Dissertation der Fakultät für Biologie der Ludwig-Maximilian-Universität München

Aspects of the Immunobiology of Myelin Oligodendrocyte Glycoprotein (MOG)-induced Experimental Autoimmune Encephalomyelitis (EAE)

vorgelegt von

Anna Schubart aus Frankfurt

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Summary

In the Dark Agouti (DA) rat, immunisation with the extracellular domain of the myelin oligodendrocyte glycoprotein (MOG-Igd) induces both an encephalitogenic T cell response and a demyelinating antibody response (Stefferl et al., 2001). These immune effector mechanisms synergise to induce a demyelinating and relapsing remitting variant of experimental autoimmune encephalomyelitis (RR-EAE), which reproduces the immuno-pathology of demyelination observed in a large proportion of multiple sclerosis (MS) patients (Storch et al., 1998; Lucchinetti et al., 2000). This model was used to investigate two facets of MS that are poorly understood; 1) the mechanism(s) responsible for relapse and disease progression, and 2) the a possible mechanistic link between milk consumption and disease prevalence.

This study demonstrates for the first time that pathogenic autoantibodies can become the driving force responsible for disease progression in RR-EAE. Although MOG-specific T cells can induce repeated episodes of CNS inflammation, this response is self-limiting and the intensity of the inflammatory response decreases with time. MOG-specific autoantibodies induce demyelination and amplify this inflammatory response, resulting in progressive neurological deficits. This has important implications for the treatment of MS, in which MOG-specific autoantibodies are implicated in the immunopathogenesis of demyelination (Storch et al., 1998; Haase et al. 2000; Reindl et al., 1999; Genain et al., 1999; Raine et al., 1999).

The second part of this thesis evolved from the observation that MOG-specific T cells can cross-react with the bovine milk protein butyrophilin (BTN) (Stefferl et al., 2000), which may explain the reported correlation between milk consumption and the prevalence of MS (Butcher, 1986; Malosse et al., 1992; Lauer, 1997). Immunological cross-reactivity or molecular mimicry is often discussed as a potential trigger to induce auto-aggression in MS and other autoimmune diseases (Fujinami and Oldstone, 1985; Wucherpfennig et al., 1995; Zhao et al., 1998; Gautam, 1998). This study demonstrates that cross-reactivity with BTN is restricted to a minor subset of a very complex, polyclonal MOG-specific T cell repertoire. Nevertheless, stimulation of this T cell population with BTN peptide dramatically suppresses the pathogenic potential of MOG reactive T cell lines. It is shown that the BTN peptide acts as an altered peptide ligand on MOG/BTN-reactive T cells, modifying cytokine synthesis in favour of a counter-inflammatory Th2-like response. This demonstrates that dietary derived peptides may have important and previously unrecognised effects on the autoimmune repertoire.

1 Introduction

1.1 Multiple sclerosis (MS) - an autoimmune syndrome?

1.1.1 Disease course

Multiple Sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterised by the presence of multiple inflammatory demyelinating lesions throughout the CNS. Depending on their topography, these lesions result in various neurological deficits, such as visual impairment, limb weakness, fatigue, cramps, parathesia, ataxia, bladder and bowel symptoms and vertigo, and in later stages paralysis and loss of renal and respiratory homeostasis, which often contribute to an early death. The clinical course of MS is no less variable than its symptoms. A high proportion of patients are diagnosed between 20 and 40 years of age and develop a relapsing-remitting course of disease, during which clinical exacerbations can be followed by intervals of more or less complete recovery. During this period the clinical deficits are a direct consequence of the inflammatory demyelinating response and recovery is associated with significant remyelination of the affected areas of the CNS. However, irreversible axonal loss does occur (Ferguson et al., 1997; Trapp et al., 1998; Kornek et al., 2000) and as disease progresses this accumulates and results in chronic disability. In later stages of MS most patients develop secondary progressive disease, in which relapses occur against a background of a steadily worsening neurological deficit. The pathophysiology of secondary progressive MS is poorly understood, and it is now recognised that clinical decline in some patients apparently continues in the absence of new episodes of CNS inflammation (Trapp et al., 1998). The duration of MS varies dramatically and while many patients will have the disease for several decades, approximately 10% develop an acute, fulminant form of MS in which clinical deterioration is rapid and the patients die within one to three years. At the other end of the clinical spectrum there are patients with very benign disease and a low relapse rate, who may never develop a secondary progressive disease.

1.1.2 Aetiology of MS

MS is the most common inflammatory demyelinating disease of the CNS in Europe and North America, where it affects about 80 to 100 per 100,000 people. However, its prevalence is not globally uniform, but decreases at lower latitudes reaching levels of only 1 to 2 per 100,000 in Africa and Asia. The aetiology of MS is unknown, but involves both genetic and environmental factors. The involvement of genetic factors is most clearly demonstrated by twin

studies, which revealed that concordance in monozygotic twins was 30%, but only 4% in dizygotic twins and sibs (Sadovnick 1993; Sadovnick and Ebers, 1995; Ochsenberg and Barcellos, 2000). Several susceptibility loci were tentatively identified by whole genome screens involving a total of several thousand patients, but the various research groups involved identified different loci and it is now generally accepted that MS is a polygenic disorder. The only consistent correlation involved the MHC locus and may reflect linkage of MS with the MHC class II allele HLA-DR2, which is over-represented in Caucasian MS patients (Martin et al., 1992; Liblau et al., 1993). Apart from the importance of genetic factors, the concordance rate of only 30% in monozygotic twins also indicates a crucial role for environmental factors in disease development. Migration studies showed that moving from a high risk to a low risk area will result in acquisition of the risk of the new region, and vice-versa (Gale and Martyn, 1995). However, this is only the case if migration occurs before the age of 15, suggesting that the impact of the environment is concentrated within a certain time window. This phenomenon has been attributed to infectious agents, but could also involve geographical as well as socio-economic factors, such as better sanitation, diet and stress, features associated with a “western life style". Among the individual factors implicated in the aetiology of MS are several dietary components, such as milk (Lauer, 1997), as well as a wide range of pathogens including human herpes virus 6 (HHV-6, Challoner et al., 1995; Soldan et al., 1997), measles virus (Brody et al., 1972; Burgoon et al., 1999), Epstein-Barr-Virus (Bray et al., 1983; Ascherio and Munch, 2000) and chlamydia (Sriram et al., 1998). However, the association between any of these agents and MS is controversial (see Derfuss et al., 2001 with respect to chlamydia) and as yet no infectious agent was reproducibly isolated from the central nervous system (CNS) of MS patients.

1.1.3 Immunopathology of MS

The heterogeneity of the clinical course of MS is paralleled by a similar variation in its pathology. Using immunopathological criteria, MS lesions were recently segregated into four distinct subtypes (Lucchinetti et al., 2000; Lassmann et al., 2001). The general pathology of MS, the formation of demyelinating lesions in the CNS associated with infiltrating $CD3^+$ T cells (Traugott et al., 1983), activated macrophages and microglia containing myelin debris (Ulvestad et al., 1994; Brück et al., 1995; Storch et al., 1998; Ferguson et al., 1997) and infiltrating B cells (Esiri et al., 1977; Lucchinetti et al., 2000) is common to all forms of the disease. However, detailed immunopathological analysis of the lesions identified differences in the cellular infiltrates, complement and immunoglobulin deposition and apoptosis indicating that at least four different pathomechanisms are responsible for lesion formation.

Type I and II lesions show a striking resemblance to the lesions seen in certain models of experimental autoimmune encephalomyelitis (EAE), an animal model of MS initiated by an autoimmune response to CNS myelin autoantigens. In these types of lesions there is a pronounced perivascular infiltrate associated with the invasion of T cells and activated macrophages into the CNS parenchyma. In addition, type II lesions are associated with immunoglobulin deposition on the myelin sheath and the local activation of the complement cascade. Type II lesions are by far the most frequent type of MS lesion and were seen in more than 50% of MS cases studied by Lucchinetti et al., 2000. The immunopathology and ultra structure of type II lesions are reproduced in rat and primate models of EAE, in which demyelination is antibody mediated (Linington et al., 1988; Genain et al., 1995; Storch et al., 1998; Genain et al., 1999; Raine et al., 1999). On the other hand, pattern I lesions are not associated with immunoglobulin and complement deposition and have a strong resemblance to lesions seen in mouse models of EAE, in which demyelination is thought to be mediated by soluble factors such as TNF-α. The resemblance of these animal models to MS has led to the concept that the pathogenesis of type I and II lesions involves an autoimmune component. Very different pathomechanisms seem to be involved in type III and type IV lesions, which display signs of DNA fragmentation in oligodendrocytes indicative of virus or toxin induced demyelination rather than autoimmunity. Intriguingly, at a single time point all the lesions in a patient are of the same type, but as yet it is unknown whether the type of lesion may change during the course of disease. These results clearly demonstrate that several different pathomechanisms can lead to the destruction of CNS myelin in MS. This may in part explain why it has proved difficult to identify any generalised susceptibility genes, pathogens or environmental factors common to all patients as well as the varying clinical responses of individual patients to different treatment strategies.

The inflammatory pathology of MS and its pathological similarity to EAE suggest that MS is immune mediated. This concept is supported by the presence of oligoclonal immunoglobulin bands in the CSF, genetic linkage to the MHC locus, responsiveness to immune-directed therapies, expansion of myelin-specific T and B cell responses as well as blood brain barrier leakage detected by MRI.

1.1.4 CNS myelin – the target organ

In the CNS the myelin sheath is formed by an extension of the oligodendrocyte plasma membrane, which enwraps axons with multiple layers (10-150) of plasma membranes (Figure 1.1). The majority of the cytoplasm is extruded, leaving compact multilamellar myelin, which is composed of approximately 30% protein and 70% lipid (by dry weight) and is also characterised by its low water content of 40%. This structure efficiently isolates the axon to enable fast, saltatory conduction. The structure of compact myelin is maintained by its major protein components, the cytoplasmic myelin basic protein (MBP) and the transmembrane proteolipid protein (PLP), which together comprise approximately 80% of the total myelin proteins. In addition, isolated myelin contains a number of minor protein components whose functions are obscure. These include 2´3´-cyclic nucleotide, 3´-phosphodiesterase (CNP), myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMG), myelin oligodendrocyte basic protein (MOBP), myelin oligodendrocyte glycoprotein (MOG), myelin /oligodendrocyte specific protein (MOSP) and members of the tetraspan-protein family (reviewed in Baumann and Pham-Dinh, 2001). Some of these proteins, such as MAG, may interact with axonal proteins and contribute to the macromolecular organisation of the paranodal loops of the sheath adjacent to the Node of Ranvier (Bartsch, 1996; Martini and Schachner, 1997; reviewed in Baumann and Pham-Dinh, 2001). As discussed below, autoimmune responses to many myelin proteins can induce an inflammatory response in the CNS, and enhanced auto-reactivity to myelin proteins is observed in the majority of MS patients suggesting that they may be involved in the pathogenesis of MS. MOG is however unique, since it is the only myelin autoantigen for which there is direct clinical evidence for a pathogenic autoimmune response in MS (Genain et al., 1998; Raine et al., 1999; Haase et al., 2000).

1.1.5 Myelin oligodendrocyte glycoprotein (MOG)

MOG is a type I membrane glycoprotein, which – in contrast to most other myelin proteins - is exclusively expressed in the CNS (Linington et al., 1984; Gardinier et al., 1992). The mature protein consists of 218 amino acids and is predicted to consist of a N-terminal, extracellular IgV-like domain (MOG-Igd; modelled in Hjelmström et al., 1998) containing a single N-linked glycosylation site at position Asn31 (Hjelmström et al., 1998), a highly hydrophobic membrane spanning domain and a cytoplasmic domain that includes a second

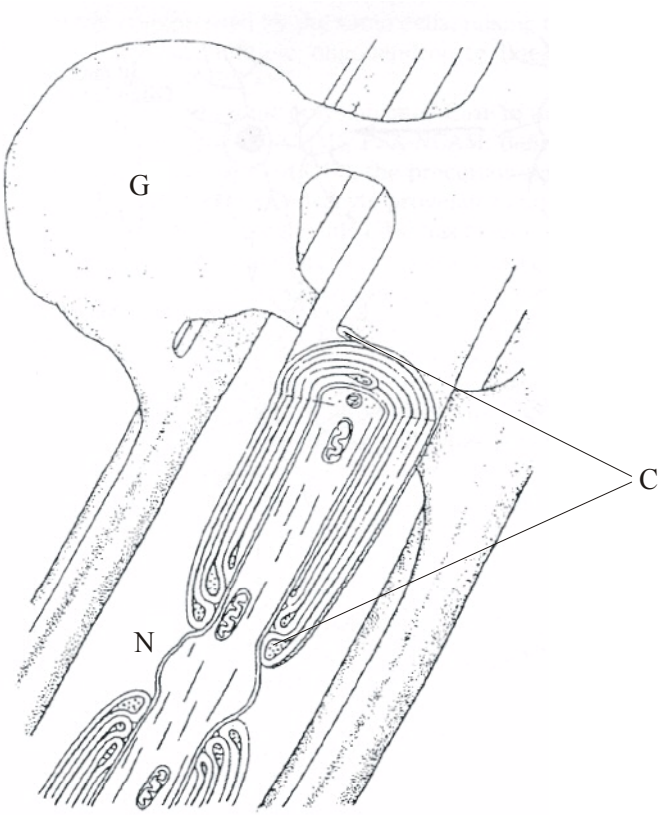


Figure 1.1 Schematic view of CNS Myelin

CNS myelin is formed by oligodendrocyte processes, which enwrap the axon with multiple lamellae of plasma membrane. The cytoplasm is extruded as the sheath compacts to form multilamellar myelin, but is retained in the paranodal loops to provide a continuous cytoplasmic channel (C) linking the myelin sheath to the oligodendrocyte cell body (G). It should be noted that in contrast to the Schwann cells of the PNS, one oligodendrocyte enwraps multiple axons. **C)** paranodal loop, **G)** oligodendrocyte, **N)** node of Ranvier (From Baumann and Pham-Dinh, 2001)

hydrophobic region. This domain is either tightly associated with or integrated into the cytoplasmic face of the membrane bilayer (Kroepfl et al., 1996; Della Gaspera et al., 1998; Figure 1.2). MOG is a member of the Ig-superfamily and its IgV-like domain is closely related to an extended family of B7- and butyrophilin (BTN)-like proteins (Pham Dinh et al., 1993; Vernet et al., 1993; Gruen et al., 1996). MOG is encoded in the distal region of the MHC and is therefore linked to the MHC class I and II loci associated with MS (Pham-Dinh et al., 1993, 1995; Lambracht et al., 1995).

Like other myelin proteins MOG is highly conserved between species (90% a.a. sequence identity between human and rat). Amino acid substitutions are concentrated within the putative

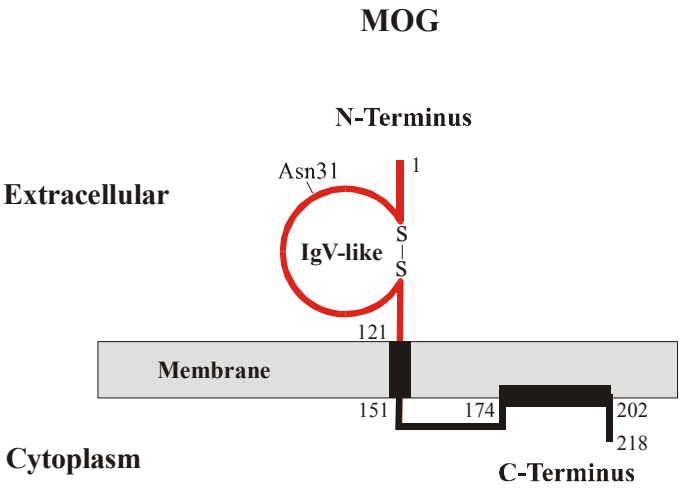


Figure 1.2 Schematic picture of MOG in the oligodendrocyte plasma membrane

The N-terminal IgV-like domain is on the extracellular side, the C-terminus intracellular (after Kröpfl et al., 1996) The numbers represent the amino acid residues at the proposed membrane boundaries.

transmembrane domain, while the cytoplasmic domain is virtually identical between mouse, rat, human and bovine species (Hilton et al., 1995) suggesting that it mediates an important interaction with other oligdendrocyte/myelin proteins, possibly components of the cytoskeleton (Dyer and Matthieu, 1994). The presence of an extracellular Ig domain certainly suggests that MOG may act as a receptor, but the putative ligand and its functional significance are obscure, a problem**,** which the development of MOG-deficient mouse mutants has failed to solve, as these animals do not develop any obvious neurological/neurobiological phenotype and myelination is completely normal (Pham-Dinh et al., submitted for publication).

1.2 MOG-induced experimental autoimmune encephalomyelitis (EAE) – a model for types I and II MS

Strikingly, there is no spontaneous animal model for MS. "EAE" was first reported in the late 19th century by Pasteur, who observed that some of the patients vaccinated against Rabies developed an encephalitis. Experiments in monkeys confirmed that this was due to contamination of the vaccine with spinal cord tissue and provided the first indication that inflammation and demyelination in MS may be autoimmune in origin (Rivers et al., 1933). The original studies, however, required multiple injections of spinal cord homogenate to induce disease in a small proportion of the experimental animals. Reproducibility was enhanced when Kabat, Wolf and Berger introduced Freund´s adjuvant (FA, 1946), an oil in water emulsion containing mycobacterium to enhance the immune response. This resulted in a highly reproducible disease, experimental autoimmune encephalomyelitis (EAE) that could be induced

in rats, mice, rabbits and guinea pigs by a single immunisation with CNS homogenates in FA. Histological examination confirmed Rivers´ original observation that disease was associated with an inflammatory infiltrate in the CNS and the formation of demyelinating plaques similar to those seen in MS.

Subsequently, single highly purified myelin proteins were identified as encephalitogenic components of this CNS homogenate, but in this case EAE was predominantly inflammatory in nature and demyelination was either absent or far less extensive (Waksman, 1954; Laatsch et al., 1962). This inflammatory pathology was finally attributed to the induction of an encephalitogenic T cell response in 1981 (Ben-Nun et al., 1981) and there is now an extensive list of myelin and non-myelin CNS autoantigens that can induce an auto-aggressive T cell response, including MBP (Ben Nun et al., 1981), PLP (Sobel et al., 1986), MOG (Linington et al., 1993), MAG (Weerth et al., 1998), myelin oligodendrocyte basic protein (MOBP, Kaye et al., 2000), as well as the astrocyte protein S100β (Kojima et al. 1994). However, although all of these antigens are capable of inducing T cell mediated encephalitis, MOG is the only myelin autoantigen that will induce demyelination in rats (Adelmann et al., 1995; Johns et al., 1995; Storch et al., 1998) and marmosets (*Calltithrix jacchus*, a small primate)(Genain et al., 1995, 1996). In these species, demyelination is strictly dependent on the presence of a MOG-specific demyelinating autoantibody response (Adelmann et al., 1995; Johns et al., 1995; Genain et al., 1995; Bernard et al., 1997; t'Hart et al., 2000), which induces a pathology reproducing that seen in type II MS lesions. In contrast, demyelination in murine EAE can also be mediated by TNF-α dependent mechanisms (Akassoglou et al., 1999) in the absence of a functional B cell response (Hjelmström et al., 1998; Eugster et al., 1998; Lyons et al., 1999), and in this species the immunopathology of autoimmune demyelination resembles that seen in type I MS lesions.

MOG was first identified as a target for antibody mediated demyelination in a guinea pig model of EAE induced by active immunization with CNS tissue homogenates in complete Freunds adjuvant (Lebar et al., 1986; Linington and Lassmann, 1987). The demyelinating potential of MOG-specific antibodies is due to the exposure of MOG-Igd at the surface of the myelin sheath and olidodendrocytes (Brunner et al., 1989) and was confirmed by the intravenous injection of a MOG-specific monoclonal antibody into rats with EAE induced by either active immunisation with MBP (Schluesener et al., 1987) or the adoptive transfer of MBP specific T cells (Linington et al., 1988; Lassmann et al., 1988). In the absence of the MOG-specific antibody, the pathology of these disease models is purely inflammatory. Intravenous injection of MOG-specific mAb at disease onset, however, induces extensive demyelination, enhances the inflammatory response in the CNS and dramatically increases disease severity.

The pathogenic potential of MOG-specific mAbs depends on their ability to fix complement or to induce antibody dependent cellular cytotoxicity (ADCC) (Linington et al., 1989 B; Piddlesden et al., 1991).

Susceptibility to MOG-Igd-induced EAE is determined mainly by the MHC class II locus, but is modified by additional MHC and non-MHC background genes. Studies of MHC congenic rats on the Lewis background revealed that the Lewis (LEW) haplotype $RT1^l$ confers low susceptibility and the Brown Norway (BN) haplotype $RT1^n$ is associated with fulminant disease, while the haplotype of Dark Agouti (DA) rats ($RT1^{av1}$) confers high susceptibility together with a tendency to develop relapsing remitting EAE (Weissert et al., 1998; Stefferl et al., 1999). This was not only seen in DA rats after immunisation with MOG, but also with spinal cord homogenates (SCH) (Lorentzen et al., 1995; Weissert et al., 1998). MOG - induced EAE in $RT1^{av1}$ rats is therefore the model system of choice for MS associated with type II lesions as it combines both an MS-like pathology and an MS-like disease course.

1.3 The immune system

The vertebrate immune system has evolved to perform the complex task of defending the organism against pathogens, while at the same time ignoring "self". The mechanisms that enable the immune system to distinguish between "self" and "non-self" and prevent autoimmune disease are still incompletely understood and their identification remains a major goal of immunological research. The major anatomical sites and mechanisms by which self-tolerance is established during the development of the immune repertoire are now however beginning to be elucidated.

1.3.1 Central tolerance

Immunological protection is generally mediated by a combination of innate and acquired mechanisms, which interact to protect the host from most pathogens. Innate immunity has a low degree of specificity and pre-dates the adaptive immune response, which evolved with the vertebrates. It is the failure to regulate or tolerise the adaptive immune response which is responsible for most autoimmune diseases. Flexibility of the adaptive immune response is ensured by the presence of a large pool of at least 10^8 -10^{10} lymphocytes that differ in their cell surface receptors and therefore antigen specificity. Antigen specificity in both naïve T and B cells is determined by receptors that mainly differ in sequence in a small region (complementarity determining region 3, CDR3) located in a loop of an immunoglobulin domain. Their diversity is generated by a complicated process of gene rearrangement, in which

different gene fragments are joined together. At this step, additional nucleotides may be inserted at the junctions. After this gene rearrangement has occurred, each lymphocyte expresses a unique receptor. In T cells the sequence of this receptor (TCR) is then fixed and undergoes no further changes. This is not the case in B cells, in which recognition of antigen is associated with somatic mutations, concentrated in the CDRs 1-3, that increase the affinity of the receptor for their target (affinity maturation) (reviewed in general immunology textbooks, e.g. Abbas, et al., 2nd edition 1994; Janeway and Travers, 1994).

This procedure invariably results in the generation of a large number of auto-reactive pre-T and B cells, the majority of which is eliminated in highly specialised immune organs (thymus for T cells and bone marrow for B cells) as soon as these gene rearrangements have taken place. This process termed negative selection is dependent on the presence of autoantigen in these organs and effectively removes maturing auto-reactive cells before they have a chance to enter the circulation (Abbas et al., 1994; Janeway and Travers, 1994). However, the efficiency of negative selection is concentration dependent. While it may completely eliminate cells recognising proteins expressed in the thymus or present in the circulation (such as C5), central tolerance may be incomplete for tissue-specific antigens even if they are also present at low levels in the thymus. (Wekerle et al., 1996).

In addition, maturation of T cells in the thymus requires positive selection by low affinity recognition of autologous MHC plus "self-peptide", a mechanism that ensures that auto-reactivity is an intrinsic component of the T cell repertoire, albeit generally restricted to low affinity recognition.

1.3.2 Peripheral tolerance

Peripheral tolerance provides a second line of defence to remove or inactivate those T and B cells that may recognise tissue-specific antigens which are not expressed in thymus or bone marrow. The mechanisms responsible for peripheral tolerance are best understood for the T cell repertoire. Full activation of T cells not only requires the identification of a specific antigen/MHC complex, but also signals provided by a variety of co-stimulatory molecules present on antigen presenting cells (APCs). Recognition of antigens in the absence of co-stimulatory signals leads to the induction of either anergy, a state of functional unresponsiveness to antigenic stimulation (Schwartz, 1996) or apoptosis, the physical elimination of the T cell from the repertoire (Miller and Basten, 1996). Whether anergy or deletion is induced depends to a large amount on the concentration of the antigen and the

affinity of the TCR for the MHC/peptide complex. In general, high concentrations favour deletion, whereas low concentrations favour the induction of anergy (Liblau et al., 1997).

All these mechanisms that may maintain self-tolerance are, however, dependent on the availability of the antigen. In the case of minor components of the CNS, such as MOG, which is <0.05% of the myelin protein, this is unlikely to be the case as they are sequestered behind the blood brain barrier (BBB). This barrier restricts the free exchange of antigen and cells between the CNS and circulation. In addition, the CNS is devoid of a classical lymphoid drainage system further reducing the probability that the immune system will ever normally be exposed to MOG. In this case self-tolerance can not be induced and potentially auto-reactive T and B cells will be a normal component of the immune repertoire. Their pathogenic potential is, however, held in check, as naïve lymphocytes are unable to cross the BBB to find their target antigen. This form of passive tolerance is termed clonal ignorance (Miller et al., 1993).

In addition to these mechanisms based on direct TCR – MHC/peptide complex interactions there is increasing evidence for the presence of regulatory or "suppressor T cells" that limit autoimmune responses. These include both natural suppressor cells and antigen-induced populations. Natural suppressor cells, characterised by the phenotype $CD4^+$ $CD25^+$, are generated in the thymus - presumably as a consequence of intermediate affinity to autoantigens (reviewed in Shevach, 2001). Although these regulatory cells require antigen-specific activation, they subsequently suppress the activation of naïve T cells in an antigen-independent manner via direct cell-cell interactions.

In contrast, antigen induced suppressor cells are generated in the periphery, for example during oral tolerance (Weiner, 1994). This mechanism is required to eliminate potentially fatal immune reactions to the diet or the symbiotic gut flora and involves a specific compartment of the immune system, the gut associated lymphatic tissue (GALT). Antigen crossing the gut mucosa is processed in the GALT to generate a non-inflammatory T cell response involving antigen induced repressor T cells. These act via the secretion of soluble, counter-inflammatory cytokines such as IL-10 and TGF-β (Weiner, 1997; Strobel and Mowat, 1998). It should be noted that immune regulation by this suppressor cell population is distinct from regulation of Th1-responses by Th2 subset T cells, which secrete IL-4 and IL-10, but not TGF-β (Liblau et al., 1995). Th1 and Th2 T cell responses are mutually antagonistic by virtue of the reciprocal regulatory effects of IFN-γ/IL-12 and IL-4/IL-10. Thus the adoptive transfer of neuroantigen specific Th2 T cells can be used to antagonise the development of EAE, a Th1 mediated autoimmune disease (Khoruts et al., 1995). Shifting the immune response from Th1 to Th2 activity or vice versa is known as immune deviation. This can be induced together with deletion

and anergy (Gaur et al., 1992; Samson and Smilek, 1995) in the periphery by treatment with high dose soluble antigen (Genain et al., 1997).

Similar mechanisms exist for B cells, but they are not as well characterised. In principle, B cell activation requires co-stimulatory signals provided by antigen-specific $CD4^+$ T cells. Antigen recognition in the absence of co-stimulatory signals induces deletion of the B cells (Russell et al., 1991). Furthermore, abundant soluble proteins can induce a state of anergy in mature B cells, characterised by reduced surface IgM and an inability to proliferate and differentiate after an immunogenic stimulus (Goodnow et al., 1989). Anergic B cells are then excluded from primary follicles in lymph nodes and spleen and are rapidly lost in the absence of T cell help (Cyster et al., 1994; Cyster and Goodnow, 1995).

1.3.3 Tolerance to MOG

The exclusive expression of MOG in the CNS led to the assumption, that the lack of reactivity against MOG is maintained by immunological ignorance, a hypothesis recently supported by the observation that MOG-deficient mice have identical immune responses to MOG as wild type animals (Pham-Dinh et al., submitted). One would therefore assume that immunisation with MOG or MOG peptides would automatically induce a highly pathogenic T cell response. Intriguingly, this is not necessarily the case. In LEW and BN rats (Adelmann et al., 1995; Stefferl et al., 1999) active immunisation with MOG peptides fails to induce a highly encephalitogenic T cell response or clinical EAE. Indeed, in these rat strains even the transfer of MOG-specific T cells only results in an asymptomatic inflammatory response in the CNS.

Is it therefore possible, that tolerance to MOG is maintained by immunological cross-reactivity/molecular mimicry with other proteins? MOG is a member of the growing butyrophilin gene family, which currently comprises 5 sub-families, each of which consists of up to three family members (Stammers et al., 2000; Rhodes et al., 2001). These proteins show a high degree of sequence identity in their N-terminal, IgV-like domains (approximately 50% between the sub-families), as well as a common predicted IgV-like structure (modelled in Hjelmström et al., 1998). Members of the family are expressed in a variety of tissues, including immune organs, suggesting that the maintenance of tolerance to these proteins must involve active processes. The high homology between MOG and these proteins suggests that at least partial tolerance to MOG may result from the deletion of those lymphocytes, which recognise cross-reactive peptides derived from MOG-homologue(s). Cross-reactivity between MOG and a xenogenic family member, bovine butyrophilin (BTN) was recently demonstrated in the DA rat, a rat strain, which develops EAE with an MS-like disease course and pathology (Stefferl et al.,

2000). This observation for the first time provided the opportunity to study the impact of a dietary antigen (environmental factor) on the MOG-specific immune repertoire.

1.4 Objectives

The aim of this study was to use MOG-induced EAE in the DA rat, a model which resembles MS both in disease course and pathology, to investigate the mechanism(s) responsible for relapse/disease progression. This first required the establishment of a highly reproducible, synchronised model of relapsing EAE. Once this model was set up, MOG-specific immune responses should be analysed at different stages of the disease in order to determine the relative contributions of T cell- and antibody-mediated pathomechanisms during the course of the disease. In detail, the following questions should be addressed:

- How does the MOG-specific T cell response develop during the course of the disease ?
- Are MOG-specific T cell responses alone capable of inducing EAE in DA rats?
- If MOG-specific T cell responses are capable of inducing EAE, are they also capable of inducing relapse activity ?
- How does the MOG-specific B cell response develop during the course of EAE ?
- Can antibodies induce relapse ?

A second part concerned the identification of the molecular basis and functional consequences of the cross-reactive immune response between MOG and bovine BTN. This involved the following questions:

- Why are DA rats not tolerant to BTN although they drink milk during suckling ?
- Does confrontation with BTN modulate MOG-specific T cell responses ? Does BTN act as an APL?
- Which T cells cross-react? – Is it possible to identify cross-reactive T cell receptors (β-chain)?
- Which BTN residues are involved in the cross-reactive response ?
- Can BTN replace MOG in the induction of antigen-specific tolerance ?
- Does the cross-reactivity extend to BTN/MOG-specific antibodies ?

2 Material and Methods

2.1 Materials

2.1.1 Animals

Female DA rats were purchased from Harlan Winkelmann, Germany and kept under conventional methods in the animal house of the Max Planck Institute for Neurobiology. All experimental procedures performed in this study are covered under the animal licence #AZ211-2531-69/94 issued by the federal government of Bavaria, Germany

2.1.2 Cell culture media

2.1.2.1 Culture medium for bacteria

LB-Medium (Luria Bertoni)	Peptone from Casein	10 g/l
	Yeast extract	5 g/l
	NaCl (Sodium chloride)	5 g/l protein expression, 10 mg/l for plasmid preparations

Autoclave, store at 4°C (months). Add selecting antibiotics (100 mg/l Ampicillin +/- 25 mg/l Kanamycin) after autoclaving (120°C¸1 atü), immediately before use after cooling to approx. 55°C.

LB agar plates	Agar	15g/l in LB medium,

autoclave, store at 4°C (months). Resolubilise in microwave (max 500 ml bottles), add antibiotics immediately before use after cooling to approx. 55°C.

