with T cells from the CNS (Figure 4.15B and D; Figure 4.16B). These results indicate that, upon EAE induction in γHV-68 mice, the T helper response is skewed towards a Th1 phenotype, whereas the Th17 response is suppressed.

Additionally, infiltrating T cells were stained for T-bet and ROR-γt (Figure 4.17). In γHV-68 EAE mice, T-bet was significantly upregulated in all CNS infiltrating T cells compared to EAE mice. Strikingly, in γHV-68 EAE mice, T cells not producing IFN-γ (Figure 4.17, upper left panels) still showed T-bet upregulation. Conversely, ROR-γt was upregulated only in IL-17 secreting T cells and the levels were comparable between EAE and γHV-68 EAE mice (Figure 4.17, upper right panel). Finally, γHV-68 T cells, in the periphery, secreted increased amounts of IFN-γ (Figure 4.18A) and exhibited an effector memory phenotype with increased expression of CD44 and downregulation of CD62L (Figure 4.18B), when compared to T cells from naïve mice either before or after EAE induction (data before EAE induction are not shown here). This demonstrates that mice latently infected with γHV-68 skew a more potent Th1 response upon immunization with suppression of the classical EAE Th17 response.

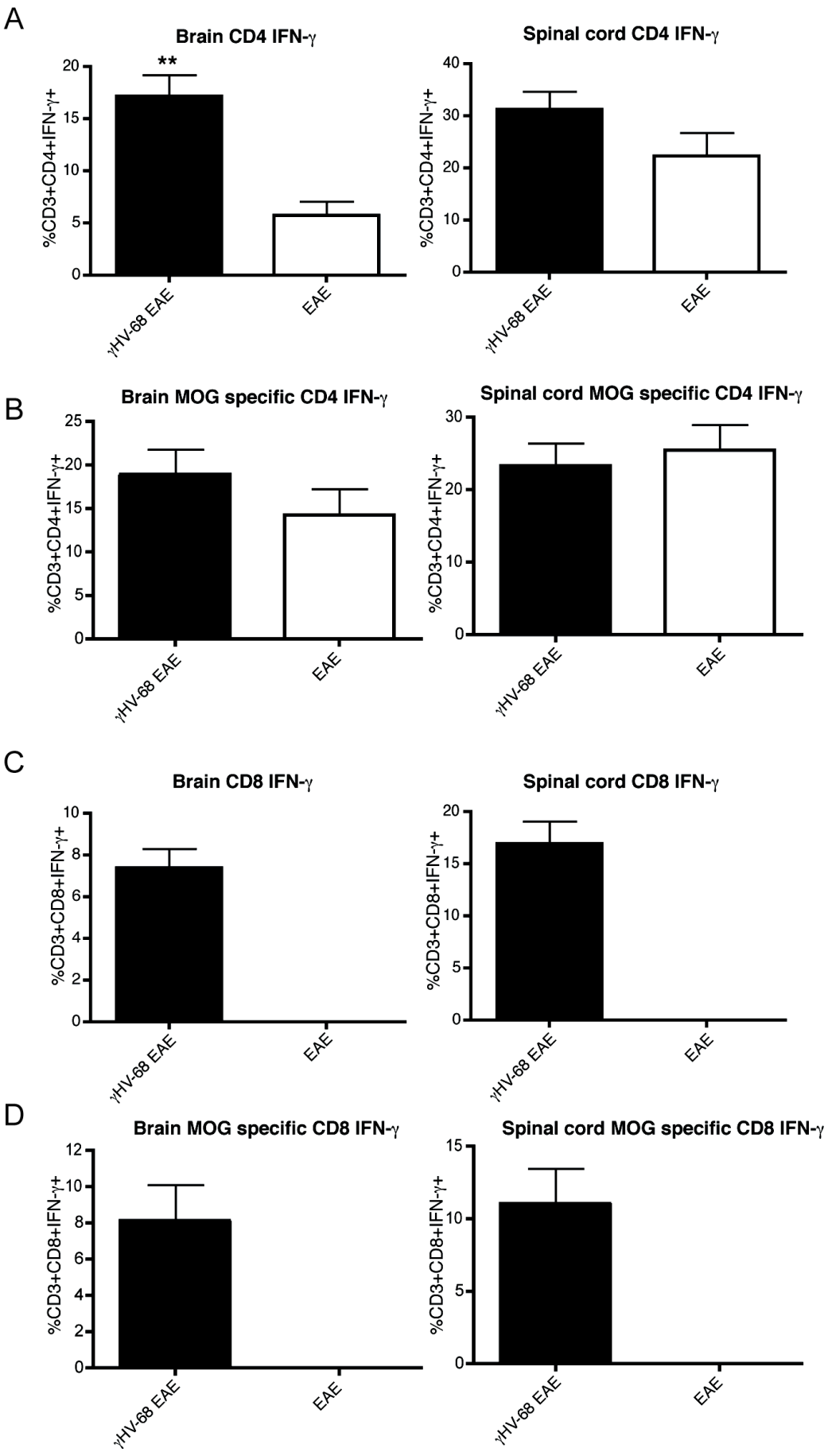

A
Brain CD4 IFN-γ
Spinal cord CD4 IFN-γ
%CD3+CD4+IFN-γ+
**
γHV-68 EAE
EAE
B
Brain MOG specific CD4 IFN-γ
Spinal cord MOG specific CD4 IFN-γ
C
Brain CD8 IFN-γ
Spinal cord CD8 IFN-γ
%CD3+CD8+IFN-γ+
D
Brain MOG specific CD8 IFN-γ
Spinal cord MOG specific CD8 IFN-γ

Figure 4.15. T cells infiltrating in the CNS of γHV-68 EAE mice produce higher amounts of IFN-γ

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains (left panels) and spinal cords (right panels) were harvested and processed to isolate immune infiltrates that were restimulated ex-vivo with PMA/ionomycin **(A-C)** or MOG **(B-D)** before performing FACS intra cellular staining. **(A-B)** Percentages of infiltrating CD3+ CD4+ IFN-γ+ lymphocytes. **(C-D)** Percentages of infiltrating CD3+ CD8+ IFN-γ lymphocytes (EAE CD8 infiltrations were not enough to perform FACS). Three separate experiments with 8-6 mice/group. Data were analyzed with t-test: ** $p<0.01$.

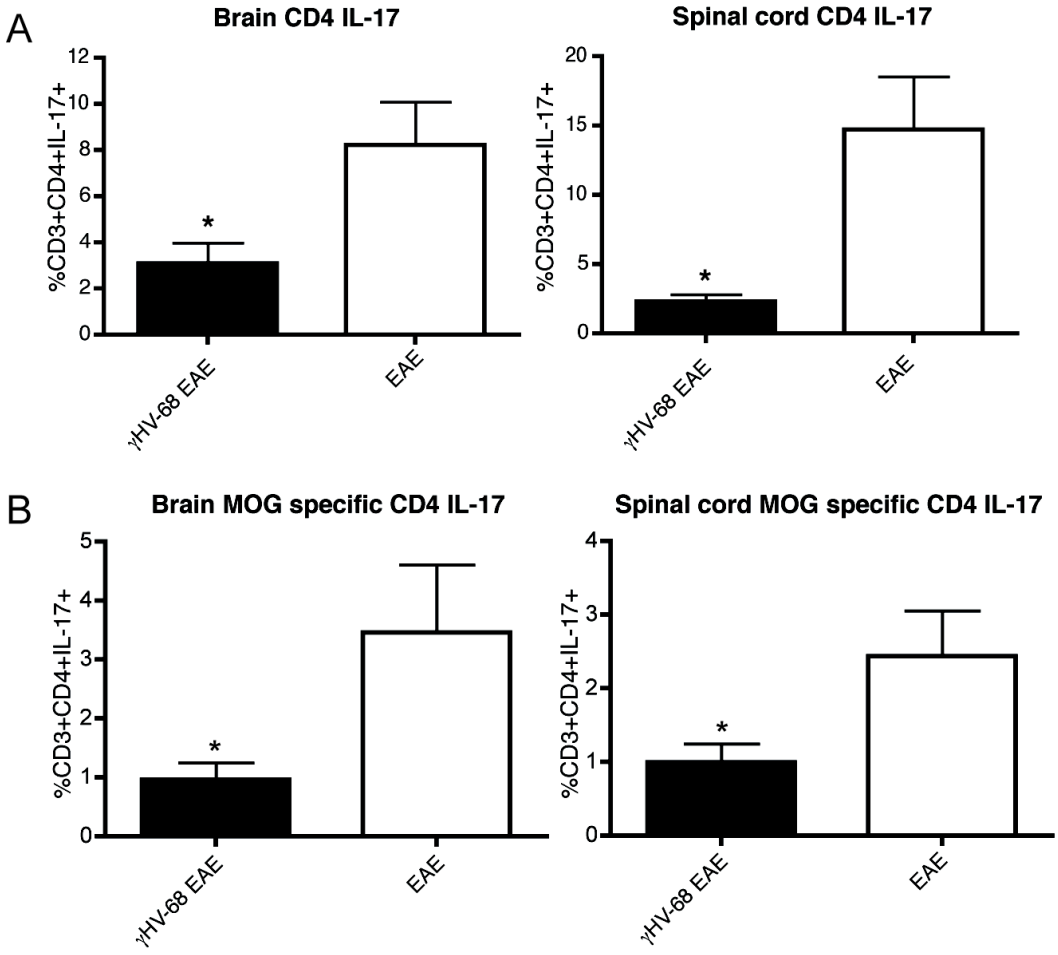


Figure 4.16. Th17 responses are suppressed in γHV-68 EAE mice

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains (left panels) and spinal cords (right panels) were harvested and processed to isolate immune infiltrates that were restimulated ex-vivo with PMA/ionomycin **(A)** or MOG **(B)** before performing FACS intra cellular staining. **(A-B)** Percentages of infiltrating CD3+ CD4+ IL-17+ lymphocytes. Three separate experiments with 8-6 mice/group. Data were analyzed with t-test: * $p<0.05$.

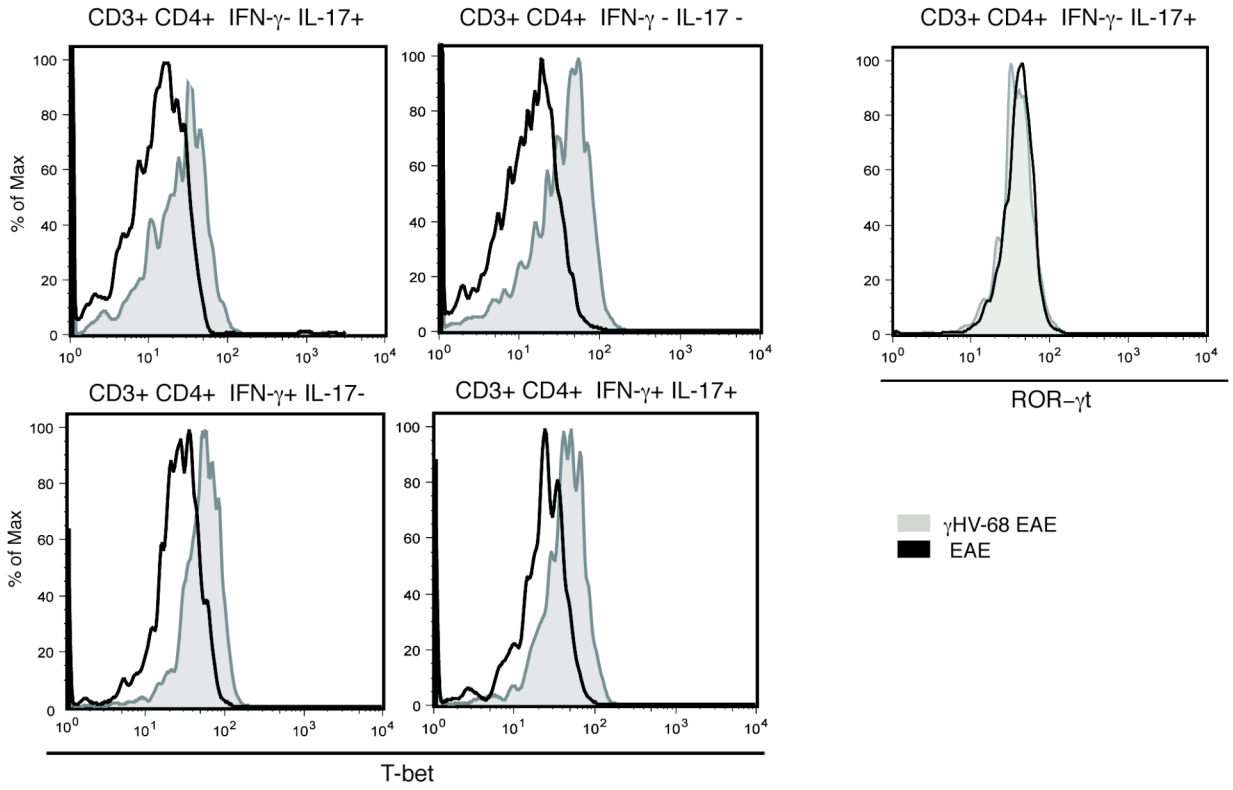
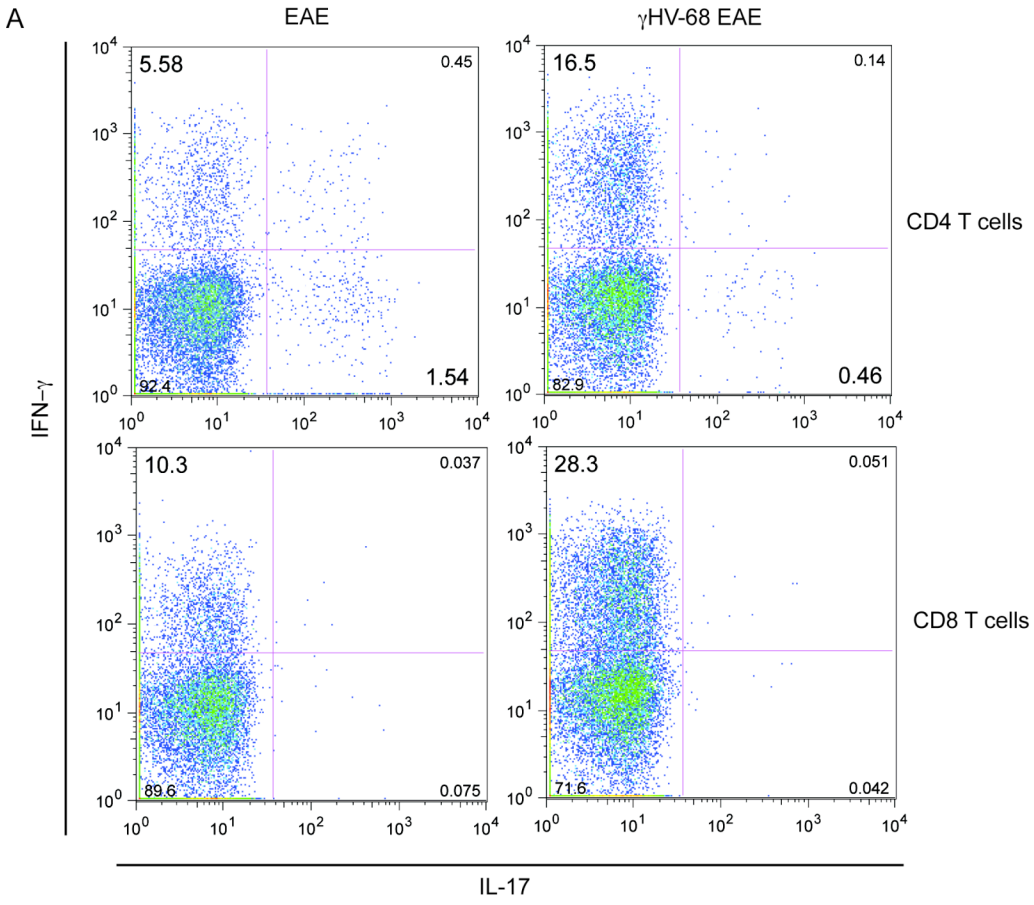


Figure 4.17. γHV-68 EAE mice upregulate T-bet in CD4 T cells

Mice were infected with γHV-68 (shaded histograms) or MEM only (open histograms). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused and spinal cords were harvested and processed to isolate immune infiltrates that were restimulated ex-vivo with PMA/ionomycin. The figure shows T-bet (left panels) and ROR-γt levels (right panel) in CD3+ CD4+ IFN-γ± IL-17± lymphocytes in the spinal cords (similar results obtained from the brains) of γHV-68 EAE mice (grey shaded histograms) or EAE mice (open histograms). Representative histograms of two separate experiments with 6-8 mice/group.


A
EAE
γHV-68 EAE
5.58
0.45
92.4
1.54
16.5
0.14
82.9
0.46
CD4 T cells
10.3
0.037
89.6
0.075
28.3
0.051
71.6
0.042
CD8 T cells
IFN–γ
IL-17

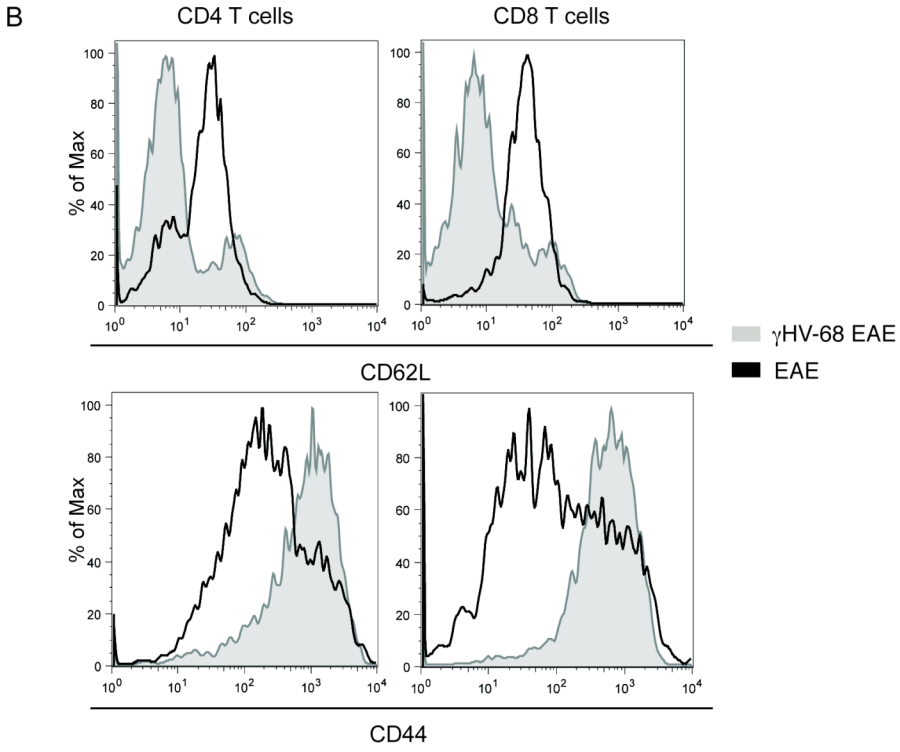

B
CD4 T cells
CD8 T cells
% of Max
CD62L
CD44
γHV-68 EAE
EAE

Figure 4.18. T cells in the periphery of γHV-68 EAE mice show increased activation and enhanced production of IFN-γ

Mice were infected with γHV-68 (shaded histograms) or MEM only (open histograms). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), spleen were harvested and processed to isolate immune cells that were either restimulated ex-vivo with PMA/ionomycin **(A)** or stained directly without any stimulation **(B)**. This figure shows data post EAE induction, similar results were obtained before EAE induction. Panel **A** shows the percentages of splenic CD4 T cells (upper panels) and CD8 T cells (lower panels) producing IFN-γ and IL-17 in EAE mice (left panels) or γHV-68 EAE mice (right panels). Panel **B** shows the levels of CD62L (upper panels) and CD44 (lower panels) in splenic CD4 T cells (left panels) or CD8 T cells (right panels) of γHV-68 EAE mice (shaded histograms) or EAE mice (open histograms). Representative data of three-four separate experiments with 6-8 mice/group.

4.2.5 γHV-68 EAE mice have increased levels of pro-inflammatory cytokines in the serum and a decreased anti-MOG IgG1/IgG2a ratio

Since γHV-68 EAE mice showed a strong Th1 response, the level of Th1 cytokines in the serum was measured. Sera was harvested at different time points post EAE induction and the following cytokines and chemokines were analyzed: IFN-γ, IL-12p70, TNF-α, IL-6, IL-10, IL-17A, GM-CSF, G-CSF, MCP-1 (CCL2), MIP-1α (CCL-3), MIP-1β (CCL-4), MIG (CXCL-9) and RANTES (CCL-5). IFN-γ and TNF-α were significantly increased in the serum of γHV-68 EAE mice, when compared to EAE mice, at day 10, 15 and 28 post EAE induction (Figure 4.19A-C). On the other hand, the chemokines RANTES (CCL-5) and MIG (CXCL-9) were statistically significant different only at day 10 post EAE induction, when mice started to develop symptoms (Figure 4.20). All the remaining cytokines tested and listed above were not differentially expressed between the sera of the two mouse groups. Levels of cytokines and chemokines were also analyzed in the supernatants obtained from brain and spinal cords homogenates (see Materials and Methods 2.16 for CNS supernatant preparation). At day 14-16 post EAE induction, MCP-1 was detected in the brains and spinal cords of both EAE and γHV-68 EAE mice at similar levels (data not shown). Increased levels of IFN-γ were detected in the brains and spinal cords of γHV-68 EAE mice when compared to EAE mice (Figure 4.19D). All the remaining cytokines analyzed in the CNS were below the detection limit. Considering the importance of type I interferons in controlling acute γHV-68 infection [273], the levels of IFN-β in the sera were also tested. IFN-β was detected at a similar level in the sera of both γHV-68 EAE mice and uninfected EAE mice (data not shown).

Since CCL-5 and CXCL-9 were found to be upregulated in the sera of γHV-68 EAE mice, the levels of their respective chemokine receptors (CCR5 for CCL-5 and CXCR3 for CXCL-9) were analyzed at day 10 post EAE induction in the spleen. No difference was detected in the expression of CCR5 in both CD4 and CD8 T cells. On the other hand, greater numbers of CD4 and CD8 T cells from γHV-68 EAE mice expressed CXCR3, compared to cells from uninfected EAE mice (Figure 4.21).

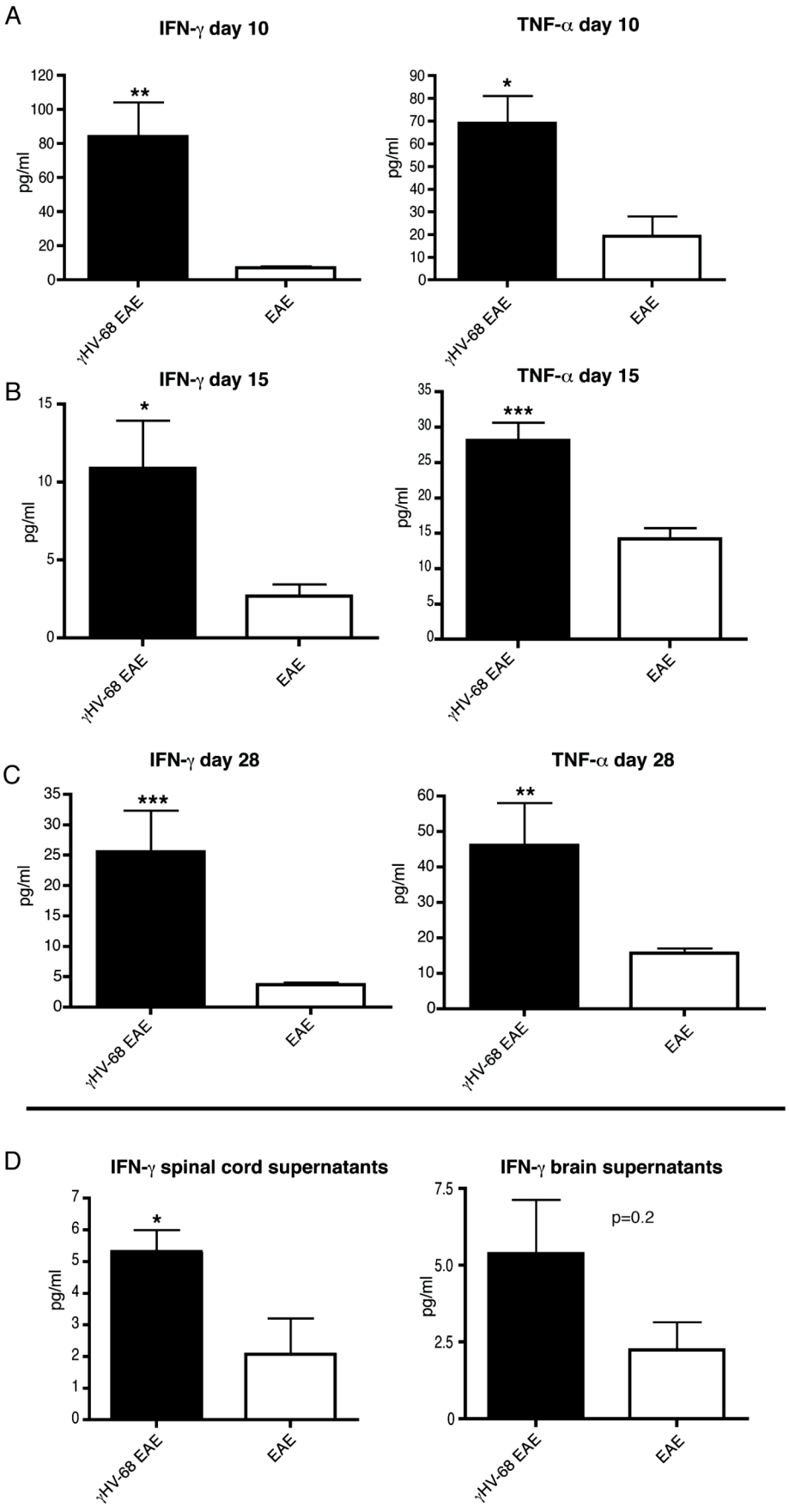

A
IFN-γ day 10
TNF-α day 10
pg/ml
**
*
γHV-68 EAE
EAE
B
IFN-γ day 15
TNF-α day 15
*
***
C
IFN-γ day 28
TNF-α day 28
***
**
D
IFN-γ spinal cord supernatants
IFN-γ brain supernatants
p=0.2

Figure 4.19. γHV-68 EAE mice have increased amounts of IFN-γ and TNF-α in the serum and in the CNS at different time points post-EAE induction

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i., EAE was induced. At day 10 **(A)**, 15 **(B)** and 28 **(C)** post EAE induction blood was harvested through a cardiac puncture; or at day 14-16 post EAE induction **(D)** (mean clinical score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused and brains and spinal cords were homogenized and the supernatants were analyzed. The levels of cytokines were evaluated using BD Cytometric Bead Array kits. Three-two separate experiment for each time point with 3-6 mice/group. Data were analyzed with t-test: *** $p<0.001$; ** $p<0.01$, * $p<0.05$.

