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Anti-Myelin T Cells in Multiple Sclerosis: Pathogenic Role and Therapeutic Strategies

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Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen,
te verdedigen door

Annegret VAN DER AA

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

2002

DOCTORAATSPROEFSCHRIFT

Universiteit Maastricht

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A little is a lot where there is little else.

Vladimir Hachinski

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ABBREVIATIONS

A: T cell receptor alpha chain
Ab: antibody
APC: antigen presenting cell
APL: altered peptide ligand
AS: autologous serum
B: T cell receptor beta chain
BDNF: brain-derived neurotrophic factor
BSA: bovine serum albumine
C: constant region of the T cell receptor alpha/beta chain
CD: cluster of differentiation
CDR 2/3: complementary determing region 2/3
CNPase: 2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS: central nervous system
Cpm: counts per minute
CP-MS: chronic progressive multiple sclerosis
CSF: cerebrospinal fluid
D: diversity region of the T cell receptor beta chain
DIG: digoxigenin
DN: double negative
EAE: experimental autoimmune encephalomyelitis
E. coli: Escherichia coli
EDSS: expanded disability status score
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunospot assay
E/T: effector-to-target ratio
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
GA: glatiramer actetate
GALT: gut-associated lymphoid tissue
Gd: gadolinium
HHV-6: human herpes virus-6
HLA: human leukocyte antigen
Hprt: hypoxanthine-guanine phosphoribosyl transferase
IFN-β/γ: interferon-beta/gamma
Ig: immunoglobulin
IL: interleukin
IL-2Rα: alpha chain of the interleukin-2 receptor
J: junctional region of the T cell receptor alpha/beta chain
LDA: limiting dilution analysis
LT: lymphotoxin
MAG: myelin-associated glycoprotein
MBP: myelin basic protein
MHC: major histocompatibility complex
MOBP: myelin oligodendrocyte basic protein
(r)MOG: (recombinant) myelin oligodendrocyte glycoprotein
MRI: magnetic resonance imaging

MS: multiple sclerosis
MSRV: multiple sclerosis-associated retrovirus
NGF: nerve growth factor
NK: natural killer cell
NS: normal subjects
OND: other non-inflammatory neurological disease
OD: optical density
OSP: oligodendrocyte specific protein
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
PCR: polymerase chain reaction
Pd: proton density
PE: phycoerytrin
PerCP: peridinin chlorophyll protein
PHA: phytohaemagglutinin
PLP: proteolipid protein
PMA: phorbol-12-myristate-13-acetate
PP-MS: primary progressive multiple sclerosis
RFLP: restriction fragment length polymorphism
rhIL-2: recombinant human interleukin-2
RR-MS: relapsing-remitting multiple sclerosis
RT-PCR: reverse transcriptase polymerase chain reaction
SEM: standard error of the mean
SI: stimulation index
SP-MS: secondary progressive multiple sclerosis
TCC: T cell clone
TCL: T cell line
TCR: T cell receptor
TCV: T cell vaccination
TGF-β: transforming growth factor-beta
Th: helper T cell
Thy: thymidine
TNF-α: tumor necrosis factor-alpha
Tr: regulatory T cell
TT: tetanus toxoid
V: variable region of the T cell receptor alpha/beta chain
VLA-4: very late antigen-4

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Chapter 1

Myelin-specific T Cells in MS: Pathogenic Role and Therapeutic Strategies - Introduction

1.1 General introduction

1.1.1 Clinical features

Multiple sclerosis (MS) is one of the most common neurological diseases in young adults. This chronic inflammatory demyelinating disease of the central nervous system (CNS) typically manifests between the age of 20 and 40 and affects women twice as often as men[1]. The prevalence is also linked to both ethnic origin and geographical localization[2]. Although the clinical manifestations of MS may be variable and unpredictable, the most common symptoms include chronic or relapsing paralysis, visual and sensory impairment and several other neurological deficits. Since there is no diagnostic test available for MS, the diagnosis is mainly based on clinical examination in combination with magnetic resonance imaging (MRI), evoked potentials and analysis of cerebrospinal fluid (cell counts and oligoclonal bands)[3].

MS is generally categorized as being either relapsing-remitting (RR-MS) or primary-progressive (PP-MS) at onset. The RR form of the disease is characterized by alternating phases of exacerbation (relapse) with (sub)acute neurologic dysfunction, followed by periods of partial or complete recovery (remission) leading slowly to accumulating disability. The primary progressive form of disease lacks the acute attacks and instead typically involves a gradual clinical decline. Over time, in about 40% of the RR-MS patients, the disease course ultimately alters into a progressive form, known as secondary progressive-MS (SP-MS).

1.1.2 Pathology

Pathologically, the disease is characterized by large, multifocal sclerotic lesions or plaques, resulting from a focal loss of CNS myelin. The lesions are dispersed throughout the white matter of the CNS, but they predominantly reside in periventricular regions, optic nerves, brain stem and spinal cord[4]. Sites of active demyelination are characterized by inflammatory infiltrates composed by CD4+ T lymphocytes, activated macrophages or microglia and small numbers of B cells[4;5]. In addition, a variety of immune related molecules such as MHC molecules, cytokines and adhesion molecules are locally expressed or produced in the lesions. Although this process suggests a pivotal role for T cell-mediated inflammation,

formation of demyelinating plaques however, seem to require additional immunological mechanisms. Based on the pathological heterogeneity of the patterns of demyelination, Lassmann and co-workers recently developed a classification system into four different patterns[6;7]. In the macrophage-associated demyelination pattern, toxic products of activated macrophages are mainly responsible for the destruction of the myelin sheath. In the antibody-associated model, demyelination is induced by co-operation between both autoreactive T cells and demyelinating antibodies, as in the MOG-induced EAE model. Lesions with primary oligodendrocyte degeneration and apoptosis are less common and restricted to PP-MS patients, whereas the distal oligodendrogliopathy is commonly found in virus-induced white matter disease. Axonal injury in MS plaques apparently occurs in two stages: first, a high incidence of acute axonal injury is found during the active stage of myelin destruction, accompanied by spontaneous remyelination forming the so called 'shadow plaques'. In addition, there is a low level of continuous axonal destruction and loss in inactive chronic lesions with a lack of remyelination[6;9]. This heterogeneity of MS pathology suggests the involvement of different pathogenic mechanisms in MS patients. This heterogeneity can have important consequences for future designs of novel therapeutic strategies which target the diverse mechanisms of demyelination[10].

1.1.3 Genetics

Evidence for genetic susceptibility to MS stems from family and twin studies. Although the familial clustering of MS cases is inconsistent with a single gene model, population-based studies have clearly documented that first-degree relatives of an affected individual have an increased risk in developing MS compared to the general population[11;12]. Furthermore, differences in clinical concordance between monozygotic (31%) and dizygotic (5%) twins definitely argues for the implication of genetic factors in the disease etiology[13;14]. However, the incomplete penetrance in MS twins reflects the contribution of several genes together with environmental factors to the susceptibility for MS.

Because MS is considered to be a T cell-mediated autoimmune disease targeting the myelin sheaths in the central nervous system, genes that encode all elements involved in T cell activation and myelin components are extensively studied in the search for predisposing genetic factors.

Genetic linkage analysis of MS to genes of the MHC cluster, showed the strongest association with the HLA-DR2 genes[15;16]. In addition, MHC class I alleles have been described to contribute to disease susceptibility, although associations were much weaker[17]. Using highly polymorphic RFLP's as markers for genetic association studies, TCR gene clusters have also been analyzed as candidate genes for MS. Several groups reported linkage between MS and TCR genes[18-20], but others failed to confirm these results[21-23]. Furthermore, linkage analysis of MS to genes of candidate myelin antigens revealed no significant effects of genes encoding MBP, PLP or MAG. However, a potential role of the MOG gene, which resides within the MHC gene cluster, could not be excluded[24;25]. In the previous decade, several genome screens were conducted for the characterization of genes involved in the susceptibility and pathogenesis of MS[26-28]. In different populations, chromosomal regions that potentially harbor MS susceptibility genes were identified. With the exception of the MHC locus on chromosome 6p21, no single locus showed convincing evidence of linkage.

Until recently, most studies of gene expression have been limited to the analysis of a few molecular targets. Some years ago, a new technology for high-throughput screening of differential gene expression has been developed. These so called 'cDNA microarrays' allow for a simultaneously analysis of thousands of genes. In MS research, expression profiles of normal white matter and acute lesions of the brain of MS patients and EAE mouse models have been compared[29-32]. Both osteopontin, an early T cell activation gene classified as a Th1 cytokine, and 5-lipoxygenase, a key enzyme in the biosynthesis of proinflammatory leukotrienes, have been found to be upregulated in MS lesions and EAE brain. These genes might play an important role in the disease pathogenesis[31;32]. Further applications of the microarray technique will most likely provide new insights in the pathogenesis of MS, leading to new targets for therapy.

1.1.4 Role of infectious agents in disease etiology

Although the etiology of MS is still not fullly understood, there is large body of evidence that it is a T cell-mediated autoimmune disease, influenced by both genetic and environmental factors. Based on the remarkable uneven geographical distribution and the occurrence of MS epidemics, it has been proposed that the disease is caused by one or more environmental infectious agents[33].

While many viruses, including rabies, human herpes virus and measles have been postulated as potential candidate viral agents in MS, no virus has been definitely shown to be associated with the disease[34;35]. Recently, much interest has focused on human herpes virus-6 (HHV-6)[36]. Based on increased HHV-6 antibody titers[37;38], demonstration of HHV-6 DNA in MS brain tissue[39] and exclusive detection of virus DNA in the CSF and sera of MS patients but not in control subjects[40], a putative role for this herpes virus has been proposed in MS. However, other studies failed to confirm these observations[41-44]. Furthermore, Perron and co-workers identified a novel retrovirus called 'MS-associated retrovirus' (MSRV)[45]. Extracellular virus particles could be isolated from both serum and CSF of about 50% of the MS patients, while only few of the control subjects were found positive[46]. However, the exact contribution of this retrovirus in the MS etiology remains unclear.

Although no MS-specific virus has been identified so far, this does not exclude the possibility that infectious agents may trigger the disease. It is possible that myelin-reactive T cells become activated in the periphery after recognition of cross-reactive viral epitopes that share sequence homologies with self-peptides of myelin antigens[47]. Alternatively, a transient viral infection in the CNS may cause minor damage to oligodendrocytes, leading to the release of myelin epitopes and subsequent activation of myelin-reactive T cells[48]. In this so called 'hit and run hypothesis', virus persistence in the CNS may not be necessary for the continuation of the disease process[49].

1.2 Immunopathogenesis of multiple sclerosis

1.2.1 Activation

Since MS is an organ-specific disease, predominant expression of autoantigens, the myelin components, occurs in the central nervous system (CNS), thereby assuming that these antigens are normally sequestered from the immune system by protection via the blood-brain barrier. Although, this hypothesis is not completely correct, since all individuals appear to harbor potentially autoreactive T cells in the periphery, these cells remain innocuous unless they become activated. Several possible mechanisms have been proposed as a way by which the autoimmune pathology may be initiated. According to the model of 'molecular mimicry', autoreactive T cells may be activated by cross-reactivity with infectious agents that share immunological epitopes with the autoantigen. Initially, sequence homology between foreign (viral or bacterial) and self proteins was considered a requirement for molecular mimicry to occur[47]. But recent studies have shown that antigen recognition is much more 'degenerate' and even completely unrelated peptide sequences may lead to cross-recognition by T cells[50-52]. This means that this mechanism most likely is a physiological and frequent event and that molecular mimicry will trigger an autoimmune disease only in the right context. Furthermore, autoreactive T cells can be activated by microbial superantigens. Based on the recognition of particular TCR BV genes, a relatively large proportion of the T cell repertoire may become activated[53;54]. A third mechanism by which myelin-specific T cells are thought to be activated is through 'bystander activation' after infectious tissue damage leading to a powerful proinflammatory microenvironment that generates novel T cell specificities to local self antigens[55].

1.2.2 Regulation

Although the presence of potentially pathogenic autoreactive T cells in normal healthy individuals is a common feature, autoimmunity occurs relatively rarely. A number of passive mechanisms have been proposed to account for the maintenance of peripheral self-tolerance, including T cell anergy and deletion and immunological ignorance[56]. In addition, a T cell-mediated dominant control mechanism also plays a key role in regulating autoimmunity[57;58].

In the animal model for MS, it has been demonstrated that the activation of regulatory Th2 cells suppresses EAE via the secretion of IL-10[59]. Sun and co-workers also characterized a MBP-reactive CD8+ T cell subset that inhibits EAE in Lewis rats[60;61] and Singh and co-workers demonstrated that mice are protected from EAE after activation of NK T cells[62].

One population of recently defined regulatory T cells are the naturally anergic and suppressive CD4+CD25+ T cells[63;64]. A number of characteristics have been described for the murine CD4+CD25+ cells that might provide insight into their mechanism of action. These regulatory cells suppress proliferation of co-cultured CD25- T cells *in vitro* by inhibiting IL-2 production, and are themselves unable to secrete IL-2. Furthermore, they constitutively express CTLA-4, a molecule that inhibits T cell activation. In different recent reports, the existence of CD4+CD25+ regulatory cells in healthy individuals was described with characteristics similar to those of murine CD4+CD25+ T cells[65;66]. In addition to this regulatory T cell population, NK T cells have also been studied for their putative regulatory function, mainly based on cytokine production. NK T cells are a discrete subset of T cells sharing characteristics of both T cells and NK cells. Their uniqueness is the expression of an invariant TCR restricted by MHC class I molecules, namely CD1d. Such NK T cells produce large amounts of various cytokines including IL-4 and IL-10[67]. Although the link between NK T cell dysfunction and autoimmune diseases has been studied most extensively for insulin-dependent diabetes mellitus, it has been shown recently that the frequency of NK T cells is reduced both in the peripheral blood and in the lesions of MS patients as compared to other neurological autoimmune inflammatory diseases[68]. Also T_R1 cells and Th3 cells usually mediate their suppressive activities through the release of the cytokines TGF-β and IL-10. Furthermore, γδ T cells and CD3+CD4-CD8- are capable of regulating self-tolerance[69;70].

Based on the results of T cell (receptor peptide) vaccination studies both in EAE and MS, it has been demonstrated that at least two types of immunoregulatory T cells are involved in the suppression of activated pathogenic T cells. Both anti-idiotypic T cells, directed against specific regions of the T cell receptor, and anti-ergotypic T cells, recognizing activated T cells in general are part of the regulatory network. The hypervariable CDR3 and the less variable CDR2 sequences of the idiotypic T cell receptor (TCR) are the major targets of both cytolytic CD8+ MHC class I restricted and cytokine producing CD4+ MHC class II restricted anti-clonotypic T cells[71-73]. Processing and presentation of peptides of cell surface molecules,

such as TCR peptide determinants, are most often presented by MHC class I molecules. Alternatively, TCR released from dying T cells could also be presented in the context of MHC class I molecules by professional antigen presenting cells after extracellular uptake and internal processing[74;75]. Although TCR determinants may be the predominant targets, additional surface molecules may also contribute to the enhancement of the peripheral regulatory networks. Immune responses directed at activation markers common to all CD4+ T cells may also play a role in the suppression of activated T cells following T cell vaccination[76].

In conclusion, it has been proposed that protection of healthy individuals involves several layers of regulation, which consists of different types of regulatory cells, each potentially operating at different stages of the T cell-mediated immune response. In MS, alterations in these immunoregulatory mechanisms could lead to the suboptimal suppression of activated pathogenic T cells and this may finally result in autoimmunity[77].

1.2.3 Migration

Once T lymphocytes are activated, they can readily penetrate the blood-brain barrier[78]. The migration process through the endothelium is promoted by the release of proinflammatory cytokines and expression of chemokines and their receptors. Both T cells and capillary endothelial cells in the CNS upregulate the expression of adhesion molecules and after a sequential interaction, myelin-specific T lymphocytes can extravasate[79-81]. Subsequently, the cells must pass a barrier of extracellular matrix before they can enter the CNS. Therefore, the production of matrix metalloproteinases is crucially important to cleave matrix proteins[82;83]. Furthermore, recent studies have extended the targets of this family of enzymes to include myelin basic protein and membrane-anchored TNF-α[84-86]. Thus, beside their key role in opening the blood-brain barrier, these enzymes can perpetuate the inflammatory responses by generating immunogenic peptides and releasing proinflammatory cytokines such as TNF-α.

Once the immune cells have spread to the white matter of the CNS, the autoreactive T cells encounter their specific myelin epitopes presented by resident microglia cells or perivascular macrophages[87]. They become reactivated and through the release of proinflammatory cytokines they give rise to a cascade of immune reactions. Subsequently,

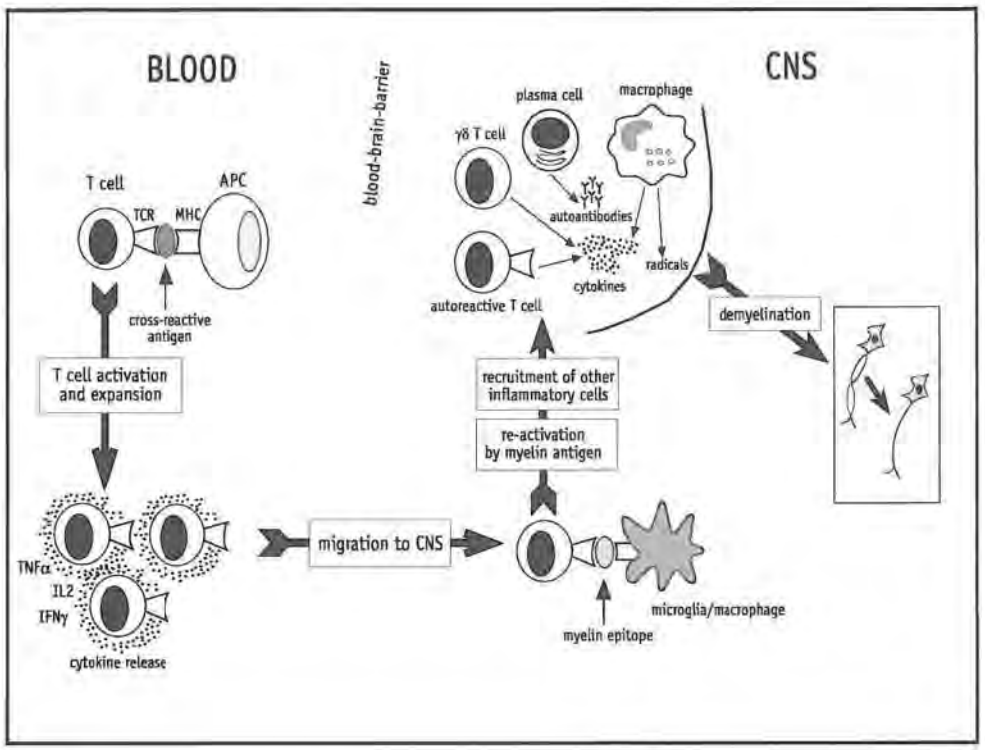

Figure 1.1. *Current concepts of the multiple sclerosis pathogenesis*[102]

T cells reactive to myelin components are postulated to become activated in the periphery. When activated, they acquire functional changes that enable them to traffic into the CNS. If they encounter an appropriate myelin antigen, T cells become reactivated with subsequent induction of inflammation and recruitment of other effector cells. Demyelination is mediated by (molecules secreted by) T cells, macrophages, plasma cells and γδ T cells.

TCR: T cell receptor; APC: antigen presenting cell; MHC: major histocompatibility complex; CNS: central nervous system; TNF-α: tumor necrosis factor alpha; IL-2: interleukin-2; IFN-γ: interferon gamma.

upregulated MHC class II molecules and adhesion molecules on astrocytes, microglia, and on the CNS endothelium, facilitate the further influx of T cells, B cells, macrophages and complement proteins to the site of inflammation. A concerted autoimmune attack by cytotoxic CD8+ and γδ T cells[88;89], demyelinating antibodies[90;91], complement activation, toxic effects of cytokines[92;93] and free radicals[94] produces areas of demyelination along the axons. In addition, along the chronic inflammation, autoreactive T cells and resident antigen presenting cells release vast amounts of glutamate, an excitatory neurotransmitter. After

binding to specific receptors on oligodendrocytes and neurons, glutamate mediates toxicity and subsequent death of the myelin-producing cells and the underlying axon leading to axon loss and atrophy of the brain and spinal cord during the degenerative phase of the disease[95].

1.2.4 Benign autoimmunity

Although CNS autoimmunity against myelin proteins is known to be a crucial factor in the pathogenesis of MS, recent studies indicate that T cell autoimmunity to specific CNS antigens may also have a protective function[96;97]. Moalem and co-workers demonstrated that anti-MBP T cells protect neurons from secondary degeneration after a partial crush of the optic nerve[98]. Furthermore, the experiments were extended to study the effects of anti-MBP T cells after spinal cord injury. Again, enhanced recovery from CNS trauma was shown[99]. One of the possible mechanisms of the neuroprotective autoimmune T cells could be the local release of neurotrophins after antigen recognition at the site of damage. Indeed, the anti-MBP T cells can secrete neurotrophins in response to MBP[100]. Moreover, a recent paper demonstrated that activated human T cells, B cells and monocytes secreted bioactive brain-derived neurotrophic factor (BDNF)[101]. The production of this neurotrophin is increased upon antigenic stimulation of T cell lines specific for both MBP and MOG, and expression of BDNF by several types of infiltrating immune cells was found at different sites in inflammatory lesions of patients with MS[101]. This concept of neuroprotective autoimmunity has obvious implications for therapy of MS. During immunomodulatory treatment, it will be important to preserve or even enhance the proposed neuroprotective function of this population of 'benign' autoreactive T cells and eliminate the autoaggressive offenders[97].

1.3 Pathogenic role of myelin-specific T cells

1.3.1 Evidence from EAE, the animal model for MS

Experimental autoimmune encephalomyelitis (EAE), the animal model of MS, is characterized by focal areas of inflammation and demyelination. Disease can be actively induced by immunization with several components derived from the myelin sheath. In different strains of rodents and nonhuman primates, MBP and PLP, the most abundant proteins of the CNS, have been extensively studied as powerful encephalitogens[103]. Moreover, adoptive transfer experiments revealed that the initiation of clinical disease in EAE is dependent on activated myelin-specific T cells, reinforcing the concept that an autoimmune T cell response to myelin is crucial in the CNS pathology[104-107].

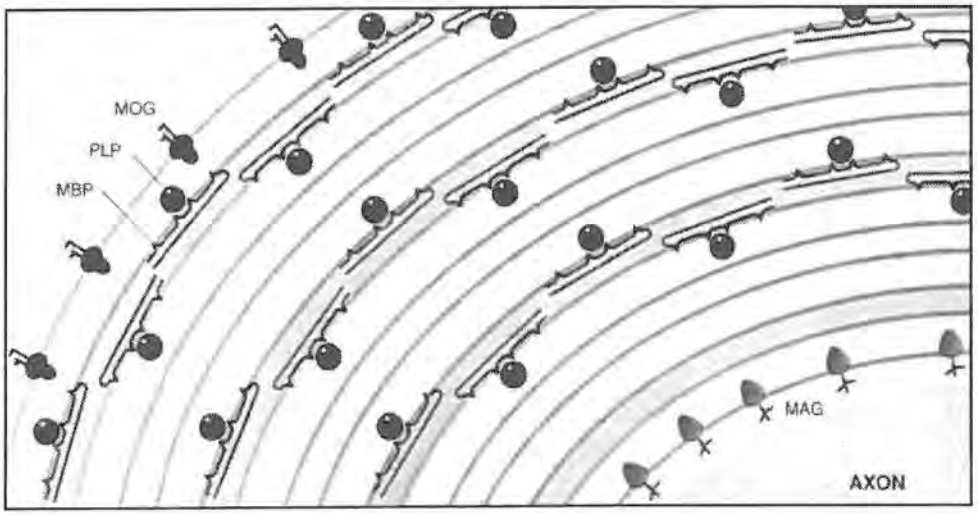

Figure 1.2. *Cross sectional representation of the myelin sheath*[108]

1.3.2 Components of the CNS myelin: candidate antigens for MS (figure 1.2)

MS is considered to be a CNS-specific disease and destruction of the myelin sheaths is a central event in the pathogenesis. The multilayer structured myelin is synthesized by oligodendrocytes[109], consists of 20 to 25% proteins and 70 to 80% lipids[110] and may contain candidate autoantigens eliciting the pathogenic autoimmune response. The cytoplasmatic myelin basic protein (MBP) and the highly hydrophobic proteolipid protein (PLP) are the

two most abundant myelin proteins, comprising approximately 80% of the CNS myelin proteins. In addition, other minor myelin proteins such as myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG)[111] may also be important in the formation and stability of the myelin structure. 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), phosphatidylinositol-specific phospholipase C and protein kinase C are cytoplasmatic proteins involved in posttranslational modifications and signal transduction. Other recently described CNS antigens include myelin oligodendrocytic basic protein (MOBP)[112], oligodendrocyte specific protein (OSP)[113], αβ crystallin[114] and S100β[115].

In the next chapters, the relevance of two of the most extensively studied myelin components, MBP and PLP, is discussed together with the potential role in the immunopathogenesis of MOG, a minor component of the myelin.

1.3.3 MBP- and PLP-reactive T cells

Frequency and activation status

Several studies have clearly demonstrated that MBP- and PLP-reactive T cells can be isolated from the peripheral blood of both MS patients and healthy individuals at similar frequencies[116-120]. Thus, the mere presence of autoreactive T cells in the periphery is insufficient for the development of autoimmune disease. If myelin-reactive T cells are involved in the MS pathogenesis, these cells must differ in some way from those found in normal controls. Zhang[121] and Chou[122] demonstrated independently higher precursor frequencies of MBP- and PLP-reactive T cells expressing the IL-2 receptor in the peripheral blood and cerebrospinal fluid of MS patients, suggesting an enhanced state of activation compared to those isolated from normal individuals. Furthermore, several other studies, using different experimental approaches, such as ELISPOT assays and identification of somatic mutations with the *hprt* marker gene, showed similar results[123-126]. Two recent reports also demonstrated that the activation of MBP-reactive T cells from the peripheral blood of MS patients is less dependent on co-stimulation by the B7.1 and B7.2 molecules, expressed on the surface of activated antigen presenting cells[127;128]. Taken together, these data indicate that myelin-specific T cells in MS patients are in an enhanced state of activation.

Epitope specificity and T cell receptor V gene usage

In contrast to the limited epitope fine specificity and the restricted TCR usage in MBP-induced EAE[129], the human T cell response to both MBP and PLP is more complex. Although multiple epitopes have been identified, MBP epitopes 84-102 and 143-168 are more frequently recognized by T cells from MS patients[130;131]. For PLP, immunodominant recognition of the epitopes 30-49, 40-60, 180-199 and 190-209 have been demonstrated in human PLP-specific T cells, both in MS patients and healthy controls[119;120;132;133]. Since their is clear evidence that MBP- and PLP-reactive T cells are activated *in vivo*, studies on TCR V gene usage might provide information on the heterogeneity of these T cell populations. Correale and co-workers reported heterogeneous PLP-specific T cell populations in MS patients and healthy controls[134]. In contrast, although the TCR V gene usage of MBP-reactive T cells has been shown to vary between patients with MS[135;136], V gene rearrangements are more restricted in individual patients. This restricted T cell repertoire is related to clonal expansion after *in vivo* activation, as demonstrated by identical CDR3 region sequences of the clones[137]. Furthermore, longitudinal studies have shown a long-term persistence of these T cells in the periphery[138-141]. Although limited clonal expansion was also observed in some healthy controls, the TCR repertoire was more heterogeneous as compared to MS patients[137;138].

In general, no correlation could be found between human TCR V gene usage and MS. However, two reports demonstrated a shared TCR sequence motif, either in plaque-derived T cells of HLA DR2+ patients[142] or in MBP 83-99-specific T cells from different MS patients[143].

Cytokine profiles

T cells mediate several effects via secretion of different cytokines. Moreover, CD4+ T cells have been classified according to the cytokine profiles that they produce upon stimulation. Proinflammatory Th1 cells produce predominantly IFN-γ, IL-2, TNF-α and lymphotoxin (LT), whereas CD4+ T cells secreting IL-4, IL-5, IL-10 and IL-13 have been defined as anti-inflammatory Th2 cells[144].

In the EAE model, there is a clear Th1/Th2 paradigm: Th1 cells specific for MBP or PLP can transfer EAE and thus are encephalitogenic. In contrast, Th2 cells are protective[145;146].

However, defining MS as a Th1 disease has been less straightforward. Two independent reports suggest that in MS patients and control subjects, the majority of myelin-reactive T cells secrete both Th1 and Th2 cytokines[147;148]. Interestingly, MBP-reactive T cells from MS patients secreted higher amounts of TNF-α and IL-2, the proinflammatory cytokines which are considered to be pathogenic in EAE. Recent studies have confirmed this Th1 bias of MBP- and PLP-specific T cells from MS patients[149-151]. Moreover, Correale and co-workers demonstrated that cytokine profiles produced by PLP-reactive T cell clones from MS patients were related to different clinical stages of disease: during exacerbations, T cell clones secreted cytokines resembling a Th1-like profile, whereas during remission the majority of the T cell clones were Th0, Th1 or Th2[134]. IFN-γ and TNF-α are also found in MS plaques, but the interpretation of these data is more complicated, since the cellular source of these cytokines is more difficult to define[92;93].

1.3.4 Myelin oligodendrocyte glycoprotein (MOG)-reactive cells

MOG: structure and possible functions

Myelin oligodendrocyte glycoprotein (MOG) is a CNS-specific quantitatively minor myelin component accounting for only 0.01-0.05% of the total myelin protein. The MOG gene is enclosed within the distal region of the MHC locus, and therefore closely linked to a gene locus associated with MS[152-154]. Cloning of cDNA for MOG from different species has revealed that the highly conserved mature protein is 218 amino acid in length[155-157]. This member of the immunoglobulin superfamily is identified as a type I membrane glycoprotein, located preferentially in the outhermost lamellae of the myelin sheaths and extracellular surface of oligodendrocytes[158-160] (figure 1.3).

Although the function of MOG is currently unknown, the extracellular location and expression late in development might suggest that MOG plays a role as an adhesion molecule, connecting neighboring myelinated fibers and subsequent compacting the myelin sheath[161]. Furthermore, Dyer and Matthieu proposed that MOG may regulate the microtubule turnover in oligodendrocytes, which is a crucial process during myelination[162]. Finally, it has been demonstrated that CNS-specific MOG can bind the C1q component of complement[163]. If this interaction implicates the activation of the complement cascade, this mechanism may also contribute to the CNS-specific demyelination process in MS.

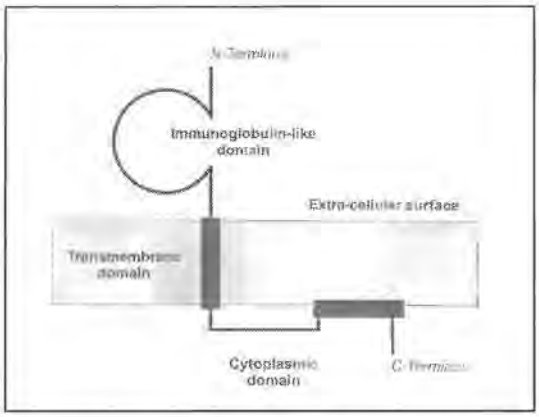

Figure 1.3. *The proposed MOG structure and organization in the myelin membrane*[158]

Autoantibody-mediated demyelination in the EAE model

Although several myelin, as well as non-myelin proteins were shown to induce EAE in experimental animals, MOG is unique in that it is the only CNS autoantigen known to induce in EAE both an encephalitogenic T cell response and a demyelinating autoantibody response. First indications for a MOG-mediated demyelination were found after induction of EAE with whole myelin in a guinea pig model[164]. These observations were subsequently confirmed by Linington and co-workers, demonstrating that injection of MOG-specific antibodies in MBP-induced EAE resulted in an enhanced inflammatory response with dramatically increased disease severity associated with an extensive demyelination[165]. However, it should be noted that the T cell response is critical in the initial stages of EAE induction, involving CNS inflammation and disruption of the blood-brain barrier. Only then, MOG-specific antibodies can penetrate the CNS and trigger effector mechanisms to attack the myelin membrane[166;167].

Encephalitogenic T cell epitopes involved in the pathogenesis have been identified in several strains of rodents and nonhuman primates[168;169]. In many different mouse strains, EAE can be induced using (the extracellular domain) of MOG, synthetic MOG peptides (MOG 35-55, MOG 79-96 and MOG 92-106) as well as MOG-specific T cells (MOG 35-55 and MOG 92-106)[161;170-176]. Lewis rats are poorly susceptible to MOG-induced EAE with a minimal peptide-specific T cell response to MOG 1-20 and MOG 35-55[177;178]. In contrast, in DA rats, MOG 74-90 and MOG 93-107 are highly encephalitogenic epitopes by T cell transfer, but only MOG 93-107 induces EAE after active immunization[179]. Both nonhuman primate models, the rhesus

monkeys and common marmosets, are susceptible to MOG-induced EAE. A study with rhesus monkeys identified three immunodominant T cell epitopes MOG 4-20, MOG 35-50 and MOG 94-116[180]. Von Büdingen and co-workers reported multiple encephalitogenic MOG epitopes in marmosets[168]. Furthermore, it has been shown that immunization with MOG 14-36 will induce EAE in all animals[181] and a recent study reported the encephalitogenic properties of T cells reactive to MOG 21-40 generated from naive marmosets[182].

In conclusion, the synergy between encephalitogenic T cells and autoantibody responses results in a chronic inflammatory disease associated with extensive CNS demyelination in different animal species[183-185]. Moreover, similar immunopathological characteristics (deposition of immunoglobulin and neo-C9 and patterns of myelin vesiculation) are also observed in a subset of MS patients, indicating that these effector mechanisms may also participate in lesion formation in the human disease[6;10;91;186].

Human B and T cell response against MOG

It has been established that anti-MOG antibodies play a significant role in mediating demyelination in various models of EAE and subsequent studies have reported anti-MOG B cell responses in MS patients. In humans, elevated levels of anti-MOG IgG antibodies have been observed in the cerebrospinal fluid (CSF) of MS patients as compared to healthy controls[187]. Furthermore, both in CSF and peripheral blood (PB), higher frequencies of anti-MOG IgG secreting B cells have been demonstrated by Sun and co-workers[188]. However, recent studies indicate that these increased frequencies of anti-MOG antibodies are not specific for MS: similar titres were found in patients with other (inflammatory) neurological diseases[189;190]. Lindert and co-workers showed an association between the clinical course and the number of anti-MOG antibody positive samples[130], but these results were not confirmed [190]. A recent report on the epitope specificity of MOG antibodies in sera of MS patients and healthy controls, demonstrated a heterogeneous response and no immunodominant epitope[191].

Two studies demonstrated an increased proliferative reactivity of circulating T cells to MOG as compared to other myelin antigens such as MBP, PLP and MAG, indicating a dominant MOG T cell response[192;193]. In 1991, Sun and co-workers observed a higher frequency of IFN-γ

secreting cells after stimulation with native MOG both in the CSF and PB of MS patients as compared to healthy controls[188]. These findings were confirmed in another study testing primary proliferative responses to recombinant MOG (rMOG), corresponding to the extracellular domain[192]. Using a panel of overlapping synthetic peptides spanning this domain, T cell reactivity was shown to be predominantly directed against three main regions 1-22, 34-56 and 64-96, which also contain encephalitogenic T cell epitopes for a number of animal models as described previously[194]. The presence of an immunodominant epitope within the region 64-96 is supported by an increased response to MOG 63-87 in DR2+ MS patients as evaluated by ELISPOT[195]. However, three recent studies could not confirm the increased anti-MOG T cell reactivity[130;193;196]. Furthermore, analysis of a panel of MOG-reactive T cell lines revealed a heterogeneous T cell response to MOG with respect to epitope reactivity and cytokine response[130].

Tabel 1.1. Schematic overview of MOG-reactive T cell responses in MS

Publication	Technical approach	Antigen	Frequency of anti-MOG T cells
Sun *et al.*, 1991	ELISPOT	Native MOG	MS > NS[1] CSF > PB[3]
Kerlero de Rosbo *et al.*,1993	Proliferation *5 days*	Native MOG MBP/PLP/MAG	MS > NS[1] MOG > MBP/PLP/MAG[4]
Kerlero de Rosbo *et al.*, 1997	Proliferation *5 days*	rMOG MOG peptides	MS > NS[1] Epitopes: 1-22, 34-56, 64-96
Wallström *et al.*, 1998	ELISPOT	MOG peptides	MS > NS[1] in DR2+ MS epitope: 63-87
Ewing and Bernard, 1998	Proliferation	Native MOG	MS = NS[2]
Diaz-Villoslada *et al.*, 1999	Proliferation *3 days*	rMOG MBP/PLP	MS = NS[2] MOG > MBP/PLP[4]
Lindert *et al.*, 1999	Proliferation *5 days* *T cell lines*	rMOG MOG peptides	MS = NS *Heterogeneous: MS = NS[2]*
Hellings *et al.*, 2001	ELISPOT	MBP/MOG (peptides)	MS = NS[2]

[1]Higher frequency in MS patients compared to normal subjects (NS); [2]Similar frequency in MS patients and normal subjects (NS); [3]Higher frequency in cerebrospinal fluid (CSF) compared to peripheral blood (PB); [4]Higher frequency of anti-MOG T cells compared to other myelin antigens.

