D O C T O R A A T S P R O E F S C H R I F T

Faculteit Geneeskunde

# Study of Anti-Myelin T Cell Reactivity and Regulatory Networks in Multiple Sclerosis

## Studie van anti-myeline T-celreactiviteit en regulatorische netwerken in multiple sclerose

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen

NIELS HELLINGS

Promotoren : Prof. dr. J. Raus
Prof. dr. P. Stinissen

2000

LIMBURGS UNIVERSITAIR CENTRUM
IN HET CENTRUM VAN DE KENNIS

## List of Abbreviations

| | |
|---|---|
| Ac | Anti-clonotypic |
| AJ/BJ | Junctional region of the TCR alpha/beta chain |
| APC | Antigen presenting cell |
| APL | Altered peptide ligand |
| AV/BV | Variable region of the TCR alpha/beta chain |
| BBB | Blood-brain-barrier |
| CD | Cluster of differentiation |
| CDR | Complementarity determining region |
| CNPase | 2', 3'-cyclic nucleotide 3'-phosphodiesterase |
| CNS | Central nervous system |
| COP-1 | Copolymer-1 |
| CPM | Counts per minute |
| CSF | Cerebrospinal fluid |
| EAE | Experimental autoimmune encephalomyelitis |
| EDSS | Expanded disability status scale |
| ELISA | Enzyme linked immunosorbent assay |
| ELISPOT | Enzyme linked immunospot assay |
| GFAP | Glial fibrillary acidic protein |
| HLA | Human leukocyte antigen |
| Hprt | Hypoxanthine-guanine phosphoribosyl transferase |
| Hsp | Heat shock protein |
| HVS | Herpesvirus saimiri |
| HVV-6 | Human herpesvirus 6 |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| IL | Interleukin |
| LDA | Limiting dilution assay |
| MAG | Myelin associated glycoprotein |
| MBP | Myelin basic protein |
| MHC | Major histocompatibility complex |
| MMP | Matrix metalloproteinase |
| MOBP | Myelin oligodendrocytic basic protein |
| MOG | Myelin oligodendrocyte glycoprotein |
| MRI | Magnetic resonance imaging |
| MS | Multiple Sclerosis |
| NK | Natural killer |
| Ols | Oligodendrocytes |
| OND | Other neurologic disease |
| PBMC | Peripheral blood mononuclear cells |
| PCR | Polymerase chain reaction |
| PHA | Phytohemagglutinin |
| PLP | Proteolipid protein |
| PNS | Peripheral nervous system |
| RFLP | Restriction fragment length polymorphism |
| RR-MS | Relapse-remitting MS |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SP-MS | Secondary progressive MS |
| TCR | T cell receptor |
| TCV | T cell vaccination |
| TGF | Transforming growth factor |
| TIMP | Tissue inhibitor of metalloproteinases |
| TNF | Tumor necrosis factor |
| TT | Tetanus toxoid |
| VCAM | Vascular intercellular adhesion molecule |

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The Universe is full of magical things
patiently waiting for our wits to grow sharper.
*Bertrand Russell, 1872-1970.*

Members of the jury:

Prof. Dr. J. Raus – Limburgs Universitair Centrum - *Promotor*
Prof. Dr. P. Stinissen – Limburgs Universitair Centrum - *Promotor*

Prof. Dr. P. Steels – Limburgs Universitair Centrum - *Chairman*
Prof. Dr. I. Lambrichts – Limburgs Universitair Centrum
Prof. Dr. H. Teuchy – Limburgs Universitair Centrum

Prof. Dr. G. Opdenakker –Katholieke Universiteit Leuven
Prof. Dr. M. Waer – Katholieke Universiteit Leuven

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# Chapter 1

## Introduction

### Insights into the Immunopathogenesis of Multiple Sclerosis

## 1.1 Multiple Sclerosis: Clinical Features, Pathology and Etiology

Multiple sclerosis (MS) is the most common neurological disease in young adults. It is a demyelinating disease of the central nervous system (CNS), which usually becomes clinically apparent in early adulthood (20-40 years). The prevalence rate in Flanders is about 89/100,000[1]. Females are more commonly affected than males, in a ratio of approximately 2:1[2].

### *Clinical features*

The clinical manifestations of MS among affected individuals are extremely variable as far as the neurological symptoms and degree of disability are concerned. Common symptoms include visual and sensory impairment, paralysis and other neurological deficits, sometimes accompanied with considerable cognitive dysfunction.

A specific laboratory-based diagnostic test for MS does not exist. The diagnosis of clinically definite MS (CD-MS) is based on clinical examination, magnetic resonance imaging (MRI), evoked potentials and laboratory tests such as cerebrospinal fluid (CSF) analysis[3].

MS is generally categorized as being either relapsing-remitting (RR-MS) or primary progressive (PP-MS) in onset. The RR form is characterized by a series of attacks that result in varying degrees of disability from which patients recover partly or completely, usually followed by a remission period of variable duration before the next exacerbation. The progressive form of the disease lacks the acute relapses and instead typically involves a gradual clinical decline. Over time, about one third of the RR patients enter a progressive form known as secondary-progressive MS (SP-MS).

### *Pathology*

Pathologically, MS is characterized by multiple sclerotic lesions or plaques found in the white matter of the central nervous system[4]. These lesions result from focal loss of CNS myelin and are localized throughout the CNS, but predominantly reside within the periventricular regions, optic nerves, brain stem and spinal cord. Sites of active demyelination are characterized by an infiltration of B cells, MHC class II expressing macrophages and activated CD4+ T cells secreting various cytokines[5,6]. Since oligodendrocytes are initially largely preserved remyelination frequently occurs[7]. In later

stages, the oligodendrocytes themselves are damaged, leading to axonal loss which is probably the main cause of sustained neurological deficits[4].

### *Genetic factors*

Evidence for the contribution of genetic factors to the pathogenesis of MS stems from family and twin studies. These studies documented that family members of affected patients have an increased risk of developing MS, with first-degree relatives and daughters of affected mothers having the highest risks[8,9]. Twin studies further provide evidence supporting the role of genetic background in MS. Clinical concordance was 31% in monozygotic (MZ) twins and 5% in dizygotic (DZ) twins[10,11]. The difference in concordance rate between DZ and MZ twins clearly argues for genetic influences. However, the concordance in MZ twins is only partial, implying that not a single gene but rather several genes contribute to MS susceptibility, together with non-genetic factors.

Because MS is thought to be a T cell mediated autoimmune disease, genes associated with the immune system have received special attention in the search for predisposing genetic factors. Particularly, genes that encode elements of the trimolecular complex (MHC-Ag-TCR), which are involved in T cell activation: major histocompatibility complex (MHC), the T cell receptor (TCR) and the encephalitogenic peptides.

The genetic linkage of human leukocyte antigen (HLA) allele has been studied extensively. The strongest association was found with the HLA-DR2 genes (DRB1*1501, DRB5*0101), that are over-expressed in Caucasian MS patients but also in some non-European ethnic groups[12-14]. In addition, HLA class I alleles have been described to contribute to MS susceptibility[15].

TCR alpha and beta germline genes were also studied as possible predisposing loci. Polymorphic RFLPs were identified within these genes and used as markers for genetic association studies. Although several studies describe a linkage between MS and TCR genes[16-18], others have failed to confirm these findings[19-21]. These conflicting data make it difficult to draw firm conclusions on the possible contribution of TCR genes to MS predisposition.

The myelin basic protein (MBP) gene has also been studied for its potential genetic linkage in MS. While some studies reported a genetic association between MS and MBP polymorphisms[22,23], others could not support these data[24,25]. Linkage analysis of MS to genes

of other candidate antigens were performed recently[26]. Genes encoding for MBP, proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) had no significant genetic effect on susceptibility to MS in the population studied. For the MOG gene, which resides within the MHC loci, a potential role could not be excluded. Several other candidate predisposing genes have been described, including: genes encoding cytokines and cytokine receptors[27-29], adhesion molecules[30,31], immunoglobulins[32] and apoptosis genes[33].
In the past few years, extensive genome screens were conducted to identify the genes involved in MS susceptibility and pathogenesis[34-36]. These studies identified discrete chromosomal regions potentially harboring MS susceptibility genes. However, with the exception of the Major Histocompatibility Complex (MHC) on 6p21, no single locus generated clear evidence of linkage[36].

In conclusion, these results indicate that no single major susceptibility gene exists for MS, but rather multiple genes are exerting moderate effects. However, genetic predisposition alone is not sufficient to develop MS. Most likely, other interacting factors – both immunological and environmental – contribute to the disease.

### *Etiology: Are infectious agents causing MS?*

Despite intensive scientific research, the etiology of MS remains largely unknown. The current working hypothesis is that MS is an autoimmune disease and that susceptibility is influenced by both genetic and exogenous factors. Epidemiological studies as well as similarities with infectious demyelinating diseases have provided circumstantial evidence supporting an infectious etiology of MS. It is well documented that the incidence of MS increases with distance from the equator[37,38]. Furthermore, migration before puberty from a high to a low prelevance area results in a reduction in the risk of developing MS[39].
In the past years, numerous potential candidate viruses have been evaluated for a possible causal association with MS. Due to contradictory reports and lack of specificity for MS, all have failed to stand the test of time. Several viruses, including rabies, human herpes virus and measles were isolated from MS brain tissue, but no clear link could be established with the onset of MS[40,41]. Recently, a novel retrovirus termed "MS-associated retrovirus" (MSRV) was identified, which produces extracellular particles, detectable in plasma and cerebrospinal fluid (CSF) of MS patients[42,43]. These virions were also detected in monocyte/macrophage cultures of MS patients together with a cytotoxic factor that targets

glial cells (gliotoxin)[44,45]. However, the exact contribution of this retrovirus as causative agent for MS remains speculative. Recently, human herpesvirus-6 (HHV-6) has drawn considerable attention. Its putative role in MS is based on immunohistochemical demonstration of HHV-6 in MS plaques[46], increased HHV-6 specific antibodies in sera and CSF of MS patients[47,48] and the detection of HHV-6 DNA in the serum of MS patients but not in normal individuals[48]. Other studies however could not reproduce some of these findings[50-53].

The lack of hard proof for the existence of an MS causing virus, does not exclude that (an) infectious agent(s) may be involved in the MS pathogenesis. Indeed, some studies suggest a correlation between viral infections and MS relapses[54,55]. Furthermore, it is possible that myelin reactive T cells become activated in the periphery by presentation of cross-reactive viral epitopes that resemble self antigens (molecular mimicry)[56]. Once activated these T cells can infiltrate the CNS and initiate a pathogenic autoimmune cascade ultimately leading to myelin destruction. Alternatively, a virus may cause minor damage to oligodendrocytes leading to the release of previously inaccessible myelin fragments[57]. These self-peptides will enable activation of myelin reactive T cells, thus amplifying the destructive inflammatory process directed at the CNS. In these scenarios, a transient infection would be sufficient to trigger MS, while viral persistence is not necessary for the further progression of the disease (Hit and run hypothesis)[58].

## 1.2 MS: a CNS specific autoimmune disease

### *1.2.1 Current hypothesis on the immunopathogenesis of MS (Figure 1.1)*

One of the first hypothetical events in the multiple sclerosis pathogenesis is the activation of myelin reactive T cells in the periphery. Once activated, these autoreactive T cells expand and traffic to the CNS[59]. The migration through the tight endothelium of the blood-brain-barrier (BBB) is promoted by the expression of adhesion molecules and release of proinflammatory cytokines by the activated T cells[60-62]. Within the CNS, the myelin reactive T cells become reactivated once they encounter their specific myelin epitope presented by resident antigen presenting cells: microglia cells or perivascular macrophages[63]. The reactivated T cells will locally produce proinflammatory cytokines such as TNF and IFN-γ, leading to the upregulation of MHC class II molecules on astrocytes and microglia and adhesion molecules on the BBB endothelium. This will facilitate the further influx of T cells, B cells and macrophages, thus contributing to the amplification of the immune inflammatory response. Demyelination will be the ultimate result of this vicious circle of events. Most probably, the myelin breakdown is brought about by the combined effects of cytotoxic cells (macrophages and γδ T cells), oxygen radicals, demyelinating autoantibodies and cytokine induced toxicity (e.g. TNF)[64-67].

Opdenakker and Van Damme proposed a model for non-specific immunity in MS[68]. In this "REGA"-model (Remnant Epitope Generates Autoimmunity), complex networks of primary and secondary cytokines (including chemokines) and matrix metalloproteinases (MMPs) are thought to be involved in the initial stages of disease. A cascade of events finally results in the release of immunogenic myelin peptides and the activation of autoreactive T cells. The authors thereby suggest that involvement of autoantigen specific T cells may occur relatively late in disease pathogenesis. Whether autoreactive T cells are the cause or consequence of the destructive inflammatory responses observed in the CNS remains a question of debate. Further investigations are needed to help clarify these issues.

Although T cells are thought to be key players in the immunopathogenesis of MS, auto-antigens may also be involved. Lassmann and co-workers recently developed a classicification system in which MS lesions were subdivided into distinct patterns of

demyelination based on their pathological characteristics[69]. Two of these patterns (I and II) showed close similarities to T cell mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, respectively. The other patterns (III and IV) were highly suggestive of primary oligodendrocyte dystrophy, reminiscent of virus-induced patterns of demyelination rather than autoimmunity. Pathogenetic characteristics of plaques were heterogeneous between patients, but homogeneous within multiple lesions from the same patient. This study indicated that the heterogeneity of MS lesions most likely reflects fundamental differences in the underlying immunological pathways leading to the disease. Pathological characterization of MS lesions may therefore be very helpful in elucidating the immunopathological mechanisms that are active in each individual MS patient.

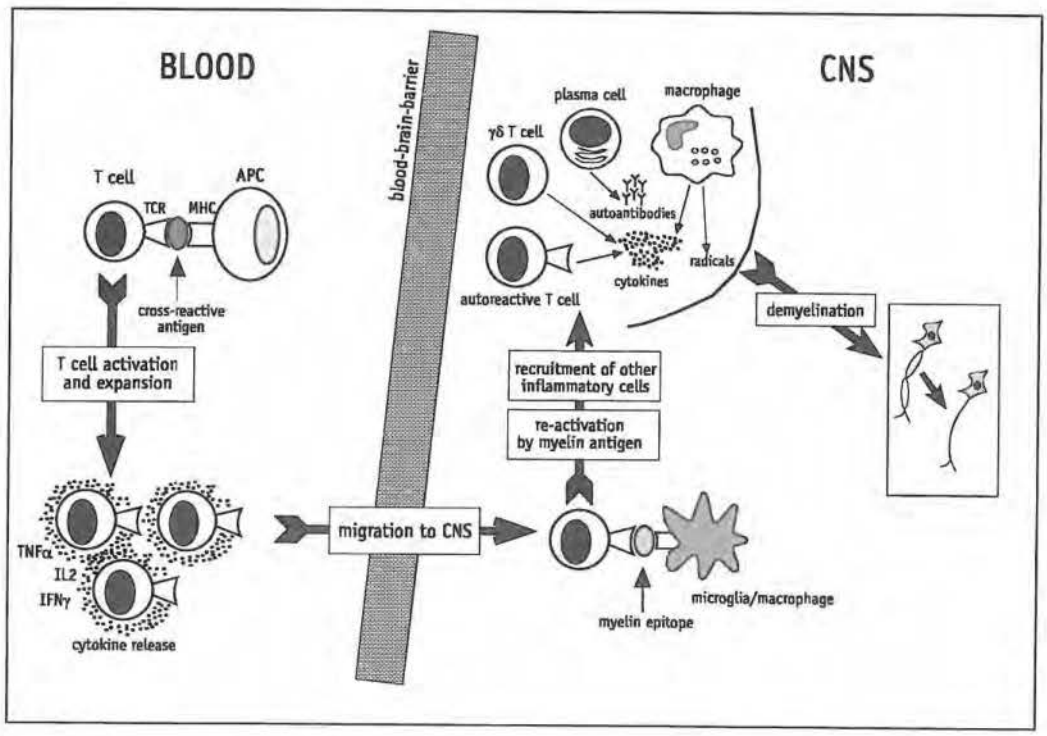


**Figure 1.1.** *Current concepts of the multiple sclerosis pathogenesis*

TCR: T cell receptor; APC: antigen presenting cell; MHC: Major histocompatibility complex CNS: central nervous system; TNF: Tumor necrosis factor; IL: Interleukin; IFN: Interferon. (Modified from Stinissen et al.[70])

### *1.2.2 Indications supporting the role of autoreactive T cells in the MS pathogenesis*

*Experimental autoimmune encephalomyelitis (EAE)*

Indirect evidence for the possible autoimmune nature of MS stems from the animal model experimental autoimmune encephalomyelitis (EAE). This experimental inflammatory disease is characterized by focal demyelination and T cell infiltration in the CNS and shares many clinical and histological features with MS[71]. EAE can be induced in a variety of animal species by injection of myelin antigens[72]. Interestingly, adoptive transfer of myelin reactive T cells to naive recipients also induces EAE, demonstrating the T cell mediated autoimmune nature of this MS model[73-76]. EAE has been shown to be a useful model to study many aspects of the autoimmune responses possibly involved in MS. Furthermore, this animal model has played an important role in testing potential therapies for MS.

*Genetic association with immune response genes*

As discussed earlier several genes involved in T cell activation have been shown to mediate susceptibility to MS. MHC-DR2 is the most strongly linked factor associated with MS. MHC may be linked to the disease by determining the ability to recognize certain epitopes of potential autoantigens. Indeed, several studies indicated that HLA-DR2 acts as major restriction element for MBP specific T cells in MS patients[77-79], and that HLA-DR2 can efficiently bind the major immunodominant epitope (84-102) of MBP. Furthermore, certain T cell receptor genes have been associated with MS predisposition. It is clear that the T cell receptor repertoire also contributes to the way autoantigens are recognized. Therefore, it is possible that individuals carrying the HLA-DR2 antigen and a particular TCR repertoire may mount a stronger response to autoantigens, leading to the expansion of autoreactive T cells and chronic disease.

*Myelin reactive T cells are detected in the blood and CSF of MS patients*

Many laboratories have succeeded in isolating myelin reactive T cells from the blood of both MS patients and healthy controls, indicating that these autoreactive T cells are part of the normal T cell repertoire[79-82]. Interestingly, T cell clones specific to myelin could also be isolated from the cerebrospinal fluid of MS patients, but not of patients with other neurological diseases (OND)[83]. Several studies addressed the question whether MBP reactive T cells present in the peripheral blood and the ones found in the CSF originate from the same precursor population. Identical oligoclonal T cells could be found in the blood and CSF

compartment of MS patients as determined by TCR usage[84,85]. Furthermore, the epitope specificity of MBP reactive T cell clones derived from the CSF of MS patients was comparable to the specificity of MBP reactive T cells isolated from paired blood samples[83]. These findings indicate that myelin reactive T cells of MS patients are present in the CNS.

*Myelin reactive T cells accumulate in CNS plaques*

Oksenberg and co-workers determined the TCR BV-D-BJ rearrangements among T cells found in MS plaques[86]. Repeated amino acid motifs were detected in the TCR junctional region of the plaque derived T cells. These CDR3 motifs shared homology with the TCR junctional regions of MBP and PLP specific T cell lines from MS patients and also with those of rodent encephalitogenic T cells[87,88], suggesting that the plaque derived TCR sequences are associated with MBP reactivity. Another study indicated that the TCR repertoire in active lesions is polyclonal and differs between plaques of the same patient, suggesting that local events influence the recruitment and expansion of T cells in the CNS[89]. In a recent report, Babbe and co-workers demonstrated that the T cell infiltrate in MS lesions was shown to be dominated by oligoclonal $CD8^+$ T cells[90]. Unfortunately, it has not been possible to obtain direct information on the antigen reactivity of the plaque-derived T cells in any of these studies.

*Myelin reactive T cells of MS patients are activated and clonally expanded in vivo*

As mentioned above, myelin specific T cells exist in the circulation of healthy individuals, indicating that they are part of the normal T cell repertoire. Thus, the mere presence of autoreactive T cells is not sufficient to invoke disease. If myelin reactive T cells are pathogenically relevant in MS, they must differ in some way from those found in normal subjects. A number of studies have recently demonstrated that MBP reactive T cells of MS patients are in an enhanced state of activation. Several studies using different experimental approaches (limiting dilution analysis after primary stimulation with IL-2, identification of somatic mutations within a marker gene and immunospot assays) showed that the frequency of *activated* myelin reactive T cells is increased in MS patients[83,91-95]. Further evidence for the *in vivo* activation of MBP reactive T cells in MS patients is provided by studies of the clonal diversity of these T cell populations. The clonal origin of a T cell can be traced by determination of its unique TCR gene rearrangements. We and others demonstrated that independent MBP reactive T cell clones from a given MS patient

frequently express identical TCR CDR3 sequences[96,97], indicating that these clones are activated and clonally expanded in vivo. Although limited clonal expansion was also observed in healthy subjects, the TCR repertoire was generally much more heterogeneous as compared to MS patients[96,98]. Together, these observations indicate that myelin reactive T cells of MS patients exist in an *in vivo* activated state, enabling them to cross the endothelial BBB, thus supporting a role of these T cells in the disease process.

*Myelin reactive T cells produce proinflammatory cytokines*

Cytokines secreted by proliferating T cells are potent effectors and modulators of immune responses. CD4+ T helper cells can be broadly categorized into different subsets based on the cytokines they produce upon activation. Th1 cells secrete proinflammatory cytokines such as IFN-γ, IL-2, TNF-α and lymphotoxin (LT), which enhance APC activation and the clearance of many intracellular pathogens. Th2 cells produce cytokines such as IL-4, IL-5 and IL-13, which inhibit Th1 responses and promote antibody responses. In EAE, encephalitogenic T cells express a Th1 phenotype, and produce IL-2, TNF-α and IFN-γ but not IL-4[99]. IFN-γ, the principle T cell derived proinflammatory cytokine, may exert its effect by inducing the upregulation of MHC class II molecules on microglia leading to an increased presentation of CNS antigens or by directly cytotoxic effect on oligodendrocytes[100]. The production of TNF-α is especially relevant because this cytokine was shown to possess demyelinating potential and was found in MS plaques[67,101].

We recently analyzed the cytokine profile of MBP reactive T cells in MS patients to find out whether these cells are also capable of producing proinflammatory cytokines[102]. Interestingly, MBP reactive clones from MS patients and control subjects generally expressed a wide range of cytokines and could not be categorized in distinct Th1 or Th2 subgroups. Hemmer and co-workers reported similar observations [103]. The MS derived MBP clones were found to secrete higher amounts of various cytokines, most significantly TNF-α, IL-2 and IL-10. Interestingly, MBP reactive T cells of MS patients expressing the disease associated DRB1*15 (DR2) allele produced increased amounts of TNF-α. TNF-α has been suggested to play an important role in inflammation and demyelination. In summary, our data indicate that MBP reactive T cell clones from MS patients produce proinflammatory cytokines such as IFN-γ, IL-2 and TNF-α, which are considered to be pathogenic in EAE.

### *1.2.3 Candidate autoantigens in MS*

*Structure of myelin*

Myelin is the multilamellar sheath necessary for saltatory conduction in the nerves. It is formed by oligodendrocytes in the CNS[104]. Because degradation of the myelin sheath is a main event in MS, proteins within the CNS myelin are considered to contain candidate antigens eliciting the pathogenic autoimmune responses. The myelin of the CNS consists of 20-25% proteins and 75-80% lipid components[105]. The formation of myelin is dependent on the expression of several myelin specific proteins, such as myelin basic protein (MBP) and proteolipid protein (PLP) (Figure 1.2). In addition, other quantitatively minor proteins are important in myelin formation and stability. 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), phosphatidylinositol-specific phospholipase C and protein kinase C are soluble proteins residing within the cytoplasm. They are involved in posttranslational modifications and signal transduction, whereas myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) are integral membrane proteins. In the next part, the possible candidate antigens for MS are discussed with emphasis on the human T cell responses against various myelin antigens (Table 1.1.).

*Myelin basic protein (MBP)*

MBP, one of the major proteins of myelin, is a water-soluble protein and is localized in the myelin cytoplasm. MBP exists in several isoforms generated by alternative splicing or post translational modifications[106,107].

Because of its relative abundance in myelin and easy isolation and purification, it was the first myelin protein to be studied and found to be encephalitogenic in EAE. Consequently, T cell reactivity to MBP in MS patients has been extensively studied. MBP reactive T cells can be isolated from most individuals – both MS patients and control subjects – indicating that MBP reactive T cells are part of the normal T cell repertoire[80,81]. In an effort to link anti-MBP reactivity with MS, MBP reactive T cells have been studied extensively with regard to their phenotypic properties, precursor frequency and epitope specificity. Much like the encephalitogenic T cells in rodents, human MBP reactive T cells exhibit the CD4+CD8-TCR$\alpha\beta$ phenotype and are cytotoxic *in vitro*[79,80]. In addition, no significant differences in precursor frequency could be found between MBP reactive T cells from MS patients and healthy individuals[80,81].

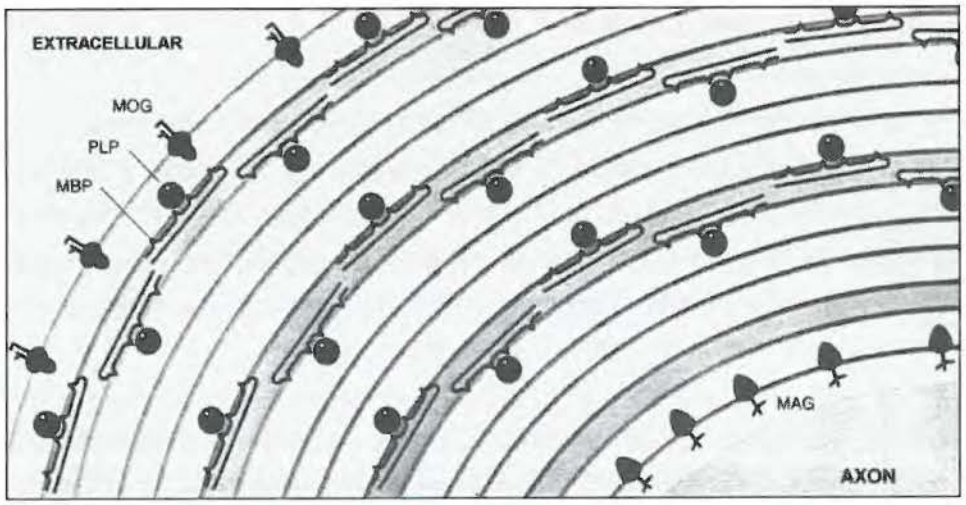


**Figure 1.2.** *Cross sectional representation of the myelin sheath*[108].

**Table 1.1.** Antigens studied in the context of MS

| Antigens | Presence in | | Location in CNS | % in CNS myelin | Manifestations in EAE | | Demyelinating antibodies | T cell reactivity in MS |
|---|---|---|---|---|---|---|---|---|
| | CNS | PNS | | | Clinical impairment | CNS inflammation | | |
| MBP | + | + | Myelin, Ols | ~30% | + | + | - | + |
| PLP | + | + | Myelin, Ols | ~50% | + | + | - | + |
| MOG | + | - | Myelin, Ols | ~0.05% | + | + | +++ | + |
| MAG | + | + | Myelin, Ols | ~1% | - | + | ± | ± |
| MOBP | + | - | Myelin, Ols | abundant | + | + | ? | + |
| OSP | + | - | Myelin, Ols | abundant | + | + | ? | ? |
| CNPase | + | + | Myelin, Ols | 3-4% | ? | ? | ? | ± |
| S100β | + | + | Astrocytes | N.A. | - | + | ? | ± |
| GFAP | + | + | Astrocytes | N.A. | - | + | ? | ? |
| Hsp | + | + | Ubiquitous | ? | ? | ? | ? | + |
| αB-crystallin | + | ? | Ols, Astrocytes | ? | ? | + | ? | + |
| Transaldolase | + | + | Myelin,Ols | ? | ? | ? | ? | + |

Ols : oligodendrocytes; CNS: central nervous system; PNS: peripheral nervous system; MBP: myelin basic protein; PLP: proteolipid protein; MOG: myelin oligodendrocyte glycoprotein; MAG: myelin-associated glycoprotein; MOBP: myelin oligodendrocytic basic protein; OSP: oligodendrocyte specific protein; CNPase: 2', 3'-cyclic nucleotide 3'-phosphodiesterase; GFAP:glial fibrillary acidic protein; Hsp :heat shock protein; N.A.: not applicable

In contrast to MBP-induced EAE, the human T cell response to MBP is much more complex with regard to epitope recognition. Indeed, several studies indicate that two MBP epitopes 84-102 and 143-168 are immunodominantly recognized by MBP reactive T cells derived from MS patients[80,82]. Other reports could not confirm these findings and demonstrated rather heterogeneous anti-MBP responses in MS patients[109,110].

Interestingly, several studies provide evidence that anti-MBP T cells undergo *in vivo* activation and expansion in the blood and accumulate in the cerebrospinal fluid of MS patients[83,91,96,111]. Two recent reports further demonstrated that MBP reactive T cells from blood of MS patients are less dependent upon B7 co-stimulation for their activation as compared to MBP T cells of normal individuals[112,113]. Together, these findings indicate that MBP-specific T cells in MS patients are in a different functional state and have less stringent requirements for their activation.

*Proteolipid protein (PLP)*

PLP is the major component of CNS myelin and is a highly hydrophobic protein embedded in the myelin membranes. In recent years it became clear that apart from MBP, PLP too has encephalitogenic properties in EAE[114]. Martin and colleagues showed that transfer of activated PLP specific T cells or immunization with PLP causes an inflammatory disease in a variety of animal species including different mice strains, rats, guinea pigs and rabbits[74,115]. PLP specific and PLP peptide specific T cells can be recovered from the peripheral blood of MS patients and healthy individuals[116,117]. Like MBP specific T cells, increased frequencies of IL-2R+ PLP specific T cells could be detected in the blood and the CSF of MS patients[83,94], and PLP specific T cells derived from MS patients exhibit increased rates of somatic mutations in the *hprt* gene, suggesting activation and proliferation of PLP reactive T cells *in vivo*[92]. The anti-PLP reactivity in MS patients is rather heterogeneous, since responses to the PLP epitopes 30-49, 40-60, 180-199 and 190-209 have been reported[116,118-120]. However, the peptides were also recognized by PLP specific T cells of controls, indicating that there is no "MS-specific" region associated with the autoimmune response to PLP. Like MBP reactive T cells, PLP specific T cells exhibited a heterogeneous TCR usage and promiscuous HLA-restriction[121].

*Myelin oligodendrocyte glycoprotein (MOG)*

MOG is a CNS myelin-specific, type I membrane protein encoded within the telomeric region of the major histocompatibility complex gene cluster(MHC). The mature protein is preferentially incorporated into the outermost surface of the myelin sheath, where a single Ig-like domain is exposed to the extracellular environment[122,123] (Figure 1.2).

Recent studies focus on the encephalitogenic potential of MOG in various models of EAE. MOG is the only antigen described so far that induces both a T cell inflammatory response and a demyelinating antibody response in animal models. MOG specific T cells as well as MOG peptide specific T cells have been shown to be encephalitogenic in various rodent strains and the common marmoset model of EAE[124-126].

The demyelinating potential of anti-MOG antibodies was first demonstrated in co-transfer experiments using MBP reactive T cells and a murine monoclonal anti-MOG antibody[122,127]. In addition, Kerlero de Rosbo and colleagues confirmed the demyelinating capacity of anti-MOG antibodies *in vitro*[128]. Interestingly, MOG-induced EAE not only differs from MBP-induced EAE with regard to lesional contribution, but also concerning the underlying immunopathology: while immune deviation therapies render animals resistant to MBP-induced EAE, oral tolerance therapy with soluble MOG in the common marmoset results in a lethal disease caused by shifting from Th1 to Th2 type cytokine patterns with a subsequent increase in anti-MOG antibody production[129].

Sun et al. (1991) demonstrated that T cells produce IFN-γ upon stimulation with MOG in MS patients, indicating the presence of MOG reactive T cells in these patients[94]. Recently, Kerlero de Rosbo and co-workers studied primary proliferative responses to several myelin antigens in MS patients and controls[130]. They observed a predominant response to MOG in MS patients, but not in control subjects. Primary responses to panels of overlapping synthetic peptides spanning the extracellular domain of MOG revealed immunodominant recognition of the epitopes 1-22, 34-56 and 64-96[131]. Another study showed that peptide 63-87 evoked the strongest response in a group of DR2+ MS patients[132]. Most recently however, a study of primary T cell reactivity to a recombinant MOG preparation as well as a detailed characterization of MOG-specific T cell lines revealed no significant differences between MS patients and healthy controls[133]. These findings are further supported by another recent report that demonstrated that MOG reactive T cells are equally present in the blood of healthy subjects[134].

*Other candidate antigens*

Tremendous efforts have been made in attempting to identify an autoantigen that can unequivocally be associated with MS. Consequently, the autoimmune response to other CNS antigens are increasingly being investigated in EAE and MS (summarized in table 1.1). These include other myelin-specific antigens like myelin-associated glycoprotein (MAG)[135], myelin oligodendrocytic basic protein (MOBP)[136] and oligodendrocyte specific protein (OSP)[137]. While MOBP and OSP were shown to be highly encephalitogenic in EAE, attempts to induce EAE with MAG reactive T cells elicited dose-dependent inflammatory responses in the CNS, without pronounced disease symptoms[138-140]. Low levels of T cell reactivity to MAG were reported in MS patients and healthy subjects[135,141]. A recent report indicated that T cells responsive to MOBP peptides are present in MS patients[142]. Whether T cell sensitization to OSP exists in MS patients remains to be investigated.

In addition, several non-myelin proteins are currently being studied for their possible role in MS. These include the S100β protein and glial fibrillary acidic protein (GFAP), both found in astrocytes. As was shown for MAG, T cell lines specific for GFAP and S100β, when transferred to naive Lewis rats, provoked a dose-dependent inflammation of the CNS, but neurological impairment was not observed[143]. While low frequencies of T cells specific to S100β were reported in MS patients and healthy subjects[144], human T-cell responses to GFAP have not been demonstrated so far.

Reactivities to non-nervous system-specific antigens such as heat shock protein (Hsp), in particular αB-crystallin[145,146], transaldolase[147] and to a lesser extent CNPase[148] have also been observed. The encephalitogenic potential of these antigens have not been demonstrated, suggesting that autoimmune responses to these antigens may represent secondary events resulting from ongoing CNS inflammation along with the course of the disease.

*Determinant spreading*

T cell reactivity to the above mentioned myelin determinants led to various and sometimes contradictory results. A possible explanation could be that anti-myelin reactivity is different from patient to patient, due to differences in genetic background (HLA). Furthermore, recent studies indicate that while a single myelin antigen may trigger the onset of MS, the subsequent disease course is accompanied by autoreactivity to other myelin antigens[149]. This so-called "determinant spreading" is most likely a result from ongoing demyelination leading to the release of previously inaccessible myelin components. Tuohy and co-workers

postulate that the progression of MS involves a shifting of T cell reactivity from primary autoantigens to defined cascades of secondary determinants that sustain the inflammatory process along disease progression. Epitope spreading has been observed in EAE and may limit the success of antigen specific therapies for MS[150].

In contrast, several authors have reported the persistence of MBP reactive T cells specific for the immunodominant 84-102 epitope in a number of DR2+ MS patients for several years, indicating that at least in some patients the anti-myelin response remains dominated by a single or a few T cell clones[98,109,109,151].

A recent study reports on the long-term dynamics of the MBP specific T cell repertoire[152]. Three distinct patterns of epitope recognition were observed: (i) persistence of a broad response with shifts and fluctuations in time (ii) broadening of an initially restricted response to a wider range of MBP epitopes and (iii) persistence of a focused anti-MBP T cell response.

Together these data indicate that T cell reactivity to myelin antigens may be largely patient dependent and that it can be influenced by factors such as disease duration and severity.

