PATHOGENIC AND THERAPEUTIC ASPECTS OF INTRATHECAL IMMUNE RESPONSES IN MULTIPLE SCLEROSIS

Doctoral thesis by

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LIST OF ABBREVIATIONS

Ag	antigen	**J**	joining
APC	antigen presenting cell	**L**	light
BBB	blood brain barrier	**LFA**	leukocyte functional antigen
BCR	B cell receptor	**mAb**	monoclonal antibody
BDNF	brain-derived neurotrophic factor	**MAG**	myelin-associated glycoprotein
C	constant	**MBP**	myelin basic protein
CD	cluster of differentiation	**MHC**	major histocompatibility complex
CDR	complementarity determining region	**MMP**	matrix metalloproteinases
CNPase	2'-3'cyclic nucleotide 3'-phosphodiesterase	**MOG**	myelin oligodendrocyte glycoprotein
CNS	central nervous system	**MS**	multiple sclerosis
CSF	cerebrospinal fluid	**OCB**	oligoclonal band
D	diversity	**OMGP**	oligodendrocyte myelin glycoprotein
DC	dendritic cell		
DMSO	dimethyl sulfoxide	**PBMC**	peripheral blood mononuclear cells
EAE	experimental autoimmune encephalomyelitis	**PCR**	polymerase chain reaction
EBV	Epstein barr virus	**PHA**	phytohemagglutinin
ELISA	enzyme-linked immunosorbent assay	**PLP**	proteolipid protein
ER	endoplasmic reticulum	**RR**	relapsing remitting
FcR	fragment crystallizable receptor	**SLE**	systemic lupus erythematosus
FR	framework region	**SP**	secondary progressive
GA	glatiramer acetate		
H	heavy	**TCR**	T cell receptor
HLA	human leukocyte antigen	**TGF**	transforming growth factor
ICAM	intracellular adhesion molecule	**Th**	T helper
Id	idiotope	**TNF**	tumour necrosis factor
IFN	interferon	**V**	variable
Ig	immunoglobulin	**VCAM**	vascular cell adhesion molecule
IL	interleukin		

LIST OF PAPERS INCLUDED IN THE THESIS

Paper I

Hestvik AL, Skorstad G, Price DA, Vartdal F and Holmoy T. Multiple sclerosis: glatiramer acetate induces anti-inflammatory T cells in the cerebrospinal fluid. *Mult Scler*. 2008; 14: 749-58.

Paper II

Holmøy T, Fredriksen AB, Thompson KM, **Hestvik AL,** Bogen B and Vartdal F. Cerebrospinal fluid T cell clones from patients with multiple sclerosis: recognition of idiotopes on monoclonal IgG secreted by autologous cerebrospinal fluid B cells. *Eur J Immunol.* 2005; 35: 1786-94.

Paper III

Hestvik AL, Vartdal F, Fredriksen AB, Thompson KM, Kvale EO, Skorstad G, Bogen B and Holmoy T. T cells from multiple sclerosis patients recognize multiple epitopes on self-IgG. *Scand J Immunol.* 2007; 66: 393-401.

Paper IV

Hestvik AL, Skorstad G, Vartdal F and Holmoy T. Idiotope-specific $CD4^+$ T cells induce apoptosis of human oligodendrocytes. *J Autoimmun.* 2009; 32: 125-32.

1. INTRODUCTION

1.1 General aspects of immunology

Our immune system protects us against and enables our coexistence with the numerous infectious microorganisms inhabiting the earth. A complex network of specialized cells, molecules, proteins and organelles comprises the immune system, which we generally divide into an innate and an adaptive arm[1]. The innate immune system provides the first line of defence against invading pathogens and provides immediate protection in a non-specific manner. One critical event in innate immunity is the inflammatory response, which is initiated by tissue resident macrophages upon their initial encounter with infectious agents. The release of cytokines and chemokines activates the vascular system and the complement system and attracts other leukocytes essential in the clearance of the infection. In addition to initiating inflammatory responses and controlling commencing infections, one of the most important tasks of the innate immune system is the priming and activation of the adaptive response. Professional antigen presenting cells (APCs) play a key role in this process. APCs engulf pathogens at the site of entry and subsequently travel via the lymphatic system into draining lymph nodes. APCs mature and acquire the ability to efficiently communicate with T lymphocytes, specialized cells of the adaptive response, which are encountered in high numbers in the lymph node. The APC presents processed antigen on major histocompatibility complex (MHC) molecules on the cell surface to T cells and enables a highly specific response against a single antigen with a resulting clonal expansion of the responding T cell. Activated T cells upregulate adhesion and costimulatory molecules, allowing efficient interaction with B cells sensitized to the same antigen. Clonal expansion of antigen specific T cells and B cells forms the basis for immunological memory, a unique feature of the adaptive immune system, which refers to the ability to "remember" a specific antigen such that on a second encounter a much faster and more efficient immune response is mounted.

The ability to mount a specific immune response against an ever-changing infectious environment while preventing immune responses against self, which is also subject to constant change, is a critical feature of the immune system. An unrestrained immune response against self-proteins can lead to tissue damage and autoimmune disease. To

prevent this, tight communication between the innate and the adaptive immune system as well as the tissue in question is warranted. The mechanisms in control are not fully understood, but rely on a conserved ability to distinguish between self and non-self combined with the release of specific danger signals[2-4].

1.1.1 T cells

T cells arise from the bone marrow and migrate to the thymus where a maturation and selection process takes place. The T cell recognizes antigen through a membrane bound heterodimeric T cell receptor (TCR) composed of an α and β chain (TCRαβ) or a γ and δ chain (TCRγδ). The receptor genes are encoded by variable (V), diversity (D) and joining (J) gene regions. In a process known as V(D)J recombination the receptor genes undergo somatic recombination events which create the vast receptor repertoire of mature T cells. T cells are divided into T helper (h) cells and cytotoxic T cells and the TCRs recognize antigens as peptide fragments bound to self-MHC molecules. Th cells express the cluster of differentiation (CD) 4 coreceptor and recognize peptides on MHC class II molecules. Peptides loaded on MHC class I molecules are recognized by cytotoxic T cells bearing the CD8 coreceptor. A positive and negative selection process in thymus, which depends on presentation of self-proteins on self-MHC molecules by thymic epithelial cells and thymic dendritic cells (DCs), defines the repertoire of circulating T cells[5-7]. While positive selection ensures survival of T cells that express a receptor with moderate affinity for self-peptides, negative selection eliminates T cells with a receptor that binds too strongly to self-peptides. Naïve T cells leave the thymus and migrate to secondary lymphoid tissues where they may encounter activated APCs.

Peripheral DCs capture antigen at the site of infection and subsequently migrate to secondary lymphoid organs. DCs, macrophages and B cells are termed professional APCs due to their expression of MHC class II molecules and their ability to present peptides to and activate naïve CD4$^+$ T lymphocytes. DCs are the most efficient APC and express high levels of MHC molecules in combination with costimulatory molecules, which are necessary for the activation and clonal expansion of naïve T cells. Upon antigen recognition, a tight interface, referred to as the immunological synapse, is formed between the T cell and the APC[8]. Thousands of TCRs in combination with CD4 or CD8 coreceptors are found in complex with peptide-bound MHC molecules clustered

in the middle of the synapse. Costimulatory molecules such as leukocyte functional antigen (LFA)-1, integrin and CD28 engaging the APC ligands, intracellular adhesion molecule (ICAM)-1 and B7, respectively, form an outer rim of the synapse. A fine balance between positive and negative signals delivered during the T cell-APC interaction decides the fate of the T cell[9,10]. In the absence of adequate costimulation a T cell may become anergic or die, whereas adequate stimulation leads to activation. Depending on the nature of activating signals and cytokines secreted by the APC, an antigen specific CD4$^+$ T cell may differentiate into one of several subsets of Th cells. So far, three major subsets have been identified: Th1, Th2 and Th17, each with different effector functions. Although no such classifications subdivide CD8$^+$ T cells, various effector functions have been described for phenotypical subsets of CD8$^+$ T cells[11].

Upon clonal expansion, one naïve T cell can give rise to approximately 1000 daughter cells of identical specificity that further differentiate into effector and memory T cells. Effector T cells upregulate cell adhesion molecules, such as ICAM, vascular cell adhesion molecule (VCAM), LFA-1 and α4β1-integrin, and chemokine receptors that allow attraction and migration into sites of inflammation. The life span of effector T cells is short and the majority will die by apoptosis during the immune response. A proportion of the activated T cells differentiate into memory T cells, which persist after the removal of antigen and form the basis for T cell memory. Alterations in the expression of several surface molecules differentiate memory T cells from naïve cells and confer enhanced survival, higher antigen sensitivity, directed migration to tissues instead of lymphoid organs and increased adhesion to APCs and endothelial cells[12].

1.1.2 B cells

B cells develop from haematopoietic stem cells in the bone marrow through the process of B cell lymphopoiesis. Membrane bound immunoglobulin (Ig) serves as the antigen specific B cell receptor (BCR) and can be secreted as an Ig molecule by B cells which have differentiated into plasma blasts and plasma cells[13]. A monomeric Ig molecule contains two identical light (L) chains and two identical heavy (H) chains, each with a V and a constant (C) region. The H chain determines the isotype and thereby the effector functions of an antibody molecule and comes in five main isotypes: IgM, IgD, IgG, IgA and IgE. L chains can either be kappa (κ) or lamda (λ). The antigen-binding site is

formed by pairing the V domains of the H and L chains. This results in a surface complementary to a specific antigen, with three highly variable loops termed the complementarity determining regions (CDRs), interspaced by three framework regions (FRs)[14]. The diversity of the Ig repertoire in naïve B cells is generated by stochastic recombination of the Ig H (V,D,J) and the Ig L (V,J) chain loci, similar to the development of the TCR. Once a functional BCR is expressed, the specificity of this receptor is tested[15]. B cells with a receptor that recognizes self-molecules will either be eliminated from the repertoire, become anergic or ignorant or undergo receptor editing. The process of receptor editing involves replacement of the L chain, which will combine with the existing H chain, and change the antigen specificity of the receptor. If the new receptor is not self-reactive, the B cell is rescued from apoptosis. Naïve B cells with a functional receptor migrate to secondary lymphoid organs where further maturation takes place through antigen recognition and T cell help.

The activation and differentiation of a naïve B cell into an antibody secreting cell requires two signals; one signal is provided through the BCR upon antigen binding, the other signal is usually provided by an armed $CD4^+$ T cell which recognizes fragments from the same molecular complex presented by MHC class II molecules on the surface of the B cell. An important part of this interaction is expression of the B cell stimulatory molecule CD40 ligand on the T cell surface, and the secretion of stimulatory cytokines[16,17]. The ensuing T-B cell collaboration, which takes place at the border between the T cell and B cell zones in secondary lymphoid organs leads to rapid expansion of both B and T cells. A proportion of the activated B cells form germinal centers where several modifications of the B cells are induced[18,19]. This includes isotype switching where the gene segment encoding the V region reshuffles to another C gene, maintaining the antigen specificity while providing a new C region with a suitable effector function. Subsequently, the rearranged BCR V gene segment undergoes hypermutation at a rate that is at least 10^3-10^4 fold greater than the normal rate of mutations across the genome[20]. When these hypermutated BCRs are "tested" against the antigen, B cells having achieved better affinity will be positively selected, while B cells with lost affinity will die from apoptosis. This ongoing process is referred to as affinity maturation and ensures selection of B cells with the highest affinity. B cells differentiate into antibody secreting plasma cells or into memory B cells. Memory B cells upregulate MHC class II molecules and costimulatory molecules enabling a highly efficient

response upon a second encounter with the antigen. Some plasma cells migrate back to the bone marrow where they provide a source of long-lasting, high-affinity antibodies. The mechanisms maintaining serological memory is poorly understood but may in part depend on polyclonal activation of memory B cells[21].