2.1.2.2 Medium and buffer for preparation of RbCl-competent bacteria

MTB medium	tryptone	20 g/l
	yeast extract	5 g/l
	KCl (Porassium chloride)	0.75 g/l

Autoclave, store at 4°C (months)

TF1	RbCl (Rubidium chloride)	0.1 M (12 g/l)
	$MnCl_2$ (Manganese chloride)	50 mM (8 g/l)
	KCH_3COO (Potassium acetate)	30 mM (3 g/l)
	$CaCl_2$ (Calcium chloride)	10 mM (1 g/l)
	Glycerol	15% (v/v)
	Set pH5.8 with acetic acid, sterile filter	

TF2	RbCl	10 mM (1 g/l)
	$CaCl_2$	75 mM (8 g/l)
	Glycerol	15% (v/v)
	MOPS (3-[N-Morpholino]butanesulfonic acid)	10 mM (2.3g/l)
	pH7, sterile filter	

2.1.2.3 Culture media for insect cells

SF9 cells	TNM-FH (Invitrogen)	
	10% FCS	
	100 U/ml Penicillin/Streptomycin	
High 5 cells	High five medium (Invitrogen)	
	100 U/ml Penicillin/Streptomycin	

2.1.2.4 Culture media for eucaryotic cells

Based on Dulbecco´s Modification of Eagle´s Medium (DMEM), 4500 mg/ml glucose with the following supplements:

Eagles Hepes	$NaHCO_3$ (Sodiumbicarbonate)		45 mM (3.7 g/l)
	HEPES (N-[2Hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid])		25 mM (6 g/l)

Sterile filter (0.22µm), store at 4°C (months)

DMEM/Asn	$NaHCO_3$		45 mM (3.7 g/l)
	L-Asparagine		0.024 mM (36 mg/l)

Sterile filter (0.22µm), store at 4°C (few months)

complete DMEM	DMEM/Asn		
	Penicillin/Streptomycin	(10,000U/ml Pen, 10,000µg/ml Strep)	1% (v/v)
	Sodium pyruvate	(100 mM)	1% (v/v)
	MEM Non-essentiel Aas	(100x)	1% (v/v)
	L-Glutamine	(200 mM)	1% (v/v)
	0.4% (v/v) ß-Mercaptoethanol		0.1% (v/v)

Sterile filter (0.22µm), store at 4°C (max. 14 days)

T cell Restimulation medium	complete DMEM	
	Rat serum	1% (v/v)

Sterile filter (0.22µm), use immediately

TCGF (T cell growth factor)	Complete DMEM	
	Heat inactivated horse serum	15% (v/v)
	MLA-supernatant (source of IL-2)	15% (v/v)

Sterile filter (0.22µm), store at 4°C for max. 14 days

Freezing medium	EH	45% (v/v)
	Heat inactivated fetal calf serum	45% (v/v)
	DMSO (Dimethylsulfoxide)	10% (v/v)

Filter first serum, then DMSO in EH (0,22µm), use immediately

Medium for Ag8-cells	Complete DMEM	
	Heat inactivated fetal calf serum	10% (v/v)

Sterile filter (0.22µm), store at 4°C for max. 14 days

Medium for MLA-cells	Complete DMEM	
	Heat inactivated horse serum	15% (v/v)

Sterile filter (0.22µm), store at 4°C for max. 14 days

2.1.3 Buffer and reagents

10 x PBS	NaCl (Sodium chloride)	1.37 M (80g/l)
	KCl (Potassium chloride)	27 mM (2g/l)
	Na_2HPO_4 (Disodium phosphate)	100 mM (14.2g/l)
	NaH_2PO_4 (Monosodium phosphate)	100 mM (13.8g/l)
	pH 7.4 with NaOH (Sodium hydroxide pellets)	

PFA, 4%	Paraformaldehyde (PFA)	4 % (w/v)
		in PBS, pH 7.3

dissolve PFA in water with a drop of NaOH, add 10x PBS after complete dissolution

2.1.3.1 Buffer for FACS-analysis

FACS-buffer	BSA (Bovine serum albumine)	0.2%
	NaN_3 (Sodium azide)	10 mM in PBS

Last wash and analysis in PBS only or PBS/azide

2.1.3.2 ACK lysis buffer for erythrocytes

ACK buffer	NH_4Cl (Ammonium chloride)	0.15 M (8.6 g/l)
	$KHCO_3$ (Potassium bicarbonate)	1 mM (1 g/l)
	Na_2EDTA (TitriplexIII)	0.1 mM (37.2 mg/l)
	set pH 7,2-7,4, sterile filter	

2.1.3.3 Buffer for purification of MOG-Igd

Buffer for Nickel-chelate chromatography

Urea buffer	Urea	6 M
	KH_2PO_4 (Potassiumdihydrogenphosphate)	0.1 M
	NaCl	0.5 M
	pH8	
Wash buffer	Urea buffer	
	Imidazole	40 mM
	pH8	
Elution buffer	Urea buffer	
	Imidazole	0.5 M
	pH8	

Buffer for gel exclusion chromatography

Urea	6 M
ß-Mercaptoethanol	10 mM
in PBS	

Acetate buffer

Sodium acetate ($NaCH_3COO$)	5 mM
pH 3	

2.1.3.4 Buffer for purification of BTN^{exo}

PBS **see 2.1.3**

TBS

Tris/Cl pH7.5	100 mM
NaCl	150 mM (0.9%)

Tris/Cl stock solution

Dissolve 121g Tris base in 800 ml distilled H_2O for 1M Tris/Cl

Adjust pH with concentrated HCl (approximately 70 ml for pH7.4, 42 ml for pH8) - **pH is temperature sensitive!!!**

Add distilled H_20 to 1 l

2.1.3.5 Buffer for SDS-PAGE

Running gel (15%, 50 ml)	1.5 M Tris/HCl pH8.8	12.5 ml
	30% Acrylamide/Bis	25 ml
	10% SDS (Sodiumdodecylsulphate)	0.5 ml
	H_2O (Aqua bidest)	12 ml
	10% APS (Ammonium persulfate)	250 µl
	TEMED(N,N,N´,N´-Tetramethylethylenediamine)	25 µl
Stacking gel (12.5 ml)	0.5 M Tris/HCl pH 6.8	3.1 ml
	30% Acrylamide/Bis	2.5 ml
	10% SDS	250 µl
	H_2O (Aqua bidest)	6.8 ml
	10% APS	125 µl
	TEMED	12.5 µl
Running buffer (Laemmli)	Tris	25 mM (3 g/l)
	Glycine	190 mM (14.4 g/l)
	SDS	0.1% (w/v) (1 g/l)
Loading buffer, 10x	Tris/HCl, pH7.5	400 mM
	Glycerol	4.5 g/l
	Bromphenoleblue	10 mg/l
	SDS	150 g/l
	ß-Mercaptoethanol	1.5 M

Stock solutions

30% Acrylamide/Bis (Roth, ready to use)	Acrylamide	30% (w/v)
	Bisacrylamide	16 g/l

Store at 4°C in the dark

1.5 M Tris, pH8.8	Tris/HCl	1.5 M (182 g/l)
10 % SDS	Sodium lauryl phosphate	100 g/l

Store at RT, not 4°C!

0.5 M Tris, pH6.8	Tris/HCl	0.5 M (60.3 g/l)
10% APS	Ammoniumpersulfate	100 mg/ml

Always prepare freshly!

TEMED	Tetramethylethylenediamine	

Butanol – water saturated

2.1.3.6 Buffer for staining proteins in SDS gels

Coomassie Blue staining solution	Coomassie brilliant blue R250	2 g/l
	Ethanol	45% (v/v) (450 ml/l)
	Acetic acid 37%	90 ml/l
Destaining solution	Ethanol	45% (v/v)
	Acetic acid 37%	10% (v/v)

2.1.3.7 Buffer for Western Blot

Blotting buffer	Tris	25 mM (3 g/l)
	Glycine	190 mM (14.3 g/l)
	SDS	0.1% (w/v)
	Methanol	20% (v/v)
Wash buffer (TBS-T)	Tris/HCl pH7.6	20mM (2.4 g/l)
	NaCl	137mM (8 g/l)
	Tween 20	0.1% (v/v)
Blocking buffer	dried milk powder	5% (w/v) in TBS-T
Antibody-solution	dried milk powder	2% (w/v) in TBS-T

2.1.3.8 Determination of protein concentration according to Lowry

All reagents were used according to the SIGMA protein assay kit

2.1.3.9 Determination of protein concentration according to Bradford

Bradford stock solution was diluted 1/5 and used according to the BIORAD-instructions

2.1.3.10 Buffer for DNA analysis

TBE	Tris base	89 mM (10.8g/l)
	Boric acid	89 mM (5.5 g/l)
	EDTA (ethylenediamine tetraacetic acid)	2mM
	pH8 (with HCl)	(4 ml of 0.5M stock)
Agarose gels	Agarose	8-20 g/l in TBE
	Ethidium bromide	0.5 µg/ml

(choose agarose content according to the size of the product (0.8-2%), boil in microwave, add ethidium bromide after boiling, immediately prior to pouring the gel (cancerogenic))

Loading buffer	EDTA	100 mM
	glycerol	30% (v/v)
	bromophenole blue	0.25% (w/v)
	xylene cyanol	0.25% (w/v)

2.1.3.11 Buffer for quick plasmid isolation

STETL	Sucrose	8% (w/v)
	Triton X-100	5% (v/v)
	Tris/HCl pH8	50 mM
	EDTA	50 mM
	Lysozyme	0.5 mg/ml

Buffer can be stored at 4°C for months, but lysozyme needs to be added freshly

TE	Tris/HCl pH8	20 mM
	EDTA pH8	1 mM
DEPC-H2O	0.01% in H_20, leave ON, then autoclave	

2.1.3.12 Buffer for ELISA (Enzyme linked immunosorbant assay)

Coating buffer	NaN_3 (Sodium azide)	0.02 % in PBS
Block buffer	BSA (Bovine serum albumine)	1% (w/v)
	NaN_3	0.1% (w/v) in PBS
Wash buffer	Tween 20	0.5% (v/v)
	NaN_3	0.02% (w/v) in PBS
Substrate buffer	Diethanolamine	1 M
	NaN_3	0.02%
	$MgCl_2$ x 6 H_2O	4 mM
	pH 9,8 Store in dark.	
Substrate for alkaline phosphatase		
	p-nitrophenyl phosphate	0.5 - 1 mg/ml

2.1.3.13 Buffer for cytokine ELISAs

All cytokine ELISAs were performed according to the manufacturer´s instructions (Biosource) using the reagents provided.

2.1.4 Peptides

2.1.4.1 Rat MOG peptides (spanning the Ig-domain)

M_{1-26} GQF RVI GPG HPI RAL VGD EAE LPC RI

M_{14-39} ALV GDE AEL PCR ISP GKN ATG MEV GW

M_{27-50} SPG KNA TGM EVG WYR SPF SRV VHL

M_{38-60} GWY RSP FSR VVH LYR NGK DQD AE

M_{50-74} LYR NGK DQD AEQ APE YRG RTE LLK E

M_{63-87} PEY RGR TEL LKE SIG EGK VAL RIQ N

M_{76-100} IGE GKV ALR IQN VRF SDE GGY TCF F

M_{89-113} RFS DEG GYT CFF RDH SYQ EEA AVE L

$M_{101-120}$ RDH SYQ EEA AVE LKV EDP FY

M_{74-90} ESI GEG LVA LRI QNV RF

M_{93-109} EGG YTC FFR DHS YQE EA

M_{92-109} DEG GYT CFF RDH SYQ EEA

2.1.4.2 Rat MOG peptides for fine mapping of the DA epitopes

These peptides cover the rat MOG-Igd T cell epitopes of the DA rat. They were synthesized on a peptide synthesiser 431A (Applied Biosystems) by Dr. N. Groome, Oxford, UK and were at least 98% pure.

Peptide	Amino acid residues	Sequence
M 26	62-76	P E Y R G R T E L L K E S I
M 27	65-79	R G R T E L L K E S I G E G
M 28	68-82	R T E L L K E S I G E G K V A
M 29	71-85	L L K E S I G E G K V A L R I
M 30	74-88	E S I G E G K V A L R I Q N V
M 31	77-91	G E G K V A L R I Q N V R F S
M 32	79-93	G K V A L R I Q N V R F S D E
M 33	84-99	R I Q N V R F S D E G G Y T C
M 34	87-101	N V R F S D E G G Y T C F F R
M 35	90-104	F S D E G G Y T C F F R D H S
M 36	93-107	E G G Y T C F F R D H S Y Q E
M 37	96-110	Y T C F F R D H S Y Q E E A A
M 38	99-113	F F R D H S Y Q E E A A V E L
M 39	102-116	D H S Y Q E E A A V E L K V E

2.1.4.3 Bovine BTN peptides (spanning the first Ig-domain, Genosys)

B_{1-26} APF DVI GPQ EPI LAV VGE DAE LPC RL

B_{14-39} AVV GED AEL PCR LSP NVS AKG MEL RW

B_{27-50} LSP NVS AKG MEL RWF REK VSP AVF VS

B_{38-60} RWF REK VSP AVF VSR EGQ EQE GE

B_{50-74} VSR EGQ EQE GEE MAE YRG RVS LVE D

B_{63-87} AEY RGR VSL VED HIA EGS VAV RIQ E

B_{76-100} IAE GSV AVR IQE VKA SDD GEY RCF F

B_{89-113} KAS DDG EYR CFF RQD ENY EEA IVH L

$B_{101-120}$ RQD ENY EEA IVH LKV AAL GS

B_{74-90} DHI AEG SVA VRI QEV KA

2.1.4.4 Alanine-substituted BTN_{74-90}

BT 1 DHI AEG SVA VRI QEV KA

BT 2 **A**HI AEG SVA VRI QEV KA

BT 3 D**A**I AEG SVA VRI QEV KA

BT 4 DH**A** AEG SVA VRI QEV KA

BT 5 DHI A**A**G SVA VRI QEV KA

BT 6 DHI AE**A** SVA VRI QEV KA

BT 7 DHI AEG **A**VA VRI QEV KA

BT 8 DHI AEG S**A**A VRI QEV KA

BT 9 DHI AEG SV**G** VRI QEV KA

BT 10 DHI AEG SVA **A**RI QEV KA

BT 11 DHI AEG SVA V**A**I QEV KA

BT 12 DHI AEG SVA VR**A** QEV KA

BT 13 DHI AEG SVA VRI **A**EV KA

BT 14 DHI AEG SVA VRI Q**A**V KA

BT 15 DHI AEG SVA VRI QE**A** KA

BT 16 DHI AEG SVA VRI QEV **A**A

2.1.4.5 Rat BTN peptides (homologous to the immunogenic MOG-peptides in the DA rat)

Rat B_{74-90} ARL LDG LAT LRI RGV RV

Rat BTN_{92-109} DQG QYR CFL KDN DDS EEA

2.1.5 Primers

2.1.5.1 Construction of BTN-MOG-fusion protein for DNA vaccination

1. BTN sense 1 cag aag ccg cca cca tgg cag tct ttc caa act cc

2. BTN sense 2 cca gca tct tgc tgc cca gaa gcc gcc acc atg gc

3. BTN sense 3 aac tcg agc cca cca gca tct tgc tgc cca gaa gc

4. BTN sense 5 aca aac tcg agc cca cca gca tct tgc

5. BTN sense 6	aca aac tcg agc cca cca gc
6. BTN as TM-MOG	acc caa tag aag ggc ctt ggg aag aag gag gct gg
7. BTN-MOG TM sense	cct tct tcc caa ggc cct tct att ggg tca acc cc
8. mouse MOG IC as1.1	cga gga tcc gtc gag gct gat cag cgg
9. mouse MOG IC as1.2	cga gga tcc gtc gag gc

2.1.5.2 Verification of the quality of cDNA: rat ß-Actin

1. ß- Actin sense	TGC TAG GAG CCA GGG CAG TAA TC
2. ß-Actin antisense	TAC AAT GAG CTG CGT GTG GCC

2.1.5.3 Sequencing of rat BTN

1. **Mouse BTN sense 1** (placed in the mouse signal sequence)

CAG TTA GCT CAG AGA TGG CAG TTC CCA CC

2. **Mouse BTN sense 2** (placed in the mouse signal sequence)
   CTC CTG CCT CCT GGT CTG TCT GC
3. **Mouse BTN as 2** (placed in the mouse transmembrane region)
   GCT ACT ATC CAG GGA GTC AGC CTT GG
4. **Rat BTN as 6** (placed in the second Ig domain, identified in the first round of PCRs)
   GCA CTC CAG CTC CAT GTC TCC

2.1.5.4 Analysis of tissue specific expression

1. **rat BTN sense 8** (placed in the N-terminal IgV-like domain)

CCG AGG AGG CCG CTG TGC

2. **rat BTN as 8** (placed in the transmembrane region)
   CGA TTG CGA GAA ACC CTA AGG C

2.1.6 Antibodies for FACS analysis

antibody	**recognised antigen**
R73	C-region of α/β TCR
W3/25	CD4 (T helper cells)
G418	CD3 (TCR associated)
OX39	IL2-R (upregulated on activated T cells)
OX40	OX40 R
OX6	MHC class II

Cy5-labelled rabbit-anti-mouse $F(ab')_2$ (DAKO, Denmark), First antibodies were purchased at Serotec

2.2 Molecular biology methods

2.2.1 Preparation of RNAse free water

All RNA manipulations were performed with twice autoclaved pipet tips and tubes. All solutions were prepared with ultrafiltered and autoclaved H_2O.

0.01% Diethylpyrocarbonate (DEPC) was added to doubly distilled water and incubated for 24 h at room temperature, after which DEPC was destroyed by autoclaving to avoid inactivation of RNA.

2.2.2 Total RNA extraction from tissues or cultured cells

Total RNA was prepared by the single-step guanidine method according to manufacturer's instructions (Trizol, Gibco BRL). Tissues were flash-frozen in liquid nitrogen and stored at –80°C until use. Frozen tissues were dropped into 1 ml Trizol in a 2 ml polypropylene tube and homogenized with a Polytron (Kinematica) homogenizer. After 5 min incubation at RT, extraction was performed with 200 µl chloroform (vortexed, 2 min RT, centrifuged 12000 g, 15 min at 4°C). The upper aqueous phase was taken and RNA precipitated with 0,5 ml isopropanol (10 min at RT). After centrifugation (12000 g, 10 min at 4°C) the pellet was washed in 1 ml 75 % ethanol, centrifuged, dried and dissolved in 20-200 µl DEPC-H_2O. RNA concentration was measured by spectrophotometer at A_{260}.

2.2.3 Extraction of mRNA from tissues or cultured cells using Dynabeads

For small-scale preparations, mRNA was isolated using oligo-dT-coated Dynabeads obtained in the "mRNA direct micro kit", according to manufacturer's instructions (Dynal, Germany). Tissues were flash-frozen in liquid nitrogen and stored at –80°C until use.

40 µg Dynabeads were aliquoted into the RNAse free Eppendorf tubes and washed twice with Lysis/binding buffer.

5 mg tissue samples were ground on dry ice using a manual tissue grinder, then 200 µl Lysis/Binding Buffer was added and tissues were ground for a further 2 minutes. The lysate was centrifuged (30-60s, 13,000 rpm, RT) and can be stored like this at –80°C. Clear lysates were mixed with the pre-washed dynabeads by pipetting and incubated under gentle rolling for 5 min at room temperature. The beads were separated from the supernatant using a Dynabead magnet and washed twice with 100 µl washing buffer A and twice with washing buffer B. After this, the beads were resuspended in 60 µl ice cold 10 mM Tris-HCl. The mRNA was eluted from the

beads by incubation for 5 mins at 50°C and separated at 50°C in the preheated magnet. The supernatant containing the mRNA was transferred into fresh tubes and stored at –80°C. RNA concentration was measured by spectrophotometer at A_{260}.

2.2.4 Reverse transcription of RNA to cDNA

Reverse transcription of RNA to cDNA was performed according to manufacturer's instructions (Gibco BRL) in a total volume of 20 µl. For 1 µg total RNA, 1 µl oligo $(dT)_{12-18}$ (500 µg/ml) and H_2O were added to a volume of 12 µl. Mixture was heated to 65°C for 10 min and chilled on ice. The following components were added:

4 µl 5x 1st strand buffer
2 µl 0.1 M DTT (Dithiothreitole)
1 µl 10 mM dNTP mix

After 2 min incubation at 42°C, 1 µl Superscript II was added and incubation was continued for 80 min. Reaction was stopped by heating at 70°C for 15 min. 1 µl were taken to perform a PCR reaction.

2.2.5 Polymerase chain reaction (PCR)

The following reagents were added for a PCR reaction:

x µl cDNA
2.5 µl 10x PCR buffer
0.5 µl 10 mM dNTP mix
0.5 µl 5' primer (20 µM)
0.5 µl 3' primer (20 µM)
0.625 units DNA polymerase
H_2O to a total volume of 25 µl

Samples were amplified in a thermocycler (Perkin-Elmer) with the following standard conditions if not otherwise stated:

4 min at 94°C (denaturation), then 25-35 cycles with
1 min at 94°C (denaturation)
1 min at 54-70°C (annealing)
0.75-1 min at 72°C (elongation)

After the last cycle elongation was continued at 72°C for a further 10 min, then samples were cooled to 4°C and 8-10 µl were loaded onto an agarose gel for electrophoresis.

The annealing temperature for each primer was calculated using the formula

$$(4n + 2m – 4)°C,$$

with n= the number of C or G in the primer, and m= the number of A or T in the primer

Pfu *(Pyrococcus furiosus)* DNA polymerase (Stratagene) was used for all cloning reactions, because of its proofreading activity, Taq *(Thermus aquaticus)* (QIAGEN) was used in the sequencing reactions and expression analysis, because of higher efficiency and lower price. All sequences were confirmed at least once on independent PCR products.

2.2.6 Separation of DNA fragments by agarose gel electrophoresis

Separation of DNA fragments was performed as described by McDonell et al., 1997 and Ausubel et al., (Current Protocols) on 0.8 -1.2 % agarose gels with 0.5 µg/ml ethidium bromide in 1 x TBE at 5-10 V/cm. 0,2 volumes of loading mix were added to the DNA before loading on the gel. The fragments were separated by electrophoresis according to size and can be visualized in UV light due to the presence of ethidium bromide intercalated between the DNA strands. Ladder Mix (MBI Fermentas) was used as size standard.

2.2.7 Determination of DNA concentration

Concentration of DNA, RNA and oligonucleotides was determined by measuring the absorption at 260 and 280 nm by spectrophotometer (GeneQuant II, Pharmacia). The maximum of absorption of nucleic acids is at 260 nm, the maximum absorption of proteins at 280 nm. At 260 nm with a 1 cm pathway, an optical density of 1.0 corresponds to 50 µg/ml of double-stranded DNA, 37 µg/ml of RNA and 20 µg/ml for oligonucleotides. Purity of DNA was determined by a 260/280 ratio, which had to be higher than 1.7. Very pure DNA solutions have a ratio 260/280 of between1.8 and 1.95, very pure RNA solutions between 1.9 and 2.0.

2.2.8 Isolation of DNA fragments from agarose gels

To isolate individual DNA fragments from mixtures, e.g. after restriction, DNA was separated by agarose gel electrophoresis. The desired fragments were cut from the gel and DNA was isolated using a QIAGEN Gel extraction kit (QIAGEN) according to manufacturer's instructions.

2.2.9 Purification of DNA

To remove contaminants and primer from DNA, PCR products were purified using QIAGEN PCR purification kit (QIAGEN) according to manufacturer's instructions.

2.2.10 Restriction of DNA

1 - 5 U of restriction enzyme(s) were added for 1 µg DNA in the appropriate restriction buffer. Volume of buffer used was at least 10 times the volume of enzyme added, as glycerol in the enzyme storage buffer may inhibit the reaction/decrease specificity. The restriction mix was incubated 2 h at 37°C or according to manufacturer´s instructions (NEB, New England Biolabs).

2.2.11 Dephosphorylation of linearised vectors

To inhibit vector religation, the 5' end phosphate group were removed from the linearised vector. 1 µl of shrimp alkaline phosphatase (USB) was added directly to the restricted DNA in 20 µl of the restriction buffer. The mix was incubated for 1 h at 37°C and DNA was then purified using QIAGEN PCR purification kit

2.2.12 Ligation

DNA fragments with compatible ends were ligated with T4 DNA ligase (Boehringer, Mannheim). The amount of insert used was approximately 3 times that of vector as judged in ethidium bromide stained agarose gels. 1 U enzyme in 20 µl ligation buffer (Boehringer Mannheim) was added to the appropriate mixture of vector and insert DNA and the reaction was incubated overnight at 16°C. Linearised, dephosphorylated vector in the absence of the insert was ligated for the negative control

2.2.13 Generation of RbCl-competent cells for transformation

A fresh *E. coli* culture (DH5α or JM109) was prepared by inoculating 5 ml MTB medium with a single colony and incubating for 37°C/200 rpm up to $O.D._{550}$ (approx. 2h). This starter culture was used to inoculate 100 ml MTB medium and grown to a density of $O.D._{550}$ 0.5. The cell suspension was chilled on ice and centrifuged at 7,000 g for 5 min at 4°C. Cells were resuspended on ice in 30 ml ice cold buffer TF1 and centrifuged again. The pellet was

resuspended very carefully on ice in 4 ml ice cold TF2 and stored in 100 µl aliquots at –80°C. The final transformation efficiency was approximately 10^6 cells/µg DNA.

2.2.14 Transformation of bacterial cells by heat shock transformation

50 µl of competent cells were thawed on ice and incubated with 10 µl ligation mix for 20 min on ice. The cells were then transferred into 37°C for 2 min, followed by incubation on ice for up to 30 min (heat shock, uptake of plasmid). After this 1 ml LB-medium (without antibiotics) was added. The cell suspension was incubated at 37°C for 1 h on a shaker in a polypropylene tube and then plated on LB-agar plates containing the appropriate selective antibiotics and incubated over night at 37 °C. Plates can be stored at 4°C.

2.2.15 Plasmid DNA preparation

2.2.15.1 Rapid plasmid purification

3 ml LB medium were inoculated with a single bacterial clone and incubated over night at 37°C. Cells were centrifuged for 1 min at 13,000 rpm and lysed in 150 µl STETL buffer for 1 min in a boiling water bath. Bacterial remains were removed using a toothpick and the plasmid DNA in the remaining supernatant was precipitated with 180 µl Isopropanol for 10 min at room temperature, after which it was centrifuged for 10 min at 13,000 rpm. Pellets were washed in 70% ethanol and taken up in 50 µl TE after drying.

2.2.15.2 Small scale plasmid preparation using QIAGEN plasmid purification kit

In order to sequence plasmids, plasmids containing inserts of the correct size were purified using the QIAGEN plasmid purification kit according to the manufacturers instructions. In short, bacteria are lysed with SDS and NaOH that denature DNA and proteins. The mixture is neutralized, causing the covalently closed plasmid DNA to reanneal rapidly. While the chromosomal DNA and proteins precipitate, the plasmid DNA in the supernatant is washed and concentrated by isopropanol and ethanol precipitation. Maximum yield was 20 µg.

2.2.15.3 Mega- and Gigapreparations

For preparing large amounts of plasmid for DNA vaccination, Endo-free Mega- and Gigapreparations (Qiagen) were performed according to the manufacturers instruction, following the same principle of alkaline lysis. Maximum yield is 2,5 mg (Megaprep) and 10 mg (Gigaprep), derived from 500 ml or 2.5 l cultures, respectively.

Quality of plasmid DNA was verified by spectrophotometer and by agarose gel after restriction with appropriate DNA endonucleases.

2.2.16 DNA sequencing

Sequencing of DNA was performed by Toplab or Willy Metzger in Martinsried, the sequencing for the TCR spectratyping was carried out by Frau Ingrid Eiglmeier in the neuroimmunology department of the Klinikum Großhadern.

2.2.17 Glycerol stocks

After verification of the insert, bacterial clones were expanded over night in 5 ml LB medium at 37 °C. The solution was diluted 1/1 in sterile cold glycerol and stored at –80°C.

2.2.18 Cloning of the BTN-MOG fusion construct

The templates for the construction of this plasmid were kindly provided by Dr. Carole Bourquin (pcDNA-MOG construct, E.J.I., 2000 encoding mouse MOG under a CMV promoter in pcDNA3.1(-), from Invitrogen) and by Dr. I. Mather and Sherry Ogg in Maryland, who provided cDNA encoding the signal sequence and the extracellular domains of bovine BTN.
A 5´non-translated region containing an optimised Kozak sequence as well as a Xho I restriction site was added to the 5´end of the BTN signal sequence by sequential PCRs using BTN sense primers 1-6 (Figure 2.1, step 1, left) and the anti-sense primer BTN as-TM-MOG. This antisense primer not only served for the amplification, but also added the first 14 nucleotides encoding the MOG-TM region to the construct, fused in-frame to the butyrophilin extracellular domains (Figure 2.1, step1, left). In an independent reaction, the sequence encoding the transmembrane and intracellular domains of mouse MOG was amplified by PCRs using primers BTN-MOG TM sense and MOG cterm as 1 and 2. Once again, the primer used for the fusion to MOG (in this case the sense primer) contained an additional sequence of 14 nucleotides, homologous to the last 14 3´nucleotides encoding the bovine BTN-extracellular domain, fused in-frame to the MOG-transmembrane sequence. The antisense primers introduced a BamHI endonuclease restriction site to the 3´region of the MOG-intracellular domain (Figure 2.1 step1, right).
The two products were fused together in a new PCR reaction of 10 cycles, in which the overlapping 28 nucleotides served as internal primers (Figure 2.1 step 2). After these 10 cycles,

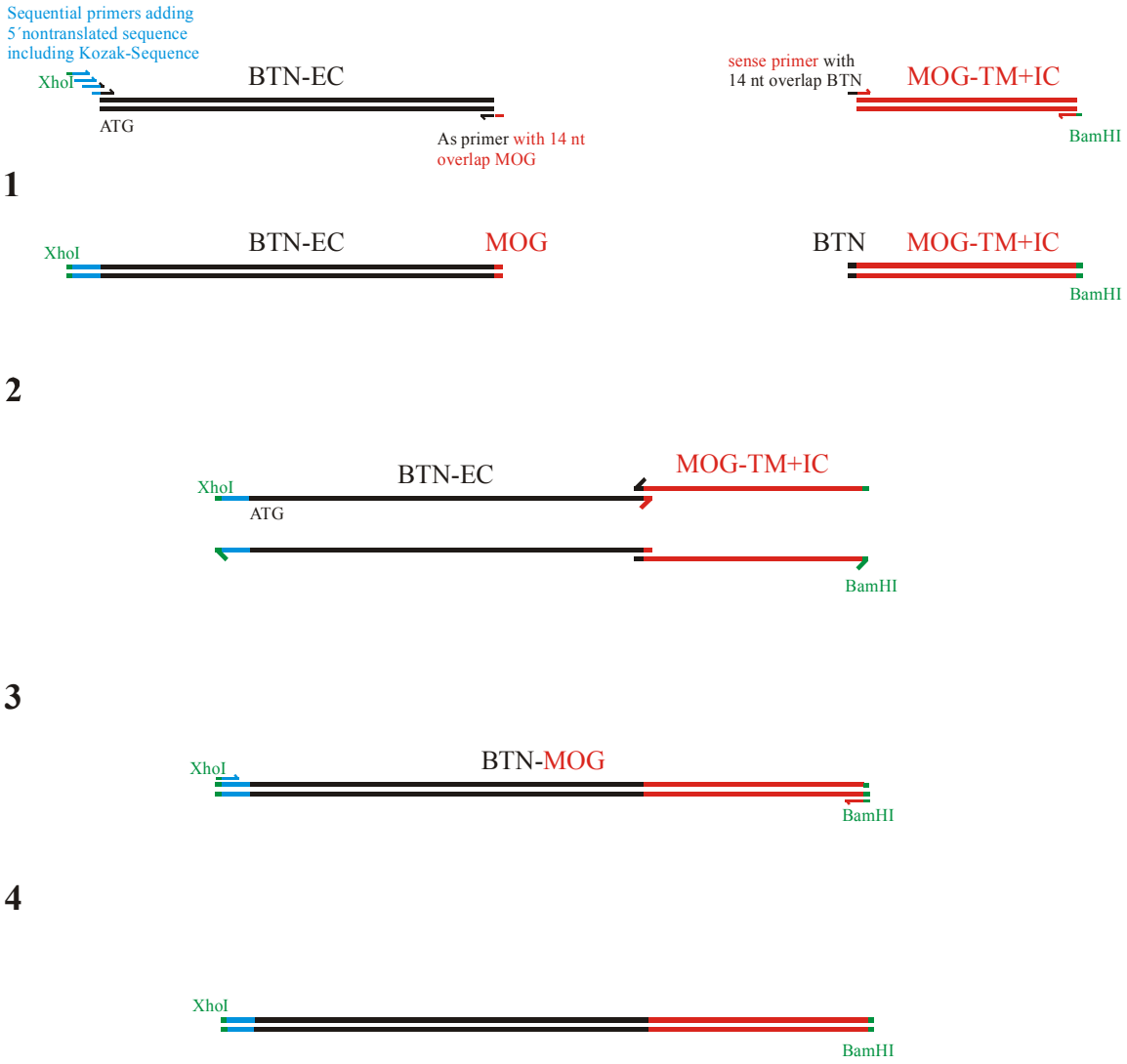


Figure 2.1 Cloning scheme for BTN-MOG fusion construct

In the first step, serial PCRs were carried out to add a 5´nontranslated sequence containing a XhoI endonuclease restriction site and an optimised Kozak sequence to the cDNA encoding the extracellular domains of bovine BTN. In addition, the 3´end of this cDNA was fused with 15 additional nucleotides, which are homologous with the 5´end of MOG-TM/IC. Simultaneously, 14 additional nucleotides were added to the 5´end of the cDNA encoding the transmembrane and intracellular domains of MOG, which are homologous to the 3´end of BTN-EC. A BamHI endonuclease restriction site was added to the 5´end of MOG-TM/IC.

In step 2, the two cDNAs were incubated together for 10 PCR cycles in the absence of additional primers. In this step, the overlapping nucleotides, which were added in step one, serve as internal primers to obtain fused templates

In step 3, primers were added to the assay of step 2.

The following PCR leads to the amplification of the fusion cDNA (step 4). The obtained product was then digested with BamH1 and XhoI and ligated into pcDNA 3.1 (-), which had also been digested with BamHI and XhoI.

primers BTN sense 6 and MOG cterm 2 were added (Figure 2.1 step 3), and the fusion product was amplified in another 30 cycles (Figure 2.1 step 4). The PCR product of 1,100 nt length was purified using the QIAGEN gel extraction kit, digested with XhoI and BamHI and ligated into pcDNA3.1(-) which had been digested with the same enzymes. Sequence and correct insertion were verified by Toplab, Martinsried, using T7 and pcDNA3.1 antisense primer, located behind the multiple cloning site.