1.4 Therapies for MS

1.4.1 Approved therapies

Beside the nonspecific immune suppressive agents that have been used since long to counter the inflammatory reaction and are considered a first line treatment for relapses, five drugs have obtained regulatory approval to modify the course of MS. Three preparations of interferon-β and glatiramer acetate have shown efficacy in relapsing-remitting (RR)-MS. Recently, mitoxantrone (Novantrone™) was approved for secondary progressive and progressive relapsing MS based on results of a randomized multicenter clinical trial[197].

Interferon-β

Interferon-β (IFN-β) is a type I interferon with antiviral properties. Currently, two forms of recombinant IFN-β are available. IFN-β1a (Avonex™ and Rebif™) is a glycosylated preparation with amino acid sequences identical to natural human IFN-β. IFN-β1b (Betaseron™) is produced by *E.coli*, is nonglycosylated and has one amino acid substitution on position 17 (Cys to Ser). Beneficial effects, based on the results of multicenter, placebo-controlled, double-blind clinical trials led to FDA approval for the treatment of RR-MS patients[198-200]. All three preparations of IFN-β induce a reduced relapse rate, slow disability progression and reduce the number of active brain lesions as measured by MRI. However, in all three phase III clinical trials, neutralizing antibodies to IFN-β and side effects (transient flu-like symptoms and skin necrosis at injection site) were reported[201]. Subsequently, several other studies have been conducted to test the long-term effect of IFN-β treatment (PRISMS[202] and Champions study) and the efficacy in patients experiencing a first clinical demyelinating episode (CHAMPS[203] and ETOMS study[204]). Furthermore, IFN-β has been tested in patients with SP-MS with varying results[205;206].

Because IFN-β induces expression of many genes, the mode of action in MS is likely complex[207]. Possible mechanisms include an inhibition of autoreactive T cells[208], inhibition of MHC class II expression with reduced antigen presentation within the CNS[209], inhibition of metalloproteases[210;211] or altered expression of cell-associated adhesion molecules leading to reduced migration to the CNS[212]. Furthermore, IFN-β treatment induces the production of

immunosuppressive cytokines[213] and inhibition of proinflammatory cytokines[214], leading to reduction of the inflammatory process.

Glatiramer acetate (GA, Copolymer-1, Copaxone™)

Glatiramer acetate (GA) is a standardized, randomized mixture of polypeptides consisting of L-glutamine, L-lysine, L-alanine and L-tyrosine, initially developed to mimic MBP, thereby presuming to induce EAE. Unexpectedly, the drug had both suppressive and protective effects in EAE-induced animals of different species[215-217]. Results of clinical trials demonstrated that treatment with GA significantly and beneficially alters the course of RR-MS in a well-tolerated fashion, leading to FDA approval in 1996[218;219]. Subsequent studies confirmed these primary trials, indicating a reduction in relapse rate, extended time to first exacerbation, a reduced number of Gd-enhancing lesions and a decrease in brain atrophy[220-223]. Currently, oral administration of GA is tested in a phase III double-blind, placebo-controlled clinical trial evaluating both clinical and MRI effects in RR-MS patients (Coral study). Furthermore, in a recent study, primary-progressive MS patients are receiving the drug daily for 3 years and efficacy, tolerability and safety will be evaluated ('Promise study').

Although the mode of action has not been completely elucidated yet, four major mechanisms have been proposed[224-226]. First, GA can compete with several myelin antigens for binding to MHC class II molecules[227]. Furthermore, competition at the level of the T cell receptor ('TCR antagonism') between the complex of MBP-derived peptides with MHC class II molecules and the complex of GA with MHC class II molecules may occur[228]. However, these two mechanisms are unlikely to play a role *in vivo*, since GA is quickly degraded to free amino acids and small oligopeptides, so it can not reach the CNS where it could compete with the relevant autoantigens for MHC binding. Partial activation after a specific TCR engagement by GA may induce anergy in MBP-specific T cells[228]. In addition, it has been clearly demonstrated that GA treatment induces a shift of Th1 to Th2 in GA-reactive cells *in vivo*. These cells act as regulatory cells after crossing the blood-brain barrier and reactivation *in situ* by MBP, thereby releasing anti-inflammatory cytokines, leading to bystander suppression[229-231]. These two latter mechanisms can occur *in vivo*, thereby contributing to the clinical effects of the drug.

Table 1.2. Overview of currently performed T cell- and antigen-based experimental treatments

Therapeutic approach	Therapeutic agent	Clinical trial phase
T cell-based		
T cell vaccination	PB-derived MBP-T cell clones	II
	PB-derived whole bovine myelin-T cell lines	I/II
	PB-derived MBP/PLP/MOG-T cell lines	I/II
	CSF-derived activated CD4+ T cells	II
TCR peptide vaccination	peptide cocktail: BV5S2 – BV6S5 – BV13S1	I/II
Other T cell target molecules	anti-CD4 antibody (cM-T412 and BF5)	II
	anti-CD40-ligand antibody (co-stimulation)	I
	CTLA-4-Ig fusion protein (co-stimulation)	NT
	anti-VLA-4 antibody (migration)	I/II
Antigen-based		
Oral tolerance	bovine myelin/glatiramer acetate	III
Altered peptide ligand	peptide based on MBP 83-99 (NBI5788)	II
	peptide based on MBP 83-99 (CPG77116)	II
Other antigen-based strategies	MBP peptides based on MBP peptide 82-99	II
	soluble HLA-DR2:MBP 84-102 complex	I
	MBP-PLP fusion protein	NT
Gene therapy	*delivery of autoantigens, TCR BV peptides, cytokines, growth or neuroprotective factors... through*	
	plasmids or viral vectors	NT
	autoreactive T cells/oligodendrocytes	NT

NT: not tested in MS clinical trials to our knowledge; TCR BV: T cell receptor variable region of the beta chain; VLA-4: very late antigen-4; HLA: human leukocyte antigen; CD: cluster of differentiation.

1.4.2 Experimental therapies (overview in table 1.2)

1.4.2.1 T cell-based therapies

T cell vaccination

The concept of T cell vaccination (TCV) is, at least partially, analogous to classical vaccination against infectious disease. However, the agents to be eliminated or neutralized are not foreign microbial agents but a pathogenic autoreactive T cell population[232]. Initial experiments in rats demonstrated that immunization with attenuated encephalitogenic T cells protects naive animals from a subsequent attempt to induce EAE[233] and induces remission of autoimmune disease[234]. Additional experiments in EAE showed that TCV induces regulatory networks that specifically suppress vaccine T cells by activating T cells specific for the TCR ('anti-idiotypic response')[235;236]. Furthermore, after immunization with attenuated (but recently activated) T cells, 'anti-ergotypic responses' were demonstrated by regulatory T cells that recognize accessory signals, which appear to be activation markers on the vaccine cells[76;237].

Based on the results of successful treatment of TCV in animal models, our group conducted a pilot trial in a small number of MS patients. Eight patients with relapsing-remitting or chronic progressive MS were immunized three times with activated and subsequently irradiated autologous MBP-specific T cell clones at intervals of two to four months[74;238;239]. This study demonstrated that subcutaneous inoculations of autologous vaccine clones are well tolerated and cause no adverse effects. Clinical data suggest a moderate clinical improvement in some of the RR-MS patients with respect to reduced rate of exacerbations, stabilization of EDSS scores and MRI data on brain lesions[239]. Administration of the vaccines induced an anti-idiotypic T cell response, specifically recognizing the vaccine clones, accompanied with a progressive depletion of circulating MBP-reactive T cells in all patients[238]. The vast majority of anti-clonotypic T cells were cytotoxic CD8+ T cells , which lyzed the vaccine cells in a MHC class I restricted fashion, recognizing the CDR2 and CDR3 regions of the TCR[73]. A long-term follow-up study revealed that in most of the patients MBP-reactive T cells remained undetectable for one to two years after vaccination. However, after an additional period of one to three years, MBP-reactive T cells reappeared in 5 MS patients, which coincided with clinical relapses in two patients. Moreover, the isolated T cell clones possessed similar functional properties with regard to cytokine profile, cytotoxic potential

and epitope reactivity but had a different clonal origin from the MBP-reactive T cells identified prior to vaccination. In subsequent rounds of TCV, these reappearing T cell clones could again effectively be depleted[240]. Interestingly, despite the reappearance of new clones in some patients, the original vaccine clones remained undetectable in all patients, indicating that TCV induces long-term anti-clonotypic immune responses.

More recently, 49 MS patients were treated in an extended open label phase I trial to study safety, clinical effects and cellular and humoral immune responses in a larger group of patients[241]. A detailed analysis of the immune response towards vaccine cells revealed that CD8+ T cells displayed direct (cytolytic) anti-idiotypic effects, while CD4+ T cells predominantly induced production of cytokines[242]. To a lesser extent, expansion of γδ T cells and NK cells was observed upon stimulation with the vaccine, suggesting that these cells also play a role in the regulatory T cell network[69]. In contrast, no major antibody responses towards the vaccine clones were detected in the vaccinated patients, although in one patient a transient humoral response was observed[242].

Zhang and co-workers carried out a similar extended preliminary clinical trial using an identical protocol in 54 MS patients (28 RR-MS, 26 SP-MS). This study confirms that vaccination with autoreactive MBP-T cells induces immune responses, resulting in the depletion or suppression of circulating MBP-reactive T cells[72;243]. Clinical results indicate that these enhanced immune responses coincided with a prolonged time to progression in both RR- and SP-MS patients as compared with the natural history of MS[244].

Correale, Weiner and co-workers performed a pilot trial on TCV treatment using bovine myelin-reactive T cell lines as vaccines in 4 SP-MS patients[245]. Immunological data showed a progressive decline of circulating whole myelin-reactive T cells. After vaccination, cytotoxic CD8+ and CD4+ T cells recognizing the inoculates were isolated from the peripheral blood of 2 MS patients. In a follow-up period (33 to 39 months), two patients showed a stable EDSS over time, one patient improved by one EDSS step, and in the remaining patient the EDSS score advanced.

Although these preliminary clinical trials provided important clinical indications in favor of the role of TCV in MS, the treatment efficacy must be evaluated in double-blind placebo-

controlled clinical trials. Currently, there are phase I and II trials ongoing or planned in Houston, Los Angeles, and Jerusalem using different vaccination protocols[248]. In our institute, a new approach for vaccine preparations, based on activated CD4+ T cells derived from the cerebrospinal fluid of MS patients, is tested in a double-blind, placebo-controlled phase II clinical trial[247].

These studies will provide further insights into the boosted regulatory mechanisms, and subsequent therapeutic effects, and may provide information on the most appropriate protocol for T cell vaccination.

TCR peptide vaccination

A second, more simplified, vaccination strategy, is a peptide-based approach in which synthetic peptides, corresponding to the target molecule on autoimmune T cells, the T cell receptor, are responsible for eliciting the regulatory immune response[75;248-250]. In several animal models for MS, encephalitogenic MBP-specific T cells preferentially utilize the TCR AV2 and BV8S2 gene elements. Immunization with TCR peptides corresponding to both the CDR2 region[251] and the CDR3 region[252] could induce anti-TCR peptide regulatory T cells and antibodies that prevented or reversed clinical paralysis in EAE.

Although MBP-specific T cells of MS patients display a much more diverse TCR V gene region expression profile, a few V genes have been associated with MS[142;253;254]. In three human pilot trials, predominantly focusing on the BV5S2 CDR2 region, a total of 45 MS patients received repeated intradermal injections of low doses of a BV5S2 38-58 peptide[71;116;255;256]. Immunization induced significant T cell responses in about half of the progressive patients. However, patients unresponsive to BV5S2 TCR peptide vaccination could be successfully immunized with CDR2 peptides from different BV gene families overexpressed in their MBP-specific T cells[71]. An inverse correlation between responsiveness to the BV5S2 peptide and responsiveness to MBP was observed, suggesting a regulatory effect of TCR-specific T cells on MBP-reactive T cells[257]. TCR-specific T cells isolated from the blood of the responders were predominantly Th2 cells, and directly inhibited MBP-specific Th1 cells *in vitro* through the release of IL-10, which may implicate a role for bystander suppression mechanisms[256]. Moreover, a significant correlation between the degree of response to the vaccinating

peptide and the lack of disease progression was observed[116]. More recently, a larger multicenter double-blind placebo-controlled clinical trial with 106 MS patients confirmed these basic findings, although T cell responses were markedly reduced[250].

In another attempt to generate a customized TCR peptide vaccine, Wilson and co-workers first characterized the T cell population infiltrating in the CNS and reported an overexpression of BV6 among the activated T cells in CSF of MS patients[258]. Based on these results, 10 MS patients, previously screened for increased BV6+ T cells in their CSF, were vaccinated in a phase I open label study with a BV6S5 39-58 CDR2 peptide[259]. The results indicated that the peptide used for vaccination was immunogenic. Moreover, patients treated with the higher vaccine dose, displayed reduced CSF cellularity, a lack of growth of activated CD4+ CSF T cells and a diminution of BV6 mRNA in these cultured T cells. However, it should be noted that no information on myelin reactivity of these T cells was provided. All patients remained clinically stable during the course of the study, and the MRI data indicated no increase of active lesions after treatment. In a follow-up study, the BV6S5 CDR2 peptide was found to be highly immunogenic, even in MS patients that were not pre-screened for the expression of BV6+ T cells in their CSF[260].

To determine the clinical efficacy of the TCR peptide-based vaccination approach, a phase I/II randomized double-blind multicenter trial is currently carried out, using a cocktail of three CDR2 peptides (BV5S2, BV6S5 and BV13S1) to immunize 60 MS patients (The Immune Response Corporation). The study will compare different ways of vaccine administration, immunogenicity of the 3 peptides and will evaluate both immune responses and MRI changes.

Other T cell target molecules

Based on the hypothesis that MS is a Th1 mediated autoimmune disease, another target molecule for therapy could be the CD4 molecule. Both phase I and II clinical trials have been performed using a chimeric monoclonal anti-CD4 antibody (cM-T412)[251-254]. All studies reported long-lasting, selective depletion of circulating CD4 lymphocytes, whereas no long-term effects on other cell subsets were observed. The antibody was well tolerated (although frequently minor side effects occurred after infusion), but no significant reduction in MRI

activity was measured. A second monoclonal antibody (BF5) tested in 2 phase II clinical trials reported similar results on efficacy, and demonstrated a reduction of cytokine secretion by Th1 cells following treatment[265;266].

Two reagents targeting co-stimulatory molecules, which are the secondary signals for T cell activation, are being tested. The first molecule is a humanized monoclonal anti-CD40L, interfering with the CD40-CD40L (gp39) co-stimulatory pathway. This antibody is currently tested in a phase I dose-finding study. Promising results with anti-CD40L antibody therapy were reported for the EAE animal model[267] and in a phase I trial for systemic lupus erythematosus (SLE)[268]. Another inhibitor of the co-stimulatory pathway is the fusion protein CTLA-4-Ig that binds CD28 and may make T cells tolerant. This protein was shown to reduce the incidence of graft-versus-host disease in patients receiving a stem cell transplantation from a non-genetically matched donor[269;270].

Finally, inhibition of leukocyte trafficking across the blood-brain barrier into the CNS may be an appealing therapeutic strategy. Inhibition of the adhesion molecule very-late antigen-4 (VLA-4) by a humanized monoclonal antibody, has been tested in 72 MS patients with active MS[271]. Short-term treatment was well tolerated and showed a reduction in the number of new active MRI lesions. Further studies are required to determine long-term effects of this treatment on MRI and clinical outcomes.

1.4.2.2. Antigen-based therapies

Oral tolerance

Since it has been shown that in MS autoreactive T cells are targeting the myelin components of the CNS, several attempts have been made to induce T cell tolerance by oral administration of myelin antigens[272].

In the EAE model, antigen feeding has been successfully used to suppress clinical disease[273;274]. Three distinct mechanisms have been proposed for the systemic antigen-specific immune suppression associated with oral tolerance, depending on the dose[275;276]. When antigen encounters the gut-associated lymphoid tissue (GALT), it may act either directly at the level of the GALT, or have an effect following absorption. High dosages of

orally administered antigen result in T cell anergy[277;278] or T cell deletion[279]. In contrast, feeding multiple low doses of antigen induces a regulatory cell-driven tolerance in the GALT, where several cells are capable of antigen presentation. This presentation results in the generation of regulatory cells, which secrete suppressive cytokines like TGF-β, IL-4 and IL-10. Migration of these cells to the target organ may suppress ongoing inflammatory reactions through 'bystander suppression'[280-282].

In an initial human phase I/II clinical trial, 30 RR-MS patients were fed daily with a preparation of crude bovine myelin, including MBP and PLP ('Myloral')[283]. Study results indicated that the peptides were well tolerated but no statistically significant clinical effect was found. Immunologically, there was no increase of MBP- or PLP-specific Th1 cells in the treated patients. In contrast, induction of regulatory myelin-specific TGF-β-secreting Th3 cells in the peripheral blood was observed[284;285]. However, a phase III clinical trial with 515 RR-MS patients did not show differences between placebo and treated groups in blocking disease progression, while a large placebo effect was noted. It has been suggested that these negative results may be due to an inadequate dose or antigen preparation[276].

Currently, a new phase III clinical trial of oral tolerance with the MBP analogue, glatiramer acetate, is in progress. This study will evaluate clinical and MRI outcomes of glatiramer acetate in 1300 RR-MS patients ('Coral study').

Altered peptide ligand (APL) therapy

Altered peptide ligands are peptides that have been modified from an autoantigenic peptide with one or a few substitutions at the amino acid positions essential for contact with the T cell receptor[286]. The original MHC binding moieties are retained, so APL's can compete for TCR binding, without full T cell activation. T cell responses can be blocked by acting as partial agonist, TCR antagonist or by inducing regulatory T cell populations that mediate 'bystander suppression'[287;288]. Substitutions of amino acid 91 in the core sequence of the immunodominant region of MBP resulted in an altered peptide MBP 83-99. A series of experiments demonstrated that *in vivo* administration of the APL could prevent or reverse clinical disease in the EAE model[289-291].

Further modifications of the peptide MBP 83-99 led to the APL sequence termed NBI5788 that has been used in an initial phase I clinical trial[292]. Results of this study showed that the APL was generally well tolerated and no clinical exacerbations or changes in EDSS were noted during treatment. Immunological analysis demonstrated that NBI5788 induced an APL-reactive response in which T cells, cross-reactive to the native MBP peptide, secrete anti-inflammatory cytokines[292].

Two larger phase II trials were initiated to test the ability of two APL's to reduce the number of MRI lesions[293;294]. However, both studies using an APL based on the MBP 83-99 peptide (NBI5788 and CPG77116) were halted because of hypersensitivity or other side reactions. Moreover, in one study, a tendency to trigger exacerbations was observed that could be linked to the encephalitogenic potential of the peptide in a subgroup of patients[294]. In contrast, the double-blind, placebo-controlled multicenter trial using NBI5788, reported no increases in either clinical relapses or new enhancing lesions, even in patients with hypersensitivity reactions. Immunological analysis indicated that the APL induced a Th2 response, initially driven by the APL itself, and spreading to the MBP autoantigen[293;295]. Although the first phase II trials showed considerable adverse effects, they also demonstrated that the APL is highly immunogenic *in vivo*. In future studies, balancing the strength of the Th2 response will be a prime consideration, in order to prevent hypersensitivity and promote beneficial effects.

Other antigen-based strategies

In another attempt to use myelin antigen peptides, MBP peptides containing the immunodominant epitope MBP 82-99 were tested in phase I clinical trials. Both intrathecal and intravenous injections reduced the rate of anti-MBP antibodies during the initial phase of acute relapses in MS patients[296;297]. Furthermore, intrathecal injection for a maximum of 5 days maintained remission for a prolonged period of one month although MBP antibodies reappeared afterwards. Intravenous administration did not prevent occurrence of future relapses after remission. No side effects were noted. In a subsequent clinical trial with chronic progressive MS patients, only intravenous (and not intrathecal) injection(s) resulted in a long-lasting MBP peptide-based tolerance induction, as measured by low or undetectable levels of anti-MBP antibodies in the CSF[298]. An extended placebo-controlled

clinical trial demonstrated a suppression of CSF anti-MBP antibody levels in the majority of the patients treated, whereas in the control group antibody levels remained elevated[299]. A phase II clinical trial has been conducted recently, but no data have been published yet.

To circumvent the disadvantage of rapid clearance of free peptide, a solubilized MHC-peptide complex was constructed. Based on the strong association of HLA-DR2 and the immunodominance of MBP peptide 84-102 in the T cell response of DR2+ MS patients, a mixture of DR2a and DR2b molecules bearing MBP 84-102 in the peptide binding cleft have been tested (AG284/AnergiX.MS). An extended bio-distribution and extended periods of tolerance induced at low doses, provide a significant advantage as compared to the peptide-based strategies. A double-blind placebo-controlled phase I/II clinical trial involving 40 CP-MS patients, pre-screened for HLA-DR2 expression and T cell reactivity to MBP 84-102, has been conducted[300]. Results indicated no significant adverse effects or generalized immune suppression[300]. Furthermore, there was some evidence of clinical response[300].

However, since multiple immunodominant epitopes of myelin have been reported, systemic administration of whole myelin antigens rather than a single peptide may be a useful immunosuppressive strategy in a complex disease as MS. A recombinant chimeric fusion protein that encodes multiple immunogenic epitopes from an MBP isoform as well as three hydrophilic portions of PLP was engineered (MP4). It has been demonstrated both in a murine and a primate model of EAE that the protein can be effectively processed *in vivo* and has potential to induce tolerance to multiple MBP and PLP peptide epitopes simultaneously[301]. However, human clinical trials are needed to test therapeutic efficacy of the MBP-PLP fusion protein.

1.4.2.3 Gene therapy, a future approach

Systemic administration of proteins modifying the ongoing autoimmune responses and subsequent target tissue destruction in a chronic disease as MS, shows limited clinical efficacy and may also cause undesirable side effects[302]. The use of gene transfer techniques as therapeutic strategies in autoimmune disease arose in response to the need to deliver therapeutic products in a sustained and site-specific manner. Both *in vivo* and *ex vivo*

approaches are being used for gene delivery after introduction in plasmids or viral vectors[303;304].

Subcutaneously or intramusculary injected plasmid DNA encoding specific genes have been shown to induce potent cellular and humoral responses that modulate clinical disease in EAE. This so called 'DNA vaccination' approach may induce tolerance to autoantigens when DNA constructs are used that encode encephalitogenic myelin proteins[305;306]. Following vaccination with plasmid DNA incorporating the sequence of overexpressed encephalitogenic T cell receptors, EAE can be reversed[307]. In addition, naked DNA construct encoding proinflammatory chemokines and cytokines induced an immunological memory and subsequent resistance to EAE[308-310].

Systemic injection of viral vectors engineered with cytokine-encoding genes has also been reported to be effective in murine EAE[311]. However, exposing multiple organs to high concentrations of the gene product, increases the risk of side effects. Therefore, local delivery in the CNS of the appropriate genes incorporated into plasmids or viral vectors may circumvent this problem. Recently, a novel system to deliver cytokine-encoding genes into the CNS was developed[312;313]. Intrathecal injection of non-replicative viral vectors encoding the IL-4 gene showed significant amelioration of clinical and pathological features of EAE both before and after the appearance of clinical signs in murine EAE[314;315]. In addition, IL-4 therapy in a nonhuman primate model showed similar results: after injecting the IL-4 encoding vector, three out of five rhesus monkeys were completely protected, whereas animals injected with a control vector developed severe disease[316].

Another strategy is based on the knowledge that activated T cells can traffic to the CNS. In this *ex vivo* approach, lymphocytes are recovered and genetically modified prior to transfer in order to deliver their transgene products at the site of inflammation[317]. It has been demonstrated that delivery of anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β, by genetically altered autoreactive (MBP/PLP-specific) T cells, can inhibit the onset and severity of EAE[318-320]. Furthermore, the concept of 'regenerative gene therapy' is currently being investigated. CNS remyelination by oligodendrocytes can be promoted either by delivering growth factors or neuroprotective factors to the target tissue as demonstrated by genetically modified autoreactive T cells expressing platelet-derived growth factor[321] or

nerve growth factor[322]. In addition, it may be possible to transfer oligodendrocyte precursor cells, transfected with regenerative factors, to enhance their remyelinating potential during autoimmune demyelinating diseases[323]. Based on the encouraging data using different strategies of gene therapy in animal models, their application in humans may be considered as a potential treatment for MS in the near future.

In conclusion, although the treatment of MS has progressed remarkably over the past years, the therapeutic success of approved therapies (interferon-β and glatiramer acetate) is only moderate. Several experimental therapies are currently tested in clinical trials, targeting different key molecules in the disease pathogenesis. Both the tolerization against myelin components and the immunosuppression of autoreactive T cells might provide new strategies for beneficial treatment of MS in the near future.

1.5 Aim of the study

Multiple sclerosis is generally considered to be a T cell-mediated autoimmune disease, targeting components of the myelin sheaths in the central nervous system. Although there is evidence indicating that autoreactive T cells are the key players in the initiation of chronic inflammation, the exact mechanisms of the onset of disease are not completely understood.

This study is aimed to further characterize the potentially pathogenic role of myelin-reactive T cells in the disease process. We focused on T cell responses towards a minor component of the myelin that recently has been suggested to play a primary role in MS. Furthermore, a new protocol for T cell vaccination, in which these autoreactive T cells, isolated from CSF, are used as therapeutic targets, was optimized. To obtain information about feasibility, safety and immunological effects of the protocol, T cells from the CSF are used as vaccine cells in a pilot clinical trial involving 5 MS patients.

Goal 1: Analysis of the frequency and functional properties of MOG-reactive T cells in MS patients and healthy controls

Autoreactive T cells, recognizing a broad spectrum of myelin components are considered to play a critical role in the immunopathogenesis of MS. However, the identity of the myelin antigen(s) that play(s) a primary role remains unknown. Although the most abundant myelin proteins have been extensively studied, there is increasing evidence that quantitatively minor components can also have a significant function. Myelin oligodendrocyte glycoprotein (MOG), comprising only 0.05% of the myelin, may fulfill the criteria for a primary target antigen. This CNS-specific, potentially accessible glycoprotein is the only myelin component that induces both an inflammatory T cell response and a demyelinating autoantibody response in EAE, the animal model for MS.

Based on either cytokine secretion (ELISPOT) or proliferation (limiting dilution analysis, LDA) upon stimulation, frequencies of MOG-reactive T cells in MS patients and healthy controls were estimated. This will reveal whether quantitative differences in anti-MOG

reactivity can be found between MS patients and control subjects and whether antigen reactivity is restricted towards one or several immunodominant epitopes.

In addition, to analyze the functional properties of this T cell population, we generated MOG-specific T cell lines/clones from 4 MS patients and 2 healthy controls. Flow-cytometric analysis provides information both on the phenotype and Th subtype of MOG-reactive T cell clones. A panel of T cell clones was characterized for their T cell receptor (TCR) CDR3 region sequences and these results give indications for *in vivo* clonal expansion after (super)antigenic stimulation. Both neuroprotective and cytotoxic properties were tested in ELISA assays measuring neurotrophin production upon antigenic stimulation and ^{51}Cr release assays respectively. Data on functional characteristics of this potentially pathogenic T cell population may have to be taken into consideration when developing new strategies for antigen-specific immunotherapies.

Goal 2: Study of activated CSF-derived T cells - optimization of a protocol for T cell vaccination in MS patients

Previous studies have shown that activated myelin-specific T cells are increased in the cerebrospinal fluid (CSF) of MS patients. This finding is particularly of interest since the CSF is the compartment that reflects best the ongoing inflammatory response in the CNS, and T cell subsets isolated from this site may be relevant for the disease process. However, since only small numbers of predominantly T cells can be isolated after a lumbar puncture, it is crucial to optimize a culturing protocol specifically expanding the activated CD4+ T cells to sufficient numbers needed for T cell vaccination.

In this part of the study, we determined the optimal culture conditions of CSF-derived CD4+ activated T cells and developed a depletion protocol using magnetic beads to exclude other (T) cell populations. Using flow-cytometric analysis, cell cultures were characterized at regular time points for their phenotype. Subsequent TCR BV gene analysis provides information about the clonal heterogeneity and stability of the CSF-T cell populations. Furthermore, the influence of freezing and subsequent thawing on the composition of the cell population was evaluated. Because only one lumbar puncture is performed in the T cell

vaccination clinical trial and MS patients receive three vaccines at two month intervals, several samples of cells need to be frozen and thawed for each immunization.

In addition, we compared the phenotype and TCR BV gene usage of CSF-derived activated (T) cells in a population of 5 MS patients and 4 patients with other non-inflammatory neurological diseases. For two MS patients, we evaluated whether similar activated T cell clones are present in the CNS and blood compartment after analysis of the clonal composition as determined by TCR BV gene usage screening and subsequent analysis of the T cell-specific hypervariable CDR3 region by CDR3 fragment length screening or sequence analysis.

Based on the results of this optimization phase, a standard protocol for the isolation and expansion of CSF-derived activated CD4+ T cells will be used in a subsequent pilot trial for T cell vaccination, an experimental immunotherapy for MS, as will be described in goal 3.

Goal 3: Evaluation of feasibility, safety and immunological effects of T cell vaccination with CSF-derived activated CD4+ T cells in a pilot clinical trial involving 5 MS patients

In previous T cell vaccination (TCV) clinical trials performed in our lab, the vaccines consisted of MBP-reactive T cell clones isolated from peripheral blood of MS patients. However, other myelin components, such as MOG and PLP, may also be targeted by pathogenic T cells. Furthermore, several reports demonstrated an accumulation of activated myelin-reactive T cells in the cerebrospinal fluid of MS patients. Based on these findings, a broader vaccine containing activated T cells derived from the CSF may be a more appropriate approach for T cell vaccination.

To test feasibility, safety and immunological effects of TCV with activated CD4+ T cells derived from the CSF, a pilot clinical trial involving 5 MS patients was carried out. Patients were monitored for at least 12 months, starting two months before the first vaccination until six months after the last vaccination. After a preparation period of about 8 weeks, three immunizations with irradiated CSF-derived vaccine cells were performed at two month intervals. All vaccines were tested for their phenotype, cytokine profile, myelin antigen

reactivity and clonal heterogeneity (TCR analysis). Patients were monitored for several clinical (adverse events, EDSS score, relapse rate, MRI) and immunological parameters at regular intervals. Before the first and after the third vaccination, frequencies of MBP-, PLP- and MOG-reactive T cells in the blood were determined in a classical limiting dilution assay (LDA). Furthermore, to gain information about the autologous immunological reaction on the vaccine T cells both anti-idiotypic (towards the vaccine cells) and anti-ergotypic (towards activated cells in general) proliferative response were measured after each vaccination and a comparison of the cellular composition of the CSF before and after vaccination was performed. Taken together, these data can provide information about the impact of TCV on the potentially pathogenic anti-myelin T cell repertoire. Evaluation of these data resulted in the initiation of a larger double-blind placebo-controlled clinical trial involving 60 MS patients.

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Chapter 2

General Materials and Methods

2.1 Cell culture-based techniques

2.1.1 Cell culture media and antigens

CSF-derived mononuclear cells obtained by lumbar puncture were isolated by centrifugation and resuspended in RPMI medium supplemented with L-glutamine, sodium pyruvate, non-essential amino acids, 10 mM HEPES buffer (Life Technologies, Paisley, Scotland) and 10% heat-inactivated autologous human serum. The protocol for the generation of CSF-derived T cell vaccines will be described in detail in chapter 5. Peripheral blood-derived mononuclear cells (PBMC) were isolated from heparinized blood using HistoPaque density centrifugation (Sigma, St. Louis, MO). The cells obtained were washed extensively and cultured in RPMI 1640 medium supplemented with L-glutamine, sodium pyruvate, non-essential amino acids, 10 mM HEPES buffer, and 10% heat-inactivated fetal bovine serum (FBS, Hyclone Europe, Erembodegem, Belgium) or autologous human serum.

Human MBP and PLP were purified from white matter of normal human brain, according to the methods of Deibler *et al.*[1] and Lees *et al.*[2]. Endotoxin-free extracellular domain of MOG (rMOG) was expressed in *E. coli* and purified to homogeneity as previously described and kindly provided by Dr. C. Bernard[3]. *Tetanus toxoid* was obtained from RIVM (Bilthoven, The Netherlands). 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 (more than 95% purity) by Severn Biotech Ltd (Worcester, UK). The amino acid sequences of the peptides are shown in Table 2.1.

Table 2.1. Amino acid sequence of synthesized myelin peptides

Myelin peptide	Amino acid sequence
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

2.1.2 Flow-cytometric phenotype analysis

Expression of cell surface proteins was assayed by flow-cytometric analysis. Cells were suspended in FACS buffer (phosphate-buffered saline with 2% FBS) and stained for 30 minutes at 4°C with fluorescein isothiocyanate (FITC) and/or phycoerythrin (PE) conjugated monoclonal antibodies specific for CD3, CD4, CD8, CD25, CD16/56, TCRαβ, TCRγδ and mouse IgG_1/IgG_{2a} as an isotype control (BD Biosciences, Erembodegem, Belgium). Cells were washed and phenotypically characterized with a FACSCalibur flow-cytometer (BD Biosciences).

2.1.3 Depletion of specific cell populations using magnetic beads

To generate pure CD4+ activated T cell vaccines derived from the cerebrospinal fluid (chapter 5), other cell subsets representing > 15% of the total cell population (CD8+ T cells, γδ T cells and natural killer (NK) cells) were depleted using immunomagnetic beads during the expansion period (Figure 2.1).

Depletion of CD8+ T cells was performed with anti-CD8 antibody coated beads (Dynal, Skoyden, Norway). Beads were mixed with the cell suspension at a ratio of 10/1 and incubated for 30 minutes at 4°C at cell concentrations of $10\text{-}20x10^6$ cells/ml and bead concentrations of $>10^7$ beads/ml. Next, suspensions were placed in a magnet (Dynal) for at least 2 minutes for a rapid and effective CD8+ cell separation. Negatively isolated CD8- cells in the supernatant were removed and resuspended after centrifugation for further culturing.

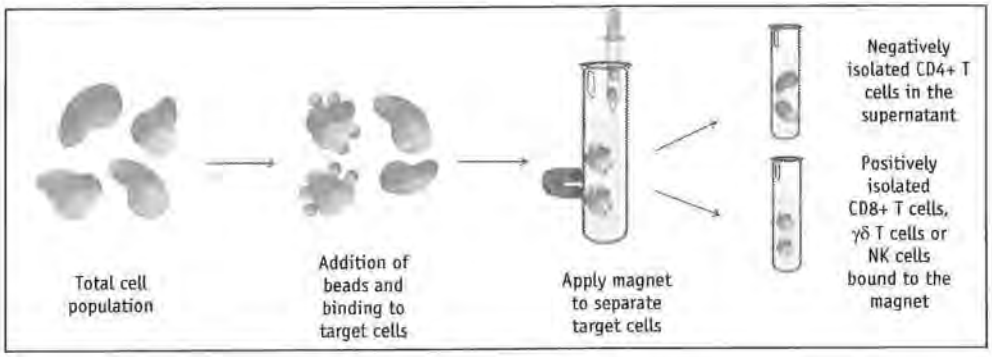

Figure 2.1. *Schematic overview of the cell depletion technique using immunomagnetic beads*

Indirect depletion of γδ T cells and NK cells requires a two-step procedure. First, mouse anti-human TCR $V\delta_{1/2}$ antibodies (Serotec, Kidlington, UK) or mouse anti-human CD16 antibodies (BD Biosciences) were pre-incubated at concentrations of 2 µg/mg beads for 30 minutes at 4°C with rat anti-mouse IgG_1 coated beads (20 mg/ml, Dynal) or rat anti-mouse IgM coated beads (20 mg/ml, Dynal), respectively. Anti-TCR $V\delta_{1/2}$ or anti-CD16 coated beads were collected after separation in a magnet for at least 2 minutes. Next, antibody coated beads were mixed with the cell suspensions at a ratio of 10/1, incubated for 30 minutes at 4°C at cell concentrations of $10\text{-}20x10^6$ cells/ml and bead concentrations of $>10^7$ beads/ml, and placed in a magnet. In this way, boured negatively isolated γδ T cells and NK cells were effectively eliminated. Depletion efficiency was monitored by flow-cytometric phenotype analysis as described in 2.1.2.