### *1.2.4 How do autoreactive T cells become activated in the periphery?*

The initial event in the hypothetical pathway of the MS immunopathogenesis is the activation of autoreactive T cells in the periphery. What can trigger this initial activation of autoreactive T cells? This question is particularly interesting in an organ-specific disease such as MS, where the target antigen(s) (myelin) may not be readily accessible to resting T cells. Indeed, since resting T cells are unable to cross the intact blood-brain-barrier an autoimmune attack against brain components is prevented in a normal situation by excluding resting T cells from being activated locally in the brain. However, when myelin reactive T cells are activated systemically in the absence of myelin, these autoreactive T cells could home into the brain and initiate an inflammatory response.

Several possible mechanisms can be put forward for the systemic activation of autoreactive T cells and are based on experimental animal studies and human *in vitro* T cell studies. One possible pathway is "molecular mimicry". According to this hypothesis, some infectious agents are comprised of peptides that mimic autoantigenic epitopes. Upon infection, presentation of these viral or bacterial peptides in the periphery by infected APC may cross-activate autoreactive T cells. Several cross-reactive viral and bacterial peptides have been identified which trigger myelin reactive T cells from MS patients[56,153]. For many years it was

believed that sequence identity is a necessity for cross-recognition of T cells. However, the emerging knowledge about the flexibility of the TCR and its degenerate recognition has changed this point of view (reviewed in Hemmer et al.[154]). While this flexibility is fundamental for efficient host defence, it may impose a potential risk by activating potentially autoagressive T cells.

Autoreactive T cells may also be activated by bacterial or viral superantigens[155]. These superantigens cross-link MHC class II molecules to a specific TCR BV segment, thereby activating T cells irrespective of their antigen specificity. Thus, it may be possible that autoimmune T cells are activated in this way.

Apart from molecular mimicry or the release of superantigens, autoreactive T cells could also be stimulated by completely nonspecific mechanisms, such as the exposure to high local concentrations of cytokines secreted in the course of unrelated immune inflammatory responses[156].

### *1.2.5 How are autoreactive T cells regulated?*

Activation of myelin reactive T cells by molecular mimicry or superantigen stimulation is probably a rather common process, which may not always lead to disease. Therefore, it is postulated that autoreactive T cells are controlled by strict regulatory immune mechanisms. Indeed, several observations support the existence of a peripheral regulatory T cell network that prevents uncontrolled expansion of potentially pathogenic T cells[157-159]. However, an imbalanced regulatory network would lead to the suboptimal suppression of activated pathogenic T cells and may finally result in autoimmunity. Our current knowledge about these immunoregulatory mechanisms are partially based on T-cell vaccination and T cell receptor vaccination studies in both animal models and MS[160-162]. This experimental therapy is aimed at enhancing the regulatory networks to specifically suppress the circulating autoreactive T cells. Several types of immune cells take part in the immunoregulatory T-T cell interactions. Anti-idiotypic T cells that recognize TCR-related structures of the autoreactive T cells play a major role. Anti-ergotypic T cells directed to an unknown marker commonly expressed by activated T cells may also be involved[161,163-165]. Apart from these two populations other lymphocytes, such as γδ T cells and NK cells may contribute to the naturally existing immunoregulatory networks[166,167].

In addition to the T-T cell interactions, other mechanisms may be involved in the regulation of autoreactive T cells. Indeed, several reports from both human and animal studies indicate

that specific suppressor cells may be responsible for the immune suppression of autoreactive T cells in the periphery[168-171].

### *1.2.6 Autoimmune T cells: not always the bad guys?*

Autoimmunity is usually considered only as a cause of disease. Nevertheless, recent reports from Schwartz, Cohen and co-workers strongly suggest that T cell autoimmunity to CNS self-antigens, if expressed at the right time and place, can have major beneficial effects[172,173]. These authors demonstrated that activated anti-MBP T cells could enhance recovery from CNS trauma in two different animal systems: optic nerve injury and spinal cord contusion[174,175]. It is not clear which mechanisms account for this neuroprotective role of anti-MBP T cells. However, since anti-MBP T cells have recently been shown to produce nerve growth factors[173], it is conceivable that myelin specific T cells are activated at the site of injury to secrete neurotrophins, which rescue the remaining nerve tissue from spreading degeneration. Also other possible mechanisms, such as transient arrest of nerve conduction may be responsible for the observed neuroprotection. In addition, preliminary findings indicate that natural autoimmunity may also contribute to wound healing elsewhere in the body[172]. These results challenge the traditional concept of autoimmunity as always being harmful, and suggest that in certain situations T cell autoimmunity may actually be beneficial. This has to be kept in mind in the further development of immunotherapeutic treatments that target autoimmune T cells.

## 1.3 Therapies for MS

The increasing understanding of the disease pathogenesis has led to the implementation of a number of immunotherapeutic approaches that are currently evaluated as treatment for MS. Table 1.2 summarizes some of these experimental immunotherapies. I will briefly describe the proven treatments and focus on the immunotherapies that aim to specifically target the myelin reactive T cells.

### 1.3.1 Approved treatments of MS

*IFN-β*

IFN-β1 is a type I interferon and has proliferation inhibiting and antiviral properties. Two forms of recombinant interferon beta, IFNβ-1a (Avonex® and Rebif®) and IFNβ-1b (Betaseron®) have been approved for the treatment of RR-MS patients, based on multicenter, placebo-controlled clinical trials[176-178]. Beneficial effects of IFN-β treatment include reduced relapse rate, slowing of disability progression and fewer active MRI brain lesions. Several possible mechanisms of action are described including downregulation of proliferation, inhibition of metalloproteinases secretion, blocking of T cell migration and stimulatory effects on the production of immunomodulatory cytokines[179-181].

*COP-1 (Glateramer acetate)*

Copolymer-1 (COP-1) is a mixture of random sequence polypeptides composed of 4 amino acids (Glu, Lys, Ala, Tyr). Subcutaneous administration of COP-1 was demonstrated to reduce clinical disease activity in patients with relapsing-remitting MS[182]. The mechanism of action of copolymer-1 has not been completely elucidated yet. One of the proposed mechanisms involves binding of COP-1 to MHC class II molecules. Recent studies suggest that this binding results in the competition with myelin antigens for T cell activation both at the MHC and the TCR level and in an induction of specific suppressor cells[183,184]. COP-1 (COPAXONE®) is now approved for general use in the clinic. Recently, a Phase III clinical trial started which aims at testing the efficacy of an oral formulation of glateramer acetate (Coral Study, TEVA Pharmaceuticals).

**Table 1.2** Overview of some immunotherapeutic approaches tested in the context of MS

| *target* | *Therapeutic strategy* | *effective in EAE* | *MS trails* |
|---|---|---|---|
| Tolerization against myelin | Oral feeding of myelin | YES | Phase III |
| | I.V. administration of MBP | YES | NT |
| | MBP-PLP fusion proteins | YES | Phase I |
| Block activation of autoreactive T cells | Altered peptide ligand (APL) | YES | Phase I/II |
| | Copolymer-1 (Glateramer Acetate) | YES | Approved |
| | Antibodies to co-stimulatory molecules | YES | NT |
| | Soluble DR2:MBP(84-102) complex | YES | Phase I |
| Block interaction of MHC-T cells | MHC blocking peptides (antagonists) | YES | NT |
| Autoreactive T cells | T cell vaccination | YES | Phase I/II |
| TCR of autoreactive T cells | TCR peptide vaccination | YES | Phase I/II |
| | TCR V gene specific antibodies | YES | NT |
| | DNA vaccination (TCR BV plasmids) | YES | NT |
| Cytokine network interactions | Interferon-beta (IFN-β) | YES | Approved |
| | Transforming growth factor beta | YES | PhaseI |
| | Genetically engineered T cells | YES | NT |
| Block T cell migration to CNS | Antibodies to adhesion molecules | YES | NT |
| | Inhibition of MMPs (TIMPs)* | YES | PhaseI/II |
| Tissue repair (remyelination) | Growth factor delivery | YES | NT |
| | Cell therapy:<br>activated macrophages: regrowth<br>autoreactive T cells : neuroprotection | YES | NT |

NT: not tested in MS trials as to our knowledge; I.V.: intravenous injection; TCR: T cell receptor; CNS: central nervous system; MHC: major histocompatibility complex; EAE: experimental autoimmune encephalomyelitis; TCR BV: variable region of the TCR beta chain; MMP: matrix metalloproteinase; TIMPs: tissue inhibitors of metalloproteinases. *MMPs are also thought to be involved in enzymatic demyelination, since gelatinase B has been shown to cleave MBP into fragments[68].

### 1.3.2 Experimental Treatments for MS: Targeting of myelin specific T cells

IFN-β and COP-1 are the first products that were shown to significantly alter the natural course of MS in RR patients. However, the moderate therapeutic success together with the side effects and the potential induction of neutralizing antibodies demonstrate the need for more effective therapies. Here we will briefly discuss some of the currently evaluated experimental therapies that specifically target the "pathogenic" T cell and its TCR.

*T cell vaccination*

Autoreactive T cells can be targeted by T cell vaccination (TCV), a procedure in which patients are immunized with attenuated pathogenic T cells. Experiments in EAE demonstrated that TCV enhances the regulatory networks to specifically suppress the eliciting autoreactive T cells rendering the animals resistant to EAE induction[160,163].

We have performed a pilot study of T cell vaccination with MBP reactive T cells in a small number of MS patients[161,185]. The patients were immunized three times with autologous irradiated MBP reactive T cell clones. The vaccinations were well tolerated and induced substantial specific anti-vaccine T cell responses, accompanied with a specific depletion of circulating MBP reactive T cells in all recipients. Our studies showed that $CD8^+$ anti-clonotypic T cells, which specifically lyse the immunized myelin reactive T cells in a class I restricted fashion may play an important role in the protective mechanisms of T cell vaccination[165]. These anti-clonotypic T cells most likely recognize a TCR related sequence expressed by the vaccine cells.

In most of the treated patients, MBP reactive T cells remained undetectable 3 years after vaccination. However, in three patients MBP reactive T cells reappeared in the circulation 2 to 5 years after vaccination, which coincided with clinical relapses in two of these patients[165]. We recently reported on the cytokine profile, cytotoxicity and epitope specificity of these reappearing clones[186]. The data suggest that the MBP clones isolated before and after TCV possess similar functional characteristics, indicating that these T cells may contribute to the further perpetuation of the disease process. These clones can however be depleted in subsequent rounds of TCV. Thus, to prevent autoreactive T cell responses at later stages of the disease, it may not be sufficient to knock-out autoreactive T cell clones at a given time. Interestingly, despite of the reappearance of new clones in some patients, the original vaccine clones remained undetected in all patients, indicating that TCV induces long-term immune responses in a clonotype specific manner.

Recently, 49 MS patients received T cell vaccination in an extended open label phase I trial to study the safety, immune responses and clinical effects in a larger group of patients[187]. The phenotype, the cytokine secretion profile and the functional properties of the T cells that respond to stimulation with the vaccine cells were studied in detail[166,167]. Our data suggest that TCR$\alpha\beta^+$ $CD8^+$ T cells display the most important direct anti-idiotypic effects towards the vaccine clones, while $CD4^+$ T cells are the predominant cytokine producers upon stimulation with the vaccine cells. Several uncommon lymphocyte populations including T cells and NK cells are also expanded upon stimulation with the vaccine, suggesting that these cells may play a role in immunoregulatory T-T cell interactions. Further studies are necessary to resolve which of these lymphocyte populations plays an active role in the T cell vaccination mechanism. In most of the patients, no upregulated antibody responses could be detected to the vaccine clones, although a transient antibody response was observed in one patient. In conclusion, immunization with attenuated autoreactive T cells induces a complex cellular response specifically targeted at the vaccine cells, but no antibody response. These data provide further insights into the mechanisms of T cell vaccination and improve our understanding of the complex regulatory networks of autoreactive T cells.
Recently, T cell vaccination projects have also been initiated in Houston, Los Angeles and Jerusalem. These studies and our ongoing projects will further help clarify the therapeutic effects of TCV. Preliminary data in our lab indicate that clinical effects are most evident in patients showing an early RR course with a low EDSS at entry[187].

*T cell receptor peptide vaccination*

A second vaccination strategy is the application of synthetic peptides representing TCR sequences. This approach was shown to be effective in EAE where encephalitogenic T cells display limited V gene diversity[188,189]. The rationale for a TCR vaccination trail in humans was based on two studies demonstrating that MBP reactive T cells from MS patients preferentially use BV6S1 and BV5S2 TCR gene products and that T cells derived from CNS lesions also expressed BV5 and BV6 genes[86,190]. In a double-blinded placebo controlled study, Vandenbark and colleagues immunized 23 HLA-DR2+ progressive MS patients with a BV5S2 CDR2 peptide vaccine. Vaccine responders had a reduced MBP response and remained clinically stable during 1 year of follow-up. TCR specific T cells isolated from the blood of the responders were Th2 and released IL-10, which may play a role in bystander suppression mechanisms. The success of this strategy largely depends on the preferential usage of this

BV5S2 gene by MBP reactive T cells which is not confirmed by other studies including ours[96,158]. It may be necessary to predetermine the BV usage of anti-MBP T cells for each patient and make a customized TCR peptide vaccine, which seriously complicates this approach. Such a pre-screening was done in a recent phase I clinical trial of TCR peptide vaccination. Wilson et al. based their peptide vaccine on the observed overexpression of BV6 among activated T cells in the CSF of MS patients[191,192]. Unfortunately, the study did not test if these activated T cells showed any reactivity to myelin antigens. The MS patients vaccinated with the high dose of peptides demonstrated immune responses to the vaccine and a decrease in CSF cellularity. So far, no clinical data on this specific trail have been reported.

*DNA vaccination*

Another approach aimed at inducing anti-TCR T cell reactivity is DNA vaccination, which was shown to be effective in EAE and may be evaluated in MS in the near future. Waisman and co-workers showed that EAE could be reversed when mice are vaccinated with DNA plasmids encoding the TCR BV8S2 region, which is overexpressed by the encephalitogenic T cells. Suppressive vaccination resulted in a shifting of the immune response to Th2, with production of the cytokines IL-4 and IL-10[193]. Recently, DNA vaccination with naked DNA encoding for other molecules including chemokines and TNF-$\alpha$ were shown to induce long-lasting protective immunity to EAE[194-196].

Other investigators used DNA constructs that encode encephalitogenic self-antigens in order to induce tolerance to these autoantigens[197-199]. The precise mechanisms leading to tolerance induced by DNA vaccination remains to be established, but may involve clonal deletion, anergy induction, immune deviation to Th2 or a combination of these events[198,200].

Another new strategy closely related to DNA vaccination, involves the administration of genetically modified autoimmune T cells[201,202]. Autoimmune T cells are thought to traffic to the CNS and accumulate at the site of inflammation. Therefore, they may serve as ideal vehicles for regulated and site-specific delivery of therapeutic transgenic factors to the autoimmune inflammatory milieu. Accordingly, anti-inflammatory factors as well as neuroprotective or regenerative factors could be delivered to the inflammatory sites. Although the above-mentioned strategies are currently studied in animal models only, the encouraging data indicate that their application in humans has to be considered as potential treatment of MS.

## 1.4 Aim of the study

Recent advances in our understanding of multiple sclerosis have led to the general belief that MS is a T cell mediated autoimmune disease of the CNS. Although circumstantial evidence indicates that autoreactive T cells are key players in the pathogenesis of MS, the causal trigger and exact cascade of events leading to this chronic inflammatory disease are not completely elucidated.

This study is aimed at further characterizing the anti-myelin T cell responses in MS patients and their possible contribution to the disease process. Another goal is to get a better insight in the mechanisms of T cell vaccination, an experimental therapy, currently performed at the Biomedisch Onderzoeksinstituut – Dr. L. Willems Instituut (Diepenbeek, Belgium). Detailed information on both topics will add to our current knowledge of MS and will be of considerable importance for the further development of immunotherapies for MS.

**Goal 1: to further characterize the molecular basis of the anti-clonotypic recognition of MBP reactive T cells in MS patients treated with T cell vaccination**

Several aspects of the anti-vaccine immune responses induced in MS patients who received T cell vaccination were studied in the past. For instance, we were able to isolate $CD8^+$ anti-clonotypic T cells from blood of vaccinated MS patients, which specifically lyse the immunizing MBP reactive T cells in a MHC class I restricted manner. Although several reports -including ours- suggest that anti-clonotypic T cells may recognize TCR determinants expressed by the vaccine cells, no direct evidence is available on the molecular basis of this T-T cell interaction.

This part of study was undertaken to further define the recognition pattern and functional properties of $CD8^+$ anti-clonotypic T cells induced by T cell vaccination. We followed three distinct strategies to investigate which epitope(s) within the T cell receptor of the vaccine clone is (are) targeted by the anti-clonotypic T cells. Identification of the TCR determinants that are involved in the regulation of myelin reactive T cells will add to our understanding of the *in vivo* regulatory networks and may be of great importance in the further development of simplified T cell (receptor) vaccines.

**Goal 2: to simultaneously analyze T cell reactivity to multiple myelin antigens in MS patients using the ELISPOT technique**

Myelin proteins, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been studied as candidate autoantigens in MS. It is not clear whether MS patients show a predominant reactivity to one or several of these myelin antigens. Several techniques have been used to measure antigen specific T cell reactivity in humans. Most of these procedures require *in vitro* culture and imply a selection of cells with preferential proliferative capacity rather than cells that respond by producing effector molecules. In addition, these classical techniques require a relatively large amount of blood lymphocytes, making it problematic to study T cell responses to various myelin antigens. The ELISPOT technique circumvents most of these problems.

In this part of the study the ELISPOT assay is used to analyze simultaneously T cell reactivities to a range of myelin antigens in MS patients and healthy controls. This will reveal whether any quantitative or qualitative differences in anti-myelin T cell reactivity can be found in MS patients as compared to healthy individuals. In addition, these data will show whether MS patients display a heterogeneous or restricted anti-myelin T cell reactivity. This part of the study can have an impact on applications of antigen-specific immunotherapies.

**Goal 3: to study temporal changes in T cell related parameters and their correlation with clinical parameters and MRI activity in patients with multiple sclerosis**

If MS is truly an immune mediated disease, and immunotherapy is effective, additional proof for this hypothesis will be the identification of immune parameters that are linked to both clinical disease course and to response to therapy. Major efforts have been made in attempting to find such surrogate markers of disease activity. However, the results of these studies have often been contradictory.

In this last part, we report on a longitudinal study in which several immunological and clinical parameters were monitored in 7 MS patients and 2 healthy controls at regular intervals for a period of 18 months. Every two months, T cell reactivity to several myelin antigens was determined in ELISPOT and proliferation assays. These data will indicate

whether in a given patient the anti-myelin reactivity is stable over time or if determinant spreading takes place. The cytokine production, measured in both culture supernatant and serum samples will further provide information on the Th subtype of the immune responses. Every six months, the frequency of MBP and PLP reactive T cells was analyzed using the classical limiting dilution assay (LDA). The MBP and PLP reactive T cell clones obtained at these time points were characterized for their TCR V gene expression. Each time, patients were subjected to a clinical examination (EDSS, number of relapses, etc). Every 4 months, MRI scans were taken to determine the number of T1 and T2 weighted and gadolinium enhancing lesions as a measure for the inflammatory status of the brain.

By interpreting these data we looked for correlations between the immunological, clinical and MRI parameters. This could lead to the identification of a para-clinical disease marker that can be used for both diagnostic and prognostic purposes.

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# Chapter 2

# Materials and Methods

## 2.1 Cell culture-based techniques

### 2.1.1 Frequency analysis of myelin reactive T cells and generation of myelin reactive T cell clones

Human MBP and PLP was purified from white matter of human brain, as described[1,2]. Endotoxin-free extracellular domain of MOG (rMOG) was kindly provided by Dr C. Bernard (La Trobe University, Bundoora, Australia)[3]. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll gradient centrifugation, counted and suspended in autologous medium (RPMI 1640 supplemented with L-glutamine, sodium pyruvate, non-essential amino acids, 10 mM Hepes buffer and 10% heat-inactivated autologous serum). Subsequently, PBMC were plated at $1x10^5$ cells per well (60 wells) in U-bottom 96-well plates (Nunc, Roskilde, Denmark) in the presence of either MBP or PLP (40 µg/ml). Seven days later, cultures were restimulated with the corresponding myelin antigen and irradiated autologous PBMC as antigen presenting cells (APC). After another week, each culture was tested for antigen specificity in proliferation assays. Briefly, each well was split into 4 aliquots and cultured in duplicate in the presence of either antigen-pulsed or unpulsed (control) irradiated APC ($10^5$ cells/well), followed by a $^3$H-thymidine incorporation assay 72 h later. A T cell line was considered to be antigen specific when the counts per minute (cpm) were greater than 1500 and exceeded the reference cpm (in the absence of antigen) by at least threefold[4,5]. The frequency of MBP and PLP reactive T cells was estimated by dividing the number of specific T cell lines by the total number of PBMC plated.

Resulting myelin specific T cell lines were cloned with phytohemagglutinin (PHA) in the presence of allogeneic accessory cells[6]. Briefly, T cells were plated out by limiting dilution at 0.3 cells/well and cultured with $10^5$ irradiated allogeneic PBMC and 2 µg/ml of PHA (Difco, Detroit, U.S.). Growth positive wells were tested for specificity in proliferation assays as mentioned above. MBP and PLP reactive T cell clones were further expanded by successive rounds of restimulation with MBP or PHA and autologous APC.

### 2.1.2 Generation of anti-clonotypic T cell clones

Anti-clonotypic T cell clones were isolated from MS patients treated with T cell vaccination, as described earlier[7,8]. Briefly, freshly isolated PBMC ($5x10^4$ cells/well) of the vaccinated patient were plated out in the presence of the irradiated vaccine clone ($5x10^4$ cells/well). Seven days later, cultures were restimulated with the irradiated immunizing cells and

expanded with rIl-2. On day 14, growth-positive wells were examined for their specific proliferation and cytotoxicity towards the original stimulator clone. Anti-vaccine T cell lines were subsequently cloned according to the same procedure used to clone myelin reactive T cell lines (2.1.1). Anti-clonotypic T cell clones were further expanded by restimulation with PHA or irradiated vaccine T cells.

### 2.1.3 Lymphocyte stimulation assays

At least triplicate aliquots of $10^5$ PBMC were stimulated for 5 days with MBP (40 µg/ml), PLP (40 µg/ml), rMOG (10 µg/ml) control antigens (anti-CD3: 2 µg/ml; PHA: 2 µg/ml TT: 2.5 Lf/ml) or medium only. After 4 days of culture, supernatant was removed from all cultures and stored at -70°C for cytokine analysis (2.1.7). Proliferation to the different antigens was measured using $^3$H-thymidine incorporation assays in which cultures were pulsed with 1 µCi $^3$H-thymidine (Amersham, Buckinghamshire, U.K.) per well for the last 16 h of culture and then harvested by an automated cell harvester (Betaplate 1295-004, Pharmacia, Uppsala, Sweden). Incorporated radioactivity was measured using a Beta-plate liquid scintillation counter (Wallac, Turku, Finland). The stimulation index (S.I.) for each antigen was calculated by dividing the mean cpm of triplicate wells by the mean cpm of the unstimulated control wells.

### 2.1.4 Cytotoxicity assays

Specific cytotoxicity was measured using a classical $^{51}$Cr-release assay. Target cells were labeled with 200 µCi $^{51}$Cr ($Na_2Cr_3O_4$, Amersham, Buckinghamshire, U.K.) at 37°C for 1 hour and washed four times with medium. Effector cells were incubated in triplicates with the labeled targets at various effector-to-target ratios in 200 µl microwells for at least 5 hours. Supernatants were then harvested and measured for radioactivity in a gamma counter (Cobra II 5002, Packard Instrument Company, Meriden, U.S.). Maximum and spontaneous release were measured in wells containing target cells in the presence of respectively detergent or medium only. The percentage of specific cytolysis was calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

### 2.1.5 Flow-cytometry

Expression of cell surface proteins was assayed by flow-cytometric analysis. Cells were suspended in FACS buffer (phosphate-buffered saline with 2% fetal calf serum) and stained with phycoerythrin (PE) and/or fluorescein (FITC) conjugated monoclonal antibodies specific for CD3, CD4, CD8, TCRαβ, TCRγδ, CD25, CD54, HLA-DR, CD19, CD16+56, CD45RA/RO (Becton-Dickinson, Erembodegem, Belgium) for 30 minutes at 4°C. Cells were washed twice and analyzed on a FACSscan flow cytometer (Becton-Dickinson).

### 2.1.6 ELISPOT assay

The number of cytokine secreting T cells in response to stimulation with myelin antigens, synthetic peptides or control stimuli was estimated using an ELISA-based technique called ELISPOT (enzyme-linked immunospot assay, Figure 2.1)[9,10].

For IFN-γ ELISPOT assays, nitrocellulose bottomed 96-well Millititer HA plates (Millipore, Brussels, Belgium) were coated overnight at 4°C with 10 µg/ml anti-IFN-γ capture Ab 1-D1K (MabTech, Stockholm, Sweden). Unbound antibody was removed by successive washings with sterile PBS and non-specific binding sites were blocked by incubation with 10 % FCS for 2 hours at 37°C. Next, freshly isolated PBMC were incubated in triplicate at a concentration of $2x10^5$cells/well in the presence of MBP (40 µg/ml), rMOG (10 µg/ml), synthetic myelin peptides (10 µg/ml) and control stimuli (PHA or anti-CD3: 2 µg/ml) or medium only in a humidified incubator (37°C, 5 % $CO_2$). After 20 hours of culture, cells were removed by washing four times with PBS 0.05 % Tween and at least once with distilled water to remove residual cells. To visualize the captured IFN-γ , biotinylated detecting Ab (1 µg/ml, 7-B6-1, Mabtech) was added and incubated for 2 hours followed by incubation with streptavidin-alkaline phosphatase (MabTech) and using BCIP/NBT (Pierce) as substrate. Spots were counted using a dissection microscope. The number of cytokine secreting cells was calculated by subtracting the number of spots in control wells (medium only) from the number of spots obtained for each stimulation.

IL-4 ELISPOT was performed in parallel following an identical protocol as described above, using the anti-IL-4 Ab pair from MabTech (Stockholm, Sweden). Because lower numbers of IL-4 secreting cells were expected, PBMC were plated at a density of $4x10^5$ cells/well.

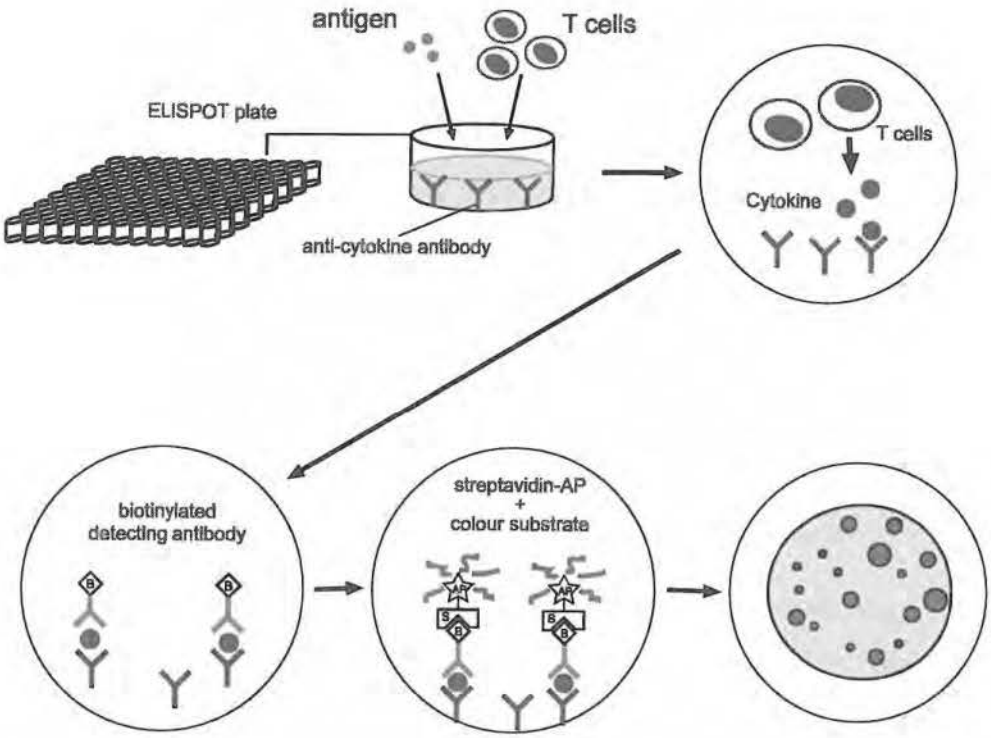


**Figure 2.1.** *Schematic overview of the enzyme-linked immunospot assay (ELISPOT)*
B: biotin, S: streptavidin, AP: alkaline phosphatase

### 2.1.7 Quantification of cytokines and soluble marker molecules by ELISA

The cytokine production in supernatants of antigen-stimulated PBMC was measured using a sandwich ELISA based on commercially available mAb-pairs (CytoSets, Biosource Europe, Nivelles, Belgium). 96-well ELISA plates (Immunosorb, Nunc) were coated overnight at 4°C with 1 µg/ml capture antibody. Non-specific binding sites were blocked with 0.5 % BSA in PBS and subsequently washed with washing solution consisting of 0.1 % Tween-20 in 0.9% NaCl solution. Fifty µl of sample or diluted standard were added together with 50 µl of the matched biotinylated detecting antibody and incubated for 2 hours at room temperature. Next, plates were washed four times and incubated for 30 min with streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch laboratories, West Grove, U.S.). To

establish the color reaction, 100 µl TMB/$H_2O_2$ citrate buffer (substrate) and 50 µl 1.8 N $H_2SO_4$ (stop solution) was added consecutively. Optical densities were measured at 450 nm and 630 nm using an ELISA-reader and sample concentrations were calculated using an 8-point standard curve of recombinant cytokine. PBMC stimulated with MBP, PLP, rMOG or PHA were analyzed for production of IL-4, IL-6, IL-10, IFN-γ and TNF-α. Net cytokine production was calculated by subtracting background levels (non stimulated PBMC) from the cytokine levels measured in the stimulated cultures.

Serum samples were drawn in collection tubes supplemented with aprotinin (BD, Vacutainer Systems). Immediately after collection, blood samples were centrifuged and serum was frozen in aliquots (-70°C). Serum levels of the soluble forms of intercellular adhesion molecule-1 (ICAM-1/CD54) and vascular intercellular adhesion molecule-1 (VCAM-1/CD106) were measured using commercially available ELISA kits according to manufacturer's instructions (Flexia, EASIA and CytoScreen kits, Biosource Europe, Nivelles, Belgium).

### 2.1.8 Generation and culture of Herpesvirus saimiri transformed T cell clones

Until present, the most commonly used viruses to transform lymphocytes are human T cell leukemia virus type I (HTLV-I) en Epstein Barr virus (EBV). However, virus-mediated transformation with these viruses has a number of disadvantages (reviewed in table 2.1). Recently, Herpesvirus saimiri (HVS) has been shown to transform human T cell lines and clones to permanent and stable growth without altering their essential properties: antigen specificity and integrity of the T cell receptor and CD markers[11,12]. Once transformed, the T cells grow independent of antigen restimulation.

**Table 2.1.** Properties of *in vitro* transformed human lymphocytes after prolonged culture

| Properties | Transforming virus | | |
|---|---|---|---|
| | HVS | HTLV-1 | EBV |
| Transformed cell type | T | T | B |
| Ag-receptor persistence | + | +/- | + |
| Viral persistence | Episomal | Integrated | Episomal |
| Release of the virus | Not seen | Spontaneously | Inducible |

From Meinl et al.[9] HVS: H. saimiri, HTLV-1: human T cell leukemia virus type 1, EBV: Epstein-Barr virus, Ag: antigen.

*Preparation of a Herpesvirus saimiri viral stock*

Epithelial owl monkey kidney cells (OMK cells) are the typical propagation system for HVS. By infecting these cells, fresh infectious virion suspensions can be made.

OMK cells were maintained in DMEM supplemented with 10% FCS, glutamine (350 µg/ml) and antibiotics (120 µg/ml penicillin/streptomycin). Once a week, cells were trypsinized and split onto a doubled area of tissue culture plastic ware. On day two to four after splitting, the culture medium of the confluent OMK monolayer cultures was removed and infectious virion suspension was added in a minimal volume (e.g. in 2 ml for a 25 $cm^2$ flask, in 5 ml for a 80 $cm^2$ flask). Adsorption was allowed to take place at 37°C for 2 hours. Subsequently, medium was added and the cultures were further incubated. After 1 to 14 days, initial cytopathic changes were detectable. Several days later the virus lysed the whole layer. Once the CPE (cytopathic effect) was completed, supernatant was harvested and used as virus stock.

*Growth transformation of human T cells by HVS*

Best results for growth transformation were obtained by culturing the T cells in 45% CG medium (Vitromex, Germany), 45% RPMI 1640, 10% FCS (Life Technologies), antibiotics and rIL-2 (20-40 U/ml, Boehringer Mannheim). Fresh infectious OMK supernatant (10% v/v) was added to a lymphocyte culture (e.g. 500 µl to 5 ml culture volume with $5x10^6$ cells). During the following weeks cells were carefully observed and medium partially exchanged twice a week.

It can take up to several months until the viral infected cells start to proliferate quickly. Transformation is indicated by several criteria: 1. Doubling of cell number once to four times a week for several months without antigen restimulation; 2. Morphology of T lymphoblasts, irregular shapes; 3. Death of control cultures.

## 2.2 Molecular biology-based techniques

### 2.2.1 RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets ($2x10^6$ cells) using the High Pure total RNA isolation kit (Boehringer Mannheim). Next, RNA was reversed transcribed into single stranded cDNA with AMV reverse transcriptase using an oligo dT primer (according to the manufacturer's protocol, Promega, Madison, U.S.). Finally, cDNA was precipitated with 3M of sodium acetate in ice-cold ethanol and resuspended in 35 µl of sterile water.

The integrity of the isolated cDNA was confirmed by performing a control PCR reaction with primers specific for the household gene β2-microglobulin. One µl of cDNA was amplified in a total volume of 25 µl. An identical PCR protocol was used as described for the amplification of TCR V genes (2.2.2).

### 2.2.2 Analysis of TCR AV and BV gene usage and direct sequencing

TCR V gene rearrangements of myelin specific T cell clones were analyzed by specific PCR amplification and direct sequencing of TCR AV and TCR BV transcripts, as previously described[13]. Briefly, cDNA derived from $2x10^6$ cells of the myelin reactive T cell clone was subjected to PCR amplification with a set of primers specific for TCR AV and TCR BV gene families. The amplified products were separated on a 0.6% agarose gel and visualized with ethidium bromide. Next, the PCR fragments were cut out of the gel and purified on a Sephadex G50-M column. The purified amplicons were sequenced with a TCR C specific primer using the dye terminator cycle sequencing kit (Perkin Elmer). PCR was performed for 25 cycles (10 sec 96°C, 5 sec 50°C, 4 min 60°C) and amplicons were purified on a Sephadex G50-M column, vacuum dried and suspended in 5µl 25 mM EDTA/formamide (1:50). DNA sequences were evaluated on a 6 % polyacrylamide gel using the 373 DNA Sequencer (ABI Systems).

### 2.2.3 DR2/MHC class II typing by PCR

PBMC of normal controls and MS patients were DR2 typed as described elsewhere[14]. Genomic DNA was isolated by suspending a pellet of $2x10^6$ PBMC in lysis buffer (10 mM Tris, 100 mM NaCl, 25 mM EDTA, 0.5 % SDS) and incubating the lysate overnight at 37°C with proteinase K (0.3 mg/ml). DNA was precipitated with 6 M NaCl and ethanol and finally suspended in TE-buffer.