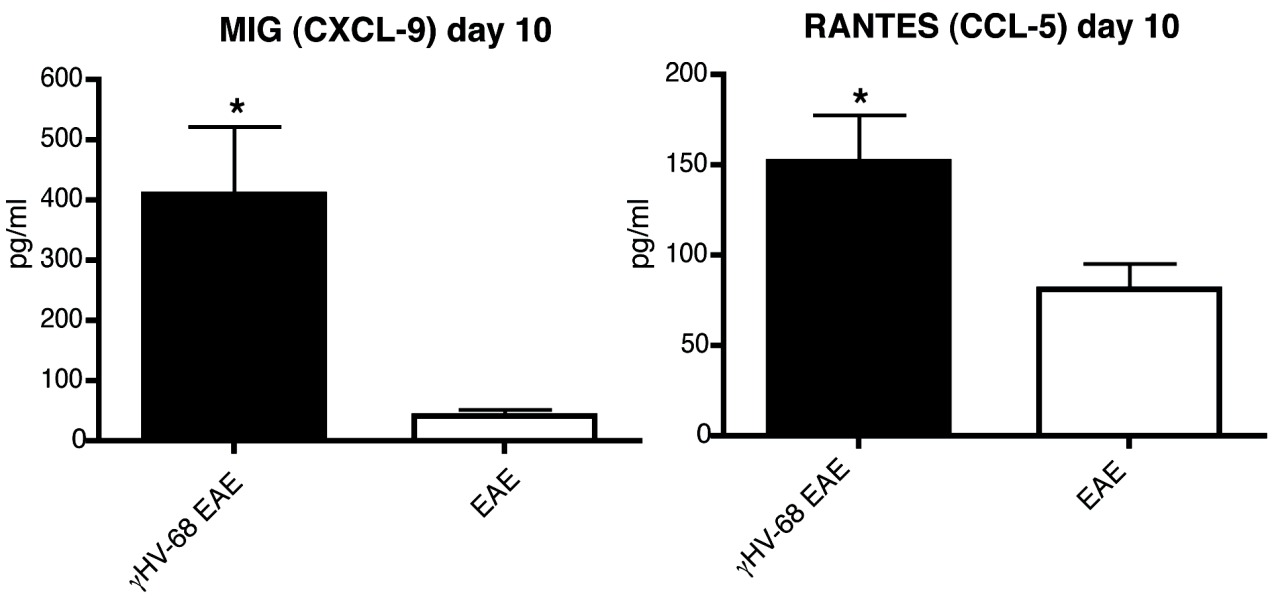


Figure 4.20. γHV-68 EAE mice show upregulation of RANTES and MIG in the serum at day 10 post EAE induction

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i., EAE was induced. At day 10, blood was harvested through a cardiac puncture. The levels of chemokines were evaluated using BD Cytometric Bead Array kits. Three-two separate experiments with 3-6 mice/group. Data were analyzed with t-test: * $p<0.05$.

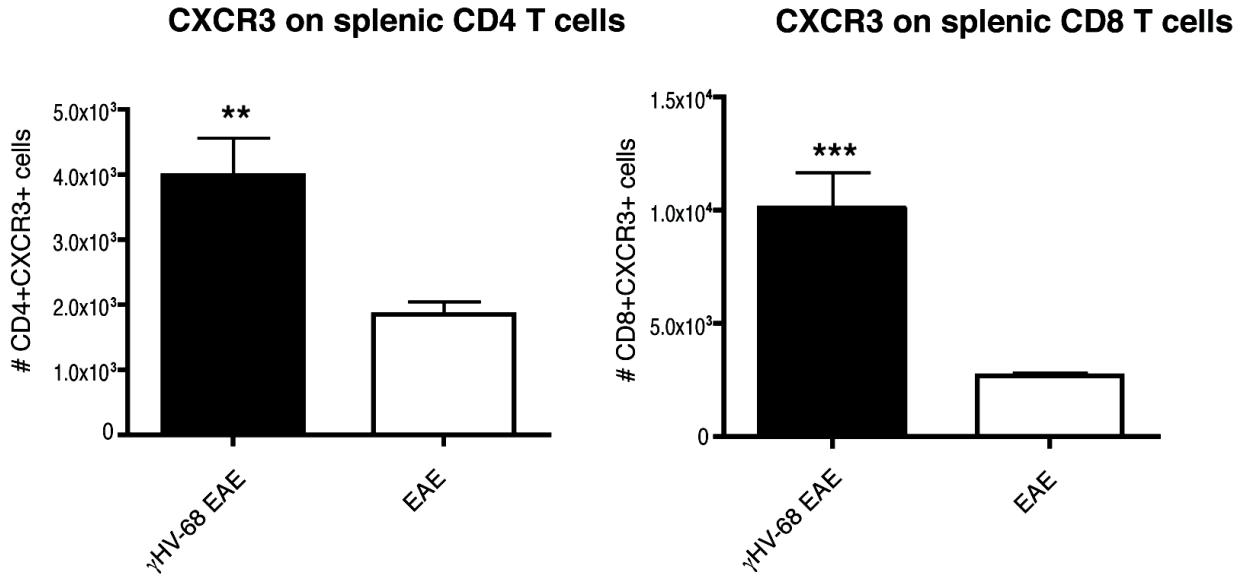


Figure 4.21. CXCR3 is upregulated on splenic T cells at day 10 post EAE induction in γHV-68 EAE mice

Mice were infected i.p. with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i., EAE was induced. At day 10 post EAE induction, spleens were harvested and the levels of CXCR3 were assessed through FACS analysis. The histograms show the numbers of CD4+CXCR3+ cells (left panel) or CD8+CXCR3+ cells (right panel) One experiment with 5-6 mice/group. Data were analyzed with t-test: *** $p<0.001$; ** $p<0.01$.

These results show that the Th1 response triggered by EAE induction in γHV-68 EAE mice is sustained even at later time points post immunization and high levels of IFN-γ and TNF-α are likely responsible for perpetuating this Th1 response. Moreover, as RANTES and MIG control leukocyte adhesion and migration into the target tissue [274], their upregulation at day 10 post EAE, coincident with the upregulation of CXCR3 on T cells, is likely responsible for the increased T cell infiltrations in γHV-68 EAE mice.

Since γHV-68 infects B cells and B cells infiltrating the CNS were not detected (results confirmed by both FACS analysis of CNS infiltrates and immunohistochemistry at day 15 and 28 post EAE using anti-CD19 antibodies), the hypothesis that the viral infection could precipitate EAE by increasing the production of anti-MOG autoantibodies was investigated. Sera harvested at day 28 were tested for the presence of both MOG-specific and brain-extract specific IgGs (Figure 4.22). There was a slight increase in the amount of anti-MOG and anti-brain extract antibody in γHV-68 EAE mice. This indicates that MOG autoantibodies are not likely playing a role in the increased clinical score of γHV-68 EAE mice and the increased presence of brain-extract specific antibodies is likely due to epitope spreading to CNS proteins due to increased CNS inflammation in γHV-68 EAE mice. Since anti-MOG IgGs were not different between infected and uninfected mice, differences in IgG isotype were tested. γHV-68 EAE mice showed a decreased anti-MOG IgG1/IgG2a ratio (Figure 4.23). This is consistent with the high concentrations of IFN-γ detected in the serum that are likely inhibiting isotype switching towards IgG1 [275], thus confirming the presence of a strong Th1 response in γHV-68 EAE mice.

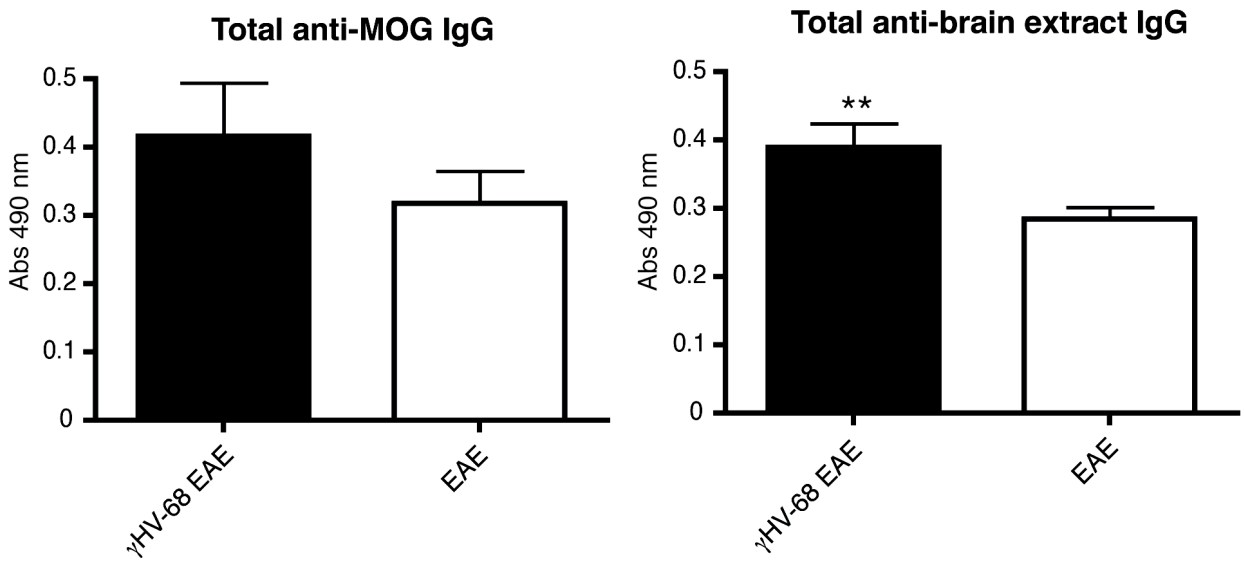


Figure 4.22. γHV-68 EAE mice secrete more brain extract specific autoantibodies but not anti-MOG autoantibodies

Mice were infected i.p. with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i., EAE was induced. Serum was harvested at day 28 post EAE and the levels of total anti-MOG IgG (left panel), total anti-brain extract IgG (right panel) were quantified through ELISA. Two separate experiments with 8 mice/group. Data were analyzed with t-test: ** $p<0.01$.

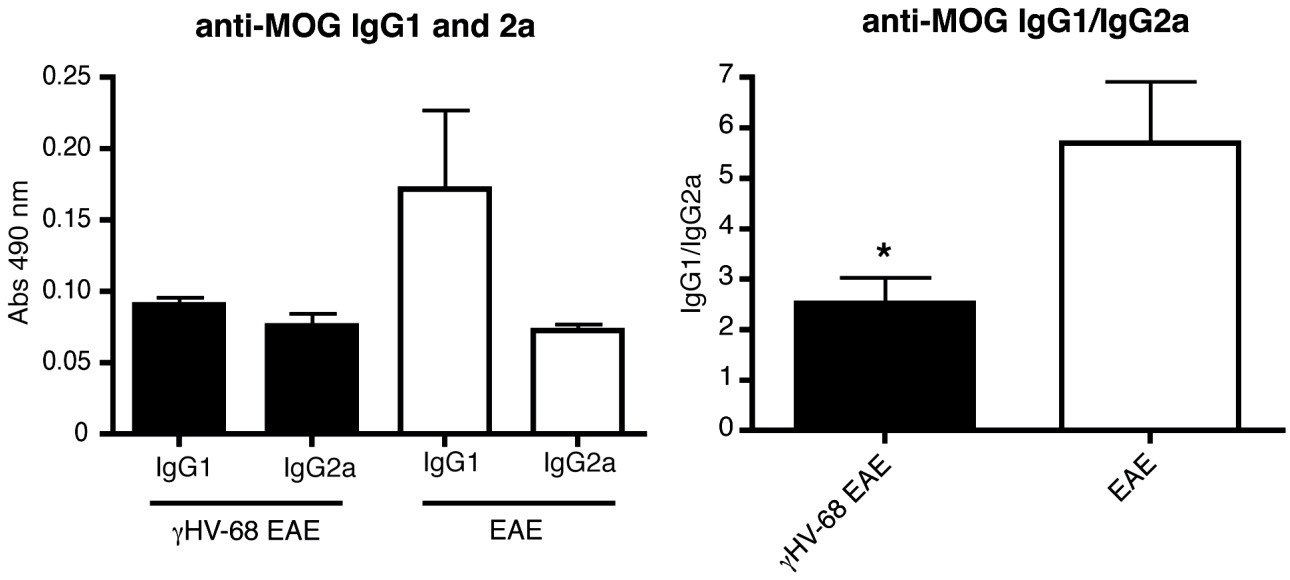


Figure 4.23. γHV-68 EAE mice have a decreased Ig1/IgG2a anti-MOG antibodies ratio

Mice were infected i.p. with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i., EAE was induced. Serum was harvested at day 28 post EAE and the levels of anti-MOG IgG1 and IgG2a were quantified through ELISA. Two separate experiments with 8 mice/group. Data were analyzed with Mann-Whitney U test: * $p<0.05$.

4.2.6 γHV-68 EAE mice are protected from EAE during acute γHV-68 infection and develop paralysis and CD8 T cell infiltrations in the brain parenchyma only upon establishment of γHV-68 latency

Next we decided to investigate if an acute infection with γHV-68 could precipitate EAE symptoms as we observed during γHV-68 latency. In order to address this, mice were infected with γHV-68, MCMV or HSV two days before EAE induction. Surprisingly, γHV-68 EAE mice were protected from EAE during acute infection (Figure 4.24). In fact, mice acutely infected with γHV-68 started to develop paralysis between day 16-20 post EAE induction, 18-22 days post γHV-68 infection when the virus is cleared and has started to establish latency. Conversely, MCMV and HSV acutely infected EAE mice developed paralysis symptoms between day 6 and 10 post EAE induction. MCMV mice displayed earlier onset and worse clinical scores than HSV mice. This indicates that γHV-68 mice are protected from EAE during acute infection and that latency is required to lead to enhanced EAE. To further corroborate this, CD8 T cell infiltrations inside the brain parenchyma were detected only in γHV-68 EAE mice after 20 days post EAE induction, at the onset of clinical disease and establishment of viral latency (Figure 4.25, left panel). The brains of mice acutely infected with either HSV (Figure 4.25, central panel) or MCMV (Figure 4.25, right panel) were free from CD8 T cells at any time points post paralysis onset.

These results indicate that CD8 T cell infiltrations inside the brain parenchyma require the establishment of γHV-68 latency. It is possible that CD8 T cells need to acquire a memory phenotype with upregulation of CD44 (see Figure 4.18) and interact with viral specific T helper CD4 T cells before they are able to infiltrate the CNS. This process (i.e. a complete adaptive immune response with generation of memory) is completed only upon resolution of acute γHV-68 infection and establishment of latency. Additionally, since it has been shown by other groups that high levels of IFN-γ are protective during EAE [276-278], it is conceivable that the immune response during acute γHV-68 protects the mice from EAE through the high levels of IFN-γ that are produced to control and clear acute infection.

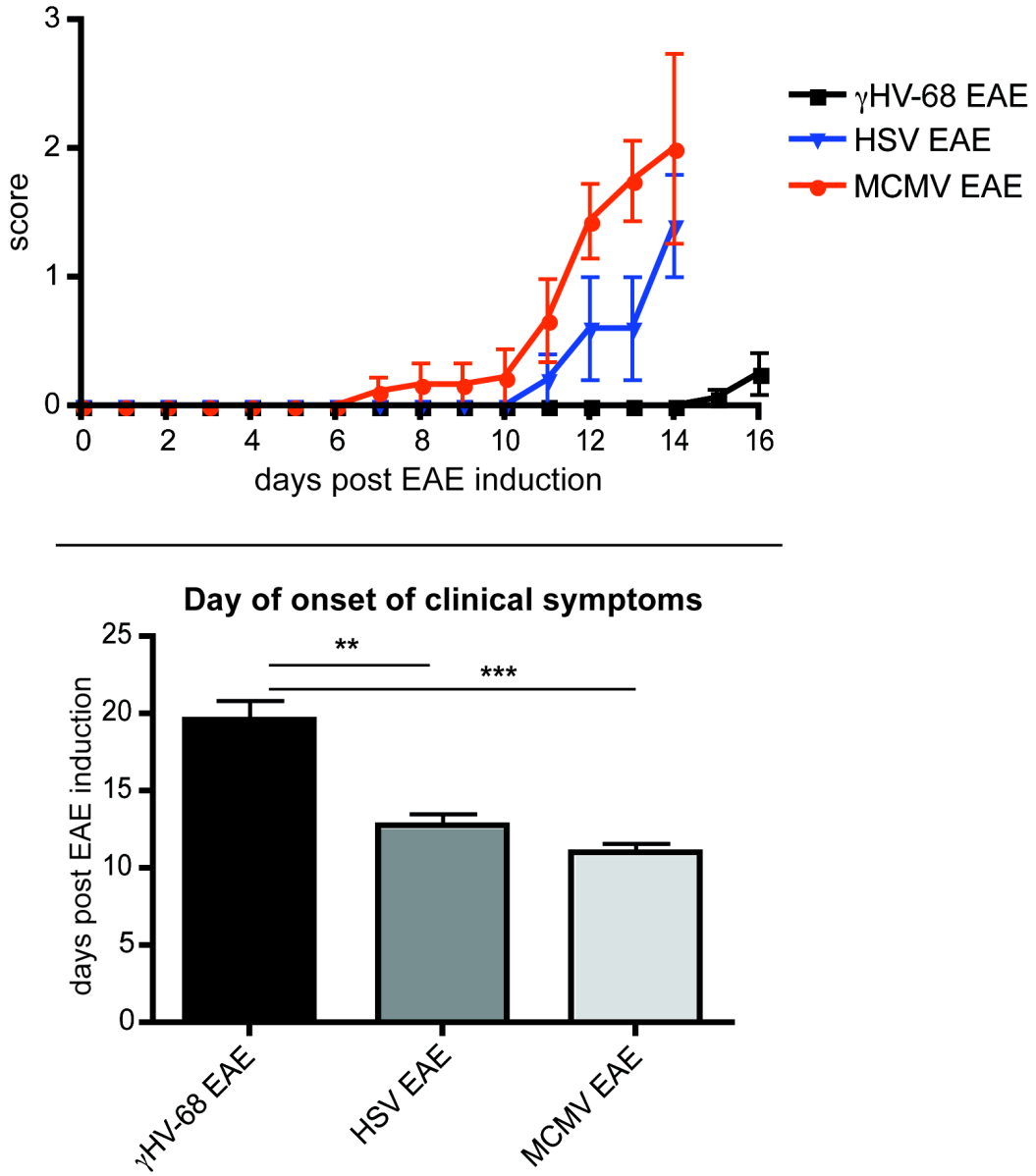


Figure 4.24. γHV-68 EAE mice are protected from EAE development during acute infection and start to develop EAE symptoms during the establishment of γHV-68 latency

Mice were infected with γHV-68 (black line) or HSV (blue line) or MCMV (red line). Two days p.i. EAE was induced (day 0 on the graph). The upper graph shows EAE scores up to day 14-16 post EAE induction. The lower graph shows the mean day of onset of clinical symptoms post EAE induction in the three different groups: γHV-68 EAE (black bar); HSV EAE (dark grey bar) and MCMV EAE (light grey bar). Two separate experiment, n=10/group. Data were analyzed with t-test comparing γHV-68 EAE with HSV EAE and γHV-68 EAE with MCMV EAE : ** $p<0.01$; *** $p<0.001$.

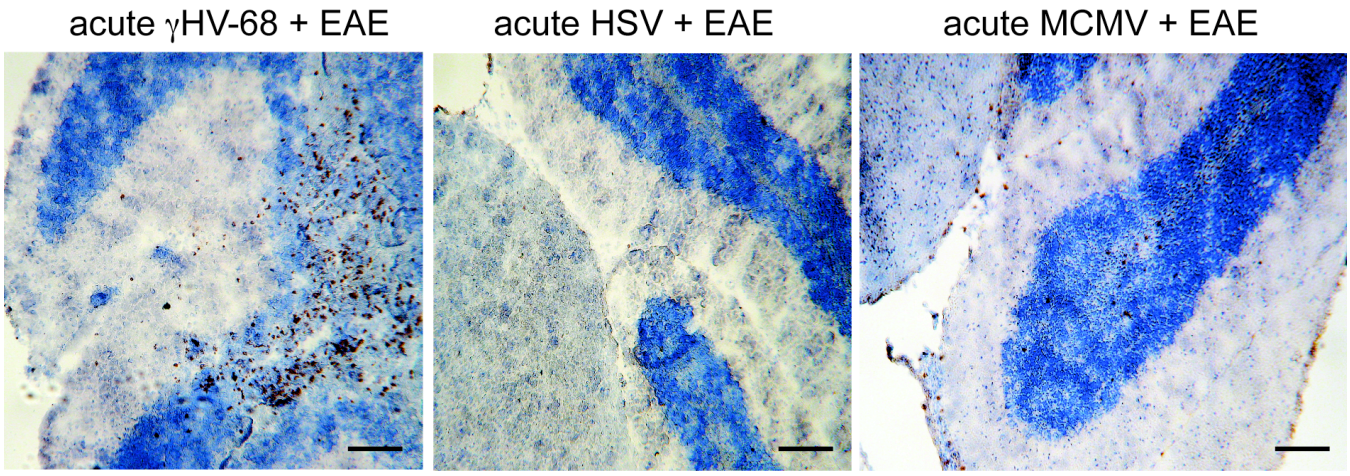


Figure 4.25. Only γHV-68 EAE mice show CD8 T cell infiltrations in the brain parenchyma at the onset of EAE symptoms, after γHV-68 acute infection clearance and establishment of γHV-68 latency

Mice were infected with γHV-68 (left panel) or HSV (middle panel) or MCMV (right panel). Two days p.i.; EAE was induced. Each group of mice was euthanized when it reached a mean clinical score between 2 and 3. Mice were perfused and brains were embedded in OCT, snap frozen, cut and stained with antibodies specific for CD8. The pictures are representative of two separate experiments (n=10/group). Scale bar: 100 μm.