1.2 Idiotopes and anti-Id responses

The ability of the immune system to mount a highly specific response against the diverse antigenic determinants present in our environment relies on the process of TCR and BCR rearrangement as well as affinity maturation of the B cells. This creates unique protein sequences that are not encoded by germline genes and can themselves be perceived by the immune system as immunological non-self, antigenic determinants. This forms the basis for the description of idiotopes (Ids), which are clonally unique antigenic determinant located in the V region of BCRs and TCRs[22,23]. One receptor carries several Ids, which collectively are referred to as the idiotype of that receptor. In 1974 Niels K. Jerne postulated the presence of an immunologic regulatory network based on anti-idiotopic antibodies, which later won him the Nobel Prize[24]. Jerne's theory included only Ids located in BCRs and can be illustrated by the following example: antibody (Ab) 1 binds an antigen. Ab2 binds the Id of Ab1 and is thereby an anti-idiotypic antibody. Ab2 displays a structural resemblance to the antigen and is therefore an internal image of the antigen. Ab2 could thus play a key role in immunological memory, preserving the epitope of encountered antigens and at the same time serve a regulatory role by immobilizing Ab1 and inhibit the immune response to the antigen. This type of B lymphocyte regulation was suggested to be partially responsible for preventing an uncontrolled immune response[24].

1.2.1 T cells in idiotypic networks

In Jerne's original network theory only antibodies and B cells were included, but it has since become clear that also T cells can participate in Id-driven regulatory networks[25,26]. Studies on Id-specific T cell responses have been facilitated by Ids expressed by plasma cell tumors[27]. Mice immunized with myeloma protein were shown to develop protective antibody responses against Ids and suppressed growth of the corresponding transplanted tumour cells[28]. The anti-Id response was suggested to be T cell dependent because T

cell deficient mice did not produce anti-Id antibodies upon immunization[29,30]. Additional evidence for the existence of Id-specific T cells was demonstrated in adoptive cell transfer experiments[25].

By employing a similar assay system, the target of an Id-specific T cell response was mapped to the λ2 Id located to the BCR L chain, and the immune response was shown to be controlled by MHC linked genes[31,32]. Three somatic mutations present in the VL region were found to be critical for the T cell response[33]. This system has been used to further investigate Id-specific T cell responses in cancer surveillance and autoimmunity. CD4^{+} T cells specific for the λ2 Id were shown to protect against a MHC class II positive and a MHC class II negative tumour, which both secreted the λ2 Id[34]. This suggested that the λ2 Id was processed and presented on MHC class II molecules by host APCs, which in turn could activate Id-specific T cells. This finding was principally important considering the tendency of tumours to down-modulate MHC class II molecules, and supported by the observation that DCs surrounding the tumours were loaded with tumour antigens and could activate tumour-specific CD4^{+} T cells[35]. These observations have paved the way for strategies involving Id-based vaccination against tumour development[36]. Several of these have been successfully applied in animal models and may hold promise for cancer treatment in humans.

As demonstrated in experimental autoimmune encephalomyelitis (EAE), Ids present in TCRs of pathogenic T cells may also hold therapeutic potential. Immunization with attenuated encephalitogenic T cells has been shown to protect mice against disease development[37]. The clinical effect was associated with the activation of T cells specific for Ids located in the TCR of encephalitogenic T cells, suppressing their pathogenic potential[38]. This has led to clinical trials in multiple sclerosis (MS) patients[39]. Patients vaccinated with expanded autologous myelin specific T cells have consistently demonstrated a reduction in T cell reactivity to myelin and a concomitant positive clinical effect has been observed in some patients[40-42].

1.2.2 Id responses in autoimmunity

The relevance of Id-specific responses in the development of autoimmune disease can be demonstrated in experimental models of systemic lupus erythematosus (SLE). SLE is associated with strong autoantibody responses against DNA and nuclear proteins[43] and

the majority of SLE patients develop anti-idiotypic antibodies against common Ids present in the VH chain of anti-DNA autoantibodies[44,45]. Experimental SLE can be induced in naïve mice of different strains following immunization with anti-DNA autoantibodies or with Id-peptides present in the H chain of these antibodies[44,46]. Prior to clinical disease lupus prone mice spontaneously develop polyclonal T cell responses to Id-peptides, suggestive of *in vivo* processing and presentation of autoantibodies on MHC molecules[47]. Two important observations underscore the relevance of T cell responses to Id in experimental lupus. First, adoptive transfer of Id-specific T cells is shown to accelerate disease[48,49] and, second, amelioration of disease can be achieved by the induction of T cell tolerance to Id-peptides[47,50,51]. This was done by weekly intravenous injections of Id-peptides in the absence of adjuvant and was associated with induction of an anti-inflammatory phenotype of responding T cells displaying a decrease in interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α production and an increase in the production of transforming growth factor (TGF)-β[51-53]. It has been demonstrated that T cell responses to Id in SLE depend on somatic hypermutation in the V region of antibodies as non-mutated V regions failed to activate T cells and non-mutated antibodies were not immunogenic in immunization experiments[54,55].

1.2.3 Presentation of Id to Id-specific T cells

As mentioned above, Ids may be presented to Id-specific T cells by APCs such as DCs. The immunogenicity of Id is particularly interesting considering the fact that B cells constitutively present endogenous Ig-derived peptides in combination with MHC molecules on the cell surface[56-58]. In B myeloma cells, this was shown to be independent of Ig secretion or expression of membrane bound Ig, suggesting processing of newly synthesized Igs in the endoplasmatic reticulum (ER)[59]. Presentation of Id may in the majority of cases induce tolerance in responding T cells[33,60,61]. However, B cells also present rare Id-peptides, which can be recognized by CD4+ T cells on MHC class II molecules[61,62] and by CD8+ T cells on MHC class I molecules[63,64]. This may lead to activation of the responding Id-specific T cells[59,65]. Moreover, the reciprocal Id-driven collaboration between T and B cells may sustain the activation, the proliferation and the differentiation of Id-bearing B cells[66,67]. These studies have shown that B cells have the ability to directly stimulate T cells in the absence of conventional antigen, contradicting

the generally accepted belief that the collaborating T cell and B cell must recognize epitopes on the same molecular complex. This concept has been termed non-linked recognition[66]. Thus, there are two ways by which an Id-specific T cell may receive cognate activation: i) An APC may phagocytose and process soluble Id-bearing Ig and present Id-peptides to an Id-specific T cell, and ii) an Id-bearing B cell may present endogenous Ids from its own receptor to Id-specific T cells (**Figure 1**).
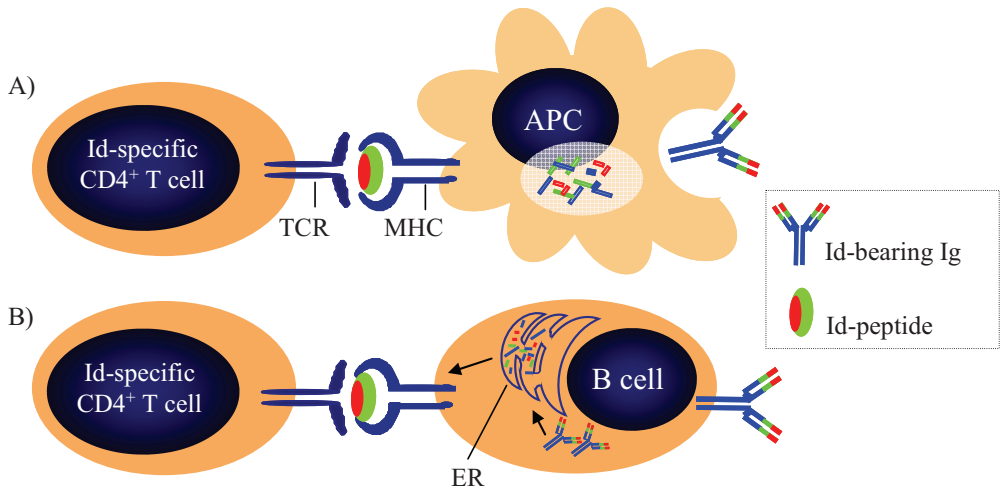
Figure 1. *Presentation of Id to Id-specific T cells.*

A) Soluble Id-bearing Ig may be taken up by an APC, which processes and presents Id-peptides to an Id-specific T cell. B) An Id-bearing B cell may process and present Id-peptides from endogenous Ig molecules in combination with MHC class II to an Id-specific CD4+ T cell.

Transgenic mice expressing λ2 positive B cells (Id+) or the corresponding TCR have facilitated the study of Id-driven T-B cell collaboration *in vivo*[66]. Id-specific T cells injected into Id+ mice were shown to stimulate B cell activation and generation of germinal centers, plasma cells and production of autoantibodies[66]. By repetitive injection of Th2 cells it was shown that Id+ mice developed lymphomas after a latency period of more than 5 months[68]. This demonstrated that Id-specific T cells not only could eliminate Id+ B cells, but also provide help and sustain tumour growth. The generation of Id-matched double transgenic mice allowed the study of Id-driven T-B cell collaboration from early ontogeny[69]. These mice were shown to develop systemic

autoimmunity with manifestations in skin, bowel and joints in addition to inflamed tissue containing foci of cells resulting from Id-driven T-B cell collaboration. Furthermore, T cells and B cells from the spleen of double transgenic mice were shown to transfer disease to mice deprived of immune cells.

1.2.4 Id responses in human disease

The contribution of Id-specific responses in the development of human disease is less clear. Id-specific T cells have been reported in SLE, rheumatoid arthritis and MS. A human anti-single stranded DNA antibody carrying the Id 16/6 Id can induce SLE in mice[70]. This Id was found to be present in anti-DNA autoantibodies of 50-60 % of patients with active SLE compared to 4 % in healthy controls[44,45,70-72]. When assessing proliferative responses in blood T cells to the same Id or peptides corresponding to the CDRs of the 16/6 Id, a significantly weaker response was detected in patients compared to healthy controls[45,70,72]. The proliferative capacity of Id-specific T cells declined along with exacerbations of the disease. Accordingly, CDR peptides specifically inhibited 16/6 induced proliferation and IL-2 production. The latter correlated with increased expression of the regulatory cytokine TGF-β, suggesting the involvement of regulatory T cells in the anti-Id response in SLE patients[73].

Joint-derived polyclonal T cells from rheumatoid arthritis patients have been found to proliferate in response to autologous IgG H chains[74,75]. The response was shown to be human leukocyte antigen (HLA) restricted, but since T cells also proliferated in response to plasma IgG, the specificity of the response was uncertain.