PCR was performed by adding 1 µl of genomic DNA to the following amplification mixture: 0.5 µl dNTP mix (10 mM), 5 µl 10X PCR buffer (25 mM $MgCl_2$, Boehringer Mannheim), 0.25 µl *Taq* DNA polymerase (5U/µl, Boehringer Mannheim), 2 µl (0.4 µM) forward primer (5'-TTCCTGTGGCAGCCTAAGAGG-3'), 2 µl (0.4 µM) reverse primer (5' CCGCTGCACTGTGAAGCTCTC-3') in a total volume of 50 µl. PCR was performed on a GeneAmp® PCR System 9600 thermal cycler (Perkin Elmer) for 30 cycles (20 s at 95°C, 20 s at 60°C, 40 sec at 72°C). For DR2 positive individuals a PCR product of 261 bp was detected on a 1% agarose gel.

### 2.2.4 Bacterial cloning of TCR chains in eukaryotic expression vectors

The genes encoding the TCR alpha and beta chains of MBP reactive T cell clones were isolated by PCR. Briefly, from a pellet of $2x10^6$ cells of the MBP reactive T cell clone total RNA was extracted and reversed transcribed into cDNA as stated in 2.2.1. T cell receptor genes were subsequently amplified by PCR, using primers located at the 5' and 3' ends of the open reading frame (Table 2.2). Unique restriction sites were incorporated into the primer sequences facilitating further subcloning of the PCR fragments. PCR amplicons were ligated into the pCR2.1 cloning vector by TA cloning (TOPO TA Cloning Kit, Invitrogen, The Netherlands). The nucleotide sequence of the cloned TCR cDNA fragments was verified by DNA sequencing (2.2.2). Next, TCR genes were subcloned into pREPx expression vectors (Invitrogen) using the appropriate restriction enzymes (Life Technologies). These EBV-based expression vectors are maintained extra-chromosomally in mammalian cells and contain marker genes for eukaryotic selection.

**Table 2.2.** Nucleotide sequence of primers specific for T cell receptor genes

| | |
|---|---|
| AV1S3-NotI | GCGGCCGC-ATG CTC CTG CTG CTC GTC CC |
| AV1S4-NotI | GCGGCCGC-ATG CTC CTG GAG CTT ATC CC |
| AV2S1-NotI | GCGGCCGC-ATG ATG AAA TCC TTG AGA GTT TTA C |
| BV3S1-NotI | GCGGCCGC-ATG GGA ATC AGG CTC CTC TGT |
| BV13S1-NotI | GCGGCCGC-ATG AGC ATC GGC CTC CTG TGC |
| BV18S1-NotI | GCGGCCGC-ATG GAC ACC AGA GTA CTC TGC TG |
| TCR AC-BamHI | GGC TGT CTT AGG ATC CTG CAG ATC TCA CG |
| TCR BC-BamHI | CCT GGG ATC CTT TTG GAG CTA GCC TC |

### 2.2.5 Transfection of autologous lymphocytes with T cell receptor constructs

EBV-transformed B cells, HVS-transformed MBP reactive T cell clones or PHA-activated T cell blasts were used as autologous target cells for the transfection of T cell receptor chain constructs. Host cells were transfected by electroporation with a BioRad Gene pulser at 250 V and 960 µFD using 400 µl of cell suspension ($10^7$ cells/ml, in culture medium) and 10-15 µg of plasmid DNA. Time constants varied from 20 to 25 ms. After the pulse, cells were incubated at room temperature for 15 min, supplemented with 5 ml of culture medium with 5U/ml rIL-2 and incubated at 37°C in 6 well culture plates. After 48-72 hours, cell cultures were selected by adding selective agents to the medium (150 µg/ml Hygromycin, 150 µg/ml L-Histidinol or 100 µg/ml G418).

At different time points before and after transfection, transfectants were tested in functional assays and cell pellets were collected for DNA and RNA extraction. Using vector specific primers the presence of the construct was evaluated (DNA level). RT-PCR with primers specific for the cloned TCR chain was applied to demonstrate the transcription of the TCR constructs.

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# Chapter 3

## Analysis of Anti-clonotypic T Cells in MS Patients Treated with T Cell Vaccination

## 3.1 Introduction

Several studies indicate that autoreactive T cells are not completely deleted in the thymus, but are part of the normal T cell repertoire[1,2]. Accidentally activated autoreactive T cells, however, may not automatically lead to autoimmune disease. Indeed, several reports support the existence of peripheral regulatory networks that prevent the activation and expansion of pathogenic T cells[3,4]. Anti-idiotypic and anti-ergotypic T cells are part of this regulatory network and are thought to control autoreactive T cells by recognition of certain clonotypic determinants[5-8]. These clonotypic networks may not function properly in patients with multiple sclerosis (MS). Immunization with attenuated autoreactive T cells (T cell vaccination) may enhance the regulatory networks to specifically suppress the autoreactive T cells as shown in experimental autoimmune encephalomyelitis (EAE), a commonly used animal model for MS[6,9]. We conducted a pilot study of T cell vaccination (TCV) with MBP reactive T cells in a small number of MS patients[10,11]. Preliminary data on an extended phase I study with 49 MS patients showed that TCV on a larger scale is feasible and safe[12]. In addition to its possible therapeutic applications, T cell vaccination provides a unique tool to study the *in vivo* network regulation of autoreactive T cells. Our studies indicated that $CD8^+$ anti-clonotypic T cells isolated from vaccinated MS patients specifically lyse the immunizing MBP reactive T cells in a MHC class I restricted way and may play an important role in the protective mechanisms of T cell vaccination[11,13]. Several reports including ours suggest that anti-clonotypic T cells may recognize TCR determinants within the hypervariable CDR3 (complementarity-determining region 3) region or less variable CDR2 regions, as predicted by characteristic sequence diversity within these regions[6,13-16]. Furthermore, previous studies suggested that T cells are capable of presenting their TCR proteins in the context of MHC molecules[17-19].

This study was undertaken to further define the recognition pattern and functional properties of $CD8^+$ anti-clonotypic T cells induced by T cell vaccination. To investigate which epitope within the T cell receptor of the vaccine clone is targeted by the anti-clonotypic T cells (Ac T cells), we followed three distinct strategies. In a first approach we determined the recognition pattern of the Ac T cells to a panel of autologous T cell clones with known TCR V gene usage and CDR3 sequence. Based on the cross-reactivity we aimed to find homologous TCR sequences that are shared by the positively recognized T cell clones. In a second strategy, specific reactivity was tested to a panel of synthetic overlapping peptides

(9-mers) corresponding to the CDR3 regions of the TCR α and β chains of the vaccine T cell clone. In a last approach, the full length TCR α and β chains were cloned in expression vectors and transfected to autologous lymphocytes. The α en β transfectants were subsequently tested for their recognition by the Ac T cells.
Identification of the TCR determinants that are involved in the regulation of myelin reactive T cells will add to our understanding of the *in vivo* regulatory networking and may be of great importance for the further development of simplified T cell (receptor) vaccines.

## 3.2 Results

### 3.2.1 Isolation and characterization of the recognition pattern of anti-clonotypic T cells from MS patients treated with T cell vaccination

MS patients were treated with T cell vaccination in an extended phase-I clinical trail[12]. Briefly, autologous MBP reactive T cell clones were isolated from the peripheral circulation by LDA and single cell cloning (see 2.1.1). After attenuation by irradiation, 10 x $10^6$ cells of each of these clones were injected subcutaneously three times at 2-4 months intervals[10,11]. In all recipients, MBP reactive T cells were depleted from the circulation after immunization, as exemplified for patient DM in Figure 3.1. Although in the majority of the patients MBP reactive T cells were undetectable for several years after vaccination, in some patients (including DM) MBP reactive T cells reappeared in the circulation after 2 to 5 years. These clones can be depleted by additional immunization[20].

To study the anti-vaccine response induced by TCV, we isolated anti-clonotypic T cells from vaccinated MS patients as described in 2.1.2. For 4 recipients, we further characterized these anti-clonotypic T cell clones (Ac clones) as to their phenotype, TCR expression, and their reactivity pattern to a panel of autologous and allogeneic targets (patient characteristics: Table 3.1).

The Ac clones from patient DM are both TCRαβ$^+$ CD8$^+$ and specifically lysed the stimulating clone, but not autologous PHA-activated T cell blasts or other MBP reactive T cells isolated from the same or a different patient (Figure 3.2). Interestingly, additional experiments revealed that DM-Ac-1 recognized another autologous MBP T cell clone (DM-2E2) that expressed identical TCR sequences as the stimulating clone (Table 3.2). These findings

indicate that the T cell receptor most likely is the target for the Ac clones. This is further supported by the fact that MBP reactive T cells that appeared in the circulation of DM three years after TCV had a different clonal origin from the T cells present before vaccination (Figure 3.1). MBP T cells included in the vaccine however stayed undetected for at least 5 years post vaccination. Our previous data further suggested that sequences within the TCR of the vaccine clone are seen as antigens by the Ac clones[11,13]. Therefore, we compared the TCR V and J gene expression of the different target clones used in the cross-reactivity assays (Table 3.2B). Interestingly, DM-Ac-1 was not capable of recognizing DM-2D5, which expressed identical AV and BV genes as DM-2D6, but distinct AJ and BJ genes. This could indicate that the V(D)J region might be targeted in the clonotypic recognition of DM-2D6. Surprisingly, DM-Ac-1 also recognized an allogeneic MBP reactive T cell clone (PAG-2F8) that shared no TCR V or J genes. However, patients DM and PAG share some of their MHC class I molecules (Table 3.1), suggesting that these restriction elements may be important in the observed clonotypic T-T cell interactions. Taken together, these data suggest that the recognition by DM-Ac-1 is directed at the hypervariable CDR3 regions of the vaccine clone DM-2D6.

Three $CD8^+$ $TCR^+$ Ac clones were isolated from patient MOA (Table 3.3). Each of the Ac clones expressed distinct TCRs, indicating that they are individual clones of unique clonal origin. We studied the cross-reactivity profile of these anti-clonotypic T cell clones and found that all three of them specifically recognized the stimulating clone (MOA-2F8), but none of the other MBP reactive T cell clones tested. Detailed analysis of the TCR V and J gene expression showed that the clones that could not be recognized express TCR molecules distinct from the positively recognized stimulating clone (MOA-2F8). This again suggests that the anti-clonotypic T cells specifically target the TCR.

A similar situation was observed for patient MIV, where independently isolated Ac clones were found to specifically lyse the stimulating clone (MIV 2G4), but not another autologous MBP reactive T cell clone expressing different V and J genes (Table 3.4). In addition, the Ac clones failed to recognize PHA-stimulated T cell blasts, which is in line with the previously observed specificity of the clonotypic recognition.

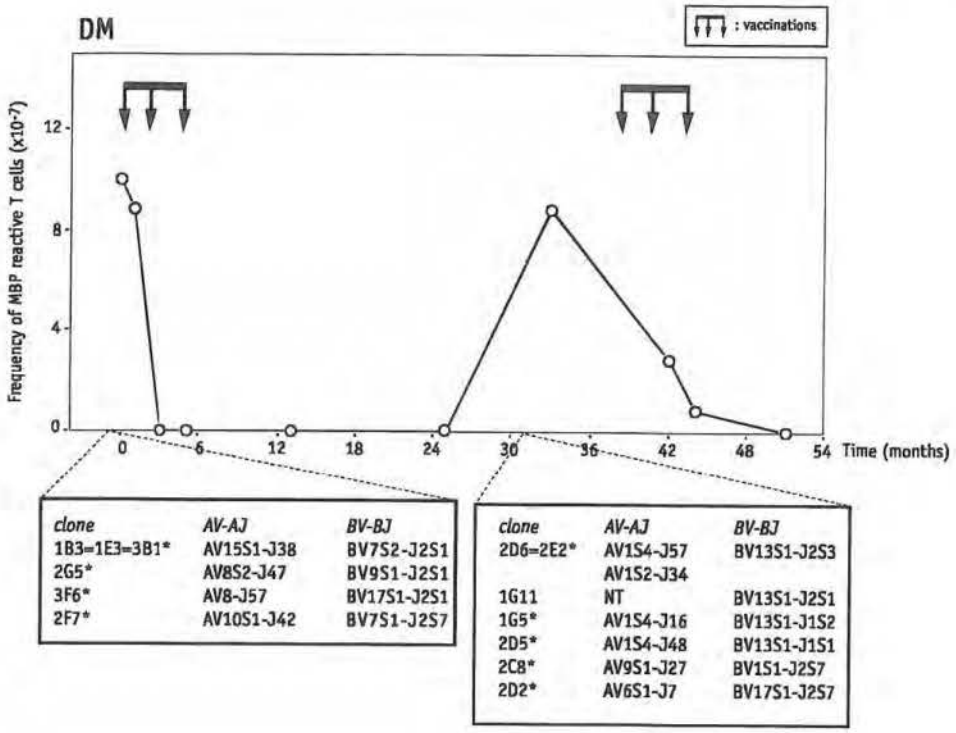


**Figure 3.1.** *TCR expression of MBP reactive T cells isolated at different times before and after vaccination*

The frequency of MBP reactive T cells was determined by limiting dilution assays. Each round of TCV consisted of three subsequent immunizations (2-4 month intervals) with $10x10^6$ cells of each autologous MBP reactive T cell clone. MBP reactive T cell clones isolated at different times were analyzed for their TCR V gene expression as described in 2.2.2. Clones marked with an asterisk (*) were used as T cell vaccine. NT: not tested.

**Table 3.1.** Patient characteristics

| Patient | Age/Sex | Duration (years) | Disease Type | EDSS | HLA A,B,C haplotype |
|---|---|---|---|---|---|
| DM | 43/M | 6 | Chronic Progressive | 6.5 | A3 A31 A19 B7 B13 Bw6 Cw7 |
| MOA | 44/F | 5 | Chronic Progressive | 3.5 | NT[1] |
| MIV | 32/F | 12 | Relapse Remitting | 5.0 | A2 A24 A9 B44 B12 Bw4 B39 B16 Bw6 |
| HEV | 52/M | 5 | Relapse Remitting | 2.0 | NT |
| PAG | 44/M | 3 | Relapse Progressive | 3.5 | A1 A31 A19 B8 B60 B40 |

[1] NT: not tested; shared MHC class I molecules are underlined

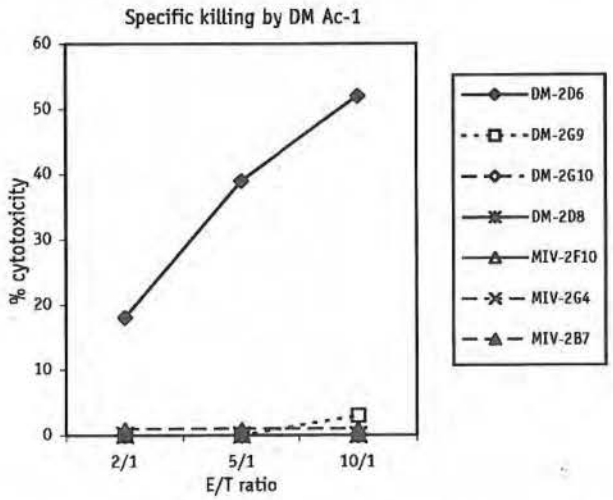


**Figure 3.2.** *Cytotoxicity pattern of Ac clone DM-Ac-1 to a panel of autologous and allogeneic targets*
Specific killing was evaluated in Cr-release assays, as depicted in 2.2.4. The x-axis displays the different effector-to-target (E/T) ratios tested. The y-axis shows the percentage of specific lysis by the anti-clonotypic T cell clone. DM-2D6 is the stimulating clone.

**Table 3.2.** Recognition pattern of Ac clones isolated from patient DM

*A. Characteristics of Ac clones*

| Ac clone | Stimulus | Phenotype | TCR expression | | Cross-reactivity Profile[1] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | AV-AJ | BV-BJ | DM-2D6 | DM-2D5 | DM-2E2 | DM-2C8 | DM-blasts | PAG-2F8 | MIV-2G4 |
| Ac-1 | DM-2D6 | CD8$^+\alpha\beta^+$ | 12S1-J13 | 13S2-J2S3 | YES | NO | YES | NO | NO | YES | NO |
| Ac-11 | DM-2D6 | CD8$^+\alpha\beta^+$ | NT | 13S1-J2S2 | YES | NO | NT | NT | NT | NT | NT |

*B. TCR expression of T cell clones used as targets for cross-reactivity assays*[2]

| Target cells | AV | AJ | BV | BJ | Recognition by DM-Ac-1 |
|---|---|---|---|---|---|
| DM-2C8 | AV9S1 | J27 | BV1S1 | J2S7 | NO |
| DM-2D5 | **AV1S4** | J48 | **BV13S1** | J1S1 | NO |
| DM-2E2 | **AV1S4** | *J57* | **BV13S1** | *J2S3* | YES |
| DM-2D6 | **AV1S4** | *J57* | **BV13S1** | *J2S3* | YES |
| | AV1S2 | J34 | | | |
| PAG-2F8 | AV5S1 | J43 | BV10S2 | J2S1 | YES |
| | AV22S1 | J29 | BV3S1 | J2S7 | |
| MIV-2G4 | AV2S1 | J41 | BV3S1 | J2S1 | NO |

[1] Anti-clonotypic T cell clones were examined for their recognition of a panel of MBP reactive T cell clones in standard Cr-release assays (scored positive if more than 20% specific killing was observed).
[2] Clones sharing identical TCR sequences are underlined. AV and BV genes expressed in several clones from DM are in bold. Shared AJ and BJ genes are in italic. NT: not tested.

**Table 3.3.** Recognition pattern of Ac clones isolated from patient MOA

*A. Characteristics of Ac clones*

| Ac clone | Stimulus | Phenotype | TCR expression | | Cross-reactivity profile[1] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | AV-AJ | BV-VJ | MOA-2F8 | MOA-2G3 | MOA-1G2 | MOA-2D2 | MOA-2F7 |
| Ac-D4 | MOA-2F8 | $CD8^+TCR\alpha\beta^+$ | 7S2-J24 | 2S1-J2S7 | YES | NO | NO | NO | NO |
| Ac-D7 | MOA-2F8 | $CD8^+TCR\alpha\beta^+$ | NT | 14S1-J2S6 | YES | NO | NO | NO | NO |
| Ac-B9 | MOA-2F8 | $CD8^+TCR\alpha\beta^+$ | 23S1-J40 | 1S1-1S3 | YES | NO | NO | NO | NO |

*B. TCR expression of T cell clones used as targets for cross-reactivity assays[2]*

| Target cells | AV | AJ | BV | BJ | Recognized by MOA-Ac |
|---|---|---|---|---|---|
| MOA-2F8 | AV1S3 | J54 | BV18S1 | J1S4 | YES |
| MOA-2G3 | **AV12S1** | J42 | BV13S1 | J1S1 | NO |
| MOA-1G2 | AV2S1 | J33 | NT | NT | NO |
| MOA-2F7 | **AV12S1** | J57 | BV8Sx | J2S7 | NO |
| MOA-2D2 | NT | NT | BV2Sx | NT | NO |

[1]Anti-clonotypic T cell clones were examined for their recognition of a panel of MBP reactive T cell clones in standard Cr-release assays (scored positive if more than 20% specific killing was observed).
[2] AV and BV genes that are expressed in several clones are in bold. NT: not tested.

The $CD8^+$ Ac clones isolated from patient HEV were able to lyse another independently isolated MBP T cell clone (HEV-1F6) in addition to the vaccine clone (Table 3.5). Analysis of the TCR expression profile and direct sequencing of the CDR3 regions revealed that HEV-2E2 and HEV-1F6 are sister clones, bearing identical TCR sequences.

Taken together, our data demonstrate that $CD8^+$ anti-vaccine T cells could be isolated from the circulation of MS patients treated with T cell vaccination. The cross-reactivity assays indicated that these anti-clonotypic T cells are highly specific for the immunizing clone, most likely by recognizing the TCR. These observations further support the idea that anti-idiotypic T cells contribute to the specific suppression of MBP reactive T cells after T cell vaccination.

**Table 3.4.** Recognition pattern of Ac clones isolated from patient MIV

*A. Characteristics of Ac clones*

| Ac clone | Stimulus | Phenotype | Cross-reactivity profile[1] | | |
|---|---|---|---|---|---|
| | | | MIV-2G4 | MIV-2B7 | MIV-blasts |
| Ac-C5 | MIV-2G4 | $CD8^+TCR\alpha\beta^+$ | YES | NO | NO |
| Ac-F3 | MIV-2G4 | $CD8^+TCR\alpha\beta^+$ | YES | NO | NO |
| Ac-B10 | MIV-2G4 | $CD8^+TCR\alpha\beta^+$ | YES | NO | NO |

*B. TCR expression of T cell clones used as targets for cross-reactivity assays*

| Target cells | AV | AJ | BV | BJ | Recognition by MIV-Ac |
|---|---|---|---|---|---|
| MIV-2G4 | AV2S1 | J41 | BV3S1 | J2S1 | YES |
| MIV-2B7 | NT | NT | BV10S1 | J1S5 | NO |

[1]Anti-clonotypic T cell clones were examined for their recognition of a panel of MBP reactive T cell clones in standard Cr-release assays (scored positive if more than 20% specific killing was observed). NT: not tested.

**Table 3.5.** Recognition pattern of Ac clones isolated from patient HEV

*A. Characteristics of Ac clones*

| Ac clone | Stimulus | Phenotype | TCR expression | | Cross-reactivity profile | | | |
|---|---|---|---|---|---|---|---|---|
| | | | AV-AJ | BV-BJ | HEV-2E2 | HEV-1D9 | HEV-1E6 | HEV-1F6 |
| Ac-F4 | HEV-2E2 | $CD8^+TCR\alpha\beta^+$ | V2S1-J23 | V7S2-J2S2 | YES | NO | NO | YES |
| Ac-B10 | HEV-2E2 | $CD8^{+/-}TCR\alpha\beta^+$ | V7S2-J38 | V9S1-J1S6 | YES | NO | NO | YES |
| Ac-E8 | HEV-2E2 | $CD8^{+/-}TCR\alpha\beta^+$ | NT | V4S1-J1S2 | YES | NO | NO | YES |

*B. TCR expression of T cell clones used as targets for cross-reactivity assays*[2]

| Target cells | AV | AJ | BV | BJ | Recognized by HEV-Ac |
|---|---|---|---|---|---|
| HEV-2E2 | **AV6S1** | *J44* | **BV7S2** | *J2S7* | YES |
| HEV-1F6 | **AV6S1** | *J44* | **BV7S2** | *J2S7* | YES |
| HEV-1D9 | AV20S1 | J26 | BV14S1 | J2S5 | NO |
| HEV-1E6 | NT | NT | BV17S1 | J1S1 | NO |

[1]Anti-clonotypic T cell clones were examined for their recognition of a panel of MBP reactive T cell clones in standard Cr-release assays (scored positive if more than 20% specific killing was observed).
[2]Clones sharing identical TCR junctional sequences are underlined. AV and BV genes expressed in several clones are in bold. Shared AJ and BJ genes are in italic. NT: not tested.

### 3.2.2 Fine specificity of Ac T cells: testing of overlapping TCR peptides

The detailed analysis of the cross-reactivity pattern of the Ac clone DM-Ac-1 provided indications that the V(D)J junctional region could harbor the anti-clonotypic epitope. However, when aligning the sequences of the clones tested in the cross-reactivity assay, no homologous amino acid sequence could be identified as target epitope (Table 3.6). Therefore, we decided to screen the complete V(D)J region for specific recognition by the Ac clone (Figure 3.3). In order to do so, a total of 88 overlapping synthetic 9-mers (offset by 1 residue) were synthesized corresponding to the TCR V(D)J-regions of the immunizing clone DM-2D6 (PEPSETS, Chiron technologies, France). Autologous targets (PHA activated blasts or EBV B cells) were preincubated at 37°C, but also at 26°C. Ljunggren and co-workers showed that culturing of cells at reduced temperature induces the expression of empty MHC class I molecules[21]. Targets were pulsed with the individual peptides for 2 hours. The TCR peptide-pulsed T cells were used as targets in $^{51}$Cr-release assays at effector-to-target ratios of 40 to 1 (2.1.4).

While DM-Ac-1 specifically lysed the positive target clone (72%), only low cytotoxicity was observed towards the different TCR peptides. Peptides that gave more than 5% lysis (Figure 3.4) were retested at different E/T ratios. For peptides p5 and p6 derived from the TCR alpha chain an increase in cytotoxicity was observed with increasing E/T ratios at both temperatures (Figure 3.5). Interestingly, p6 shares the amino acids P and N with the corresponding region of the positively recognized PAG alpha-chain, but not with the other clones (Table 3.6). These residues may be essential in the clonotypic T-T cell interaction. Still, the obtained cytotoxic values were quite low. In additional experiments, no significant killing could be discovered for any of the target peptides (data not shown).

In conclusion, none of the screened TCR peptides could induce a high cytotoxic response by the Ac clone DM-Ac-1. Therefore, we cannot rule out that the target epitope may be located in other less variable regions of the TCR, which were not screened in this part of the study. Furthermore, technical limitations might be of influence (e.g. efficient peptide binding, availability of empty MHC I molecules...) and have to be taken into account when interpreting these results.

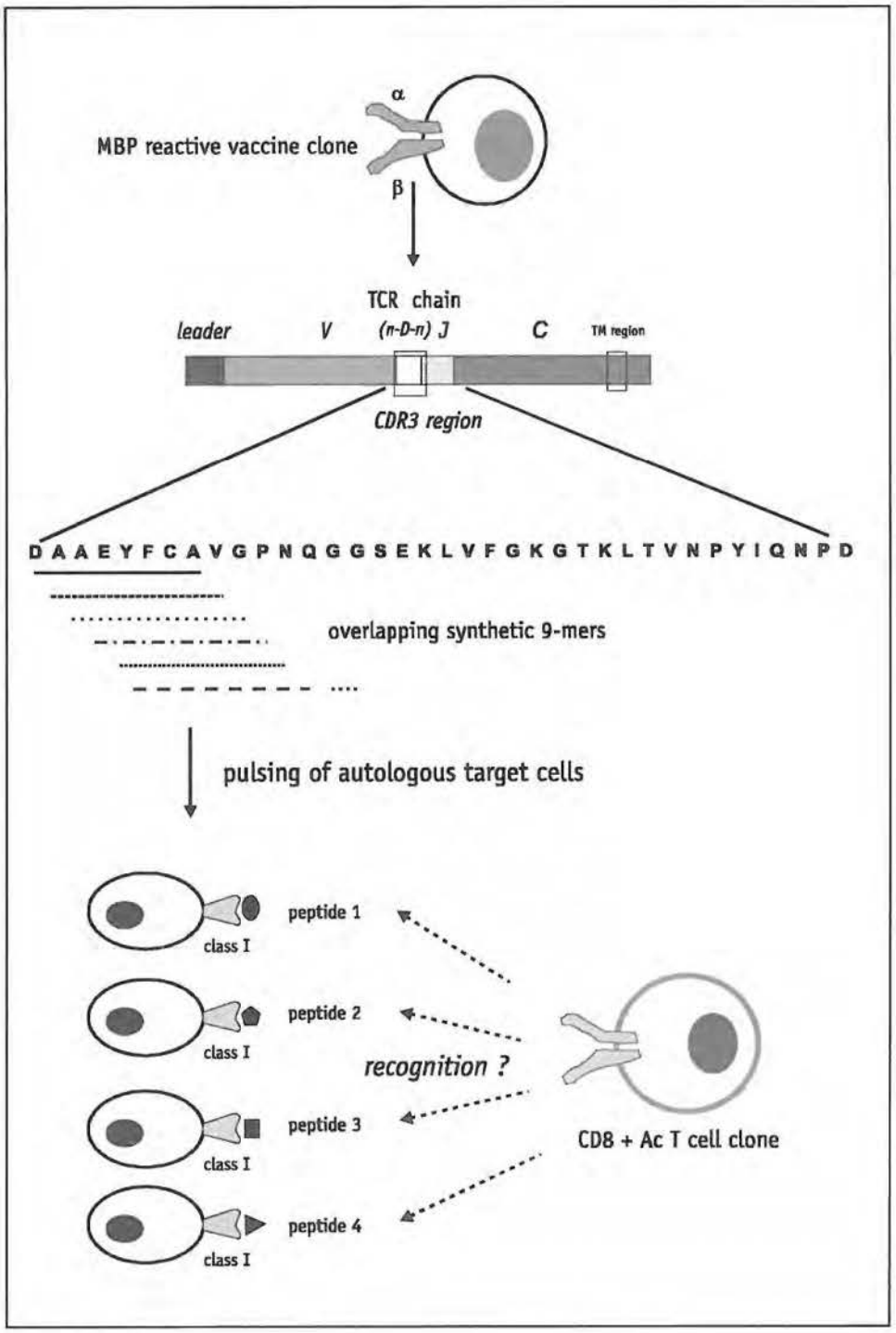


**Figure 3.3.** *Experimental strategy to test reactivity to TCR peptides derived from the vaccine clone.*

**Table 3.6.** Amino Acid Sequence alignment of DM and PAG clones[1]

| *Clone* | *V(D)J Sequence* | | | | *Recognized by DM-Ac-1* |
|---|---|---|---|---|---|
| **α-chain** | V | N | J | C | |
| DM-2D8 | DSAVYFCAL | ISVGXGGYQ | KVTFGTGTKLQVIP | NIQNPDPAV | NO |
| DM-2G9 | DSAVYFCAA | PPGYGN | KLVFGAGTILRVKS | YIQNPDPAV | NO |
| DM-2D5 | DAAEYFCAV | DGGNE | KLTFGTGTRLTIIP | NIQNPDPAV | NO |
| DM-2D6 chain 1 | DAAEYFCAV | GPNQGGSE | KLVFGKGTKLTVNP | YIQNPDPAV | YES |
| DM-2D6 chain 2 | DAAEYFCAV | GGWTD | KLIFGTGTRLQVFP | NIQNPDPAV | YES |
| PAG-2F8 chain 1 | DSAVYFCAL | PNSGNT | PLVFGKGTRLLVIA | NIQNPDPAV | YES |
| PAG-2F8 chain 2 | DSATYLCAL | GGDNNN | DMRFGAGTRLTVKP | NIQNPDPAV | YES |
| **β-chain** | V | N-D-N | J | C | |
| DM-2G9 | TSVYFCASS | PTAN | NSPLHGNGTRLTVT | EDLNKVFPP | NO |
| DM-2G10 | TSVYFCAIS | KDFADTD | YFGPGTRLTVL | EDLKNVFPP | NO |
| DM-2D8 | SAVYLCASS | PSGGPL | YGYTFGSGTRLTVV | EDLNKVFPP | NO |
| DM-2C8 | SALYFCASS | PSEGTGSH | EQYFGQGTRLTVT | EDLNKVFPP | NO |
| DM-2D5 | TSVYFCASS | YGAPG | TEAFFGQGTRLTVV | EDLNKVFPP | NO |
| DM-2D6 | TSVYFCASS | YAVGG | DTQYFGPGTRLTVL | EDLKNVFPP | YES |
| PAG-2F8 chain 1 | TALYFCASS | KDAQGLAEXGKTR | EQFFGPGTRLTVL | EDLKNVFPP | YES |
| PAG-2F8-chain 2 | TSMYLCASS | FWGGTN | FGPGTRLTVT | EDLKNVFPP | YES |

[1] homologous sequences are in color. 9-mers were synthesized for the underlined sequences. A potential recognition site (peptide 6) is marked in yellow.


**Figure 3.4.** *Reactivity to overlapping TCR peptides corresponding to the V(D)J junctional regions of clone DM-2D6* Peptides with > 5 % killing at 26° or 37°C is shown as full or dashed lines respectively. V: variable; C: constant region.

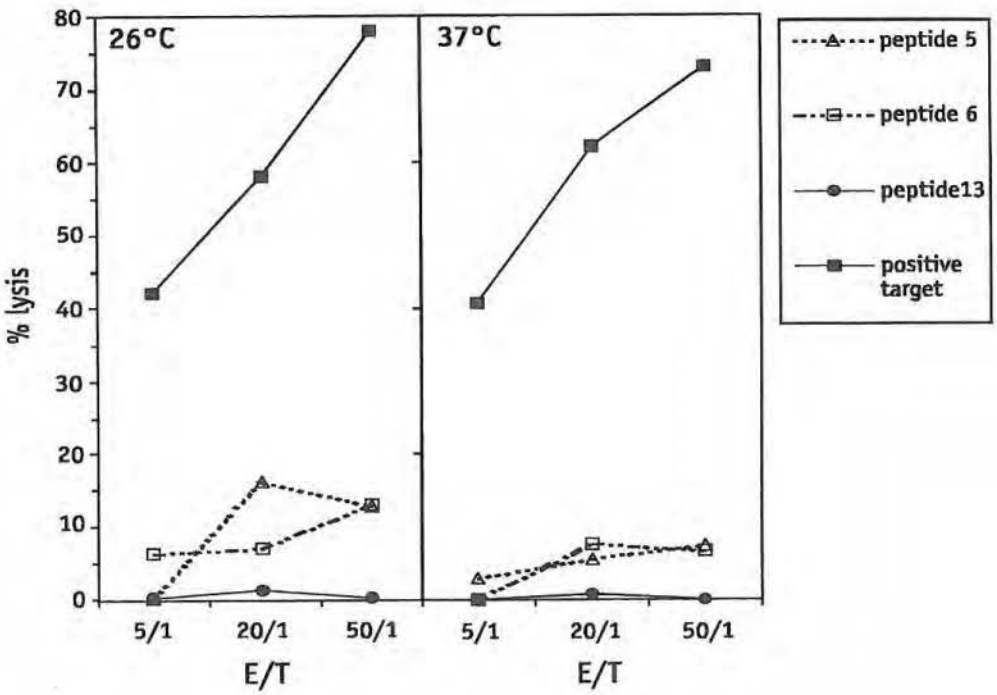


**Figure 3.5.** *Specific lysis of TCR peptide pulsed target cells by DM- Ac-1*
Peptides showing more than 5 % killing (Figure 3.4) were tested for different E/T ratios. Peptide 5 (AEYFCAVGP) and peptide 6 (EYFCAVGPN) represent peptides with more than 5 % killing in the pilot experiment; peptide 13 (PNQGGSEKL) less than 5 % killing. Clone PAG-2F8 was used as positive target.

### 3.2.3 Fine specificity of Ac T cells: transfection of T cell receptor genes

In a second attempt to identify the T cell receptor as molecular target for the anti-clonotypic T cells, the TCR alpha and beta genes were cloned into expression vectors and transfected to target cells. The resulting transfectants were then tested for recognition by the Ac clones (Figure 3.6). In the first part, I will explain which target cells are useful for the transfection of TCR constructs. The second part summarizes the results on the transfection experiments for 3 MS patients treated with TCV.

**HVS transformed human T cell clones: an efficient target for DNA transfections**

Autoantigen specific T cell clones are an important tool to study the molecular and cellular mechanisms of autoimmunity. To culture autoreactive T cells they need to be stimulated with antigen and antigen presenting cells (APC) at regular intervals. Even then, the life span of human T cells in culture is finite. For these reasons human T cell clones are a difficult target for transfection experiments. However, antigen specific T cell clones can be transformed to continuous growth by Herpesvirus saimiri (HVS)[22]. The obtained immortalized T cells still require IL-2, but no longer depend on antigen stimulation. We have tested whether the HVS-immortalized T cell clones are an efficient target for transfection experiments.