4.2.7 CD11b+ CD11c+ cells from γHV-68 EAE mice are able to prime an enhanced Th1 response both in vitro and in vivo

Collectively our results indicate that γHV-68 EAE mice are polarizing a distinct T helper response post MOG immunization in comparison with typical EAE. We hypothesized that a subset of antigen-presenting cells (APCs), as a result of a direct infection or under the influence of other signals received by other immune cells during the infection, was able to skew a Th1 phenotype upon MOG presentation. The levels of different cytokines (IFN-γ, TNF-α, IL-12, IL-10 and IL-6) produced by B cells, CD11b+ and CD11c+ cells were assessed during the antigen presentation phase of EAE (day 4 post EAE, Figure 4.26). In γHV-68 EAE mice, CD11b+ CD11c+ cells from the inguinal lymph nodes produced IFN-γ at day 4 post EAE (7-8%); conversely, CD11b+CD11c+ cells from uninfected EAE mice produced less IFN-γ (3-4%). No differences were detected for the other cytokines analyzed. To test the ability of these antigen-presenting cells to prime a Th1 phenotype during MOG presentation, T cells from 2D2 T cell receptor (TCR) transgenic mice, that express a MOG- specific TCR, were isolated and incubated with MOG peptide and CD11b+ CD11c+ cells isolated either from an uninfected EAE mouse or a γHV-68 EAE mouse at day 4 post EAE induction. γHV-68 EAE CD11b+ CD11c+ cells induced an increased production of IFN-γ in these MOG specific transgenic T cells (Figure 4.27), while IL-17 was not detectable. Further, a nested PCR on CD11b+ CD11c+ cells isolated from γHV-68 mice did not find any γHV-68 DNA, indicating that the ability of these antigen-presenting cells to prime a Th1 response was not dependent on direct γHV-68 infection or replication. Additionally, the expression levels of co-stimulatory molecules and MHC class I- II on CD11b+ CD11c+ cells were analyzed at day 4 post EAE induction. Splenic CD11b+CD11c+ cells isolated from γHV-68 EAE mice expressed higher levels of CD40, MHC I and MHC II when compared to EAE mice (Figure 4.28), whereas the levels of CD80 and CD86 were similar between the two groups. Finally, to assess if these CD11b+CD11c+ cells were able to prime a stronger Th1 response also in vivo, CD11b+CD11c+ cells isolated from either γHV-68 EAE mice or EAE mice were adoptively transferred into naïve mice and 24 hours later EAE was induced. Mice transferred with γHV-68 EAE CD11b+CD11c+ cells presented with increased percentages of infiltrating CD4+ IFN-γ+ T cells into the

CNS and decreased percentages of CD4+ IL-17+ T cells (Figure 4.29 shows data obtained from the spinal cords, a similar trend was observed in the brains).

These results indicate that a subset of CD11b+ CD11c+ antigen-presenting cells found in γHV-68 EAE mice, without being directly infected by the virus, induced the production of increased levels of IFN-γ in T cells both in vivo and in vitro. This cell subset is likely responsible for the polarization of the potent Th1 response that is observed in γHV-68 EAE mice.

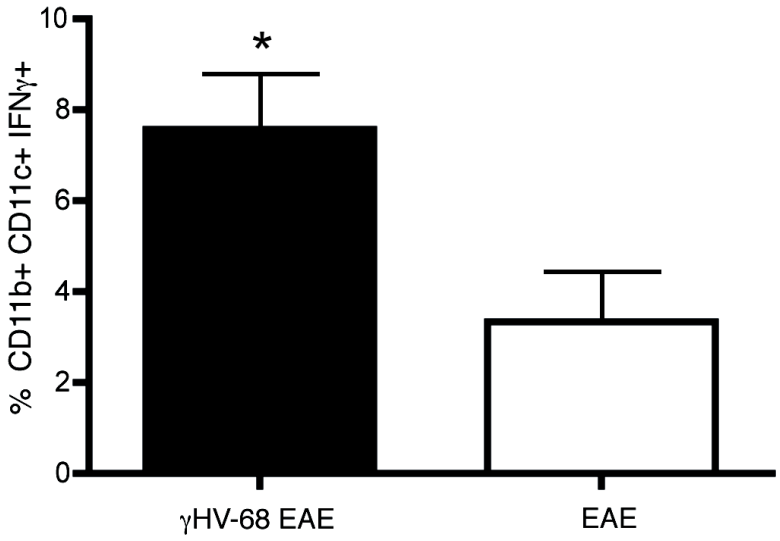


Figure 4.26. CD11b+CD11c+ cells in the periphery of γHV-68 EAE mice produce more IFN-γ at day 4 post EAE induction

Mice were infected with γHV-68 (black bar) or MEM only (open bar). Five weeks p.i. EAE was induced. At day 4 post EAE induction, mice were injected i.v. with brefeldin A. Six hours later, inguinal lymph nodes were harvested and immune cells were stained for cytokine production without any further restimulation. Three separate experiments, n=10/group, data were analyzed with t-test: * $p<0.05$.

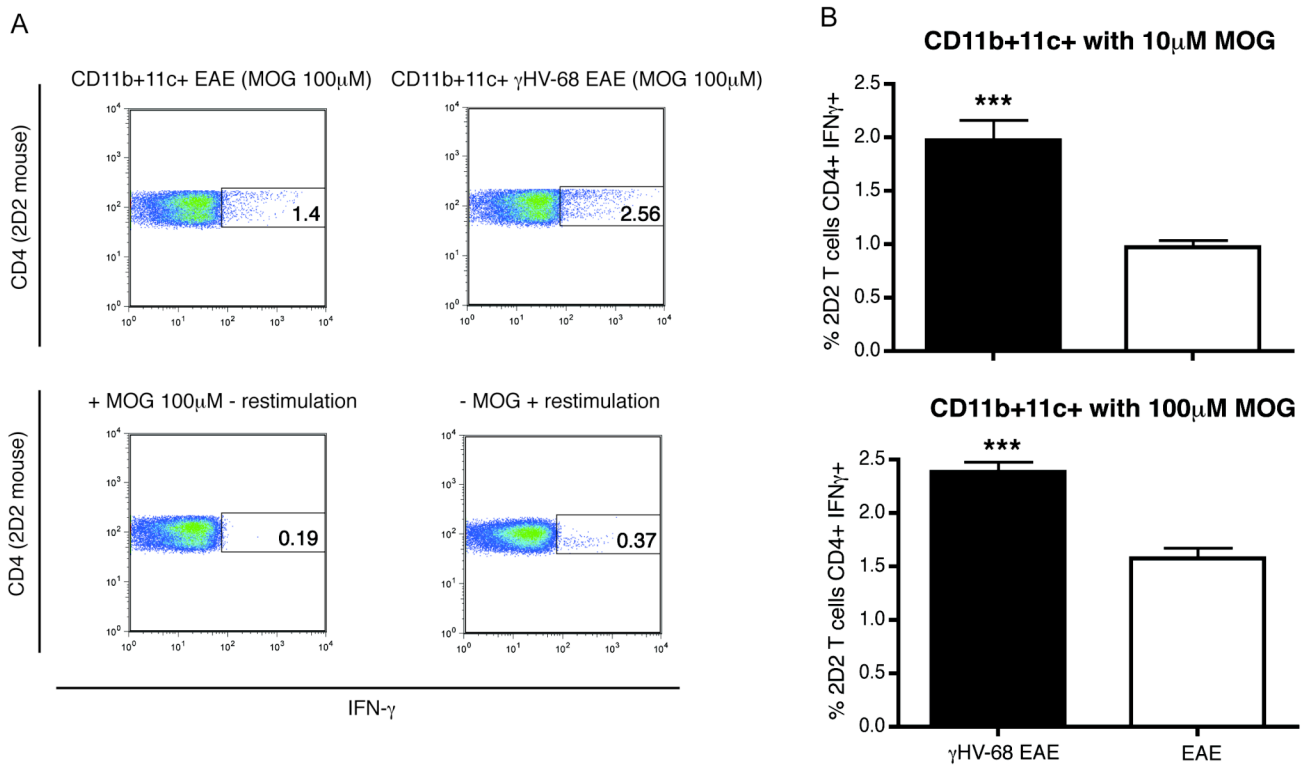


Figure 4.27. CD11b+ CD11c+ cells isolated from γHV-68 EAE mice are able to prime an enhanced IFN-γ response in 2D2 CD4+ T cells in vitro

Mice were infected with γHV-68 or MEM only. Five weeks p.i., EAE was induced. At day 4 post EAE induction, spleens and lymph nodes were harvested and CD11b+CD11c+ cells were isolated. CD4 T cells from 2D2 mice were isolated at the same time. CD11b+CD11c+ were incubated with 2D2 CD4 T cells and different concentrations of MOG peptide for 72 hours. T cells were restimulated and stained to assess the production of IFN-γ. **(A)** Representative FACS plot showing IFN-γ production in 2D2 CD4 T cells after incubation with CD11b+CD11c+ cells isolated from a γHV-68 EAE mouse (upper right panel) or a naïve EAE mouse (upper left panel) and MOG peptide (100 μM). Lowers panels show the negative controls with/without MOG and with/without restimulation. **(B)** Histograms showing the percentages of 2D2 CD4 T cells producing IFN-γ after incubation with CD11b+CD11c+ cells from a γHV-68 EAE mouse (black bars) or a naïve EAE mouse (open bars) and 10 μM (upper panel) or 100 μM (lower panel) MOG peptide. Three separate experiments with triplicate wells for each group. Data were analyzed with t-test: *** $p<0.001$.

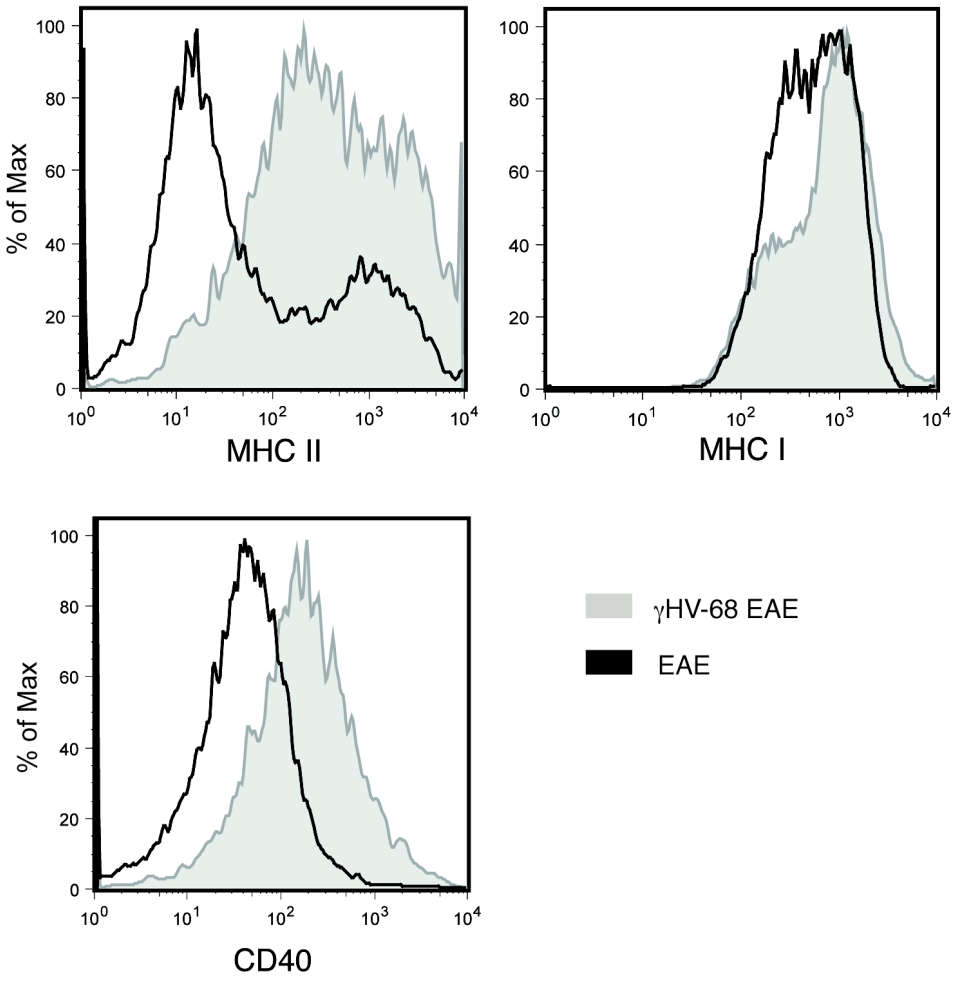


Figure 4.28. CD11b+CD11c+ cells isolated from γHV-68 EAE mice express higher levels of CD40 and MHC II

Mice were infected with γHV-68 or MEM only. Five weeks p.i., EAE was induced. At day 4 post EAE induction, spleens were harvested and CD11b+CD11c+ cells were isolated. The figure shows the levels of MHC II, MHC I and CD40 expressed on CD11b+CD11c+ isolated from the spleens of γHV-68 EAE mice (grey shaded histograms) or a naïve EAE mice (open histograms). Two separate experiments.

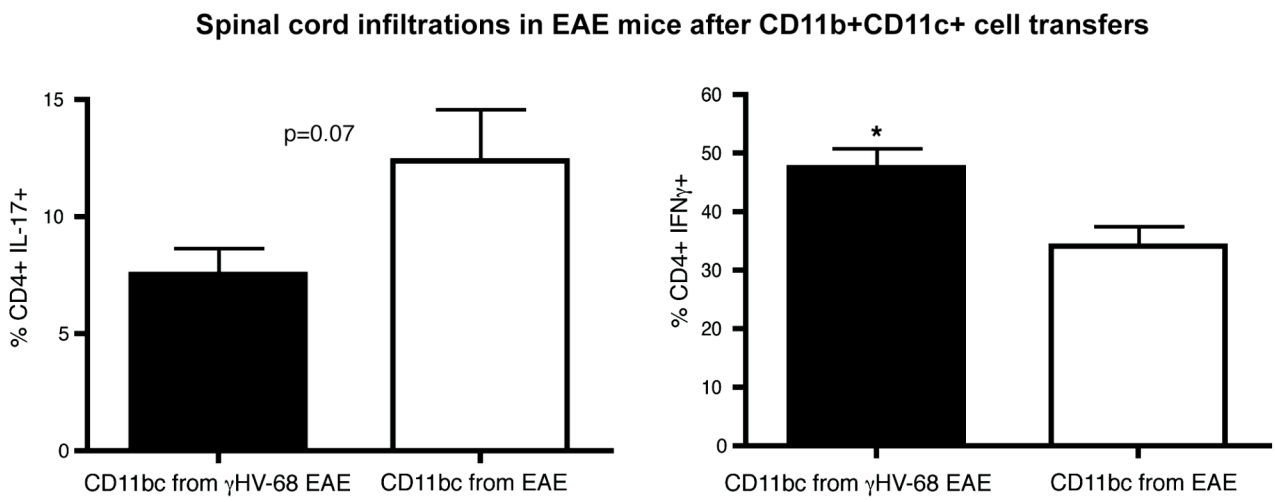


Figure 4.29. CD11b+ CD11c+ cells isolated from γHV-68 EAE mice are able to prime an enhanced Th1 response in vivo.

CD11b+CD11c+ isolated from both γHV-68 EAE and naïve EAE mice at day 4 post EAE were transferred into naïve mice. Twenty-four hours later EAE was induced in both groups. At day 14-16 post EAE induction (mean clinical score between 2 and 3 for both groups) brains and spinal cords were harvested and the production of IFN-γ and IL-17 by CD4 T cells in the CNS of mice that received CD11b+CD11c+ cells from γHV-68 EAE mice (black bars) or from naive EAE mice (open bars) was assessed after in-vitro restimulation with PMA and ionomycin. Results obtained from the spinal cords are shown, a similar trend was observed in the brain. Three separate experiments with 5 mice/group. Data were analyzed with t-test: * $p<0.05$.

4.2.8 Regulatory T cells are decreased in both the CNS and the periphery of γHV-68 EAE mice even before EAE induction

It has been previously shown that mice infected with γHV-68 present with decreased expression of Foxp3 and diminished T cell regulatory activity up to day 15 post γHV-68 infection [279]. Additionally, we detected increased expression of CD40 on APCs and CD40 has been shown to control regulatory T cells (Tregs) development [280, 281]. For these reasons, we decided to investigate the frequency of Tregs both in the periphery and the CNS before and after EAE induction. Spleens from latently infected γHV-68 mice were harvested before or after EAE induction. The frequencies of Tregs were decreased in the spleens of γHV-68 mice at 40-50 days post acute infection before and after EAE induction when compared to naïve and EAE mice (Figure 4.30, upper and lower left panels). Conversely, the percentages of Tregs found in the cervical lymph nodes after EAE induction were similar between γHV-68 EAE and EAE mice (Figure 4.30, upper right panel). Despite this accumulation in the cervical lymph nodes, the frequencies of Tregs in the CNS of γHV-68 EAE mice were decreased (Figure 4.31), thus indicating that Tregs lost the ability to migrate at the site of inflammation in γHV-68 EAE mice or converted to an inflammatory phenotype when they enter the CNS of γHV-68 EAE mice, losing Foxp3 expression.

These results show for the first time that γHV-68 mice have decreased splenic percentages of Tregs at late time points post primary infection, during latency (day 40-50 p.i.). Additionally, these results demonstrate that γHV-68 EAE mice have decreased frequencies of Tregs in both brains and spinal cord during EAE, despite their accumulation in the cervical lymph nodes.

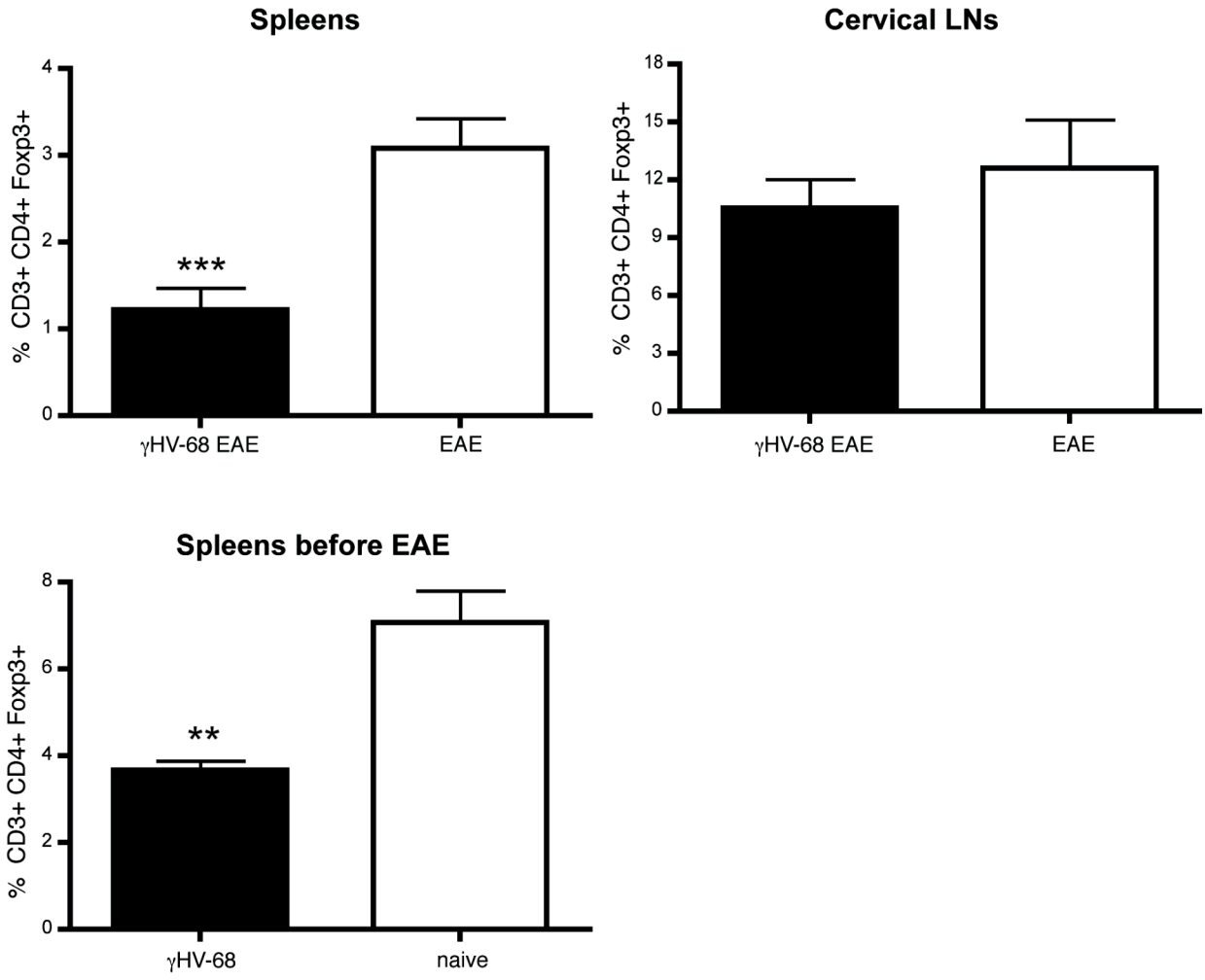


Figure 4.30. Regulatory T cells are decreased in the spleens but not in the cervical lymph nodes of γHV-68 mice both during EAE and before EAE induction

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced (upper panels) or not (lower panel). At day 14-16 post EAE induction (mean score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, cervical lymph nodes (right upper panel) and spleens (left upper and lower panel) were harvested and stained. The graphs show the percentage of regulatory T cells (CD4+ CD3+ Foxp3+). Two separate experiments with 8-6 mice/group; data were analyzed with t-test: ** $p<0.01$; *** $p<0.001$.

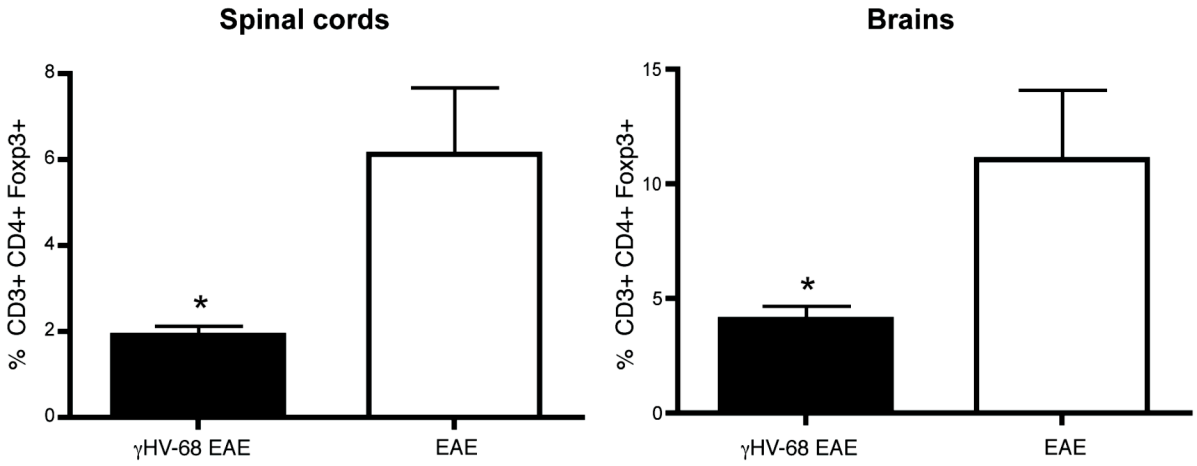


Figure 4.31. Regulatory T cells are decreased in brains and spinal cords of γHV-68 mice during EAE

Mice were infected with γHV-68 (black bars) or MEM only (open bars). Five weeks p.i. EAE was induced. At day 14-16 post EAE induction (mean score of 3 for γHV-68 EAE mice, EAE mice were harvested at the same time), mice were perfused, brains (right panel) and spinal cords (left panels) were harvested and processed to isolate immune infiltrates. The graphs show the percentage of infiltrating regulatory T cells (CD4+ CD3+ Foxp3+). Two separate experiments with 8-6 mice/group; data were analyzed with t-test: * $p<0.05$.

4.3 Discussion

The study of the “viral etiology of MS” has been challenging because of the absence of a mouse model that could be exploited to dissect the relationship between candidate MS viruses and development of CNS autoimmunity. EBV has been strongly linked to MS development even if the mechanisms by which EBV triggers autoimmunity are not understood [256]. We demonstrated that a latent infection with a gammaherpesvirus changes the polarization of the myelin specific T cell response after EAE induction leading to a potent Th1 response with more severe paralysis, atypical neurological symptoms, different composition and localization of the CNS infiltrates and myelin lesions in the white matter reminiscent of human MS.

Brain inflammation accompanied by T cell infiltrations within the brain parenchyma is rarely detected in EAE, although it is a critical characteristic of human MS [109]. Further, during EAE, CD4 T cells are the predominant T cell type to invade the CNS, whereas in humans, CD8 T cells are equally present in the MS plaques [109, 189]. In γHV-68 EAE mice, CD8+ IFN-γ+ granzyme+ cells infiltrate the brain parenchyma and a minority of these is specific for γHV-68 proteins. Thus, activated CD8 T cells that are not specific for CNS epitopes are able to enter the brain parenchyma and likely participate in sustaining a pro-inflammatory loop that recruits additional immune cells. These findings are similar to those recently shown by Matullo et al. in which a polymicrobial challenge leads to the recruitment of LCMV specific T cells in the CNS despite the absence of LCMV infection in the CNS [282]. Interestingly, this phenotype is dependent on the establishment of latency. In fact, acute infection with γHV-68 protects mice from EAE induction and CD8 T cell infiltrations inside the brain parenchyma appear only when acute infection is cleared and latency is established. We suggest that our model could potentially be used to study the behavior of CD8 T cells in CNS autoimmunity and elucidate the signals that guide CD8 infiltrations inside the brain during demyelinating diseases.