2.1.4 Frequency analysis by ELISPOT assay

The frequency of cytokine secreting T cells in response to stimulation with myelin antigens, synthesized myelin peptides or control stimuli was estimated using an ELISA-based technique called ELISPOT (enzyme-linked immunospot assay, Figure 2.2).
Optimal cell densities and antigen (peptide) concentrations were calculated from dose response curves or based on previous reports[4-8].

For IFN-γ ELISPOT assays, nitrocellulose bottomed 96-well Millititer HA plates (Millipore Corp., Bedford, MA) were coated overnight at 4°C with 10 µg/ml anti-IFN-□γ capture Ab 1-D1K (MabTech, Nacka, Sweden). Unbound antibody was removed by successive washings with sterile PBS and non-specific binding sites were blocked by incubation with 10% FBS for 2 hours at 37°C. Next, either CSF-derived vaccine cells in the presence of 10^5 irradiated autologous PBMC as antigen-presenting cells or freshly isolated PBMC in culture medium supplemented with 10% FBS were incubated in triplicate in a humidified incubator at a concentration of $2x10^4$ cells/well or $2x10^5$ cells/well, respectively, in the presence of rMOG (10 µg/ml), synthetic myelin peptides (10 µg/ml) and control stimuli (PHA or anti-CD3, 2 µg/ml) or without antigen. After 20 hours of culture, cells were removed by washing and captured IFN-□γ was visualized by adding 1µg/ml biotinylated Ab (MabTech) for 2 hours followed by incubation with streptavidin-alkaline phosphatase (MabTech) and BCIP/NBT (Pierce, Rockford, IL) 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 (without antigen) from the number of spots obtained in the presence of each stimulating agent.

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

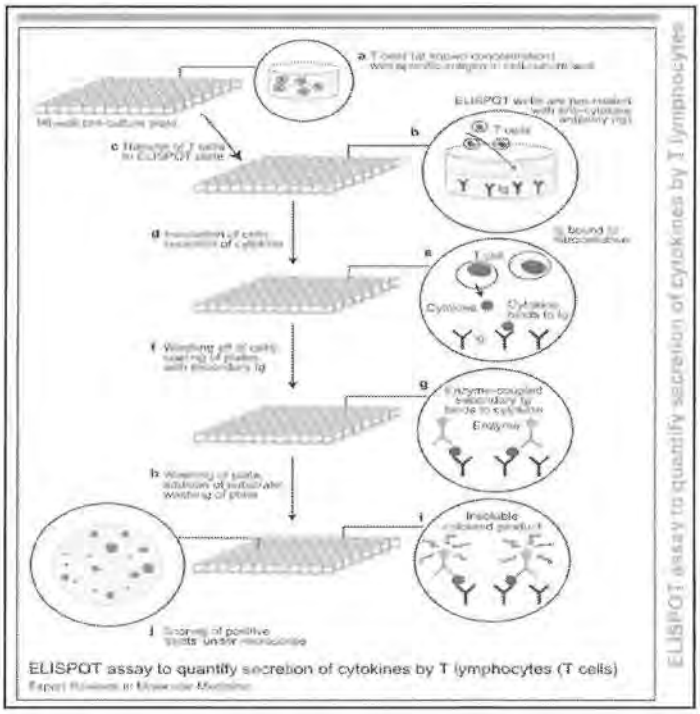

Figure 2.2. *Schematic overview of the enzyme-linked immunospot assay (ELISPOT)*[9]

2.1.5 Frequency analysis by limiting dilution analysis and generation of myelin-reactive T cell clones

Freshly isolated PBMC were cultured at $1x10^5$ cells per well (30 or 60 wells) in U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Medium was supplemented with 10% heat-inactivated autologous serum and contained either MBP (40 µg/ml), a mixture of the 3 PLP peptides (10 µg/ml each) or a mixture of the 4 MOG peptides (10 µg/ml each). After 7

days, cultures were restimulated with 10^5 irradiated autologous PBMC, pulsed with the corresponding myelin antigen and supplemented with 2 U/ml recombinant human IL-2 (rhIL-2, Roche Diagnostics, Brussels, Belgium). After one week, myelin-reactive T-cells were identified using the split-well technique. Briefly, each cultured well was split into 4 aliquots and restimulated in duplicate with either 10^5 irradiated antigen-pulsed or non-pulsed autologous PBMC. After 3 days, proliferation capacities were measured using a classical ^{3}H-Thy incorporation assay. During the last 16 hours of culture, cells were pulsed with 1 µCi ^{3}H-Thymidine (Amersham, Buckinghamshire, UK) and subsequently harvested with an automated cell harvester (Pharmacia, Uppsala, Sweden). Incorporated radioactivity was measured with a Beta-plate liquid scintillation counter (Wallac, Turku, Finland). A T cell line was considered to be antigen-reactive when mean counts per minute (cpm) in the presence of antigen were larger than 1,000 cpm and the stimulation index (mean counts with antigen / mean counts in control wells) was larger than three[10;11]. The frequency of myelin-reactive T cells was estimated by dividing the number of reactive T cell lines by the total number of PBMC plated.

T-cell clones were established using a previously described cloning procedure[12]. Briefly, myelin-reactive T-cell lines were plated out by limiting dilution at 0.3, 0.5 and 1 cell per well and stimulated with 10^5 irradiated allogeneic feeder cells and phytohaemagglutinin (PHA, 2 µg/ml, Difco, Detroit, MI). Cultures were refreshed every 3 days with medium containing 5 U/ml rhIL-2. After 14 days, growth positive clones were examined for their antigen specificity in a proliferation assay as described above. In addition, myelin-reactive T cell clones were further expanded by successive rounds of restimulation with myelin-pulsed autologous PBMC.

2.1.6 Evaluation of cytokine profiles (and neurotrophins) using ELISA: quantitative analysis

Triplicate aliquots of $2x10^4$ CSF-derived vaccine cells or MOG-reactive T cell clones were stimulated with 10^5 MOG-peptide-pulsed autologous irradiated PBMC (only for MOG-T cell clones), PHA (2 µg/ml) and 10^5 autologous irradiated feeder cells or 10^5 irradiated autologous feeder cells alone. After 72 hours, cell supernatants were harvested and the cells were subjected to a proliferation assay as described above.

The cytokine production in supernatants of (un)stimulated cell cultures was measured using a sandwich ELISA based on commercially available monoclonal Ab pairs (CytoSets, Biosource Europe, Nivelles, Belgium) in 96-well microtiter plates (MaxiSorp, Nunc). Wells were coated overnight at 4°C with capture antibody. Non-specific binding sites were blocked with 0.5% BSA in PBS and subsequently washed in washing solution consisting of 0.1% Tween-20 in 0.9% NaCl solution. Fifty µl of test samples or of serialy diluted standard solutions 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 with streptavidin-conjugated horse radish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes. A color reaction was established using a TMB/H_2O_2 citrate buffer as substrate and 1.8 N H_2SO_4 to stop the enzymatic reaction. Optical densities were measured at 450 nm and 630 nm using an ELISA-reader (ICN Biomedicals, Asse, Belgium) and cytokine concentrations were calculated using standard curves. Net production of TNF-α, IFN-γ, IL-4, IL-6 and IL-10 was calculated by subtracting background levels (no stimulation) from the cytokine levels measured in the stimulated cultures.

For the analysis of the neurotrophins brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (chapter 3), commercially available ELISA kits were used according to the manufacturer's instructions (NGF/BDNF Emax ImmunoAssay System, Promega). For enhanced detection, supernatants were acid-treated 15 minutes before the assay.

2.1.7 Evaluation of cytokine profiles using flow-cytometry after intracellular cytokine staining: semi-quantitative analysis (Figure 2.3)

Intracellular cytokine production was determined by flow-cytometry after stimulating the MOG-specific T cell lines/clones at concentrations of $2x10^6$ cells/ml with phorbol-12-myristate-13-acetate (PMA, 25 ng/ml, Sigma) and ionomycin (1µg/ml, Sigma) in the presence of brefeldin A (10 µg/ml, PharMingen, San Diego, CA) for 4 hours at 37°C (chapter 3). Subsequently, cells were washed in staining buffer (phosphate-buffered saline with 1% FBS and 0.09% Na azide) and surface-stained with anti-CD3-PerCP (peridinin chlorophyll protein, BD Biosciences) for 15 minutes at room temperature. After an additional washing step, cells were fixed with paraformaldehyde and permeabilized with saponin during an incubation period of 20 minutes at 4°C in Cytofix/cytoperm solution (100µl, PharMingen). Next, cells were washed and resuspended in a Perm/wash buffer containing 0.1% saponin

before intracellular labeling with an anti-IFN-□γ-FITC/anti-IL-4-PE antibody panel (BD Biosciences) or the appropriate intracellular isotype control (anti-mouse IgG_{2a}-FITC/IgG_1-PE, BD Biosciences) for 30 minutes at 4°C. Cells were washed twice and analyzed with a FACSCalibur flow-cytometer (BD Biosciences).

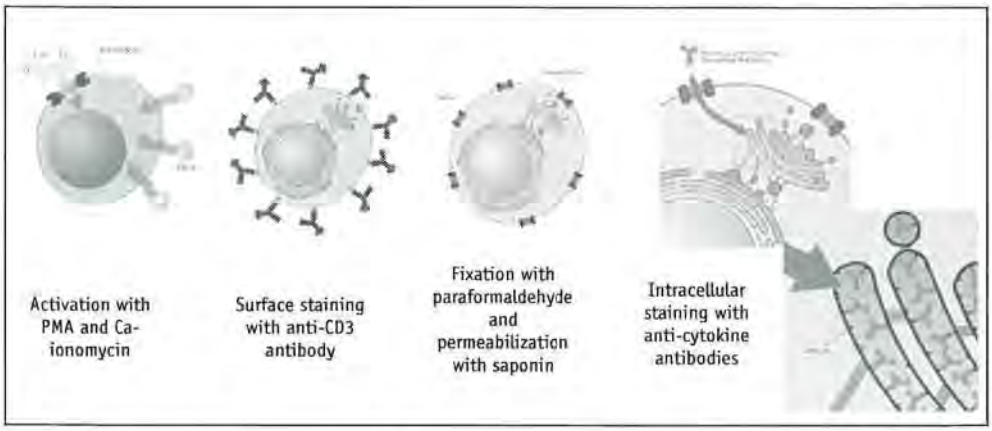

Figure 2.3. *Schematic overview of the intracellular cytokine staining procedure*

2.1.8 Cytotoxicity assays

Cytotoxicity was measured in a ^{51}Cr-release assay using MOG-reactive T cell clones as effector cells and autologous MOG-pulsed PBMC and K562 cells as targets (chapter 3). First, PBMC were pulsed for 2 hours with the corresponding MOG peptide (10 µg/ml) and subsequently labeled with 200 µCi ^{51}Cr ($Na_2Cr_3O_4$, Amersham) at 37°C for 1 hour. Labeled target cells were washed extensively and incubated with the MOG-reactive T cell clones at various effector-to-target ratios (5/1, 10/1 and 20/1) in 200 µl microwells. After 6 hours, the supernatants were harvested and released radioactivity was measured in a gamma counter (Cobra II 5002, Packard Instrument Company, Meriden, CT). Maximum and spontaneous release of ^{51}Cr were determined in wells containing target cells in the presence of respectively detergent or medium only. The percentage of specific cytolysis was calculated as (experimental release - spontaneous release/maximum release - spontaneous release) x 100.

2.1.9 Proliferative responses to activated cells (anti-vaccine response)

The proliferative response to both vaccine cells (anti-clonotypic) and activated cells in general (anti-ergotypic), was measured at various time points before, during and after the immunization procedure with the CSF-derived T-cell vaccines in the T cell vaccination pilot study (chapter 5).

Freshly isolated PBMC ($5x10^4$ cells/well) were co-cultured in triplicate with $5x10^4$ irradiated stimulator T cells for 72 hours as described previously[12]. The immunizing CSF-derived T cells or PHA-activated non-specific T cells were used as stimulators. To prepare activated non-specific T cells, PBMC were cultured in the presence of 1 µg/ml PHA and 5 U/ml IL-2 for 7 days and washed extensively. As a control, PBMC and irradiated stimulator T cells were cultured in parallel. Cell proliferation was measured in a classical proliferation assay using ^{3}H-thymidine incorporation as described above and stimulation indices (SI) were calculated as follows: (counts per minute (cpm) of PBMC co-cultured with irradiated stimulator T cells / (cpm of PBMC cultured alone) + (cpm of irradiated stimulator T cells alone)).

2.2 Molecular biology-based techniques

2.2.1 RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets using the High Pure total RNA isolation kit (Roche Diagnostics). Next, RNA was reverse transcribed into single stranded cDNA with AMV reverse transcriptase using an oligo dT primer according to the manufacturer's protocol (Promega, Madison, WI). Finally, cDNA was precipitated with sodium acetate in ice-cold ethanol and resuspended in 35 µl of sterile water.

A control PCR amplification was performed with primers specific for either the household gene β2-microglobin to confirm the integrity of the isolated cDNA, or the constant region of the TCR beta chain gene to check whether a sufficient amount of T cell-specific mRNA was present in samples with low cell numbers. One µl of cDNA was amplified in a total volume of 25 µl and identical PCR conditions were used as described for the amplification of TCR V genes in 2.2.3.

2.2.2 HLA DR2 typing by PCR

PBMC of healthy controls and MS patients were DR2 typed as described elsewhere[13]. Genomic DNA was isolated by suspending a pellet of $2x10^6$ PBMC in lysis buffer (10 mM Tris, 100 mM NaCl, 25 mM EDTA and 0.5% SDS) and incubating the lysate overnight at 37°C with proteinase K (0.3 mg/ml). DNA was precipitated with 3 M NaCl and ethanol and finally suspended in TE-buffer.

PCR was performed by adding 1 µl of genomic DNA to an amplification mixture containing 0.5 µl dNTP mix (10 mM, Perkin Elmer, New Yersey, USA), 5µl 10x PCR buffer (25 mM MgCl, Boehringer Mannheim), 0.25 µl *Taq* DNA polymerase (5U/µl, Boehringer Mannheim), 2 µl forward primer (0.4 µM, 5'-TTCCTGTGGCAGCCTAAGAGG-3') and 2 µl reverse primer (0.4 µM, 5'-CCGCTGCACTGTGAAGCTCTC-3') in a total volume of 50 µl. The PCR reaction consisted of 30 cycles of 20 seconds at 95°C, 20 seconds at 60°C and 40 seconds at 72°C. For DR2 positive individuals a PCR product of 261 bp was visualized with ethidium bromide on a 1% agarose gel.

2.2.3 Semi-quantitative TCR BV gene repertoire screening using PCR-ELISA (Figure 2.4)

PCR amplification was performed with each of 21 TCR BV gene specific primers as forward primer and a digoxigenin (DIG) labeled TCR BC specific primer as reverse primer[14]. 0.7 µl cDNA was added to an amplification mixture composed of 2.5 µl 10x PCR buffer (25 mM MgCl, Boehringer Mannheim), 0.175 µl *Taq* DNA polymerase (5U/µl, Boehringer Mannheim), 0.2 pmol DIG labeled TCR BC primer and 0.2 pmol of a TCR BV gene specific reverse primer in a total volume of 25µl. PCR was performed in microtiter plates on a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer) for 35 cycles of 20 seconds denaturation at 94°C, followed by 20 seconds at 55°C for primer annealing and 40 seconds for primer extension at 72°C. For low cell numbers (< $1.5x10^6$ cells), the sensitivity of the technique was increased using DIG labeled dNTP (Boehringer Mannheim) in the PCR reaction at different concentrations according to the available cell number based on earlier performed pilot experiments[15].

Subsequently, PCR amplicons were denatured for 3 minutes at 95°C and hybridized to a FITC labeled TCR BC probe (50 µl, 3.75 pmol, 5'-FITC-CCGAGGTCGCTGTGTTTGAGCCAT-3') for 30 minutes at 55°C in a PCR thermal cycler. Thirty µl of the DNA hybrids were transferred to a microtiter plate, which was precoated with 1/500 diluted anti-FITC monoclonal Ab (1 mg/ml, Eurogenetics, Tessenderlo, Belgium) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3) and then blocked with PBS containing 3% (w/v) bovine serum albumine (BSA, Sigma). Hybridisation was performed in hybridisation buffer (0.15 M NaCl, 15 mM sodium citrate, 0.04% (w/v) Ficoll, 0.02% (w/v) polyvinyl-pyrolidone, 0.04%(w/v) BSA, 1 mM EDTA, 14 mM trihydroxymethyl-aminomethane, pH 7.5) for 2 hours at room temperature. After four washing steps in washing buffer (1.5 mM KH_2PO_4, 4.5 mM Na_2HPO_4, 0.13 M NaCl, 0.5% Tween-20), captured DNA hybrids were visualized by staining with 100 µl 1/5000 diluted anti-DIG peroxidase conjugate (150 U/ml, Boehringer Mannheim) in conjugate buffer (1.5 mM KH_2PO_4, 4.5 mM Na_2HPO_4, 0.13 M NaCl, 0.05% Tween-20). Microtiter plates were washed four times and 100 µl substrate solution containing color A (0.1 M Na_2HPO_4, 0.05 M citric acid, 0.002% (w/v) gentamycine (Life Technologies), 0.05% (v/v) H_2O_2, pH 5.0) and color B (0.05 M citric acid, 0.01 M tetramethylbenzidine, 4% (v/v) dimethylsulfoxide, pH 2.4) mixed at 1 :1 was added. DIG labeled amplicons were incubated for 30 minutes at 37°C and the color reaction was terminated with 50 µl 1 M H_2SO_4.

Colorimetric detection of specific PCR products was performed at 450 nm using an automated ELISA-reader.

Relative expression of TCR BV genes in the total TCR BV gene repertoire were presented as fractions of the total TCR BV gene expression using the following formula: % BV_x = (OD_{450} (BV_x) x 100)/ Σ OD_{450} (BV_n) after subtracting the OD_{450} values in control wells where PCR amplification was performed without cDNA. Overrepresented TCR BV genes were defined as exceeding an arbitrarily defined cut-off value based on the mean TCR BV gene expression levels in the blood of 10 healthy controls + 3 standard deviations[15] (chapters 4 and 5).

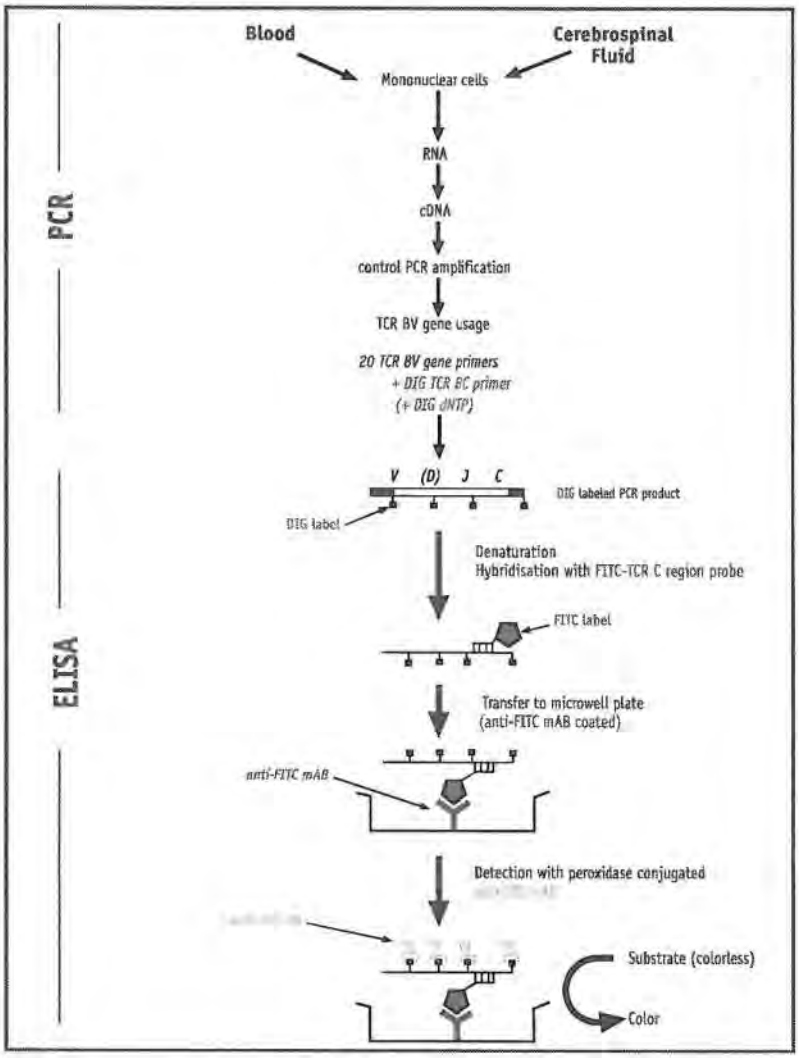

Figure 2.4. *Schematic overview of the PCR-ELISA technique*

2.2.4 Analysis of clonal heterogeneity : bacterial cloning and TCR B CDR3 region sequencing

cDNA of overrepresented TCR BV genes was amplified using the TCR BV region specific and a TCR BC region specific primer as described before. Purified PCR amplicons were ligated in the pCR2.1 cloning vector and transformed by heat shock in *E.coli* cells following the manufacturer's instructions (TOPO TA Cloning Kit, Invitrogen, Leek, The Netherlands). Subsequently, plasmid DNA was isolated from 15 to 25 recombinant plasmids. After an additional round of amplification of the inserts with TCR BV and TCR BC region specific primers, the amplicons were sequenced with a TCR BC region specific primer using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). PCR conditions were: 30 seconds at 96°C followed by 5 seconds at 50°C and 4 minutes at 60°C for 25 cycles. Fluorescently labeled PCR amplicons were purified on a sephadex G-50 M column, vacuum dried and resuspended in 5 µl 1 :50 25 mM EDTA/formamide. DNA sequences were evaluated on a ABI Prism 310 Genetic Analyzer (Applied Biosystems) using specific sequencing software.

2.2.5 Analysis of clonal heterogeneity: TCR B CDR3 region fragment length screening

cDNA of overrepresented TCR BV genes was amplified using the TCR BV region specific primer and a TCR BC region specific primer as described before. PCR amplicons were reamplified in a nested PCR for 25 cycles using the BV gene family specific primer as forward primer and a FAM labeled TCR BC region specific primer (5'-FAM-GTGGCCAGGCACACCAGTGTGGCC-3', Perkin Elmer, New Yersey, USA) as reverse primer. Fluorescently labeled nested PCR products were diluted 1/30 in loading buffer (24:1 formamide / 25 mM EDTA) and separated on a 6% polyacrylamide, 8 M urea gel in 90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3 on the 373 ABI DNA sequencer (Perkin Elmer). Fragment sizes of TCR BV gene products were calculated using an internal Genescan-1000 ROX labeled standard (Perkin Elmer) and fragment length analysis was performed with 672 Genescan Software (Perkin Elmer).

2.2.6 Sequence analysis of TCR rearrangements in myelin-reactive T cell clones

CDR3 sequence analysis of MOG-specific T cell clones (chapter 3) was performed by specific PCR amplification followed by direct sequencing of TCR AV and TCR BV transcripts as previously described. cDNA from $2x10^5$ to $2x10^6$ cells were PCR-amplified using 19 TCR AV and 21 TCR BV gene specific primers as forward primer and a TCR C region-specific reverse primer. The PCR amplification reaction consisted of 35 cycles of 20 seconds at 94°C, 20 seconds at 55°C and 40 seconds at 72°C. Detectable TCR AV/BV gene amplicons were further expanded in a second round of PCR and, after purification, sequenced with a TCR C region-specific primer as described above (2.2.4).

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Chapter 3

Frequency and Functional Properties of MOG-reactive T Cells in MS Patients and Healthy Controls

Based on:

Functional properties of myelin oligodendrocyte glycoprotein-reactive T cells in Multiple Sclerosis patients and controls

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Submitted for publication

Abstract

Autoimmune T cell reactivity to myelin components may be implicated in the initiation or maintenance of the inflammation leading to myelin destruction in multiple sclerosis (MS). Myelin oligodendrocyte glycoprotein (MOG), a quantitatively minor myelin protein, is an important candidate autoantigen in MS. We studied T cell responses to recombinant MOG (extracellular domain, rMOG) and a panel of four peptides within this domain (amino acids 1-22, 34-56, 64-86 and 74-96) in MS patients and healthy controls (NS). Frequency analysis of T cells reactive to rMOG as measured by IFN-□γ ELISPOT did not reveal significant differences between MS patients and controls. MOG-reactive T cell lines and clones (TCL/TCC) were generated by stimulating PBMC of four MS patients and three healthy subjects with a cocktail of the four MOG peptides. The functional properties of 50 MOG peptide-reactive TCL/TCC obtained were studied. All TCL were TCR□□αβ+ CD4+, 20 TCL showed reactivity to MOG peptide 1-22, 13 to 34-56, 1 to 64-86 and 16 to 74-96. No significant differences in peptide recognition were observed between MS patients and controls. The T cell receptor (TCR) hypervariable regions of MOG-reactive TCL/TCC showed a heterogeneous usage of various TCR V(-D)-J elements. The data provide no evidence for clonal expansions within the MOG-reactive T cell repertoire of the two study groups. Intracellular cytokine analysis demonstrated predominantly Th1-TCC (IFN-□γ+/IL-4-) in MS patients, while most MOG-reactive TCC of control subjects had a mixed Th0/Th1 phenotype. The MS-derived MOG-reactive TCC did not secrete brain-derived growth factor and/or nerve growth factor upon stimulation, but produced increased levels of TNF-□α as compared to controls. Most of the MS-derived MOG-TCC induced specific cytolysis of autologous MOG-pulsed PBMC (9/11) while none of the MOG-TCC isolated from control subjects showed this cytotoxicity (0/8). In conclusion, although the frequency of anti-MOG T cells was similar in MS patients and controls, our data indicate potential differences in the functional properties of MOG TCL in MS patients versus healthy controls which may relate to their role in the disease process.

Key words: *myelin oligodendrocyte glycoprotein, multiple sclerosis, T cell repertoire*

3.1 Introduction

Myelin basic protein (MBP) and proteolipid protein (PLP), the most abundant proteins of CNS myelin, have been extensively studied as putative target autoantigens in MS (reviewed in Hohlfeld[1] and Schmidt[2]). There is increasing evidence that autoimmune reactivity against the quantitatively minor myelin components can also take part in the disease pathogenesis. Recent data indicate that myelin oligodendrocyte glycoprotein (MOG) is an important candidate autoantigen in MS. MOG is the only myelin antigen described so far that induces both an inflammatory T cell response and a demyelinating antibody response upon immunization in different strains of mice, rats and nonhuman primates[3-5] (reviewed in Von Büdingen[6]). MOG-induced EAE results in CNS inflammatory lesions that resemble typical MS lesions. Demyelination is accompanied by the deposition of MOG-specific autoantibodies and patterns of myelin vesiculation[7-9].

MOG is a CNS-specific, quantitatively minor myelin component (0.01 - 0.05%) expressed on oligodendrocyte surfaces and the outermost lamellae of the myelin sheaths[10;11]. In the membrane topology model, proposed by Kroepfl and co-workers, a single immunoglobulin-like domain is exposed to the extracellular environment[12]. Most of the studies performed in animals and humans, investigated B or T cell responses against (peptides of) this extracellular domain because it is potentially accessible to an autoimmune attack. Despite the difficulties in purifying the highly hydrophobic MOG, several reports have demonstrated T and B cell responses against this minor myelin component in MS patients. Elevated autoreactive B cell responses in cerebrospinal fluid (CSF) and peripheral blood (PB) of MS patients were described by several groups[13-15]. In contrast, Reindl and co-workers reported similar frequencies of anti-rMOG IgG antibodies in patients with MS and other neurological inflammatory diseases[16].

Other reports demonstrated an increased proliferative reactivity of circulating T cells to MOG as compared to other myelin antigens such as MBP, PLP and MAG[17;18]. Sun and co-workers described a higher frequency of IFN-γ secreting MOG-reactive T lymphocytes in MS patients as compared to controls[14]. These findings were confirmed in another study in which primary proliferative responses to MOG were evaluated[17]. Using a panel of overlapping synthetic

peptides spanning the extracellular domain of MOG, T cell reactivity was shown to be predominantly directed against three main regions 1-22, 34-56 and 64-96[19]. Wallström and co-workers described a dominant MOG epitope (peptide 63-87) in DR2+ MS patients[20]. Recent studies could not confirm the increased anti-MOG T cell reactivity in MS, and reported similar frequencies of anti-MOG T cell responses in both MS patients and healthy controls[15;18;21;22]. Lindert and co-workers isolated MOG-reactive T cell lines from MS patients and healthy control subjects[15]. This study revealed a heterogeneous T cell response to MOG with respect to epitope specificity and cytokine production[15].

In this report, the T cell receptor hypervariable CDR3 region sequences and cytotoxic potential of MOG-reactive T cell lines and clones isolated from the blood of patients with MS and healthy control subjects were analyzed. In addition, the frequency, phenotype, peptide reactivity, T helper (Th) type and secretion of BDNF, NGF and TNF-α of MOG-reactive T cells in MS patients and control subjects were evaluated. Our data demonstrate similar frequencies of MOG (peptide)-reactive T cells in MS patients and healthy controls. The analysis of the functional properties of MOG-reactive T cells revealed some interesting differences between MOG-reactive T cells from MS patients and controls. MOG-reactive T cells from MS patients were almost exclusively Th1-like cells, while those derived from healthy controls most often had a mixed Th1/Th0-like phenotype. In addition, MOG-reactive T cells from MS patients produced significantly more TNF-α as compared to the anti-MOG T cells isolated from controls. Finally, the majority of CD4+TCRαβ□□+ MOG-reactive T cells isolated from MS patients showed specific cytotoxic reactivity to MOG-pulsed autologous PBMC. This cytotoxicity was not observed for the MOG-reactive clones isolated from controls. Together, our results demonstrate for the first time important differences in the functional properties of MOG-reactive T cells from MS patients as compared to healthy control subjects. Our results provide further support for a pathogenic role of MOG-reactive T cells in MS and therefore could have implications for the development of novel immunotherapies for MS.

3.2 Materials and methods

3.2.1 Patients and healthy controls

Heparinized venous blood samples were collected from 12 women and 6 men with clinically definite MS. Fourteen patients had a relapse-remitting form of MS, the remaining four showed a progressive disease course. In addition, blood was drawn from eight randomly selected healthy control subjects (NS). Nine out of 18 MS patients and 2 out of 8 NS were HLA DR2+ as determined by PCR as described in 2.2.2. Table 3.1 provides an overview of the characteristics for the MS patients and controls. Informed consent was obtained from all subjects volunteering for this study.

Table 3.1. Characteristics of MS patients and healthy controls

Subject	Sex	Age	HLA-DR2[1]	Disease type[2]	Treatment[3]
MS patients					
AMH	F	37	+	RR	-
ANM	F	36	+	RR	-
BAC	F	29	-	RR	IFN-□β
BLG	F	54	-	RR	IFN-□β
DEG	M	40	-	RR	IFN-□β
DIA	F	31	+	SP	IFN-□β
EUG	M	39	-	RR	IFN-□β
FRW	M	32	-	RR	
GEJ	M	59	+	SP	-
GOP	M	50	+	SP	-
HIC	F	30	-	RR	IFN-□β
HUM	F	34	+	RR	IFN-□β
JEL	F	43	+	CP	-
JOV	M	35	-	RR	-
KEM	F	32	+	RR	-
LIB	F	52	-	RR	-
MJD	F	62	+	RR	-
SCI	F	32	-	RR	-
VEL	F	25	+	RR	-
VOJ	F	48	-	RR	-

Subject	Sex	Age	HLA-DR2[1]	Disease type[2]	Treatment[3]
Healthy controls					
DEA	F	50	+		
DEC	F	27	-		
HAH	F	41	-		
HEN	M	28	+		
KOI	F	39	-		
MIL	M	43	-		
RUI	F	40	-		
VAA	F	26	-		

[1]: MS type at time of sampling: RR, relapsing-remitting; SP, secondary progressive; CP, chronic progressive.
[2]: Subjects were scored positive (+) or negative (-) for the HLA-DR2 haplotype, as determined by PCR.
[3]: Treatment type at time of blood sampling: IFN-□β, interferon beta-1a (Rebif™); -: none.

3.3 Results

3.3.1 Enumeration of MOG-reactive T cells by IFN-□γ ELISPOT

To study and compare the frequency of MOG-reactive T cells, we analyzed reactivity to rMOG in 16 MS patients and 8 healthy subjects (NS) using an IFN-□γ ELISPOT assay. The characteristics of patients and controls are listed in Table 3.1. As shown in Figure 3.1, IFN-γ secreting rMOG-reactive cells were detected in 15/16 MS patients and all healthy controls (8/8). The mean frequency of rMOG-reactive cells was higher in MS patients ($14.7x10^{-5}$) compared to healthy controls ($12.0x10^{-5}$), but the difference was not statistically significant (Figure 3.1). No significant differences were found in MS-subgroups (relapsing *versus* progressive), DR2+ *versus* DR2- and treated *versus* untreated patients.

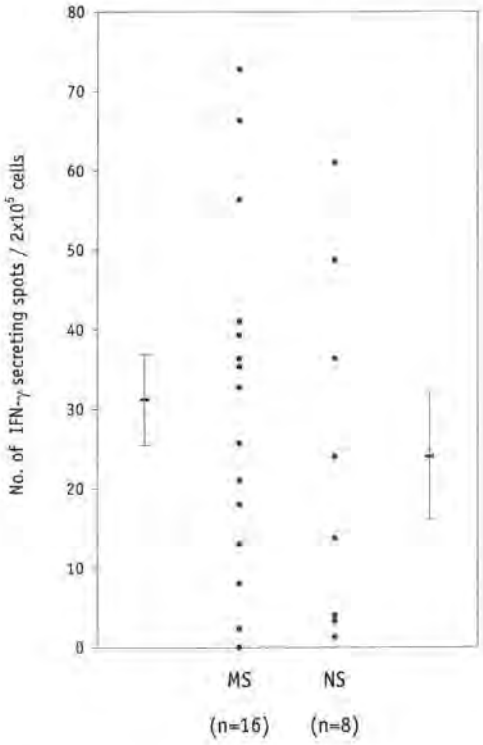

Figure 3.1. *T cell reactivity to rMOG in MS patients and normal subjects (NS)*

Black dots represent specific numbers of IFN-□γ secreting cells per $2x10^5$ cells for each subject. Specific numbers were obtained after subtraction of background values from control wells without stimulus. Horizontal lines for each study group represent mean and standard errors.

3.3.2 Generation of MOG-reactive T cell lines

To study the functional properties of MOG-reactive T cells, PBMC of 9 MS patients and 5 healthy controls were stimulated at limiting dilution conditions with a mixture of four synthetic MOG peptides. These peptides were selected on the basis of their reported immunodominance in MS patients and/or EAE animals[19]. They span most of the extracellular domain of the native antigen[20]. PBMC were stimulated with the MOG peptide mix at $1x10^5$ cells per well in 60 wells. After 14 days of culture, each well was tested for the presence of MOG-reactive cells by a ^{3}H-Thy incorporation assay. MOG-reactive T cell lines were identified in 8/9 MS patients and in all of the control subjects (5/5) (data not shown). Although the frequency of MOG-reactive T cells was much lower than in the ELISPOT analysis, the mean frequency of MOG-reactive T cells was similar in both study groups (MS: $13.3x10^{-7}$ cells, NS: $14.2x10^{-7}$ cells). Most of the MOG-reactive T cell lines were then cloned by PHA at 0.3, 0.5 or 1 cell per well. However, since some of these T cell cloning experiments were not successful, we used both MOG-reactive T cell lines (TCL) and clones (TCC) in subsequent experiments. In all experiments independent TCC or TCL were used, implying that they were generated from different primary MOG-reactive T cell lines obtained at limiting dilution conditions.

3.3.3 Peptide reactivity and phenotype of MOG-reactive TCL

Fifty MOG-reactive T cell lines (TCL) or clones (TCC) obtained from 4 MS patients and 3 healthy controls were tested for their phenotype and reactivity to the four MOG peptides. All TCL were CD3+, CD4+ and TCR$\alpha\beta\square\square$+. None of the TCL expressed markers of natural killer cells (CD16/CD56) (data not shown). Three MS patients and three controls had TCL reactive to at least two different MOG peptides (Table 3.2). The two DR2+ MS patients GEJ and HUM showed a heterogeneous peptide response. Patient HIC is the only patient with a skewed reactivity to a single peptide (peptide 1-22). Out of the 50 TCL tested, only one TCL was reactive to peptide 64-86. There was no significant difference in the overall reactivity profile to the four tested MOG peptides in the MS patients as compared to the control subjects. In conclusion, in most MS patients and controls, TCL reactive to one of the tested MOG peptides were present at similar frequencies.