As will be discussed thoroughly in this thesis, Id-specific responses including Id-driven T-B cell collaboration may contribute to the chronic inflammation in MS.

1.3 Autoimmunity; protective and pathogenic aspects

Autoimmunity can be defined as an adaptive immune response directed against the body's own tissues. At the beginning of the twentieth century, the German physician Paul Ehrlich coined the term "horror autotoxicus" arguing that the normal body would never mount an immune response against its own tissue. According to this view, any autoimmune reaction was destructive and connected to human disease. We now know

that the causal relationship between autoimmune reactions and autoimmune diseases is more complex.

It is estimated that about 20-50 % of the TCRs and BCRs created during VDJ recombination are self-reactive[76,77]. Although most T and B cells carrying such self-reactive receptors are deleted during maturation[78,79], a high frequency of autoreactive T cells, B cells and autoantibodies is present in the normal repertoire without causing disease[77,80]. Indeed, it is proposed that recognition of self is essential for survival of naïve lymphocytes and that it can enhance reactivity to foreign antigen[81], that it may regulate the extent and duration of immune responses[24], and that autoantibodies can contribute to the clearance of damaged tissue[82]. Furthermore, recognition of self-proteins in the absence of costimulation is important for the maintenance of immunological tolerance[83-85].

1.3.1 Protective autoimmunity in the central nervous system

Studies in rodents have shown that the same myelin basic protein (MBP) specific T cells that cause EAE in mice also can have a protective function by reducing secondary degeneration of neurons after primary injury to the optic nerve or spinal cord[86,87]. The effect was shown to be specific for T cells reactive with central nervous system (CNS) antigens, as T cells with other specificities did not confer protection even though they were shown to home to the injury site[86]. A follow-up study demonstrated that suppression of the autoimmune reaction by tolerance induction to MBP or by the injection of CD4$^+$CD25$^+$ regulatory T cells reduced the protective effect of MBP specific T cells[88]. Thus, a degree of autoimmunity was required to confer protection. These observations have implicated T cells as important mediators of CNS neurogenesis, the process by which neuronal precursor cells give rise to new neurons in certain areas of the brain[89,90]. Accordingly, it was shown that T cell deficient mice displayed decreased neurogenesis from endogenous precursor cells compared to normal mice, and that this could be partly restored by reconstitution of the T cell pool[91].

The protective effect of CNS reactive T cells most likely involves activation of local APCs present at the injury site[92,93]. It was demonstrated that activation of microglia by either the Th1 cytokine IFN-γ or the Th2 cytokine IL-4 induced neuronal and oligodendroglial differentiation from adult neuronal precursor cells[94]. It was accordingly

hypothesized that the release of cytokines may serve a function in the recruitment of neuronal precursor cells to the injury site. In support of this, neuronal precursor cells layered onto hippocampal slice cultures were shown to migrate towards sites treated with inflammatory stimuli, such as TNF-α or IFN-γ[95]. This was dependent upon cytokine-induced upregulation of chemo-attractants. Differentiation of progenitor cells arriving at the injury site may in part be mediated by growth factors. Activated microglia and macrophages have been shown to secrete a variety of growth factors *in vivo*[96], and may reciprocally induce the production of growth factors in other cells[91]. Furthermore, activated human T cells, B cells and monocytes in inflammatory MS lesions as well as myelin oligodendrocyte glycoprotein (MOG) reactive T cells from healthy individuals express nerve growth factors[97,98].

1.3.2 Autoimmune diseases

The relation between benign autoimmunity and the progression and establishment of an autoimmune disease is unclear. An autoimmune disease can be organ specific, such as in type 1 diabetes and MS or systemic such as in SLE and Sjogren's syndrome and is characterized by a chronic adaptive immune response directed against self-tissue. About 3-5 % of the world's population is affected by an autoimmune disease[99] with women accounting for 78 % of cases[100]. It is believed that a combination of risk-associated polymorphisms in immunoregulatory genes, infectious agents and other environmental triggers contribute to the initiation and propagation of disease[101,102]. Thus, a few examples of human autoimmune diseases induced by defined microbes exist, such as rheumatic fever[103] and Guillain-Barré syndrome[104]. These are thought to arise from antigenic mimicry between the infectious agent and human tissue triggered during the initial inflammatory response. For the majority of human autoimmune diseases, however, the link to infection remains circumstantial. This may be due to the time lag between infection and establishment of disease and the potential contribution of subclinical infections.

1.4 Immune surveillance of the central nervous system

The CNS is comprised of the brain and spinal cord, surrounded by three layers of meningeal membranes[105]. The blood brain barrier (BBB) is a feature of the cerebral vasculature, which restricts access of ions and other solutes present in the blood into the brain parenchyma. The anatomical structure of the BBB comprises two cell layers, which are separated by the perivascular space. One is formed by endothelial cells lining the brain capillaries and an underlying basement membrane, and the other is formed by astrocytic foot processes and their parenchymal basement membrane. Unlike in other tissues, the endothelial cells of the BBB display no fenestration and are connected by tight junctions, which efficiently restrict the traffic of molecules and cells in and out of the brain. The cerebrospinal fluid (CSF) baths the brain and is produced from arterial blood by the choroid plexus[106]. It flows from the ventricles of the brain into the subarachnoid space located between the arachnoid and the pial membrane and is eventually absorbed into the venous circulation[107]. The CSF communicates with the interstitial fluid of the brain through the perivascular spaces. Due to the lack of tight junctions in the ependymal linings of the ventricles, small hydrophilic molecules as well as proteins diffuse freely between the CSF and brain interstitium[108].

Under physiological conditions, immune cells enter the CNS at a very low level for the purpose of immune surveillance[109-111]. In contrast, during inflammatory diseases such as MS, activated cells readily traverse the inflamed BBB[110]. CNS fluids continuously drain into cervical lymph nodes, ensuring communication with the peripheral lymphoid system[112], but the absence of secondary lymphatic structure, the low expression of MHC class II molecules and the lack of DCs in the CNS have questioned how immune surveillance of the brain takes place under physiologic conditions[113,114].

Perivascular cells within the subarachnoid space probably play a key role in immune surveillance. It is suggested that activated memory cells enter the CSF from the systemic circulation and monitor the subarachnoid space under physiologic conditions[105,111]. This is strongly supported by observations in EAE where parenchymal inflammation and disease onset is preceded by inflammation and accumulation of Th17 polarized CD4$^+$ T cells in the subarachnoid space[115-117]. Furthermore, it was recently demonstrated that Th17 cells expressing the chemokine receptor CCR6 were allowed

access into the perivascular space through the choroid plexus by interaction with the CCR6 ligand, CCL20[118]. This step triggered and was indispensable for a second wave of inflammation mediated by T cell infiltration through the BBB. CCR6 was found to be constitutively expressed by cells of the choroid plexus also in humans, and the entry of Th17 cells into the CSF was suggested to control immune surveillance of the CNS during physiologic condition.

1.4.1 Migration of cells across the blood brain barrier

The migration of mononuclear cells across an inflamed BBB is a two-step process, which first requires entry across the endothelial cell layer and its basement membrane into the perivascular space. Several adhesion molecules, including activated leukocyte cell adhesion molecule, ICAM, VCAM-1, α4-integrin and laminins seem to be selectively involved in the adhesion and transmigration of T cells[119-123]. Adhesion molecules are believed to aggregate in microdomains on the endothelium, so-called transmigratory cups, which guide the migration of lymphocytes across inflamed cerebral vessels. The monoclonal antibody (mAb) natalizumab, used in the treatment of MS, efficiently inhibits the infiltration of lymphocytes into the brain by blocking the VCAM-1-ligand, α4β1-integrin[124]. Furthermore, T cell transmigration could be selectively inhibited by laminin-α5, an adhesion molecule expressed on the endothelial basement membrane[125].

To reach the brain parenchyma from the perivascular space leukocytes must traverse the parenchymal basement membrane and the glia limitans, a thick layer of astrocytic processes that seals the entire surface of the CNS. The molecular mechanisms facilitating this step are less defined, but thought to rely on the secretion of matrix metalloproteinases (MMPs) by perivascular macrophages or DCs. Hence, mice were made resistant to EAE by deletion of MMP-2 and MMP-9, and T cells were trapped in the perivascular space[126]. Thus, cells that gain access to the perivascular space through interaction with inflamed brain endothelium may only traverse the glia limitans into the parenchyma if they recognize their cognate antigen presented by perivascular APCs[127,128]. Also the strength of lymphocyte reactivation in the perivascular space may determine migration into the brain parenchyma[129]. MHC expression by endothelial cells may also play a role in the recruitment of antigen specific T cells as was recently

demonstrated for the migration of CD8$^+$ T cells across the BBB[130]. Finally, T cells that infiltrate the brain parenchyma may interact with resident microglia, which in response to CNS inflammation acquire a macrophage-like phenotype with increased expression of costimulatory and adhesion molecules[131].

1.5 Multiple Sclerosis

MS was first described in 1868 by Jean-Martin Charcot[132], but early reports of people suffering distinct neurological symptoms analogous to MS date back to the middle ages[133,134]. Today MS has a prevalence that generally ranges from 2-150 per 100,000, although this can be significantly higher in certain regions[135]. The etiology of MS is complex and involves genetic and environmental factors[136-138].

1.5.1 Clinic

MS usually presents with a clinically isolated syndrome, a neurological episode suggestive of inflammation and demyelination, but not sufficient by itself to qualify for an MS diagnosis[139]. In the majority of patients, disease typically evolves with irregular relapses followed by more or less complete remission. The use of magnetic resonance imaging allows visualization of affected CNS sites. Radiological evidence of demyelination episodes disseminated in time and space is part of the revised diagnostic criteria for MS[140]. At the onset of disease, the majority of patients follow a relapsing-remitting (RR) course, whereas in about 15 % of patients, disease progresses without intermittent relapses in what is referred to as primary progressive MS[141]. Progression of disease in most RRMS patients will over time also be devoid of remissions and evolve into a secondary progressive course[142].

1.5.2 Etiology

Clustering of MS cases within families and the sharp decline in concordance with increasing genetic distance demonstrate the genetic contribution to MS[136]. The strongest genetic association to MS is found within the HLA complex. The *HLA-DRB1*1501* allele is thought to confer the primary association in Caucasians and Afro-Americans[143,144]. Lately, genome wide association studies have pointed out an

association between MS and immunoregulatory genes encoding IL-2 and IL-7 receptor α chains, which are associated with activation and homeostasis of T cells[145,146].

Several observations demonstrate the environmental contribution to MS risk: i) the relative low concordance rate in monozygotic twins, ii) the influence on MS risk by migration to areas of low or high MS prevalence, and iii) the increase in female: male sex ratio observed over time[137,138]. Both infectious and non-infectious factors, such as vitamin D and smoking, have been implicated by epidemiological evidence. It seems that environmental factors in childhood contribute to MS risk and a study on adoptees have demonstrated that environmental factors in MS operate on a population basis and not in the microenvironment[147].

