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Christina Lomparski

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STUDY OF THE INFLAMMATORY AND IMMUNOLOGICAL ACTIONS OF RETROVIRUSES

a dissertation submitted to the
University Joseph Fourier - Grenoble 1 - UFR Biology
to obtain the degree of Doctor of Biology

presented by

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defended the 21st july 2009

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The present manuscript represents a piece of work that was rather difficult for me to accomplish, both materially and mentally. Thinking about the past five years in terms of pure thesis work does not inspire me to thank anybody in particular, except my friends that are like a second family to me and that made me want to stay in France when times were tough.

Anyway, the people that have been surrounding me during these five years and that I am supposed to thank here will recognize themselves, since everybody knows perfectly which side he or she is on. And for those who would have expected to be acknowledged more explicitly, here is at least the explanation *why* I do not feel like thanking, and *why* nobody in particular will be mentioned.

After graduating in Germany, my decision to go to France came rather spontaneously and did not have any professional or career goals. But still, I left Germany thinking that five years of studies had prepared me enough to consider myself as a researcher.

Is it really so pretentious to believe that five years of university education made me competent enough to contribute to research in an autonomous way, at least to have developed my personal expertise, to decide and to speak for myself, to feel worthy being listened? Arriving in France, I had to learn painfully that this state of mind – that I personally had considered and still consider as an important step in individual development – was rather unwelcome.

One important obstacle to what I would have considered a successful PhD thesis has been the absence of a person to *talk science* with, I mean, somebody else than those unwilling to consider PhD students as scientists. I think that without being listened, one simply cannot learn; I understood that the suitable condition for “learning”, “progressing”, or more explicitly for *being useful* would have been to completely restart at Zero, to admit my ignorance and the profound unimportance of my

opinion, to become the unwritten page I would have needed to be in the eyes of some.

The fact that I did not really agree to do so can be seen, in a certain way, as my fault, because I precisely knew about the consequences; however, it is my responsibility that I am willing to assume. If the choice I had to make was to *sink or swim*, I chose to swim *against the current*, having perfect conscience of the obstacles this attitude of resistance and the interpersonal conflicts I did not try to avoid would engender for my future work.

Indeed, being surrounded by people considering me as a rebelling alien that did not learn how to behave was everything else but stimulating, and the fact that superiors preferred to direct their speech towards higher-grade colleagues in my presence when talking about my work was nothing else but humiliating. I guess that the exercise of *thanking* at this point of the way may come spontaneously under different circumstances, at least it may not seem as artificial as it seems to me now. I guess that the numerous “helpers” that may usually be there to *accompany* a PhD student in his or her difficult work – nothing but the fact to recognise the difficulty of this work ! – surely deserve being acknowledged; the idea to thank should come rather naturally, or should not come at all.

I must admit having worked much less efficiently and accurately than I would have liked to work myself. All in all, I am highly dissatisfied by the work I present here because I know that I could have *done* better if I would have *felt* better, which means if I would have been allowed to appropriate my thesis work instead of executing in vain the orders of a never satisfied higher instance.

Most of the experiments I performed during my thesis did not work (hence they are not shown here); maybe this is “normal” for natural sciences. But what does not seem so “normal” to me is the fact that *negative* results are equivalent to *no* results. Since the classification scheme applicable to researchers – indexing of impact factors and quantitative scoring of publications (bibliometrics) – hangs like the sword of Damocles above the heads of PhD students already, I would have had to recognise the value of my “failed” work by myself, but for a long time I was not able to do so. I almost started to believe in the currently omnipresent dogma that everything is quantifiable and *quality* becomes something secondary exactly because it is not *quantitatively* measurable. In the absence of any encouragement, I finally managed to stop searching for the error on my side and concentrated on not going crazy and not transforming into a marionnette.

Maybe this experience cannot be generalised, and the circumstances I had to deal with may be exceptional; all I know is that I needed to oppose myself to the ongoing infantilisation that, in my eyes, everybody should have noticed... this infantilisation that I was supposed to undergo if I wanted to fully integrate a classical laboratory's hierarchy. Having the illusion to be good for something else than a technician's work may be considered as naïve by some, but against all odds, today I choose to assume that I was right to see myself as autonomous instead of apologising to those who felt offended in their supremacy by my behaviour. At any time, my behaviour was necessary to me personally to preserve my integrity.

If I was religious, I would possibly thank God to have made me endure this trial without losing my mind, but since I am not, I guess that I simply have to thank myself for having been strong enough to save my tears.

Maybe I wrote this having a dark day. But maybe every day is important.

Grenoble, 21st july 2009

Contents

Acknowledgements	i
Table of Contents	iv
List of Figures	viii
List of Tables	xi
Abbreviations	xiii
1 Introduction	1
1.1 The Immune System	1
1.1.1 Innate Immunity	3
1.1.2 Adaptive Immunity	6
1.1.3 Cytokines	7
1.1.4 Autoimmune Disorders	8
1.2 Multiple Sclerosis	10
1.2.1 Aetiology and Diagnostics	10
1.2.2 Pathophysiology	12
1.2.3 Treatment	13
1.3 Endogenous Retroviruses	14
1.3.1 The HERVs	14
1.3.2 MSR/V	15
1.4 Animal Models	19
1.4.1 Experimental Autoimmune Encephalomyelitis (EAE)	19
1.4.2 Humanized SCID Mice	20
1.4.3 Transgenic Mice	21
1.4.4 Animal Models used in this Work	21
1.4.5 Ethical Considerations	22
1.5 Thesis Objectives	25

2	Introduction	29
2.1	Le système immunitaire	29
2.1.1	L'immunité innée	31
2.1.2	L'immunité adaptative	35
2.1.3	Les cytokines	36
2.1.4	Les maladies auto-immunes	37
2.2	La Sclérose en Plaques	39
2.2.1	Étiologie et Diagnostic	40
2.2.2	Physiopathologie	41
2.2.3	Traitement	42
2.3	Les rétrovirus endogènes	44
2.3.1	Les HERV	44
2.3.2	MSRV	45
2.4	Modèles animaux	49
2.4.1	Encéphalomyélite Auto-immune Expérimentale (EAE)	49
2.4.2	Souris SCID humanisées	50
2.4.3	Souris transgéniques	51
2.4.4	Modèles animaux utilisés dans ces travaux	52
2.4.5	Considérations Éthiques	52
2.5	Objectifs du travail	56
3	Material and Methods	59
3.1	ENV-SU	59
3.1.1	Different Batches of ENV-SU	61
3.1.2	Neutralising Antibodies	62
3.2	Cell Culture	63
3.2.1	Human PBMCs	63
3.2.2	Murine Splenocytes	63
3.2.3	Murine Lymph Node Cells	63
3.2.4	Murine Dendritic Cells	64
3.2.5	Stimulation Assays	64
3.3	Dosage of Cytokines (ELISA)	65
3.3.1	Cytokines	65
3.3.2	Samples and Dilutions	65
3.4	Flow Cytometry	66
3.4.1	Antibodies and Staining	66
3.5	Mice	67
3.5.1	Experimental Autoimmune Encephalomyelitis (EAE)	67

3.5.2	Systemic ENV Injection	69
3.5.3	Transgenic ENV Mice	70
Digression		71
4	Results	89
4.1	Inflammatory Properties of ENV	89
4.1.1	Studies on Human Cells <i>in vitro</i>	89
4.1.2	Study of Transposability on Mouse Cells <i>in vitro</i>	91
4.1.3	TLR4 and CD14 in Mouse	94
4.1.4	Neutralising Antibodies	96
4.1.5	<i>In vivo</i> Systemic Effects of ENV-SU on Mice	98
4.2	The <i>in vivo</i> Model, Murine EAE	111
4.2.1	Symptoms	111
4.2.2	Autoreactive T Cells	119
4.2.3	MRI	122
4.3	Transgenic Mouse Model	126
4.3.1	Validation of the Promoters	126
4.3.2	Localisation of ENV	128
4.3.3	Sequence Shuffling	128
5	Discussion	141
5.1	Inflammatory Properties of ENV-SU – from Men and Mice	143
5.2	Murine Experimental Autoimmune Encephalomyelitis (EAE) – From Mice to Men?	153
5.3	Transgenic Mice	161
6	Conclusion and Perspectives	167
Appendices		173
A	Publications	175
A.1	Recherche, Éthique, Citoyenneté	175
A.2	Article in Preparation	185
B	International Communications	187
B.1	European Congress of Immunology (ECI) 2006	187
B.2	Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) 2007	191
B.3	European BioAlpine Convention (EBC) 2007	195

TABLE OF CONTENTS

C MRI Protocol	199
D Cytokine Table	213
Bibliography	217

List of Figures

1.1	Haematopoiesis in humans.	2
1.2	TLRs and their ligands.	5
1.3	TLRs and their signalling pathways.	6
1.4	Theoretical mechanism by which HERVs can cause disease.	15
1.5	Molecular characterisation of the MSR/V ENV protein.	17
2.1	Hématopoïèse chez l'Humain.	30
2.2	Les TLR et leurs ligands.	33
2.3	Les TLR et leurs voies de signalisation.	34
2.4	Mécanismes théoriques par lesquels les rétrovirus endogènes peuvent causer des maladies chez l'homme et l'animal.	45
2.5	Caractérisation moléculaire de la protéine ENV de MSR/V.	47
3.1	Amino acid sequence of the MSR/V envelope protein fraction ENV-SU.	60
3.2	SDS-PAGE and MALDI-TOF analysis of the MSR/V ENV-SU.	61
4.1	IL-6 production (ELISA) in human PBMCs; test of different batches of ENV.	90
4.2	IL-6 production (ELISA) in murine BMDCs upon ENV stimulation.	91
4.3	Mean IL-6 production (ELISA) in murine BMDCs upon ENV stimulation (five experiments).	92
4.4	IL-12p70 and TNF production (ELISA) in murine BMDCs upon ENV stimulation.	99
4.5	Cell maturation (flow cytometry) of murine BMDC induced by ENV.	100
4.6	IL-6 production (ELISA) in murine BMDCs; test of different batches of ENV.	101
4.7	IFN- γ production (ELISA) in murine splenocytes upon ENV stimulation.	102
4.8	Cell maturation of murine Tlr4 ^{-/-} BMDCs (flow cytometry) induced by ENV.	103

LIST OF FIGURES

4.9	Cell maturation of murine Tlr4 ^{-/-} BMDCs (flow cytometry) induced by ENV; graphic representation of percentages of double positive cells.	104
4.10	Cell maturation of murine Cd14 ^{-/-} BMDCs (flow cytometry) induced by ENV; graphic representation of percentages of double positive cells.	105
4.11	IL-6 and IL-12p70 production (ELISA) in murine Tlr4 ^{-/-} BDMCs upon ENV stimulation.	106
4.12	IL-6 and TNF production (ELISA) in murine Cd14 ^{-/-} BDMCs upon ENV stimulation.	107
4.13	Antibody neutralisation of ENV-induced IL-6 production (ELISA) in murine BMDCs.	108
4.14	Lymphocyte populations (flow cytometry) in splenocytes of ENV-injected mice.	109
4.15	Lymphocyte populations (flow cytometry) in abdominal lymph node cells of ENV-injected mice.	110
4.16	Clinical Score follow-up of ENV/IFA-induced EAE (10 or 50 µg). . .	112
4.17	Clinical Score follow-up of ENV/IFA-induced EAE (10, 25 or 50 µg of ENV/IFA or ENV/IFA w/o MOG).	116
4.18	Mean body weights and corresponding mean CS.	118
4.19	IFN-γ production (ELISA) in murine splenocytes; reactivity of freshly collected or frozen cells.	119
4.20	IFN-γ production (ELISA) in pooled murine splenocytes from ENV/IFA-induced EAE mice.	120
4.21	IFN-γ production kinetics (ELISA) in murine splenocytes from ENV/IFA-induced EAE mice.	121
4.22	IFN-γ production (ELISA) in murine splenocytes from one ENV/IFA-induced EAE mouse and one IFA mouse.	122
4.23	Coronal MRI of EAE mice compared to healthy mice; cervical medulla, hindbrain, pons + cerebellum.	123
4.24	Coronal MRI of EAE mice compared to healthy mice; hindbrain, pons + cerebellum.	123
4.25	Coronal MRI of EAE mice compared to healthy mice; midbrain. . . .	124
4.26	Coronal MRI of EAE mice compared to healthy mice; forebrain and cerebrum.	124
4.27	Coronal MRI of EAE mice compared to healthy mice; olfactory bulbs.	124
4.28	Molecular sequence of the MSRV-ENV and ENV-SU; genetic constructions including the promoters pCMV, pGFAP and pMHCII. . . .	130

4.29	Confocal microscopy of HEK293T cells transfected with the pCMV envelope constructs.	131
4.30	Confocal microscopy of J774 cells transfected with the pMHCII envelope constructs.	132
4.31	Schematic representation of the pGFAP constructs including GFP, MSR-ENV or HERV7q-ENV.	133
4.32	Confocal microscopy for the localisation of the MSR-ENV in HEK293T cells.	134
4.33	Confocal microscopy for the localisation of the HERV7q-ENV in HEK293T cells.	135
4.34	Amino acid sequences of MSR-ENV and HERV7q-ENV (cDNA plasmids).	136
4.35	Genetic constructions shuffling the MSR and HERV7q envelopes including the pCMV, pMHCII and pGFAP promoters.	137
4.36	Confocal microscopy of HEK293T cells transfected with pCMV-MSR-ENV/HERV7q-TM.	138
4.37	Confocal microscopy of HEK293T cells transfected with pCMV-HERV7q-ENV/MSR-TM.	139

LIST OF FIGURES

List of Tables

3.1	Composition of the mixtures injected to healthy C57BL/6 mice for EAE induction.	67
3.2	EAE Clinical Score (CS) Scale.	68
4.1	Cell maturation (flow cytometry) induced by ENV in Tlr4 ^{-/-} and Cd14 ^{-/-} compared to wild-type C57BL/6 BMDCs.	95
4.2	IL-6 production (ELISA) induced by ENV in Tlr4 ^{-/-} and Cd14 ^{-/-} compared to wild-type C57BL/6 BMDCs.	97
4.3	Antibody neutralisation of ENV-induced IL-6 production (ELISA) in murine BMDCs; percentages of inhibition.	98
4.4	EAE Clinical Score (CS) Scale for evaluation of symptoms.	111
4.5	Individual cumulative scores calculated for ENV/IFA-induced EAE (10 or 50 μ g).	113
4.6	Mean cumulative scores calculated for ENV/IFA-induced EAE (10 or 50 μ g).	114
4.7	Individual cumulative scores calculated for ENV/IFA-induced EAE (10, 25 or 50 μ g of ENV/IFA or ENV/IFA w/o MOG).	115
4.8	Mean cumulative scores calculated for ENV/IFA-induced EAE (10, 25 or 50 μ g of ENV/IFA or ENV/IFA w/o MOG).	117

LISTE OF TABLES

Abbreviations

APC	antigen-presenting cell (CPA en français)
ATTC	American type culture collection
BBB	blood brain barrier (BHE en français)
BHE	barrière hémato-encéphalique (BBB in English)
BMDC	bone marrow-derived dendritic cell
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CMH	complex majeur d'histocompatibilité (MHC in English)
CMV	cytomegalovirus
CNS	central nervous system (SNC en français)
CPA	cellule présentatrice d'antigène (APC in English)
CS	clinical score
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethylsulfoxide
E. coli	Escherichia coli
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EC	ectodomain / extracellular domain
EDSS	expanded disability status scale
ELISA	enzyme-linked immunosorbent assay
ENV	viral envelope protein
ENV-SU	surface unit of the viral envelope protein ENV
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum

ABBREVIATIONS

FOXP3	forkhead box P3
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEK	human embryonic kidney (cells)
HERV	human endogenous retrovirus
HERV-W	human endogenous retrovirus W family
HLA	human leukocyte antigen
HSC	haematopoietic stem cell
HSV	herpes simplex virus
IC	intracellular domain
IFN	interferon
IL	interleukin
IMDM	Iscove's modified Dulbecco's media
ITGAX	Integrin Alpha X (CD11c)
LAL	Lymolus amebocyte lysate
LPS	lipopolysaccharide
LTR	long terminal repeat
MALDI-TOF	matrix-assisted laser desorption ionisation - time of flight
MBP	myelin basic protein
MDC	myeloid dendritic cell
MDDC	monocyte-derived dendritic cell
MHC	major histocompatibility complex (CMH en français)
MLV	murine leukaemia virus
MMTV	mouse mammary tumour virus
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MS	Multiple Sclerosis
MSRV	Multiple Sclerosis- associated retroviral element
MT	Mycobacterium tuberculosis
MyD	myeloid differentiation factor
NEAA	non-essential amino acids
ORF	open reading frame

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDC	plasmacytoid dendritic cell
PFA	para-formaldehyde
PHA	phytohaemagglutinin
PI	propidium iodide
PLP	proteolipid protein
PPMS	primary progressive MS
PRMS	progressive relapsing MS
PRR	pattern recognition receptor
PSB	pansorbine
PTX	Bordetella pertussis toxin
RRMS	relapsing-remitting MS
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEP	Sclérose en Plaques
SEP	somatosensory evoked potential
SNC	système nerveux central (CNS in English)
SNP	single nucleotide polymorphism
SNS	self-non-self model
SPMS	secondary progressive MS
SU	(soluble) subunit (of ENV)
TCR	T cell receptor
TGF	transforming growth factor
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adapter protein
TLR	toll-like receptor
TM	transmembrane domain
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter inducing interferon-beta
VEP	visual evoked potential

ABBREVIATIONS

Chapter 1

Introduction

1.1 The Immune System

The mammalian immune system consists of a variety of different actors and organization levels that play together to eliminate foreign agents such as bacteria, viruses and fungi, as well as modified or mutated endogenous structures such as tumour cells. It comprises a cell-mediated and a humoral response. Two major components can be distinguished: the innate immunity that represents the first, non-specific line of defence, and the adaptive immunity that is able to recognize dangerous pathogenic agents in a specific manner and to memorise pathogen contact.

The actors of the innate and the adaptive immune system share a high number of different molecules called cytokines to communicate with each other. A variety of different immune cell types can produce and respond to cytokines; by this means they are able to polarise the response of the immune system towards inflammation or to shut down its reactivity. All immune cells originate from omnipotent haematopoietic stem cells (HSCs) that are located in the bone marrow. These HSCs give rise to the precursors of two different lineages: the myeloid and the lymphoid lineage.

The cells of the myeloid lineage can develop into platelets, monocytes/macrophages, granulocytes (neutrophil, eosinophil and basophil granulocytes) and dendritic cells of myeloid origin; the cells of the lymphoid lineage continue differentiation and maturation in the primary lymphatic organs (in the bone marrow to become B cells and in the thymus to become T cells) before they are released into the blood circulation

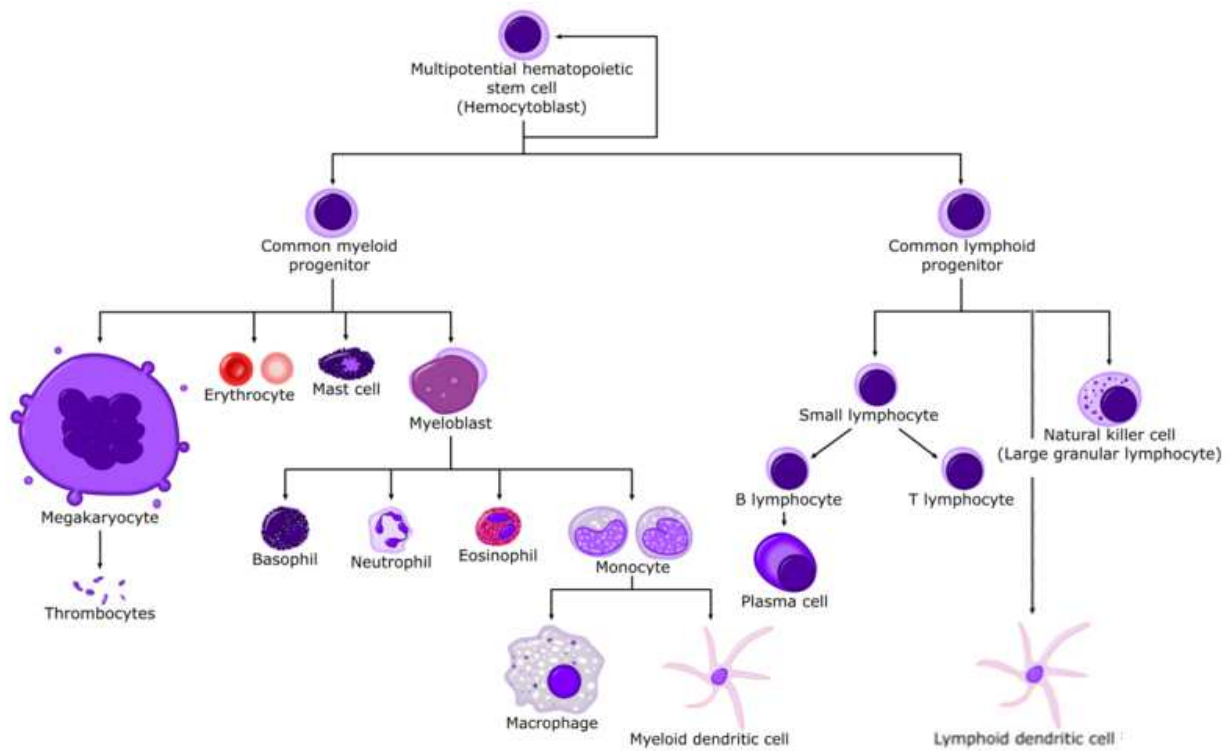


Figure 1.1: *Haematopoiesis in humans.*

to reach the secondary lymphatic organs (spleen, lymph nodes).

1.1.1 Innate Immunity

The innate immunity constitutes the first non-specific barrier for any external intruder. It comprises the complement system and several specialised cell types like macrophages, mast cells, natural killer cells, granulocytes and dendritic cells.

The complement system consists of plasma proteins that can induce a biochemical cascade in order to assign pathogens to other immune cells by opsonising (coating) them, or to perforate and lyse them directly. These two elements contribute to the first vague of clearance immediately after pathogen contact. The inflammatory reaction caused by the mediators of this first barrier also involves cytokine release and recruitment of the actors of the adaptive immune system by presentation of captured antigens.

Dendritic cells

Dendritic cells (DCs) like other phagocytic cells can engulf pathogens by endocytosis and present digested peptidic fragments by the MHC-II system. The MHC-II (major histocompatibility complex class II) is a molecule complex that is expressed by all so-called antigen-presenting cells (APCs) that are mainly B lymphocytes, monocytes/macrophages and dendritic cells; it interacts with the CD4 molecule expressed on the CD4⁺ subpopulation of T lymphocytes (so-called T helper cells). These “professional APCs” carrying MHC-II also possess accessory molecules that facilitate the contact with CD4⁺ T lymphocytes; other cell types can occasionally express MHC-II molecules and become temporarily APCs, like brain glial cells, thymus epithelial cells or vascular endothelial cells.

The function of MHC-II is to present degraded fragments of biomolecules (exogenous antigens) that entered into the cell by endocytosis. In contrast, MHC molecules of class I are expressed on all body cells. They enable infected or damaged cells to present antigenic molecule fragments of the pathogen or degradation products of their own metabolism released by the proteasome (endogenous antigens) in order to induce the recruitment of CD8⁺ T lymphocytes (so-called T killer cells).

In mature and activated DCs, the expression of MHC-II molecules is increased, which makes them highly potent APCs. They also dispose of a large number of T co-stimulatory molecules and are capable of producing inflammatory cytokines to induce and polarise T cell response, so they can be considered as the interface between innate and adaptive immunity. The MHC-II presentation of antigens to T cells will give rise to distinct T cell subpopulations that will specifically recognise and memorise the antigen and initiate a concerted response of the adaptive immune system.

DCs can arise from both the myeloid and the lymphoid lineage. After leaving the bone marrow, they can differentiate and mature under the influence of chemokines and cytokines according to their environment. DCs of myeloid origin (MDCs) can develop into skin Langerhans cells and interstitial cells present in most of the tissues; DC of lymphoid origin can migrate to the secondary lymphoid organs and become plasmacytoid DCs (PDCs) with antiviral properties. For *in vitro* studies, human DCs can be derived from circulating blood monocytes by cytokines and growth factors (MDDCs - monocyte-derived dendritic cells) but are difficult to obtain and to handle because of the low number of monocytes available; for murine DCs cultures,

femur bone marrow-derived monocytes can be artificially differentiated into DCs (BMDCs - bone marrow-derived dendritic cells) and cultivated to obtain abundant quantities.

Toll-like receptors

The toll-like receptors (TLRs) belong to the highly conserved family of pattern recognition receptors (PRRs) and are expressed on the surface of APCs and other cells. In humans, 13 different TLRs (named TLR1 to TLR13) have been identified; with exception of TLR10, homologues to all the other TLRs are known also in mice. TLRs recognize a variety of microbial patterns that are conserved among many pathogens (see figure 1.2) but that are not present in the host.

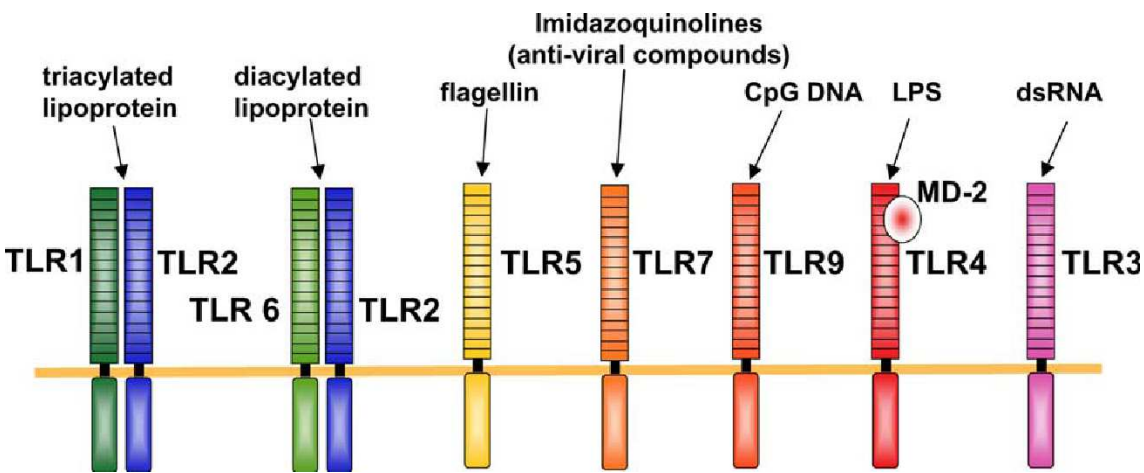


Figure 1.2: *TLRs and their ligands* [113].

One of the pathogen-associated molecular patterns (PAMP) recognized by TLRs is the bacterial endotoxin LPS (lipopolysaccharide) that is a major component of the outer membrane of gram-negative bacteria. It can interact with the TLR4 receptor and stimulate TLR4-expressing cells, e. g. DCs, to produce inflammatory cytokines like interleukin-6 (IL-6) or interleukin-12 (IL-12) and to undergo phenotypical changes that reflect DC maturation and activation, e. g. enhanced surface expression of MHC-II, and thereby intensify interaction with T cells.

The different TLRs share all in all four adaptor molecules that are called MyD88, TIRAP, TRIF and TRAM. These molecules located on the inside of the cell are part

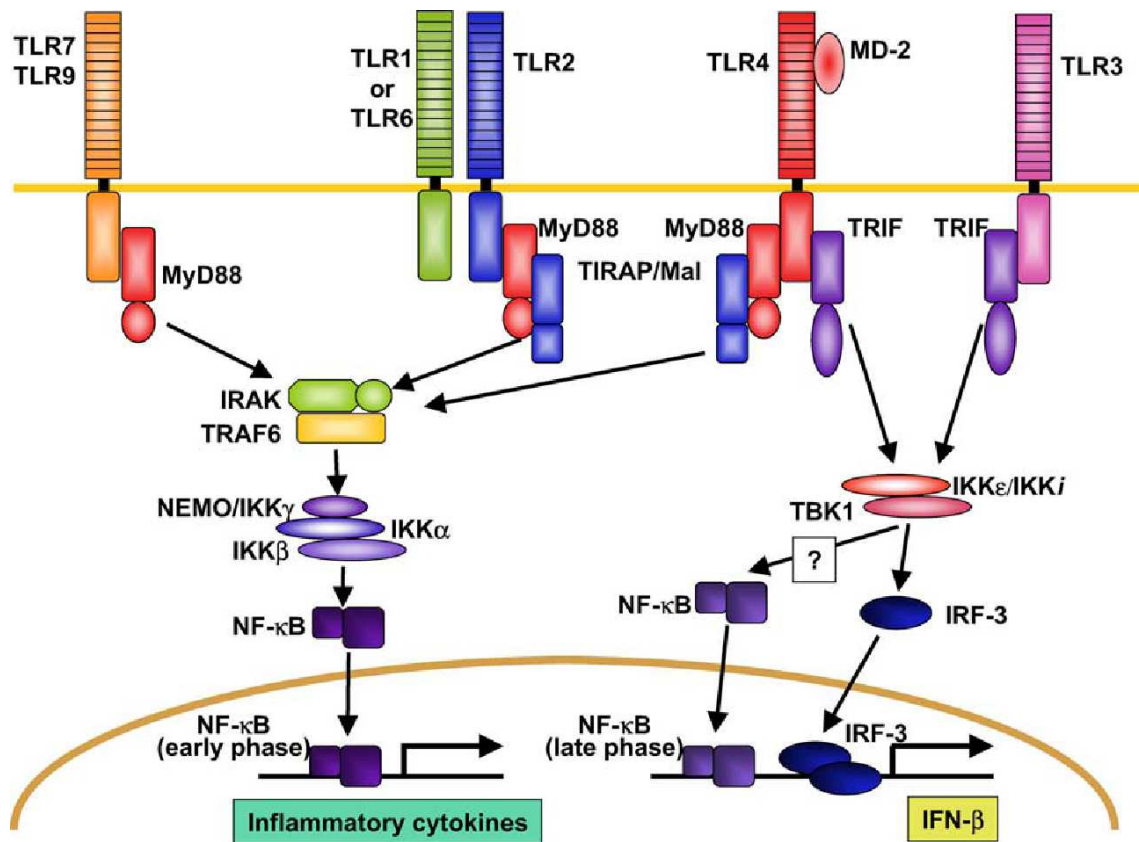


Figure 1.3: *TLRs and their signalling pathways* [113].

of the downstream signalling cascade that can lead to the activation of different genes. To interact with T cells and induce an adaptive immune response, the TLR4 signalling pathway activated by bacterial LPS requires the T co-stimulatory molecule CD14 that is spatially co-located to TLR4 on the cell surface.

1.1.2 Adaptive Immunity

Unlike the innate immune system, the adaptive immune system is able to specifically recognize antigens, to combat them in a focalised concerted manner, and to keep specific memory of earlier immune events. Its effector cells are mainly T and B lymphocytes. T lymphocytes can recognize a high number of antigenic epitopes presented either by APCs via MHC-II ($CD4^+$ helper T cells) or by non-immune somatic cells via MHC I ($CD8^+$ killer T cells). The variability of epitopes that T lymphocytes can specifically recognize is the result of a genetic recombination

process that takes place in the thymus and that gives rise to a large repertoire of T cell receptors (TCRs). Together with CD4 or CD8 as accessory (non-specific) recognition molecules, each individual TCR recognizes one particular epitope. T lymphocytes that have been stimulated by their respective antigen epitope will respond with a strong immune reaction involving cytokine production, B lymphocyte activation and memorisation of the encountered antigen.

The main role of B lymphocytes is the production of antibodies that are antigen-specific; the immune defence via antibodies is part of the humoral immune response. Antibodies released by B cells can bind to their respective antigen to form a so-called antigen-antibody complex or immune complex; this immune precipitate can be degraded by proteases or eliminated by phagocytes. In contrast to T cells, B cells are capable of recognizing soluble antigens, i. e. that do not have to be presented on the surface of another cell. Engulfed antigens can be processed and exposed on the surface via MHC-II; the contact with a T cell that has been “primed” on the same antigen leads to cytokine release by the T cell that is thereby activating the B cell which will proliferate, produce specific antigens, and generate memory B cells. Beside this T cell-dependant B cell activation, B cells can also be activated by stimulation of their PRRs or by other APCs that present the same antigen.

1.1.3 Cytokines

The different cell types of the immune system communicate with each other by molecules called cytokines. Cytokines drive the immune response either to cellular or to humoral response which means that they polarise the immune response either to inflammation mediated by Th1 helper cells (Th1 response), or to antibody production mediated by Th2 helper cells that coordinate B cell activation (Th2 response). There is a third possibility of response that can be called Th17 response and that induces a type of T lymphocytes called Th17 helper cells [37, 38, 119]; Th17 cells are able to regulate an ongoing immune response, and their function is likely to be implicated in chronic pathologies and autoimmune disorders [80].

T lymphocytes are polarised to Th1, Th2 or Th17 by the cytokines secreted by themselves or other cells of their environment; in reaction to these cytokines they differentiate, undergo phenotypical changes and produce cytokines in order to propagate the respective type of response. Th1 cells secrete Th1 cytokines like IL-2, interferon-beta (IFN- β), and transforming growth factor-beta (TGF- β) to activate

macrophages, IL-3 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate leukogenesis by the bone marrow, or IFN-gamma (IFN- γ) to inhibit Th2 response. Typical Th2 cytokines are IL-4, IL-5, IL-6 and IL-10 which stimulate antibody production by B cells; IL-10 can also inhibit the secretion of IL-2 and IFN- γ by Th1 cells. The response called Th17 is mediated by Th17 cells that secrete IL-17 and IL-21; the immune response can be polarised towards Th17 by the influence of TGF- β , IL-6 and IL-23; Th17 cells are negatively regulated by IL-4 and IFN- γ .

A fourth T cell population has recently been identified: the CD8⁺CD4⁺CD25⁺ regulatory T cells (Treg) that express the transcription factor FOXP3 and that regulate the immune functions via the cytokines TGF- β and IL-10. T reg have been reported to be implicated in systemic immune tolerance [4, 30] and their number is diminished in autoimmune diseases [116, 114].

An overview on the most important cytokines, their target cells and their biological functions is given in appendix D.

1.1.4 Autoimmune Disorders

Immune reactions against self-antigens are driven by autoreactive T cells that have been primed to recognize the concerned antigen as *foreign* or *dangerous*. During the clonal selection of T lymphocytes in the thymus, positive screening for cells responding to *non-self* and negative selection for those responding to *self* antigens takes place to avoid auto-reactivity and to induce self-tolerance; autoreactive T lymphocytes are cells that escaped to this selection process and thus represent an abnormality in the system.

In the *danger model* described by Polly MATZINGER that goes beyond the conventional model of *self-non-self* (SNS) discrimination, we understand that an endogenous molecule can be presented to an APC as *dangerous* when it is presented in a context of strong inflammation setting free “hidden” antigens that are not yet *known* to the immune system and thus evoke rejection [67]; another possibility is that a pathogen, which would be seen as *foreign*, can use *molecular mimicry* to prime T cells to recognize a structurally resembling *self* antigen [78]. In both cases, the result would be the breakdown of tolerance towards the antigen.

In type I Diabetes, insulin-producing Langerhans cells are eliminated during embryogenesis and early childhood by autoreactive T cells; in the same way, the loss of glial cells and neurons in Multiple Sclerosis is caused by a chronic inflammatory autoimmune reaction against components of the myelin sheath; what remains unclear is the fact that the onset of Multiple Sclerosis symptoms generally takes place between late adolescence and early adulthood, and therefore cannot be solely explained by a genetic deregulation of T lymphocyte selection. According to the *danger model*, a strong local inflammatory event in the central nervous system (CNS) could set free myelin-antigens that are usually not exposed to APCs and thus escaped to (positive or negative) immune education; the exposure in an inflamed tissue would create auto-reactivity, and the localisation of the inflammation centre would decide about the nature of the target antigen set free [67]. It remains to elucidate if priming of autoreactive T cells can occur in the CNS; in any case the breakdown of the blood brain barrier (BBB) is necessary to allow lymphocytes to massively invade the brain to cause tissue damage. The antigen contact is more likely to take place in the periphery, presuming at least temporal BBB rupture that would lead to peripheral exposure of the antigen.

The link between environmental factors, innate immunity and autoimmunity is discussed in several articles [106, 97, 109, 103, 93].

1.2 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic demyelinating inflammatory disease of the central nervous system in which infiltration of activated macrophages and autoreactive T lymphocytes is at the origin of glial and neuronal loss [110]; both the brain and the spinal cord white matter are affected. Demyelination consists in damages of myelin-producing oligodendrocytes which are a type of glial cells that envelop and maintain neuronal axons in the CNS and allow them a higher transmission speed than in natively unmyelinated axons.

As the localisation of the inflammation determines what part of the brain is damaged, several types of symptoms are occurring. The most common initial symptoms are visual problems, changes in sensation (*hypoesthesia*) and motor deficits like weakness and balance problems. In later phases, other symptoms appear: muscle spasms, difficulty in moving and coordination (*ataxia*), problems in speech (*dysarthria*) or swallowing (*dysphagia*), problems in vision like nystagmus, partial or total loss of vision (*optic neuritis*) or double vision (*diplopia*), fatigue, acute or chronic pain, bladder and bowel difficulties, cognitive impairment and emotional symptomatology such as depression. The clinical measure of disability progression and severity of the symptoms is the Expanded Disability Status Scale (EDSS). There are several clinical forms of MS that show different patterns of disease progression over time: *relapsing-remitting MS (RRMS)*, *secondary progressive MS (SPMS)*, *primary progressive MS (PPMS)* and *progressive relapsing MS (PRMS)*; depending on the diagnosis of one of the respective subtypes, prognosis and medication are adapted.

Like other autoimmune diseases, MS is more common in women than in men; its overall prevalence ranges between 2 and 150 out of 100,000, depending on the geographical zone. Zones of high prevalence are f. ex. industrial zones in Europe, Canada and the north of the U.S.A.; prevalence is significantly lower f. ex. in the mediterranean area and in Mexico; almost no cases are reported from sub-saharan Africa and Japan.

1.2.1 Aetiology and Diagnostics

MS is a multifactorial disease. Because of the different prevalence values and the unequal distribution of MS cases even inside one continent or country, environmen-

tal factors like pollution, viral or bacterial infections have been evoked. There is evidence for the influence of viral infections during childhood and during pregnancy of the mother. One hypothesis suggests the implication of a viral infection in the initiation of the early inflammatory phase. According to the *danger model* [67], endogenous myelin antigens could be set free by an acute and local inflammation, taken up by local DCs and presented to T cells which would become reactive against the myelin auto-antigen. Another possibility is the transactivation of endogenous retroviral superantigens by actively infecting retroviruses like the *Epstein-Barr virus (EBV)* [111]. In the same manner, viruses of the herpesviridae family can activate integrated genomic sequences of viral origin by interacting with their long terminal repeats (LTRs), possibly leading to the production of viral particles causing chronic inflammation observed in autoimmune disorders like MS [52]; the chronic inflammation localized in the brain and spine areas of retroviral expression would set free auto-antigens in a permanent manner that would aliment the autoimmune response to the antigens released.

Beside, genetic implications are of importance since f. ex. the concordance in monozygotic twins is significantly higher than in dizygotic twins (20-40% compared to 5%); however, differential twin concordance by the latitude of the birthplace has been evoked, pointing to a link between genetics and environment. Studies on *single nucleotide polymorphisms (SNPs)* have shown that the genes coding for the human leukocyte antigen (HLA) or MHC complex situated on chromosome 6 as well as those coding for the IL-2 and IL-7 receptors are implicated in a genetic predisposition for MS and other autoimmune diseases [112, 1]. In addition, geographically or socially isolated populations, f. ex. habitants of Sardinia, show prevalence rates that are significantly higher than average [95].

Genetic predisposition to MS is hardly identifiable because of the variability of the potential risk genes in the normal population. When physical and neurological findings point to MS, further diagnostics is rather done by *magnetic resonance imaging (MRI)* or *magnetic resonance spectroscopy (MRS)* to visualize abnormalities in the brain and the spinal cord; demyelinated *MS plaques* are detectable but can be confounded with plaques caused by different other demyelinating pathologies, so complementary methods are required to confirm diagnostics. The presence of so-called *oligoclonal bands* in the cerebrospinal fluid (CSF) drawn by lumbar puncture is a reliable criteria in most of the cases of MS; oligoclonal bands reveal high amounts of immunoglobulins that reflect inflammation in the brain or spinal cord, caused by MS or other CNS disorders of infectious origin. Another means is the measurement

of neuronal conduction speed in the optic and sensory nerves by *visual evoked potentials (VEPs)* and *somatosensory evoked potentials (SEPs)*; because of glial damage, the transmission speed is reduced in patients and thus indicates MS. In general, these methods together allow a more reliable diagnostics of MS than one of them alone that could be ambiguous.

In addition to these conventional methods, novel and innovative approaches are studied. One of them has been developed by a team we are collaborating with; it targets the expression of endogenous retroviral sequences that is increased in MS patients [23, 88] and whose level seems to be correlating with the disease evolution.

1.2.2 Pathophysiology

The chronic inflammation observed in MS has been evidenced by the detection of high titers of anti-myelin antibodies and autoreactive Th1 lymphocytes; it is sustained by massive invasion of blood-borne leukocytes across the BBB into the perivascular spaces and the brain parenchyma. In the healthy brain, the BBB is reluctant to the permeation of immune cells, with exception of a limited number of cells that are able to transmigrate and that constitute the *immune surveillance* [9, 40]. In MS, leukocyte trafficking is significantly increased, and perivascular inflammatory spots give rise to demyelination *plaques*. Enhanced cellular adhesion in the BBB capillaries allows entrance of T and B lymphocytes, plasma cells, activated macrophages and microglia which can release cytokines and toxic molecules and thereby create tissue damage [16].

DCs have been demonstrated to be present in the CNS [66] and are thought to play a role in CNS inflammation and autoimmunity [68]; since they are highly potent APCs, they are supposed to initiate the formation of encephalitogenic T lymphocytes. Their participation in immune invasion in an animal model of MS has been shown in mice [35].

The brain possesses limited capacities to renew damaged myelin sheaths in the early phase of disease, even though rebuilt oligodendrocyte layers are mostly thinner than original ones; in this stage, damage remains reversible and patients are able to recover and can be virtually free of symptoms. In progressive stages of MS, axonal injury occurs, and the neuropathological manifestations are irreversible and lead to gradual loss of motor and sensory functions.

1.2.3 Treatment

Conventional MS treatment is restricted to palliative approaches that are able to attenuate disease progression and to alleviate symptoms. Steroids are used to restrain acute inflammation during relapses. To control chronic inflammation that causes relapses, modulators or suppressors of immune function can be applied; f. ex. the cytokine IFN- β can limit disease progression and frequency of relapses; unfortunately, many patients are not able to endure the treatment with IFN- β because of its secondary effects such as inflammatory reactions near the injection site, extreme fatigue, fever and depression. In general, immunomodulatory or immunosuppressive drugs can be difficult to support because of their systemic effects.

Innovative approaches of treatment exist but are not yet fully developed and therefore no alternative treatment is available today. They are based on new insights in the fundamental aspects of MS aetiology, and the idea to be able to remedy the cause rather than the symptoms of MS is promising. Following the hypothesis of endogenous retroviral expression and their involvement in autoimmune diseases like MS, neutralisation of these permanently released retroviral particles or proteins could be of interest.

1.3 Endogenous Retroviruses

Endogenous retroviruses are genomic sequences that originate from viral infections during which a provirus (i. e. the DNA of a virus) has been integrated into the genome of the host; if the host survives the infection, the integrated provirus can be transmitted to its progeny if it has been incorporated in the DNA of germ cells.

1.3.1 The HERVs

Human endogenous retroviruses (HERVs) represent 8% of the human genome; these ancient retroviral sequences are supposed to have entered the germ line about 25 million years ago, at the divergence of hominids and old world monkeys [118]. The HERV-W family is a multicopy endogenous family of HERVs that comprises about 1% of the human genome and that is present in great apes and humans [11]. Like exogenous infectious retroviruses, integrated homologues carry the *gag* (encoding structural proteins), *pol* (viral enzymes) and *env* (surface envelope proteins) sequences as well as enclosing LTRs. The *gag*, *pol* and *env* genes are often found to be mutated because they do not have biological functions and are not vital for the host organism; nevertheless, some sequences kept intact open reading frames (ORFs), i. e. remained functional and therefore might be physiologically useful for the host [48, 54, 69, 89, 74].

Expression of HERVs has been argued to be implicated in human diseases (see figure 1.4) like MS, Diabetes and other autoimmune diseases, as well as in neuropathological syndromes like Schizophrenia and Bipolar disorder [121, 49, 7, 22]. In addition, the presence of a gliotoxic factor combined with reverse transcriptase activity in patients' CSF and monocytes/macrophages has been attributed to the expression of the HERV homologue MSRv and is correlated to relapsing disease symptoms [72, 71, 70, 73].

There is evidence for a physiological expression of the GAG, POL and ENV proteins of members of the HERV-W family that is pathophysiologically modulated in MS [88, 76]. The HERV-W envelope glycoproteins Enverin [2] and Syncytin-1 are physiologically expressed and implicated in syncytiotrophoblast fusion and placental development; in MS brains, the increased expression of Syncytin-1 contributes to demyelination [6], modulates the inflammatory cascade and alters astrocyte function

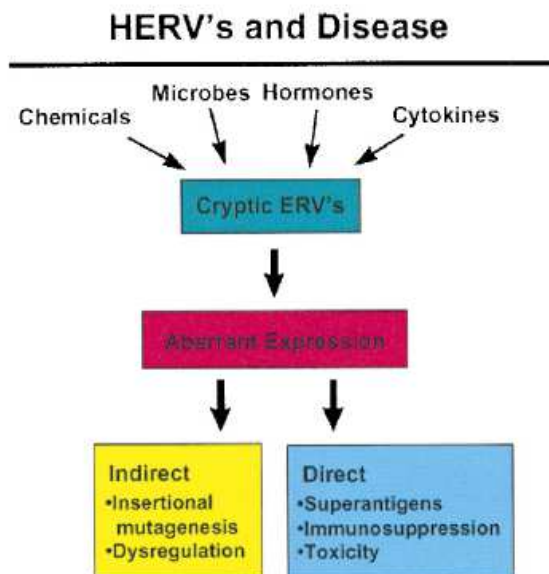


Figure 1.4: *Theoretical mechanisms by which human endogenous retroviruses (HERVs) can cause disease in humans and animals [123].*

[5].

(For a more complete synthesis of the findings linking HERVs and human (autoimmune) diseases, see the review articles by GRIFFITHS [36], PORTIS [94], DOLEI [22], PERRON et al. [90] and PERRON & LANG [91]; for the HERVs database project, see PACES et al. [81].)

1.3.2 MSR/V

The Multiple Sclerosis-associated RetroViral element (MSRV) is a virus that has been repeatedly isolated from cerebrospinal fluid (CSF) of MS patients [85]. Extracellular viral particles have been observed in monocyte and leptomeningeal cell cultures as well as in peripheral blood mononuclear cells (PBMCs) of these patients [105]; the isolated particles were able to infect healthy cell cultures. Hence MSRV can possibly be considered as an exogenous, infectious retrovirus, although the criteria of spontaneous transmissibility might not be met.

The RNA sequence of MSRV is homologous to a sequence belonging to the HERV-W family that is situated on chromosome 7 [89] (other MSRV homologues have been detected on chromosome 14 [3] and on the X gonosome [48, 55]); more precisely, the HERV-W family has been described in order to molecularly classify

MSRV [11]. Even if the HERV-W-7q sequence is not entirely transcriptionally functional [89], MSRV could be the exogenous form of a replication-competent, virion-producing provirus. There is evidence for transactivation of endogenous proviruses of the HERV-W family by *influenza virus* and *herpes simplex virus (HSV)-1* infection [101, 75].

It still remains unclear whether the transcriptional activation and the appearance of MSRV particles are causally correlated to MS symptoms; it could be at the origin of CNS inflammation as well as its side effect, considering that demyelination and axonal injury could lead to expression of MSRV RNA. Nevertheless, expression of retroviral RNA [33, 108, 104] (as well as its copy numbers [61]) and the presence of retroviral particles [8] are significantly correlated to MS and could thus provide a diagnostic tool [124, 107, 62].

Not all the members of the HERV-W family possess intact ORFs, whereas a functional promoter and a possibly transcription-potent ORF for the envelope (ENV) protein have been identified [11]. The *gag*, *pol* and *env* genes of MSRV are related to type C and D oncoviruses and their sequences have been cloned [50]. To simplify, the MSRV envelope protein that is homologous to the HERV-ENV will here be referred to as “ENV”.

The MSRV envelope protein ENV is composed of three major domains: the ectodomain (EC), corresponding to the extracellular component, a transmembrane unit (TM), and an intracytoplasmic domain (IC) (see figure 1.5). Our interest is focused on ENV and more particularly on its EC domain; interactions of pathogens with host cells in general are supposed to be mediated by their extracellular components that are exposed to their environment and constitute the first point of contact between the host cell and the pathogen.

MSRV virions have been repeatedly extracted from B cell cultures of MS patients [86, 85, 26] and are capable to infect healthy blood cell cultures [87]; the viral particles as well as *Escherichia coli*-produced recombinant ENV show superantigen effects *in vitro* by inducing abnormal immune response and polyclonal expansion of the T cell subset Vbeta16 [87]. Injection of MSRV particles in humanised SCID (*severe combined immunodeficiency*) mice (huSCID) grafted with human PBMCs leads to brain haemorrhage and T lymphocyte-dependant death [26].

The soluble subunit of ENV, ENV-SU, corresponds to a fragment that is sup-

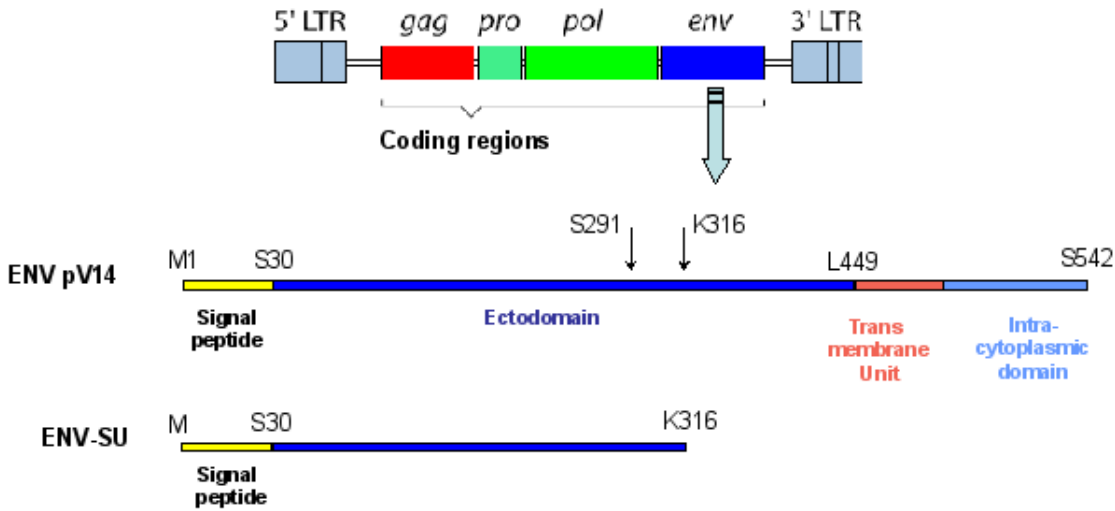


Figure 1.5: *Molecular characterisation of the MSR V ENV protein. The pV14 plasmid has been created from MSR V mRNA and includes a signal peptide and three major domains: ecto-, transmembrane and intracytoplasmic domain. ENV-SU is a 293 amino acid fraction, representing the extracellular soluble unit cleaved at K316 of the full length ENV pV14 protein. Both natural cleavage sites are presented (arrows).*

posed to be naturally generated by cleavage at K316 1.5. *In vitro*, the recombinant protein ENV-SU can be produced in *E. coli*, and its property to activate innate immunity *in vitro* has been demonstrated. On human PBMCs, purified blood monocytes and MDDCs, ENV-SU exerts inflammatory properties by activation of the CD14/TLR4 pathway and thereby is likely to promote Th1-like immune response [100].

In PBMCs isolated from MS patients' blood samples, cytokine-release mediated by ENV-SU is significantly higher than in PBMCs of normal healthy donors; in addition, the quantities of the cytokines IL-6 and IL-12p40 produced in response to ENV-SU correlate with the disease severity score (*EDSS, expanded disability status scale*) of the patients [99]. Recent findings confirm the ENV-induced cytokine production in PBMCs of RRMS patients [102].

1.4 Animal Models

Animal models are used to understand diseases and to test the application of drugs in pre-clinical studies. Diseases like MS that affect the CNS are difficult to study without animal models because of the limited access to biological material taken from patients. Easily available material like blood and CSF samples do not allow the same insights than tissue biopsies or brain slices would; experimental medicine as a research method would implicate taking high risks because MS symptoms and side effects of conventional drugs are already difficult to bear for MS patients. On the other hand, cell culture assays have limited importance because of the complexity of the physiological implications in MS that involve both the CNS and the immune system; a wide range of anatomical, pathophysiological and biomedical studies need therefore to be done in animals.

1.4.1 Experimental Autoimmune Encephalomyelitis (EAE)

Experimental Autoimmune Encephalomyelitis (EAE) is the classical animal model of CNS demyelinating diseases like MS; it is applicable on laboratory rodents and primates. EAE is widely used to simulate neuroimmunological events in the CNS occurring during disease, like inflammation, demyelination and axonal injury, and to test the impact and the posology of immunomodulatory drugs on the disease course. To cause demyelination, an autoimmune response is induced by immunisation with antigenic myelin components like *myelin oligodendrocyte glycoprotein (MOG)*, *myelin basic protein (MBP)* or *proteolipid protein (PLP)*, and adjuvants containing cell wall components of *Mycobacterium tuberculosis (MT)*, like the *complete Freund's adjuvant (CFA)*. To rupture the BBB in order to permit immune invasion into the brain and the spinal cord, *Bordetella pertussis toxin (PTX)* can be co-injected.