Table 3.2. Peptide specificity of MOG-reactive T cell lines

		MOG peptide				
		1-22	34-56	64-86	74-96	Total
MS	GEJ	2[1]	0	1	2	5
	HIC	3	0	0	0	3
	HUM	4	2	0	4	10
	VOJ	1	3	0	1	5
	Total (%)	10 (43%)	5 (22%)	1 (4%)	7 (31%)	23
NS	DEC	0	1	0	1	2
	DEA	7	1	0	2	10
	MIL	3	6	0	6	15
	Total	10 (37%)	8 (30%)	0 (0%)	9 (33%)	27

[1]: Number of TCL/TCC within a given subject that are reactive to this peptide.
NS: healthy controls.

3.3.4 T helper profile of MOG-reactive TCL

To study the T helper profile of the MOG-reactive T cells, flow-cytometric intracellular cytokine analysis was performed with anti-IL-4 and anti-IFN-γ antibodies. After stimulation with PMA and ionomycin the intracellular staining was evaluated in 12 independent TCC from 4 MS patients and 13 independent TCC from 2 healthy controls (Table 3.3). Figure 3.2 shows a typical intracellular cytokine profile of a MOG specific T cell clone of a MS patient (left, GEJ-4) and a healthy control (right, DEA-1).

After stimulation the majority of the MS-derived MOG-reactive TCC (83.3%, 10/12) consisted of pure Th1 cells, producing IFN-γ but little or no IL-4. One MS-derived TCC (VOJ-1) was Th0-like (IFN-γ+□□/IL-4+), while one TCC (VOJ-2) consisted of a mixed Th0/1 population (IFN-γ+ and IL-4+/IFN-γ+). MOG-specific TCC derived from healthy controls frequently contained mixed T helper subtypes (Table 3.3). For instance, TCC DEA-5 contained one population with a Th1-like profile (IFN-γ□+/IL-4-: 38%) and one population of Th0 subtype (IFN-γ□+/IL-4+: 62%). Interestingly, Th0 populations were observed significantly more frequently in NS

Table 3.3. Intracellular cytokine expression profiles of MOG-reactive T cell lines/clones

		% IFN-γ +	% IL-4 +	% IFN-γ and IL-4 +	DN[1]	Th phenotype[2]
MS	GEJ-1	**93**	0	5	2	Th1
	GEJ-3	**98**	0	2	0	Th1
	GEJ-4	**99**	0	1	0	Th1
	GEJ-5	**98**	0	0	2	Th1
	GEJ-8	**78**	0	0	22	Th1
	GEJ-9	**95**	0	1	4	Th1
	HIC-1	**84**	0	13	3	Th1
	HUM-1	**95**	0	1	4	Th1
	VOJ-1	15	11	**60**	14	Th0
	VOJ-2	**74**	0	**22**	4	Th1/0
	VOJ-5	**92**	0	0	8	Th1
	VOJ-6	**91**	0	5	4	Th1
NS	DEA-1	**30**	8	**53**	9	Th0/1
	DEA-5	**38**	0	**62**	0	Th0/1
	DEA-6	**63**	0	**36**	1	Th1/0
	DEA-7	7	**45**	**41**	7	Th0/2
	DEA-9	11	1	**88**	0	Th0
	MIL-1	**50**	0	**50**	0	Th0/1
	MIL-2	**29**	15	**46**	10	Th0/1
	MIL-3	**98**	0	1	1	Th1
	MIL-4	**91**	0	2	7	Th1
	MIL-5	**89**	3	5	3	Th1
	MIL-6	**91**	0	2	7	Th1
	MIL-10	**41**	1	**52**	6	Th0/1
	MIL-11	**37**	1	**48**	14	Th0/1

[1] DN: double negative cells, producing no cytokines after stimulation; NS: healthy controls.
[2]: T helper (Th) subtype was determined as follows: Th_1 :predominant production of IFN-γ alone; Th_2: predominant production of IL-4 alone and Th_0: production of both IFN-γ and IL-4. Predominant production (by the majority of the T cells) is printed in bold.

derived TCC (9/13) than in MS-derived TCC (2/12) (Mann-Whitney U-test, $p<0.01$). One NS-derived TCC (DEA-7) contained a Th2-like population (IL-4+/IFN-γ□-). Taken together, we observed a marked difference in the T helper profiles of MS-derived *versus* NS-derived MOG-reactive TCC. While MS-derived TCC were predominantly Th1-like cells, the TCC isolated from healthy controls predominantly had a mixed phenotype of Th0- and Th1-like cells. This difference was mainly due to the more frequent production of IL-4 in the NS-derived TCC as compared to the MS-derived TCC.

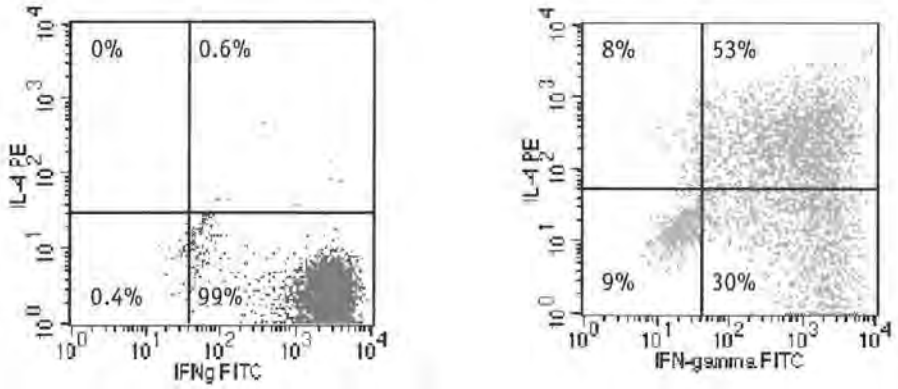

Figure 3.2. *Cytokine expression profile of MOG-reactive T cell clones*

Dot plots represent flow-cytometric analysis of intracellular cytokine production by MS-TCC GEJ-4 (left dot plot) and a NS-TCC DEA-1 (right dot plot). Percentages of T cells producing IFN-γ alone (lower right), IL-4 alone (upper left), both IFN-γ and IL-4 (upper right) or none of the two cytokines (lower left) upon stimulation are calculated.

3.3.5 Cytotoxic properties of MOG reactive T cell clones

To examine the possible cytotoxic properties of CD4+ MOG-reactive T cells, a panel of 19 TCC isolated from 3 MS patients and 2 healthy controls were tested for their cytotoxic reactivity towards MOG peptide-pulsed autologous mononuclear cells and natural killer-sensitive K562 cells (Figure 3.3). None of the T cell clones tested induced a natural killer-like cytolysis of the K562 cells (data not shown). The panel of 8 TCC from the healthy controls consisted of independent clones reactive towards three different MOG peptides (1-22, 34-56 and 74-96). None of the NS-derived clones tested could induce specific killing (>10%) of MOG peptide-pulsed autologous target cells (Figure 3.3 B). In contrast, as demonstrated in Figure 3.3 A, 9/11 T cell clones from 3/3 MS patients, reactive to different MOG-peptides (1-22 and 74-

96), induced specific cytolysis of the peptide-pulsed PBMC (but not of non-pulsed cells). Remarkably, 2 TCC from one MS patient (GEJ-7 and GEJ-8) were not able to specifically provoke lysis of the target cells, although 3 other TCC from this patient were cytotoxic. There was no correlation between peptide reactivity and cytotoxic potential. Taken together, these data indicate that cytotoxic CD4+ T cells are present within the MOG-reactive T cell repertoire of MS patients. In contrast, no cytotoxic MOG-reactive TCC were not observed in the NS-derived TCC in this study.

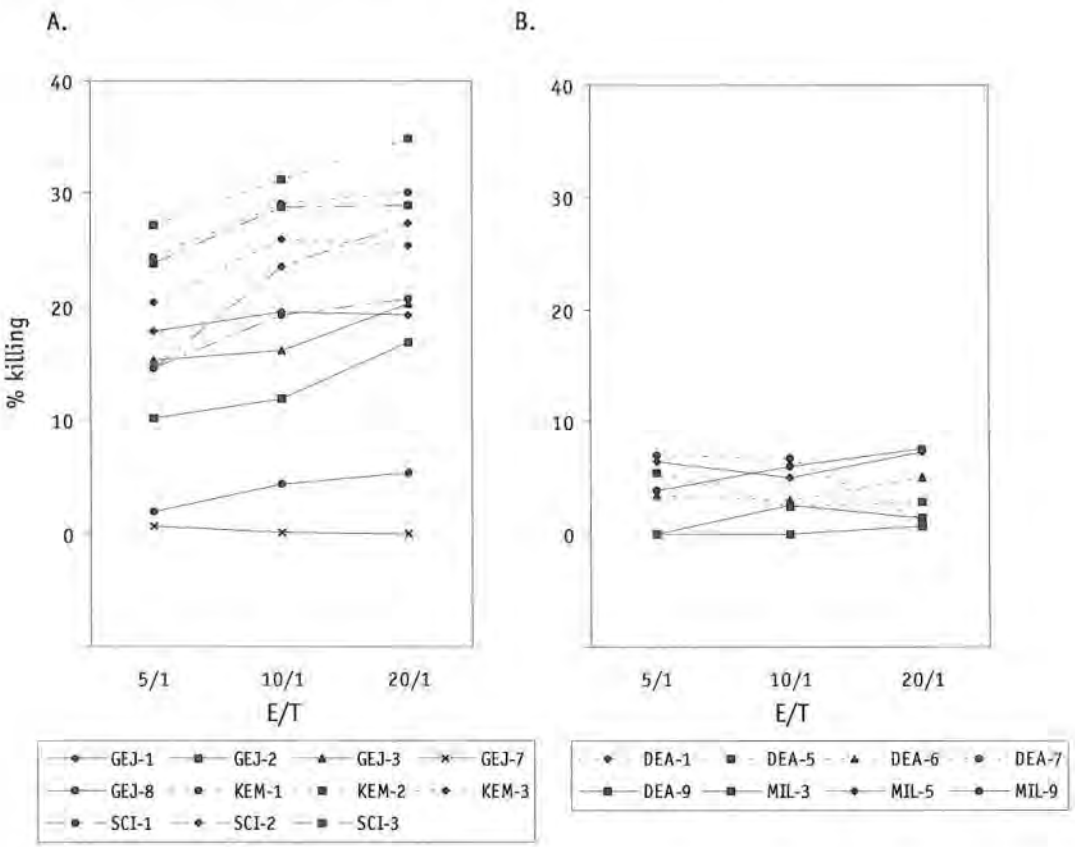

Figure 3.3. *Cytotoxic properties of MOG-reactive T cell clones from MS patients and controls*

Cytotoxic properties of MOG-reactive CD4+ T cell clones to MOG peptide-pulsed autologous mononuclear cells. Specific killing was evaluated in a standard ^{51}Cr-release assay. Line graphs display the percentage of specific cytolysis (y-axis) at increasing effector-to-target ratios (as shown in the x-axis) for different T cell clones. Figure A shows the results for 11 T cell clones generated from 3 MS patients, whereas figure B displays the data obtained from 8 T cell clones from 2 healthy controls.

3.3.6 Secretion of TNF-α, BDNF and NGF protein by MOG-reactive TCC

We then explored whether MOG-reactive TCC could secrete brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and tumor necrosis factor alpha (TNF-α). Sixteen MOG-reactive TCC (9 from 2 MS patients, 7 from 2 NS) were cultured in the presence of PBMC alone; PHA and autologous PBMC; or MOG peptide-pulsed PBMC for a period of 72 hours. The cell supernatants were used for ELISA quantification while the remaining cells were subjected to a proliferation assay to measure the PHA-induced and antigen-induced proliferation. MOG-derived TCC from both MS patients and healthy controls produced low constitutive mean levels NGF (MS: 90 ± 24 pg/ml, NS: 125 ± 14 pg/ml) but no detectable amounts of BDNF (Figure 3.4). No increased levels of BDNF and NGF were seen after stimulation with PHA or MOG peptide, although these stimuli induced significant proliferation (Figure 3.4). In marked contrast, TNF-α levels were significantly elevated both after stimulation with PHA and after stimulation with the MOG peptide (Figure 3.4). Interestingly, MS-derived MOG-reactive TCC produced higher levels of TNF-α as compared to NS-derived TCC. This difference was notable after PHA stimulation (although not statistically significant), but was even more pronounced after stimulation with the specific MOG peptide (unpaired t-test, $p<0.05$). MOG stimulated the levels of TNF-α secretion more than 10 fold in MS-derived TCC (8.5 ± 3.3 ng/ml) as compared to the NS clones (0.6 ± 0.2 ng/ml). The increased TNF-□α production was predominantly observed in the TCC from the DR2+ MS patient (data not shown). The MOG-induced proliferation rate was also higher in MS-derived TCC (about 3X). Interestingly, using the same technical approach we have previously reported TNF-α levels of 0.8 ± 0.2 ng/ml and 0.4 ± 0.07 ng/ml in the supernatant of stimulated MBP-reactive TCC from MS patients and controls respectively[23].

Taken together, our data indicate that MOG-reactive TCC do not produce BDNF and NGF after antigen stimulation, but produce high levels of the proinflammatory cytokine TNF-□α. The TNF-α secretion is significantly higher in MS-derived as compared to NS-derived MOG-reactive TCC and appears to be higher in MOG-reactive clones as compared to MBP-reactive clones.

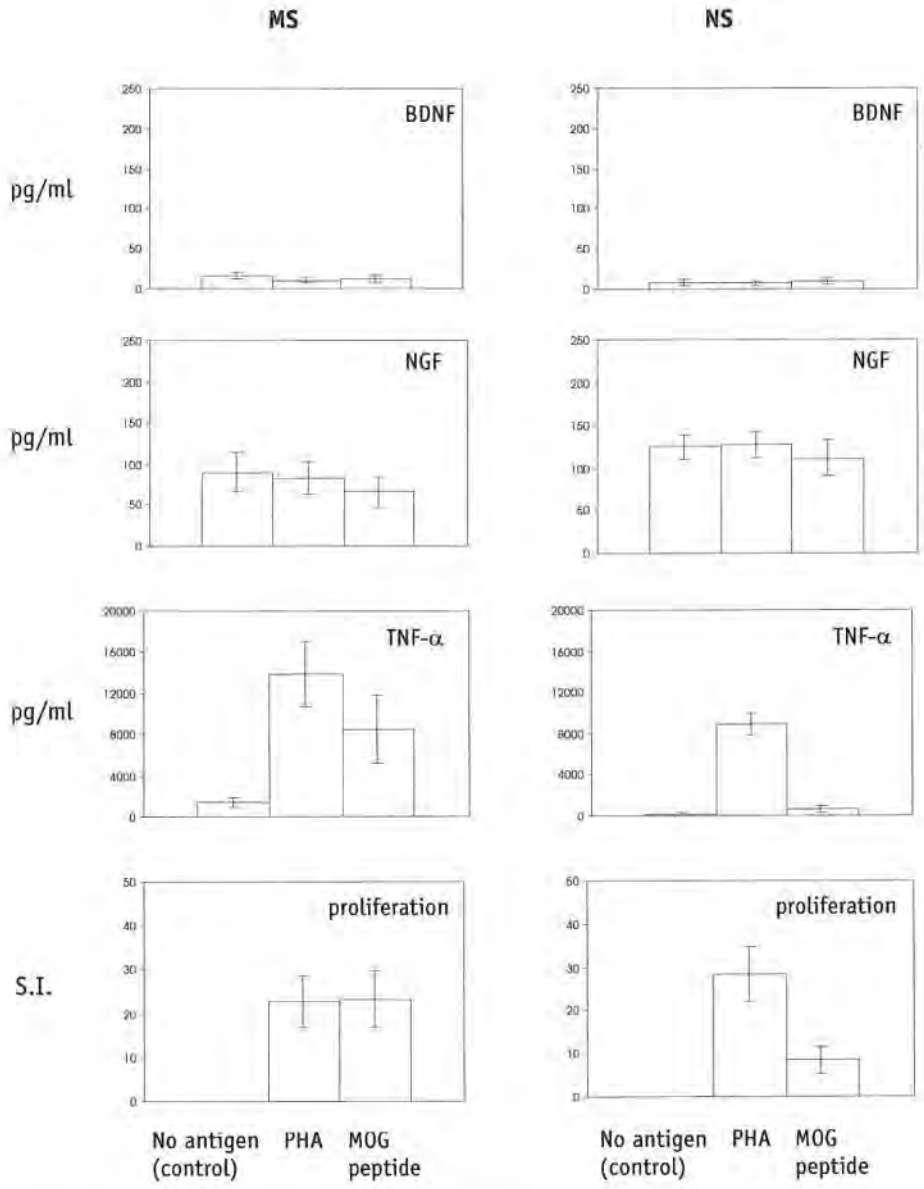

Figure 3.4. *Mean production of BDNF, NGF and TNF-α by MOG-reactive T cell clones*

Mean protein production of 16 MOG-reactive CD4+ T cell clones, 9 from 2 MS patients and 7 from 2 healthy controls. Mean concentrations in pg/ml of BNDF, NGF and TNF-□α as measured by ELISA in supernatants of non-stimulated (no antigen), PHA-stimulated and MOG peptide-stimulated T cell clones after 72 hours. Proliferative response is also determined after 72 hours using a ^{3}H-thymidine incorporation assay. Stimulation index (SI) is calculated as (mean counts after stimulation / mean counts without stimulation).

Table 3.4 A Sequence analysis of TCR rearrangements in T cell lines/clones of MS patients

Patient		TCL	TCC	Epitope	BV[1]	BnDn[1]	BJ[1]	AV[1]	An[1]	AJ[1]
VOJ	-1		X	34-56	13.3	VAE	2.5	8.1	SES	49
	-2		X	34-56	3.1	PRRDSGI	2.7	2.3	RG	39
	-3		X	34-56	13.2	RDSN	1.6	-	-	-
	-4		X	1-22	7.2/3	GAIRA	1.1	30.1	VQRH	42
	-5		X	74-96	9.1	RASVD	2.1	23.1	VPG	8
GEJ	-1		X	74-96	6.6	AGS	2.5	15.1	-	40
	-5		X	74-96	13.1	STLPD	2.2	23.1	L	30
	-6	X		1-22	6.5	ASISGSRA	2.3	5.1	LDDA	33
					10.1	NIPDQPA	1.4	20.1	VGAPF	37
					17.1	IMQGN	1.2			
HIC	-1		X	1-22	12.1	DAEDRARQVG	1.5	2.3	RG	15
					17.1	GHEG	2.7			
HUM	-1		X	1-22	6.5	VVTTRTP	2.3	5.1	LDV	6
	-2	X		1-22	2.1	PPRLAGGA	2.3	2.1	RYDD	43
					3.1	RLDSGGS	2.2			
					6.5	RVQMT	2.1			
	-3	X		1-22	2.1	LTGTGGD	2.7	2.1	RY	43
					6.1	TGG	2.7	8.2	GE	6
					7.2/3	EVRR	2.7	11.1	EGPE	23
	-4	X		34-56	2.1	RSSV	2.7	5.1	F	30
					4.1	VRRD	2.1	7.2	VIIGGD	7
					8.1/2	RMDPA	1.3	22.1	PARH	12
	-5	X		34-56	7.2/3	PLAGAR	2.7	4.1	LV	53
					13.2	QTGTSGSFG	2.7	8.1	SI	29
					13.6	PGQAH	1.6	10.1	GAG	52
	-6	X		74-96	8.1/2	QEGPAMA	1.1	2.1	RY	43
					13.1	SSGGAD	2.3	4.1	LRDG	53
					13.2	RKSAE	2.5	11.1	EDA	12
	-7	X		74-96	6.3	ALLAGR	2.7	3.1	TDEG	53
					13.1	TGAL	2.3	4.1	LRDD	30
					21.3	KTFWREN	2.1	22.1	YS	37
	-8	X		74-96	2.1	DRWRVT	2.1	2.3	G	23
					6.1	TDRGHG	1.5	18.1	FSIGD	49
					13.1	SAGGA	2.3			
	-9	X		74-96	3.1	RTSSGP	1.6	7.2	VG	39
					6.3	RGPN	2.7	8.1	GVG	23
					13.2	YAVG	1.5	17.1	KE	57

[1]: Sequence analysis of the junctional region of the α (AV-An-AJ) and □β (BV-BnDn-BJ) chain of the T cell receptor of T cell lines (TCL) or T cell clones (TCC) reactive to a specific MOG epitope (1-22, 34-56, 74-96).

Table 3.4 B Sequence analysis of TCR rearrangements in T cell lines/clones of controls

Patient		TCL	TCC	Epitope	BV[1]	BnDn[1]	BJ[1]	AV[1]	An[1]	AJ[1]
DEA	-1		X	1-22	6.5	AGWG	2.1	1.4	A	37
					10.1	AKGRA	2.6	18.1	FPP	17
	-2		X	1-22	2.1	IEVAWG	2.5	22.1	LM	8
					8.1	EATGR	1.2			
	-3	X		1-22	1.1	DSGGAL	2.3	10.1	GGR	37
					17.1	QQGPGG	2.3			
	-4	X		1-22	2.1	G	1.5	8.2	FY	10
					13.6	TSRGVA	1.4	17.1	K	28
					14.1	YKTS	2.1			
	-5		X	34-56	8.1/2	FTEG	1.2	-	-	-
	-6		X	34-56	13.5	DSL	1.1	23.1	VRD	34
	-7		X	74-96	9.1	PMDRDLL	1.1	-	-	-
	-8		X	74-96	5.1	ATQG	1.4	3.1	TEG	53
					13.3	VV	2.1	4.1	LP	49
					17.1	VGVT	2.7	17.1	RK	8
MIL	-1		X	34-56	20.1	FRTRVH	2.1	11.1	EDPPG	10
	-3		X	34-56	7.2/3	PGGV	1.3	17.1	KE	36
	-5		X	34-56	8.1/2	DEQGG	1.5	1.1	HAW	22
	-7	X		34-56	8.1/2	GAGTGD	2.7	4.1	RPL	49
					13.2	QDRLE	1.5	5.1	VLGDPS	37
					17.1	RRGLD	2.1	8.1	Y	12
	-8	X		34-56	8.1/2	QGQGIA	2.2	2.3	IV	40
					13.1	WGL	2.3	19.1	VKY	58
					17.1	GGVSSGG	2.3			
	-9		X	74-96	2.1	DRPGGP	2.3	-	-	-
					18.1	RETGL	1.1	-	-	-
	-10	X		74-96	3.1	HAGGIS	2.7	6.1	N	28
					9.1	TNQ	2.1	7.1	SV	23
								12.1	LSEDD	9
	-11	X		74-96	13.6	RLYGTGN	2.1	2.1	MG	39
					17.1	PRQRTT	2.3	6.1	ASR	13
					25.1	GGYD	1.2	8.2	ST	44
	-12	X		74-96	1.1	DGRG	2.7	2.1	GG	9
					7.2/3	DKSRGQGR	2.7	6.1	GG	50
					16.1	VGDLI	2.1	19.1	VD	48
	-13	X		74-96	9.1	EVH	1.2	1.4	DSA	28
								11.1	EATGG	20
								22.1	F	20
	-14		X	1-22	8.1/2	GGGP	1.2	5.1	LLE	36
	-15	X		1-22	2.1	SNSGAP	1.1	2.1	D	23
					13.3	NEG	2.1	2.2	AQS	27
					17.1	GYSGLAG	2.3	3.1	TDAP	28
	-16	X		1-22	2.1	SRQGL	2.4	2.1	LE	12
					8.1/2	GGA	1.1	6.1	GRGV	47
					13.3	RFQGVG	2.6	8.1	SAHFLRG	26

[1]: Sequence analysis of the junctional region of the α (AV-An-AJ) and □β (BV-BnDn-BJ) chain of the T cell receptor of T cell lines (TCL) or T cell clones (TCC) reactive to a specific MOG epitope (1-22, 34-56, 74-96).

3.3.7 Sequence analysis of TCR rearrangements

To reveal whether a skewed TCR V gene usage can be observed among these T cells, the TCR expression of MOG-reactive TCC was evaluated. In addition, TCR analysis may indicate possible clonal expansion of the MOG-reactive TCC in patients or controls. Clonal expansion can be 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*[24]. TCR rearrangements were analyzed by direct sequencing of 39 independent TCL or TCC from 2 healthy controls and 4 MS patients. Tables 3.4 A and B list the TCR AV and BV gene usage and CDR3 amino acid sequences of these T cells. The MOG-reactive TCC and TCL expressed a wide range of BV and AV gene families. Interestingly, 41% of all TCC/TCL expressed a BV13 element. However, this BV gene was not differently expressed among MS-derived TCC/TCL as compared to NS-derived T cells. Some BV families were found at increased or reduced levels in MS *versus* NS-derived TCC/TCL. The BV6 family was found in 44% of the MS-derived MOG-reactive TCC/TCL but in 4.7% of the NS-derived TCC/TCL only. BV8 and BV17, on the other hand, were less frequently observed in MS-derived TCC/TCL as compared to NS-derived T cells.

Within a given patient or control subject, T cells reactive to a MOG peptide do not share a particular TCR AV and BV rearrangement and do not express identical CDR3 junctional sequences. Together, the data do not support clonal expansion of T cells recognizing a specific MOG epitope in MS patients and healthy controls.

3.4 Discussion

Although MOG is only a minor component of the myelin sheath, there are several incentives to study this protein as a candidate autoantigen for MS. MOG is a CNS-specific glycoprotein that is expressed on the outhermost lamellae of the myelin sheath where it is accessible to an autoimmune attack especially by autoantibodies[25]. In EAE, MOG induces both an inflammatory T cell response and a demyelinating autoantibody response[26]. Therefore, MOG fulfills the criteria of a potential primary target antigen, and a detailed study of this autoimmune T cell reactivity is of particular interest for a better understanding of disease development. So far, only one report addressed the characteristics of MOG-reactive T cell lines in MS[15]. We analyzed the frequency and functional properties of MOG-reactive T cells in MS patients and controls. Our results provide some interesting observations.

Our data show that MOG-reactive T cells are present in the majority of MS patients and healthy controls, but that there is no difference in the frequency between these study groups. These results are consistent with earlier findings[15;18;21], but are in contrast with results from other groups showing increased anti-MOG T cell reactivity in MS[14;17;19]. It remains to be studied whether these contrasting data are related to the use of different technologies to estimate the frequency of antigen-reactive T cells. We analyzed the frequency of MOG-reactive cells using two techniques. ELISPOT is based on the detection of cytokine secreting cells upon antigen stimulation, whereas limiting dilution analysis is based on the proliferative capacities of MOG-responsive T cells. Although both techniques did not reveal differences between MS patients and controls, absolute numbers of cells responsive to rMOG are underestimated by limiting dilution analysis. In contrast to mean frequencies of 1 in 10^6 cells as measured by LDA, using the ELISPOT technique we found frequencies of MOG-reactive IFN-□γ secreting cells as high as 1 in 3000. Similar discrepancies between proliferation-based frequency assays and ELISPOT were reported in our previous report for MBP-reactive T cells[22]. This observation should be taken into account when comparing T cell frequency data that were obtained using different approaches. In addition, it should be noted that we analyzed the response towards the extracellular domain of MOG (rMOG) only. We can therefore not exclude reactivity to transmembrane or cytoplasmatic domains of the protein. However, a previous study has reported no significant differences in proliferative responses to whole native human MOG and recombinant MOG protein

(extracellular domain), suggesting that the extracellular regions of MOG comprise most T cell epitopes in humans[18].

We also explored whether MOG-reactive T cells in MS patients and controls show different reactivity to four selected MOG peptides. These peptides were selected based on a previous report by Kerlero de Rosbo and co-workers demonstrating dominant T cell reactivity to three main regions of MOG: 1-22, 34-56 and 64-96[19]. These regions also correspond to epitopes shown to be encephalitogenic in several strains of rodents and nonhuman primates[6]. We generated T cells by stimulating PBMC with a cocktail of the four MOG peptides at limiting dilution conditions, and then analyzed the reactivity of each cocktail-reactive TCL to the four peptides. No differences were detectable in the overall peptide reactivity profile between MS patients and control subjects. All but one person had TCL reactive to at least two different peptides, indicating that most individuals show reactivity to several MOG epitopes. Interestingly, only one out of 50 TCL tested recognized the MOG peptide 64-86. Wallström and co-workers demonstrated preferentially T cell reactivity to MOG peptide 63-87 in HLA-DR2+ MS patients[20]. However, in the two DR2+ patients tested in our study one patient only showed reactivity to this peptide. Our results therefore indicate that 63-87 is not a dominant MOG epitope in the patients and control subjects analyzed in the present study. Not enough DR2+ patients were included in the study to address whether a possible increased response to the 63-87 region is present in these patients.

Analysis of the TCR rearrangements of independent MOG-reactive T cells showed expression of a wide range of BV and AV gene families in MS and controls. Interestingly, the T cells did not share any CDR3 region sequence, indicating that these T cells did not clonally expand *in vivo* in MS patients nor healthy controls. These data are in contrast to earlier observations that MBP-specific T cells are clonally expanded in MS patients[24;27]. This may imply that, in contrast to MBP-reactive T cells, MOG-reactive T cells are not present in an *in vivo* activated state in MS patients. Alternatively, clonally expanded MOG-reactive T cells may have been lost during *in vitro* culture, or may not be found due to rapid apoptosis after antigenic stimulation *in vivo*. Longitudinal analysis of anti-MOG T cells in patients and controls may answer some of these questions. Interestingly, some BV genes, including BV13, were found at increased frequency in MOG-reactive T cells of patients and controls, while BV6 was found at increased levels in the MS-derived TCC only. BV-specific expansion of T cells may indicate

stimulation of these T cells by superantigens. Further studies are needed to explore this possibility. Interestingly, our data are consistent with an earlier report showing a diverse BV gene usage of T cells reactive to MOG peptide 35-55 in MOG-induced EAE[28]. Our TCR analysis of MOG-reactive T cells indicates that TCR-based therapeutic approaches should incorporate a wide range of TCR BV gene elements to be successful in depleting the anti-MOG T cell repertoire in MS patients.

An important observation in our study is the biased Th1 profile of MOG-reactive T cells in MS patients, whereas MOG-reactive T cells from control individuals expressed predominantly a mixed Th0/Th1-like profile as shown by intracellular cytokine analysis. This is of particular interest since previous studies have shown that the encephalitogenicity of myelin-reactive T cells in EAE is associated with the Th1 phenotype[29]. The present data indicate that MOG-reactive T cells in healthy subjects often produce IL-4, a Th2 cytokine. It is tempting to speculate whether the production of IL-4 may help to control these autoreactive T cells in healthy subjects. The loss of IL-4 production may correlate with the triggering of autoimmune disease in susceptible individuals. Wilson and co-workers have recently shown that the loss of IL-4 production in a subset of T cells (Vα24JαQ T cells) is involved in susceptibility to autoimmune diabetes[30]. In addition, the MOG-reactive T cells produced increased levels of TNF-α upon stimulation with their specific MOG peptide as compared to controls. TNF-□α production may especially be relevant to MS, since this cytokine has been shown to damage myelin and oligodendrocytes and has also been observed in MS plaques[31]. The TNF-α □□production of MOG-reactive T cells evaluated in this present study was higher than the production reported for MBP-reactive T cells in our previous studies[23]. In addition, the TNF-α production of MOG-reactive T cells of the HLA-DR2+ patient was higher than that of DR2- patient. An association between DR2 and levels of TNF-□α production has been reported before[23]. Taken together, this first observation of a Th1 bias and high TNF-□α production of MOG-reactive T cells of a limited number of MS patients may correlate with their potential pathogenic role in MS.

A striking observation of our study is the cytotoxic potential of MOG-reactive T cells derived from MS patients. Nine out of eleven clones from three MS patients were able to induce a specific cytolysis of MOG peptide-pulsed autologous target cells. Surprisingly, none of the CD4+ TCC generated from two healthy controls showed any specific cytotoxicity towards

autologous target cells. Furthermore, no association between cytotoxic activity and epitope specificity was observed. It seems likely that this cytotoxicity correlates with the cytokine profile (including TNF-α production) of MOG-reactive T cells of MS patients. However, it is unclear whether this observed cytotoxicity plays an important role in the inflammatory response in MS. Cytotoxic MOG-reactive T cells may induce lysis of oligodendrocytes that present MOG epitopes in the context of MHC class II molecules. However, current information suggests that oligodendrocytes in MS lesions do not express class II molecules. Alternatively, MOG could be taken up and presented by resident microglia and thus activate the infiltrating MOG-reactive T cells. On the other hand, in EAE there is some evidence that encephalitogenic CD4+ myelin-reactive T cells posses cytotoxic potential[32]. Previously, cytotoxicity was observed for MBP- and PLP-specific CD4+ TCC[33-35]. Vergelli and co-workers have shown that the cytotoxic MBP-reactive T cells induce cytotoxicity of autologous antigen-pulsed targets through Fas-dependent and perforin-dependent pathways[35].

Finally, the potential neuroprotective role of MOG-reactive T cells was evaluated by analysis of the secretion of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) upon stimulation with PHA or the MOG-specific peptide. No detectable amounts of BDNF and low constitutive levels of NGF were measured. None of the MOG-TCC produced elevated concentrations of BDNF and NGF after stimulation. Our data are in contrast with the observations of Kerschensteiner and co-workers who recently reported an increased production of BDNF in several CD4+ myelin-reactive T cell lines upon stimulation[36].

In conclusion, the present study has revealed some interesting functional differences between MOG-reactive T cells of MS patients and NS. MOG-reactive T cells of MS patients have a Th1-like cytokine profile, produce highly increased levels of TNF-α, and frequently show cytotoxic reactivity. These T cells recognize various epitopes and express diverse TCR V gene elements and hypervariable CDR3 regions. On the other hand, MOG-reactive T cells of healthy controls mostly have a mixed Th1/0 profile and do not have cytotoxic potential. Interestingly, MOG-reactive T cells from MS patients resemble the encephalitogenic MOG-reactive T cells in EAE[6;37]. Recently, it was shown that MOG-reactive T cells with similar characteristics are present at a high frequency in the circulation of naive healthy marmosets[38]. Upon adoptive transfer, these T cells are capable of triggering CNS autoimmunity[38]. Our study provides further support for a pathogenic role of MOG-reactive T

cells in MS. Although it should be stressed that only a limited number of donors have been included in this study, these preliminary data could be important for the design of novel immunotherapies that are designed to target these autoreactive T cells.

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Chapter 4

Study of Activated T Cells in the Cerebrospinal Fluid – Optimization of a Protocol for CSF-based T Cell Vaccination

4.1 Introduction

Characterization of abnormalities in the T cell repertoire of patients with this chronic inflammatory disease of the central nervous system (CNS) could provide insights in the etiology of MS and may lead to the development of selective immunotherapies[1;2]. To study the myelin-reactive T cell repertoire in MS patients, most reports have focused on the peripheral blood compartment and only limited data of the CNS are available. Since it has been hypothesized that the population of lymphocytes in the cerebrospinal fluid (CSF) reflects the inflammatory cells infiltrating the parenchyma, the CSF can be considered as the best available source of cells infiltrating the CNS. Different studies on antigen-reactivity have demonstrated a high frequency of myelin-reactive T cells in the CSF of MS patients. Based on cell proliferation assays, two independent reports demonstrated high numbers of CSF-derived activated MBP- and PLP-specific T cells[3;4]. Soderstrom and co-workers showed an enriched myelin-reactive IFN-γ secreting T cell population recognizing different myelin antigens in the CSF compartment as compared to the peripheral blood of MS patients[5;6]. Based on results on the pathogenicity of these myelin-reactive T cells in the EAE model[7;8], the high frequency of this T cell population in the CSF of MS patients and reports illustrating the presence of myelin-reactive T cells in MS lesions[9;10], we hypothesize that these T cells may be particularly relevant to the pathogenesis of the disease.

When developing new therapeutic strategies for MS involving T cell(receptor peptide)s as vaccines, an accurate characterization of the T cell repertoire is necessary. Wilson and co-workers conducted a phase I clinical trial of a TCR BV6 peptide vaccine based on an analysis of the restricted TCR usage of CSF-derived T cells[11;12]. However, other studies reported different clonally expanded T cell subpopulations in the CSF varying from one patient to another[13;14]. In addition, heterogeneous BV gene expression profiles of CSF-derived T cells have been described[15-17]. The present controversial data may be related to different strategies used to study the CSF-T cell repertoire. However, these studies indicate that the CSF compartment may be a useful source of pathogenic cells involved in the disease development. Furthermore, it has been demonstrated that dominant T cell clones may persist in the CSF for extended periods of time, which may also be an indication for the relevance of these T cells[11].