Of many possible infectious agents suggested to confer MS risk, Epstein-Barr virus (EBV) is supported by the strongest epidemiological evidence. MS risk is higher in individuals with a past history of infectious mononucleosis, and a temporal increase in serum titres of antibodies to EBV has been shown to correlate with the onset of MS later in life[148]. The functional relevance of EBV in MS is supported by a higher frequency of EBV specific T cell in MS patients[149] and by the demonstration of MBP specific T cells that cross react with EBV-proteins[150,151]. Furthermore, strong CD8$^+$ T cell responses to EBV can be detected in cases of early MS[152]. After the identification of EBV-infected B cells in white matter lesion in MS[153], the importance of EBV and EBV specific T cells in MS have gained new ground. However, this observation, which may prove seminal in terms of understanding MS pathogenesis, awaits confirmation, and the specificity of CD8$^+$ T cells found in the vicinity of EBV-infected B cells has not been identified.

1.5.3 Pathogenesis

Lesson from EAE have guided much of the research in MS and formed the long-held view that myelin specific CD4$^+$ cells play a key role in MS. EAE is caused by a direct attack on myelin proteins mediated by myelin specific CD4$^+$ T cells[154]. EAE can be induced in susceptible strains of rodents and nonhuman primates through immunization with myelin proteins or peptides emulsified in complete Freund's adjuvant. Alternatively, adoptive transfer of activated myelin specific T cells can induce EAE. Although the EAE model may reflect important pathogenic mechanisms in MS, observations such as a dominance of clonally expanded CD8$^+$ T cells in active MS

lesions, perpetual intrathecal production of oligoclonal IgG fractions in MS patients, and the failure to firmly establish myelin proteins as target antigens in MS underscore critical differences in the pathogenesis of human MS and animal EAE[155].

The clinical benefit of blocking migration of lymphocytes into the CNS or of strong immunosuppression with mAbs has for the first time provided conclusive evidence for the detrimental effect of the immune response in MS[156,157]. However, whether the immune response in MS is primarily autoimmune or secondary to neurodegeneration elicited by other factors is still not settled. According to the autoimmune hypothesis of MS, it is assumed that effector CD4$^+$ and CD8$^+$ T cells are primed in secondary lymphoid organs through antigen specific T cell-APC interactions[158]. In view of recent findings, it may be proposed that reactivation of antigen specific Th17 cells in the perivascular space plays a crucial role for the transmigration of other T cell subsets[117,118]. The upregulation of adhesion molecules and corresponding ligands by brain epithelium and activated lymphocytes allows the migration of activated T cells and B cells across the BBB. It is believed that only those T cells that recognize cognate antigen will be retained in the brain[110]. Reactivation may occur by cross recognition of myelin or neuronal antigens, by recognition of an original infectious agent[153] or of other antigens present in the brain, such as Ids[159]. Reactivation of T cells triggers parenchymal inflammation, which recruits T cells, B cells, DCs and microglia to the site of inflammation. The release of pro-inflammatory cytokines, direct damage mediated by MHC class I restricted CD8$^+$ T cells and indirect damage by MHC class II restricted CD4$^+$ T cells, complement deposition and local activation of microglia and macrophages[160] may all have a role in the inflammatory response. The reason why the immune response becomes chronic remains unknown, but could be explained by site-specific expression of autoantigens, persistence of latent infections, a permissive CNS environment or a combination of all.

1.5.4 The intrathecal immune response in MS

A hallmark of the immune response in MS is the formation of isolated areas of inflammation called lesions or plaques. Lesions can appear throughout the brain, both in the white and in the grey matter and are often found around the ventricles, in the optic nerve, in the brain stem and in the spinal cord[161]. Within lesions the most characteristic

pathological feature is demyelination. Axonal damage is probably present from early in the disease process and numerous transected axons can be visualized in active lesions[162]. Mononuclear infiltrates of CD4^{+} T and CD8^{+} T cells, B cells and macrophages are present to various extents and are thought to be critical for disease development and progression. Much effort has been devoted to analyzing the phenotype of T and B lymphocytes dominating in the intrathecal compartment as a means to identify potential antigens and to understand the underlying disease process.

1.5.4.1 T cells

Within the active lesion clonally expanded CD8^{+} T cells outnumber CD4^{+} T cells, which are more polyclonal and often found in the periphery of the lesion[163,164]. By analyzing 22 tissue blocks from patients and healthy controls, the majority of T cells in active MS lesions were found to express IL-17[165]. Unlike what is described for EAE, both CD4^{+} and CD8^{+} T cells from MS lesions stained positive for IL-17. Their expression of cytolytic granules and their ability to kill neurons *in vitro*[166] have pointed out Th17 cells as a critical subset of T cell in MS[167].

T cells within MS lesions display a restricted receptor repertoire[168,169], suggesting that a limited number of clones participate in local immune reactions. Furthermore, central memory T cells within MS lesions have been shown to lack CCR7[170], indicating that they had differentiated into effector T cells upon restimulation with antigen. However, the antigen-specificity of T cells in MS remains unclear. In light of EAE, the search for an MS target antigen has revolved around myelin peptides, but the role of myelin specific T cells in MS is uncertain. The frequency of MBP specific T cells is overlapping in MS patients and controls, although T cells from MS patients seem to display a higher frequency of activation markers and tend to belong to the memory pool of T cells[171]. A phase II clinical trial where MS patients were immunized with an altered peptide ligand based on an immunodominant MBP epitope, resulted in clinical exacerbations associated with an increase in the frequency of T cells specific for the MBP epitope[172]. This suggests that MBP specific T cells may cause encephalomyelitis also in humans, but does not pinpoint MBP specific T cells as culprits in MS. In a young patient suffering from hyperacute MS, T cell infiltrates were shown to display reactivity to myelin proteins[173]. However, earlier attempts to chart the specificity of T cell clones isolated from MS lesions have not detected reactivity to MBP or proteolipid

protein (PLP)[174]. The pathogenic role of myelin specific T cells is further complicated by the therapeutic potential these cells may have[175].

The search for alternative antigens in MS has suggested the stress protein αB-crystallin as a potential target. αB-crystallin is a small heat shock protein and one of the most abundantly expressed proteins that is found in active MS lesions, but not in normal brain[176,177]. In comparison to a variety of other myelin proteins, which included MBP, PLP and MOG, αB-crystallin elicited strong proliferative responses in peripheral blood lymphocytes from MS patients. Thus, other potential T cell targets in MS may be proteins expressed uniquely in MS brains and not in normal brain[178].

1.5.4.2 B cells

Several observations demonstrate that B cells are involved in the disease process of MS. B cells as well as deposits of Ig and complement are found within MS lesions[179] and more than 95 % of MS patients display a perpetual intrathecal synthesis of oligoclonal Ig, which can be visualized as distinct oligoclonal bands (OCBs) by isoelectric focusing or agarose gel electrophoresis. The OCBs of MS patients are predominantly IgG1. OCBs can also be observed in infectious diseases of the CNS where the antigenic target is the infectious etiologic agent[180,181].

Analyses of transcribed BCR V genes from CSF and MS lesions have revealed a population of B cells that is clonally expanded, displays a limited H chain repertoire and that contains numerous replacement mutations[182-187]. These observations strongly suggest that B cells in MS are the result of an antigen-driven T cell dependent process. Prominent clonal expansion of CSF B cells is an early feature of MS, suggesting that antigen-specific B cell responses may be implicated at the onset of disease[188,189]. Short-lived plasma blasts are probably the major antibody-secreting cell in the CSF and described as the main effector population[190,191]. A comparison of the Ig transcriptome of B cells with the corresponding Ig proteome in the CSF of four MS patients established that CSF B cells were at least one possible source of the OCBs[186]. However, OCB formation in CSF may result from B cell activation within the CNS parenchyma and whether B cells present in the CSF correlate with CNS B cells is not clear. Efforts to identify the specificity of the main oligoclonal IgG in MS remains a challenge[192].

Antibodies with reactivity against myelin proteins can readily be detected in the CSF of MS patients[193,194]. IgG from CNS tissue was found to contain anti-MOG antibodies in seven of 14 MS patients[195], and although debated, serum antibodies specific for MBP and MOG have been suggested as an early diagnostic marker for MS[196,197]. Also, it was recently demonstrated that nine out of 10 antigen binding fragments from clonally expanded CSF B cells from four MS patients recognized MBP[198]. However, the pathogenic relevance of myelin specific antibodies in MS remains uncertain.

A curious feature of MS is the perpetual intrathecal production of virus specific antibodies[199,200]. These antibodies are typically directed towards measles, varicella zoster, rota and mumps viruses[199,201,202]. They mainly display IgG1 subclass restriction[203] and are also present in vaccinated individuals[204]. Virus specific antibodies display an oligoclonal pattern, but are not part of the main OCBs and constitute only a small fraction of intrathecally synthesized IgG[181,199]. Moreover, clonally expanded CSF B cells cultured *in vitro* were shown to display specificity for the same viruses as that described for intrathecally produced antibodies[205]. The fact that some of these antibodies are directed against RNA-viruses that most probably do not persist in the CNS, suggests that they are not a result of an ongoing virus specific immune response.

Whether the intrathecal humoral immune response in MS is pathogenic or represents an epiphenomenon has been unclear. However, there are several indications that B cells in MS may play a role in the disease process beyond their capacity to produce antibodies. Ectopic lymphoid follicles enriched with B cells and plasma cells have been observed in the meninges of patients with secondary progressive MS[206], compatible with an ongoing B cell differentiation at least in late stages of the disease. The formation of local germinal center-like structures has also been described in other autoimmune conditions[207] and may be a common feature of chronic inflammatory responses. In the case of MS, this may suggest that maturation of B cells takes place in the intrathecal compartment. This view is supported by the presence of centroblasts, a B cell population typical of secondary lymphoid organs, in the CSF[208]. In addition, antigen dependent short-lived plasma blasts are common in the CSF[191]. These observations are intriguing in light of the therapeutic potential of rituximab[209]. Rituximab targets CD20, which is carried by B cells at all stages of B cell differentiation, except for pro-B cells and plasma cells. A near complete deletion of CD20 expressing B cells in the CSF and

blood was observed in treated patients, who concomitantly experienced a marked reduction in clinical attacks and a decrease in the number of lesions. Moreover, following 24 weeks of rituximab treatment the number of CD3^{+} T cells in the CSF was significantly reduced in the majority of patients[210]. However, the IgG concentration in CSF, the IgG index, the IgG synthesis rate and the number of OCBs were not affected[210]. This may suggest that the therapeutic effect of rituximab is independent of antibody production and that other B cell effector functions are involved, such as bystander activation through cytokine secretion or the ability to present antigen to T cells[171].

1.6 Glatiramer acetate in the treatment of MS

Currently seven drugs are officially approved for the treatment of MS, while many new therapies are emerging. For the most part these include agents that either interfere with lymphocyte migration, activation or proliferation, or that modulate neurotransmission[211]. Included are also antigen-specific therapies, which may be promising candidates considering the often-observed limited effect and potential serious adverse effects of currently approved and upcoming drugs. Glatiramer acetate (GA) is one of the first-line drugs for the treatment of RRMS and its immunomodulatory potential has been a topic in this thesis.