It has long been described that MS patients have a potent CNS Th1 response [283, 284]. The potent Th1 response observed in γHV-68 EAE mice not only includes higher amounts of IFN-γ but also an upregulation of T-bet in all T cells, including those not actively producing any cytokines. Interestingly T cells from MS patients exhibit preferentially a Th1 phenotype [283, 284], T-bet levels are predictive of IFN-β therapy

efficacy in MS patients [285] and T-bet is upregulated in patients with active disease and is downregulated during remission [286].

Our data demonstrate that, upon MOG peptide presentation, CD11b+CD11c+ cells from γHV-68 EAE mice are able to induce, both ex vivo and in vivo, an increased production of IFN- γ in T cells with a suppression of the Th17 response. Intriguingly, CD11b+CD11c+ cells from γHV-68 EAE mice have increased expression of MHC class I and II as well as CD40. It has been shown that the strength of signal upon antigen presentation dictates the fate of the T helper response [287]. A weaker signal preferentially primes a Th17 response. Thus, increased expression of MHC II and CD40 on the surface of CD11b+CD11c+ cells likely provides a strong activation signal and could be responsible for the suppression of the Th17 response and the skewing towards the observed Th1 phenotype found in γHV-68 EAE mice. Additionally, it has been shown that CD40 is important for cytotoxic CD8 T cell activation [288-291], suggesting that increased CD40 expression in γHV-68 EAE mice may have a role in CD8 T cell enhanced activation and CNS infiltration. Finally, CD40 upregulation could lead to decreased percentages of Tregs in γHV-68 EAE mice since CD40 signaling suppresses the development of Tregs [280, 281].

CD11b is found mainly on the surface of monocytes/macrophages, but it can also be co-expressed, along with CD11c, on the surface of dendritic cells and NK cells [292, 293]. Moreover, IFN-γ is mainly produced by T cells and NK cells but macrophages [294] and dendritic cells [295] have also been shown to produce IFN-γ in response to both endogenous and exogenous stimuli. So it is conceivable that CD11b+ CD11c+ IFN-γ+ cells isolated from the inguinal LNs of γHV-68 mice (see Figure 4.26) could be NK cells. Further analysis of surface markers on this cell subset is ongoing to better characterize it. Interestingly, γHV-68 activates macrophages and this phenotype protects mice from lethal infections by another intracellular pathogen, Listeria [103]. This mechanism is independent from T cells but dependent on IFN-γ, showing that a latent infection with γHV-68 confers a broad innate cross protection that does not require IFN-γ produced by memory T cells. In our model, it is possible that macrophages and/or dendritic cells with an activated phenotype that are secreting more pro-inflammatory Th1 skewing cytokines are responsible for the priming of the potent Th1 response in γHV-68 EAE mice.

Lastly and significantly, the ability to discover EBV in the brains of MS patients has

been controversial [165, 167-169]. In the γHV-68 EAE mouse model, γHV-68 is no longer detectable in the CNS tissue (by PCR) during disease and yet, it is indirectly influencing the autoimmune response and immune cell polarization. It is easy to imagine that EBV may well be acting similarly as the first trigger of the second hit hypothesis. It is intriguing that mice latently infected with the murine homologue of EBV are developing an EAE that is more reminiscent of human MS. From this model, experiments can be designed to ask how γHV-68 is acting to allow T cells to preferentially enter the brain during MS, specifically addressing the signals that are required and where they arise.

In conclusion, we propose that EBV latent infection in patients is influencing the development of disease following a second hit. In this case, EAE induction results in a stronger disease as the result of polarization of the adaptive T cell response and a decrease in Tregs. In MS patients, a second trigger also leads to the skewing of the immune system towards a Th1 biased phenotype and an increased activation status resulting in MS lesions. As such, a patient's history of infection and, specifically, EBV latent infection are as important as genetics in determining an individual's susceptibility to autoimmune diseases.

5 Gammaherpesvirus latency affects plasma cells and anti-nuclear autoantibodies production in a spontaneous mouse model of systemic lupus erythematosus[4]

[4] A version of chapter 5 will be submitted for publication: Costanza Casiraghi, Iryna Shanina and Marc S. Horwitz. Gammaherpesvirus latency affects plasma cells and anti-nuclear autoantibodies production in a spontaneous mouse model of systemic lupus erythematosus.

5.1 Introduction

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease. It affects between 2 and 4 million people worldwide [191]. SLE patients, 90% of whom are women, develop autoantibodies against nuclear self-antigens (DNA, histones, chromatin). Deposition of self reactive antibodies bound to nuclear material starts an immune response in the target organ leading to tissue inflammation and damage [192]. Genetic predisposition and environmental factors have both been shown to be important contributors to SLE. However, the trigger that leads to the production of autoantibodies and immune dysregulation is currently unknown [192, 194]. Different viruses have been indicated as environmental triggers of SLE [197, 198] and Epstein-Barr virus (EBV) is the most closely associated with SLE development.

EBV is a DNA virus of the γ-herpesvirus family. It establishes a life-long latent infection in B cells [12]. It infects 90% of the human population and it is usually acquired during childhood. When primary infection takes place later in life, it leads to infectious mononucleosis [5].

EBV seropositivity is higher in SLE patients than in the general population in both adult and pediatric cohorts [196, 199, 200]. SLE patients have from 15 fold [203] to 40 fold [204] higher EBV viral loads than healthy individuals. Intriguingly, increases in EBV viral loads always occur one week or more after the onset of a SLE flare [205]. This impaired control of EBV replication could be due to an impaired ability of the EBV specific T cell response to control viral reactivation in SLE patients. EBV specific CD8 responses have been documented to be decreased in number and impaired in cytotoxic function in SLE patients [204, 205, 216]. Finally cross-reactive antibodies between nuclear self-antigens and EBV proteins have been isolated from SLE patients [208-211]. These cross-reactive antibodies are typical of both SLE patients and mononucleosis patients [211]. However anti-EBV cross-reactive antibodies disappear after mononucleosis resolution in healthy individuals but they persist in patients that later develop SLE [211]. Additionally some reports have shown that mononucleosis can induce the onset of SLE symptoms [212-214]. It is conceivable that, after EBV infection, SLE patients cannot control the production of autoantibodies that increase in titer and expand their specificities to other nuclear epitopes until the onset of clinical disease. Despite all these findings, it is still not clear how EBV would trigger SLE.

New Zealand black/white hybrid (NZBW) mice are a F1 hybrid obtained from the crossing of a New Zealand black (NZB) with a New Zealand white (NZW) mouse and it is a well studied spontaneous model of human SLE. NZBW hybrids develop an autoimmune disease that includes: lymphadenopathy, splenomegaly, elevated antinuclear autoantibodies and immune complex-mediated glomerulonephritis that closely resemble the kidney pathology observed in SLE patients [220, 221]. As for SLE patients, disease in NZBW mice is biased in favor of females. Starting around 4-5 months of age, NZBW mice start to develop anti-nuclear antibodies that subsequently switch from IgM (less pathogenic) to IgG (more pathogenic) isotype. Glomerulonephritis develops in females around 6 months of age [225].

EBV does not infect rodents but murine gamma herpesvirus-68 (γHV-68) is considered the murine homolog of EBV and it has been extensively employed to study the relationship between the immune system and latent γ-herpesvirus infection in mice [50]. EBV's and γHV-68 genomes are different, however both viruses elicit a similar immune response. They are both capable of establishing a life long infection in B cells and they both modulate the immune response of the host through the expansion of a viral specific CD8 response similar to that detected in humans during mononucleosis [270]. We have previously shown that mice latently infected with γHV-68, after EAE induction, develop a clinical disease that resembles human MS and it is driven by a potent Th1 response, indicating that γHV-68 is a good model to study the interaction between autoimmunity development and γ-herpesvirus latency. Because of these similarities, we decided to test if a γHV-68 latent infection had an impact on the development of SLE in NZBW mice. NZBW mice latently infected with γHV-68 presented with a different composition of anti-nuclear autoantibodies driven by an enhanced Th1 response.

5.2 Results

5.2.1 γHV-68 NZBW mice have a decreased number of plasma cells and produce different types of anti-nuclear autoantibodies

Female NZBW mice were infected intraperitoneally with γHV-68 between 8-10 weeks of age. An equal number of uninfected mice were used as a control. Around day 200 post infection (8 months of age), NZBW mice started to develop proteinuria and kidney failure (mean value of 1g/L in both groups). The development of autoreactive antibodies was monitored over time. Sera were harvested before infection and every two-three weeks subsequently. The levels of both anti-ssDNA and anti-dsDNA IgGs and IgMs were measured before infection and at days 30, 70, 100 post infection (p.i.). No differences between the groups were detected at these earlier time points (data not shown). Conversely, at 200 days p.i. (endpoint), γHV-68 NZBW mice presented with decreased amounts of anti-dsDNA, ssDNA and histones IgM antibodies and similar levels of anti-dsDNA, ssDNA and histones IgG antibodies (Figure 5.1).

Since similar levels of anti-nuclear IgGs were detected in both γHV-68 and naïve NZBW mice, we further characterized the IgG autoreactive response by analyzing the amount of IgG1 and IgG2a produced against the same nuclear antigens. The levels of autoreactive IgG1 and IgG2a before infection and at days 50, 90 and 130 p.i. were similar between the groups (data not shown). In contrast, at the endpoint (200 days p.i.), γHV-68 NZBW mice presented with an increased, but not statistically significant, IgG2a/IgG1 ratio of anti-dsDNA and ssDNA autoantibodies (Figure 5.2). No differences were found when anti-histone antibodies were analyzed (data not shown).

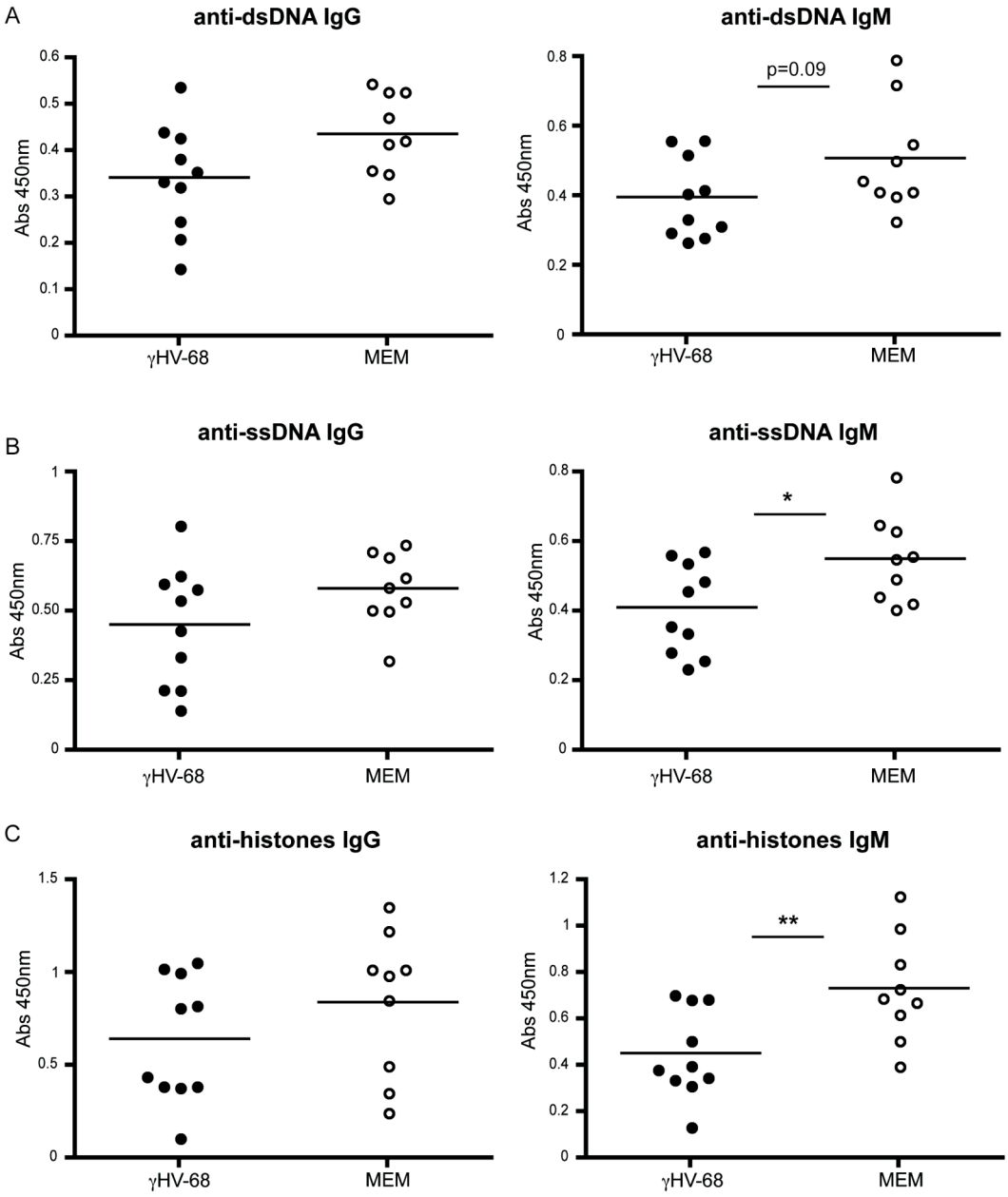


Figure 5.1. γHV-68 NZBW mice secrete less IgM anti-nuclear antibodies but similar amounts of IgG anti-nuclear antibodies at 8 months of age

Mice were infected with γHV-68 (black circles) or MEM only (open circles) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), serum was harvested through cardiac puncture. The levels of IgG (left panels) and IgM (right panels) specific for dsDNA **(A)**, ssDNA **(B)** and histones **(C)** were quantified by ELISA. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$; ** $p<0.01$.

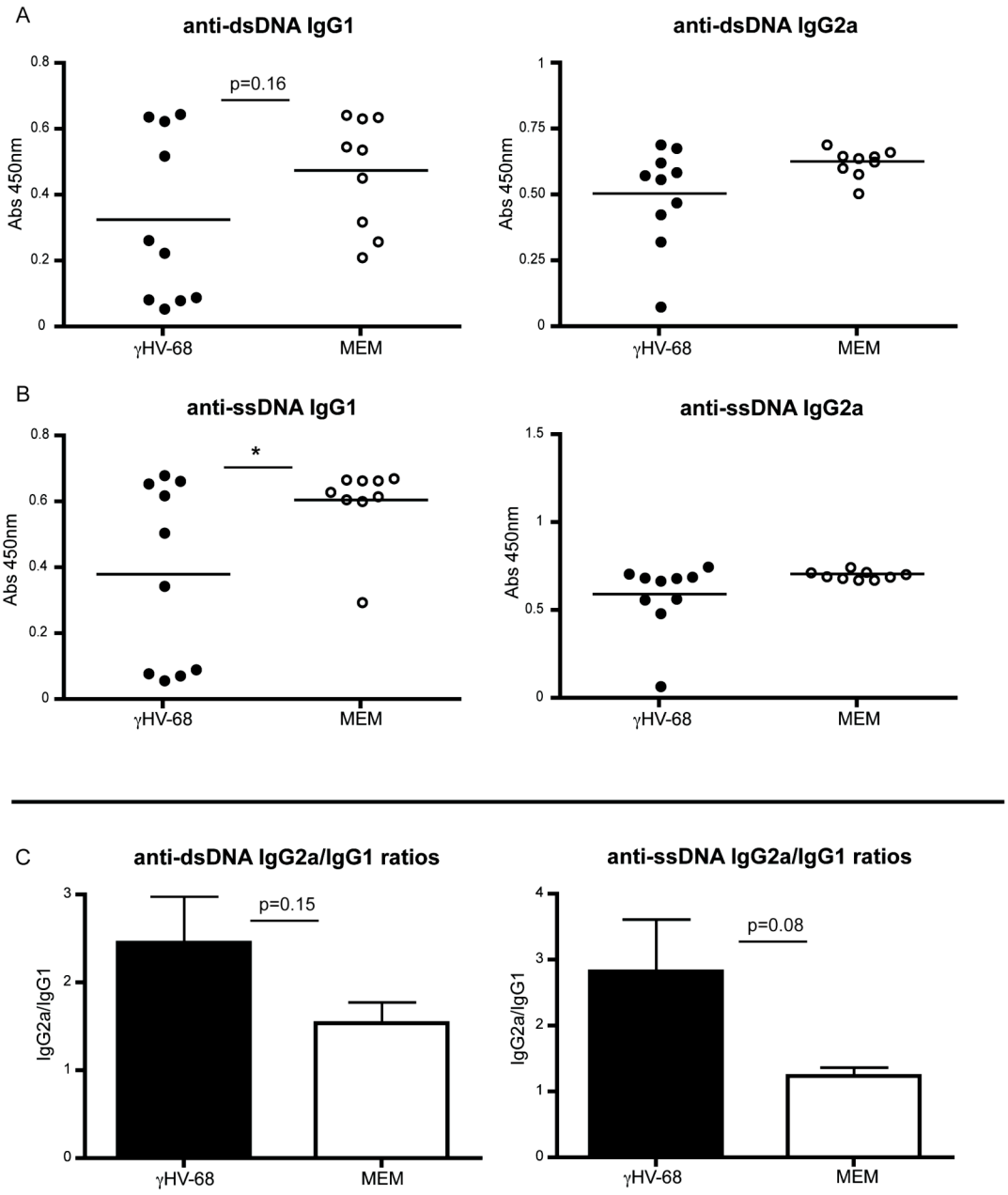


Figure 5.2. γHV-68 NZBW mice have an increased IgG2a/IgG1 anti-DNA antibodies ratio at 8 months of age

Mice were infected with γHV-68 (black circles) or MEM only (open circles) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), serum was harvested through cardiac puncture. The levels of IgG1 (left panels) and IgG2a (right panels) specific for dsDNA **(A)**, ssDNA **(B)** were quantified by ELISA. Panel **(C)** shows IgG2a/IgG1 ratios for either γHV-68 infected (black bars) or naïve (open bars) NZBW mice. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$.

Since we found a difference in autoantibody production between γHV-68 and naïve NZBW mice, the composition of B cell subsets in the spleen was analyzed at the endpoint (200 days p.i.). No differences in the percentages of marginal zone B cells (CD19+ IgM^{high} $CD21^{high}$ CD23-), follicular B cells (CD19+ IgM^{high} $CD21^{low}$ $CD23^{high}$), T1 B cells (CD19+ IgM^{high} CD21- CD23-) and T2 B cells (CD19+ IgM^{high} CD21+ $CD23^{high}$) were found between the spleens of γHV-68 and naïve NZBW mice (data not shown). Surprisingly, γHV-68 NZBW mice presented with decreased percentages of plasma cells (CD138+) and decreased B cell activation (CD19+ CD69+) in the spleen (Figure 5.3).

These results indicate that at earlier time points p.i., there are no differences in autoantibody production if mice are infected with γHV-68. Conversely, at later time points, at the onset of kidney damage and after five months of γHV-68 interaction with the host's immune system, γHV-68 NZBW mice are producing different types of anti-nuclear antibodies and have decreased frequencies of plasma cells and activated B cells in the spleen.

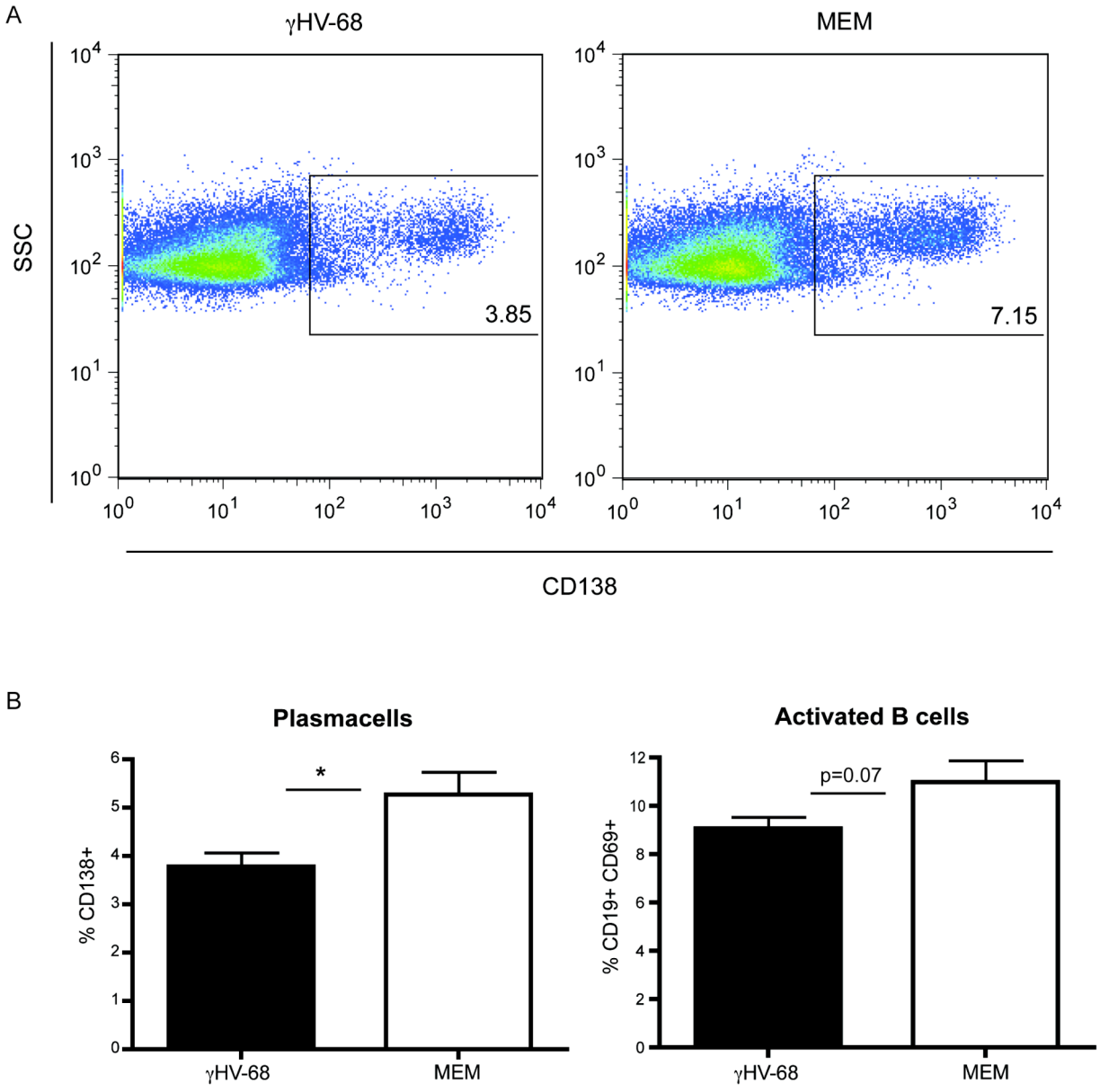


Figure 5.3. γHV-68 NZBW mice show decreased numbers of plasma cells and activated B cells at 8 months of age

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), spleens were harvested. FACS analysis was performed to quantify the percentages of CD138+ plasma cells **(A-B)** and the percentages of activated B cells (CD19+ CD69+; **(B)**) in either γHV-68 infected (black bars) or naïve (open bars) NZBW mice. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$.

5.2.2 The extent of lupus nephritis and IgGs deposition in the kidneys are comparable between γHV-68 latently infected and uninfected NZBW mice

IgG2a antibodies are more pathogenic in SLE than IgG1 and IgM. Additionally, IgG2a antibodies deposit preferentially in the kidneys leading to glomerulonephritis. Since we found an increased ratio of IgG2a/IgG1 anti-nuclear antibodies in γHV-68 NZBW mice, we asked if this difference was enough to lead to enhanced kidney damage and glomerulonephritis, hallmarks of both human SLE and NZBW SLE. At the endpoint (200 days p.i.), the levels of protein in the urine, a parameter used to assess kidney failure, were comparable between γHV-68 and naive NZBW mice (Figure 5.4), indicating that the extent of kidney damage was similar between the two groups.