2.2.19 Sequencing of rat butyrophilin

The extracellular domain of rat butyrophilin was sequenced using primers derived from the published mouse sequence. Primers were placed in the signal sequence and the transmembrane regions, because these are regions, which are highly conserved between the mouse, rat and bovine gene. Primer rat BTN6 was homologous to a previously identified rat sequence. All sequences were confirmed at least twice in independent PCR and sequencing reactions.

2.2.20 Sequence comparisons

Sequence comparisons were performed using the BLAST program provided by the NIH (www.ncbi.nlm.nih.gov).

2.2.21 Analysis of the tissue specific expression of rat BTN

To verify tissue-specific expression of rat BTN, RT-PCR was carried out in a variety of DA rat tissues using primers placed in the N-terminal IgV-like domain (rat BTN sense 8) and in the transmembrane region (rat BTN as 8) of the rat-BTN-sequence. This results in the amplification of a 393 nt fragment of the mRNA and covers (parts of) the exons encoding the IgV-like domain, the IgC-like domain and the transmembrane region (amino acids 105-236). The genomic sequence would therefore presumably contain two introns (As seem in the mouse, gene accession number U67065). This excludes the amplification of genomic DNA, which would be several thousand nucleotides long. The PCR was done in the presence of 1 mM $MgCl_2$ and run for 35 cycles. Each step (denaturation, primer annealing (58°C) and elongation) lasted 1 minute.

2.2.22 TCR spectratyping

This is a RT-PCR technique based on the length polymorphism in the CDR3 (complementarity determining region) of the TCR and the fact that each T cell expresses a single β chain. Primers are placed in the individual Vβ regions and the constant domain of the TCR and thus span the CDR3, which is the area in which the TCR rearrangement occurs during T cell maturation in the thymus (joining of different D and J regions with a V region, combined with junctional diversity). Fluorescence-labelled nucleotides are used for the PCR reactions. The PCR products are then separated on polyacrylamide gels (Long ranger single packs; Biozym, Hess. Oldendorf) (Figures 3.2.7). To facilitate analysis, size of product and intensity of fluorescence are represented as histograms (Figure 3.2.8 and 3.2.9). TCR spectratyping was performed by I. Eiglmeier and Dr. N. Goebels, Klinikum Großhadern, Munich, Germany

2.3 Biochemical methods

2.3.1 SDS PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) and Ausubel (Current Protocols in mol. biology chap 10.2 A) with the Mini-Protean II system (Bio-Rad) with 1.5 mm spacers. Electrophoresis was performed in Laemmli buffer at 150 V for 1 to 2 h. All samples were mixed with 0.2 Vol. 5 x loading buffer and incubated at 95°C for 5 min before loading. Molecular weight markers used were prestained and unstained broad range (Bio-Rad).

2.3.2 Staining of proteins in polyacrylamide gel

The gels were stained with Coomassie Blue staining solution for at least 60 min, then incubated for at least 60 min in destaining solution to visualise the separated proteins in polyacrylamide gels

2.3.3 Western blotting

2.3.3.1 Electrotransfer of protein from a polyacrylamide gel onto a nitrocellulose membrane

The electrotransfer of proteins onto a nitrocellulose membrane was performed according to Towbin et al., 1979 and Ausubel et al., Current Protocols in mol. biology chap 10.8). After separation of proteins by SDS-PAGE, the gel was placed on a nitrocellulose membrane

(Hybond ECL, Amersham) soaked in TBE buffer. Gel and membrane were surrounded on both sides by buffer saturated Whatman paper No I. Electrotransfer was performed in a blotting chamber (Bio-Rad) for 1 h at 100 V and 4°C. To verify successful transfer of protein and note the position of the marker, the membrane was stained for 5-15 min in 0,2 % Ponceau solution (Sigma) and destained with H_2O.

2.3.3.2 Detection of membrane-bound protein by chemoluminescence

To detect the presence of membrane-bound protein with specific antibodies, unspecific binding sites on the membrane were first blocked with blocking buffer for 1 h at room temperature (RT). The membrane was then incubated with the specific unlabelled antibody or serum at the appropriate concentration for 1 h at RT. After washing 3 x in TBS-T, the membrane was incubated in the appropriate concentration of the second peroxidase-conjugated antibody for 1 h. The membrane was again washed 3 x and detection by chemiluminescence was performed according to manufacturer's instructions (ECL, Amersham).

2.3.4 Determination of protein concentration according to Lowry

In this assay, tyrosine residues in the soluble protein react with a Folin-Ciocalteu reagent to form a protein-dye complex with a maximal absorption at 600 nm. The assay was performed according to manufacturer's instructions (Sigma) with different dilutions of protein solution and absorption was measured at 600 nm. A calibration curve was established using bovine serum albumine (BSA, SIGMA) and concentration of the test sample was calculated from the absorption value.

2.3.5 Determination of protein concentration according to Bradford

In this assay, the Coomassie brilliant blue G250 forms a protein/dye complex with arginine residues of proteins, with a maximal absorption at 595 nm. 1-10 µl of the protein solution were incubated with 1 ml Bradford reagent (1/5 diluted from the stock solution) for 10-30 min at room temperature (RT). A calibration curve was established using bovine serum albumine (BSA, SIGMA) and concentration of the test sample was calculated from the absorption value. The method can be used for end concentrations of 1-10 µg/ml.

2.3.6 Purification of recombinant MOG-Igd from bacteria

The expression of recombinant MOG-Igd was performed in the *Escherichia coli* - strain DH5α Fiq, which had been transformed with pQE12 (Qiagen) containing the cDNA coding for the extracellular domain of rat MOG (amino acids 1-125). Gene expression in pQE 12 is under regulation of the IPTG inducible lac promoter. The plasmid also codes for 6 C-terminal histidines, allowing purification by nickel-chelate chromatography (Amor et al., 1994) and contains an ampicillin resistance gene. Transformed bacteria are selected with kanamycin (25 mg/l) and ampicillin (100 mg/l). Kanamycin resistance is encoded on the Fiq episome.

2.3.6.1 Expression of recombinant protein in bacteria

Culture medium (1.2 l) was inoculated with a transformed *E. coli* glycerol stock and incubated over night at 37°C on a shaker. After centrifugation at 5,000 rpm for 20 min, the bacterial pellet was resuspended in 12 l of selective culture medium without glucose (glucose is a repressor of the lac promoter) and shaken at 37°C. When the absorption at 600 nm reached 0.7, 10 ml of 0.1 M IPTG (BioTech Trade) were added per liter of culture to induce MOG-Igd expression. Incubation was continued for a further 3-4 h, after which the bacteria were centrifuged (60 min, 5,000 rpm (max. 10,000rpm), 4°C), resuspended in 200 ml PBS and centrifuged again. The pellet was stored over night at –20°C.

2.3.6.2 Lysis of bacteria

The bacterial pellet was resuspended in 65 ml PBS and sonicated for 30 min on ice (Branson Sonifier 450) to lyse the cells. Like most recombinant proteins, MOG is sequestered in inclusion bodies in E coli and is insoluble in PBS. After centrifugation (4°C, 16,000 rpm in SS34 rotor, 30 min) the pellet containing the MOG-Igd inclusion bodies and other insoluble proteins was resuspended in urea buffer and sonicated for 30 min to dissolve the inclusion bodies. The suspension was again centrifuged and the supernatant containing the dissolved MOG was used for affinity chromatography, while the pellet was re-sonified in 8 M urea buffer before adding it onto the same column.

2.3.6.3 Nickel-Chelate Affinity Chromotography

The column (40 ml chelating sepharose, Pharmacia) was washed sequentially with distilled water and with 1% EDTA/0.05% Tween 20 to remove the 70 % ethanol used for storage and any remaining nickel, followed by another wash with water. The column was loaded with a 1% nickel chloride ($NiCl_2$) solution, washed with dH_2O and equilibrated with urea buffer. The column was then loaded with the sample at a rate of 0.5 ml/min and washed with at least 200 ml wash buffer. The elution was performed with an increasing imidazole gradient (40 mM – 500 mM over 200 ml). 2 ml fractions were collected and analysed by SDS-

PAGE. The protein concentration in the eluate was monitored by photometer (280 nm). The column was then washed first with 1% EDTA, 0.05% Tween 20 to remove all nickel and then with H_2O and stored in 70% ethanol.

2.3.6.4 Dialysis

After analysis by SDS-PAGE, protein-containing fractions were pooled and transferred into dialysis tubes (exclusion 3,500 Da, Biomol). Dialysis was performed for 48 h against 2 x 5 l 10 mM acetate buffer pH3 at 4°C. Protein concentration was determined and purity was verified by SDS-PAGE. Protein was then concentrated to 2 mg/ml using Macrosep centrifugal concentrators (PALL FILTRON, cut off: 10 kDa)

2.3.7 Expression of bovine BTN^{exo} using the Baculovirus system

The extracellular domains of bovine butyrophilin were expressed using the baculovirus system in Sf9 (derived from pupal ovarian tissue of the fall army worm, *Sporodoptera frugiperda*) and HiFive (derived from ovarian cells of the cabbage looper, *Trichoplusia ni*) insect cells (Invitrogen). This system allows the production of native, glycosylated proteins, fused to a hexa-histidine tag. BTN is secreted into the cell culture medium and thus easily purified. Unfortunately the yield of the proteins was low in this system, but attempts to express BTN^{exo} in bacteria were unsuccessful both in Munich and Maryland.

The Baculovirus construct (derived from *Autographa California* multiple nuclear polyhedrosis virus (AcMNPV)) was kindly provided by Sherry Ogg and Dr. Ian Mather at the University of Maryland. It contains the cDNA encoding the signal sequence and the two extracellular domains of bovine butyrophilin under the control of the polyhedrin promoter (an important protein component of the membrane of occluded viruses, therefore highly expressed. Since the occluded virus is a replicative form, which is not essential in cell culture, the polyhedrin gene could be replaced by the BTN-cDNA), leading to strong expression of the transgene.

Protein was expressed according to the Invitrogen "MaxBac 2.0 Expression and Purification Manual" at 27°C, using TNM-FH or High 5 media purchased from Invitrogen and completed with Penicillin/Streptomycin and Serum. High titre virus stock was prepared in adhesive cultures of Sf9 cells, protein was obtained from suspension cultures of High five cells (2 x 10^6cells/ml) after 1/10 dilution of the high titre stock in the culture medium. The protein was harvested 2-3 days after transfection of the High five cells with the high titre virus stock. Cells were centrifuged (3,000 rpm,10 min, 4°C), and the supernatant was dialysed for 48 h against at least 10 l of first TBS and then PBS.(exclusion size 12-14 kd). Protein was then

purified via Nickel-Chelate chromatography, as described for MOG-Igd, but using PBS as the buffer (elution with 500 mM Imidazol in PBS, pH8). Eluate fractions were analysed by SDS-PAGE, and protein-containing fractions were pooled and concentrated using a Macrosep centrifugal concentrators (PALL FILTRON, cut off 10kDa).

2.3.8 Enzyme-linked immunosorbent assay (ELISA)

This technique (Engvall and Perlmann, 1971) allows the detection of antibodies that bind to a plate-bound antigen or antibody by an enzymatic reaction and was used to determine serum antibody titers and specificities.

96-well ELISA plates (polyvinyl coating, Costar) were coated for 1 h at 37°C or over night at 4°C with 5 µg/ml antigen in coating buffer (100 µl/well). The plates were washed 3x with wash buffer (ELISA-washer, NUNC), and unspecific binding sites on the plate were blocked with 200 µl/well blocking buffer for at least 1 h at 37°C or over night at 4°C. After this, the plates were washed again and 100 µl rat serum were added to each well at an appropriate dilution in coating buffer (often serial dilutions) and incubated at least 1 h at 37°C, followed by washing. Bound antibodies were then detected using 100 µl/well anti-rat antibodies (1h, 37°C), which were either directly coupled to alkaline phosphatase (anti-rat-IgG(H+L), anti-rat-IgG and anti-rat IgM (Southern Biotechnology Associates, Birmingham, Al, USA)) or biotinylated (IgG1, 2A, B and C)(Pharmingen), and which were diluted 1/2000 - 1/4000 in coating buffer. Plates were washed 3 times. For the biotinylated antibodies, an additional incubation step was required with 100 µl/well Streptavidin-alkaline phosphatase conjugate in coating buffer (Pharmingen, 1/2000), again 1h at 37°C, followed by washing.

To develop a colored reaction, p-nitrophenyl phosphate was used as substrate for the alkaline phosphatase. 100 µl substrate buffer were added in each well and reaction was developed for 5 to 60 min. The plate was read at 405 nm in a spectrophotometer (MR 4000 ELISA Reader, Dynatech) at different time points.

2.3.9 Cytokine ELISA

The measurement of cytokines (IL-10, IFN-γ, TNF-α and TGF-β) in T lymphocyte culture supernatant was also performed by ELISA. In this assay cytokines were detected by sandwich ELISA, in which plates were coated with an anti-cytokine antibody. Binding of secreted cytokines was detected by a biotinylated anti-cytokine antibody followed by streptavidin coupled to peroxidase. Assays were performed according to manufacturer's instructions (kits from R & D Systems (TGF-β) and Biosource (IL-10, IFN-γ and TNF-α)). In

each case, a calibration curve was determined for standard concentrations and titer of the sample was calculated from the absorption value at 450 nm.

2.4 Animal experimentation

2.4.1 Active immunisation of rats with Freund's adjuvant

The antigen solution was mixed with an equal volume of complete or incomplete Freund's adjuvant (Gibco, BRL). The mixture was forced through a 24G connector (Hamilton, 86509, FEM. HUB, GA24, 50 mm) fixed between 2 syringes until a thick emulsion was obtained. Rats were injected subcutaneously into the tail after ether anesthesia with 100 µg of antigen in 100 µl of emulsion.

2.4.2 Clinical evaluation of EAE

After immunisation, rats were weighed and clinically assessed on a daily basis. The clinical signs of EAE were evaluated according to the following scale:

0: no signs of disease

1: loss of muscle tone of tail

2: paraplesia of hind limbs

3: paralysis of hind limbs

4: moribund

5: dead

Half-points were also given.

In accordance with federal guidelines, animals with a score of 3.5 for 2 consecutive days or reaching a score of 4 were sacrificed.

2.4.3 EAE-induction via transfer of MOG- or MOG-peptide specific T cells

MOG- or MOG-peptide specific T cell lines were restimulated for three days in the presence of their selecting antigen and irradiated thymocytes, which were used as antigen presenting cells (APCs). After this, the cells were centrifuged for 12 min at 4°C and 12,000 rpm and washed twice in EH. 1.5 – 50 x10^6 T cell blasts were injected i.p. in 2 ml EH or alternatively in 1 ml EH into the tail vain.

2.4.4 DNA vaccination of rats

For DNA vaccination, the plasmid DNA was injected intramuscularly into the tibialis anterior muscle. To avoid a too deep insertion of the needle, a tubing was inserted over the 27G canula, covering all but 4-5 mm of the tip. Female rats (age 4-5 weeks) were anesthesized with ether and injected in each tibialis anterior muscle with 200 µg DNA in PBS (1 mg/ml). MOG-specific antibody titers in serum were verified by ELISA 2-8 weeks after immunisation. For vaccination with two different constructs, 400 µg of each plasmid were injected into a single muscle.

2.4.5 Intranasal application of peptides

6-8 week old female DA rats were treated for 10 consecutive days with 50 µg peptide (1 mg/ml), i.e. 25 µl per nostrils, applied using a micropipette. Three days after the last treatment, the animals were immunised with 100 µg MOG-Igd in IFA.

2.4.6 Intravenous application of serum or peptides

For HDSA treatment, 0.5 ml of a 2 mg/ml peptide solution were injected into the tail vein 2 and 4 d.p.t. of freshly restimulated T cell blasts (1.5 - 50 x 10^6). For serum transfer, 1 - 2 ml of serum were injected at various d.p.i. into the tail vein.

2.4.7 Treatment with Minocycline

Minocycline (SIGMA) was dissolved to 45 mg/ml in PBS. Animals were treated daily i.p. with 45 mg/kg body weight (ca 150-180 µl).

2.4.8 Preparation of serum

500-1,000 µl of blood were collected in 1.5 ml polypropylene tubes from the tail vein. Blood was left to coagulate a few hours at room temperature (RT) or overnight at 4°C. It was then centrifuged 10 min at 1,200 rpm, supernatant was taken and centrifuged again. This step was repeated until supernatant was clear. The serum obtained was stored at –20°C. Alternatively, blood was collected after dissection of the vena cava superior if the animals were to be killed

2.4.9 Perfusion of rats

For histopathological examinations rats were perfused with paraformaldehyde (PFA) solution (4% in PBS). Under ether narcosis rats were thoracotomised and the upper vena cava was sectioned. 50 ml of PFA solution were injected slowly into the left ventricle. Brain and vertebral column were isolated, postfixed overnight in PFA solution, then stored in PBS at 4°C. Histopathological examination was carried out on paraffin embedded sections by Drs. R Höftberger, M. Storch and A, Stefferl in the group of Prof. H. Lassmann, Vienna.

2.5 Cell biology methods

2.5.1 Preparation of a single cell suspension from rat tissues

Thymus was derived from naïve animals, superficial inguinal lymph nodes and/or spleen were isolated from immunised rats at different time points after immunisation d.p.i.s in aseptic conditions and immediately placed in EH medium on ice. Single cell suspensions were prepared by passing the tissues through a glass homogeniser, after which they were washed twice in 35 ml EH.

2.5.2 Establishment of antigen-specific T cell lines

2.5.2.1 Primary culture of T lymphocytes

Lymph node cells or splenocytes in single cell suspension were incubated in restimulation medium in the presence of selecting antigens for 3 days at 37°C with 10 % CO_2 at a density of 1 x 10^7 cells/ml. T cell specificity assays were carried out in parallel (section 2.5.2.4). For cytokine ELISA, cells were incubated at a density of 6 x 10^6 cells in 2 ml restimulation medium in 12-well plates in the presence or absence (background) of selecting antigen. Supernatant was taken at 72 h and stored at –20°C.

2.5.2.2 Expansion of the T cells in IL-2-containing medium

After three days of primary culture, the cells were centrifuged and washed with EH and transferred into the IL-2 containing TCGF-medium at a concentration of approximately 5 x 10^5 cells/ml. 10 ml of this suspension were plated into 10 cm culture plates. The cells were split in two whenever they reached a density of about 2 x 10^6 cells/ml.

2.5.2.3 Restimulation of the T cells

Four to seven days after the transfer into TCGF, the size and proliferation rate of the cells was dramatically reduced and the cells had to be restimulated in the presence of freshly prepared irradiated thymocytes serving as antigen presenting cells (APCs) and their selecting antigens. After washing twice, the thymocytes were resuspended in 5 ml EH and irradiated with 4,000 rad in order to inhibit their proliferation. The cells were then counted and 10^8 thymocytes were mixed with 2-4 x 10^6 T cells. This mixture was then centrifuged and resuspended in 5 ml restimulation medium/10^8 thymocytes. 5 ml were plated into 6 cm culture dishes or alternatively diluted ¼ for specificity tests in 96-well plates and incubated at 37°C, 100% humidity and 10% CO_2. For cytokine analysis, 2 to 5 x 10^5 T cells were cultured with 9-11 x 10^7 thymocytes in 2 ml restimulation medium in 12-well plates.

2.5.2.4 T cell specificity assay

In this assay, which is performed in parallel with each primary culture and restimulation, T lymphocytes are incubated in the presence of different antigens and/or various concentrations of antigen. Tritium-labelled thymidine is incorporated into the newly synthesized DNA of dividing lymphocytes, thus giving a measure of antigen-specific proliferation of T lymphocytes. 5 x 10^5 lymph node cells or splenocytes (or 2-4 x 10^4 T cells with 10^6 irradiated thymocytes) were added in 200 µl of restimulation medium per well into a 96-well plate in the presence of specific antigen, the mitogen Con A or in the absence of antigen. Cells were incubated for 2 days at 37°C with 10 % CO_2. 20 µl of ^{3}H-thymidine stock solution were added (final concentration 1 µCi/well) and cells were incubated for a further 16-18 h. Cells were then transferred to a fiberglass filter, washed and specific activity was measured in a beta-counter (Matrix 96 Direct Beta Counter, Packard). The stimulation index (SI) is determined as the ratio of the proliferative responses (c.p.m.) in the presence and absence of antigen. An SI larger than 2 is considered significant.

2.5.2.5 Isolation of T cell blasts by density gradient centrifugation

After the three days of restimulation, T cell blasts were isolated by density gradient centrifugation. The cells were centrifuged and the content of 2-3 6 ml plates was resuspended in 3 ml Lymphoprep (1.077g/ml density, 280 mOsmol, contains 9.6% sodium-metrizoate and 5.6% polysaccharide) and carefully overlayed with 3 ml EH. The cells were then centrifuged for 30 min at 4°C and 3,000 rpm (2,500 g)(breaks off!), after which T cell blasts could be found in

the interphase, while small cells or debris were in the pellet. Blasts were washed in serum containing medium to avoid clumping, then propagated for 4-7 days in TCGF.

2.5.3 Determination of cell numbers

Trypan Blue staining differentiates between live and dead cells, as the membrane of dead but not of live cells is permeable to the stain. The cell suspension was mixed with Trypan Blue at the appropriate dilution (1/2 – 1/10) and 10 µl were transferred to a Neubauer hemocytometer chamber. 100-200 live cells (unstained, round and bright) were counted under the microscope. Cell number was calculated as follows:

[Cells in one big square] x [dilution factor] x 10^4 = cells/ml

2.5.4 Freezing and thawing of cells

Cells were taken up in ice-cold freezing medium and stored at –18°C for 1 h and then transferred to –80°C. All cell types were thawed rapidly and immediately transferred into serum containing medium. T cells, Ag8 and MLA cells were then centrifuged to get rid of all DMSO, resuspended in their culture medium and incubated at 37°C, 10% CO_2 and 100% humidity.

2.5.5 Culture of the monkey cell line MLA

The monkey cell line MLA (Chen et al., 1985) was grown in MLA culture medium at 37°C, 100% humidity and 10 % CO_2. Cells were diluted 1/2 in fresh medium whenever the medium had turned yellow. Cell supernatant was aliquoted and stored at –18°C.

2.5.6 Culture of insect cell lines High five and SF9

Insect cells were kept according to the Invitrogen manual " Growth and Maintenance of Insect Cell Lines". In short, insect cells were thawed and first kept as adherent cultures in their appropriate medium at 27°C. Cells were monitored daily and split upon reaching confluency in order to keep them in log-phase. SF9-cells were infected with low titre of Bacculovirus for the creation of high titre stocks. High Five^R cells were transferred into suspension culture (to start with in the presence of 10 I.E. heparin) and kept at a concentration of 1-2 x 10^6 cells/ml. Once they reached log phase proliferation, the 2 10^6 cells/ml were infected with 1/10 volume of the high titre virus stock (ca. 10 MOI). Virus-infected cells were autoclaved twice before disposal. Supernatant was collected for the isolation of BTN^{exo}.

2.5.7 Culture of a myeloma cell line (Ag8)

The myeloma cell line Ag8 was grown in Ag8 culture medium at 37°C and 10 % CO_2. Cells were diluted 1/3 in fresh medium every 3-5 days. MOG-transfected Ag8-cells were kept in the presence of 1.5 mg/ml geneticin (G418)

2.5.8 Flow cytometry

Cell surface antigens can be recognized by specific antibodies coupled to fluorescent markers and detected by flow cytometry. A fluorescence-activated cell sorter (FACS) measures the light emission generated by single cells flowing through a laser beam (excitation wavelength 488 nm). Several parameters can be measured: size (forward scatter, FSC) and granularity (side scatter, SSC) of cells, as well as fluorescence emitted from surface-bound antibodies coupled to fluorochromes. The following fluorochromes were used: fluorescein isocyanate (FITC, emission at 530 nm, FL1), phycoerythrein (PE, emission at 578 nm, FL2) and RPE-Cy5 (emission at 667 nm, FL3). Dead cells were stained with propidium iodide as the membrane of live cells is impermeable to this compound.

2.5.8.1 FACS analysis of Ag8-cells

10^5-10^6 cells from a single cell suspension were added in 50 µl FACS buffer per well of a 96-well plate. Cells were washed twice by centrifugation (1200 rpm, 6 min) in 100 µl FACS buffer and resuspended in 50 µl FACS buffer containing an optimal concentration of the first antibody (unlabelled or coupled to biotin or to FITC) or rat serum to be tested. Cells were incubated on ice for 20-45 min and washed twice with FACS buffer. To detect an unlabeled first antibody, a second FITC or PE-labelled antibody directed against the Ig of the first antibody species was added. To detect biotin in the first staining, PE-coupled streptavidin (Amersham) was added. Cells were again incubated on ice for 20-45 min, washed twice and transferred to FACS tubes in 100-200 µl FACS buffer. Propidium iodide was added at a concentration of 10 µg/ml. Fluorescence was measured with a FACS Scan (Becton-Dickinson) and results were analysed with Becton-Dickinson software (Lysis).

2.5.8.2 FACS-analysis of T cells

Cell types as well as their activation status are characterised by a specific pattern of cell surface molecules. These were measured by FACS analysis, using non-conjugated first antibodies (mouse) and RPE-Cy-5-labelled second antibodies (rabbit anti-mouse). The antibodies were used in 1/100 dilutions of a 1 mg/ml stock. After three days of antigen-specific

restimulation, 10^4-10^5 T cell blasts were isolated by density gradient centrifugation and washed three times with FACS buffer. T cells were then incubated for 1 h on ice with 100 µl non-conjugated cell surface marker-specific antibodies. After this, unbound antibody was washed off (3x with FACS buffer) and the cells were incubated with Cy5-labelled second antibody. Again, unbound antibody was washed off (3x with FACS buffer) and binding was detected using FACS.

2.5.9 Statistical analysis

Data are generally represented as average and standard deviation, and in some cases with standard error (always marked in the figure legend). Significance was calculated using Student´s t-test and Duncan´s post-hoc test.

3 Results

3.1 The impact of antibody dependent effector mechanisms on disease progression in a model of relapsing remitting EAE

There is no spontaneous animal model for MS. However, in many species the immunisation with spinal cord homogenates (SCH) or several different myelin proteins induces EAE, an autoimmune disease which resembles MS and has provided the basis for most of the current knowledge about this disease. It should be noted that EAE, like MS, is not a homogeneous disease, but varies in disease course, intensity and pathological features. These are influenced by a variety of factors, such as nature and dose of antigen, adjuvant, species, (inbred) strain/genotype, age and gender, as well as external stress factors. This leads to a wide range of different disease models, all of which are initiated by the peripheral activation of myelin-specific T cells, an event, which is also thought to initiate MS (Hohlfeld, 1997).

However, little is known about the mechanisms responsible for the classical clinical course of MS, an initial period of relapsing-remitting disease followed by a progressive worsening of the clinical deficit. In mice (SJL/J and Biozzi) (Brown and McFarlin, 1981; Baker et al., 1990), EAE often follows a similar relapsing remitting disease course (RR-EAE). In these animals disease is T cell mediated, since the adoptive transfer of encephalitogenic $CD4^+$ T cells alone can induce relapsing remitting disease and demyelination (Mokhtarian et al., 1984). Relapse activity is associated with epitope spreading, the sequential switch/diversification of the autoimmune T cell response to additional CNS target autoantigens as disease progresses (reviewed in Tuohy et al., 1998). Pathologically, these mouse model develop lesions mimicking type I lesions, which are seen in a minor subgroup of MS patients (Lucchinetti et al., 2000; Lassmann et al., 2001; section 1.1.3).

Most patients, however, develop lesions which are characterised by the deposition of antibody and complement on the myelin sheath in addition to inflammatory infiltrates (type II lesions, Lucchinetti et al., 2000; Lassmann et al., 2001), indicating that antibodies play a major role in disease pathogenesis. This concept is supported by the observation that plasmapheresis often induces remission in patients with acute, steroid-non-responsive relapses (Weinshenker et al., 1999).

Clearly more insight is required into the mechanisms responsible for driving disease progression in MS, in particular the potential role of autoantibodies. RR-EAE induced by active immunisation with MOG-Igd in the rat is an obvious candidate for such studies. As in the

mouse, the clinical course is genetically determined by multiple loci (Weissert, 1998). DA rats are particularly susceptible to develop RR-EAE after active immunisation with either SCH (Lorentzen et al., 1995) or MOG-Igd (Weissert et al., 1998). Crucially, the immunopathology reproduces type II-antibody mediated demyelination (Storch et al., 1998) suggesting that this strain will provide a deeper insight into the pathomechanisms involved in this large subset of MS patients. However, any studies of RR-EAE are complicated by inter-animal variation in disease progression. Analysis of the mechanisms responsible for relapse and disease progressions requires a highly predictable time frame. Preliminary studies indicated that this may be possible in DA rats following immunisation with MOG-Igd in IFA, a protocol that also avoids adjuvant-induced arthritis (AA). The first experiments revealed that while low doses of MOG-Igd in IFA (10-50µg) would induce relapsing-remitting disease, a higher dose (100 µg) was required to synchronise the onset of relapse.

3.1.1 Sensitisation with MOG-Igd in IFA results in a predictable pattern of disease remission and relapse.

Immunisation with 100 µg MOG-Igd in IFA resulted in a synchronised biphasic disease course in female DA rats (Figure 3.1.1). The first clinical symptoms appeared 7-10 d.p.i. (days post immunisation), the severity of the clinical deficit then increased rapidly to reach a peak 10 to 11 d.p.i., after which the animals began to recover. Clinical recovery was complete in approximately 50% of the animals by 15 d.p.i., whereas the remainder showed some degree of residual disease activity (grade 0.5 to 1.5). This remission lasted for 2 to 3 days, but irrespective of the extent of recovery this was followed by a relapse characterised by a progressive increase in disease severity. The majority of the rats developed severe hind limb paralysis with fore limb paraplegia by 20 to 25 d.p.i. and were sacrificed in accordance with the animal regulations of the federal government of Bavaria.

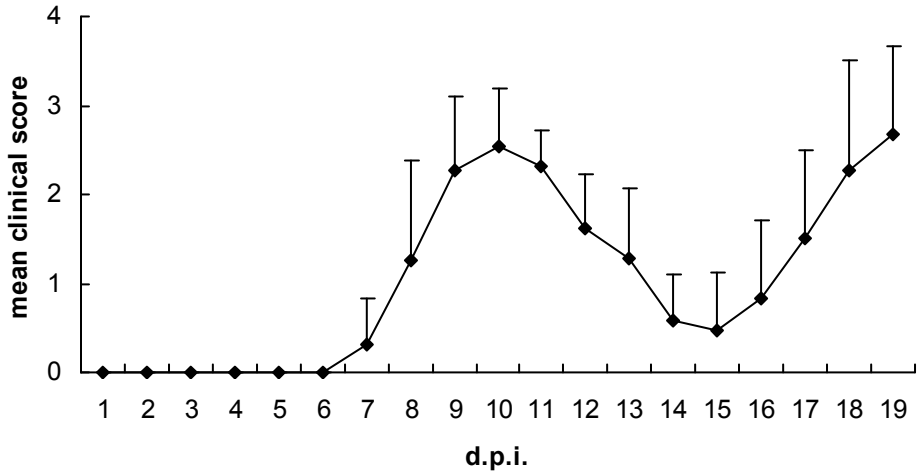


Figure 3.1.1 Disease course in DA rats after immunisation with 100 µg MOG-Igd/IFA.
Depicted is the average and standard deviation of the clinical course of 23 rats, of which 5 were euthanised 10 d.p.i; 9 rats 15 d.p.i. and the last 9 rats 19 d.p.i.