The symptoms of EAE are classified according to a *clinical score (CS)* scale ranging in mice from tail weakness with loss of muscle tone to limb paralysis and death. The evolution of EAE symptoms depends on the mouse strain as well as the antigen used; f. ex., in C57BL/6 mice, a chronic, but remitting type of EAE can be induced by MOG immunisation [10].

The demyelination observed in EAE is mediated by autoreactive T lymphocytes

[65]; their invasion into the brain and spinal cord parenchyma can be followed by chemical staining methods, specific antibody-immunostaining and MRI; in addition, anti-myelin antibodies can be used as serological markers as they induce demyelination in MS as well as in EAE [53]. Around the BBB vessels formed by endothelial cells and coated by astrocytes, strong inflammation recruiting lymphocytes, microglia and phagocytes can be observed before and during relapses of EAE [25]. Once primed to recognize a myelin auto-antigen such as MOG, the autoreactive T cells can arouse an immune response against a larger spectrum of structural myelin epitopes; this phenomenon called *epitope spreading* [117] can be observed in MS as well as in some cases of EAE.

EAE induction by myelin immunisation is called *active EAE induction*. To illustrate the autoimmune character of the encephalitogenic T cells found in EAE plaques, splenocytes of actively induced EAE mice can be challenged *ex vivo* with the same myelin antigen as used for the immunisation, and inflammatory cytokine production can be measured; isolated splenocytes can also be injected to healthy mice, causing demyelination and EAE symptoms (*adoptive transfer EAE induction*).

Polarisation of T lymphocytes to Th1 response and autoimmunity in EAE is likely to be mediated by DCs [120]; by responding to the conserved patterns (PAMPs) of the MT cell walls and by priming of naïve T lymphocytes to the encountered myelin antigen, they constitute the link between innate and adaptive immunity that is thought to play an important role in the induction of autoimmunity.

1.4.2 Humanized SCID Mice

Severe combined immunodeficiency (SCID) is a heritable genetic immunodeficiency disorder that occurs in several mammalian species like humans, horses and mice. Individuals suffering from SCID are deprived of humoral and cellular immunity because B and T lymphocytes fail to mature. SCID animals can be used to study the genetics and physiology of the human immune system, to elucidate the mechanisms of tumour or transplant rejection, and to experiment possible drug applications to remedy or to deal with human SCID. SCID mice can be grafted with human PBMCs after elimination of residual immune components, e. g. natural killer cells, by irradiation. In this model called *huSCID*, it is possible to study the grafted human immune cells and their implication in the immune response in an *in vivo* system. One of the advantages of huSCID mice is that “humanized” therapeutic products

acting on the human immune system can be tested in mice before going into clinical studies; as the main immune cells are not natively existing but replaceable by human cells, the system is compatible with drugs designed for humans.

1.4.3 Transgenic Mice

Genetic manipulation on laboratory animals is done to study the properties of genes in a physiologically complex and authentic environment, and to study genes that are not naturally present in the available laboratory animal. Basically, two types of genetic manipulation aimed at changing the genotype of the experimental animal can be distinguished: invalidation of an existing gene (*knock-out mutagenesis*) and addition of a new gene (*knocking-in* or *transgenesis*).

Knock-out mice are mice lacking one particular gene product encoded by a target gene that has been experimentally manipulated. Elimination or *nonsense mutation* of the target gene causes lack of its product from birth on. As innate genetic deficiencies often lead to developmental disturbances or death, *conditional knocking-out* is necessary to study these lethal mutations; by introducing an inducible promoter in the gene of interest, the gene can be “switched off” at any time to induce physiological consequences.

Transgenesis is the process of introducing an exogenous gene – called *transgene* – into a living organism; the integrated transgene will confer to the organism a new property that will be transmitted to its descendents if the transgene is integrated into the gametes. The addition of a gene can be achieved either randomly or by targeting a given locus. Similar to the situation in knock-out mice, introduction of a foreign gene can alter the physiological situation in these *knock-in* mutants and possibly be lethal. Analogically, *conditional knocking-in* is possible to “switch on” the gene at a chosen moment.

1.4.4 Animal Models used in this Work

Pathophysiological studies on the implication of ENV in MS are hampered by the lack of an animal model expressing ENV since MSR/V and the family of HERVs are found only in great apes and humans. The results we obtained on wild-type C57BL/6

mice indicate that ENV injected in combination with MOG provokes a disorder similar to classical EAE induced by injection of MOG and Freund's adjuvant (CFA); these data suggest that mice are intrinsically susceptible to neuroinflammatory diseases induced by ENV. This EAE model that is elaborated in the second part of this work is based on the injection of ENV in association with other products, which is very invasive as a method and does not reflect reality in terms of exposition and availability of ENV in the hypothetical physiological context. One means to come as close as possible to the situation in MS is to create mice expressing ENV directly, that is to set up a transgenic model for ENV, as developed in the third part of this thesis.

1.4.5 Ethical Considerations

Animal Experimentation - Exploiting the Living

Animal experimentation is a delicate issue that is worth being considered because it brings up questions of ethics, conscience and responsibility. The ethical dimension of animal experimentation engages both a personal (daily-life) and a philosophical (ideological) level of acceptance and justifiability of our acting as researchers. The following text represents an extract of an exposé that has been elaborated during the optional interdisciplinary doctoral education unit "Applied Ethics and Responsibility in the Performance of Research" (GIERE - Groupe Inter-Universitaire d'Éthique de la Recherche, Universities of Grenoble) whose aim was to discuss the research projects of the participants in the light of deontological ethics.

Immediate confrontations, *applied ethics*. To find oneself actor of experiences whose primary visible effects - doing harm to a living creature - are destructive and deleterious provokes spontaneous reflexion and raises several questions: Are we ourselves entirely responsible for our actions and the actions of others that are under our influence? Does this responsibility towards the Living imply the duty to actively minimize any nocuous effect beyond the inevitable effects? On the other hand, do existing codes of behaviour that tackle technical questions restrain our personal responsibility in a way that might incite us to "hide behind the rules" in order to ease our conscience? The improvement of conditions, the optimisation of protocols, and a critical evaluation of experimental procedures concerning both the

immediate contact with the animal and the maximisation of scientific results are important issues to address, without regard to existing rules that should not be considered as ultimate or universal. Individual particularity and the uniqueness of every human being reveals on the intellectual, mental, moral and emotional level; this particularity can distinguish us from others and is not necessarily respected by a uniform behavioural codex.

Extended reflexions, *normative ethics*. Beyond the spontaneous level of reflexion, the use of animals for scientific experimentation evokes deeper questioning that requires justification in front of ourselves and in front of the society. The *anthropocentric* approach developed by Immanuel KANT¹ comprises individual responsibility of the human being on the level of our unique capacity of morality: the standards that we apply to our human societies must not be lowered by atrocities done to other living beings; considering animals as inferior to humans, it is though by principle that we are obliged to keep up morality in our behaviour. Furthermore, the *biocentric* view relieved by A. SCHWEITZER, G.M. TEUTSCH and T. REGAN¹ commits us to respect every living creature since the putative differences between humans and other living entities - based on arbitrary criteria like conscience or consciousness - can be considered as factually inexistent. The duty to “respect life” is of universal validity and does not require any justification or explication. In this context, a critical viewpoint should lead us to conscientious evaluation of the necessity to choose *in vivo* rather than *in vitro* models (f. ex., blood cell cultures or permanent cell lines can be used instead of experimental animals, most notably in preliminary studies). But, confronted with his global responsibility, the benefits of *in vivo* models for the researcher are not negligible: Aren’t we responsible also for the good that we do *not* do, for the bad that we do *not* fight? If we become aware of patients’ suffering and the absence of adequate treatment, aren’t we obliged to capitalise our knowledge to help, by all means that we dispose of? On the side of drawbacks, the general notion of “respect of life” as such represents a heavy weight that can be disburdened or weakened by the *pathocentric* idea (P. SINGER, M. St. DAWKINS¹) that is based on the more or less gradual differences of suffering capacities in animals. These differences can be balanced in order to “measure” the good and bad in animal experimentation, but this approach - that refers to a *utilitarian* point of view (L. BENTHAM¹) - is limited in its applicability because it implies the

¹Metzler Philosophie-Lexikon, Begriffe und Definitionen, PRECHTL P. und BURKARD F.-P. (Herausgeber) (1996)

judgement of animal suffering which is objectively impossible.

Personal questions. The central dilemma a researcher in biomedical research is confronted with is the question of his attitude towards animal experimentation, a question that is permanently present. It is difficult or even impossible to solve this problem in a definite way because the necessity of animal models should undergo critical evaluation each time anew, and even if the *in vivo* decision has been made for one particular case, daily confrontation rests a challenge. Uncomfortable reflexions of this kind might produce a separation of professional research reality and our private sphere in which a distinct scale of values would be applied. The creation of a fictitious “double life” is a phenomenon that can occur and “help” to avoid moral confrontation: the actions and decisions of our professional life would no longer have to agree with our conscience that dominates our private life, which means that they would not need to be justified any longer. But it is indispensable to address to ourselves the question whether the loss of sensibility is really preferable or not, or if it is not rather this sensibility that enables us to morally and ethically decide about the sense of our actions, and to behave conscientious in delicate situations. At this level, recommendations and laws on animal experimentation made by “specialists” can be dangerous because they represent frontiers of our personal and civic responsibility, and because they can be comfortable at first sight for a researcher that is caught in the dilemma, but they can also make him passive and blind for the real moral questions that he should stay aware of in each and every moment. In the same way I consider as dangerous the role of ethics committees because at present their judgement can have *consultative* value at best, and their evaluations and recommendations are never *decisive*; their existence might contribute to our tendency to refuse individual responsibility. After all, the daily exploitation of the Living and of natural resources in general is too often unconsidered and represents an abuse that we may too easily banish from our arrogant minds.

1.5 Thesis Objectives

The aim of the studies presented in this thesis was to create an appropriate animal model for MS that includes the MSR_V ENV protein in order to study its inflammatory properties. The *in vivo* model represents a dynamic system that takes into account the complexity of the physiological circumstances in MS and is necessary to understand the systemic implications of the different elements, including the immune system, the pathogenic factors and possible therapeutic agents.

After decades of MS research, the enigma of its aetiology remains unresolved. Even though several risk factors like viral infections, vaccination, genetic predisposition and diverse environmental factors like industrial pollution have been identified, mainstream MS research is concentrating on palliative strategies that do not tackle the origin of the problem and therefore have limited impact on the disease course.

Our approach to the MS problem is not a palliative but a curative one; it implicates the understanding of the fundamental processes that are at the origin of the MS pathology. Even though our ambition is clearly therapeutical, our work can be considered as fundamental research as well because basic information on the initial events in MS onset is still missing.

The project of this thesis consisted in three major parts. As the molecular and inflammatory properties of the MSR_V envelope protein have been established by our group in *in vitro* assays on the human system, the first step was to study the applicability of these previous findings on the murine system **(1)**. The second step was to induce Experimental Autoimmune Encephalomyelitis (EAE), the classical animal model of MS, by integrating ENV in the experimental protocol to study its properties as an adjuvant and its immunological actions **(2)**. In the continuity of this thesis, further studies on the effects of systemic presence of ENV and on the effects and the posology of innovative treatment methods are scheduled. Transgenic mice carrying ENV are about to be created, the target cells of its expression being brain astrocytes and antigen-presenting cells (APC). A transgenic mouse model introducing ENV into the mouse genome is necessary since homologous copies of HERV ENV proteins are present only in great apes and humans. The choice of the mouse strain utilised in the EAE induction experiments presented in this work reflects the necessity of applicability of the obtained results and the established methods on the future transgenic ENV mice, and the coherence of the experimental proceedings.

The preliminary studies preparing the creation of transgenic ENV mice including the steps of genetic vector construction and transfection into target cells were part of this thesis and form its third main part (**3**). (In further experiments, selected constructions will be used to derive transgenic mice for physiological studies and for *in vivo* assays. The pathophysiological consequences will be evaluated by analysis of the immune components (inflammation, lymphocyte functions) and analysis of the behaviour of the animals.)

The work was focused on the following points:

(1) Inflammatory properties of ENV-SU

- Studies on human cells *in vitro*: reproducibility of the previously established cellular activation by ENV
- Study of transposability on mouse cells *in vitro*: dendritic cell maturation
- Reproduction of the neutralizing properties of monoclonal anti-ENV antibodies
- *In vivo* systemic effects of ENV-SU on the murine organism

(2) The *in vivo* model: murine EAE

- Modification and adaptation of the classical EAE induction protocol using ENV as adjuvant
- Classification and comparison of EAE symptoms caused by the classical and the adapted induction method
- Neuropathophysiological studies on diseased mice:
 - Detection of autoreactive T cells
 - Magnetic Resonance Imaging (MRI) of brain and spine

(3) Transgenic ENV mice

The transgenic mice we want to create will allow for the expression of the envelope proteins MSR_V-ENV and HERV7_q-ENV under the control of the MHC-II promoter to target its expression in immune cells such as APCs including DCs, and under the control of GFAP (glial fibrillary acidic protein) to drive its expression in astroglial cells of the nervous system, which are precisely the cells expressing ENV in humans. These genetic constructions have been transfected in culture cells for *in vitro* assays and for functional validation.

Chapter 2

Introduction

2.1 Le système immunitaire

Le système immunitaire mammifère se compose d'une variété d'acteurs et de niveaux d'organisation différents qui collaborent pour éliminer des agents étrangers comme les bactéries, les virus et les champignons, ainsi que des structures endogènes modifiées ou mutées comme les cellules tumorales. Il comprend une réponse cellulaire et une réponse humorale. Deux composantes principales peuvent être distinguées : l'immunité innée qui représente la première barrière non-spécifique, et l'immunité adaptative qui est capable de reconnaître des pathogènes dangereux d'une manière spécifique et de mémoriser le contact avec ces pathogènes.

Les acteurs des systèmes immunitaires inné et adaptatif partagent un grand nombre de molécules différentes appelées cytokines pour communiquer entre eux. Une grande variété de types de cellules immunitaires peut produire et répondre à des cytokines ; par ce moyen, ils sont capables de polariser la réponse immunitaire vers une inflammation ou d'atténuer sa réactivité. Toutes les cellules immunitaires proviennent de cellules souches hématopoïétiques omnipotentes (HSCs, haematopoietic stem cells) qui sont localisées dans la moelle osseuse. Ces HSCs donnent naissances aux précurseurs de deux lignées différentes : les lignées myéloïde et lymphoïde.

Les cellules de la lignée myéloïde peuvent se différencier en plaquettes, monocytes/macrophages, granulocytes (neutrophiles, éosinophiles et basophiles) et cellules dendritiques d'origine myéloïde ; les cellules d'origine lymphoïde continuent

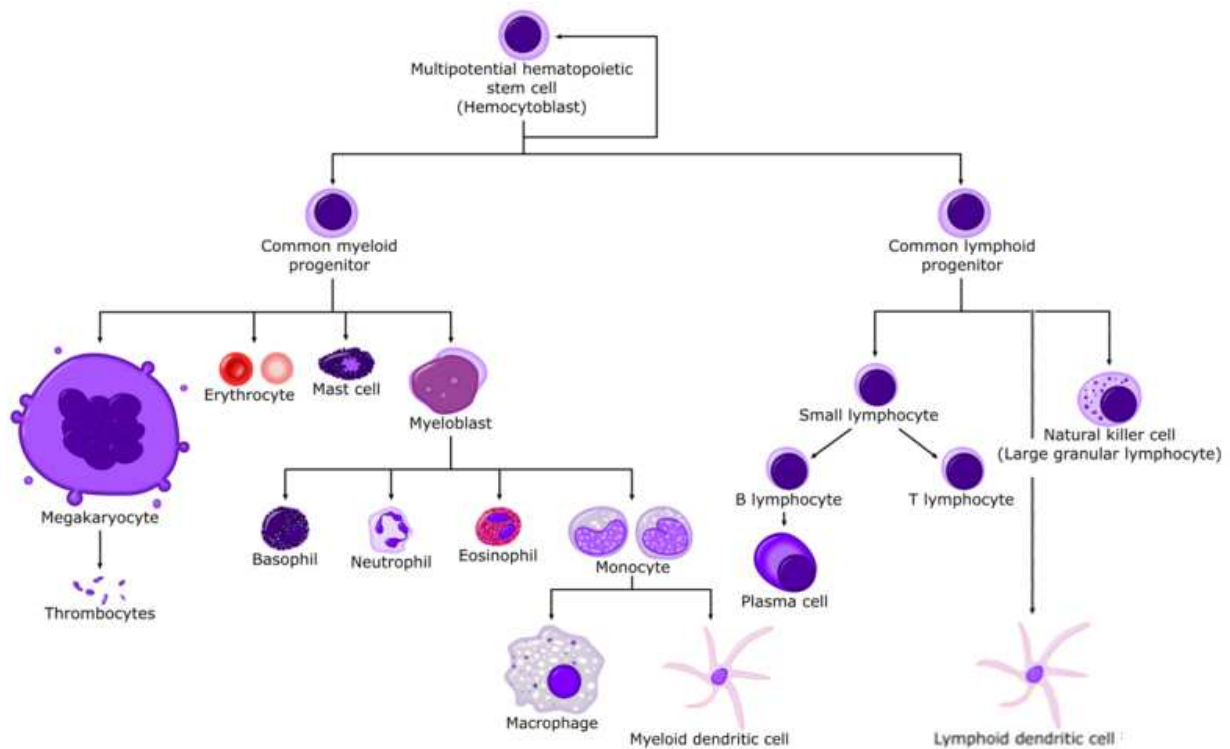


Figure 2.1: *Hématopoïèse chez l'Humain.*

leur différenciation et maturation dans les organes lymphatiques primaires (dans la moelle osseuse pour devenir des cellules B et dans le thymus pour devenir des cellules T) avant d'entrer dans la circulation sanguine pour atteindre les organes lymphatiques secondaires (rate, ganglions lymphatiques).

2.1.1 L'immunité innée

L'immunité innée constitue la première barrière non-spécifique empêchant une intrusion externe. Elle comprend le système du complément et divers types de cellules spécialisées comme les macrophages, mastocytes, lymphocytes NK (cellules tueuses naturelles), granulocytes et cellules dendritiques.

Le système du complément se constitue de protéines plasmatiques qui peuvent induire une cascade biochimique afin d'assigner à d'autres cellules des pathogènes en les opsonisant, ou de les perforer et de les lyser directement. Ces deux éléments contribuent à la première vague de clairance immédiatement après contact avec le

pathogène. La réaction inflammatoire causée par les médiateurs de cette première barrière implique également la sécrétion de cytokines et le recrutement des acteurs du système immunitaire adaptatif par la présentation d'antigènes capturés.

Les cellules dendritiques

Les cellules dendritiques (DC) comme d'autres cellules phagocytiques peuvent capturer des pathogènes par endocytose et présenter des fragments peptidiques digérés par le système du CMH-II (complexe majeur d'histocompatibilité). Le CMH-II est un complexe de molécules qui est exprimé par toutes les cellules présentatrices d'antigènes (CPA) qui sont majoritairement lymphocytes B, monocytes/macrophages et cellules dendritiques ; il interagit avec la molécule CD4 exprimée par la sous-population CD4⁺ de lymphocytes T (nommés cellules T helper). Ces « CPA professionnelles » portant le CMH-II possèdent également des molécules accessoires qui facilitent le contact avec les lymphocytes T CD4⁺ ; d'autres types de cellules peuvent exprimer le CMH-II occasionnellement et devenir temporairement des CPA, comme les cellules gliales du cerveau, les cellules épithéliales du thymus ou les cellules de l'endothélium vasculaire.

La fonction du CMH-II est de présenter des fragments dégradés de biomolécules (antigènes exogènes) entrées à l'intérieur de la cellule par voie d'endocytose. Cependant, les molécules CMH du type I sont exprimées sur toutes les cellules somatiques. Elles permettent aux cellules infectées ou endommagées de présenter des fragments moléculaires antigéniques du pathogène ou des produits de dégradation de leur propre métabolisme libérés par le protéasome (antigènes endogènes) afin d'induire le recrutement des lymphocytes T CD8⁺ (appelés cellules T killer).

Chez les DC matures et activées, l'expression des molécules CMH-II est augmentée, ce qui en fait de très puissantes CPA. Elles disposent également d'un nombre important de molécules co-stimulatrices T et sont capables de produire des cytokines inflammatoires qui induisent et polarisent la réponse T. Elles peuvent donc être considérées comme interface entre l'immunité innée et l'immunité adaptative. La présentation d'antigènes aux cellules T par le CMH-II génère des sous-populations distinctes de cellules T qui reconnaîtront et mémoriseront l'antigène et initieront une réponse concertée du système immunitaire adaptatif.

Les DC peuvent provenir de la lignée myéloïde ou de la lignée lymphoïde. Après

avoir quitté la moelle osseuse, elles peuvent se différencier et atteindre la maturité cellulaire sous l'influence de chémokines et de cytokines, selon leur environnement. Les DC d'origine myéloïde (MDC) peuvent se différencier en cellules de Langerhans de la peau et en cellules interstitielles présentes dans la plupart des tissus ; les DC d'origine lymphoïde peuvent migrer dans les organes lymphatiques secondaires pour devenir des DC plasmacytoïdes (PDC) dotées de propriétés antivirales. Pour des études *in vitro*, DC humaines peuvent être dérivées de monocytes du sang circulant à l'aide de cytokines et de facteurs de croissance (MDDC – monocyte-derived DC) mais sont difficiles à obtenir et à manipuler à cause des faibles quantités de monocytes disponibles ; pour créer des cultures de DC murines, monocytes dérivés de moelle osseuse de fémur peuvent être différenciés en DC (BMDC – bone marrow-derived DC) et cultivés afin d'obtenir des quantités abondantes.

Les récepteurs Toll-like

Les récepteurs Toll-like (TLR) appartiennent à la famille très conservée des PRR (pattern recognition receptors) et sont exprimés à la surface des CPA et d'autres cellules. Chez l'Homme, 13 différents TLR (nommés de TLR1 à TLR13) ont été identifiés ; à l'exception de TLR10, des homologues à tous les autres TLR sont connus également en souris. Les TLR reconnaissent une variété de structures microbiennes qui sont conservées entre beaucoup de pathogènes (voir figure 2.2) mais qui ne sont pas présentes dans l'hôte.

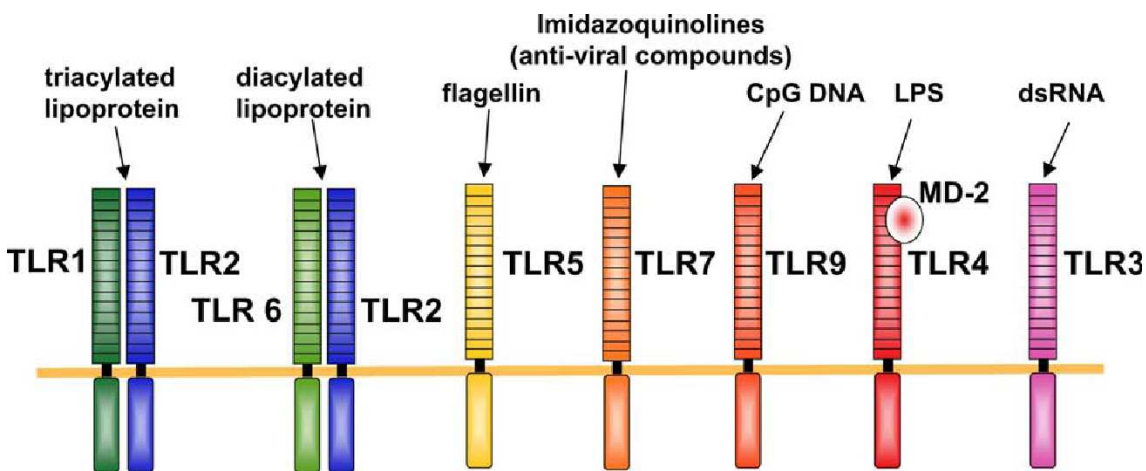


Figure 2.2: Les TLR et leurs ligands [113].

Une de ces structures moléculaires associées aux pathogènes (PAMP – pattern-associated molecular pattern) reconnues par les TLR est l'endotoxine bactérienne LPS (lipopolysaccharide) qui est le composant majeur de la membrane extérieure des bactéries gram-négatives. Elle peut interagir avec le récepteur TLR4 et stimuler les cellules exprimant TLR4, par exemple les DC, à produire des cytokines inflammatoires comme l'interleukine-6 (IL-6) ou l'interleukine-12 (IL-12) et à subir des changements phénotypiques qui reflètent la maturation et l'activation des DC, par exemple l'augmentation de l'expression du CMH-II à la surface cellulaire, et ainsi intensifier l'interaction avec les cellules T.

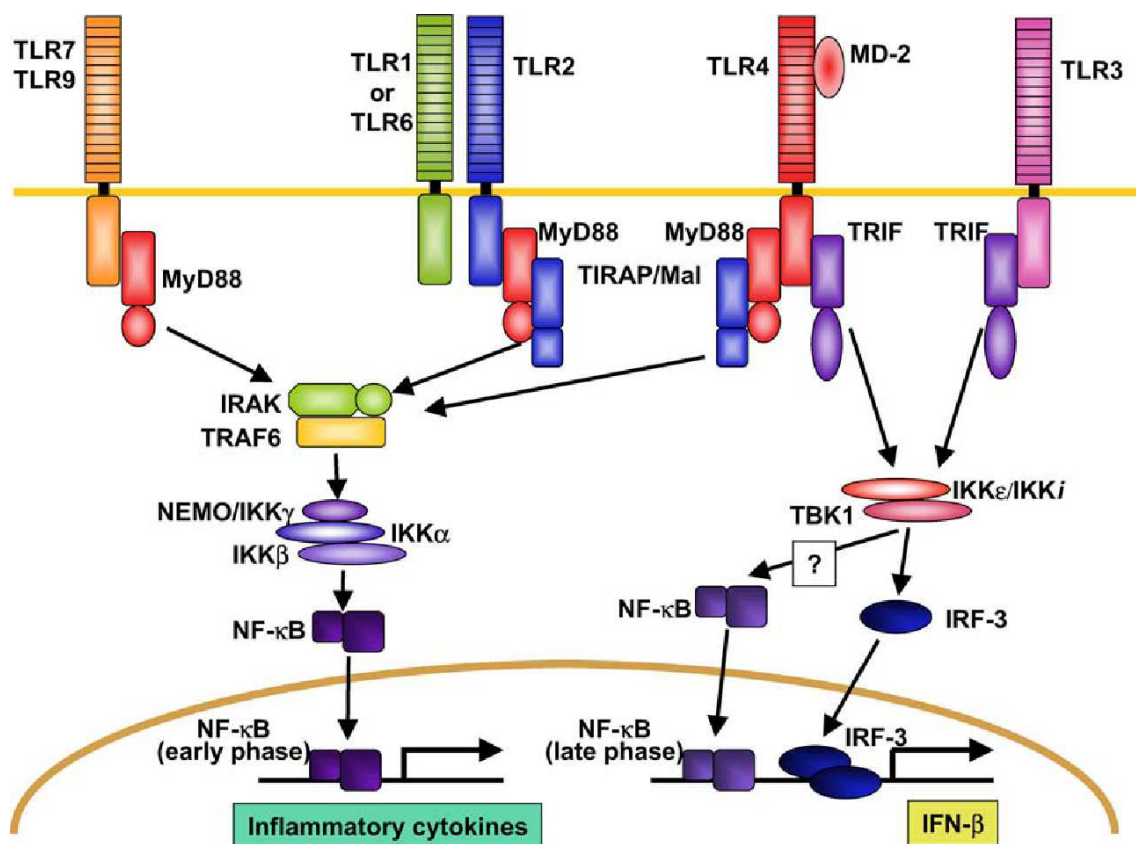


Figure 2.3: Les TLR et leurs voies de signalisation [113].

Les différents TLR partagent en tout quatre molécules adaptatrices qui sont MyD88, TIRAP, TRIF et TRAM. Ces molécules localisées à l'intérieur de la cellule font partie de la cascade de signalisation « downstream » qui peut mener à l'activation de différents gènes. Pour interagir avec les cellules T et induire une réponse du système immunitaire adaptatif, la voie de signalisation TLR4 activée par le LPS requiert la molécule co-stimulatrice CD14 qui est co-localisée avec TLR4

à la surface de la cellule.

2.1.2 L'immunité adaptative

Contrairement au système immunitaire inné, le système immunitaire adaptatif est capable de reconnaître des antigènes de façon spécifique, de les combattre d'une manière focalisée et concertée, et de mémoriser spécifiquement les événements immunologiques précédents. Ses cellules effectrices sont principalement les lymphocytes T et B. Les lymphocytes T peuvent reconnaître un nombre important d'épitopes antigéniques présentés soit par les CPA via le CMH-II (cellules T helper CD4⁺) soit par des cellules somatiques non-immunitaires via le CMH I (cellules T killer CD8⁺). La variabilité des épitopes que les lymphocytes T peuvent reconnaître spécifiquement est le résultat d'un processus de recombinaison génétique qui a lieu dans le thymus et qui donne naissance à un large répertoire de récepteurs T (TCR). Avec CD4 et CD8 comme molécules accessoires (non-spécifiques) de reconnaissance, chaque TCR reconnaît un épitope particulier. Les lymphocytes T stimulés par leur épitope antigénique respectif vont répondre avec une réaction immunitaire forte qui impliquera la production de cytokines, l'activation de lymphocytes B et la mémorisation de l'antigène rencontré.

Le rôle principal des lymphocytes B consiste en la production d'anticorps qui sont spécifiques aux antigènes ; la défense immunitaire via les anticorps fait partie de la réponse immunitaire humorale. Les anticorps sécrétés par les cellules B peuvent entrer en liaison avec leurs antigènes respectifs et ainsi former un complexe appelé complexe antigène-anticorps ou complexe immun ; ce précipité immun peut être dégradé par les protéases ou éliminé par les phagocytes. Contrairement aux cellules T, les cellules B sont capables de reconnaître des antigènes solubles, c'est-à-dire qui ne doivent pas nécessairement être présentés à la surface d'une autre cellule. Les antigènes phagocytés peuvent être procédés et exposés sur leur surface via le CMH-II ; le contact avec une cellule T qui a été « primée » au même antigène induit la sécrétion de cytokines par la cellule T, celle-ci activant et incitant la cellule B à proliférer, à produire des antigènes spécifiques et à générer des cellules B mémoire. Indépendamment de cette activation dépendante des cellules T, les cellules B peuvent être également activées par une stimulation de leurs récepteurs PRR ou par d'autres CPA qui présentent le même antigène.

2.1.3 Les cytokines

Les différents types de cellules du système immunitaire communiquent entre eux à l'aide de molécules appelées cytokines. Les cytokines dirigent la réponse immunitaire soit vers une réponse cellulaire soit vers une réponse humorale, ce qui veut dire qu'elles polarisent la réponse soit vers une inflammation médiée par les cellules Th1 helper (réponse Th1), soit vers la production d'anticorps médiée par les cellules Th2 helper qui coordonnent l'activation des cellules B (réponse Th2). Il y a une troisième possibilité de réponse qui peut être appelée réponse Th17 et qui induit un type de cellules T appelé cellules Th17 helper [37, 38, 119]; les cellules Th17 sont capables de réguler une réponse immunitaire en cours, et leur fonction est probablement impliquée dans les pathologies chroniques et les maladies auto-immunes [80].

Les lymphocytes T sont polarisés vers des réponses de type Th1, Th2 ou Th17 par les cytokines secrétées par eux-mêmes ou par d'autres cellules de leur environnement ; en réaction à ces cytokines, ils se différencient, subissent des changements phénotypiques et produisent des cytokines afin de propager le type de réponse respectif. Les cellules Th1 secrètent des cytokines Th1 comme l'IL-2, l'interferon-beta (IFN- β) et le transforming growth factor-beta (TGF- β) afin d'activer les macrophages, l'IL-3 et le granulocyte-macrophage colony-stimulating factor (GM-CSF) pour activer la leucogenèse par la moelle osseuse, ou l'interferon-gamma (IFN- γ) pour inhiber la réponse Th2. Les cytokines typiques Th2 sont l'IL-4, l'IL-5, l'IL-6 et l'IL-10 qui stimulent la production d'anticorps par les cellules B ; l'IL-10 peut également inhiber la sécrétion d'IL-2 et d'IFN- γ par les cellules Th1. La réponse Th17 est médiée par les cellules Th17 qui secrètent de l'IL-17 et l'IL-21 ; la réponse immunitaire peut être polarisée vers Th17 sous l'influence de TGF- β , d'IL-6 et d'IL-23 ; les lymphocytes Th17 sont régulés négativement par l'IL-4 et l'IFN- γ .

Une quatrième population de lymphocytes T a été identifiée récemment : les cellules CD8⁺CD4⁺CD25⁺ régulatrices (Treg) qui expriment le facteur de transcription FOXP3 et qui régulent les fonctions immunitaires via les cytokines TGF- β et IL-10. L'implication des Treg dans la tolérance immunitaire systémique a été démontrée [4, 30] et leur nombre est diminué dans les maladies auto-immunes [116, 114].

Pour une vue d'ensemble des cytokines les plus importantes, leurs cellules cibles et leurs fonctions biologiques, voir annexe D.

2.1.4 Les maladies auto-immunes

Les réactions immunitaires contre des auto-antigènes sont dirigées par des cellules T autoréactives qui ont été « primées » à la reconnaissance de l'antigène en question comme *étranger* ou *dangereux*. Pendant la sélection clonale de lymphocytes T dans le thymus, une sélection positive des cellules répondant aux antigènes du *non-soi* et une sélection négative de celles répondant aux antigènes du *soi* a lieu afin d'éviter une autoréactivité et pour induire une auto-tolérance ; les cellules T autoréactives sont des cellules qui ont échappé à ce processus de sélection et de ce fait représentent une anomalie du système.

Le « *danger model* » décrit par Polly MATZINGER qui va au-delà du modèle conventionnel de discrimination *soi-non-soi* (SNS), nous apprend qu'une molécule endogène peut être présentée à une CPA comme *dangereuse* quand elle est présentée dans un contexte d'inflammation aiguë libérant des antigènes « cachés » qui ne sont pas encore *connus* au système immunitaire et ainsi provoquent un rejet [67] ; une autre possibilité est que le pathogène, qui serait reconnu comme *étranger*, peut se servir de mimétisme moléculaire pour « primer » des cellules T à reconnaître un auto-antigène à structure ressemblante [78]. Dans les deux cas, le résultat serait la rupture de la tolérance envers l'antigène.

Dans le cas du Diabète de type I, les cellules Langerhans productrices d'insuline sont éliminées pendant l'embryogenèse et le début de l'enfance par des cellules T autoréactives ; de la même manière, la perte gliale et neuronale dans la Sclérose en Plaques (SEP) est causée par une réaction inflammatoire chronique auto-immune dirigée contre des composants de la gaine de myéline ; ce qui reste incompris est le fait que le début des symptômes de la SEP se situe en général entre la fin de l'adolescence et l'âge adulte, ce qui ne peut pas être expliquée uniquement par une dérégulation génétique de la sélection lymphocytaire T. Selon le « *danger model* », un évènement inflammatoire fort dans le système nerveux central (SNC) pourrait libérer des antigènes myéliniques qui ne sont pas habituellement exposés aux CPA et qui par conséquent avaient échappés à la sélection immunitaire (positive ou négative) ; l'exposition dans un tissu inflammé créerait une auto-immunité, et la localisation du centre de l'inflammation déciderait de la nature de l'antigène cible libéré [67]. Il reste à élucider si le « priming » des cellules T autoréactives peut avoir lieu dans le SNC ; dans tous les cas, la rupture de la barrière hémato-encéphalique (BHE) est nécessaire pour permettre aux lymphocytes d'envahir massivement le cerveau et de causer des dégâts tissulaires. Le contact avec l'antigène se passe plus probablement

dans la périphérie, présumant une rupture de la BHE au moins temporaire qui mènerait à l'exposition périphérique de l'antigène.

Le lien entre les facteurs environnementaux, l'immunité innée et l'auto-immunité est discuté à travers de nombreux articles [106, 97, 109, 103, 93]

2.2 La Sclérose en Plaques

La Sclérose en Plaques (SEP) est une maladie chronique inflammatoire démyélinisante du système nerveux central dans laquelle l'infiltration de macrophages activés et de lymphocytes T autoréactifs et à l'origine de pertes gliales et neuronales [110] ; la matière blanche et du cerveau et de la moelle épinière est affectée. La démyélinisation relève de l'endommagement des oligodendrocytes producteurs de myéline qui sont un type de cellules gliales qui recouvrent et maintiennent les axones neuronales dans le SNC et leur permettent une vitesse de transmission supérieure à celle des axones naturellement non-myélinisés.

La localisation de l'inflammation, déterminant quelle partie du cerveau est endommagée, engendre différents types de symptômes. Les symptômes initiaux les plus fréquents sont des troubles de la vue, des changements sensoriels (*hypoesthésie*), et des déficits moteurs comme la faiblesse et les problèmes d'équilibre. Dans les phases suivantes, d'autres symptômes apparaissent : spasmes musculaires, difficultés motrices et de coordination (*ataxie*), problèmes du langage (*dysarthrie*) ou de déglutition (*dysphagie*), troubles visuels comme le *nystagmus*, perte partielle ou totale de vue (*névrite optique*) vision dédoublée (*diplopie*), fatigue, douleurs aiguës ou chroniques, troubles génito-sphinctériens, atteintes cognitives et symptomatologie émotionnelle (dépression, ...). L'échelle clinique de la progression de l'invalidité et de la gravité des symptômes est l'EDSS (Expanded Disability Status Scale). Il y a plusieurs formes cliniques de la SEP qui présentent différents profils pathologiques évolutifs : *relapsing-remitting* (RRMS), *secondary progressive* (SPMS), *primary progressive* (PPMS) et *progressive relapsing* (PRMS) ; en fonction du diagnostic d'un de ces sous-types respectifs, le pronostic et la médication seront adaptés.

Comme d'autres maladies auto-immunes, la SEP est plus fréquente chez les femmes que chez les hommes ; sa prévalence globale se situe entre 2 et 150 sur 100.000, dépendant de la zone géographique. Les zones de prévalence importante sont par exemple les régions industrialisées de l'Europe, du Canada et des Etats-Unis ; la prévalence est sensiblement réduite dans la zone méditerranéenne et au Mexique ; de très rares cas ont été rapportés de l'Afrique sub-saharienne et du Japon.

2.2.1 Étiologie et Diagnostic

La SEP est une maladie multifactorielle. À cause des valeurs différentes de prévalence et de la distribution inégale des cas de SEP même à l'intérieur d'un continent ou d'un pays, des facteurs environnementaux comme la pollution, les infections bactériennes et virales ont été évoquées. Il y a des indices mettant en lumière l'influence d'infections virales pendant et la grossesse de la mère l'enfance. Une hypothèse suggère l'implication d'une infection virale dans le déclenchement de la phase inflammatoire initiale. Selon le *danger model* [67], des antigènes myéliniques endogènes pourraient être libérés par une inflammation locale aiguë, internalisés par les DC locales et présentés aux cellules T qui deviendraient réactives envers l'auto-antigène myélinique. Une autre possibilité est la transactivation de super-antigènes viraux endogènes par des rétrovirus ayant une activité infectieuse comme le virus d'*Epstein-Barr* (EBV) [111]. De la même manière, les virus de la famille des herpesvirus peuvent activer des séquences génomiques intégrées d'origine virale en interagissant avec leurs séquences LTR (long terminal repeat - séquence terminale longue répétée), ce qui mènerait potentiellement à la production de particules virales qui causeraient une inflammation chronique comme on l'observe dans les maladies auto-immunes comme la SEP [52] ; une inflammation chronique localisée dans les zones d'expression virale au niveau du cerveau et de la moelle épinière libérerait d'une manière permanente des auto-antigènes qui alimenteraient la réponse auto-immune envers l'antigène libéré.

Par ailleurs, l'implication génétique est considérée comme importante ; ainsi la concordance entre jumeaux monozygotes est considérablement élevée comparée à celle entre jumeaux dizygotes (20-40% contre 5%) ; néanmoins, une concordance différentielle de jumeaux liée à la latitude du lieu de naissance a été évoquée et pointerait un lien entre les facteurs génétiques et environnementaux. Des études sur les *single nucleotide polymorphisms* (SNP) ont montré que les gènes codant pour l'antigène des leucocytes humains (HLA – human leukocyte antigen) ou le complexe CMH situés sur le chromosome 6 aussi bien que ceux codant pour les récepteurs d'IL-2 et d'IL-7 sont impliqués dans la prédisposition génétique de la SEP et d'autres maladies auto-immunes [112, 1]. En outre, des populations isolées géographiquement ou socialement, par exemple les habitants de Sardaigne, ont un taux de prévalence considérablement élevé par rapport à la moyenne [95].

La prédisposition génétique pour la SEP est difficilement identifiable parce qu'il existe une grande variabilité dans les gènes à risque dans la population normale.

Quand les symptômes physiques et neurologiques indiquent une SEP, un diagnostic plus profond sera effectué plutôt par IRM (imagerie par résonance magnétique) ou par SRM (spectroscopie à résonance magnétique) pour visualiser d'éventuelles anomalies au niveau du cerveau et de la moelle épinière ; les plaques démyélinisantes typiques de la SEP sont détectables mais peuvent être confondues avec des plaques venant de différentes autres pathologies démyélinisantes, ce pourquoi des méthodes complémentaires sont requises pour confirmer le diagnostic. La présence de bandes oligoclonales dans le liquide céphalo-rachidien (CSF – cerebrospinal fluid) prélevé par ponction lombaire est un critère fiable dans la plupart des cas de la SEP ; les bandes oligoclonales relèvent de quantités importantes d'immunoglobulines qui reflètent une inflammation du cerveau ou de la moelle épinière causée par la SEP ou d'autres atteintes du SNC d'origine infectieuse. Une autre méthode est la mesure de la vitesse de conduction neuronale dans les neurones optiques et sensoriels par les potentiels évoqués visuels (VEP - visual evoked potentials) et les potentiels évoqués somato-sensoriels (SEP - somatosensory evoked potentials) ; à cause d'endommagements gliales, la vitesse de transmission est réduite chez les malades de la SEP. En général, l'usage combiné de ces méthodes permet un diagnostic de la SEP plus fiable qu'avec l'emploi d'une seule qui mène souvent à des résultats ambigus.

En plus de ces méthodes conventionnelles, des approches novatrices sont étudiées. Une de ces méthodes a été développée par une équipe avec laquelle nous sommes en collaboration ; elle cible l'expression des séquences rétrovirales endogènes qui est élevée chez les patients de la SEP [23, 88] et dont le taux semble être corrélé avec l'évolution de la maladie.

2.2.2 Physiopathologie

L'inflammation chronique que l'on observe dans la SEP a été mise en évidence par la détection de taux élevés d'anticorps anti-myéliniques et de lymphocytes Th1 auto-réactifs ; il est appuyé par l'invasion massive de lymphocytes B d'origine sanguine à travers la BHE dans les espaces péri-vasculaires et le parenchyme cérébral. Dans le cerveau sain, la BHE empêche la perméation de cellules immunitaires, à l'exception d'un nombre limité de cellules qui sont capables de transmigrer et qui constituent la *surveillance immunitaire* [9, 40]. Dans la SEP, le *trafic leucocytaire* est significativement élevé et les plaques inflammatoires péri-vasculaires génèrent des plaques démyélinisantes. L'adhésion cellulaire élevée dans les capillaires de la BHE permet

l'entrée de lymphocytes T et B, plasmocytes, macrophages activés et microglie qui peuvent libérer des cytokines et des molécules toxiques et ainsi endommager le tissu [16].

La présence de DC dans le SNC a été démontrée [66] ; elles pourraient jouer un rôle dans les processus d'inflammation et d'auto-immunité [68]. Étant des CPA puissantes, elles sont supposées initier la formation de lymphocytes T encéphalitogènes. Leur participation dans l'invasion immunitaire dans un modèle animal de la SEP a été démontrée chez la souris [35].

Le cerveau possède une capacité limitée à renouveler les gaines de myéline endommagées dans la première phase de la maladie, même si les couches « réparées » d'oligodendrocytes sont en majorité plus fines que les couches initiales ; à ce stade, l'endommagement reste réversible et les patients peuvent connaître des phases asymptomatiques. Dans les stades avancés de la SEP, des endommagements axonaux apparaissent, et les manifestations neuropathologiques sont irréversibles et mènent à la perte graduelle des fonctions motrices et sensorielles.

2.2.3 Traitement

Le traitement conventionnel de la SEP est limité aux approches palliatives qui peuvent atténuer la progression de la maladie et alléger les symptômes. Les stéroïdes sont utilisés pour restreindre l'inflammation aiguë pendant les poussées. Pour contrôler l'inflammation chronique qui cause les poussées, des modulateurs ou supprimeurs de la fonction immunitaire peuvent être appliqués ; ainsi la cytokine IFN- β peut limiter la progression de la maladie et la fréquence des poussées ; malheureusement, beaucoup de patients ne sont pas capables d'endurer le traitement à l'IFN- β à cause de ses effets indésirables comme les réactions inflammatoires du site d'injection, la fatigue extrême, la fièvre et la dépression. En général, le traitement aux immunomodulateurs et immunosuppresseurs peut être difficile à supporter à cause de leurs effets systémiques.

Des approches innovatrices de traitement existent mais ne sont pas encore pleinement développées, ce pourquoi aucun traitement alternatif n'est disponible à ce jour. Elles sont basées sur la découverte de nouveaux aspects fondamentaux de l'étiologie de la SEP, et l'idée d'être capable de lutter contre la cause plutôt que les symptômes est prometteuse. Suivant l'hypothèse d'expression de rétrovirus endogènes et leur

2. INTRODUCTION

implication dans les maladies auto-immunes comme la SEP, la neutralisation de ces particules rétrovirales libérées de manière permanente pourrait avoir un intérêt.

2.3 Les rétrovirus endogènes

Les rétrovirus endogènes sont des séquences génomiques qui proviennent d'infections virales pendant lesquelles un provirus (soit l'ADN d'un virus) a été intégrée dans le génome de l'hôte ; si l'hôte survit à l'infection, le virus intégré peut être transmis à sa descendance s'il a été incorporé dans l'ADN des cellules germinales.

2.3.1 Les HERV

Les rétrovirus endogènes humains (HERV – human endogenous retroviruses) représentent 8% du génome humain ; ces séquences rétrovirales ancestrales sont supposées être entrées dans la ligne germinale il y a environ 25 millions d'années, à la divergence des hominidés et des primates de l'Ancien monde [118]. La famille HERV-W est une famille d'HERV endogène à copies multiples qui comprend à peu près 1% du génome humain et qui est présente chez les grands singes et chez l'Homme [11]. Comme les rétrovirus exogènes infectieux, les homologues intégrés portent les séquences *gag* (codant pour des protéines structurales), *pol* (enzymes virales) et *env* (protéines de la surface de l'enveloppe) ainsi que les LTR qui les entourent. Les gènes *gag*, *pol* et *env* sont souvent trouvés mutés parce qu'ils n'ont plus de fonction biologique et ne sont pas vitaux pour l'organisme de l'hôte ; néanmoins, quelques séquences ont gardé des ORF (open reading frame – phase ouverte de lecture) intactes, c'est-à-dire sont restées fonctionnelles et pourraient donc être utiles à l'hôte [48, 54, 69, 89, 74].

L'implication de l'expression des HERV dans les maladies humaines (voir figure 2.4) comme la SEP, le Diabète et autres maladies auto-immunes, ainsi que dans les syndromes neuropathologiques comme la Schizophrénie et les troubles bipolaires a été affirmée [121, 49, 7, 22]. De plus, la présence d'un facteur gliotoxique en combinaison avec une activité de reverse transcriptase dans le liquide céphalo-rachidien et les monocytes/macrophages de patients a été attribuée à l'expression de la séquence MSR-V, homologue de HERV, qui s'avère corrélée avec les poussées de symptômes [72, 71, 70, 73].

Il y a des indices pour une expression physiologique des protéines GAG, POL et ENV de la famille des HERV-W qui est altérée de façon physiopathologique dans la SEP [88, 76]. Les glycoprotéines d'enveloppe Enverin [2] et Synytin-1 de la famille HERV-W sont physiologiquement exprimées et impliquées dans la fusion

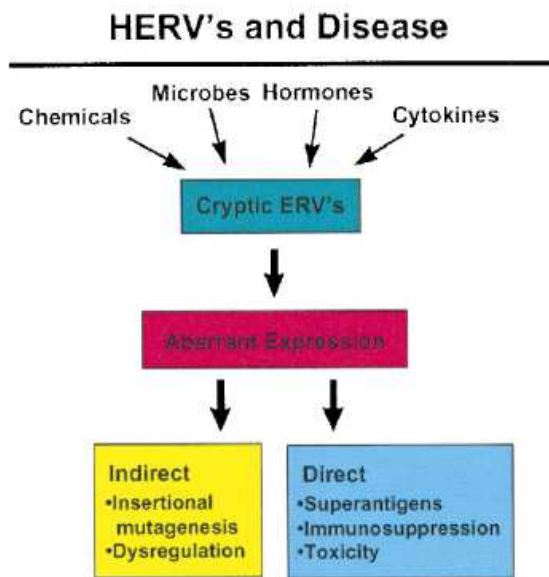


Figure 2.4: Mécanismes théoriques par lesquels les rétrovirus endogènes peuvent causer des maladies chez l'homme et l'animal [123].

du syncytiotrophoblast et dans le développement du placenta ; dans le cerveau de patients de SEP, l'expression élevée de Syncytin-1 est impliquée dans le processus de démyélinisation [6], intervient dans la cascade inflammatoire et altère la fonction des astrocytes [5].

(Pour une synthèse plus complète des données renforçant le lien entre les HERV et les maladies (auto-immunes) humaines, voir les articles « review » de GRIFFITHS [36], PORTIS [94], DOLEI [22], PERRON et al. [90] et PERRON & LANG [91] ; pour le projet de base de données HERV, voir PACES et al. [81].)

2.3.2 MSRVS

Le *Multiple Sclerosis-associated RetroViral element* (MSRV) est un virus qui a été isolé à plusieurs reprises du liquide céphalo-rachidien (CSF) de patients de SEP [85]. Des particules virales extracellulaires ont été observées dans des cultures de monocytes, de cellules leptoméningées et de PBMC (peripheral blood mononuclear cells) de ces patients [105] ; les particules isolées étaient capables d'infecter des cultures saines. En conséquence, le MSRV pourrait être considéré comme un virus exogène infectieux, même s'il ne répond pas au critère de transmission spontanée.

La séquence ARN de MSRV est homologue à une séquence appartenant à la

famille HERV-W qui se situe sur le chromosome 7 [89] (d'autres séquences homologues ont été détectées sur le chromosome 14 [3] et sur le gonosome X [48, 55]) ; autrement dit, la famille HERV-W a été décrite dans le but d'une classification moléculaire de MSR/V [11]. Même si la séquence HERV-W-7q n'est pas entièrement fonctionnelle au niveau transcriptionnel [89], MSR/V pourrait être la forme exogène d'un provirus compétent à la reproduction et producteur de virions. Il a été démontré qu'une transactivation de rétrovirus endogènes de la famille HERV-W peut être initiée par une infection au *virus de grippe* et au *HSV-1* (*herpes simplex virus*) [101, 75].

Il reste à faire la lumière sur un éventuel lien de causalité entre l'activation transcriptionnelle, l'apparition de particules MSR/V et les symptômes de la SEP ; la production de virions pourrait être à l'origine de l'inflammation cérébro-spinale aussi bien qu'elle pourrait en être l'effet secondaire, considérant que la démyélinisation et les dommages axonaux pourraient mener à l'expression d'ARN du MSR/V. Néanmoins, l'expression d'ARN rétrovirale [33, 108, 104] (et le nombre de leurs copies [61]) et la présence de particules virales [8] sont sensiblement corrélées à la SEP et pourraient, de ce fait, représenter un outil diagnostique [124, 107, 62].

Tous les membres de la famille HERV-W ne possèdent pas des ORF intacts, mais en revanche, un promoteur fonctionnel et une ORF probablement transcriptionnelle ont été identifiés pour la protéine d'enveloppe (ENV) [11]. Les gènes *gag*, *pol* et *env* de MSR/V sont apparentés aux oncovirus de type C et D, et leurs séquences ont été clonées [50]. Pour simplifier, l'abréviation « ENV » sera utilisée ici pour désigner la protéine d'enveloppe ENV du MSR/V qui est l'homologue de la protéine d'enveloppe HERV-ENV.

La protéine d'enveloppe ENV du MSR/V est composée de trois domaines principaux : l'ectodomaine (EC) qui correspond à la composante extracellulaire, l'unité transmembranaire (TM) et le domaine intracytoplasmique (IC) (voir figure 2.5). Notre intérêt se focalise sur l'ENV et plus particulièrement sur son domaine EC ; les interactions entre pathogènes et cellules hôtes en générale sont supposées être médiées par leurs composantes extracellulaires qui sont exposées à leur environnement et constituent le premier point de contact entre cellule hôte et pathogène.