This result was confirmed through hematoxylin and eosin (H&E) staining of formalin fixed, paraffin embedded kidney sections (Figure 5.5) and IgGs staining of frozen kidney sections (Figure 5.6). H&E staining showed a similar extent of glomerulonephritis between the two groups with an equivalent percentage of glomeruli showing hyperproliferation and infiltrations (Figure 5.5). Immunohistochemistry for IgGs deposits showed a similar number of positive glomeruli (Figure 5.6, upper panels) with a comparable stain intensity (Figure 5.6 lower panels) between γHV-68 and naive NZBW mice. Finally, immunohistochemical staining to detect CD8 and CD4 T cell, B cell and macrophage infiltrations were performed on kidney sections. Few mice showed diffused T cell and macrophage infiltrations in the kidneys, however these immune infiltrations were observed in both γHV-68 and naive NZBW mice (data not shown).

This indicates that differences in autoantibody production with predominance of more pathogenic IgG2a antibodies in γHV-68 NZBW mice are not enough to precipitate more extensive kidney damage.

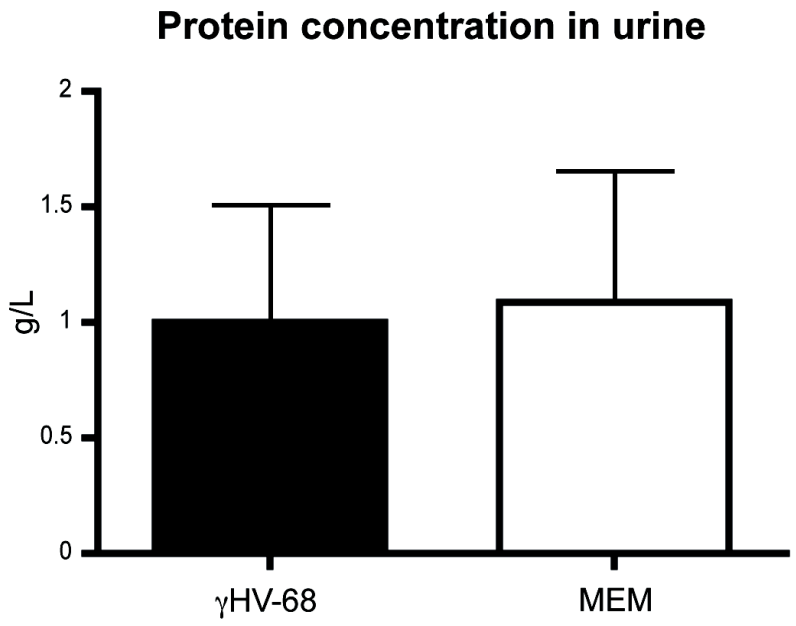


Figure 5.4. Naïve and γHV-68 NZBW mice have similar levels of proteins in the urine at 8 months of age

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At 8 months of age, mice were euthanized and protein levels in the urine were measured using Chemstrips GP (visual readings: 0 g/L, 0.3 g/L, 1g/L, 5 g/L).

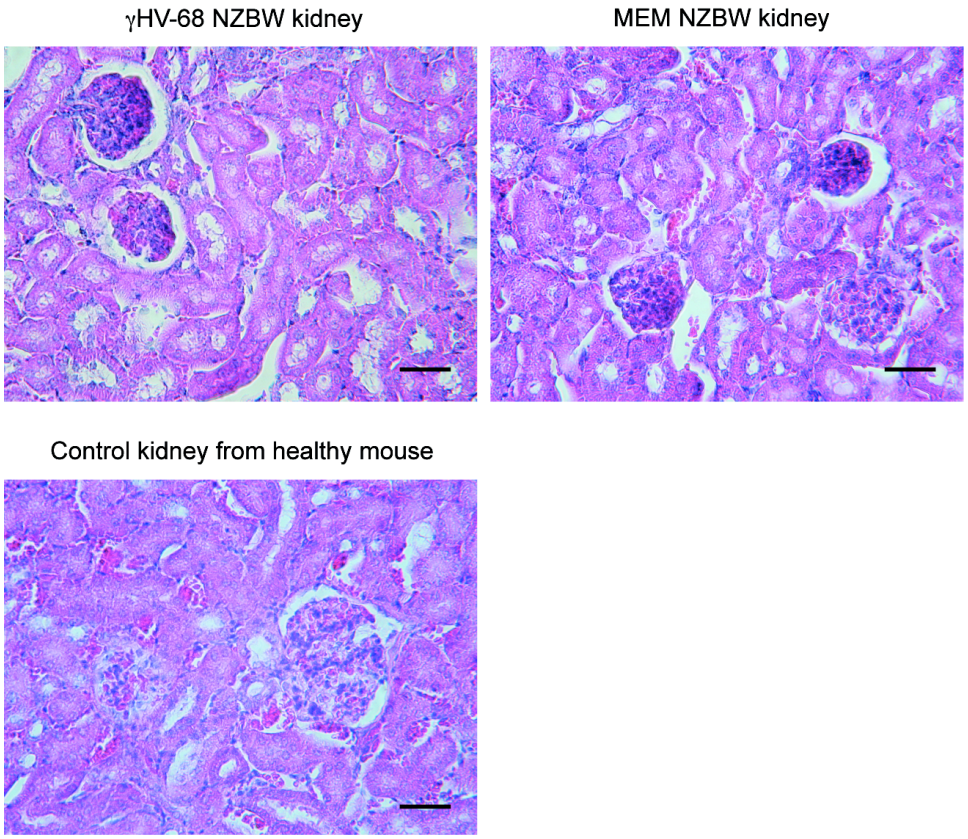


Figure 5.5. Lupus nephritis is similar between γHV-68 NZBW mice and naïve NZBW mice

Mice were infected with γHV-68 (upper left panel) or MEM only (upper right panel) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), kidneys were harvested, formalin fixed, paraffin embedded and stained with hematoxylin and eosin. Upper panels show glomerulonephritis in NZBW mice, lower panel show a normal glomeruli from a healthy mouse (C57Bl/6 strain). Scale bar = 50 μm. Two separate experiments with 5 mice/group.

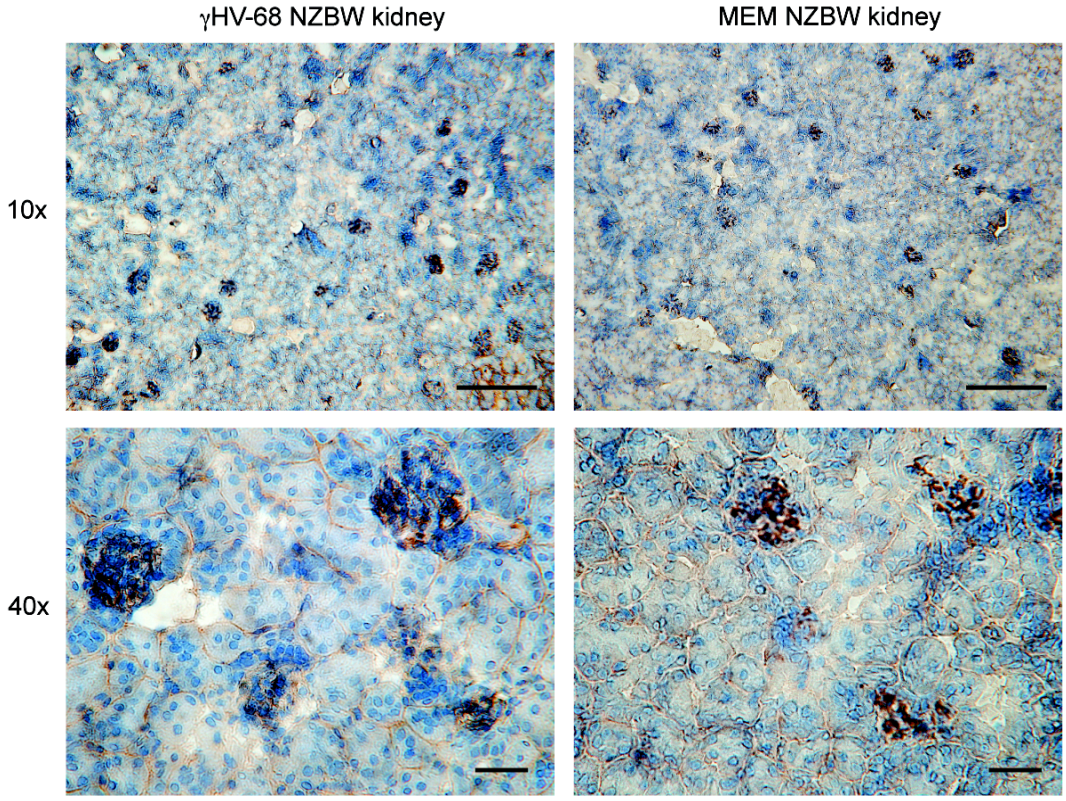


Figure 5.6. The levels of IgGs deposition are similar between γHV-68 NZBW mice and naïve NZBW mice

Mice were infected with γHV-68 (left panels) or MEM only (right panels) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), kidneys were harvested and frozen in OCT. Sections were stained with anti-mouse IgG antibody. The pictures show IgGs deposition in several glomeruli (brown spots) at both lower magnification (upper panels, scale bar = 100 μm) and higher magnification (lower panels, scale bars = 50 μm). Two separate experiments with 5 mice/group.

5.2.3 T cells isolated from γHV-68 NZBW mice have an effector phenotype and secrete higher levels of IFN-γ and TNF-α

Other groups have shown that the T helper response in NZBW mice is skewed neither towards a Th1 phenotype, nor towards a Th2 phenotype before SLE development [236-238]. Since we previously detected a potent Th1 response in γHV-68 EAE mice and in γHV-68 NZBW we found an increased IgG2a/IgG1 ratio, indicative of a Th1 response, we decided to further characterize the T cell response in both γHV-68 and naïve NZBW mice. First we analyzed the levels of different cytokines (IL-6, IL-10, IL-12, TNF-α, IFN-γ, MCP-1) in the serum at day 15, 30 and 70 post γHV-68 infection and at the endpoint (200 days p.i.). γHV-68 NZBW mice showed increased amounts of IL-6 and IL-12 in the serum 15 days post γHV-68 infection (Figure 5.7). Then IL-12 and IL-6 levels decreased in γHV-68 NZBW mice at day 30 and day 70 p.i., reaching the same levels detected in naïve NZBW mice (data not shown). However γHV-68 NZBW mice again upregulated IL-12 at the onset of lupus clinical symptoms and this upregulation remained present until the endpoint (200 p.i., Figure 5.8). Additionally, MCP-1 and TNF-α were both increased at the same level in both groups at the endpoint (data not shown). Overall, this indicates that γHV-68 NZBW have a T helper response skewed towards a Th1 phenotype. To further address this, splenocytes isolated at the endpoint (200 days p.i.) were re-stimulated in vitro and stained for IFN-γ, IL-17, IL-4 and TNF-α (Figure 5.9). γHV-68 NZBW mice T cells, both CD4 and CD8, were capable of producing higher levels of both IFN-γ (Figure 5.9 A-B) and TNF-α (Figure 5.9 C-D) when compared to naïve NZBW mice. In contrast no IL-17 or IL-4 production were detected.

Additionally, activation markers on T cells were analyzed at the endpoint. γHV-68 NZBW mice presented with increased percentages and increased numbers of effector and memory CD8+CD62L^{low}CD44^{high} T cells (Figure 5.10), thus confirming what we previously observed in γHV-68 C57Bl/6 mice.

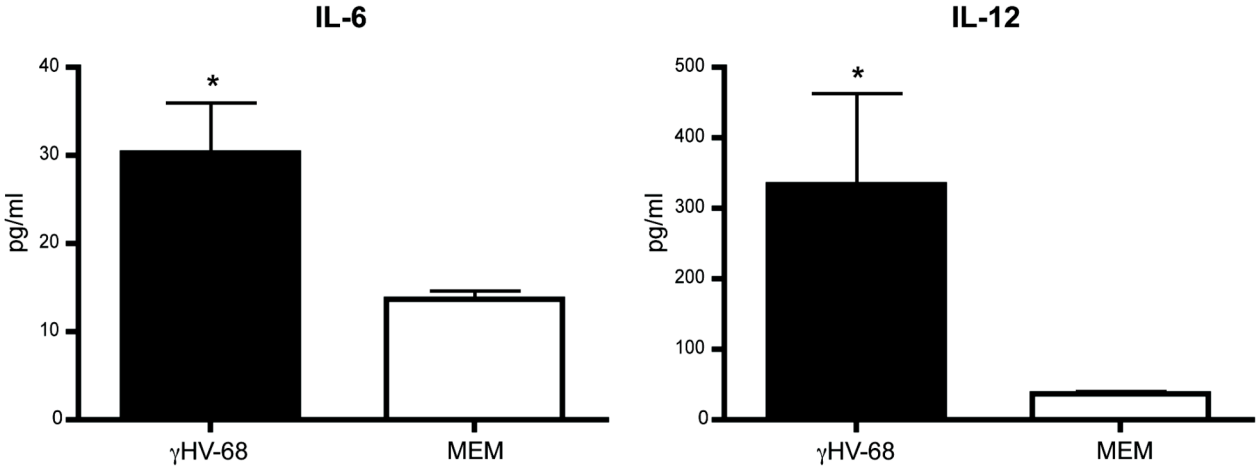


Figure 5.7. γHV-68 NZBW mice produce more IL-6 and IL-12 two weeks after γHV-68 infection

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At day 15 post infection, serum was harvested through saphenous vein bleeding. The levels of cytokines were evaluated using BD Cytometric Bead Array kits. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$.

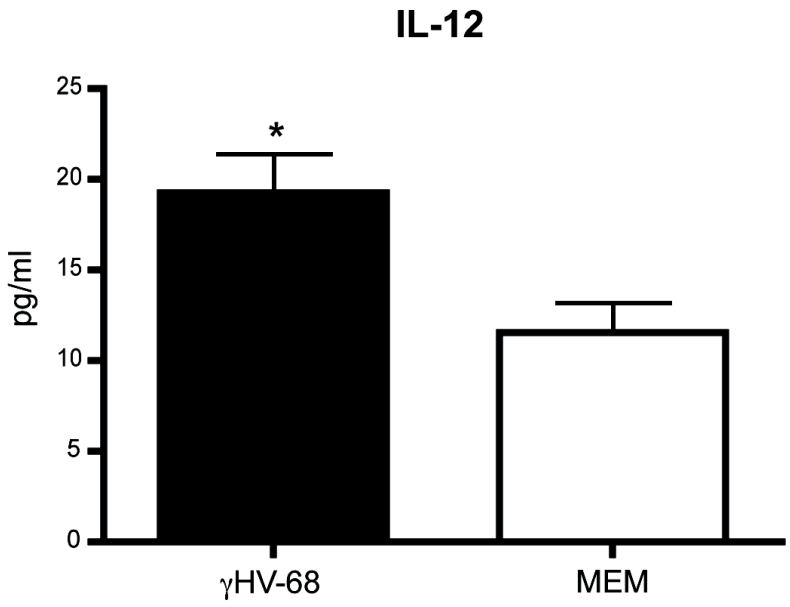


Figure 5.8. γHV-68 NZBW mice are still producing more IL-12 at 8 months of age, 200 days post γHV-68 infection

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), serum was harvested through cardiac puncture. The levels of cytokines were evaluated using BD Cytometric Bead Array kits. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$.

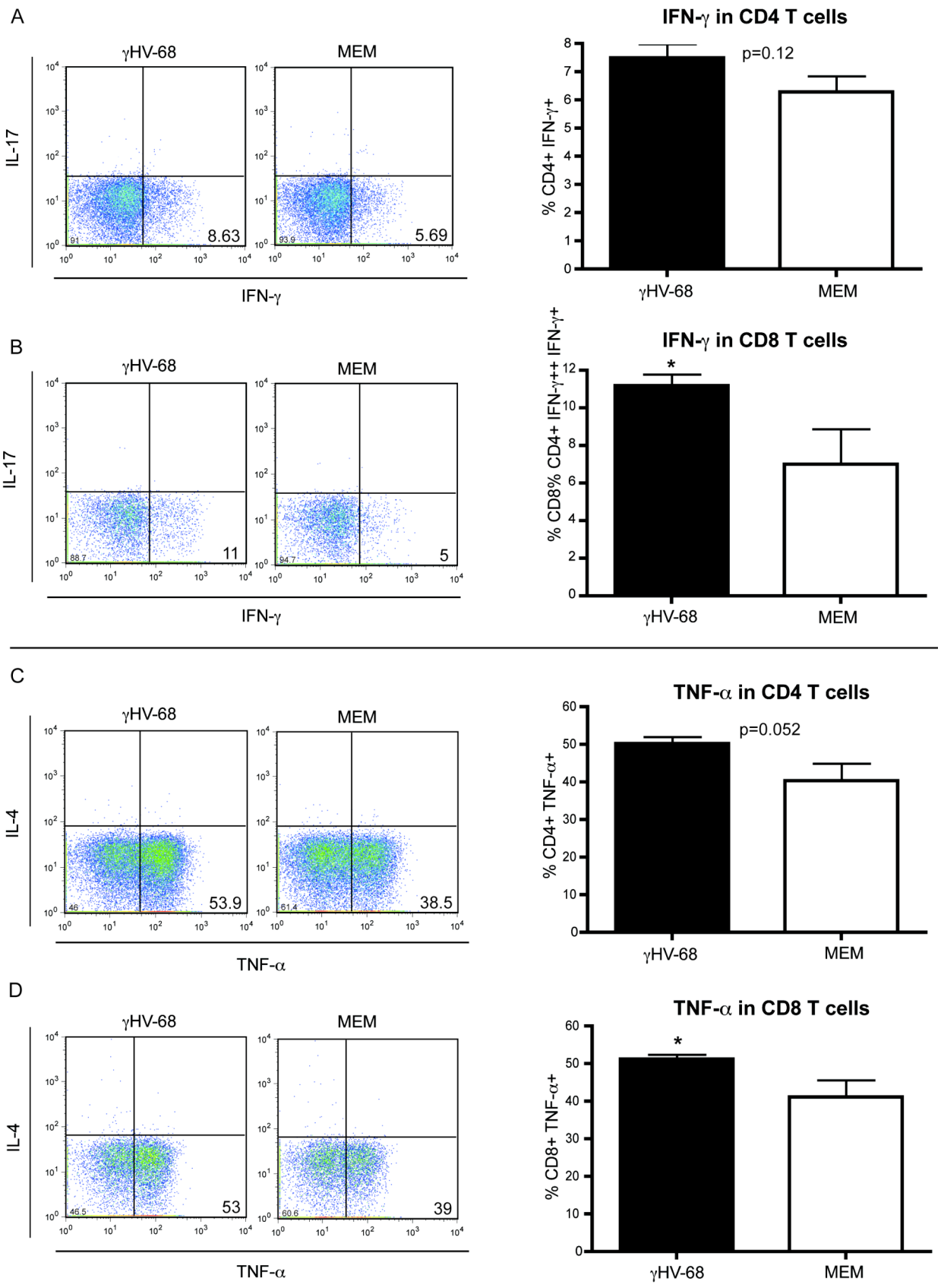

A
γHV-68
MEM
IL-17
IFN-γ
8.63
5.69
IFN-γ in CD4 T cells
p=0.12
% CD4+ IFN-γ+
γHV-68
MEM
B
γHV-68
MEM
IL-17
IFN-γ
11
5
IFN-γ in CD8 T cells
*
% CD8% CD4+ IFN-γ++ IFN-γ+
γHV-68
MEM
C
γHV-68
MEM
IL-4
TNF-α
53.9
38.5
TNF-α in CD4 T cells
p=0.052
% CD4+ TNF-α+
γHV-68
MEM
D
γHV-68
MEM
IL-4
TNF-α
53
39
TNF-α in CD8 T cells
*
% CD8+ TNF-α+
γHV-68
MEM

Figure 5.9. γHV-68 NZBW mice have increased percentages of T cells capable of secreting IFN–γ and TNF-α at 8 months of age

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), spleens were harvested. The production of IFN-γ and IL-17 **(A-B)** or IL-4 and TNF-α **(C-D)** by CD4 and CD8 T was assessed after in-vitro restimulation with PMA and ionomycin. Representative FACS plots and histograms showing percentages of CD4+IFN-γ+/ CD8+IFN-γ+ or CD4+TNF-α+/CD8+TNF-α+ are displayed here. Two separate experiments with 5 mice/group, data were analyzed with t-test: * $p<0.05$.

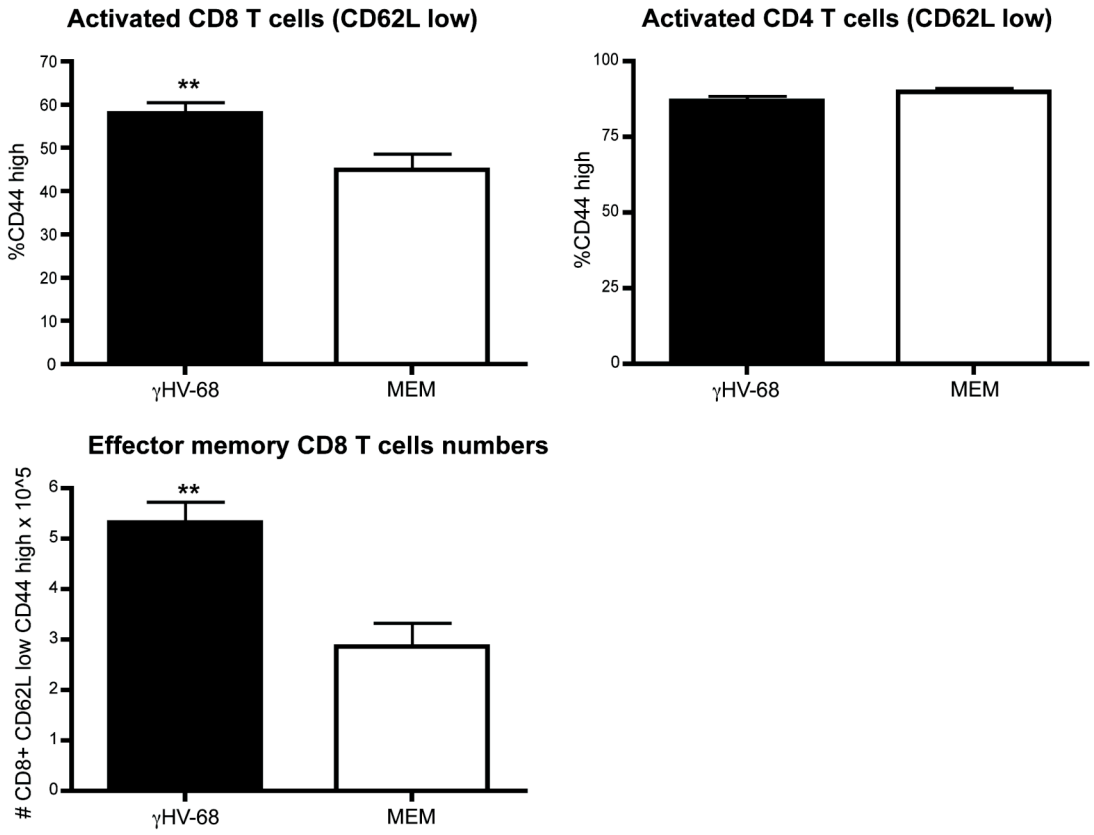


Figure 5.10. γHV-68 NZBW mice have increased numbers of CD8 T cells with an effector/memory phenotype at 8 months of age

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), spleens were harvested. FACS analysis was performed to quantify the percentages and the numbers of effector memory T cells. The histograms show the percentages and the numbers of CD4 and CD8 T cells that are CD62L-low and CD44-high. Two separate experiments with 5 mice/group, data were analyzed with t-test: ** $p<0.01$.

Finally, since the strong Th1 response driven by γHV-68 infection can have an impact on regulatory T cell (Tregs) numbers as previous assessed by our work (see chapter 4) and the work of others, the numbers and the percentages of CD4+Foxp3+ regulatory T cells were quantified in the spleens of γHV-68 and naïve NZBW mice at the endpoint (Figure 5.11). γHV-68 NZBW mice displayed a trend towards decreased frequencies of Tregs in comparison to naive NZBW mice, even though this difference was not statistically significant.

In summary these results confirm that γHV-68 NZBW have a T helper response skewed towards a Th1 phenotype, even at late time points post viral infection (200 days), with increased production of IFN-γ and TNF-α in T cells, increased levels of IL-12 in the serum and a decrease in the frequency of Tregs. This phenotype likely explains the increased IgG2a/IgG1 ratio of anti-nuclear autoantibodies.