In this study, we optimized a culturing protocol to identify disease-related activated CD4+ T cells from the CSF-derived mononuclear cell population obtained after a lumbar puncture. To selectively enrich for T cells, recently activated *in vivo* and therefore expressing high-affinity interleukin-2 (IL-2) receptors, we cultured CSF-derived mononuclear cells in autologous medium containing low concentrations of recombinant human IL-2 (rhIL-2, 2U/ml). To obtain information about the heterogeneity of the activated (T) cell population in the CSF, IL-2-expanded CSF cell cultures obtained from 5 MS patients and 4 patients with other non-inflammatory neurological diseases were analyzed for their phenotype and TCR BV gene usage. TCR expression profiles from 2 MS patients were also determined in parallel in directly *ex vivo* CD4+ PBMC and in short term IL-2-expanded activated T cells derived from the peripheral blood. Further clonal analysis of overrepresented BV genes may indicate whether T cell clones present in the periphery and the central nervous system are identical. In addition, the effects of long time culturing of these CSF-T cells on the composition of the mixture of cells was evaluated. This is important for the possible application of the cultured T cells as vaccines in T cell vaccination as discussed in chapter 5. To test the stability of the cultures over longer periods of time both phenotype and clonal composition of CSF T cells were determined at regular time points. Furthermore, the effect of freezing and thawing of these cell cultures on the phenotype was evaluated. Based on the results of this study, an optimized culturing protocol for the expansion of CSF-derived activated CD4+ T cells was established. This protocol was used in a pilot clinical trial of T cell vaccination involving 5 MS patients as described in chapter 5.

4.2 Materials and methods

4.2.1 Study design: expansion of CSF-derived activated T cells

Ten to 15 ml of cerebrospinal fluid was obtained from 5 relapsing-remitting MS patients (1 male/4 female) by lumbar puncture and from 4 patients with non-inflammatory neurological diseases (4 male/0 female). Cells were spinned down and resuspended in autologous medium (2.1.1.) in the presence of 10^5 irradiated autologous feeder cells and low concentrations of recombinant human interleukin-2 (2U/ml rhIL-2, Roche Diagnostics, Brussels, Belgium) at a concentration of 20,000 cells/well maximum. These culture conditions were optimized in pilot experiments with peripheral blood mononuclear cells (PBMC) as described in 4.3.1. When more than 30,000 mononuclear cells were isolated, a depletion of CD8+ T cells was performed on day 0 with anti-CD8 antibody coated immunomagnetic beads as described in 2.1.3. Cells were cultured for 7 days and restimulated with rhIL-2 and 10^5 irradiated autologous feeder cells. For the characterization of cultured activated CSF-derived cells, cells were harvested and counted after a total culturing period of 16 to 22 days and analyzed for their phenotype and clonal composition. To compare the heterogeneity of the activated T cell population in the blood and the CSF, PBMC from 2 MS patients were cultured using an identical protocol. To study the stability of CSF-derived T cell populations, cell cultures were expanded for an extended period of 2 months. Every two to three weeks, cells were restimulated with rhIL-2 and irradiated autologous feeder cells, and autologous medium containing rhIL-2 was refreshed every 3 days. At different time points during culturing, cells were phenotypically characterized and analyzed for their TCR expression profile. To study the influence of freezing and thawing on the phenotype, cells from one MS patient were frozen, thawed and cultured again for an extended period.

4.3 Results

4.3.1 Pilot experiments: optimal culturing conditions for expansion of activated T cells

To optimize a culturing protocol for the selective expansion of activated CD4+ T cells from CSF-derived mononuclear cells, pilot experiments using low numbers of peripheral blood mononuclear cells (PBMC) were performed. Activated cells, expressing the IL-2 receptor, were selectively expanded for 7 days at different concentrations of recombinant human IL-2 (rhIL-2). Based on proliferation assays and phenotype analysis, a standard concentration of 2U/ml rhIL-2 was used for all following experiments. Lower concentrations (1U/ml) were not sufficient to expand activated T cells, and higher concentrations (5U/ml) resulted in lower numbers of CD4+ T cells and higher percentages of CD8+ T cells and natural killer (NK) cells after culturing. As illustrated in Figure 4.1 A, after 7 days, significantly higher cell numbers were found in cell cultures with autologous serum (10%) added (460,000 cells/well) as compared to fetal bovine serum (10%) (210,000 cells/well). Furthermore, addition of autologous irradiated PBMC ('feeder cells', 10^5) to wells with low cell numbers also provided a better condition for cell expansion (no feeder cells added: 90,000 cells/well; feeder cells added: 414,000 cells/well) (Figure 4.1 B).

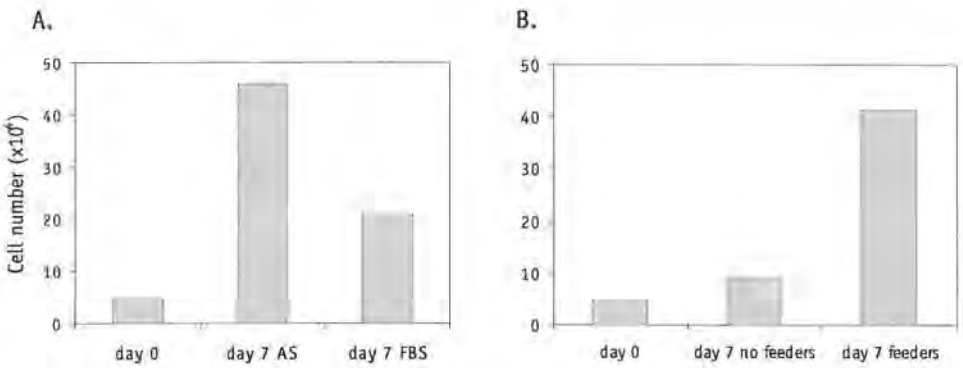


Figure 4.1. *Optimal culturing conditions for the expansion of activated T cells*

Bars represent cell numbers before and after 7 days of culturing with different sera (A) and with or without feeder cells (B). Peripheral blood mononuclear cells (PBMC) were cultured under different conditions in medium with low concentrations of recombinant human interleukin-2 (rhIL-2).
AS: medium + 10% autologous serum + IL-2 + 10^5 autologous irradiated PBMC; FBS: medium + 10% fetal bovine serum + IL-2 + 10^5 autologous irradiated PBMC; No feeders: medium without 10^5 irradiated autologous PBMC + IL-2 + 10% autologous serum; Feeders: medium + 10^5 irradiated autologous PBMC + IL-2 + 10% autologous serum.

To develop depletion protocols for the selective elimination of non-CD4+ T cells from low numbers of cultured mononuclear cells, PBMC were used. Procedures to effectively deplete CD8+ T cells, NK cells and γδ T cells were optimized and described in detail in 2.1.3.

4.3.2 Study of CSF-derived activated T cells

4.3.2.1 Expansion and phenotype analysis of activated CSF-derived cells

Most often, cell numbers in the CSF of control subjects (PRE NS: 8,225 ± 3,988) were lower than those in MS patients (PRE MS: 24,600 ± 6,652). After an expansion period of 16 to 22 days, high cell numbers were observed both in the control group (POST NS: 510,000 ± 39,648) and the MS group (POST MS: 1,174,200 + 270,501) although significant differences in expansion capacities were observed among individual patients (Figure 4.2).

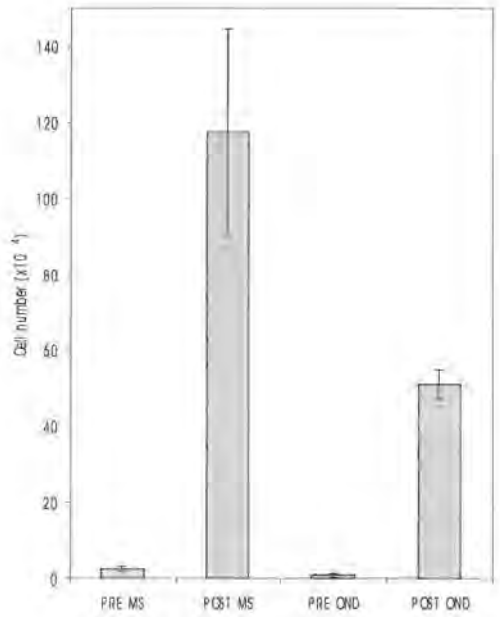


Figure 4.2. *Expansion of CSF-derived cells after culturing*

Bars represent mean cell numbers before and after culturing (16 to 22 days) for 5 MS patients and 4 patients with non-inflammatory neurological diseases. Standard errors of the mean (SEM) are shown in vertical lines.
PRE: before culturing; POST: after 16 to 22 days of culturing; MS: group of 5 MS patients; OND: group of 4 patients with non-inflammatory neurological diseases.

Flow-cytometric analysis demonstrated that CD3+ lymphocytes bearing the TCRαβ constituted the vast majority of CSF-derived cells in all patients (MS: 85.4 ± 4.5%; NS: 95.0 ± 1.7%). As illustrated in figure 4.3, little or no NK cells (MS: 4.9 ± 3.6%; NS: 0.6 ± 0.2%) or γδ T cells (MS: 4.4 ± 2.6%; NS: 2.4 ± 1.7%) were observed. However, in 2 MS patients either an elevated number of NK cells (MS-JOV: 19%) or γδ T cells (MS-HIC: 13%) was found. Although in the control group higher percentages of CD8+ T cells were present (MS: 5.3 ± 1.5%; NS: 35.8 ± 9.3%), it should be noted that for 4 out of 5 MS patients a CD8+ T cell depletion was performed before culturing. For only one control subject (NS-JAB), sufficient cell numbers could be obtained at day 0 (> 30,000 cells) to deplete CSF-derived mononuclear cells from CD8+ T cells. All the CSF samples that were depleted from CD8+ T cells before culturing showed low percentages of CD8+ T cells after expansion. These data demonstrate that CD8+ cell depletion before expansion is useful for the specific generation of CD4+ activated T cell cultures when sufficient cell numbers are available.

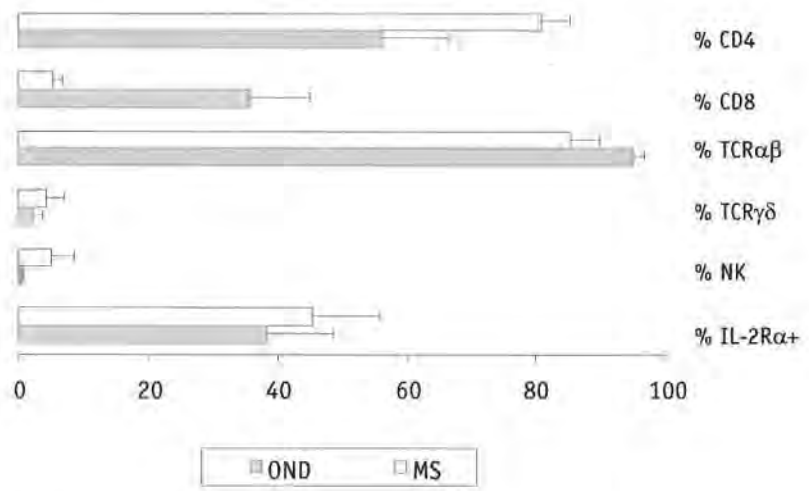


Figure 4.3. *Phenotypic expression profile of cultured CSF-derived cells*

Bars represent the mean percentages of cellular subsets. Standard errors of the mean (SEM) are shown in horizontal lines. Mean percentages of T helper cells (CD4), cytotoxic T cells (CD8), TCRαβ+ T cells (TCRαβ), γδ T cells (TCRγδ), natural killer cells (NK) and activated T cells (IL-2Rα+) are shown for 5 MS patients and 4 control patients with other non-inflammatory neurological diseases (OND).

4.3.2.2 TCR BV gene usage in activated CSF-derived T cells

To assess whether the TCR BV gene repertoire in CSF-derived activated T cells varied between MS patients and patients with other non-inflammatory neurological diseases (OND), using a PCR-ELISA assay, a semi-quantitative analysis of the TCR BV gene expression repertoire was performed. As demonstrated in figure 4.4, CSF-derived activated CD4+ T cells showed a rather restricted TCR BV gene expression profile in all patients as compared to unstimulated PBMC of a panel of healthy controls. Within a given patient, a limited number of overrepresented TCR BV genes (exceeding the mean TCR BV gene expression levels in the blood of 10 healthy subjects + 3 standard deviations) was present (Table 4.1). However, both in the MS and the non-MS group, different BV genes were overrepresented among different patients and no 'MS-specific' TCR BV gene could be identified. TCR BV2, 4, 7 and 9 showed an increased expression in three out of nine patients. TCR BV6, 17 and 18 were found in two patients and TCR BV3 was exclusively overexpressed in two out of four patients with other non-inflammatory neurological diseases (LUC and URM), but in none of the MS patients (Table 4.1). In conclusion, although the TCR expression profile is restricted in activated CSF-derived CD4+ T cells, different TCR BV genes are overrepresented among individual patients.

Table 4.1. Overview of overrepresented TCR BV genes in CSF-derived activated CD4+ T cells

Patient	Overrepresented BV genes[1]
MS-DOV	6 - 7 - 8 - 13.1
MS-HIC	17 - 18
MS-JOV	2 - 4 - 9
MS-MJD	9 - 13.2
MS-PAR	4 - 7 - 9 - 19
OND-HEG	2 - 5
OND-JAB	1 - 2 - 9
OND-LUC	3 - 6 - 17 - 18
OND-URM	3 - 4 - 7 - 9

[1]: Overrepresented TCR BV genes were defined as exceeding an arbitrarily defined cut-off value based on the mean TCR BV gene expression levels in the blood of 10 healthy subjects + 3 standard deviations. TCR BV gene expression levels as determined by PCR-ELISA are shown in Figure 4.4.
OND: patients with other non-inflammatory neurological diseases.

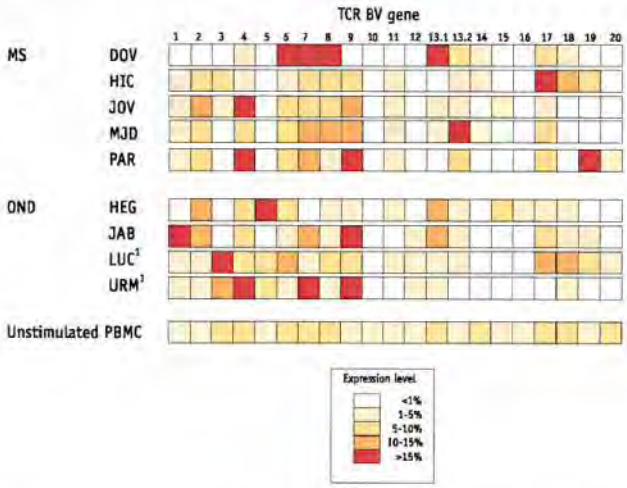


Figure 4.4. *TCR BV gene expression profiles of CSF-derived activated CD4+ T cells*

[1]: For 2 samples (LUC and URM), cell cultures were depleted from CD8+ T cells using anti-CD8 antibody coated immunomagnetic beads prior to analysis because <50% of the cells were CD4+.
After 16 to 22 days, CSF-derived T cell cultures were submitted to semi-quantitative PCR-ELISA analysis to determine the TCR BV gene expression repertoire. As a comparison, the mean TCR BV gene expression profile of unstimulated PBMC from 10 healthy controls is shown, to illustrate the skewed BV gene usage of the CSF-derived cultured T cells. The expression of each individual TCR BV gene ($A_{450}(BV_x)$) is presented as a percentage of the total BV gene expression ($\Sigma A_{450}(BV_n)$).
OND: patients with other non-inflammatory neurological diseases.

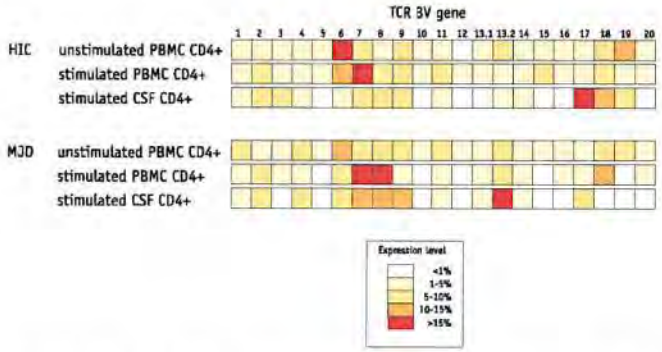


Figure 4.5. *Comparison of TCR BV gene expression profiles in blood and cerebrospinal fluid of 2 MS patients*

For 2 MS patients, HIC and MJD, directly *ex vivo* (unstimulated) CD8+ T cell depleted peripheral blood mononuclear cells (PBMC) as well as IL-2 stimulated mononuclear cells from the blood and the cerebrospinal fluid (CSF) were submitted to PCR-ELISA analysis to determine the TCR BV gene expression repertoire. The expression level of each individual BV gene ($A_{450}(BV_x)$) is presented as a percentage of the total BV gene expression ($\Sigma A_{450}(BV_n)$).

4.3.2.3 Comparison of clonal composition of PB- and CSF-derived (activated) T cells

To compare the clonal composition of T cells in blood and CSF, TCR BV gene expression profiles of unstimulated (CD8+ T cell depleted) and activated CD4+ T cells in blood and of activated CSF-derived CD4+ T cells of 2 MS patients were analyzed. Semi-quantitative PCR-ELISA analysis showed a rather heterogeneous TCR BV gene usage in the unstimulated CD4+ T cell population of both MS patients (Figure 4.5). After IL-2 expansion, both blood- and CSF-derived activated CD4+ T cells had a more restricted TCR BV gene expression profile but different BV genes were overrepresented (HIC: BV7 in blood; BV17 and 18 in CSF; MJD: BV7 and 18 in blood; BV7, 9 and 13.2 in CSF).

To determine whether overrepresented TCR BV gene families had a limited clonal origin, the clonal composition of respectively 2 and 4 overrepresented TCR BV gene families of patient HIC and MJD were analyzed using CDR3 spectratype analysis. The heterogeneity of the CDR3 spectratype profiles provides an indication about the clonality of T cell populations: monoclonal with one single peak, oligoclonal with 2 to 4 peaks and polyclonal with more than 4 peaks. The height of the peaks corresponds to the relative contribution of a clonotype within a specific T cell population and identical peak lengths strongly indicate the presence of identical T cell clones in different samples. Table 4.2 demonstrates that for all TCR BV gene families tested, polyclonal T cell populations were found in the unstimulated CD4+ T cells of the blood. However, in the activated blood- and CSF-derived T cell populations, the clonal composition was more restricted. Mono- and oligoclonal profiles were found in the CSF-T cells, whereas in the blood T cell populations with a slightly broader oligoclonal origin are present. As an example, CDR3 spectratype profiles of TCR BV18 of patient HIC are shown in figure 4.6. The single peak in the monoclonal profile of the activated CSF-T cells (Figure 4.6 C) could also be found in the oligoclonal profile of the activated blood-derived T cells (Figure 4.6 B) and was also the most dominant clonotype in the unstimulated polyclonal CD4+ T cell population of the blood (Figure 4.6 A). We confirmed these data with CDR3 sequence analysis. To this end, the TCR BV18 PCR products of the three samples were cloned in plasmid vectors and 24 to 36 randomly selected recombinant clones were sequenced. Table 4.3 provides an overview of the CDR3 sequences of the TCR BV18 gene family in the different samples. For the CSF-derived T cells, the vast majority of sequenced plasmids showed the CDR3 sequence 'GRGG' (33/36) and corresponds to a monoclonal spectratype profile. Interestingly, this T cell clone was also dominant in

the unstimulated blood ('GRGG', 11/29) and showed the highest peak in the polyclonal CDR3 fragment length profile. Furthermore, the CDR3 sequence 'GRGG' could also be found in the activated blood-derived T cell population but with a lower frequency ('GRGG', 02/24). This is in line with the CDR3 spectratype profile, where this clonotype could be demonstrated, but where another T cell clone was dominant ('YRE', 14/29). These T cells were also present before stimulation in the blood, but at a lower frequency ('YRE', 03/29) (Table 4.3).

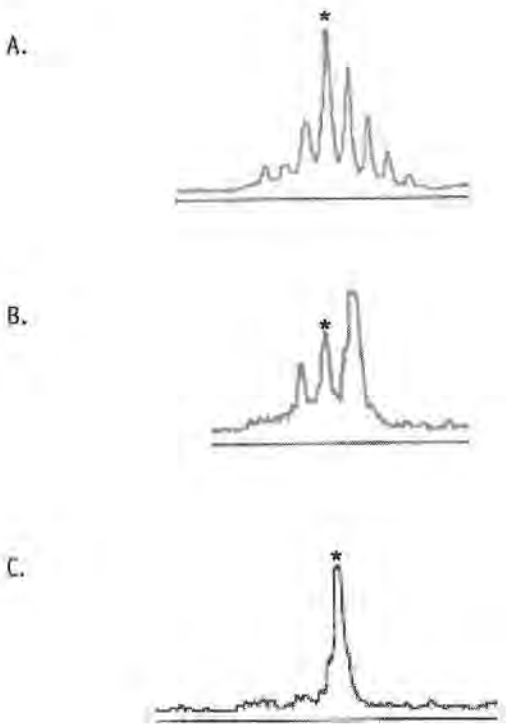


Figure 4.6. *CDR3 spectratype analysis of TCR BV 18 gene family in blood and cerebrospinal fluid of a MS patient*

For MS patient HIC, the clonal composition of TCR BV 18 gene family was determined in directly *ex vivo* (unstimulated) CD8+ T cell depleted peripheral blood mononuclear cells (PBMC) (A) as well as IL-2 stimulated mononuclear cells from the blood (B) and the cerebrospinal fluid (CSF) (C). After nested PCR amplification with a fluorescently labeled TCR BC region specific primer of BV gene family amplicons and separation on a polyacrylamide gel, CDR3 fragment lengths were calculated using the Genescan-1000 ROX size standard and the 672 Genescan Software. Peaks with an identical CDR3 length in different samples are marked with *.

Table 4.2. CDR3 spectratype analysis of relevant TCR BV genes in the blood and cerebrospinal fluid of 2 MS patients

Patient	BV gene	Sample					
		Unstimulated CD4+ PBMC		Stimulated CD4+ PBMC		Stimulated CD4+ CSF	
		% BV[1]	Clonality[2]	% BV	Clonality	% BV	Clonality
HIC	BV17	<5	Polyclonal	<5	Oligoclonal (2)	>15	Oligoclonal (3)
	BV18[3]	5-10	Polyclonal	5-10	Oligoclonal (3)	10-15	Monoclonal
MJD	BV7	5-10	Polyclonal	>15	Oligoclonal (2)	10-15	Oligoclonal (2)
	BV8	5-10	Polyclonal	>15	Oligoclonal (2)	10-15	Oligoclonal (2)
	BV9	5-10	Polyclonal	<5	Polyclonal	10-15	Oligoclonal (2)
	BV13.2	5-10	Polyclonal	5-10	Oligoclonal (4)	>15	Oligoclonal (2)

[1]: % BV: TCR BV gene expression level as determined by PCR-ELISA.
[2]: Clonality: The CDR3 fragment length profile was considered polyclonal, oligoclonal or monoclonal when respectively more than 4, between 2 and 4, or only a single peak was observed.
[3]: For BV18 gene family of patient HIC, CDR3 spectratype profiles are shown in Figure 4.6 and CDR3 sequence analysis results are listed in Table 4.3.

In conclusion, data on CDR3 fragment length screening and sequence analysis both showed that the clonal composition of activated T cells is different but highly restricted in the CSF and the blood. Furthermore, heterogeneous T cell populations are present in the blood but dominant T cell clones from the activated T cell fraction are found at higher frequencies.

4.3.3 Stability of CSF-derived T cell cultures

To generate sufficient amounts of activated CD4+ T cells from the cerebrospinal fluid of MS patients for vaccination, long-term expansion periods are needed. In the CSF-based T cell vaccination (TCV) protocol, as described in chapter 5, MS patients received 3 subsequent T cell vaccines consisting of 10 million cells each. For ethical reasons, CSF was obtained only once before vaccination. Since immunizations were performed three times at two month intervals, T cell vaccines had to be frozen and subsequently thawed prior to vaccination. To study whether T cell cultures are stable during extended periods of expansion and after freezing, we analyzed both phenotype and TCR BV gene usage in IL-2 expanded CSF-derived T cell cultures of different MS patients.

Table 4.3. CDR3 sequence analysis of TCR BV18 gene family in blood and cerebrospinal fluid of an MS patient

Sample	Freq[1]	Amino acid sequence			
		BV[2]	BnDn[2]	BJ[2]	BC[2]
Unstimulated CD4+ PBMC	11/29	YFCASSP	GRGG	QPQHFGDGTRLSIL	EDLNK
	07/29	YFCASS	LLPEGN	EQYFGPGTRLTVT	EDLKN
	03/29	YFCASSP	YRE	SYNEQFFGPGTRLTVL	EDLKN
	02/29	YFCASSP	SRLG	TEAFFGQGTRLTVV	EDLNK
	01/29	YFCASS	LIQGDS	TEAFFGQGTRLTVV	EDLNK
	01/29	YFCASSP	SRFG	TEAFFGQGTRLTVV	EDLNK
	01/29	YFCASSP	DRD	YGYTFGSGTRLTVV	EDLNK
	01/29	YFCASS	SRTGS	NSPLHFGNGTRLTVT	EDLNK
	01/29	YFCASS	VP	STDTQYFGPGTRLTVL	EDLKN
	01/29	YFCASSP	DRF	QETQYFGPGTRLLVL	EDLKN
Stimulated CD4+ PBMC	14/24	YFCASSP	YRE	SYNEQFFGPGTRLTVL	EDLKN
	02/24	YFCASSP	GRGG	QPQHFGDGTRLSIL	EDLNK
	02/24	YFCASS	LLPEGN	EQYFGPGTRLTVT	EDLKN
	02/24	YFCASSP	AAY	QETQYFGPGTRLLVL	EDLKN
	02/24	YFCAS	TPTDG	YNEQFFGPGTRLTVL	EDLKN
	01/24	YFCASSP	TAGA	NTGELFFGEGSRLTVL	EDLKN
	01/24	YFCASS	DSI	NQPQHFGDGTRLSIL	EDLNK
Stimulated CD4+ CSF cells	33/36	YFCASSP	GRGG	QPQHFGDGTRLSIL	EDLNK
	01/36	YFCASS	RVQGRT	AKNIQYFGAGTRLSVL	EDLKN
	01/36	YFCASSP	DRD	YGYTFGSGTRLTVV	EDLNK
	01/36	YFCASSP	VAAK	NTGELFFGEGSRLTVL	EDLKN

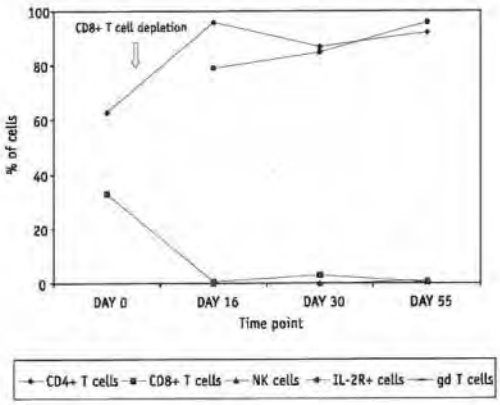
For MS patient HIC, the clonal composition of TCR BV18 gene family was analyzed in directly *ex vivo* (unstimulated) CD8+ T cell depleted peripheral blood mononuclear cells (PBMC) as well as IL-2 stimulated mononuclear cells from the blood and the cerebrospinal fluid (CSF) by subcloning TCR BV18 gene amplification products in recombinant plasmids.

[1]Freq: frequency; number of plasmids with a particular CDR3 amino acid sequence as a fraction of the total number of plasmids sequenced for a given TCR BV gene in a specific sample.

[2]BV: variable region of the TCR beta chain; BnDn: diversity region of the TCR beta chain; BJ: junctional region of the TCR beta chain; BC: constant region of the TCR beta chain.

Identical CDR3 sequences are presented in the same color.

A.



B.

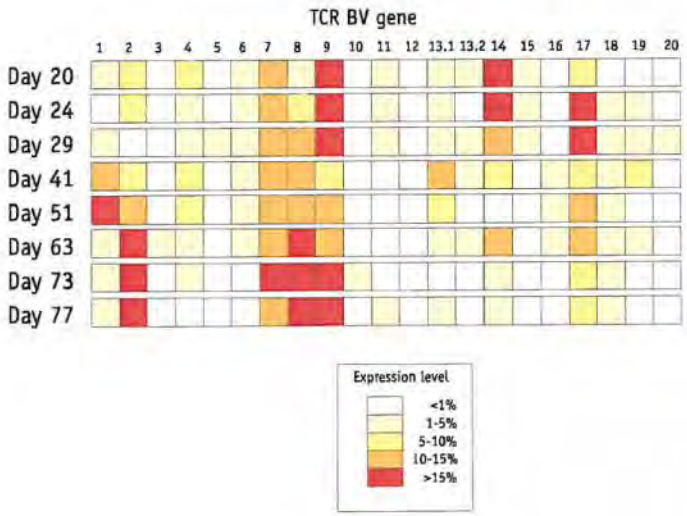


Figure 4.7. *Stability of CSF-derived T cell cultures after long-term culturing*

A. Evolution of the phenotypic expression profile of CSF-derived cells during culturing.
Percentages of T helper cells (CD4), cytotoxic T cells (CD8), natural killer cells (NK), γδ T cells (gd T cells) and activated T cells (IL-2Rα+) are shown at different time points during expansion for a CSF-derived cell culture of a MS patient as determined by flow-cytometric analysis.

B. Evolution of TCR BV gene expression profiles of CSF-derived cells during culturing.
CSF-derived T cell cultures of a MS patient were submitted to semi-quantitative PCR-ELISA analysis at different time points during expansion to determine the stability of the TCR BV gene expression repertoire. The expression level of each individual BV gene (A_{450} (BV_x)) is presented as a percentage of the total BV gene expression (Σ A_{450} (BV_n)).

4.3.3.1 Phenotypic analysis and TCR BV gene usage

The stability of a CSF-derived cell cultures is shown in figure 4.7 A. The phenotypic expression profiles at different time points during expansion were determined by flow-cytometric analysis. After a CD8+ T cell depletion using antibody coated immunomagnetic beads at day 0, the vast majority of cells consisted of activated CD4+ T cells. After a culturing period of approximately 2 months, the mean period of time needed for the generation of sufficient cell numbers for three vaccinations, a limited number of other cell populations were detected in the cell culture.

To obtain information about the clonal stability of the T cell populations, TCR BV gene expression profiles were analyzed at regular time points during culturing. As shown in Figure 4.7 B, in general, similar TCR BV gene families were overexpressed over time. For this particular MS patient, TCR BV2, 7, 8, 9, 14 and 17 showed an increased expression level in the majority of the samples taken at different time points, although fluctuations during a culturing period of 11 weeks were observed.

4.3.3.2 Effects of freezing

We also studied whether it was possible to freeze and subsequently expand the CSF-derived T cell cultures. The phenotype of the cells after freezing was similar to the original T cell population and remained stable for up to 12 weeks (Figure 4.8). It should be noted that there was a slight increase of CD8+ T cells after 49 days of culturing. These small numbers of CD8+ T cells can efficiently be removed by CD8+ T cell depletion as described in 2.1.3.

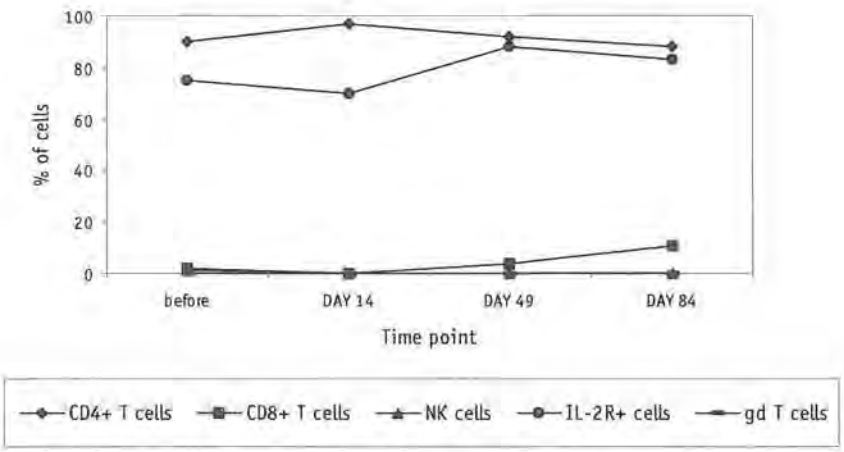


Figure 4.8. *Phenotypic expression profile of cultured CSF-derived cells before and after freezing*

Flow-cytometric analysis was performed on cultured CSF-derived cells of a MS patient before freezing and at 3 different time points after thawing. Percentages of T helper cells (CD4+ T cells), cytotoxic T cells (CD8+ T cells), natural killer cells (NK cells), γδ T cells (gd T cells) and activated T cells (IL-2Rα+ cells) are shown.

4.4 Discussion and conclusion

Because of its proximity to the central nervous system (CNS), the target organ in MS, the CSF represents an important source of T lymphocytes that potentially could mediate the inflammatory response in the disease. Mainly due to the small number of inflammatory cells that can be sampled from CSF, little is known about the properties of activated T cells in the CNS. One approach for the characterization of potentially relevant T cells in MS is to identify recently *in vivo* activated T cells. Based on the expression of high affinity receptors for IL-2, these cells can be specifically expanded in the presence of IL-2.

In the present study, the phenotype and TCR repertoire of IL-2 expanded CSF-derived cell cultures from 5 MS patients and 4 control patients with other non-inflammatory neurological diseases were analyzed. High numbers of (activated) CD4+ T cells were found in both study groups after expansion. Examination of the TCR BV gene expression profiles by semi-quantitative PCR-ELISA revealed that in all patients a bias in the BV gene usage of *in vivo* activated T cells in the CNS compartment was detected. Different overrepresented BV genes were found among patients although some TCR BV genes were overexpressed in more than one patient. In contrast to previous reports demonstrating TCR BV gene biases for different BV genes[18], we were not able to define a 'MS-specific' TCR BV gene[11;19]. Other groups also reported an overexpression of different TCR BV genes varying from one patient to another[13;16]. However, this difference in overrepresented BV genes in individual patients does not necessarily mean that CSF-derived activated T cells are not relevant for the disease. Previously, it has been shown that both MBP-specific and PLP-specific T cells display a heterogeneous TCR BV gene repertoire[20-22]. In chapter 3, similar results were obtained for the MOG-reactive T cell population in MS patients. Differences observed in the TCR BV gene repertoire could reflect different myelin antigen specificities of the autoreactive T cells involved in the inflammatory process in the CNS of an individual MS patient.

The clonal composition of T cells of 2 MS patients in peripheral blood were compared with CSF. TCR BV gene repertoire analysis of directly *ex vivo* CD8+ T cell depleted PBMC showed a heterogeneous profile, although an elevated expression of TCR BV6 and to a lesser extend of TCR BV19 for patient HIC were found. Skewing of the TCR BV gene repertoire in unstimulated PBMC has been demonstrated previously[23], but in that particular study

polyclonal, thus rather unrestricted, T cell subpopulations were reported for all overrepresented BV gene families[23]. Furthermore, our data show a less heterogeneous profile after an enrichment for activated T cells. After the analysis of overrepresented TCR BV gene families, a limited clonal origin was observed as demonstrated by oligoclonal profiles in spectratype analysis and less different clonotypes in CDR3 sequence analysis of TCR BV18 in patient HIC. In contrast, polyclonal profiles were found in unstimulated T cells. In addition, a restricted T cell population was found in the activated T cells of the CSF, although other TCR BV gene families were overrepresented, and within a specific TCR BV family, other clonotypes were dominant as demonstrated both by spectratype analysis and CDR3 sequence analysis. These data are in agreement with other reports analyzing paired samples of activated T cells in the blood and the CSF[13;16]. However, analysis of the clonal composition of the overrepresented TCR BV 18 gene family by CDR3 sequence analysis demonstrated that the dominant clonotype in the activated CSF-derived T cells was also present with an increased frequency in the unstimulated PBMC and to a lesser extent in the activated T cell fraction, suggesting that this T cell clone was *in vivo* activated in the periphery and subsequently migrated towards the CNS where it could have contributed to the inflammatory response. Although this study did not provide any information about the antigen specificity of T cells in the CSF of MS patients, the presence of recently activated CD4+ T cells with a limited clonal composition also suggest their relevance to the disease.