Des virions MSR/V ont été isolés plusieurs fois de cultures de cellules B de patients de SEP [86, 85, 26] et sont capables d'infecter des cultures saines [87] ; ces particules virales ainsi que l'ENV recombinante produite en *Escherichia coli* présentent

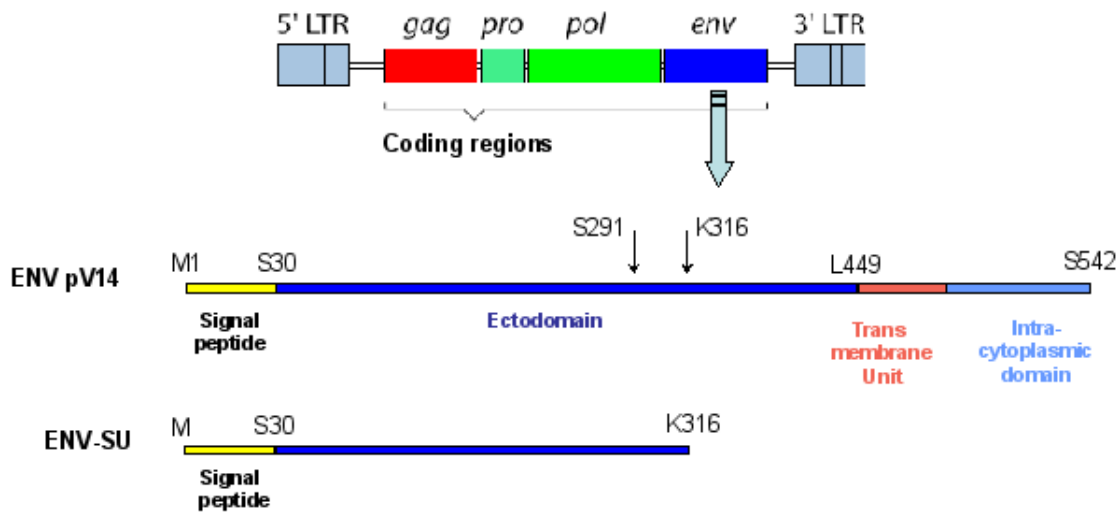


Figure 2.5: *Caractérisation moléculaire de la protéine ENV de MSRV. Le plasmide pV14 a été créé à partir d'ARNm de MSRV et inclut un peptide signal et trois domaines principaux : extracellulaire, transmembranaire et intracytoplasmique. ENV-SU est une fraction de 293 acides aminés qui représente l'unité extracellulaire soluble clivée à K316 de la protéine entière ENV de pV14. Les deux sites naturels de clivage sont présentés (flèches).*

des effets de type superantigène *in vitro* en induisant une réponse immunitaire anormale et une expansion polyclonale de la sous-population de lymphocytes T Vbeta16 [87]. L'injection de particules MSRV à des souris SCID (severe combined immunodeficiency) humanisées (huSCID) greffées de cellules PBMC humaines cause des hémorragies cérébrales et une mort T-dépendante des souris [26].

La sous-unité soluble d'ENV, ENV-SU, correspond à un fragment qui est probablement généré naturellement par clivage à K316. *In vitro*, la protéine recombinante ENV-SU peut être produite en *E. coli*, et ses propriétés activatrices de l'immunité innée ont été démontrées *in vitro*. Sur PBMC humaines, monocytes purifiés et MDDC, l'ENV-SU agit de façon inflammatoire en activant la voie de signalisation CD14/TLR4 et de ce fait est susceptible de promouvoir la réponse immunitaire de type Th1 [100].

Sur des échantillons de PBMC isolées de patients de SEP, la libération de cytokines médiée (relayée) par ENV-SU est considérablement augmentée, comparée à celle observée dans les PBMC de donneurs sains ; de plus, les quantités des cytokines IL-6 et IL-12p40 produites en réponse à ENV-SU sont en corrélation avec l'atteinte

des patients quantifiée selon l'échelle EDSS (expanded disability status scale) [99]. Une étude récente confirme la production de cytokines induite par ENV dans les PBMC de patients atteints de RRMS [102].

2.4 Modèles animaux

Des modèles animaux sont utilisés pour comprendre les maladies et pour tester l'application de médicaments dans des études pré-cliniques. Des maladies comme la SEP qui affectent le SNC sont difficiles à étudier sans modèle animal à cause d'un accès limité au matériel biologique prélevé chez les patients, le matériel facilement accessible comme le sang et les échantillons de CSF ne permettant pas les mêmes investigations que des biopsies de tissu ou des coupes de cerveau ; la médecine expérimentale comme méthode de recherche impliquerait une prise de risque importante car les symptômes de la SEP et les effets indésirables des médicaments conventionnels sont déjà difficiles à supporter pour les patients. D'un autre côté, des essais de culture cellulaire ont une pertinence limitée en raison de la complexité des implications physiologiques dans la SEP qui associent autant le SNC que le système immunitaire ; un large éventail d'études anatomiques, physiopathologiques et biomédicales doit pour cela être effectué en modèle animal.

2.4.1 Encéphalomyélite Auto-immune Expérimentale (EAE)

L'encéphalomyélite auto-immune expérimentale (EAE) est le modèle animal classique des maladies démyélinisantes du SNC comme la SEP ; il est applicable aux rongeurs de laboratoire et aux primates. L'EAE est utilisé de manière courante pour simuler les événements neuro-immunologiques du SNC se manifestant pendant la maladie, comme l'inflammation, la démyélinisation et l'endommagement axonal, et pour tester l'impact et la posologie de médicaments immunomodulateurs sur le cours de la maladie. Pour causer une démyélinisation, une réponse auto-immune est induite par immunisation avec les composantes antigéniques de la myéline comme le *myelin oligodendrocyte glycoprotein* (MOG), le *myelin basic protein* (MBP) ou le *proteolipid protein* (PLP), et des adjuvants contenant des composantes de la paroi cellulaire de *Mycobacterium tuberculosis* (MT) comme l'adjuvant complet de Freund (CFA). Pour créer une rupture de la BHE afin de permettre l'invasion immunitaire dans le cerveau et la moelle épinière, la toxine de *Bordetella pertussis* (PTX) pouvant être co-injectée.

Les symptômes de l'EAE sont classés selon l'échelle du score clinique (CS) qui pour la souris s'étend d'une faiblesse de la queue avec perte de tonus musculaire jusqu'à une paralysie des membres et la mort de l'animal. L'évolution des symptômes

EAE dépend de la souche de souris ainsi que de l'antigène utilisé ; par exemple en souris C57BL/6, une forme chronique mais récurrente d'EAE peut être induite par immunisation à la MOG [10].

La démyélinisation que l'on observe dans l'EAE est médiée par les lymphocytes T autoréactifs [65] ; leur invasion dans le parenchyme du cerveau et de la moelle épinière peut être suivie par des méthodes de coloration chimique, d'immunomarquage aux anticorps et par IRM ; de plus, des anticorps anti-myéliniques peuvent servir de marqueurs sérologiques car ils induisent la démyélinisation dans la SEP et l'EAE [53]. Autour des vaisseaux de la BHE formés par des cellules endothéliales et couverts d'astrocytes, une forte inflammation recrutant des lymphocytes, de la microglie et des phagocytes peut être observée avant et pendant les poussées d'EAE [25]. Une fois « primées » à reconnaître un auto-antigène myélinique comme la MOG, les cellules T autoréactives peuvent susciter une réponse immunitaire contre un spectre plus large d'épitopes structurels myéliniques ; ce phénomène appelé *epitope spreading* [117] peut être observé dans la SEP ainsi que dans quelques cas d'EAE.

L'induction d'EAE par immunisation myélinique s'appelle *induction active d'EAE*. Pour illustrer le caractère auto-immun des cellules T encéphalitogènes observées dans les plaques EAE, les splénocytes des souris atteintes d'une EAE induite activement peuvent être stimulés *ex vivo* avec le même antigène myélinique utilisé pour l'immunisation, et la production de cytokines inflammatoires est mesurée ; des splénocytes isolés peuvent également être injectés en souris saines pour provoquer une démyélinisation et des symptômes d'EAE (*induction d'EAE par transfert adoptif*).

La polarisation des lymphocytes T vers une réponse Th1 et une auto-immunité dans l'EAE est portée par les DC [120] ; en répondant aux motifs conservés (PAMP) de la paroi du MT et en « primant » les lymphocytes T naïfs à l'antigène myélinique rencontré, elles constituent le lien entre les systèmes immunitaires inné et adaptatif qui joue un rôle important dans l'induction de l'auto-immunité.

2.4.2 Souris SCID humanisées

L'immunodéficience sévère combinée (SCID – severe combined immunodeficiency) est une maladie génétique héréditaire qui existe chez plusieurs espèces mammifères, par exemple chez l'Homme, chez la souris et chez le cheval. Les individus souffrant

de SCID sont privés d'immunité humorale et cellulaire parce que leurs cellules B et T ne parviennent pas à maturité. Les animaux SCID peuvent être utilisés pour étudier la génétique et la physiologie du système immunitaire humain, pour élucider les mécanismes de rejet de tumeurs ou de transplants, et pour expérimenter les applications médicamenteuses possibles afin de guérir ou traiter la SCID humaine. Les souris SCID peuvent être greffées avec des PBMC humaines après élimination de composants immunitaires résiduels, comme les cellules tueuses naturelles, par irradiation. Dans ce modèle appelé *huSCID*, il est possible d'étudier les cellules humaines greffées et leur implication dans la réponse immunitaire dans un système *in vivo*. Un des avantages des souris huSCID est que les produits thérapeutiques « humanisés » qui agissent sur le système immunitaire humain peuvent être testés sur des souris avant d'entrer en phase clinique. Comme les cellules immunitaires principales n'existent pas naturellement mais peuvent être remplacées par des cellules humaines, le système est compatible avec les médicaments destinés aux humains.

2.4.3 Souris transgéniques

La manipulation génétique sur les animaux de laboratoire est faite pour étudier les propriétés des gènes dans un environnement physiologiquement complexe et authentique, et pour étudier des gènes qui ne sont pas naturellement présents dans l'animal de laboratoire. Globalement, deux types de manipulation génétique visant à changer le génotype de l'animal expérimental peuvent être distingués : l'invalidation d'un gène existant (mutagenèse *knock-out*) et l'ajout d'un nouveau gène (*transgénèse*).

Les souris knock-out sont des souris manquant d'un produit génétique particulier codé par un gène cible qui a été manipulé expérimentalement. L'élimination ou la mutation non-sens (*nonsense mutation*) du gène cible cause l'absence de son produit dès la naissance ; de même, la mutation *missense* altère la fonctionnalité du produit. Puisque les déficiences génétiques innées mènent souvent à des perturbations du développement ou à la mort, le *knock-out conditionnel* est nécessaire pour étudier ces mutations létales ; en introduisant un promoteur inductible dans le gène en question, le gène peut être « désactivé » à tout moment pour induire les conséquences physiologiques.

La transgénèse est le processus d'introduction d'un gène exogène – appelé transgène – dans un organisme vivant ; le transgène intégré confère à l'organisme de nouvelles propriétés qui seront transmises à ses descendants si le transgène a été

intégré dans les gamètes. L'ajout d'un gène peut être réalisé soit de façon aléatoire soit en ciblant un locus donné. De manière similaire à la situation des souris *knock-out*, l'introduction d'un gène étranger peut altérer la situation physiologique dans ces mutantes *knock-in* et potentiellement être létal. Par analogie, le *knock-in conditionnel* est possible pour « activer » le gène à un moment choisi.

2.4.4 Modèles animaux utilisés dans ces travaux

Les études physiopathologiques sur l'implication d'ENV dans la SEP sont restreintes par le manque de modèle animal exprimant l'ENV puisque le MSRV et la famille des HERV sont présentes uniquement chez l'Homme et chez les grands singes. Les résultats que nous avons obtenus en souris C57BL/6 sauvages indiquent que l'ENV injectée en combinaison avec de la MOG provoque un trouble similaire à l'EAE classique induite par l'injection de MOG et d'adjuvant de Freund (CFA) ; ces données suggèrent que la souris est intrinsèquement susceptible aux maladies neuro-inflammatoires induites par ENV. Ce modèle d'EAE qui est développé dans la deuxième partie de ces travaux est basé sur l'injection ENV en association avec d'autres produits, ce qui représente une méthode très invasive et ne reflète pas la réalité quant à l'exposition et à la disponibilité d'ENV dans le contexte physiologique hypothétique. Un des moyens d'approcher le plus possible la situation dans la SEP est de créer des souris qui expriment l'ENV directement, soit d'établir un modèle transgénique d'ENV, comme cela sera développé dans la troisième partie de cette thèse.

2.4.5 Considérations Éthiques

Expérimentation animale – L'exploitation du Vivant

L'expérimentation animale est un sujet délicat qui mérite d'être considéré parce qu'il soulève des questions d'éthique, de conscience et de responsabilité. La dimension éthique de l'expérimentation animale engage en même temps un niveau personnel (quotidien) et un niveau philosophique (idéologique) d'acceptation et de justification de nos actes en tant que chercheur-euse-s. Le texte suivant représente un extrait d'un exposé élaboré dans le cadre de l'unité de formation doctorale interdisciplinaire « Éthique appliquée et responsabilité dans la conduite de la recherche » (GIERE –

Groupe Inter-Universitaire d'Éthique de la Recherche, Universités de Grenoble) dont l'objectif était de discuter les projets de recherche des participant-e-s sous un angle d'éthique déontologique.

Confrontations immédiates, *Éthique appliquée.* Le fait de se retrouver acteur d'expériences dont les effets primaires visibles – nuire à une créature vivante – sont destructeurs et nocifs provoque une réflexion spontanée et soulève plusieurs questions : Sommes-nous nous-mêmes entièrement responsables de nos actions et des actions d'autres qui sont sous notre influence ? Cette responsabilité devant le Vivant, implique-t-elle le devoir de minimiser activement tout effet nocif au-delà des effets inévitables ? D'un autre côté, est-ce que les codes de comportement existants qui abordent des questions techniques restreignent notre responsabilité personnelle d'une façon qui pourrait nous inciter à nous « cacher derrière les règles » afin de soulager notre conscience ? L'amélioration des conditions, l'optimisation des protocoles, et une évaluation critique des procédures expérimentales concernant à la fois ce contact immédiat avec l'animal et la maximisation de résultats scientifiques sont d'importants points à analyser, sans tenir compte des règles existantes qui ne devraient pas être considérés comme ultimes ou universelles. La particularité de chaque individu-e s'affirme au niveau intellectuel, mental, moral et émotionnel ; un code de comportement uniforme ne laisse pas la place à cette particularité qui pourtant peut nous distinguer des autres.

Réflexions étendues, *éthique normative.* Au-delà du niveau spontané de réflexion, l'utilisation d'animaux dans l'expérimentation scientifique évoque un questionnement plus profond qui exige une justification vis-à-vis de nous-mêmes et vis-à-vis de la société. L'approche *anthropocentrique* développée par Immanuel KANT¹ comprend la responsabilité individuelle de l'être humain de par notre capacité unique de moralité : les standards que nous appliquons à nos sociétés humaines ne doivent pas être abaissés par des atrocités infligées à d'autres êtres vivants ; considérant les animaux comme inférieurs, il faut cependant se souvenir que, par principe, nous sommes obligé-e-s de préserver notre intégrité morale dans notre comportement. En outre, le point de vue *biocentrique* développé par A. SCHWEITZER, G.M. TEUTSCH and T. REGAN¹ nous engage à respecter toute créature vivante car les différences putatives entre les Hommes et d'autres entités vivantes – basées sur des critères

¹Metzler Philosophie-Lexikon, Begriffe und Definitionen, PRECHTL P. une BURKARD F.-P. (Herausgeber) (1996)

arbitraires comme la conscience – peuvent être considérées comme matériellement inexistantes. Le devoir de « respecter la vie » est de validité universelle et ne requiert pas de justification ni d'explication. Dans ce contexte, une démarche critique devrait nous mener à une évaluation consciencieuse de la nécessité de choisir des modèles *in vivo* plutôt qu'*in vitro* (par exemple, des cultures de cellules sanguines ou des lignées permanentes peuvent être utilisées à la place d'animaux expérimentaux, notamment dans les études préliminaires). Mais, confronté à sa responsabilité globale, les bénéfices des modèles *in vivo* pour le/la chercheur-euse ne sont pas négligeables : Ne sommes-nous pas aussi responsables du bien que nous ne faisons *pas*, du mal que nous ne combattons *pas* ? Si nous prenons conscience de la souffrance des patients et de l'absence de traitement adéquat, ne sommes-nous pas obligé-e-s de capitaliser notre savoir pour aider, par tous les moyens dont nous disposons ? Du côté des inconvénients, la notion générale du « respect de la vie » en tant que telle représente un poids lourd qui peut être soulagé ou affaibli par l'idée *pathocentrique* (P. SINGER, M. St. DAWKINS¹) qui est basée sur les différences plus ou moins graduelles dans la capacité de souffrance chez les animaux. Ces différences peuvent être pesées afin de « mesurer » le bien et le mal dans l'expérimentation animale, mais cette approche – qui fait référence à la pensée *utilitariste* (L. BENTHAM¹) – est limitée dans son applicabilité parce qu'elle implique le jugement de la souffrance animale, ce qui est objectivement impossible.

Questions personnelles. Le dilemme central auquel un-e chercheur-euse dans la recherche biomédicale est confronté est la question de son attitude face à l'expérimentation animale, une question qui est présente en permanence. Il est difficile voire impossible de résoudre ce problème d'une manière définitive parce que la nécessité du modèle animal devrait être à chaque fois soumise à une évaluation critique, et même si la décision de l'*in vivo* a été prise pour un cas particulier, la confrontation quotidienne reste un défi. De telles réflexions inconfortables peuvent nous amener à séparer notre réalité scientifique professionnelle de notre sphère privée dans laquelle une échelle de valeurs différente serait appliquée. La création d'une « double vie » fictive est un phénomène qui peut se produire et nous « aider » à éviter la confrontation morale : les actions et décisions de notre vie professionnelle ne doivent plus être en accord avec la conscience qui domine notre vie privée, ce qui veut dire qu'elles ne doivent désormais plus être justifiées. Mais il est indispensable que nous nous posions la question qui est de savoir si la perte de sensibilité est vraiment préférable ou pas, ou si ce ne serait pas plutôt cette sensibilité qui nous permet de décider moralement et éthiquement du sens de nos actions, et de nous comporter conscien-

cieusement dans des situations délicates. À ce niveau, les recommandations et lois sur l'expérimentation animale faites par des « spécialistes » peuvent être dangereuses parce qu'elles représentent des limites à notre responsabilité personnelle et civique, et parce qu'elles peuvent sembler utiles au premier regard pour un-e chercheur-euse qui se retrouve invariablement face à ce dans le dilemme, mais elles peuvent aussi le/la rendre passif-ve et aveugle aux vraies questions morales dont il/elle devrait avoir conscience à tout moment. De la même manière, je considère comme dangereux le rôle des comités d'éthique car jusqu'à présent non seulement leur jugement peut au mieux avoir une valeur *consultative*, leurs évaluations et recommandations n'étant jamais *décisives*, mais leur existence pourrait même contribuer à amplifier notre tendance à refuser la responsabilité individuelle. Après tout, l'exploitation quotidienne du Vivant et des ressources naturelles en général reste trop souvent sans considération éthique et représente un abus que nous bannissons trop facilement de nos pensées arrogantes.

2.5 Objectifs du travail

Le but des travaux présentés dans ce mémoire était la création d'un modèle animal approprié de la Sclérose en Plaques (SEP) qui inclut la protéine d'enveloppe ENV de MSRV afin d'étudier ses propriétés inflammatoires. Le modèle *in vivo* représente un système dynamique qui tient compte de la complexité des circonstances physiologiques de la SEP, et il est nécessaire pour comprendre les implications systémiques des différents éléments, y compris le système immunitaire, les facteurs pathogéniques et de possibles agents thérapeutiques.

Après des décennies de recherche sur la SEP, l'énigme de son étiologie reste irrésolue. Bien que plusieurs facteurs de risque comme les infections virales, les vaccinations, les prédispositions génétiques et divers facteurs environnementaux comme la pollution industrielle ont été identifiés, l'approche dominante dans la recherche sur la SEP se concentre sur des stratégies palliatives qui n'attaquent pas l'origine du problème et qui de fait n'ont qu'un impact limité sur la course de la maladie.

Notre approche de la problématique de la SEP n'est pas de nature palliative mais curative ; elle implique la compréhension des processus fondamentaux qui sont à l'origine de la pathologie de la SEP. Même si notre ambition est clairement thérapeutique, notre travail peut en même temps être considéré comme de la recherche fondamentale parce que les données élémentaires sur les événements initiaux de la SEP ne sont toujours pas acquises.

Le projet de ces travaux consistait en trois parties principales. Comme les propriétés moléculaires et inflammatoires de la protéine d'enveloppe de MSRV ont été établies par notre laboratoire dans des essais *in vitro* sur le système humain, la première étape était l'étude de l'applicabilité de ces résultats préalables au système murin **(1)**. La deuxième étape consistait en l'induction de l'Encéphalomyélite Auto-immune Expérimentale (EAE), le modèle animal classique de la SEP, en intégrant ENV dans le protocole expérimental afin d'étudier ses propriétés d'adjuvant et ses actions immunologiques **(2)**. Dans la continuité de cette thèse, des travaux supplémentaires sur la présence systémique d'ENV et sur les effets et la posologie de méthodes innovatrices de traitement sont prévues. Des souris transgéniques porteuses d'ENV seront créées, les cellules cibles de son expression étant les astrocytes cérébrales et les cellules présentatrices d'antigène (CPA). Un modèle transgénique murin introduisant ENV dans le génome murin est nécessaire parce que des copies

homologues aux protéines HERV ENV ne sont présentes que chez les grands singes et chez l'Homme. Le choix de la souche de souris utilisée dans les expériences d'induction d'EAE présentées dans ce mémoire reflète la nécessité de pouvoir appliquer les résultats obtenus et les méthodes établies aux futures souris transgéniques ENV, et la cohérence des procédures expérimentales. Les études préliminaires préparant la création de souris transgéniques ENV incluant les étapes de construction des vecteurs génétiques et de transfections dans les cellules cibles faisaient partie de cette thèse et constituent sa troisième partie **(3)**. (Par la suite, des constructions sélectionnées seront utilisées pour dériver des souris transgéniques pour des études physiologiques et pour des essais *in vivo*. Les conséquences physiopathologiques seront évaluées par des analyses des composantes immunitaires (inflammation, fonctions lymphocytaires) et analyses comportementales des souris.)

Le travail focalisait sur les points suivants :

(1) Propriétés inflammatoires d'ENV-SU

- Étude sur cellules humaines *in vitro* : reproductibilité de l'activation cellulaire par ENV établie auparavant
- Étude de la transposabilité sur cellules murines *in vitro* : maturation de cellules dendritiques
- Reproduction des propriétés neutralisantes des anticorps monoclonaux anti-ENV
- Effets systémiques *in vivo* d'ENV-SU sur l'organisme murin

(2) Le modèle *in vivo* : EAE murine

- Modification et adaptation du protocole classique d'induction d'EAE utilisant ENV comme adjuvant
- Classification et comparaison des symptômes EAE induits par les méthodes d'induction classique et adaptée
- Études neurophysiopathologiques sur souris atteintes :
 - Détection de cellules T autoréactives

- Imagerie par Résonance Magnétique (IRM) du cerveau et de la moelle épinière

(3) Souris transgéniques ENV

Les souris transgéniques que nous voulons créer permettront l'expression des protéines d'enveloppe MSR-V-ENV et HERV7q-ENV sous le contrôle du promoteur CMH-II pour cibler son expression dans des cellules immunitaires comme les CPA dont les cellules dendritiques (DC), et sous le contrôle de GFAP (glial fibrillary acidic protein) pour déclencher son expression dans des cellules astrogliales du système nerveux, qui sont précisément les cellules exprimant ENV chez l'humain. Ces constructions génétiques ont été transfectées dans des cellules en culture pour des essais *in vitro* et pour la validation fonctionnelle.

Chapter 3

Material and Methods

3.1 ENV-SU

ENV-SU is a 33-kDa and 293-aa fraction of the full-length MSRV envelope protein (ENV pV14; GenBank accession no. AF331500). ENV-SU and ENV pV14 organisations are presented in figure 1.5, page 17, and amino acid sequence of ENV-SU is shown in 3.1.

```
MALPYHTFLFTVLLPPFALTAPPPCCCTSSSPYQEFLWRTRLPGNIDAPSYRSLSKGNSTFTAHTHMPRNCY  
NSATLCMHANTHYWTGKMINPSCPGGLGATVCWTYFTHTSMSDGGGIQQQAREKQVKEAISQLTRGHSTPS  
PYKGLVLSKLNHETLRTHRLVSLFNNTLTRLHEVSAQNPTNCWMCLPLHFRPYISIPVEQWNNFSTEINTTSV  
LVGPLVSNLEITHTSNLTVCVKFSNTIDTTSSQCIRWVTPPTRIVCLPSGIFFVCGTSAYHCLNGSSESMCFLSFL  
VPPMTIYTEQDLYNHVVPKPHNK
```

Figure 3.1: *Amino acid sequence of the MSRV envelope protein fraction ENV-SU.*

Recombinant ENV-SU protein was produced and purified by Protein'eXpert (Grenoble, France) by using the pET-15b expression vector (Novagen, Merck) and AD494 (DE3) *Escherichia coli* cells. Briefly, isopropyl-beta-D-thiogalactopyranoside-treated cultures grown at 37°C were centrifuged and the pellet was washed. After centrifugation, the pellet was resuspended in wash buffer in the presence of a mixture of protease inhibitors (Roche Diagnostics) and subjected to sonication. The cell lysate was then centrifuged, and the pellet, containing the inclusion bodies,

3. MATERIAL AND METHODS

was resuspended in buffer plus protease inhibitors. After centrifugation, the pellet was washed in the presence of 2% Triton X-100, then washed again to remove detergent. Inclusion bodies were solubilised by resuspension in the presence of 8 M urea before centrifugation. The supernatant was diluted in Tris-HCl (pH 8), 1 mM 2-ME, and loaded onto a Chelating Sepharose Fast Flow column (Amersham Biosciences) for purification under denaturing conditions. ENV-SU was refolded by dialysis against 50 mM Tris (pH 7), 300 mM NaCl, 1 mM 2-ME, 2% sucrose, 2% glycerol, and 2 M urea. Under these conditions, the protein remained soluble. Amino acid sequence determination of purified ENV-SU based on Edman degradation was in accordance with the known sequence. Fifty-microgram aliquots/per millilitre were flash frozen into liquid nitrogen and stored at -80°C. SDS-PAGE and mass spectrometry (MALDI-TOF) analysis profiles of rENV-SU (Protein'eXpert) are shown in 3.2. The ENV-SU protein was tested for the presence of endotoxins by *Limulus ameobocyte lysate* (LAL) test performed by CleanCells, and all fractions were below the detection level of 5 UI/ml.

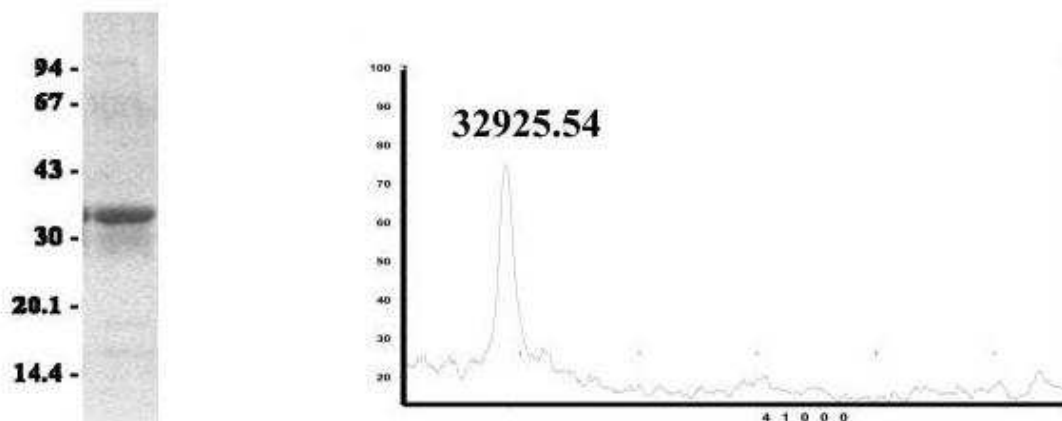


Figure 3.2: *SDS-PAGE and MALDI-TOF analysis of the MSR V ENV-SU.*

3.1.1 Different Batches of ENV-SU

The batches 4A, 4B, 4C, 5A and 5B of recombinant ENV-SU that have been utilised in this work were produced, purified and controlled in the same way by Protein'eXpert (as described above). Even if the characterisation of the potential differences between these batches was not part of the objectives, slightly different reactions have been observed and documented.

3.1.2 Neutralising Antibodies

To determine the specificity of the inflammatory properties of ENV-SU, before cell stimulation, ENV-SU was preincubated with monoclonal antibodies (mAbs) directed against recombinant ENV-SU (monoclonal mouse IgG1: clone 13H5A5 and monoclonal mouse IgG2a: clone 3B2H4; BioMérieux, Marcy l'Étoile, France).

3.2 Cell Culture

3.2.1 Human PBMCs

Blood was obtained by venous puncture and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation on Ficoll Paque (GE Healthcare). Cells at the interphase were collected and washed three times in PBS supplemented with 2% foetal calf serum (FCS) (BioWest). Cells were either utilised directly or frozen at -80°C or -180°C (liquid nitrogen) in FCS + 10% DMSO. Prior to use, frozen cells were thawed and washed three times in RPMI 1640 (GIBCO, Invitrogen) supplemented with 1% L-glutamine (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 1% NEAA (non-essential amino acids, Sigma-Aldrich), and 10% heat inactivated FCS (BioWest). Cell viability was then assessed by Trypan blue exclusion and viable cells were resuspended in complete medium at 10^6 cells/ml and 1 ml of cell suspension was plated for each assay condition tested.

3.2.2 Murine Splenocytes

Fresh splenocytes were obtained by teasing the spleen under aseptic conditions. Erythrocytes were removed by osmotic lysis with ammonium chloride solution. Remaining unfractionated nucleated spleen cells were washed twice and counted. Cells were adjusted to a density of 2×10^6 cells/ml of complete culture medium (RPMI 1640 (GIBCO, Invitrogen), 10% FCS (BioWest), 1% NEAA (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich)) and 1 ml of cell suspension per condition was plated in culture dishes. Remaining cells were frozen in DMSO/FCS in liquid nitrogen (-180°C). For one experiment, whole spleens were frozen in liquid nitrogen and cells were isolated and cultured after unfreezing.

3.2.3 Murine Lymph Node Cells

Abdominal lymph nodes were collected under aseptic conditions and cells were isolated and washed in complete culture medium (see medium for splenocytes). Cells were then counted and plated in 24-well dishes at 2×10^6 cells/ml.

3.2.4 Murine Dendritic Cells

Dendritic cells (DCs) were generated from bone marrow (BM) as previously described [40]. Briefly, BM cells were isolated by flushing cells from the femurs. Erythrocyte cells and Gr1⁺ cells were removed by magnetic cell sorting. Negatively selected cells were resuspended at 5×10^5 cells/ml in complete Iscove's modified Dulbecco's media (IMDM) (GIBCO, Invitrogen) supplemented with 1% of the GM-CSF-transfected J558 cell line supernatant, 40 ng/ml recombinant FLT-3 L, and 5 ng/ml mouse recombinant IL-6. Every 3 days, cells were resuspended at 5×10^5 cells/ml in complete IMDM. By day 6, IL-6 was removed and FLT-3 L was used at 20 ng/ml. For stimulation assays, cells were counted and adjusted to 10^6 cells/ml and 1 ml of cell suspension was plated per assay condition.

3.2.5 Stimulation Assays

Cells were plated in 24-well plates in 1 ml of the appropriate culture medium and incubated at 37°C in 5% CO₂ in humidified atmosphere before and during stimulations. Recombinant ENV-SU, Lipopolysaccharide (LPS, Sigma-Aldrich), Phytohaemagglutinin (PHA, Sigma-Aldrich) or Pansorbine (PSB, Calbiochem) were added undiluted or diluted either in culture medium or in phosphate-buffered saline (PBS). For the neutralizing assays using the anti-ENV antibodies 3B2H4 and 13H5A5, 0.5-1 μ g of ENV-SU was preincubated for 45 min at 4°C with 30 μ g/ml of the respective monoclonal antibody, and the mixture was subsequently added to the cell culture samples. Cells and culture supernatant were harvested after 24, 48, and 72h and centrifuged at 500 \times g to separate cells for flow cytometry analysis. If only supernatant for ELISA analysis was required, cells were discarded after centrifugation. The supernatant was then frozen at -20°C prior to use.

3.3 Dosage of Cytokines (ELISA)

The culture supernatants were harvested at 24h and/or 48h and/or 72h, frozen at -20°C and unfrozen immediately before use. To measure the cytokine release in the supernatant of cell cultures, Enzyme-Linked Immuno-Sorbent Assay (ELISA) sets including cytokine standards and antibodies (OptEIA, BD Pharmingen) for the respective cytokines were used. All procedures were carried out according to the manufacturer's instructions. Samples were developed with TMB (BD Pharmingen) and measured at 450 nm wavelength in a Victor 3 spectrophotometer (1420 multilabel counter, Perkin Elmer).

3.3.1 Cytokines

The cytokines tested for were basically human IL-6 to represent general inflammation measured on PBMCs, murine IFN-gamma to represent T cell-mediated auto-reactivity on splenocytes of EAE mice, and murine IL-6 to show maturation of DCs and to compare the stimulation-induced maturation capacities of DCs of knock-out and wild-type mice. Other cytokines like murine IL-12p40/70, TNF, IL-10 and IL-4 were measured occasionally on DCs to get an overview of the effects of the stimulations.

3.3.2 Samples and Dilutions

The supernatants were diluted according to the expected amounts of cytokines depending on the stimulation type and the nature of the secreting cells. In general, unstimulated samples were used undiluted to avoid calculation-induced false-positive values, and dilutions of stimulated samples ranged between 1/2 and 1/500. The cytokine standards were used according to the user's manual, and the range of the standard curve was modified when necessary to fit the range of secreted cytokines expected in the samples.

3.4 Flow Cytometry

For flow cytometry analysis, cells were harvested, washed in PBS complemented with 3% FCS and 0.16% sodium azide and immunostained for surface expression of distinct markers for 30 min at 4°C. The conjugated antibodies used for staining were all obtained from BD Biosciences: anti-I-A^b/classII-PE, anti-CD11b-FITC, anti-CD11c-APC for murine DCs and anti-CD3, anti-CD8, anti-CD4 and anti-B220 for murine lymph node cells and splenocytes. Data acquisition and analysis were performed with a FACS Aria flow cytometer and the software FACSDiva5 (BD Biosciences).

3.4.1 Antibodies and Staining

Cells were stained with propidium iodide (PI) which intercalates in the DNA that becomes accessible in dead or dying cells. PI-positive cells are excluded by gating during acquisition or analysis. On DCs, the antibodies anti-CD11b and anti-CD11c are utilised to visualise the overall population of DCs, and the anti-I-A^b/classII-stained cells represent mature DCs expressing the class II molecule inside this DC population. The cells that show double positive for CD11b/c and I-A^b/classII can be mathematically isolated to compare their percentages between the different stimulation conditions. On lymph node cells and splenocytes, the antibodies anti-CD3, anti-CD4, anti-CD8 and anti-B220 were used to distinguish the B (B220⁺) and T (CD3⁺) lymphocyte populations, the latter being divided in CD4⁺ helper and CD8⁺ killer T cells.

3.5 Mice

Pathogen-free 6 weeks-old wild-type C57BL/6 mice were purchased from Charles River. Tlr4^{-/-} mice, Cd14^{-/-} mice and Myd88^{-/-} mice were obtained from Dr. Bernard RYFFEL from the Centre National de la Recherche Scientifique/Centre de Distribution, Typage et Archivage Animal (Orléans, France).

3.5.1 Experimental Autoimmune Encephalomyelitis (EAE)

The classical induction method of active EAE that consists in immunising healthy mice with a myelin component has been modified to fit our purpose, which is to study the inflammatory and immunological actions of ENV-SU in the context of murine EAE.

Injections

The antigen chosen was MOG 35-55 (NeoMPS), a peptide component of the Myelin Oligodendrocyte Glycoprotein (MOG) that is situated in the outer myelin sheath. IFA (incomplete Freund's adjuvant, Sigma-Aldrich) and CFA (complete Freund's adjuvant, Sigma-Aldrich) containing 1 mg/ml of *Mycobacterium tuberculosis* (H37 RA) were purchased from DIFCO (Invitrogen). The injected amount corresponds to 100 μg of *M. t.* per mouse; for some experiments, CFA was complemented to 5 mg/ml of *M. t.*, and 500 μg per mouse were injected. The following groups of mice received intramuscular (scoring and recall experiments) or subcutaneous (MRI experiments) injections with the compositions listed in table 3.1.

Table 3.1: *Composition of the mixtures injected to healthy C57BL/6 mice for EAE induction.*

	antigen	adjuvant	supplement
IFA	MOG 150-200 μg	incomplete (IFA)	-
CFA	MOG 150-200 μg	complete (CFA)	-
ENV	MOG 150-200 μg	incomplete (IFA)	ENV 10-50 μg

When injected intramuscularly, mice were anaesthetised by intraperitoneal injection of 10 mg/kg Xylazine (Rompun, Bayer) and 100 mg/kg Kétamine (Imalgène, Merial). According to the recommendations, anaesthetised mice were kept at 30-35°C under a lamp or on a heating pad during the injections and the recovery phase.

In addition to the immunisation mixture, all mice received intraperitoneal injections of 200 ng PTX (Calbiochem, Merck) containing the Pertussis toxin on day 0 and day 2 of injections. PTX is used to rupture the blood brain barrier to allow the immune cells that have been primed to recognize the MOG antigen to enter the brain.

Clinical Score and evaluation of symptoms

The evaluation of symptoms was done every day according to the clinical score (CS) scale shown in table 3.2.

Table 3.2: *EAE Clinical Score (CS) Scale.*

0	no symptoms
1	tail weakness
2	clumsy gait, hind limb weakness
2.5	hind limb weakness, tremor, loss of balance
3	hind limb paresis
4	hind limb paralysis
5	hind limb paralysis, sphincter dysfunction, nasal and ocular haemorrhages, forelimb paresis or paralysis
5.5	moribund stage without immediate death
6	moribund stage without immediate death, respiratory dysfunctions, death

Furthermore, in one experiment, mice were weighed once a day in order to establish a correlation between the course of the disease in terms of symptoms and the body weight of the mice. In general, diseased mice were sacrificed at the highest possible CS values, taking into account the relapsing-remitting character of the disease course. It is important to note that, according to ethical recommendations, mice showing CS values higher than CS 4 should be sacrificed in order to avoid suffering. In the EAE experiments presented in this work, CS values of 4 or higher were not reached.

Sacrifice and perfusion

Mice were sacrificed either by cervical dislocation or by terminal anaesthesia and subsequent intracardial perfusion, the latter being applied in order to fix the brain tissue with para-formaldehyde (PFA, Merck) for histological analysis of brain slices. Before intracardial perfusion, spleens were cut or clamped to avoid dilution or fixing; the blood circuit of the mice was then rinsed with PBS prior to PFA perfusion in order to eliminate blood residues.

Magnetic Resonance Imaging (MRI)

MRI measurements were done in the ANIMAGE animal facility of the neurological hospital of Lyon (Bron, France). The detailed protocol established by Jean-Baptiste LANGLOIS (ANIMAGE) is documented in appendix C.

Antigen Recall

To show the specificity of the antigen recognition and the resulting auto-reactive T cell response, splenocytes were isolated and cultured as described in 3.2.2, page 63, and stimulated with 1 μg to 20 μg of the MOG 35-55 peptide for 24h, 48h or 72h. Supernatants were harvested and IFN-gamma production was tested as described in 3.3, page 65.

3.5.2 Systemic ENV Injection

These experiments were designed to study the systemic effect of ENV intraperitoneal injection on healthy C57BL/6 mice. Unlike in the EAE experiments, ENV-SU was injected in an aqueous phase (PBS) and not in mineral oil. The injected amounts ranged between 1 μg and 50 μg of ENV-SU. Mice were sacrificed after 24h, 48h or 72h post injections, and spleens as well as visibly swollen lymph nodes (normal lymph nodes for control animals) were extracted. Cells were prepared as described in 3.2.2 and 3.2.3, page 63, and flow cytometry analysis as described in 3.4, page 66, was performed.

3.5.3 Transgenic ENV Mice

The creation of transgenic mice comprises several steps of genetic and cellular manipulation *in vitro* prior to the introduction of the transgene into the animal. The steps of vector construction, cell transfection and validation by confocal microscopy have been accomplished in collaboration with Hei-lanne REYNAUD-DOUGIER who is pursuing the transgenesis project as a Post-doc researcher.

Gene targeting and vector constructions

On one hand, the plasmids containing the MSRV or Herv7q ENV fragment (F.-L. COSSET, ENS Lyon) were digested and the ENV sequences (including ENV-SU and -TM) were cloned under the control of the promoters pCMV (ubiquitous human promoter, F.-L. COSSET, ENS Lyon), pMHCII (murine monocyte and dendritic cell promoter (D. MATHIS, USA)) or pGFAP (human astroglial cell promoter; described as functional in mice (H. KETTENMAN, Germany)). On the other hand, the MSRV-ENV and Herv7q-ENV fragments were digested in order to interchange their transmembrane (TM) domains (sequence shuffling); the cleavage is located on the KpnI site. The obtained genetic combinations (MSRV+TM of Herv7q and Herv7q+TM of MSRV) were cloned in the same way under the control of the promoters of interest (pCMV, pMHCII or pGFAP).

Culture and transfection of cell lines

HEK-293T human kidney cells (ATCC) were cultured in Dulbecco's modified Eagle's medium DMEM (GIBCO, Invitrogen) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. GL26 murine glial cells (F. BERGER, Grenoble) were cultured in the same medium without antibiotics. The murine macrophage J774 cell line (ATTC) was maintained in complete medium consisting of RPMI-1640 medium (GIBCO, Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were cultured in 24 well plates and transfected according to Invitrogen™ life technologies procedures: volume of culture medium (500 μ l), quantity of pDNA (0.8 μ g), volume of lipofectamine™2000 (2 μ l) and Opti-MEM®-I reduced Serum Medium as solution to dilute the transfection agent.

Confocal microscopy

Cells were fixed for 10 min in 3% para-formaldehyde (PFA) at room temperature, washed 3 times with PBS, and permeabilised with 0.2% saponin and 2% BSA in PBS (PBS-SB) for 20 minutes. Cells were then incubated for 1h with primary antibody (anti-ENV 3B2H4: 1 μ g/ml or Golgin97 (Invitrogen): 1/100 dilution), washed 3 times with PBS-SB, followed by anti-mouse secondary antibody anti-IgG2a Alexa 488 (BD Biosciences) or anti-IgG1 Alexa 555 (BD Biosciences) in 1:500 dilution for 1h, and washed 3 times with PBS-SB. All antibodies were diluted in PBS-SB. The nuclei of fixed cells were labelled with DAPI. Images were taken with a Zeiss 510 inverted confocal microscope (64 \times , oil immersion).

Digression

This chapter exposing some personal reflexions of its author is meant to be digressive, which means that it does not pretend to be of scientific character and thus does not require to be approved by the reader. It stands per definitionem out of context and is entirely dispensable to the comprehension of the manuscript.

In my eyes, embedding scientific research in a more global, political and social environment is, or should be, part of a scientist's work. The following paragraphs are born of the double necessity I feel to integrate my scientific activity into society as a whole: firstly, I personally feel responsible for my acting as a citizen, and I am willing to cultivate a critical awareness of what one could call instrumentalisation of science for economic purposes; secondly, I consider insufficient the efforts undertaken up to now – by the scientific community in general and by its actors in particular – in terms of public concertation, anticipation of risks, responsibility and moral conscience. I perceive the current (political) climate as repressive and hostile to the expression of personal doubts and criticism, and I accuse all those who are able but not willing to change things, whatever their level of influence or their societal impact.

Priorities and Decision-making in Capitalist Societies

Nowadays, economic valorisation dominates political decisions, which implicates that benefits, and not public welfare, are the centre of interest in research and development in our occidental societies. The notions of productivity and profitability invade and control our lives in almost every possible manner; competitiveness rules out morality while possession, personal welfare and abundance are more and more gaining in importance. A conflict of interest is emerging that divides those who

defend the idea to share knowledge and resources equally from those who sacrifice their individual ideals to gain integrity and to meet normativity. In this sense, economic pressure is threatening independency of research, and patents are attacking our liberties because they deny us the choice to share scientific attainments to the benefit of less privileged societies.

In a world where researchers revealing harmful impacts of novel technologies on nature and human health are psychologically and materially persecuted and silenced by those holding economical power, liberty of research is seriously threatened. The question remains what political challenge would explain the prevailing climate of aggressive repression, surveillance and censorship exerted by governmental institutions on ethically motivated citizen initiatives - if it was not agreements with the global economic players. The paradigm of infinite economic growth in a world of limited resources seems a logical paradox, but it is though widely accepted. While mainstream thinking is tending to the “*after us the flood*” attitude, we may ask ourselves if the responsibility of decision-making authorities (that remains too often unquestioned) does actually free us from personal, moral responsibilities.

Science - for the Progress of Humankind?

The priorities in research today, particularly in biomedical sciences, are designed by a diminishing number of actors concentrating more and more power - public participation in decision-making is virtually inexistent although we live in a so-called *democratic* system, and the ongoing social and intellectual infantilisation creates a gap between science and society and propagates the image of unaware and “profane” citizens faced with omniscient experts that decide in their - our - place. Monopolisation of knowledge is exerted by the “competent” value-creating class towards the voiceless class of consumers inside one society. But it exists in the same way between economically and/or politically strong nations and economically and/or politically weaker ones; this tendency is continuously growing and reinforces the global imbalance of power.

There is an economical and a political dimension of power that are inseparably linked with each other and that determine the position of a nation in the global equilibrium of forces; when politicians participate in the management of private industry and industrialists become political deciders, it is difficult to judge if it is industry that is at the service of politics or if it is rather politics that is taken in

hostage by industry - as it is difficult to decide if it was hen or egg that started the perpetual circle. Anyway, the duality of politically justified economic exploitation and economically justified political decisions creates a situation in which nobody - neither individuals nor institutions - is free of ambition and neutrality does not exist anymore. It should be possible to imagine politics and economy as strictly separated - as are the executive, legislative and judicial organs in classical societal models.

I consider that the major part of research as it is today - and as it is likely to develop tomorrow - does not deal with problems of general interest for mankind, at least it does not accord them reasonable priority. Scientific progress does not serve the amelioration of the living conditions of the global population; it serves those that can afford to participate actively in the economic development by being able to financially contribute. Financial pressure is at the origin of the necessity to patent ideas, inventions and discoveries, and the notion of intellectual property has become a keyword in the struggle for economic valorisation of scientific progress. There was a time when researchers contented themselves with publications of the milestones in their research, and the tendency to publish small pieces of the puzzle in the ongoing era of the internet - instead of accumulating data secretly until the final breakthrough worth a Nobel price - represented a menace to those “old school” researchers. Maybe it was this frustration or fear not to be recognised personally by the scientific community that made them more easily accept patents as a means to assure the immortalisation of their *own* work. The second reason is of economic nature: nowadays, private financing of research and the steadily growing participation of private funding even in the public sector are indispensable for the survival of most laboratories since public funding is not sufficient to guarantee the perpetuity of long-term research projects. Meanwhile, the private investors orientate research towards application and new technologies, and the new field of *research & development* clearly outcompetes *fundamental* research because economic profitability outcompetes curiosity and the simple admiration of the beauty of things. Louis PASTEUR² believed that there are no *applied sciences*, but only *applications* of science or scientific results, that is to say, the utilisation of results as a consequence and not as the purpose of research. It is obvious enough that fundamental research *a priori* is necessary to make us understand how nature works, and that without fundamental knowledge no application *a posteriori* is conceivable. In the same time, empiric research is more and more replaced by experimental, manipulative and invasive research that, in my eyes, has lost the spirit that animated the first researchers,

²1822-1895

in the old times when scientists were philosophers and scientific achievements were born of contemplation.

Privatisation of Knowledge

Patents are dangerous for two main reasons. The first one is that they privilege research promising direct applications to the detriment of fundamental research - the latter being simply not *profitable* enough to be exploited in terms of economy. The second and even more obvious one is that they forbid free exchange of material and intellectual resources between research entities because the financial barrier is excluding those that cannot afford to *buy* the patent or the right to use its content. One consequence of this second point is that patents *de facto* paralyse research because progress is attenuated in every respect when communication of results is impossible. There seems to be no limit to human blindness towards the fact that collaboration and sharing of ideas and competences are essential for any kind of development. The more important consequence that we arrogantly forget is that poor or developing countries - that our occidental countries unscrupulously exploit and though still pretend to support - will never be able to take part in the benefits; a striking example are diseases that since decades are easily curable and factually eradicated in our part of the world, but that kill and will continue killing millions of people elsewhere on the globe. Our notions of *need* or *comfort* are born of the ridiculous point of view of a spoiled child that prefers to destroy its fellows rather than to share, whatever the stake.

Democracy - Way or Goal?

As researchers we should keep in mind that things are what they are only because *people* are creating and supporting them; the idea of “society” as something exterior that imposes universal rules is a cowardly attitude and does not take into account that society consists of intelligent, responsible individuals. There is no reason to believe that we have to carry on reproducing the same schemes in order to keep up the *status quo*. In this sense, the Aristotelian³ vision of every human being as a *zoon politicon* (“political animal”) actively participating in society as a dynamic organism seems crucial to me for the understanding of societal phenomena that *emerge* at a

³Aristotle, 384-346 B. C.

certain level of *complexity*. The notion of *emergence* in natural sciences as well as in philosophy describes the process of self-organisation and “spontaneous” generation of new properties arising from the fusion of individual parts inherently carrying their value for the *whole*⁴. When the system as a *whole* becomes *more than the sum of its parts*, the value and the contribution of each individual part are essential.

Lessons from History

The feeling of social and political powerlessness as a phenomenon becomes a prophecy fulfilling itself while spreading inside society; but we must not forget that, as citizens, we *are* society and that it is in our hands to shape it. Already during World War II, social philosophers like Erich FROMM described the link between the feeling of social uselessness diffusing inside society and people’s susceptibility to manipulation and takeover by a totalitarian regime like the national socialist dictatorship⁵. The sociopsychological background situation causing the feeling of personal insignificance might not have been exactly the same in the 1930s and today (E. FROMM describes the sequels of the Lutheran⁶ ideology and the influence of the new economic individualism arising and growing since late Middle Age and beginning Renaissance), but the effects are quite similar in both cases: the lack of personal aims (and thus the adoption of “fake values”, nowadays mostly created by media) is a symptom of our modern times and makes us susceptible to any kind of psychological manipulation and propaganda. Furthermore, Erich FROMM identifies the two faces of freedom - positive freedom (*to be free to*) and negative freedom (*to be free of*) - and diagnoses that the average citizen - especially the predominating *authoritarian* character - is unable to use its “new” freedom (acquired by the struggle for economic independence that was certainly necessary in the past) in a constructive way that he calls *spontaneous activity* and that would represent the positive face of freedom. Instead of empowering us to realise our own conceptions, the new individualism pushes us into egoism, isolation and loss of social contacts; the resulting lack of imagination and the missing awareness of our personal perception of the world reinforces the necessity to blindly adopt values created by the *public opinion*.

⁴Introduction à la pensée complexe, Edgar MORIN (1990)

⁵Die Furcht vor der Freiheit (Escape from Freedom), Erich FROMM (1941)

⁶Martin LUTHER, 1483-1546

Citizens as Administrators or Administrated Citizens?

It is important to remember that society as an organism - comprising also the institutions that govern society - should be at the service of every citizen to simplify exchanges and to administer goods equally, as is defined its primary function. Unfortunately, notions like *democracy*, *social progress* and *liberty* have become meaningless in our habitual use of language; they have been appropriated by the new playwrights of society that praise the bellicose lord of global competition and redefine society's function as an economic challenge. (If there was a time when *religion* could be considered as the *opiate of the people*⁷, nowadays *consumption* is the opiate for the masses; the tragic difference is that *religion* was meant to appease people that more or less *appropriated* religious thinking to deal with their personal problems in a spiritual way, whereas the phenomenon of *mass consumption* is entirely invented and propagated by the economic machinery, with the unique intention of perpetuating economic growth and making people accept - and even desire - technological progress.) We are thus developing towards a society that has lost its original function as an instance of social justice. Society today justifies its existence and its functioning by itself; it has become an entity detached from individual needs. Being citizen today morally binds us to contribute to this higher instance and to serve the ideals of a society that do not necessarily match the ideals of each individual any more. This means that the initial idea of *society* has been betrayed, perverted and literally turned into the opposite.

Capitalism as an economic system is in line with the values propagated by television, radio, newspapers, publicity, brands etc. The daily flood of meaningless information continuously pouring down on us keeps our senses busy and our minds away from real, sensible preoccupations. The new mentality forged by the media is based on mass consumption, leisure and superficiality, and therefore perfectly serves the economic interests of our capitalist leaders: indifference and ideological hollowness are the ideal ground for the brainwashing propaganda that incites us to consume excessively, regardless of necessity and quality; those who choose to join the vicious circle of productivity, competition and consumption may thus ignore that they are deliberately offering their liberties for sale, and that in doing so they are approving our throwaway society. Individual participation in society is restricted to financial contribution based on our new and exclusive role as consumers; to counter this ongoing development and to regain personal autonomy and responsibility, we

⁷Karl MARX (1843)

need to cut the strings of our puppet masters - we need to re-appropriate society and to participate actively in the processes that are shaping our reality, implicating and applying our individual ideas in order to deliver moral, ideological and creative *input*.

Digression (français)

Ce chapitre exposant quelques réflexions personnelles de son auteure a vocation à être digressif, ce qui veut dire qu'il ne prétend pas être de nature scientifique et ne requiert donc pas être approuvé par le lecteur ou la lectrice. Il se situe per definitionem en dehors du contexte, sa lecture est entièrement facultative et n'entraîne pas de conséquence pour la compréhension du manuscrit.

À mon avis, l'insertion de la recherche scientifique dans un cadre plus global, politique et social fait, ou devrait faire, partie du travail d'un scientifique. Les paragraphes suivants sont nés de la double nécessité que je ressens d'intégrer mon activité scientifique dans la société dans son ensemble : premièrement, je me sens personnellement responsable de mes actes en tant que citoyenne, et j'ai la volonté de cultiver une conscience critique de ce que l'on pourrait appeler instrumentalisation de la science à des fins économiques; deuxièmement, je considère insuffisants les efforts entrepris jusqu'à présent – par la communauté scientifique en général et par chacune de ses acteurs particuliers – en termes de concertation publique, anticipation des risques, responsabilité et conscience morale. Je perçois le climat (politique) actuel comme répressif et hostile à l'expression de doutes et de critiques personnelles et j'accuse tous ceux et toutes celles qui sont capables mais qui n'ont pas la volonté de changer les choses, quel que soit leur niveau d'influence ou leur impact sociétal.