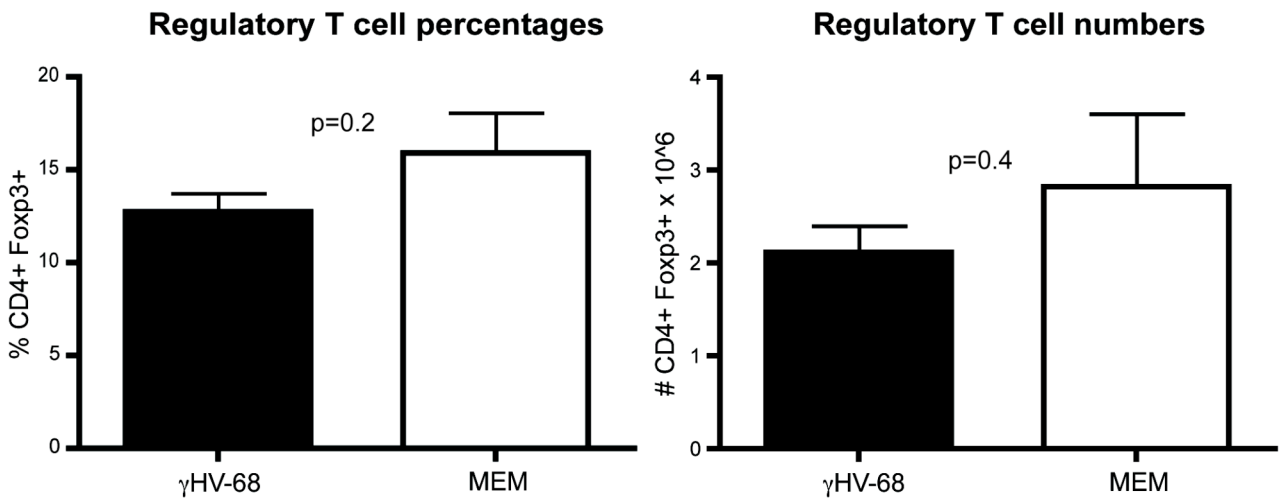


Figure 5.11. Naïve and γHV-68 NZBW mice show similar numbers of regulatory T cells

Mice were infected with γHV-68 (black bars) or MEM only (open bars) between 8-10 weeks of age. At the onset of kidney failure (8 months of age, 150-200 days post infection), spleens were harvested. The graphs show the percentages and the total numbers of regulatory T cells (CD4+ CD3+ Foxp3+). Two separate experiments with 5 mice/group, data were analyzed with t-test.

5.3 Discussion

EBV has been implicated as a possible cause of SLE for many years but proving a direct link has been challenging due to both the high prevalence of EBV infection in the general population and the lack of a small animal model to study the interaction between herpesviruses and the development of autoimmunity. Here we demonstrate that, in a mouse strain that spontaneously develops SLE and thus it is already prone to autoimmunity, latent infection with γHV-68 is able to further skew the immune response towards an autoimmune phenotype with a strong Th1 response and a trend towards decreased frequencies of Tregs.

The NZBW model was preferred because it has several advantages in comparison with the two other available spontaneous mouse model of SLE (MRL/lpr and BXSB). In fact, the NZBW model is the only one that is biased in favor of females as human SLE and it is not characterized by a pronounced lymphoproliferation as MRL/lpr and BXSB mice [296]. This is an important aspect, considering that γHV-68 is a lymphotropic virus that could lead to further uncontrolled lymphoproliferation in MRL/lpr and BXSB mice, leading to confusing results.

The T cell response in NZBW mice is normally not biased towards any T helper subset [236-238]. Upon γHV-68 infection, the T cell response is skewed towards a Th1 phenotype. Interestingly, SLE patients exhibit preferentially a Th1 response [297, 298] and IL-12, that we detected at higher levels in the serum of γHV-68 NZBW mice, has been shown to be pathogenic in SLE patients [297]. Additionally T-bet, a transcription factor gatekeeper of the Th1 response, is found at higher levels in the urine sediments and in the kidney tissue of SLE patients with active nephritis [299]. Finally, there is a significant correlation between kidney expression of T-bet and IFN-γ, serum levels of anti-dsDNA antibodies, the extent of glomerulonephritis and the amount of kidney immune cell infiltrations [300, 301].

γHV-68 infection does not alter the frequencies of B cell populations in the spleen as previously shown by others [67]. In agreement with these findings, we did not detect an alteration in the percentages of marginal zone B cells, follicular B cells or T1-T2 B cells in γHV-68 NZBW mice. In contrast, we found a decrease in plasma cell frequencies. Plasma cell differentiation is linked to γHV-68 reactivation from latency and

the vast majority of B cells that reactivate γHV-68 are plasma cells [67]. The decreased percentages of plasma cells found in γHV-68 NZBW mice could be explained by the γHV-68 specific CD8 T cells cytotoxic activity that clear cells that reactivate γHV-68. A decreased amount of plasma cells in lupus could lead to protection or amelioration of kidney damage. In fact, higher numbers of plasma cells correlates with disease activity in SLE patients and lower numbers with remission [302]. However, we detected a comparable degree of kidney damage in both γHV-68 and naïve NZBW mice, indicating that plasma cells in γHV-68 NZBW mice, even if lower in frequencies, are likely producing higher levels of autoantibodies or a different type of autoantibodies. In fact, decreased amounts of IgM and IgG1 autoantibodies were detected in γHV-68 NZBW mice that also presented with an increased IgG2a/IgG1 ratio. Interestingly, the onset of glomerulonephritis in NZBW mice is concomitant with the appearance of anti-nuclear IgG2a antibodies, which are the most pathogenic [222-224]. The switching towards IgG2a autoantibodies is dependent on IFN-γ [226-228]. In γHV-68 NZBW mice, the increased productions of IL-12 and IFN-γ are likely responsible for the suppression of autoantibodies with an IgG1 isotype in favor of an IgG2a one, as described in other animal models of autoimmunity and γHV-68 infection [67, 275].

Despite the fact that γHV-68 NZBW mice seem to be biased towards enhanced autoimmunity and production of more pathogenic autoantibodies, these differences are not enough to worsen lupus nephritis in our model. So, the results shown here only partially support our hypothesis that γHV-68 could have the potential, like EBV, to act as an environmental trigger on a lupus susceptible genetic background. There are different reasons that can explain this: NZBW mice were infected with γHV-68 at 8 weeks of age to mimic EBV infection in humans that occurs early in life. It is possible that infection with γHV-68 is required to occur later in the life of NZBW mice to accelerate lupus. Additionally, it is conceivable that a second hit (i.e. a second infection) that further activates B cells is needed to lead to SLE acceleration in γHV-68 NZBW mice. As such, future work will be aimed at assessing if a second infection or exogenous stimulus (i.e. TLR ligands) that activates B cells has the potential to accelerate SLE development in γHV-68 NZBW mice. Intriguingly it has been shown that TLR-9 is important for the production of autoantibodies. Activation of TLR-9 leads to enhanced disease [243], conversely TLR-9 KO have decreased autoantibody levels [244]. TLR-9 ligands will be

tested on γHV-68 NZBW mice.

In conclusion, considering that both genetic susceptibility and EBV latency seem to be linked to SLE development, γHV-68 mice NZBW mice could be a useful model to understand how other environmental factors and thus, a patient's history of infection, can accelerate SLE in genetically susceptible individuals that have been infected by EBV.

6 General discussion, future directions and conclusions

6.1 Discussion

The aim of my thesis was to identify how a latent infection with gammaherpesvirus can interact with the host's immune system to lead to the development of autoimmunity. The first research chapter tests the hypothesis that EBV is directly inducing MS by infecting a precise subset of cells in the CNS, while the two remaining research chapters analyze more broadly how a latent gammaherpesvirus infection is skewing T cell responses through dysregulated antigen presentation.

Two approaches were used in my thesis research. The first was to use human primary cell lines in vitro to be able to study directly EBV infection that is restricted to humans. The second involved the use of a mouse model in which the murine equivalent of EBV, murine gamma herpesvirus 68, was employed. While the first approach allows the study of the actual human pathogen, the second one is critical to have a better understanding of the interactions between the host's immune system and the pathogen.

6.1.1 EBV infection of the blood brain barrier

My results demonstrated that EBV is able to infect endothelial cells of the blood brain barrier leading to increased production of CCL-5 and ICAM-1. This upregulation of pro-inflammatory mediators supports increased adhesion of immune cells on the surface of an EBV infected brain endothelial monolayer [303].

The first evidence that EBV was able to infect the MS brain was published in 2007 by Aloisi's group [165]. Other groups were not able to reproduce these findings and they did not find evidence of EBV infection in the MS brain [167-169]. It is still not clear how these differences can be reconciled. Diffuse EBV infection was found only in patients that had ectopic B cell follicles in the meninges. Aloisi's group argues that preserving meninges in frozen and paraffin embedded tissue section might be challenging and meninges can be lost during tissue sectioning [304]. They also pointed out that there are issues regarding RNA preservation: in fact, other groups did not detect EBER signal despite positive results for viral protein and viral DNA and this could indicate that RNA might have been degraded [304]. Finally other groups used RNA isolated from an entire section without performing any EBV cDNA pre-enrichment prior

to PCR analysis. Whole section approaches could be less sensitive and well below the detection limit [166, 304]. Aloisi's tissue sections were selected from patients with prominent B cell infiltrations and high levels of inflammation. On the other hand, the other groups were looking at less well-selected tissues that are not enriched in B cells infiltrations or ectopic follicles structures.

Our model proposes a different reason for these discrepancies. In fact I hypothesize that reactivation of latent EBV infection in brain endothelial cells could locally upregulate pro-inflammatory mediators that would attract the first wave of autoreactive lymphocytes into a small area of the brain. In this manner, EBV would only need to infect a small population of HBMECs and reactivation would require to occur within a minority of these cells. This proposed model would serve to explain the inconsistent detection of EBV infection in MS brains. This mechanism would also explain why, even if there is a lack of detectable virus in the MS plaques, both viral and myelin specific lymphocytes are infiltrating inside the CNS and also why anti-viral treatments, like interferon-β, are effective in preventing MS relapses. Further work will need to address if EBV infection can be detected in vivo in the BBB of MS patients.

Finally, this model may work in concert with other mechanisms including EBV influence on B cells or APCs. As such, the γHV-68 EAE model was developed to address this possibility.

6.1.2 Latent gammaherpesvirus infection affects antigen presentation and skews the T cell response leading to enhanced disease in two different mouse models of autoimmunity

Previous works have shown that infection with γHV-68 could precipitate autoimmunity or chronic inflammation [97, 98, 100-102]. However, the mechanisms that the virus exploit to cause enhanced disease have not been addressed by these articles.

I demonstrated that latent infection with γHV-68 skews the T helper response towards a strong Th1 phenotype with decreased frequencies of regulatory T cells and increased CD8 T cell activation. When mice are stimulated with a second pro-inflammatory stimulus, i.e. EAE or a second infection (data not shown), that normally give rise to other T helper responses, besides Th1, a strong Th1 response is still

triggered in γHV-68 mice. Additionally, in a spontaneous SLE model, γHV-68 latency is also able to skew the T helper response towards a long-lasting Th1 phenotype (up to 200 days post γHV-68 infection). Overall, these in vivo results indicate that a gammaherpesvirus is indeed able to tip the balance towards autoimmunity and also indicate that an indirect mechanism (i.e. modulation of the immune response in the periphery and not direct infection in the organ where autoimmunity develops) could be involved in EBV-induced autoimmunity.

In γHV-68 EAE mice, a subset of APCs that express increased amounts of MHC II and CD40, without being infected by γHV-68, is responsible for Th1 skewing and Th17 suppression. Blocking of CD40 signaling could be a possible treatment to revert the phenotype towards tolerance and this is currently under investigation. However, upregulation of IFN-γ and downregulation of IL-17 upon transfer of these APCs in naïve mice, are not as strong as the ones observed in a γHV-68 infected mouse. This indicates that other pro-inflammatory mediators or possibly other cell types are contributing in keeping the immune system biased towards a Th1 phenotype. Interestingly, T cells isolated from a γHV-68 EAE mouse and directly transferred into a naïve mouse lose the Th1 phenotype (data not shown, experiment repeated twice with 5 mice/group/experiment: 1-2 million CD4 and/or CD8 T cells isolated from γHV-68 mice or naïve mice were transferred into naïve hosts. EAE was induced in the recipients 24 hours post transfer. At the development of EAE signs, brains and spinal cords were collected to assess CD4 T helper [Th1] and CD8 CTL responses). This indicates that the long-lasting interaction between the latent infection and the host is required to bias the immune system towards a Th1 phenotype.

Importantly, the observed Th1 skewed phenotype and CD8 enhanced activation are specific for gammaherpesvirus only. In fact, infections with beta or alpha herpesviruses or unrelated viruses, like LCMV, that do not establish latency, do not alter the course of EAE. It is interesting to note that recent findings have concluded that γHV-68 is the most immunomodulatory virus when compared to either MCMV or HSV-1 [305]. These observations parallel human MS and SLE studies in which it has been shown that both MS and SLE are associated with EBV infection only but not with other herpesvirus infections. Additionally, MS and SLE patients have a dysregulated immune response towards EBV only, but, again, not towards other human herpesvirus. My work

thus confirms these human findings and indicates that an in vivo model that is using a virus closely related to EBV, but not EBV itself, has still a good correlation to observations made in humans. It is also of interest to point out that, in both EAE and NZBW mice, latent infection with γHV-68 leads to a disease phenotype that mirrors more closely the human disease in comparison to naïve mice, as I discussed in chapters 4 and 5. Thus γHV-68 is a valuable tool to study, in the mouse, the long-lasting mutual interactions between EBV and the immune system.

My work suggests a possible new mechanism that EBV might be exploiting to cause autoimmunity in humans: EBV affects APCs leading to Th1 skewing, CD8 T cell activation and decreased Tregs frequencies. Since both MS and SLE are heterogeneous diseases, it is conceivable that they do not have only one etiologic agent. It is likely that EBV could be the trigger of the disease in only a subset of patients. To address this, the activation status of APCs in humans affected by different sub-types of MS or SLE could be investigated and linked to both EBV infection/mononucleosis history and T helper responses/CD8 activation status.

6.2 Future directions

6.2.1 Establishment of latency and reactivation of EBV infection in HBMECs

I showed that EBV can infect HBMECs and that the viral genome, along with expression of some viral genes, is still present in these endothelial cells up to two weeks post infection (data not shown). However, our hypothesis also requires the ability of EBV to establish latency in HBMECs and this has not been addressed yet. As such, it would be interesting to keep infected HBMECs in culture for longer (up to 60 days) to fully address if EBV is able to establish true latency. If this is possible, different stimuli could then be used in latently infected HBMECs to confirm the ability of endothelial cells to reactivate latent EBV infection. If reactivation occurs, upregulation of pro-inflammatory cytokines, adhesion molecules and immune cell adhesion-migration could be investigated to assess if endothelial cells that reactivate a latent infection could also promote the passage of immune cells.

Additionally, co-immunofluorescence approaches could be used to test for co-localization between brain endothelial cell markers and EBV viral proteins. If only a small number of endothelial cells are infected by EBV in humans or latently infected cells are not expressing any viral product, a second approach could be the use of in-situ PCR, to amplify the presence of EBV genome. These experiments could be performed using tissue samples from MS patients and from patients suffering from other neurological inflammatory diseases. If EBV is also found in the BBB of MS patients, this could be additional evidence of the validity of our hypothesis.

6.2.2 Modulation of CD40 signaling during EAE in γHV-68 latently infected mice

My results show that CD40 is increased on the surface of CD11c+CD11b+ cells isolated from a γHV-68 mouse. CD40 is a key pro-inflammatory molecule that has the ability to fully activate T helper responses, CD8 T cell cytolytic responses [288, 289] and downregulate Treg responses [280]. It is possible that chronic CD40 expression has a key role in keeping the Th response skewed towards a Th1 phenotype with suppression of Tregs generation and in activating CD8 T cell responses during EAE. In order to fully

assess the role of CD40, two different monoclonal antibodies that either inhibit CD40 (MR-1) or act as a CD40 agonist (FGK-45) will be tested in parallel in γHV-68 latently infected mice and naïve mice. I will then measure the frequencies of Tregs and the potency of the Th1 response under different pro-inflammatory stimuli such as EAE induction or a second infection. If CD40 is a key mediator of the γHV-68 phenotype, blocking CD40 signaling should revert the "autoimmune" phenotype of the immune system towards a more tolerogenic one.

Additional work to better characterize CD11b+CD11c+ cells will be performed. I have already shown that these APCs are capable of producing increased amounts of IFN-γ during the antigen presentation phase of EAE. Interestingly NK cells can co-express CD11b and CD11c and they are the major source of innate IFN-γ. It is conceivable that a percentage of these CD11c+ CD11b+ cells could be NK cells. To test this, a more comprehensive analysis of the surface markers expressed by CD11b+ CD11c+ cells will be performed.

6.2.3 γHV-68 EAE as a model to study CD8 T cell infiltrations inside the CNS

I have shown that CD8 T cells become activated in γHV-68 EAE mice and infiltrate inside the CNS. CD8 T cells are found only in low percentages during EAE in naïve mice. It would be interesting to further understand why and how CD8 T cells infiltrate in the CNS of γHV-68 EAE mice. It is possible that CD8 T cells expressing CXCR3 are following the chemokine MIG (CXCL-9), as I showed in chapter 4, to gain access to the brain parenchyma. Additional experiments can be performed to block CXCL-9 and assess if CD8 T cell infiltrations are still present. Moreover kinetic experiments can be performed to verify if CD8 T cells require a first wave of CD4 T cells and macrophages before they are able to infiltrate inside the CNS. In order to test this, brains and spinal cords can be isolated at different time points after EAE induction (day 7-10-15) and stained for CD4, CD8 and F4/80.

Since EAE in naïve mice does not lead to CD8 T cells infiltrations, but MS patients present with CD8 T cells along with CD4 T cells in demyelinating plaques, we propose that our model could be exploited to address which signals guide CD8 T cells when they infiltrate the CNS.

6.2.4 B cell activation in γHV-68 NZBW mice as a trigger to accelerate lupus

In chapter 5, I have shown that γHV-68 NZBW mice have an immune phenotype skewed towards a Th1 response and they produce more pathogenic autoantibodies. Thus γHV-68 NZBW mice should be more prone to develop kidney inflammation. However, these differences are not enough to lead to enhanced kidney damage.

It is conceivable that γHV-68 NZBW mice need a second pro-inflammatory trigger to precipitate SLE. In order to test this, a second infection or an injection of TLR ligands, that can reactivate B cells, will be administered to γHV-68 NZBW mice. The level of damage of the kidneys will be monitored by measuring the levels of protein present in the urine. Two weeks after secondary infection/injection of TLR ligands, kidneys will be harvested and analyzed for immune infiltrations, IgGs depositions and glomerulonephritis.

6.2.5 Is latency required?

A mutant γHV-68 that is not able to establish latency and it is cleared after acute infection is resolved will be employed to fully address if latency is required to maintain an hyper activated immune response, or if acute infection per se is able to trigger this long-lasting Th1 biased phenotype (up to 200 days post infection in SLE mice). Preliminary data in the EAE model, using acute γHV-68 infection, indicate that establishment of latency is required to obtain stronger CD4 and CD8 T cell responses (see chapter 4). The use of a latency deficient γHV-68 will fully address this issue. It is important to understand if latency is necessary to maintain this phenotype for the development of therapies. If latency is not required for enhanced autoimmune pathology, an EBV vaccine or drug that is designed to prevent or better control mononucleosis, but does not prevent the establishment of latency, may be sufficient to avoid the development of autoimmunity later in life. Conversely, if latency is required to lead to increased susceptibility to autoimmunity, a vaccine or drug that prevents the establishment of latency, or it is able to eradicate latency once established, has to be designed instead.

6.3 Concluding remarks

In conclusion, the results presented in this thesis identify two different mechanisms that EBV can exploit to trigger autoimmunity. We confirmed that our two initial hypotheses are both valid and they could act in concert or alone to lead to the development of MS or SLE. This work extends our understanding of the mechanisms that a pathogen can employ to lead to the development of autoimmunity. Particularly, we identify a subset of APCs that might be responsible of keeping the immune system hyper activated during gammaherpesvirus latency. The future experiments proposed here will help in the further dissection of the interactions between these APCs and the other arms of the immune system, particularly the role of CD40. Understanding these mechanisms can provide insights that might reveal new therapeutic targets to re-establish peripheral tolerance during autoimmunity.

The data that I have presented here also suggest that gammaherpesviruses have a profound and long-lasting effect on the host immune system. Therefore a reductionist approach that study autoimmune diseases, inflammation and response to infections in naïve, clean mice may not be always ideal. A more complete understanding of “normal” immune function has to take into account the effect of latent infections. The study of mice latently infected with herpesviruses could lead to a better understanding of human immunology, considering that EBV, but also other herpesviruses, infect close to 90% of the human population.

References

1. Paludan, S.R., et al., *Recognition of herpesviruses by the innate immune system.* Nat Rev Immunol, 2011. **11**(2): p. 143-54.
2. Pellet, P.E. and B. Roizman, *The Family: Herpesviridae. A Brief Introduction*, in *Fields Virology*. 2007, Lippincott, Williams and Wilkins: Philadelphia. p. 2479-2500.
3. de Oliveira, D.E., G. Ballon, and E. Cesarman, *NF-kappaB signaling modulation by EBV and KSHV.* Trends Microbiol, 2010. **18**(6): p. 248-57.
4. Elgui de Oliveira, D., *DNA viruses in human cancer: an integrated overview on fundamental mechanisms of viral carcinogenesis.* Cancer Lett, 2007. **247**(2): p. 182-96.
5. Klein, E., L.L. Kis, and G. Klein, *Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions.* Oncogene, 2007. **26**(9): p. 1297-305.
6. Hutt-Fletcher, L.M., *Epstein-Barr virus entry.* J Virol, 2007. **81**(15): p. 7825-32.
7. Savard, M., et al., *Infection of primary human monocytes by Epstein-Barr virus.* J Virol, 2000. **74**(6): p. 2612-9.
8. Jones, K., et al., *Infection of human endothelial cells with Epstein-Barr virus.* J Exp Med, 1995. **182**(5): p. 1213-21.
9. Xiong, A., et al., *Epstein-Barr virus latent membrane protein 1 activates nuclear factor-kappa B in human endothelial cells and inhibits apoptosis.* Transplantation, 2004. **78**(1): p. 41-9.
10. Miller, G., et al., *Lytic cycle switches of oncogenic human gammaherpesviruses.* Adv Cancer Res, 2007. **97**: p. 81-109.
11. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.* Lancet, 1964. **1**(7335): p. 702-3.
12. Bornkamm, G.W. and W. Hammerschmidt, *Molecular virology of Epstein-Barr virus.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1408): p. 437-59.
13. Allen, M.D., L.S. Young, and C.W. Dawson, *The Epstein-Barr virus-encoded LMP2A and LMP2B proteins promote epithelial cell spreading and motility.* J Virol, 2005. **79**(3): p. 1789-802.
14. Tsurumi, T., M. Fujita, and A. Kudoh, *Latent and lytic Epstein-Barr virus replication strategies.* Rev Med Virol, 2005. **15**(1): p. 3-15.
15. Benoit, L., et al., *Defective NK cell activation in X-linked lymphoproliferative disease.* J Immunol, 2000. **165**(7): p. 3549-53.
16. Tangye, S.G., et al., *Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome.* J Immunol, 2000. **165**(6): p. 2932-6.
17. Steven, N.M., et al., *Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response.* J Exp Med, 1997. **185**(9): p. 1605-17.
18. Callan, M.F., et al., *Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo.* J Exp Med, 1998. **187**(9): p. 1395-402.
19. Tan, L.C., et al., *A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers.* J Immunol, 1999. **162**(3): p. 1827-35.