GA is a synthetic copolymer comprised of the four amino acids most frequent in MBP; glutamine, alanine, lysine and tyrosine. The idea was that GA would be sufficiently identical to MBP in terms of sequence and antigenicity that it could be used as a substitute for the induction of EAE. Administration of GA unexpectedly showed the opposite effect; mice were protected against the development of clinical disease[212]. A phase III, multicenter, double blind, placebo-controlled trial, which included 251 RRMS patients, demonstrated a 29 % reduction in relapse rate compared to placebo in patients who received GA for two years[213,214]. This result was the main basis for the subsequent approval by the US federal drug administration of GA for the treatment of MS.

1.6.1 Effects on the immune system

GA binds with high affinity to HLA class II molecules of the DR isotype, and can do so without prior processing[215,216]. GA seems to have a number of effects on APC function, such as a reduction in the secretion of pro-inflammatory cytokines and an increase in the secretion of anti-inflammatory cytokines[217-219]. This may in turn induce an anti-inflammatory phenotype in GA reactive T cells[220,221]. A GA-induced shift in the cytokine profile of GA reactive T cells towards an anti-inflammatory phenotype has been demonstrated in patients by comparing the cytokine profile of GA reactive blood T cell lines before and after treatment[222,223]. Additionally, GA may lead to anergy-induction of pathogenic lymphocytes[224,225], and restoration of function and frequency of regulatory T cells[226-228].

1.6.2 Cross reactivity

Due to similarities in amino acid composition to MBP recognition of MBP has been thought to account for the reactivation of GA reactive T cells in the CNS[229]. This has been based upon the observations that GA reactive T cells accumulated in the CNS of GA-treated mice[230,231] in combination with studies demonstrating cross reactivity between MBP and GA as assessed by cytokine secretion[229,231,232]. In mice, GA reactive T cells were shown to react to MBP by secretion of IL-4, IL-6 and IL-10[229] and to target the immunodominant epitope 82-100 of MBP by TCR antagonism[233]. The secretion of anti-inflammatory cytokines is thought to mediate bystander suppression of nearby pathogenic T cells within the CNS[231]. However, GA is not only efficient in MBP-induced EAE, but also in PLP and MOG-induced disease[234,235], suggesting either that cross reactivity with MBP is not essential for the therapeutic effect or that cross reactivity is a more general phenomenon.

Studies using human T cell lines have either failed to detect cross reactivity between GA and myelin proteins[236,237] or reported it as a low frequency or unspecific event[222,223,238,239]. GA reactive T cell lines from six of seven patients displayed cross reactivity to random combinatorial peptide libraries, including peptides from MBP. Cross reactivity was determined by cytokine secretion and was consistent with a degenerative response as no dominantly cross reactive peptide emerged[222]. Two other studies reported cross reactivity to MBP by cytokine secretion in 10-25 % of GA

reactive cell lines[223,239]. Cross recognition assessed by proliferation was observed for three of 18 GA reactive cell lines against MBP in a patient treated with GA for 6 years[238]. Together these observations suggest that cross reactivity may occur, but that other mechanisms probably contribute to the efficacy of GA reactive T cell inside the CNS.

1.6.3 Neurotrophic effect

The therapeutic effect of GA is postulated to involve neuroprotection[240]. GA reactive T cell lines from MS patients and healthy controls have been shown to display low basal secretion of brain-derived neurotrophic factor (BDNF), which increased upon stimulation with GA[241,242]. In mice, *in situ* secretion of BDNF by GA reactive T cells correlated with reduced neuronal damage as well as increased neuronal proliferation[243], and GA treatment was recently shown to induce remyelination in EAE[244]. Secretion of BDNF is not restricted to GA reactive T cells, but seems to be a more general feature of activated cells. T cells, B cells and monocytes have all been demonstrated to secrete BDNF *in vitro* and in inflammatory brain lesions[97]. This may suggest that the daily injection of GA promotes BDNF secretion as a result of a continuous activation of GA reactive peripheral T cells, which subsequently gain access to the CNS[245].

Taken together, studies in mice suggest that GA has the ability to support growth of nervous tissue[240,243,244]. To which extent this applies also in humans is less clear and needs further attention using human cells and tissue. However, the observed long-term clinical efficacy of GA may support both an anti-inflammatory and a neurotrophic effect of GA[246].

2. AIMS OF THE STUDY

Although the intrathecal immune response in MS is predominantly detrimental to the CNS, there seems to be protective elements as well. In contrast to animal studies where the effect of T cell responses against specific antigens can be studied directly, the pathogenic significance of any particular T cell response in humans is usually unknown. Thus, T cell responses to Ids could be either irrelevant or pathogenic, or they could be part of a regulatory and anti-inflammatory network. GA provides an exceptional opportunity to study a T cell response with a proven beneficial clinical effect. The overall aim of this thesis has been to explore the detrimental and beneficial effects of intrathecal T cell responses, by using GA and Id as model antigens. The specific aims of the thesis were:

- To establish whether GA reactive T cells gain access to the intrathecal compartment of MS patients, and if so, then characterize their cytokine profile

- To characterize the T cell epitopes on autologous CSF IgG molecules

- To identify the target antigens of CSF B cells potentially involved in Id-driven T-B cell collaboration

- To develop an *in vitro* model to study the pathogenic potential of Id-specific T cells

3. METHODOLOGICAL CONSIDERATIONS

3.1 The generation of T cell lines and clones

T cell lines and clones employed in this thesis have been generated from the CSF and blood of MS patients. Patients involved in this thesis are described in Table 1.

Patient	Age at study onset	Diagnosis at study onset	Paper
MS 1	38	SPMS	II, III and IV
MS 2	39	RRMS	II and III
Patient 1	20	RRMS	I
Patient 2	32	RRMS	I
Patient 3	29	RRMS	I
Patient 4	32	RRMS	I and IV
Patient 5	38	RRMS	I

Table 1. *Patients involved in the study.* SP = secondary progressive

For the generation of CSF T cells approximately 20-25 ml of CSF was obtained from patients by lumbar puncture. The first two ml of CSF was always discarded because even minute amounts of blood would render the CSF sample unrepresentative. Due to the low number of cells present in the CSF, T cells were expanded to allow subsequent study. T cells from blood and CSF were cultured in parallel following identical procedures to minimize the differences in the cell populations as a result of *in vitro* manipulations.

For the first two weeks of culture T cells were expanded in the presence of autologous peripheral blood mononuclear cells (PBMCs), which had been preincubated with antigen overnight. Previous experiments have shown that the presence of antigen in the absence of mitogens in the culture medium is important for the initial propagation of antigen specific T cells from the CSF[151]. To avoid propagation of allospecific T cells, auotologus serum was used for the first two weeks of culture. Since activated T cells express high affinity IL-2 receptors, T cells were further expanded with the addition of IL-2. This favours expansion of T cells with strong proliferative capacities that have been triggered by antigen *in vitro*. Further expansion of antigen-specific T cells was

done in the presence of the T cell mitogen phytohemagglutinin (PHA), IL-2 and heterologous feeder cells. Antigen responsive T cell lines were cloned by limiting dilution by seeding 0.1-1 cell per well. The cloning frequency was 3-27 %, which makes it more than 95 % likely that each cell culture is monoclonal[247]. As IL-15 has been shown to upregulate anti-apoptotic proteins in activated T cells[248] it was included in the culture to increase survival of resting cells.

An important consideration when comparing T cells from CSF and blood is that *in vitro* expansion may differently affect the two cell populations due to heterogeneity in their putative receptors and activation status. Antigen priming in combination with cytokine stimulation may expand both naïve and memory T cells. This is most relevant when expanding cells from blood as a great proportion of these are of a naïve phenotype. In contrast, the majority of T cells present in CSF are of an activated memory phenotype[249-251]. However, it may be argued that the *in vitro* conditions employed preferentially expand T cells of a memory phenotype from both compartments as these will respond faster to antigen stimulation and therefore dominate the cell culture.

Also, *in vitro* expansion may preferentially expand different phenotypes or affect the cytokine profile of expanding cells. We were, however, able to expand both Th1 and Th2 polarized GA reactive T cell lines from both blood and CSF (Paper I), indicating that the *in vitro* procedure is well suited for expansion of several phenotypes of CD4$^+$ T cells.

Because CD4$^+$ T cells have been the focus in this study, *in vitro* cell culture conditions that preferentially expand this subset have been employed throughout the study. Considering the high number of clonally expanded CD8$^+$ T cells both in CSF and CNS of MS patients[163,252], this would clearly be an interesting subset to expand and investigate. However, studies on CD8$^+$ T cell in CSF mostly rely on direct analysis and the expansion of antigen-specific CD8$^+$ T cells from the CSF is challenging and not well described.

3.1.1 Characterization of T cell epitopes on CSF mAbs

The CSF mAbs were generated from EBV-transformed B cell clones isolated from the CSF of patient MS1 and MS2. The purification procedure and testing for potential contaminants are outlined in paper II, but will not be discussed here, as this was not part of the work performed in this thesis.

The nucleotide sequences of the VL and VH regions of the two CSF mAbs were obtained from polymerase chain reaction (PCR) amplified complimentary DNA of the EBV-transformed B cells. By comparing the CSF mAb sequences to the closest germline encoded sequences, the frequency and distribution of amino acid replacements were identified. To chart T cell epitopes of CSF mAb specific T cell clones, a panel of peptides (>70 % pure) covering mutated sequences and the highly variable CDR3s of the VL and VH chains of the CSF mAbs was synthesized. Lyophilized peptides were stored at -70° C and dissolved in dimethyl sulfoxide (DMSO) before they were added to the culture medium. The final concentration of DMSO in the cell culture medium was always far below toxic concentrations.

3.1.2 Testing of cross reactivity of GA specific T cell clones.

The ability of GA reactive T cells to cross react with myelin proteins is thought to account for the reactivation of GA reactive T cell in the CNS[230]. We tested 20 GA specific T cells clones against recombinant human MBP and a panel of 531 15-mer overlapping peptides spanning the complete amino acid sequences of MBP, PLP, MOG, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMGP), αβ-cystallin, s100β and 2'-3'cyclic nucleotide 3'-phosphodiesterase (CNPase). Potential cross reactivity was assessed by proliferation and by cytokine secretion. The peptides were synthesized at a purity of 44-100 %. Although only 0.5 % of the peptides had a purity of less than 50 %, it may be criticized that the peptide fractions could contain contaminants. However, as no cross reactivity was detected the problem of false positives was not relevant. T cell clones were tested against pools of 8-10 peptides at a concentration of 2.5 μM. Higher concentrations were not used due to the potential toxic effect of the dissolvent, DMSO. Bearing in mind the low dose of GA needed to stimulate GA specific T cell clones (responded to 160 ng/ml of GA), and the

significantly lower concentration of individual stimulating peptides in the GA mixture, 2.5 μM was considered a sufficient dose to identify cross reactivity. A shortcoming of this assay is that myelin specific T cell clones were not included as positive controls. The same peptide panel was, however, used in a previous study and shown to elicit proliferative responses of CD4$^+$ T cells from MS patients[253].

3.2 Testing of T cell responses

3.2.1 T cell proliferation assays

T cell proliferation was assessed by [^{3}H] thymidine incorporation, which was chosen for its robustness and ease by which large numbers of wells can be assayed simultaneously. T cells were stimulated to proliferate by receptor engagement, either by specific recognition of antigen in the presence of APCs or by addition of monoclonal anti-CD3 and anti-CD28 antibodies. This assay was ideal for screening and monitoring the large number of cell lines and clones established from each patient. A weakness of assaying initial cell cultures with proliferation alone is that cells with a non-proliferative phenotype may have been missed.