Priorités et prise de décisions dans les sociétés capitalistes

De nos jours, la valorisation économique domine les décisions politiques, ce qui implique que les bénéfiques et non pas le bien public soient au centre de l'intérêt

dans le domaine de la recherche et du développement dans nos sociétés occidentales. Les notions de productivité et de rentabilité envahissent et contrôlent presque tous les aspects de nos vies. Pendant que la compétitivité supplante la morale, le bien-être personnel et l'abondance gagnent de plus en plus en importance. Un conflit d'intérêts survient, séparant celles et ceux qui défendent l'idée de partager le savoir et les ressources équitablement de celles et ceux qui sacrifient leurs idéaux individuels pour gagner en intégration et atteindre la normativité. En ce sens, la pression économique menace l'indépendance de la recherche, et les brevets attaquent nos libertés car ceux-ci nous privent du choix de partager les avancées scientifiques, désavantageant d'autant plus les sociétés moins privilégiées.

Dans un monde où des chercheur-euse-s dévoilant des effets nocifs de nouvelles technologies sur la nature et la santé humaine sont persécuté-e-s psychologiquement et matériellement, réduit-e-s au silence par ceux et celles qui détiennent le pouvoir économique, la liberté de la recherche semble sérieusement menacée. Une question subsiste : Quelle logique politique expliquerait le climat prédominant de répression agressive, de surveillance et de censure exercé par les institutions gouvernementales sur des initiatives citoyennes de motivation éthique, si ce n'est celle des accords avec les acteurs de l'économie mondiale ? Le paradigme de la croissance économique infinie dans un monde de ressources limitées semble paradoxal, mais il est pourtant communément accepté. La pensée dominante tend vers l'attitude de l'« *après nous le déluge* », alors que nous devrions nous demander si la responsabilité des autorités décisives (qui reste trop souvent incontesté) nous libère réellement de nos responsabilités personnelles et morales.

La science – pour le progrès de l'humanité ?

Les priorités dans la recherche d'aujourd'hui, particulièrement dans les sciences biomédicales, sont fixées par un nombre décroissant d'acteurs qui concentrent de plus en plus de pouvoir, la participation publique dans la prise de décisions est virtuellement inexistante, bien que nous vivions dans un système prétendument *démocratique*. L'infantilisation sociale et intellectuelle en cours crée un écart entre la science et la société et propage l'image du citoyen inconscient et « profane » face à des experts omniscients qui décident à leur – notre – place. La monopolisation du savoir est exercée par la classe « compétente » créatrice de valeurs sur la classe de consommateurs sans voix à l'intérieur d'une société. Mais elle existe de

la même manière entre les nations économiquement et/ou politiquement fortes et celles économiquement et/ou politiquement plus faibles ; cette tendance augmente continuellement et renforce le déséquilibre mondial des pouvoirs.

(Il y a une dimension économique et une dimension politique du pouvoir qui sont inséparablement liées l'une à l'autre et qui déterminent la position d'une nation dans l'équilibre mondial des forces ; quand les politiques participent à la direction de l'industrie privée et que les industriels deviennent décideurs politiques, il est difficile de juger si l'industrie serait au service de la politique ou si plutôt la politique serait prise en otage par l'industrie – comme il est difficile de décider si la poule ou l'œuf serait à l'origine du cercle perpétuel. D'une façon ou d'une autre, la dualité de l'exploitation économique justifiée par la politique et les décisions politiques elles-mêmes justifiées par l'économie crée une situation dans laquelle personne – ni les individu-e-s ni les institutions – n'est libre d'ambitions ; la neutralité n'existe plus. Il devrait être possible d'imaginer la politique et l'économie comme strictement séparées – comme le sont les organes exécutifs, législatifs et judiciaires dans les modèles sociétaux classiques.)

Je considère que la partie majeure de la recherche comme elle existe aujourd'hui – et comme elle évoluera probablement demain – ne traite pas les problèmes d'intérêt général pour l'humanité, du moins elle ne leur accorde pas une priorité raisonnable. Le progrès scientifique ne sert pas l'amélioration des conditions de vie de la population mondiale ; elle sert à ceux et celles qui ont les moyens de participer activement au développement économique en étant capables d'y contribuer financièrement. La pression financière est à l'origine de la nécessité de breveter les idées, inventions et découvertes, et la notion de la propriété intellectuelle est devenue un mot-clé dans la lutte pour la valorisation économique du progrès scientifique. Il fût un temps où les chercheur-euse-s se contentaient de la publication des avancées fondamentales de leur recherche. La tendance à publier de petits morceaux du puzzle dans l'ère de l'internet – au lieu d'accumuler des données secrètement jusqu'à la percée finale digne d'un prix Nobel – représentait alors une menace pour ces chercheur-euse-s de la « vieille école ». Peut-être que cette frustration ou peur de ne pas être reconnu-e-s personnellement par la communauté scientifique les amenait à accepter plus facilement les brevets comme moyen d'assurer l'immortalité de leurs *propres* travaux. La deuxième raison est de nature économique : de nos jours, le financement privé de la recherche et la participation croissante du financement privé dans le secteur public sont indispensables à la survie de la plupart des laboratoires car le financement public n'est pas suffisant pour garantir la perpétuation des

projets de recherche au long terme. Cependant, les investisseurs privés orientent la recherche vers les applications et les nouvelles technologies, et le nouveau domaine *Recherche & Développement* supplante clairement la recherche *fondamentale* parce que la rentabilité économique supplante la curiosité et la simple admiration de la beauté des choses. Louis PASTEUR² croyait qu'il n'y a pas de *science appliquée*, mais seulement des *applications* de la science ou des résultats scientifiques, c'est-à-dire l'utilisation des résultats comme une conséquence et non pas comme l'objectif de la recherche. Il est assez évident que la recherche fondamentale *a priori* est nécessaire pour nous faire comprendre comment « fonctionne » la nature, et que sans le savoir fondamental aucune application *a posteriori* n'est concevable. En même temps, la recherche empirique est de plus en plus remplacée par la recherche expérimentale, manipulatrice et invasive qui, à mes yeux, a perdu l'esprit qui animait les premier-e-s chercheur-euse-s, dans les temps anciens quand les scientifiques étaient philosophes et les acquis scientifiques étaient nés de contemplation.

Privatisation du Savoir

Les brevets sont dangereux pour deux raisons principales. La première est qu'ils privilégient la recherche promettant des applications directes au détriment de la recherche fondamentale – cette dernière n'étant simplement pas assez *rentable* pour être exploitée en termes économiques. La deuxième raison plus flagrante est qu'ils interdisent le libre échange de ressources matérielles et intellectuelles entre les entités de la recherche parce que la barrière financière exclut ceux et celles qui n'ont pas les moyens d'*acheter* le brevet ou le droit d'utiliser son contenu. Une des conséquences de ce deuxième point est que les brevets paralysent *de facto* la recherche parce que le progrès est atténué à tous égards quand la communication des résultats est impossible. Il ne semble pas y avoir de limite à l'aveuglement humain devant le fait que la collaboration et le partage d'idées et de compétences soient essentiels pour tout développement. La conséquence la plus importante, que nous oublions orgueilleusement, est que les pays pauvres et/ou en voie de développement – que les pays occidentaux exploitent sans scrupule et tout en prétendant les soutenir – ne pourront pas bénéficier de la plupart des avancées ; prenons l'exemple frappant des maladies qui depuis des décennies sont facilement traitables et pratiquement éradiquées dans notre partie du monde, mais qui tuent et continueront à tuer des millions de personnes ailleurs sur la planète. Nos notions de *besoins* ou de *confort*

²1822-1895

sont nées du point de vue ridicule d'un enfant gâté qui préfère écraser ses pairs plutôt que de partager, quelque soit l'enjeu.

La démocratie – moyen ou finalité ?

En tant que chercheur-euse-s, nous devrions garder à l'esprit que les choses sont comme elles sont seulement parce qu'elles sont créées et approuvées par des *hommes* et des *femmes* ; l'idée de la « société » comme quelque chose d'extérieur qui impose des règles universelles est une forme de lâcheté et ne prend pas en compte le fait que la société se compose d'individu-e-s intelligent-e-s et responsables. Il n'y a pas de raison de croire que nous devons continuer à reproduire les mêmes schémas afin de maintenir le *statu quo*. Dans ce sens, la vision aristotélicienne³ de chaque être humain comme un *zoon politicon* (« animal politique ») participant activement à la société en tant qu'organisme dynamique me semble crucial pour la compréhension de phénomènes sociétaux qui *émergent* à un certain niveau de *complexité*. La notion d'*émergence* dans les sciences naturelles ainsi que dans la philosophie décrit le processus d'auto-organisation et de génération « spontanée » de propriétés nouvelles résultant de la fusion des parties individuelles qui portent, de manière inhérente, une valeur pour l'ensemble⁴. Quand le *tout* devient *plus que la somme de ses parties*, la valeur et la contribution de chaque partie individuelle sont essentielles.

Leçons d'Histoire

L'impression d'impuissance sociale et politique en tant que phénomène général devient une prédiction auto-réalisatrice en se répandant à l'intérieur de la société ; mais nous ne devons pas oublier qu'en tant que citoyen-ne-s, nous *sommes* la société et que sa construction est entre nos mains. Déjà pendant la Seconde Guerre mondiale, des socio-philosophes comme Erich FROMM décrivaient le lien entre la sensation de l'inutilité sociale qui s'étendait dans la société et la susceptibilité de la population à la manipulation et à la prise de pouvoir par un régime totalitaire comme la dictature des national-socialistes⁵. Le contexte socio-psychologique expliquant le sentiment d'insignifiance personnelle n'était peut-être pas exactement le même

³Aristote, 384-346 B. C.

⁴Introduction à la pensée complexe, Edgar MORIN (1990)

⁵Die Furcht vor der Freiheit (La peur de la liberté), Erich FROMM (1941)

dans les années 1930 qu'aujourd'hui (E. FROMM décrit les séquelles de l'idéologie Luthérienne⁶ et l'influence du nouvel individualisme économique apparaissant et croissant depuis le bas Moyen Age et le début de la Renaissance), mais les effets sont tout à fait semblables dans les deux cas : le manque d'objectifs personnels (et par conséquent l'adoption de « fausses valeurs », de nos jours créés principalement par les médias) est un symptôme de nos temps modernes et nous rend susceptible à toute sorte de manipulation psychologique et de propagande. En outre, Erich FROMM identifie les deux faces de la liberté – la liberté positive (être libre *à*) et la liberté négative (être libre *de*) – et pose le diagnostic que le/la citoyen-ne moyen-ne – particulièrement le caractère *autoritaire* prédominant – est incapable d'utiliser sa nouvelle liberté (acquise par la lutte pour l'indépendance économique qui était certainement nécessaire dans le passé) d'une manière constructive qu'il appelle *activité spontanée* et qui représenterait la face positive de la liberté. Au lieu de nous permettre de réaliser nos propres conceptions, le nouvel individualisme nous conduit à l'égoïsme, l'isolement et la perte de contacts sociaux ; le manque d'imagination et de conscience dans notre perception personnelle du monde qui en découle renforce la nécessité d'adopter aveuglément les valeurs créées par (et pour) l'*opinion publique*.

Administration citoyenne ou citoyen-ne-s administré-e-s ?

Il est important de se rappeler que la société en tant qu'organisme – qui comprend également les institutions qui gouvernent la société – devrait être au service de tout-e citoyen-ne pour simplifier les échanges et pour administrer les biens équitablement, comme est définie sa fonction primaire. Malheureusement, des notions telles la *démocratie*, le *progrès social* et la *liberté* sont devenues insignifiantes dans l'usage habituel de notre langage. Les nouveaux dramaturges de la société, qui louent les dieux belliqueux de la compétition mondiale et qui redéfinissent la fonction de la société comme étant un défi économique, se les appropriées. (S'il fût un temps où la *religion* pouvait être considérée comme l'*opium du peuple*⁷, aujourd'hui la *consommation* est l'*opium des masses* ; la différence tragique est que la *religion* était destinée à apaiser les hommes et les femmes qui *s'appropriaient* plus ou moins la pensée religieuse afin de gérer leurs problèmes personnels de façon spirituelle, alors que le phénomène de *consommation de masse* est entièrement inventé et propagé par la machinerie économique, dans l'intention unique de perpétuer la croissance

⁶Martin LUTHER, 1483-1546

⁷Karl MARX (1843)

économique et d'amener les masses à accepter – et même désirer – le progrès technologique.) Nous sommes alors en train d'évoluer vers une société qui a perdu sa fonction originelle d'instance de justice sociale. La société d'aujourd'hui justifie son existence et son fonctionnement par elle-même ; elle est devenue une entité détachée des besoins individuels. Être citoyen-ne aujourd'hui nous engage moralement à contribuer à cette instance supérieure et à servir les idéaux d'une société qui ne coïncident plus obligatoirement avec les idéaux de chaque individu-e. Cela veut dire que l'idée de *société* a été pervertie et littéralement détournée de son but initial.

Le capitalisme comme système économique est en phase avec les valeurs propagées par la télévision, la radio, les journaux, la publicité, les marques etc. Le flot quotidien d'informations futiles déversé sur nous de façon permanente occupe nos sens et éloigne nos esprits des vraies préoccupations intelligentes. La nouvelle mentalité forgée par les médias est basée sur la consommation de masse, les loisirs et la superficialité, et sert, de ce fait, parfaitement les intérêts économiques de nos dirigeants capitalistes : l'indifférence et le vide idéologique sont un terrain idéal pour la propagande qui nous « lave le cerveau » et qui nous incite à consommer excessivement, sans considérer la nécessité ni la qualité ; ceux et celles qui choisissent de rejoindre le cercle vicieux de rentabilité, compétition et consommation ne savent peut-être pas pour autant qu'ils/elles vendent leurs libertés au plus offrant, et qu'en faisant ainsi ils/elles approuvent notre société jetable. La participation individuelle dans la société est restreinte à la contribution financière basée sur notre nouveau rôle exclusif de consommateurs ; pour contrer ce développement en cours et pour retrouver notre autonomie personnelle et notre responsabilité, nous devons couper les fils à nos marionnettistes – nous devons nous réapproprier la société et participer activement aux processus qui déterminent notre réalité, en s'impliquant et en appliquant nos idées individuelles afin d'apporter notre contribution morale, idéologique et créative.

Chapter 4

Results

4.1 Inflammatory Properties of ENV

The inflammatory properties of ENV-SU on human PBMCs, monocytes and monocyte-derived dendritic cells (MDDC) have been shown by our group; the specificity of the stimulation has been proven by utilising monoclonal anti-ENV antibodies to block ENV-mediated cytokine production, and the TLR4/CD14 pathway has been identified to be induced by ENV-SU to mediate cell activation. The following experiments were done to reproduce these findings with different production batches of the ENV-SU recombinant protein, to understand more about the molecular action of ENV-SU and to evaluate the applicability of our findings on the mouse system.

4.1.1 Studies on Human Cells *in vitro*

Human PBMCs were incubated with 0.5-2 μg of different production batches of the ENV-SU recombinant protein produced in *E. coli* and the IL-6 secretion in the culture supernatants was tested after 24h of incubation.

All the batches of ENV-SU (3B, 4A, 4B, 5A and 5B) are able to induce secretion of significant amounts of IL-6 compared to unstimulated controls. In this experiment, no dose-dependency is visible; all ENV stimulations (0.5-2 $\mu\text{g}/\text{ml}$) lead to production of high amounts of IL-6 that are comparable to the strong TLR4 stimu-

4. RESULTS

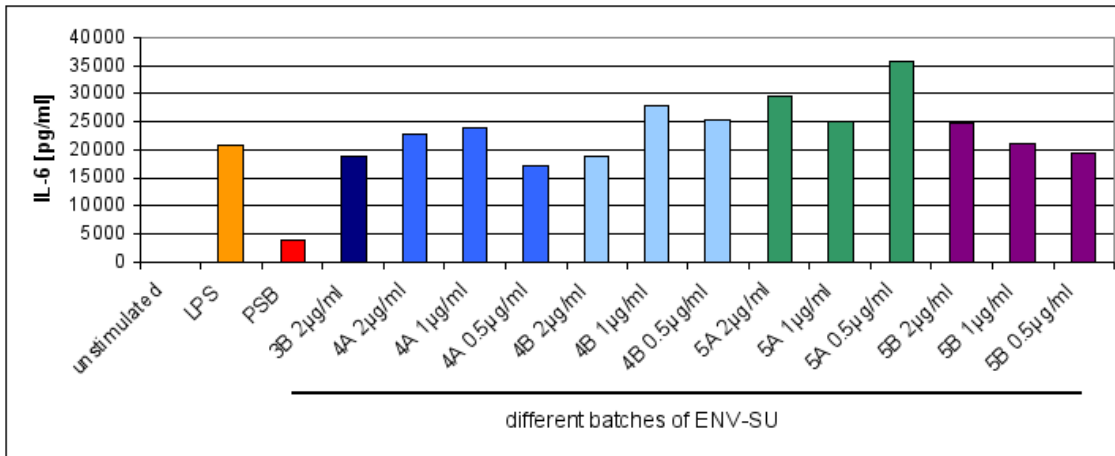


Figure 4.1: *IL-6* production in human PBMC cultures upon stimulation with lipopolysaccharide (LPS), pansorbine (PSB) and different batches of ENV-SU after 24h of stimulation, measured by ELISA (ELISA standard deviations <5%).

lator LPS and could therefore attain the saturation limit. The capacity to respond to the TLR2 stimulator PSB is limited.

IL-6 has been chosen here because previous work of our laboratory has demonstrated that ENV induces a parallel production of IL-6, IFN- γ , TNF- α and other major pro-inflammatory cytokines in the human system ([100], data not shown); to compare the different batches of ENV, IL-6 has thus been chosen as the indicator cytokine for the inflammatory effect of ENV.

4.1.2 Study of Transposability on Mouse Cells *in vitro*

In order to confirm the applicability of these results on the mouse model, several cell culture assays have been done on murine dendritic cells and splenocytes to reproduce ENV-mediated cytokine secretion and cell activation.

Inflammation and cell maturation in wild-type DCs

Cultures of bone-marrow derived dendritic cells (BMDC) were stimulated with ENV-SU in different concentrations between 0.25 $\mu\text{g}/\text{ml}$ and 2 $\mu\text{g}/\text{ml}$ for 24h (cells were cultured at $1 \times 10^6/\text{ml}$). To monitor inflammatory effects, production of IL-6, IL-

12p70 and TNF were assessed by ELISA of cell culture supernatants and surface expression markers for DC maturation (class II) were measured by flow cytometry analysis.

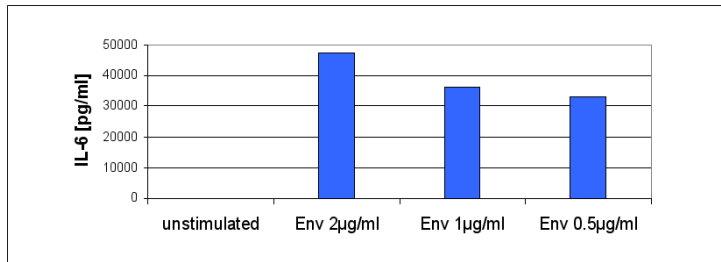


Figure 4.2: *IL-6 secretion by murine dendritic cell cultures (BMDC) upon stimulation with ENV-SU, assessed by ELISA of culture supernatants after 24h of culture (ELISA standard deviations < 5%).*

ELISA cytokine assay of supernatants shows significant dose-dependant production of IL-6 in wild-type C56BL/6 dendritic cells upon 24h incubation with ENV-SU and no production in the unstimulated control.

To resume five different stimulation experiments of DCs evaluated by ELISA for IL-6 and to confirm statistically relevant dose-dependency of the ENV-induced stimulation, mean values for each stimulation type are shown in figure 4.3.

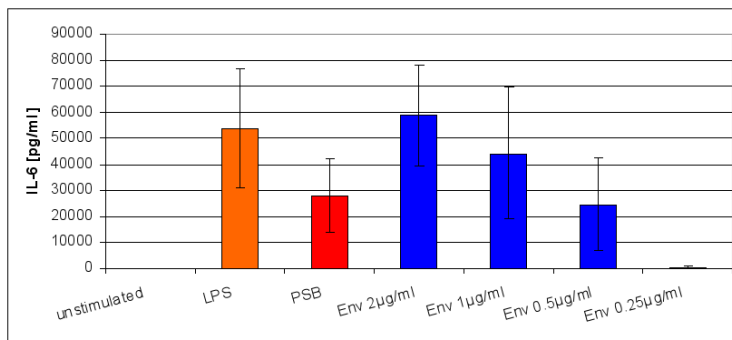


Figure 4.3: *Statistical evaluation of five stimulation experiments including LPS, PSB and different concentrations of ENV-SU, evaluated by ELISA for IL-6 after 24h of culture (unstimulated: n=5, LPS: n=4, PSB: n=3, ENV 2 µg/ml: n=3, ENV 1 µg/ml: n=5, ENV 0.5 µg/ml: n=5, ENV 0.25 µg/ml: n=2).*

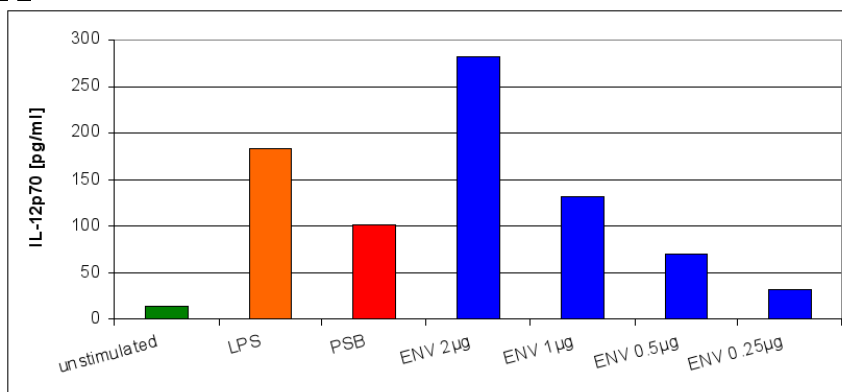
A strong dose-dependency of the ENV-mediated stimulation is clearly visible; 2 µg/ml of ENV stimulate DCs at a slightly higher level than 2 µg/ml of LPS which is the reference stimulation for the TLR4 pathway in DCs. Standard deviations point to strong differences between the absolute IL-6 values in the different experiments that are due to variations in the health and maturation status of the DC cultures

4. RESULTS

before stimulation; nevertheless, the results of the different experiments are highly reproducible.

To further characterise the cytokine response of DCs upon ENV stimulation, IL-12p70 and TNF were measured in DC culture supernatants of the same type of experiment, using LPS and PSB as positive stimulation controls (see figure 4.4).

A



B

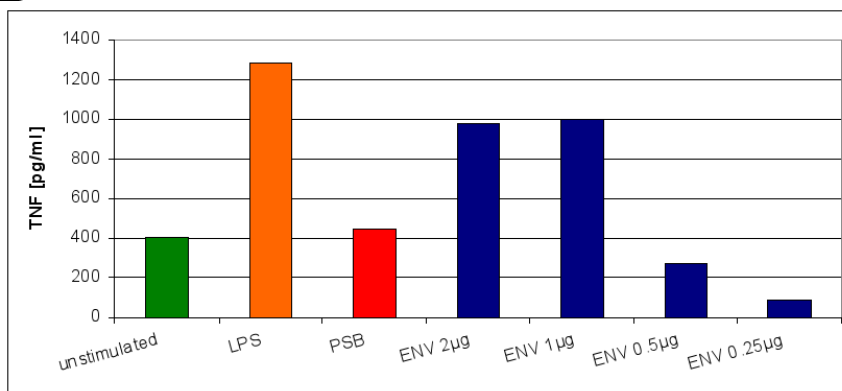


Figure 4.4: Production of IL-12p70 (A) and TNF (B) by murine BDMC cultures upon stimulation with ENV, compared to LPS- and PSB-induced stimulation. Quantities were assessed by ELISA of culture supernatants after 24h of incubation (ELISA standard deviations <5%).

The quantities of IL-12p70 produced by BDMC cultures upon stimulation with ENV, LPS or PSB are significant compared to unstimulated controls; we observe a dose-dependant effect of ENV stimulation and 2 µg of ENV stimulate stronger than both LPS and PSB. Regarding TNF, unstimulated BDMC cultures show a baseline secretion that stays higher than secretion caused by low concentrations of ENV (0.5 and 0.25 µg/ml); in the range of 400 pg/ml, differences can be neglected since the highest secretion levels (LPS) reach more than 1200 pg/ml. Other cytokines like IL-4 and IL-10 have been tested in ENV-stimulated cultures, but measured amounts were not significant or situated below the detection limit (data not shown).

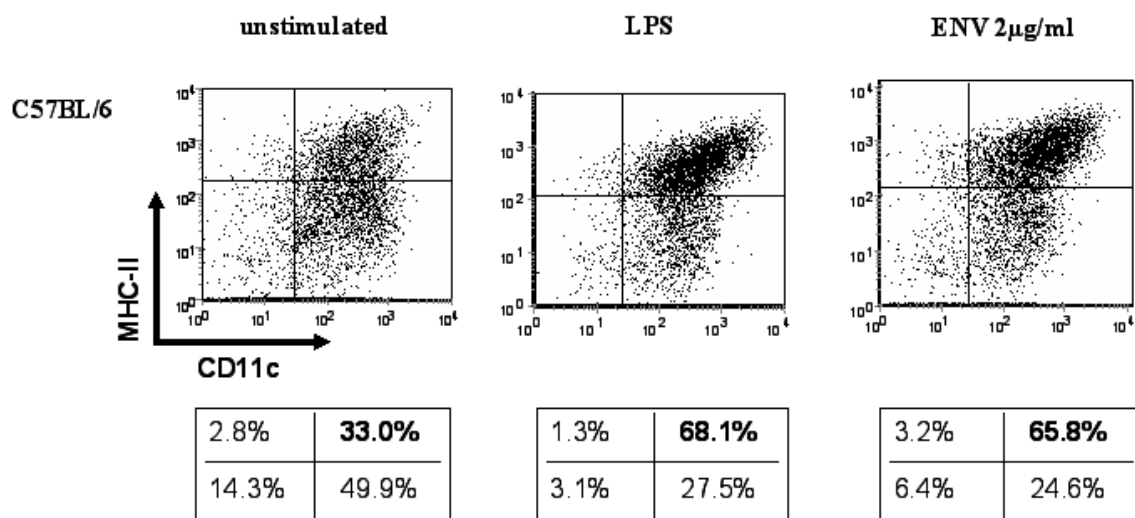


Figure 4.5: Flow cytometry analysis of cell maturation in murine dendritic cell cultures (BMDC) upon stimulation with LPS and ENV-SU for 24h. Double positive cells for CD11c and MHC-II (upper right quadrant) represent mature BMDCs.

For flow cytometry analysis of cell maturation induced by ENV, the antibodies anti-CD11c and anti-class II were chosen (see figure 4.5). The APC-labelled anti-CD11c antibody recognizes all (immature and mature) DC. Maturation is characterized by an augmentation of the class II MHC molecule expression level (called I-A^b in mouse), as assessed with the PE-labelled anti-I-A^b/class II antibody.

The representation in figure 4.5 shows double positive cells for CD11c and I-A^b/class II in the upper right quadrant. Both cell culture samples stimulated with LPS as well as with ENV-SU show significant level of maturation.

Furthermore, we tested different batches of the ENV-SU recombinant protein on mouse DCs. Quantities of secreted IL-6 were assessed by ELISA of the supernatants after 24h of incubation.

Figure 4.6 shows that the batches 3B and 4A induce significant IL-6 production levels, whereas the batch 5A fails to induce IL-6 production on murine DCs. For the further experiments in the murine system, only the batches 4A, 4B and 4C have been utilised because the batch 5A has shown no activating capacities on mouse DCs; the batch 5B has not been utilised in the murine system either because it has been derived from the same production batch than 5A.

4. RESULTS

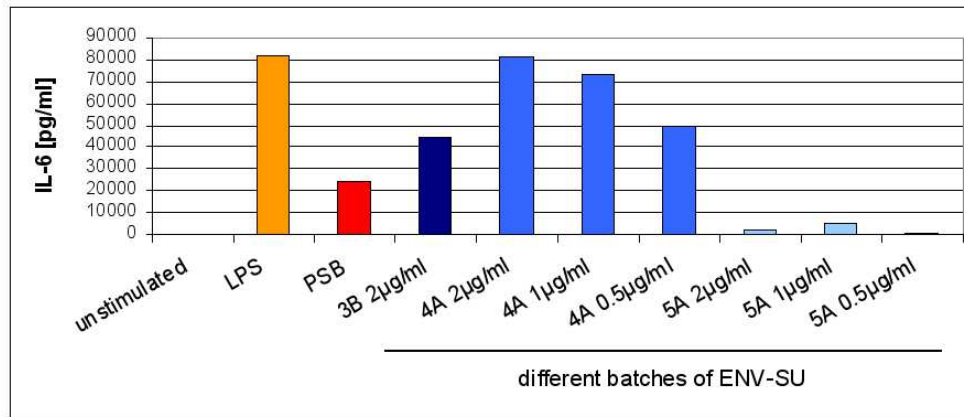


Figure 4.6: Stimulation of BMDC cultures with LPS, PSB and different batches of ENV-SU. IL-6 production was measured by ELISA of supernatants after 24h of stimulation (ELISA standard deviations <5%).

IFN- γ production by murine splenocytes

Splenocytes of wild-type C57BL/6 mice were incubated with different concentrations of LPS or ENV-SU and IFN- γ secretion was measured after 24h, 48h, and 72h of incubation (figure 4.7).

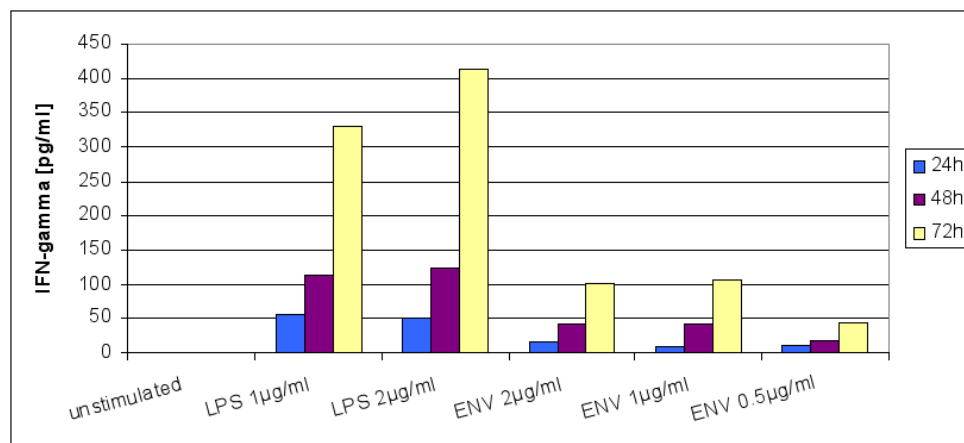


Figure 4.7: ELISA for IFN- γ production in murine splenocyte culture supernatants after 24h, 48h and 72h of stimulation with LPS and ENV-SU (ELISA standard deviations <5%).

The splenocyte cultures secrete high amounts of IFN- γ upon stimulation with

LPS and smaller but significant amounts upon stimulation with ENV-SU compared to unstimulated controls, notably after 72h of incubation.

4.1.3 TLR4 and CD14 in Mouse

To study the molecular actions of ENV and to confirm the activation of the TLR4/CD14 pathway shown before on human cells, stimulation of DCs of Tlr4^{-/-} and Cd14^{-/-} mice were carried out. The evaluation of the stimulation experiments was done on two levels: flow cytometry analysis for the expression of surface maturation markers and ELISA assay for cytokine production.

Induction of cell maturation in Tlr4^{-/-} and Cd14^{-/-} mice

Cell maturation was evaluated by flow cytometry analysis (see figure 4.8). Staining antibodies are anti-CD11c-APC to stain all dendritic cells, and anti-I-A^b/class II-PE to distinguish mature from immature cells (dead cells were excluded by IP staining).

The percentages displayed in diagram 4.9 have been calculated by gating on CD11c-positive dendritic cells and by counting those positive for I-A^b/class II among this dendritic cell population (dp, double positive).

Diagram 4.9 shows that the class II molecule is constitutively expressed at low level in resting/immature DC, and that stimulation with LPS, PSB or ENV-SU can initiate an increase in class II expression in wild-type C57BL/6. Concerning the Tlr4^{-/-} mice we can conclude that also the capacity to augment the class II expression upon TLR4 challenging is impaired, and that TLR2 is not affected.

The same evaluation has been made for Cd14^{-/-} mice (figure 4.10). The only stimulation that activates Cd14^{-/-} DC at the same level (or more) than the wild-type cells is the TLR2 activator PSB. As shown for the IL-6 production, the class II response upon TLR4 stimulation is less strong in Cd14^{-/-} than in wild-type dendritic cells. To compare the percentages of double positive (dp) cells of Tlr4^{-/-}, Cd14^{-/-} and wild-type mice, relative values are represented in table 4.1.

The calculation of percentages of the Tlr4^{-/-} and Cd14^{-/-} DC response compared to the wild-type DC response confirms that both TLR4 and CD14 are required for

4. RESULTS

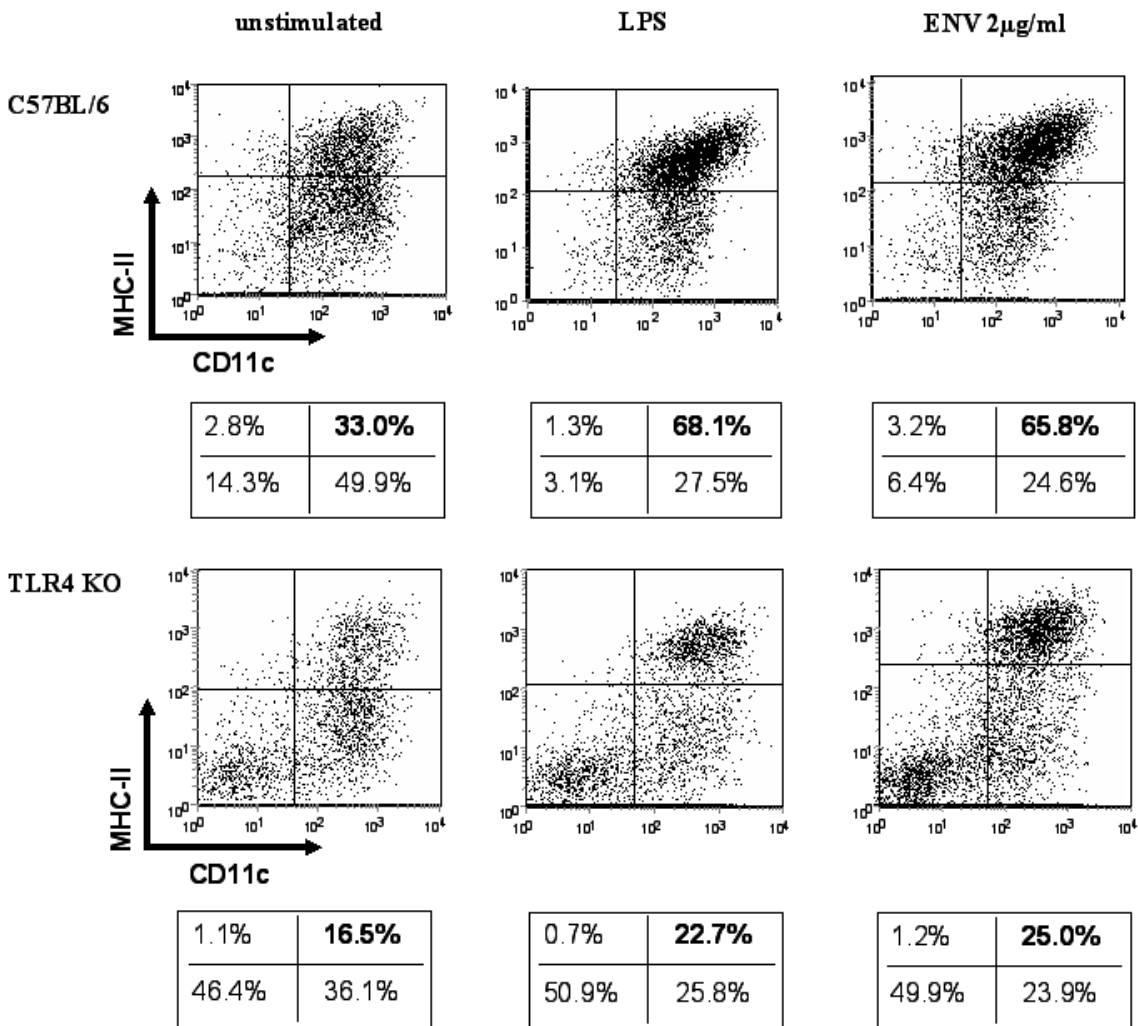


Figure 4.8: *Flow cytometry analysis of BMDC cultures derived from wild-type C57BL/6 and Tlr4^{-/-} mice after 24h of stimulation with LPS and ENV. Mature DCs are double positive for CD11c and MHC-II (upper right quadrant).*

complete TLR4 signalling in mouse DC.

In conclusion, ENV-SU is stimulating mouse dendritic cells to produce IL-6 and to express higher levels of the surface molecule class II/I-A^b. This stimulation depends on the TLR4 pathway in which the co-receptor CD14 participates as an accessory molecule, as shown before on human cells by our group.

To further elucidate the TLR4-CD14 interaction and the importance of CD14 in the TLR4 signalling pathway, it would be interesting to create Tlr4^{-/-} Cd14^{-/-} hybrid mice to study their TLR4 response and the signalling behaviour of their dendritic

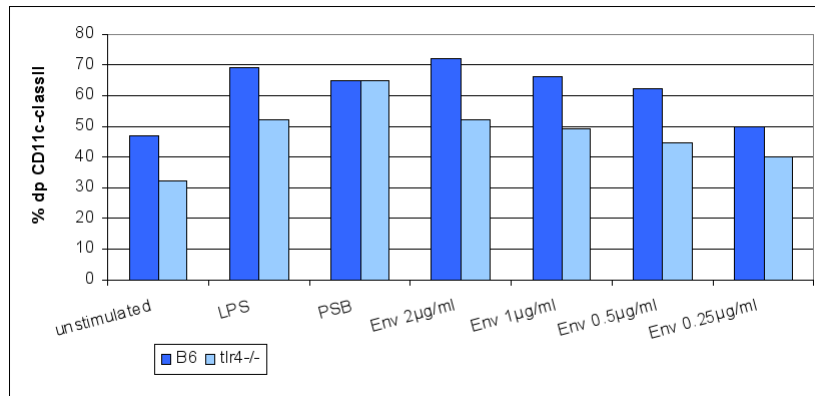


Figure 4.9: *Flow cytometry analysis of C57BL/6 and Tlr4^{-/-} BMDC upon stimulation with LPS, PSB and ENV for 24h. The displayed percentages represent mature DCs (double positive cells for CD11c and MHC-II staining) after exclusion of IP stained dead cells.*

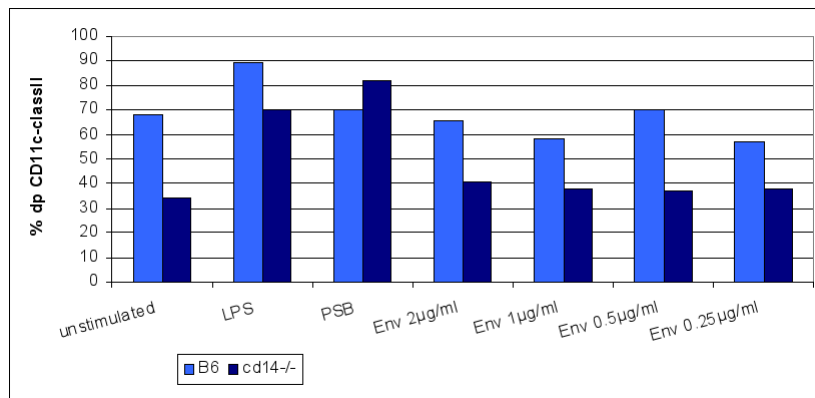


Figure 4.10: *Flow cytometry analysis of C57BL/6 and Cd14^{-/-} BMDC stained for CD11c and MHC-II. Cells were stimulated with LPS, PSB and ENV for 24h and dead cells were excluded by IP staining. Percentages of double positive cells represent mature DCs.*

cells.

4. RESULTS

Table 4.1: Comparison of cell maturation induced by LPS, PSB and ENV on BMDC cultures derived from $Tlr4^{-/-}$, $Cd14^{-/-}$ and wild-type C57BL/6 mice. Percentages represent mature DCs (double positive for CD11c and MHC-II) after exclusion of dead cells (IP staining) and were calculated relative to the response of wild-type mice ($\% = 100 - ((\% \text{ dp } CD11c^+ I-A^{b+} \text{ knock-out mice}) / (\% \text{ dp } CD11c^+ I-A^{b+} \text{ wild-type C57BL/6}) * 100)$).

dp CD11c ⁺ I-A ^{b+}	Tlr4^{-/-}	Cd14^{-/-}
LPS	-25%	-21%
PSB	0	+17%
ENV 2 $\mu\text{g/ml}$	-18%	-38%
ENV 1 $\mu\text{g/ml}$	-26%	-34%
ENV 0.5 $\mu\text{g/ml}$	-27%	-47%
ENV 0.25 $\mu\text{g/ml}$	-20%	-33%

Inflammatory cytokine response in DCs of $Tlr4^{-/-}$ and $Cd14^{-/-}$ mice

The diagram 4.11 shows the same type of experiment including dendritic cells from wild-type C57BL/6, $Tlr4^{-/-}$ and $Cd14^{-/-}$ mice stimulated with LPS, PSB or ENV-SU. Dendritic cells of $Tlr4^{-/-}$ and $Cd14^{-/-}$ mice were cultured and stimulated in the same way than those of wild-type C57BL/6. Production of IL-6, IL-12p70 and TNF were assessed in culture supernatants after 24 h of stimulation.

Diagram 4.11 shows that LPS-induced production of IL-6 and IL-12p70 is significantly decreased in $Tlr4^{-/-}$ mice compared to wild-type mice, whereas PSB induces the same IL-6 production and significantly higher IL-12p70 production. These two samples prove as controls that the TLR4 pathway (induced by LPS) is indeed impaired in $Tlr4^{-/-}$ mice, and that the TLR2 pathway (induced by pansorbine) is still functioning.

The findings for ENV-SU are similar to those of LPS which means that ENV-SU as well is using the TLR4 pathway in mouse dendritic cells, as already demonstrated for several human cell types.

Diagram 4.12 reveals that the production of both IL-6 and TNF in response to LPS or ENV-SU is diminished in $Cd14^{-/-}$ mice compared to wild-type mice, but the capacity to produce IL-6 in general does not seem to be affected since stimulation with the TLR2 activator PSB leads to similar IL-6 levels and even higher TNF levels

A

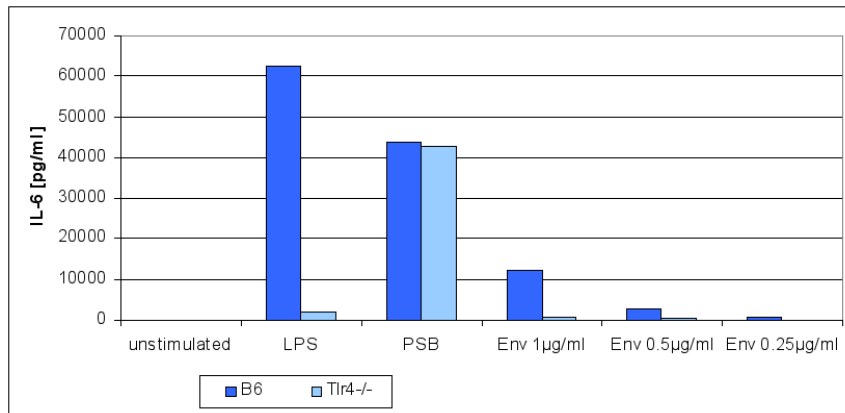
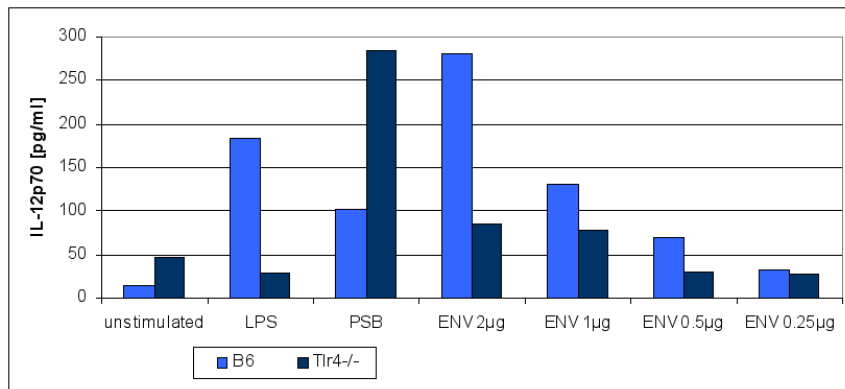


Figure 4.11: ELISA for IL-6 (A) and IL-12p70 (B) secretion in supernatants of BMDC cultures of C57BL/6 and Tlr4^{-/-} mice upon stimulation with LPS, PSB and different concentrations of ENV for 24h (ELISA standard deviations <5%).

B



in Cd14^{-/-} mice.

To show the decreased response to LPS and ENV-SU in Tlr4^{-/-} and Cd14^{-/-} mice relative to wild-type mice, we calculated percentages of IL-6 response (see table 4.2); the displayed values represent absolute values (pg/ml) obtained in one representative experiment, as well as the diminution of IL-6 release (%) compared to C57BL/6.

Table 4.2 shows almost no measurable difference in IL-6 production for pansorbine-stimulated (PSB) cells in Tlr4^{-/-} mice and a slight increase in Cd14^{-/-} mice, whereas for LPS- and ENV-SU-stimulated cells IL-6 production is significantly decreased in both Tlr4^{-/-} and Cd14^{-/-} mice.

These results suggest that CD14 seems to be essential for TLR4 signalling initiated by LPS or ENV-SU, whereas there is no influence of CD14 on the PSB-induced TLR2 pathway.

4. RESULTS

Table 4.2: *IL-6* production in BMDC culture supernatants of *Tlr4*^{-/-} and *Cd14*^{-/-} mice compared to wild-type C57BL/6 mice after 24h of stimulation with LPS, PSB and ENV. Absolute *IL-6* values (pg/ml) are shown for *Tlr4*^{-/-} (A) and *Cd14*^{-/-} (B) as obtained in one representative experiment, respectively; percentages of *IL-6* secretion were calculated relative to the *IL-6* secretion in wild-type BMDC cultures under the same conditions ($\% = 100 - (([IL-6] \text{ knock-out mice}) / ([IL-6] \text{ wild-type C57BL/6}) * 100)$) (C).

A	IL-6 [pg/ml]	wt	<i>Tlr4</i> ^{-/-}
	unstimulated		96
LPS		62336	1916
PSB		43596	42829
ENV 1 $\mu\text{g/ml}$		12275	796
ENV 0.5 $\mu\text{g/ml}$		2640	317
ENV 0.25 $\mu\text{g/ml}$		528	83

B	IL-6 [pg/ml]	wt	<i>Cd14</i> ^{-/-}
	unstimulated		21
LPS		39411	24986
PSB		16506	18375
ENV 1 $\mu\text{g/ml}$		66307	991
ENV 0.5 $\mu\text{g/ml}$		21396	480
ENV 0.25 $\mu\text{g/ml}$		827	199

C	IL-6 [% wt]	<i>Tlr4</i> ^{-/-}	<i>Cd14</i> ^{-/-}
	LPS		-97%
PSB		-2%	+11%
ENV 1 $\mu\text{g/ml}$		-94%	-99%
ENV 0.5 $\mu\text{g/ml}$		-88%	-98%
ENV 0.25 $\mu\text{g/ml}$		-84%	-76%

A

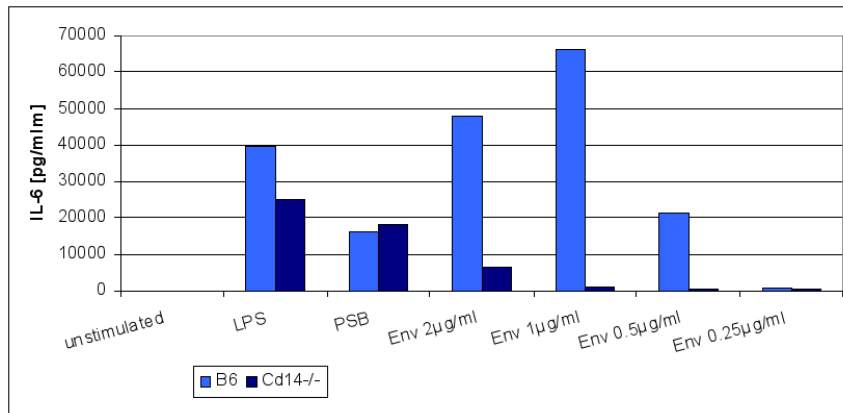
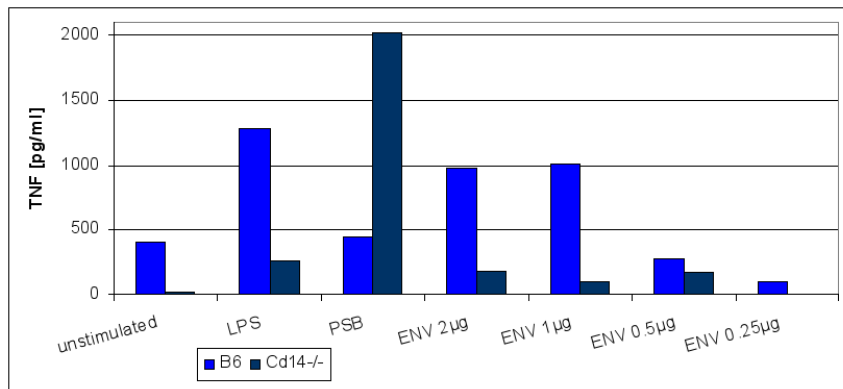


Figure 4.12: Secretion of IL-6 (A) and TNF (B) in BMDC culture supernatants of C57BL/6 and Cd14^{-/-} mice upon stimulation with LPS, PSB and ENV during 24h, assessed by ELISA (ELISA standard deviations <5%).

B



4.1.4 Neutralising Antibodies

The monoclonal antibody 3B2H4 has been designed to neutralize the inflammatory properties of ENV-SU. Its neutralising action had been shown before on human PBMC and monocytes. Here dendritic cells have been incubated with either 0.5 $\mu\text{g}/\text{ml}$ or 1 $\mu\text{g}/\text{ml}$ of ENV-SU and 30 $\mu\text{g}/\text{ml}$ of antibody for 24 h. Before incubation with the cells, ENV-SU is pre-incubated with the antibodies during 1 h at 4°C in PBS. Control samples are incubated with ENV-SU alone, with LPS or without stimulation (NA).

Cultures incubated with ENV-SU and neutralizing antibody show significantly decreased IL-6 production 24h after stimulation compared to cells incubated with ENV-SU alone (see figure 4.13).

Table 4.3 shows calculated percentages of IL-6 production in cells incubated with ENV-SU and 3B2H4 compared to the IL-6 production in cells stimulated with ENV-

4. RESULTS

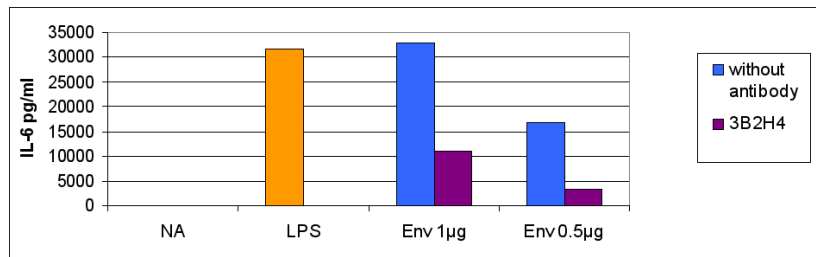


Figure 4.13: *Murine BMDC* were incubated with *LPS*, *ENV* and neutralising monoclonal antibodies (clone *3B2H4*) ; *IL-6* production was measured by *ELISA* in supernatants after 24h of incubation (*ELISA* standard deviations <5%).

Table 4.3: *Inhibition of the ENV-induced IL-6 secretion by wild-type murine BMDCs by the monoclonal antibody 3B2H4. Percentages displayed were calculated in relation to the wild-type IL-6 response (% inhibition = 100 - ((([IL-6] with antibody)/([IL-6] without antibody))*100)).*

	antibody	inhibition
ENV 1 µg/ml	+3B2H4	-66%
ENV 0.5 µg/ml	+3B2H4	-81%

SU alone. The ENV-mediated cellular activation is highly specific and not due to contaminations, since it can be blocked efficiently by anti-ENV antibodies.

4.1.5 *In vivo* Systemic Effects of ENV-SU on Mice

Different concentrations of ENV-SU were administered to healthy C57BL/6 mice by intraperitoneal injection in order to study its systemic effects. Mice were sacrificed after 48h and 72h, the spleens as well as visibly enlarged abdominal lymph nodes (normal lymph nodes for control animals) were isolated and cell analysis was performed by flow cytometry. Total numbers of cells stained by anti-B220, anti-CD3, anti-CD4 and anti-CD8 antibodies were calculated and are displayed in the figures 4.14 and 4.15.