20. Silins, S.L., et al., *A functional link for major TCR expansions in healthy adults caused by persistent Epstein-Barr virus infection.* J Clin Invest, 1998. **102**(8): p. 1551-8.
21. Cohen, J.I., *The biology of Epstein-Barr virus: lessons learned from the virus and the host.* Curr Opin Immunol, 1999. **11**(4): p. 365-70.
22. Levitsky, V. and M.G. Masucci, *Manipulation of immune responses by Epstein-Barr virus.* Virus Res, 2002. **88**(1-2): p. 71-86.
23. Sun, Q., et al., *CD4(+) Epstein-Barr virus-specific cytotoxic T-lymphocytes from human umbilical cord blood.* Cell Immunol, 1999. **195**(2): p. 81-8.
24. Munz, C., et al., *Human CD4(+) T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1.* J Exp Med, 2000. **191**(10): p. 1649-60.
25. Nagy, N., et al., *Soluble factors produced by activated CD4+ T cells modulate EBV latency.* Proc Natl Acad Sci U S A, 2012. **109**(5): p. 1512-7.
26. Brooks, J.M., et al., *Cytotoxic T-lymphocyte responses to a polymorphic Epstein-Barr virus epitope identify healthy carriers with coresident viral strains.* J Virol, 2000. **74**(4): p. 1801-9.
27. Yao, Q.Y., et al., *Frequency of multiple Epstein-Barr virus infections in T-cell-immunocompromised individuals.* J Virol, 1996. **70**(8): p. 4884-94.
28. Levin, L.I., et al., *Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis.* JAMA, 2005. **293**(20): p. 2496-500.
29. Lunemann, J.D., et al., *Broadened and elevated humoral immune response to EBNA1 in pediatric multiple sclerosis.* Neurology, 2008. **71**(13): p. 1033-5.
30. Liu, M.T. and C.Y. Yeh, *Prognostic value of anti-Epstein-Barr virus antibodies in nasopharyngeal carcinoma (NPC).* Radiat Med, 1998. **16**(2): p. 113-7.
31. Lynn, T., et al., *Prognosis of nasopharyngeal carcinoma by Epstein-Barr virus antibody titer.* Arch Otolaryngol, 1977. **103**(3): p. 128-32.
32. Magrath, I., et al., *Prognostic factors in Burkitt's lymphoma: importance of total tumor burden.* Cancer, 1980. **45**(6): p. 1507-15.
33. Levitskaya, J., et al., *Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1.* Nature, 1995. **375**(6533): p. 685-8.
34. Gottschalk, S., et al., *An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs.* Blood, 2001. **97**(4): p. 835-43.
35. Khanna, R., et al., *Evolutionary dynamics of genetic variation in Epstein-Barr virus isolates of diverse geographical origins: evidence for immune pressure-independent genetic drift.* J Virol, 1997. **71**(11): p. 8340-6.
36. Devergne, O., M. Birkenbach, and E. Kieff, *Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin.* Proc Natl Acad Sci U S A, 1997. **94**(22): p. 12041-6.
37. Devergne, O., et al., *Expression of Epstein-Barr virus-induced gene 3, an interleukin-12 p40-related molecule, throughout human pregnancy: involvement of syncytiotrophoblasts and extravillous trophoblasts.* Am J Pathol, 2001. **159**(5): p. 1763-76.
38. Larochelle, B., et al., *Epstein-Barr virus infects and induces apoptosis in human neutrophils.* Blood, 1998. **92**(1): p. 291-9.
39. Beaulieu, A.D., R. Paquin, and J. Gosselin, *Epstein-Barr virus modulates de novo protein synthesis in human neutrophils.* Blood, 1995. **86**(7): p. 2789-98.

40. McColl, S.R., et al., *EBV induces the production and release of IL-8 and macrophage inflammatory protein-1 alpha in human neutrophils.* J Immunol, 1997. **159**(12): p. 6164-8.
41. Roberge, C.J., et al., *Epstein-Barr virus induces GM-CSF synthesis by monocytes: effect on EBV-induced IL-1 and IL-1 receptor antagonist production in neutrophils.* Virology, 1997. **238**(2): p. 344-52.
42. Li, L., et al., *Epstein-Barr virus inhibits the development of dendritic cells by promoting apoptosis of their monocyte precursors in the presence of granulocyte macrophage-colony-stimulating factor and interleukin-4.* Blood, 2002. **99**(10): p. 3725-34.
43. Bejarano, M.T. and M.G. Masucci, *Interleukin-10 abrogates the inhibition of Epstein-Barr virus-induced B-cell transformation by memory T-cell responses.* Blood, 1998. **92**(11): p. 4256-62.
44. Takayama, T., et al., *Mammalian and viral IL-10 enhance C-C chemokine receptor 5 but down-regulate C-C chemokine receptor 7 expression by myeloid dendritic cells: impact on chemotactic responses and in vivo homing ability.* J Immunol, 2001. **166**(12): p. 7136-43.
45. Davis, J.E., et al., *Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1.* Cell Death Differ, 2000. **7**(10): p. 973-83.
46. Chen, G., et al., *CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection.* Blood, 2001. **98**(1): p. 156-64.
47. Faint, J.M., et al., *Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics.* J Immunol, 2001. **167**(1): p. 212-20.
48. Strowig, T., et al., *Priming of protective T cell responses against virus-induced tumors in mice with human immune system components.* J Exp Med, 2009. **206**(6): p. 1423-34.
49. Blaskovic, D., et al., *Isolation of five strains of herpesviruses from two species of free living small rodents.* Acta Virol, 1980. **24**(6): p. 468.
50. Olivadoti, M., et al., *Murine gammaherpesvirus 68: a model for the study of Epstein-Barr virus infections and related diseases.* Comp Med, 2007. **57**(1): p. 44-50.
51. Virgin, H.W.t., et al., *Complete sequence and genomic analysis of murine gammaherpesvirus 68.* J Virol, 1997. **71**(8): p. 5894-904.
52. Fowler, P., et al., *ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency.* J Gen Virol, 2003. **84**(Pt 12): p. 3405-16.
53. Moorman, N.J., D.O. Willer, and S.H. Speck, *The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency.* J Virol, 2003. **77**(19): p. 10295-303.
54. van Dyk, L.F., et al., *The murine gammaherpesvirus 68 v-cyclin gene is an oncogene that promotes cell cycle progression in primary lymphocytes.* J Virol, 1999. **73**(6): p. 5110-22.
55. Roy, D.J., et al., *Murine gammaherpesvirus M11 gene product inhibits apoptosis and is expressed during virus persistence.* Arch Virol, 2000. **145**(11): p. 2411-20.

56. Wang, G.H., T.L. Garvey, and J.I. Cohen, *The murine gammaherpesvirus-68 M11 protein inhibits Fas- and TNF-induced apoptosis.* J Gen Virol, 1999. **80 (Pt 10)**: p. 2737-40.
57. Stevenson, P.G., et al., *Inhibition of MHC class I-restricted antigen presentation by gamma 2-herpesviruses.* Proc Natl Acad Sci U S A, 2000. **97**(15): p. 8455-60.
58. Evans, A.G., et al., *A gammaherpesvirus-secreted activator of Vbeta4+ CD8+ T cells regulates chronic infection and immunopathology.* J Exp Med, 2008. **205**(3): p. 669-84.
59. Coscoy, L. and D. Ganem, *Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 8051-6.
60. Ishido, S., et al., *Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein.* Immunity, 2000. **13**(3): p. 365-74.
61. Sutkowski, N., et al., *Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen.* Immunity, 2001. **15**(4): p. 579-89.
62. Parry, C.M., et al., *A broad spectrum secreted chemokine binding protein encoded by a herpesvirus.* J Exp Med, 2000. **191**(3): p. 573-8.
63. van Berkel, V., et al., *Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action.* J Virol, 2000. **74**(15): p. 6741-7.
64. Herskowitz, J.H., M.A. Jacoby, and S.H. Speck, *The murine gammaherpesvirus 68 M2 gene is required for efficient reactivation from latently infected B cells.* J Virol, 2005. **79**(4): p. 2261-73.
65. Jacoby, M.A., H.W.t. Virgin, and S.H. Speck, *Disruption of the M2 gene of murine gammaherpesvirus 68 alters splenic latency following intranasal, but not intraperitoneal, inoculation.* J Virol, 2002. **76**(4): p. 1790-801.
66. Siegel, A.M., J.H. Herskowitz, and S.H. Speck, *The MHV68 M2 protein drives IL-10 dependent B cell proliferation and differentiation.* PLoS Pathog, 2008. **4**(4): p. e1000039.
67. Liang, X., et al., *Gammaherpesvirus-driven plasma cell differentiation regulates virus reactivation from latently infected B lymphocytes.* PLoS Pathog, 2009. **5**(11): p. e1000677.
68. Barton, E., P. Mandal, and S.H. Speck, *Pathogenesis and host control of gammaherpesviruses: lessons from the mouse.* Annu Rev Immunol, 2011. **29**: p. 351-97.
69. Flano, E., et al., *Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages.* J Immunol, 2000. **165**(2): p. 1074-81.
70. Sunil-Chandra, N.P., S. Efstathiou, and A.A. Nash, *Interactions of murine gammaherpesvirus 68 with B and T cell lines.* Virology, 1993. **193**(2): p. 825-33.
71. Weck, K.E., et al., *Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells.* J Virol, 1999. **73**(4): p. 3273-83.
72. Weck, K.E., et al., *B cells regulate murine gammaherpesvirus 68 latency.* J Virol, 1999. **73**(6): p. 4651-61.
73. Flano, E., et al., *Gamma-herpesvirus latency is preferentially maintained in splenic germinal center and memory B cells.* J Exp Med, 2002. **196**(10): p. 1363-72.

74. Willer, D.O. and S.H. Speck, *Long-term latent murine Gammaherpesvirus 68 infection is preferentially found within the surface immunoglobulin D-negative subset of splenic B cells in vivo.* J Virol, 2003. **77**(15): p. 8310-21.
75. Guggemoos, S., et al., *TLR9 contributes to antiviral immunity during gammaherpesvirus infection.* J Immunol, 2008. **180**(1): p. 438-43.
76. Gargano, L.M., J.M. Moser, and S.H. Speck, *Role for MyD88 signaling in murine gammaherpesvirus 68 latency.* J Virol, 2008. **82**(8): p. 3853-63.
77. Gargano, L.M., J.C. Forrest, and S.H. Speck, *Signaling through Toll-like receptors induces murine gammaherpesvirus 68 reactivation in vivo.* J Virol, 2009. **83**(3): p. 1474-82.
78. Gregory, S.M., et al., *Toll-like receptor signaling controls reactivation of KSHV from latency.* Proc Natl Acad Sci U S A, 2009. **106**(28): p. 11725-30.
79. Barton, E.S., et al., *Alpha/beta interferons regulate murine gammaherpesvirus latent gene expression and reactivation from latency.* J Virol, 2005. **79**(22): p. 14149-60.
80. Hwang, S., et al., *Conserved herpesviral kinase promotes viral persistence by inhibiting the IRF-3-mediated type I interferon response.* Cell Host Microbe, 2009. **5**(2): p. 166-78.
81. Hahn, A.M., et al., *Interferon regulatory factor 7 is negatively regulated by the Epstein-Barr virus immediate-early gene, BZLF-1.* J Virol, 2005. **79**(15): p. 10040-52.
82. Wang, J.T., et al., *Epstein-Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway.* J Virol, 2009. **83**(4): p. 1856-69.
83. Gredmark-Russ, S., et al., *The CD8 T-cell response against murine gammaherpesvirus 68 is directed toward a broad repertoire of epitopes from both early and late antigens.* J Virol, 2008. **82**(24): p. 12205-12.
84. Liu, L., et al., *Lytic cycle T cell epitopes are expressed in two distinct phases during MHV-68 infection.* J Immunol, 1999. **163**(2): p. 868-74.
85. Freeman, M.L., et al., *Two kinetic patterns of epitope-specific CD8 T-cell responses following murine gammaherpesvirus 68 infection.* J Virol, 2010. **84**(6): p. 2881-92.
86. Stevenson, P.G., et al., *Changing patterns of dominance in the CD8+ T cell response during acute and persistent murine gamma-herpesvirus infection.* Eur J Immunol, 1999. **29**(4): p. 1059-67.
87. Cush, S.S., et al., *Memory generation and maintenance of CD8+ T cell function during viral persistence.* J Immunol, 2007. **179**(1): p. 141-53.
88. Loh, J., et al., *Granzymes and caspase 3 play important roles in control of gammaherpesvirus latency.* J Virol, 2004. **78**(22): p. 12519-28.
89. Topham, D.J., et al., *Perforin and Fas in murine gammaherpesvirus-specific CD8(+) T cell control and morbidity.* J Gen Virol, 2001. **82**(Pt 8): p. 1971-81.
90. Cardin, R.D., et al., *Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells.* J Exp Med, 1996. **184**(3): p. 863-71.
91. Stevenson, P.G., J.P. Simas, and S. Efstathiou, *Immune control of mammalian gamma-herpesviruses: lessons from murid herpesvirus-4.* J Gen Virol, 2009. **90**(Pt 10): p. 2317-30.
92. Stuller, K.A., S.S. Cush, and E. Flano, *Persistent gamma-herpesvirus infection induces a CD4 T cell response containing functionally distinct effector populations.* J Immunol, 2010. **184**(7): p. 3850-6.

93. Sangster, M.Y., et al., *Analysis of the virus-specific and nonspecific B cell response to a persistent B-lymphotropic gammaherpesvirus.* J Immunol, 2000. **164**(4): p. 1820-8.
94. Stevenson, P.G. and P.C. Doherty, *Kinetic analysis of the specific host response to a murine gammaherpesvirus.* J Virol, 1998. **72**(2): p. 943-9.
95. Kim, I.J., et al., *Antibody-mediated control of persistent gamma-herpesvirus infection.* J Immunol, 2002. **168**(8): p. 3958-64.
96. Tibbetts, S.A., et al., *Effective vaccination against long-term gammaherpesvirus latency.* J Virol, 2003. **77**(4): p. 2522-9.
97. Peacock, J.W., et al., *Exacerbation of experimental autoimmune encephalomyelitis in rodents infected with murine gammaherpesvirus-68.* Eur J Immunol, 2003. **33**(7): p. 1849-58.
98. McMillan, T.R., et al., *Exacerbation of established pulmonary fibrosis in a murine model by gammaherpesvirus.* Am J Respir Crit Care Med, 2008. **177**(7): p. 771-80.
99. Alber, D.G., P. Vallance, and K.L. Powell, *Enhanced atherogenesis is not an obligatory response to systemic herpesvirus infection in the apoE-deficient mouse: comparison of murine gamma-herpesvirus-68 and herpes simplex virus-1.* Arterioscler Thromb Vasc Biol, 2002. **22**(5): p. 793-8.
100. Alber, D.G., et al., *Herpesvirus infection accelerates atherosclerosis in the apolipoprotein E-deficient mouse.* Circulation, 2000. **102**(7): p. 779-85.
101. Nelson, D.A., C.C. Petty, and K.L. Bost, *Infection with murine gammaherpesvirus 68 exacerbates inflammatory bowel disease in IL-10-deficient mice.* Inflamm Res, 2009. **58**(12): p. 881-9.
102. Cadwell, K., et al., *Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine.* Cell, 2010. **141**(7): p. 1135-45.
103. Barton, E.S., et al., *Herpesvirus latency confers symbiotic protection from bacterial infection.* Nature, 2007. **447**(7142): p. 326-9.
104. Haque, A., et al., *Co-infection of malaria and gamma-herpesvirus: exacerbated lung inflammation or cross-protection depends on the stage of viral infection.* Clin Exp Immunol, 2004. **138**(3): p. 396-404.
105. Nguyen, Y., et al., *Gammaherpesvirus modulation of mouse adenovirus type 1 pathogenesis.* Virology, 2008. **380**(2): p. 182-90.
106. Posnett, D.N., *Herpesviruses and autoimmunity.* Curr Opin Investig Drugs, 2008. **9**(5): p. 505-14.
107. Niller, H.H., H. Wolf, and J. Minarovits, *Regulation and dysregulation of Epstein-Barr virus latency: implications for the development of autoimmune diseases.* Autoimmunity, 2008. **41**(4): p. 298-328.
108. Pender, M.P., *Infection of autoreactive B lymphocytes with EBV, causing chronic autoimmune diseases.* Trends Immunol, 2003. **24**(11): p. 584-8.
109. Sospedra, M. and R. Martin, *Immunology of multiple sclerosis.* Annu Rev Immunol, 2005. **23**: p. 683-747.
110. Clanet, M., *Jean-Martin Charcot. 1825 to 1893.* Int MS J, 2008. **15**(2): p. 59-61.
111. Fraser, K.B., et al., *Increased tendency to spontaneous in-vitro lymphocyte transformation in clinically active multiple sclerosis.* Lancet, 1979. **2**(8145): p. 175-6.
112. Craig, J.C., M. Haire, and J.D. Merrett, *T-cell-mediated suppression of Epstein-Barr virus-induced B lymphocyte activation in multiple sclerosis.* Clin Immunol Immunopathol, 1988. **48**(3): p. 253-60.

113. Craig, J.C., et al., *Immunological control of Epstein-Barr virus-transformed lymphocytes in multiple sclerosis.* Clin Immunol Immunopathol, 1983. **29**(1): p. 86-93.
114. Craig, J.C., et al., *Subsets of T lymphocytes in relation to T lymphocyte function in multiple sclerosis.* Clin Exp Immunol, 1985. **61**(3): p. 548-55.
115. Bray, P.F., et al., *Epstein-Barr virus infection and antibody synthesis in patients with multiple sclerosis.* Arch Neurol, 1983. **40**(7): p. 406-8.
116. Myhr, K.M., et al., *Altered antibody pattern to Epstein-Barr virus but not to other herpesviruses in multiple sclerosis: a population based case-control study from western Norway.* J Neurol Neurosurg Psychiatry, 1998. **64**(4): p. 539-42.
117. Sumaya, C.V., L.W. Myers, and G.W. Ellison, *Epstein-Barr virus antibodies in multiple sclerosis.* Arch Neurol, 1980. **37**(2): p. 94-6.
118. Martin, R., et al., *Myelin basic protein-specific T-cell responses in identical twins discordant or concordant for multiple sclerosis.* Ann Neurol, 1993. **34**(4): p. 524-35.
119. Warner, H.B. and R.I. Carp, *Multiple sclerosis and Epstein-Barr virus.* Lancet, 1981. **2**(8258): p. 1290.
120. Warner, H.B. and R.I. Carp, *Multiple sclerosis etiology--an Epstein-Barr virus hypothesis.* Med Hypotheses, 1988. **25**(2): p. 93-7.
121. Wucherpfennig, K.W. and J.L. Strominger, *Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein.* Cell, 1995. **80**(5): p. 695-705.
122. Vaughan, J.H., et al., *An Epstein Barr virus-related cross reactive autoimmune response in multiple sclerosis in Norway.* J Neuroimmunol, 1996. **69**(1-2): p. 95-102.
123. Esposito, M., et al., *Human transaldolase and cross-reactive viral epitopes identified by autoantibodies of multiple sclerosis patients.* J Immunol, 1999. **163**(7): p. 4027-32.
124. Ascherio, A. and M. Munch, *Epstein-Barr virus and multiple sclerosis.* Epidemiology, 2000. **11**(2): p. 220-4.
125. Alotaibi, S., et al., *Epstein-Barr virus in pediatric multiple sclerosis.* JAMA, 2004. **291**(15): p. 1875-9.
126. Banwell, B., et al., *Clinical features and viral serologies in children with multiple sclerosis: a multinational observational study.* Lancet Neurol, 2007. **6**(9): p. 773-81.
127. Pohl, D., et al., *High seroprevalence of Epstein-Barr virus in children with multiple sclerosis.* Neurology, 2006. **67**(11): p. 2063-5.
128. Handel, A.E., et al., *An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis.* PLoS One, 2010. **5**(9).
129. Nielsen, T.R., et al., *Effects of infectious mononucleosis and HLA-DRB1*15 in multiple sclerosis.* Mult Scler, 2009. **15**(4): p. 431-6.
130. Ramagopalan, S.V., et al., *Association of infectious mononucleosis with multiple sclerosis. A population-based study.* Neuroepidemiology, 2009. **32**(4): p. 257-62.
131. Levin, L.I., et al., *Primary infection with the Epstein-Barr virus and risk of multiple sclerosis.* Ann Neurol, 2010. **67**(6): p. 824-30.
132. Lindsey, J.W., S. Patel, and J. Zou, *Epstein-Barr virus genotypes in multiple sclerosis.* Acta Neurol Scand, 2008. **117**(2): p. 141-4.
133. Simon, K.C., et al., *EBNA1 and LMP1 variants in multiple sclerosis cases and controls.* Acta Neurol Scand, 2011.

134. Brennan, R.M., et al., *Strains of Epstein-Barr virus infecting multiple sclerosis patients.* Mult Scler, 2010. **16**(6): p. 643-51.
135. Wandinger, K., et al., *Association between clinical disease activity and Epstein-Barr virus reactivation in MS.* Neurology, 2000. **55**(2): p. 178-84.
136. Lindsey, J.W., et al., *Quantitative PCR for Epstein-Barr virus DNA and RNA in multiple sclerosis.* Mult Scler, 2009. **15**(2): p. 153-8.
137. Wagner, H.J., K.L. Munger, and A. Ascherio, *Plasma viral load of Epstein-Barr virus and risk of multiple sclerosis.* Eur J Neurol, 2004. **11**(12): p. 833-4.
138. Rand, K.H., et al., *Molecular approach to find target(s) for oligoclonal bands in multiple sclerosis.* J Neurol Neurosurg Psychiatry, 1998. **65**(1): p. 48-55.
139. Rand, K.H., et al., *Epstein-Barr virus nuclear antigen-1 (EBNA-1) associated oligoclonal bands in patients with multiple sclerosis.* J Neurol Sci, 2000. **173**(1): p. 32-9.
140. Franciotta, D., et al., *Cerebrospinal BAFF and Epstein-Barr virus-specific oligoclonal bands in multiple sclerosis and other inflammatory demyelinating neurological diseases.* J Neuroimmunol, 2011. **230**(1-2): p. 160-3.
141. Jafari, N., et al., *No evidence for intrathecal IgG synthesis to Epstein Barr virus nuclear antigen-1 in multiple sclerosis.* J Clin Virol, 2010. **49**(1): p. 26-31.
142. Pohl, D., et al., *Intrathecal antibody production against Epstein-Barr and other neurotropic viruses in pediatric and adult onset multiple sclerosis.* J Neurol, 2010. **257**(2): p. 212-6.
143. Ascherio, A., et al., *Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study.* JAMA, 2001. **286**(24): p. 3083-8.
144. Sundstrom, P., et al., *An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study.* Neurology, 2004. **62**(12): p. 2277-82.
145. DeLorenze, G.N., et al., *Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up.* Arch Neurol, 2006. **63**(6): p. 839-44.
146. Nociti, V., et al., *Epstein-Barr virus antibodies in serum and cerebrospinal fluid from multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy and amyotrophic lateral sclerosis.* J Neuroimmunol, 2010. **225**(1-2): p. 149-52.
147. Lunemann, J.D., et al., *Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis.* Ann Neurol, 2010. **67**(2): p. 159-69.
148. Farrell, R.A., et al., *Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI.* Neurology, 2009. **73**(1): p. 32-8.
149. Zivadinov, R., et al., *Epstein-Barr virus is associated with grey matter atrophy in multiple sclerosis.* J Neurol Neurosurg Psychiatry, 2009. **80**(6): p. 620-5.
150. Lang, H.L., et al., *A functional and structural basis for TCR cross-reactivity in multiple sclerosis.* Nat Immunol, 2002. **3**(10): p. 940-3.
151. Holmoy, T., E.O. Kvale, and F. Vartdal, *Cerebrospinal fluid CD4+ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein.* J Neurovirol, 2004. **10**(5): p. 278-83.
152. Hollsberg, P., H.J. Hansen, and S. Haahr, *Altered CD8+ T cell responses to selected Epstein-Barr virus immunodominant epitopes in patients with multiple sclerosis.* Clin Exp Immunol, 2003. **132**(1): p. 137-43.

153. Gronen, F., et al., *Frequency analysis of HLA-B7-restricted Epstein-Barr virus-specific cytotoxic T lymphocytes in patients with multiple sclerosis and healthy controls.* J Neuroimmunol, 2006. **180**(1-2): p. 185-92.
154. Cepok, S., et al., *Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis.* J Clin Invest, 2005. **115**(5): p. 1352-60.
155. Jilek, S., et al., *Strong EBV-specific CD8+ T-cell response in patients with early multiple sclerosis.* Brain, 2008. **131**(Pt 7): p. 1712-21.
156. Jaquiery, E., et al., *Intrathecal immune responses to EBV in early MS.* Eur J Immunol, 2010. **40**(3): p. 878-87.
157. Jaquiery, E., et al., *Cytokine mRNA profile of Epstein-Barr virus-stimulated highly differentiated T cells in multiple sclerosis: a pilot study.* J Neuroimmunol, 2010. **225**(1-2): p. 167-70.
158. Pender, M.P., et al., *Decreased T cell reactivity to Epstein-Barr virus infected lymphoblastoid cell lines in multiple sclerosis.* J Neurol Neurosurg Psychiatry, 2009. **80**(5): p. 498-505.
159. Lindsey, J.W. and L.M. Hatfield, *Epstein-Barr virus and multiple sclerosis: cellular immune response and cross-reactivity.* J Neuroimmunol, 2010. **229**(1-2): p. 238-42.
160. Lunemann, J.D., et al., *Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis.* Brain, 2006. **129**(Pt 6): p. 1493-506.
161. Lunemann, J.D., et al., *EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2.* J Exp Med, 2008. **205**(8): p. 1763-73.
162. Hilton, D.A., et al., *Absence of Epstein-Barr virus RNA in multiple sclerosis as assessed by in situ hybridisation.* J Neurol Neurosurg Psychiatry, 1994. **57**(8): p. 975-6.
163. Opsahl, M.L. and P.G. Kennedy, *An attempt to investigate the presence of Epstein Barr virus in multiple sclerosis and normal control brain tissue.* J Neurol, 2007. **254**(4): p. 425-30.
164. Morre, S.A., et al., *Is Epstein-Barr virus present in the CNS of patients with MS?* Neurology, 2001. **56**(5): p. 692.
165. Serafini, B., et al., *Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain.* J Exp Med, 2007. **204**(12): p. 2899-912.
166. Serafini, B., et al., *Epstein-Barr virus latent infection and BAFF expression in B cells in the multiple sclerosis brain: implications for viral persistence and intrathecal B-cell activation.* J Neuropathol Exp Neurol, 2010. **69**(7): p. 677-93.
167. Peferoen, L.A., et al., *Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis.* Brain, 2010. **133**(Pt 5): p. e137.
168. Sargsyan, S.A., et al., *Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis.* Neurology, 2010. **74**(14): p. 1127-35.
169. Willis, S.N., et al., *Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain.* Brain, 2009. **132**(Pt 12): p. 3318-28.
170. Ota, K., et al., *T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis.* Nature, 1990. **346**(6280): p. 183-7.