The main goal of this part of the study was to determine whether it is feasible to specifically expand activated CD4+ T cells from the cerebrospinal fluid (CSF) mononuclear cells to sufficient cell numbers for T cell vaccination as described in chapter 5 without taking into account their antigen reactivity. In pilot experiments using low numbers of peripheral blood mononuclear cells (PBMC), we determined the optimal culturing conditions for the efficient expansion of predominantly CD4+ T cells. Cells were preferentially cultured in autologous medium with low concentrations of IL-2 in the presence of irradiated autologous PBMC. In addition, protocols were optimized for the efficient depletion of non-CD4+ T cell populations. Based on previous studies on TCV using autologous irradiated MBP-specific T cell clones[24], CSF-based TCV should be performed three times with 10^7 irradiated CSF-T cells at two month intervals. To this end to obtain sufficient cell numbers, CSF cultures need to be expanded for an extended period of time. In CSF-derived cell cultures of 5 MS patients, we found relatively stable T cell populations during culturing in

terms of phenotype and TCR BV gene usage profiles. Based on these promising results on the specific expansion of activated CD4+ T cells from the cerebrospinal fluid of several MS patients, a protocol was established for the generation of CSF-derived T cell vaccines. Safety, feasibility and immunological effects of this procedure in a pilot T cell vaccination clinical trial involving 5 MS patients are described in chapter 5.

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Chapter 5

T Cell Vaccination in MS with CSF-derived Activated T Cells: Results from a Pilot Study

Based on:

T cell vaccination in MS with CSF-derived activated T cells: results from a pilot study

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Submitted for publication

Abstract

Myelin-reactive T cells are considered to play an essential role in the pathogenesis of multiple sclerosis (MS), an autoimmune disease of the central nervous system. We have previously studied the effects of T cell vaccination (TCV), a procedure by which MS patients are immunized with attenuated autologous myelin basic protein (MBP)-reactive T cell clones. However, since several myelin antigens are described as potential autoantigens for MS, T cell vaccines incorporating a broad panel of anti-myelin reactivities may have therapeutic effects. Furthermore, previous reports showed an accumulation of activated T cells recognizing multiple myelin antigens in the cerebrospinal fluid (CSF) of MS patients.

Based on these observations, we conducted a pilot clinical trial of TCV with activated CD4+ T cells derived from the CSF involving 5 MS patients to study safety, feasibility and immune effects of TCV. Patients were immunized three times at 2 month intervals. Vaccines consisted of almost pure TCR$\alpha\beta$+ CD4+ T cells of the Th0/1 subtype, showed reactivity towards different myelin antigens and had a restricted clonality. Vaccinations were well tolerated and no toxicity or adverse effects were reported. All patients remained clinically stable with no relapses during and shortly after vaccination. Vaccine T cells induced both a specific anti-idiotypic response in the majority of the patients and an anti-ergotypic proliferative response in all MS patients. Anti-myelin reactivity in blood remained low or was further reduced in all patients.

Based on these encouraging results, to study the efficacy of vaccination with CSF-derived T cells in a larger population, a double-blind placebo-controlled clinical trial involving 60 early RR-MS patients was initiated recently.

Keywords: Multiple Sclerosis, T cell vaccination, activated CD4+ T cells, cerebrospinal fluid, pilot clinical trial

5.1 Introduction

Several immunotherapeutic strategies based on the potential pathogenicity of myelin reactive T cells have been designed to specifically inactivate these T lymphocytes (reviewed by Hohlfeld[1]). One possible therapeutic approach involves immunization with attenuated myelin-reactive T cells. This so called 'T cell vaccination' (TCV) has been shown previously to prevent disease initiation and to induce remission in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, by enhancing the existing peripheral regulatory network[2]. The protective effect involves both an anti-idiotypic T cell response, recognizing T cell receptor (TCR) determinants[3] and an anti-ergotypic regulatory T cell network based on interactions with activation markers[4]. The successful demonstration of T cell vaccination in animal studies resulted in human clinical trials conducted to evaluate the therapeutic effect of TCV as a treatment strategy for MS[5-7].

To assess safety of and immunological responses to T cell vaccination, a pilot trial was performed in a small number of MS patients[6;8;9]. Patients were immunized three times with autologous irradiated MBP-reactive T cell clones. Subcutaneous inoculations of the vaccine cells were well tolerated and caused no adverse effects. Clinical data suggested a moderate improvement in some relapsing-remitting (RR)-MS patients[8]. Furthermore, vaccinations induced an effective anti-clonotypic T cell response in all patients, associated with a specific depletion of MBP-reactive T cells[6]. These results were confirmed in an extended phase I open label trial with 49 MS patients[10;11]. Further analysis of the anti-vaccine response revealed that CD8+ T cells display a direct cytolytic anti-idiotypic effect, whereas CD4+ T cells are the predominant cytokine producers. In addition, other cell populations are expanded upon stimulation with the vaccine clones including γδ T cells and NK cells, which may also play a role in the peripheral regulatory network[11]. Furthermore, it was shown that a significant anti-clonotypic T cell response was still present several years after vaccination[12]. However, in 5 patients MBP-reactive T cells reappeared in the circulation and this coincided with clinical relapses in two of these patients[13]. Reappearing T cells belonged to a different clonal origin and were successfully depleted in subsequent rounds of vaccination[12;13]. In conclusion, these pilot studies indicated that T cell vaccination with attenuated autologous MBP-reactive T cell clones is safe and feasible, and that this experimental treatment induces

a specific anti-vaccine response thereby enhancing the peripheral immunoregulatory networks.

However, there is increasing evidence that T cells recognizing other myelin components also contribute to the disease process in MS. Experiments in the EAE model and studies on human T cell reactivity demonstrated that PLP and MOG may play an important role as candidate myelin antigens in the autoimmune mediated demyelination[14-19]. Incorporating these T cell populations in the vaccines may improve the effectiveness of the current TCV protocol, but technically it is almost impossible to generate T cell clones specific for three different myelin antigens with this protocol design. It has also been shown that a higher frequency of (activated) myelin-reactive T cells is present in cerebrospinal fluid (CSF) as compared to peripheral blood[20-24]. Furthermore, the population of CSF lymphocytes reflects best the repertoire of inflammatory cells infiltrating the parenchyma and may contain infiltrating pathogenic cells relevant to the disease process because of its proximity to the target organ in MS[25].

Based on these observations and a prior study of CSF-derived activated T cells as described in chapter 4, a protocol was developed to expand activated CD4+ T lymphocytes from CSF of MS patients. We were able to grow these T cells to sufficient numbers for vaccination (10^7 T cells) after stimulation with low doses of recombinant human interleukin-2 in the presence of irradiated autologous feeder cells[20]. Using immunomagnetic beads, other mononuclear cell populations were successfully depleted from the CSF cell cultures. In this pilot trial of CSF-based T cell vaccination, 5 MS patients were immunized subcutaneously three times with 10^7 irradiated activated CD4+ T cells at two month intervals. We characterized the T cells used for vaccination and studied safety, feasibility and immune effects following immunization. T cell vaccines consisted predominantly of activated Th1/0 TCR□□αβ+ CD4+ T cells, showed reactivity towards at least two out of three myelin antigens tested and had a restricted clonality as determined by TCR analysis. The vaccinations were well tolerated and all patients remained clinically stable on EDSS without relapse during and at least 4 months after treatment. Another 6 to 12 months later, 2 patients worsened on EDSS (1 RR-MS and 1 CP-MS), and in the RR-MS patient this was accompanied by a relapse. Both anti-idiotypic responses against the vaccine cells and anti-ergotypic responses were observed after vaccination in the majority of the patients. Myelin reactivities in the peripheral blood

towards MBP, PLP and MOG remained low or were further reduced in all patients. In conclusion, these preliminary data illustrate that T cell vaccination with CSF-derived CD4+ activated T cells is feasible and safe and induces a specific anti-vaccine response.

5.2 Materials and methods

5.2.1 Study design

Five patients with clinically definite MS received 3 subcutaneous vaccinations containing 10 million CSF-derived activated CD4+ T cells at two month intervals. A lumbar puncture was performed at time 0, patients were immunized after month 2, 4 and 6 and followed up until 10 to 15 months after the last vaccination. Four months after the last vaccination, a second CSF sample was obtained for post-vaccination analysis. For each patient, MRI-scans were performed before the first and after the third vaccination. During the whole procedure, patients were monitored monthly for safety parameters and changes in clinical or immunological status.

5.2.2 Patients

Table 5.1 provides an overview of the patient characteristics. The five patients (4 female/1 male) participating in this study all had clinically definite MS: 4 of the relapsing-remitting (RR) type and one of the chronic progressive (CP) type. The mean age was 36.8 (range 24-51) years, mean disease duration at the time of study entry was 7.4 (range 1-28) years and baseline EDSS scores varied from 1.0 to 6.5. The 4 RR-MS patients showed a relapse rate from 1 to 3 during the last 2 years before entering the study. None of the patients used immunosuppressive or immunomodulatory drugs within 3 months before study entry. All patients signed a letter of informed consent.

Table 5.1. Patient characteristics at study entry

Subject	Sex	Age	Disease type[1]	Disease duration	EDSS	Relapse rate[2]
AMH	F	36	RR	3	2.5	3
VEL	F	24	RR	2	1.0	1
FRW	M	31	RR	3	3.5	2
LIB	F	51	RR	1	3.5	2
JEL	F	42	CP	28	6.5	NA

[1]MS type: RR: relapsing-remitting; CP: chronic progressive.
[2]Number of relapses 2 years prior to study entry.

5.2.3 Monitoring safety and clinical status variables

During the entire study period, all patients were monitored for safety using standard toxicity assays and were observed for adverse effects following the immunization protocol. To determine expanded disability status score (EDSS) and relapse rate, neurological examinations were performed. Relapses were defined clinically as an increase of at least one point on the Kurtzke scale. MRI of the brain were obtained two times, before the first vaccination and after the third vaccination using a 1.5 Tesla instrument (Magneton Symphony, Siemens, Erlangen, Germany). Using two interleaved series of turbo spin-echo dual echo sequences, proton density (Pd, TR: 2560 ms, TE: 11 ms) and T2-weighted (T2w, TR: 600 ms, TE: 12 ms) images were obtained. These sequences are followed by two interleaved series of T1-weighted spin-echo sequences (T1w, TR: 600 ms, TE: 12 ms) after administration of Gadolinium (Gd). All these sequences were acquired with a slice thickness of 3 mm, an inter-slice gap of 3 mm, a field of view of 250 and 190/256 matrix for Pd and T2w images and 192/256 matrix for T1w images. The combination of the two interleaved series covers the whole brain. The total number of T1w and T2w lesions and the number of Gd enhanced T1w 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 also Gd enhanced on T1w images were counted only once as an active lesion.

5.2.4 Generation of CSF-derived activated CD4+ T cell vaccines

Fresh CSF-derived mononuclear cells were isolated after centrifugation of the cerebrospinal fluid obtained by lumbar puncture (10-15 ml). Cells are resuspended in autologous medium, counted and cultured for 10 to 12 days at cell densities of $2x10^4$ cells/well, in the presence of 10^5 irradiated autologous PBMC as feeder cells and low concentrations of recombinant human IL-2 (2U/ml rhIL-2, Roche Diagnostics, Brussels, Belgium). In parallel, freshly isolated PBMC were plated out. Cell cultures were phenotypically analyzed using flow-cytometry as described below. Lymphocyte subsets other than CD4+ T cells (CD8+ T cells, TCR$\gamma\delta$ T cells or NK cells) representing > 15% of the total cell population were depleted using immunomagnetic beads either directly (anti-CD8 beads, Dynal, Skoyen, Norway) or indirectly, after pre-incubation of rat anti-mouse IgG_1 coated beads (Dynal) with mouse anti-human TCR $V\delta_{1/2}$ antibody (Serotec, Kidlington, UK) or after pre-incubation of rat anti-mouse IgM coated beads (Dynal) with mouse anti-human CD16 antibody (Beckton

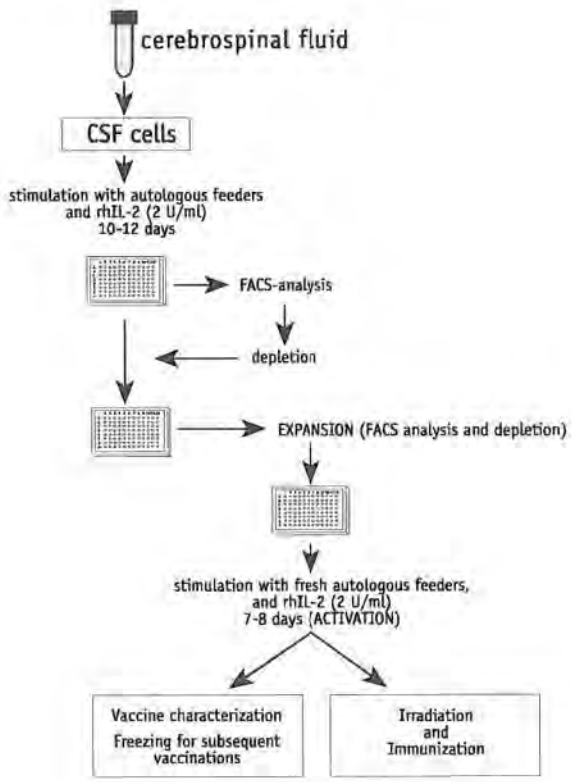

Figure 5.1. *Schematic overview of the procedure for the generation of CSF-derived T cell vaccines*

CSF-derived mononuclear cells are cultured in the presence of rhIL-2 and irradiated autologous feeder cells. After 10 to 12 days, a flow-cytometric phenotype analysis is performed and non-CD4+ T cells are depleted using immunomagnetic beads. After an expansion period, CSF cultures are restimulated one week prior to vaccination. 10^7 CSF-derived T cells are used for vaccination and remaining cells are aliquoted for subsequent vaccinations and characterized in detail.

Dickinson, Erembodegem, Belgium) as described in detail in 2.1.3. Cells were further expanded by repeated stimulation with autologous feeder cells and rhIL-2. At regular time points, T cell cultures were analyzed for their phenotype and, if necessary, depleted from non-CD4+ T cell subsets during the expansion period. One week prior to the first vaccination, T cells were activated with freshly isolated irradiated autologous feeder cells and rh IL-2. Before immunization, 10^7 attenuated (6000 rad, Cs-source) autologous CSF-derived activated T cells were used for vaccination. The remaining cells were aliquoted and

frozen for subsequent immunizations and used for detailed vaccine characterization as described below. Figure 5.1 provides a schematic overview of the procedure for the preparation of CSF-derived T cell vaccines.

5.3 Results

5.3.1 Vaccine preparation

For 4 out of 5 MS patients, we were able to expand CSF-derived mononuclear cells to sufficient numbers to perform three immunizations. Most patients received 10^7 vaccine cells in each of the three immunizations. Due to culturing difficulties, the first vaccines of patient FRW and JEL contained 2 to 3 millions cells only. For one patient (VEL), cells from the CSF could not be cultured. Instead, we performed three vaccinations using rhIL-2 expanded mononuclear cells from peripheral blood.

5.3.2 Vaccine characterization

5.3.2.1 Phenotypic analysis

Flow-cytometric analysis demonstrated that vaccines were composed of TCR□□αβ+ CD4+ T cells (93.5 ± 0.9%) with a variable expression of the IL-2 receptor (69.7 ± 5.7%). Little or no CD8+ T cells (3.5 ± 1.1%), γδ T cells (5.7 ± 2.3%) or natural killer cells (0.16 ± 0.08%) were present in the vaccines (Figure 5.2). Figure 5.3 shows a typical phenotypic profile of a T cell vaccine.

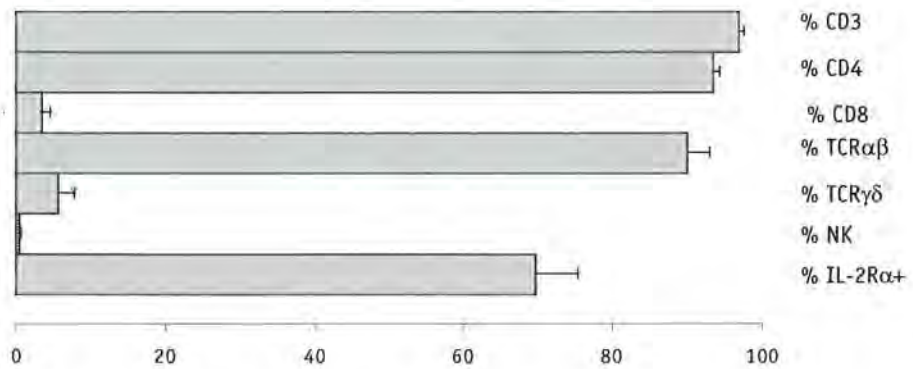

Figure 5.2. Mean phenotypic expression profiles of T cell vaccines

Bars represent the mean percentages of cellular subsets. Standard errors of the mean (SEM) are shown in horizontal lines. Mean percentages of total T cells (CD3), T helper cells (CD4), cytotoxic T cells (CD8), TCRαβ+ T cells, γδ T cells, natural killer cells (NK) and activated T cells (IL-2Rα+) are shown for the 3 vaccines of the 5 MS patients.

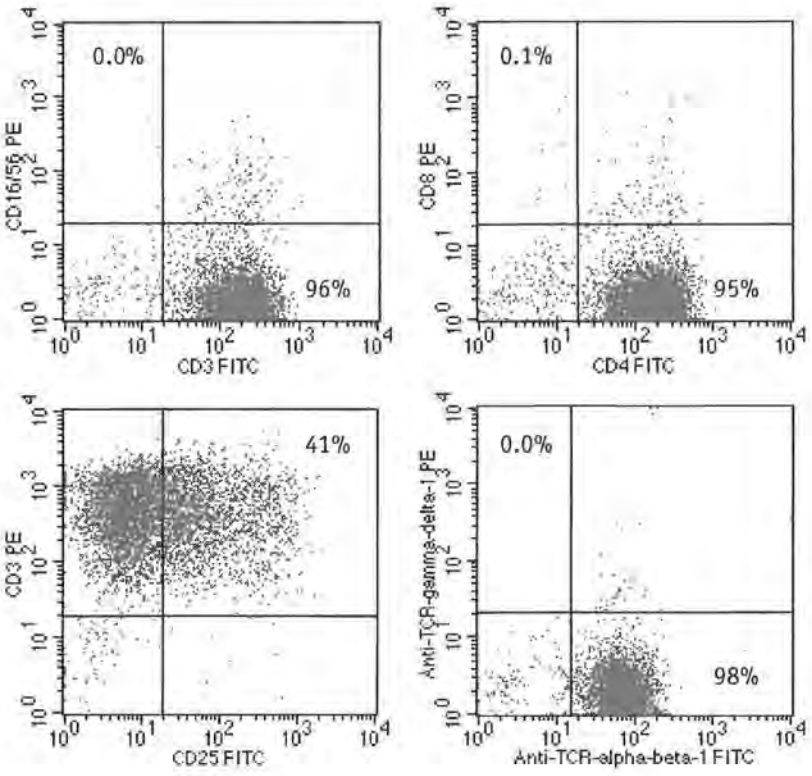

Figure 5.3. *Phenotypic expression profile of a typical T cell vaccine*

Flow-cytometric analysis of T cell cultures was performed prior to each vaccination for each MS patient. Four dot plots representing staining for different surface markers are shown. Upper left graph shows the percentage of CD3+ T lymphocytes (lower right), and the absence of natural killer cells (CD3-CD16/56+; upper left). The upper right graph represents the percentage of CD4+ T cells (lower right) and CD8+ T cells (upper left). Activated T cells (CD25+CD3+) are shown in the lower left graph in the upper right quadrant. Distinction between T cell receptor (TCR) surface expression are demonstrated in the lower right graph with percentage of TCRαβ+ cells in the lower right part and γδ T cells in the upper left part of the figure.

5.3.2.2 Cytokine profile

Next, the cytokine secretion profiles of T cells used for the first vaccination were analyzed after *in vitro* stimulation with phytohaemagglutinin (PHA). Both pro-inflammatory (TNF-α□, IFN-□γ and IL-6) and anti-inflammatory cytokines (IL-4 and IL-10) were measured by ELISA in the supernatants of the cell cultures. In the 4 vaccines tested, high levels of TNF-α,

IFN-γ and IL-6 were measured. CSF-derived T cells from two patients (AMH and FRW) also produced considerable amounts of IL-4 and IL-10, and were subtyped as Th0-type cells (Figure 5.4). In contrast, anti-inflammatory cytokines were not present in the cell culture supernatants of the 2 other patients (VEL and JEL). These T cell vaccines consisted predominantly of Th1-like cells (Figure 5.4).

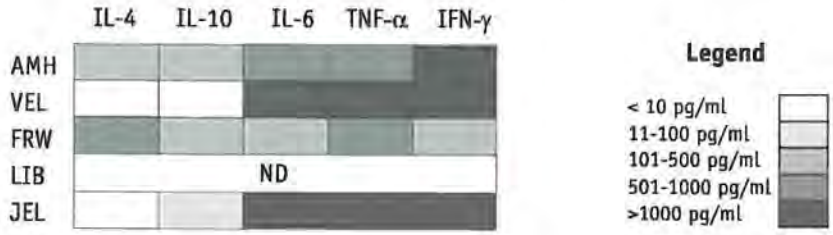

Figure 5.4. *Cytokine production of T cell vaccines*

T cell vaccines used for the first vaccination were stimulated with PHA. After 3 days, cell supernatants were harvested and cytokine levels were measured by ELISA. Net cytokine production (in pg/ml) of IL-4, IL-10, IL-6, TNF-α and IFN-γ was calculated by subtracting background levels (without PHA) from the cytokine levels determined in the PHA-stimulated cultures. ND: not determined.

5.3.2.3 Myelin reactivity

Myelin specificity of the vaccine cells was tested at the time of the first vaccination. The recognition pattern of immunodominant peptides of MBP, PLP and MOG was analyzed by IFN-γ and IL-4 ELISPOT using $2x10^4$ vaccine cells in the presence of 10^5 irradiated autologous PBMC as antigen presenting cells. After subtracting the number of spots in the control wells (no antigen), no IL-4 secreting cells were detected after stimulation with the myelin peptides, although a high reactivity was found against the control stimuli PHA and anti-CD3. As shown in Table 5.2, IFN-γ secreting vaccine cells displayed a heterogeneous reactivity towards different peptides of the three myelin antigens tested. In 3 out of 4 cell cultures we found relatively high reactivity towards MOG. All four T cell populations recognize one or more PLP peptide, but to a lesser extent. Although in only two patients MBP-specific T cells were demonstrated, we can not exclude the possibility that T cells recognizing other MBP epitopes are present, since only two immunodominant peptides of the MBP protein were tested. Furthermore, pilot experiments were performed using low numbers of pure MBP- or MOG-specific Th1 cell clones demonstrating that the number of

spots does not always correspond to the number of myelin-reactive T cells. Theoretically, every myelin-specific T cell should produce IFN-γ upon stimulation with the peptide it is recognizing. However, counting the number of spots showed that only 1/10 of the T cells secreted IFN-γ after stimulation with the myelin peptide or a control stimulus (anti-CD3) (Figure 5.5).

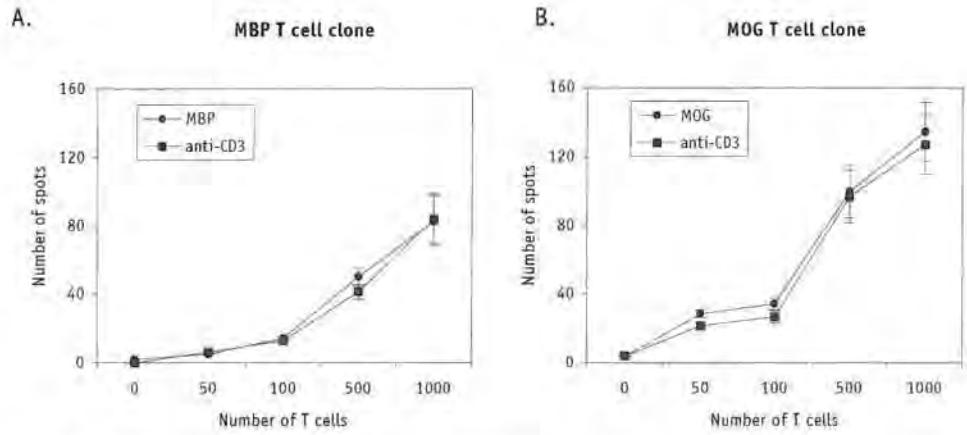

Figure 5.5. Pilot experiments of ELISPOT using myelin-specific T cell clones

A. T cell reactivity was tested to the specific MBP peptide (10μg/ml) or a control stimulus anti-CD3 (2μg/ml) in the presence of 10^5 antigen presenting cells with increasing cell numbers per well.
B. T cell reactivity was tested to the specific MOG peptide (10μg/ml) or a control stimulus anti-CD3 (2μg/ml) in the presence of 10^5 antigen presenting cells with increasing cell numbers per well.
Black dots represent the number of spots (cytokine secreting T cells) after subtraction of background values from non-stimulated control wells.

Table 5.2. Myelin reactivity of T cell vaccines

Patient	Antigen reactivity[1]			
	MBP[1]	PLP[1]	MOG[1]	TT[2]
AMH	8	5	17	4
VEL	9	8	25	2
FRW		NI		
LIB	0	2	1	0
JEL	0	3	17	0

[1]T cell vaccines used for the first vaccination were tested for myelin reactivity by ELISPOT. $2x10^4$ vaccine cells in the presence of 10^5 antigen presenting cells were incubated with different myelin peptides of MBP (84-102 and 143-168), PLP (41-58, 84-99 and 196-209) and MOG (1-22, 34-56, 64-86 and 74-96). The sum of the positive spots is shown for each myelin antigen. TT: tetanus toxoid, control antigen; NI: not identified, for patient FRW, the ELISPOT assay failed twice.

5.3.2.4 Clonal composition

To study the clonal composition of the three subsequent vaccines for each patient, the TCR BV gene expression profiles were analyzed by a semi-quantitative PCR-ELISA assay. The TCR BV gene expression in the vaccine cells is rather restricted and only a limited number of BV genes are overexpressed (Figure 5.6). The three vaccines from one patient had a rather stable BV gene expression profile. For example in patient LIB, BV7 and BV13.2 are overrepresented in all three vaccines. In addition, an increased expression of BV6, BV17 and BV19 was observed in the three cell populations. Despite the limited number of overexpressed TCR BV genes in all patient samples, overrepresented BV genes definitely varied among different patients. In general, the BV gene expression pattern in the vaccines was different among patients (Figure 5.6).

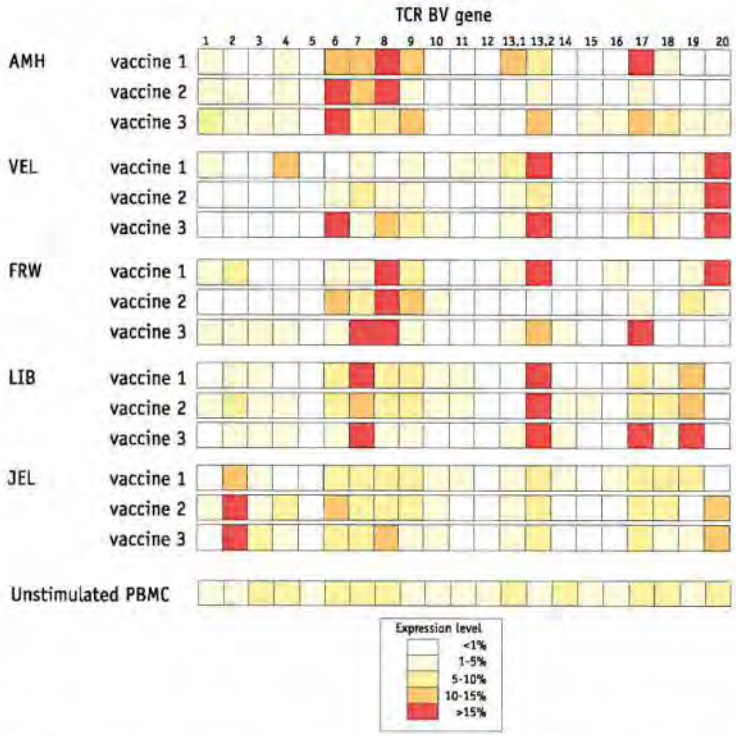

Figure 5.6. *TCR BV gene expression profiles in 3 T cell vaccines of 5 MS patients*

T cell vaccines were submitted to semi-quantitative PCR-ELISA analysis to determine the TCR BV gene expression repertoire. As a comparison to illustrate the skewed BV gene usage of the cultured vaccine T cells, the mean TCR BV gene expression profile of unstimulated PBMC from 10 healthy controls is shown,. The expression of each individual TCR BV gene ($A_{450}(BV_x)$) is presented as a percentage of the total BV gene expression ($\Sigma A_{450}(BV_n)$).

CDR3 spectratype analysis provides information about the clonal composition of specific BV gene families. Identical fragment lengths of PCR amplified CDR3 regions, which are depicted as peaks in the profile, strongly suggest that identical T cell clones are present in different samples. Furthermore, the height of the peaks correspond to the frequency of a specific clonotype within a given BV gene family. Polyclonal T cell populations show a Gaussian distribution profile with at least 4 peaks. A less heterogeneous profile with two to four peaks represents an oligoclonal T cell population, whereas a single peak in the CDR3 spectratype profile strongly suggests a monoclonal T cell population. In general, the majority of the analyzed TCR BV gene families in the T cell vaccines showed a rather restricted clonal origin as demonstrated by predominantly mono- or oligoclonal CDR3 spectratype profiles (Figure 5.7). Furthermore, comparison of the different clonotypes of the 3 vaccine samples within a given BV gene family also indicates that identical T cell clones are present in different samples. Although, the frequency (determined by peak height) may vary at different time points (Figure 5.7). For example, CDR3 fragment length analysis of the TCR BV6 gene family in patient AMH shows an oligoclonal profile for all 3 vaccines as illustrated in figure 5.7 A. In vaccine 1, 3 different peaks are present and the dominant clonotype also persist in vaccine 2. The oligoclonal T cell population of vaccine 3 is represented by 3 different clonotypes but the frequency of the dominant clonotype from vaccine 1 and 2 is much lower in this sample. Interestingly, in the unstimulated PBMC a restricted oligoclonal profile, with the same dominant clonotype was also observed (Figure 5.7 A). Figure 5.7 B illustrates the persistence of a given clonotype in the BV7 T cell populations of the 3 vaccines of patient FRW. Although at time point 2 and 3 a monoclonal CDR3 spectratype profile is observed, this clonotype is also present in vaccine 1 but at a low frequency. Furthermore, the unstimulated PBMC were polyclonal with an increased frequency of one peak. The CDR3 fragment length of this band corresponds to the dominant peak in the CSF-derived vaccine samples. This may indicate an increased expression level in the peripheral blood after *in vivo* activation and subsequent clonal expansion of this specific clonotype (Figure 5.7 B).

In conclusion, TCR BV gene expression profiles and subsequent CDR3 region analysis of the 3 vaccines used for immunization showed a restricted but stable heterogeneity with different overrepresented BV genes between different patients.

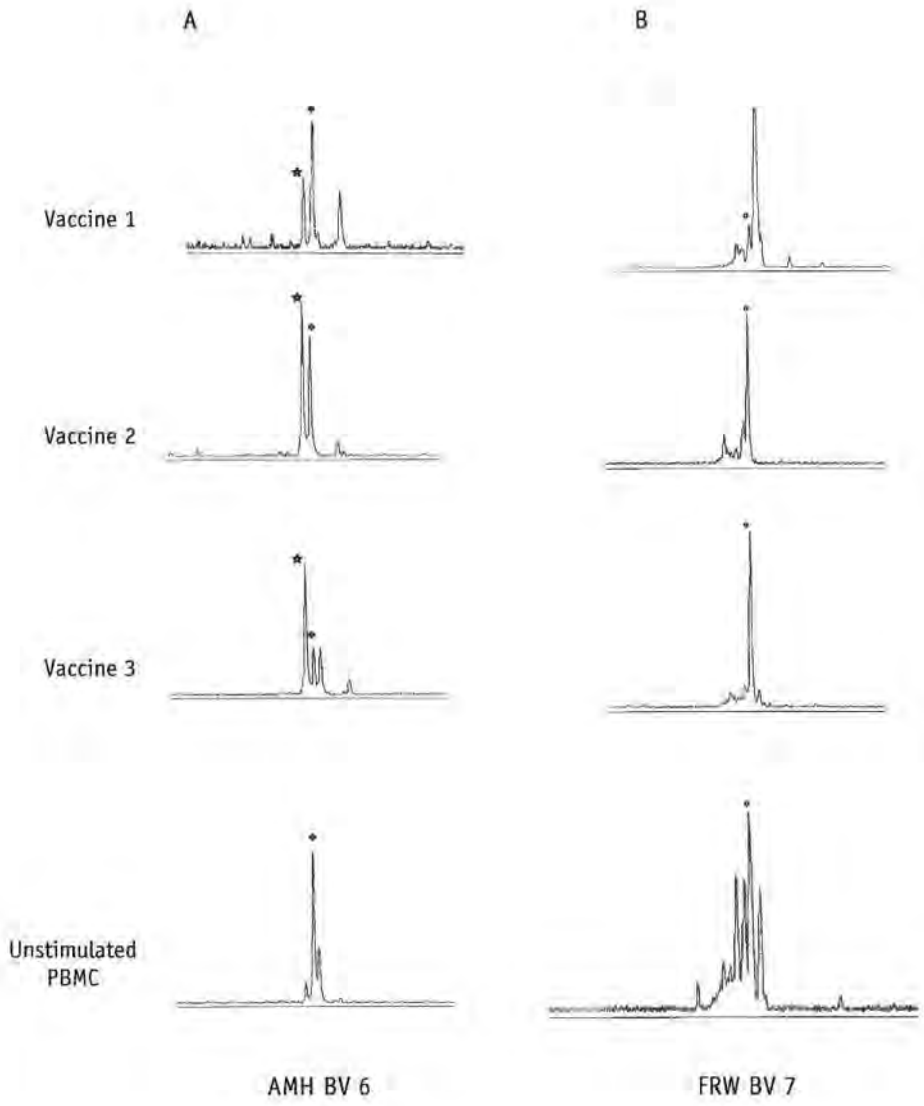

Figure 5.7. *CDR3 fragment length screening of 2 selected TCR BV genes in 3 CSF-derived T cell vaccines from 2 MS patients*

The clonal composition of 2 TCR BV gene families from two different MS patients was determined after nested PCR amplification with a fluorescently labeled TCR BC region specific primer of BV gene family amplicons. CDR3 fragment lengths were calculated using the Genescan-1000 ROX size standard and the 672 Genescan Software.
Peaks with an identical CDR3 length in different samples are marked with a symbol (* ,* and *).

5.3.2.5 CDR3 sequence analysis of overrepresented BV genes in patient AMH

For one patient (AMH), we also determined the clonal composition of different BV gene families in the 3 vaccine samples using CDR3 sequence analysis. To this end, the PCR products were cloned in a plasmid vector and 15 to 25 randomly selected recombinant clones were sequenced. Table 5.3 provides an overview of the CDR3 sequences of 3 BV gene families obtained in the 3 vaccine samples of patient AMH.