3.2.2 Detection of cytokine production

In addition to [^{3}H] thymidine incorporation several of the T cell lines and clones were analyzed for the secretion of cytokines, which correlated well with proliferative responses.

Cytokine production can be detected at the mRNA level with PCR or with *in situ* hybridization techniques. Intracellular cytokines can be detected by flow cytometry, whereas the detection of secreted cytokines often relies on enzyme-linked immunosorbent assay (ELISA)-based protocols[254]. In our studies we employed a bead-based multiplex system carried out on the Luminex 100 technology platform, which measured the presence of cytokines in supernatants of cells. T cells were stimulated or not and supernatants were harvested after 48 hours and kept frozen at -70° C prior to analysis. Several populations of beads in suspension were each covalently linked to a unique antibody and could be separated from each other based on internal fluorescent

dyes. The beads could be mixed in the same assay allowing simultaneous detection of multiple cytokines. This system was chosen due to the relative ease by which a large number of samples could be analysed simultaneously for the presence of multiple cytokines. Detection of intracellular cytokines is superior in terms of identifying individual cytokine-producing cells, but does not provide a quantitative measure of actual cytokine secretion, which was most relevant in our study. Comparison of assays has shown high correlation between the detection of intracellular and secreted cytokines[255].

3.3 Human oligodendrocyte cell lines

Two human oligodendrocyte cell lines, HOG and Human Oligos, were used to study the pathogenic potential of T cells. The HOG cell line is derived from an oligodendroglioma and expresses markers characteristic of immature oligodendrocytes, such as galactosyl cerebroside and CNPase[256]. The Hum Oligos is a cell line immortalized by the persistent infection of Bornea virus[257]. To induce differentiation of oligodendrocytes, cells were cultured in low serum conditions. This has previously been shown to enhance the expression of cell surface markers characteristic of mature oligodendrocytes and to increase susceptibility to apoptosis[258]. The use of differentiated cells was considered relevant because the majority of myelinating oligodendrocytes in the brain are terminally differentiated[259,260].

The two cell lines showed consistency in terms of growth rate and morphological appearance, and displayed comparable levels of apoptosis in response to T cells in *in vitro* assays. Immortalized cell lines may, however, respond differently to stress signals and cytokine milieu compared to cells in tissues or primary and short-term cell cultures, and results must be considered with this in mind. We did make an attempt to obtain oligodendrocytes from biopsy material, but were not successful. The use of immortalized cell lines in research is nevertheless a valuable tool for the investigation of cellular mechanisms. The ability to proliferate makes HOG and Human Oligos suitable for proliferation-based assays, such as the JAM assay employed in this study. Moreover, both HOG and Human Oligos have been employed in previous studies on the immunobiology of oligodendrocytes[258,261].

3.3.1 Detection of apoptosis

The interaction between T cells and oligodendrocytes was studied in a coculture system, and the JAM assay[262] was employed to assess oligodendrocyte apoptosis. The JAM assay specifically measures the amount of DNA fragmentation, a hallmark of apoptosis, and represents a robust system for the detection of apoptosis. Oligodendrocytes were prelabeled with thymidine prior to T cell exposure. Washing steps removed fragmented DNA from the filter onto which the cell suspension was harvested. Apoptosis was then determined based on remaining intact DNA measured as counts per minute in a β-plate liquid scintillation counter. The JAM assay was well suited for our coculture system as we could easily detect apoptosis of prelabeled oligodendrocytes without interference of apoptotic T cells present in the culture. As a validation of the JAM assay, annexin V positive oligodendrocytes could be detected by flow cytometry after coculture with T cells and confirmed that oligodendrocytes indeed died by apoptosis.

3.4 Searching for target antigens of CSF mAbs

Several attempts were made to identify potential target antigens for the two mAbs, CSF mAb1 and CSF mAb2.

A multitude of candidate targets have been described for the Ig in the CSF of MS patients, including myelin proteins and viral proteins[199,263]. A first attempt to identify the specificity of the CSF mAbs was performed in an ELISA against MBP, αB-crystallin, cytomegalovirus, varicella-zoster virus, *Borrelia burgdorferi*, Toxoplasma gondii and EBV nuclear antigen. Furthermore, antibody reactivity against purified OMGP was tested in dot blot assays.

Solid-phase assays may be better than solution-phase assays for the detection of low-affinity interactions[264]. However, immobilization of antigens on a plastic surface or membrane may conceal critical epitopes or result in loss of epitopes due to denaturation[265].

A search for potential antigens in soluble phase was performed by using monodisperse magnetic particles (Dynabeads) coated with the CSF mAbs in immunoprecipitation assays. The surface tosyl groups allow covalent linking of antibodies via primary

amino- or sulphydryl groups and assure correct orientation of the antibodies to the bead surface. The beads were coated with CSF mAb1, CSF mAb2 or a control mAb in the presence of an alkaline buffer. Coated beads were incubated with cell lysate from differentiated HOG or Hum Oligos or lysate from human brain. Bound antigen was eluted from the beads by the use of an acidic buffer. Eluted proteins were visualized by gel electrophoresis and protein bands that differed between CSF mAbs and control mAbs were examined using mass spectrometry.

4. SUMMARY OF RESULTS

Paper I:

Multiple sclerosis: glatiramer acetate induces anti-inflammatory T cells in the cerebrospinal fluid.

We investigated the presence and phenotype of GA reactive T cells from the CSF and blood in three short-time treated (3-6 months) RRMS patients before and after onset of GA treatment and in two patients who had been treated with GA for two and three years. From the short-time treated patients we established a total of 22 cell lines from the CSF and 36 cell lines from the blood before treatment, and 28 T cell lines from CSF and 31 T cell lines from blood during treatment. Ratios between pro-inflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-5, IL-13 and IL-10) were used to define the phenotype of GA reactive T cells. The principal findings were: i) GA treatment induced an anti-inflammatory cytokine profile of GA reactive T cells from blood and CSF, and ii) following treatment, the anti-inflammatory shift was more pronounced for T cells present in the CSF compared to T cells present in the blood. A total of 20 GA specific T cell clones were generated from the two long-term treated patients. GA specific T cell clones and T cell lines were screened against recombinant human MBP and against 15-mer overlapping peptides spanning the complete sequences of MBP, PLP, MOG, MAG, OMGP, αβ-cystallin, s100β and CNPase. No cross reactivity was detected. Four out of 10 GA reactive T cell clones from the CSF were restricted by HLA-DP whereas the remaining CSF T cell clones were restricted by HLA-DR molecules. All 10 GA reactive T cell clones from blood were restricted by HLA-DR molecules.

Paper II:

Cerebrospinal fluid T cell clones from patients with multiple sclerosis: recognition of idiotopes on monoclonal IgG secreted by autologous cerebrospinal fluid B cells.

In this paper we generated two EBV-transformed CSF B cell lines from patients MS1 and MS2 and two IgG1λ mAbs were derived from the CSF B cell lines. Sequencing of the complimentary DNA encoding the V regions of the CSF mAbs revealed a ratio and

distribution of replacement to silent mutations suggesting that the B cells had undergone somatic hypermutation as part of a T cell dependent antigen-driven process *in vivo*. Attempts to identify the specificity of the CSF mAbs included ELISA against MBP, αB-crystallin and several virus proteins, Western blotting against OMGP, and finally immunoprecipitation of lysate from human brain and human oligodendrocyte cell lines. No target antigen was identified. T cell lines and clones from CSF and blood of the two MS patients were found to recognize autologous, but not heterologous CSF mAbs. The T cell clones were CD4$^+$ and responses were restricted by HLA-DR molecules. The T cell clones were found to be Th1 polarized and secreted predominantly IFN-γ. T cell responses were dependent on heat-aggregation of the CSF mAbs. Blocking of fragment crystallizable γ receptors (FcγRs) by preincubation of APCs with aggregated IgG from normal donors inhibited the response. CSF and blood T cell clones were tested against a panel of synthetic peptides spanning all amino acid replacements and CDR3s of the CSF mAbs. Two CSF T cell clones from one of the patients responded against a peptide from the FR, which contained two replacement mutations. For one of the CSF T cell clones the response was critically dependent on the mutations, as the T cell did not recognize the corresponding germline encoded peptide. The CSF T cell clones were found to secrete predominantly IFN-γ.

Paper III:

T cells from multiple sclerosis patients recognize multiple epitopes on self-IgG.

The results in this paper extend the findings in paper II by showing that the idiotype of one single CSF mAb derived from an MS patient carries several antigenic Ids, which are able to elicit a broad polyclonal T cell response. PBMCs stimulated with synthetic peptides spanning the V regions of the CSF mAbs (the same peptide panel as that employed in paper II) showed strong proliferation against two VH and two VL peptides in patient MS1 and against two VH peptides in patient MS2. From the CSF of patient MS1 four T cell lines recognizing either a VL CDR3 peptide or a VH CDR1/FR2 peptide were generated. From the blood of the same patient, six T cell lines recognizing the same VL CDR3 peptide, a VH FR3/CDR3 peptide or a VH FR1/CDR1/FR2 peptide were generated. T cell clones established from blood recognized a mutated VH FR1/CDR1/FR2 peptide (MS1-VH1) containing two amino acid substitutions compared to the germline encoded sequence. T cell responses were dependent on both mutations

as responses were lost when one or both mutations had been reverted to the germline encoded amino acids. The same T cell clones proliferated in response to HLA-matched PBMCs preincubated with high concentrations of CSF IgG (80-360 μg/ml) overnight indicating that APCs process CSF IgG and present the MS1-VH1-peptide to Id-specific T cells. These T cell clones were CD4^{+} and restricted by HLA-DRB1*1302 encoded molecules. One T cell clone was shown to recognize both the mutated MS1-VH1-peptide and the Id-bearing B cell clone suggesting that the B cells process and present Ids from its own BCR. The T cell clones were shown to secrete predominantly IFN-γ.

Paper IV:

Idiotope-specific CD4^{+} T cells induce apoptosis of human oligodendrocytes.

This study shows that Id-specific CD4^{+} T cells can induce apoptosis of oligodendrocytes *in vitro*. Two human oligodendrocyte cell lines were used to study the interaction between T cells and oligodendrocytes. Id-specific T cells stimulated either by Id-bearing B cells, by APCs preincubated with Id-peptides overnight or by anti-CD3/anti-CD28 in the absence of accessory cells were shown to induce DNA fragmentation and killing of oligodendrocytes in an *in vitro* coculture system. The ability of the T cells to kill oligodendrocytes rapidly peaked upon their activation. Induction of apoptosis required contact between the T cells and the oligodendrocytes as no killing was observed when effector and target cells were separated by a transwell membrane (0.4 μm pore size). Microscopy showed that activated T cells clustered around the oligodendrocyte cell body and lined up along its processes. Oligodendrocytes exposed to various concentrations and combinations of the pro-inflammatory cytokines IFN-γ and TNF-α showed no signs of apoptosis. Induction of oligodendrocyte apoptosis was independent of the T cells' cytokine profile and required no cognate interaction between T cells and oligodendrocytes. Apoptosis could be inhibited by a general caspase inhibitor or by an anti-Fas mAb. Finally, T cells were shown to undergo apoptosis upon contact with oligodendrocytes.