171. Tejada-Simon, M.V., et al., *Aberrant T cell responses to myelin antigens during clinical exacerbation in patients with multiple sclerosis.* Int Immunol, 2000. **12**(12): p. 1641-50.
172. Brabb, T., et al., *Triggers of autoimmune disease in a murine TCR-transgenic model for multiple sclerosis.* J Immunol, 1997. **159**(1): p. 497-507.
173. Millward, J.M., et al., *IFN-gamma-induced chemokines synergize with pertussis toxin to promote T cell entry to the central nervous system.* J Immunol, 2007. **178**(12): p. 8175-82.
174. Correale, J. and A. Villa, *The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting.* Autoimmunity, 2007. **40**(2): p. 148-60.
175. Olitsky, P.K. and R.H. Yager, *Experimental disseminated encephalomyelitis in white mice.* J Exp Med, 1949. **90**(3): p. 213-24.
176. Kamradt, T., et al., *Pertussis toxin prevents the induction of peripheral T cell anergy and enhances the T cell response to an encephalitogenic peptide of myelin basic protein.* J Immunol, 1991. **147**(10): p. 3296-302.
177. Shive, C.L., et al., *The enhanced antigen-specific production of cytokines induced by pertussis toxin is due to clonal expansion of T cells and not to altered effector functions of long-term memory cells.* Eur J Immunol, 2000. **30**(8): p. 2422-31.
178. Trotter, J.L., et al., *Myelin proteolipid protein induces demyelinating disease in mice.* J Neurol Sci, 1987. **79**(1-2): p. 173-88.
179. Schreiner, B., F.L. Heppner, and B. Becher, *Modeling multiple sclerosis in laboratory animals.* Semin Immunopathol, 2009. **31**(4): p. 479-95.
180. Pettinelli, C.B. and D.E. McFarlin, *Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes.* J Immunol, 1981. **127**(4): p. 1420-3.
181. Waldor, M.K., et al., *Reversal of experimental allergic encephalomyelitis with monoclonal antibody to a T-cell subset marker.* Science, 1985. **227**(4685): p. 415-7.
182. Sriram, S. and L. Steinman, *Anti I-A antibody suppresses active encephalomyelitis: treatment model for diseases linked to IR genes.* J Exp Med, 1983. **158**(4): p. 1362-7.
183. Jameson, B.A., et al., *A rationally designed CD4 analogue inhibits experimental allergic encephalomyelitis.* Nature, 1994. **368**(6473): p. 744-6.
184. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* J Exp Med, 2005. **201**(2): p. 233-40.
185. Stromnes, I.M., et al., *Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells.* Nat Med, 2008. **14**(3): p. 337-42.
186. Seamons, A., A. Perchellet, and J. Goverman, *Immune tolerance to myelin proteins.* Immunol Res, 2003. **28**(3): p. 201-21.
187. Tompkins, S.M., et al., *De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis.* J Immunol, 2002. **168**(8): p. 4173-83.
188. Hickey, W.F. and H. Kimura, *Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo.* Science, 1988. **239**(4837): p. 290-2.
189. Johnson, T.A., F.R. Jirik, and S. Fournier, *Exploring the roles of CD8(+) T lymphocytes in the pathogenesis of autoimmune demyelination.* Semin Immunopathol, 2010. **32**(2): p. 197-209.

190. Mix, E., H. Meyer-Rienecker, and U.K. Zettl, *Animal models of multiple sclerosis for the development and validation of novel therapies - potential and limitations.* J Neurol, 2008. **255 Suppl 6**: p. 7-14.
191. Ruiz-Irastorza, G., et al., *Systemic lupus erythematosus.* Lancet, 2001. **357**(9261): p. 1027-32.
192. Tsokos, G.C., *Systemic lupus erythematosus.* N Engl J Med, 2011. **365**(22): p. 2110-21.
193. Manson, J.J., et al., *Relationship between anti-dsDNA, anti-nucleosome and anti-alpha-actinin antibodies and markers of renal disease in patients with lupus nephritis: a prospective longitudinal study.* Arthritis Res Ther, 2009. **11**(5): p. R154.
194. Gualtierotti, R., et al., *Updating on the pathogenesis of systemic lupus erythematosus.* Autoimmun Rev, 2010. **10**(1): p. 3-7.
195. Deapen, D., et al., *A revised estimate of twin concordance in systemic lupus erythematosus.* Arthritis Rheum, 1992. **35**(3): p. 311-8.
196. Harley, J.B. and J.A. James, *Epstein-Barr virus infection may be an environmental risk factor for systemic lupus erythematosus in children and teenagers.* Arthritis Rheum, 1999. **42**(8): p. 1782-3.
197. Doria, A., et al., *Infections as triggers and complications of systemic lupus erythematosus.* Autoimmun Rev, 2008. **8**(1): p. 24-8.
198. Zandman-Goddard, G. and Y. Shoenfeld, *Infections and SLE.* Autoimmunity, 2005. **38**(7): p. 473-85.
199. James, J.A., et al., *An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus.* J Clin Invest, 1997. **100**(12): p. 3019-26.
200. James, J.A., et al., *Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure.* Arthritis Rheum, 2001. **44**(5): p. 1122-6.
201. Yu, S.F., et al., *Detecting Epstein-Barr virus DNA from peripheral blood mononuclear cells in adult patients with systemic lupus erythematosus in Taiwan.* Med Microbiol Immunol, 2005. **194**(3): p. 115-20.
202. Gross, A.J., et al., *EBV and systemic lupus erythematosus: a new perspective.* J Immunol, 2005. **174**(11): p. 6599-607.
203. Moon, U.Y., et al., *Patients with systemic lupus erythematosus have abnormally elevated Epstein-Barr virus load in blood.* Arthritis Res Ther, 2004. **6**(4): p. R295-302.
204. Kang, I., et al., *Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus.* J Immunol, 2004. **172**(2): p. 1287-94.
205. Larsen, M., et al., *Exhausted cytotoxic control of Epstein-Barr virus in human lupus.* PLoS Pathog, 2011. **7**(10): p. e1002328.
206. Maddison, P.J. and M. Reichlin, *Quantitation of precipitating antibodies to certain soluble nuclear antigens in SLE.* Arthritis Rheum, 1977. **20**(3): p. 819-24.
207. Arbuckle, M.R., et al., *Shared early autoantibody recognition events in the development of anti-Sm B/B' in human lupus.* Scand J Immunol, 1999. **50**(5): p. 447-55.
208. James, J.A., et al., *Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity.* J Exp Med, 1995. **181**(2): p. 453-61.
209. McClain, M.T., et al., *Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry.* Nat Med, 2005. **11**(1): p. 85-9.

210. McClain, M.T., et al., *An altered immune response to Epstein-Barr nuclear antigen 1 in pediatric systemic lupus erythematosus.* Arthritis Rheum, 2006. **54**(1): p. 360-8.
211. McClain, M.T., et al., *Infectious mononucleosis patients temporarily recognize a unique, cross-reactive epitope of Epstein-Barr virus nuclear antigen-1.* J Med Virol, 2003. **70**(2): p. 253-7.
212. Bhimma, R., M. Adhikari, and H.M. Coovadia, *Epstein-Barr virus-induced systemic lupus erythematosus.* S Afr Med J, 1995. **85**(9): p. 899-900.
213. Dror, Y., et al., *Systemic lupus erythematosus associated with acute Epstein-Barr virus infection.* Am J Kidney Dis, 1998. **32**(5): p. 825-8.
214. Verdolini, R., et al., *Systemic lupus erythematosus induced by Epstein-Barr virus infection.* Br J Dermatol, 2002. **146**(5): p. 877-81.
215. Tsokos, G.C., I.T. Magrath, and J.E. Balow, *Epstein-Barr virus induces normal B cell responses but defective suppressor T cell responses in patients with systemic lupus erythematosus.* J Immunol, 1983. **131**(4): p. 1797-801.
216. Berner, B.R., et al., *Phenotypic and functional analysis of EBV-specific memory CD8 cells in SLE.* Cell Immunol, 2005. **235**(1): p. 29-38.
217. Caldwell, R.G., et al., *Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals.* Immunity, 1998. **9**(3): p. 405-11.
218. Merchant, M., et al., *The effects of the Epstein-Barr virus latent membrane protein 2A on B cell function.* Int Rev Immunol, 2001. **20**(6): p. 805-35.
219. Helyer, *Positive lupus erythematosus tests in a cross-bred strain of mice NZB/BL-NZY/BL.* Proc Univ Otago Med School, 1961. **39**: p. 3-4.
220. Dubois, E.L., et al., *NZB/NZW mice as a model of systemic lupus erythematosus.* JAMA, 1966. **195**(4): p. 285-9.
221. Theofilopoulos, A.N. and F.J. Dixon, *Murine models of systemic lupus erythematosus.* Adv Immunol, 1985. **37**: p. 269-390.
222. Baudino, L., et al., *Molecular and cellular basis for pathogenicity of autoantibodies: lessons from murine monoclonal autoantibodies.* Springer Semin Immunopathol, 2006. **28**(2): p. 175-84.
223. Gonzalez, M.L. and F.J. Waxman, *Glomerular deposition of immune complexes made with IgG2a monoclonal antibodies.* J Immunol, 2000. **164**(2): p. 1071-7.
224. Steward, M.W. and F.C. Hay, *Changes in immunoglobulin class and subclass of anti-DNA antibodies with increasing age in N/ZBW F1 hybrid mice.* Clin Exp Immunol, 1976. **26**(2): p. 363-70.
225. Theofilopoulos, A.N., et al., *A comparative immunologic analysis of several murine strains with autoimmune manifestations.* Clin Immunol Immunopathol, 1980. **15**(3): p. 258-78.
226. Haas, C., B. Ryffel, and M. Le Hir, *IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB x NZW)F1 mice.* J Immunol, 1998. **160**(8): p. 3713-8.
227. Jacob, C.O., P.H. van der Meide, and H.O. McDevitt, *In vivo treatment of (NZB X NZW)F1 lupus-like nephritis with monoclonal antibody to gamma interferon.* J Exp Med, 1987. **166**(3): p. 798-803.
228. Peng, S.L., J. Moslehi, and J. Craft, *Roles of interferon-gamma and interleukin-4 in murine lupus.* J Clin Invest, 1997. **99**(8): p. 1936-46.

229. Adachi, Y., et al., *Effects of administration of monoclonal antibodies (anti-CD4 or anti-CD8) on the development of autoimmune diseases in (NZW x BXSB)F1 mice.* Immunobiology, 1998. **198**(4): p. 451-64.
230. Carteron, N.L., et al., *F(ab')2 anti-CD4 and intact anti-CD4 monoclonal antibodies inhibit the accumulation of CD4+ T cells, CD8+ T cells, and B cells in the kidneys of lupus-prone NZB/NZW mice.* Clin Immunol Immunopathol, 1990. **56**(3): p. 373-83.
231. Chen, S.Y., et al., *The natural history of disease expression in CD4 and CD8 gene-deleted New Zealand black (NZB) mice.* J Immunol, 1996. **157**(6): p. 2676-84.
232. Oliveira, G.G., P.R. Hutchings, and P.M. Lydyard, *Anti-CD4 treatment of NZB mice prevents the development of erythrocyte autoantibodies but hastens the appearance of anaemia.* Immunol Lett, 1994. **39**(2): p. 153-6.
233. Oliveira, G.G., et al., *Production of erythrocyte autoantibodies in NZB mice is inhibited by CD4 antibodies.* Clin Exp Immunol, 1994. **96**(2): p. 297-302.
234. Wofsy, D. and W.E. Seaman, *Successful treatment of autoimmunity in NZB/NZW F1 mice with monoclonal antibody to L3T4.* J Exp Med, 1985. **161**(2): p. 378-91.
235. Borchers, A., et al., *The pathogenesis of autoimmunity in New Zealand mice.* Semin Arthritis Rheum, 2000. **29**(6): p. 385-99.
236. Chen, Y.C., Y.L. Ye, and B.L. Chiang, *Establishment and characterization of cloned CD4- CD8- alphabeta-T cell receptor (TCR)-bearing autoreactive T cells from autoimmune NZB x NZW F1 mice.* Clin Exp Immunol, 1997. **108**(1): p. 52-7.
237. Naiki, M., et al., *Generation and characterization of cloned T helper cell lines for anti-DNA responses in NZB.H-2bm12 mice.* J Immunol, 1992. **149**(12): p. 4109-15.
238. Ofosu-Appiah, W., et al., *Isolation and functional characterization of IL-2 responsive T cell clones from NZB x NZW F1 mice.* J Autoimmun, 1996. **9**(5): p. 617-27.
239. Yoshida, S., J.J. Castles, and M.E. Gershwin, *The pathogenesis of autoimmunity in New Zealand mice.* Semin Arthritis Rheum, 1990. **19**(4): p. 224-42.
240. Jyonouchi, H. and P.W. Kincade, *Precocious and enhanced functional maturation of B lineage cells in New Zealand Black mice during embryonic development.* J Exp Med, 1984. **159**(4): p. 1277-82.
241. Jyonouchi, H., et al., *B lymphocyte lineage cells in newborn and very young NZB mice: evidence for regulatory disorders affecting B cell formation.* J Immunol, 1983. **131**(5): p. 2219-25.
242. Viglianti, G.A., et al., *Activation of autoreactive B cells by CpG dsDNA.* Immunity, 2003. **19**(6): p. 837-47.
243. Hasegawa, K. and T. Hayashi, *Synthetic CpG oligodeoxynucleotides accelerate the development of lupus nephritis during preactive phase in NZB x NZWF1 mice.* Lupus, 2003. **12**(11): p. 838-45.
244. Christensen, S.R., et al., *Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus.* J Exp Med, 2005. **202**(2): p. 321-31.
245. Dorovini-Zis, K., R. Prameya, and P.D. Bowman, *Culture and characterization of microvascular endothelial cells derived from human brain.* Lab Invest, 1991. **64**(3): p. 425-36.
246. Quandt, J. and K. Dorovini-Zis, *The beta chemokines CCL4 and CCL5 enhance adhesion of specific CD4+ T cell subsets to human brain endothelial cells.* J Neuropathol Exp Neurol, 2004. **63**(4): p. 350-62.

247. Jebbink, J., et al., *Development of real-time PCR assays for the quantitative detection of Epstein-Barr virus and cytomegalovirus, comparison of TaqMan probes, and molecular beacons.* J Mol Diagn, 2003. **5**(1): p. 15-20.
248. Luo, B., et al., *Expression of Epstein-Barr virus genes in EBV-associated gastric carcinomas.* World J Gastroenterol, 2005. **11**(5): p. 629-33.
249. Shannon-Lowe, C., et al., *Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification.* J Virol, 2009. **83**(15): p. 7749-60.
250. Wong, D., R. Prameya, and K. Dorovini-Zis, *In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1.* J Neuropathol Exp Neurol, 1999. **58**(2): p. 138-52.
251. Dal Canto, A.J., H.W.t. Virgin, and S.H. Speck, *Ongoing viral replication is required for gammaherpesvirus 68-induced vascular damage.* J Virol, 2000. **74**(23): p. 11304-10.
252. Mora, A.L., et al., *Control of virus reactivation arrests pulmonary herpesvirus-induced fibrosis in IFN-gamma receptor-deficient mice.* Am J Respir Crit Care Med, 2007. **175**(11): p. 1139-50.
253. Weck, K.E., et al., *Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68.* J Virol, 1996. **70**(10): p. 6775-80.
254. Liu, F. and J.L. Whitton, *Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections.* J Immunol, 2005. **174**(10): p. 5936-40.
255. Ascherio, A. and K.L. Munger, *Environmental risk factors for multiple sclerosis. Part I: the role of infection.* Ann Neurol, 2007. **61**(4): p. 288-99.
256. Lunemann, J.D. and C. Munz, *EBV in MS: guilty by association?* Trends Immunol, 2009. **30**(6): p. 243-8.
257. Valbuena, G. and D.H. Walker, *The endothelium as a target for infections.* Annu Rev Pathol, 2006. **1**: p. 171-98.
258. Ransohoff, R.M., *Immunology: In the beginning.* Nature, 2009. **462**(7269): p. 41-2.
259. Ban, S., et al., *Systemic granulomatous arteritis associated with Epstein-Barr virus infection.* Virchows Arch, 1999. **434**(3): p. 249-54.
260. Guarner, J. and E.R. Unger, *Association of Epstein-Barr virus in epithelioid angiomatosis of AIDS patients.* Am J Surg Pathol, 1990. **14**(10): p. 956-60.
261. Jarvis, M.A. and J.A. Nelson, *Human cytomegalovirus tropism for endothelial cells: not all endothelial cells are created equal.* J Virol, 2007. **81**(5): p. 2095-101.
262. Gosselin, J. and P. Borgeat, *Epstein-Barr virus modulates 5-lipoxygenase product synthesis in human peripheral blood mononuclear cells.* Blood, 1997. **89**(6): p. 2122-30.
263. Prat, A., et al., *Migration of multiple sclerosis lymphocytes through brain endothelium.* Arch Neurol, 2002. **59**(3): p. 391-7.
264. Chui, R. and K. Dorovini-Zis, *Regulation of CCL2 and CCL3 expression in human brain endothelial cells by cytokines and lipopolysaccharide.* J Neuroinflammation, 2010. **7**: p. 1.
265. Mrass, P. and W. Weninger, *Immune cell migration as a means to control immune privilege: lessons from the CNS and tumors.* Immunol Rev, 2006. **213**: p. 195-212.

266. Feederle, R., et al., *Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection.* Int J Cancer, 2007. **121**(3): p. 588-94.
267. van Veen, T., et al., *CCL5 and CCR5 genotypes modify clinical, radiological and pathological features of multiple sclerosis.* J Neuroimmunol, 2007. **190**(1-2): p. 157-64.
268. Simpson, J.E., et al., *Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions.* J Neuroimmunol, 1998. **84**(2): p. 238-49.
269. Dietrich, J.B., *The adhesion molecule ICAM-1 and its regulation in relation with the blood-brain barrier.* J Neuroimmunol, 2002. **128**(1-2): p. 58-68.
270. Flano, E., D.L. Woodland, and M.A. Blackman, *A mouse model for infectious mononucleosis.* Immunol Res, 2002. **25**(3): p. 201-17.
271. Saxena, A., et al., *Cutting edge: Multiple sclerosis-like lesions induced by effector CD8 T cells recognizing a sequestered antigen on oligodendrocytes.* J Immunol, 2008. **181**(3): p. 1617-21.
272. Yang, Y., et al., *T-bet is essential for encephalitogenicity of both Th1 and Th17 cells.* J Exp Med, 2009. **206**(7): p. 1549-64.
273. Dutia, B.M., et al., *Type I interferons and IRF-1 play a critical role in the control of a gammaherpesvirus infection.* Virology, 1999. **261**(2): p. 173-9.
274. Ure, D.R., et al., *Neutralization of chemokines RANTES and MIG increases virus antigen expression and spinal cord pathology during Theiler's virus infection.* Int Immunol, 2005. **17**(5): p. 569-79.
275. Kaplan, C., et al., *Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis.* Arthritis Res, 2002. **4**(1): p. 54-8.
276. Krakowski, M. and T. Owens, *Interferon-gamma confers resistance to experimental allergic encephalomyelitis.* Eur J Immunol, 1996. **26**(7): p. 1641-6.
277. Billiau, A., et al., *Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma.* J Immunol, 1988. **140**(5): p. 1506-10.
278. Duong, T.T., et al., *Effect of anti-interferon-gamma monoclonal antibody treatment on the development of experimental allergic encephalomyelitis in resistant mouse strains.* J Neuroimmunol, 1994. **53**(1): p. 101-7.
279. Gasper-Smith, N., I. Marriott, and K.L. Bost, *Murine gamma-herpesvirus 68 limits naturally occurring CD4+CD25+ T regulatory cell activity following infection.* J Immunol, 2006. **177**(7): p. 4670-8.
280. Martin, S., et al., *CD40 expression levels modulate regulatory T cells in Leishmania donovani infection.* J Immunol, 2010. **185**(1): p. 551-9.
281. Ferrer, I.R., et al., *Antigen-specific induced Foxp3+ regulatory T cells are generated following CD40/CD154 blockade.* Proc Natl Acad Sci U S A, 2011. **108**(51): p. 20701-6.
282. Matullo, C.M., et al., *CNS recruitment of CD8+ T lymphocytes specific for a peripheral virus infection triggers neuropathogenesis during polymicrobial challenge.* PLoS Pathog, 2011. **7**(12): p. e1002462.
283. Olsson, T., et al., *Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma.* J Clin Invest, 1990. **86**(3): p. 981-5.
284. Pelfrey, C.M., et al., *Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production.* J Immunol, 2000. **165**(3): p. 1641-51.

285. Drulovic, J., et al., *Expression of Th1 and Th17 cytokines and transcription factors in multiple sclerosis patients: does baseline T-bet mRNA predict the response to interferon-beta treatment?* J Neuroimmunol, 2009. **215**(1-2): p. 90-5.
286. Frisullo, G., et al., *pSTAT1, pSTAT3, and T-bet expression in peripheral blood mononuclear cells from relapsing-remitting multiple sclerosis patients correlates with disease activity.* J Neurosci Res, 2006. **84**(5): p. 1027-36.
287. Purvis, H.A., et al., *Low-strength T-cell activation promotes Th17 responses.* Blood, 2010. **116**(23): p. 4829-37.
288. Bennett, S.R., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-80.
289. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-3.
290. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell.* Nature, 1998. **393**(6684): p. 474-8.
291. Lanzavecchia, A., *Immunology. Licence to kill.* Nature, 1998. **393**(6684): p. 413-4.
292. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.
293. Schleicher, U., A. Hesse, and C. Bogdan, *Minute numbers of contaminant CD8+ T cells or CD11b+CD11c+ NK cells are the source of IFN-gamma in IL-12/IL-18-stimulated mouse macrophage populations.* Blood, 2005. **105**(3): p. 1319-28.
294. Romero, C.R., et al., *The role of interferon-gamma in the pathogenesis of acute intra-abdominal sepsis.* J Leukoc Biol, 2010. **88**(4): p. 725-35.
295. Frucht, D.M., et al., *IFN-gamma production by antigen-presenting cells: mechanisms emerge.* Trends Immunol, 2001. **22**(10): p. 556-60.
296. Perry, D., et al., *Murine models of systemic lupus erythematosus.* J Biomed Biotechnol, 2011. **2011**: p. 271694.
297. Gomez, D., et al., *Th1/Th2 cytokines in patients with systemic lupus erythematosus: is tumor necrosis factor alpha protective?* Semin Arthritis Rheum, 2004. **33**(6): p. 404-13.
298. Masutani, K., et al., *Predominance of Th1 immune response in diffuse proliferative lupus nephritis.* Arthritis Rheum, 2001. **44**(9): p. 2097-106.
299. Chan, R.W., et al., *Imbalance of Th1/Th2 transcription factors in patients with lupus nephritis.* Rheumatology (Oxford), 2006. **45**(8): p. 951-7.
300. Chan, R.W., et al., *Intrarenal cytokine gene expression in lupus nephritis.* Ann Rheum Dis, 2007. **66**(7): p. 886-92.
301. Uhm, W.S., et al., *Cytokine balance in kidney tissue from lupus nephritis patients.* Rheumatology (Oxford), 2003. **42**(8): p. 935-8.
302. Dorner, T., C. Giesecke, and P.E. Lipsky, *Mechanisms of B cell autoimmunity in SLE.* Arthritis Res Ther, 2011. **13**(5): p. 243.
303. Casiraghi, C., K. Dorovini-Zis, and M.S. Horwitz, *Epstein-Barr virus infection of human brain microvessel endothelial cells: a novel role in multiple sclerosis.* J Neuroimmunol, 2011. **230**(1-2): p. 173-7.
304. Aloisi, F., et al., *Detection of Epstein-Barr virus and B-cell follicles in the multiple sclerosis brain: what you find depends on how and where you look.* Brain, 2010. **133**(Pt 12): p. e157.

305. White, D.W., R. Suzanne Beard, and E.S. Barton, *Immune modulation during latent herpesvirus infection.* Immunol Rev, 2012. **245**(1): p. 189-208.