The flow cytometry analysis of both splenocytes and lymph node cells shows an augmentation of the B cell marker B220 in ENV-injected mice 72h after injection

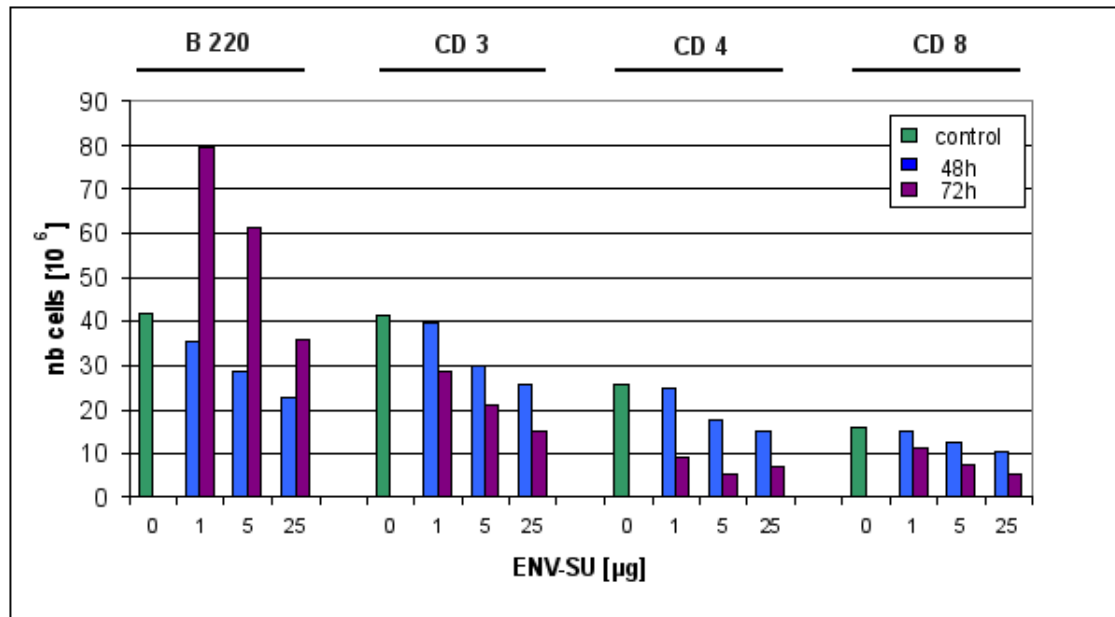


Figure 4.14: Flow cytometry analysis of lymphocyte populations (surface expression of B220, CD3, CD4 and CD8) in splenocytes from ENV-injected C57BL/6 wild-type mice. Mice were sacrificed 48h and 72h after injection of 0-25 µg of ENV.

(see figure 4.14). Compared to control mice, the B cell populations are significantly enlarged after 72h upon ENV injection.

4. RESULTS

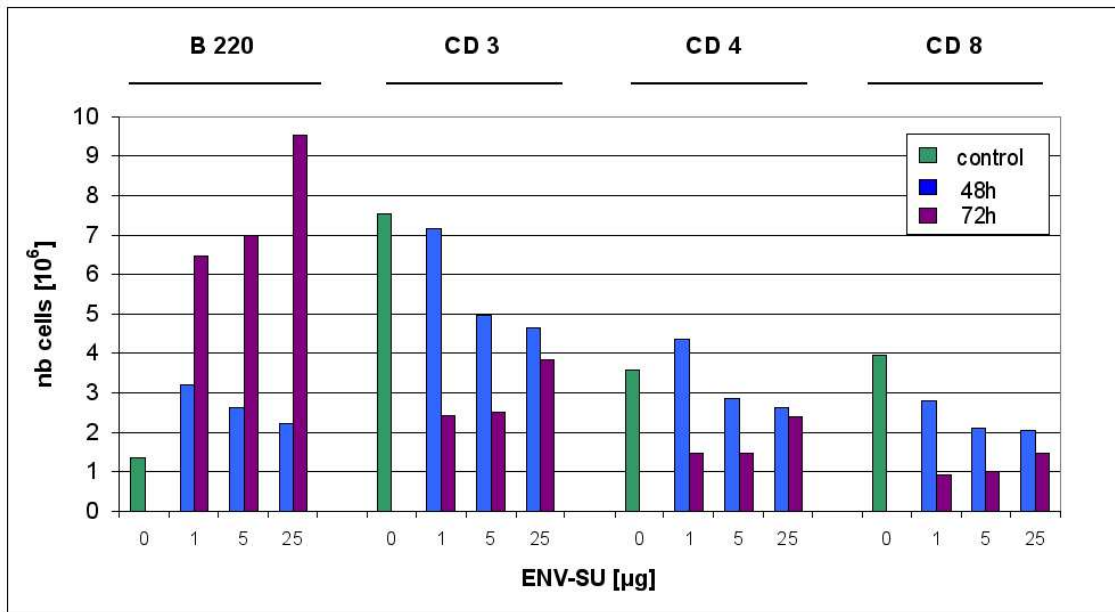


Figure 4.15: *Flow cytometry analysis of lymphocyte populations (B220, CD3, CD4 and CD8 expression) in abdominal lymph node cells of C57BL/6 wild-type mice upon systemic ENV injection. Mice were sacrificed 48h and 72h after injection.*

Regarding T cells, 72h after systemic injection of 1 μg , 5 μg or 25 μg of ENV-SU, the CD3-stained overall T lymphocyte populations are decreased compared to control mice both in spleens and in lymph nodes, whereas the number of B220-stained B lymphocytes is augmented.

Table 4.4: *EAE Clinical Score (CS) Scale for evaluation of symptoms.*

0	no symptoms
1	tail weakness
2	clumsy gait, hind limb weakness
2.5	hind limb weakness, tremor, loss of balance
3	hind limb paresis
4	hind limb paralysis
5	hind limb paralysis, sphincter dysfunction, nasal and ocular haemorrhages, forelimb paresis or paralysis
5.5	moribund stage without immediate death
6	moribund stage without immediate death, respiratory dysfunctions, death

4.2 The *in vivo* Model, Murine EAE

In order to study the *in vivo* effects of ENV-SU, mice were treated for experimental allergic encephalitis (EAE) induction, the mouse model for MS, which consists in antigenic myelin peptide MOG 35-55 immunisation either with complete Freund's adjuvant (CFA) or ENV-SU in incomplete Freund's adjuvant (IFA).

4.2.1 Symptoms

Clinical scoring (table 4.4) showed significant EAE symptoms in both mice but no significant symptoms in control mice, i. e. mice injected with myelin antigen but neither CFA nor ENV/IFA. The two experiments shown in the following included 23 mice each. Preliminary experiments were done to design the appropriate quantity of antigen to inject for immunisation; these experiments are not presented here.

Clinical scores were documented every day for each mouse individually and mean values for each group of mice (IFA, CFA, ENV/IFA 50 μg and ENV/IFA 10 μg in this experiment) were calculated (see figure 4.16).

The clinical score follow-up during 45 days after initial injections shows that disease onset is the earliest and symptoms mount to the highest level in mice injected with 50 μg ENV-SU/IFA (individual disease score 3, hind limb paresis).

4. RESULTS

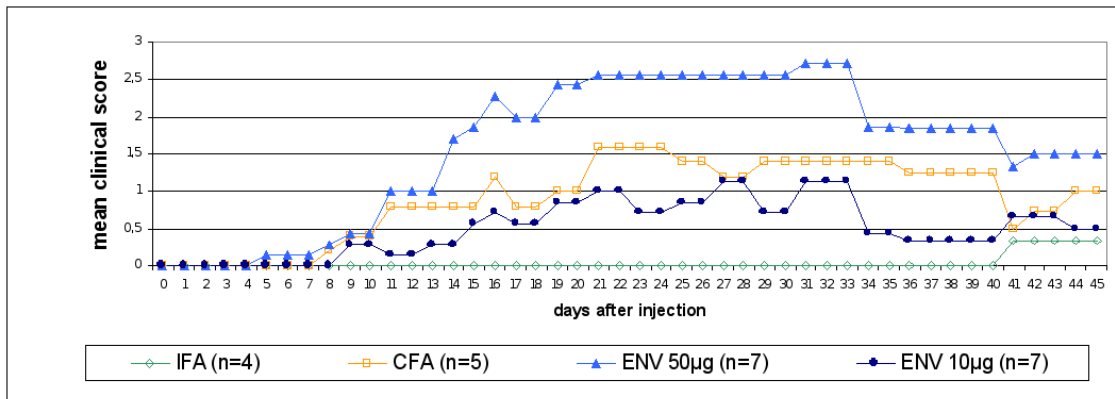


Figure 4.16: *Clinical Score follow-up of C57BL/6 mice injected with MOG 35-55 and IFA, CFA or ENV/IFA. Mean values were calculated for each group of mice.*

In table 4.5 disease onset, disease score at day 35, maximum score and cumulative score at day 35 of each mouse are shown. The cumulative score was calculated by additionning the clinical score values of each day during a period of 35 days.

To compare groups of mice, mean values of disease onset, mean scores at day 35, mean maximum scores and mean cumulative scores at day 35 were calculated and are displayed in table 4.6. Disease incident was recorded on day 35 and no IFA control mice showed EAE symptoms.

The comparison shows that all mice injected either ENV/IFA or CFA develop EAE symptoms according to the clinical score table. Disease onset is earlier and scores are higher in mice injected with 50 μg of ENV/IFA (mean maximum CS 2.9) compared to mice injected with CFA that represent the positive EAE control (mean maximum CS 1.8). Mice injected with 10 μg of ENV/IFA develop symptoms later and scores are lower (mean maximum CS 1.6).

In the following EAE experiment that reproduces the same groups of mice, we added a group of mice injected with 25 μg of ENV/IFA as well as a group of mice injected with 50 μg of ENV in IFA without the MOG peptide. Mice were scored every day individually and follow-up was pursued until day 50 after injections (see figure 4.17).

The dose-dependency of the action of ENV was reproduced in this experiment, even if only slight differences are visible between the 25 μg and the 50 μg group.

Table 4.5: *Disease onset, clinical score at day 35, maximum clinical score and cumulative score at day 35 for each mouse. Cumulative scores represent sums of individual daily scores of each mouse.*

		disease onset (d)	score d35	max score	cumulative score d35
IFA	1	—	0	0	0
	2	—	0	0	0
	3	—	0	0	0
	4	—	0	0	0
CFA	5	9	2	2	34
	6	11	1	2	32
	7	11	2	2	43
	8	9	1	2	32
	9	21	1	1	15
ENV 50 μg	10	11	3	3	59
	11	11	1	3	58
	12	11	1	3	45
	13	9	3	3	74
	14	9	2	3	64
	15	5	2	3	71
	16	8	1	2	27
ENV 10 μg	17	16	0	1	10
	18	19	0	1	2
	19	17	1	2	32
	20	19	0	2	16
	21	9	2	2	34
	22	9	0	2	29
	23	13	0	1	8

4. RESULTS

Table 4.6: *Disease incident, mean disease onset, mean clinical score at day 35, mean maximum score and mean cumulative score at day 35 were calculated for each group of mice to resume this EAE experiment.*

	dis. incident (nb)	mean dis. onset (d)	mean CS d35	mean max CS	mean cum. score d35
IFA	0/4	—	0	0	0
CFA	5/5	12.2	1.4	1.8	31.2
ENV 50 μg	7/7	9.1	1.9	2.9	56.9
ENV 10 μg	7/7	14.6	0.4	1.6	18.7

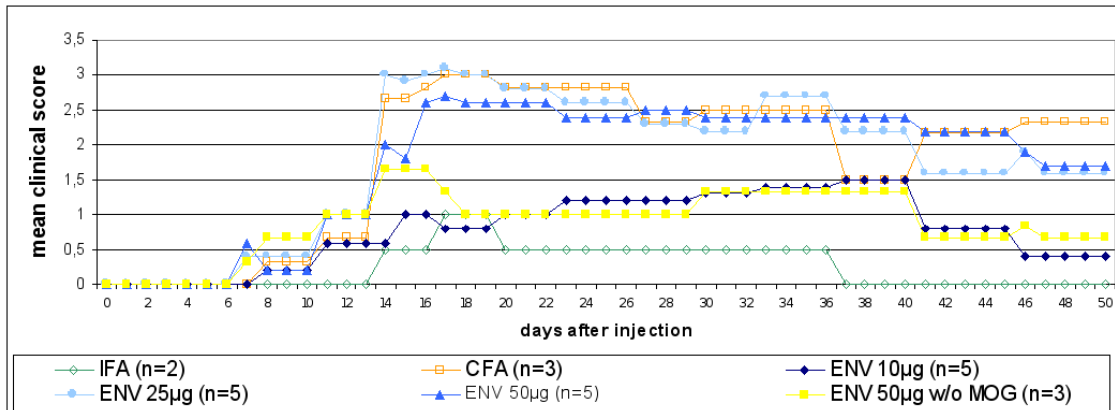


Figure 4.17: *Clinical score follow-up of C57BL/6 mice injected with IFA, CFA, different concentrations of ENV/IFA and ENV/IFA without the MOG peptide. Mean scores per group until day 50 are displayed.*

Mice injected with 50 μg of ENV/IFA alone (w/o MOG) display EAE symptoms according to the clinical score scale.

IFA injected control mice temporarily developed weak symptoms (CS 1, tail weakness) that disappeared in the course of the experiment. The impairments we observed are not necessarily classified as EAE symptoms and might be due to local complications at the injection site.

The individual follow-up (table 4.7) shows that the highest maximum scores were reached in the groups injected with 25 μg or 50 μg of ENV/IFA (CS 4, hind limb paralysis), whereas the highest cumulative score was calculated in the CFA group.

Table 4.7: Individual follow-up of injected mice including disease onset, clinical score at day 50, maximum score and cumulative score at day 50. Cumulative scores were calculated as the sum of daily individual scores up to day 50.

	disease onset (d)	score d50	max score	cumulative score d50	
IFA	1	17	0	1	20
	2	14	0	1	6
CFA	3	14	2	3	78
	4	8	2.5	3	109
	5	11	2.5	3	92
ENV 10 μg	6	11	2	2.5	64.5
	7	11	0	2.5	58
	8	14	0	1	28
	9	11	0	1	26
	10	8	0	1	25
ENV 25 μg	11	11	1	3	64.5
	12	11	3	4	136
	13	7	1	2.5	78.5
	14	7	1	4	87
	15	11	2	4	88
ENV 50 μg	16	11	1	1	40
	17	7	2.5	3	104
	18	11	2	3	102.5
	19	7	1	4	95
	20	7	2	3	106
ENV 50 μg w/o MOG	21	8	0	2	38
	22	11	2	2.5	43.5
	23	7	0	2	54

4. RESULTS

Table 4.8: *Disease incidence and mean values per group of mice for disease onset, clinical score at day 50, maximum score and cumulative score at day 50.*

	dis. incident (nb)	mean dis. onset (d)	mean CS d50	mean max CS	mean cum. score d50
IFA	2/2	15.5	0	1	13
CFA	3/3	11	2.3	3	93
ENV 10 μg	5/5	11	0.4	1.6	40.3
ENV 25 μg	5/5	9.4	1.8	3.5	90.8
ENV 50 μg	5/5	8.6	1.7	2.8	89.5
ENV 50 μg w/o MOG	3/3	8.7	0.7	2.2	45.2

In this experiment, the daily follow-up of the mice included determination of body weight between day 7 and day 50 to monitor a potential relation between disease course and loss of body weight. In figure 4.18 are presented the mean weights of each group of mice (IFA, CFA, ENV/IFA 10 μg , ENV/IFA 25 μg , ENV/IFA 50 μg and ENV/IFA 50 μg w/o MOG) and the corresponding clinical scores (same colours).

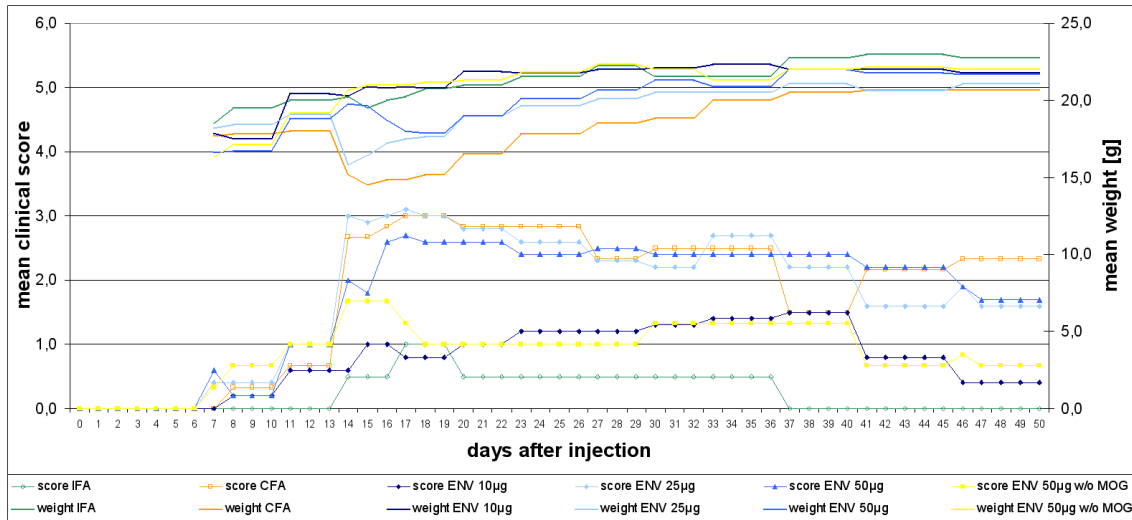


Figure 4.18: Mean body weights and corresponding mean clinical scores per group of mice (same colours).

The groups of mice showing the highest disease scores were CFA, ENV/IFA 25 μg and ENV/IFA 50 μg . For these three groups of mice a loss of body weight can be noticed that coincides with the appearance of strong symptoms higher than score 2 (clumsy gait, hind limb weakness).

4.2.2 Autoreactive T Cells

Cultures of splenocytes from either ENV-SU/IFA or adjuvant treated mice recalled with the myelin antigen led to IFN- γ production in some mice, suggesting T lymphocyte reactivity towards the myelin antigen.

In a first step, to validate experimental procedures, the capacity of splenocytes to secrete IFN- γ upon stimulation as well as the reactivity of naïve splenocytes towards MOG was tested on frozen and freshly collected splenocytes (see figure 4.19).

Phytohaemagglutinin (PHA) induces significant IFN- γ secretion both in fresh and in frozen splenocytes, whereas the MOG 35-55 peptide does not show any effect. The IFN- γ response upon PHA stimulation is decreased in frozen cells compared to freshly collected cells, but the same dose-dependant effect with saturation at 10 $\mu\text{g}/\text{ml}$ (and possible cell death induction at 20 $\mu\text{g}/\text{ml}$) is visible.

4. RESULTS

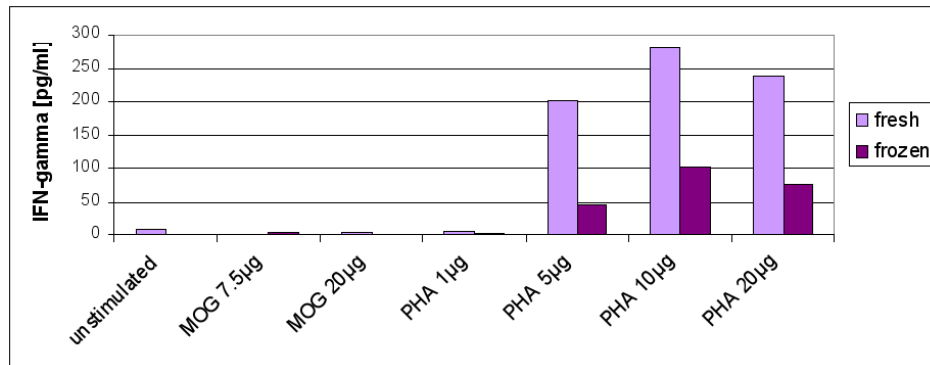


Figure 4.19: *Reactivity of freshly collected and frozen splenocytes upon incubation with MOG or PHA for 24h. IFN- γ secretion in culture supernatants was assessed by ELISA (ELISA standard deviations <5%).*

Splenocytes from EAE mice were collected and IFN- γ production upon MOG stimulation has been tested by ELISA. Figure 4.20 represents IFN- γ secretion of pooled splenocytes from 2 mice for the IFA group and 3 mice for the ENV-SU/IFA group.

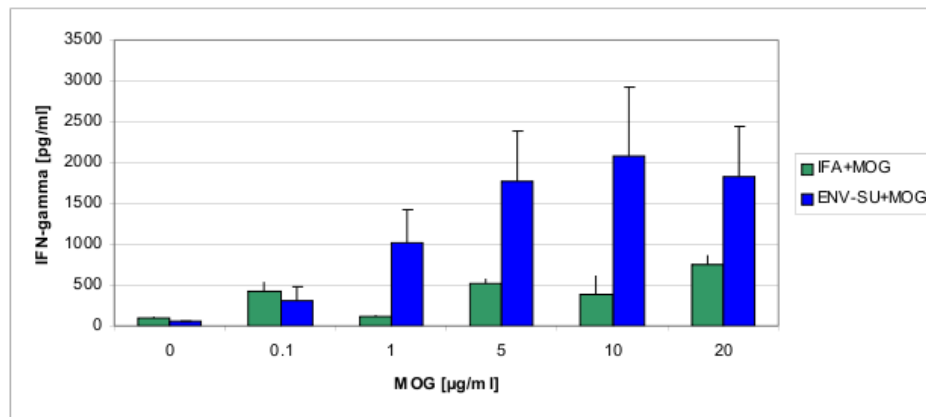


Figure 4.20: *IFN- γ secretion of pooled splenocytes of EAE mice assessed by ELISA of culture supernatants 24h after stimulation (2 mice for the IFA group and 3 mice for the ENV-SU/IFA group).*

Pooled splenocytes of mice injected with IFA and MOG show low levels of IFN- γ secretion, whereas splenocytes of ENV-SU/IFA mice produce significant amounts of IFN- γ in a dose-dependant manner with a plateau at 10 $\mu\text{g/ml}$ of MOG.

The kinetics of IFN- γ production has been tested for the same pools of splenocytes during 72h of incubation with 20 $\mu\text{g}/\text{ml}$ of MOG; samples were taken after 24h, 48h and 72h (see figure 4.21).

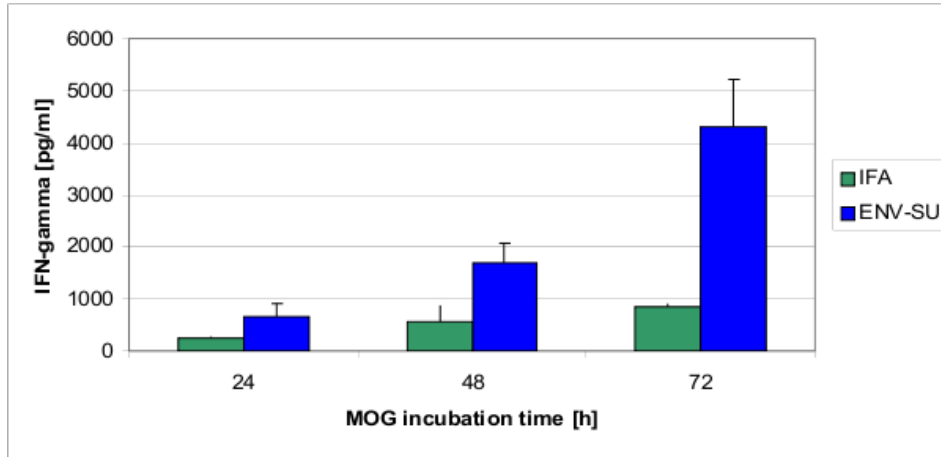


Figure 4.21: Kinetics of IFN- γ production upon MOG recall on pooled splenocytes of IFA and ENV-SU/IFA injected EAE mice. IFN- γ secretion was assessed in culture supernatants after 24h, 48h and 72h of incubation (2 mice for the IFA group and 3 mice for the ENV-SU/IFA group).

In another experiment, splenocytes of individual mice have been tested for IFN- γ production upon MOG recall stimulation (figure 4.22).

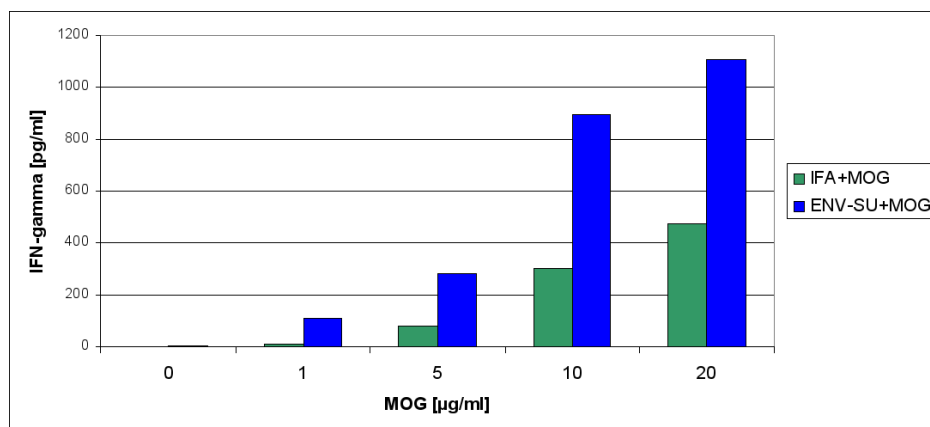


Figure 4.22: MOG recall of EAE splenocytes of one IFA mouse and one ENV-SU/IFA mouse. IFN- γ production was measured by ELISA of culture supernatants after 24h of incubation with MOG (ELISA standard deviations <5%).

4. RESULTS

These results are representative for several experiments of the same kind; in each experiment, we observed diseased mice that did respond upon MOG stimulation and others that did not.

4.2.3 MRI

MRI imaging has been done to visualize the effects of classical and ENV/IFA-mediated EAE immunisation on C57BL/6 wild-type mice. The following images (figures 4.23 to 4.27, representing one healthy IFA/MOG mouse, 3 diseased CFA/MOG mice and 6 diseased ENV-IFA/MOG mice) have been acquired by Magnetic Resonance Imaging (MRI) at the animal facility ANIMAGE in Bron/Lyon (see appendix C).

Since MRI shows hydrogen atoms, white zones that differ between healthy and injected diseased animals are due to accumulation of extracellular water and point to demyelinating lesions with lymphocyte infiltration and inflammation.

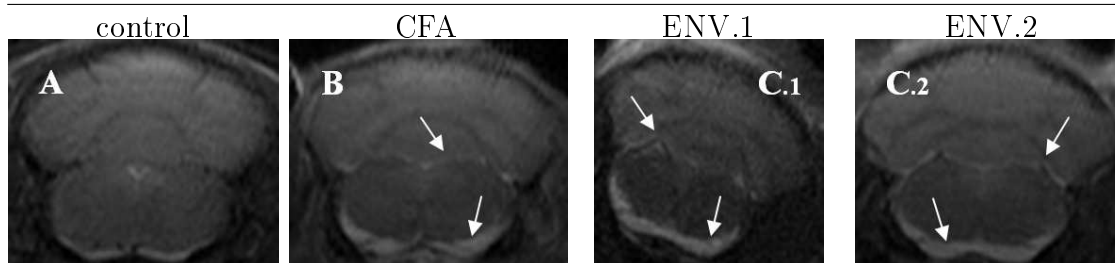


Figure 4.23: *Coronal Magnetic resonance imaging (MRI) of the cervical medulla, hindbrain, pons and cerebellum of a healthy control mouse (A), a diseased EAE mouse injected with CFA+MOG (B) and two diseased EAE mice injected with ENV-SU/IFA+MOG (C.1 and C.2). White zones correspond to liquid accumulation and thus represent infiltration and inflammation (arrows).*

Figure 4.23 compares the cervical part of the medulla and the beginning hind-brain of an IFA+MOG injected control mouse that was without symptoms (A) with two diseased mice injected with CFA+MOG (B) and ENV/IFA+MOG (C). The white zones in CFA and ENV/IFA mice are located in the central area between pons and cerebellum and in the ventral area (indicated with arrows); they represent accumulation of liquid pointing to immune infiltration probably corresponding to demyelination spots.

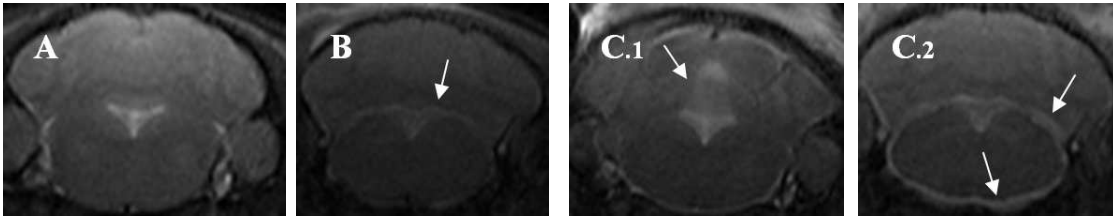


Figure 4.24: *Coronal MRI of the hindbrain, pons and cerebellum of a healthy control mouse (A) and diseased EAE mice injected with CFA (B) or ENV/IFA (C.1 and C.2).*

The acquisition of MRI images was successively advancing from caudal to rostral; the infiltration zones shown in figure 4.24 are situated in the hindbrain and indicate that the infiltrates observed in the cervical medulla (figure 4.23) are not artefacts or punctual anomalies but spread continuously throughout different brain areas. Around the lateral ventricles bordering the fourth ventricle in the shown cross section, liquid accumulation is visibly stronger in CFA and ENV/IFA mice.

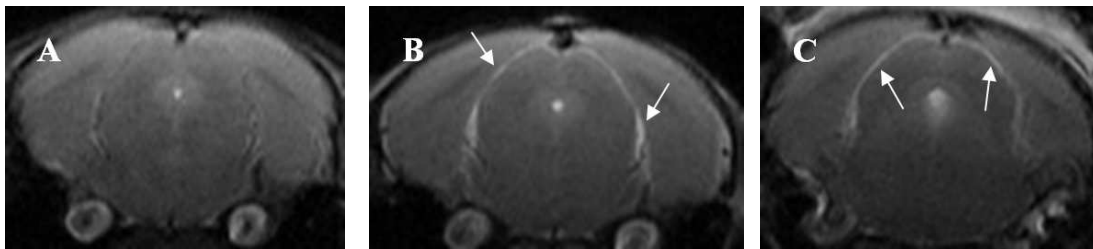


Figure 4.25: *Coronal MRI of the midbrain of a healthy control mouse (A) and diseased EAE mice injected with CFA (B) or ENV/IFA (C).*

Reaching midbrain, the same observations can be made (figure 4.25). In both CFA (B) and ENV/IFA mice (C), white zones appear in both cerebral hemispheres that are not present in control IFA mice (A). The infiltration is localised around the lateral ventricles that in this section cross the brain from dorsal to ventral.

As shown in figure 4.26, significant liquid accumulation representing immune invasion (lymphocyte infiltration) is detectable in the hippocampal area that lies between the adjacent lateral ventricles and the rostral brain stem.

The coronal MRI section of the olfactory bulbs (figure 4.27) shows that the inner surfaces of both bulbs are affected in CFA (B) and ENV/IFA mice (C), whereas in the control IFA mouse (A), no infiltration is visible.

4. RESULTS

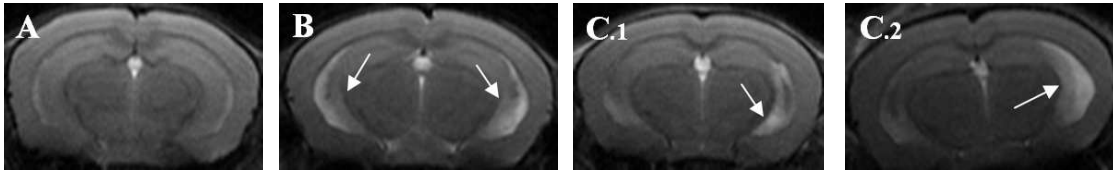


Figure 4.26: *Coronal MRI of the forebrain and cerebrum of a healthy control mouse (A) and diseased EAE mice injected with CFA (B) or ENV/IFA (C.1 and C.2).*

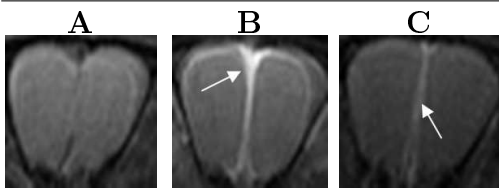


Figure 4.27: *Coronal MRI of the olfactory bulbs of a healthy control mouse (A) and diseased EAE mice injected with CFA (B) or ENV/IFA (C).*

By acquiring successive MRI images throughout the mouse brain from caudal to rostral, we discover that both in CFA and ENV/IFA mice diseased with EAE different regions are affected, and that our findings are not due to visual artefacts. Regarding the intensity and the localisation of the spots, we observe close similarity between CFA and ENV/IFA mice and therefore conclude that ENV/IFA-induced EAE causes brain damage similar to classical CFA-induced EAE.

4.3 Transgenic Mouse Model

In order to generate a mouse model expressing ENV on different levels of distribution inside the organism, different genetic constructions including different promoters have been created:

- pCMV: ubiquitous human promoter
- pGFAP: human astroglial cell promoter that has been reported to be functional in mice, astrocytes being the cells expressing ENV in the human system
- pMHCII: murine monocyte and dendritic cell promoter.

As a second step, the coding sequences for MSRV-ENV and HERV7q-ENV were cloned under the control of these three promoters (figure 4.28). The resulting constructions were tested *in vitro* to validate the transgenes before utilising them to derive transgenic ENV mice.

In a third step, transmembrane (TM) sequences of MSRV-ENV and HERV7q-ENV were interchanged in order to the structural and functional differences of the two ENV proteins.

4.3.1 Validation of the Promoters

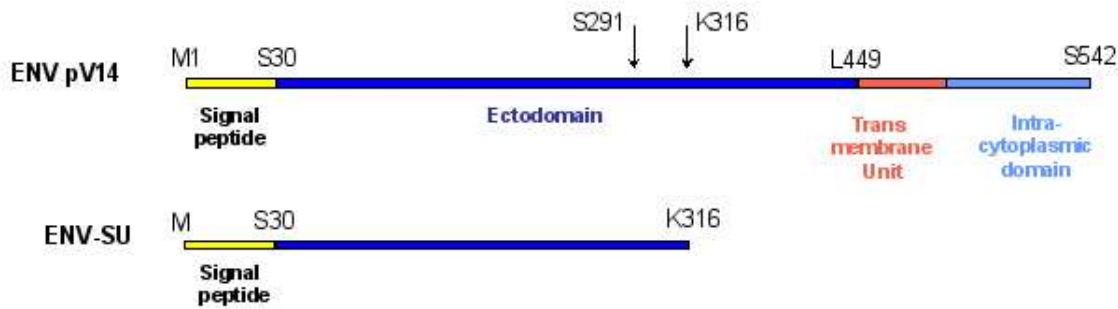
Several experiments of transfection into different cell lines have been performed in order to validate the different genetic constructions. For the pCMV constructs, HEK293T cells were transfected, for pMHCII murine J774 macrophages were chosen, and pGFAP constructs were transfected into murine glial GL26 cells. All cells were chemically permeabilised in order to allow an intracellular staining with the monoclonal anti-ENV antibody.

pCMV

Lipofectamine 2000 transfections of HEK293T cells with pCMV-MSRV-ENV and pCMV-HERV7q-ENV are shown in figure 4.29. In both cases, the ENV protein revealed with anti-ENV antibody is present.

4. RESULTS

A



B

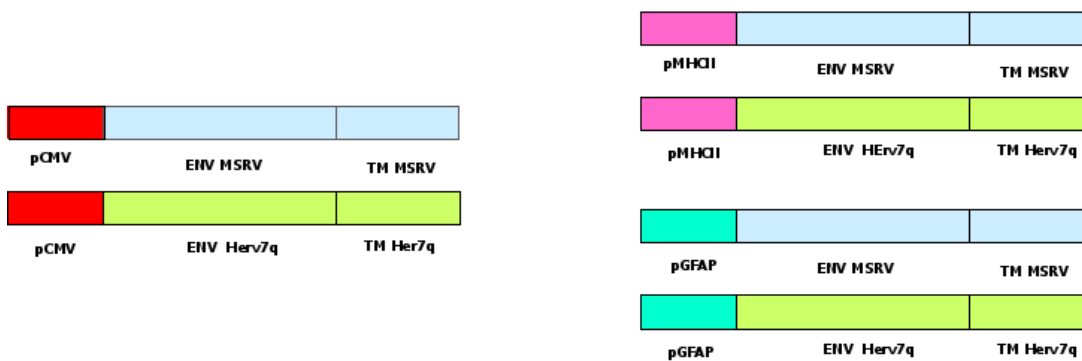


Figure 4.28: Sequences of the MSRV envelope proteins ENV and ENV-SU derived from human cDNA (A) and schematic representation of the genetic constructions including the promoters pCMV, pGFAP and pMHCII as well as the proteins MSRV-ENV and HERV7q-ENV (B). The sequences of the ENV proteins are composed of ENV-SU (soluble subunit, main part) and ENV-TM (transmembrane unit).

Figure 4.29 shows that both in pCMV-MSRV-ENV and pCMV-HERV7q-ENV transfected cells ENV is expressed under the control of the pCMV promoter (green fluorescence). The HERV7q-ENV (figure 4.29 B) is diffusely distributed all over the cytoplasm, whereas MSRV-ENV (figure 4.29 A) is accumulating around distinct structures.

These results validate the genetic constructions and the structural integrity of the ENV protein, or at least of the epitope recognized by the anti-ENV antibody.

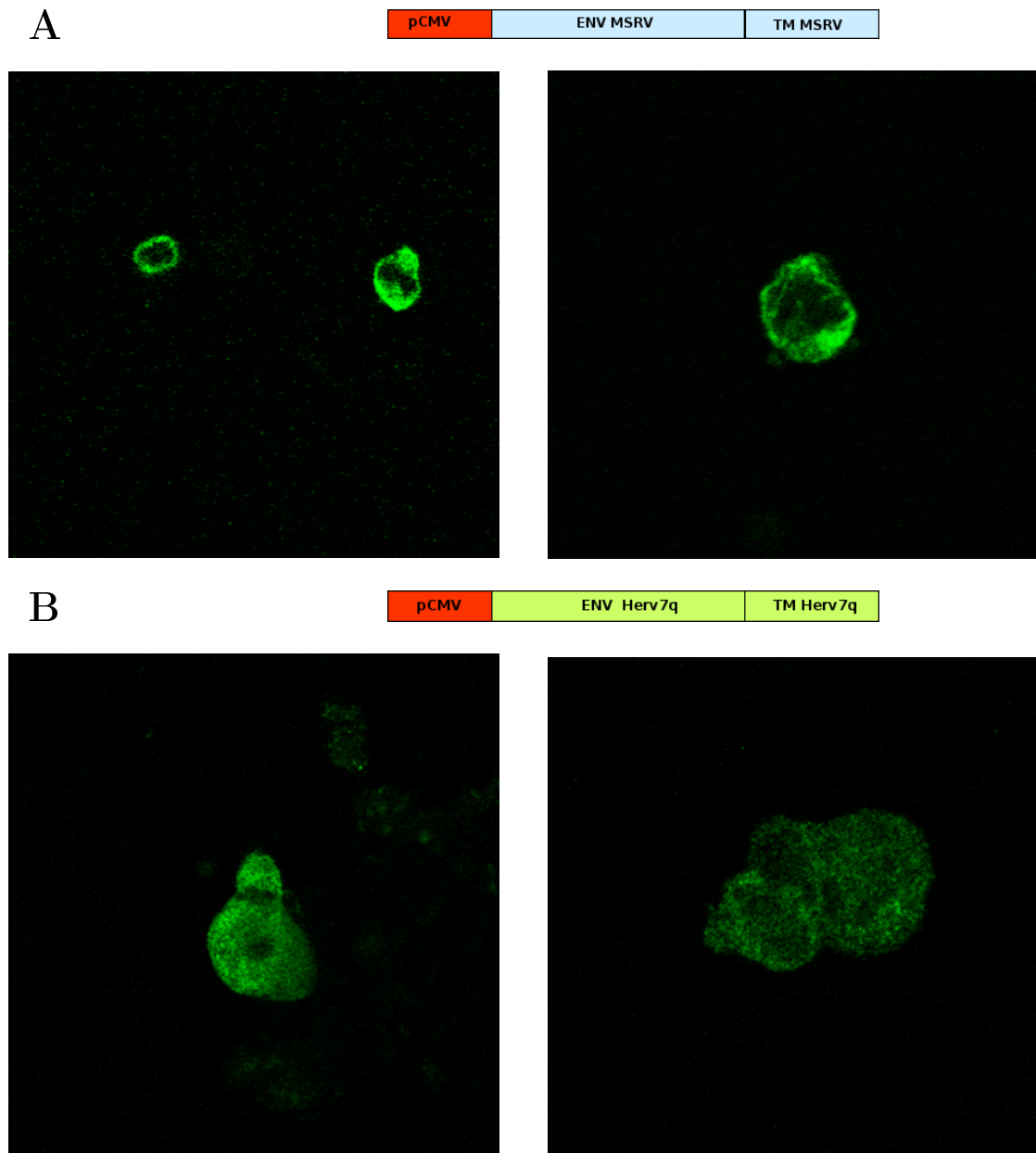


Figure 4.29: Confocal microscopy of HEK293T cells transfected with (A) pCMV-MSRV-ENV and (B) pCMV-HERV7q-ENV, revealed with the monoclonal anti-ENV antibody 3B2H4 and a green-fluorescent secondary antibody.

pMHCII

The functionality of the pMHCII promoter has been studied by transfecting the constructions pMHCII-MSRV-ENV and pMHCII-HERV7q-ENV into the murine macrophage cell line J774. The ENV protein could be evidenced in both cases

4. RESULTS

by antibody staining and confocal microscopy (see figure 4.30).

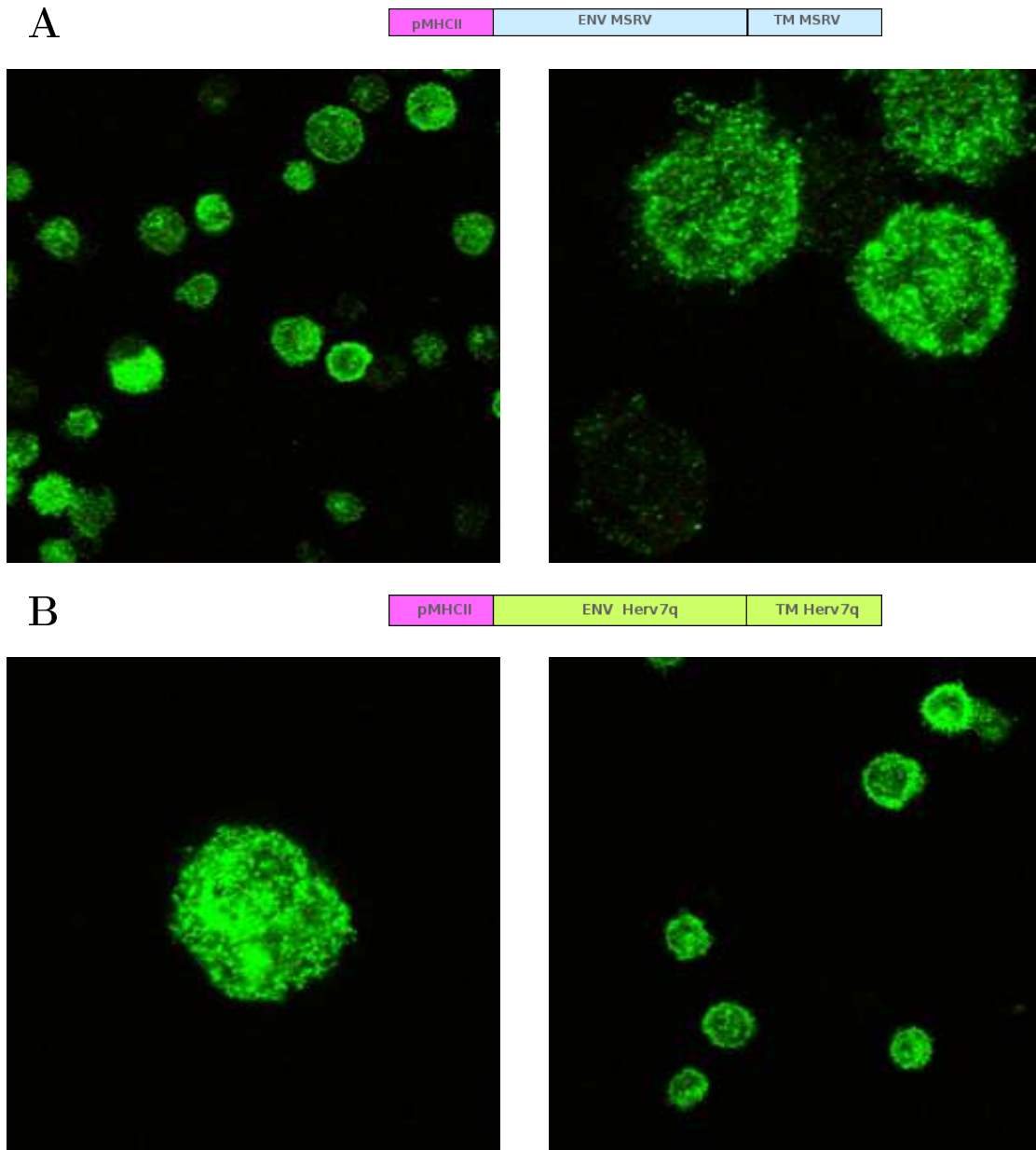


Figure 4.30: *Transfection of pMHCII-MSRV-ENV (A) and pMHCII-HERV7q-ENV (B) into the murine macrophage cell line J774, visualized by confocal microscopy after antibody staining with the anti-ENV 3B2H4 and a green-fluorescent secondary antibody.*

Both pMHCII-MSRV-ENV and pMHCII-HERV7q-ENV transfected cells displaying ENV expression are detectable, suggesting that both pMHCII constructs are functional in murine macrophages J774. In both cases, the ENV signal is spread



Figure 4.31: *Schematic representation of the pGFAP constructs including GFP, MSR V-ENV or HERV7q-ENV.*

diffusely inside the cell (figure 4.30).

These two transgenes have been sent to the SEAT platform (Service d'Expérimentation Animale et de Transgénése, CNRS, Villejuif) for the creation of the transgenic animals. To date, 6 mice carry the transgene pMHCII-HERV7q-ENV and 16 are positive for pMHCII-MSRV-ENV. These founders transmitted the transgene in their offsprings. Mice are screened for ENV expression by several assays on peritoneal cells and splenocytes including immunofluorescent labelling with anti-ENV antibodies to detect the ENV protein and PCR to measure the transcription of ENV.

pGFAP

Transfections with pGFAP-GFP (green fluorescent protein), pGFAP-MSRV-ENV and pGFAP-HERV7q-ENV have been carried out to validate the pGFAP promoter. The constructs shown in figure 4.31 have been transfected into murine glial GL26 and cells by Eugene HD transfection, but their functionality has not yet been established because of difficulties of reproducibility.

4.3.2 Localisation of ENV

The confocal microscopy images of the different transfection assays confirm the functionality of the constructs and suggest a slightly different cellular repartition of those carrying MSR V-ENV and those carrying HERV7q-ENV. Further localisation assays with the pCMV constructs have been realized on HEK293T cells; confocal microscopy was carried out after antibody staining for ENV (green) and the Golgi apparatus (red), and DAPI staining for the nucleus (blue) (figures 4.32 and 4.33).

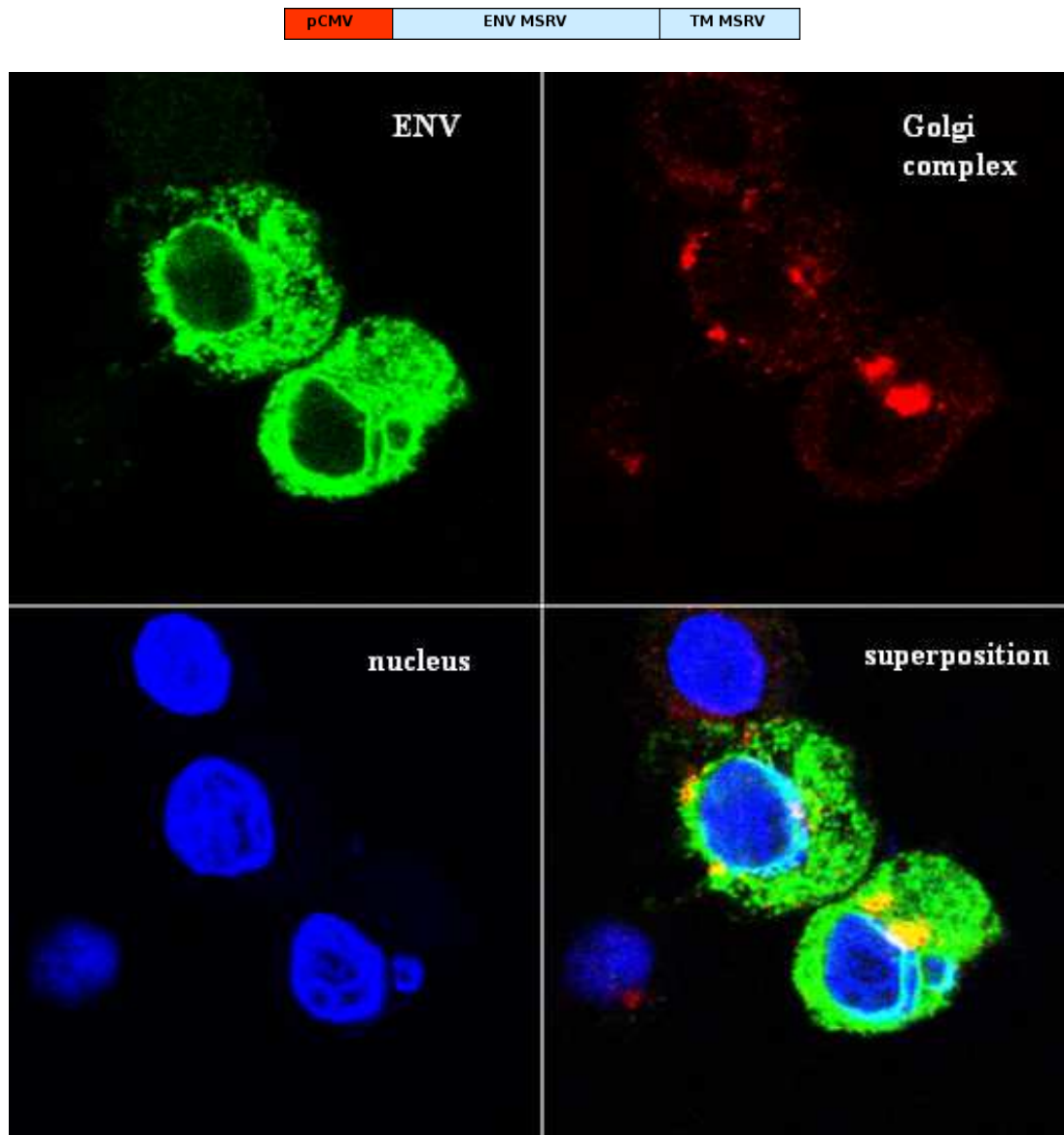


Figure 4.32: *Localisation of the MSRV-ENV inside HEK293T cells. Confocal microscopy was done after antibody staining targeting ENV (green fluorescence) and the Golgi apparatus (red fluorescence); DAPI staining was used for the nucleus (blue fluorescence) and superposition of green, red and blue fluorescence is displayed.*

For MSRV-ENV, signals are pointing to an accumulation in the peri-membrane area, and a clear co-localisation with the Golgi complex is visible (figure 4.32), whereas HERV7q-ENV shows diffuse distribution in the cytoplasm and no co-localisation with any particular structure of the cell (figure 4.33). Furthermore, as described in the literature, HERV7q-ENV forms syncytia after 24h of culture.

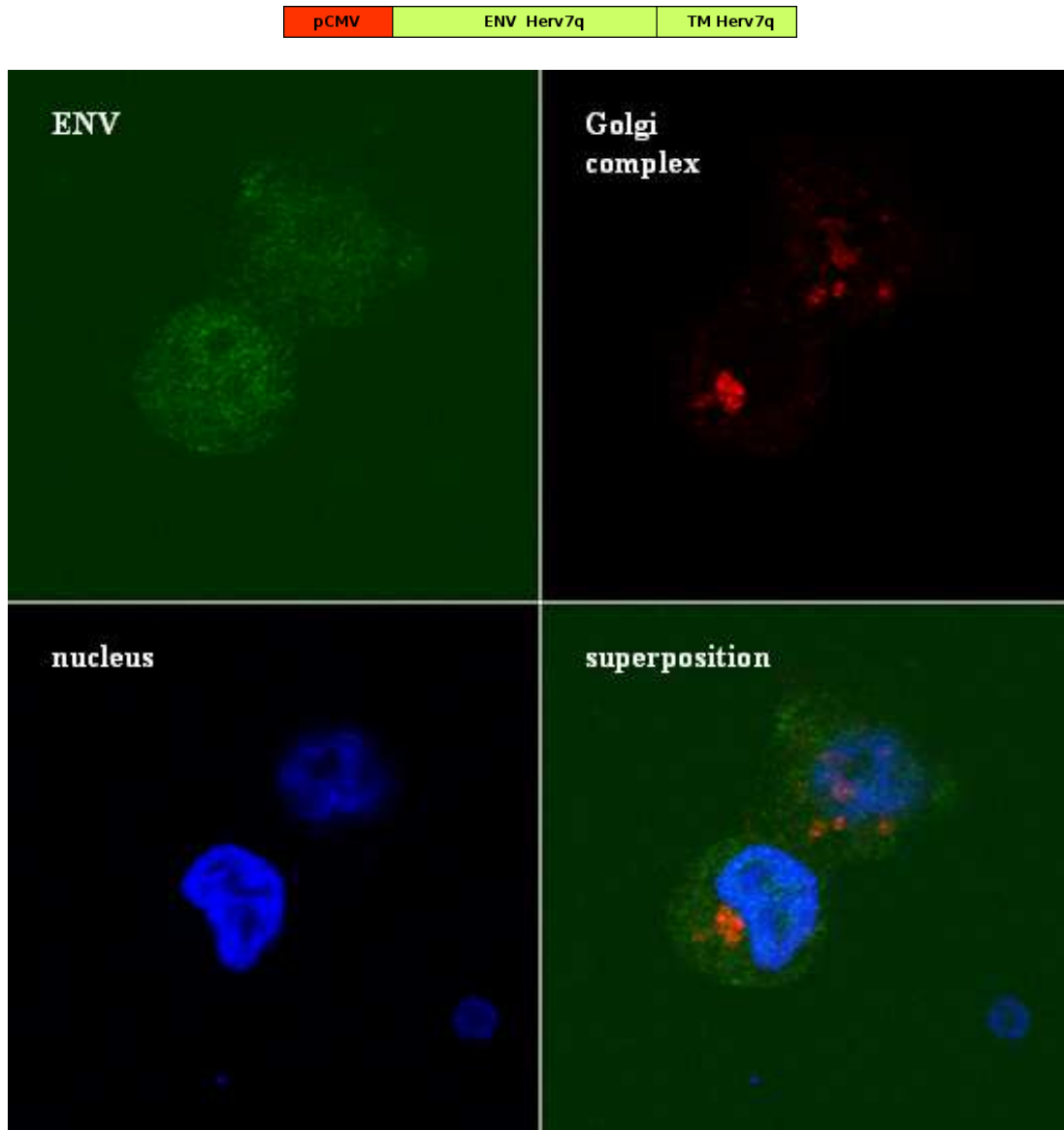


Figure 4.33: Localisation of the *HERV7q-ENV* inside *HEK293T* cells. Confocal microscopy was done after antibody staining targeting *ENV* (green fluorescence) and the Golgi apparatus (red fluorescence); DAPI staining was used for the nucleus (blue fluorescence) and superposition of green, red and blue fluorescence is displayed.