5. GENERAL DISCUSSION

A major part of the work in this thesis is based upon the study of B cells and T cells originating from the CSF. The underlying thought is that the CSF provides a "window to the brain" and that cells present in this compartment more accurately reflect the population of cells relevant for the disease process in MS than cells from blood. This is supported by observations in celiac disease showing that T cells from the gut display a more stringent HLA restriction pattern and recognize other antigens than T cells present in the blood[266]. As long as viable lymphocytes from MS lesions are not accessible, lymphocytes from the CSF were considered the best alternative in our study.

The majority of lymphocytes in the CSF are CD4$^+$ T cells of a memory phenotype, which most likely reflect the disease process. The population of disease relevant cells present in the CSF will be highly concentrated compared to disease relevant cells in the blood. Accordingly, when CSF fluid drains into the systemic circulation mononuclear cells present in the CSF will be diluted several 1000-fold making the disease relevant T cells in the blood difficult to detect. Comparison of T cells from blood and CSF can therefore point out important differences which may bear relevance for the disease process in CNS.

5.1 The therapeutic potential of GA

The entry of GA reactive T cells into the intrathecal compartment is considered a prerequisite for the therapeutic effect of GA reactive T cells[230,245]. GA is quickly degraded upon subcutaneous administration making it highly unlikely that it is present in the CNS in sufficiently high concentrations to have an immunologic effect[267]. Thus, GA reactive T cells which have been activated by GA experienced APCs outside of the CNS probably migrate to the CNS where they exert their therapeutic effect. Another possibility may be that GA experienced APCs migrate to the brain and stimulate T cells within the CNS[217]. Prior to our study, the ability of GA reactive T cells to access the brain had to some extent been established in mice[230,231]. The evidence for this in humans was limited to the observation that GA reactive T cells could migrate across an artificial BBB[268]. Our first question was therefore whether GA reactive T cells are present in the CSF. Our findings show that GA reactive T cells are present in blood and CSF both

prior to and after the onset of treatment, suggesting that GA reactivity exists independently of prior immunization. This is in agreement with other studies showing a high frequency of GA reactive T cells in treatment-naïve individuals as well as in the cord blood of newborns[222,223,238]. All T cell lines generated from both blood and CSF with primary GA stimulation were GA reactive. The low number of isolated CSF cells precludes direct calculation of the frequency of GA reactive T cells in the original sample. However, a rough estimate can be made based on the number of cells seeded per well and the resulting number of GA reactive T cell lines. Since the maximum number of CSF cells seeded per well was 5000 and all T cell lines turned out to be GA reactive, this suggests that the precursor frequency of GA reactive T cells in CSF both before and after initiation of GA treatment exceeded 1:5000. This frequency is much higher than normally observed with primary antigens and is more comparable to a recall response[269].

The daily subcutaneous injection of 20 mg GA is probably associated with an active priming of both naïve and memory T cells in secondary lymphoid organs. In agreement with several other studies[222,223,238], we showed that GA treatment induced a shift in the cytokine profile of responding T cells towards an anti-inflammatory phenotype. Recent findings suggest that GA acts primarily on the APC population and that the effect on T cells may be secondary and dictated by the GA-induced cytokine profile of APCs[217,220,221]. When generating and testing T cell lines from patients before and after treatment, we used fresh autologous PBMCs as APCs and could therefore not separate the GA-induced effect on T cells from the effect on APCs. However, at least two observations indicate that there is a difference between GA reactive T cells in blood compared to CSF: i) the anti-inflammatory profile was more pronounced for T cells present in the CSF compared to T cell present in the blood, and ii) the GA induced shift towards a more anti-inflammatory phenotype was more pronounced in CSF than in blood, even though the same APCs were used in both blood and CSF. This suggests that *in vivo* peripheral priming of GA reactive T cells is followed by homing of a selected subset of GA reactive T cells to the intrathecal compartment. In addition, GA specific T cell clones from the CSF were restricted by either HLA-DR or HLA-DP molecules, whereas only HLA-DR restricted T cell clones were detected in the blood. This could be coincidental as only ten clones were investigated from each compartment, but is nevertheless remarkable as DP-restricted T cell clones have rarely been reported and to

our knowledge never in the context of GA reactivity. Thus, this finding may further suggest a difference between CSF cells and blood cells.

Reactivation of GA reactive T cells in the brain by cross recognition of myelin proteins is one of the proposed mechanisms of action of GA and could potentially explain the recruitment of a selected population of GA reactive T cells into the intrathecal compartment[229,231]. Reports showing cross reactivity with myelin proteins have to the best of our knowledge only been investigated with the use of GA reactive T cell lines. By cloning GA reactive T cells we had for the first time the opportunity to investigate cross reactivity at clonal level with human T cells. Cross reactivity was tested against complete MBP protein and a panel of peptides that spanned the majority of the candidate autoantigens in MS. We were unable to detect cross reactivity for any of our T cell clones or lines, neither by proliferation nor by cytokine secretion. Our results cannot exclude the possibility that GA reactive T cells may cross recognize myelin proteins. Importantly, a limited number of clones were tested and we relied on secreted cytokines as this was considered most relevant, and did not attempt to detect changes in the pattern of intracellular cytokine production.

The high frequency of GA reactive T cells in the blood and CSF of treatment-naïve individuals observed by us and others, and the demonstration that the majority of these are recruited from the memory pool of T cells[223,238], suggest that GA mimics recall antigens to which the patient has been exposed previously. This is not unexpected considering the multitude of epitopes which may arise from the random composition of GA. Furthermore, the well-defined degeneracy of TCRs[270,271] implies that a low level of random cross reactivity may be expected. Such random cross reactivity may account for reactivation of GA reactive cells in a variety of tissues and perhaps contribute to the therapeutic potential of GA in inflammatory diseases outside of the CNS[272,273]. Also, it may be hypothesized that a persistent inflammatory environment triggers a transient reactivation of GA reactive T cells in a TCR independent manner, which results in release of anti-inflammatory cytokines. This may contribute to bystander suppression of local inflammation in MS and other inflammatory diseases.

5.2 T cell responses to CSF IgG

Paper II and III in this thesis describe T cell responses to Id present in CSF IgG. The foundation for this work was provided by the observation that MS patients display increased T cell responses to autologous IgG compared to patients with other neurological diseases[159], which suggested that responses to IgG could be relevant for the disease process in MS. Paper II describes the establishment of EBV-transformed B cells and the purification of monoclonal CSF IgG from two MS patients, which greatly facilitated the study of T cell responses to IgG in the absence of potential contaminants. Strong proliferative T cell responses were recorded towards autologous, but not heterologous CSF mAbs in both MS patients. Based on the considerations mentioned for GA reactive T cell lines a similar estimate can be done for the frequency of CSF T cells recognizing CSF IgG. The overall frequency of CSF T cells recognizing CSF mAbs or CSF mAb peptides was thus estimated to be approximately 1:10000 or higher. This is a high frequency indicating that the CSF mAb responsive T cells were not recruited from the naïve repertoire, but rather represent memory T cells expanded during an *in vivo* immune response. Also supporting this was the ability to generate antigen-specific T cell lines with only one round of antigen stimulation and the vigorous proliferative responses observed in PBMCs.

The accumulation and distribution of replacement to silent mutations in the V regions of the CSF mAbs suggest that the B cells had undergone somatic hypermutation during T cell dependent germinal center reactions. Somatic hypermutation is a hallmark of affinity-matured B cells both from the CSF[182,183,186,189] and brain tissue of MS patients[185,274], suggesting that the B cells producing the CSF mAbs are representative of disease relevant B cells in MS. The search for T cell epitopes in the CSF mAbs was restricted to regions containing amino acid substitutions and to the highly variable CDR3s. This was in part due to the shortage of CSF T cells and therefore a need to focus on the most probable immunogenic determinants. T cell responses to Ig in other model systems have consistently shown that amino acid substitutions are essential for the immunogenicity of Ids and that T cells generally are tolerant towards germline encoded sequences[61,275,276]. In agreement with this, our study shows that T cell responses to a mutated peptide containing two replacement mutations were lost when the amino acid substitutions were reverted to the germline encoded amino acids. However, most of the CSF mAb responsive T cell clones did not recognize any of the

mutated peptides. This may suggest that they were specific for epitopes located outside of the sequences covered by the peptide panel employed in this study. Supporting this is the observation that germline encoded IgM has been shown to be immunogenic in syngeneic mice[277]. An alternative explanation is that the number of peptides was too small to ensure correct positioning of the mutated amino acids in relation to MHC binding and T cell recognition.

The finding of T cell responses directed against several Ids in the V region of one CSF mAb is compatible with a diversification of the immune response through the process of epitope spreading (paper III). Epitope spreading is a typical feature of chronic immune responses and is defined as the dissemination of epitope specificity from an initial dominant epitope to subdominant or cryptic ones[278]. This is thought to be important in the progression of EAE and Theiler's murine encephalomyelitis[278]. Furthermore, epitope spreading to Ids on autoantibodies is thought to be important in experimental lupus[279]. Epitope spreading is, however, difficult to verify in human disease because the identity of the initiating epitope is hard to determine.

CD4$^+$ T cells can only respond to Ids when presented in combination with MHC class II molecules. Microglia and blood derived DCs are present in active MS lesions[128] and may present Id to Id-specific T cell *in vivo*. Paper II shows that T cell responses to CSF mAbs are dependent on FcγR mediated uptake and required prior heat aggregation of the IgG molecules. This may raise a concern regarding the immunogenicity of single soluble IgG molecules. However, antibodies in the CSF of MS patients have been shown to form immune complexes[280], and strong upregulation of FcγR I, II and III on microglia in MS lesions suggests that facilitated uptake of IgG may occur in MS[281]. Moreover, Id-specific T cells may become activated by Id-bearing B cells. Thus, B cells constitutively process and present peptide fragments from their endogenous BCRs in combination with MHC class II and can activate Id-specific T cells that are specific for rare Id sequences[26]. In this study we show that autologous B cells from the CSF have the capacity to specifically activate an Id-specific T cell clone. This was demonstrated by incubating Id-specific T cells in the presence of Id-bearing and several non-Id-bearing EBV-transformed B cell lines, and by inhibiting the response with anti-HLA-DR antibodies (Paper III and IV).

The observation that the CSF mAb1 and 2 are IgG with a high ratio of silent to replacement mutations suggests that the B cells producing these mAbs had been subject to somatic hypermutation in a T cell driven germinal center reaction. Studies on Id-driven T-B cell collaboration in mice have shown that ligation of the BCR may be necessary for isotype switching and affinity maturation to occur[66]. It was therefore of interest to search for potential CNS targets of the CSF mAbs. However, we have not been able to identify the target for any of our CSF mAbs, despite several attempts employing both solution-phase and solid-phase assays (Paper II). The potential pathogenic effect of the CSF mAbs was also tested *in vivo* by Claudia Sommers' research group in Wuerzburg, Germany. Here, the CSF mAbs were injected into the intrathecal compartment of rats, which were monitored for signs of EAE. Injected animals displayed no clinical signs of neurological disease and no signs of immunoreactivity in slices of brain or spinal cord. Also, no specific binding was detected when the CSF mAbs were incubated with brain or spinal cord tissue from human, rat or mouse. It is, however, fully possible that the CSF mAbs target other antigens, such as inflammation-induced proteins or infectious agents that have not been tested by us or our collaborators.