3.1.2 The pathology of MOG-Igd/IFA induced EAE.

Animals were immunised with 100 µg MOG-Igd/IFA and perfused for immuno/histo-pathological analysis of the CNS 4, 10, 15 and 21 d.p.i. (Figure 3.1.2: collaboration with Professsor H. Lassmann and Dr. R. Höftberger, University of Vienna, Vienna, Austria). This revealed that CNS pathology changed dramatically during the course of the disease (Figure 3.1.2). Four d.p.i., i.e. three to four days before the onset of any clinical signs of EAE, no obvious pathological changes were seen in the CNS (Figure 3.1.2 1a-e). However, six days later (10 d.p.i.), coincident with the first peak of disease, an intense inflammatory response had developed in the CNS, characterised by large numbers of $ED1^+$ monocytes (Figure 3.1.2 2C) and $W3/13^+$ T cells and polymorphonuclear cells (PMNs) (Figure 3.1.2 2E) infiltrating into the perivascular space and CNS parenchyma. At this time point demyelination was minimal (Figure 3.1.2 2B) and restricted to small areas around perivascular/sub-pial infiltrates. Five days later, when the animals were in clinical remission, the inflammatory infiltrate was greatly reduced with respect to $W3/13^+$ T cells (Figure 3.1.2 3E) as well as $ED1^+$ macrophages/microglia (Figure 3.1.2 3C). There was still no obvious evidence of demyelination (Figure 3.1.2 3B). This was in striking contrast to the situation 21 d.p.i., when the rats were in relapse and had developed severe clinical disease. At this late time point disease was associated with extensive demyelination throughout the CNS (spinal cord, fore brain and cerebellum) (Figure 3.1.2 4B) accompanied by a diffuse inflammatory infiltrate of $ED1^+$ and $W3/13^+$ cells (Figure 3.1.2 4C

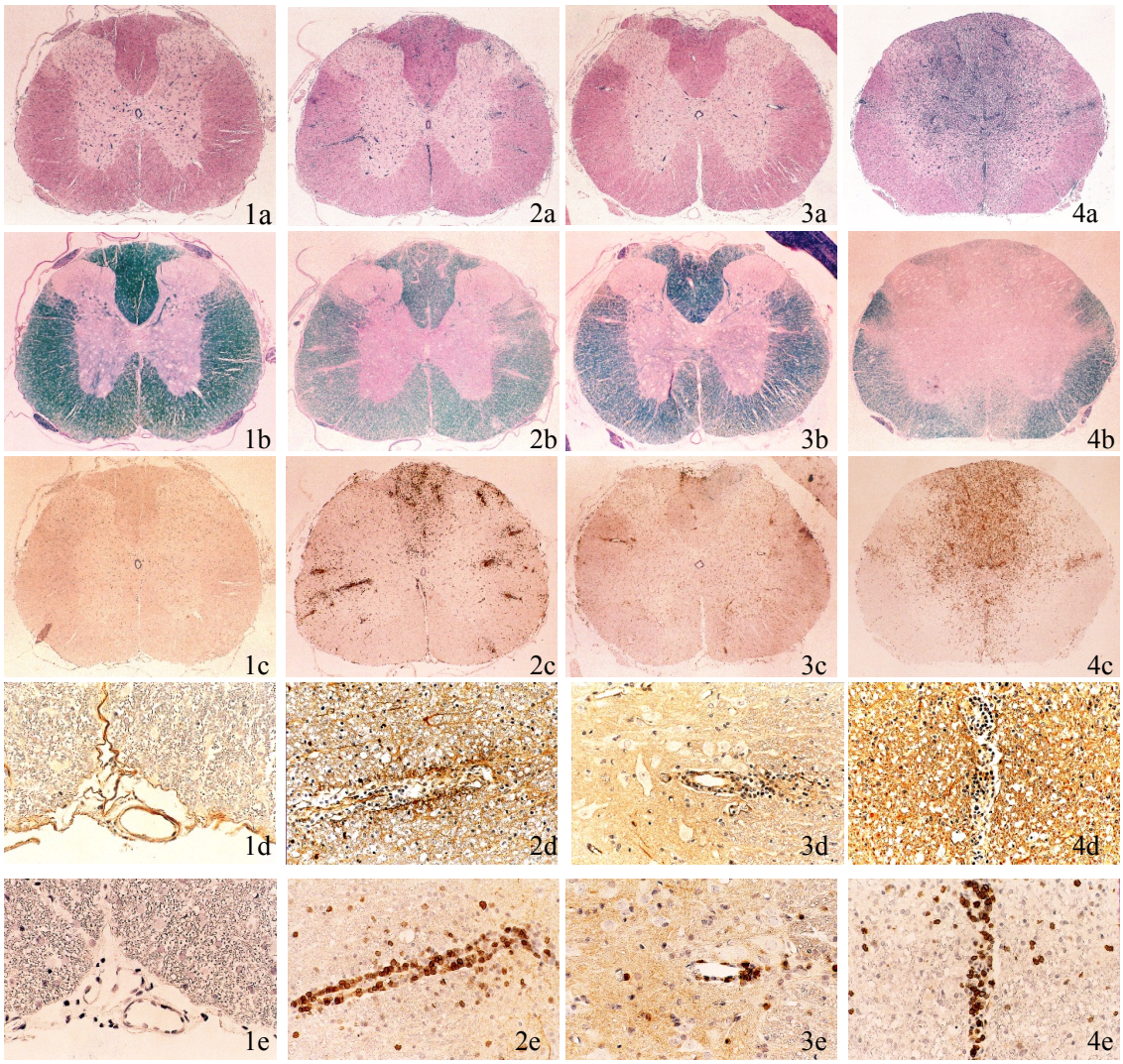


Figure 3.1.2. Histopathology in the different stages of MOG-Igd/IFA induced EAE.

DA rats were immunised with 100 µg MOG-Igd/IFA and perfused 4 d.p.i. **(1)**; 10 d.p.i. **(2)**; 15 d.p.i. **(3)** and 21 d.p.i. **(4)**. Serial sections were stained for **a)** inflammatory infiltrates (hematoxylin eosin); **b)** myelin (Luxol fast blue); **c)** activated microglia and macrophages (ED1, mouse mAb); **d)** complement deposition (rabbit anti-C9) and **e)** T cells and polymorphonuclear cells (W3/13, mouse mAb). Amplification is 40x in **a) - c)** and 400x in **d)** and **e)**. Photos by Dr. R. Höftberger, Vienna.

and 4E). These active demyelinating lesions were associated with the deposition of antibodies and C9 (Figure 3.1.2 4D) in the affected white and grey matter, characteristic features of antibody- mediated demyelination in the rat (Linington et al., 1988; Linington et al., 1989 A; Storch et al., 1998).

As summarised in Table 3.1.1, relapse is clearly associated with the onset of extensive demyelination, while the initial phase of EAE is purely inflammatory.

Phase of disease	Inflammation	Demyelination
Preclinical (4 d.p.i.)	-	-
First peak of disease (10 d.p.i.)	+++	+/-
Remission (15 d.p.i.)	+	+/-
Relapse (19 d.p.i.)	++	++++

Table 3.1.1 Summary of the pathological analysis.

The symbols represent: - absent; +/- questionable; + to ++++ relative increase in severity

3.1.3 Is there a progressive change in the immune effector mechanisms responsible for disease activity in this model of MS ?

The observation that the initial phase of disease induced following immunisation with MOG-Igd/IFA was associated with CNS inflammation whereas clinical relapse was characterised by inflammation *plus* extensive demyelination suggests that there is a progressive change in the immune effector mechanisms responsible for disease pathogenesis in this model. Previous studies have shown that in the rat the inflammatory response in EAE is triggered by a $CD4^+$ Th1 T cell response, which induces the recruitment and activation of $ED1^+$ macrophages into the CNS and blood brain barrier (BBB) dysfunction (Ben-Nun et al., 1981). However, this T cell mediated response alone is unable to induce demyelination (Linington et al., 1988). Demyelination in EAE in both rats and marmosets (*Callithrix jacchus*, a non-human primate) requires the participation of a myelin-specific antibody response (Lassmann et al., 1988; Linington et al., 1988; Genain et al., 1995). This is associated with the deposition of immunoglobulin and complement on the myelin sheath, myelin vesiculation and the phagocytosis of opsonised myelin debris by macrophages (Linington et al., 1988; Storch et al., 1998 b; Genain et al., 1999; Raine et al., 1999). In view of the obvious changes that occur in the immunopathology of MOG-Igd-induced EAE during the course of the disease, we anticipated that the first, inflammatory phase of disease was driven by a MOG-specific $CD4^+$ T cell

response, while MOG-specific antibodies evolved to become the dominant pathomechanism responsible for driving disease activity in relapse. In order to test this hypothesis, we investigated the characteristics of the MOG-specific T cell- and antibody-response at the following time points:

I. Preclinical (4 d.p.i.)

II. First peak of disease (10/11 d.p.i.)

III. Remission (15 d.p.i.)

IV. Relapse (19/21 d.p.i.)

3.1.3.1 Characterisation of the MOG-Igd-specific T cell response

3.1.3.1.1 MOG-specific T cell proliferation is maximal during the first peak of disease

A MOG-Igd-specific proliferative response was detected in the draining lymph nodes as early as 4 d.p.i. The stimulation index increased over the next six days, reaching a maximum 10 d.p.i., concurrent with the first peak of disease, after which it decreased, but remained significantly increased over baseline levels (Figure 3.1.3).

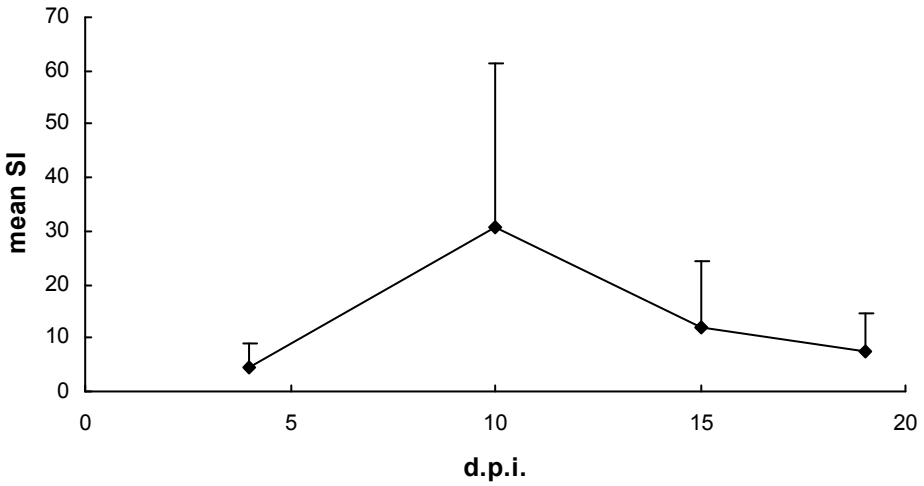


Figure 3.1.3 Time course of MOG-Igd-specific T cell proliferation in the draining lymph nodes
Shown is the mean stimulation index and standard deviation obtained after immunisation with 100 µg MOG-Igd/IFA, using three animals per time point, which were analysed individually in quadruplicate

A similar, but less pronounced response was observed in the spleen (data not shown). It should be noted that no significant increase in antigen-specific proliferation in the peripheral immune organs was associated with clinical relapse.

3.1.3.1.2 The MOG-specific T cell response recognises two distinct epitopes after sensitisation with MOG-Igd/IFA

Previous studies of MOG-Igd/CFA induced EAE in the DA rat identified two encephalitogenic T cell epitopes (Stefferl et al., 2000; Schubart et al., 2001; Iglesias et al., 2001). The relative contribution of these epitopes to the IFA model was analysed 10 d.p.i. by epitope mapping using a panel of nine overlapping peptides spanning the complete Ig-domain. Cells derived from both the draining lymph nodes and spleen proliferated in response to MOG-Igd, and a dominant epitope was identified within the amino acid sequence M_{89-113} (Figure 3.1.4 A). A minor response was observed to the overlapping peptides M_{63-87} and M_{76-100}. The fine specificity of this response was determined using MOG-Igd-specific T cell lines (TCLs) and a panel of shorter overlapping 15-mer peptides (Figure 3.1.4 B). This analysis identified M_{93-109} as the immunodominant MOG-Igd-epitope and confirmed the presence of a second epitope within M_{74-90}.

3.1.3.1.3 Characterisation of MOG-Igd and MOG-peptide specific T cell lines

In order to test the hypothesis that the first phase of MOG-Igd-induced EAE may be driven by a pathogenic $CD4^+$ T cell response, we analysed the surface phenotype and cytokine spectrum of MOG-, M_{93-109} and M_{74-90}-specific T cell lines (TCLs) derived from the draining lymph nodes 10 d.p.i. and then tested their pathogenicity by adoptive transfer into naive syngeneic recipients. After three to four rounds of *in vitro* selection, all three TCLs were highly specific for their selecting antigen. In addition, the peptide-specific TCLs retained their responsiveness to MOG-Igd, and the MOG-Igd selected TCLs continued to respond to both peptide epitopes (Table 3.1.2).

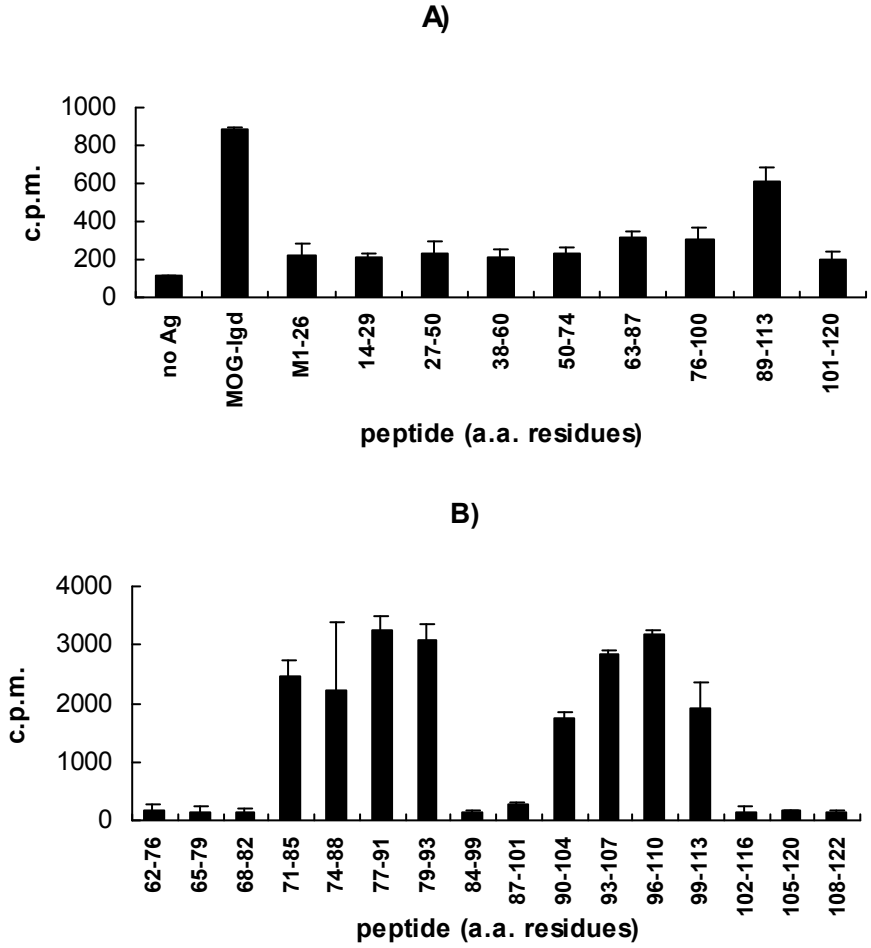


Figure 3.1.4 Epitope mapping of MOG-Igd-specific T cells.

A) Peptide specificity of lymph node cells pooled from 2 rats 10 d.p.i. with 100 µg MOG-Igd/IFA; **B)** Data for a MOG-specific T cell line, which was analysed using smaller peptides that spanned the sequences identified in A). Depicted are the average c.p.m. (counts per minute) and standard deviation of quadruplicate measurements.

Antigen T cell line	**Proliferation (c.p.m.)**			
	No antigen	**MOG**	**M_{93-109}**	**M_{74-90}**
MOG-Igd	72.5 +/- 2	2403 +/- 192	1383 +/- 90	1078 +/- 68
M_{93-109}	38 +/- 11	3017 +/- 486	3710 +/-564	20 +/-5
M_{74-90}	155 +/- 76	1126 +/- 100	164+/- 36	2284 +/- 132

Table 3.1.2 Proliferative response of the MOG-Igd or MOG-peptide specific T cell lines (TCLs) to MOG and the two MOG epitopes.

Shown are mean counts per minute (c.p.m.) and standard deviation of quadruplicate or triplicate analysis of representative restimulation assays.

FACS analysis revealed that these TCLs consisted of pure $CD4^+$ T cell populations (>99% $CD3^+$, >99% $\alpha\beta TCR^+$, >98% $CD4^+$, see Figure 3.1.5 for a representative analysis). When stimulated with their selecting, cognate antigen, all TCLs secreted large amounts of IFN-γ (30-60 ng/ml), IL-10 (0.5-1.8 ng/ml) and TNF-α (1.5 - 6 ng/ml), as described previously for *in vitro* selected Th1 subset rat T cell lines (Stefferl et al., 1999; Lenz et al., 1999).

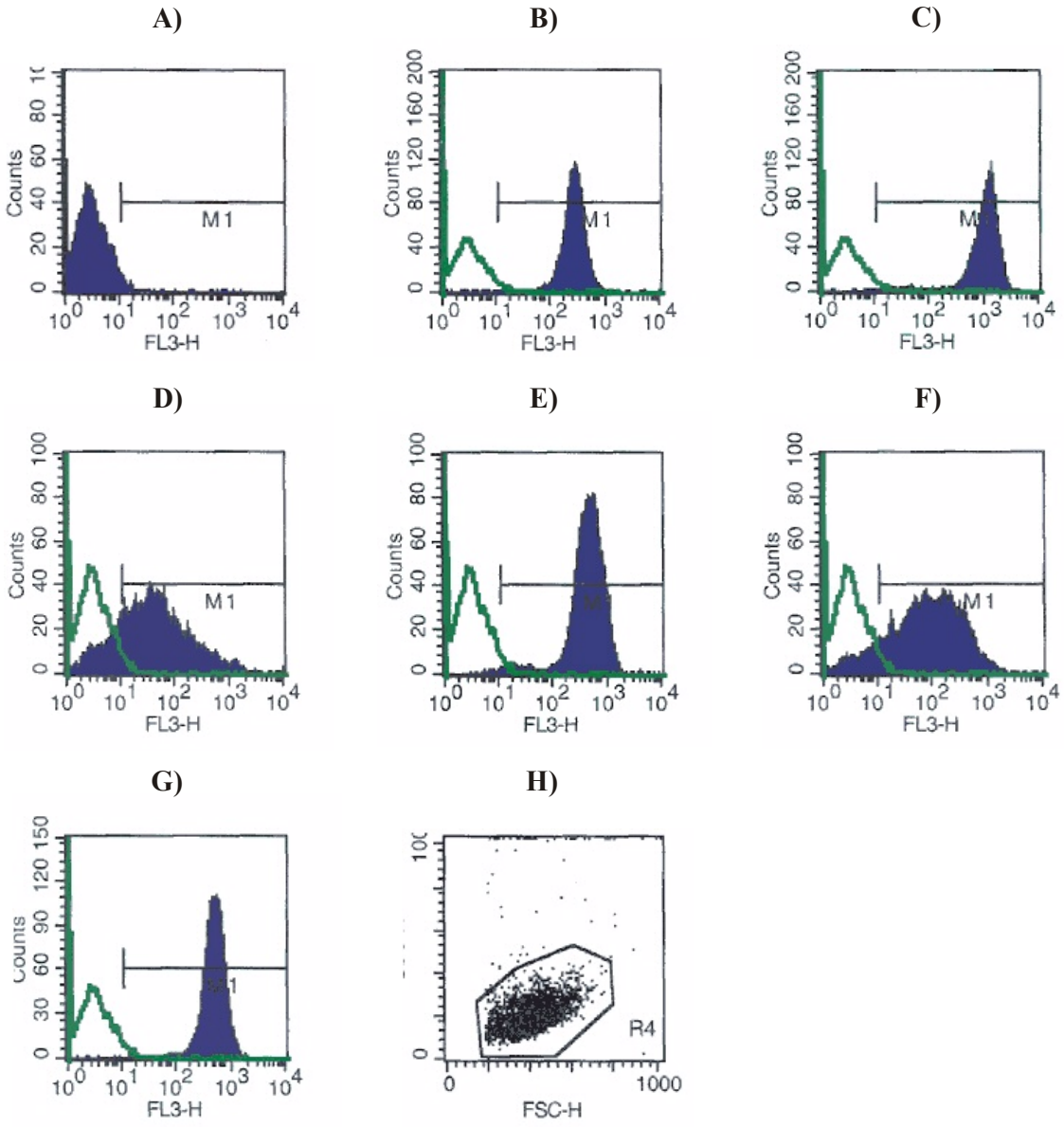


Figure 3.1.5 Representative FACS analysis of a M_{74-90}-specific T cell line.

Histograms show **A)** isotype control; **B)** CD3; **C)** IL-2R; **D)** MHC class II; **E)** CD4; **F)** OX 40 antigen; **G)** TCR in blue and the isotype control overlay in green; **H)** dot plot illustrating the cell population gated for analysis.

To investigate their pathogenicity, MOG-Igd- or MOG-peptide (M_{93-109} or M_{74-90}) - specific T cell blasts were harvested after 72 hours of antigen-driven restimulation *in vitro* and adoptively transferred into naïve syngeneic recipients. All three cell lines were pathogenic and induced a monophasic disease, which started 4-5 d.p.t. (days post transfer, see Figure 3.1.6 for a representative disease course). The severity of disease increased rapidly for the next 24 to 48 hours, after which the animals recovered. Clinical recovery was complete by 10 - 11 d.p.t.. Histopathological investigations revealed that disease was associated with an intense inflammatory response in the CNS that was dominated by macrophages. There was no evidence of demyelination. (Figure 3.1.7)

The pathogenicity of the MOG-specific T cell response in DA rats was striking as this was not the case in either LEW and BN rats (Stefferl et al., 1999; Linington et al., 1993; Adelmann et al., 1995). In these other rat strains the adoptive transfer of MOG-specific $CD4^+$ Th1 T cells fails to induce clinical disease although they trigger the recruitment of T cells into the CNS. The observation that the DA T cell response to MOG is highly pathogenic, but fails to initiate demyelination supports the hypothesis that this response alone may be responsible for the first, inflammatory phase of disease in MOG-Igd/IFA induced EAE in DA rats.

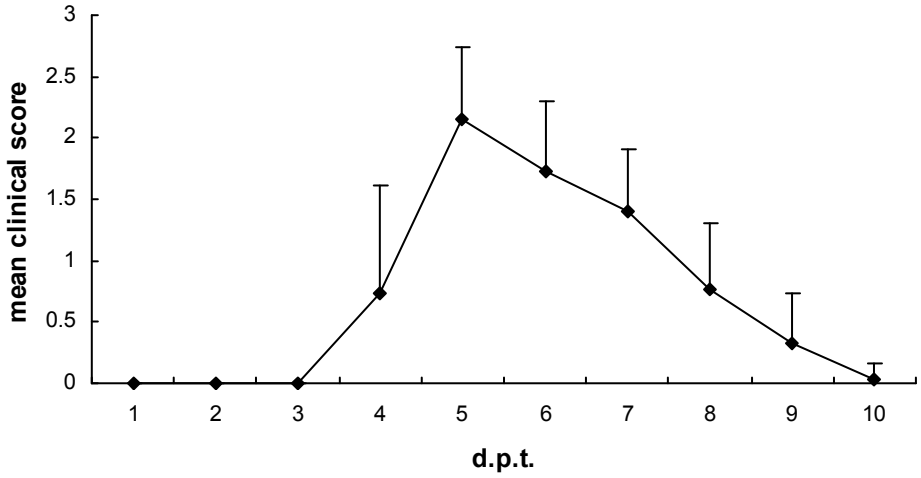


Figure 3.1.6 Clinical course of EAE induced by adoptive transfer of M_{93-109}-specific T cells.
Depicted is the mean and standard deviation of the clinical course of 15 DA rats injected with 1.7 x 10^6 M_{93-109} - specific T cells.

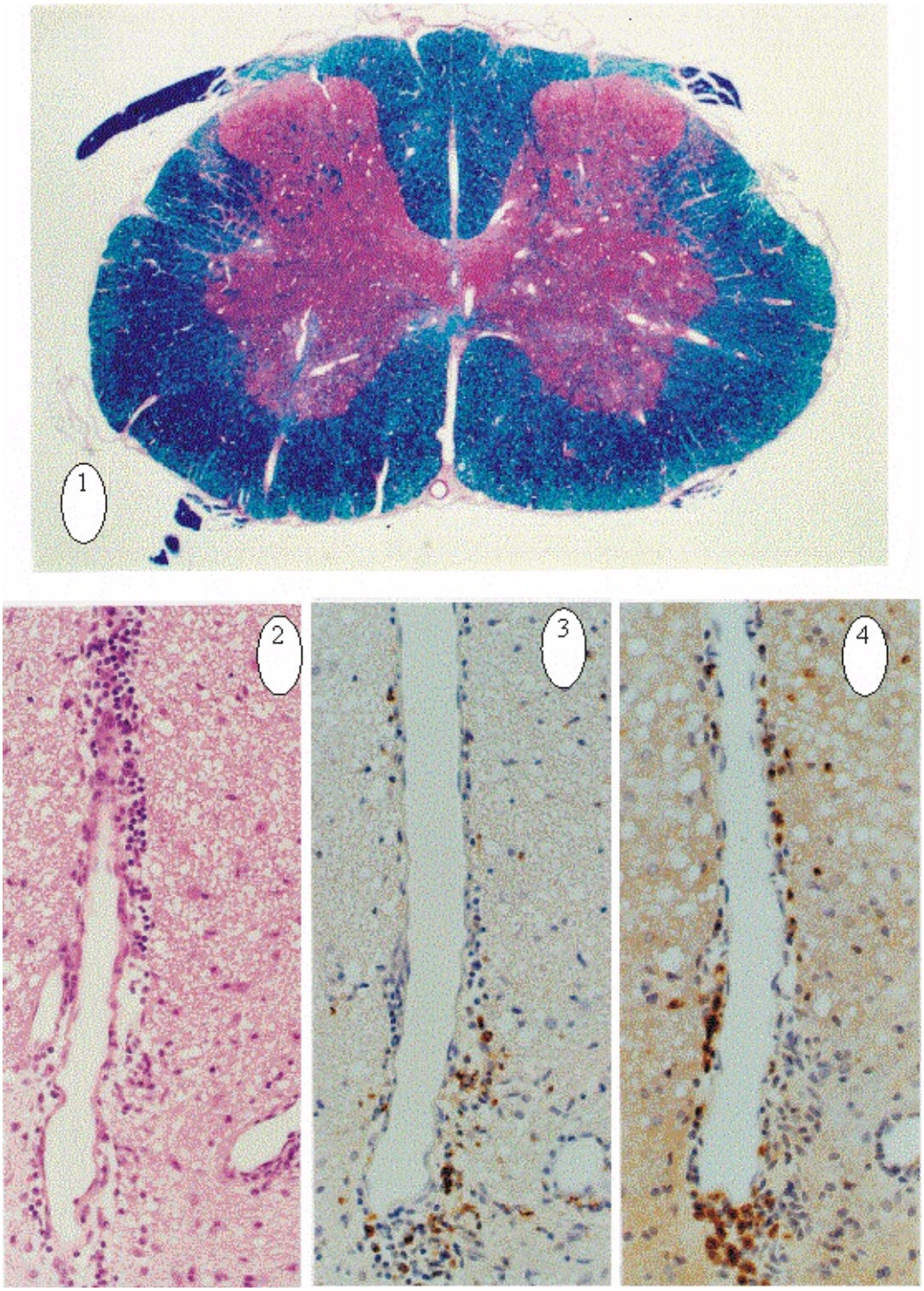


Figure 3.1.7 CNS pathology 6 d.p.t. of 5 x 10^6 MOG-Igd-specific T cells

Serial sections were stained with **1)** Luxol fast blue (LFB), revealing a complete absence of demyelination (32x); **2)** Hemytoxylin eosin (cellular infiltrates); **3)** ED1 (activated microglia and macrophages) and **4)** W3/13 (T cells and granulocytes). 2) – 4) 300x. Stainings reveal strong perivascular infiltrates of macrophages, T cells and granulocytes. The rat had grade 4 disease at the day of perfusion. Photo provided by Dr. M. Storch, University Med School Graz, Austria

3.1.3.2 Analysis of the antibody response

If the hypothesis is correct that the first phase of MOG-Igd induced EAE in the DA rat is purely T cell mediated, the MOG-specific antibody response should be minimal during the initial phase of disease and only start to increase in remission.

Analysis by ELISA using sera taken at four time points (Figure 3.1.8 A) demonstrated that this was indeed the case. MOG-Igd-specific antibodies were absent 4 d.p.i., and only low levels were detected during the first peak of disease (10 d.p.i.). The antibody titre then increased rapidly, 3.5-fold in the next 5 days, and then another nine fold in the following 4 days, so that during relapse the total MOG-specific antibody titre was approximately 30-fold higher than during the first phase of disease.

Analysis of the isotype-composition of the MOG-Igd-specific antibody response revealed that MOG-specific IgM dominated the early response (10 d.p.i.) and increased slightly over the next nine days (Figure 3.1.8 B). In contrast, the levels of MOG-specific IgG, IgG1, Ig2A, IgG2B and IgG2C were very low during the first acute phase of disease, but increased dramatically between 15 and 19 d.p.i.. This reflects the normal course of maturation of an antibody response. While early adaptive immune responses are generally characterised by the secretion of IgM (IgM pentamers have a high avidity to their antigen, even if the affinity of the antibodies is still low), a cytokine-mediated recombination of DNA takes place in parallel with affinity maturation, leading to isotype switching.

The epitope specificity of MOG-Igd-specific antibodies was characterised using a panel of nine overlapping synthetic 18 - 25-mer peptides. During the first phase of disease (10 d.p.i.), when the antibody titre was still low, we observed an immunodominant response to the N-terminal peptide M_{1-26} (Figure 3.1.8 C). Nine days later, during relapse, the response to M_{1-26} was still dominant, but was now accompanied by a response to a second peptide, M_{50-74} (Figure 3.1.8 D). This demonstrates that not only titre and isotype distribution of the antibody response, but also its specificity and complexity develops with time. The rapid increase in the MOG-specific antibody titre and complexity occurs as the animals relapse and is associated with the onset of widespread demyelination.

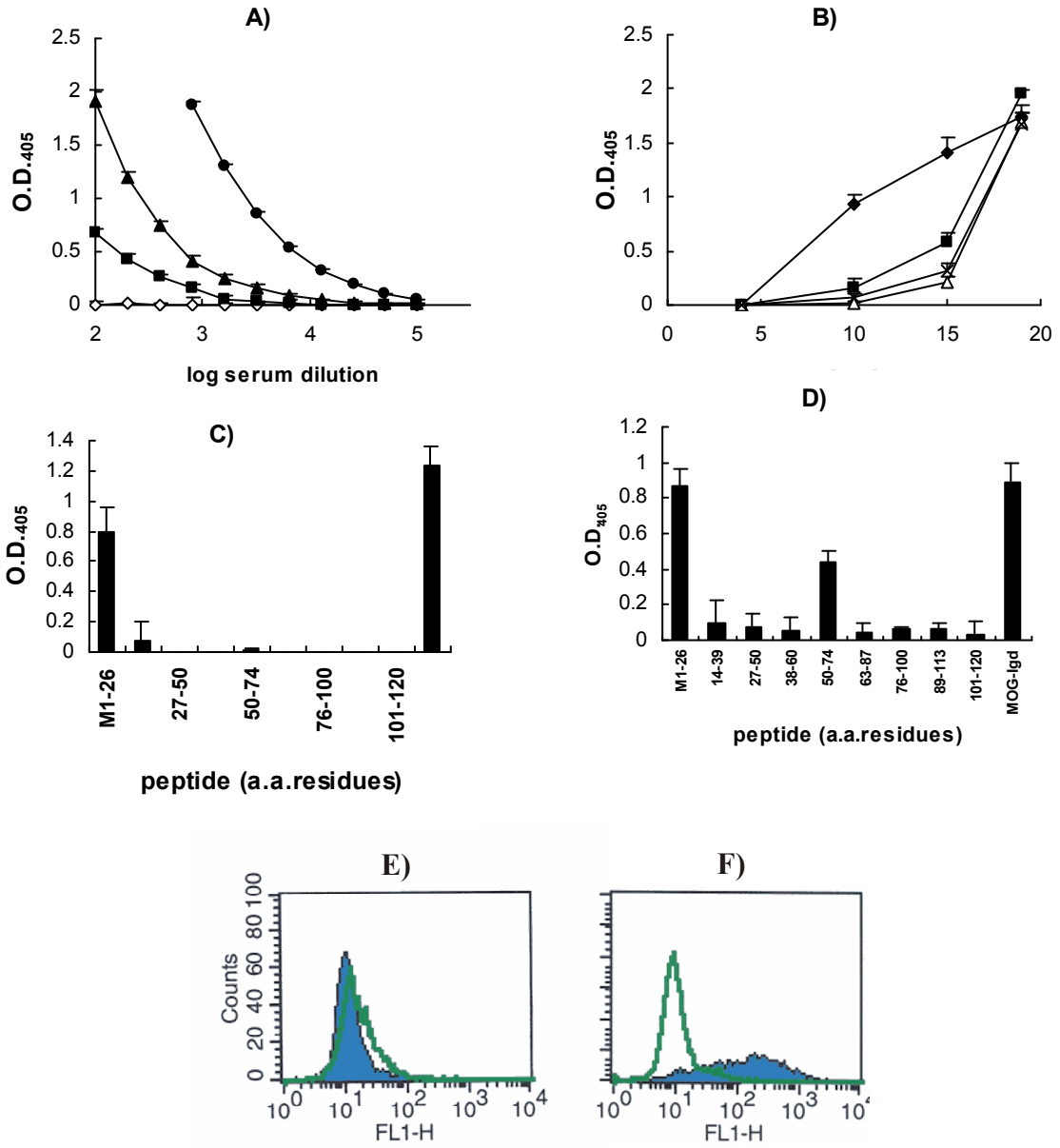


Figure 3.1.8 Analysis of the antibody response after immunisation with 100 µg MOG-Igd in IFA.

A) Time course of the total MOG-Igd-specific antibody response (IgG + IgM). Diamonds: 4 d.p.i., squares 10 d.p.i., triangles 15 d.p.i., circles 19 d.p.i. Shown are the mean $O.D._{405}$ values and standard deviations obtained using sera pooled from 2-3 rats per time point and analysed in quadruplicate. **B)** Time course of the isotype composition of the MOG-Igd-specific antibodies. Depicted are the mean $O.D._{405}$ values and standard deviations obtained using sera pooled from 2-3 rats per time point and analysed in quadruplicate. Sera were diluted 1/300 for IgM (diamond); 1/900 for IgG (square); 1/300 for IgG1(triangle) and 1/100 for IgG2A (cross). IgG2B- and IgG2C-responses were similar to the IgG2A response. **C), D)** Analysis of the epitope specificity 10 d.p.i. **(C)** and 19 d.p.i. **(D)** revealed that two linear epitopes were recognised during relapse, but only one at the earlier time point. Shown are the mean $O.D._{405}$ values and standard deviations of 3 individually analysed rats 10 d.p.i. and 6 individually analysed rats 19 d.p.i.. The serum of each rat was analysed in quadruplicate. Note that the two ELISAs were developed for different times in order to give a comparable response to peptide 1-26 and MOG-Igd. **E, F)** representative FACS analysis of sera derived 19 d.p.i. with MOG-Igd/IFA. Shown is the binding to MOG-transfected (blue) or to untransfected (green) Ag8-cells. **E)** Serum derived 19 d.p.i with MOG-Igd/IFA; **F)** positive control using 8-18C5, a monoclonal MOG-specific antibody. No specific binding was detectable 19 d.p.i.. Serum was pooled from 3 rats derived 19 d.p.i. and analysed in a 1/40 dilution. 8-18C5 was analysed at 25 µg/ml. Figures E and F) provided by Dr. C. Bourquin, Martinsried.