4.3.3 Sequence Shuffling

To know more about the differences between *MSRV-ENV* and *HERV7q-ENV* that could explain their different cellular distribution, we studied their sequences (see figure 4.34) and performed sequence shuffling experiments.

4. RESULTS

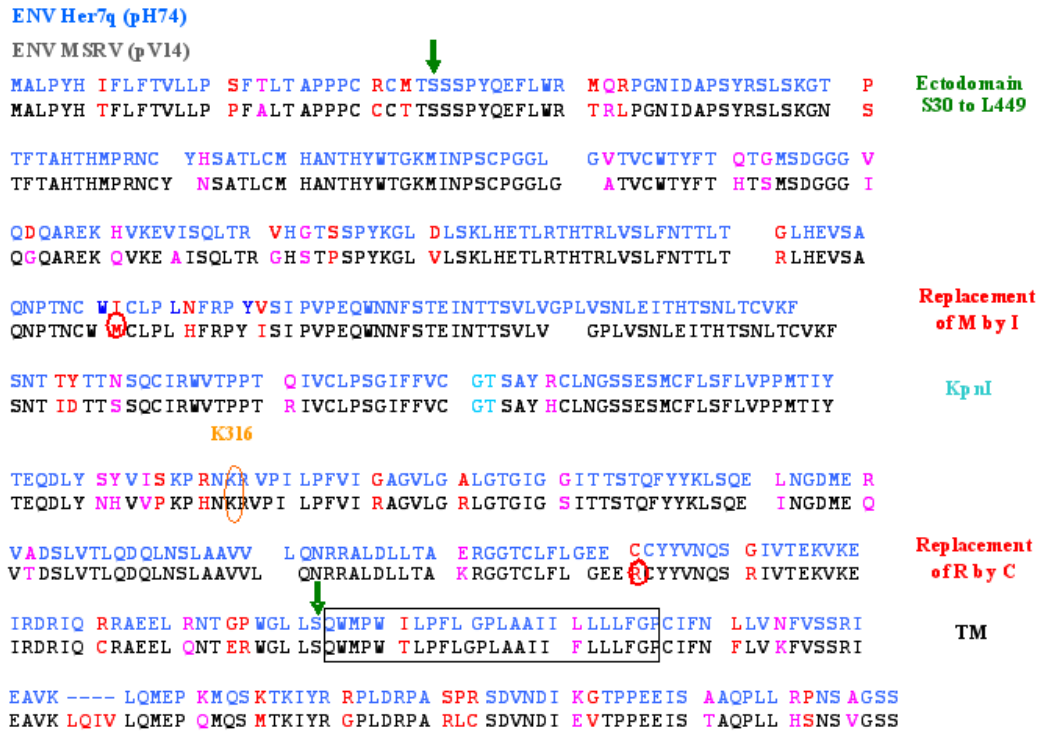


Figure 4.34: Amino acid sequences of MSRV-ENV (cDNA plasmid pV14, black) and HERV7q-ENV (cDNA plasmid pH74, blue), comprising the ectodomain (green arrows) and the transmembrane unit (TM, black box). The natural cleavage site K316 is shown in orange, conservative mutations are pink and mutations altering the biochemical properties are red; the two encircled sites (red) participate in disulfide bridge formation and these mutations are thus suspected to alter the structural integrity of the protein.

The sequences that we compare in figure 4.34 comprise the ectodomain and the transmembrane domain of MSRV-ENV and HERV7q-ENV. The most important differences between the two sequences are two mutation sites (encircled in red) concerning two cysteines in the HERV7q copy that are involved in the formation of disulfide bridges. The KpnI restriction site (light blue) that has been chosen for the exchange of the TM units (sequence shuffling) is located between these two mutations.

The constructions pCMV-MSRV-ENV/HERV7q-TM and pCMV-HERV7q-ENV/MSRV-TM shown in figure 4.35 were transfected into HEK293T cells and confocal microscopy was performed to evidence distribution of ENV inside the cell (see figures 4.36 and 4.37).

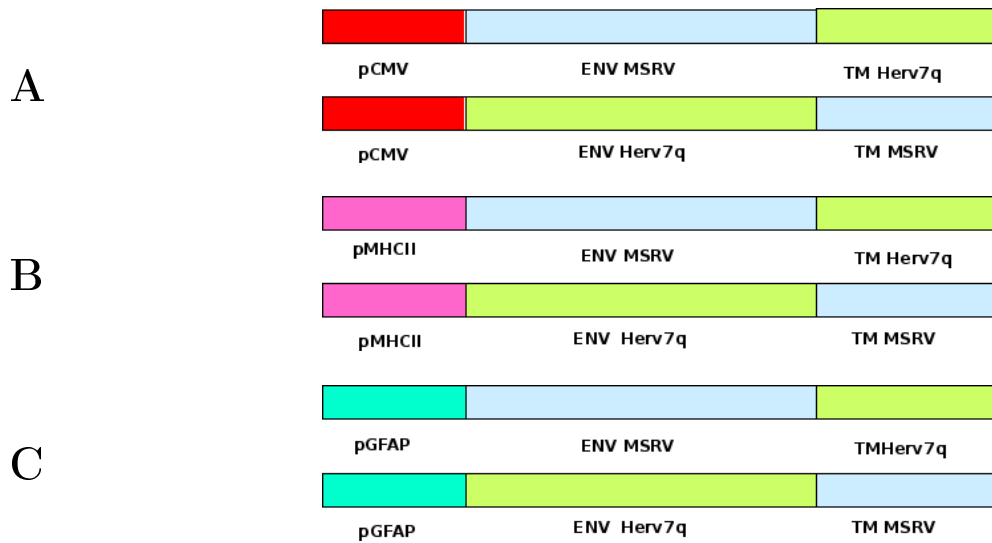


Figure 4.35: *Genetic constructions including the three promoters pCMV (A), pMHCII (B) and pGFAP (C). The transmembrane domains (TM) of the MSRV-ENV and HERV7q-ENV sequences have been interchanged at their common KpnI restriction site.*

In the case of pCMV-MSRV-ENV/HERV7q-TM (figure 4.36), the repartition of ENV inside the cell (green) is diffuse, cells are forming syncytia carrying several nuclei and no co-localisation with any distinct structure is visible. pCMV-HERV7q-ENV/MSRV-TM transfected cells give an image similar to those transfected with the full MSRV-ENV protein (figure 4.37), since ENV is co-localised with the Golgi complex (red) around the nucleus (blue), and also because no syncytium formation is detectable.

To resume the results concerning all transfections done on HEK293T cells, the HERV7q-TM domain (in combination with both MSRV-ENV or HERV7q-ENV) allows for syncytium formation and a diffuse distribution of ENV inside the cell without association to distinct structures, whereas the MSRV-TM confers to the full protein the properties of peri-membrane distribution and co-localisation with the Golgi apparatus around the nucleus.

4. RESULTS

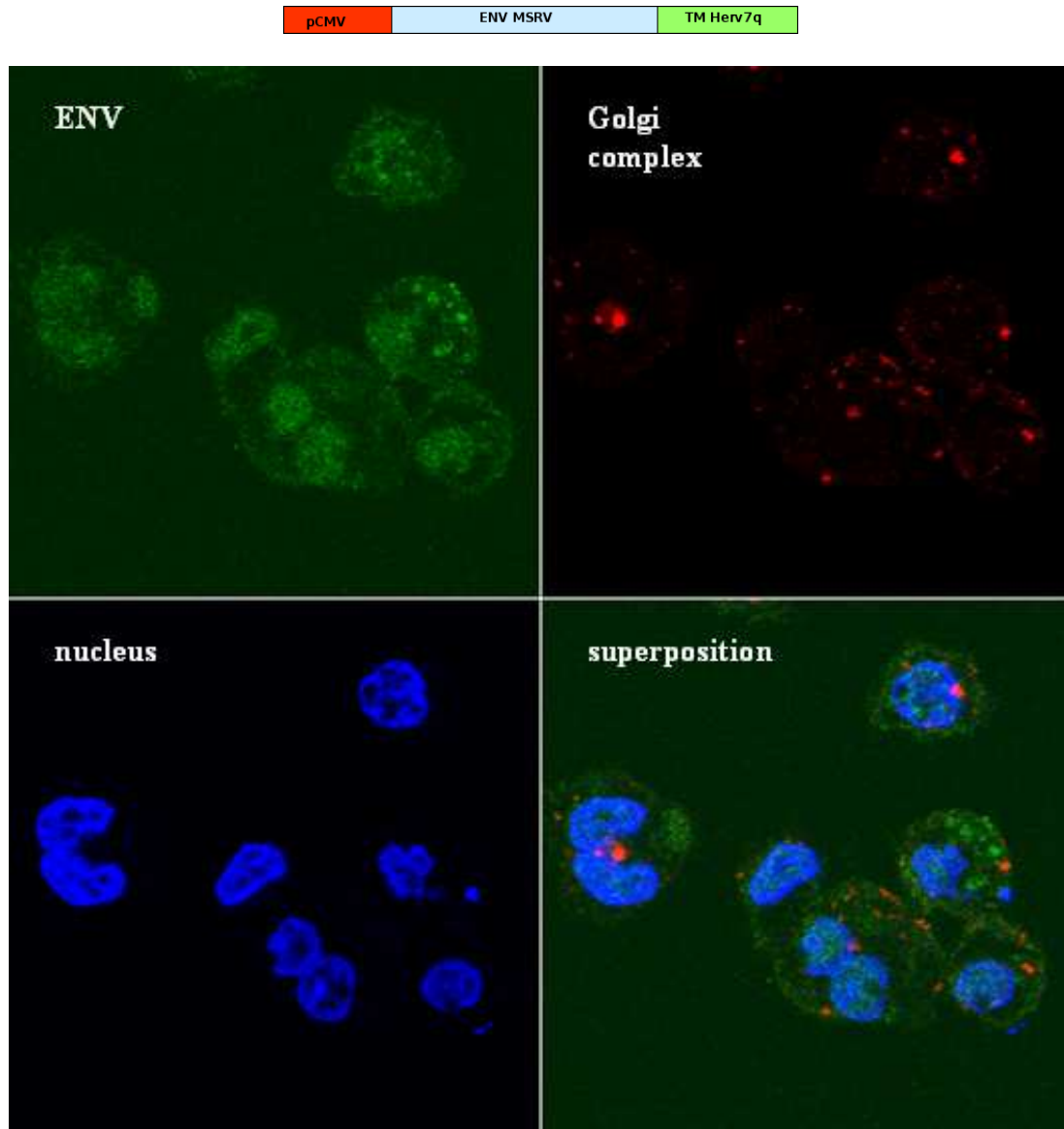


Figure 4.36: *Confocal microscopy of HEK293T cells transfected with pCMV-MSRV-ENV/HERV7q-TM; antibody staining for ENV (green) and the Golgi apparatus (red), DAPI staining for the nucleus (blue); superposition of green and red fluorescence shows yellow.*

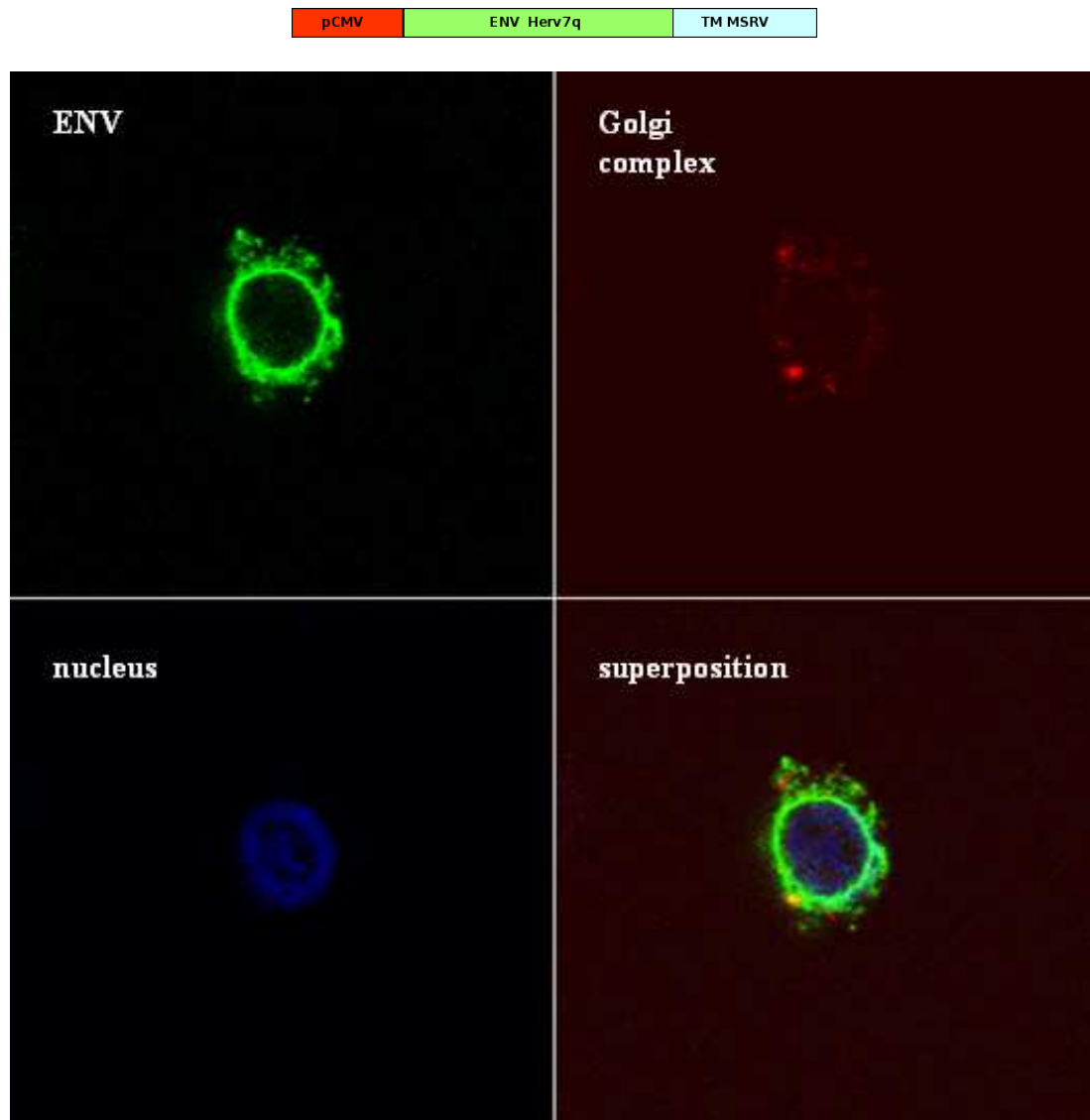


Figure 4.37: Confocal microscopy of HEK293T cells transfected with pCMV-HERV7q-ENV/MSRV-TM; antibody staining for ENV (green) and the Golgi apparatus (red), DAPI staining for the nucleus (blue); superposition of green and red fluorescence shows yellow.

4. RESULTS

Chapter 5

Discussion

The objective of this work being the study of the properties of the MSRV envelope protein ENV *in vitro* and *in vivo*, three parts will be discussed:

1. The inflammatory properties of ENV in the system of cellular assays *in vitro*, as assessed by ELISA for cytokines and flow cytometry analysis for expression of cellular surface markers, as well as the preliminary *in vivo* assay of systemic injection of ENV into the peritoneum of healthy mice whose effects on the immune cells are measured by flow cytometry analysis,
2. The modified mouse model of Experimental Autoimmune Encephalomyelitis (EAE) relying on the use of ENV as an adjuvant, evaluated by scoring of symptoms, antigen recall assays to detect autoreactive T cells and MRI of brain and spine, and
3. The establishment of a transgenic mouse model expressing ENV under the control of different promoters targeting specific cell types, whose preliminary results – genetic vector constructions and successful transfection into cell cultures – are shown here.

The more general aim resuming the challenge of all experiments taken together is to elucidate the actions of the envelope protein ENV in its physiological context – or at least to come as close as possible to the conditions that allow for conclusions on its role in diseases like Multiple Sclerosis (MS). Considering that some envelope proteins of the HERV-W family are expressed as soluble molecules in the fluids of

the healthy central nervous system (CNS) [88, 2] and can be pathophysiologically modified in CNS diseases [121, 5], our studies on the soluble subunit comprising the sequence downstream of a natural cleavage site (see figure 1.5, page 17) are in line with the fact that envelope protein fragments can be present without being necessarily associated to a complete viral particle.

5.1 Inflammatory Properties of ENV-SU – from Men and Mice

Previous studies of Rolland et al. performed in our laboratory have shown that ENV-SU possesses inflammatory properties on human peripheral blood mononuclear cells (PBMCs), monocytes and monocyte-derived dendritic cells (MDDCs) by stimulating cells to produce inflammatory cytokines and to express surface markers representing cell maturation [99, 100]. In the continuity of this work, different batches of the recombinant ENV-SU protein produced in *E.coli* have been tested in order to guarantee the reproducibility of the cellular stimulation assays and experimental injections *in vivo*. The batches called 4B, 5A and 5B have been tested on human PBMCs and compared to the reference batch 4A that has been utilised in previous works. As shown in figure 4.1 (page 90), all four batches stimulate the production of high amounts of Interleukine-6 (IL-6), as does the reference stimulator lipopolysaccharide (LPS). The fact that no dose-dependency is visible in the shown experiment is due to the variability in the cellular capacities: the excitability and the ability of isolated cells *ex vivo* to survive strong stimulation and to produce cytokines can differ from one experiment to another, as naturally does the quantity of produced cytokines; in PBMCs, stimulations between 0.5 and 2 $\mu\text{g}/\text{ml}$ can cause the same state of excitation, which means that the cells are at the limit of saturation towards the stimulus.

Tested on murine dendritic cells (DCs), the same quantities of the recombinant protein batches 5A and 5B do not cause any stimulation in terms of IL-6 production, compared to the batches 4A and 4B (the comparative experiment for 5A and 4A is shown in figure 4.6, page 101). We conclude that slight differences in structure or folding are responsible for their failure to stimulate murine DCs; all four batches being produced in the same place, starting from the same cDNA plasmid, using the same protocols for production, purification and quality controls, these differences in their biological activity are not yet palpable for us. Nevertheless, the batches 5A and 5B have not been used for further experiments in the murine system.

The *in vitro* experiments on murine DCs were important for us since envelope proteins of the HERV-W family like the MSR_V ENV are not naturally present in mice; their capacity to excite the murine immune system needed to be validated with regard to later *in vivo* experiments. Dendritic cells have been chosen because of their role in the immune system: they are highly potent antigen-presenting cells

(APCs) and crucial for the capture and presentation of antigens most notably in the CNS [66, 68], and they are capable to strongly stimulate T lymphocytes that are the key effector cells in auto-immunity, but they also participate in T cell homeostasis in the absence of nominal antigens [14]. However, their position as the interface between the innate and the adaptive immune system and their multiple functions in T cell interaction made them interesting candidates for us, supposing they could play an important role in the response to envelope exposure. In addition, DCs express the Toll-like receptor 4 (TLR4) that has been shown to mediate the inflammatory action of ENV on the human immune system [100]; TLRs in general have been evidenced to play a key role in the induction of adaptive immune responses, and their deregulation is likely to lead to autoimmune pathologies [82, 77].

As shown in the figures 4.2 to 4.4 (pages 91 to 99), murine DCs produce the pleiotropic cytokine IL-6 (IL-6 can be a pro- or anti-inflammatory Th2 modulator and is basically produced after bacterial or viral infection), the typical DC cytokine IL-12p70 (IL-12p70 is mainly produced after viral contact, activates Th1 and attenuates Th2) and the mediator of chronic inflammation TNF (tumour necrosis factor; TNF can stimulate the proliferation of astrocytes and microglia) in a dose-dependant manner in response to ENV; no production of the Th2 cytokine IL-4 and the anti-inflammatory Th2 cytokine IL-10 has been detected. Our results show that ENV-SU is capable of activating murine DCs in a way that is comparable to the effect on human PBMCs which has been previously shown. DCs are susceptible to stimulation by ENV *in vitro*, pointing to their possible capacity to become *in vivo* immune effectors and T cell stimulators under the influence of stimuli like the presence of ENV.

The dose-dependant IL-6 production shown in figure 4.3 (page 92) resuming several experiments gives a hint to the specificity of the ENV-induced cellular stimulation. The remarkably high standard deviations are due to a variation in viability and maturation state of the DCs that is an inherent problem of bone-marrow derived dendritic cell (BMDC) cultures: differentiation into DCs is provoked by means of cytokine and growth factor addition to pluripotent bone marrow-isolated progenitor cells, and even if a distinct time point in their development is chosen for each experiment, differences in their reactivity to stimuli persist and become manifest in high variations of total cytokine values. For most of the following experiments, IL-6 has been chosen to represent the response to the inflammatory challenge by ENV because the amounts produced were higher than for TNF and IL-12p70, and because the production of IL-6 after 24h of stimulation was the most reproducible

of all cytokines tested.

To measure the inflammatory effect of ENV and other stimulators on the DC phenotype, flow cytometry analysis for surface markers has been performed. Visualisation of the cellular maturation state by detecting the phenotypical changes DCs undergo upon immunological challenge is one method that monitors their capacity to be functional immunological effector cells. Since the role of a mature DCs is to communicate with T cells and to present captured antigens to them (APC function), one analysable phenotypical adaptation is the expression of an increased number of MHC-II molecules. Class II MHC molecules are constitutively expressed at low levels in immature DCs and their number is increased during maturation and upon immunological challenge to become more efficient in their role of antigen presentation to lymphocytes. Besides the secretion of cytokines, accomplished maturation is the second condition for DCs to act as functional APCs in the immunological context *in vivo*.

A common DC marker that stains immature as well as mature DCs – independently from the number of MHC-II molecules – is CD11c, a transmembrane protein also called Integrin Alpha X (ITGAX) that is expressed mainly on DCs but also on monocytes, macrophages, neutrophils and some B cells; more precisely, its expression is restricted to myeloid DCs (MDCs), as distinct from those of lymphoid origin, also called plasmacytoid DCs (PDCs) that are CD11c-negative. CD11c has recently been found to facilitate activation of CD4⁺ and CD8⁺ lymphocytes by DCs [51]. The BMDCs utilised here were (artificially) derived from femur bone marrow; they can therefore be considered as close to CD11c-positive myeloid DCs.

As shown in figure 4.5 (page 100), double staining for CD11c and I-A^b (murine MHC-II) reveals a shift in the CD11c-positive overall DC population towards maturation upon stimulation with LPS or ENV: we observe 68.1% of mature DCs for LPS and 65.8% for ENV (double positive cells for CD11c and I-A^b), compared to 33% in unstimulated control samples. Constitutive MHC-II expression in DCs reflects the necessity of class II molecules for DC functions other than antigen presentation and priming of T cells upon pathogen contact: during thymic development, DCs drive tolerance induction by inducing negative selection of autoreactive CD4⁺ lymphocytes and clonal deletion; in the periphery, class II expressing DCs play a major role in maintaining survival of mature peripheral CD4⁺ T cells [14]. The value of about 30% observed here as baseline MHC-II expression level is not absolute but relative; variations are commonly observed when working with BMDCs and can be

due to fluctuations in the culture conditions and experimental procedures. BMDCs are generally delicate to handle and susceptible to many kinds of putatively exciting stimuli that can alter their viability and maturation state, and consequently their excitability upon experimental stimulation.

To draw a more complete picture of the immunological activation of the murine immune system caused by ENV, we investigated the reactivity of total splenic immune cells towards ENV and other immune stimulators. Freshly isolated total splenocytes of healthy wild-type C57BL/6 mice were incubated with ENV and LPS and their activation was measured by ELISA for the lymphocyte cytokine Interferon-gamma (IFN- γ) after 24h, 48h and 72h of culture (see figure 4.7, page 102). Compared to unstimulated controls which do not secrete any IFN- γ , 2 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ of ENV activate the production of small but significant amounts of IFN- γ after 72h of culture. The level of IFN- γ secretion is clearly inferior in response to ENV than in LPS-stimulated samples which reach comparable levels already after 48h and much higher amounts after 72h, but we must take into account that LPS is a very potent stimulator of many cell types and touches a wider range of target cells than ENV possibly does.

To resume these first results obtained in the murine system in order to outline the reactivity of murine cells to ENV as an immunological stimulator, we can conclude that the observed effects of ENV are clearly of immunological nature since ENV activates the murine system in a way that is similar to classical pathogen stimulation: murine DCs as the interface between innate and adaptive immunity were successfully challenged to produce inflammatory T-stimulatory cytokines and to express increased amounts of MHC class II molecules representing cellular maturation, these two conditions enabling them to efficiently polarise T lymphocytes towards specific defence and, if the antigen is endogenous, towards auto-immunity. The experiments performed on murine splenocytes give a hint to general activation of multiple cell types present in the spleen including T lymphocytes that produce IFN- γ upon challenge with ENV. The measured reactivity of splenocytes towards ENV enables us to utilise IFN- γ as a representative cytokine in subsequent experiments to measure T cell activation in the murine system.

Signalling via TLR4 and associated molecules in murine DCs

To elucidate the signalling pathway initiated by ENV, we studied knock-out mice for TLR4 and CD14 since these two molecules have been demonstrated to be susceptible to interact with ENV in humans. Studies on knock-out mice deficient for receptors and signalling molecules are useful to study their role in a physiologically coherent environment, i. e. in an authentic *in vivo* situation where all components but the molecule of interest are functional and the interactions between the remaining actors are supposed to be unchanged. Nevertheless, we have to consider that in manipulating one component of a complex system like the mammalian immune system, we might introduce secondary changes resulting from the primary modification. Interpretations must therefore be done with reservation unless the entire physiological consequences of each particular knock-out are understood.

To molecularly characterise the way DCs are activated and respond to stimulation by ENV, stimulation assays were carried out on BMDCs derived from knock-out mice for the pattern recognition receptor (PRR) TLR4 (Tlr4^{-/-}) and its accessory molecule CD14 (Cd14^{-/-}) as compared to wild-type C57BL/6 mice. The TLR4 cascade has been identified by our laboratory to mediate the ENV-induced inflammatory effects on human total blood PBMCs, monocytes and dendritic cells [100]; stimulation experiments in which the cellular activation capacities of ENV were successfully attenuated utilising neutralising antibodies directed against TLR4 and CD14 have demonstrated the necessity of both TLR4 and CD14 for significant cellular stimulation as evidenced by assessment of cytokine production. On the background of this work, the Tlr4^{-/-} and Cd14^{-/-} knock-out mice were chosen to confirm the role of TLR4 and CD14 for the ENV-mediated cellular activation in the murine system.

Evaluation by flow cytometry analysis (figures 4.8 to 4.10, pages 103 to 105, and table 4.1, page 95) reveals one aspect of this cellular stimulation involving TLR4 and CD14: the augmented expression of the major histocompatibility complex (MHC) class II molecule (I-A^b in mouse) on the cellular surface upon ENV stimulation represents cellular maturation of DCs under the influence of ENV. If TLR4 and CD14 are crucial for ENV-triggered signalling, cellular maturation is supposed to be attenuated in knock-out mice for TLR4 or CD14. Again, the TLR4 agonist LPS has been chosen as a reference for strong cellular stimulation that drives murine DCs into maturation i. e. expression of high levels of the class II MHC molecules. In addition, the TLR2 stimulator pansorbine (PSB) was added to ensure that cellular activata-

bility and reactivity towards other (non-TLR4 or non-CD14) stimuli is undisturbed in these knock-out mice and that the isolated DCs possess the capacity to produce significant amounts of MHC-II molecules despite the impaired functionality of the TLR4 or CD14 pathway.

Figure 4.8 (page 103) shows the effects of ENV and LPS stimulation on BMDCs of Tlr4^{-/-} mice compared to wild-type C57BL/6 mice: analysis of cellular maturation measured by double staining for CD11c and I-A^b (murine MHC class II) reveals significantly less strong activation of TLR4^{-/-} DCs upon both LPS and ENV stimulation (22.7% and 25.0%) compared to wild-type DCs (68.1% and 65.8%). As we expected, the capacity of the Tlr4^{-/-} DCs to respond to LPS and ENV is dramatically disturbed. In figure 4.9 (page 104), percentages of double positive cells of the same type of experiment are displayed, including PSB and different concentrations of ENV, showing that Dcs from both C57BL/6 and Tlr4^{-/-} mice respond to PSB. This finding indicates that, as we supposed, TLR2 signalling is undisturbed by the TLR4 deficiency. Regarding ENV, differences in the response of Tlr4^{-/-} cells compared to wild-type cells are detectable for each concentration (0.25 - 2 μ g); nevertheless, a slight dose-dependency is visible for wild-type cells as well as for Tlr4^{-/-} cells, pointing to a remaining reactivity of the Tlr4^{-/-} cells towards ENV that might be due to a different pathway activated by ENV independently from TLR4.

In Cd14^{-/-} mice, the same tendency can be observed (figure 4.10, page 105): whereas DC reactivity towards ENV and LPS are impaired, PSB activates cellular maturation even stronger for Cd14^{-/-} than for wild-type C57BL/6 cells. One possible explanation could be the increase of cellular sensibility towards other stimuli in the absence of one particular stimulus. Table 4.1 (page 95) resumes the experiments done on Tlr4^{-/-} and Cd14^{-/-} BMDCs and shows the variations in the percentages of cellular maturation (double positive cells for CD11c and MHC class II staining) compared to the wild-type C57BL/6 control BMDCs. Considering that unstimulated controls display a certain level of maturation that is variable, the calculated percentages show more clearly that for the TLR4 stimulators LPS and ENV, excitation and induction of maturation is remarkably higher in BMDCs from wild-type mice than in those from knock-out mice for both Tlr4^{-/-} and Cd14^{-/-}, and that stimulation with the TLR2 agonist PSB causes comparable or slightly higher maturation in BMDCs of these knock-out mice.

To compare cytokine production levels, IL-6, IL-12p70 and TNF were measured in Tlr4^{-/-}, Cd14^{-/-} and wild-type C57BL/6 mice upon stimulation with LPS or ENV.

As already developed above, BMDCs derived *in vitro* may exhibit variable properties in terms of excitability and viability from one assay to another, which means that cytokine expression levels in general have to be considered as relative values, and not all cytokine assays are representative. In some assays, cells were capable of producing mainly TNF and IL-6 in response to the given stimuli, in others IL-12p70 and IL-6 were most representative.

Figure 4.11 (page 106) shows a comparative stimulation experiment of DCs of Tlr4^{-/-} and wild-type C57BL/6 mice in which LPS, PSB and ENV cause production of significant amounts of IL-6 and IL-12p70 in wild-type mice, but considerably lower levels of both cytokines in Tlr4^{-/-} mice concerning LPS and ENV stimulation, confirming that the action of ENV is based on TLR4 signalling; the synchronicity of the findings concerning LPS and ENV point out the fact that the lack of response is actually due to the deficiency in TLR4 signalling of the Tlr4^{-/-} DCs. On the other hand, the production of IL-6 by Tlr4^{-/-} DCs in response to the TLR2 agonist PSB is as high as for wild-type C57BL/6 mice, and the IL-12p70 value is even higher in Tlr4^{-/-} mice; the principal outcome of this result is that the capacity of cytokine production in general is maintained in Tlr4^{-/-} mice, that is to say, the lack of TLR4 receptors does not influence the reactivity towards the agonists of other receptors. The unexpectedly higher amounts of IL-12p70 induced by PSB in Tlr4^{-/-} DCs could be explained by a compensational augmentation in TLR2 signalling due to the diminution of TLR4 reactivity; since TLR2 is participating in the recognition of bacterial lipopeptides and mycobacterial PAMPs (pattern-associated molecular patterns) [64], compensating the loss of TLR4 by the upregulation of TLR2 would help to maintain a functional host defence, at least against certain pathogens; however, this possibility has not been confirmed experimentally and stays hypothetical.

Concerning the accessory molecule CD14, the same experiments have been performed utilising BMDCs of the Cd14^{-/-} knock-out mouse. As CD14 is unlikely to be expressed on the cellular surface of dendritic cells, recruitment of soluble CD14 is thought to take place during initiation of the TLR4 pathway, as it has been established for the LPS signalling cascade in general [29] and the MyD88-independent pathway in particular [43]. However, CD14 was found to play a role in the ENV-induced pathway in human PBMC, monocytes and MDSCs as evidenced by antibody blocking of CD14 [100]. The experiments on murine DCs performed in this work confirm the crucial role of CD14 for the ENV-activated inflammatory cascade. ELISA dosage of the cytokines IL-6 and TNF demonstrate that both LPS and ENV cause lower cytokine levels in Cd14^{-/-} mice than in wild type C57BL/6 mice (see

figure 4.12, page 107). Regarding IL-6, the difference is less striking than for TNF, pointing to the possibility of simultaneous activation of the CD14-independent pathway by LPS – putatively implicating heat shock proteins, chemokine receptors and growth differentiation factors – that would explain the persistence of a signal in the absence of CD14 [115, 84]. Meanwhile, the PSB-induced TNF secretion is remarkably higher in $Cd14^{-/-}$ mice compared to wild-type mice, which might be due to the same phenomenon that we propose to explain the enhanced IL-12p70 production in $Tlr4^{-/-}$ mice in response to PSB: redirection of the signal to the remaining functional receptors could lead to an augmentation in their reactivity that would counterbalance the lack of response via the classical way.

To resume the cytokine responses of $Tlr4^{-/-}$ and $Cd14^{-/-}$ cells, absolute cytokine values and percentages calculated relative to wild-type cells are shown in table 4.2 (page 97).

In addition to CD14 which displays co-stimulatory properties, the TLR4 signalling pathway also implies the downstream adaptor molecules MyD88, TIRAP and TRIF, sharing MyD88 with the TLRs 2, 7 and 9, TIRAP with TLR2 and TRIF with TLR3. It would thus be interesting to consider the role of these adaptor molecules for the ENV-activated cascade. Especially MyD88 as the common adaptor molecule for four different TLRs has an important role in host defence, as evidenced by studies on MyD88 knock-out mice showing its crucial function for endotoxin response [46]. Preliminary experiments on BMDCs derived from $MyD88^{-/-}$ and $TIRAP^{-/-}$ mice bone marrow were carried out in our laboratory but no results are available up to now because these cells are difficult to culture and survival upon stimulation was not yet sufficient to obtain conclusive results.

Our results concerning the knock-out mice for TLR4 and CD14 confirm the role of the TLR4 pathway for dendritic cell signalling initiated by ENV. TLRs are well known to be the main actors in recognition of bacterial PRRs, but they have also been demonstrated to be implicated in innate sensing of viruses [13]. TLR4 in particular has been found to mediate activation and cellular maturation of murine dendritic cells *in vitro* and *in vivo* by the retrovirus MMTV (mouse mammary tumour virus) by direct binding to TLR4 [15].

Specificity of the ENV-induced DC activation

The specific action of the recombinant MSR_V ENV-SU has been established on human PBMCs by utilising monoclonal anti-ENV antibodies which neutralise the inflammatory cytokine production initiated by ENV; the blocking effect of the anti-ENV antibodies was specific for ENV since antibodies failed to block LPS-induced cytokine production [100]. To confirm the specificity of the ENV-mediated inflammatory effect in the murine cellular system, we reproduced the neutralisation assay utilising the same antibody on BMDC cultures of wild-type C57BL/6 mice. As illustrated in figure 4.13 (page 108) and table 4.3 (page 98), 30 μg of the monoclonal antibody clone 3B2H4 pre-incubated with ENV-SU significantly inhibits the ENV-induced IL-6 production: 66% inhibition for 1 $\mu\text{g}/\text{ml}$ and 81% inhibition for 0.5 $\mu\text{g}/\text{ml}$ of ENV. As the recombinant ENV is produced in *E. coli*, experiments of this kind are necessary to ensure its purity and to exclude potential contaminations by bacterial endotoxins like LPS, and also because therapeutical applications of the neutralising antibody are an interesting target to study.

Systemic injection of ENV-SU

Taking into account the inflammatory effects of ENV on murine dendritic cells and splenocytes previously demonstrated, we injected different concentrations of ENV (1, 5 and 25 μg) emulsified in PBS into healthy wild-type C57BL/6 mice. Mice were sacrificed to harvest spleens and lymph nodes after 48 and 72h of culture. Flow cytometry analysis of isolated cells using the lymphocyte markers B220 for B lymphocytes, CD3 for total T lymphocytes, CD4 for helper T lymphocytes and CD8 for cytotoxic T lymphocytes highlights augmentation of total numbers of splenic B lymphocytes after 72h, as shown in figure 4.14 (page 109). Analysis of the lymph node cells reveals enlargement of the B220-positive B lymphocyte population and diminution of the CD3-positive overall T lymphocyte population 72h after injection (see figure 4.15, page 110).

It is unclear whether the observed lymphatic T cell decrease is causally related to the coincident B cell increase or not. Furthermore, the inversed dose-dependency of the splenic and lymphatic B cell increase as well as of the lymphatic T cell decrease remains unexplained. However, direct peritoneal injection of high doses of ENV into mice could cause local necrotic effects altering immune cell functions that might not

be as pronounced when lower concentrations are injected.

B lymphocytes are antigen-presenting cells (APCs) expressing, like dendritic cells, the Toll-like receptor TLR4. The importance of TLRs for B cell immune signalling has been shown studying Myd88^{-/-} mice [83], and the role of TLR4 in particular for B cell maturation has been demonstrated *in vitro* [39]. As ENV-SU is initiating the TLR4 pathway in dendritic cells, we might suppose that the B cell augmentation observed when injecting ENV-SU to healthy mice is due to TLR4 triggering. This hypothesis is supported by studies on the Mouse Mammary Tumour Virus (MMTV) and the Murine Leukaemia Virus (MLV): retroviral MMTV particles fail to activate B cells of mice carrying a naturally mutated TLR4 gene, and isolated envelope proteins of both MMTV and MLV co-immunoprecipitate with TLR4 in transfected 297T cells [96].

These findings consolidate the inflammatory properties of ENV. Together with our previous results indicating TLR4 as the key receptor for the inflammatory action of ENV on murine DCs, we can conclude that ENV is capable of initiating a complex inflammatory immune response including DCs and total splenocytes, as shown *in vitro*, and B cells, as shown *in vivo* by systemic injection of ENV.

5.2 Murine Experimental Autoimmune Encephalomyelitis (EAE) – From Mice to Men?

The results we obtained in the *in vitro* system of murine dendritic cells (DCs) allow us to conclude that ENV triggers the murine immune response via the TLR4 pathway, initiating an inflammatory cytokine response as well as phenotypical adaptations augmenting DCs capacity of antigen presentation. Intraperitoneal injection into the murine organism gives a hint to a putative implication of B cells in the systemic activation we identify, and activation of splenic T lymphocytes adds one possible field of action to the functional “pleiotropy” of ENV.

Consequently, we can suppose that immunisation using ENV as an adjuvant – as done here for Experimental Autoimmune Encephalomyelitis (EAE) induction – is based on the actual immunological actions of ENV, and that the observed activation of multiple immune effectors is not due to molecular toxicity, as it would be the case for other adjuvants of organic origin like the *Mycobacterium tuberculosis* cell wall components contained in Freund’s complete adjuvant (CFA). Here we compare classical CFA-induced EAE induction with ENV-induced EAE induction, both being based on the myelin oligodendrocyte glycoprotein (MOG) peptide, for the latter using an adapted protocol where ENV replaces the *M. tuberculosis* fragments. To obtain the required formula for immunisation, i. e. an oil-in-water emulsion allowing for optimal and long-lasting antigen exposition, we solubilised ENV and MOG in the aqueous phase and mixed it with the oil phase (Freund’s incomplete adjuvant (IFA), mineral oil without *M. tuberculosis*). An emulsion of the same texture was obtained by mixing CFA and the aqueous phase containing MOG for the classical CFA-MOG immunisation that represented our “positive control”. We injected an IFA-MOG emulsion as “negative control” to exclude “false” symptoms due to the injection mode or effects of the vehicle (IFA) or the antigen (MOG).

Clinical Score (CS) – Evaluation of symptoms

The evaluation of EAE symptoms was done by daily scoring of each individual mouse according to the Clinical Score (CS) scale (see table 4.4, page 111). The same or similar scales are used by several groups for classical EAE scoring on mice or rats, among them Joseph BOUCRAUT’s team (Marseille) with whom we were collaborating and who gave us helpful advice on handling EAE experiments and

calculation of cumulative scores [21].

The figures 4.16 and 4.17 (pages 112 and 116) show the evolution of EAE symptoms (clinical scoring) per group of mice (mean values) over time. Calculation of mean scores per group and graphic representation in curves was chosen to illustrate the unsteadiness of the symptoms that points to a form of EAE similar to the relapsing-remitting form of Multiple Sclerosis (RRMS). On the other hand, looking at the end of each experience (CS at day 45 / day 50, see tables 4.5 to 4.8, pages 113 to 117), even if symptoms tend to a level inferior to the maximum (maximum CS), they persist on a certain level and indicate a possible chronicity as it can be observed in primary / secondary progressive MS (PPMS / SPMS), suggesting altogether an intermediate form of EAE, showing characteristics of progressive relapsing MS (PRMS). However, comparison of EAE and MS symptoms is not always concluding and stays hypothetical; extrapolation from rodent EAE to MS has often been successful but must be done with precaution since wrong parallels have been made in some cases [31, 34].

A second possibility to compare the injected mice individually or in groups is to calculate the so-called *cumulative scores* corresponding to the severity of the EAE symptoms (see tables 4.5 to 4.8, pages 113 to 117). Cumulative scores are based on daily individual scoring and addition of the daily scores (per mouse or per group) from the first day after injection until sacrifice of all mice in the end of the experiment. Using cumulative scores has the advantage that the severity of symptoms can be compared between EAE experiments of different types, e. g. using different peptides / proteins for immunisation or different mouse strains with different genetic background, whose development over time can be too different to compare them *qualitatively*. Cumulative scores represent an efficient tool to describe disease symptoms *quantitatively* in order to be able to compare EAE forms with weak but chronic symptoms with those displaying strong but punctually occurring symptoms.

In the first of the two presented EAE experiments (shown in figure 4.16 (page 112), tables 4.5 and 4.6 (pages 113 and 114)), 4 groups of healthy C57BL/6 mice were injected: IFA-MOG, CFA-MOG, 10 or 50 μg of ENV.

In the IFA group, we observe that none of the animals shows clinical symptoms until the end of the experiment, proving the inoffensiveness of the IFA-MOG emulsion and also of the injection technique. Since the injections were done intra-

muscularly into the hind limbs and hind limb paresis or paralysis are part of the expected EAE symptoms, we needed to assure that the injection alone did not exert an influence on the motor capacities of the mice.

For the three other groups, disease incident was 100%; the highest mean maximum clinical score was reached in the 50 μg group (2.9), which also shows the earliest mean disease onset (9.1) and the highest cumulative score (56.9), compared to the CFA group with a mean maximum CS of 1.8, disease onset at 12.2 days and a cumulative score of 31.2, and the 10 μg group where the maximum CS was 1.5, disease onset at 14.6 days and a cumulative score of 18.7. These findings confirm the dose-dependency of the action of ENV on the murine organism, and show that ENV in higher doses (here 50 μg) has stronger effects than the *M. tuberculosis* contained in CFA.

To further differentiate the action of ENV, we performed the second EAE experiment shown in figure 4.17 (page 116) and the tables 4.7 and 4.8 (pages 115 and 117) in which we added two groups of mice that were not present in the first experiment discussed above: a first one where mice were injected 25 μg of ENV and a second one where we injected 50 μg of ENV in an IFA emulsion without the MOG antigen.

This time, both animals of the IFA group show periodically slight symptoms (tail weakness, CS 1) from day 14 or day 17 on, which leads to cumulative scores of 20 and 6, respectively, but no symptoms persist until the end of the experiment (CS 0 at day 50). Compared to mean cumulative scores of 93 for CFA animals, 40.3 for ENV 10 μg , 90.8 for ENV 25 μg and 89.5 for ENV 50 μg , their mean cumulative score of 13 stays insignificant. Nevertheless, we must conclude that the injection of IFA-MOG represents an invasion to the murine organism and caused tissue damage (due to the injection itself) and / or systemic damage (due to the MOG peptide).

We also remark that 25 μg of ENV is sufficient to cause strong symptoms, and that by injecting higher quantities (50 μg), neither mean disease onset, nor mean maximum CS, nor CS at day 50, nor the mean cumulative score is augmented.

In addition, we observe that for all animals, symptoms are attenuated in the disease course, but that for most of the mice in the groups 10 μg and 50 μg w/o MOG, symptoms completely disappear before the end of the experiment (4 mice out of 5, and 2 mice out of 3, respectively), which is not the case for the CFA nor for the other ENV groups.

Of special interest is the 50 μg group consisting of 3 animals that did not receive any MOG peptide but only the highest dose of ENV in IFA. In the absence of an antigen, we do not expect immunisation of the animals, yet the symptoms we observe can be classified EAE symptoms (CS 2.5, hind limb weakness, tremor, loss of balance). The explanation we suggest for this phenomenon is that ENV alone triggers systemic effects – as described in 4.1.5 (page 98) and discussed in 5.1 (page 151) – which in this case could be responsible for EAE-like symptoms. One hypothesis could be that ENV acts both as an adjuvant and as the antigen itself, inducing immunisation against ENV. This hypothesis needs to be confirmed by further studies.

In addition to the daily scoring for EAE motor symptoms, in this experiment we also measured the body weight of each mouse. Figure 4.18 (page 118) shows the curves of mean body weight per group, starting at disease onset (about one week after injections), as an overlay to the graphic representation of symptoms. Taking into account that the average age of mice in the beginning of the experiment was about 2 months, a slight but constant augmentation in body weight can be noticed throughout all the experiment. This augmentation is interrupted and body weight even decreases at disease onset for all animals of the groups ENV 25 μg , ENV 50 μg and CFA. Regarding the mean clinical scores of the groups, we observe that loss of body weight occurs when clinical scores climb above 2: whereas in the IFA and ENV 50 μg w/o MOG groups, no change in weight (IFA) or correlation between weight and symptoms (ENV w/o MOG) can be noticed, the augmentation of EAE symptoms in the CFA, ENV 50 μg and ENV 25 μg groups is clearly associated to a loss of body weight, supporting the mice's supposed state of systemic disease.

Another means to record the disease state of animals is to measure their body temperature. Unlike systemic immune reactions that are in general accompanied by fever, the body temperature of EAE mice is often found to be decreased. In Multiple Sclerosis patients, a neuroprotective effect of *hypothermia* has been observed, and *hyperthermia* on the contrary exacerbates symptoms and is most significantly correlated with disease onset and relapses. The electrophysiological explanation to this phenomenon is the relation between temperature and neuronal conductivity: low temperatures facilitate signal conduction, whereas high temperatures hamper or even block the neuron's capacity to conduct the electrical excitation.

We did not perform temperature measurements for the simple reason that we did not dispose of the adequate equipment to do so. For small animals, a special

thermometer is required and measurements need to be done in a very accurate way, taking into account fluctuations in body temperature during the day-and-night cycle which can influence the precision of the results.

Immunological memory and antigen recall *ex vivo*

To illustrate the immunising character of the EAE injections and to prove that the immune system of the animals had been challenged successfully, *ex vivo* antigen recall experiments were done on the isolated splenocytes. The spleen is, after the bone marrow, the second site of haematopoiesis in humans. It is a very active organ in early childhood and keeps its faculty to produce lymphocytes throughout life; in the adult, the spleen is of importance when analysing the immune system because it represents the body's lymphocyte "arsenal" which is constantly adapting its lymphocyte populations to encountered pathogens. If an autoimmune reaction has been triggered by EAE induction, we should be able to evidence autoreactive T cells in the spleen of the immunised animals.

In a first step, we measured the reactivity of naïve splenocytes towards the MOG peptide that was used for the immunisation, and towards the lymphocyte activator phytohaemagglutinin (PHA). Figure 4.19 (page 119) shows the interferon-gamma (IFN- γ) production by fresh splenocytes, compared to splenocytes that were frozen prior to the culture assays, after 24h of culture. The experiment reveals three important points: firstly, fresh as well as frozen naïve splenocytes are capable of secreting IFN- γ upon stimulation with PHA, and unstimulated cells do not respond to PHA; this indicates that the isolation method does not cause a baseline excitation of cells (as it can be expected when extracting cells from biological material *ex vivo*), and that the protocol preserves the cells' capacity to secrete significant amounts of cytokines. Secondly, freshly collected cells produce much higher quantities of IFN- γ upon PHA stimulation; as a consequence, we stimulated cells directly after extraction in the following recall experiments. Thirdly, incubation with MOG does not evoke any IFN- γ production in naïve splenocytes; this point allows us to exclude an intrinsic cellular activation effect by the MOG peptide.

As shown in the figures 4.20 to 4.22 (pages 120 to 122), splenocytes from diseased EAE mice injected with ENV produce significant quantities of IFN- γ in response to MOG after 24h, and the secreted IFN- γ quantities augment after 48 and 72h, whereas splenocytes from IFA-injected animals do not produce significant IFN- γ lev-

els. IFN- γ has been chosen as representative for inflammatory cytokines produced by lymphocytes, and more particularly by T lymphocytes. T lymphocytes being the cells that carry out the memory function of response towards an antigen, IFN- γ secretion is a sign for us that the mice have been successfully immunised against MOG and that their splenic T lymphocytes “remember” this antigen. When evidencing the splenocytes’ cytokine response against MOG in culture, we suggest that this response points out autoreactivity of these T cells directed against the MOG peptide utilised for immunisation, but putatively also against other myelin epitopes, in analogy to the situation in “natural” autoimmunity, caused by the phenomenon called *epitope spreading* [117]. In any case, we suppose that the lymphocyte reactivity we observe here is responsible for the long-lasting character of the EAE symptoms in diseased mice.

The dose-dependency of the response to MOG is clearly visible in figure 4.20 that represents the IFN- γ secretion by splenocytes that have been pooled from different animals of the same group (2 animals for the IFA group, 3 animals for the ENV group); significant amounts are secreted by splenocytes from ENV mice when 1 $\mu\text{g}/\text{ml}$ of MOG or more are added to cultures, and a plateau appears between 10 and 25 $\mu\text{g}/\text{ml}$.

In figure 4.22 (page 122) we show the IFN- γ production for one single ENV mouse compared to one single IFA mouse. The same dose-dependency is visible when looking at this ENV mouse in particular, but also for the IFA mouse which was without EAE symptoms, a slight augmentation in the IFN- γ level is detected. The ENV mouse represents several mice of its group that displayed the same response towards MOG; though, we observed several ENV mice whose splenocytes in culture did not respond to MOG. We suggest that this effect is due to the special character of the EAE symptoms observed in our C57BL/6 mouse model: taking into account the relapsing-remitting profile of symptoms, it is not surprising that not all of the splenocyte cultures showed IFN- γ production upon MOG stimulation; the day of sacrifice we chose to stop the experiment for all mice might not always coincide with an imminent relapse of each individual animal, and T reactivity might be temporarily impaired in those cases.

Murine EAE brains – MRI analysis

In the two preceding paragraphs, EAE symptoms and physiological consequences of the immunisation on the level of immune cells were evaluated by scoring of motor symptoms and detection of autoreactive anti-myelin T lymphocytes. Since EAE has been conceived to mimic neurodegenerative diseases like Multiple Sclerosis (MS), demyelinating lesions as found in MS patients are supposed to occur in the central nervous system (CNS) of diseased mice.

With the help of Jean-Baptiste LANGLOIS of the ANIMAGE animal facility at the neurological hospital of Lyon (Bron, France), we performed Magnetic Resonance Imaging (MRI) to evidence the neuronal demyelination we expect to observe in the brains and spinal cords of the mice showing EAE symptoms (for the MRI protocol see appendix C). Given the fact that demyelination can be reversible if EAE symptoms follow the relapsing-remitting scheme and mice are about to recover partially or entirely, we chose to analyse only animals that were diseased with at least CS 2 for a period of several days. Irreversible demyelination can be supposed if animals do not recover rapidly after reaching scores higher than 2 (clumsy gait, hind limb weakness).