Another possibility that may explain the failure to identify target antigens for the CSF mAbs is that they display very low affinity or have lost affinity for the original antigen. B cells that loose their affinity during affinity maturation will normally not be selected and die from apoptosis[282]. However, such B cells may be rescued if a loss-of-affinity mutation creates an Id-peptide recognized by germinal center Id-specific T cells. This appeared to be the case for a dominant chromatin specific B cell clone associated with the induction of experimental lupus[283]. Two replacement mutations in FR1 and CDR2 were traced back to the original single precursor cell giving rise to the B cell clone. None of these mutations were shown to increase the binding affinity for the antigen, but instead created an immunodominant epitope recognized by MHC class II restricted Id-specific T cells. Mutations in the FR usually do not confer stronger binding affinity, but rather lead to detrimental structural changes in the antibody molecule and are therefore not prone to be selected[284]. It was therefore hypothesized that the FR mutation had created an immunogenic Id, which selected the B cell due to its receptor presentation capabilities instead of increased affinity for an antigen[283]. In support of such processes also playing a role in the maturation of the Id-bearing B cells in our study[155], we

encountered an Id-specific T cell clone that recognized an Id located in the FR of the CSF mAb (paper II). If low affinity B cells are really rescued in such a process, it may explain the difficulties in identifying the target antigen of the CSF mAbs. Moreover, an intrathecal B cell response sustained by Id-specific T cells may offer an explanation for the persistent production of both the main oligoclonal IgG of unknown specificity and the virus-specific antibodies in the CSF of MS patients.

In paper IV we demonstrate that CD4^{+} Id-specific T cells induce apoptosis of oligodendrocytes, suggesting a potential role of Id-specific T cells in MS pathogenesis. The relevance of apoptosis as a result of T cell mediated inflammation in MS has been debated. Two of four patterns of demyelination which were identified in autopsies and biopsies from 83 MS patients suggested T cell mediated inflammation as a secondary event to primary myelin damage[285]. Also, apoptosis of oligodendrocytes was observed in the absence of T cell infiltration in patients who died of acute MS[286]. However, biopsy and autopsy material from acute or lethal MS may not be fully representative for common MS. Moreover, it is possible that T cell mediated apoptosis of oligodendrocytes may prevail during a short time span in early stages of MS and be less apparent in late stages of the disease[286,287].

Inflamed brain endothelium upregulates expression of adhesion molecules, which allow entrance of CD4^{+} T cells into the CNS irrespective of antigen-specificity[109]. It is believed that only those T cells that recognize their cognate antigen will be retained and expanded intrathecally[110]. Accordingly, CD4^{+} T cells without specificity for any CNS antigen have been shown to enter the brain, but caused no damage to glial cells[288]. Importantly, the B cells producing Id-bearing mAbs were derived from the intrathecal compartment. Thus, Id-specific T cells may be pathogenic in MS because they may be activated and retained intrathecally when encountering CNS residing Id-bearing B cells.

We chose a reductionistic approach and studied the potential pathogenic effect of Id-specific T cells towards oligodendrocytes in the absence of accessory cells. It is possible that direct killing of oligodendrocytes by T cells mainly is an *in vitro* phenomenon and perhaps not as relevant *in vivo*. Indeed, differences between *in vitro* and *in vivo* systems have been demonstrated for the killing of Id-bearing myeloma cells by Id-specific T cells in mice. While *in vitro* killing was mediated by Fas/Fas ligand interactions[289], killing *in vivo* was dependent on macrophages activated by T cell derived IFN-γ[290].

Macrophage activation was also apparent during inflammatory responses associated with Id-driven T-B cell collaboration in mice[291]. This implies that the cytokine profile of T cells may be critical *in vivo* in contrast to our findings where both Th2-polarized GA reactive T cells and Th1-polarized T cells killed oligodendrocytes with equal efficiency. Lastly, establishment of T-B cell interaction *in vivo* may recruit other inflammatory cells and direct killing of oligodendrocytes by T cells may be only one of several mechanisms operative *in vivo*.

5.2.1 A hypothesis on Id-driven T-B cell collaboration in MS

Oligoclonal expansion of T cells and B cells with unknown antigen specificity is a prominent feature of MS[164,182,184,188]. We have shown that B cells, which bear similarities to clonally expanded B cells in the CSF of MS patients, carry several immunogenic Ids. The high frequency of Id-reactive T cells in the CSF and the observation that Id-bearing B cells and Id-specific T cells may reciprocally activate and sustain each other[66] suggest that Id-driven T-B cell collaboration may contribute to the intrathecal immune response in MS. In this context it is interesting to note the finding of B cell follicles in the brain of MS patients[206] and the clinical benefit of B cell depletion[209] that in a new way have underscored the importance of B cells in the pathogenesis of MS.

Based on the concept of Id-driven T-B cell collaboration and the observations on this phenomenon made by us and others, the following scenario may be proposed[155]: The initial trigger of MS could be an infectious agent that leads to germinal center reactions in secondary lymphoid organs and triggers activation of T and B cells. During the process of affinity maturation, somatic hypermutation creates immunogenic Ids, which are recognized by Id-specific T cells. Id-bearing B cells and Id-specific T cells may by chance re-encounter in the CNS, resulting in Id-driven T-B cell collaboration and the establishment of inflammatory foci. Id-specific T cell activation may lead to unspecific bystander killing of oligodendrocytes and other CNS cells (**Figure 3**). This model could explain the persistent cellular and humoral immune response in the CNS and the ongoing inflammation in MS in the absence of a conventional target antigen. It may explain how normal immune responses against infectious agents may eventually lead to an immune mediated disease in the brain. Moreover, this model does not have to imply

breaking of tolerance against self-molecules, as Id-specific T cells target an immunological "non-self" on a self-molecule.
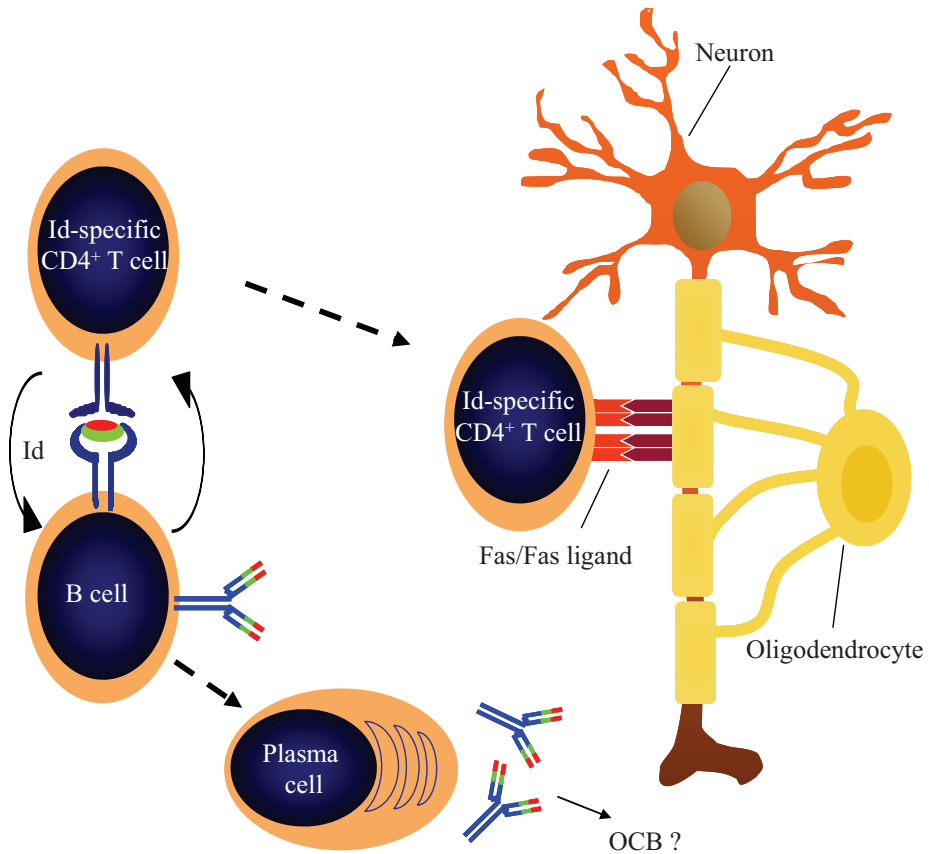
Figure 3. A schematic view of Id-driven T-B cell collaboration in MS. *Id-specific T cells and Id-bearing B cells activated during germinal center reactions in peripheral lymphoid organs may re-encounter intrathecally. Reactivation may lead to establishment of inflammatory foci with a T cell sustained differentiation of the B cell into an Ig-secreting plasma cell and stimulation of T cell effector mechanisms. The Ig may be a source of OCBs in MS and Id-specific T cells may induce apoptosis of oligodendrocytes via Fas/Fas ligand interactions.*

6. FUTURE STUDIES

6.1 Id-driven T-B cell collaboration in MS

The results presented in this thesis suggest that Id-driven T-B cell collaboration may contribute to the immune mediated plaque-wise destruction of the CNS. However, as all results are based on *in vitro* observations on a limited number of cells from two MS patients, they may not bear relevance *in vivo*. To approach this issue, a modified model of the previously mentioned transgenic mice expressing Id$^+$ B cells and the corresponding Id-specific TCR will be employed[69]. The new model includes an NF-κB-responsive luciferase reporter transgene, which allows *in vivo* visualization of inflammation in addition to clinical recordings and conventional histopathologic studies[292]. We wish to use this system to examine whether T-B cell collaboration in the brain correlates with the development of neurological symptoms and MS-like disease. In support of this, four animals out of about 200 of the double transgenic mice did develop signs of neurological disease with partial hind limb paralysis, suggestive of CNS inflammation (personal communication L. Munthe, Rikshospitalet University Hospital).

6.2 Production of growth factors by GA reactive T cells

With the establishment of GA reactive T cell clones from the CSF and blood of GA treated patients we have the possibility to assess the secretion of growth factors at a clonal level and compare GA reactive cells from the CSF and blood with T cells of other specificities. So far, we have tested BDNF secretion by two GA specific T cell clones from the CSF stimulated by GA in the presence of autologous PBMCs or by anti-CD3/anti-CD28 antibodies. Preliminary results indicate that production of BDNF was mainly a feature of PBMCs as no BDNF was detected in the supernatant of anti-CD3/anti-CD28 stimulated T cell clones in the absence of accessory cells.

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8. ERRATUM

Paper III:

Hestvik AL, Vartdal F, Fredriksen AB, Thompson KM, Kvale EO, Skorstad G, Bogen B and Holmoy T. T cells from multiple sclerosis patients recognize multiple epitopes on self-IgG. *Scand J Immunol.* 2007; 66: 393-401:

Page 398; Figure 6: A = A + C