3.1.3.2.1 The titre of demyelinating antibodies is low in the periphery

A prerequisite for antibody-mediated demyelination is that antibodies bind to native antigen expressed on the surface of the myelin sheath (Brehm et al., 1999). Recognition of native MOG can be assayed *in vitro* by FACS analysis using MOG-transfected target cells (Brehm et al., 1999; Bourquin et al., 2000; Haase et al., 2000). Previous studies using this approach suggest that the pathogenic component of the MOG-specific antibody response is conformation dependent (Bourquin et al., 2000; Haase et al., 2000). This response can not be distinguished from the response to linear epitopes by ELISA, as the recombinant antigen used in these studies is partially denatured and will therefore bind antibodies directed against both types of determinants. We therefore tested the capability of MOG-Igd-specific antibodies to bind to native antigen expressed on the surface of a myeloma cell line (Ag8) that had been transfected with full length MOG via FACS analysis (Bourquin et al., 2000).

Intriguingly, even 19 d.p.i. we were unable to detect any significant binding of MOG-specific antibodies to the transfectants (3.1.8 E), suggesting that the level of pathogenic antibodies in the periphery is very low. In view of the intense demyelinating pathology associated with antibody and C9 deposition in the CNS during relapse, it is probable that the pathogenic component of the antibody response is rapidly absorbed within the CNS. Absorption of MOG-specific antibodies within the CNS was already suggested to occur in a guinea pig model of CR-EAE (Linington and Lassmann, 1987) and this was subsequently confirmed experimentally in the course of this study (see section 3.1.3.4)

3.1.3.3 Combined T cell- and antibody-mediated immune effector mechanisms reproduce the biphasic course of MOG-Igd/IFA-induced EAE

The different kinetics of the MOG-Igd-specific T- cell and antibody responses support the concept that different effector mechanisms are responsible for the clinical deficits associated with the initial phase of disease and relapse. Disease was initially associated with a highly encephalitogenic T cell response, while the antibody titre was low. This correlated with the observation that early lesions were purely inflammatory and demyelination minimal, similar to the pathology observed after the transfer of activated MOG-Igd- or MOG-peptide-specific T cell blasts. In relapse, this situation was reversed. The T cell response was reduced, while the antibody response had greatly increased in titre and had matured with respect to antigen specificity and isotype usage. Correspondingly, the pathology was dominated by extensive demyelination in the context of a diffuse inflammatory infiltrate.

If the hypothesis that relapse is mediated by pathogenic autoantibodies is correct, it should be possible to reproduce the biphasic disease course seen after immunisation with MOG-

Igd by actively inducing an encephalitogenic T cell response and then adoptively transferring demyelinating autoantibodies after clinical recovery.

3.1.3.3.1 Immunisation with the immunodominant peptide induces attenuated disease

As a first step to ”reconstruct” the clinical course and pathology of MOG-Igd/IFA induced EAE, we studied the pathogenicity of the response induced by active immunisation with the immunodominant T cell epitope $M_{93\text{-}109}$. Previous studies indicated that the antibody response to this linear peptide would not bind to the native protein and fail to induce demyelination (Brehm, 1999b). DA rats were therefore immunised with 100 µg $M_{93\text{-}109}$ in IFA. This induced a monophasic disease resembling the first phase of MOG-induced EAE in 89% of the animals (Figure 3.1.9 A). The first clinical symptoms were seen 7-11 d.p.i., disease peaked 2-3 days later, after which the animals recovered slowly. The disease was usually monophasic, but in one rat (n=27), which had failed to recover completely, clinical relapses were observed 20 and 27 d.p.i.. Pathology at the peak of disease was dominated by infiltrating $ED1^+$ macrophages, comparable to the pathology observed in the early phases of MOG-Igd-induced EAE. There was no evidence of demyelination.

Increasing the immunogenicity of the emulsion by adding 25µg *Mycobacterium smegmatis* per rat (CFA-) did not greatly influence disease onset, but increased the incidence to 100% and resulted in a relapsing remitting disease course in the majority of the rats (10/11)(Figure 3.1.9 B). In this model, the clinical severity of relapses tended to decrease with time and their onset became progressively less synchronised, a first relapse occurring between 18 and 25 d.p.i. and a third bout of disease activity starting between 26 and 39 d.p.i.. Disease was associated with scattered lesions containing activated $ED1^+$ macrophages/microglia and $W3/13^+$ T cells (Figure 3.1.10). Strikingly, although antibodies derived 20 and 40 d.p.i. recognised both $M_{93\text{-}109}$ and MOG-Igd in ELISA (Figure 3.1.9 C), no demyelination was detected in the CNS, and sera from healthy $M_{93\text{-}109}$ immunised rats failed to bind to native MOG expressed on MOG-transfected Ag8 cells (Figure 3.1.9 D, E). These results not only demonstrate that $M_{93\text{-}109}$-specific antibodies are incapable of binding to the native protein and mediate demyelination, but also that multiple episodes of CNS inflammation fail to induce demyelination in this disease model.

In the absence of a demyelinating antibody response, $M_{93\text{-}109}$ –peptide induced EAE is self-limiting and the animals eventually recover, a situation very different to the lethal disease induced by approximately $1/10^{th}$ of the molar dose of MOG-Igd in IFA.

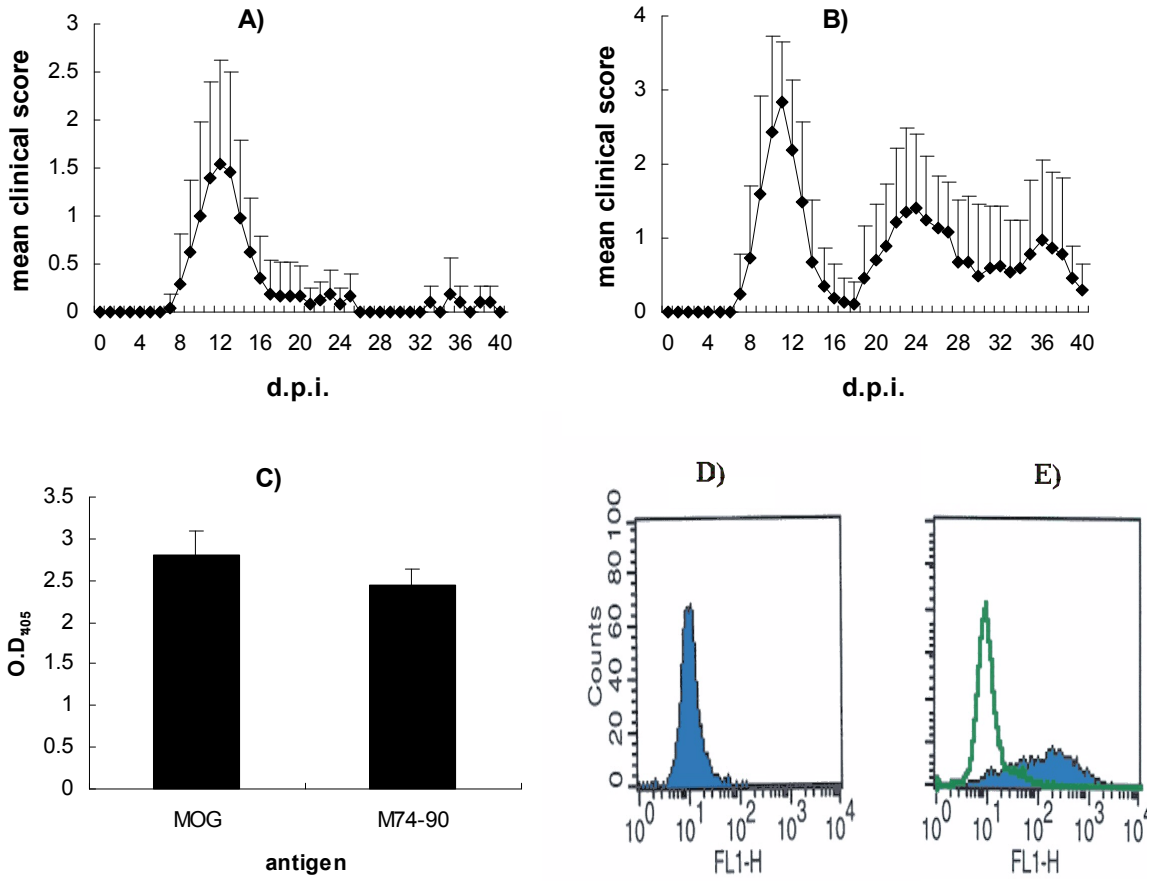


Figure 3.1.9 Disease course of M_{93-109}-induced EAE.

A) Female DA rats were immunised with 100 µg M_{93-109} in IFA. Represented is the mean clinical score and standard deviation of 26 rats; **B)** Female DA rats were immunised with 100 µg M_{93-109} in CFA. Shown is the mean clinical course and standard deviation of 11 rats. **C)** ELISA analysis of sera taken 20 d.p.i. after immunisation with M_{93-109} in IFA. Shown is the average $O.D._{405}$ and standard deviation derived from 3 rats, analysed individually in quadruplicate. **D**) FACS analysis of serum derived 40 d.p.i. with M_{93-109}; **E)** positive control using 8-18C5, a monoclonal MOG-specific antibody. Shown is the binding to MOG-transfected (blue) or to untransfected (green) Ag8-cells. No specific binding was detectable. Serum was pooled from 3 rats and analysed in a 1/40 dilution. 8-18C5 was used at 25 µg/ml. Figures D) and E) provided by Dr. C. Bourquin.

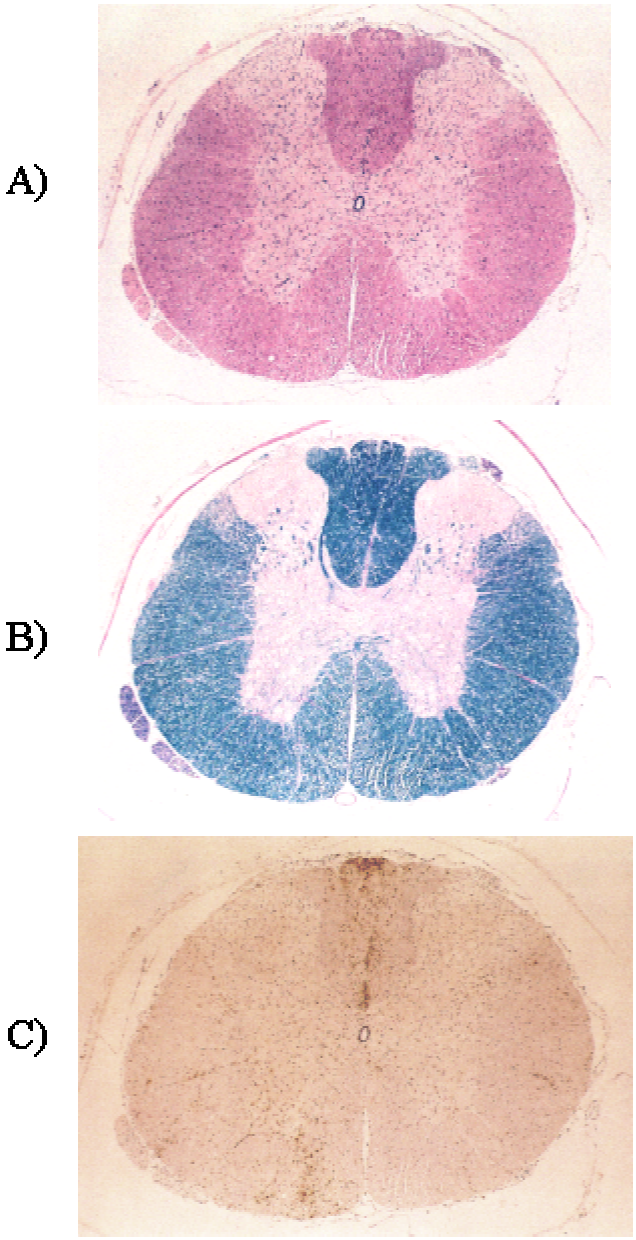


Figure 3.2.10 Pathology 29 d.p.i. with M_{93-109} in IFA

DA rats were immunised with 100 µg M_{93-109} in IFA and perfused 29 d.p.i. Serial sections were stained for **A)** inflammatory infiltrates (hematoxylin eosin); **B)** myelin (Luxol fast blue); **C)** activated microglia and macrophages (ED1). There is no evidence of demyelination. Photos provided by Dr. R. Höftberger, Vienna, Austria

3.1.3.3.2 DNA vaccination induces high titres of demyelinating MOG-specific antibodies

Since the titre of pathogenic antibodies in the sera of animals immunised with MOG-Igd was effectively undetectable, it was apparent that very large numbers of donors would be required to obtain sufficient antibody for adoptive transfer experiments. We therefore investigated whether DNA vaccination with a plasmid encoding MOG could induce high titres of pathogenic antibodies to MOG-Igd in DA rats, as reported for mice (Bourquin et al., 2000).

DA rats aged 4-5 weeks were injected intramuscularly with 200 µg of a plasmid encoding full length MOG (Bourquin et al., 2000) into each tibialis anterior muscle. A MOG-Igd-specific antibody-response could be detected 11 days post vaccination (d.p.v.) and this increased steadily over the following 4 weeks, while no response was detected in control animals vaccinated with the original "empty" plasmid pcDNA3.1 (Figure 3.1.11 A). As observed in mice, none of the rats developed EAE, presumably due to the blood brain barrier, which inhibits the entry of antibodies into the CNS (Litzenburger et al., 1998).

Analysis of antibody isotypes 6 weeks after vaccination revealed the presence of IgM, IgG2A and IgG2B-isotypes. IgG1 was absent, indicating a purely Th1-associated antibody response (Figure 3.1.11 B). The titre of MOG-Igd specific antibodies detected by ELISA was approximately hundred-fold lower than 19 d.p.i. with MOG-Igd/IFA. However, in contrast to the response after active immunisation, the antibody-response after DNA vaccination was purely conformation dependant, as ELISA revealed a complete lack of binding to linear MOG peptides (Figure 3.1.11 C), while FACS analysis demonstrated specific binding to the surface of MOG-transfected Ag8 cells (Figure 3.1.11 D, E).

The pathogenicity of this established immune response was confirmed by the transfer of 2 x 10^6 M_{93-109}-specific T cells into MOG- or control-vector-vaccinated rats 4 weeks after vaccination. MOG vaccinated animals developed a rapidly lethal disease with early onset, compared to the relatively mild disease in control animals (Figure 3.1.12 A). Pathological analysis revealed extensive demyelination, deposition of immunoglobulins and C9 as well as a PMN infiltrate, findings characteristic for antibody mediated demyelination (Linington et al., 1988; Linington et al., 1989; Storch et al., 1998) and similar to those seen in relapse of MOG-induced EAE (Figure 3.1.12 B).

Serum of DNA-vaccinated animals therefore seemed to be an ideal source for passive transfer studies. In addition, DNA vaccinated animals provide an appropriate model system to investigate the specific absorption of MOG-specific antibodies into the CNS (see 3.1.3.4).

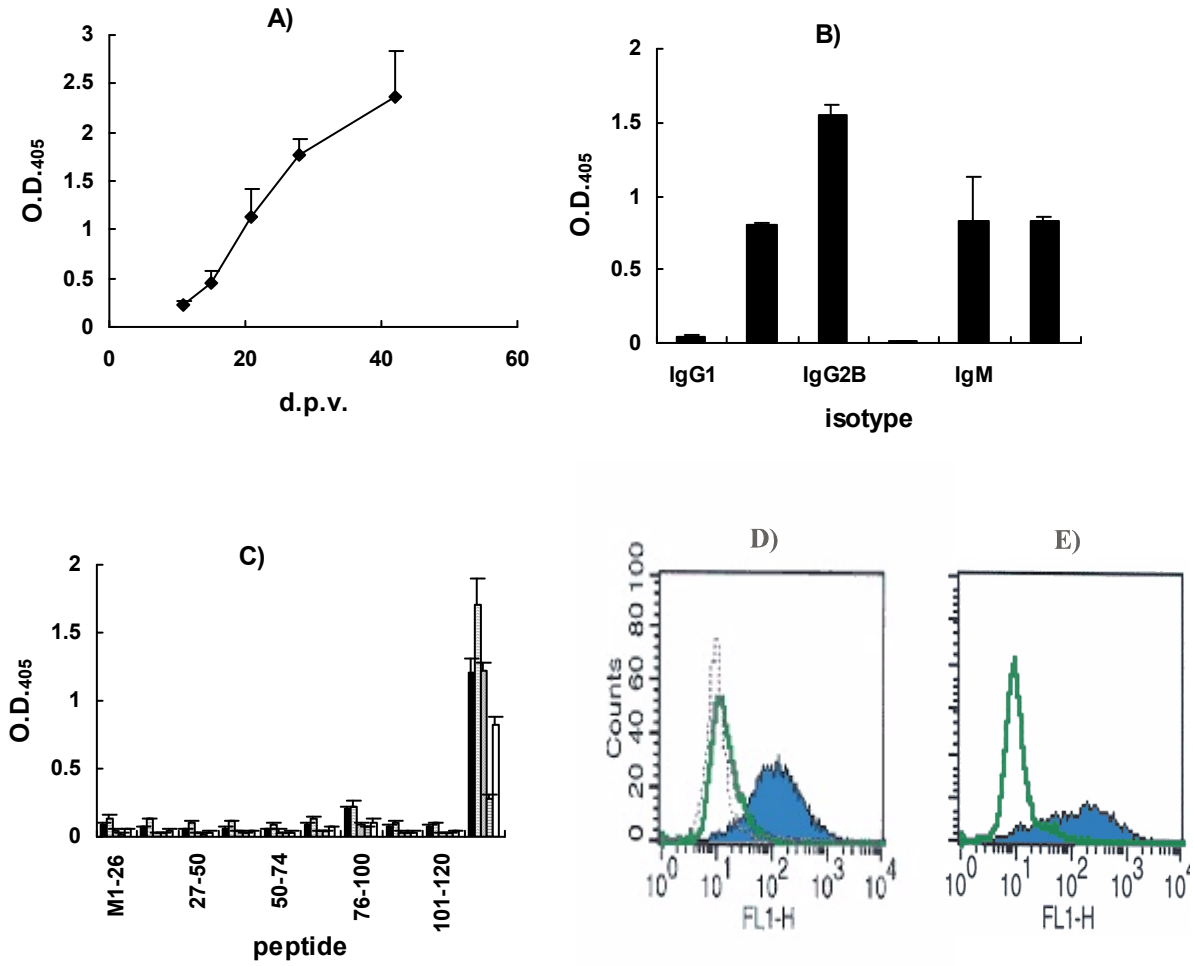


Figure 3.1.11 Analysis of the antibody response induced by vaccination with a plasmid encoding MOG.

A) Development of the antibody response after vaccination with pcDNA-MOG. Shown is the mean $O.D._{405}$ and standard deviation of serum derived from 4 rats bled at different times post vaccination and analysed individually in quadruplicate. Serum was analysed at a dilution of 1/100. Control animals vaccinated with pcDNA3.1 developed no MOG-specific antibody response; **B)** analysis of the isotype distribution 28 d.p.v.. Note that the values are not directly comparable, since different secondary antibodies were used for the detection. However, this response was significantly different to the response after immunisation with MOG-Igd in IFA, where all isotypes were clearly detectable and the response was "dominated" by IgG1. **C), D)** Epitope specificity of the antibody response after vaccination with pcDNA-MOG. **C)** DNA vaccination induced no response to linear peptides. Shown is the data derived from 5 rats analysed individually in quadruplicates at a dilution of 1/100. **D)** FACS analysis clearly demonstrates specific binding of sera from MOG-vaccinated DA rats to the MOG-transfected Ag8 cells (blue). In contrast, no binding was detected using naïve control serum (black) or untransfected Ag8-cells (green). Sera were used at a dilution of 1/40. **E)** Positive control using 8-18C5 a monoclonal MOG-specific antibody. 8-18C5 was used at a dilution of 25 µg/ml. Figures D) and E) provided by Dr. C. Bourquin.

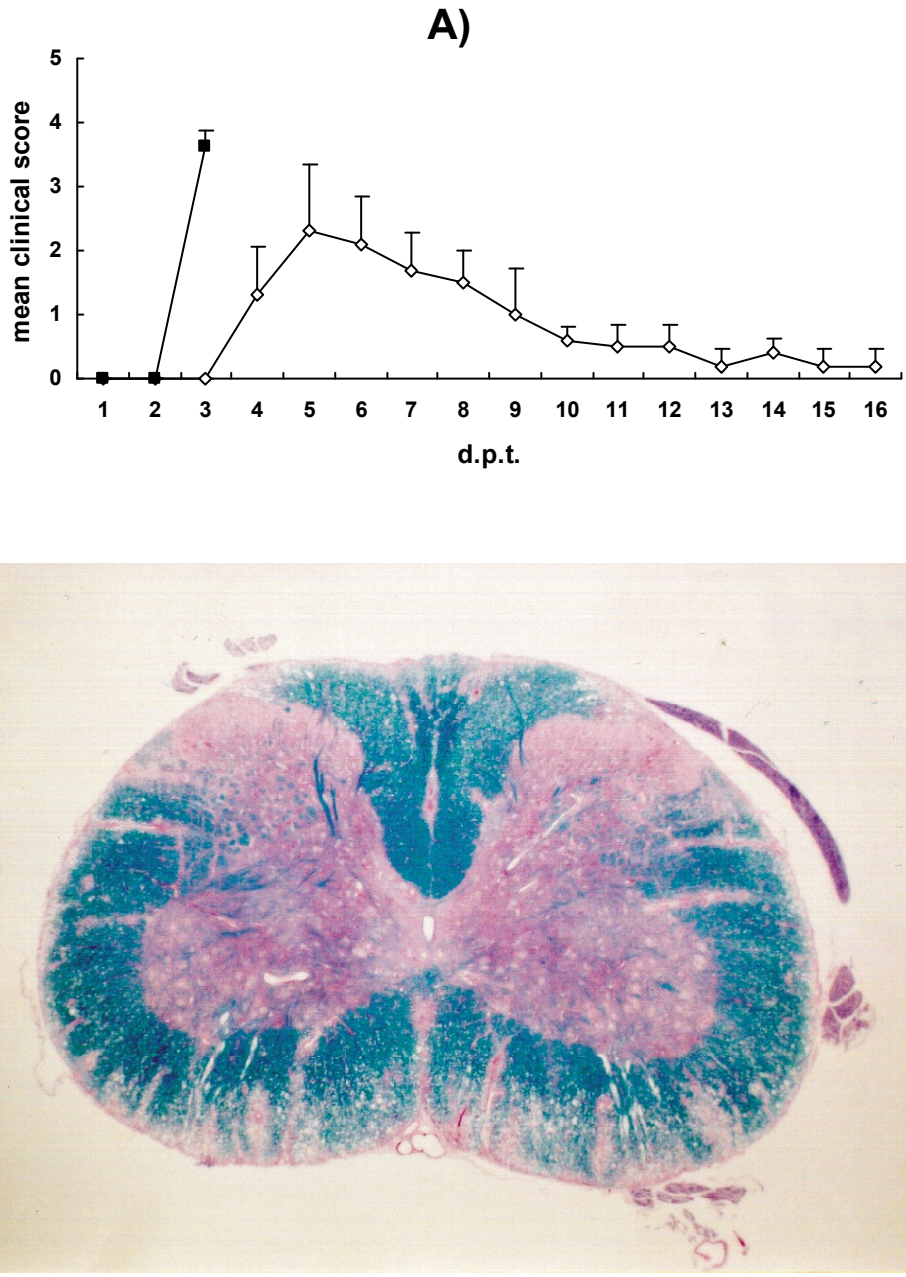


Figure 3.1.12 DNA vaccination with pcDNA-MOG enhances severity of tEAE and induces a demyelinating pathology

2 x 10^6 $M_{93\text{-}109}$-specific T cells were transferred into MOG- or control-vector-vaccinated DA rats 4 weeks after vaccination**. A)** Shown is the average and standard deviation of the clinical scores of 4 (pcDNA-MOG) or 5 rats (control vector). **B)** Enhanced disease activity is associated with enhanced demyelination/inflammation in the CNS. Represented is a spinal cord cross section from a pcDNA-MOG-vaccinated rat 3 d.p.t., stained for myelin (LFB). Note that demyelination is extensive considering the early time point after diesase onset. Photo by Dr. A. Stefferl, Vienna

3.1.3.3.3 The transfer of demyelinating autoantibodies can induce relapse

In order to test the hypothesis that antibodies are the driving force for the relapse in MOG-Igd in IFA-induced EAE, we investigated whether a combination of immunisation with the immunodominant MOG peptide M_{93-109} in IFA and antibody co-transfer would replicate the biphasic disease course seen after immunisation with MOG-Igd in IFA. DA rats were immunised with 100 µg M_{93-109} in IFA and then after complete recovery, 20 and 21 d.p.i. injected i.v. with 1 and 2 ml, respectively, of serum derived from MOG-DNA vaccinated rats.

Strikingly, the injection of anti-MOG-serum induced severe disease within 48 hours of transfer in all animals tested (Figure 3.1.13 B). In contrast, no relapse activity was observed in animals injected with serum derived from naïve animals (Figure 3.1.13 A). This clearly demonstrates that MOG-specific autoantibodies are capable of initiating relapse in peptide primed animals if they are available during the period in which relapse activity is seen in animals immunised with either MOG-Igd/IFA, or M_{93-109}/CFA (and occasionally even in IFA).

Histopathological analysis demonstrated that relapse induced by the transfer of anti-MOG sera was associated with extensive demyelination and a diffuse inflammatory infiltrate. In contrast, inflammation in the control animals had resolved by the time they were perfused (24 d.p.i.) (Figure 3.1.14 A).

However, would the passive transfer of MOG-specific antibodies also induce relapse during remission? To test this, 2 ml of serum derived from MOG-DNA vaccinated rats were injected 15 and 16 d.p.i. i.v.. At this time one animal had fully recovered, while the remaining two animals showed residual disease activity (grades 0.5 and 1.5). Within 72 hours of the first injection, those rats with residual disease relapsed and developed severe EAE (grade 3 and 3.5), while the other animal, which had fully recovered only developed mild clinical symptoms (grade 1) (Figure 3.1.13 C). Therefore, pathogenic antibodies were capable of inducing relapse during early remission, even when the recipient had fully recovered. However, the clinical effect of serum transfer at this time point was not as dramatic and more variable compared to the transfer 20/21 d.p.i., suggesting that the blood brain barrier may be less compromised at this time point and/or that the tissue itself may be less vulnerable to antibody-mediated damage.

This interpretation is supported by the observation that demyelination was less pronounced after serum transfer at the earlier time point (Figure 3.1.14 B). Strikingly, even in the absence of antibody-transfer large inflammatory infiltrate were detected in the CNS four days later, when the rats were perfused (20 d.p.i.).

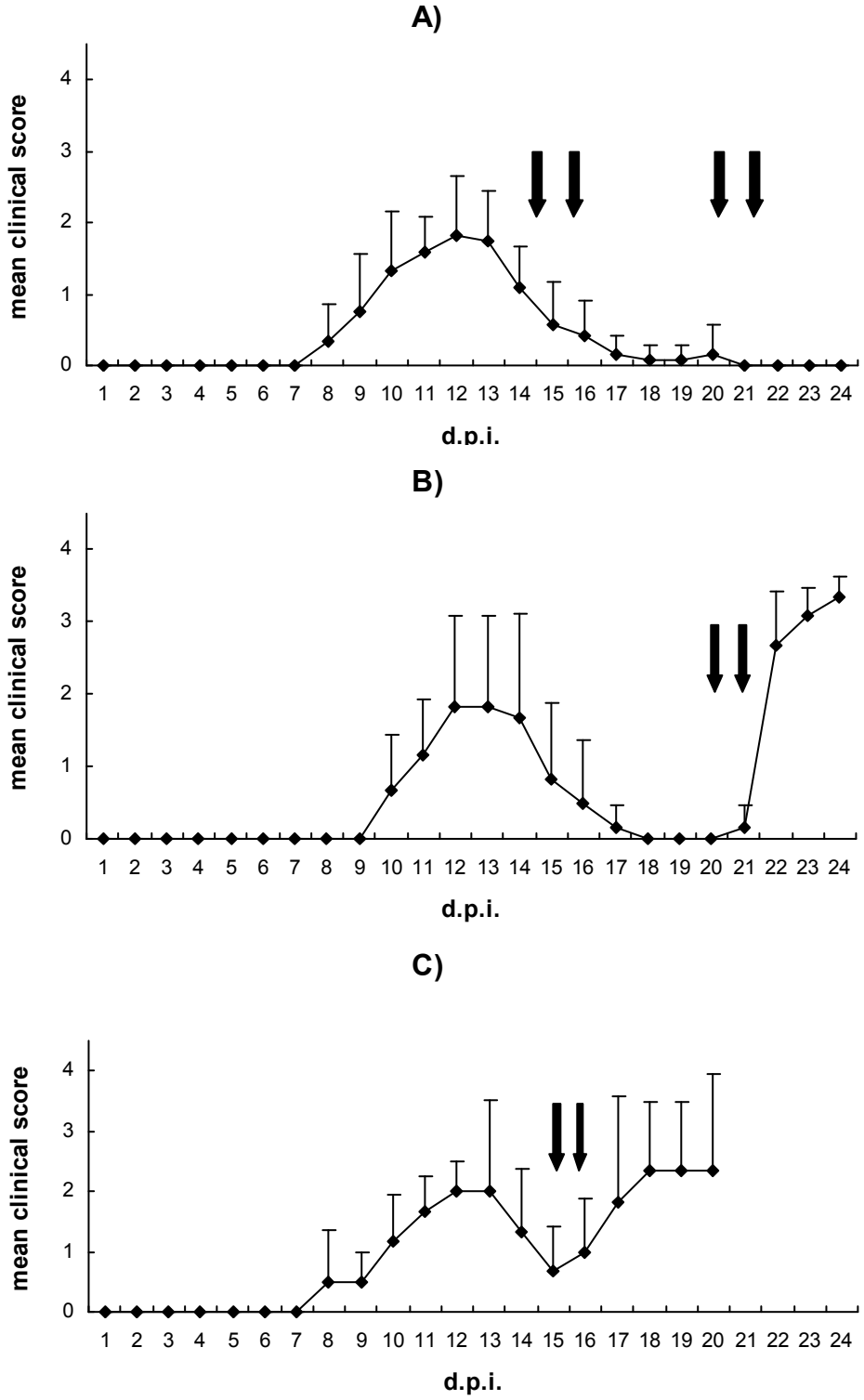


Figure 3.1.13 Co-transfer of sera derived from MOG-vaccinated DA rats into rats immunised with M_{93-109} induces severe relapse.

A) Rats injected with control serum at either 20/21 or 15/16 d.p.i.; **B**) rats injected with two doses of serum of pcDNA-MOG vaccinated rats 20 and 21d.p.i.; **C**) rats injected with two doses of serum of pcDNA-MOG vaccinated rats 15 and 16 d.p.i.. Shown are the average and standard deviation for 3 rats in B), and C) and for 6 rats in A). The arrows indicate the time of antibody transfer. In A) this was either 15/16 d.p.i. or 20/21 dp.i..