MRI evidences demyelination spots as zones of liquid accumulation; more precisely, the infiltration of lymphocytes and other immune cells in the areas of inflammatory EAE plaques represents an augmentation of the extracellular water and becomes visible as white zones in the MRI images. We compare MRI recordings from ENV- and CFA injected mice with those from IFA-injected mice that are the MRI “negative control” for us since these mice did not show any symptoms and their MRI images are without white spots. It is necessary to compare images of the same depth to make sure to distinguish real inflammatory plaques with lymphocyte infiltration (in diseased mice) from technical artefacts on one hand, and from normal white-appearing structures of the healthy mouse brain on the other hand.

The coronal MRI images shown in the figures 4.23 to 4.27 (pages 123 to 124) were acquired from caudal to rostral. Advancing from the cervical medulla through the hindbrain with pons and cerebellum, through the midbrain and the forebrain with cerebrum to the olfactory bulbs, a series of images has been taken and the most representative ones are shown here. White zones indicating demyelinating spots have been detected all along the acquisition (plaques pointed by arrows) in both the CFA and the ENV animals; in both cases, plaques are focussed mainly around the

different brain cavities visible in the different cross sections: the figures 4.23 and 4.24 show white spots around the lateral ventricles that border the fourth ventricle; in the midbrain (4.25), the lateral ventricles are crossing the brain from dorsal to ventral, and the same white infiltration areas can be observed. In figure 4.26, white spots are localised in the hippocampal area between the adjacent lateral ventricles and the rostral brain stem, and figure 4.27 shows affectation of the inner surface of the olfactory bulbs.

We have evidence to conclude that ENV-induced EAE causes not only the same symptoms than classical CFA-induced EAE, but also that physiological properties are similar: splenic T cells show reactivity against the MOG peptide (as shown by IFN- γ production *ex vivo*) which could be a sign of autoimmunity induced by immunisation against a myelin component, and immune cell infiltration into the brain and spinal cord (as shown by MRI).

ENV is in this case acting as an adjuvant, as do the *Mycobacterium tuberculosis* cell wall fragments that compose the classical CFA adjuvant. We suppose that ENV can be considered as a “soft” adjuvant with less toxic side effects like local tissue necrosis and cutaneous overreactions that can occur on the injection site when using bacterial adjuvants. ENV is of eukaryotic origin and we can therefore hypothesise a better acceptability and compatibility for mammals. However, we cannot totally exclude yet the persistence of slight side effects, since IFA-injected animals are not always entirely free of symptoms, as shown in the second EAE experiment.

All animals received repeated injections of the Pertussis Toxin (PTX) in the beginning of the experiment (days 0 and 2). We use PTX to open the blood brain barrier (BBB) in order to permit leukocyte trafficking into the brain, and it has been argued that the action of PTX was closely linked to the activation of the TLR4 pathway [47]. Taking into account the fact that experimental induction of neurodegenerative diseases is always highly invasive, we still presume that the effect of PTX is not influencing the action of ENV, and that the effects we observe are not due to PTX alone, firstly because all animals, including IFA animals, received the same quantities of PTX, and secondly because EAE induction without PTX has been done in some mouse strains [32].

5.3 Transgenic Mice

As described above, the EAE induction method based on experimental immunisation and artificial rupture of the BBB brings up several problems. Besides the fact that anaesthetising the animals and injecting them with relatively big volumes of emulsion (and anaesthesia) is highly aggressive and may cause local complications or influence the health of the animals in general, we also need to take into account the physiological divergence of mice and humans which has sometimes led to misinterpretation when comparing rodent EAE and human MS. Two reviews by FRIESE et al. and GOLD et al. critically discuss this problem [31, 34].

In spite of the valuable results we obtained, given the invasiveness of the techniques employed for active EAE induction, we need to consider alternative approaches to study neurodegenerative diseases on animals in order to avoid the unwanted side effects that cannot be excluded when doing physical harm to the manipulated animals. In our case, we chose genetic manipulation (transgenesis) because we tackle the question of MS aetiology (and of neurodegenerative diseases in general) by its genetic component, the presence of multicopy retroviral envelope sequences in the human genome. Since the retroviral envelope protein ENV is not naturally expressed in animals other than hominids, we need to create transgenic animals carrying ENV if we want to continue doing *in vivo* experiments. (The necessity of using animal models for studying neuronal diseases and their use for this work in particular are discussed in 1.4 and 1.4.4 (pages 19 to 21).)

The first part of the transgenesis project, which is exposed in this manuscript, is restricted to the genetic construction of vectors carrying the transgenes and their *in vitro* validation by transfection of cell cultures and analysis of the expression of the transgene. The further steps leading to the creation of transgenic animals have been / are about to be accomplished by Hei-lanne REYNAUD-DOUGIER.

Promoters and cellular targeting

The choice of the promoters is necessary to selectively express ENV in certain cell types. It is important to target the cells in which we want the transgene to be expressed because we need to distinguish the action of ENV that is supposed to be specific for certain cell types, and to avoid systemic effects of ubiquitous expres-

sion that could influence the general condition of the transgenic mice. Besides the ubiquitous human pCMV promoter (originating from the *cytomegalovirus*, infecting immune cells as well as muscle tissue, glands and endothelial cells), we therefore chose the pGFAP promoter to target human astroglial cells which are the cells that physiologically express ENV; this promoter has been reported to be functional in mice. The third promoter we chose is pMHCII which targets murine monocytes and dendritic cells.

We realised genetic vector constructions to bring under control of these three promoters the sequences coding for the two envelope proteins MSR-ENV and HERV7q-ENV, both containing the full-length coding sequences for the extracellular component – called ENV here since the extracellular domain is the principal domain of the envelope to interact with surrounding cells or molecules – and the transmembrane subunit – here called TM – whose function is to anchor the ENV protein in the cellular membrane. (To simplify the terminology, we use "ENV" to design the full-length protein ENV-TM.) All resulting constructs are represented schematically in figure 4.28 B (page 130).

The interest for the MSR-ENV comes from the discovery of its implication in MS. Its physiologically expressed endogenous homologue HERV7q-ENV (syncytin-1) is not necessarily involved in human disease; the genetic sequences of both MSR-ENV and HERV7q-ENV being known, HERV7q-ENV is though of interest for us because the putatively differential pathogenic and infectious character of these two proteins when expressed in mice would enable us to link their sequences to their functional protein domains and to identify the domains that are crucial for pathogenicity.

For the pCMV constructs (pCMV-MSR-ENV and pCMV-HERV7q-ENV), we chose HEK293T cells which are easy to culture and to transfect. Figure 4.29 (page 131) shows confocal microscopy images of successfully transfected HEK293T cells, both ENV proteins being revealed with the monoclonal primary anti-ENV antibody 3B2H4 and a green-fluorescent secondary antibody. The distribution inside the cells does not show the same profile for the two ENV proteins: the MSR-ENV is accumulating around distinct structures on the inside of the cellular membrane and in the cytosol, whereas the HERV7q-ENV is diffused all over the cytoplasm.

When transfecting cells of the murine macrophage cell line J774 with the pMHCII constructs (pMHCII-MSR-ENV and pMHCII-HERV7q-ENV), using the same combination of antibodies to reveal ENV, we obtain a different picture: in both cases,

ENV is densely spread all over the cell body.

For both pCMV and pMHCII constructs, validity of the genetic vectors and structural integrity of the envelope proteins has been evidenced using the monoclonal 3B2H4 antibody which is also the antibody used for neutralisation assays blocking the inflammatory properties of ENV, as discussed in 5.1 (page 151). Given that the epitope recognised by 3B2H4 in both the MSR-ENV and the HERV7q-ENV is also the crucial epitope exerting an inflammatory action on cells, we can presume that both proteins have conserved their “infectious” character and that both genetic ENV constructs are valid for being used in the transgenesis project.

The fact that chemical permeabilisation of the transfected cells was necessary to obtain the observed anti-ENV staining which is, as a consequence, only intracellular, gives an insight into the structural features of the protein and into the nature of the exposed epitopes. Since the intracellular staining (after permeabilisation) with the 3B2H4 anti-ENV antibody is detectable both for the MSR-ENV and the HERV7q-ENV constructs, the recognised epitope seems to be intact in both cases. If we do not measure any extracellular staining signal, we can consider that the epitope of interest must though be hidden in case of membrane expression of ENV.

Incorrect folding, missing glycosylation and defects in other post-translational modifications are sensible problems that can occur when producing eukaryotic proteins in prokaryotic organisms (as *E. coli* in our case), especially when producing complex and insoluble membrane proteins. The problems that we encounter when using the *E. coli*-produced ENV (e. g. agglomeration) are due to the lack of a post-translational processing machinery (Endoplasmatic Reticulum, Golgi apparatus) in prokaryotes; analogically, in the case of eukaryotic cells transfected with the ENV constructs, organ-, tissue- or cell-specific post-translational processing could be required to ensure correct folding and membrane exportation of the protein. However, recognition of the two ENVs by the monoclonal anti-ENV antibody reveals structural integrity of the recognised epitope at least when ENV is expressed inside the cell, but does not give a hint on membrane expression. In contrast, the syncytia formation observed for the HERV7q-ENV clearly proves that ENV must be present on the surface of the cell (other aspects of syncytia formation will be discussed below).

The transgenes pMHCII-MSR-ENV and pMHCII-HERV7q-ENV have been sent to the Service d'Expérimentation Animale et de Transgénése (SEAT platform, CNRS, Villejuif, France) where the creation of transgenic mice has been performed

for both constructs. To date, 2 mice out of 6 are carrying the pMHCII-HERV7q-ENV transgene and 15 out of 34 the pMHCII-MSRV-ENV transgene. The transmissibility of the two genes to the F1 generation has not yet been confirmed, but crossing experiments are about to be done.

Concerning the promoter pGFAP targeting the *glial fibrillary acidic protein*-expressing glial cells, constructions associating MSRV-ENV, HERV7q-ENV or GFP (*green fluorescent protein*) have been realised. First successful transfections into murine GL26 cells can be reported (data not shown here), but difficulties of reproducibility do not allow us yet to establish the functional validity of the constructs.

Intracellular localisation of ENV

To study the localisation of the expressed transgenes MSRV-ENV and HERV7q-ENV inside the cell soma and around the cytoplasmic compartments, we performed confocal microscopy highlighting the antibody-stained Golgi apparatus (red fluorescence) and the DAPI-coloured nucleus (blue fluorescence), co-localising the 3B2H4-revealed ENV (green fluorescence). In the figures 4.32 and 4.33 (pages 134 and 135) showing pCMV-MSRV-ENV- and pCMV-HERV7q-ENV-transfected HEK293T cells, respectively, all three stainings are shown separately and overlaid.

The MSRV-ENV construct shows an accumulation in the peri-membrane area with co-localisation to the Golgi apparatus (yellow), but strong signals for ENV are distributed also all over the cell body. For the HERV7q-ENV, signals are weaker and no particular co-localisation is detectable. Furthermore, the HERV7q-ENV transfected cells formed syncytia after 24h of culture. For the human HERV-W glycoproteins of the syncytin family, placental expression has been evidenced [59], and a crucial role in syncytiotrophoblast fusion has been shown for syncytin [28] and more particularly for syncytin-1 and syncytin-2 [42]. Transfection of several cell lines with HERV-W cDNA led to formation of syncytia in primate and pig cells upon interaction with mammalian retrovirus receptors [12]. Its fusogenic properties have also been demonstrated on mouse cells, although syncytia formation is weaker than in human cells and different receptors are involved [56].

Our results are in line with these previous findings and confirm the functionality of our HERV7q-ENV constructs, since the physiological function of membrane fusion has been preserved for the HERV7q-ENV. The fact that the MSRV-ENV does not

lead to syncytia formation must though be due to structural changes resulting from the mutations highlighted in figure 4.34 (page 136). Among the mutations altering the biochemical properties of the protein, two mutated sites can be identified that participate in disulfide bridge formation and are thus likely to be responsible for drastic structural differences between the two proteins.

Functional characterisation of the envelope domains – Genetic shuffling

To further investigate the function of the two major domains (ENV-SU (here referred to as “ENV”) and TM) and the differences between MSRV and HERV7q, we interchanged their TM sequences (genetic shuffling). As shown in figure 4.35 (page 137), 6 constructs associating the three promoters pCMV, pMHCII and pGFAP with the two shuffled proteins (MSRV-ENV/HERV7q-TM and HERV7q-ENV/MSRV-TM) have been cloned.

The point of recombination between ENV and TM is a common KpnI restriction site that separates two mutations participating in disulfide bonds (see figure 4.34, page 136); these mutations implicate for the MSRV copy that the chemical environment of the first cysteine is changed (mutation situated in the ENV part) and that the second cysteine responsible for the disulfide bridge is missing (mutation situated in the TM part). These differences may explain the divergence we observe in terms of in cellular distribution of the expressed proteins in the transfection assays discussed above.

Transfection of HEK293T cells with the plasmids pCMV-MSRV-ENV/HERV7q-TM and pCMV-HERV7q-ENV were carried out, and confocal microscopy superposing ENV (green), Golgi (red) and DAPI staining for the nucleus (blue) reveals co-localisation of HERV7q-ENV/MSRV-TM to the Golgi and distribution around the nucleus (4.37, page 139) without formation of syncytia, whereas MSRV-ENV/HERV7q-TM is not co-localised to any distinct structure and polynuclear syncytia are identifiable (4.36, page 138).

We can conclude that the transfer of the HERV7q TM domain on MSRV-ENV confers to the MSRV-ENV protein the same properties than the full HERV7q protein; in other words, the fusogenic character we observed for the full HERV7q-ENV is likely to be due to the second disulfide bond in the TM domain of HERV7q that is missing in the MSRV TM domain (mutated cysteine). For the placental

syncytins, the fusogenic properties have been localised in the TM [98]. The fusogenicity observed for syncytin-1 and syncytin-2 is genetically independent from the immunosuppressive activity observed only for syncytin-2 [63].

Furthermore, our studies show that the MSR_V-TM seems to be responsible for the more specific co-localisation of ENV to the Golgi apparatus around the nucleus, a property that may also result from the structural changes introduced by the second cysteinic mutation.

It remains to elucidate how the expression of the two ENVs would modify the function of astrocytes, monocytes, dendritic cells and other target cells of the pGFAP and pMHCII promoters. For instance, these assays are about to be performed but no results are yet available.

Chapter 6

Conclusion and Perspectives

The idea to study the action of the MSR/V envelope protein ENV was born of the discovery of the physiological expression of intact HERV-W envelopes in the healthy human body, and the pathophysiological expression of the MSR/V ENV in MS patients [88, 60]. Starting from the recombinant ENV protein – more precisely its soluble extracellular subunit ENV-SU – produced in *E. coli*, its inflammatory actions on the immune system have been evidenced, *in vitro* for human blood cells, *ex vivo* for murine splenocytes and dendritic cells, and *in vivo* for the murine organism. Its way of action has been shown to be TLR4-dependant and CD14 seems to interact positively; these findings are supporting the crucial role of dendritic cells in the pathogenesis of neuroinflammatory diseases, and the pathogenicity of ENV as an activator of dendritic cells which are at the interface between innate and adaptive immunity.

To study the effect of ENV on the blood brain barrier (BBB), a study on endothelial BBB cells in culture is currently in progress in our laboratory. The first results available show that ENV induces inflammatory cytokine production by these cells and adhesion protein expression on their surface. These findings reinforce our hypothesis of ENV as a pleiotropic factor.

Furthermore, the capacity of at least one monoclonal anti-ENV antibody to block cellular stimulation by ENV has been proven. The neutralising action of the anti-ENV antibodies rises hope for a therapeutic application as an early phase treatment of MS or other neurodegenerative diseases implicating endogenous retroviruses. But, the importance of ENV in MS aetiology remains uncertain and the consequences of

blocking ENV in an early stage of disease remains to elucidate. Taking into account the presence of other pathogenic factors whose importance is unclear, the effect of eliminating one factor is difficult to prognosticate. Likewise, there is no evidence that ENV could become effective in terms of stopping MS in a phase where myelin damage has become irreversible.

The piece of work we add to the MS puzzle certainly helps progressing in understanding MS, but it also reminds the necessity to develop more powerful diagnostic tools in order to tackle the therapeutic question as early as possible. However, the approach of neutralising an inflammatory viral protein in order to *prevent* the disease is on the curative side of the problem, and we do not bring forward palliative treatments that are certainly important and all the more urgent for the well-being of patients.

On the side of systemic effects of ENV on the organism of healthy mice, we have evidenced implication of splenocytes and lymph nodes, conducting us to consider that the effects observed in myelin immunisation experiments (EAE) are due to a multiple action of ENV rather than being the effect of its simple adjuvant properties. Besides physical symptoms and brain injury monitored by MRI, we have shown the auto-immune character of the induced EAE by recalling the immunological memory of anti-myelin effector cells. In similar experiments on humanised SCID mice realised by our partners, treatment with monoclonal anti-ENV antibodies successfully prevents EAE symptoms before disease onset and even restores physical health of the animals when given during ongoing EAE. These experiments can be seen as the first step to clinical trials, and humanisation of the antibodies is in progress.

In parallel, the transgenic mouse model will allow us to observe the actions of ENV in a physiological context without invasive experimental procedures; moreover, by performing directed mutagenesis and genetic shuffling with the two envelopes, we will be able to determine their functional domains in relation to their molecular properties. To express ENV physiologically comes closer to the *in vivo* situation observed in the body fluids of MS patients, and the role of the soluble extracellular part of ENV – as naturally occurring when cleaved at its intact natural cleavage site – can be studied. Furthermore, expressing the ENVs – as well as their shuffled or mutated variants – in eukaryotic cell cultures could be a means to avoid using bacterial cultures, which are still widely employed for the production of biomolecules despite their numerous drawbacks (contamination, incorrect folding, problems expressing complex molecules).

All in all, this study confirms the inflammatory actions of the MSR/V envelope protein and its implication in MS pathogenesis, and the results we obtained rise hope for future therapeutic applications. The implication of retroviruses in other CNS diseases and complex functional psychosis disorders like Schizophrenia has been argued [17, 18, 57] and retroviral RNA homologues of the HERV-W envelopes have been detected in the CSF [44], plasma [45, 41, 122] and *post mortem* brain samples [27] of Schizophrenia patients. Antibodies directed against non-primate retroviruses [58] and against the HERV-W proteins *gag* and *pol* [92] have been detected in the serum of patients. In several studies, genetic polymorphisms of HERV-W and HERV-K sequences have been evidenced to be associated to Schizophrenia susceptibility [79] and genome screening for retroviral sequences in patients has lead to identification of MSR/V homologues that have been baptised *SZR/V* (*Schizophrenia-associated retrovirus*) [19, 20]. By elucidating the mechanism of action of the endogenous MSR/V envelope and exploring possible therapeutic approaches, we contribute to the more general quest for understanding this type of CNS diseases implicating both genetic and environmental factors acting on the immune system, as it is the case for adult-onset auto-immune diseases like MS [24].

6. CONCLUSION AND PERSPECTIVES

Conclusion et perspectives

L'idée d'étudier l'action de la protéine d'enveloppe ENV de MSRV est née de la découverte de l'expression physiologique d'enveloppes intactes de la famille HERV-W chez l'humain, et de l'expression physiopathologique de l'ENV de MSRV chez les patients atteints de SEP [88, 60]. Partant de la protéine recombinante ENV – plus précisément de sa sous-unité extracellulaire soluble ENV-SU – produite dans *E. coli*, ses actions inflammatoires sur le système immunitaire ont été mises en évidence, *in vitro* sur cellules sanguines humaines, *ex vivo* sur splénocytes et cellules dendritiques murines, et *in vivo* pour l'organisme murin. Son mode d'action dépend de la voie TLR4, et CD14 semble interagir positivement ; ces résultats confirment le rôle crucial des cellules dendritiques dans la pathogénie des maladies neuro-inflammatoires, et la pathogénicité de l'ENV comme activatrice des cellules dendritiques se situant à l'interface entre l'immunité innée et adaptative.

Pour étudier l'effet de l'ENV sur la barrière hémato-encéphalique (BHE), une étude utilisant des cellules endothéliales de la BHE en culture menée par notre laboratoire est actuellement en cours. Les premiers résultats disponibles montrent que l'ENV induit la production de cytokines inflammatoires par ces cellules et l'expression de protéines adhésives sur leur surface. Ceci renforce notre hypothèse de l'ENV comme facteur pléiotropique.

De plus, la capacité de bloquer la stimulation cellulaire par ENV a été démontrée pour au moins un des anticorps monoclonaux anti-ENV. L'action neutralisante de ces anticorps alimente l'espoir d'une application thérapeutique dans les phases précoces de la SEP et d'autres maladies neurodégénératives impliquant les rétrovirus endogènes. Mais l'importance de l'ENV dans l'étiologie de la SEP reste incertaine, et les conséquences d'un blocage de l'ENV pendant le stade initial de la maladie reste à élucider. Compte tenu de la présence d'autres facteurs pathogènes dont le rôle n'est pas clairement identifié, l'effet de l'élimination d'un facteur est difficile

à pronostiquer. De la même façon, nous ne pouvons pas présumer que l'anticorps anti-ENV agirait de manière efficace contre la SEP aux stades avancés où les lésions myéliniques deviennent irréversibles.

La pièce que nous ajoutons au « puzzle » de la SEP aide certainement à avancer dans la compréhension de la SEP, mais il rappelle également la nécessité de développer des outils diagnostiques plus puissants afin de pouvoir aborder la question thérapeutique le plus tôt possible. Toutefois, l'approche de neutralisation d'une protéine virale inflammatoire pour *prévenir* la maladie se situe du côté curatif du problème, et nous ne faisons pas avancer les traitements palliatifs qui sont certainement importants et d'autant plus urgents pour le bien-être des patients.

Du côté des effets systémiques de l'ENV sur l'organisme murin sain, nous avons mis en évidence l'implication de splénocytes et de cellules ganglionnaires, ce qui nous amène à considérer que les effets observés dans les expériences d'immunisation myélinique (EAE) sont dûs à l'action multiple de l'ENV plutôt qu'à ses simples propriétés adjuvantes. En plus des symptômes physiques et des lésions cérébrales visualisées par IRM, nous avons démontré le caractère auto-immun de l'EAE en « rappelant » la mémoire immunologique des cellules effectrices anti-myéline. Lors d'expériences similaires sur souris SCID humanisée réalisées par nos partenaires (Hervé PERRON, GeNeuro SA, Genève, Suisse), le traitement avec les anticorps monoclonaux anti-ENV avant apparition des symptômes s'est avéré prévenir efficacement l'EAE ; lorsqu'injecté en cours de maladie, l'anticorps rétablit même l'intégrité physique des souris atteintes. Ces expériences peuvent être considérées comme un premier pas vers des études cliniques, et l'humanisation des anticorps par GeNeuro SA est en cours.

En parallèle, le modèle de souris transgéniques nous permettra d'observer les actions de l'ENV dans un contexte physiologique sans procédures expérimentales invasives ; qui plus est, avec la mutagenèse dirigée et le « shuffling » génétique des deux enveloppes, nous serons capables de déterminer leurs domaines fonctionnels en relation avec leurs propriétés moléculaires. Le fait d'exprimer ENV physiologiquement est par ailleurs plus proche de la situation *in vivo* observée dans les fluides corporels de patients de SEP, et le rôle de la partie extracellulaire soluble de l'ENV – qui existe naturellement quand l'ENV entière est clivée au niveau de son site naturel de clivage – pourra être étudié. En outre, l'expression des ENVs – et également de leurs variantes mutées ou « shuffled » – en culture cellulaire eucaryote peut représenter un moyen d'éviter les cultures bactériennes, pourtant largement util-

isées dans la production de biomolécules, en dépit de leurs nombreux inconvénients (contamination, pliage incorrect, difficultés à exprimer des molécules complexes).

En résumé, cette étude confirme les actions inflammatoires de la protéine d'enveloppe de MSR/V et l'implication de cette dernière dans la pathogénèse de la SEP ; de plus, les résultats que nous avons obtenu soulèvent l'espoir d'une future application thérapeutique. L'implication des rétrovirus dans d'autres maladies du SNC et dans les psychoses fonctionnelles complexes comme la Schizophrénie a été évoquée [17, 18, 57] et des homologues de l'ARN rétroviral des enveloppes HERV-W ont été détectés dans le liquide céphalo-rachidien [44], le plasma [45, 41, 122] et dans des échantillons de cerveau *post mortem* [27] de patients atteints de Schizophrénie. Des anticorps dirigés contre des rétrovirus de « non-primates » [58] et contre les protéines HERV-W *gag* and *pol* [92] ont été observés dans le sérum de patients. De plus, des polymorphismes génétiques des séquences HERV-W et HERV-K ont été associés à la susceptibilité pour la Schizophrénie [79], et le « screening » génomique de ces patients pour des séquences rétrovirales a par ailleurs mené à l'identification d'homologues de MSR/V baptisés *SZR/V* (*Schizophrenia-associated retrovirus*) [19, 20]. En élucidant les mécanismes d'action de l'enveloppe MSR/V endogène et en explorant de possibles approches thérapeutiques, nous contribuons dans un cadre plus global à relever le défi que suscite la compréhension de ce type de maladie du SNC qui implique à la fois des facteurs génétiques et environnementaux agissant sur le système immunitaire, comme on l'observe pour les maladies auto-immunes se déclarant à l'âge adulte telle que la SEP [24].

Appendix A

Publications

A.1 Recherche, Éthique, Citoyenneté

RECHERCHE, ETHIQUE, CITOYENNETE

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Résumé

Nos sociétés européennes souhaitent une recherche ouverte et des citoyens éclairés. Les pouvoirs publics et politiques se doivent donc de proposer des outils permettant de renforcer ce lien, c'est à dire d'aller vers une recherche citoyenne. Cet article présente l'initiative de la mise en place d'un outil de recherche citoyenne, une « Boutique des Sciences » (BdS), dans la région Grenobloise. Les BdS visent à faciliter l'accès aux outils et au savoir-faire de la recherche, par des groupements de citoyens (e.g. associations) s'emparant de questions émanant de la société civile. Le besoin sociétal d'une BdS à Grenoble est mis en évidence par une enquête. La création d'une association (ADReCA - Association pour le Développement d'une Recherche Citoyenne Active) initie la mise en place de la future BdS de Grenoble.

Abstract

Our European societies wish for an open research and informed citizens. In consequence, authorities and politics have the duty to offer the means to do so, that is to direct towards civil research. This article presents the initiative of establishing a civil research tool, a "Science Shop", in the region of Grenoble. Science Shops aim at facilitating access to the tools and the know-how of research, for citizen groups (e.g. NGOs) that want to appropriate the questions arising from civil society. The society's need for an institution such as a Science Shop has been evidenced by a survey performed in Grenoble. The creation of an association (ADReCA – Association for the Development of Civil and Active Research) starts the establishment of the Science Shop in Grenoble.

1. Introduction

Depuis une trentaine d'années, la relation de nos sociétés européennes aux pouvoirs décisionnaires a évolué de façon profonde. Il apparaît un nouveau désir de construction de relations raisonnées entre nos vies quotidiennes et les politiques publiques. Les sciences ne sont pas exemptes de cette reconstruction de son lien aux sociétés. Dans son nouvel engagement de 1999 pour la science du XXI^{ème} siècle, l'UNESCO invite « à ne ménager aucun effort pour promouvoir le dialogue entre la communauté scientifique et la société (...), agir en coopération et dans le respect des règles éthiques, dans nos sphères de responsabilité respectives (...) »

(UNESCO, 1999). Si le fonctionnement actuel de production des savoirs n'est aucunement remis en cause, les politiques de recherche et la diffusion des connaissances acquises doivent évoluer pour pleinement intégrer la dimension sociale de l'entreprise de recherche.

L'attente d'une recherche plus citoyenne, *i.e.* d'une recherche qui s'ouvre aux questions de la société et qui permet à celle-ci de bénéficier des connaissances acquises, est de plus en plus importante. Devant cette attente, il est donc du devoir des pouvoirs public et scientifique (universités et laboratoires) de proposer des outils qui permettent de favoriser la participation de la société civile à la production des connaissances, mais également de diversifier les sources potentielles d'innovation en mobilisant les initiatives citoyennes.

2. Outils de recherche citoyenne

Nous distinguons 4 voies d'implication de la société dans les projets de recherche :

La consultation des acteurs de la société civile. Le citoyen est alors un observateur de la recherche et n'a aucune garantie sur son influence effective. Par exemple, les CCSTI (Centres de Culture Scientifique, Technique et Industrielle) ont pour mission la diffusion de la culture scientifique. Depuis peu, le CCSTI de Grenoble participe également à l'organisation d'une forme de débat public, pouvant être contestée, autour des sciences (*e.g.* Forum Science et Démocratie). L'association des « Petits débrouillards » s'inscrit également dans cette démarche. Organisés en réseau, ils contribuent à former des citoyens acteurs de la construction du monde, et en particulier sur les problèmes de sciences. Des outils, dit participatifs, tels que les conférences de consensus se mettent également en place en France sur des thématiques de recherche précises.

L'évaluation a posteriori de la recherche. L'évaluation a posteriori s'effectue en pratique par une acceptation ou un refus des résultats de la recherche (*e.g.* refus de cultures OGM). A notre connaissance, aucun outil pratique ne permet de faciliter les liens sciences/sociétés par ce biais.

Participation à la construction de la prospective. La Fondation Sciences Citoyennes (FSC), association créée en 2002, a pour objectif de favoriser et prolonger le mouvement actuel de réappropriation citoyenne et démocratique de la science, afin de la mettre au service du bien commun. En faisant la promotion de l'élaboration démocratique des choix scientifiques et techniques, la FSC réunit les actions qui permettront aux sociétés d'influer sur les perspectives de recherche.

Instigation des recherches. La capacité de la recherche à s'emparer des questionnements de la société passe par l'implication concrète de celle-ci dans le déroulement de la recherche. Dans cet axe, les PICRI (Partenariats Institutions Citoyens pour la Recherche et l'Innovation) du Conseil Régional Île-de-France visent à promouvoir des programmes de recherche reposant sur une collaboration étroite entre laboratoires de recherche publics et organisations de la société civile à but non lucratif. Cette initiative s'approche du concept de Boutique des Sciences présenté dans la section suivante.

Il convient de bien définir ces outils et de les évaluer. D'abord pour éviter qu'ils ne soient que des simulacres d'un idéal participatif, et ensuite pour que les relations créées entre recherche et société soient du type gagnant-gagnant. En effet, les

attentes sociales qui sont exprimées envers le monde de la recherche peuvent être également bénéfiques à la science, par exemple en apportant de nouveaux sujets de recherche. L'expérience éprouvée de pays tels que le Danemark et les Pays-Bas permet de préciser les outils à utiliser et leurs conditions d'efficacité.

3. Les Boutiques des Sciences (BdS)

3.1. Concept de Boutique des Sciences

Selon (Gnaiger et Martin, 2001), une Boutique des Sciences (BdS) « fournit un support de recherche indépendant et participatif qui répond aux préoccupations de la société civile ». Toutes ont leur spécificité, leur approche, leur public. Contrairement à ce que l'appellation « Boutique » laisse penser, une BdS est un organisme à but non lucratif qui se veut être l'interface entre des groupements de citoyens (associations, syndicats, ...) qui ne peuvent pas se payer des études indépendantes, et des institutions scientifiques (Neubauer, 2002). Elle propose une source de production de savoirs, complémentaire à celles des demandes de l'état et du marché, répondant à une demande sociale.

Une BdS n'entreprend pas elle-même une recherche mais contribue à traduire une telle question en des termes suscitant une démarche scientifique. La recherche, nécessitant parfois uniquement un travail bibliographique, est ensuite effectuée par un(e) étudiant(e) dans le cadre de sa formation universitaire ou par un(e) chercheur(e) d'un laboratoire à même de traiter la question posée. Dans les grandes lignes, une BdS réalise donc le lien entre l'organisation demandeuse et ce laboratoire compétent. Le fonctionnement exact d'une BdS à Grenoble, et en particulier le financement des projets, est encore à préciser (*cf.* section 5.2).

3.2 Expériences étrangères des Boutiques des Sciences

Le concept de BdS a vu le jour au sein des mouvements étudiants et contre-culturels du début des années 1970, lorsqu'un groupe d'étudiants néerlandais a décidé d'aider des personnes à titre gracieux. Aidés par le personnel de l'université, ils avaient pour but d'accroître l'influence de la société civile dans les cercles académiques et de constituer de meilleurs liens entre groupes de citoyens et scientifiques. En l'espace de dix ans, l'idée s'était étendue à toutes les universités néerlandaises qui instituèrent une ou plusieurs BdS. Inspirés par cette expérience, d'autres BdS firent leur apparition en Europe. À mesure que l'idée s'est diffusée, elle a évolué et s'est adaptée aux conditions et aux besoins locaux.

Aujourd'hui, on compte près d'une centaine de BdS en Europe. La France est malheureusement peu représentée, faute de soutien institutionnel. Depuis quelques années, ces BdS se sont mises en réseau (Living Knowledge Network) au sein du programme Européen TRAMS (Training & Mentoring of Science Shops) qui vise à diffuser le concept de BdS, mais aussi à appuyer la création de nouvelles structures et à former de futurs administrateurs de BdS.

Deux constantes sont observées dans les BdS européennes en fonctionnement : elles possèdent un fort appui des institutions et sont ancrées dans des problématiques régionales. Lorsqu'en 2002, le ministre Belge, F.-X. De Donnea, lance la question de l'expertise scientifique au service du citoyen, le questionnement fait naître la BdS de Bruxelles. La Queen's University (Royaume-Uni) constatant le

rôle important de la BdS de Belfast a repris son financement. Au cours des cinq dernières années, la Boutique a oeuvré sur plus de 400 projets de recherche.

L'Europe est de ce point de vue une source d'inspiration au plan international. Le programme Community-University Research Alliances (CURA) a été créé pour soutenir des partenariats entre chercheurs et organismes communautaires Canadiens. Aux États-unis, l'Institut Loka a été l'instigateur, il y a huit ans, du Community Research Network (réseau de recherche en milieu communautaire) pour accroître la capacité des centres de recherche en milieu communautaire et ainsi répondre au manque de financement alloués pour les besoins de la collectivité.

3.3. Expériences françaises des Boutiques des Sciences

Au début des années 80, 16 boutiques existaient dans l'hexagone, mais ont progressivement disparues. C'est notamment le cas de la Boutique créée à Grenoble. Cette BdS créée avec le CCSTI et le soutien moral des universités, n'a fonctionné que deux ans (Frumy, 1983). Deux raisons majeures ont participé à l'échec de cette BdS : d'une part la difficulté des acteurs à traiter les demandes qui dépassaient les compétences du noyau dur de la BdS, et d'autre part le manque de soutien des institutions.

Plus récemment, une BdS s'est ouverte à Cachan sous l'impulsion d'étudiants de l'ENS Cachan. Après trois ans d'existence, le bilan est mitigé : plusieurs demandes de projets ont pu être traitées, mais la pérennisation de la structure est menacée par la dispersion de ses acteurs principaux. Suite à la création de cette BdS à Cachan, un mouvement de création de BdS est apparu en France. Soutenues par la FSC, plusieurs initiatives ont vu le jour en France, notamment à Avignon et Montpellier.

4. Étude de la faisabilité d'une BdS à Grenoble

Grenoble est riche des différents acteurs que relie une BdS: 1) un large spectre de thèmes de recherches, 2) une importante population universitaire, 3) des institutions publiques impliquées dans des orientations de la recherche (*e.g.* financement du projet Minatec) et 4) un questionnement social fort sur le développement des sciences (en particulier sur l'environnement, les neurosciences et les nanotechnologies). La région Grenobloise nous semble donc être un terreau idéal dans lequel une BdS pourrait recueillir des questionnements et les traiter.

Le questionnaire (Chevalier et al., 2007) vise à étudier la faisabilité d'une BdS dans le tissu Grenoblois, ainsi que son adéquation au projet de création d'une plus forte relation entre science et société. L'enquête s'est déroulée pendant la Fête des Sciences, les 7, 8 et 14 octobre 2006, à Grenoble sur les sites du chapiteau des sciences (Place V. Hugo), du polygone scientifique et de l'INRIA. Les questionnaires ont été administrés par 7 enquêteurs, en suivant le protocole suivant : dans un premier temps, l'enquêteur expliquait le concept d'une BdS, et dans un second temps, le sondé pouvait répondre aux 12 questions et à tout moment demander des précisions sur les BdS. Le nombre réduit d'enquêtes (203) ne permet pas de présenter de conclusions toujours statistiquement significatives mais autorise néanmoins de mettre en évidence des tendances.

La conclusion de l'enquête présente un intérêt estimé fort par la population de la région Grenobloise pour une telle structure (avec une priorité pour le traitement de questions environnementales et de santé). En effet, les sondés pensent quasi-

unanimement (94.6%) qu'une BdS est un moyen intéressant pour les impliquer plus largement dans les décisions de recherche. Le plébiscite est tout aussi clair (97.0% favorable ou très favorable) pour la création d'une BdS sur Grenoble. Avec de tels résultats, on peut s'attendre à ce qu'une BdS puisse être utilisée et reconnue par les citoyens. De plus, les sondés sont favorables à son financement par les pouvoirs publics.

L'enquête a mis en évidence que l'intérêt est estimé plus important par les personnes des catégories socio-professionnelles inférieures (CSP-) que celles des CSP+, bien qu'elles ne se sentent pas moins impliquées dans les décisions de recherche. En l'absence de plus d'information, nous avons émis l'hypothèse d'un lien avec une compétence scientifique estimée ou vécue : les CSP- ont peut être un accès plus difficile à l'information scientifique. La BdS permettant de résoudre ce problème, un intérêt concret est perçu, et positivement estimé.

5. ADReCA : La Boutique des Sciences de Grenoble

5.1 Présentation de ADReCA

L'association pour le Développement d'une Recherche Citoyenne Active, ADReCA, constitue une interface entre groupements de citoyens (associations, syndicats □) et institutions scientifiques (universités, laboratoires □). Elle a deux objets. Premièrement, elle vise à faciliter la production de connaissances scientifiques *à partir de et au bénéfice de* la demande sociale. Les moyens pour mener à bien cette mission sont de susciter et permettre la réalisation de projets de recherche indépendants, et de répondre aux préoccupations de la société civile. Deuxièmement, elle permettra la diffusion des résultats de ces projets de recherche ou études scientifiques en garantissant un accès libre et gratuit, et en facilitant leur compréhension auprès du public.

ADReCA a vu le jour grâce à la convergence des points de vue de moniteurs du CIES de Grenoble et de participants au programme Sokori venant du milieu associatif (éducation populaire) et du CNRS. L'envie commune de voir la parole citoyenne occuper un réel espace au sein des instances décisionnelles de la recherche, ainsi que l'enquête réalisée, nous ont amenées à croire en la concrétisation et la viabilité d'une Boutique des Sciences dans la région Grenobloise. Cependant, les expériences de BdS française montrent que ADReCA a besoin pour assumer ses missions de nouveaux membres, représentant des milieux plus diversifiés que ceux dont les fondateurs émanent. La réussite du projet de BdS sera conditionnée par sa pluridisciplinarité, ainsi que par l'indépendance qu'ADReCA saura conserver vis-à-vis des institutions partenaires locales.

5.2 Vers la définition d'un fonctionnement d'ADReCA

La première activité de l'association ADReCA consiste en l'organisation d'un *scenario workshop* (SWS), concept développé par le FBI Centre d'Innsbruck, qui aura pour but la définition du fonctionnement d'ADReCA dans le contexte régional.

Des représentants des quatre types d'acteurs permettant le fonctionnement d'ADReCA (associations, scientifiques, étudiants, décideurs politiques) seront réunis et interviendront dans la réalisation de projets « test ». Nous souhaitons ainsi formaliser les démarches et les étapes à suivre lors de la réalisation d'un projet par

l'association, et faire ressortir les problèmes qui pourraient surgir en situation réelle (communication entre les acteurs, poids de l'enjeu politique/économique, notion de neutralité, encadrement, financement, etc.). Ce SWS s'inscrit dans un programme de plusieurs SWS sur le thème des BdS (en Île-de-France et PACA courant 2007), soutenu par la Fondation Sciences Citoyennes et en partenariat avec la BdS de Cachan (association PRACCIS).

6. Conclusions/Perspectives

L'atelier-projet « Recherche, Ethique, Citoyenneté » nous a conduits à proposer de renforcer concrètement le lien entre sciences et société. Parmi un large choix d'outils de recherche citoyenne qui ont été expérimentés en Europe et dans le monde, nous nous sommes intéressés aux Boutiques des Sciences, *i.e.* une interface qui fournit un support de recherche participatif. Une première étude de faisabilité pertinente sur la demande sociale nous a incité à créer l'association ADReCA, support de cette BdS. L'organisation d'un *scenario workshop* s'inscrit dans la continuité d'une relation construite entre les différents partenaires. Il permettra également, par des échanges croisés, d'inscrire cette BdS dans les réseaux de BdS européennes (Living Knowledge) et françaises (Partenariat FSC, PRACCIS). Afin de poursuivre cette démarche, des outils méthodologiques (enquête qualitative par exemple) devront être mis en place pour informer et sensibiliser les acteurs de la recherche et la société sur le fonctionnement de la BdS.

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Les auteurs

Les auteurs de cet article sont doctorants, moniteurs du CIES à Grenoble. F. Chevalier (Lab. PDC) effectue une thèse en biologie cellulaire. T. Guyet et L. Maisonnasse (Lab. LIG) réalisent leurs travaux en informatique. C. Lomparski (Institut Albert Bonniot) achève une thèse en immunologie. E. Mondoloni (Lab. GRESEC) effectue une thèse en communication. F. Ong (Institut Neel) travaille sur la physique des basses températures. Enfin, T. Sainte-Luce Banchelin (Lab. SERCO) travaille dans le domaine de la chimie organique. Ce travail a été encadré par C. Lartigue (Lab. LMGP) et A.-M. Benoit (IEP Grenoble, PACTE).

Cet article est issu du projet « Recherche, Éthique, Citoyenneté », atelier de troisième année de monitorat né de l'initiative d'un groupe de moniteurs.

Étant publié dans les *Annales des ateliers du CIES de l'académie de Grenoble* (volume 1, 2007), l'article résume l'avancée du projet initial dans le cadre du monitorat et mentionne la création de l'association ADReCA (Association pour le Développement d'une Recherche Citoyenne et Active) en février 2007. Depuis sa création, plusieurs activités ont été menées par l'association ADReCA :

- Organisation d'un « Scenario Workshop (Atelier Scénario) » (Quaix-en-Chartreuse, juin 2007) (descriptif voir article)
- Participation à la 3^{ième} Conférence du réseau international des Boutiques de Sciences Living Knowledge (Paris, août 2007)
- Animation d'un atelier et tenue d'un stand aux Rencontres Mondiales de la Démocratie Participative – Région Rhône-Alpes (Lyon, décembre 2007)
- Réunion publique organisée par l'ADReCA, présentation et discussion ouverte sur le concept de Boutique de Sciences et le projet de développement local (EVE, Grenoble, Janvier 2008)
- Conférence-débat et table ronde « les Boutiques de Sciences – ouvrir la recherche à la société civile » (INSA, Lyon, 27 mars 2008 ; MSH, Grenoble, 28 mars 2008) ; Invitation de Henk Mulder (chercheur à l'université de Groningue, co-président des Science Shops des Pays-Bas)
- Présentation d'un poster « Les Boutiques de sciences : une démarche de co-construction des savoirs ouverte à la société civile » aux 29^{èmes} Journées Internationales sur la communication, l'éducation et la culture scientifiques, techniques et industrielles (Chamonix, mai 2008)
- Participation à l'organisation du relais local du Forum Social Mondial (Bélem, Brésil, janvier 2009) dans le cadre de la « EVE Maison Citoyenne » au Campus Universitaire de Grenoble ; organisation d'une journée d'information et de débats sur le thème « Marchandisation des Savoirs »
- Proposition de sujets de stages dans le cadre de l'unité d'ouverture « Projet avec un tiers » au DLST (département des licences en Sciences et Technologies) de l'université Joseph Fourier Grenoble (2^{ième} semestre de l'année universitaire 2008-2009, stages en cours)

A. PUBLICATIONS

- Proposition de projet international associant 25 partenaires européens en réponse à l'appel d'offre « Science en Société » de la Commission Européenne (7^{ième} PCRD) (en attente de réponse, ayant franchi le seuil d'éligibilité)

A.2 Article in Preparation

The article in preparation provisionally entitled “MSRV (HERV-W) ENV ACTIVATES INNATE IMMUNITY AND CAUSES EAE IN MICE” was meant to be shown here. Unfortunately, not all of the relevant data is available – or at least it is not accessible to me.

The article will treat some of the experimental data shown in the present manuscript, notably concerning dendritic cells (DC) and the Multiple Sclerosis animal model EAE (Experimental Autoimmune Encephalomyelitis). Additional data acquired by or in collaboration with the co-authors will complete the article that will be finalised and submitted soon.

The following elements will be presented:

- Inflammatory properties of ENV:
 - ENV-induced cellular maturation of murine DC (BMDC) assessed by flow cytometry
 - ENV-induced inflammatory cytokine production by murine DC assessed by ELISA
 - Neutralisation of the ENV-induced cytokine production by monoclonal anti-ENV antibodies
- Innate immune receptors:
 - ENV-induced cellular maturation of murine DC of Tlr4^{-/-} and Cd14^{-/-} mice as compared to wild-type C57BL/6
 - ENV-induced inflammatory cytokine production by murine DC of Tlr4^{-/-} and Cd14^{-/-} mice as compared to wild-type C57BL/6
- EAE:
 - Clinical Scoring of ENV/IFA/MOG-induced EAE as compared to CFA/MOG-induced EAE
 - Magnetic Resonance Imaging (MRI) of EAE brains
 - Immunohistochemistry of EAE brains

A. PUBLICATIONS

- Cytokine production by splenocytes of immunised animals in response to MOG (MOG recall)

Its authors, in alphabetical order, are:

Corinne BERNARD

Rachel COUDERC

Evelyne JOUVIN-MARCHE

Jean-Baptiste LANGLOIS

Christina LOMPARSKI

Patrice MARCHE

Hervé PERRON

Iuliana POPA

Jacques PORTOUKALIAN

Hei-lanne REYNAUD-DOUGIER

Alexandre ROLLAND

Kamel SANHADJI

Christian VILLIERS

Appendix B

International Communications

B.1 European Congress of Immunology (ECI) 2006

1st Joint Meeting of European National Societies of Immunology under the Auspices of EFIS, 16th European Congress of Immunology, September 6-9, 2006 - Paris, France

Multiple Sclerosis Retroviral Envelope causes inflammation by monocyte, DC and T cell activation

C. Lomparski¹, H.L. Dougier¹, A. Rolland¹, C. Bernard², C. Viret¹, E. Jouvin-Marche¹, H. Perron², P. N. Marche¹

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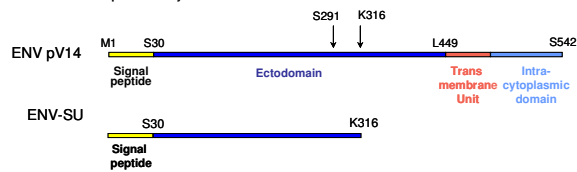


Introduction

Abstract: The Multiple Sclerosis RetroViral element (MSRV) isolated from MS patients cerebrospinal fluid (Perron et al., 1991) belongs to the Human Endogenous RetroVirus (HERV) W family which comprises about 2% of the human genome.

MSRV recombinant envelope protein (ENV) may contribute to initiate and/or exacerbate MS. 1) The soluble subunit of ENV (ENV-SU, see figure below) activates PBMCs from MS patients blood to produce inflammatory cytokines such as IFN- γ , IL-12p40 and IL-6, in relation with disease severity score (Rolland et al., 2005). 2) ENV-SU induces human monocytes and dendritic cells to produce inflammatory cytokines through engagement of CD14 and TLR4. Anti-CD14, anti-TLR4 and anti-ENV antibodies block the activation by ENV-SU (Rolland et al., 2006). In this study we have further characterized the pro-inflammatory properties of the soluble fraction of the envelope protein of MSRV (ENV-SU).

For *in vivo* validation in the Experimental Allergic Encephalitis (EAE) model, mice are injected with antigenic myelin peptide and adjuvant (Complete Freund) or ENV protein. Clinical score shows significant EAE symptoms both in mice injected with ENV or adjuvant, and no symptoms in control mice. Evaluation of myelin damage in brain and of immune responses from ENV injected mice will provide clue to validate if disease promoted by ENV is similar to conventional EAE.



Organizations of the envelope: ENV-pV14 (full-length MSRV envelope protein) and ENV-SU. ENV-SU is a 293-aa fraction, representing the extracellular surface unit cleaved at K316 of the full-length ENV pV14 protein.

Conclusions and Perspectives

In the present study we show that MSRV, a retrovirus often found in MS patients, encodes an envelope protein with the ability to directly activate cells of the innate immune system such as monocytes through pattern recognition receptors TLR4/CD14.

This activation is also characterized by an over-production of IL-6 and IL-12p40 in MS patients when compared with healthy controls. This production was correlated with the patients' clinical score (EDSS).

The role of infections and the resulting activation of the innate immune system together with the over-production of cytokines such as IL-6 and IL-12p40 has been argued in the initiation of inflammation in MS pathogenesis. MSRV and the pro-inflammatory properties of its ENV-SU could thus play a major role in MS.

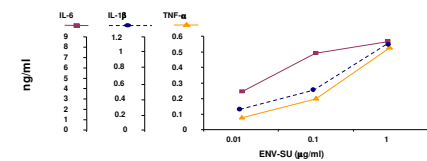
Considering the potent pro-inflammatory properties of ENV-SU, it appears that blocking monoclonal antibodies directed against ENV-SU or its receptors could have beneficial effects in diseases expressing MSRV such as MS.

Preliminary experiments show that anti-ENV antibodies block encephalitis in EAE model when given together with ENV. In conclusion, ENV can promote *in vivo* inflammation leading to disease similar to EAE.

Thus further evaluation in animal models could ultimately provide us with the information regarding the role of ENV-SU in MS pathogenesis and will allow us to evaluate therapeutic applications for anti-ENV-SU.

Results

1) ENV-SU induces a dose-dependant production of pro-inflammatory cytokines in human PBMC cultures

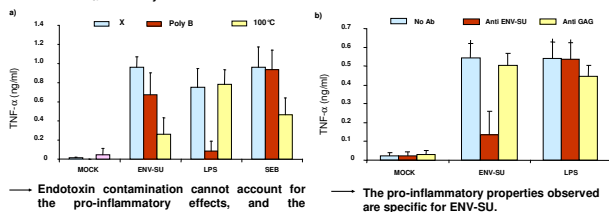


Human Peripheral Blood Mononuclear Cells (PBMC) were stimulated with graded doses of ENV-SU for 24h and cytokine productions were analyzed by ELISA.

NB: No cytokine production was obtained with a mock control protein synthesized and purified under the same conditions as ENV-SU.

2) ENV-SU pro-inflammatory properties are specifically blocked by monoclonal antibodies

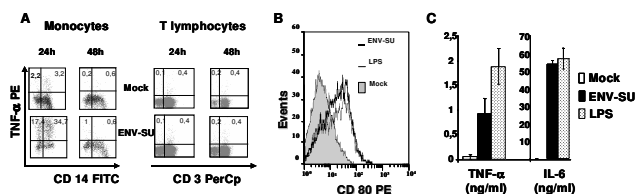
Human PBMCs were stimulated for 24 h with mock control, ENV-SU, LPS (bacterial endotoxin) or SEB (bacterial SAg) all at 1 μ g/ml. a) Where indicated, cells were treated with Polymyxin B an endotoxin inhibitor or incubated with proteins and toxins boiled for 30 min. b) Where indicated, cells were pre-incubated with monoclonal antibodies directed against ENV-SU or Mock. Culture supernatants were then harvested and tested for TNF- α release by ELISA.



Endotoxin contamination cannot account for the pro-inflammatory effects, and the component responsible for these effects is the protein ENV-SU.

The pro-inflammatory properties observed are specific for ENV-SU.

3) ENV-SU directly activates purified monocytes



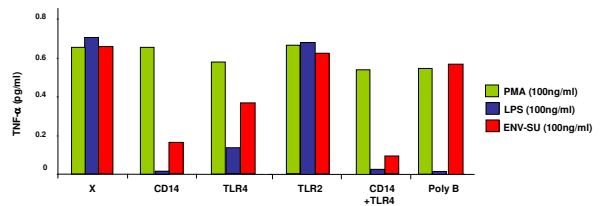
A - PBMC were stimulated with Mock or ENV-SU for 24 and 48h and intracellular TNF- α production was then assessed by flow cytometry on monocytes and lymphocytes. ENV-SU preferentially targets monocytes for cytokine secretion.

B - Monocytes were purified from human PBMCs and stimulated for 24h with Mock, ENV-SU or LPS. Cells were harvested and the expression of the activation marker CD80 was assessed by flow cytometry. ENV-SU is able to directly activate purified monocytes as assessed by CD80 expression.

C - Supernatants from stimulated monocytes were harvested and analyzed for cytokine release. ENV-SU induces the secretion of the pro-inflammatory cytokines TNF- α and IL-6 by purified monocytes.

4) ENV-SU pro-inflammatory properties are mediated through CD14/TLR4

Purified monocytes (~95%) were pre-incubated with neutralizing mAbs directed against CD14, TLR4, or TLR2, or with Polymyxin B for 1h prior to stimulation with PMA, LPS or ENV-SU. Supernatants were then harvested and tested for TNF- α production by ELISA.



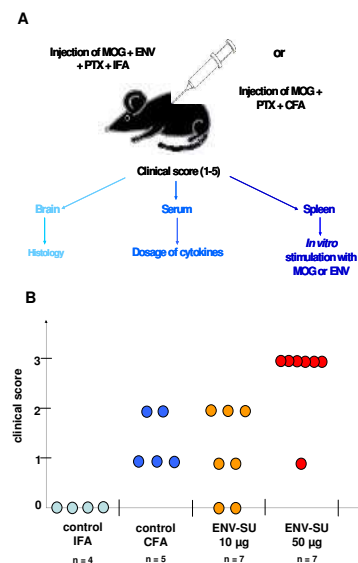
5) Experimental Allergic Encephalopathy Model

A - For *in vivo* validation in the Experimental Allergic Encephalitis (EAE) model, mice are injected with Pertussis Toxin (PTX), antigenic myelin peptide and adjuvant (Complete Freund) or ENV-SU protein.