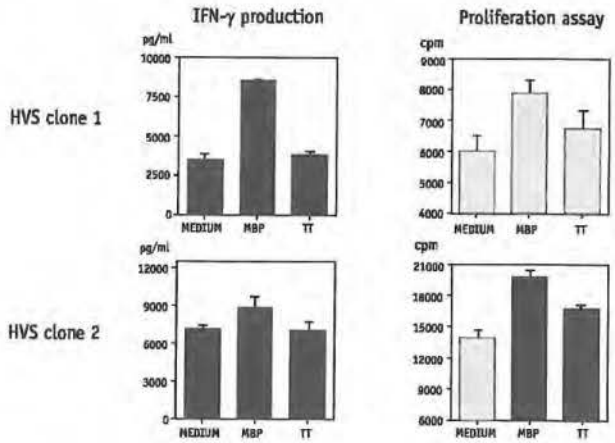


**Figure 3.7.** *Antigen response of HVS transformed T cell clones*

MBP reactive T cell clones 1 and 2 were transformed with Herpesvirus saimiri and tested for their specific response to MBP. Reactivity was measured as increased production of IFN-γ (left) and in proliferation assays ($^3$H thymidine uptake). cpm: counts per minute; TT: tetanus toxoid: control antigen.

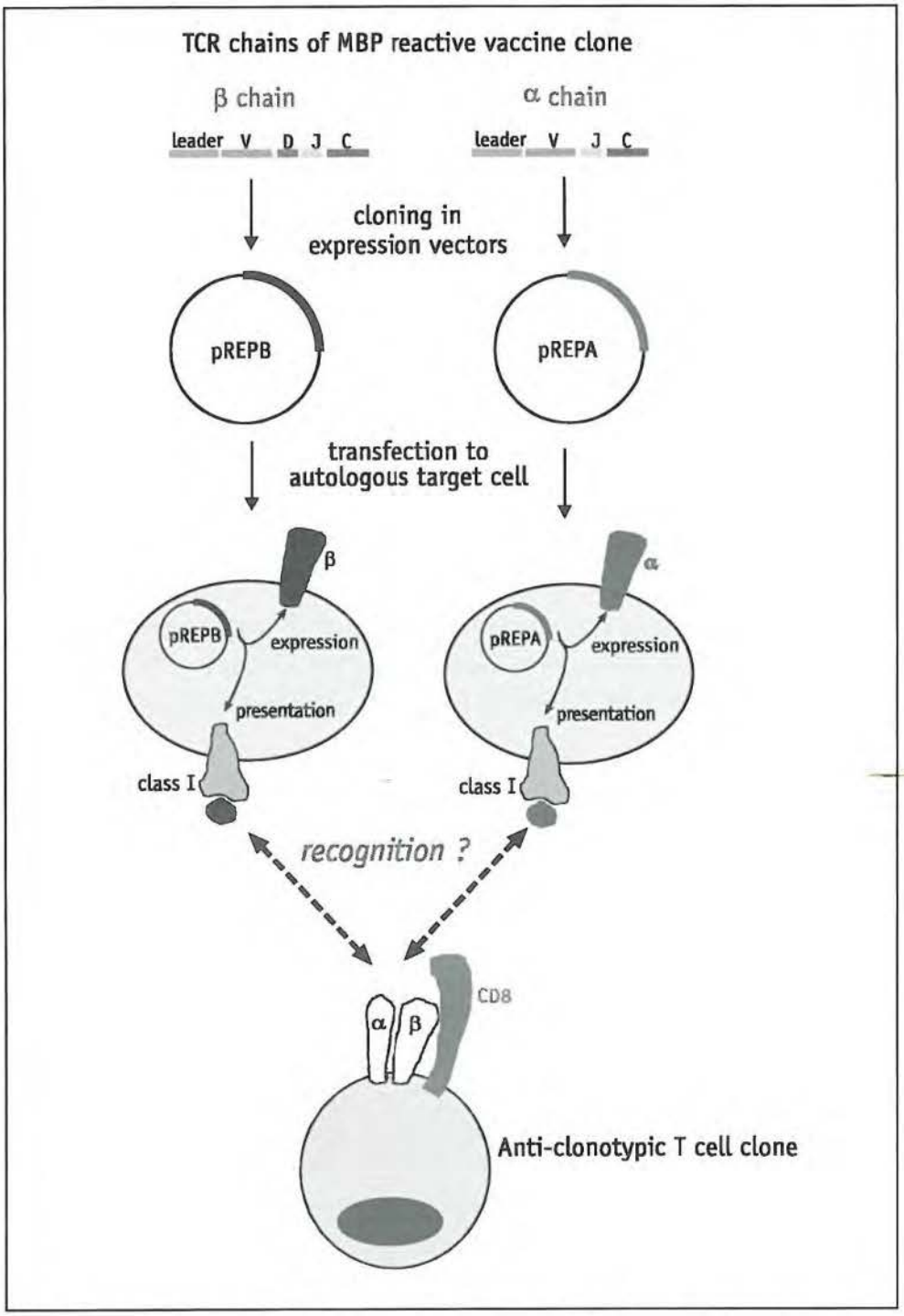


**Figure 3.6.** *Experimental approach for the transfection of vaccine clone derived TCR α and β chains*

A panel of 10 MBP specific T cell clones from 4 MS patients were virus-transformed (2.1.8) and characterized as to their antigen reactivity, T cell receptor V gene usage, and phenotypic expression. The HVS clones kept their antigen specificity (Figure 3.7) and are phenotypically identical (TCR expression; $CD4^+/TCR\alpha\beta^+$, data not shown) to the respective nontransformed clones. The high background signals observed in our proliferation assays are consistent with the fact that HVS-transformed T cells grow via an autocrine mechanism, in response to activation signals mediated by their CD2 molecules during mutual cell-cell contact[23]. Weber et al. showed that this background proliferation can be reduced in the presence of anti-CD2 mAbs[22]. Next, we examined whether the HVS transformed T cell clones can be easily transfected with plasmid constructs. Therefore, the T cell receptor $\alpha$- and $\beta$-chain of a myelin basic protein (MBP) reactive T cell clone derived from an MS patient were cloned in pREP4, pREP8 or pREP9 as stated in 2.2.4. Next, these TCR constructs were transfected to an unrelated HVS T cell clone (2.2.5). Three to four weeks after transfection, cell pellets were collected for DNA and RNA extraction. Using pREP specific primers we found that the construct is still present after 4 weeks (DNA level, Figure 3.8). RT-PCR with primers specific for the cloned TCR chain demonstrated the transcription of the TCR constructs (Figure 3.9). Similar results were found for other control target cell lines: EBV transformed B cells and the Jurkat cell line.

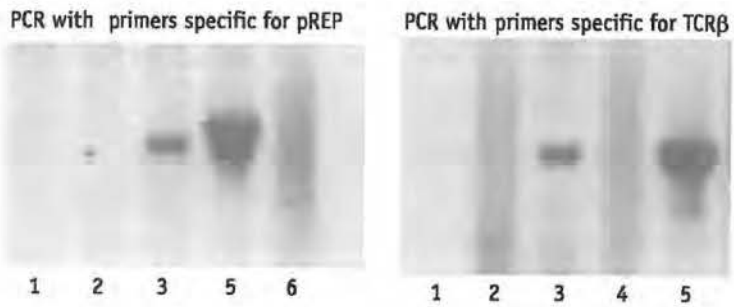


**Figure 3.8.** *PCR on DNA of a transfected HVS clone*

A HVS transformed MBP reactive T cell clone was transfected with TCR receptor constructs. After three to four weeks of culture, pellets were taken and DNA was extracted from these pellets. PCR was performed with pREP vector- or insert-specific primers. The insert (TCRβ) is 942 bp.

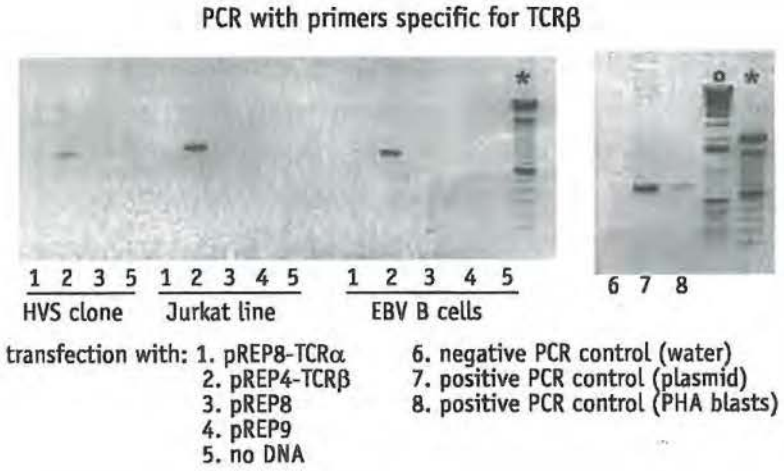


**Figure 3.9.** *RT-PCR on TCR transfectants*

An HVS immortalized T cell clone was transfected with TCR receptor constructs. After three to four weeks of culture, pellets were taken and RNA was extracted from these pellets. RNA was reverse transcribed into cDNA (2.2.1). PCR was performed with primers specific for the beta-insert (TCRβ: 942 bp). As control target cells EBV transformed B cells and the Jurkat cell line were used for transfection. *:100 bp and °:1 kb DNA marker

Our data demonstrate that Herpesvirus saimiri transformed T cell clones can be readily transfected. Transfection of these HVS T cell clones with autoreactive TCR constructs will therefore be a useful tool to study the interactions between autoreactive T cells and their anti-idiotypic counterparts.

### Transfection of TCR chains and recognition by Ac T cell clones

From the vaccine clones of three MS patients (DM, MOA, MIV) the TCR chains were amplified by PCR and cloned into pREPx expression vectors as described in 2.2.4 (Table 3.7). The DNA sequences of the recombinant plasmids were verified and sufficient amounts of plasmid DNA were prepared and purified for subsequent use in the transfection experiments.

**Table 3.7.** Overview of the T cell receptor chain constructs[1]

| *clone* | *TCR chain* | *TCR construct* | *V-J expression* |
|---|---|---|---|
| DM-2D6-15 | alpha | p8DMA | AV1S4-J57 |
| | beta | p4DMB | BV13S1-J2S3 |
| MOA-2F8-31 | alpha | p8MOAA | AV1S3-J54 |
| | beta | p4MOAB | BV18S1-J1S4 |
| MIV-2G4-5 | alpha | p9MIVA | AV2S1-J41 |
| | beta | p8MIVB | BV3S1-J2S1 |

[1] p4: pREP4; p8: pREP8; p9: pREP9

For patient MOA, PHA activated blasts were used as target cells for transfection with TCR constructs. Eighty-four hours post transfection pellets were taken from the transfected cultures for RNA isolation. Transcription of the recombinant TCR gene was demonstrated by PCR as illustrated in Figure 3.10. At the same time, the transfectants were tested for specific recognition by the Ac clone. However, the anti-clonotypic T cell clone MOA-Ac(2F8) only recognized and specifically lysed the vaccine clone MOA-2F8-31, but not the TCR alpha or beta transfectants.

Different target cells (HVS-clone, EBV B cells, PHA blasts) were used to transfect the TCR constructs derived from patient DM. Although the different alpha and beta transfectants expressed the recombinant TCR chain (data not shown), significant killing could only be demonstrated for the positive target (Table 3.8).

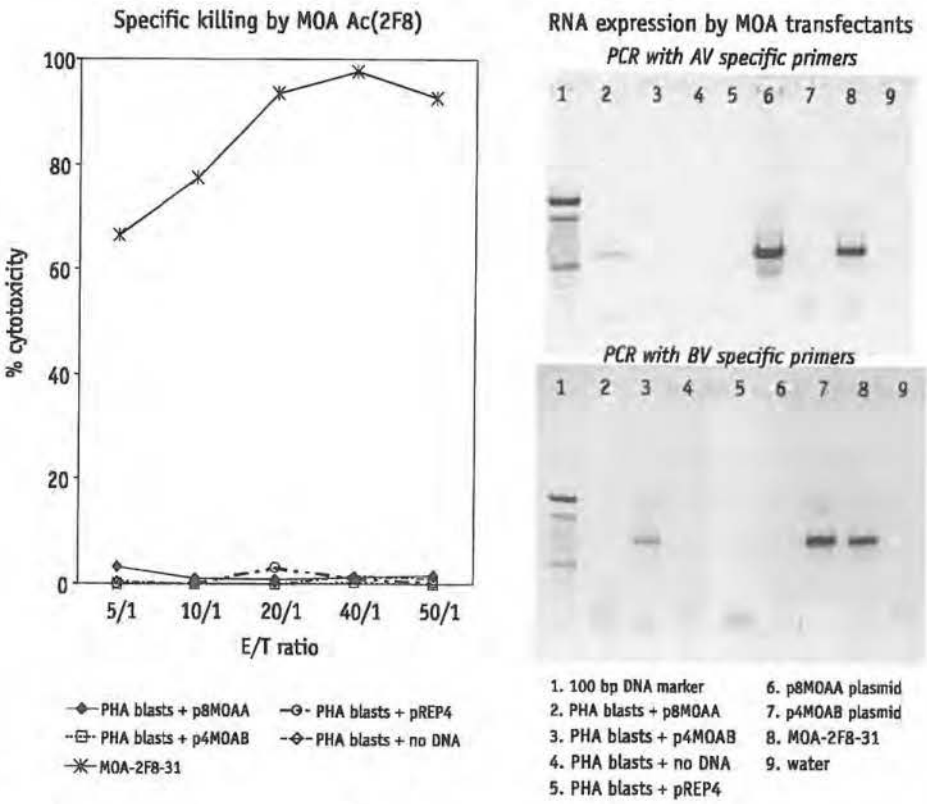


**Figure 3.10:** *Transfection experiments for patient MOA*
MOA derived PHA blasts were transfected with p8MOAA and p4MOAB. 48 hours later, transfectants were tested for recognition by MOA-Ac(2F8) in $^{51}$Cr-release assays. In parallel mRNA expression was evaluated by PCR.

**Table 3.8.** Transfection experiments for patient DM

| *Target cells* | *TCR construct* | *Specific killing by Dm-Ac(2D6) (%)* | | |
|---|---|---|---|---|
| | | 10/1[1] | 20/1 | 40/1 |
| HVS-DM-2C8 | p8DMA | 9 | 7 | 15 |
| | p4DMB | 0 | 6 | 5 |
| | no DNA | 2 | 0 | 0 |
| PHA blasts | p8DMA | 2 | 4 | 3 |
| | p4DMB | 0 | 2 | 16 |
| | no DNA | 0 | 0 | 1 |
| EBV B cells | p8DMA | 0 | 3 | 7 |
| | p4DMB | 1 | 5 | 6 |
| | no DNA | 3 | 2 | 4 |
| positive clone | N.A. | 41 | 62 | 73 |

[1]Specific killing was tested for different E/T ratios; mRNA expression was confirmed by PCR (data not shown)

A similar picture was found for patient MIV, where the Ac clone did not specifically recognize autologous lymphocytes transfected with the vaccine-derived TCR chains (data not shown).

In conclusion, although the original vaccine clone is thought to present its endogenously produced T cell receptor to its anti-clonotypic counterparts, we were not able to mimic this situation *in vitro*.

### 3.2.4 Results: conclusions

The cross-reactivity experiments described in this chapter further support the idea that the TCR is the major target for the anti-clonotypic T cells induced by T cell vaccination. It still remains elusive however which mechanisms account for the presentation and recognition of the TCR. Using two *in vitro* models we attempted to further unravel the molecular basis of the clonotypic T-T cell interactions. However, we could not provide clear-cut evidence that support our previous assumptions. Further investigations are needed to better understand the precise mechanisms involved in the *in vivo* clonotypic regulation of autoreactive T cells.

## 3.3 Discussion

T cell vaccination has been shown to effectively prevent various experimental autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), an animal model for MS[6,24,25]. Although the exact mechanisms by which T cell vaccination ameliorates autoimmune disease remain unclear, indirect evidence suggests that the anti-vaccine T cell responses in these animals specifically target the immunizing T cells by recognition of their T cell receptor (TCR). First, the protection against the disease is restricted to the T cell clones selected for immunization. Vaccination with MBP reactive T cells protects only against EAE but not against adjuvant arthritis induced by *Mycobacterium tuberculosis* specific T cells, and vice versa[26]. Second, anti-idiotypic $CD8^+$ T cells isolated from immunized rodents specifically lyse the immunizing T cell clones but not T cells expressing structurally distinct T cell receptors[6,25,27]. In addition to the anti-idiotypic T cell responses, other regulatory mechanisms are thought to contribute to the protective immunity induced by T cell vaccination. Cohen and colleagues observed that anti-clonotypic T cells might possibly induce autoreactive T cells to shift from Th1 to Th2[28]. Lohse et al. further demonstrated that anti-ergotypic T cell responses directed at activation markers may partially account for the suppression of activated autoreactive T cells after vaccination[7]. In addition, T cell vaccination in EAE was shown to induce humoral responses that inhibited the proliferation of vaccine cells[29]. Together, these findings reveal that TCV in EAE induces a complex immune response that results in the neutralization of pathogenic T cells.

In phase I clinical trials, we observed substantial and long-term anti-vaccine and anti-clonotypic responses, accompanied by a specific depletion of circulating MBP T cells in MS patients vaccinated with irradiated MBP reactive T cell clones[11,30,31]. Upon characterization of the anti-vaccine responses, we illustrated that apart from anti-idiotypic $CD8^+$ $\alpha\beta^+$ T cells, other lymphocyte populations such as $CD4^+$ cytokine secreting T cells, $\gamma\delta^+$ T cells and NK cells take part in the complex immune response elicited by TCV[32,33].

In this report, we made an attempt to define the idiotypic determinants responsible for triggering the $CD8^+$ cytotoxic T cell responses in TCV treated MS patients. Therefore, $CD8^+$ $TCR\alpha\beta^+$ Ac T cells were isolated from the vaccinated patients and tested for their recognition of a panel of autologous and allogeneic T cell clones. We found that the $CD8^+$ Ac T cell clones only lysed the inducing clone, but not other autologous clones with distinct

TCRs. Interestingly, Ac T cells also displayed specific cytotoxicity towards independently isolated MBP T cell clones that expressed identical T cell receptors as the stimulating clone. In patient DM, we demonstrated that the target epitope possibly resides within the hypervariable V(D)J region, since the Ac T cell clone could not recognize the autologous MBP clone DM-2D5, which expressed identical AV and BV genes as the immunizing clone (DM-2D6), but different CDR3 structures. This is consistent with our previous findings which showed that Ac T cell lines did not recognize a panel of control clones with irrelevant antigen specificity[13]. Surprisingly, the Ac clones from patient DM also recognized an allogeneic MBP reactive T cell clone (PAG-2F8) that shared no V or J genes with the original stimulator clone (DM-2D6). When aligning the CDR3 sequences of the clones DM-2D6 and PAG-2F8, no clear amino acid sequence could be identified as the target epitope. However, several reports of Hemmer and co-workers provide evidence that antigen recognition by T cells is highly degenerate[34,35]. They indicated that not all amino acid residues within a T cell epitope equally contribute to Ag recognition[36]. To further define the fine specificity of the anti-clonotypic recognition for patient DM, a panel of overlapping 9-mers spanning the V(D)J regions of the DM-2D6 clone were screened in cytotoxicity assays. In general, the TCR peptides induced little or no killing by the Ac T cells. In spite of the low values, we observed an increase in cytotoxicity with increasing E/T ratios for two peptides (p5 and p6) derived from the TCR alpha-chain. Strikingly, when comparing peptide 6 with the corresponding region within the CDR3 of the positively recognized PAG-2F8 clone, two identical amino acid residues (P and N) were found. These residues were not present in the CDR3 sequences of the clones that were not recognized by the Ac T cells. These observations together with the fact that patients PAG and DM share some of the HLA class I restriction elements may indicate that the CDR3 region is involved in the observed clonotypic interactions.

It is difficult to find out why the TCR peptide pulsed target cells could not induce high cytotoxic responses. Possibly, the PHA activated T cell blasts used for pulsing are not efficient in presenting the peptides in the context of HLA class I molecules. A recent study demonstrated that $CD8^+$ anti-idiotypic T cells only efficiently recognized $CD4^+$ T cells clones when they were activated[37]. This indicates that although the TCR may be the predominant target, additional surface antigens contribute in some extent to the recognition process. Possibly, other surface markers that are not expressed by PHA blasts, could be needed as a "second signal" for enhanced T cell recognition. A previous report stated that Ag specific

activation induces the expression of surface molecules different from those induced by PHA stimulation[38]. The precise composition of these molecules is not known. Ware et al. proposed that an increased expression of adhesion molecules on the target CD4+ T cells might account for selective recognition[39].

There are several models that explain how the idiotypic determinants of target TCR are presented to and recognized by anti-clonotypic T cells. Recent reports demonstrated that endogenous TCR peptides can be presented by self-MHC (I and II) to anti-idiotypic T cells[18,40]. There is experimental evidence indicating that peptides of surface molecules are often presented by MHC class I molecules[41]. Accordingly, TCR-peptides would be generated during normal protein turnover of endogenously produced clonotypic T cell receptors. Alternatively, TCR proteins could be taken up from degenerating T cells by professional antigen-presenting cells, such as macrophages, processed and displayed on the macrophage surface in MHC class II-bound form. Both of these presentation pathways may be involved since our data and previous reports suggested that T cell (receptor) vaccination induces both CD8+ MHC class I restricted and CD4+ MHC class II restricted anti-idiotypic T cells[11,30,42]. For three patients, we isolated the cDNA of the TCR receptor chains of the vaccine clone and cloned them into expression vectors. The resulting TCR plasmids were tranfected to autologous T cells (either PHA blasts, HVS T cells or EBV T cells). Positive transfectants transcribed the recombinant TCR chain as shown by RT-PCR. However, none of the alpha or beta transfectants caused significant killing by the CD8+ Ac T cell clones. The success of this experimental strategy depends on critical factors that are difficult to control: the transfection efficiency and the expression levels, processing and class I presentation of the recombinant TCR proteins. Indeed, although transcription of the recombinant gene is a first prerequisite, this does not automatically mean that the protein is functionally expressed, processed and presented. Moreover, since transient transfections were used we could not provide information on the actual percentage of cells that expressed the recombinant protein. Possibly, the number of cells expressing the TCR was too low to be recognized in the cytotoxicity assays. To circumvent this problem, we attempted to generate stable monoclonal transfectants, but did not succeed in doing so. Furthermore, it remains speculative if the target cells used for transfection possess the necessary cell machinery and pathways that are responsible for the MHC class I presentation of self-peptides. Even then, these pathways may be inhibited in the virus-transformed targets. Recently, Ploegh

illustrated that several viruses including EBV and human Herpes virus (HSV-1 and 2) can inhibit MHC-restricted antigen presentation to escape from immune recognition and eradication[43]. It remains unknown whether Herpesvirus saimiri may also have inhibitory potential on antigen-presentation. Therefore, virus-transformed lymphocytes may not be the ideal tools for *in vitro* models of antigen presentation. Finally, the same argument as in the peptide approach can be mentioned: a second signal may be necessary for the efficient T cell recognition.

Although the above described *in vitro* approaches could not confirm our previous indications, this does not necessarily mean that the TCR is not the major target in the anti-idiotypic T-T cell interactions. The presentation of self-TCR peptides may follow unconventional pathways in which other "helper" cells that are present *in vivo*, but not *in vitro* play a vital role. Kozovska and co-workers demonstrated that MBP specific $CD4^+$ T cells showed an outstanding ability to present TCR peptide due to the presence of a unique population of $CD4^-$ $CD8^-$ T cells[44]. Still, a recent study strongly supports the hypothesis that $CD8^+$ CDR3-specific T cells play a predominant role in the immune responses induced by T cell vaccination[45]. This study reported for the first time that TCR peptide reactive T cell lines isolated from vaccinated MS patients cross-react with the original immunizing MBP T cells.

In conclusion, based on our experimental *in vitro* approaches we could not provide convincing evidence that TCR determinants are indeed the major target for the anti-clonotypic $CD8^+$ T cells induced by TCV. However, we emphasized that an additional factor may be necessary for enhanced T cell recognition, which might be present *in vivo* but not in our *in vitro* models. Further investigations are needed to prove this assumption and study the nature of this second signal.

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# Chapter 4

## Analysis of T Cell Reactivity to Myelin Antigens in MS Patients and Healthy Controls by ELISPOT

Based on:

**T Cell Reactivity to Multiple Myelin Antigens in Multiple Sclerosis Patients and Healthy Controls.**

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## Abstract

*Myelin proteins, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) are candidate autoantigens in MS. It is not clear whether MS patients show a predominant reactivity to one or several myelin antigens. We evaluated the IFN-γ production induced by MBP and MOG and selected MBP-, MOG- and PLP-peptides in MS patients and healthy controls using the IFN-γ ELISPOT assay. Most MS patients and healthy controls showed a heterogeneous anti-myelin T cell reactivity. Interestingly in MS patients a positive correlation was found between the anti-MOG and anti-MBP T cell responses. However no myelin peptide was preferentially recognized among the peptides tested (MBP 84-102, 143-168, MOG 1-22, 34-56, 64-86, 74-96, PLP 41-58, 184-199, 190-209). In addition the frequency of IL-2R$^+$ (CD25$^+$) MBP reactive T cells was significantly increased in blood of MS patients as compared to healthy subjects, indicating that MBP reactive T cells exist in an in vivo activated state in MS patients. Most of the anti-MBP T cells were of the Th1-type since reactivity was observed in IFN-γ but not in IL-4 ELISPOT-assays. Using Th1 (IL-12) and Th2 (IL-4) promoting conditions we observed that the cytokine secretion pattern of anti-MBP T cells still is susceptible to alteration. Our data indicate that precursor frequency analysis of myelin reactive T cells by proliferation-based assays may underestimate the true frequency of myelin specific T cells significantly. Our findings further implicate a role for myelin reactive T cells in the pathogenesis of MS and are of potential relevance for the development of myelin specific immunotherapies in MS.*

*Keywords: Multiple Sclerosis, myelin, myelin reactive T cells, myelin oligodendrocyte glycoprotein, myelin basic protein, proteolipid protein, ELISPOT*

## 4.1 Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by focal demyelination and infiltration of macrophages and lymphocytes in the CNS lesions. Autoreactive T cell responses against CNS myelin components are considered to play a primary role in the pathogenesis of MS[1]. Various myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been studied as putative target autoantigens in MS.

MBP and PLP reactive T cells are present in MS patients and control subjects, indicating that they are part of the normal T cell repertoire[2-5]. Previous studies showed that MBP and PLP reactive T cells undergo *in vivo* activation and expansion in the blood, and accumulate in the cerebrospinal fluid in MS patients[6-9]. The pathogenic properties of MBP and PLP reactive T cells are well defined in experimental autoimmune encephalomyelitis (EAE). Adoptive transfer of activated MBP and PLP specific T cells causes EAE in a variety of animal species including different mice strains, rats, guinea pigs and rabbits[10-14].

MOG is the only antigen described so far that induces both a T cell inflammatory response and a demyelinating antibody response in animal models. MOG specific T cells as well as MOG peptide specific T cells have been shown to be encephalitogenic in various rodent strains and the common marmoset model of EAE[15-17]. The demyelinating potential of anti-MOG antibodies was first demonstrated in co-transfer experiments using MBP reactive T cells and a murine monoclonal anti-MOG antibody, and later confirmed in *in vitro* studies[18,19]. Interestingly, MOG-induced EAE not only differs from MBP-induced EAE as far as lesions are concerned, but also with respect to the underlying immunopathology. While immune deviation therapies render animals resistant to MBP-induced EAE, oral tolerance therapy in the common marmoset with soluble MOG leads to a lethal disease caused by shifting from a Th1 to Th2 type cytokine pattern with a subsequent increase in anti-MOG antibody production[20]. MOG-reactive T cell responses were also found in MS patients. Sun et al. (1991) observed T cells that produce IFN-γ upon stimulation with MOG in MS patients[21]. Kerlero de Rosbo and coworkers studied primary proliferative responses to several myelin antigens in MS patients and controls and found a predominant response to MOG in MS

patients, but not in control subjects[22]. Primary responses to panels of overlapping synthetic peptides spanning the extracellular domain of MOG revealed an immunodominant recognition of the epitopes 1-22, 34-56 and 64-96[23]. Another study showed that peptide 63-87 induced the strongest response in a group of $DR2^+$ MS patients[24]. A recent study of primary T cell reactivity to recombinant MOG and characterization of MOG-specific T cell lines however revealed no significant differences between MS patients and healthy controls[25]. This is consistent with another study that reported no significant differences in anti-MOG T cell responses in MS patients and healthy subjects[26].

Several techniques have been used to measure antigen specific T cell reactivity in humans. The traditional ways to calculate the precursor frequency of antigen responsive T cells include $^3$H-thymidine incorporation as a reflection of antigen-induced proliferation, and related limiting dilution assays (LDA). However, these procedures require *in vitro* culture and imply a selection of cells with preferential proliferative capacity rather than cells that respond by producing effector molecules. Therefore, the frequency of anti-myelin reactive T cells *in vivo* is potentially much higher than the one appreciated from the proliferation-based techniques. Indeed, while the anti-MBP T cell frequency as measured by LDA most often is in the range of 1 to 10 MBP T cells in 1 million PBMC, Bieganowska et al. (1997) observed an anti-MBP T cell frequency of 1 in 300 PBMC using a T cell receptor sequence-based approach[27]. In addition, the LDA technique requires a relatively large amount of blood lymphocytes. It becomes therefore quite difficult to study T cell frequencies of various myelin reactive T cell populations in individual patients. The ELISPOT technique may circumvent some of these problems. ELISPOT allows for a quantification of T cells that secrete IFN-γ when stimulated with antigen[28]. A major advantage of this assay is that ELISPOT does not require the *in vitro* proliferation of T cells and may therefore better reflect the *in vivo* T cell frequency. In addition, it requires limited amounts of blood only, making it possible to analyze simultaneously T cell reactivity to a whole range of myelin antigens. The ELISPOT assay also provides information on the cytokine profile or Th phenotype of the antigen specific T cells. Measurement of the IFN-γ production as a read out for T cell reactivity may especially be relevant for MS studies as this cytokine is thought to play an essential proinflammatory role in the disease[29]. Immunopathological features of MS such as macrophage activation and upregulation of MHC class II molecules are possibly attributable to IFN-γ.

In this study, we analyzed T cell reactivity to MBP, MOG and a panel of synthetic MBP, MOG and PLP peptides in MS patients and healthy subjects using ELISPOT. We did not observe significant differences in reactivity between MS patients and control subjects to any of these myelin antigens and peptides. In addition, the frequency of MBP-reactive T-cells in IL-2 expanded lymphocytes was analyzed and an increased frequency of MBP-reactive T cells was found among IL-2 expanded lymphocytes from MS patients as compared to healthy controls. These data further support the view that MBP reactive T cells are activated *in vivo* in MS patients. We also analyzed the Th-phenotype of the anti-MBP T cells in MS and studied whether the cytokine phenotype of these T cells could be altered in Th1 and Th2 biasing conditions. This study provides further information about the anti-myelin T cell reactivity in MS, and can be useful for the development of antigen specific immunotherapies for MS.

## 4.2 Materials and Methods

### 4.2.1 Patients and healthy controls

Peripheral blood was collected from a total of 23 women and 13 men with definite MS. Twenty-four patients had the relapse-remitting form of MS; the remaining 12 showed a progressive disease course. The age of the patients ranged from 26 to 60, with a mean age of 43. The mean EDSS was 4.5 (range: 1 to 9) and the mean disease duration was 9 years (range 1 to 25 years). Table 4.1 provides an overview of the patient characteristics. In addition, blood was drawn from 31 randomly selected healthy control subjects (NS), with a mean age of 32 years. 13/36 MS patients and 5/31 NS were HLA $DR2^+$, as determined by PCR[30]. PBMC were either tested freshly or kept frozen in liquid nitrogen in fetal bovine serum (FBS, Hyclone) containing 10% DMSO. Informed consent was obtained from all subjects volunteering for this study.

### 4.2.2 Cell culture media and antigens

Cells were cultured in RPMI 1640 medium supplemented with L-glutamine, sodium pyruvate, non-essential amino acids and 10 mM HEPES buffer (Life Technologies, Gent, Belgium) and 10 % heat-inactivated FBS (Hyclone Europe, Erembodegem, Belgium). Human MBP was purified from white matter of human brain, as described[31]. Endotoxin-free extracellular domain of MOG (rMOG) was expressed in *E. coli* and purified to homogeneity as previously described[32]. Tetanus toxoid was obtained from RIVM (Bilthoven, The Netherlands). The synthetic myelin peptides MBP (84-102), MBP (143-168), MOG (1-22), MOG (34-56), MOG (64-86), MOG (74-96), PLP (41-58), PLP (184-199) and PLP (190-209) were synthesized and HPLC purified (>95% purity) by Severn Biotech Ltd (Worcester, UK). The amino acid sequences of the peptides are shown in Table 4.2.

**Table 4.2.** Amino acid sequence of synthetic myelin peptides

| | |
|---|---|
| MBP(84-102) | NPVVHFFKNIVTPRTPPPS |
| MBP(143-168) | GVDAQGTLSKIFKLGGRDSRSGSPMA |
| MOG(1-22) | GQFRVIGPRHPIRALVGDEVEL |
| MOG(34-56) | GMEVGWYRPPFSRVVHLYRNGKD |
| MOG(64-86) | EYRGRTELLKDAIGEGKVTLRIR |
| MOG(74-96) | DAIGEGKVTLRIRNVRFSDEGGF |
| PLP(41-58) | GTEKLIETYFSKNYQDYE |
| PLP(184-199) | QSIAFPSKTSASIGSL |
| PLP(190-209) | SKTSASIGSLCADARMYGVL |

**Table 4.1.** Patient characteristics

| Subject | Sex | Age | Disease type[a] | Duration | EDSS | HLA-DR2[b] | Medication[c] |
|---|---|---|---|---|---|---|---|
| MS1 | F | 51 | RR | 13 | 2.0 | - | IFN-β |
| MS2 | F | 28 | RR | 12 | 1.0 | + | IFN-β |
| MS3 | M | 48 | RR | 13 | 4.0 | - | IFN-β |
| MS4 | M | 35 | RR | 5 | 3.0 | - | IFN-β |
| MS5 | F | 26 | RR | 2 | 1.5 | - | no |
| MS6 | M | 47 | RR | 4 | 5.0 | + | no |
| MS7 | M | 36 | RR | 6 | 4.5 | - | no |
| MS8 | F | 36 | RR | 3 | 7.5 | - | IFN-β |
| MS9 | F | 46 | SP | 7 | 4.0 | - | IVIg |
| MS10 | F | 41 | RR | 11 | 3.0 | - | IFN-β |
| MS11 | M | 45 | SP | 16 | 9.0 | - | no |
| MS12 | M | 39 | SP | 3 | 7.0 | - | no |
| MS13 | F | 49 | SP | 17 | 8.0 | - | no |
| MS14 | F | 46 | RR | 17 | 4.0 | - | no |
| MS15 | F | 36 | RR | 5 | 3.0 | + | IFN-β |
| MS16 | F | 45 | RR | 4 | 2.0 | + | IFN-β |
| MS17 | F | 47 | CP | 12 | 6.5 | - | IVIg |
| MS18 | F | 44 | RR | 1 | 1.0 | - | no |
| MS19 | F | 40 | CP | 6 | 6.0 | - | no |
| MS20 | M | 36 | RR | 7 | 5.0 | + | no |
| MS21 | F | 39 | CP | 5 | 6.0 | + | no |
| MS22 | F | 59 | SP | 24 | 8.5 | + | no |
| MS23 | M | 34 | RR | 5 | 4.5 | + | no |
| MS24 | M | 32 | RR | 5 | 5.0 | - | no |
| MS25 | F | 60 | CP | 25 | 8.5 | - | no |
| MS26 | M | 33 | RR | 5 | 2.5 | - | no |
| MS27 | F | 54 | CP | 14 | 6.0 | - | no |
| MS28 | F | 38 | SP | 13 | 6.5 | + | no |
| MS29 | M | 51 | RR | 9 | 2.0 | + | no |
| MS30 | F | 36 | RR | 5 | 4.5 | + | no |
| MS31 | F | 50 | RR | 2 | 1.5 | - | no |
| MS32 | M | 58 | SP | 4 | 4.5 | - | no |
| MS33 | M | 52 | RR | 5 | 2.0 | - | no |
| MS34 | F | 44 | RR | 22 | 4.5 | - | no |
| MS35 | F | 48 | RR | 16 | 4.5 | + | IVIg |
| MS36 | F | 60 | RR | 8 | 2.5 | + | no |

[a] MS type at time of sampling: RR: relapse remitting; SP: secondary progressive; CP: chronic progressive.