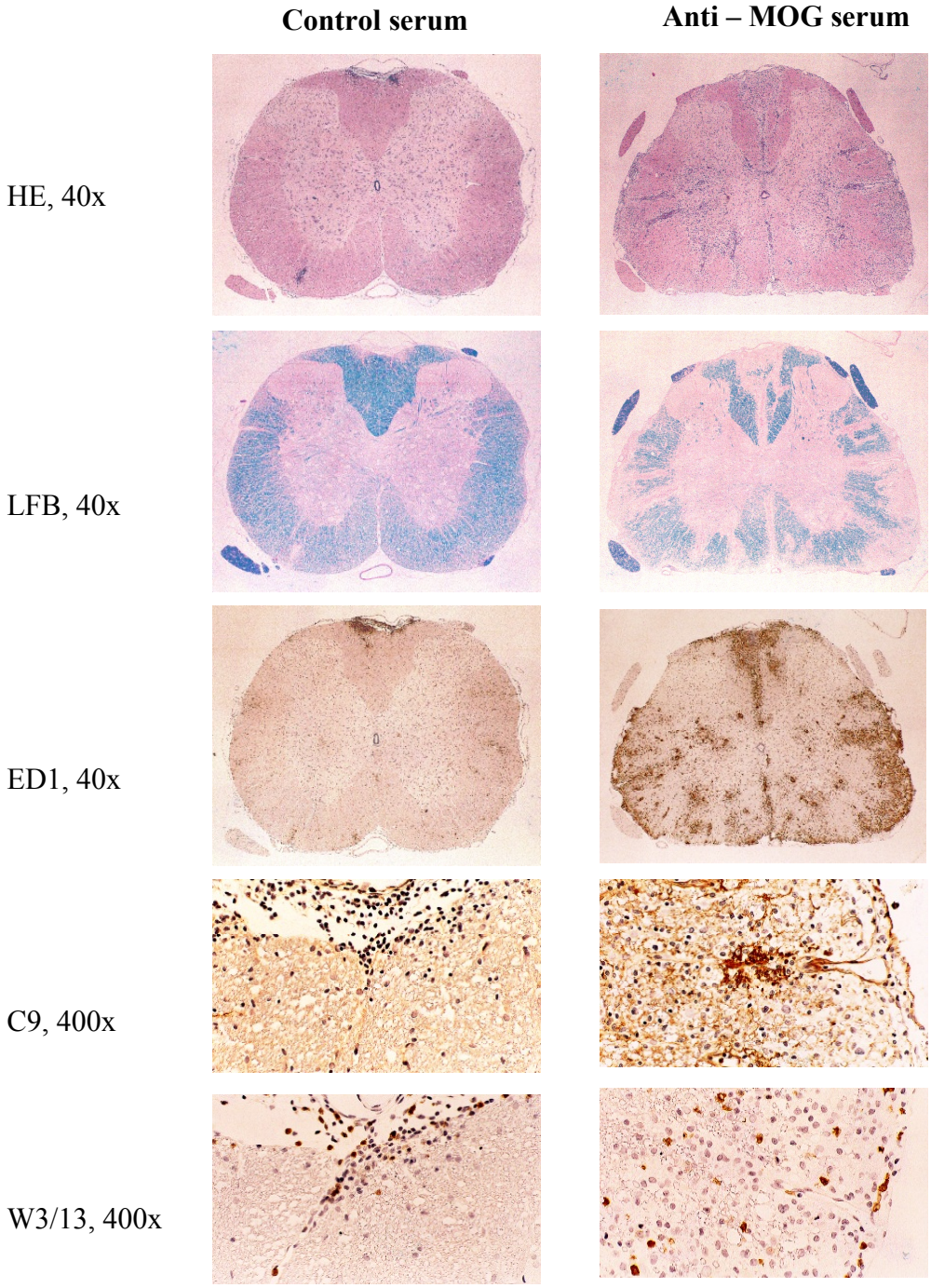


Figure 3.1.14 A) Pathological analysis after co-transfer of MOG-specific antibodies 20 and 21 d.p.i. into DA rats immunised with M_{93-109} in IFA.

Serial sections derived 24 d.p.i. from representative DA rats, which were immunised with M_{93-109} in IFA and co-injected with control- or MOG-serum 20 and 21 d.p.i.. Note that inflammation at the time of perfusion (24 d.p.i.) is minimal in control animals. Sections were stained for inflammatory infiltrates (HE); myelin (LFB); activated microglia or macrophages (ED1); complement deposition (C9) or T cells (W3/13). Photos by Dr. R. Höftberger, Vienna, Austria

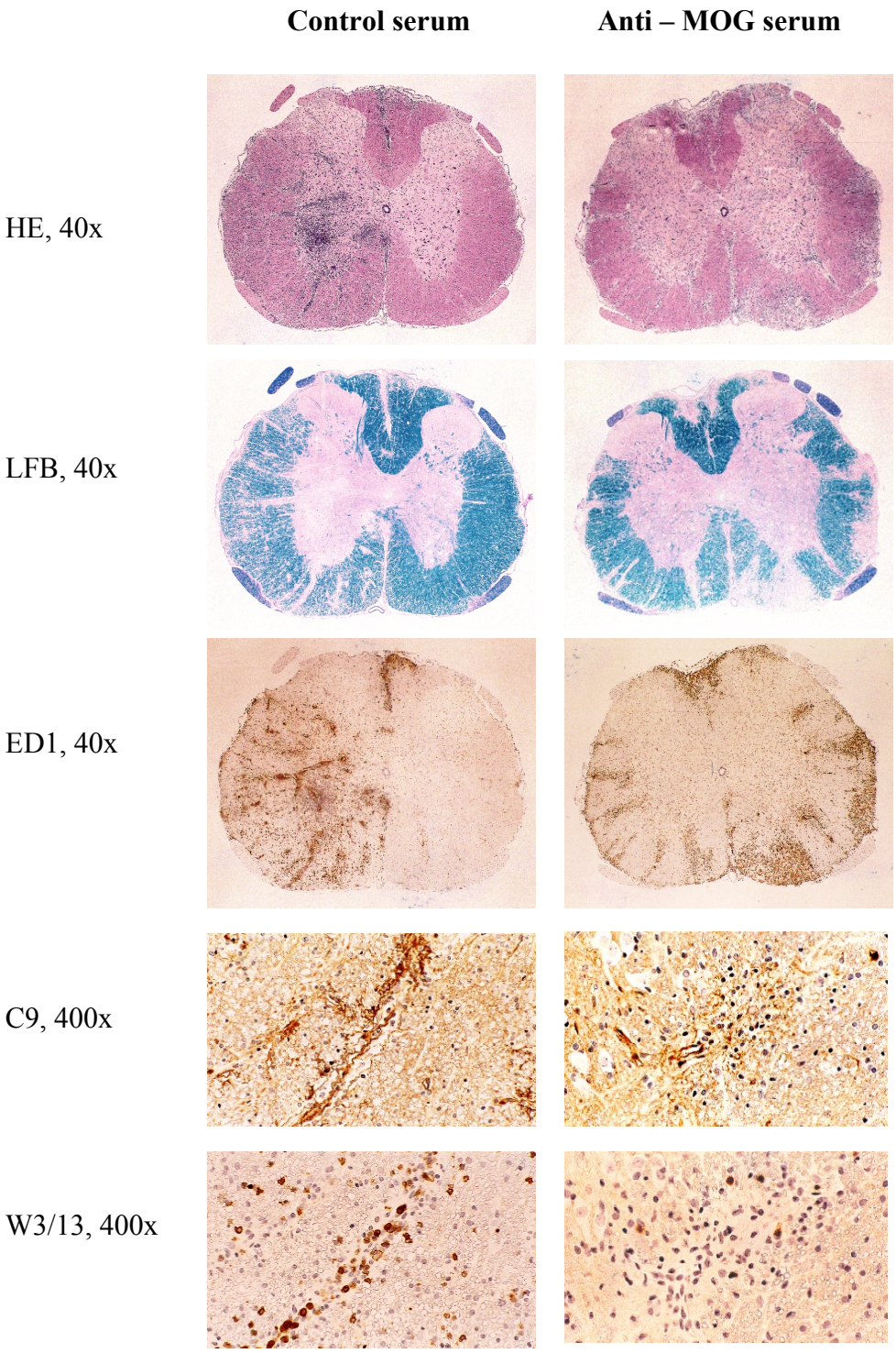


Figure 3.1.14 B) Pathological analysis after co-transfer of MOG-specific antibodies 15 and 16 d.p.i. into DA rats immunised with M_{93-109} in IFA.

Serial sections derived 20 d.p.i. from representative DA rats, which were immunised with M_{93-109} in IFA and co-injected with control- or MOG-serum 15 and 16 d.p.i.. Note that in comparison to the control animals at 24 d.p.i.. inflammation at this time of perfusion (20 d.p.i., figure 3.1.14 A) is very pronounced in the controls. Sections were stained for inflammatory infiltrates (HE); myelin (LFB) and activated microglia or macrophages (ED1). Photos by Dr. R. Höftberger, Vienna, Austria

3.1.3.4 Pathogenic antibodies are selectively absorbed into the inflamed CNS

As mentioned above (Section 3.1.3.2.4), previous studies in the guinea pig suggested that the opening of the BBB in response to CNS inflammation is correlated with a decrease in the MOG-specific antibody titre due to selective absorption of MOG-specific antibodies in the CNS (Linington and Lassmann, 1987). We proposed that this could be the reason why we were unable to detect pathogenic MOG-specific antibody in the serum of DA rats with MOG-Igd-induced EAE. Having established a method to produce a demyelinating MOG-specific antibody response, which is normally excluded from the CNS by the BBB, it was now possible to determine, whether a damage of the BBB would lead to selective absorption of this antibody response. DA rats were vaccinated with a combination of two plasmids, pcDNA-MOG and a control vector (pcDNA-BTN/MOG). This vector encodes a fusion protein, whose N-terminal IgV like domain is homologous to MOG and which induces a non-demyelinating antibody response after vaccination (see section 3.2.7). 28 d.p.v. all animals had developed antibody responses to both MOG and BTN, and were then injected with 2.8 x 10^6 freshly activated MOG-specific T cells to induce EAE. The rats were then bled on a daily basis and the levels of MOG and BTN-specific antibody determined by ELISA. As the animals started to develop disease, the MOG-specific antibodies in the periphery decreased dramatically in all animals (to 67% +/- 11; range 20-44% at 4 d.p.t., compared to 1 d.p.t.; $p<0.01$). In contrast, the titre of peripheral BTN-specific antibodies was virtually unchanged (mean 102% +/- 17; range 89-128%, not significant) (Figure 3.1.15). This differential decrease in the two responses in the periphery demonstrates that MOG-specific antibodies are selectively absorbed within the CNS once the BBB is disrupted. As suggested previously (Linington and Lassmann, 1987; Haase et al., 2000), in the case of a very low titre demyelinating response to MOG this effect may reduce the level of circulating antibodies below detection limits of the FACS assay.

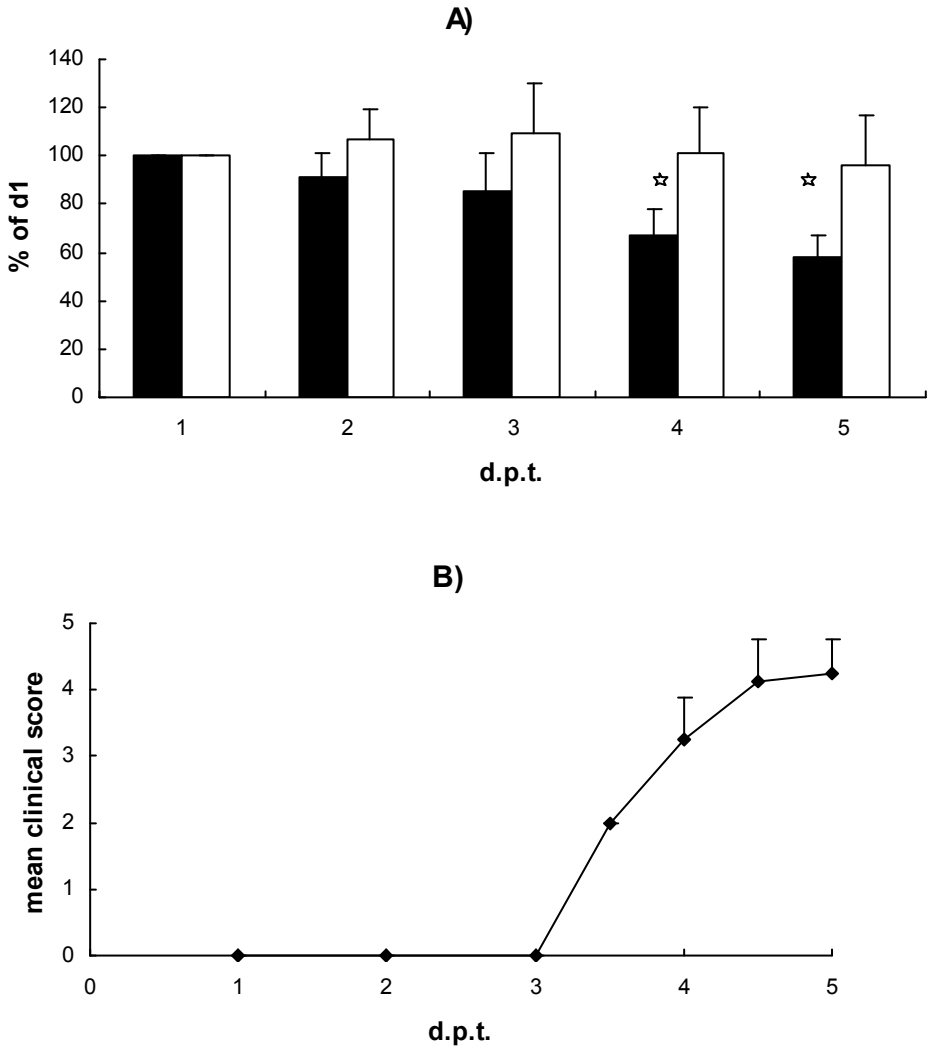


Figure 3.1.15 The transfer of MOG-specific T cells induces a selective reduction of MOG-specific antibodies in the periphery

Female DA rats were vaccinated with both pcDNA-MOG and pcDNA-BTN/MOG and injected with 2.8 x 10^6 MOG-specific T cells 28 d.p.v.. **A)** Analysis of the titre of MOG-Igd- and BTN^{exo}-specific antibodies. Sera were taken daily after the transfer and analysed by ELISA at a dilution of 1/100. Shown is the percent reduction compared to 1 d.p.i. and standard deviation of the four rats (three rats at 5 d.p.i.) analysed individually in quadruplicate. Standard deviation in the individual rats was around 5-10%. **Black:** MOG-specific antibodies, **white:** BTN-specific antibodies. The titre of MOG-specific antibodies was significantly reduced 4 and 5 d.p.i. ($p<0.01$, using one-tailed Student´s t-test, comparison to the titre 2 d.p.i.). In contrast, the titre of BTN-specific antibodies was unsignificantly reduced; **B)** Clinical course of the same rats after the T cell transfer. Shown is the mean and standard deviation of 4 rats (n=3 at 5 d.p.i.). The titre of MOG-specific antibodies dropped significantly concurrent with the onset of clinical symptoms.

3.1.4 Discussion

Traditionally, EAE studies in the rat have mainly dealt with monophasic models of T cell mediated EAE and investigated questions pertaining to the cellular and molecular basis of induction and recovery rather than relapse. Recovery from acute inflammatory CNS disease in the rat is attributed to multiple cellular (Sun et al., 1988; Lenz and Swanborg, 1999; Karpus and Swanborg, 1991; Schwartz and Cohen, 2000), humoral (McPhee et al., 1990; Asakura et al., 1998) and hormonal (Mason et al., 1990; Hohlfeld et al., 2000) immunoregulatory mechanisms, all of which contribute to the resolution of clinical disease and the development of tolerance to a second encephalitogenic challenge with the same antigen. However, unlike these acute, T cell mediated models of EAE, MS normally presents as a relapsing remitting disease, which eventually becomes chronic progressive with no obvious remissions. Virtually nothing is known about the mechanisms responsible for either triggering relapses early in disease, or the switch to a chronic progressive disease course.

Relapse is commonly attributed to either the failure of one or more of the mechanisms mentioned above, or alternatively to epitope spreading, the development of encephalitogenic responses to additional CNS specific T cell epitopes as disease progresses (Tuohy et al., 1998; Vanderlukt et al., 1998). However, irrespective of the mechanisms involved, relapse activity in EAE is determined by multiple genetic loci. (Lorentzen et al., 1997 ; Olsson et al., 2000). This is very clearly seen in MOG-Igd-induced EAE in the rat. DA rats are highly susceptible to relapse, while LEW rats show a very low relapse activity (Weissert et al., 1998; Olsson et al., 2000; Lorentzen et al., 1997). This is strongly influenced by the DA MHC locus ($RT1^{av1}$ haplotype), but also by other, yet ill-defined non-MHC "background" genes. (Weissert et al., 1998; Lorentzen et al., 1997). The current study demonstrates for the first time that in the context of this susceptible genotype, clinical relapse and the initiation of chronic progressive disease can be driven by the development of a pathogenic, demyelinating antibody response.

As demonstrated in a transgenic mouse model, the presence of circulating MOG-specific autoantibodies alone is insufficient to induce EAE (Litzenburger et al., 1998). Disease induction not only requires a dysfunctional BBB, which is essential for the entry of antibodies into the CNS, but in all probability also a susceptible milieu within the CNS, since inflammatory factors such as IFN-γ are vital for significant amplification of disease activity by the antibody response (Vass et al., 1992). Only when these two criteria are fulfilled will antibodies, which can bind to the myelin surface, be able to efficiently induce demyelination, amplify the inflammatory response in the CNS and induce a severe episode of EAE.

Previous studies have shown that the adoptive transfer of MOG-specific antibodies can induce relapse after incomplete recovery in chronic relapsing EAE in SJL/J mice (Schluesener et al., 1987) and amplify the clinical deficit when co-transferred with MBP-specific T cells in LEW rats. In addition, the MOG-specific antibody response potentiates disease activity in BN and LEW rats. In these two rat strains, the MOG-specific T cell response alone is insufficient to induce clinical symptoms of EAE, as shown by adoptive transfer studies of MOG-specific T cells (Adelmann et al., 1995; Stefferl et al., 1999) or immunisation with MOG-peptides (Adelmann et al., 1995). Immunisation with MOG-Igd, on the other hand, induces fulminant (BN) or chronic progressive (LEW) EAE. In contrast, the MOG-specific T cell response alone is highly pathogenic in the DA rat and the adoptive transfer of 1.7 to 5 x 10^6 MOG-specific T cells induces severe clinical EAE associated with a strong inflammatory response in the CNS (Figures 3.1.6/3.1.7). The pathogenicity of this response is determined by the $RT1^{av1}$ MHC haplotype (Stefferl, pers. comm.) and may reflect the high affinity of this peptide to the MHC (Weissert et al., 2001; De Graaf, pers. comm.).

This inflammatory pathology is reproduced when DA rats are actively immunised with the immunodominant peptide $M_{93\text{-}109}$ in IFA, a protocol which generally induces a single episode of clinical EAE. Earlier studies using CNS homogenates to induce disease demonstrated that DA rats are susceptible to develop relapsing remitting EAE (Lorentzen et al., 1995). Relapse activity was also seen in 91% of the animals immunised with $M_{93\text{-}109}$ in CFA (Figure 3.1.9 B), although here the disease was self-limiting and histopathological analysis revealed an absence of demyelination. These observations indicate that although the T cell response to MOG can induce multiple relapses, regulatory mechanisms eventually develop to suppress their clinical impact. Relapse activity was not normally seen in $M_{93\text{-}109}$-Igd/IFA immunised animals, but inflammatory activity was observed in CNS tissues of apparently healthy animals 20 d.p.i. (Figure 3.1.14 B, control) suggesting that waves of inflammation also occur in $M_{93\text{-}109}$/IFA-immunised animals. However, after the initial episode of disease, the intensity of the inflammatory insult fails to cross the threshold required to induce an obvious clinical deficit. The adoptive transfer of demyelinating MOG-specific antibodies 20 and 21 d.p.i. with $M_{93\text{-}109}$/IFA confirms that the BBB is compromised during this episode of subclinical inflammation and that the CNS milieu is exquisitely sensitive to the pathogenic effects of antibody mediated demyelination.

It is no coincidence that the onset of relapse and irreversible disease progression in MOG-Igd/IFA-induced EAE occurs after a rapid increase of the antibody titre, at a time, when a second sub-clinical wave of inflammation is developing within the CNS. As demonstrated in

animals immunised with MOG_{93-109} in either IFA or CFA, the T cell mediated component of the disease would be self-limiting. The presence of a demyelinating MOG-specific antibody response, however, amplifies this subclinical response, leading to a secondary progressive disease from which the animals fail to recover.

Interestingly, antibody transfer during the remission phase of MOG-peptide induced EAE was less efficient at inducing relapse, but still potentiates disease activity, even in a clinically fully recovered animal (Figure 3.1.14 B). In contrast, in a mouse model of RR-EAE antibody transfer has no effect once the animals had clinically recovered (Schluesener et al., 1987), suggesting a species specific difference in the recovery of the BBB after CNS inflammation. In DA rats the inflammatory response in the CNS is dramatically reduced, but still present during remission (Dr. R. Höftberger, pers. communications; Stefferl et al., 2001). It is yet unknown whether the lower efficiency of the antibody to induce relapse at this time is due to recovery of BBB function and exclusion of antibodies from the CNS (Dore-Duffy et al., 1996), or the development of a transient "counter-inflammatory" CNS environment. The latter possibility is supported by the local down regulation of IL-1β expression in the CNS during remission (Stefferl et al., 2001) which suggests that the inflammatory milieu in the CNS is less "permissive" for antibody-mediated augmentation of EAE (Vass et al., 1992). Nevertheless, pathogenic autoantibodies antibodies are able to override the regulatory mechanisms responsible for the transient resolution of clinical disease activity and enhance both inflammation and demyelination. However, if antibody can only promote relapse by amplifying an existing inflammatory response in the CNS, it remains to be determined why the DA rat fails to control the T cell mediated inflammatory reaction in the CNS after the initial immune attack.

Relapsing remitting EAE induced in the DA rat using MOG_{93-109} in CFA provides a purely inflammatory model of CNS disease characterised by a high incidence (>90%) of well synchronised relapse activity (in the first relapse), which can be used to answer this question. In particular, it will be possible to determine the relative importance of peripheral antigen depot, epitope spreading and defects in the neuroendocrine axis/immunoregulation. The suppression of disease activity in a guinea pig model of RR-EAE after excision of the injection site indicates that the peripheral antigen depot may be essential to continue driving relapses in EAE (Tabira et al., 1984). A role for the peripheral depot, as opposed to secondary response to autoantigen derived from the inflamed CNS i.e. epitope spreading is also supported by the observation that even 30 d.p.i. no detectable response to other encephalitogens e.g. MBP has been detected (A. Stefferl, pers. communication)

In contrast, the importance of neuroendocrine control in determining relapse activity was recently demonstrated in the MOG/IFA paradigm (Stefferl et al., 2001). This study revealed that relapse was associated with a desensitisation of the HPA axis to immunological stress, resulting in a corresponding inability of the affected animals to mount a regulatory, counter-inflammatory corticosteroid response as they entered the second phase of disease. However, considering the complexity of the effector mechanisms involved in disease pathology and the fact that multiple genetic loci determine whether the disease follows a relapsing remitting course, it is to be expected that more than one mechanism is involved in the regulation of this model of MS.

The induction of relapse due to the development of a pathogenic antibody response in clinically healthy animals is a novel mechanism that may help to understand disease progression in those cases of MS associated with a demyelinating autoantibody response (Genain et al., 1999; Haase et al., 2000; Lucchinetti et al., 2000), and supports the use of plasmapheresis to treat selected patients (Weinshenker, 1999 and 2001). However, this study does not eliminate the role of T cells, but stresses that clinical progression - while T cell dependent - may be driven by antibody mediated mechanisms. It should not be forgotten that in the case of MS, this antibody response might not be specific for MOG, but may also be directed against other yet unidentified targets on the surface of the myelin sheath.

Despite the high proportion of MS-cases with type II lesions (indicative of antibody mediated immune mechanisms, Lucchinett et al., 2000), only a small proportion of MS patients have serum antibodies capable of binding native MOG expressed on the cell surface of transfected cell lines (Haase et al., 2000). In contrast, MOG-Igd-specific antibodies recognising linear peptide epitopes are present in 30 - 60% of patients (Reindl et al., 1999; Lindert et al., 1999; Haase et al., 2000). This discrepancy between the high number of patients with "antibody-mediated" type II lesions and the low frequency of patients with "pathogenic" antibody responses to MOG was attributed to either the existence of other target antigens or to a selective absorption of demyelinating antibodies into the brain (Haase et al., 2000). Using DNA vaccination in rats (reviewed in Gurunathan et al., 2000), this study has clearly demonstrated that such an absorption can have a pronounced effect on the level of circulating antibodies. This will have to be taken into account for the development of serological assays, which will identify patients with antibody-mediated effector mechanisms at an early time point.

The pathology of this model of highly synchronised relapsing remitting EAE closely resembles that of the major subset of MS patients (type II lesions) with respect to microglial activation, the recruitment of immune effector cells into the CNS, deposition of antibody and C9 on the myelin sheath, myelin degradation and vesiculation, the presence of macrophages

carrying immunoglobulin, C9 and myelin debris, and finally axonal loss. This model, which lasts only 3-4 weeks provides the opportunity not only to test immune directed therapies, but also remyelination strategies or neuroprotection (with respect to the axonal loss). One such investigation is presented in Appendix I.

3.2 Molecular mimicry with the milk protein butyrophilin modulates MOG-Igd - induced EAE

Little is known about the aetiology of multiple sclerosis (MS), but it is generally agreed that it involves the interplay of both genetic and environmental factors. This concept is supported by twin and familial studies, which demonstrate that the concordance rate for disease is about 30% in monozygotic twins but only about 4% in dizygotic twins (Sadovnick et al., 1993; Sadovnick and Ebers, 1995; Oksenberg and Barcellos, 2000). These observations indicate that while genetic factors play an important role in determining disease susceptibility, other as yet unidentified "environmental" factors must act as disease triggers, possibly by disrupting self-tolerance to CNS myelin antigens (Gale et al., 1995).

One mechanism by which environmental factors may disrupt self-tolerance to tissue specific autoantigens is molecular mimicry, an amino acid sequence homology between two unrelated antigens which leads to a cross-reactive immune response (Fujinami and Oldstone, 1985). In this situation, a protective immune response induced by a pathogen may also recognise epitopes derived from a tissue specific autoantigen and mediate tissue specific auto-aggression. Molecular mimicry in the context of EAE was first described in rabbits with respect to an encephalitogenic T cell epitope of MBP and a homologous peptide present in hepatitis B virus polymerase (Fujinami and Oldstone, 1985). This concept was later investigated in man using human MBP-specific T cell clones, which were found to cross-react with different synthetic viral peptides *in vitro* (Wucherpfennig et al., 1995). Moreover, in experimental animals immunisation with synthetic microbial peptides can induce an auto-aggressive T cell response *in vivo* (Fujinami and Oldstone, 1985; Gautam et al., 1998; Ufret-Vincenty et al., 1998).

Molecular mimicry can lead to a cross-reactive T cell response because recognition of MHC/peptide complexes by the T cell receptor is degenerate. This results in the recognition of multiple, possibly thousands of agonistic and antagonistic peptide ligands by a single T cell receptor (Mason, 1991; Gran et al., 1999). It was recently demonstrated that molecular mimicry involving an epitope of HSV-1 was directly involved in the aetiology of a murine model of herpes stromal keratitis (HSK) by inducing an autoimmune response to a yet unidentified retinal antigen (Zhao et al., 1998). However, whether or not mimicry involving microbial pathogens and myelin autoantigens leads to CNS auto-aggression during the natural course of any infection remains unknown.

Microbial antigens are not the only candidates to trigger auto-aggression by molecular mimicry, but theoretically any antigen may induce a cross-reactive immune response, including components of the diet. Indeed, dietary exposure to a bovine albumin peptide has been proposed as a trigger for autoimmune diabetes by inducing a cross-reactive antibody-response to the β-cell surface protein p69 (Karjalainen et al., 1992; reviewed in Berdanier et al., 1995).

However, dietary antigens normally stimulate a very different immune response to that induced following challenge with an infectious agent. Infections generally induce a Th1 dominated inflammatory response in order to effectively eliminate the pathogen, a situation mimicked in EAE and other models of autoimmune diseases by immunisation with protein/adjuvant emulsions. These inflammatory responses are, however, dangerous as they may damage surrounding tissue and disrupt normal physiological function. Specific mechanisms have therefore evolved in the gastro-intestinal tract to suppress potentially lethal inflammatory responses to components of the diet and symbiotic gut flora. This is termed oral tolerance (Weiner et al., 1994; Chen et al., 1994; Chen et al., 1996; Strobel 1996; Weiner, 1997, Gutgemann, 1998; Strobel and Mowat, 1998) and is maintained by multiple mechanisms, including the induction of apoptosis (programmed cell death) and anergy (unresponsiveness) of the potential effector cells and/or the generation of suppressor responses (reviewed in Weiner 1997 and Strobel and Mowat, 1998). Intriguingly, although the induction and activation of suppressor cells by oral tolerance is antigen specific, these cells act via the secretion of soluble mediators (IL-10 and TGF-β) and are hence capable of inducing bystander suppression (Weiner, 1997). The relative importance of the individual mechanisms in oral tolerance is determined by nature and dose of antigen, the frequency of exposure as well as genetic factors. The redundancy of mechanisms involved in oral tolerance provides a robust system leading to long lasting, systemic tolerance.

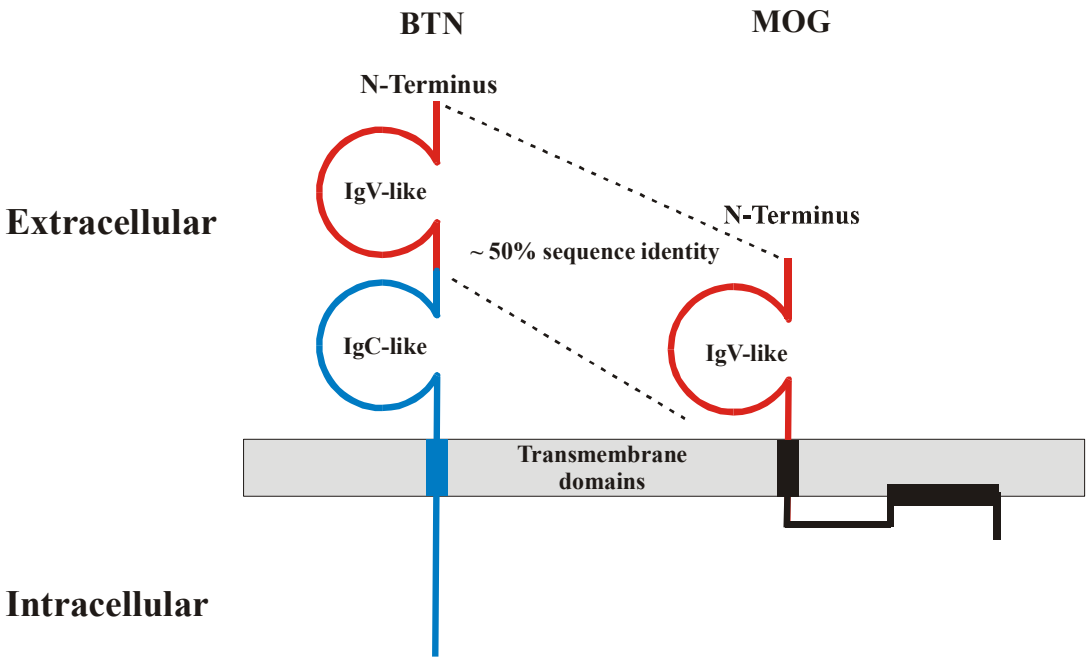
However, soluble factors secreted by pathogens within the gastro-intestinal tract can disrupt oral tolerance. This is well documented for cholera toxin, which when fed with an unrelated antigen abrogates oral tolerance and induces immune responses (Hornquist and Lycke 1993; reviewed in Weiner, 1997; Hänninen et al., 2000). In addition, oral application of soluble proteins in neonates (under 4 - 6 days of age in mice) may prime the immune system rather than inducing tolerance (Miller et al., 1994; reviewed in Strobel and Mowat, 1997). This demonstrates that in specific circumstances molecular mimicry between a dietary antigen and self can result in a potentially auto-aggressive Th1 response and trigger autoimmune disease.

It was therefore interesting to find extensive sequence homology between MOG-Igd and the N-terminal Ig-domain of the milk protein butyrophilin (BTN) (Gardinier et al., 1992)(Figure

3.2.1). As BTN is a major component of the milk fat globule membrane (Jack and Mather, 1990), this observation immediately raised the question, whether exposure to BTN in the diet could influence the composition or function of the MOG-reactive repertoire in MS patients. This is an intriguing possibility in the light of epidemiological studies that identified an association between the consumption of milk and dairy produce and the prevalence of MS (Butcher 1986; Malosse et al., 1992; reviewed in Lauer, 1997).

Indeed, in an initial study we demonstrated that immunisation with bovine BTN or purified bovine milk fat globule membrane could induce a sub-clinical inflammatory response in the CNS in the context of a permissive MHC-haplotype (e.g. $RT1^{av1}$, DA rat) (Stefferl et al., 2000). This was due to the induction of a T cell response that cross-reacted with the peptide $M_{74\text{-}90}$, a subdominant MOG-T cell epitope in the DA rat. The pathogenicity of this response was confirmed by adoptive transfer of BTN/MOG cross-reactive T cell lines, which induced an inflammatory response in the CNS and mild clinical symptoms of EAE in naïve syngeneic recipients (Stefferl et al., 2000). Interestingly, the CNS lesions induced by the transfer of $B_{74\text{-}90}$-specific T cells remained restricted to the perivascular space and failed to recruit macrophages into the CNS. This correlated with a low pathogenicity of this T cell response, resembling EAE induced by the adoptive transfer of T cells specific for the astrocyte protein S100β in the Lewis rat (Kojima et al., 1994) rather than disease induced by TCLs specific for MOG in DA (section 3.1.3.1.3). These experiments demonstrated that molecular mimicry can occur between MOG and BTN, but left a number of important questions unanswered.