Table 5.3. CDR3 sequence analysis of 3 TCR BV gene families in the 3 vaccines of MS-AMH

TCR BV gene	Sample	Freq[1]	Amino acid sequence			
			BV[2]	BnDn[2]	BJ[2]	BC[2]
BV6	Vaccine 1	08/20	YLCASS	VRGD	QPQHFGDGTRLSIL	EDLNK
		05/20	YLCASSL	YGPLAGVG	EQFFGPGTRLTVL	EDLKN
		03/20	YRCAS	RPGGG	GYTFGSGTRLTVV	EDLNK
		03/20	YLCASS	DPGH	YGYTFGSGTRLTVV	EDLNK
		01/20	YLCASS	VRGD	QPQHFGDGTRLSIL	EDLNK
	Vaccine 2	20/22	YLCASS	VRGD	QPQHFGDGTRLSIL	EDLNK
		02/22	YLCASSL	YGPLAGVG	EQFFGPGTRLTVL	EDLKN
	Vaccine 3	24/24	YLCASS	VRGD	QPQHFGDGTRLSIL	EDLNK
BV13.2	Vaccine 1	06/20	YFCAS	RDPPT	YNEQFFGPGTRLTVL	EDLKN
		03/20	YFCASS	PFAGR	TEAFFGQGTRLTVV	EDLNK
		03/20	YFCASSY	RGTLG	NEQFFGPGTRLTVL	EDLKN
		02/20	YFCAS	RSPT	NTEAFFGQGTRLTVV	EDLNK
		01/20	YFCASSY	SGTA	NYGYTFGSGTRLTVV	EDLNK
		01/20	YFCAS	RNRGF	SYNEQFFGPGTRLTVL	EDLKN
		01/20	YFCASSY	KGSG	TEAFFGQGTRLTVV	EDLNK
		01/20	YFCAS	RLQGN	SNQPQHFGDGTRLSIL	EDLNK
		01/20	YFCAS	GLQGN	SNQPQHFGDGTRLSIL	EDLNK
		01/20	YFCAS	THQEYG	NQPQHFGDGTRLSIL	EDLNK
	Vaccine 2	07/22	YFCAS	RNRGF	SYNEQFFGPGTRLTVL	EDLKN
		06/22	YFCASSY	KGSG	TEAFFGQGTRLTVV	EDLNK
		04/22	YFCAS	RDPPT	YNEQFFGPGTRLTVL	EDLKN
		03/22	YFCASSY	RGTLG	NEQFFGPGTRLTVL	EDLKN
		01/22	YFCASS	PFAGR	TEAFFGQGTRLTVV	EDLNK
		01/22	YFCASSY	SGRTYD	EQFFGPGTRLTVL	EDLKN
	Vaccine 3	10/21	YFCASSY	KGSG	TEAFFGQGTRLTVV	EDLNK
		09/21	YFCASSY	SGTA	NYGYTFGSGTRLTVV	EDLNK
		02/21	YFCAS	RNRGF	SYNEQFFGPGTRLTVL	EDLKN
BV17	Vaccine 1	21/24	YLCASSI	RMD	TEAFFGQGTRLTVV	EDLNK
		01/24	YLCASS	GHTGDN	NSPLHFGNGTRLTVT	EDLNK
		01/24	YLCASSI	VPG	GANVLTFGAGSRLTVL	EDLKN
		01/24	YLCASSI	PRGGSG	YGYTFGSGTRLTVV	EDLNK
	Vaccine 2	18/20	YLCASSI	RMD	TEAFFGQGTRLTVV	EDLNK
		02/20	YLCASSI	VPG	SGANVLTFGAGSRLTVL	EDLKN
	Vaccine 3	05/09	YLCASSI	RMD	TEAFFGQGTRLTVV	EDLNK
		03/09	YLCASSI	GG	NEQFFGPGTRLTVL	EDLKN
		01/09	YLCASSI	VPG	SGANVLTFGAGSRLTVL	EDLKN

[1]Freq: frequency; number of plasmids with a particular CDR3 amino acid sequence as a fraction of the total number of plasmids sequenced for a given TCR BV gene in a specific sample.
[2]BV: variable region of the TCR beta chain; BnDn: diversity region of the TCR beta chain; BJ: junctional region of the TCR beta chain; BC: constant region of the TCR beta chain.
Identical CDR3 sequences within a specific TCR BV gene family are presented in the same color.

These results demonstrate that TCR BV gene families consisted of a limited number of different T cell clones and that identical clonotypes are persistent in the 3 vaccines, although the frequency may vary. This finding is consistent with the previously described data on CDR3 fragment length analysis. For BV6, CDR3 sequence 'VRGD' represents the dominant clone in vaccine 1, although several other clonotypes are present. In vaccine 2 and 3, this T cell clone accounts for > 90% (20/22) and even 100% (24/24) of the randomly selected TCR BV6 gene products. Within the BV17 gene family, high frequencies of the dominant T cell clone with CDR3 sequence 'RMD' are found in vaccine 1 (21/24), vaccine 2 (18/20) and vaccine 3 (5/9). Although the clonal composition of the less expressed BV13.2 gene family is more heterogeneous, similar CDR3 sequences are found in the 3 different samples (Table 5.3). In conclusion, CDR3 sequence analysis of T cell clones present in a given TCR BV gene family demonstrated a stable and restricted clonal composition of vaccine T cell populations in the 3 vaccines of patient AMH.

5.3.3 Immunological follow-up

5.3.3.1 Frequency of myelin-reactive T cells

To study the effect of CSF-based T cell vaccination on circulating myelin-reactive T cells in the peripheral blood, we determined the frequency of anti-myelin T cells before and after vaccination by limiting dilution analysis. PMBC were plated out in the presence of MBP, PLP or MOG. *Tetanus toxoid* was used as a control antigen. After 14 days, using a classical proliferation assay, the number of antigen-specific T cell lines was determined. Frequencies of anti-myelin T cells before and after vaccination are shown in Table 5.4. In two patients (FRW and JEL), the frequency of myelin-specific T cells was rather low before vaccination and remained stable after vaccination. However, in 3/5 patients, T cell lines reactive towards the three myelin antigens tested are present in the peripheral blood before vaccination. Interestingly, after vaccination, no myelin-reactive T cells were found in one patient (LIB). Furthermore, for patients AMH and VEL, who had a high frequency of anti-MBP, anti-PLP and anti-MOG T cells before vaccination, a significant decline of circulating myelin-specific T cells was observed after vaccination (Table 5.4). In contrast, no significant change in the immune response to the control antigen *tetanus toxoid* was observed. In conclusion, these data indicate that following T cell vaccination anti-myelin reactivity in the peripheral blood remained low or was further reduced in all patients.

Table 5.4. Frequency of myelin-reactive T cells before and after vaccination

Patient	Antigen	Number of antigen-specific T cell lines[1]	
		before TCV[2]	after TCV[2]
AMH	MBP (60 wells)	5	1
	PLP (30 wells)	2	0
	MOG (30 wells)	14	0
	Tetanus toxoid (30 wells)	4	7
VEL	MBP (60 wells)	16	0
	PLP (30 wells)	3	2
	MOG (30 wells)	7	0
	Tetanus toxoid (30 wells)	18	17
FRW	MBP (60 wells)	2	3
	PLP (30 wells)	0	0
	MOG (30 wells)	1	1
	Tetanus toxoid (30 wells)	NT	NT
LIB	MBP (60 wells)	3	0
	PLP (30 wells)	1	0
	MOG (30 wells)	1	0
	Tetanus toxoid (30 wells)	0	0
JEL	MBP (60 wells)	1	0
	PLP (30 wells)	0	1
	MOG (30 wells)	0	2
	Tetanus toxoid (30 wells)	0	0

[1]Frequency of myelin-specific T cells was determined by limiting dilution analysis. Reactivity towards MBP, PLP and MOG was tested. In addition, a control antigen (tetanus toxoid) was incorporated to provide information about the fluctuations in the immune response in general.
[2]Frequency analysis was performed with freshly isolated PBMC shortly before the first vaccination and 4 months after the third vaccination.
TCV : T cell vaccination ; NT : not tested.

5.3.3.2 Proliferative anti-vaccine response

To study the cellular immune response induced by T cell vaccination, PBMC of vaccinated patients were stimulated with irradiated vaccine cells or irradiated autologous PHA stimulated T cell blasts (PHA blasts). The proliferative responses were evaluated at day 4 after stimulation using a classical ^{3}H-thymidine assay. Figure 5.8 summarizes the proliferative responses, induced by T cell vaccines or PHA blasts for the 5 MS patients one month after the first or second and third vaccination. Significant proliferative responses (SI >3) towards the vaccine cells were observed in 4/5 patients. For 2 patients (LIB and JEL), the proliferative response was more pronounced after the third vaccination (SI of 10 and 32 respectively). Patient VEL showed the highest anti-vaccine response after the second vaccination, and high stimulation indices were calculated after each vaccination for patient FRW. In addition, strong proliferative responses were induced after stimulation with autologous PHA blasts in all 5 patients. This anti-ergotypic response was more pronounced after the third vaccination (Figure 5.8). In conclusion, these data demonstrate that immunization with activated T cells induced a specific anti-idiotypic response (towards the vaccine T cells) in the majority of the patients. Furthermore, anti-ergotypic proliferative responses were observed in all patients, predominantly after the third vaccination.

5.3.3.3 Comparison of cultured CSF cells before and after vaccination

To obtain information about differences in CSF cellularity, IL-2 expanded CSF cultures were compared before and after vaccination. Cell numbers after lumbar puncture (10-15 ml) varied significantly between patients (Table 5.5), both before (range: 5,225 - 157,000 cells) and after vaccination (range 11,250 - 105,000 cells) and comparison of mean cell numbers showed a slight decrease of CSF cells after vaccination (before: 59,910 ± 28,708; after: 44, 940 ± 16,910). Before vaccination, we were able to grow CSF-derived cells from 4/5 MS patients, and after depletion of CD8+ T cells and γδ cells, expanded CSF cultures consisted predominantly of CD4+ TCRαβ□□+ T cells after approximately 8 weeks of culture (Table 5.5). Remarkably, although similar cell numbers were obtained by lumbar puncture after the third vaccination, we could not grow CSF-derived T cells in 2/5 patients (LIB/JEL). Furthermore, CD8+ and γδ T cell populations were more persistent and could not always be depleted efficiently as is demonstrated by the phenotype expression profiles after approximately 8 weeks of culture (Table 5.5).

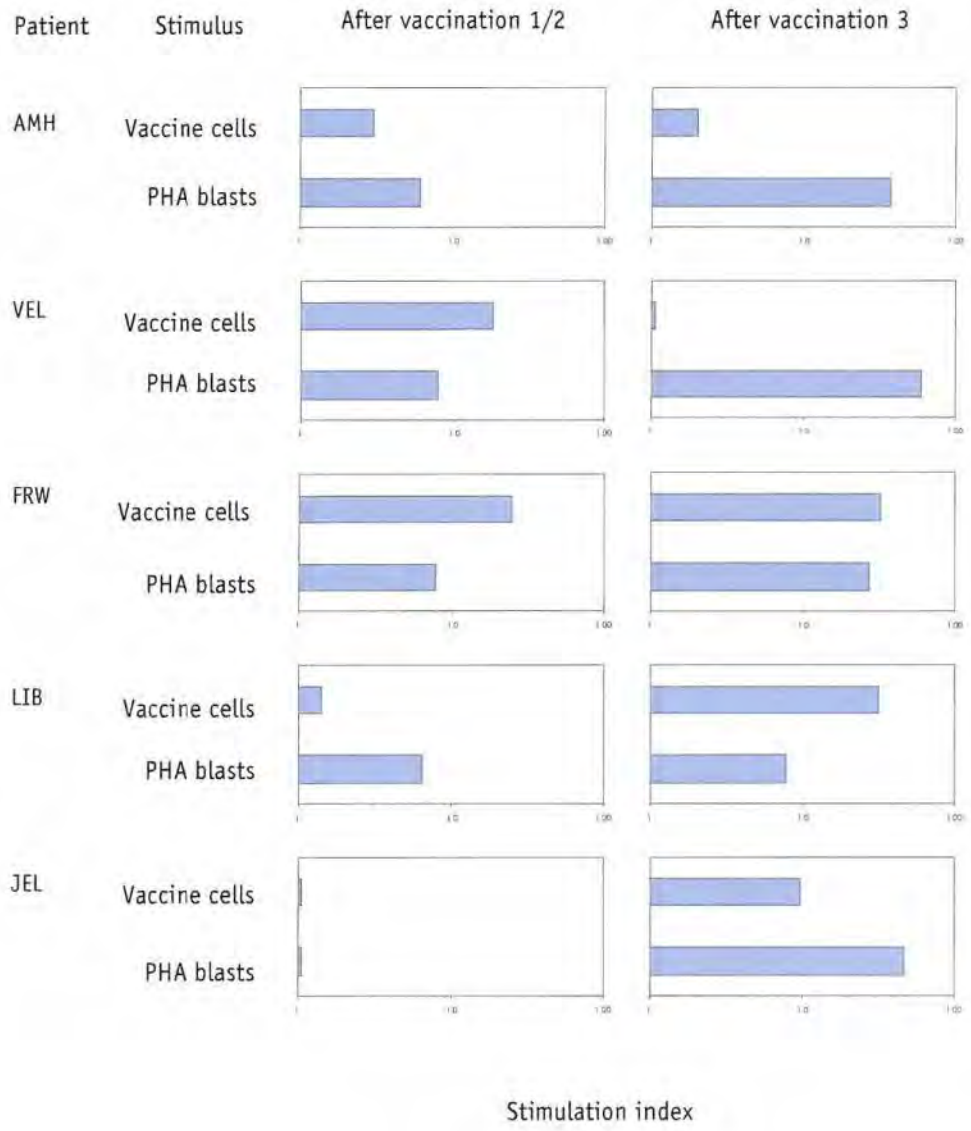

Figure 5.8. *Proliferative response to T cell vaccines and autologous PHA stimulated cells after vaccination*

Freshly isolated PBMC from vaccinated patients were stimulated with irradiated vaccine cells or irradiated autologous PHA-stimulated PBMC one month after each vaccination. After 4 days, cells were harvested and proliferative responses were evaluated in a ^{3}H-thymidine incorporation assay. Based on the incorporated radioactivity, stimulation indices were calculated.

Table 5.5. CSF cellularity before and after vaccination

Patient		Cell number[1]	Expansion[2]	Phenotype[3]		
				CD4+	CD8+	γδ+
AMH	before	40,000	+	95	5	21
	after	23,900	+	9	35	62
VEL	before	157,000	-	ND	ND	ND
	after	58,400	+	9	39	88
FRW	before	90,000	+	90	8	12
	after	105,000	+	80	1	19
LIB	before	6,925	+	90	8	1
	after	11,250	-	ND	ND	ND
JEL	before	5,625	+	95	0	2
	after	26,150	-	ND	ND	ND

[1]Cell number counted after lumbar puncture (10-15 ml) before expansion.
[2]Expansion of CSF-derived cells using low doses of rhIL-2 was successful for 4/5 MS patients before vaccination, and for 3/5 MS patients after vaccination.
[3]Phenotype of cultured CSF-derived cells as determined after 8 weeks of expansion and depletion of non-CD4+ T cell populations using immunomagnetic beads. In the CSF cultures after vaccination, large populations of CD8+ and/or γδ cells could not always be successfully depleted.
ND: not determined.

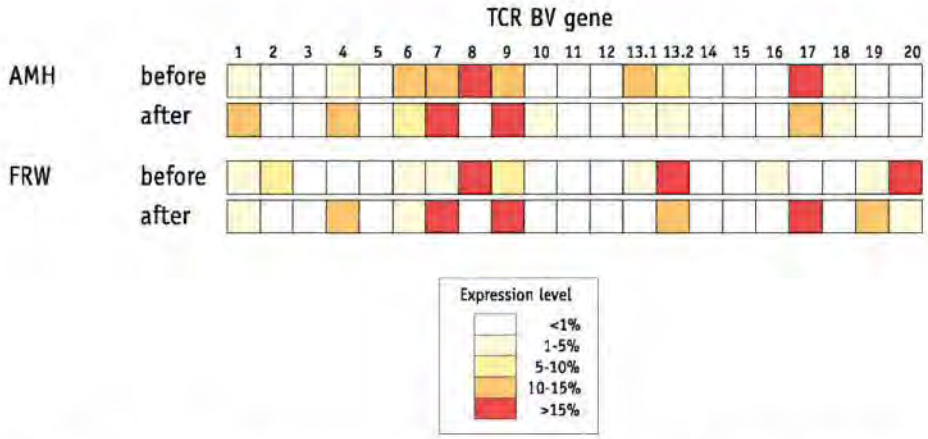

Figure 5.9. *TCR BV gene expression profiles of cultured CSF-derived T cells before the first and after the third vaccination*

CSF T cells obtained before the first and after the third vaccination by lumbar puncture were cultured for 8 weeks and depleted from non-CD4+ T cells prior to PCR-ELISA analysis.
The expression of each individual TCR BV gene ($A_{450}(BV_x)$) is presented as a percentage of the total BV gene expression ($\Sigma A_{450}(BV_n)$).

Table 5.6. CDR3 sequence analysis of 4 TCR BV gene families in cultured CSF T cells and unstimulated PBMC before and after vaccination for patient AMH

TCR BV gene	Sample	Amino acid sequence[1]	Frequency[2]		Evolution[3]
			before	after	
BV6	CSF	VRGD	8/20	8/19	=
		YPLAGVG	5/20	0/20	↓
		IPPAGA	0/20	6/19	↑
	PBMC	VRGD	11/20	16/17	↑
		YPLAGVG	0/20	1/17	=
		VGEQ	7/20	0/17	↓
BV9	CSF	RTNN	19/22	4/21	↓
		PATLA	0/22	8/21	↑
	PBMC	RTNN	0/19	1/21	=
		PATLA	8/19	14/21	↑
BV13.2	CSF	RDPPT	6/20	6/21	=
		KGSG	1/20	12/21	↑
		SGTA	1/20	1/21	=
	PBMC	KGSG	3/18	6/21	↑
		SGTA	15/18	14/21	↓
BV17	CSF	RMD	21/24	1/17	↓
		GG	0/24	5/17	↑
		LEYRGQ	0/24	6/17	↑
	PBMC	RMD	1/16	1/26	↓
		GG	13/16	13/26	↓

[1]Amino acid sequence of the hypervariable diversity region of the TCR beta chain (BnDn) from dominant T cell clones within a specific BV gene family.
[2]The frequency is the number of plasmids with a particular CDR3 amino acid sequence as a fraction of the total number of plasmids sequenced for a given TCR BV gene in a specific sample.
[3]Evolution: comparison between frequency of a specific T cell clone before and after vaccination: increased (↑), decreased (↓) or similar (=).
CSF T cells cultured for about 8 weeks and depleted from non-CD4+ T cells were obtained before the first and after the third vaccination.

For two patients, AMH and FRW, we compared the TCR BV gene repertoire of cultured CSF-derived CD4+ T cells before and after vaccination (Figure 5.9). In these patients, differences in TCR BV gene expression profiles were seen at the two time points. Some TCR BV gene families were only preferentially expressed before or after vaccination (AMH BV1, 4, 8 and 13.1; FRW BV4, 7, 8, 17, 19 and 20) whereas for other T cell populations fluctuations in the TCR BV gene expression were observed. In addition, the clonal composition of 4 TCR BV gene families were analyzed for patient AMH in CSF cultures and unstimulated PBMC before and after vaccination (Table 5.6). Although for both the TCR BV6 and 13.2 gene family, a reduced expression was observed in the cultured CSF (Figure 5.9), the clonal composition of the CSF remained rather stable. For BV6, the dominant clone 'VRGD' persisted in the CSF after treatment and was even more frequent in peripheral blood (VRGD, 16/17). A new dominant clonotype was found in CSF at the second time point (IPAGGA, 6/19), whereas another clone was no longer detectable after vaccination (YGPLAGVG). For BV13.2, the dominant clone 'KGSG' after vaccination (12/21), was also part of the more heterogeneous T cell population at the first time point (KGSG, 1/20) and could also be detected in the blood at both time points (before: 3/18; after: 6/21). The most predominant clone in the CSF before vaccination (RDPPT, 6/20) persisted in the CSF after treatment (RDPPT, 6/21). Although the dominant clone from the blood (SGTA, 15/18) was found with a lower frequency after vaccination (SGTA, 14/21), no increased frequency of this T cell clone was found in the CSF afterwards (SGTA, 1/21). In contrast to the BV6 and 13.2 gene family, a decreased expression of BV17 was demonstrated by PCR-ELISA. In addition, the dominant clone before vaccination for this BV gene family (RMD, 21/24) was after treatment only present in the CSF (RMD, 1/17) and in the blood (RMD, 1/26) at a very low frequency. However, although the dominant clone in the blood (GG, 13/16) was detected at lower frequency after vaccination (GG, 13/26), this clone was one of the two new and most abundant clonotypes in the CSF after vaccination (GG, 5/17; LEYRGQ, 6/17). Similar observations were reported for the TCR BV9 family. The overrepresented T cell clone before vaccination (RTNN, 19/22), was found at lower frequency in the CSF cultures after vaccination (RTNN, 4/21). Furthermore, the dominant clone from the unstimulated PBMC before vaccination (PATLA, 8/19) persisted in the blood after vaccination and was also found at high frequency in the cultured CSF at the second time point (PATLA, 8/21). This might indicate that some T cells have migrated from the periphery to the CNS.

In conclusion, although no clear differences in the number of cells obtained after lumbar puncture could be found before and after vaccination, we were not able to isolate CD4+ T cells in 4/5 MS patients after vaccination, because of large percentages of non-CD4+ T cell populations or poor expansion after IL-2 culturing. Furthermore, we found fluctuations in the TCR BV gene expression profiles and characterization of clonotypes in 4 BV gene families showed that some clones persisted after vaccination, others were found at significantly lower frequency and some T cells migrated from the peripheral blood into the CSF. Although this analysis allows to follow individual T cell clonotypes in CSF and blood before and after vaccination, it does not provide any information about a pathogenic or regulatory role of these T cells.

5.3.4 Safety and clinical parameters

Vaccinations were well tolerated and no toxicity or adverse effects were reported following administration of vaccine T cells. Patients were monitored for changes in clinical status variables at several time points before, during and after vaccination (Table 5.7). As demonstrated by EDSS scores, patients remained clinically stable during and at least 4 months after treatment. After a longer period of ten to 15 months after the last vaccination, one patient (FRW) showed a remarkable improvement of 2.0 points on the EDSS scale after treatment, whereas in two patients the EDSS score worsened. The remaining two patients were stable for the entire follow-up period. In addition, a reduced relapse rate was observed in all RR-MS patients. The mean relapse rate was 2.0 ± 0.4 during the 2 years prior to TCV and 0.3 ± 0.3 in a period of 14 to 20 months after the first vaccination. MRI scans were obtained before the first and after the third vaccination. We observed active lesions in 3/5 MS patients after the last vaccination (2 to 8 months) but thus far, this observation did not result in a clinical exacerbation. For the two remaining patients, no active lesions on MRI were detected at both time points.

Table 5.7. Overview of the clinical data

Patient	EDSS score[1]			Relapse rate[2]		Active lesions[3]	
	before TCV	after TCV		before TCV	after TCV	before TCV	after TCV
		4m	10-15 m				
AMH	2.5	2.5	4.5	3	1	0	0
VEL	1.0	1.0	1.0	1	0	1	8
FRW	3.5	2.0	1.5	2	0	0	3
LIB	3.5	3.5	3.5	2	0	1	1
JEL	6.5	6.5	7.5	NA	NA	0	0

[1]EDSS scores as determined at study entry (before TCV), 4 months after the third vaccination (4 m) and 10 to 15 months after the third vaccination (10-15 m).
[2]Relapse rates were calculated for a period of 2 years prior to TCV treatment (before TCV) and calculated for a period of 14-20 months beginning at the date of the first vaccination (after TCV).
[3]Active lesions were determined by MRI at one time point before vaccination and at one time point (2 to 8 months) after the last vaccination. An active lesion is defined as a T1w Gd enhanced lesion and new or enlarging T2w lesion. Lesions that appeared on T2w scans and were Gd enhanced on T1w were counted only once.
NA: not applicable.

5.4 Discussion

Our previous study with TCV using MBP-reactive T-cells has shown that this procedure leads to an upregulation of the regulatory anti-clonotypic networks, and a specific suppression of the MBP-specific T cells in the periphery[6;26]. However, there are some limitations associated with this protocol of TCV using only MBP-specific T cell clones. Indeed, it has been demonstrated that the autoimmune response in MS is also directed to several other myelin antigens like PLP and MOG[17;18;27]. This diverse T cell reactivity pattern might be present from disease onset and may persist along the disease progression as was shown in a study by Soderstrom and coworkers[22]. In contrast, recognition of multiple myelin antigens could also be the result of inter- or intramolecular epitope spreading after breakdown of the blood-brain barrier and subsequent demyelination and release of myelin fragments[28]. These two mechanisms may account for the heterogeneous reactivity against multiple myelin antigens and should be taken into consideration when developing an improved T cell-specific therapy for MS. In addition, it has been shown that the frequency of myelin-reactive T cells is increased in the cerebrospinal fluid of MS patients[20-22;25]. Other studies have demonstrated a high frequency of activated CD4+ T cells in CSF and showed that certain T cell clones persist for a long time in the CSF, further supporting their relevance for the immunopathogenesis of MS[29;30]. In EAE studies, it has been demonstrated that, although the phenotype of T cells in the target organ diversifies as the disease progresses, disease-associated T cells are preserved throughout the course of the disease[31;32]. Furthermore, also bystander CSF-derived cells attracted to the site of inflammation may be potentially important in the disease process.

We carried out a CSF-derived T cell vaccination protocol based on the concept that CSF activated CD4+ T cells, recognizing a broad spectrum of myelin or other currently unidentified CNS antigens and bystander cells may be pathologically important in the disease process. We cultured CSF-derived mononuclear cells obtained from 5 MS patients with low doses of rhIL-2 to specifically expand activated T cells, bearing the IL-2 receptor[29]. Other cell populations were depleted using immunomagnetic beads (CD8+ T cells and γδ□□□□ cells). Using this protocol CSF-based vaccines were generated from 4/5 MS patients. For one patient, CSF-derived T cells could not be expanded and instead we used IL-2 expanded T cells from the peripheral blood.

The majority of the CSF-T cell cultures consisted of activated cells, which was previously shown to be necessary for an efficient recognition by anti-idiotypic T cells in EAE. The T cells in the vaccines phenotypically resemble pathogenic myelin-reactive T cells previously described in EAE and MS: CD4+ T helper cells of Th1- or mixed Th0-subtype[33-35]. All CSF-derived T cells produced high levels of the pro-inflammatory cytokines TNF-α and IFN-γ□. Their production may be important since these cytokines have been shown to damage the myelin-producing oligodendrocytes, facilitate the recruitment of inflammatory cells to the site of inflammation by upregulating the expression of adhesion molecules and lead to an increased presentation of CNS antigens after upregulation of MHC class II molecules[36;37].

What is the composition of the CSF-based vaccines in terms of myelin-reactivity and clonal heterogeneity? As demonstrated by IFN-γ ELISPOT, all CSF-derived T cell vaccines displayed a heterogeneous reactivity towards MBP-, PLP- and MBP- peptides. However, we only tested reactivity to a limited number of immunodominant peptides of three myelin antigens. It remains possible that T cells, recognizing other epitopes of MBP, PLP and MOG or even other (unidentified) CNS antigens, may also be present. In addition, we demonstrated that the true frequency of cytokine-secreting T cells upon specific antigen stimulation may be underestimated using the ELISPOT-technique, as illustrated for pure CD4+ Th1 MBP- and MOG reactive T cell clones. Semi-quantitative analysis of the TCR BV gene repertoire revealed that only a limited number of BV genes is overexpressed, although the identity of the predominant BV gene families varied between different MS patients. These findings do not fully agree with a previous study that reported a marked bias of TCR BV6 in expanded CSF samples for the majority of the MS patients screened[29]. CDR3 fragment length screening and CDR3 sequence analysis of specific BV genes demonstrated a restricted clonal composition with only one or a few dominant clonotypes within a given BV family. In addition, our data illustrate that clonal composition of the vaccines remained rather stable and dominant clones persisted during further culturing. In conclusion, our results demonstrate that the vaccines where composed of activated CD4+ Th1/0 cells with a limited clonal origin and with reactivity to different myelin antigens but also unidentified antigens.

Our data demonstrate a cellular proliferative response to the vaccines after immunization. Although the exact mechanism by which T cell vaccination ameliorates the disease course is still not completely resolved, several types of regulatory cells have been identified to

contribute to disease suppression. From animal studies, we have learned that the T cell receptor (TCR) is the major target of both CD8+ MHC class I restricted and CD4+ MHC class II restricted anti-idiotypic T cells[38;39]. Based on T cell (receptor) vaccination studies in humans, it has been shown that the anti-idiotypic T cell responses are preferentially directed at the hypervariable CDR3 or the less variable CDR2 sequences of the idiotypic TCR[12;40-42]. Although TCR determinants may be the predominant targets, additional surface molecules may also contribute to the enhancement of the peripheral regulatory networks. Immune responses directed at activation markers common to all CD4+ T cells may also play an important role in the suppression of activated T cells following vaccination[4]. Although the targets for these T-T cell interactions remain unidentified, cytokine receptors have recently been proposed as candidate molecules[43]. Our data indicate that CSF-derived T cell vaccines are immunogenic since both anti-clonotypic and anti-ergotypic responses are present after vaccination. Indeed we observed proliferative responses to irradiated vaccine cells but also against irradiated autologous PHA stimulated T cell blasts. These data correspond to our previous studies of TCV with MBP-reactive T cell clones: anti-clonotypic T cell lines isolated from immunized patients were predominantly CD8+ cytolytic T cells that specifically recognized and lyzed the immunizing T cell clones in the context of MHC class I molecules[7] and CD4+ T cells were the major cytokine producing cells in the anti-vaccine cell population after TCV[12;11]. Furthermore, anti-ergotypic T cell responses have been demonstrated in almost all patients[9].

Interestingly, we found a significant reduction of MBP, PLP and MOG-reactive T cells in the periphery after TCV in 2 MS patients. In the other 3 patients the anti-myelin was rather low before vaccination and remained stable or was further reduced. Our results suggest that by enhancing the regulatory networks (both anti-clonotypic and anti-ergotypic), the frequency of myelin-reactive T cells can be reduced by TCV. Furthermore, we were not able to successfully expand activated CD4+ T cells from the CSF after vaccination in the majority of the patients. It is tempting to speculate that CSF cultures either are depleted from activated T cells or are dominated by other cell subsets involved in the anti-clonotypic and anti-ergotypic regulation of the pathogenic T cells. These findings are consistent with another T cell receptor vaccination study, where MS patients were immunized with a TCR BV6 peptide[44]. This BV gene family was overexpressed in the CSF-derived activated T cell population of the majority of the MS patients screened previously[29]. After vaccination, CSF

cultures of some patients failed to expand in cytokine supplemented conditions and the authors proposed that the lack of cell growth might imply the absence of activated T cells in the CSF of these patients[44]. However, CDR3 sequence analysis to compare the clonal heterogeneity of cultured CSF and non-stimulated PBMC before and after vaccination in 1 MS patient revealed that T cell clones comprised in the CSF vaccines were still detectable: some clones persisted in the CSF and PB, although the frequency of other dominant T cell clones significantly diminished after vaccination. Despite the fact that this patient (AMH) showed an anti-vaccine response following vaccination, the number or immunogenicity of certain T cell clones in the CSF vaccines might be to low to induce a sufficient anti-clonotypic response to (completely) eliminate these pathogenic T cells. These observations are in line with a previous report on TCR peptide vaccination: immunization with a low dosage of the TCR BV6 peptide did not reduce the frequency of TCR BV6 specific T cells after treatment. In contrast, the higher dose vaccination was more effective[44]. Another possibility to circumvent this problem is to perform repeated vaccinations (more than 3) to obtain efficient immune responses towards all vaccine cells over time. It should also be noted that for one MS patient, we were not able to generate a CSF-derived T cell vaccine. Instead we used activated T cells from the blood, and both anti-clonotypic and anti-ergotypic responses were detected after vaccination. In addition, we found a significant reduction of MBP, PLP and MOG-reactive T cells in the periphery. Although we presume that the frequency of pathogenic T cells is higher in the CSF, we have also demonstrated in patient AMH that activated T cell clones in the blood before vaccination can be detected in the CSF afterwards. Therefore, using these T cells as vaccines might prevent their migration to the CNS and subsequent pathological effects. The procedure for the generation of blood-derived activated CD4+ T cell vaccines is less complicated, but the relevance of these activated T cells to the disease is less clear.

In conclusion, we demonstrated a significant immunological response both to the vaccine cells (anti-idiotypic) as well as to activated cells in general (anti-ergotypic), although T cell clones used in the CSF-derived vaccines were not completely eliminated after vaccination.

This pilot trial was not designed to draw conclusions about treatment efficacy, but preliminary data suggest some degree of clinical benefit for MS patients in terms of a reduced relapse rate and a stabilization of disease scores shortly after vaccination. Based on

the promising results on feasibility and safety of this approach, together with the immune effects, a double-blind placebo-controlled clinical trial involving 60 early RR-MS patients was recently initiated to study the efficacy of vaccination with CSF-derived T cells in a larger population.

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Chapter 6

Summary and Discussion

Summary and discussion

The final section represents a brief overview and discussion of the main results of this thesis. For convenience, the considerations are arranged according to the goals described in chapter 1. Furthermore, the implications of the most important findings, together with some general considerations concerning the design of immunotherapies, and T cell vaccination in particular, are pointed out.

Goal 1: Analysis of the frequency and functional properties of MOG-reactive T cells in MS patients and healthy controls

Although the identity of the myelin antigen(s) that play(s) a primary role in the initiation of the inflammation in MS remains unknown, the quantitatively minor myelin oligodendrocyte glycoprotein (MOG) fulfills some criteria of a potential primary target antigen. This CNS-specific myelin component is located at the outhermost lamellae of the myelin sheath and is the only antigen that induces both an inflammatory T-cell and demyelinating B-cell response in EAE. In chapter 3, we evaluated the frequency and functional properties of MOG-reactive T cells in MS patients and healthy controls.

We analyzed the frequency of MOG-specific T cells using two different techniques based on either the detection of cytokine secreting cells (IFN-γ ELISPOT) or proliferative capacities of antigen-reactive T cells (limiting dilution analysis). Both in MS patients and healthy controls MOG-reactive T cells are present but no significant differences in frequency were found between the two groups. These results are consistent with earlier findings[1-3] but are in contrast to results from other groups showing an increased anti-MOG reactivity in MS patients[4-6]. These differences may be due to the use of different technologies to estimate the frequency of antigen-reactive T cells.

Furthermore, we analyzed the functional properties of this T cell population in a panel of 50 MOG-specific T cell lines/clones from 4 MS patients and 2 healthy controls. All T cell lines were TCRαβ+ CD4+. Epitope specificity was tested using a panel of 4 MOG peptides, which were previously shown to be immunodominant[5,7]. Most individuals displayed a

heterogeneous reactivity to several MOG epitopes and no significant differences in the overall peptide recognition were observed between MS patients and controls. Interestingly, only one out of fifty T cell lines tested recognized the MOG peptide 64-86, although a preferential T cell reactivity to this peptide was reported in HLA-DR2+ MS patients previously[7].

Analysis of the TCR rearrangements showed expression of a wide range of TCR BV and AV gene families in MS and controls. Although, some BV genes, including BV13, were found with an increased frequency in both groups or predominantly in the MS patients tested as demonstrated for BV6. This BV-specific expansion of T cells may indicate a superantigenic stimulation. Interestingly, none of the T cells tested shared any CDR3 region sequence, so no evidence for *in vivo* clonal expansion could be provided. However, these cells may also have been lost during *in vitro* culturing or may have been eliminated by rapid apoptosis after antigenic stimulation. Our results are consistent with an earlier report showing a diverse BV gene repertoire of MOG-reactive T cells in MOG-induced EAE[8].

As demonstrated by intracellular cytokine analysis, a biased profile of MOG-reactive T cells in MS patients was observed, whereas in healthy controls a mixed Th0/Th1-like profile was expressed. Apart from the high percentage of cells producing predominantly IFN-γ, increased levels of TNF-α were produced by MS-derived T cells after stimulation with their specific MOG-peptide as compared to controls, and this observation was even more pronounced in one HLA-DR2+ patient tested. An association between DR2 and high levels of TNF-α production has been reported previously for MS-derived MBP-specific T cell clones[9].

Another striking observation is the cytotoxic potential of the majority of T cell clones from all three MS patients. Induction of specific cytolysis of MOG peptide-pulsed autologous target cells was not seen for any of the CD4+ MOG-reactive T cell clones from the two healthy controls. Previously, cytotoxicity was also observed for MBP- and PLP-specific T cells[10-12], although it is unclear whether this mechanism plays an important role in the inflammatory response in MS. However, in EAE, it has been shown that encephalitogenic CD4+ T cells possess cytotoxic characteristics[13].

Based on a recent report on the increased production of brain-derived neurotrophic factor (BDNF) by different CD4+ myelin-specific T cell lines upon stimulation[14], we evaluated the secretion of two neurotrophins, BDNF and nerve growth factor (NGF) after stimulation with the specific MOG peptide. No indications for a potential neuroprotective role of MOG-reactive T cells were found since none of the T cell clones tested produced elevated levels of these neurotrophins upon stimulation.

In conclusion, although the frequency of anti-MOG T cells was similar in MS patients and healthy controls, our data indicate potential differences in their functional properties, with regard to cytokine production and cytotoxic reactivity. These differences may relate to their role in the disease process. Furthermore, our observations could be important for the design of novel immunotherapies targeting these autoreactive T cell population.

Goal 2: Study of activated CSF-derived T cells - optimization of a protocol for T cell vaccination in MS patients

In chapter 4, we optimized a culturing protocol to identify disease-related activated CD4+ T cells from CSF-derived mononuclear cells obtained after a lumbar puncture. Previous studies have shown that activated myelin-reactive T cells are accumulated in the CSF of MS patients. These cells may be relevant for the disease process since the CSF is the compartment that reflects best the ongoing inflammatory response in the CNS. Selective enrichment of recently *in vivo* activated T cells, expressing high-affinity interleukin-2 (IL-2) receptors, was achieved by expansion of the cells in medium containing low concentrations of IL-2.