[b] Patients were scored positive (+) or negative (-) for the HLA-DR2 haplotype, as determined by PCR.

[c] Medication during the last three months before sampling: IVIg: intravenous immunoglobulin G, IFN-β: interferon beta.

### 4.2.3 Stimulation of PBMC and enumeration of IFN-γ secreting T cells

PBMC were tested for their ability to secrete IFN-γ in response to MBP, rMOG, synthetic myelin peptides and control antigens in ELISPOT assays as described in 2.1.6. Optimal cell densities and concentrations for MOG and MBP were determined in pilot experiments. For the myelin peptides, the concentrations used are based on previous reports[28,33].

Th1 or Th2 biasing conditions were mimicked by adding 50 U/ml rIL-12 (PeproTech Ltd) or 20 U/ml rIL-4 (Sigma) respectively along with the stimulating agent.

To study the frequency of IL-2 receptor positive cells, PBMC were incubated with 2U/ml of rIL-2 (Roche Diagnostics, Brussels, Belgium) at $1x10^6$ cells/ml in a 12 well plate. After 24 h, cells were washed and assayed for antigen reactivity by IFN-γ ELISPOT.

### 4.2.4 Enumeration of IL-4 secreting T cells

For a subgroup of MS patients and healthy controls, IL-4 ELISPOT was performed in parallel as described in 2.1.6.

### 4.2.5 Lymphocyte proliferation assay

PBMC were tested for specific proliferation to rMOG and MBP in stimulation assays as stated in 2.1.3.

## 4.3 Results

### 4.3.1 Optimization of the ELISPOT technique

The optimal antigen concentrations for ELISPOT were determined by stimulating triplicate wells of $2x10^5$ cells with increasing concentrations of MBP (10, 25, 50 and 100 µg/ml), or rMOG (2, 5, 10, 20, 50 µg/ml). Antigen dose response curves are shown in Figure 4.1 A and B. Concentrations of 40 µg/ml for MBP and 10 µg/ml for rMOG were used for all subsequent experiments. These concentrations reside within the linear phase of the curve and are known to induce T cell proliferation as observed in pilot studies.

To define the optimal cell densities for the ELISPOT assay, an increasing number of PBMC (range 0.5 - 4 x $10^5$ cells/well) were stimulated with rMOG (10 µg/ml), MBP (40 µg/ml) and TT (as control antigen) (Figure 4.1C). An almost linear correlation between the number of PBMC plated and the number of IFN-γ secreting spots was found. Because the spots were easy to count while the background values (no antigen added) remained low, $2x10^5$ cells/well were used in all subsequent experiments.

Assaying fresh PBMC at the time of receipt is sometimes not feasible. Furthermore, it can be advantageous to confirm the results of an assay at a later date or assay multiple samples at the same time. Therefore, responses of cryopreserved and fresh PBMC were compared. PBMC of three healthy volunteers were first tested freshly. A fraction of the PBMC was cryopreserved in liquid nitrogen, thawed and assayed again after one week. As exemplified for one donor in Figure 4.1C, nearly identical results were obtained for MBP reactivity in frozen and fresh PBMC.

Finally, the inter-assay variability was tested by setting up two identical ELISPOT assays in parallel. Figure 4.1D shows small variations only in the number of MBP specific IFN-$\gamma^+$ spots indicating an acceptable reproducibility of the assay.

### 4.3.2 T cell reactivity to MBP and MOG

T cell reactivity to MBP and rMOG was tested in 33 MS patients (MS1-16, MS18-34, Table 4.1) and 9 MS patients (MS1-9) respectively, and in healthy control subjects (NS) by an IFN-γ ELISPOT assay. IFN-γ secreting MBP reactive T cells were detected in 31/33 MS patients and 28/28 healthy controls (Fig. 4.2A). The mean frequency of MBP reactive T cells is comparable for MS patients ($4.1x10^{-5}$) and NS ($4.4x10^{-5}$). MOG reactivity was detected in all MS patients (n=9) and healthy controls (n=10) tested.

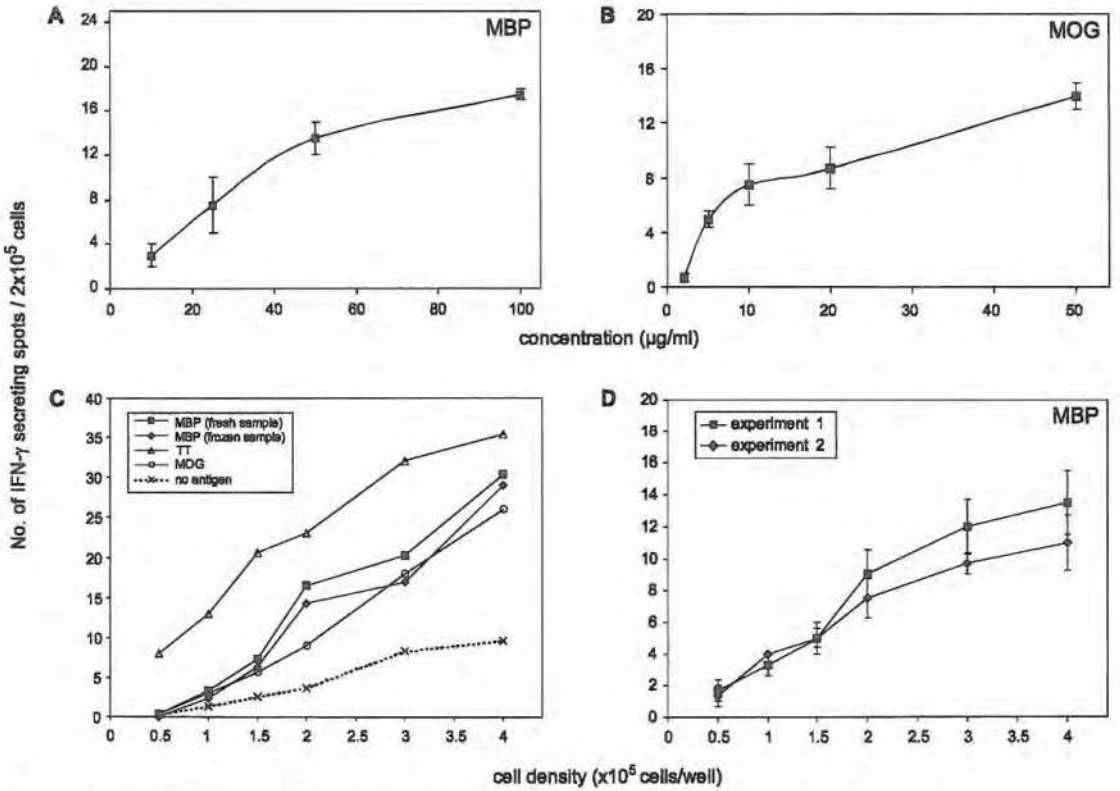


**Figure 4.1.** *Optimization of the ELISPOT technique*

A and B. Antigen dose response curves for MOG and MBP: an ELISPOT assay was set up with $2x10^5$ cells/well and increasing concentrations of MOG and MBP. Mean values and standard errors are shown for each concentration tested.

C. Reactivity was tested to MOG (10 µg/ml), MBP (40 µg/ml), TT (10 Lf/ml) or no antigen with increasing numbers of cell per well. MBP reactivity was tested both on freshly isolated PBMC and after thawing the same PBMC after cryopreservation.

D. To evaluate the inter-assay variability two identical ELISPOT assays were set up in parallel. PBMC were plated at different cell densities and tested for reactivity to MBP.

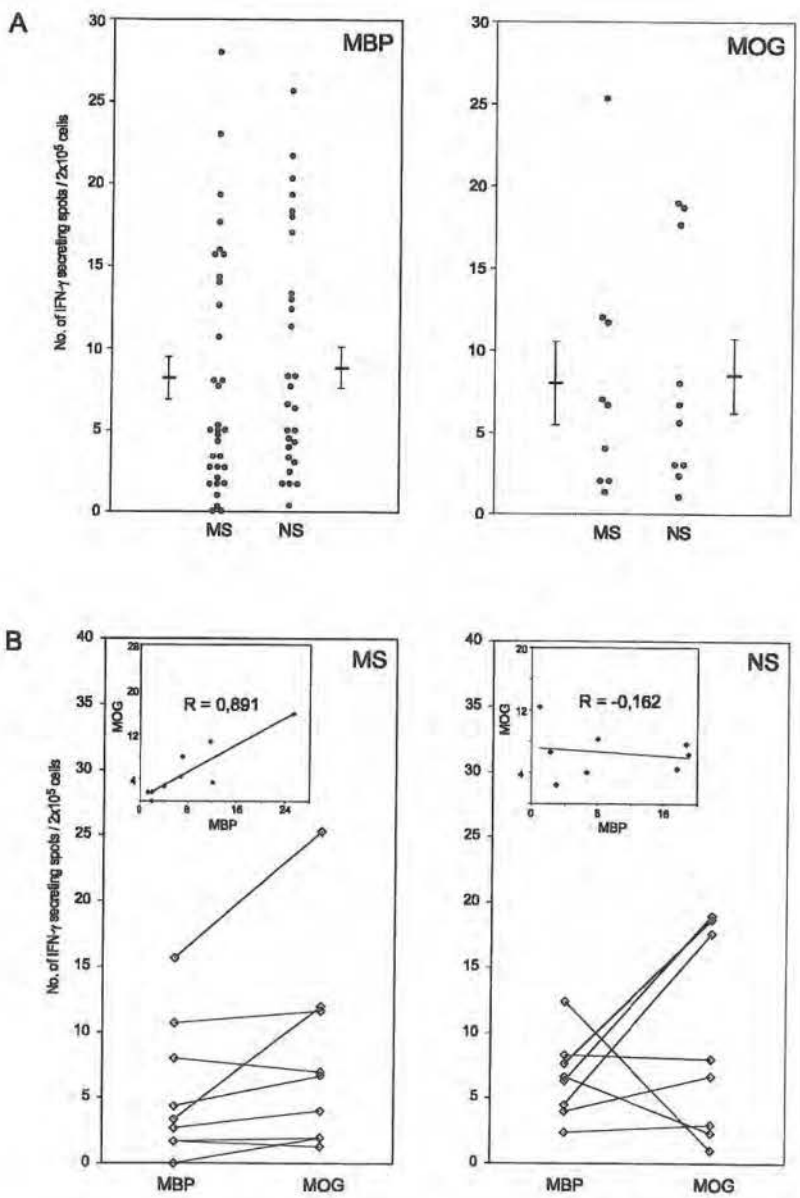


**Figure 4.2.** *T cell reactivity to MBP and MOG in MS patients and healthy subjects.*

A. Black dots represent specific numbers of IFN-γ secreting cells per $2x10^5$ for each subject. Specific numbers were obtained after subtraction of background values (from non-stimulated control wells). Negative values were arbitrarily set to zero. Mean and standard errors are represented by horizontal lines for each study group.

B. MBP and MOG reactivity in individual subjects. Paired values for each subject (NS, n= 9; MS, n= 9) are connected with a line. Graphics in the left-hand corner show the correlation between reactivity to MOG and MBP for both study groups. R stands for the correlation coefficient.

Similar mean frequencies for anti-MOG T cells were found for both groups studied (MS: $4.0x10^{-5}$, NS: $4.2x10^{-5}$). A positive correlation (r=0.891, p=0.0013) was found between the number of MOG and MBP reactive T cells in MS patients (Fig. 4.2B), but not in healthy controls (r=-0.162, p=0.752). No significant differences were found in MS subgroups (relapsing versus progressive), in the HLA-DR2$^+$ versus DR2$^-$ subgroups and in treated versus untreated patients.

We subsequently tested whether the anti-MOG and anti-MBP responses as determined by ELISPOT could also be identified in a bulk proliferation assay. To this end, bulk stimulation assays were set up in parallel with the ELISPOT assays with PBMC of 7 MS patients (MS1-7, Fig. 4.3). MOG T cell reactivity as measured by the proliferation assay was positively correlated with the MOG reactivity as measured by ELISPOT (r=0.834, p=0.019). This correlation was not observed for the MBP reactivity. While MBP reactivity was clearly detected by the ELISPOT technique, the bulk stimulations showed little MBP reactivity (r=-0.186, p=0.728).

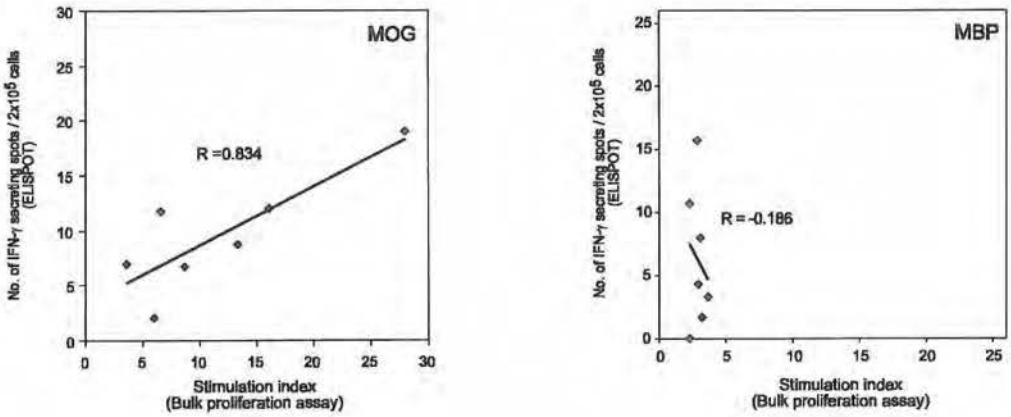


**Figure 4.3.** *Comparison of ELISPOT and proliferation assay.*

In parallel with the ELISPOT assay, bulk stimulations cultures were set up for 7 MS patients. Proliferation was measured using a classical $^3$H-thymidine uptake assay. Stimulation index is the ratio of incorporated cpm in antigen-stimulated wells to cpm in control wells (no antigen added). R stands for the correlation coefficient.

### 4.3.3 Reactivity towards MBP, MOG and PLP peptides

We then tested whether there was a difference in recognition of a set of potential immunodominant peptides of MBP, MOG and PLP (Table 4.2) in MS patients (n=16, MS1-16) and healthy controls (n=11). The selection of peptides was based on previous reports[3,23,34,35]. The tested MOG and PLP peptides span most of the extracellular domain of the antigens.

T cell reactivity against one or more of the MOG peptides was observed in 12/16 MS patients and 10/11 NS (Fig. 4.4). The four MOG peptides included in this study stimulated comparable numbers of T cells in the MS group, indicating that none of these peptides is immunodominant. A similar picture was seen in the NS group, although peptide MOG (64-86) was slightly more frequently recognized than the other peptides tested, but this difference was not statistically significant. When analyzing the MOG peptide-induced responses in individual cases, 4 MS patients showed reactivity to 1 MOG peptide only, while 8 MS patients showed reactivity to two or more MOG peptides. Reactivity to 1 MOG peptide was detected in 3 healthy subjects, while the remaining 7 controls recognized 2 or more MOG peptides.

T cell reactivity towards the MBP peptides 84-102 and 143-168, and PLP peptides 41-58, 184-199, 190-209 was slightly increased in the MS group as compared to the control group, but again the differences were not statistically significant (Fig. 4.4). The anti-MBP peptide 84-102 and 143-168 response represented a rather low fraction of the total MBP response in MS patients (no more than 20 %, data not shown). PLP peptide reactive T cells were detected in 4/8 NS and in 4/9 MS patients tested (MS1-9). The reactivity to the three PLP peptides was similar in the MS group. No differences were found in the myelin peptide reactivity pattern when comparing $DR2^+$ to $DR2^-$ MS patients, in the clinical subgroups (relapsing versus progressive) and in treated versus untreated patients (data not shown).

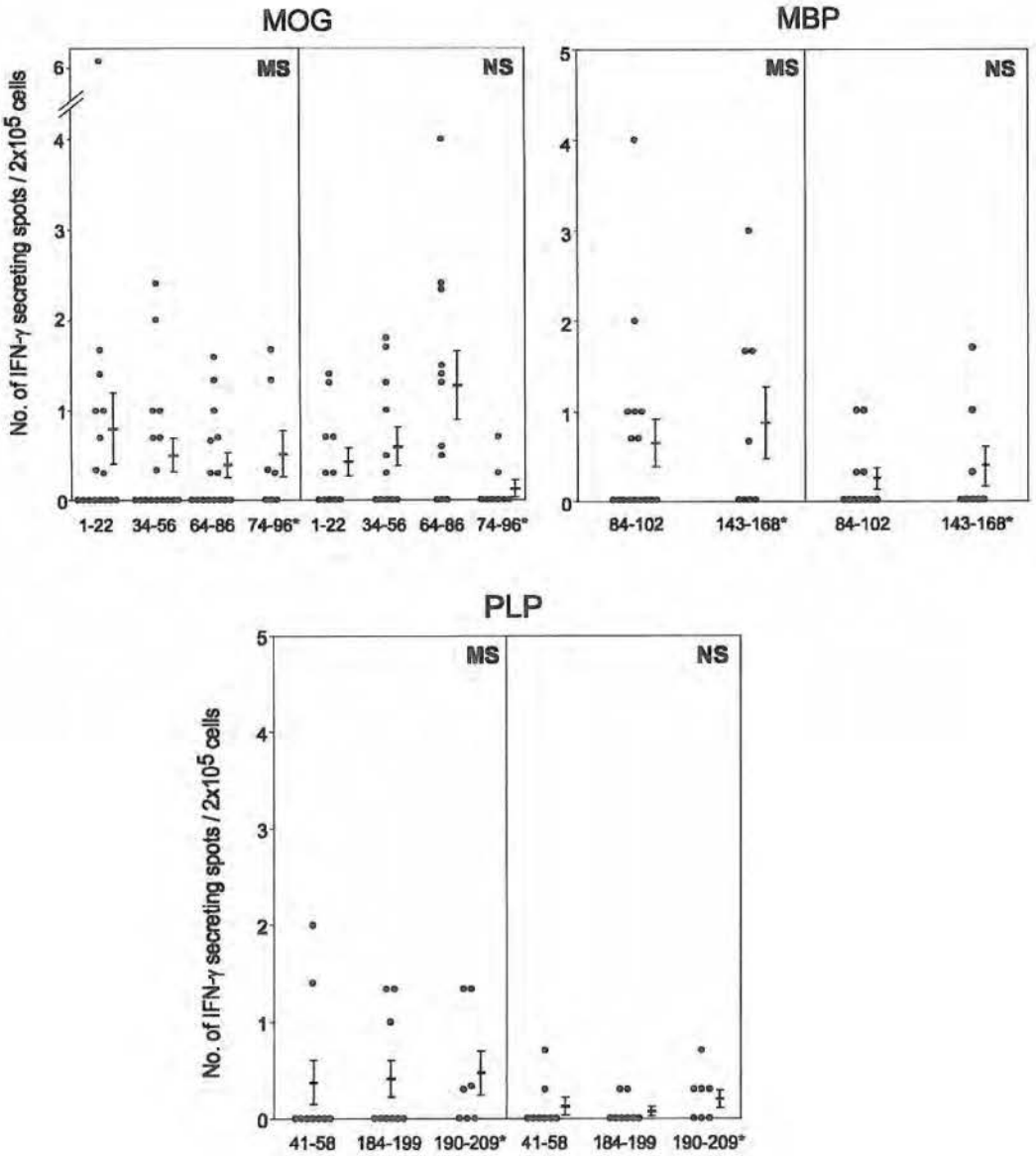


**Figure 4.4.** *T cell reactivity to potential immunodominant epitopes of MOG, MBP and PLP in MS patients and healthy subjects.*

Black dots represent specific numbers of IFN-γsecreting cells per $2x10^5$ cells for each subject. Negative values were arbitrarily set to zero. Mean and standard errors are shown with horizontal lines for each peptide. Asterisks indicate that reactivity to this peptide was not tested for all study subjects.

### 4.3.4 Frequency of IL-2 responsive MBP reactive T cells

To study the frequency of IL-2 responsive MBP reactive T cells by ELISPOT, PBMC were pre-incubated with a low amount of IL-2 (2U/ml) and subsequently analyzed for their MBP reactivity by IFN-γ ELISPOT. PBMC samples from 17 MS patients (MS18-34) and 17 healthy controls were tested before and 24h after an *in vitro* stimulation with rIL-2 (2U/ml). The culturing time was based on a pilot experiment showing a profound increase (20%) of $CD25^+$ T cells in the absence of NK cells ($CD16^+CD56^+$) as analyzed by flow-cytometry (data not shown). As previously shown, similar frequencies of MBP T cells were observed in both MS patients ($5.0x10^{-5}$) and NS ($5.9x10^{-5}$). After IL-2 stimulation, the mean frequency of MBP specific T cells was not altered in the MS patients ($4.9x10^{-5}$) (Fig. 4.5A). In contrast, in the healthy control group a significantly reduced number of MBP reactive T cells were found after IL-2 stimulation ($1.3x10^{-5}$). The frequency of MBP reactive T cells after primary IL-2 stimulation is significantly higher in MS patients as compared to healthy individuals ($p=0.0002$). Thus, while the frequency of MBP reactive T cells remained the same after IL-2 stimulation in the MS patients, a significant reduction was seen in control subjects. We then tested whether a similar observation was found for the control antigen tetanus toxoid. The frequency of tetanus toxoid reactive T cells decreased significantly after primary IL-2 stimulation in both MS patients (n=5, MS30-34) and healthy controls (n=4) (Fig. 4.5B).

### 4.3.5 T-helper profile of the anti-MBP response and susceptibility to alteration in Th1 / Th2 biasing conditions

To study the T-helper profile of the MBP reactive T cells, a comparison was made between the number of IFN-γ secreting (Th1 marker) and IL-4 secreting (Th2 marker) MBP reactive T cells. IL-4 and IFN-γ ELISPOT assays were set up in parallel for 9 MS patients (MS9-17) and 9 healthy control subjects. The anti-MBP T cells were predominantly Th1 cells since reactivity was observed in the IFN-γ, but not in the IL-4 ELISPOT assays (Fig. 4.6).

We then explored whether the cytokine secretion profile of the anti-MBP T cell response in MS patients (n=11; MS9-17, MS33-34) and NS (n=10) can be altered *in vitro* using Th1 or Th2 biasing conditions. Therefore, IL-12 or IL-4 was added along primary stimulation with MBP. As shown in Figure 4.7, the number of IFN-γ secreting MBP reactive T cells significantly increased when IL-12 was added, but decreased in the presence of IL-4 for

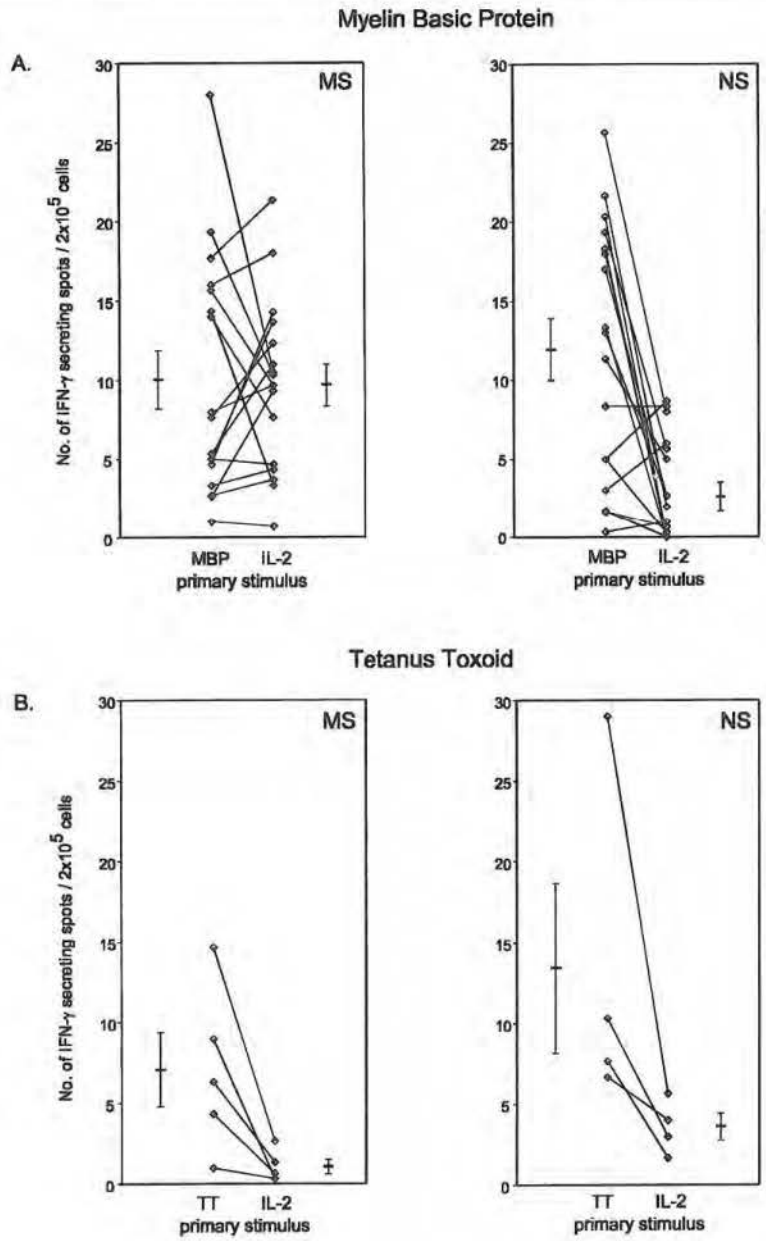


**Figure 4.5.** Frequency analysis of TT and MBP T cells after primary IL-2 stimulation.

PBMC samples of MS patients and NS were pre-incubated with IL-2 and analyzed for A. MBP reactivity (NS, n=17; MS n=17) or B. TT reactivity (NS, n=4; MS, n=5) by IFN-γELISPOT. For each individual subject, the numbers of IFN-γ secreting T cells observed before or after IL-2 stimulation are connected with a line (paired observations). The x-axis shows the primary stimuli. Negative values were arbitrarily set to zero. Mean values and standard errors are represented by horizontal lines.

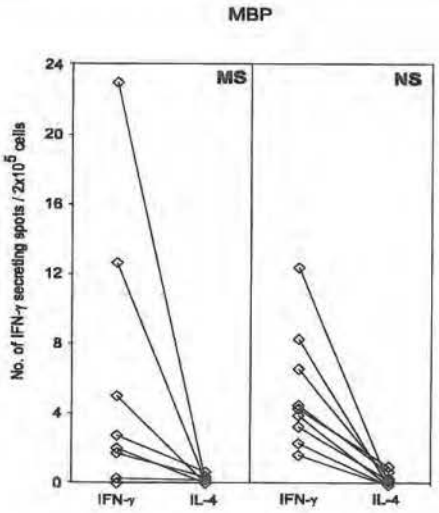


**Figure 4.6.** *T helper phenotype of the anti-MBP T cell response.*

Numbers of MBP reactive T cells detected in the IL-4 and IFN-γγELISPOT for each individual subject (NS, n=9; MS, n=9) are connected with a line (paired observations). Negative values were arbitrarily set to zero.

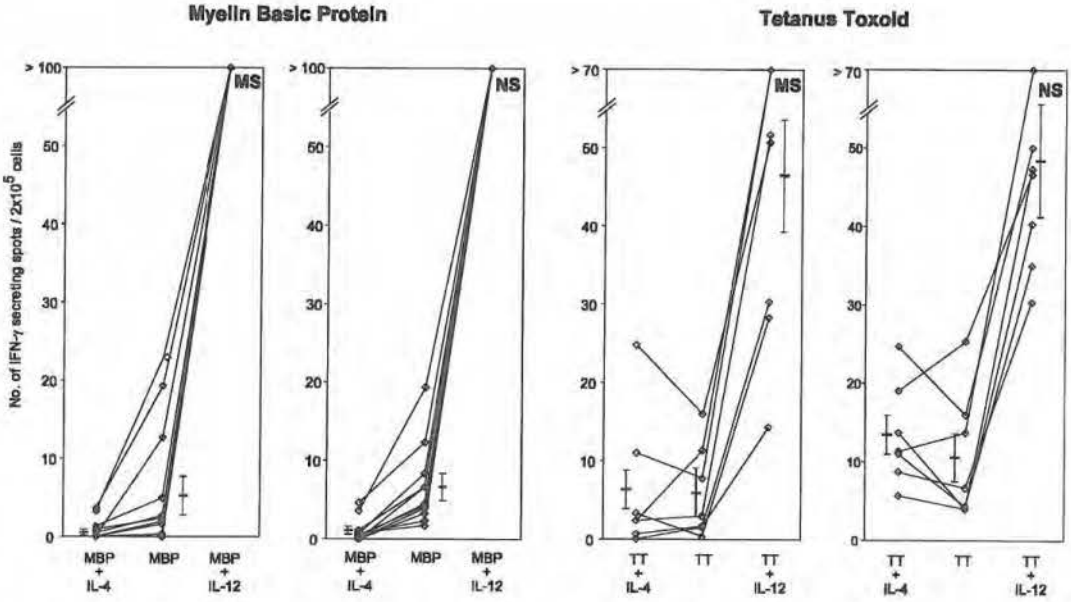


**Figure 4.7.** *T cell response in Th1/Th2 inducing conditions.*

To mimic Th1 or Th2 biasing conditions IL-4 or IL-12 was added together with the antigen (MBP/TT). Paired values for each subject are connected with a line. The number of spots in control wells stimulated with IL-4 or IL-12 in the absence of antigen was used as background signal.

both MS patients and NS. Little or no IFN-γ spots were detected in control wells containing PBMC in the presence of IL-4 or IL-12 alone. FACS analysis showed no alteration in the number of $CD16^+CD56^+$ NK cells when comparing IL-12 stimulated PBMC with non stimulated PBMC (data not shown), suggesting that the rise in IFN-γ secreting cells was not simply due to an increase in the number of IFN-γ secreting NK cells. To investigate if this *in vitro* alteration of cytokine secretion is specific for MBP, reactivity towards tetanus toxoid in Th1/Th2 conditions was tested in 7 MS patients (MS30-36) and 7 NS, using the same protocol. A significant rise in the number of IFN-γ secreting tetanus toxoid reactive cells was detected in the presence of IL-12. Adding IL-4 did not cause a decrease in the number of IFN-γ secreting tetanus toxoid reactive cells (Figure 4.7).

## 4.4 Discussion

Studies of T cell reactivity to myelin antigens have led to various and contradictory results. A possible explanation could be that anti-myelin reactivity differs among individual patients due to disease heterogeneity or differences in genetic background (HLA). Furthermore, recent studies indicate that while a single myelin antigen may trigger the onset of MS, the subsequent disease course is accompanied by reactivity to other myelin antigens[36]. This so-called "determinant spreading" is most likely a result from ongoing demyelination leading to the release of previously inaccessible myelin components.

To determine whether T cell responses in individual MS patients were directed at a few or a wide range of myelin epitopes T cell reactivities to MBP, MOG and MBP-, PLP, and MOG-peptides were studied by ELISPOT assay. This assay allows for a simultaneous analysis of T cell reactivities to a broad range of myelin antigens. Comparable numbers of MBP reactive T cells were found in MS patients and healthy controls, which is consistent with previous reports based on proliferation assays[2,3]. A Th1-type anti-MBP response was found both in MS patients and controls. The frequency of MOG reactive T cells was comparable in MS patients and control subjects. The level of the anti-MBP and anti-MOG T cell responses was similar in the two groups tested. No differences were found between subgroups in the MS population (relapsing versus progressive, HLA-DR2$^+$ versus DR2$^-$, treated versus untreated). Interestingly, a correlation was found between T cell reactivity to MOG and MBP in MS patients, but not in control subjects. Thus while in individual MS patients T cell responses were found to both MBP and MOG, healthy subjects recognized one of the myelin antigens. It is possible that the dual reactivity to MBP and MOG as observed in MS patients may represent the intermolecular spreading of an immune response that initially arose against a single antigen only. Another possibility could be that the initial autoimmune response in MS patients is already directed to several myelin antigens and that this T cell reactivity pattern persists along the disease progression. This second explanation is in line with the study of Söderström and co-workers who demonstrated that optic neuritis, a common first manifestation of multiple sclerosis, is characterized by a T cell repertoire similar to clinically definite MS[37].

Our data did not reveal differences in the frequency of myelin reactive T cells in blood of MS patients versus healthy subjects. It is known that only activated T cells are able to infiltrate the brain compartment and eventually mediate an autoimmune process in the CNS[38]. Therefore, the frequency of activated MBP reactive T cells was also analyzed in blood of MS patients and controls by ELISPOT. IL-2 receptors (IL-2R) are transiently expressed after T cell stimulation and are associated with T cell activation. Thus, *in vivo* activated T cells may therefore expand preferentially upon stimulation with IL-2. PBMC isolated from MS patients and healthy controls were pre-incubated with a low dose of IL-2 to specifically expand IL-2 receptor-bearing T lymphocytes. A higher fraction of IL-2 expanded T cells was MBP reactive in MS patients as compared to healthy controls. The increased frequency of MBP reactive T cells among the IL-2 expanded lymphocytes in MS patients versus controls was not observed for the control antigen tetanus toxoid. Our findings therefore suggest that MBP reactive but not TT reactive T cells are activated *in vivo* in MS patients but not in healthy subjects. This observation is in line with the findings of Zhang et al. (1994) and Chou et al. (1992) who provided evidence that MBP reactive T cells are activated *in vivo* in MS patients but not in control subjects[7,8]. Together, these data further support the view that MBP reactive T cells may be relevant to the disease process in MS patients.

Our observation of a comparable anti-MOG and anti-MBP T cell reactivity in MS patients seems to be in contrast with an earlier study in which proliferation assays were used to determine the MOG, MBP and PLP reactivity in MS patients and controls[22]. These authors observed proliferative MOG reactivity in 50% of the MS patients but not in healthy controls. In addition, only low MBP and PLP responses were found in MS patients and healthy subjects in that study, suggesting that MOG could be a primary target antigen in MS. These authors used standard short-term bulk proliferation assays that have a rather low sensitivity. We found that anti-MOG T cell reactivity as measured by bulk proliferation positively correlated with the ELISPOT results. However no such correlation was found for the MBP reactivity. While MBP reactivity was clearly detected by the ELISPOT technique, the parallel bulk stimulations showed a very low level of MBP reactivity only. It is not clear why no correlation was found for MBP between proliferative reactivity and ELISPOT reactivity. This may relate to a different state of activation of MBP reactive T cells in MS patients. Indeed, our data and previous studies demonstrate that MBP reactive T cells are activated *in vivo* in MS patients[8]. Activated T cells may undergo activation-induced apoptosis upon

stimulation with antigen leading to reduced proliferation[39]. Since antigen reactivity as analyzed by ELISPOT is not dependent on proliferation, these cells may still be scored by this method. Our findings therefore indicate that bulk proliferation assays may not be the best method to measure antigen specific T cell reactivity. In addition, our results also indicate that the precursor frequency of myelin reactive T cells in patients with MS as measured by limiting dilution analysis (LDA) may seriously underestimate the true frequency of myelin-reactive T cells. In contrast to frequencies of one in $10^5$ to $10^6$ as measured by LDA, we found frequencies of MBP reactive IFN-γ secreting cells as high as 1 in 8000. A recent study in which a direct *ex vivo* analysis was used to quantify MBP reactive T cells, shows that the actual frequency may even be as high as 1 in 300[27]. This indicates that the estimated frequencies may largely depend on the technique used to test T cell reactivity. Therefore, caution has to be taken when comparing T cell frequency data that were obtained using different approaches.