Particularly intriguing was the question, why $BTN_{74\text{-}90}$-selected T cells were significantly less pathogenic than those cells selected using the homologous MOG peptide, although both recognise the same pair of antigens. Several explanations could possibly account for this observation. First, it could be due to oral tolerance induced by autologous BTN during suckling. Alternatively, the two antigens may either select different but partially overlapping T cell populations that differ in their pathogenicity or the two peptides may elicit different cytokine responses in the cross-reactive T cell population and thereby modify T cell function.




```
Rat MOG    GQFRVIGPGHPIRALVGDEAELPCRISPGKNATGMEVGWYRSPFSRVVHLYRNGKDQDAEQA
Bov BTN    AP-D----QE--L-V--ED------L--NVS-K---LR-F-EKV--A-FVS-E-QE-EGEEM
           1                                                             62

Rat MOG    PEYRGRTELLKESIGEGKVALRIQNVRFSDEGGYTCFFRDHSYQEEAAVELKVED
Bov BTN    A-----VS-VEDH-A--S--V---E-KA--D-E-R----QDENY---I-H---AA
          63                                                    117
```

Fig. 3.2.1 Structural and amino acid sequence homologies between BTN and MOG

Upper panel: Structural comparison of BTN and MOG. BTN contains two extracellular Ig domains (Ω-shaped in this figure) as well as a transmembrane domain and intracellular domains (Jack and Mather, 1990). MOG consists of a single extracellular Ig domain, one hydrophobic transmembrane domain and the cytoplasmic tail containing a second hydrophobic region (Kroepfl et al., 1996; Della Gaspera et al., 1998). Note that only the N-terminal IgV-like domain of BTN has a significant sequence identity to MOG. **Lower panel**: Amino acid sequence comparison between rat MOG-Ig (Gardinier et al., 1992, accession number: M99485) and the N-terminal Ig-domain of bovine butyrophilin (BTN) (Jack and Mather, 1990, accession number: M35551). Amino acids are represented in the one-letter-code, amino acids that are identical in the two proteins are represented as dashes in the BTN sequence. The immunodominant T cell epitope of rat MOG (a.a. 93-109) is printed in red, the subdominant epitope (a.a. 74-90) in blue. The numbers represent the amino acid residues in the mature protein

3.2.1 Rat BTN fails to influence the bovine BTN/rat MOG cross-reactive T cell response

The observation that T cell responses to the bovine BTN peptide B_{74-90} are potentially encephalitogenic in DA rats (Stefferl et al., 2000), albeit less than the cross-reactive MOG TCLs indicates that the ingestion of BTN via the mothers milk fails to induce complete oral tolerance to the xenogenic BTN in the pups. This is in direct contradiction to experiments using ovalbumin as a control antigen, in which repeated feeding of rat pups resulted in a firm state of long-term tolerance (Brown et al., 1994; Wald et al., 1987; Hall et al., 1988). However, one simple explanation for this observation could be that the amino acid sequence of the N-terminal IgV-like domain of BTN is not highly conserved between species. In this case tolerance would be established to the autologous protein, but would not extend to BTN derived from other species – in this case the bovine protein. Comparison of the published BTN sequences, which were available for *Homo sapiens* (Vernet et al., 1994; Accession number NM_001732), *Bos taurus* (Jack and Mather, 1990; accession number M35551) *and Mus musculus* (Ogg et al., 1996; accession number NM_013483), suggested that this may indeed be the case, the amino acid sequence conservation between the N-terminal IgV-like domains of mouse and bovine BTN being only 56%.

To confirm that lack of sequence identity was responsible for the failure of the rat to develop tolerance to bovine BTN, the deduced amino acid sequence of the extracellular domains of rat BTN was obtained by RT-PCR sequencing. This allowed a direct comparison with the published bovine and mouse sequences and enables the analysis of immune responses to the autologous protein. Several primer pairs were selected from regions of mouse BTN which had the highest levels of conservation between species and used to generate RT-PCR products from lactating rat mammary gland mRNA (Materials and Methods, 2.2.19). This approach identified products of the appropriate sizes, which were then sequenced.

In order to confirm that the amplified cDNA encoded for butyrophilin rather than a BTN homologue (Henry et al., 1999; Stammers et al., 2000; Rhodes et al., 2001), we analysed its expression pattern in various organs by RT-PCR using primers placed in the exons encoding the N-terminal IgV-region and the transmembrane region (Materials and Methods 2.2.20). As predicted for butyrophilin, expression was restricted to the lactating mammary gland. (Figure 3.2.2)

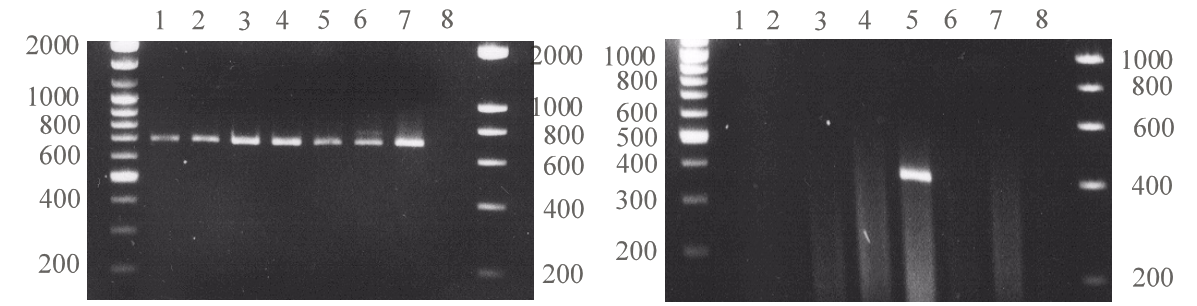


Figure 3.2.2 RT-PCR analysis of rat BTN expression in different tissues

Left) β-actin control; **right)** rat BTN. 1. Liver; 2. Thymus; 3.Spleen; 4. Brain; 5. Mammary gland; 6. Heart; 7. Lung and 8. negative control in the absence of cDNA. The BTN primers used amplified a fragment of 393 nts, corresponding to the sequence encoding amino acids 105-236. The expected size of the β-Actin product was 700 nts.

```
----------------------------------------------------------------------------------------------------------------
Rat BTN    1   APFNVTAPQESVLAVVGSDAELTCRFSPNASSEQMELLWFRQTRSPAVLLYRAGQEQEGE
Mouse BTN      ---D------P---L---------G--------Y-----------T------D------Q
Bovine BTN     ---D-IG---PI-----E----P--L---V-AKG---R---EKV----FVS-E-------

Rat BTN      QMTEYHGRATLVTARLLDGLATLRIRGVRVSDQGQYRCFLKDNDDSEEAAVHLKVAA 117
Mouse BTN    -----R-----A--G----R---L--D-------E---LF-----F-----Y----- 84%
Bovine BTN   E-A--R--VS--EDHIAE-SVAV--QE-KA--D-E----FRQDENY---I------- 58%
----------------------------------------------------------------------------
```

Figure 3.2.3 Amino acid sequence comparison between rat, mouse and bovine BTN.

Amino acids are represented in the one-letter-code, amino acids identical to rat BTN are represented as bars. The sequence homologous to the immunodominant MOG peptide (a.a. residues 93-109) is printed in red, the part corresponding to the subdominant MOG-peptide (a.a. residues 74-90) in blue. The sequence was confirmed at least once for each residue using different PCR reactions for the each sequencing reaction.

Comparison of the predicted sequence of the N-terminal IgV-like domain of rat BTN with the homologous mouse and bovine BTN domains identified a relatively strong conservation of the rat and mouse sequences (84% sequence identity in the first Ig-domain), which is significantly higher than sequence identity with either of the known mouse BTN homologues (Henry et al., 1999; Stammers et al., 1999; Rhodes et al., 2001) or with bovine BTN (58% sequence identity in the first Ig-domain, Figure 3.2.3). Intriguingly, the homology between rat and bovine BTN was not evenly distributed throughout this Ig domain. An area of low homology was found within the a.a. residues 73-90, exactly the sequence containing the epitope responsible for the cross-reactive T cell response between rat MOG and bovine BTN. In this peptide the sequence identity was only 23.5% (Figure 3.2.3, in blue).

```
Rat MOG    GQFRVIGPGHPIRALVGDEAELPCRISPGKNATGMEVGWYRSPFSRVVHLYRNGKDQDAEQA
Bov BTN    AP-D----QE--L-V--ED------L--NVS-K---LR-F-EKV-PA-FVS-E-QE-EGEEM
Rat BTN    AP-N-TA-QESVL-V--SD---T--F--NASSEQ--LL-F-QTR-PA-L---A-QE-EGE-M

Rat MOG    PEYRGRTELLKESIGEGKVALRIQNVRFSDEGGYTCFFRDHSYQEEAAVELKV
Bov BTN    A-----VS-VEDH-A--S--V---E-KA--D-E-R----QDENY---I-H--- 51%
Rat BTN    T--H--AT-VTARLLD-LAT---RG--V--Q-Q-R--LK-NDDS-----H--- 44%
```

Figure 3.2.4 Amino acid comparison between rat MOG and bovine and rat butyrophilin.
Amino acids are represented in the one-letter-code, amino acids identical to rat MOG are represented as bars. The immunodominant MOG peptide (93-109) is printed in red, the subdominant MOG peptide (74-90) in blue. Homology of rat MOG to rat BTN (44%) was slightly lower than that to bovine BTN (52%)

Comparison of rat MOG and the autologous milk protein revealed an overall sequence identity of 44% between the N-terminal Ig domains, which is only slightly lower than the homology between rat MOG and bovine BTN (51%). The homology in the "epitope of interest" (a.a. residues 74-90) was reduced from a 53% identity between bovine BTN and rat MOG to only 35% identity between autologous BTN and MOG (Figure 3.2.4).

Having obtained the rat BTN sequence, it was now possible to investigate directly whether the cross-reactivity between rat M_{74-90} and bovine B_{74-90} extended to the corresponding rat BTN peptide. Strikingly, synthetic rat-BTN_{74-90} was unable to induce either a proliferative response or cytokine secretion (IL-10, IFN-γ, TNF-α or TGF-β) in M_{74-90}/B_{74-90}-reactive TCLs (data not presented). This lack of a response could have several explanations. The peptide could be incapable of binding to the DA-MHC class II molecules or even if bound fail to stimulate the TCR of the M_{74-90}/B_{74-90}-reactive cells. On the other hand, although rat-BTN_{74-90} may not directly cross-react with the M_{74-90}/B_{74-90}-reactive T cells, the peptide may still be immunogenic, in which case ingestion of rat BTN during suckling would have resulted in a "suppressor" cell population characterised by antigen-specific secretion of IL-10 and TGF-β (Weiner, 1997). Splenocytes derived from naïve rats were therefore restimulated with rat-BTN_{74-90}, and rat-BTN_{93-109}. Neither peptide induced proliferation or the secretion of IL-10, IFN-γ, TNF-α or TGF-β, demonstrating that DA rats fail to induce a T cell response to these two rat-BTN peptides.

This clearly demonstrates that the neonatal exposure to rat BTN does not modulate the cross-reactive T cell response between bovine BTN and MOG. The reduced pathogenicity of

BTN-selected TCLs is therefore not due to a modulation by autologous BTN, but must involve other mechanisms.

3.2.2 Is B_{74-90} an altered peptide ligand (APL) for M_{74-90}-specific T cells?

A reasonable explanation for the failure of the BTN-specific T cells to induce macrophage recruitment could be that TCR signalling induced by the MHC/BTN peptide complex may differ from that induced by the autologous MOG peptide/MHC complex, possibly due to differences in affinity. Such effects are well documented for the response of MBP- or PLP-specific T cell clones to peptide "mimics" or "altered peptide ligands" (APL) (Nicholson et al., 1995; Smilek et al., 1991; Gaur et al., 1997) and can dramatically influence cytokine production. This may lead to reduced secretion of Th1-associated proinflammatory cytokines such as IFN-γ, while enhancing the secretion of Th2-associated counter-inflammatory cytokines such as IL-4 and IL-10 in encephalitogenic T cells (Nicholson et al., 1995). *In vivo*, this APL mediated effect on cytokine synthesis (immune deviation) can be exploited to suppress CNS inflammation and thereby disease activity in EAE (Nicholson et al., 1995).

The possibility that bovine BTN could provide a naturally occurring APL, which modifies the MOG-specific T cell response, is intriguing. In order to determine whether this was the case, proliferation, cytokine secretion, cell surface phenotype and finally the pathogenicity of M_{74-90}-specific TCLs were compared following restimulation with either M_{74-90} or B_{74-90} *in vitro*.

3.2.2.1 Restimulation with B_{74-90} reduces proliferation of M_{74-90}-specific T cells

The two M_{74-90}-selected TCLs used in this study proliferated in response to both MOG and their cognate ligand $M_{74-90,}$ but not to the second encephalitogenic MOG-epitope M_{93-109} (Figure 3.2.5 A and Table 3.2.1 for one of the lines). Proliferation was also observed in response to the homologous BTN peptide, B_{74-90}. However, over a wide range of peptide concentrations B_{74-90} induced less proliferation than M_{74-90}. Interestingly, the two TCLs varied in their relative responsiveness to B_{74-90}. In one line, B_{74-90} only induced 50% of the response seen with M_{74-90}, whereas in the other line this was approximately 80%.

3.2.2.2 Restimulation with B_{74-90} fails to alter the cell surface phenotype of M_{74-90} - specific T cells

Optimal restimulation of T cells with their cognate antigen induces an increase in cell size as well as a number of activation-associated changes in the surface phenotype. This includes the down-modulation of the CD3/TCR complex and the up-regulation of IL-2R and OX40 antigen-

receptor (CD 134)(Flügel et al., 2001; Nohara et al., 2001). We therefore compared the surface expression of these molecules on M_{74-90}-specific T cell blasts following restimulation with either M_{74-90} or B_{74-90} to determine whether both peptides induced similar levels of activation.

M_{74-90}-specific T cells were restimulated with M_{74-90} or B_{74-90}. After 72 hours of restimulation, T cell blasts were analysed for the expression of IL-2R, TCR, CD3, CD4, OX40 antigen or MHC class I by FACS analysis. We were unable to detect any significant, reproducible differences in the expression of these molecules following restimulation with cognate or xenogenic ligand (Figure 3.2.6). This demonstrates that all M_{74-90}-specific T cells which cross-react with B_{74-90} are fully activated and express the same pattern of cell surface markers seen on other encephalitogenic T cell lines (Flügel et al., 2001; Kojima et al., 1994)

3.2.2.3 Restimulation with B_{74-90} modulates the cytokine spectrum of M_{74-90}-specific T cell lines

Many T cell functions are mediated by secreted cytokines, and the ratio of individual cytokines has a major influence on the outcome of the immune response (Liblau, et al., 1995). Some cytokines, like IFN-γ and TNF-α, are characteristic for inflammatory responses mediated by Th1 T cells, while others, such as IL-10 and TGF-β, are generally considered as mediating counter-inflammatory functions and are associated with the Th2 T cell subset. Nevertheless, these divisions are not absolute and encephalitogenic rat T cell lines secrete both IFN-γ and IL-10 (Stefferl et al., 1999; Lenz et al., 1999). In order to determine whether B_{74-90} acts as an APL and affects the cytokine balance of the M_{74-90}-specific T cells, M_{74-90}-specific T cell lines were restimulated with a range of concentrations of B_{74-90} and M_{74-90} and cytokine secretion assayed by ELISA (Figure 3.2.5 B, C, D). Intriguingly, despite the difference of the two lines in terms of their proliferative response to B_{74-90}, restimulation with this peptide induced a consistent shift in cytokine secretion in favour of IL-10 relative to TNF-α and IFN-γ. For the TCL shown in Figure 3.2.5 A-D, despite its reduction of the proliferative response, restimulation with B_{74-90} did not modify the secretion of IL-10, while secretion of IFN-γ was decreased by 40% and secretion of TNF-α reduced by 80%. No significant secretion of TGF-β was detected (data not shown). However, the presence of TGF-β in the serum added to the restimulation medium generated a high background, which may significantly reduce the detection limit of the method.

These results demonstrate that restimulation with the cross-reactive BTN peptide is associated with immune deviation, in this case away from a “pro-inflammatory” Th1-like towards a counter-inflammatory Th2-like response.

3.2.2.4 Restimulation with B_{74-90} reduced the pathogenicity of M_{74-90} specific T cells

The counter-inflammatory properties of IL-10 have been exploited previously to suppress disease activity in EAE (Rott et al., 1994), and increased IL-10 production is generally associated with a reduction in the pathogenicity of auto-aggressive Th1 T cell lines. It was therefore anticipated that the relative increase in the synthesis of IL-10 by the M_{74-90} selected T cell lines following restimulation with B_{74-90} would be associated with a decrease of the pathogenic potential of the TCLs.

M_{74-90}-selected T cell lines were therefore restimulated with either M_{74-90} or B_{74-90} for 72 hours and 5 x 10^6 T cell blasts transferred into naïve DA rats. Strikingly, although the same number of T cell blasts was transferred, disease onset was delayed and disease severity strikingly reduced in those rats injected with T cell blasts that had been restimulated with the BTN-derived peptide (Figure 3.2.5 E).

Bovine BTN therefore acts as an APL for M_{74-90}-specific TCLs and provides a model system in which APL-mediated effects can modulate the autoimmune repertoire. The generation of TCR transgenic rats over-expressing the appropriate cross-reactive TCR(s) would open the door to analyse the *in vivo* effect of a dietary APL on the cross-reactive T cells as well as facilitating the generation of T cell clones for the analysis of the *in vitro* effects on a clearly defined T cell population.

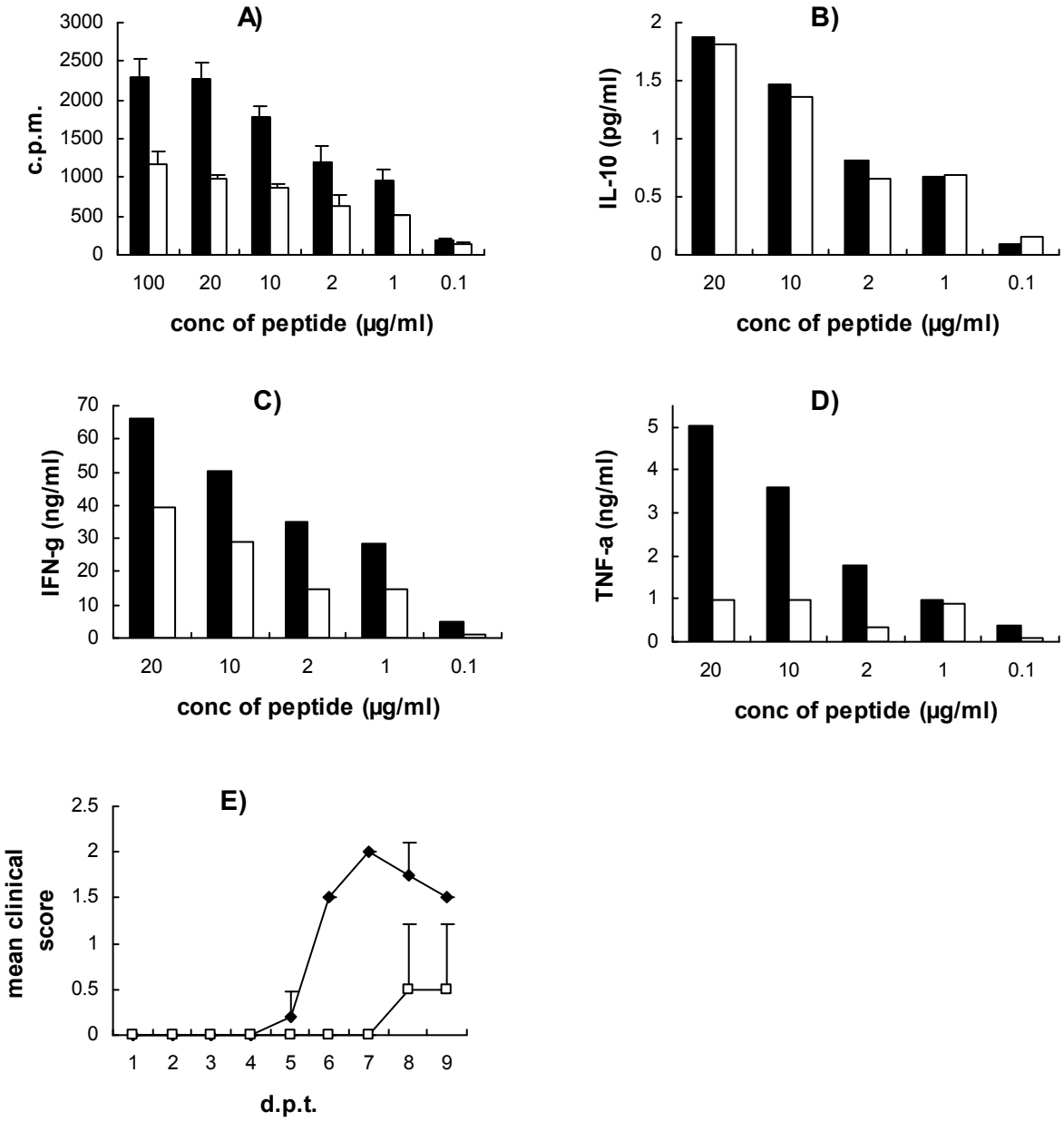


Figure 3.2.5 Analysis of the phenotype of M_{74-90}-selected T cells in response to M_{74-90} or B_{74-90}

A) Proliferation of M_{74-90} selected T cells to a range of concentrations of M_{74-90} (black columns) or B_{74-90} (white columns). Shown are the mean c.p.m. and standard deviations of a quadruplicate analysis. **B - D)** ELISA analysis of the secreted cytokines of the cells restimulated in A). **B)** IL-10, **C)** IFN-γ and **D)** TNF-α. Shown are the averages of duplicate analyses. Cells were restimulated in the presence of M_{74-90} (black columns) or B_{74-90} (white columns). **E)** Pathogenicity of 5 x 10^6 T cell blasts. M_{74-90} selected T cells were restimulated for 72 hours with M_{74-90} (diamonds) or B_{74-90} (squares). Shown is the average of two animals per group of a representative experiment.

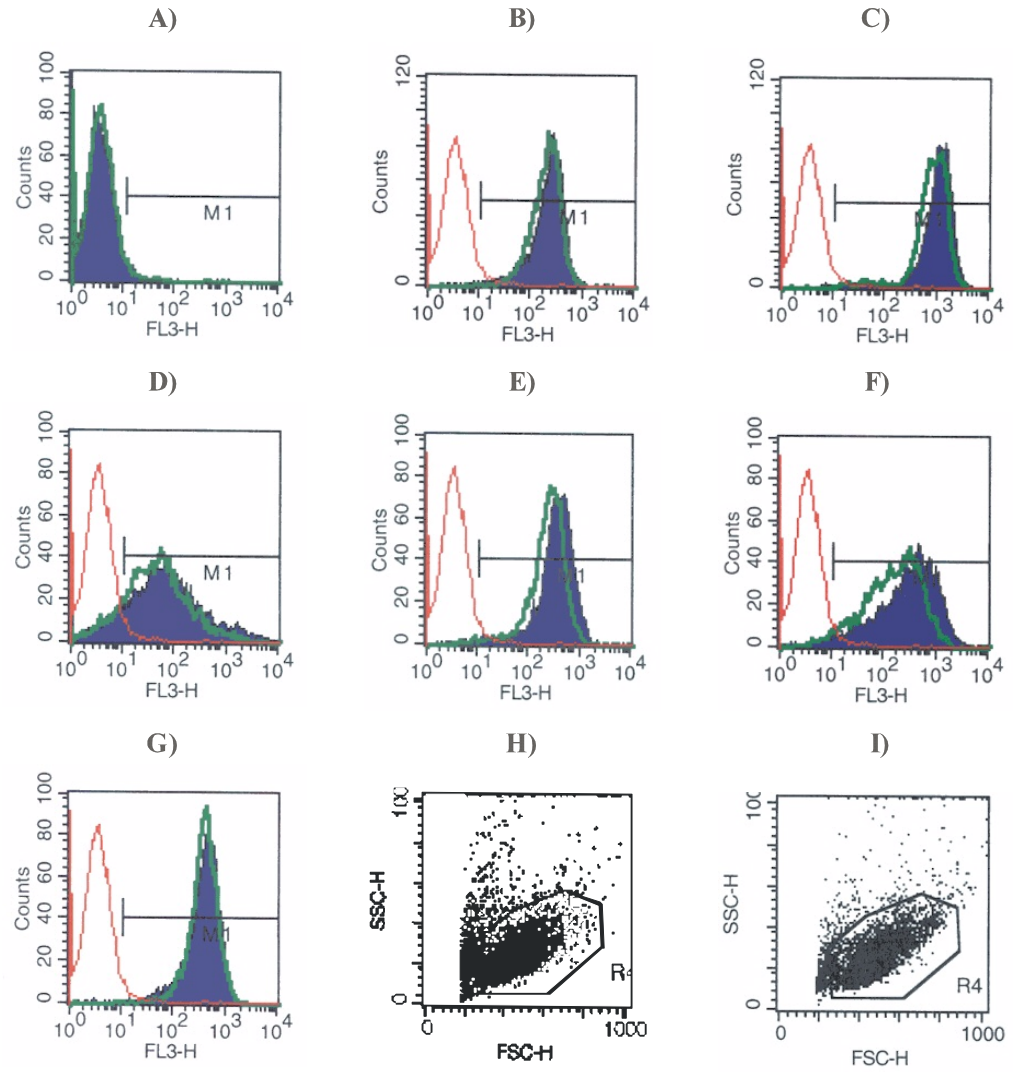


Figure 3.2.6 FACS analysis of M_{74-90} selected T cells after restimulation with the two peptides.
M_{74-90} selected TCLs were restimulated in the presence of irradiated thymocytes and either M_{74-90} or B_{74-90}. 3 days later, T cell blast were analysed for the expression of cell surface markers. **Blue**: Restimulation with M_{74-90}; **green**: restimulation with $B_{74-90;}$ **red overlay** represents the negative control. Shown is a representative analysis. Shifts are minimal and did not always follow the same trend. Cells are stained for **A)** isotype control; **B)** CD3; **C)** IL-2R; **D)** MHC class II; **E)** CD4; **F)** OX40; **G)** α/β TCR. **H)** and **I)** represent the gated populations.

3.2.3 Repeated selection with B_{74-90} or M_{74-90} results in the expansion of T cell clones with identical TCR usage

A pre-requisite for any future studies involving TCR transgenic rats is a full molecular characterisation of the TCR(s) used by the cross-reactive T cell response. Previous studies indicate that encephalitogenic T cell responses are associated with a limited number of clonal expansions (Chluba et al., 1989; Burns et al., 1989; Wucherpfennig et al., 1990). These can be

identified by spectratyping of the β chain of the T cell receptor (TCR spectratyping) (in collaboration with Dr. N. Goebels and I. Eiglmeier, Klinikum Großhadern, Munich).

This is a RT-PCR technique based on the length polymorphism in the CDR3 (complementarity determining region) of the TCR and the fact that each T cell expresses a single β chain. Primers are placed in the individual Vβ regions and the constant domain of the TCR and thus span the CDR3, which is the area in which the TCR rearrangement occurs during T cell maturation in the thymus (joining of different D and J regions with a V region, combined with junctional diversity) (see section 2.2.21). The PCR products are then separated on polyacrylamide gels (Figures 3.2.7). To facilitate analysis, size of product and intensity of fluorescence are represented as histograms (Figure 3.2.8). Spectratyping analysis of a mixed T cell culture will result in a gaussian distribution of PCR products of various lengths. In contrast, if a clonal expansion of T cells occurs, this gaussian distribution of the corresponding Vβ family is disrupted. In a monoclonal expansion this results in a single band, allowing the product to be sequenced by RT-PCR. This technique provides detailed information on the clonality and complexity of an antigen-specific T cell response.

In order to clearly identify the clonal expansions associated with the cross-reactive response, TCR usage was compared in TCLs derived from MOG-immunised rats and selected by repeated restimulation with either M_{74-90} or the cross-reactive BTN peptide B_{74-90}. As controls, cells were selected with MOG-Igd or M_{93-109}, the immunodominant MOG-peptide which does not cross-react with either M_{74-90} or B_{74-90} and which should therefore expand a different sub-population of the MOG-Igd-specific T cell repertoire. In addition, thymus from naïve DA rats was used as a source for a polyclonal T cell population.

Proliferation assays confirmed the antigen specificity of the TCLs (Table 3.2.1). Within two cycles of restimulation, TCLs selected with M_{93-109} lost all responsiveness to M_{74-90} and B_{74-90}. Conversely, selection with M_{74-90} or B_{74-90} resulted in a loss of responsiveness to M_{93-109}, while the responsiveness to MOG was retained. Interestingly, the B_{74-90}–selected cell lines always proliferated better in response to M_{74-90} than to their selective peptide B_{74-90} (Figure 3.2.5), a phenomenon, which has been described for altered peptide ligands previously (Nicholson et al., 1995; Nicholson et al., 2000). After six restimulation cycles T cell blasts were isolated and analysed by spectratyping.

Antigen / T cell line	Proliferation (c.p.m.)				
	No antigen	MOG	M_{93-109}	M_{74-90}	B_{74-90}
MOG - selected	48 +/- 14	3268 +/- 263	1387 +/-135	724 +/- 82	318 +/- 54
M_{93-109}-selected	4 +/- 4	3206 +/- 486	2936 +/- 343	2 +/- 2	2 +/- 2
M_{74-90} - selected	19 +/- 2	501 +/- 293	28 +/- 3	1665 +/- 177	901 +/- 65
B_{74-90} - selected	24 +/- 7	1801 +/- 154	17 +/- 8	3805 +/- 176	3554 +/- 192

Table 3.2.1 Representative proliferative response of the four different T cell lines to MOG and peptides.
TCLs were restimulated in the presence of irradiated thymocytes and antigens. Shown are the mean c.p.m. and standard deviation of quadruplicate measurements

Analysis of mRNA derived from DA thymus confirmed that all of the primer pairs used in this study generated a typical gaussian distribution of PCR products for each Vβ family (Figure 3.2.7 A). In contrast, spectratyping of the MOG-Igd-specific TCL and its three peptide-selected derivatives demonstrated the presence of multiple clonal expansions. This indicates that unlike the response of Lewis rats to MBP, which is dominated by a monoclonal expansion of Vβ8.2 expressing T cells (Chluba et al., 1989), the MOG-Igd-specific response in the DA rat is oligoclonal, even when specificity is restricted to the single peptide epitopes (Figure 3.2.7 B-D). Nevertheless, despite this complexity some of the TCLs shared TCR β-chains with CDR3 expansions of the same size, suggesting the presence of identical T cell clones (Table 3.2.2). However, this can only be confirmed by sequence analysis.

In detail, analysis of the MOG-Igd-selected TCL revealed multiple expansions in 21 of 22 Vβ families. In nine of these Vβ families, dominant expansions could be identified as single or clearly prominent products (judged by fluorescence intensity), suggesting that they can be sequenced. One of these (Vβ4) was also dominant in the TCL selected with the immuno-dominant peptide epitope M_{93-109} (Figure 3.2.8 A), which had expansions in 18/22 Vβ families (Table 3.2.2). Sequence analysis confirmed the presence of an identical TCR β-chain in the two TCLs (MOG and M_{93-109}-selected, Figure 3.2.8 B).

As expected, selection with M_{93-109} failed to generate any overlap with the M_{74-90}- or B_{74-90}-selected T cell lines and vice versa. The M_{93-109}-selected TCL was therefore used as a control for the sequencing reactions to demonstrate that expansions identified in more than one of the other TCLs are specific and not due to contamination.

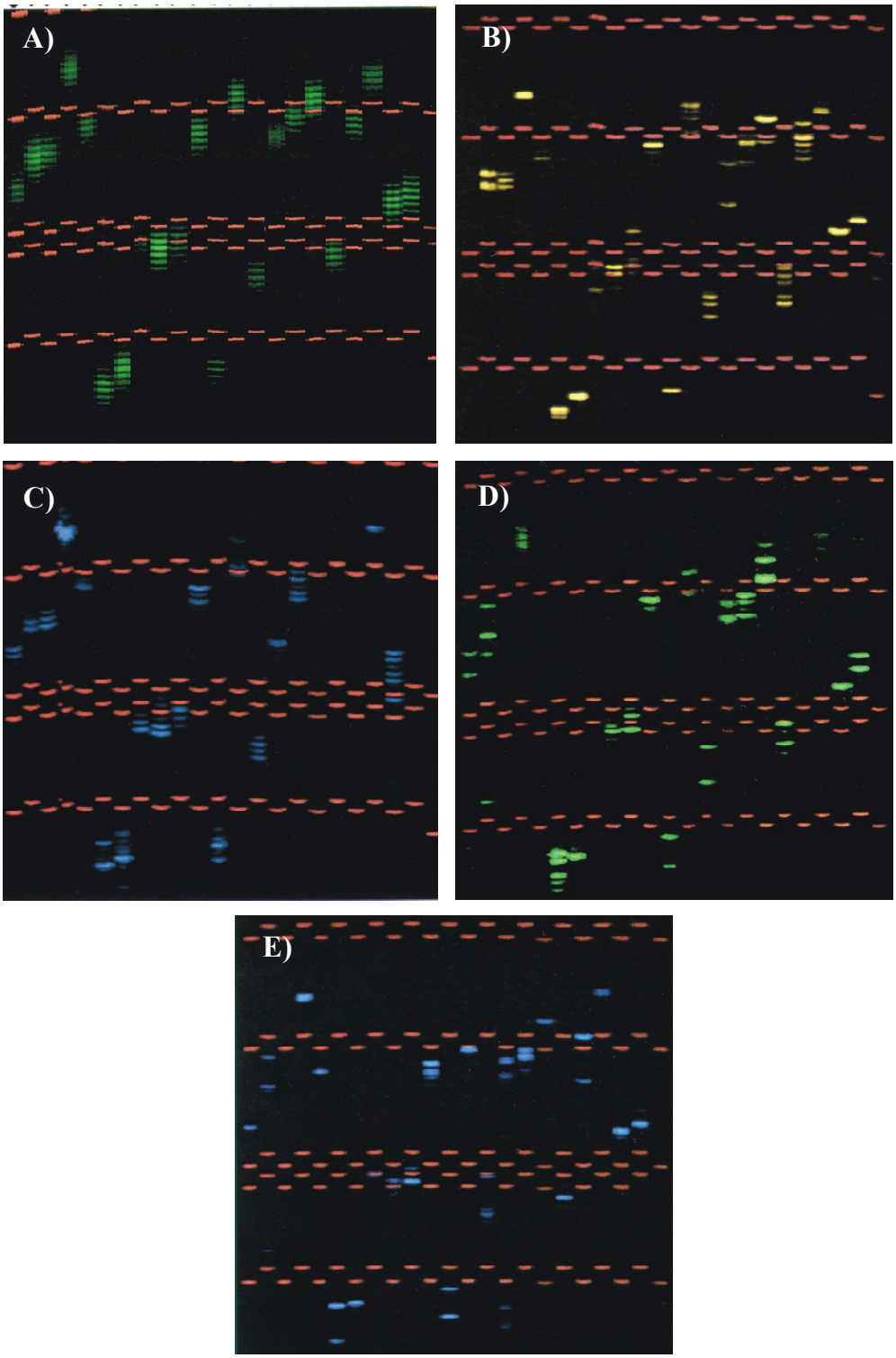


Figure 3.2.7 Spectratypes of rat thymus and T cell lines

A) Thymus control; **B-E)** T cell lines selected with **B)** MOG**; C)** $M_{93\text{-}109}$; **D)** $M_{74\text{-}90}$ and **E)** $B_{74\text{-}90}$. Each lane represents a single Vβ-family, analysed sequentially from Vβ1 to Vβ20 (s. Table 3.2.2). The horiyontal double/or quadruple bands are molecular size markers. Note that there are multiple expansions in most of the Vβ families, but these differ from the gaussion distribution observed in the thymus

	MOG	M_{93-109}	M_{74-90}	B_{74-90}
Vβ1	_	+	+	**365**
Vβ2	+	+	+	+
Vβ3	+	+	-	-
Vβ4	**415**	**415**	+	417
Vβ5	+	395	+	390
Vβ6	(280)	(272)	**(285)**	**(285)**
Vβ7	**287**	(278)	**287**	292
Vβ8.1/2	+	+	-	340
Vβ8.5	+	+	+	+
Vβ8.6	+	+	(338)	335
Vβ9	**398**	+	**398**	393
Vβ10	287	+	+	+
Vβ11	+	+	**(398)**	**398**
Vβ12	+	+	+	+
Vβ13	+	375	+	+
Vβ14	+	+	+	+
Vβ15	(410)	-	(405)	415
Vβ16	+	-	(339)	335
Vβ17	+	-	-	(405)
Vβ18	(405)	418	**(420)**	**420**
Vβ19	**360**	+	**360**	365
Vβ20	360	-	**363** and 370	**363**

Table 3.2.2 TCR β-chain spectratyping revealed expansions in 21/22 Vβ families in MOG, 18/22 families in M_{93-109}, 18/22 families in M_{74-90} and 21/22 in B_{74-90}.