B - In preliminary studies, clinical score shows significant EAE symptoms both in mice injected with ENV or adjuvant, and no symptoms in control mice.

Evaluation of myelin damage in brain and of immune responses from ENV injected mice will provide clue to validate if disease promoted by ENV is similar to conventional EAE.

NB: Cultures of splenocytes from either ENV or adjuvant treated mice with the myelin antigen lead to IFN- γ production, suggesting a T lymphocyte reactivity towards the myelin antigen (data not shown).



A - For *in vivo* validation in the Experimental Allergic Encephalitis (EAE) model, mice are injected with Pertussis Toxin (PTX), antigenic myelin peptide and adjuvant (Complete Freund) or ENV-SU protein.

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NB: Cultures of splenocytes from either ENV or adjuvant treated mice with the myelin antigen lead to IFN- γ production, suggesting a T lymphocyte reactivity towards the myelin antigen (data not shown).

B.2 Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) 2007

12th Annual Conference of Rehabilitation in MS, October 11-14, 2007 - Prague, Czech Republic

Multiple Sclerosis Retroviral Envelope causes inflammation by DC and T cell activation in humans and mice

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2 – Geneuro, Genève, Switzerland



Introduction

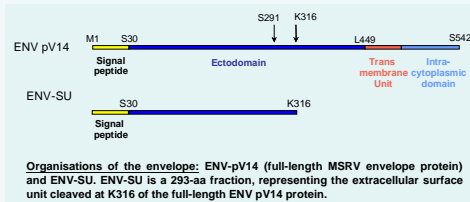
Several lines of evidence support that the envelope protein (ENV) of the multiple sclerosis retroviral element (MSRV) or its soluble extracellular subunit (ENV-SU) contributes to Multiple Sclerosis (MS). MSRV isolated from MS patients cerebrospinal fluid belongs to the human endogenous retrovirus (HERV) W family which comprises about 1% of the human genome.

Previous work of our group showed that MSRV ENV / ENV-SU may initiate and/or exacerbate MS : 1) ENV stimulates peripheral blood mononuclear cells (PBMC) which display polyclonal expansion of TCR Vb16 T cells supporting a possible superantigen activity, 2) ENV-SU activates PBMC from MS patients to produce inflammatory cytokines in relation with disease severity score (Rolland et al., 2005), 3) ENV-SU induces human monocytes and dendritic cells (DC) to produce inflammatory cytokines through engagement of CD14 and TLR4 (Rolland et al., 2006).

In order to study *in vivo* effects, mice were treated for experimental allergic encephalitis (EAE) induction, the mouse model for MS, after antigenic myelin peptide immunisation either with complete Freund's adjuvant (CFA) or ENV-SU. Clinical score showed significant EAE symptoms in both mice but no symptoms in control mice (no CFA or ENV-SU). Administration of anti-ENV-SU antibodies blocked EAE symptoms induced only in ENV-SU treated mice. Cultures of splenocytes from either ENV-SU or CFA-treated mice, recalled with the myelin antigen, led to IFN- γ production, suggesting T lymphocyte reactivity towards the myelin antigen.

To characterise the mode of action of ENV in mice, DC were cultured from C57BL/6 bone marrow and incubated with ENV-SU. DC secreted amounts of IL-6 dose-dependent on ENV-SU. DC derived from *Tlr4*^{-/-}, *Cd14*^{-/-} and *Myd88*^{-/-} knock out mice were unable to respond to ENV-SU stimulation, arguing that, as found in humans, signalling through TLR4/CD14 pathway via MyD88 is involved in DC response in mice.

In conclusion, by promoting inflammatory response through CD14/TLR4 pathway, the envelope of MSRV/HERV contributes to EAE in mice and MS in humans.



Conclusions and Perspectives

In the present study we show that MSRV, a retrovirus often found in MS patients, encodes an envelope protein with the ability to directly activate cells of the innate immune system such as dendritic cells through pattern recognition receptors TLR4/CD14.

This activation is also characterised by an over-production of IL-6 and IL-12p40 in MS patients when compared with healthy controls. This production was correlated with the patients' clinical score (EDSS).

The role of infections and the resulting activation of the innate immune system together with the over-production of cytokines such as IL-6 and IL-12p40 has been argued in the initiation of inflammation in MS pathogenesis. MSRV and the pro-inflammatory properties of its ENV-SU could thus play a major role in MS.

Considering the potent pro-inflammatory properties of ENV-SU, it appears that blocking monoclonal antibodies directed against ENV-SU or its receptors could have beneficial effects in diseases expressing MSRV such as MS.

Mouse dendritic cells are activated *in vitro* by ENV-SU through the TLR4/CD14 pathway as in humans ; mice injected with ENV-SU and myelin antigen develop EAE symptoms ; in conclusion, ENV can promote *in vivo* inflammation leading to disease similar to EAE.

Thus further evaluation in animal models could ultimately provide us with the information regarding the role of ENV-SU in MS pathogenesis and will allow us to evaluate therapeutic applications for anti-ENV-SU.

Methods and Results

1) Experimental Allergic Encephalopathy Model

A - For *in vivo* validation in the Experimental Allergic Encephalitis (EAE) model, mice are injected with Pertussis Toxin (PTX), antigenic myelin peptide and adjuvant (Complete Freund) or ENV-SU protein.

B - In preliminary studies, clinical score shows significant EAE symptoms both in mice injected with ENV or adjuvant, and no symptoms in control mice.

Evaluation of myelin damage in brain and of immune responses from ENV injected mice will provide clue to validate if disease promoted by ENV is similar to conventional EAE.

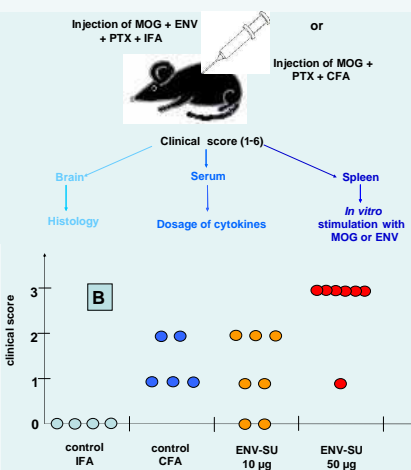
NB: Cultures of splenocytes from either ENV or adjuvant treated mice with the myelin antigen lead to IFN- γ production, suggesting a T lymphocyte reactivity towards the myelin antigen (data not shown).

CLINICAL SCORE :

0	no symptoms
1	tail weakness
2	clumsy gait, hind limb weakness
2.5	hind limb weakness, tremor, loss of balance
3	hind limb paresis
4	hind limb paralysis
5	hind limb paralysis, sphincter dysfunction, nasal and ocular haemorrhages, forelimb paresis or paralysis
5.5	moribund stage without immediate death
6	moribund stage without immediate death, respiratory dysfunctions, death

Cumulative scores representation of two EAE experiments.

	disease incident (nb)	mean disease onset (d)	mean score d35	mean max score	mean cumulative score d35
IFA	0 / 4	-	0	0	0
CFA	5 / 5	12.2	1.4	1.8	31.2
ENV 50 μ g	7 / 7	9.1	1.9	2.9	56.9
ENV 10 μ g	7 / 7	14.6	0.4	1.6	18.7
IFA	2 / 2	15.5	0	1	13
CFA	3 / 3	11	2.3	3	93
ENV 10 μ g	5 / 5	11	0.4	1.6	40.3
ENV 25 μ g	5 / 5	9.4	1.8	3.5	90.8
ENV 50 μ g	5 / 5	8.6	1.7	2.8	89.5
ENV 50 μ g without MOG	3 / 3	8.7	0.7	2.2	45.2

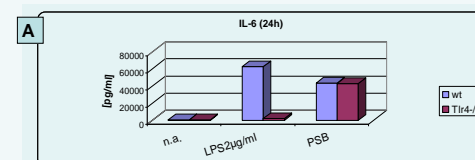


2) Dendritic cell culture essays

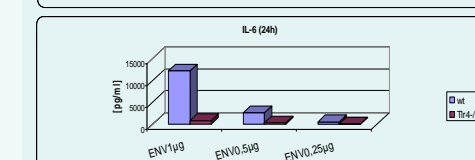
Haematopoietic precursor cells were isolated from mouse femur bone marrow and differentiated to dendritic cells by adding appropriate growth factors. Precursor cells were isolated from C57BL/6 wild-type, *Tlr4*^{-/-}, *Cd14*^{-/-} and *Myd88*^{-/-} mice.

Bone-marrow derived dendritic cells (BMDC) were cultured with ENV-SU or other immune activators to elucidate the inflammatory capacity of the ENV-SU-mediated pathway via TLR4. Antibodies anti-ENV-SU were utilised to block the inflammatory action of ENV-SU.

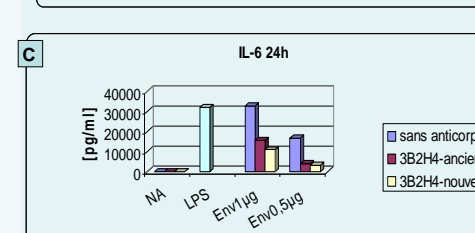
A - Control activation of BMDC by LPS and PSB ; comparison of ENV-SU-mediated activation of wild-type and *Tlr4*^{-/-} BMDC by assessment of IL-6.



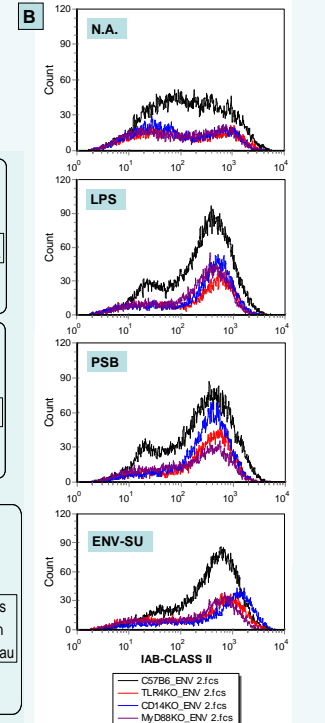
B - Comparison of ENV-SU-mediated activation of wild-type, *Tlr4*^{-/-}, *Cd14*^{-/-}, *Myd88*^{-/-} BMDC by FACS analysis of surface maturation markers. CD11c is a specific marker for DC. Mature DC are CD11c⁺ and lab/classII⁺.



C - Neutralisation of the inflammatory activity of ENV-SU in BMDC cultures by monoclonal anti-ENV-SU antibodies assessed by ELISA for IL-6.



NB : Neutralisation of ENV-SU by monoclonal antibodies has been confirmed on cultures of brain and endothelial cells (data not shown).



B.3 European BioAlpine Convention (EBC) 2007

2nd Edition - Inflammation and Autoimmunity: Bridging Public and Private Research, November 6-7, 2007 - Turin, Italy

(Poster Price)

Multiple Sclerosis Retroviral Envelope promotes EAE-like pathology in mice



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¹ – IAPC, Inserm U823, Institut Albert Bonniot, Grenoble, France
² – Geneuro, Genève, Switzerland



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Introduction

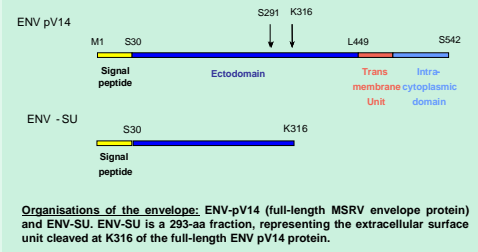
Several lines of evidence support that the envelope protein (ENV) of the multiple sclerosis retroviral element (MSRV) or its soluble extracellular subunit (ENV-SU) is associated to multiple sclerosis (MS). MSRV was initially isolated from MS patients cerebrospinal fluid. It belongs to the human endogenous retrovirus (HERV) W family which comprises about 1% of the human genome.

Our previous *in vitro* studies on human cells showed that MSRV ENV / ENV-SU may initiate and/or exacerbate inflammation by its superantigen-like properties, and the activation of monocytes and dendritic cells, via the TLR4/CD14 pathway.

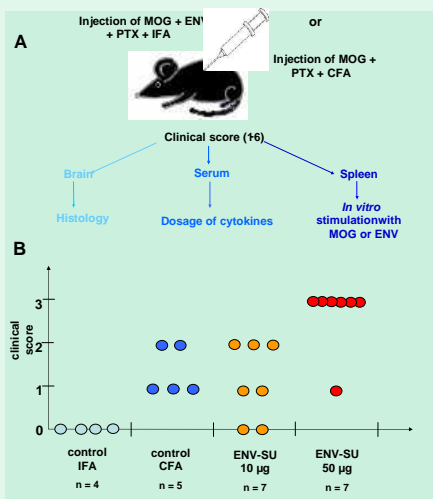
In order to seek for *in vivo* effects, we tested whether ENV can promote Experimental Autoimmune Encephalomyelitis (EAE), the mouse model for MS. Mice were treated for EAE induction, by immunisation against the antigenic myelin peptide MOG 35-55, in presence of either complete Freund's adjuvant (CFA) or ENV-SU in incomplete Freund's adjuvant (IFA). Clinical score showed significant EAE symptoms in both mice but no symptoms in control mice, i. e. mice injected with myelin antigen only.

Cultures of splenocytes from either ENV-SU or CFA treated mice, recalled with the myelin antigen, led to IFN- γ production, suggesting T lymphocyte reactivity towards the myelin antigen (data not shown).

Together with the findings that ENV-SU is stimulating DC maturation via the CD14/TLR4 pathway in humans (Rolland et al, 2006, J.Immunol 176:7636) and in mice (see poster of H-L Reynaud-Dougier), we conclude that the envelope of MSRV/HERV promotes EAE in mice and thus may contribute to MS in humans by promoting inflammatory response through CD14/TLR4 pathway.



Methods and Results



Cumulative scores representation of two EAE experiments.

	disease incident (nb)	mean disease onset (d)	mean score d35	mean max score	mean cumulative score d35
IFA	0 / 4	-	0	0	0
CFA	5 / 5	12,2	1,4	1,8	31,2
ENV 50µg	7 / 7	9,1	1,9	2,9	56,9
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ENV 10µg	5 / 5	11	0,4	1,6	40,3
ENV 25µg	5 / 5	9,4	1,8	3,5	90,8
ENV 50µg	5 / 5	8,6	1,7	2,8	89,5
ENV 50µg without MOG	3 / 3	8,7	0,7	2,2	45,2

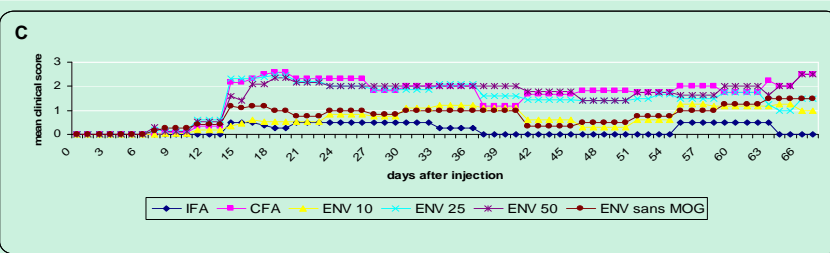
A For *in vivo* validation in the Experimental Allergic Encephalitis (EAE) model, mice are injected with Pertussis Toxin (PTX), antigenic myelin peptide and adjuvant (Complete Freund) or ENV-SU protein.

B In preliminary studies, clinical score shows significant EAE symptoms both in mice injected with ENV or adjuvant, and no symptoms in control mice.

C clinical score follow-up of 6 groups of mice during 70 days after injection

Evaluation of myelin damage in brain and of immune responses from ENV injected mice will provide clue to validate if disease promoted by ENV is similar to conventional EAE.

NB: Cultures of splenocytes from either ENV or adjuvant treated mice with the myelin antigen lead to IFN- γ production, suggesting a T lymphocyte reactivity towards the myelin antigen (data not shown).



CLINICAL SCORE :

0	no symptoms
1	tail weakness
2	clumsy gait, hind limb weakness
2,5	hind limb weakness, tremor, loss of balance
3	hind limb paresis
4	hind limb paralysis
5	hind limb paralysis, sphincter dysfunction, nasal and ocular haemorrhages, forelimb paresis or paralysis
5,5	moribund stage without immediate death
6	moribund stage without immediate death, respiratory dysfunctions, death

Conclusions and Perspectives

In the present study we show that MSRV, a retrovirus found in MS patients, encodes an envelope protein with the ability to activate T cell antigen response in mice that leads to Experimental Allergic Encephalopathy symptoms:

- > Mouse dendritic cells are activated *in vitro* by ENV-SU through the TL4/CD14 pathway as in humans ;
- > Mice injected with ENV-SU and the myelin antigen MOG develop EAE symptoms;
- > ENV can promote *in vivo* inflammation leading to disease similar to EAE.

The role of infections and the resulting activation of the innate immune system together with the over-production of cytokines such as IL-6 and IL-12p40 has been argued in the initiation of inflammation in MS pathogenesis. MSRV and the pro-inflammatory properties of its ENV-SU could thus play a role in MS.

Considering the potent pro-inflammatory properties of ENV-SU, it appears that blocking monoclonal antibodies directed against ENV-SU or its receptors could have beneficial effects in diseases expressing MSRV such as MS.

Thus further evaluation in animal models could ultimately provide us with the information regarding the role of ENV-SU in MS pathogenesis and will allow us to evaluate therapeutic applications for anti-ENV-SU.

Appendix C

MRI Protocol

Acquisitions on brain and spinal cords

MR Imaging: BRUKER Biospec 7 Tesla

Coils:

- Transmitting body coil, Bruker BioSpin MRI GmbH, Model: 1P T9974 (o.d. = 112 mm and i.d. = 72 mm)
- 15 mm receive-only coil, Bruker BioSpin MRI GmbH, Model: 1P T5795

Monitoring:

- Monitoring breathing rate on an oscilloscope with a pression sensor under the abdomen

Animal handling:

- The mouse is placed in prone position on the dedicated mouse bed
 - Equipped with stereotactic attachment (tooth bar and ear pins), integrated warm water bed for circulating water (37°C)
 - Equipped with moveable anesthesia mask to accommodate differing weights of mouse
- (see Fig 1)

Anesthesia :

- Gazous anesthesia

In an induction box, 3% isoflurane with 0.6 l/min air flow

After induction, during scanning the % of anesthesia is decreased at 1.5% with 0.6 l/min air flow

Images :

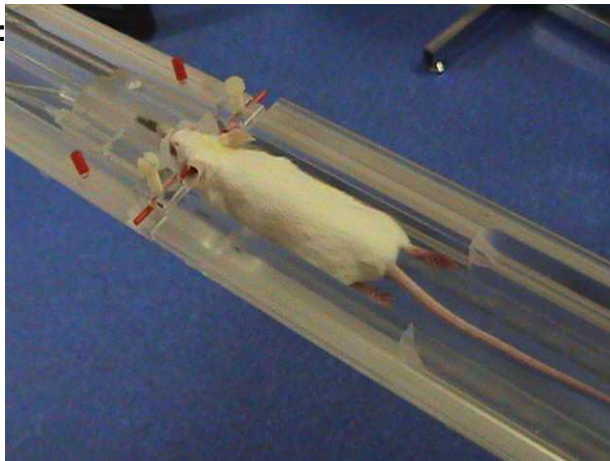


Figure 1

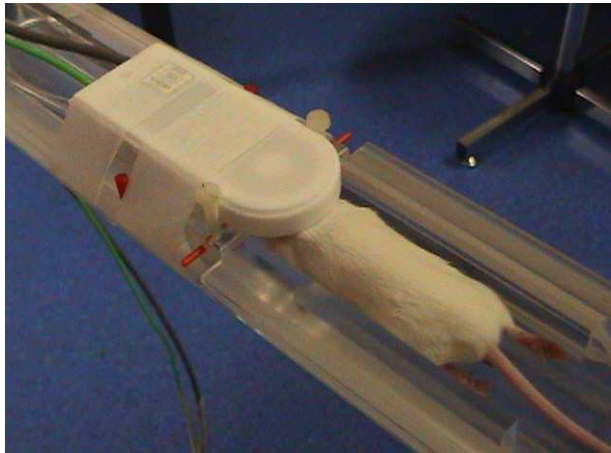
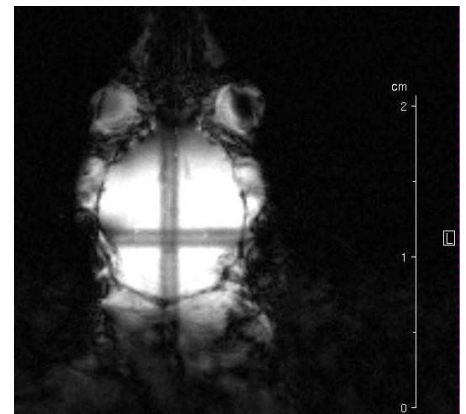
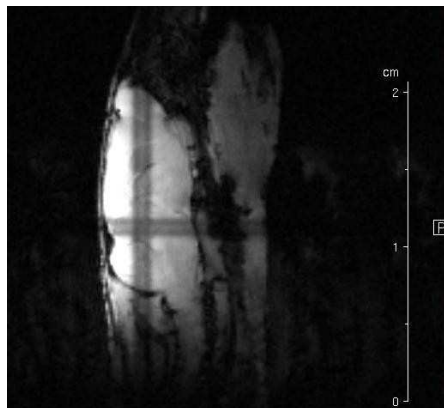
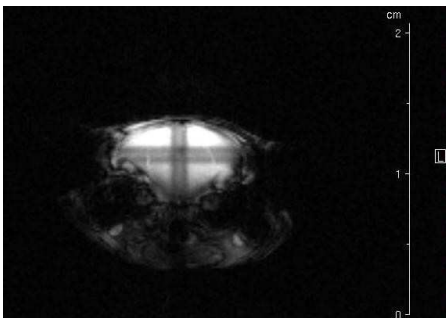


Figure 2: Coil positioning
for brain acquisition

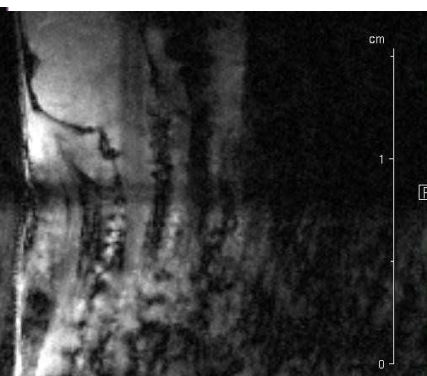
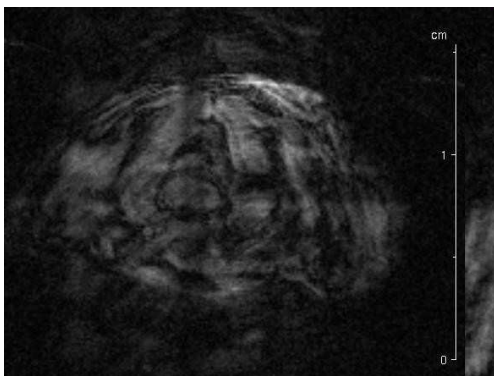
Sequence 1.1 : Localizer Tri pilot (Tri_Pilot_BR)

- Quick gradient-echo localizer with three orthogonal orientations used to identify the region of interest (ROI) and allowed graphic prescriptions for following scans (Figure 2)

Brain acquisition



Spinal cords



FOV = 4 x 4 cm, Matrix = 256 x 256 Resolution 156 x 156 μ m
Echo Time (ET) / Repetition Time (RT) = 36/4200 ms Nb slices = 3
Bandwidth = 50505 Hz
Time acquisition = 30 sec

Sequence 2.1 : T2 weight (TurboRARE_T2_BR)

- First T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m
ET/RT = 36/4200 ms
17 slices (800 μ m thickness)
Bandwidth = 35714 Hz / Fat Suppression = ON
Time acquisition = 2 mins

Sequence 2.2 : T2 weight (TurboRARE_T2_BR)

- Second T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m
TE1 = 17ms TE2 = 51 ms TR = 3000 ms
17 slices (800 μ m thickness)
Bandwidth = 55555 Hz / Fat Suppression = ON
Time acquisition = 2 mins

Sequence 3.1: Localizer Tri pilot (Tri_Pilot_BR) (for spinal cords)

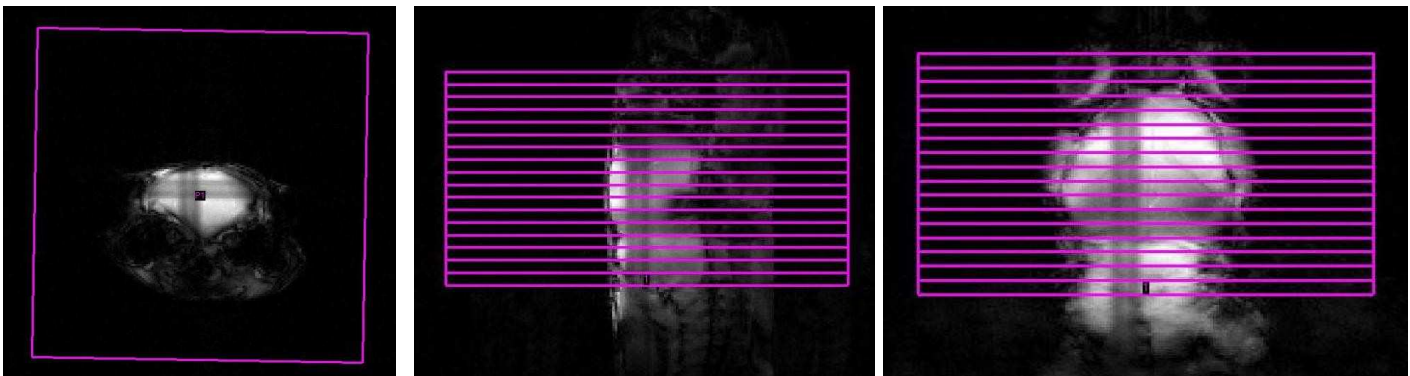
Sequence 4.1: T2 weight (TurboRARE_T2_BR) (for spinal cords)

Sequence 4.2: T2 weight (TurboRARE_T2_BR) (for spinal cords)

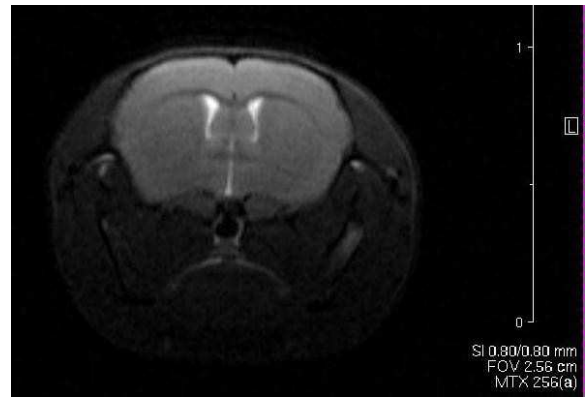
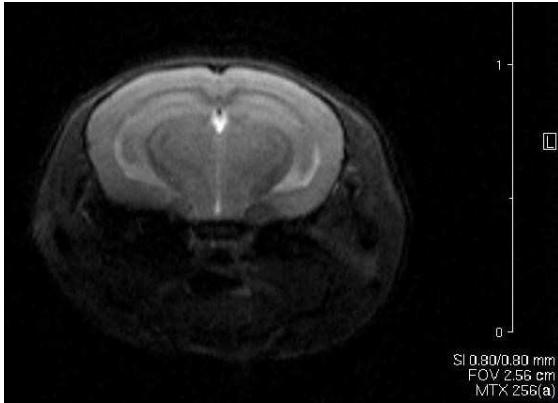
TOTAL TIME 40 minutes

EXAMPLES

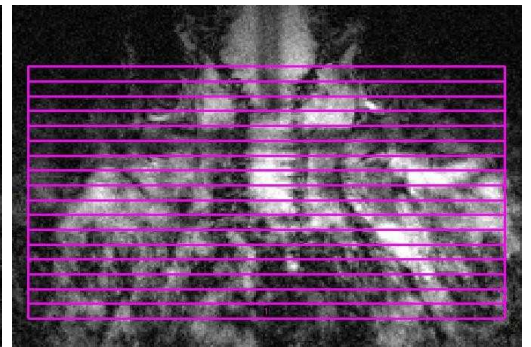
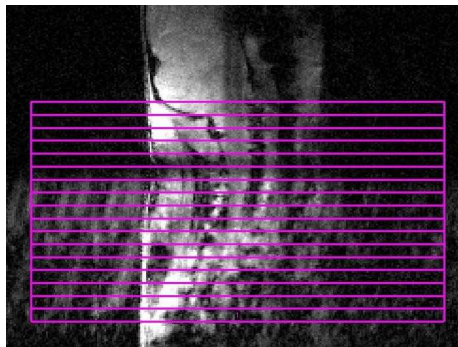
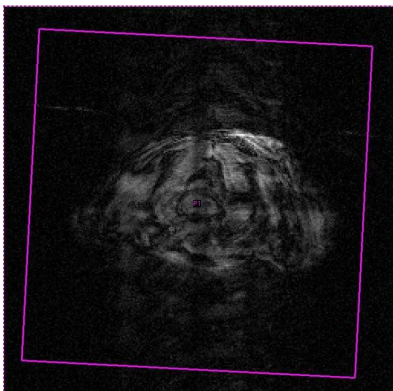
Images from 1st ROI: Brain acquisition (three orthogonal orientations)



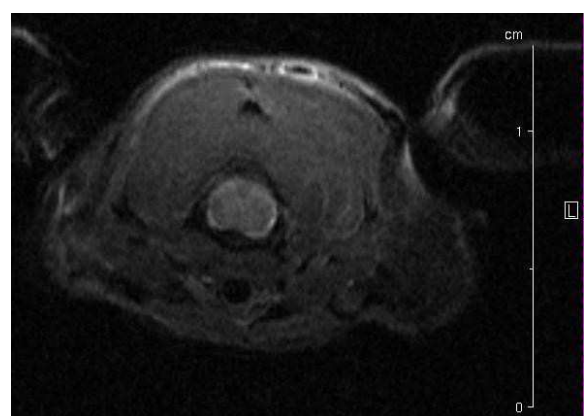
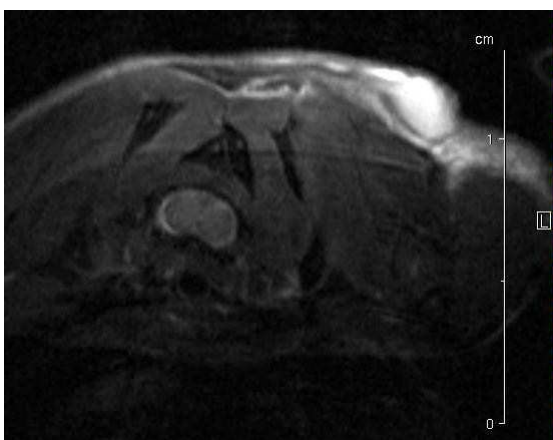
Brain images (Seq 2)



Images from 2nd ROI: Spinal cords acquisition (three orthogonal orientations)



Spinal cords images (Seq 4)



Protocole d'IRM

Sclérose en plaques : acquisition anatomique cerveau et moelle épinière

Appareil d'imagerie : BRUKER Biospec 7 Tesla

Antennes :

- Antenne volumique Bruker pour l'émission du signal RMN (diamètre interne de 72 mm)
- Antenne de surface Bruker pour la réception du signal RMN (diamètre de 15 mm)

Monitoring :

- Monitoring de la fréquence respiratoire en utilisant un capteur de pression placé sur l'abdomen, visualisation sur oscilloscope

Positionnement de l'animal :

- L'animal est placé en décubitus ventral sur le lit d'examen (lit souris Bruker) dans lequel est placée une circulation d'eau, thermorégulée, 37°C
- Le nez de la souris est maintenu dans le cadre stéréotaxique à l'aide de la barre de dents
- La tête de la souris est maintenue à l'aide de barres d'oreilles (voir Fig 1)

Anesthésie :

- Utilisation de l'anesthésie gazeuse
Après une induction de l'anesthésie dans la boîte d'induction (% Isoflurane entre 2 et 3), la souris est placée dans le lit d'examen (% Isoflurane entre 1,5 et 2,5) avec un débit d'air entre 0,6 et 1 L/min

Images :

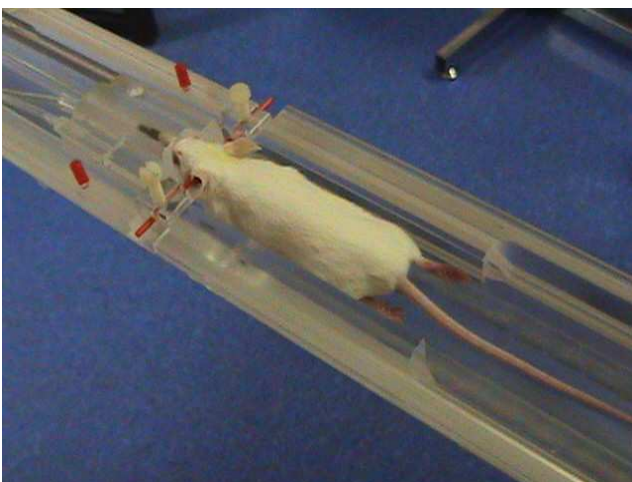


Fig 1 : Souris dans le lit en décubitus ventral

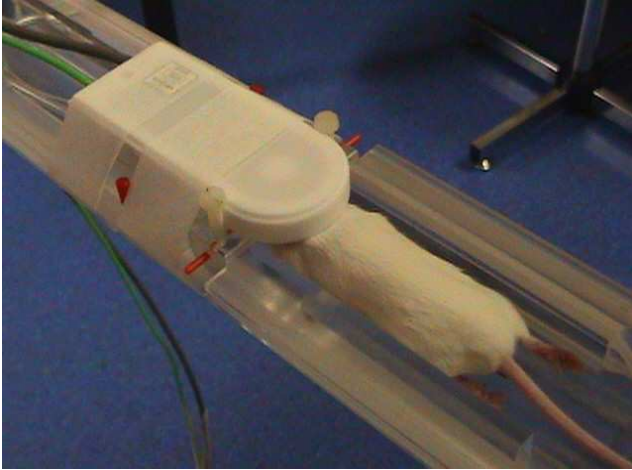


Fig 2 : Positionnement de l'antenne de surface pour une acquisition cerveau

Enregistrement de l'animal :

Etude : Projet_SEP_Souris

Registration : Souris + _n°souris

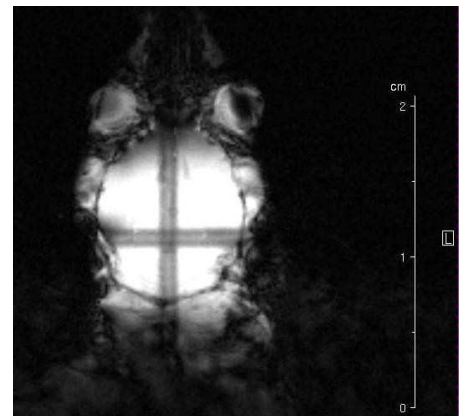
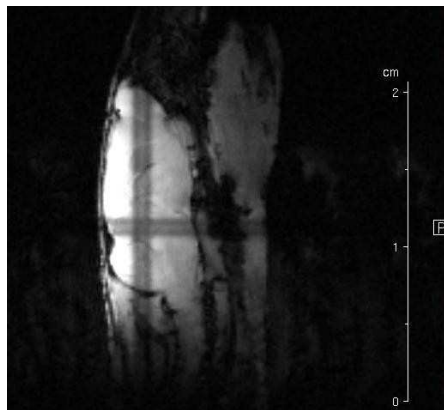
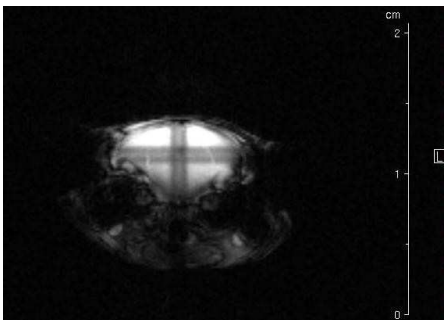
Study : Date d'examen JJ/MM_Cerveau_Moelle

Protocole : A_Borg

Position : feet first / prone

Séquence 1 : Localizer Tri pilot (axiale, sagittale, coronale) (1_Tri_Pilot_BR)

- La première séquence réalisée est une « séquence rapide » de positionnement
- Les images obtenues permettent de vérifier le bon positionnement de l'animal
- La zone d'acquisition est le cerveau, l'antenne de surface est positionnée de façon à avoir un maximum de signal entre le bulbe olfactif et le centre du cerveau (voir Fig 2)



FOV = 4 x 4 cm, Matrice = 256 x 256 d'où résolution de 156 x 156 μ m

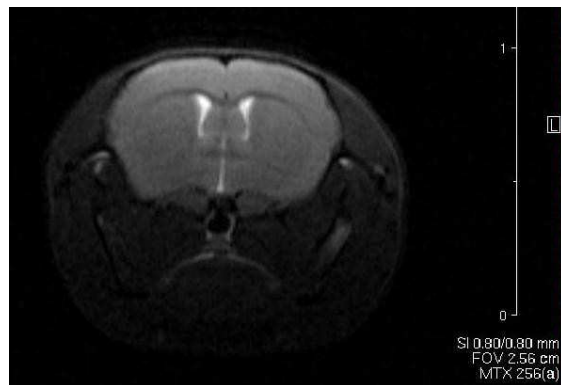
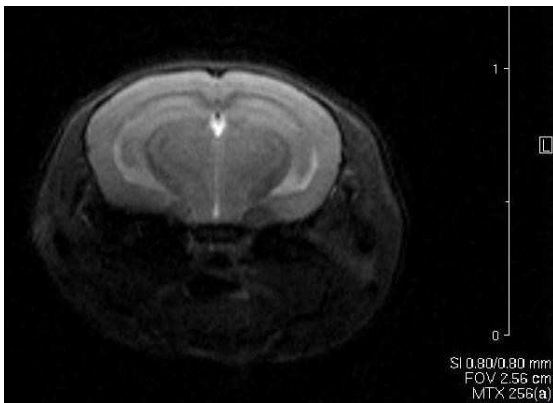
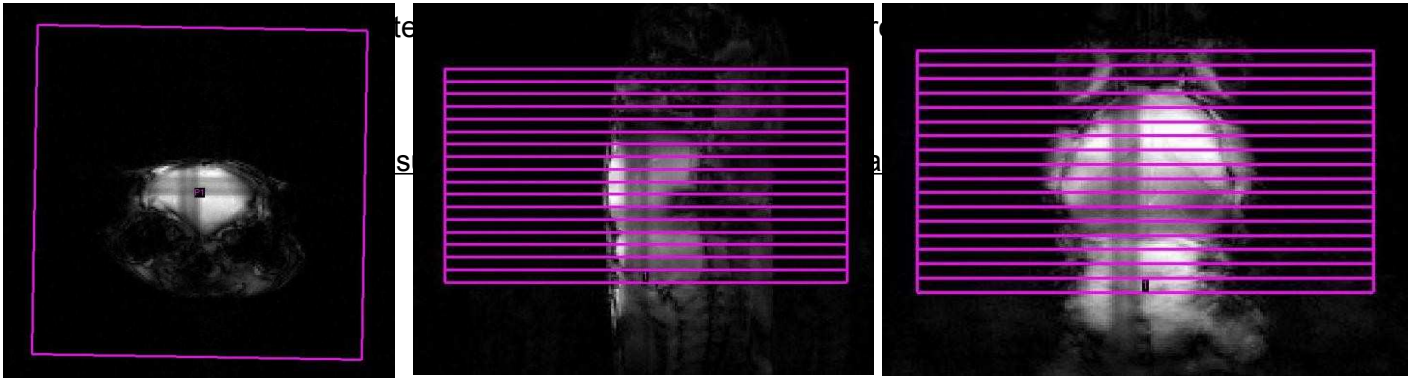
TE/TR = 5,4/100 ms, α = 30° Nb coupes = 3

Bande passante = 50505 Hz

Durée d'acquisition = 30 sec

Séquences 2 : Séquence axiale T2 (TurboRARE_T2_BR)

- Deux séquences pondérées T2 sont réalisées, coupes axiales du cerveau
- Les séquences sont des spin echo RARE



Sequence 2.1 : T2 weight (TurboRARE_T2_BR)

- First T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m

ET/RT = 36/4200 ms

17 slices (800 μ m thickness)

Bandwidth = 35714 Hz / Fat Suppression = ON

Time acquisition = 2 mins

Sequence 2.2 : T2 weight (TurboRARE_T2_BR)

- Second T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m

TE1 = 17ms TE2 = 51 ms TR = 3000 ms

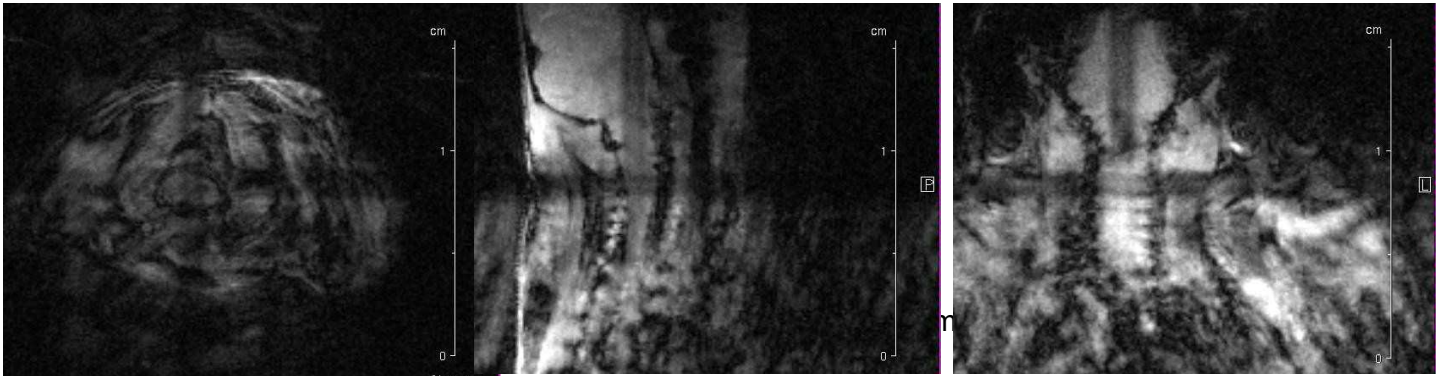
17 slices (800 μ m thickness)

Bandwidth = 55555 Hz / Fat Suppression = ON

Time acquisition = 5 mins

Séquence 3 : Localizer Tri pilot (axiale, sagittale, coronale) (1_Tri_Pilot_BR)

- La zone d'acquisition est la moelle épinière, l'antenne de surface est positionnée de façon à avoir un maximum de signal après le crâne

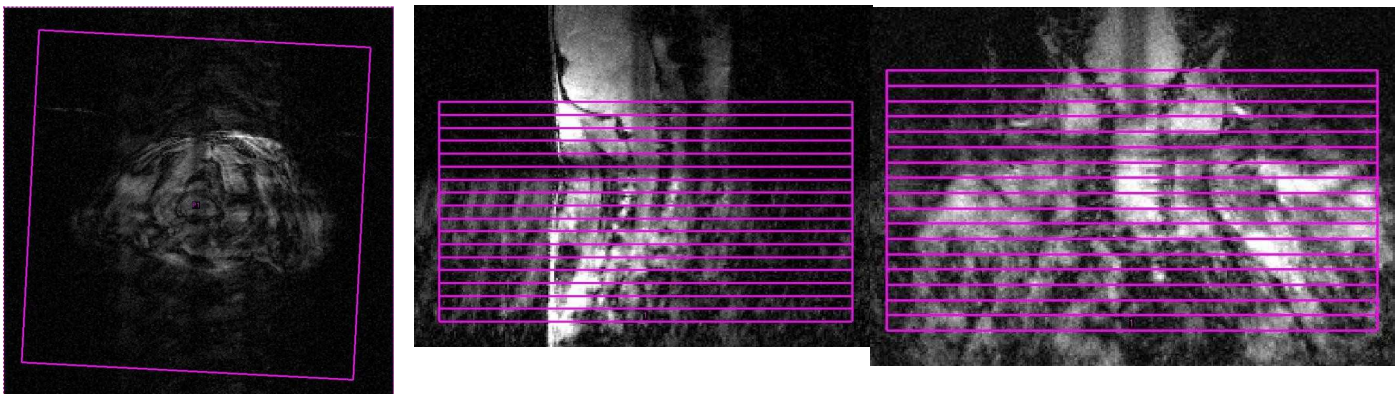


Bande passante = 50505 Hz

Durée d'acquisition = 30 sec

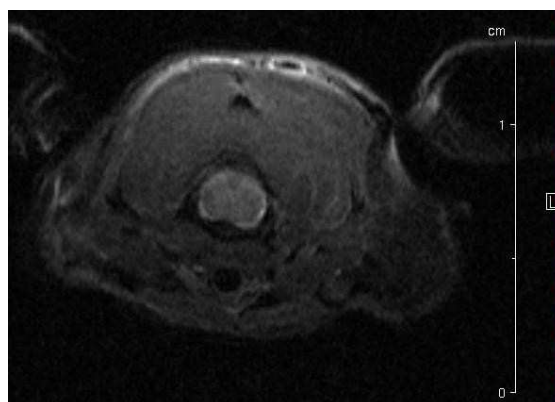
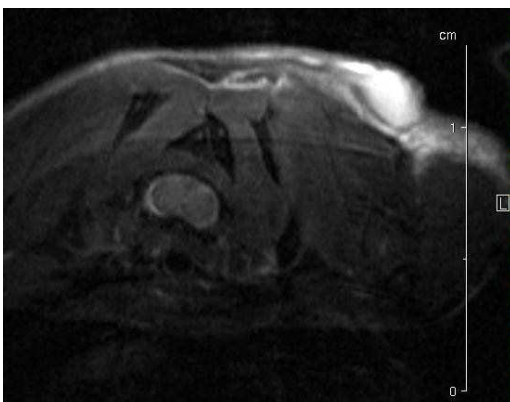
Séquences 4 : Séquence axiale T2 (TurboRARE_T2_BR)

- Deux séquences pondérées T2 sont réalisées, coupes axiales de la moelle épinière
- Les séquences sont des spin echo RARE
- Les images sont obtenues en positionnant selon les 3 directions une ROI à partir des images du Tri pilot



Géométrie : visualisation de la zone d'intérêt (vue axiale, coronale et sagittale)

Images types



Sequence 4.1 : T2 weight (TurboRARE_T2_BR)

- First T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m

ET/RT = 36/4200 ms

17 slices (800 μ m thickness)

Bandwidth = 35714 Hz / Fat Suppression = ON

Time acquisition = 2 mins

Sequence 4.2 : T2 weight (TurboRARE_T2_BR)

- Second T2-weighted, spin echo sequence : rapid acquisitions with relaxation enhancement (RARE)

FOV = 2.56 x 2.56 cm, Matrix = 256 x 256, Resolution 100 x 100 μ m

TE1 = 17ms TE2 = 51 ms TR = 3000 ms

17 slices (800 μ m thickness)

Bandwidth = 55555 Hz / Fat Suppression = ON

Time acquisition = 5 mins

Appendix D

Cytokine Table

D. CYTOKINE TABLE

cytokine	producing cell	target cell	function
IL-1a IL-1b	monocytes macrophages B cells DC	Th cells	co-stimulation
		B cells	maturation and proliferation
		NK cells	activation
		various	inflammation, acute phase response, fever
IL-2	Th1 cells, NK cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-3	Th cells NK cells	stem cells	growth and differentiation
		mast cells	growth and histamine release
IL-4	Th2 cells, mast cells	activated B cells	proliferation and differentiation, IgG ₁ and IgE synthesis
		macrophages	MHC Class II
		T cells	proliferation
IL-5	Th2 cells, mast cells	activated B cells	proliferation and differentiation, IgA synthesis
IL-6	monocytes, macrophages, Th2 cells, stromal cells	activated B cells	differentiation into plasma cells
		plasma cells	antibody secretion
		stem cells	differentiation
		various	acute phase response
IL-7	marrow stroma, thymus stroma	stem cells	differentiation into progenitor B and T cells
IL-8	macrophages, endothelial cells, stroma cells	neutrophils, T cells	chemotaxis
IL-9	T cells	various	haematopoiesis, thymopoiesis, apoptosis
IL-10	Th2 cells, CD8+ T and B cells, macrophages	macrophages	cytokine production
		B cells	activation
IL-11	stroma cells	various	haematopoiesis, thrombopoiesis
IL-12	macrophages, B cells	activated Tc cells	differentiation into CTL (with IL-2)
		NK cells	activation
IL-13	Th2 cells, B cells, macrophages	B cells	growth and proliferation
		macrophages	inflammatory cytokine production
IL-14	T cells, malignant B cells	B cells	regulation of growth and proliferation
IL-15	virus infected macrophages, mononuclear phagocytes	NK progenitors	differentiation of NK cells
IL-16	eosinophils, CD8 ⁺ T cells, lymphocytes, epithelial cells	CD4 ⁺ cells	chemoattraction
IL-17	T cell subsets	various	inflammatory cytokine production, angiogenesis
IL-18	macrophages	NK cells	NK cell activity, INF- γ production
IL-23	T cells	T cells	inflammation, angiogenesis, Th17 differentiation, IFN-g production
GM-CSF	Th cells	progenitor cells	growth and differentiation of monocytes and Dcs
IFN-a IFN-b	leukocytes, fibroblasts, macrophages, neutrophils	various	viral replication , MHC I expression
		NK cells	activation
		macrophages	activation, MHC II expression
IFN-g	Th1 cells, Tc cells, NK cells	various	viral replication , MHC I expression
		macrophages	MHC expression, pathogen elimination
		activated B cells	Ig class switch to IgG _{2a}
		Th2 cells	proliferation
MIP-1a	macrophages	monocytes, T cells	chemotaxis
MIP-1b	lymphocytes	monocytes, T cells	chemotaxis
TGF-b	T cells monocytes	monocytes, macrophages	chemotaxis
		activated macrophages	IL-1 synthesis
		activated B cells	IgA synthesis
		various	proliferation
TNF-a	macrophages mast cells NK cells	macrophages	CAM and cytokine expression
		tumour cells	cell death
TNF-b	Th1 cells Tc cells	phagocytes	phagocytosis, NO production
		tumour cells	cell death

Crossed-out activities are inhibited.

D. CYTOKINE TABLE

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RÉSUMÉ

Les rétrovirus endogènes (de la famille HERV-W) représentent près de 8% (1%) de notre génome. Leurs formes endogènes et exogènes (MSRV, Multiple Sclerosis-associated RetroVirus) peuvent altérer la régulation du système immunitaire et être impliquées dans des pathologies auto-immunes (Sclérose en Plaques) et inflammatoires. La protéine d'enveloppe (ENV) du MSRV a une action stimulatrice sur les lymphocytes T de type superantigène. Elle interagit également avec les cellules monocytaires et dendritiques via des récepteurs membranaires, ce qui provoque la production de cytokines pro-inflammatoires. Nos études reposent sur la caractérisation de la cascade immunologique menant de l'interaction de l'enveloppe virale avec son récepteur à la réaction inflammatoire pathologique. Le travail présenté dans cette thèse combine une approche cellulaire et moléculaire *in vitro* avec une validation sur modèle animal (souris) *in vivo*. Le modèle animal choisi est l'Encéphalomyélite Auto-immune Expérimentale (EAE) dans lequel l'adjuvant complet de Freund peut être remplacé par ENV. Ses effets sur l'organisme murin sont évalués à plusieurs niveaux : analyse comportementale (score clinique), analyse du cerveau (IRM), analyse cellulaire et moléculaire du système immunitaire. Par ailleurs, nous voulons générer un modèle de souris transgéniques exprimant différentes ENV (MSRV/HERV) sous le contrôle de différents promoteurs car MSRV/HERV ne sont trouvés que chez les grands singes. Ce modèle, dont les premières étapes d'élaboration font partie de ces travaux, nous permettra d'étudier le comportement des animaux surexprimant ENV ainsi qu'une analyse du cerveau et des effets sur le système immunitaire.

Mots-clés : rétrovirus endogènes, Sclérose en Plaques, auto-immunité, Multiple Sclerosis-associated RetroVirus (MSRV), enveloppe virale, cellules dendritiques, récepteurs Toll-like, Encéphalomyélite Auto-immune Expérimentale (EAE)

ABSTRACT

Endogenous retroviruses (of the HERV-W family) represent about 8% (1%) of our genome. Their endogenous and exogenous forms (MSRV, Multiple Sclerosis-associated RetroVirus) can alter the regulation of the immune system and be involved in inflammatory and autoimmune pathologies (Multiple Sclerosis). The MSRV envelope protein (ENV) stimulates T lymphocytes by acting as a superantigen. It also interacts with monocytes and dendritic cells via membrane receptors, thereby provoking inflammatory cytokine production. Our studies are based on the characterisation of the immunological cascade leading from the interaction of the viral envelope with its receptor to the pathological inflammatory reaction. The work presented in this thesis combines an *in vitro* cellular and molecular approach with an *in vivo* validation using an animal model (mouse). The chosen animal model is Experimental Autoimmune Encephalomyelitis (EAE) in which the complete Freund's adjuvant can be replaced by ENV. Its effects on the murine organism are evaluated on several levels: analysis of behaviour (clinical score) and brain (IRM), cellular and molecular analysis of the immune system. Furthermore, we want to generate a transgenic mouse model expressing different ENVs (MSRV/HERV) under the control of different promoters since MSRV/HERV are found only in great apes. This model, of which the first steps of elaboration are part of this work, will allow us to study the behaviour of the ENV over-expressing animals as well as their brain and the effects on the immune system.

Keywords: endogenous retroviruses, Multiple Sclerosis, Autoimmunity, Multiple Sclerosis-associated RetroVirus (MSRV), viral envelope, dendritic cells, Toll-like receptors, Experimental Autoimmune Encephalomyelitis (EAE)