Our data did not reveal major differences in T cell reactivity towards the tested MOG-, PLP- and MBP-peptides in MS patients and healthy donors. None of the peptides tested was preferentially recognized in either one of the study groups. No significant differences were found in peptide-reactivity patterns when comparing $DR2^+$ and $DR2^-$ MS patients. Although the tested MBP 84-102 and 143-168 peptides and the PLP 41-58, 184-199 and 190-209 peptides were slightly more frequently recognized in MS patients, the differences with the control group were not significant. Interestingly, the two tested MBP peptides (84-102 and 143-168) that were found to be immunodominant in previous studies[2,3] represented no more than 20% of the anti-MBP reactivity in the subjects studied in our report. Our data are in line with previous reports showing rather heterogeneous anti-MBP responses in MS patients and controls[33,40]. Although the majority of the MS patients (75%) responded to at least one MOG peptide, none of the tested MOG peptides appeared to be immunodominant in this group. Approximately 70% of the MOG responsive subjects showed reactivity to two or more MOG peptides. In contrast, only 44% of the MS patients responded to any of the PLP peptides. In general, our study did not reveal any dominant peptide reactivity in the myelin antigens tested.

Some of our observations are potentially relevant for the application of experimental antigen specific immunotherapies for MS. We observed a correlation between the anti-MOG

and anti-MBP T cell response in individual MS patients, indicating that antigen directed therapies might need to be targeted to different myelin antigens. The heterogeneous myelin peptide reactivity observed in this study may limit the applicability of peptide-specific immunotherapeutic approaches. These approaches may however still become successful when they induce bystander suppression in the CNS by shifting to a Th2-type response. To investigate whether MBP reactive T cells from MS patients are committed in their differentiation pathway to a stable cytokine phenotype or whether the cytokine secretion could still be altered, T cells were stimulated in the presence of Th1 (IL-12) or Th2 (IL-4) promoting conditions and tested for their MBP reactivity by IFN-γ ELISPOT. Addition of IL-12 significantly increased the number of MBP reactive IFN-γ secreting cells, while IL-4 caused a reduction in IFN-γ secreting MBP reactive T cells. In contrast, IL-4 could not inhibit IFN-γ secreting T cells specific for tetanus toxoid (TT), indicating that T cell responses to TT are more polarized to a Th1 phenotype. Unexpectedly, in the presence of IL-12 a much higher frequency of IFN-γ producing MBP and TT reactive T cells was found. It could be argued that these observations are due to an increased frequency of IFN-γ producing NK cells. However, little or no IFN-γ spots were detected in control wells containing PBMC in the presence of IL-12 alone and FACS analysis showed no alteration in the number of $CD16^{+}CD56^{+}$ NK cells when comparing IL-12 stimulated PBMC with non-stimulated PBMC. Manetti et al. (1994) showed that IL-12 promotes the generation of Th1 T cell responses *in vitro* and is even capable of inducing transient IFN-γ secretion by Th2 T cell clones[41]. Further studies are necessary to resolve whether the observed IFN-γ secreting cells after IL-12 incubation are truly antigen specific. Our data indicate that IFN-γ producing MBP reactive T cells of MS patients are still susceptible to Th2 biasing conditions. These findings are consistent with a study of Windhagen and co-workers who demonstrated that the cytokine conditions in which MBP reactive T cell lines are generated *in vitro* strongly influence the secretion of IFN-γγ even in cases of longstanding autoimmune disease[42].

In conclusion, using ELISPOT assays to analyze the frequency of IFN-γ producing myelin reactive T cells, we could not detect any quantitative differences between MS patients and healthy subjects. However, an increased frequency of IL-2 responsive MBP reactive T cells was found in the blood of MS patients, indicating a functional difference in activation

status of MBP reactive T cells in MS. Our data lend further support to the view that these cells are relevant to the disease process. Our findings provide further information about the anti-myelin reactivity and its role in the disease process of MS.

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# Chapter 5

## Longitudinal Study of T Cell Related Parameters and Correlation with Disease Activity in Relapsing-Remitting MS

Based on:

**Anti-Myelin T Cell Reactivity Correlates with Brain Inflammatory Activity in Relapsing-Remitting Multiple Sclerosis: A Longitudinal Study**

Niels Hellings, Geert Gelin, Robert Medaer, Yvan Palmers, Jef Raus and Piet Stinissen

Submitted for publication

## Abstract

*Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), and is widely believed to be an autoimmune disease that results from aberrant immune responses to CNS antigens. T cells are considered to be crucial in orchestrating an immunopathological cascade that culminates in damage of the myelin sheath. This study was aimed to analyze whether clinical disease activity or brain inflammatory activity as measured by magnetic resonance imaging (MRI) was associated with changes in autoreactive T cell reactivities in MS patients. To this end, we performed a longitudinal study in which T cell immune parameters and clinical parameters (including MRI) were monitored in seven relapsing-remitting (RR) MS patients and two healthy controls at bimonthly intervals over a period of 18 months.*

*Changes in several T cell related immune variables were found to coincide with MRI activity and preceded clinical relapses. These alterations include: increased myelin-reactive IFN-γ secreting T cells as measured by ELISPOT, detection of clonally expanded myelin reactive T cells, elevated pro-inflammatory and decreased anti-inflammatory cytokine production, upregulation of ICAM-1 membrane expression and increased serum levels of soluble VCAM-1. Some of the observed immune alterations were also detected in healthy controls, indicating that additional regulatory mechanisms - which may be defective in MS - play a role in the down-regulation of potentially pathological T cell responses. The serial evaluation of anti-myelin T cell responses revealed highly dynamic shifts and fluctuations from one autoreactive pattern to another in a patient-specific manner.*

*In conclusion, this study provides further support for an important role of myelin reactive T cells in the pathogenesis of MS. The observed dynamic changes in the anti-myelin T cell reactivity pattern may be a major obstacle for the development of antigen-specific immunotherapies. In addition, our data indicate that some immune markers may be helpful in predicting clinical exacerbations in MS patients. The analysis of a combination of these immune markers may provide more accurate information on disease activity in MS.*


*Keywords: Multiple Sclerosis, myelin reactive T cells, myelin antigens, cytokines, adhesion molecules, ELISPOT, MRI*

## 5.1 Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) of presumed autoimmune origin. There is evidence that autoimmune T cells recognize components of the myelin sheath and trigger a cascade of events leading to demyelination[1]. The potential pathogenic role of myelin-reactive T cells in MS is largely based on studies in experimental autoimmune encephalomyelitis (EAE), the animal model of MS[2]. However, several important questions related to the role of autoreactive T cells in the disease process of MS remain unanswered. For instance, it is not known which components within the CNS myelin are the major targets of the pathological autoimmune response. Some of the candidate autoantigens include myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte protein (MOG)[1]. Moreover, it remains unclear whether anti-myelin T-cell responses are stable or change over time as a result of determinant spreading or shifting[3]. Also, it is not clear whether meaningful functional differences exist between myelin reactive T cells of MS patients and healthy controls. MBP reactive T cells have been shown to be activated *in vivo* in blood of patients with MS[4,5]. However, it remains unknown whether these activated T cells are actually involved in inducing clinical exacerbation. Other T cell related events including production of inflammatory cytokines and expression of adhesion molecules have also been indicated to contribute to the disease pathogenesis[6]. One strategy to correlate T cell related immune events with clinical disease activity, and thus to implicate their direct role in the disease process, is to look for possible correlations between this immune event and disease activity in a longitudinal study.

New treatments including interferon-beta and copolymer-1 were recently approved for MS[7,8]. There is however still a need for more effective therapies. Clinical studies in MS have been hampered by the lack of a paraclinical or laboratory marker of disease activity. Such surrogate markers of disease activity would be extremely helpful to study the efficacy of new treatments[9]. Although brain lesions as identified by magnetic resonance imaging (MRI) are now used as standard outcome measures in clinical studies of MS, it is known that brain MRI activity does not always correlate well with clinical disease progression[10]. In addition, the correlation between disease activity and immune related parameters may provide further clues about the autoimmune pathogenesis of the disease.

Previous reports indicated that some immune parameters may potentially be related to disease activity. A number of cytokines were found to be increased in different clinical stages of relapsing-remitting MS (RR MS). Increased levels of the pro-inflammatory cytokines IFN-γ and TNF-α were correlated with clinical exacerbations, whereas elevated levels of the anti-inflammatory cytokines IL-10 and TGF-β were found to be related to remissions[11]. The expression of various adhesion molecules and the levels of circulating soluble adhesion molecules are indicators for cell trafficking and blood brain barrier abnormalities and may correlate with clinical and MRI activity[12,13]. In addition, some other biological markers were proposed to be of potential use for predicting disease activity in RR MS, including markers of cellular activation, matrix-metalloproteinases and T lymphocyte calcium fluxes[14,15]. Although myelin reactive T cells are considered to play an important role in the pathogenesis of MS, few studies only addressed the question whether anti-myelin T cell responses may correlate with disease activity[1]. While one study found a correlation between anti-myelin T cells responses and disease activity, another group did not find a correlation with clinical variables [16,17].

Studies designed to correlate disease activity with a laboratory parameter have often led to conflicting results. Several explanations could account for these discrepancies. First, it may be possible that different pathological changes occur in the clinical subtypes of MS: relapsing-remitting (RR), primary and secondary progressive disease. In addition, many of these studies analyzed a single biological marker only. Several studies were based on single measurements in cross-sectional studies but not on longitudinal studies. Since immune markers may significantly fluctuate over time in individual patients, single measurements would lead to a one-dimensional view, limiting the success of finding a reliable disease marker.

We performed a longitudinal analysis of T cell related parameters to provide further information about the role of T cells in the MS pathogenesis. Several immunologic and clinical parameters were monitored in 7 RR MS patients and 2 healthy controls for a period of 18 months. The patients were examined clinically (EDSS, number of relapses) every 2 months. To determine the number of T1 and T2 weighted and gadolinium enhancing lesions, MRI scans were taken every 4 months. Serial analyses were performed to follow-up the myelin-specificity, phenotype and cytokine profile of T cell responses in the blood of the

patients and control subjects. T cell reactivity to several myelin antigens was determined by IFN-γ ELISPOT assays to test whether the anti-myelin reactivity is stable over time in a given patient or may shift to other antigens or epitopes due to determinant or epitope spreading. The cytokine production of *in vitro* stimulated PBMC further provided information on the Th-subtype of the immune responses. Every six months, MBP and PLP reactive T cells were isolated and characterized for their TCR V gene expression to determine if clonally expanded or persisting myelin reactive T cell clones are present in the blood of MS patients. Serum levels of soluble adhesion molecules were measured in ELISA assays. The immunological, clinical and MRI parameters were correlated to provide further information about the role of anti-myelin T cell responses in MS, and to identify potential paraclinical disease markers that can be used for diagnostic or prognostic purposes.

## 5.2 Materials and methods

### 5.2.1 Patients and healthy controls

Three female and four male patients with relapsing-remitting MS (RR-MS) were included in the present study (Table 5.1). The age of the patients ranged from 26 to 51, with a mean age of 38.5 years. The mean EDSS was 3.0 (range: 1 to 5) and the mean disease duration was 8 years (range 2 to 13 years). Four out of seven patients had at least one relapse along the study period. Two of these patients (MS6, MS7) received short courses of i.v. methylprednisolone at the time of exacerbation. All but one patient were treated with IFN-β-1a (Rebif®) during the study. In addition, 2 healthy volunteers (NS) were enrolled in this study (Table 5.1). 2/7 MS patients and none of the NS were HLA-DR2$^+$, as determined by PCR (2.2.3). Blood was collected from the subjects at two-month intervals for a total period of 18 months. Informed consent was obtained from all subjects volunteering for this study.

**Table 5.1.** Study subject characteristics

| Subject | Sex (F/M) | Age | Disease type[a] | Disease duration | EDSS at entry | Relapses | DR2[b] | Medication[c] |
|---|---|---|---|---|---|---|---|---|
| MS1 | F | 51 | RR | 13 | 2.0 | 0 | - | IFN-β |
| MS2 | F | 28 | RR | 12 | 1.0 | 0 | + | IFN-β |
| MS3 | M | 47 | RR | 13 | 4.0 | 1 | - | IFN-β |
| MS4 | M | 35 | RR | 5 | 3.0 | 0 | - | IFN-β |
| MS5 | F | 26 | RR | 2 | 1.5 | 1 | - | IFN-β[d] |
| MS6 | M | 47 | RR | 4 | 5.0 | 2 | + | IFN-β[e]/ IVMP |
| MS7 | M | 36 | RR | 6 | 4.5 | 1 | - | IVMP |
| NS1 | F | 38 | N.A. | N.A. | N.A. | N.A. | - | N.A. |
| NS2 | F | 39 | N.A. | N.A. | N.A. | N.A. | - | N.A. |

[a] MS type: RR: relapsing-remitting form of MS.

[b] Subjects were scored positive (+) or negative (-) for the HLA-DR2 haplotype, as determined by PCR.

[c] Medication: IFN-β: interferon beta-1a (Rebif®); Treatment for these patients started at month 5[d] and 7[e]; IVMP: intravenous methylprednisolon (at time of relapse); N.A.: not applicable.

### 5.2.2 Clinical examinations and MRI scans

To determine the EDSS score and relapse rate, bimonthly neurological examinations were performed. Brain MRI were obtained every four months with a 1.0 Tesla Siemens Magnetom (Erlangen, Germany). Proton density (Pd) and T2-weighted (T2w) images were obtained using a dual-echo spin-echo sequence (TR: 2500 ms, TE: 20/80 ms, one acquisition). The second set of images was obtained before and after administration of gadolinium (Gd) by applying a T1-weighted (T1w) spin-echo sequence (TR: 572 ms, TE: 12 ms, 2 acquisitions). For each sequence, contiguous 5-mm thick axial slices were acquired, with a 25 mm field of view and a 250 x 250 image matrix. The total number of T1w and T2w lesions and the number of Gd enhanced T1 lesions were counted. T1w Gd enhanced lesions and new or enlarging T2w lesions were considered to be active lesions. Lesions that appeared on T2w scans and were Gd enhanced on T1 were counted only once as an active lesion.

### 5.2.3 Cell culture media and antigens

Cell culture media and antigens used in this chapter are described in 4.2.2.

### 5.2.4 Flow-cytometry

Expression of cell surface proteins was assayed as described in 2.1.5.

### 5.2.5 IFN-γ ELISPOT assay

Freshly isolated PBMC were tested for their ability to secrete IFN-γ in response to MBP, MOG, PLP and MBP-, PLP- and MOG-peptides in ELISPOT assays as described in 2.1.6.

### 5.2.6 Generation of myelin reactive T cell clones and analysis of TCR expression

MBP and PLP specific T cell clones were isolated as indicated in 2.1.1. A fraction of each myelin reactive T cell clone was pelleted and frozen for subsequent analysis of TCR expression (as described in 2.2.2).

### 5.2.7 Lymphocyte stimulation assay

Triplicate aliquots of $10^5$ PBMC were stimulated for 5 days with different stimuli (MBP, PLP rMOG, and PHA). At day 4, supernatant was collected and frozen for cytokine analysis.

Proliferation to the different antigens was measured using a classical $^{3}$H-Thy incorporation assay (2.1.3).

### 5.2.8 Quantification of cytokines and soluble marker molecules by ELISA

The cytokine production in supernatants of antigen-stimulated PBMC was measured as stated in 2.1.7. Serum levels of the soluble forms of intercellular adhesion molecule-1 (ICAM-1/CD54) and vascular intercellular adhesion molecule-1 (VCAM-1/CD106) were measured as described in 2.1.7.

## 5.3 Results

### 5.3.1 Clinical and MRI measure changes

Patients were monitored for changes in EDSS and exacerbation rate every 2 months, and studied by MRI (T1, Gd enhanced, T2, Pd) every 4 months (Figure 5.1). Three patients were clinically stable with no exacerbations, three patients had one relapse and one patient had two relapses during the study period (18 months). In 3/4 patients (MS3, 5, 6) with a relapse, active MRI lesions were detected prior to the clinical exacerbation, while in patient MS6 no increased MRI activity or active lesions were observed before the two relapses. On the other hand, patients MS2 and MS4 experienced no disease worsening although active lesions were observed.

Serial MRI analysis has the potential of monitoring lesion formation and evolution *in vivo*. It is known that the majority of new lesions (80%) show a similar pattern of evolution, in which new T2-weighted signal abnormalities are accompanied by focal Gd enhancement on T1 scans[18]. This is illustrated in Figure 5.2 for patient MS5, where a newly appearing lesion on T2w and Pdw images simultaneously showed Gd enhancement on T1. Consecutive scans demonstrated that the lesion reduced in size and stayed detectable on T2, while Gd enhancement diminished and subsequently disappeared. This particular lesion also persisted as unenhanced hypointensity on T1 scans.

### 5.3.2 Phenotyping of peripheral blood mononuclear cells (PBMC)

PBMC were phenotypically characterized to investigate if changes in lymphocyte subsets are indicative for disease activity in patients with MS. Figure 5.3A shows the typical relative changes in phenotypic expression of blood cells for one MS patient and one healthy subject (NS). No significant alterations were observed during the study period for most of the cell subsets tested: T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($CD16+56^+$), helper and cytotoxic T cells ($CD4^+/CD8^+$), TCR $\alpha\beta$ and $\gamma\delta$ bearing T cells, naive and memory T cells ($CD45RA^+/CD45RO^+$) and $HLA\text{-}DR^+$ T cells. However, relatively large fluctuations of ICAM-1 (CD54) expression on T cells were detected in all of the subjects (Figure 5.3B). Interestingly, in all patients with a relapse a consistent decrease in CD54 expressing T cells was detected when comparing the time point prior to relapse with the time point nearest to the exacerbation ($p<0.01$) (Figure 5.3B). In all of the cases, this decrease was preceded by a gradual increase of $CD54^+$ T cells.

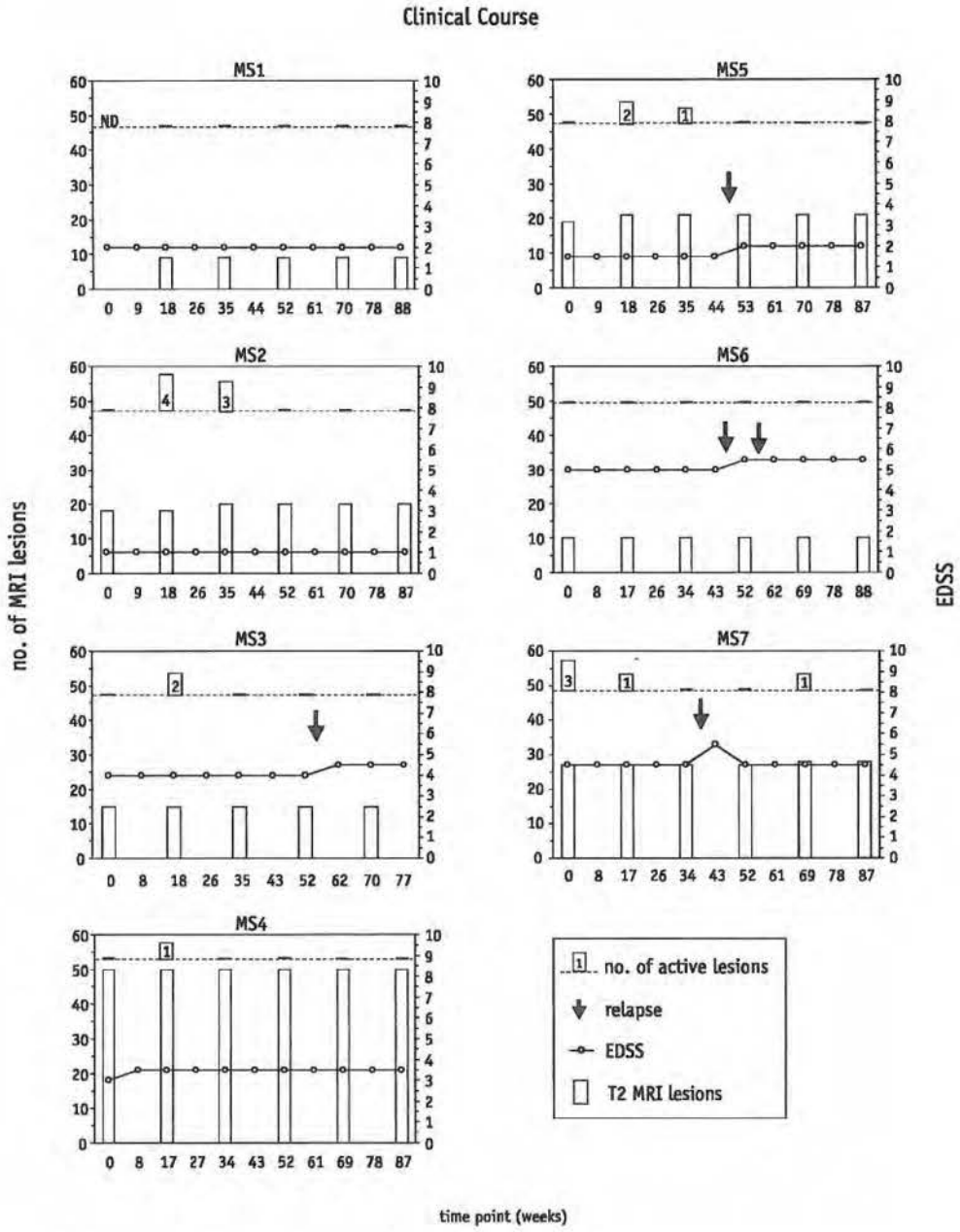


**Figure 5.1.** *EDSS, clinical exacerbations and active MRI lesions.*

The EDSS was determined for all 7 MS patients at bimonthly visits. MRI scans (T1, Gd-enhanced, T2, Pd) were performed at 4 month intervals. Open bars represent the total number of T2w lesions. Active lesions are depicted at the top of each graph. A lesion was designated to be 'active' when it was new or enlarging on T2 scan or Gd enhanced on T1 scan. ND: not determined; EDSS: Expanded Disability Status Scale.

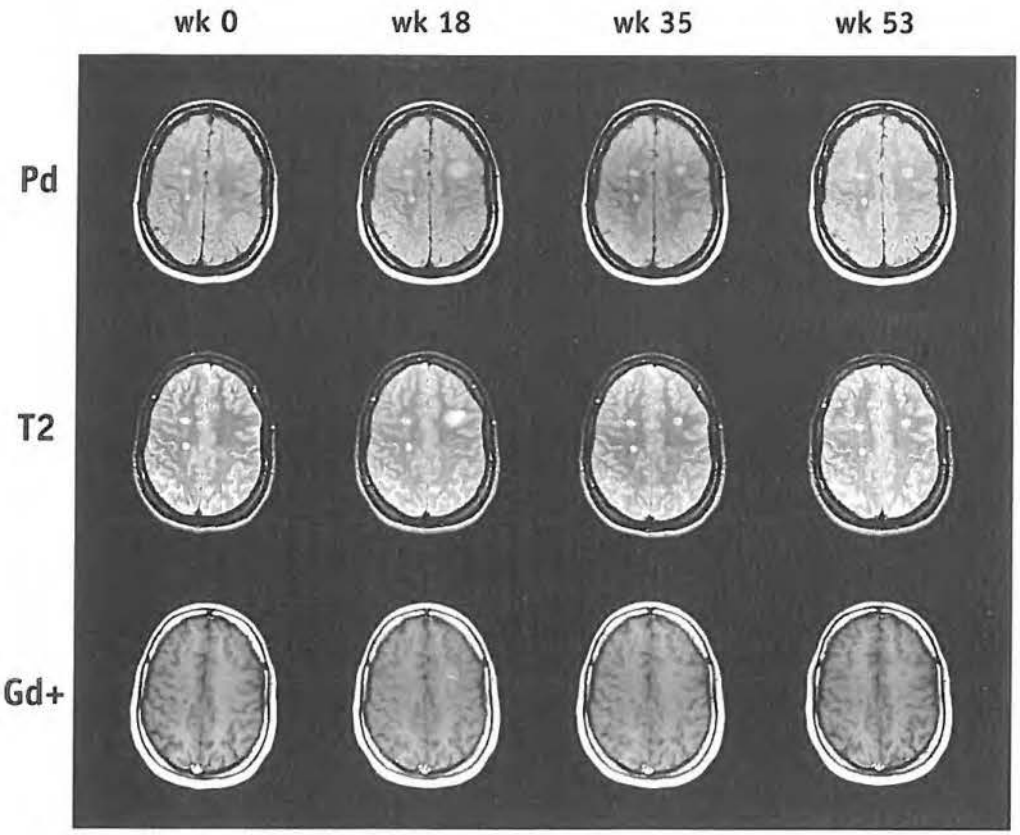


**Figure 5.2.** *MR images of a MS patient (MS5) at four different time points*

The bottom row demonstrates a section at the supraventricular level on gadolinium enhanced (Gd-enhanced) T1-weighted images. A large ring-enhancing lesion, which was not present at study entry (week 0), is apparent at week 18. Pd and T2 images taken at the same anatomical level demonstrate the appearance and shrinkage of this lesion, which remains visible on subsequent Pd and T2w images. Gd-enhancement decreased at week 35 and disappeared at week 53.

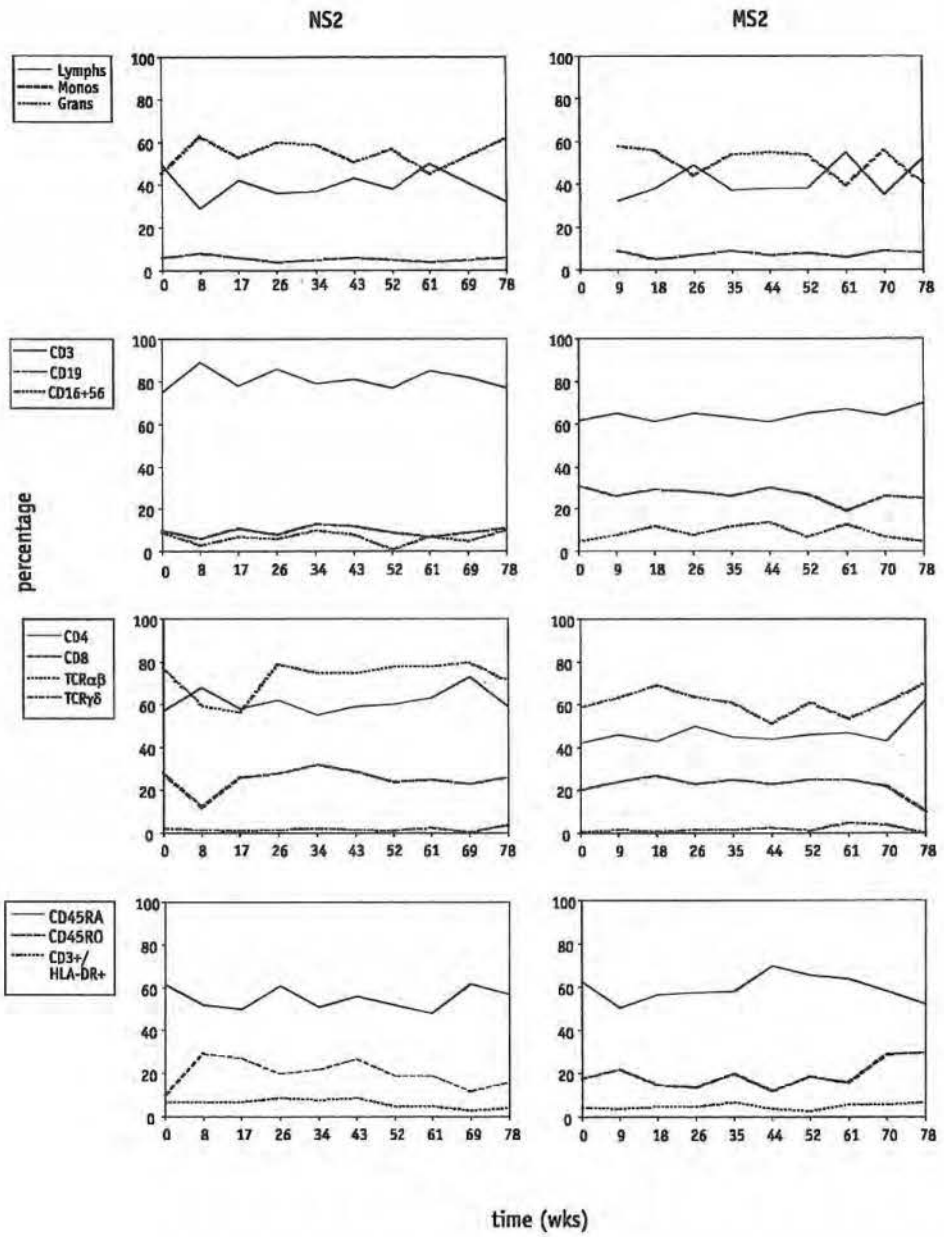


**Figure 5.3.** *Phenotypic expression of peripheral blood mononuclear cells.*

A. Phenotypic profile of PBMC from an MS patient and a healthy subject (NS).
The top drawings show the general distribution of monocytes, lymphocytes and granulocytes as analyzed by leucoGATE staining (CD14/45). The other graphs show the percentage in the lymphocyte gate of total T cells (CD3), B cells (CD19), T helper cells (CD4), cytotoxic T cells (CD8), NK cells (CD16+56), TCR $\alpha\beta^+$ and $\gamma\delta^+$ T cells, naive and memory T cells (CD45RA/RO) and activated T cells (CD3/HLA-DR). Lymphs: lymphocytes; Monos: monocytes; Grans: granulocytes.

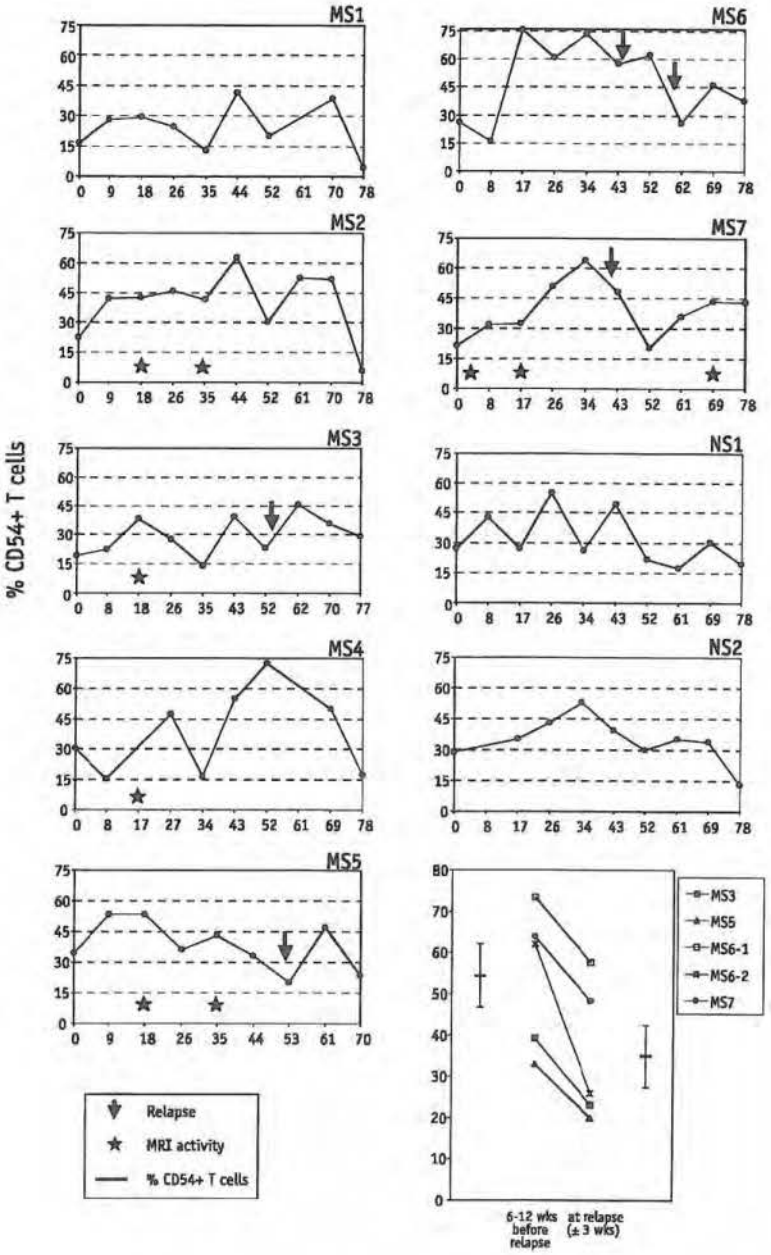


**Figure 5.3. (continued)** *Phenotypic expression of peripheral blood mononuclear cells.*

B. Expression of ICAM-1 (CD54) by circulating T cells
The percentage of $CD54^+$ T cells was calculated by correcting the percentage of $CD54^+$ $CD3^+$ cells with the total percentage of $CD3^+$ cells (x100). Arrows represent clinical relapses and stars depict the presence of active MRI lesions. The bottom-right graphic shows the percentage of ICAM-$1^+$ T cells prior to and at the time of a clinical relapse. Horizontal lines represent mean and standard errors. Mean values prior to relapse and at relapse are significantly different (t-test, $p<0.01$).

It should be noted however that some patients (e.g. MS4) show similar patterns of ICAM-1 expression changes without the consequence of a clinical relapse.

### 5.3.3 T cell reactivity to MBP, PLP and MOG

To study possible correlations with the disease course and MRI activity, T cell reactivity to MBP, PLP and MOG was serially analyzed by IFN-γ ELISPOT. Fluctuating reactivities to MBP, PLP and MOG were observed in almost all MS patients but also in healthy controls (Figure 5.4). In MS patients with a relapse along the study, anti-myelin T cell responses followed distinct patterns. For example, patient MS7 initially showed reactivity to MBP and MOG (at the time of MRI activity) but not to PLP. After the relapse, the anti-MBP and anti-MOG reactivity declined while an increased reactivity to PLP was detected (with new MRI activity). A similar shifting in T cell reactivity from MBP and MOG to PLP coinciding with a clinical relapse (but not with new MRI activity) was observed in patient MS3. In contrast, MS6 who experienced two attacks showed a broadening of the T cell reactivity from initial responses to MBP and MOG only to reactivity to all three myelin antigens. A persistent response to MBP and MOG with fluctuations over time without significant anti-PLP reactivity was seen in patient MS5. Patients MS2 and MS4 had MRI activity but no relapses during the study period. Interestingly, these patients had an increased anti-MBP and anti-MOG response at the time of MRI activity, but little anti-PLP reactivity. Note that fluctuating and relatively high anti-MBP and anti-MOG responses but little anti-PLP reactivity was also found in the healthy control subjects.

In conclusion, anti-myelin reactivities were found in most patients and healthy controls. These reactivities fluctuated in a patient-specific and dynamic manner in most patients. In some patients increased MRI activity (active lesions) correlated with an increased reactivity to one or more myelin antigens.

### 5.3.4 T cell reactivity to myelin peptides

We then tested whether clinical relapses were associated with possible shifting of the epitope reactivities of anti-myelin T cells in the MS patients. To this end, the recognition pattern of a set of potential immunodominant peptides of MBP, MOG and PLP were analyzed by ELISPOT at three time points: (i) before the relapse; (ii) at sampling closest to the relapse; (iii) after the relapse (at remission).