We compared the phenotype and TCR repertoire of IL-2 expanded CSF-derived cell cultures from 5 MS patients and 4 control patients with other non-inflammatory diseases. In all 9 patients, we observed predominantly (CD4+) T cells after expansion and a biased TCR BV gene usage. No indications for a general 'MS-specific' overrepresented TCR BV gene were found. However, this observation does not necessarily mean that these activated T cells from the CSF are not relevant to the disease. It has been demonstrated previously that both MBP- and PLP-specific T cells (and also MOG-specific T cells as shown in chapter 3) display a

heterogeneous TCR BV gene repertoire. Our data are in line with other reports illustrating different overrepresented BV genes between patients[15;16] although some groups demonstrated similar TCR BV gene biases in different MS patients[17-19].

For two MS patients, we analyzed the TCR BV gene usage and the clonal composition of a selected number of overrepresented TCR BV genes of (activated) CD4+ T cells in the peripheral blood and the CSF. Directly *ex vivo* CD4+ T cells showed a heterogeneous TCR BV gene repertoire profile, although for some BV genes an elevated expression was found. This skewing of the unstimulated T cell population in blood of MS patients has also been demonstrated previously, but in that study polyclonal T cell subpopulations were reported for each overexpressed TCR BV gene family[20]. However, after selective enrichment with IL-2, we found a more restricted TCR BV gene profile, both in the CD4+ activated T cells in the blood and the CSF. Spectratype analysis of overrepresented TCR BV gene families in the two MS patients showed a limited clonal origin in the activated T cell subpopulations (mono- or oligoclonal profiles), whereas polyclonal T cell populations were found in the unstimulated CD4+ T cell fraction in the blood. Both CDR3 fragment length and sequence analysis of an overrepresented TCR BV gene family illustrated that similar T cell clones can be found in the CSF and the blood, but in the two compartments, other clonotypes are dominant. These dominant T cell clones could also be detected in the unstimulated CD8+ T cell depleted mononuclear cells in the blood (PBMC). Similar observations were reported in studies analyzing paired samples of activated T cells in the two compartments[15;16]. Based on our results, we suggest that *in vivo* activated T cell clones in the periphery can migrate to the CNS, where they may contribute to the inflammatory response.

The main goal of this part of the study, was to determine whether it is feasible to specifically expand activated CD4+ T cells from the CSF-derived mononuclear cells for T cell vaccination in MS patients, as described in chapter 5. Since only a limited number of cells can be obtained after a lumbar puncture, we conducted pilot experiments using low numbers of mononuclear cells from the blood to determine the optimal culturing conditions for the specific expansion of predominantly activated CD4+ T cells. Cells were preferentially cultured in autologous medium supplemented with low doses of IL-2 in the presence of irradiated autologous feeder cells. Furthermore, protocols for the efficient depletion of other mononuclear cell populations were optimized. Since CSF cultures need to be expanded

for an extended period of time to obtain sufficient number of CD4+ T cells for T cell vaccination, the stability of the CSF cultures during expansion was evaluated. Based on the results of phenotype and TCR BV gene usage at different time points during culturing, we concluded that T cell populations remained relatively stable. Furthermore, we evaluated the effect of freezing on the CSF cultures, since vaccinations are performed three times at two month intervals. After thawing, we were able to specifically expand CSF-derived T cell cultures. Based on these promising results, a protocol for the generation of CSF-derived T cell vaccines was established. This protocol was evaluated in a pilot clinical trial involving 5 MS patients (goal 3 – chapter 5).

Goal 3: Evaluation of feasibility, safety and immunological effects of T cell vaccination with CSF-derived activated CD4+ T cells in a pilot clinical trial involving 5 MS patients

In our previous clinical trials on T cell vaccination (TCV) using MBP-reactive T cell clones, all MS patients showed an upregulation of the regulatory anti-clonotypic networks together with a specific suppression of MBP-specific T cells in the periphery after vaccination[21;22]. A heterogeneous autoimmune response directed at multiple myelin antigens has been demonstrated, either from disease onset or after epitope spreading[23;24]. Furthermore, several studies reported an accumulation of activated CD4+ (myelin-reactive) T cells in the cerebrospinal fluid (CSF) of MS patients[25-28]. Persistence of certain T cell clones for a long period of time in the CSF may support their relevance for the disease process[17;29].

We conducted a pilot clinical trial to study feasibility, safety and immunological effects of TCV with activated CD4+ T cells from the CSF in 5 MS patients (4 RR/1 CP). Activated CSF-derived CD4+ T cells recognize a broad spectrum of myelin and other currently unidentified CNS antigens and bystander cells, and accumulate in the CSF. Therefore, we cultured CSF-mononuclear cells with low doses of interleukin-2, to specifically expand activated T cells. Other cell populations were depleted using immunomagnetic beads. After an expansion period of approximately 8 weeks, sufficient numbers of T cells were generated for three subcutaneous immunizations with 10^7 autologous irradiated CSF-derived activated T cells at two month intervals for four out of five MS patients. For one patient, CSF-derived T cells could not be cultured and instead IL-2 expanded T cells from peripheral blood were used.

Vaccine cells consisted predominantly of CD4+ T helper cells of a Th1 or mixed Th0 subtype, resembling pathogenic myelin-reactive T cells in EAE and MS[9;30;31]. High levels of the pro-inflammatory cytokines IFN-γ and TNF-α may be pathologically important for the recruitment of inflammatory cells to the CNS, increased presentation of myelin antigens and direct damage to the myelin(-producing oligodendrocytes)[32;33]. Furthermore, T cell vaccines displayed a heterogeneous reactivity towards a limited number of MBP, PLP and MOG peptides tested. T cells recognizing other epitopes of MBP, PLP and MOG or even other (unidentified) CNS antigens may also be present. Although the identity of predominant TCR BV genes varied between MS patients, within a given patient, the clonal composition of the vaccines remained rather stable. Dominant T cell clones persisted during further culturing as demonstrated by CDR3 fragment length screening and sequence analysis.

Patients were also monitored for immunological effects of TCV before, during and after vaccination. Our data demonstrate a cellular proliferative response to the vaccines which indicates that the CSF-derived activated T cells are immunogenic. Both anti-clonotypic (to irradiated vaccine cells) and anti-ergotypic (to irradiated activated T cells in general) responses are present after vaccination. These observations are in line with our previous TCV studies where predominantly CD8+ cytolytic anti-clonotypic T cell lines were isolated from immunized patients[34]. In addition, cytokine producing CD4+ T cells recognizing the vaccine clones were also found together with anti-ergotypic T cell populations[35;36]. Based on T cell (receptor) vaccination studies in EAE and humans, we learned that the TCR is the major target of anti-idiotypic T cells with a preferential recognition of the hypervariable CDR3 or less variable CDR2 sequence and that anti-ergotypic immune responses are directed at common activation markers like cytokine receptors[37-43].

Interestingly, in 2 MS patients we found a significant reduction of T cells reactive to all three myelin antigens tested in the periphery. In the other 3 patients, the anti-myelin response was rather low before vaccination and remained stable. Our results suggest that by enhancing the anti-clonotypic and anti-ergotypic regulatory networks, the frequency of MBP-, PLP- and MOG-reactive T cells can be reduced by TCV. In addition, we were not able to expand activated CD4+ T cells from the CSF after vaccination, which may further indicate that also CSF cultures are depleted from activated T cells and/or are dominated by other regulatory cell subsets. These observations correspond to the lack of cell growth in CSF

cultures after T cell receptor BV6 peptide vaccination[44]. However, evaluation of the clonal composition of CSF after TCV in one MS patient showed that T cell clones, comprised in the vaccines, might still be detectable. Although a significant anti-vaccine response was present, we argued that the number or immunogenicity of certain T cell clones might be too low to induce a sufficient response to suppress those pathogenic T cells. These observations are in line with a previous report on TCR peptide vaccination where a low dosage of the TCR BV 6 peptide did not reduce the frequency of TCR BV6-specific T cells after vaccination in the CSF[44]. A higher dose was much more effective[44].

For one MS patient, activated T cells from the blood were used as vaccines. Both anti-clonotypic and anti-ergotypic responses were detected after vaccination, together with a specific depletion of myelin-reactive T cells in the periphery. Although this procedure for the generation of blood-derived T cell vaccines is less complicated, the relevance of the activated T cells to the disease is less clear compared to those isolated in the CSF.

Immunizations with CSF-derived T cell vaccines were well tolerated and no toxicity or adverse effects were reported following vaccination. Although this pilot trial was not designed to draw conclusions about treatment efficacy, some degree of clinical benefit was observed in terms of reduced relapse rate and stabilization of disease scores until at least 4 months after vaccination. For 3 out of 5 patients, an active MRI scan was reported after vaccination, but thus far, this observation did not result in a clinical exacerbation.

In conclusion, these preliminary data illustrate that T cell vaccination with CSF-derived activated T cells is feasible and safe, and induces a specific anti-vaccine response. Based on the promising results, a double-blind placebo-controlled clinical trial involving 60 early RR-MS patients was recently initiated to study the efficacy of this approach in a larger population.

General remarks for the design of T cell (receptor peptide)-based immunotherapies

A major goal in the treatment of T cell-mediated autoimmune diseases like MS is the establishment of therapeutic strategies that selectively target pathogenic T cells leaving the remainder of the immune system intact[45]. For a long time, it was believed that identification of 'the' MS autoantigen may be the key for an effective treatment of the disease. However, recent data, including the observations in this study, demonstrate that a broad panel of myelin antigens must be considered as potential candidate autoantigens. Furthermore, there is a wide variability of myelin recognition between MS patients and, during the disease course, this recognition pattern may significantly change over time within patients. In a recent longitudinal study of the myelin-specific T cell repertoire, Goebels and co-workers observed three distinct patterns of MBP-specific T cell repertoire dynamics: a broad epitope response that persists over time with some fluctuations, a restricted response that becomes more diverse (epitope spreading) and a strikingly persistent focused response[46]. These observations have some important consequences for the design of T cell-based immunotherapies.

This heterogeneity of the myelin-specific T cell repertoire implicates that several T cell(s) receptor peptides might be required to suppress the ongoing inflammatory response. Therefore, it is likely that for TCR peptide vaccination a cocktail of different TCR peptides, rather than a single TCR peptide, will be more appropriate to obtain an effective regulation of a higher percentage of pathogenic T cell clones in a larger population of MS patients. The results of a recently initiated clinical trial using a cocktail of TCR BV5, BV6 and BV13 peptides, might provide some answers on the efficiency of the induced response. Upregulation of TCR-specific T cells creates a bystander suppression through the release of anti-inflammatory cytokines[38;47]. However, homologous TCR motifs within the CDR2 loops of different BV and AV gene families have been recently described[48]. This might suggest some cross-reactivity of certain TCR peptides for different anti-TCR-specific T cells, although this also implicates a less selective and less specific triggering of those regulatory cells[48]. Furthermore, in a recent study by Zipp and co-workers, it was shown that *in vitro* expanded T cell lines specific for a synthetic TCR peptide did not recognize the native (processed) TCR[49]. Therefore, these authors suggested that prior selection of TCR peptides on the basis

of their recognition of the naturally processed TCR epitopes may be more appropriate for their use in clinical trials[49].

Initial experiments in EAE animals and pilot clinical trials in MS patients with T cell vaccination (TCV) used irradiated autologous MBP-reactive T cell clones as vaccines[21;50]. Although this procedure has been shown to induce a specific suppression of MBP-specific T cells in the periphery together with an enhancement of regulatory anti-clonotypic networks, other myelin-reactive T cell populations remain unaffected and could be involved in the perpetuation of the disease[21;34]. Therefore, modifications of the initial TCV vaccination protocol may also further improve the effectiveness. Both the selection of myelin antigens targeted, dosage and frequency of vaccinations are major issues that are crucial in the development of an effective TCV procedure. Currently, several groups in Jerusalem, Los Angeles and Houston are using different TCV approaches in order to obtain additional information on the most suitable protocol to be used in future studies[51]. However, in all the ongoing clinical trials, the concept of a heterogeneous autoimmune T cell response has been taken into account since T cells recognizing different myelin antigens have been included in the vaccines.

In our CSF-based TCV protocol, we attempted to incorporate an even broader panel of potentially disease-related T cells by expanding T cells isolated from the CSF without taking into account their antigen specificity. Previous studies have demonstrated that this compartment may contain pathogenic T cells that are highly relevant to the disease process in the CNS because of its vicinity to the site of inflammation. We selectively expanded recently *in vivo* activated T cells, based on the expression of high-affinity IL-2 receptors (CD25). However, it should be kept in mind, that a regulatory CD4+ T cell subpopulation has been identified that also expresses this CD25 surface antigen[52-54]. Thus far, no specific cell surface molecule could be identified that is exclusively expressed on this regulatory CD4+ T cell population. Immunizing with irradiated regulatory T cells may lead to a suppression of the regulatory response and therefore may be harmful in patients with autoimmune diseases. However, based on the cytokine profiles, myelin reactivity and the ability to induce a proliferative response after vaccination, we believe that the CSF-derived T cell vaccines used for TCV are not part of the regulatory CD4+ CD25+ T cell subpopulation.

One concept that also should be kept in mind when using T cell-based immunotherapies, is the recently described theory of 'benign autoimmunity'[55]. In different animal models it has been demonstrated that autoreactive T cells recognizing specific CNS antigens may also have a protective function. Anti-MBP T cells have been shown to protect neurons from secondary degeneration after a partial crush of the optic nerve and to enhance recovery from CNS trauma after spinal cord injury[56;57]. One possible mechanism of the neuroprotective effect could be the local release of neurotrophins, which have recently been demonstrated to be secreted by several immune cells, including myelin-reactive T cells[14;58]. However, we were not able to confirm these data with our MOG-specific T cell clones (chapter 3). In conclusion, this concept of neuroprotective autoimmunity could have important implications for TCV. It would not be appropriate to incorporate these benign myelin-specific T cells in the vaccines. This population of autoreactive T cells rather should be preserved or even enhanced. Therefore, in ongoing clinical trials of TCV, the vaccines should be analyzed in detail and patients should be monitored for potential toxic and adverse effects after vaccination.

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Nederlandse Samenvatting

Overzicht van de Experimentele Resultaten

Nederlandse samenvatting

Multiple sclerose (MS) is één van de meest voorkomende neurologische aandoeningen bij jonge volwassenen. Door een chronische ontsteking ter hoogte van het centrale zenuwstelsel (CZS) wordt de myeline schede, die de zenuwbanen omhult en zorgt voor een optimale impulsgeleiding, aangetast. Dit leidt tot de gekende symptomen van verminderde zenuwfunctie en progressieve verlamming. Hoewel de etiologie tot op heden nog niet volledig opgehelderd is, wordt algemeen aangenomen dat MS een autoimmune aandoening is. Inflammatoire cellen die lichaamseigen myeline herkennen spelen een cruciale rol, maar daarnaast zouden ook genetische en omgevingsfactoren belangrijk kunnen zijn.

Onder normale omstandigheden zullen deze myeline-reactieve T-cellen, die ook bij gezonde personen aanwezig zijn in het bloed, geen schade aanrichten. Maar wanneer ze geactiveerd worden en niet meer onder controle gehouden worden door andere regulatoire cellen, zijn ze in staat om door de bloed-hersen barrière te migreren. Hierdoor kunnen ze de myeline schede aantasten en zo een algemene inflammatie ter hoogte van het CZS initiëren. Op basis van dierexperimenten, waarbij door toediening van myeline-reactieve T-cellen een ziekte kon geïnduceerd worden die vergelijkbaar is met MS, en op basis van functionele gelijkenissen van deze pathogene cellen met humane myeline-reactieve T-cellen, werd een pathogene rol voor deze autoreactieve cellen in het ziekteproces gesuggereerd. Grote vraag blijft natuurlijk welke myeline componenten het primair doelwit zijn van deze T-cellen. Initieel werden voornamelijk de twee meest voorkomende eiwitten in het myeline, *myelin basic protein* (MBP) en *proteolipid protein* (PLP), naar voor geschoven als potentiële autoantigenen voor MS. Maar de laatste jaren wordt steeds meer aandacht geschonken aan een mogelijke rol voor andere, slechts zeer sporadisch voorkomende myeline-eiwitten. Door hun unieke lokalisatie of specifieke eigenschappen zouden deze myeline componenten ook een prominente functie kunnen vervullen.

Een van deze 'nieuwe' potentiële autoantigenen voor MS is *myelin oligodendrocyte glycoprotein* (MOG), een transmembranair glycoproteïne dat slechts 0.01 tot 0.05% van de myeline schede uitmaakt. Door zijn unieke lokalisatie in de buitenste lamellen van de schede is het eiwit meer toegankelijk voor het immuunsysteem. Daarenboven blijkt MOG tot

op heden de enige myeline component te zijn die in het diermodel voor MS zowel een inflammatoire T-cel respons kan induceren als een demyeliniserende anti-lichaamrespons.

In het eerste deel (hoofdstuk 3) werd de mogelijke functie van MOG als potentieel autoantigen onderzocht. Bij MS patiënten en gezonde controles werden zowel kwantitatieve als kwalitatieve verschillen in de anti-MOG T-cel reactiviteit bestudeerd. Met behulp van twee verschillende technieken, gebaseerd op enerzijds de productie van cytokines (ELISPOT) en anderzijds op de proliferatieve capaciteit van de cellen (*limiting dilution analysis*) na antigen stimulatie, werd de frequentie van MOG-reactieve T-cellen in het bloed bepaald. De resultaten waren eenduidig : zowel bij MS patiënten als bij gezonde personen werden hoge maar gelijkaardige anti-MOG responsen waargenomen. Hoewel er dus geen verschillen in de frequentie van deze specifieke T-cel populatie kon aangetoond worden, werd ook gezocht naar mogelijke verschillen in functionele eigenschappen van deze MOG-reactieve T-cellen in MS patiënten. Hiervoor werd een panel van 50 MOG-specifieke T-cel clones, afkomstig van 4 MS patiënten en 3 gezonde personen, geanalyseerd. De epitoopspecificiteit van deze T-cel populatie bleek heterogeen te zijn, en T-cellen in beide studiegroepen herkenden verschillende MOG peptiden. Op basis van de T-cel receptor (TCR) sequentie analyse konden geen aanwijzingen gevonden worden voor *in vivo* activatie en clonale expansie van bepaalde MOG-specifieke T-cellen. Anderzijds werd wel een opmerkelijk verschil waargenomen in het cytokine expressie profiel: bij gezonde personen bestond de MOG-T-cel populatie zowel uit T helper 0 (Th0), Th1 en Th2 cellen. In tegenstelling hiermee produceerden bijna alle MOG-T-cellen van de verschillende MS patiënten uitsluitend interferon-γ (IFN-γ) en behoorden dus tot het Th1 subtype. Na stimulatie bleken deze cellen ook zeer grote hoeveelheden tumor necrosis factor-α (TNF-α) te secreteren, een ander pro-inflammatoir (Th1) cytokine. Daarenboven induceerden de meeste CD4+ MOG-reactieve T-cellen van MS patiënten ook een zeer specifieke lyse van autologe cellen die gepulst waren met het relevante MOG peptide. Desondanks bleek geen van de geteste MOG-reactieve T-cellen van de gezonde controles deze cytotoxische eigenschappen te bezitten. Tenslotte werd ook gekeken of deze T-cellen in staat zijn om neurotrofines te produceren na stimulatie. Er werden geen aanwijzingen gevonden voor een mogelijke protectieve rol van deze T-cel populatie. Immers, geen van de T-cel clones secreteerde een verhoogde concentratie van de twee geteste neurotrofines na stimulatie met het specifiek herkende MOG peptide.

Uit deze studie kunnen we besluiten dat het waarschijnlijk niet de frequentie van MOG-reactieve T-cellen is die een rol speelt in de pathogenese van MS. Het zullen eerder verschillen in functionele eigenschappen zijn, zoals de productie van pro-inflammatoire cytokines en de mogelijkheid tot specifieke inductie van cellyse, die zouden kunnen bijdragen tot een pathogene rol van deze T-cel populatie in het ziekteproces. Deze bevindingen zijn van belang bij de ontwikkeling van nieuwe immuuntherapieën die specifiek gericht zijn op de uitschakeling van deze T-cel populatie.

Uitgaande van de hypothese dat MS een T-cel gemedieerde autoimmune aandoening is waarbij de inflammatoire reactie gericht is tegen componenten van de myeline schede, zijn diverse experimentele behandelingen voor deze ziekte dan ook gericht op enerzijds de inactivatie van deze autoreactieve T-cellen en anderzijds het opwekken van een zekere tolerantie tegen myeline antigenen. Voor de eliminatie van deze pathogene cellen worden twee verschillende strategieën aangewend. Ofwel kunnen MS patiënten 'gevaccineerd' worden met bestraalde autologe myeline-reactieve T-cellen, een procedure die T-cel vaccinatie (TCV) genoemd wordt. In het lichaam van de patiënten wordt een specifieke reactie tegen deze pathogene cellen opgewekt. Daarnaast is het ook mogelijk om zeer gericht te gaan immuniseren met synthetische peptiden die gebaseerd zijn op een uniek deel van de TCR. Deze structuur wordt beschouwd als de vingerafdruk van een (pathogene) T-cel, waartegen dan ook een zeer specifieke respons zal opgewekt worden. Tenslotte worden ook in diverse klinische studies experimentele therapieën uitgetest die gebaseerd zijn op de inductie van tolerantie voor myeline door toediening van (gemodificeerde) myeline antigenen of peptiden in MS patiënten.

In het tweede deel (hoofdstuk 4) werd vooral gewerkt aan de optimalisatie van een protocol voor de specifieke expansie en karakterisatie van geactiveerde CD4+ T-cellen uit het cerebrospinale vocht (CSV) in het kader van TCV. In de literatuur werd aangetoond dat in het CSV van MS patiënten een hoge frequentie geactiveerde T-cellen voorkomt, die verschillende myeline antigenen herkennen en die vaak gedurende een lange periode in het CSV aanwezig blijven. Deze cellen zouden zo een pathogene rol kunnen spelen in het ziekteproces.

In eerste instantie werd gezocht naar eventuele verschillen in de geactiveerde (T-)cel populaties opgekweekt uit het CSV van MS patiënten en patiënten met niet-inflammatoire neurologische aandoeningen. In beide groepen bleken de CSV-culturen na interleukine-2 (IL-2) expansie voornamelijk uit CD4+ T-cellen te bestaan. Uit de analyse van de genexpressie profielen van het variabele gebied van de T-cel receptor (TCR VB) kunnen we besluiten dat in alle patiënten de T-cel populatie weinig heterogeen is. Er werden echter geen indicaties gevonden voor specifieke TCR VB genfamilies die preferentieel voorkomen in het CSV van MS patiënten. Verder werd voor 2 MS patiënten ook een vergelijking gemaakt tussen de CD4+ T-cel populatie in het bloed en het CSV na expansie met IL-2. Uit de TCR VB genprofielen blijkt een duidelijke restrictie na IL-2 expansie van T-cellen in het bloed (en het CSV), terwijl T-cellen direct *ex vivo* (zonder kweek) een meer heterogeen patroon vertonen. Uit de analyses van de clonale samenstelling van preferentieel voorkomende TCR VB genfamilies blijkt dat de geactiveerde T-cel populaties in het bloed en het CSV slechts uit één (monoclonaal) of enkele (oligoclonaal) T-cel clones bestaan en dat in beide compartimenten andere dominante T-cell clones aanwezig zijn. Daarnaast zien we dat deze geëxpandeerde T-cellen ook terug te vinden zijn in de heterogene (polyclonale) T-cel populaties in het bloed.

Zoals beschreven in hoofdstuk 5, werd in een piloot studie de veiligheid en haalbaarheid van T-cel vaccinatie met geactiveerde CD4+ T-cellen uit het CSV van MS patiënten bestudeerd. Hoewel slechts zeer kleine hoeveelheden cellen na een lumbale punctie uit het CSV geïsoleerd worden, zijn we toch in staat om uit de stalen selectief de geactiveerde CD4+ T-cellen te expanderen door de cellen op te kweken in aanwezigheid van lage concentraties (IL-2). Andere mononucleaire celpopulaties die initieel ook in het CSV terug te vinden zijn, kunnen efficiënt gedepleteerd worden met behulp van immunomagnetische beads. Hoewel dus voldoende grote celaantallen gegenereerd konden worden, was het ook belangrijk om na te gaan of de T-cel populatie gedurende de expansie stabiel bleef. Op basis van de resultaten van fenotype analyses en T-cel receptor profielen van verschillende CSV-culturen die gedurende lange tijd in kweek gehouden werden, kunnen we besluiten dat de T-cel populatie inderdaad vrij stabiel blijft. Omdat in het vaccinatie protocol de patiënten drie maal geïmmuniseerd worden met een tussenperiode van telkens twee maanden, werd ook gekeken of deze cellen na invriezen nog opnieuw specifiek geëxpandeerd konden worden. Ook dit bleek geen probleem te vormen, zodat in de piloot studie T-cellen steeds één week

voor vaccinatie ontdooid en gereactiveerd werden. Op basis van de resultaten van deze optimalisatie studie werd een protocol vastgelegd voor de aanmaak van T-cel vaccins uit CSV stalen dat in een piloot studie geëvalueerd werd.

In eerdere klinische studies werden de effecten van T-cel vaccinatie, een procedure waarbij MS patiënten geïmmuniseerd worden met MBP-reactieve T-cellen bestudeerd. Bij alle gevaccineerde patiënten werd een depletie van MBP-specifieke T-cellen waargenomen en werd een respons tegen de vaccin cellen geïnduceerd. Maar naast MBP worden ook andere myeline antigenen naar voren geschoven als potentiële autoantigenen voor MS. Daarenboven blijken in het CSV van MS patiënten hoge aantallen geactiveerde T-cellen voor te komen die een brede waaier van myeline antigenen herkennen en gedurende een lange periode aanwezig blijven.

Na een optimalisatie fase, waarbij de expansiecapaciteit en karakteristieken van CSV-cellen bestudeerd werden, werd een piloot studie uitgevoerd bij 5 MS patiënten (4 *relapsing-remitting*/1 chronisch progressieve) om zowel de veiligheid, haalbaarheid en immunologische effecten van T-cel vaccinatie op basis van geactiveerde CD4+ T-cellen afkomstig uit het CSV te bestuderen (hoofdstuk 5). Na een gemiddelde expansieperiode van 8 weken, konden voldoende grote celaantallen worden gegenereerd voor immunisatie. Met behulp van immunomagnetische beads werden andere mononucleaire celpopulaties efficiënt uit de celculturen gedepleteerd tijdens de expansie periode. Vervolgens werden in de patiënten drie maal 10^7 bestraalde geactiveerde CD4+ T-cellen subcutaan ingespoten telkens met een tussenperiode van 2 maanden. De T-cel vaccins werden in detail gekarakteriseerd en bestonden voornamelijk uit Th0 of Th1 CD4+ T-cellen die reactief waren tegen minstens twee van de drie geteste myeline antigenen. Uit het T-cel receptor onderzoek bleek dat de clonaliteit van de T-cel populatie gerestricteerd is en stabiel voor de drie vaccins voor één bepaalde patiënt. Bij alle patiënten werd een cellulaire proliferatieve respons tegen de vaccin cellen (anti-clonotypische respons) en/of tegen geactiveerde cellen in het algemeen (anti-ergotypische respons) waargenomen na vaccinatie. Na de laatste vaccinatie werd ook de frequentie van myeline-specifieke T-cellen in het bloed bepaald: voor 3 patiënten was deze frequentie eerder laag voor vaccinatie en bleef deze stabiel. Maar in de 2 resterende patiënten, waar voor vaccinatie een hoge myelin-reactiviteit gedetecteerd werd, kon na vaccinatie een specifieke daling van zowel MBP-, PLP- en MOG-specifieke T-cellen

vastgesteld worden en niet van het controle antigen. Deze resultaten suggereren dat door inductie van regulatoire netwerken (zowel anti-clonotypisch als anti-ergotypisch), de frequentie van myeline specifieke T-cellen kan verlaagd worden. Daarenboven bleek het ook voor het merendeel van de patiënten onmogelijk om geactiveerde CD4+ T-cellen uit het CSV na vaccinatie te expanderen. Dit kan een bijkomende aanwijzing zijn voor de onderdrukking van deze cellen door andere regulatoire cellen die ook naar het CSV migreren. Uit de klinische opvolging blijkt dat TCV veilig is en geen nevenwerkingen vertoont. Daarenboven wijzen de klinische data (EDSS score en aantal opflakkeringen) op een stabilisatie van het ziekteproces tijdens en kort na de vaccinaties.

Op basis van deze resultaten werd recent gestart met een dubbel-blinde placebo-gecontroleerde klinische studie waarbij de klinische effecten van vaccinatie met T-cellen uit het cerebrospinale vocht geëvalueerd wordt in een populatie van 60 vroege *relapsing-remitting* MS patiënten.

Uit verschillende studies, waaronder ook deze, blijkt dat een brede waaier van myeline antigenen beschouwd kan worden als potentiële kandidaat autoantigenen voor MS. Daarenboven is ook aangetoond dat bij verschillende MS patiënten verschillende patronen van myeline herkenning waargenomen worden en dat ook binnen één patiënt dat patroon significant kan wijzigen gedurende het ziekteverloop. Deze bevindingen moeten ook in acht genomen worden bij de ontwikkeling van nieuwe immunotherapieën die gericht zijn op de uitschakeling van deze pathogene T-cel populaties.

Enerzijds impliceert dit heterogene myeline-specifieke T-cel repertoire dat voor (TCR) peptide vaccinatie een cocktail van verschillende synthetische peptiden vereist is voor een efficiënte onderdrukking van de inflammatoire respons. Ondanks deze heterogeniteit werd onlangs toch enige homologie beschreven tussen TCR motieven in verschillende TCR VB en VA families. Hierdoor zou kruisreactiviteit van bepaalde peptiden voor meerdere anti-TCR-specifieke T-cellen kunnen optreden, wat ook een minder specifieke respons kan impliceren. Daarenboven is het bij deze manier van vaccineren belangrijk dat regulatoire anti-TCR peptide T-cellen ook in staat zijn om, naast het synthetische peptide, ook daadwerkelijk de natieve of bewerkte vorm van de TCR te herkennen.

Anderzijds heeft de aanwezigheid van pathogene T-cellen die gericht zijn tegen een brede waaier van myeline antigenen ook gevolgen voor TCV. In eerste instantie werden voor deze strategie enkel MBP-specifieke T-cellen geïncorporeerd in de vaccins. Aanpassingen aan dit initiële protocol, met betrekking tot de uitbreiding van de myeline antigenen waartegen de T-cellen gericht zijn, maar ook de optimale dosis en frequentie van de immunisaties, zijn cruciaal in de ontwikkeling van de meest efficiënte procedure voor TCV.

In ons aangepaste TCV protocol vertrekken we van cellen afkomstig uit het cerebrospinale vocht in plaats van bloed en expanderen we ziekte-gerelateerde T-cellen op basis van hun activatie toestand (oppervlakte merker CD25). Hoewel we er vanuit gaan dat deze *in vivo* geactiveerde T-cellen relevant zijn voor het ziekteproces dat zich afspeelt in het CZS, moeten we rekening houden met het feit dat recent een regulatoire T-cel subpopulatie geïdentificeerd werd met gelijkaardige fenotypische kenmerken. Op basis echter van de gedetailleerde vaccin karakterisaties kunnen we vermoeden dat de CSV-T-cel vaccins die in de piloot studie gebruikt werden geen deel uitmaken van deze CD4+ CD25+ regulatoire T-cel subpopulatie.

Tenslotte dient opgemerkt te worden dat recent in het diermodel beschreven werd dat myeline-specifieke T-cellen ook neurprotectief kunnen zijn, waarschijnlijk mede door de produktie van neurotrofines. Ook deze 'goedaardige' autoreactieve T-cellen moeten geweerd worden uit de vaccins voor TCV omdat een suppressie van deze T-cellen het demyelinisatie proces eerder zal bevorderen.

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T Cell Reactivity against Myelin Oligodendrocyte Glycoprotein in MS and Controls.
A. Van der Aa, C. Bernard, J. Raus and P. Stinissen
09/11/2001: *Sixth National Symposium BVC/ABC*, Edegem
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Annegret, mei 2002.

Curriculum Vitae

Annegret Van der Aa werd geboren op 2 oktober 1975 te Duffel. In 1993 behaalde ze het diploma algemeen secundair onderwijs (A.S.O.) Latijn-Wetenschappen aan het Regina Pacis Instituut te Hove. Datzelfde jaar vatte ze haar universitaire studies aan in de richting Biomedische Wetenschappen aan de Faculteit Geneeskunde van het Rijksuniversitair Centrum te Antwerpen (RUCA). Haar licentiaatjaren volbracht ze in het Departement Biochemie, richting Medische Biochemie aan Universitaire Instelling Antwerpen (UIA) en in juni 1997 behaalde ze met grote onderscheiding het diploma van Licentiaat Biochemie, optie Medische Biochemie. Haar eindwerk, getiteld 'Effect van cytokinine-analogen op de proliferatie van kwaadaardige bloedvormende cellen' volbracht ze in het labo Experimentele Hematologie van de UIA/UZA waar ze tot begin 1998 verder wetenschappelijk onderzoek verrichtte. Sinds 1 februari van dat jaar is ze verbonden aan het Biomedisch Onderzoeksinstituut van het Limburgs Universitair Centrum (LUC) en de Transnationale Universiteit Limburg (tUL). Ter voorbereiding van dit doctoraatsproefschrift verrichtte ze gedurende vier jaar onderzoek in het kader van Multiple Sclerose op de afdeling Autoimmune Aandoeningen. Tijdens deze periode volgde ze een doctoraatsopleiding, waarvoor begin 2002 een certificaat werd uitgereikt.

Abbreviations

A: T cell receptor alpha chain
Ab: antibody
APC: antigen presenting cell
APL: altered peptide ligand
AS: autologous serum
B: T cell receptor beta chain
BDNF: brain-derived neurotrophic factor
BSA: bovine serum albumine
C: constant region of the T cell receptor alpha/beta chain
CD: cluster of differentiation
CDR 2/3: complementary determing region 2/3
CNPase: 2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS: central nervous system
Cpm: counts per minute
CP-MS: chronic progressive multiple sclerosis
CSF: cerebrospinal fluid
D: diversity region of the T cell receptor beta chain
DIG: digoxigenin
DN: double negative
EAE: experimental autoimmune encephalomyelitis
E. coli: Escherichia coli
EDSS: expanded disability status score
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunospot assay
E/T: effector-to-target ratio
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
GA: glatiramer actetate
GALT: gut-associated lymphoid tissue
Gd: gadolinium
HHV-6: human herpes virus-6
HLA: human leukocyte antigen
Hprt: hypoxanthine-guanine phosphoribosyl transferase
IFN-β/γ: interferon-beta/gamma
Ig: immunoglobulin
IL: interleukin
IL-2Rα: alpha chain of the interleukin-2 receptor
J: junctional region of the T cell receptor alpha/beta chain
LDA: limiting dilution analysis
LT: lymphotoxin
MAG: myelin-associated glycoprotein
MBP: myelin basic protein
MHC: major histocompatibility complex
MOBP: myelin oligodendrocyte basic protein
(r)MOG: (recombinant) myelin oligodendrocyte glycoprotein
MRI: magnetic resonance imaging

MS: multiple sclerosis
MSRV: multiple sclerosis-associated retrovirus
NGF: nerve growth factor
NK: natural killer cell
NS: normal subjects
OND: other non-inflammatory neurological disease
OD: optical density
OSP: oligodendrocyte specific protein
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
PCR: polymerase chain reaction
Pd: proton density
PE: phycoerytrin
PerCP: peridinin chlorophyll protein
PHA: phytohaemagglutinin
PLP: proteolipid protein
PMA: phorbol-12-myristate-13-acetate
PP-MS: primary progressive multiple sclerosis
RFLP: restriction fragment length polymorphism
rhIL-2: recombinant human interleukin-2
RR-MS: relapsing-remitting multiple sclerosis
RT-PCR: reverse transcriptase polymerase chain reaction
SEM: standard error of the mean
SI: stimulation index
SP-MS: secondary progressive multiple sclerosis
TCC: T cell clone
TCL: T cell line
TCR: T cell receptor
TCV: T cell vaccination
TGF-β: transforming growth factor-beta
Th: helper T cell
Thy: thymidine
TNF-α: tumor necrosis factor-alpha
Tr: regulatory T cell
TT: tetanus toxoid
V: variable region of the T cell receptor alpha/beta chain
VLA-4: very late antigen-4