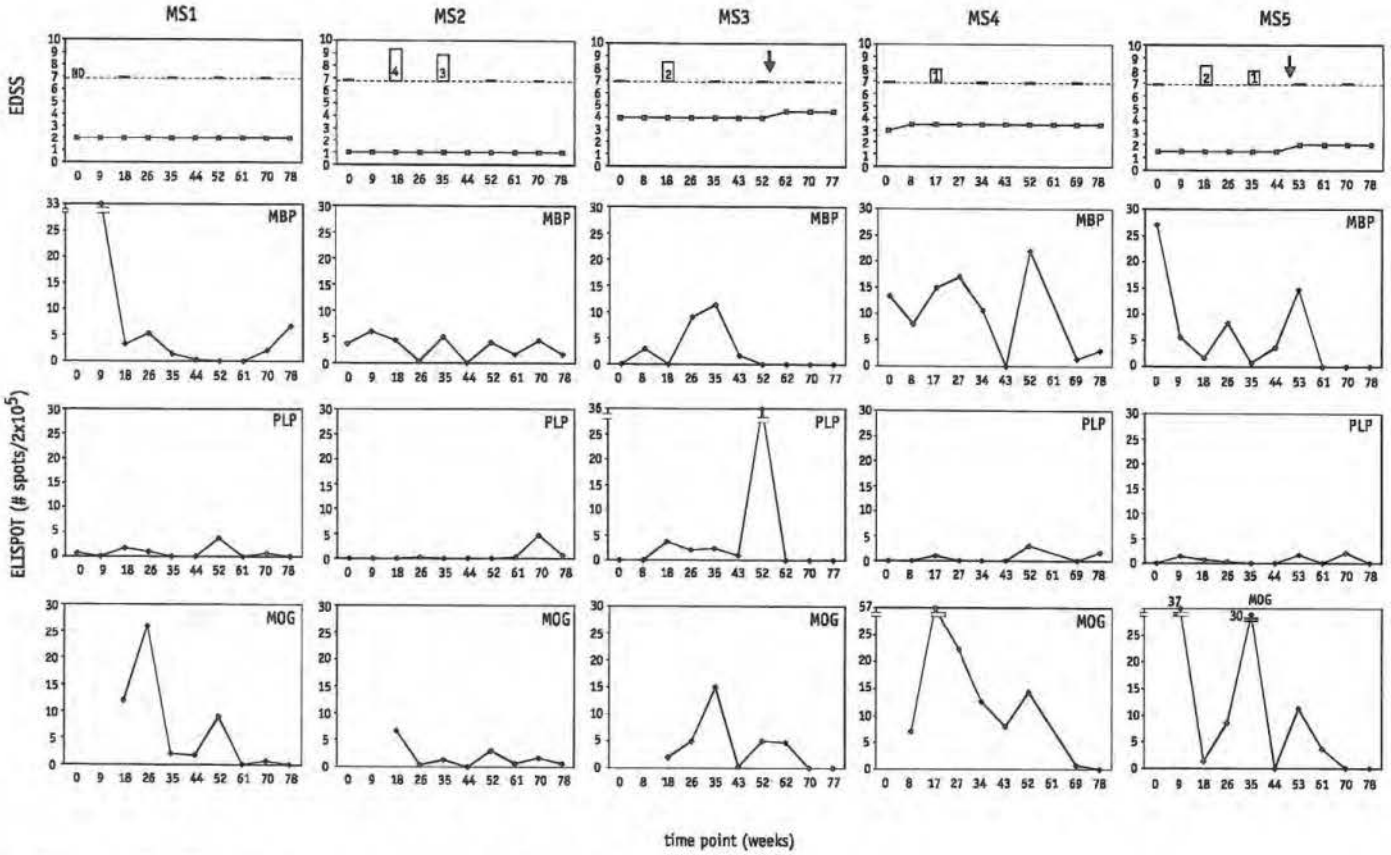


**Figure 5.4.** *T cell reactivity to MBP, PLP and MOG as determined by ELISPOT*

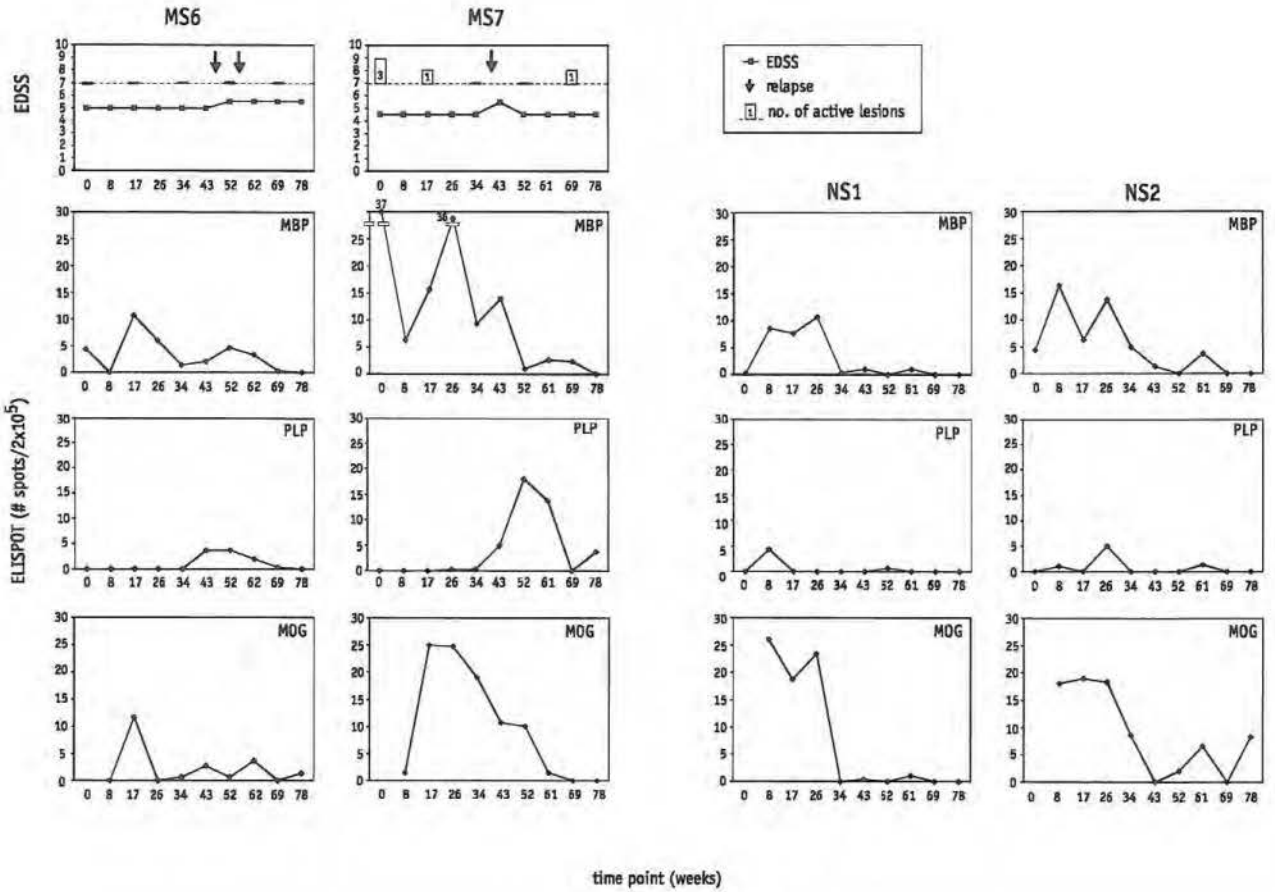


**Figure 5.4 (continued):** *T cell reactivity to MBP, PLP and MOG as determined by ELISPOT*
At each blood drawing PBMC were tested for their reactivity to MBP, PLP and MOG in an IFN-γ ELISPOT. y-axis shows the specific number of IFN-γ secreting cells per $2x10^5$ cells plated. Specific values were obtained after subtraction of background counts (PBMC in medium only). Negative values were arbitrarily set to zero. Graphs at the top show the clinical course for each of the patients.

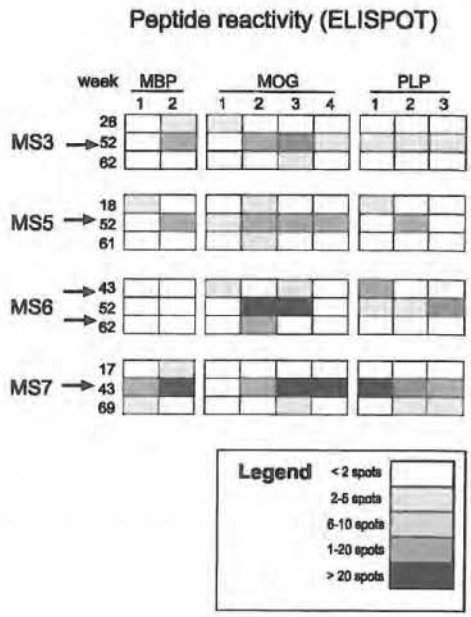


**Figure 5.5.** *T cell recognition pattern to myelin peptides*

T cell reactivity to a panel of MBP- PLP- and MOG-peptides were determined using an IFN-γ ELISPOT assay. For each of the clinically active MS patients the reactivity profiles are shown at a time point before, during and after the relapse (remission). Specific number of spots were obtained after subtraction of background counts (PBMC without antigen added). Reactivity levels were arbitrarily classified according to the specific number of spots per $2x10^5$ cells plated (see legend). Arrows represent clinical relapses. MBP 1-2: MBP (84-102) and (143-168); MOG 1-4: MOG (1-22), (34-56), (64-86) and (74-96); PLP 1-3: PLP (41-58), (184-199) and (190-209).

The selection of peptides was based on previous reports that suggested their predominant recognition in MS[19-22]. The tested MOG and PLP peptides spanned most of the extracellular domains of the antigens. As shown in Figure 5.5, alterations in the epitope recognition profile occurred at the time of relapse in all patients. Remarkably, while only one or a few myelin peptides were recognized before the relapse and at remission, a heterogeneous reactivity towards most of the peptides tested was apparent at time of exacerbation. When comparing the pre- and post-relapse recognition profiles, epitope shifting was evident in patient MS3 (MOG1 to MOG3), MS6 (MOG1-3 to MOG2) and MS7 (MBP1 to MBP2). Note that the peptides tested do not encompass the total sequence of the antigens. Other peptides may also have a role in the observed myelin responses. For instance, patient MS6 did not show reactivity to the two MBP peptides (84-102) and (143-168) (Fig. 5.5), although reactivity to MBP was observed at these samplings (Fig. 5.4). In these cases the anti-MBP response is most likely targeted at other epitopes of MBP. In conclusion, we found a broadening of the anti-myelin peptide response at times of clinical activity in MS.

### 5.3.5 Clonal expansion and persistence of MBP and PLP reactive T cells

An alternative method to study dynamic changes in anti-myelin T cell responses is by studying the T cell receptor sequences of myelin reactive T cell clones. During the course of this study MBP and PLP reactive T cell clones were isolated every six months and characterized for their TCR usage. Based on their TCR expression profile we investigated whether these clones persisted over time and whether they were clonally expanded in the circulation. Clonal expansion is demonstrated by the presence of independent myelin reactive T cell clones that have identical TCR CDR3 sequences, indicating that they are sister clones of the same clonal origin and suggesting their activation and expansion *in vivo*[5].

Interestingly, the clonally expanded anti-MBP and anti-PLP clones did not persist over time in most MS patients. In one patient however (MS5) an MBP reactive T cell clone found at week 1 was also isolated from the blood of this patient two years before this sampling, indicating that this T cell clone persisted over time (Table 5.2). Clonal persistence was also found in a healthy control subject (NS1) for a PLP reactive T cell clone, which was identified at week 1 and week 26. However, in contrast to the healthy subject, the persistent MBP reactive T cell clone in MS5 was clonally expanded at both time points, suggesting that this MBP clone may be important in the ongoing disease process. This was further supported by the simultaneous detection of brain activity and the subsequent clinical relapse (Table 5.2).

Although no one-to-one correlation was found between the dynamic changes in TCR repertoire and clinical measures, clonal expansions of T cells seemed to coincide with the appearance of active MRI lesions in most of the patients with active MRI scans (MS2, 3, 4, 5). For instance, clonally expanded MBP and PLP T cells were detected together with MRI activity in patient MS3. Furthermore, clonally expanded MBP or PLP reactive T cells were detected prior to exacerbation in 3 out of 4 patients with a relapse.

In conclusion, clonally expanded anti-MBP and anti-PLP T cell clones were detected in MS patients but also in healthy controls. In some patients clonally expanded anti-myelin T cells were found to be correlated with increased brain lesion activity and/or clinical disease activity.

**Table 5.2**: Overview of MBP and PLP reactive T cell lines

| | | MBP | | | PLP | | |
|---|---|---|---|---|---|---|---|
| | | wk 0 | wk 26 | wk 52 | wk 0 | wk 26 | wk 52 |
| **MS1** | No. of antigen reactive lines | 0 | 5 | 1 | 0 | 0 | 1 |
| | Clonally expanded T cells[1] | - | yes | - | - | - | - |
| | Persisting clones[2] | - | - | no | - | - | - |
| **MS2** | No. of antigen reactive lines | 4 | 0 | 0 | 2 | 0 | 0 |
| | Clonally expanded T cells | yes * | - * | - | no * | - * | - |
| | Persisting clones | - | - | - | - | - | - |
| **MS3** | No. of antigen reactive lines | 8 | 5 | 5 | 5 | 1 | 0 |
| | Clonally expanded T cells | yes * | yes | no ↓ | yes * | - | - ↓ |
| | Persisting clones | - | no | no | - | no | - |
| **MS4** | No. of antigen reactive lines | 8 | 0 | 1 | 0 | 0 | 0 |
| | Clonally expanded T cells | yes * | - | - | - * | - | - |
| | Persisting clones | - | - | no | - | - | - |
| **MS5** | No. of antigen reactive lines | 2 | 1 | 0 | 0 | 0 | 0 |
| | Clonally expanded T cells | yes * | - * ↓ | - | - * | - * ↓ | - |
| | Persisting clones | yes[3] | no | - | - | - | - |
| **MS6** | No. of antigen reactive lines | 3 | 1 | 0 | 0 | 0 | 8 |
| | Clonally expanded T cells | yes | - ↓ | - ↓ | - | - | ↓ yes ↓ |
| | Persisting clones | - | no | - | - | - | - |
| **MS7** | No. of antigen reactive lines | 0 | 2 | 4 | 0 | 0 | 0 |
| | Clonally expanded T cells | * - * | no ↓ | yes * | * - * | - ↓ | - * |
| | Persisting clones | - | - | no | - | - | - |
| **NS1** | No. of antigen reactive lines | 2 | 4 | 4 | 1 | 2 | 0 |
| | Clonally expanded T cells | no | yes | yes | - | yes | - |
| | Persisting clones | - | no | no | - | yes[4] | - |
| **NS2** | No. of antigen reactive lines | 8 | 3 | 1 | 0 | 0 | 0 |
| | Clonally expanded T cells | yes | yes | - | - | - | - |
| | Persisting clones | - | no | no | - | - | - |

[1]Myelin reactive T cells were defined to be clonally expanded when at least 2 independent T cell clones expressed identical TCR CDR3 sequences; [2]T cell lines identified at two different times that share identical TCR genes were termed persisting lines; [3] One clone (MF3) was present and clonally expanded at week 0 and 2 years before this sampling; [4] One clone (PG6) was present at week 0 and week 26; "-" stands for not applicable; arrows(↓) represent clinical relapses; MRI activity is marked with an asterisk (*)

### 5.3.6 Cytokine profiles

Next, we tested whether cytokine secretion profiles of T cells were correlated with disease activity and MRI activity. We analyzed the cytokine production of freshly isolated PBMC after *in vitro* stimulation with phytohemagglutinin (PHA). Both pro-inflammatory (TNF-α, IFN-γ and IL-6) and anti-inflammatory cytokines (IL-4, IL-10) were measured in the culture supernatant by ELISA. High levels of TNF-α, IFN-γ and IL-6 and varying amounts of IL-10 were detected in most of the cultures (Figure 5.6A). Interestingly, prior to clinical exacerbation a transient increase of the pro-inflammatory cytokines IFN-γ TNF-α and IL-6 was detected in all MS patients with a relapse (MS3, MS5, MS6, MS7). During this temporal increase, active lesions were identified on brain MRI scans in MS3, MS5 and MS7. Furthermore, a decrease of IL-10 production consistently preceded the occurrence of the clinical attack, most notably in patients MS3, MS6 and MS7. An upregulation of this anti-inflammatory cytokine was detected after relapse (at remission) in patients MS4 and MS5. IL-4 production was detected in 2 of 7 MS patients only (MS6 and MS7).

Cytokine production was also analyzed after short-term stimulation with MBP, MOG and PLP. MBP and MOG stimulation induced high levels of the pro-inflammatory cytokines IFN-γ, IL-6 and TNF-α and low levels of IL-10 but no detectable amounts of IL-4 (Figure 5.6B). The absolute levels of IFN-γ production were generally lower after MOG and MBP stimulation than after PHA stimulation. PLP stimulation induced lower levels of IL-6, TNF-α and IL-6 and no detectable amounts of IFN-γ. The low cytokine levels in PLP stimulated cultures were probably due to the low level of proliferation induced by PLP (data not shown). Changes in cytokine patterns of PBMC stimulated with MBP and MOG were comparable with those of PHA stimulated cells. In short, increased production of IL-6, TNF-α and IFN-γ after MBP or MOG stimulation coincided with the appearance of active lesions and preceded clinical relapses.

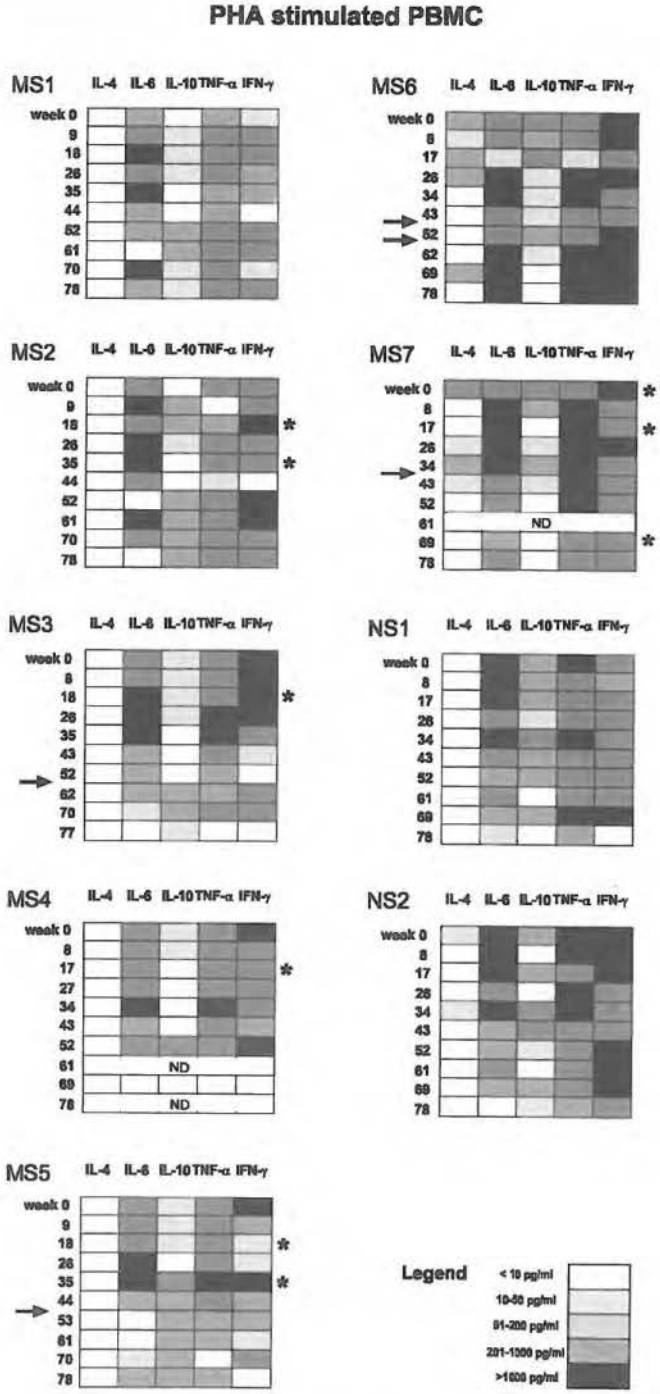


**Figure 5.6** *Cytokine production by stimulated PBMC*

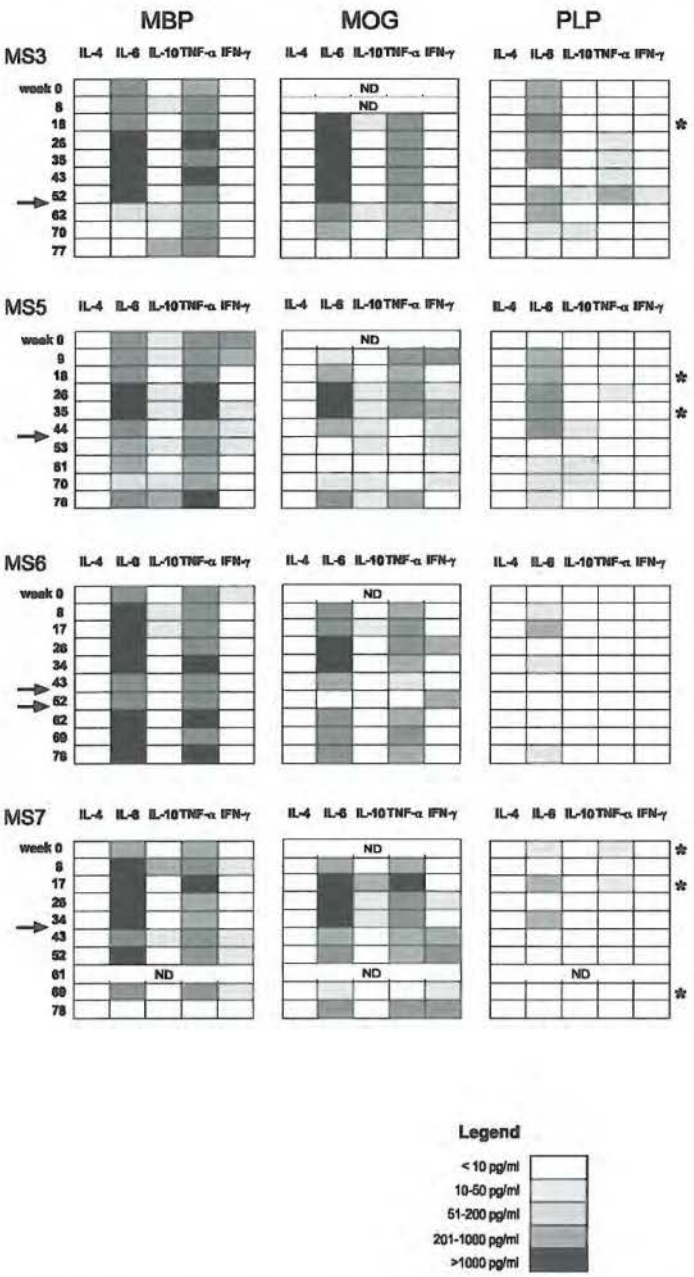


**Figure 5.6 (continued)** *Cytokine production by stimulated PBMC*

Every two months, PBMC were stimulated with PHA (shown in panel A) and MBP, PLP and MOG (shown in panel B). After 3 days, supernatants were collected for the analysis of the levels of the cytokines IL-4, IL-6, IL-10 , TNF-α and IFN-γ. The net cytokine production was calculated by subtracting background levels (non-stimulated PBMC) from the cytokine levels measured in the stimulated cultures. Arrows represent clinical relapses; MRI activity is marked by an asterisk (*); ND: not determined.

### 5.3.7 Serum markers: adhesion molecules

Circulating forms of VCAM-1 and ICAM-1 may be released from cells as a consequence of activation and may be useful markers for inflammation[6]. As reported in Table 5.3, sICAM-1 was significantly higher in MS than in NS ($p<0.03$). In addition, increased levels of sVCAM-1 were detected in MS patients (not statistically significant). Interestingly, sVCAM and sICAM were increased in patients with stable disease, but not in clinically active MS patients (Table 5.3). Figure 5.7 illustrates that no significant temporal fluctuations were found in serum adhesion molecule concentrations of healthy controls values. Remarkably, a significant increase (at least 400% compared to baseline) of sVCAM-1 preceded the occurrence of clinical relapse, most notably in patients MS5, MS6 and MS7. This sVCAM-1 peak coincided with the appearance of active MRI lesions in both MS5 and MS7. Levels of sVCAM-1 dropped to baseline levels after the relapse. No major fluctuations in sICAM-1 serum levels were found in any of the subjects. The levels of sICAM and sVCAM followed a similar pattern in MS3 and MS6, indicating that these molecules may be simultaneously upregulated in some MS patients. In summary, we found that a marked increase in sVCAM coincided with the detection of brain activity and preceded clinical relapses in MS patients.

**Table 5.3: Serum levels of soluble adhesion molecules in MS patients and healthy controls[a]**

| | $n$[b] | sICAM-1[c] | sVCAM-1[c] |
|---|---|---|---|
| NS | 17 | 112.4 ± 3.1 | 1061 ± 34 |
| MS (total) | 63 | 130.1 ± 4.3* | 1138 ± 81 |
| Active MS[d] | 36 | 112.3 ± 5.4 | 953 ± 105[¶] |
| Stable MS[d] | 27 | 153.8 ± 3.5[§] | 1386 ± 112[&] |

[a] longitudinal serum levels from 2 normal subjects (NS; 17 samples) and 7 MS patients (MS; 63 samples); [b] total number of samples; [c] mean ± SEM in ng/ml; [d] MS patients with and without a relapse during the study period were classified as active and stable MS respectively.

Non-parametric Mann-Whitney U-test was used to compare the mean levels of the different groups.

* $p=0.03$ versus MS patients; § $p<0.0001$ versus NS and active MS; ¶ $p=0.03$ versus NS; & $p=0.007$ versus active MS.

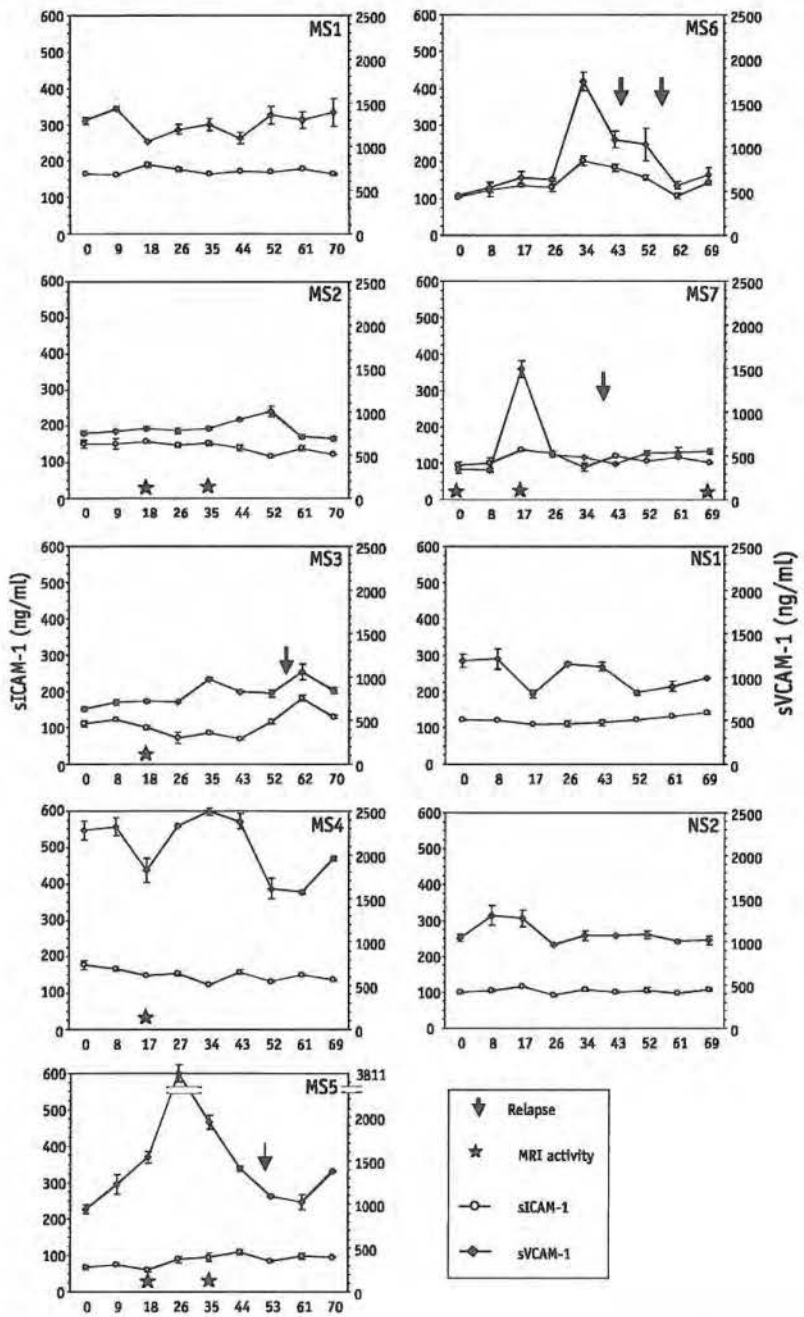


**Figure 5.7**: *Longitudinal serum levels of soluble ICAM-1 and VCAM-1*

Serum concentrations of sICAM-1 and sVCAM-1 were analyzed in ELISA assays. Curves represent the mean values ± SEM (ng/ml) of duplicate wells measured at different timepoints. Note the scale difference between the left and right Y-axis.

## 5.4 Discussion

The goal of the present study was to provide additional information about the T cell mediated pathogenesis of MS through a longitudinal analysis of T cell related parameters in a group of relapsing-remitting MS patients. The study may thus allow to identify possible correlations between T cell markers and disease activity. Such correlations are also useful for the development of a paraclinical marker of disease activity. In addition to clinical parameters such as EDSS and relapse rate we also analyzed brain inflammatory activity by MRI. Consistent with previous publications, our results indicate that new or enhancing brain lesions correlate with subsequent disease activity[23,24]. The time difference between brain activity and clinical relapse as seen in our study most likely reflects the multi-step pathway between inflammation and accumulation of clinical activity. Increased brain activity does however not always lead to clinical relapses as exemplified in two of the studied patients. Indeed, active lesions are seen 5 to 10 times more often than clinical relapses, as reported previously[24]. MRI activity may therefore be a more sensitive measure of disease activity as compared to clinical measures such as relapses. In one patient (MS6) however, no active lesions were detected while the patient had two relapses. The active lesions in this patient may be located in the spinal cord, which was not monitored by MRI in our study. Another possibility could be that active lesions were apparent and later disappeared in the period between two consecutive MRI scans[10].

Our data indicate that some of the tested immunological parameters may correlate with brain inflammatory activity as measured by MRI: expression of adhesion molecules on leucocytes and levels of soluble adhesion molecules; T cell responses to myelin antigens and cytokine production profile of T lymphocytes.

### *Adhesion molecules*

We observed a decrease of ICAM-1-expressing lymphocytes in the blood of MS patients shortly before a clinical relapse. Adhesion molecules, including ICAM-1, facilitate binding of activated peripheral blood lymphocytes to brain endothelial cells before crossing the blood brain barrier[25]. Our findings may reflect the up-regulation of ICAM-1 on activated circulating T cells and the subsequent infiltration into the CNS, leading to a decrease of $CD54^+$ T cells in the blood. This is in line with Kraus and co-workers, who reported significantly decreased

expression levels of cell surface ICAM-1 in the blood of patients undergoing a relapse as compared to patients experiencing remission[12]. It should be noted however that some patients (e.g. MS4) show similar changes in the patterns of ICAM-1 expression without a clinical relapse, indicating that altered expression of adhesion molecules is not a reliable parameter to predict clinical relapses. In addition, although soluble ICAM-1 levels were higher in the serum of MS patients as compared to healthy controls, we observed no correlations between levels of sICAM-1 and disease activity.

Interestingly, our data indicate that sVCAM serum levels are a potential marker of inflammation and possible predictor of clinical activity in MS as shown in three out of four patients with a clinical relapse. These data are in line with previous reports[26,27]. Levels of sVCAM were also found to be higher in MS sera as compared to sera of healthy controls as reported before[13]. Interestingly, sVCAM and sICAM levels were increased in patients with stable disease, but not in clinically active MS patients. These findings may be compatible with one of the proposed functions of soluble adhesion molecules. Indeed, circulating adhesion molecules are thought to promote de-adhesion and block adhesion of leucocytes to cerebral endothelial cells[27]. Thus, the high levels of sICAM-1 and sVCAM-1 in stable MS patients may block the migration of activated autoreactive T cells into the CNS. Further research is needed to resolve whether sVCAM-1 serum levels, perhaps in combination with expression levels of ICAM-1 on lymphocytes, can be used as a reliable marker of disease activity in RR MS.

### *Anti-myelin T cell reactivity*

Some of the important unresolved issues in current MS research relate to the antigen reactivity profile of potentially pathogenic autoreactive T cells; which myelin antigen is the predominant autoantigen, does the reactivity profile of myelin reactive T cells remain stable or change during the course of the disease,..? We studied the reactivity to three myelin antigens during the course of the disease using ELISPOT as a fast directly *ex vivo* technique. Our serial evaluation of myelin-recognition revealed highly dynamic shifts and fluctuations from one autoreactivity pattern to another in a patient-specific manner. These findings are in line with work of Tuohy et al., which indicated that the progression of MS is characterized by highly diverse patterns of self-reactivity caused by determinant spreading and shifting along the course of the disease[3]. Determinant and epitope spreading may also

account for the temporal broadening of the anti-myelin peptide response at times of clinical activity as seen in 4 tested patients. The mechanisms involved in epitope shifting and spreading are still unclear, but may reflect a dynamic selection/inhibition process in which autoreactive T cells respond sequentially to different determinants.

Our data further indicate that at least in some patients new anti-myelin reactivity coincided with MRI activity, which eventually resulted in the occurrence of a clinical relapse. Peaks of reactivity to MBP and MOG coincided with brain activity in most of the patients with active MRI scans (MS3, MS5, MS7). Dynamic responses to myelin antigens were also detected in the healthy controls (NS1, NS2). Immune responses to self-antigens have been demonstrated in previous studies[19,28]. Possibly, regulatory mechanisms are involved in the down-regulation of these anti-myelin responses. In several patients reactivity to a given myelin antigen appeared and subsequently gradually decreased. These autoreactive T cells may undergo peripheral clonal deletion, perhaps due to apoptosis as a result of chronic self-stimulation[3]. Alternatively, autoreactive T cells may be present but unreactive as a result of T cell anergy or suppression[29,30]. In addition, since the ELISPOT evaluates IFN-γ secretion, it cannot be ruled out that the marked decrease merely reflects the inhibition of IFN-γ secretion by the myelin reactive T cells, rather than actual clonal deletion. While these mechanisms may predominate in healthy subjects and patients with stable disease course, clinically active patients may show a decrease of self-reactive T cells in their peripheral blood due to trapping of autoreactive T cells in the CNS compartment. Our data support this view, since partially sustained autoreactivity preceded the detection of active (new and enhancing) brain lesions.

Clonally expanded MBP and PLP reactive T cells may indicate *in vivo* activation of T cells and was found at several time points in all MS patients but also in healthy controls. The presence of clonally expanded anti-MBP or anti-PLP clones was found to correlate with MRI activity in all patients having active MRI scans. Such expansions were however also identified in stable MS patients and even in healthy controls. Persistence of anti-MBP and anti-PLP T cell clones was only found in one MS patient, but also in one healthy control subject. Whether clonally expanded and/or persisting myelin reactive T cells have a pathogenic relevance in MS remains a question of debate. Clonally expanded myelin reactive T cells were previously also observed in healthy individuals[5], while persisting anti-myelin T

cells were found in both healthy controls and MS patients irrespective of disease activity[20,31,32]. Goebels et al. recently suggested that the long-term persistence of MBP reactive T cells is a relatively common feature of the human T cell repertoire and proposed that these long-lived T cells in some cases may have a regulatory or protective function[33]. However, in chronic relapsing EAE clonally expanded MBP reactive T cells were found in blood and spinal cord during acute stages but not in the remission phase, indicating that these T cells play a direct pathogenic role in this model[34]. In addition, a clear correlation between new anti-MBP T cell reactivity, clonal expansion and disease activity was observed in our T cell vaccination pilot study. After immunization with autologous irradiated MBP reactive T cell clones, anti-MBP T cells were no longer detected in blood of the treated patients. In three patients however new anti-MBP T cell reactivity could be found some time after treatment and this autoreactivity correlated with clinical exacerbations and MRI activity, indicating that these three events were induced by (a) common event(s)[35].

Taken together, dynamic fluctuations in the myelin T cell reactivity pattern were observed in MS patients and this pattern may correlate with MRI activity in some patients indicating that new anti-myelin reactivities due to determinant spreading/shifting may play a role in brain inflammatory activity. Studies in the murine and primate EAE model also illustrated that determinant spreading within the same myelin protein (intramolecular) as well as between myelin proteins (intermolecular) causes disease relapses[36,37]. We observed clonally expanded T cell populations in periods of increased MRI activity. Whether these T cell activities are the cause or consequence of brain inflammatory activity remains unknown. Interestingly, fluctuating anti-myelin T cell activity was also found in healthy controls. Potential mechanisms leading to the peripheral activation and clonal expansion of myelin reactive T cells in MS may involve superantigen stimulation and cross-activation by viral peptides (molecular mimicry)[38,39]. Autoreactive T cells of MS patients and healthy subjects are perhaps equally susceptible to activation. Therefore, additional mechanisms may contribute to the further *in vivo* expansion of myelin reactive T cells in MS patients. Improper functioning of the anti-idiotypic network regulation is a possible mechanism[40,41].

### *Cytokine profile of T lymphocytes*

We observed increased production of IL-6, TNF-$\alpha$ and IFN-$\gamma$ by MBP or MOG stimulated mononuclear cells at the time of appearance of active lesions and preceding clinical

relapses in most MS patients. These data are in agreement with previous reports which showed an increase of the *in vitro* production of TNF-α and IFN-γ prior to relapse[42,43]. The production of TNF-α may especially be relevant to MS, since this cytokine was shown to possess demyelinating potential and in addition was found in MS plaques[44,45]. Other biological effects include up-regulation of the expression of adhesion molecules, thereby facilitating the infiltration of autoreactive T cells into the CNS[46]. Our data support this view, since the elevation of TNF-α coincided with the detection of active lesions on MRI. IFN-γ is predominantly produced by $CD4^+$ T cells of the Th1 phenotype and may exert its effect by inducing the upregulation of MHC class II molecules on microglia, leading to an increased presentation of CNS antigens or by cytotoxic effects on oligodendrocytes[47,48].

Although IL-6 was shown to be elevated in the CSF and in MBP and PLP stimulated cultures of MS patients[49], its role in MS remains unclear. IL-6 is a potent B-cell stimulator and could lead to increased differentiation into plasma cells that are involved in the production of potentially tissue-destructive autoantibodies[50]. However IL-6 may also promote neural repair and mediate inhibition of TNF-α production[51,52]. IL-10 may down-regulate immune responses, which is in agreement with our observation of declining IL-10 levels prior to relapse, while increased levels were found during remission phases in some of the patients. Similar observations were reported by Rieckmann and co-workers[43]. It is clear that the above-mentioned cytokines are not independent effector molecules, but instead take part in a complex network of inter- and counteracting cytokines. Therefore, it seems evident that not the absolute quantities but rather the (im)balance between pro-inflammatory and immunoregulatory cytokines may determine the outcome of an autoimmune attack in MS.

Most of the studied MS patients received IFN-β, which is thought to have cytokine-modulating effects. Although the exact mode of action of IFN-β is not completely resolved, several reports suggest an inhibitory effect on IFN-γ and TNF-α secretion and an upregulated production of IL-10, IL-4 and TGF-β[53,54]. In contrast, a recent report demonstrated that treatment with IFNβ-1a downregulates both pro-inflammatory and anti-inflammatory cytokines, rather than causing a shift in cytokine profile[55]. Without underscoring the effect of IFN-β treatment on the cytokine production, we believe that it is still eligible to compare cytokine patterns since almost all patients received IFN-β.

Moreover, no significant differences were observed between the cytokine profile of the IFN-β-treated subjects and patient MS7, who did not receive IFN-β. Patients MS6 and MS7 also received 3 day courses of intravenous methylprednisolone (IVMP) at relapse time, which might also influence the cytokine production. However, we expected no major effects of IVMP treatment in these patients, since samples in these patients were taken more than 1 month after the end of treatment while the effects of IVMP on cytokine production are moderate and short-lived[56].

## 5.5 Conclusion

Taken together, temporal changes in several immunologic measures were found to coincide with brain activity and to precede clinical relapses in most of the MS patients studied. These changes include: an increase in myelin reactive IFN-γ secreting T cells as measured by ELISPOT, detection of clonally expanded myelin reactive T cells, elevated pro-inflammatory and decreased anti-inflammatory cytokine production, upregulation of ICAM-1 membrane expression and highly increased serum levels of soluble VCAM-1. Since these events coincided with the detection of active lesions it is tempting to speculate that these immunologic changes may be related to the observed MRI activities and the subsequent occurrence of clinical exacerbations. How do these data fit into a hypothetical pathway that would lead to the autoimmune responses in the CNS? Myelin specific T cells may become activated in the peripheral compartment, leading to an increased number of IFN-γ secreting myelin reactive T cells and the clonal expansion of some of these self-reactive T cell clones. Upon activation these T cells produce high levels of pro-inflammatory cytokines, leading to the downregulation of anti-inflammatory cytokines. Furthermore, ICAM-1 (CD54) is upregulated on activated T cells facilitating the adhesion of these T cells to the endothelial membrane of the blood-brain-barrier (BBB) and their migration into the CNS. This event may be responsible for the observed decrease of CD54 expressing T cells in blood of MS patients prior to relapse. The ongoing inflammation may lead to the release of soluble forms of adhesion molecules reflecting the disturbances of the BBB. Soluble adhesion proteins may also promote de-adhesion in order to block the further migration of blood cells into the CNS. Remarkably, most of these alterations only partially sustain and subsequently gradually disappear. In MS patients this abrogation may reflect the decrease of autoreactive T cells in their peripheral blood compartment due to trapping of these self-reactive T cells in the CNS compartment. Alternatively, this abrogation may be caused by regulatory mechanisms aimed at downregulating the potentially pathogenic immune responses in the blood. While these regulatory mechanisms may be highly efficient in healthy subjects, they may malfunction in MS patients. This could explain why some of the observed immune alterations could also be detected in healthy controls.

The observed heterogeneity and dynamic changes in myelin T cell reactivity may be a major obstacle for the effective application of antigen and peptide-specific immunotherapies.

Since these experimental approaches are aimed at tolerizing T cells specific for one myelin determinant, they would not be protective to newly emerging autoreactive T cell populations directed at different myelin antigens. These approaches may however still become successful when they induce bystander suppression in the CNS by shifting to a Th2-type response.

In conclusion, the present study indicates that autoreactive immune responses in MS are highly dynamic and provide supporting evidence for a hypothetical pathway in which anti-myelin reactive T cells play an important role. Our data also emphasize the importance of evaluating immune responses longitudinally and indicate that some immune markers may be useful as para-clinical disease marker for both diagnostic and prognostic purposes. In the future, a combination of these markers may provide more accurate information on disease activity in MS.

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# Chapter 6

## Summary and Discussion

## Summary and Discussion

This final section presents a brief overview of the results. The most important findings and their implications for the design of MS immunotherapies are further discussed. For convenience, these considerations are arranged according to the goals postulated in the aim of the study (Chapter 1).

**Goal 1: to further characterize the molecular basis of the anti-clonotypic recognition of MBP reactive T cells in MS patients treated with T cell vaccination (TCV)**

In Chapter 3, we made an attempt to define the idiotypic determinants responsible for triggering the $CD8^+$ cytotoxic T cell responses in TCV treated MS patients. Therefore, $CD8^+$ $TCR\alpha\beta^+$ Ac T cells were isolated from the vaccinated patients and tested for their recognition of a panel of autologous and allogeneic T cell clones. The cross-reactivity assays indicated that these anti-clonotypic T cells are highly specific for the immunizing clone, most likely by recognizing CDR3 sequences within the TCR. These observations further support the idea that anti-idiotypic T cells contribute to the specific suppression of MBP reactive T cells seen after T cell vaccination.

Although several reports -including ours- suggest that anti-clonotypic T cells may recognize TCR determinants expressed by the vaccine cells, no direct evidence is available on the molecular basis of this T-T cell interaction. Using two *in vitro* models we attempted to unravel the precise molecular mechanisms involved in the anti-clonotypic recognition of the vaccine clones. However, we could not provide additional evidence that supports our previous assumptions. The success of these experimental strategies depends on critical factors that are difficult to control. In addition, it remains speculative whether the used target cells possess the necessary cell machinery and pathways for the efficient processing and MHC class I presentation of self-peptides. We empathized that an additional factor may be necessary for enhanced T cell recognition and activation, which might be present *in vivo* but not in our *in vitro* models. The precise nature of this second signal is not known. Ware et al. proposed that an increased expression of adhesion molecules on the target CD4+ T cells might account for selective recognition[1]. Alternatively, the presentation of self-TCR peptides may follow unconventional pathways in which other "helper" cells play a pivotal

role. Kozovska and co-workers demonstrated that MBP specific $CD4^+$ T cells showed an outstanding ability to present TCR peptide due to the presence of a unique population of $CD4^-$ $CD8^-$ T cells[2]. Moreover, we recently demonstrated that TCV induces a complex cellular response in which several uncommon lymphocyte populations including $\gamma\delta^+$ T cells and NK cells may be involved in the immunoregulatory T-T cell interactions[3].

In conclusion, based on our experimental *in vitro* approaches we could not provide additional evidence that TCR determinants are the major target for the anti-clonotypic $CD8^+$ T cells induced by TCV. This seems to be in contrast with our observations in the cross-reactivity assays and may be explained by either technical limitations or the need for a "second signal". Nevertheless, a recent study strongly supports the hypothesis that $CD8^+$ CDR3-specific T cells play a predominant role in the immune responses induced by T cell vaccination[4]. This study reported that TCR peptide reactive T cell lines isolated from vaccinated MS patients cross-react with the original immunizing MBP T cells. Whether additional co-stimulatory molecules or cell populations may contribute in some extent to the anti-clonotypic recognition process requires further investigation. This may lead to a better understanding of the precise mechanisms involved in the *in vivo* clonotypic regulation of autoreactive T cells.

**Goal 2: to simultaneously analyze T cell reactivity to multiple myelin antigens in MS patients using the ELISPOT technique**

In Chapter 4, we evaluated the IFN-γ production induced by MBP and MOG and selected MBP-, MOG- and PLP-peptides in MS patients and healthy controls using the IFN-γ ELISPOT assay. This allowed us to look for any quantitative or qualitative differences in anti-myelin T cell reactivity between MS patients and healthy individuals.

Most MS patients and healthy controls showed a heterogeneous anti-myelin T cell reactivity. Interestingly, a correlation was found between T cell reactivity to MOG and MBP in MS patients, but not in control subjects. Thus while in individual MS patients T cell responses were found to both MBP and MOG, healthy subjects predominantly recognized one of the myelin antigens. It is possible that the dual reactivity to MBP and MOG as observed in MS patients may represent the intermolecular spreading of an immune response that initially

arose against a single antigen only ("determinant spreading"). Another possibility could be that the initial autoimmune response in MS patients is already directed to several myelin antigens and that this T cell reactivity pattern persists along the disease progression. This latter explanation is in line with the study of Söderström and co-workers who demonstrated that optic neuritis, a common first manifestation of multiple sclerosis is characterized by a highly heterogeneous anti-myelin T cell repertoire[5]. To justify one of the above-mentioned explanations, longitudinal studies are needed that evaluate myelin T cell reactivity over time in individual patients (see Chapter 5).

No preferentially recognized epitope could be identified among the myelin peptides tested (MBP 84-102, 143-168, MOG 1-22, 34-56, 64-86, 74-96, PLP 41-58, 184-199, 190-209) in both the MS and NS group. Recently, Pelfrey et al. further demonstrated that anti-myelin T cell reactivity in MS is highly diverse and unfocused by illustrating that up to 22 different PLP epitopes were recognized in individual patients[6].

Although our study did not reveal any quantitative differences in anti-myelin T cell reactivity in MS patients as compared to healthy individuals, functional differences could be detected. The frequency of $IL2R^+$ MBP reactive T cells was significantly increased in blood of MS patients, indicating that MBP reactive T cells exist in an *in vivo* activated state in MS patients[7]. This was not the case for TT reactive T cells, demonstrating that our observations do not merely reflect a general activation of the immune system in MS patients. It may be useful to further investigate by ELISPOT if increased frequencies of MOG- and PLP- (peptide) reactive T cells exist among the IL-2 expanded T cell fractions. By using other techniques (LDA after primary stimulation with IL-2 and screening of hrpt mutations), several reports found that MBP and PLP reactive T cells are activated *in vivo* in MS patients, suggesting their relevance in the disease[8,9].

Most of the anti-MBP T cells were of the Th1-type, since reactivity was observed in IFN-γ but not in IL-4 ELISPOT-assays. Using Th1 (IL-12) and Th2 (IL-4) promoting conditions we observed that the cytokine secretion pattern of anti-MBP T cells still is susceptible to alteration. The ability to alter the cytokine phenotype of MS derived autoreactive T cells indicates that even at later stages of the disease, immunomodulatory therapies might have

therapeutic benefit by switching the function of myelin reactive T cells in such a way that they become less or even non-pathogenic.

In conclusion, using ELISPOT assays to analyze the frequency of IFN-γ producing myelin reactive T cells, we did not observe any quantitative differences between MS patients and healthy subjects. However, an increased frequency of IL-2 responsive MBP reactive T cells was found in the blood of MS patients, indicating a functional difference in activation status of MBP reactive T cells in MS. Our data lend further support to the view that these cells are relevant to the disease process.

**Goal 3: to study temporal changes in T cell related parameters and their correlation with clinical parameters and MRI activity in patients with multiple sclerosis**

In Chapter 5, we report on a longitudinal study in which several immunological and clinical parameters were monitored in 7 relapse-remitting MS patients and 2 healthy controls at regular intervals for a period of 18 months. This may add to our understanding of the MS pathogenesis and may lead to the identification of biological markers predictive for disease activity.

We recorded temporal changes in several immunologic parameters coinciding with brain activity and preceding clinical relapses. From the data obtained, we can postulate that myelin specific T cells become activated in the peripheral compartment, resulting in an increased number of IFN-γ secreting myelin reactive T cells (ELISPOT) and the clonal expansion of some of these self-reactive T cell clones (TCR expression). Upon activation these T cells produce high levels of pro-inflammatory cytokines, leading to the downregulation of anti-inflammatory cytokines (cytokine ELISA). Furthermore, ICAM-1 is upregulated on activated T cells facilitating the adhesion of these T cells to the endothelial membrane of the blood-brain-barrier (BBB) and the migration into the CNS (phenotypic analysis by flow cytometry). This event may be responsible for the observed decrease of CD54 expressing T cells in blood of MS patients prior to relapse. The ongoing inflammation may lead to the release of soluble forms of adhesion molecules reflecting the disturbances of the BBB (serum ELISA). At the same time, new brain lesions are formed as evidenced by MRI.

However, some of the observed immune alterations could also be detected in healthy controls without any disease activity, indicating that additional regulatory mechanisms – that may be defective in MS- could play a role in the down-regulation of these potentially pathological responses. It therefore remains to be shown which of the observed immune abnormalities are effectively involved in the pathogenesis of MS. Evaluation of the influence of therapies on each of these parameters can provide useful information on these issues.

The serial evaluation of anti-myelin T cell responses revealed highly dynamic shifts and fluctuations from one pattern to another. Both determinant spreading and shifting were observed in individual patients. These observations may be a major obstacle for the development of antigen-specific immunotherapies as discussed below.

In conclusion, we demonstrated that autoreactive immune responses are highly dynamic in MS, thereby emphasizing the importance of evaluating immune responses at different time points. Several immune markers could be useful as para-clinical disease marker for both diagnostic and prognostic purposes. A combination of these markers, instead of one single marker may provide more accurate information on disease activity in MS.

**Implications of these findings for T cell vaccination and other immunotherapies**

For many years it was believed that identification of "the" autoantigen in MS would be the key to understand and treat the disease. However, recent data – including ours reported here - indicate that T cells recognizing multiple myelin antigens are found in the blood of MS patients. This may have implications for therapies directed at eliminating specific cells that recognize one myelin antigen only or that have a unique TCR.

*Antigen (peptide) specific immunotherapies*

Since MS is presumed to be a final result of an antigen-driven immune response, several groups have tried to inhibit this improper response by eliminating T cells specific for a given myelin antigen. One way of achieving this goal is by antigen feeding, termed "oral tolerance", which was proven to be successful in EAE[10]. Alternatively, the depletion of autoantigen specific T cells may also be accomplished by the administration of altered

peptide ligands (APLs), that compete with the native myelin epitopes for binding to the T cell receptor[11,12].

The observed heterogeneity and dynamic changes in myelin T cell reactivity reported in Chapter 4 and 5 may be a major obstacle for the application of these antigen-specific immunotherapies. Since these experimental approaches are aimed at eradicating T cells specific for one myelin determinant, they would not be protective to newly emerging autoreactive T cell populations or T cells specific for other myelin components. However, recent reports indicated that the mode of action of oral tolerance and APL therapy involves the induction of Th2 and Th3 type myelin reactive T cells[13,14]. It could be postulated that by selectively stimulating these regulatory T cells, and not the "pathogenic" Th1 cells, a detailed knowledge of the antigen-reactivity may not be required for these therapeutic approaches. Indeed, an upregulation of Th2/Th3 T cells might render the pathogenic Th1 type cells in the near vicinity harmless by the secretion of suppressive cytokines such as TGF-β and IL-10 (bystander suppression)[15,16]. It should be kept in mind however that the induction of Th2 cells by oral tolerance may augment the humoral immune responses including anti-MOG antibody production, which has been shown to cause a lethal demyelinating disease in the marmoset model of MS[17]. Until now, clinical trials of oral tolerance induction through myelin feeding could not demonstrate any treatment efficacy[18].

*T cell receptor specific immunotherapies*

The reported heterogeneous myelin reactivity may also be a drawback for therapies such as T cell vaccination and TCR peptide vaccination, which are directed at (the TCR of) a specific myelin reactive T cell clone. Indeed, although these experimental treatments have shown to effectively deplete T cells specific for MBP, other anti-myelin T cells (including anti-MOG and anti-PLP T cells) will remain in the circulation and may further be involved in the perpetuation of the disease[19,20].

Some authors provided evidence that determinant spreading in the MS animal model followed a well-defined predictable pattern along the disease progression, and that interference at any point in this cascade would stop epitope spreading[21]. In contrast, we found that T cell vaccination did not seem to interfere with determinant spreading. Indeed, re-emerging MBP T cells in some of the vaccinated patients displayed TCR genes different

from those of the vaccine clones and recognized additional MBP epitopes[22]. We demonstrated that these newly appearing T cell clones could further be depleted by an additional round of TCV. This illustrates that some of the MS patients may need to be vaccinated several times in order to down-regulate all the potentially pathogenic T cell clones.

Moreover, the vaccine composition which consists of MBP reactive T cell clones isolated from the peripheral blood could be optimized. The currently used T cell vaccine is composed of IL-2 expanded CD4+ T cells isolated from the CSF of MS patients. This vaccine will be tested in a double-blinded placebo-controlled study. The CSF derived vaccine has several important advantages as compared to the original vaccine. The CSF is the compartment closest to the ongoing inflammatory response in the CNS and may therefore harbor T cell subsets that are highly relevant to the disease. Furthermore, it has been shown that not only MBP reactive T cells, but also other myelin reactive T cells are increased in the CSF of MS patients[5,8,23]. This means that a larger group of potentially pathogenic T cells could be included in the vaccine. Ongoing T cell vaccination trials in Diepenbeek and other centres (Israel, Houston, Los Angeles) may point out which of the approaches is the most appropriate protocol for TCV.

Similarly, the applicability of TCR peptide vaccination is limited by the diversity of TCR V gene sequences expressed by myelin reactive T cells and the lack of knowledge of the immunogenic regions of these V genes. Therefore, it is difficult to decide which TCR peptides need to be included in the vaccine. However, an extensive study recently identified identical TCR motifs within AV and BV gene families of anti-myelin T cells, strongly suggesting the possibility that these peptides could be cross reactive for anti-TCR specific T cells[24]. It seems evident that vaccination with peptides harboring these TCR motifs would result in a more sensitive but less selective triggering of regulatory TCR-specific T cells. This expanded potential for T cell activation combined with a bystander suppression mechanism mediated by release of inhibitory cytokines, might make TCR peptide therapy generally applicable for treating autoimmune diseases such as MS and arthritis since they likely involve many pathogenic T-cell specificities. Further clinical studies will point out if this approach is truly efficient in treating MS.

Upon developing T cell (receptor) based therapies, it should be kept in mind that persistent myelin reactive T cell clones have been detected in healthy subjects also(Chapter 5). Several studies identified persisting myelin reactive T cells in both healthy controls and MS patients[25-29]. It is tempting to speculate that the persistence of these clones in MS patients point towards an involvement in the pathogenesis of MS. However, Goebels et al. recently suggested that long-term persistence of MBP reactive T cells is a relatively common feature of the human T cell repertoire and proposed that these long-lived T cells in some cases may exert an unknown regulatory or protective function[30]. In this view, it is of interest that MBP reactive T cells were shown to protect CNS neurons in animal systems of optic nerve an spinal cord injury[31,32]. Obviously, it would not be desirable to eliminate these potentially neuroprotective T cells by means of T cell (receptor) vaccination therapies. Further investigations are needed to establish whether persisting MBP reactive T cell clones play a harmful or protective role in course of the disease.

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# Nederlandse samenvatting

## Overzicht van experimentele resultaten

# Nederlandse samenvatting

## Hoofdstuk 1: Inleiding

Multiple sclerose (MS) is een chronische inflammatoire aandoening van het centrale zenuwstelsel (CZS), die leidt tot demyelinisatie. De etiologie en onderliggende pathogenese zijn nog niet volledig opgehelderd. Onderzoek toont echter aan dat een destructief auto-immuun proces centraal staat in de pathogenese, en dit in associatie met genetische en omgevingsfactoren. Dit auto-immuun proces wordt vermoedelijk gemedieerd door auto-reactieve T-celresponsen gericht tegen myeline antigenen. Het blijft echter onduidelijk wélke myeline antigenen het doelwit zijn van de primaire auto-immuunrespons. Vooral *myelin basic protein* (MBP), maar ook *proteolipid protein* (PLP) en *myelin oligodendrocyte glycoprotein* (MOG) komen in aanmerking als potentieel autoantigen. De heterogeniteit in de auto-immune T-celrespons zou kunnen wijzen op een verschuiving in T-celreactiviteit van het primair autoantigen naar secundaire determinanten die de inflammatoire respons onderhouden (determinant spreiding). Het is eveneens mogelijk dat, mede door het verschil in immunogenetische (HLA) achtergrond, de primaire autoantigenen patiënt gebonden zijn en dat de T-celrespons gericht blijft tegen dit ene dominante myeline antigen. Vele andere bevindingen wijzen erop dat myeline reactieve T-cellen in MS een mogelijke pathogene rol spelen. MBP-reactieve T-cellen worden aangetroffen in hersenlaesies en accumuleren in het cerebrospinaal vocht (CSV) van MS-patiënten. Klonaal geëxpandeerde MBP-reactieve T-cellen komen voor bij de meeste MS-patiënten en zijn meestal afwezig bij gezonde personen. Bovendien blijven deze T-cellen langdurig aanwezig in het bloed van MS-patiënten, wat erop wijst dat deze cellen *in vivo* geactiveerd werden.

Diverse nieuwe experimentele behandelingen voor MS richten zich dan ook op de inactivatie of eliminatie van deze pathogene T-cellen. Een aantal van deze therapieën worden in hoofdstuk 1 besproken met de nadruk op T-celvaccinatie (TCV), waarbij een immunisatie wordt uitgevoerd met autologe geïnactiveerde MBP-reactieve T-cellen.

## Hoofdstuk 2: Materialen en methoden

Dit hoofdstuk geeft een overzicht van alle materialen en methoden die werden aangewend in de verschillende hoofdstukken van deze thesis. Zowel de moleculair biologische als de op celkweek gebaseerde technieken worden uitvoerig beschreven.

## Hoofdstuk 3: Karakterisatie van de anti-klonotypische T-cellen bij MS-patiënten die behandeld werden met T-celvaccinatie

In het Biomedisch Onderzoeksinstituut - Dr. L. Willems-Instituut werd een fase I studie van T-celvaccinatie uitgevoerd. Bij alle gevaccineerde patiënten werd een depletie van de MBP-reactieve T-cellen waargenomen, samen met de inductie van CD8+ anti-klonotypische T-cellen (anti-vaccin T-cellen). Vroegere experimenten suggereren dat deze anti-klonotypische T-cellen (Ac T-cellen) vermoedelijk een deel van de T-celreceptor van de vaccinkloon herkennen en dit op een MHC klasse I gerestricteerde wijze.

In dit hoofdstuk hebben we geprobeerd te achterhalen welke idiotype determinanten verantwoordelijk zijn voor de inductie van de Ac T-celresponsen in gevaccineerde MS-patiënten. Hiervoor werden van een aantal gevaccineerde patiënten Ac T-celklonen geïsoleerd en vervolgens getest op hun herkenning van een panel van autologe en allogene T-celklonen. Deze kruisreactiviteitstesten wezen uit dat Ac T-cellen zeer specifiek zijn voor de vaccinkloon en vermoedelijk CDR3 sequenties van de T-celreceptor herkennen.

Totnogtoe is er echter geen informatie voorhanden over de moleculaire basis van deze T-T celinteractie. Met behulp van twee *in vitro* modellen hoopten we meer inzicht te krijgen in de precieze moleculaire mechanismen die instaan voor de anti-klonotype herkenning. In een eerste aanpak werden autologe PHA-geactiveerde T-celblasten gepulst met een panel van overlappende TCR-peptiden, die het hele CDR3 gebied van de TCR-ketens overspannen. In de andere strategie werden de TCR $\alpha$- en de $\beta$-ketens van de vaccinkloon getransfecteerd naar autologe lymfocyten. Telkens werd nagegaan of deze doelwitten herkend werden door de anti-klonotype T-cellen. De resultaten van deze experimenten konden echter niet bevestigen dat TCR-determinanten de voornaamste doelwitten van de Ac T-cellen zijn. Dit is in tegenstelling met onze aanwijzingen uit de kruisreactiviteitsproeven en kan deels verklaard worden door technische beperkingen. Anderzijds is het mogelijk dat bij de herkenning van TCR-epitopen een secundair signaal nodig is dat wel aanwezig is *in vivo*, maar niet in onze *in vitro* systemen. Verder onderzoek is nodig om deze veronderstelling te bevestigen en de aard van dat secundair signaal verder te bestuderen.

## Hoofdstuk 4: Analyse van de T-celreactiviteit tegen myeline antigenen in MS-patiënten en gezonde controles met behulp van de ELISPOT techniek

De technieken die we tot op heden gebruikt hebben om antigen reactieve T-cellen te isoleren en de frequentie ervan te bepalen (*limiting dilution assay*) vergen relatief veel bloed, waardoor het vrijwel uitgesloten is om de T-celreactiviteit tegen verschillende autoantigenen (MBP, PLP, MOG) simultaan te bestuderen. Er bestaat echter een alternatieve aanpak waarbij de antigen specifieke T-cellen niet worden gedetecteerd op basis van hun proliferatieve capaciteit ten opzichte van het antigen, maar via verhoogde productie van cytokines. Deze techniek is gebaseerd op een ELISA-methode en wordt ELISPOT genoemd. Het voordeel bij ELISPOT is dat er niet alleen informatie wordt bekomen over de frequentie van de T-cellen, maar ook over hun cytokineprofiel of Th-fenotype (Th1/Th2).

In dit hoofdstuk werd de T-celreactiviteit t.o.v. MBP, PLP en MOG en afgeleide synthetische peptiden bepaald via ELISPOT bij 36 MS-patiënten en 31 gezonde personen. Uit de resultaten blijkt dat de T-celreactiviteit ten opzichte van de bestudeerde myeline antigenen en peptiden heterogeen is bij MS-patiënten en gezonde controles. Er werd een correlatie gevonden tussen de graad van MOG en MBP T-celreactiviteit bij de MS-patiënten, maar niet bij de gezonde controles. Mogelijk is deze heterogene respons in de MS-patiënten het gevolg van determinant spreiding. De anti-MBP respons werd vooral waargenomen in de IFN-γ, maar niet in de IL-4-ELISPOT, wat betekent dat deze anti-MBP T-cellen hoofdzakelijk van het Th1 type zijn. We konden bovendien aantonen dat het cytokinepatroon van MBP-reactieve T-cellen nog beïnvloed kan worden in Th1 en Th2 inducerende condities, wat mogelijkheden biedt voor immuunmodulerende therapieën.

Hoewel er geen kwantitatieve verschillen werden gevonden tussen de anti-myeline T-celreactiviteit van MS-patiënten en gezonde donors, waren er wel functionele verschillen merkbaar. We toonden namelijk aan dat de frequentie van *in vivo* geactiveerde (IL-2R+) MBP T-cellen significant verhoogd is in het bloed van MS-patiënten. Hieruit kunnen we besluiten dat MBP-reactieve T-cellen in een geactiveerde toestand verkeren in het bloed van MS-patiënten, maar niet in dat van gezonde controles, wat wijst op een mogelijke relevantie van deze cellen in het MS-ziekteproces.

## Hoofdstuk 5: Opvolging van immunologische karakteristieken in functie van het ziekteproces bij MS-patiënten

In dit laatste hoofdstuk werden verschillende immunologische en klinische parameters opgevolgd in 7 *relapsing-remitting* MS-patiënten en 2 gezonde donors gedurende een periode van 18 maanden. Enerzijds kan dit ons verder inzicht verschaffen in de immuunpathogenese van MS en anderzijds kan dit leiden tot de identificatie van (een) paraklinische parameter(s) voor ziekteactiviteit.

Veranderingen in verschillende immunologische parameters gingen gepaard met een verhoogde MRI hersenactiviteit en resulteerden meestal in klinische exacerbaties. Zo vonden we onder meer een verhoogd aantal IFN-γ secreterende myeline reactieve T-cellen, klonale expansie van bepaalde myeline specifieke T-celklonen, een verhoogde productie van pro-inflammatoire cytokines, een gedaalde productie van anti-inflammatoire cytokines, een verhoogde membraanexpressie van ICAM-1 en een gestegen serum concentratie van oplosbaar VCAM-1. Hoe passen deze resultaten in een hypothetisch model dat aanleiding geeft tot een pathogene auto-immuunrespons in het CZS? Myeline reactieve T-cellen kunnen geactiveerd worden in het bloed, wat resulteert in een verhoging van IFN-γ-secreterende myeline reactieve T-cellen en de klonale expansie van sommige van deze autoreactieve T-celklonen. Na activatie produceren deze T-cellen grote hoeveelheden pro-inflammatoire cytokines, die vervolgens de productie van anti-inflammatoire cytokines inhiberen. Bovendien zal de activatie van deze T-cellen leiden tot een verhoogde membraanexpressie van ICAM-1, wat de binding aan het endotheliale membraan van de bloed-hersen-barrière (BBB) en de migratie naar het CZS mogelijk maakt. In het CZS kunnen de myeline reactieve T-cellen dan opnieuw geactiveerd worden en een inflammatoire auto-immuunreactie opstarten. Hierdoor kunnen oplosbare adhesiemoleculen vrijgezet worden, die een maat zijn voor de beschadiging van de BBB. Toch moet vermeld worden dat sommige van deze immunologische veranderingen ook optreden in de gezonde controle personen. Dat wijst erop dat regulatorische mechanismen - die mogelijk defect zijn bij MS-patiënten - een rol spelen bij het onderdrukken van deze potentieel pathogene responsen. Daarom is verder onderzoek noodzakelijk om na te gaan welke van de waargenomen immunologische veranderingen effectief betrokken zijn bij de pathogenese van MS.

Onze resultaten tonen verder aan dat de anti-myeline T-celreactiviteit zeer heterogeen is en gekenmerkt wordt door fenomenen als determinant spreiding en shifting. Deze bevindingen hebben belangrijke implicaties voor de ontwikkeling van antigenspecifieke therapieën voor MS.

Met deze studie toonden we aan dat de autoreactieve immuunrespons zeer dynamisch is bij MS-patiënten en dat verschillende immunologische parameters zouden kunnen aangewend worden als paraklinische merker. Een combinatie van meerdere van deze merkers kan mogelijk leiden tot accuratere informatie over de ziekteactiviteit bij MS.

## Implicaties van onze bevindingen voor T-celvaccinatie

De bevindingen beschreven in dit werk kunnen van groot belang zijn voor de verdere ontwikkeling van therapieën voor MS, zoals de T-celvaccinatie. Vroeger werd algemeen aangenomen dat de identificatie van "het" MS autoantigen onontbeerlijk is voor de ontwikkeling van een specifieke en doeltreffende behandeling voor MS. Recente data en de hier beschreven resultaten tonen echter aan dat niet één, maar meerdere autoantigenen een rol kunnen spelen in de immunopathogenese van de ziekte.

Er werd aangetoond dat T-celvaccinatie leidt tot de specifieke onderdrukking van MBP reactieve T-celklonen. T-lymfocyten die specifiek zijn voor andere myeline antigenen (zoals MOG en PLP) zullen echter vermoedelijk aanwezig blijven in het bloed van de MS-patiënten. Deze cellen kunnen betrokken zijn bij de verderzetting van de ziekte. Daarom werd recent besloten om de samenstelling van het T-celvaccin te optimaliseren. Het nieuwe T-celvaccin bestaat uit IL-2 geëxpandeerde CD4+ T-cellen, die geïsoleerd worden uit het cerebrospinaal vocht (CSV) van de MS-patiënten. Het CSV vaccin heeft enkele belangrijke voordelen in vergelijking met het klassieke T-celvaccin. Zo bevat het CSV vermoedelijk T-celpopulaties die actief betrokken zijn in het ziekteproces, vermits dit het compartiment is dat het dichtst in de buurt ligt van de lokale inflammatie in het centrale zenuwstelsel. Studies toonden bovendien aan dat in het CSV niet enkel MBP reactieve, maar ook PLP en MOG reactieve T-cellen terug te vinden zijn. Hierdoor zal het vaccin een grotere groep van mogelijk pathogene T-cellen bevatten.
Het geoptimaliseerde vaccin wordt binnenkort in het Biomedisch Onderzoeksinstituut – Dr. L. Willemsinstituut getest in een dubbelblinde placebo-gecontroleerde studie.

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## Curriculum Vitae

Niels Hellings werd geboren op 9 september 1973 te Hasselt. In 1992 behaalde hij het diploma middelbaar onderwijs Latijn-wiskunde aan het P.H.H.I. te Hasselt, en begon datzelfde jaar zijn universitaire studies aan de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen te Leuven. Zijn eindwerk getiteld "Structurele analyse van de gastheer-specifieke *nodPQ* genen van *Rhizobium* sp BR816" volbracht hij aan het "Centro de Investigación sobre Fijación de Nitrógeno" (U.N.A.M., Cuernavaca, Mexico), in samenwerking met het F.A. Janssens Laboratorium voor Genetica (KULeuven). In juni 1996 verwierf hij het diploma Bio-ingenieur in de Cel- en Genbiotechnologie. Sinds augustus van dat jaar is hij werkzaam aan het Biomedisch Onderzoeksinstituut- Dr. L. Willems-Instituut van het Limburgs Universitair Centrum. Ter voorbereiding van dit doctoraatsproefwerk verrichtte hij gedurende vier jaar onderzoek in het kader van MS op de afdeling Auto-immune Aandoeningen. Tijdens deze periode volgde hij een doctoraatsopleiding, waarvoor begin 2000 een certificaat werd uitgereikt.