Oligoclonal expansions are presented as + or, if clearly prominent expansions were present, by the size of the PCR products. Monoclonal expansions are without brackets, dominant bands with brackets. Similarly sized CDR3s are printed in red.

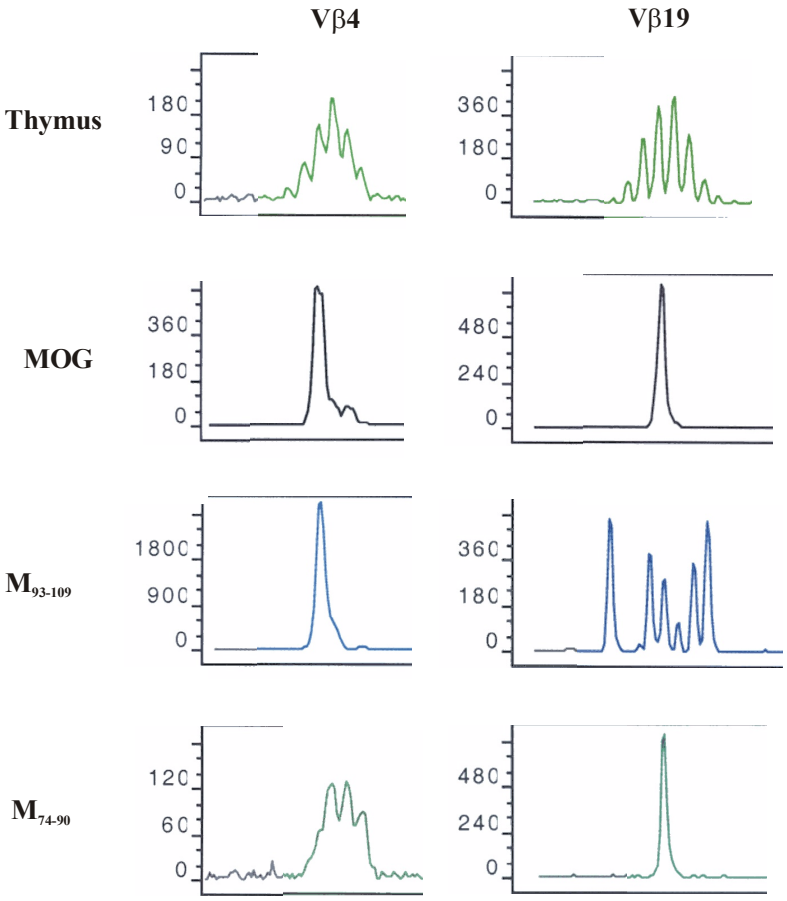
Selection with the subdominant MOG-epitope M_{74-90} (which had expansions in 19/22 Vβ families) generated dominant expansions of a similar size to dominant expansions in the MOG-Igd-selected cell line in Vβ7, Vβ9 and Vβ19 (Examples in Figure 3.2.8). The presence of identical CDR3 regions was subsequently confirmed for Vβ19 and Vβ7 by sequencing (Figure 3.2.7 B). In contrast, there was no sequence identity in Vβ9.

Strikingly, the spectratypes of the M_{74-90}- and B_{74-90}- selected TCLs were more closely related than any other two pairings. The B_{74-90}-selected TCL contained dominant expansions with five different Vβ chains, which had the same size as expansions – but not necessarily dominant expansions - in the M_{74-90}-selected T cells (Examples in Figure 3.2.9). Sequencing confirmed that the two lines used identical TCR β-chain rearrangements in at least three of these expansions (Vβ6, Vβ18 and Vβ20). However, in Vβ20 this involved only a sub-population of the M_{74-90} selected T cells (Figure 3.2.9) and therefore required an additional PCR amplification step using primers in Vβ20 and Jβ2.3, the Jβ element which was identified in the corresponding B_{74-90} sequence. Intriguingly, sequencing of the MOG and M_{93-109}-selected TCLs using primers in Vβ20 and Jβ2.3 revealed a second identical TCR β-chain in those two cell lines. This TCR β-chain was different to the one used by the M_{74-90} and B_{74-90} selected cell lines.

It should also be noted that spectratyping/sequencing of the TCL selected with MOG-Igd failed to identify any of the expansions shared by the M_{74-90} and B_{74-90} –selected TCLs (Vβ6, Vβ18 and Vβ20). This confirms the concept that the M_{74-90}/B_{74-90}-cross-reactive T cells are only a minor component of the total MOG-specific T cell repertoire in the DA rat.

To test whether the Vβ-chains, which were shared between the M_{74-90}- and B_{74-90}-selected cell lines and therefore presumably cross-reactive, might be pathogenic, we then analysed whether cells expressing TCRs would be able to invade the CNS. M_{74-90}- and B_{74-90}-selected T cell blasts were transferred into naïve syngeneic recipients and 5 d.p.t. the spinal cords were analysed for the expression (transcription) of Vβ6, Vβ18 and Vβ20. At this time point, the animals that had received the M_{74-90}-selected T cell showed the first symptoms of EAE, while the other animals were completely healthy. Using Jβ-specific primers, the shared Vβ6 and Vβ20 could be detected in animals injected with either cell line.

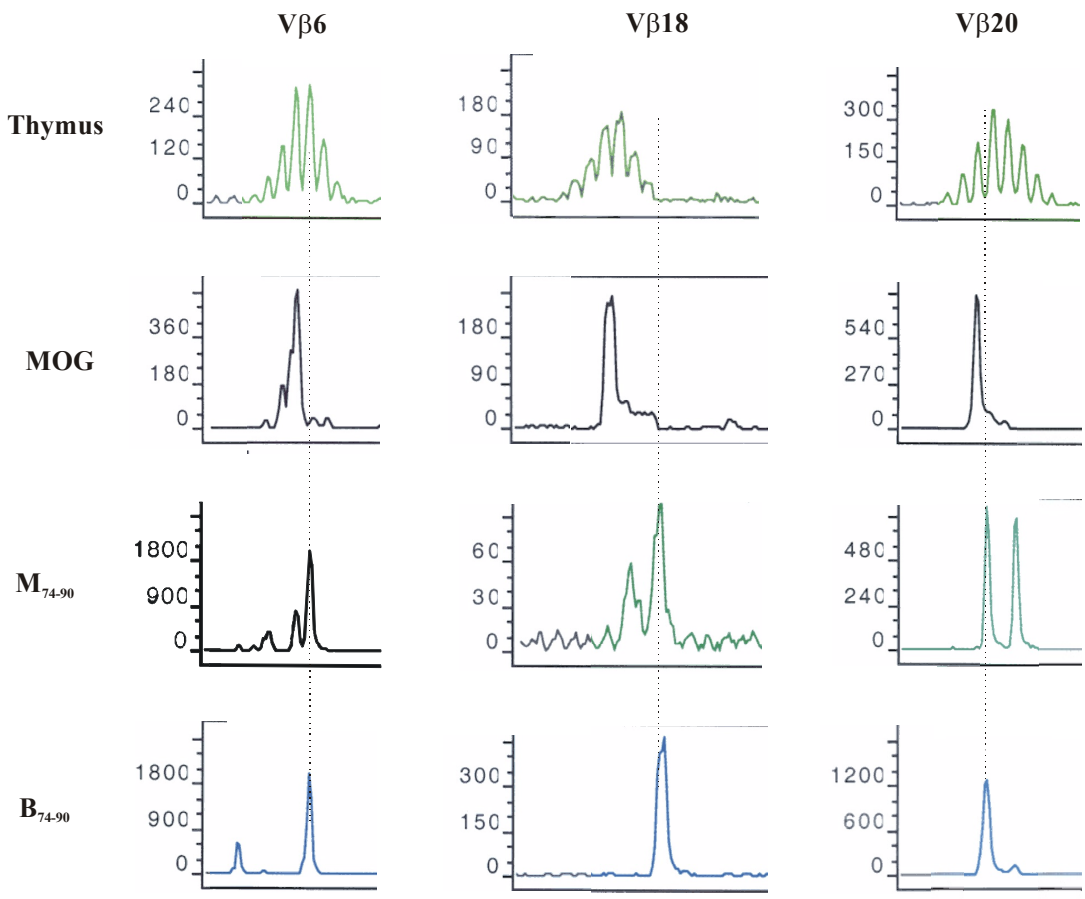
This study demonstrates that the BTN/MOG cross-reactive T cell response is complex and involves at least three different T cell clones. This is in part disappointing, as this will complicate further molecular analysis and suggests that specific TCR-directed therapies are unlikely to be effective in inhibiting MOG-induced EAE (Acha-Orbea, 1988; Vandenbark et al., 1993; Wraith et al., 1989; Anderton et al., 1998).



Vβ4	MOG	CASS – QDRD - TGQLYFGEGS-Jβ2.2
	M_{93-109}	CASS – QDRD - TGQLYFGEGS-Jβ2.2
Vβ7	MOG	CASS – DDRGDTE - VFFGKGT-Jβ1.1
	M_{74-90}	CASS – DDRGDTE - VFFGKGT-Jβ1.1
Vβ19	MOG	CASS – HTGGSYE - QYFGPGT-Jβ2.6
	M_{74-90}	CASS – HTGGSYE - QYFGPGT-Jβ2.6

Figure 3.2.8 Identification of TCR β-chain CDR3 regions, which are identical in MOG- or MOG-peptide selected TCLs.

Upper panel: Comparison of spectratypes of Vβ4 and Vβ19 in MOG- and MOG-peptide selected TCLs. Histograms showing the relative amount of PCR product (intensity of fluorescence, y-axis) with respect to the product size (x-axis). Thymocytes show a gaussian distribution of peaks, which is disrupted in all TCLs. In some Vβ families dominant expansions were observed as single peaks (e.g. Vβ19 in the MOG- and M_{74-90} - selected TCLs). Identical location of the peaks in MOG and M_{93-109}-selected lines (Vβ4, left) or MOG and M_{74-90}-selected lines (Vβ19, right) suggests identical expansions in the two TCLs, while the selection with the other peptide induced multiple expansions in these Vβ families. **Lower panel: Amino acid sequence comparison of Vβ4, Vβ7 and Vβ19.** Amino acid sequences were deduced from the sequences obtained from RT-PCR products and are represented in the one-letter-code. This revealed that cells with the same TCR β chains were expanded in the MOG or MOG-peptide selected TCLs. CDR3-regions are printed in red, surrounding Vβ and Jβ region in black. Jβ families were identified after Williams et al., 1991 after sequencing of the product.



Vβ6 $M_{93\text{-}109}$ CASS – IEKG – ERLFFGHGT-Jβ1.4

$M_{74\text{-}90}$ CASS – PPLGGH – SQNTLFFGAGT-Jβ2.4

$B_{74\text{-}90}$ CASS – PPLGGH – SQNTLFFGAGT-Jβ2.4

Vβ18 $M_{74\text{-}90}$ CSS – GDFHAF – TTDKIYFGSGT - Jβ2.3

$B_{74\text{-}90}$ CSS – GDFHAF – TTDKIYFGSGT - Jβ2.3

Vβ20 $M_{74\text{-}94}$ CSS - CQGQWG – DKIYFGSGT - Jβ2.3

$B_{74\text{-}90}$ CSS - CQGQWG – DKIYFGSGT - Jβ2.3

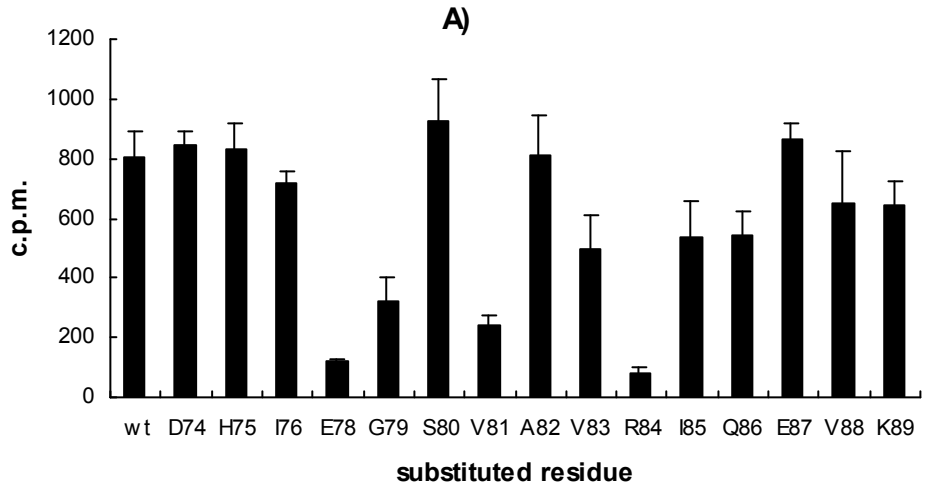
Figure 3.2.9 Identification of clones with identical TCR Vβ-usage in the BTN/MOG cross-reactive T cells

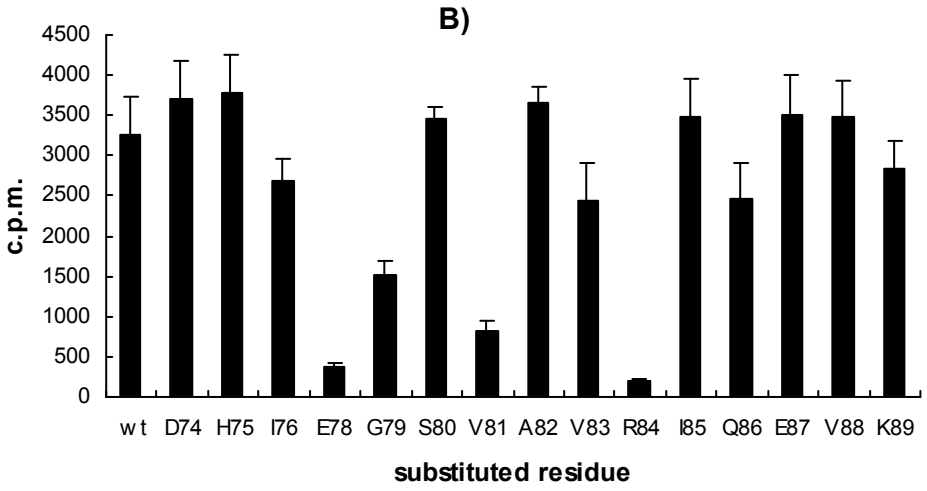
Upper panel: Comparison of spectratypes of Vβ6, Vβ18 and Vβ20 of $M_{74\text{-}90}$- and $B_{74\text{-}90}$-selected TCLs. Histograms showing relative amount of PCR products (intensity of fluorescence, y-axis) with respect to the product size (x-axis). Thymocytes show a gaussian distribution of peaks. In contrast, monoclonal expansions were observed in the $B_{74\text{-}90}$-selected TCLs. Note that the product size (location of the peak) is identical in $M_{74\text{-}90}$- and $B_{74\text{-}90}$-selected TCLs, but different in the MOG-selected line. **Lower panel: Amino acid sequence comparison of Vβ6, Vβ18 and Vβ20.** Amino acid sequences were deduced from the sequences obtained from RT-PCR products and are represented in the one-letter code. Note that for Vβ6, the clone expanded in the $M_{93\text{-}109}$-selected TCL uses a different CDR3 as well as a different Jβ fragment. CDR3-regions are printed in red, surrounding Vβ and Jβ region in black. Jβ families were identified after Williams et al., 1991 after sequencing of the product. Sequencing of Vβ20 in the $M_{74\text{-}90}$ selected TCL required a second round of PCR using Jβ-specific primer.

3.2.4 Four conserved amino acid residues are essential for the BTN/MOG cross-reactive T cell response

Clearly the identification of cross-reactive TCRs is vital for subsequent analysis of the MOG/BTN cross-reactive T cell response. At the same time it is important to identify those amino acid residues, which are necessary for this cross-reactivity. In order to define the amino acid residues contributing to the cross-reactive T cell response, M_{74-90}- or B_{74-90}-selected TCLs were restimulated with M_{74-90}, B_{74-90}, or a panel of 15 alanine substituted versions of B_{74-90}, in which single amino acids were replaced by alanine residues (alanine in position 9 was replaced by glycine). Substitution of residues E78 and R84 dramatically reduced the proliferative response (>80%) of M_{74-90}-selected TCLs (Figure 3.2.10 A), and a similarly pronounced loss of proliferation was observed when residues G79 and V81 were substituted (approximately 60%). In addition, substitutions at a further five sites (I85, Q86, I76, V88 and V83) had a consistent but low effect on the proliferation of M_{74-90}-selected T cells. (Figure 3.2.10 A). None of the substitutions resulted in significantly enhanced proliferation. An identical pattern emerged when the study was performed using B_{74-90}-selected TCLs. The same four residues were essential for significant proliferation (E78, R84, G79 and V81) (Figure 3.2.10 B). Additionally, some of the less important residues (I76, V83 and Q 86) also had an effect on the proliferation of these cell lines, while no additional residues were involved.

This analysis demonstrates that the substitution of any of four amino acids (E78, R84, G79 and V81) by alanine dramatically suppressed the proliferative response, implying that these residues are crucial to maintain the cross-reactive response between MOG and BTN. In addition, modifications at five other sites also induced a significant reduction of the cross-reactive T cell response. It should be noted that one of these a.a. residues (V83) is not conserved between MOG and BTN (Figure 3.2.10 C). However, at this position the difference between MOG and BTN is conservative, involving a switch between two bulky aliphatic amino acids (valine and leucine). In contrast, although alanine is also hydrophobic, it is much smaller. Two of the four residues which are very important for the cross-reactivity between rat MOG and bovine BTN (E78 and V81) were not conserved in rat BTN, supporting the hypothesis that the lack sequence identity in this peptide is responsible for the failure of rat BTN to modulate the M_{74-90}-specific immune response in the DA rat.





C)

	M_{74-90}	ESIGEGKVALRIQNVRF
bov	B_{74-90}	DH-A--S--V---E-KA
Rat	B_{74-90}	ARLLD-LAT---RG--V

Figure 3.2.10 Proliferative response of M_{74-90} or B_{74-90}-selected T cell lines to alanine substituted versions of B_{74-90}

A) Representative proliferative response of M_{74-90} selected TCLs to the alanine substituted B_{74-90} peptides. **B)** Representative proliferative response of B_{74-90} selected TCLs to the alanine substituted B_{74-90} peptides. **C)** Graphic presentation of the residues which are important for the cross-reactive response in the rat MOG and the bovine and comparison with the homologous rat BTN peptide. Amino acids are represented in the one-letter-code, amino acids identical to rat MOG are represented as bars. Amino acid residues, which are vital for the cross-reactive response are printed in red, less important residues in blue. Irrelevant residues are printed in black. For E78, G79, V81 and R84, significance was very high ($p<0.001$, using two-tailed student´s T-test for samples with equal variance).

3.2.5 High dose soluble antigen treatment with butyrophilin - peptide suppresses EAE mediated by M_{74-90} specific T cells

This study has demonstrated that a peptide derived from bovine BTN is a naturally occurring APL for MOG-Igd-specific T cells in the DA rat, but cross-reactivity only involves a minor subset of the MOG-Igd-specific T cell repertoire. In order to test the physiological importance of these effects in an *in vivo* system, we decided to investigate the extent by which B_{74-90} could replace the MOG-peptide in inducing antigen-specific tolerance. This was tested for two paradigms, one of which investigated the effect on an established, M_{74-90}-specific T cell response and the other the effect on the developing MOG-Igd-specific immune response.

A well-established method to induce antigen specific tolerance of T cells is treatment with high dose soluble antigen (HDSA) (reviewed in Liblau et al., 1997). This induces activation induced T cell death and/or anergy and results in a transient suppression and/or deletion of antigen-specific T cells (Bercovici et al., 1999). Since cross-reactivity between MOG and butyrophilin in DA rats is restricted to only one of the two encephalitogenic T cell epitopes (Stefferl et al., 2000), we investigated whether treatment with the homologous butyrophilin peptide (B_{74-90}) could suppress EAE induced by the transfer of M_{74-90}-specific T cells.

The transfer of 5 x 10^6 M_{74-90}-specific T cells induced severe clinical disease with a maximal score of 2.5 +/- 0.3 in naïve syngeneic recipients (Figure 3.2.11). Clinical symptoms were completely suppressed when the animals were treated intravenously with 1 mg M_{74-90} two and four d.p.t.. A protective effect was also observed after treatment with the homologous BTN peptide B_{74-90}. However, in this case suppression of disease was less efficient and all animals developed mild symptoms of EAE. This indicates that B_{74-90} cannot fully replace the tolerogenic effect of M_{74-90}. This reflects that the cross-reactive T cell response involves only a subset of the M_{74-90}-specific T cells and suggests that the non-cross-reactive T cells escape “tolerisation” and persist to induce an attenuated form of disease.

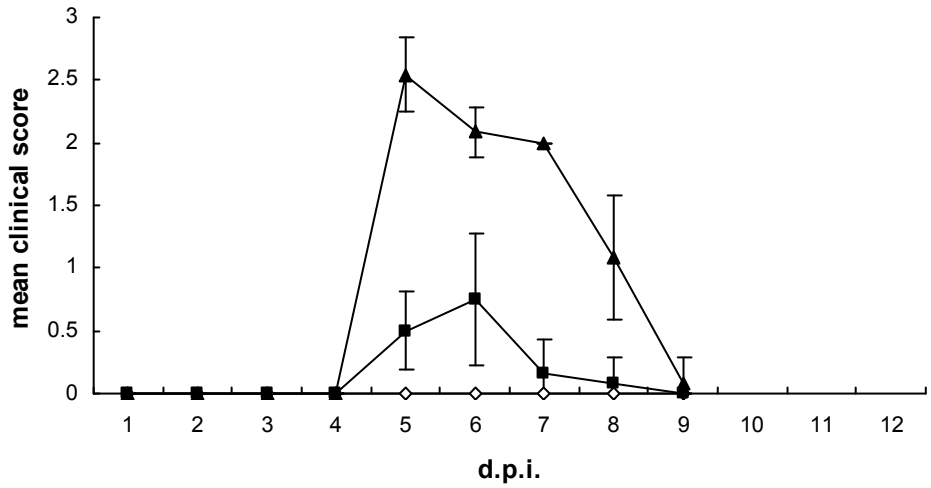


Figure 3.2.11 Treatment with HDSA suppresses T cell mediated EAE

Shown is the mean clinical course and standard deviation after adoptive transfer of 7 x 10^6 M_{74-90}-specific T cells into naïve DA rats. Animals were treated i.v. with 1 mg Ova (triangles, n=6), B_{74-90} (squares, n=6) or M_{74-90} (diamonds, n=5) at 2 and 4 d.p.t.. Disease suppression was significant ($p<0.05$, Duncan´s post hoc test)

3.2.6 Intra-nasal application of BTN-peptide can fully replace the homologous MOG-peptide in suppressing MOG-Igd-induced EAE

The effect of B_{74-90} on the developing immune response was investigated by intra-nasal induction of tolerance. Nasal tolerance duplicates many of the immunological features of oral tolerance (Strobel and Mowat, 1998), but avoids digestion in the gastro-intestinal tract and requires minimal amounts of peptide (Metzler and Wraith, 1993). For the induction of nasal tolerance, a protocol was employed that had previously been shown to inhibit MBP-induced EAE in the DA rat (Bai et al., 1997).

As described in section 3.1.3.1.2, immunisation of DA rats with MOG-Igd induces pathogenic T cell responses against two distinct epitopes, M_{74-90} and M_{93-109}. Since only one of these responses cross-reacts with BTN, we examined the influence of the individual peptides as well as combinations of peptides on MOG-Igd-induced EAE. Female DA rats were treated intra-nasally with either M_{74-90}, B_{74-90} or M_{93-109} alone, or alternatively with a combination of the immunodominant peptide M_{93-109} with either M_{74-90} or B_{74-90} for 10 consecutive days prior to immunisation with MOG-Igd. Ovalbumin-treated control rats developed biphasic disease comparable to that seen in untreated animals (Figure 3.1.1) and this was only slightly suppressed by pre-treatment with the any of the individual peptides (Figure 3.2.12 A-D). Strikingly, treatment with combinations of peptides was far more efficient (Figure 3.2.12 E, F). The combination of B_{74-90} and M_{93-109} resulted in complete suppression of the first phase of disease and a strongly reduced severity of relapse, similar to the effect of treatment with the

combination of the two MOG-peptides. These results indicate that bystander suppression does not play a major role in this disease model, since both T cell epitopes need to be targeted for full suppression of disease. Intriguingly, $B_{74\text{-}90}$ can fully replace $M_{74\text{-}90}$ in the induction of intra-nasal tolerance in this model, although we have demonstrated previously that not all of the $M_{74\text{-}90}$-specific T cells cross-react.

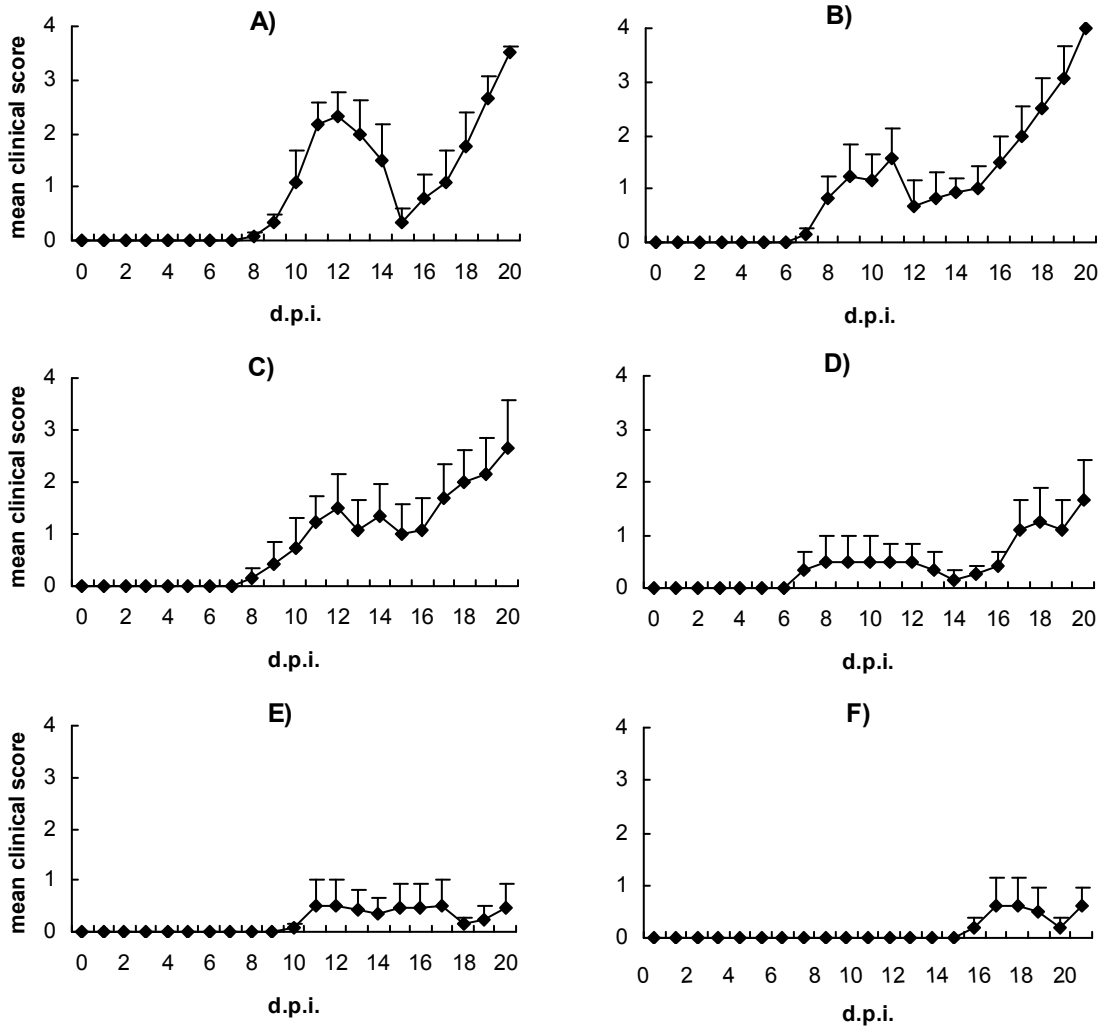


Figure 3.2.12 Intranasal treatment with MOG- or BTN-peptides can suppress MOG-Igd-induced EAE
Female DA rats were treated intranasally for 10 consecutive days with **A)** 50 µg Ova (n=6); **B)** $M_{74\text{-}90}$ (n=6); **C)** $B_{74\text{-}90}$ (n=6); **D)** $M_{93\text{-}109}$ (n=6); **E)** $M_{74\text{-}90}$ + $M_{93\text{-}109}$ (n=6) or **F)** $B_{74\text{-}90}$ + $M_{93\text{-}109}$ (n=5) prior to induction of MOG-Igd/IFA-EAE. Shown are the mean clinical course of groups of 5 - 6 rats and SEM.

3.2.7 Cross-reactivity of antibodies

3.2.7.1 The antibody response to MOG-Igd does not cross-react with BTN

The unique feature of MOG as a target for EAE is that the MOG-specific antibodies are capable of mediating demyelination in rats and primates (Linington et al., 1988; Lassmann et al., 1988; Genain et al., 1995, 1999; t´Hart et al., 2000). Since rat MOG and bovine BTN share approximately 50% sequence identity as well as a conserved IgV-like structure, it was thought to be probable that molecular mimicry would extend to the B cell response. In consequence, BTN/MOG cross-reactive antibodies may be pathogenic and contribute to disease pathogenesis.

However, analysis of the antibody response in DA rats 19 d.p.i. with 100 µg MOG-Igd in IFA by ELISA identified only a minimal level of cross-reactivity to BTN^{exo} that was restricted to peptide B_{89-113}. No cross-reactivity was detected with the peptides B_{1-26} and B_{50-74}, which are homologous to the immunodominant MOG peptides (Figure 3.2.13 A).

3.2.7.2 No cross-reactivity of antibodies after DNA vaccination with BTN or MOG

One possible explanation for the low cross-reactivity to BTN^{exo} is that the animals were immunised with denatured, non-glycosylated MOG-Igd, while their serum was tested for a cross-reactive antibody response using recombinant insect-derived BTN^{exo}, which is glycosylated and also believed to be in its native conformation. We therefore used a DNA vaccination approach to generate antibody responses to the "native" IgV-like domains of both proteins and then retested their cross-reactivity by ELISA.

In order to minimise the influence of the intracellular domains of butyrophilin on protein expression, the cDNA encoding the extracellular domain of bovine BTN was fused to the cDNA encoding the trans-membrane and intra-cellular domains of mouse MOG and cloned into pcDNA3.1(-), the same vector which had been used for the MOG construct (See section 2.2.18). Vaccination with pcDNA-BTN/MOG induced a BTN specific antibody response in DA rats, which – in contrast to the MOG-response - was not purely conformation dependent, but also recognised peptide B_{1-26} (Figure 3.2.13 B). However, no binding could be detected to either MOG-Igd or any MOG peptide (Figure 3.2.13 C). Conversely, the serum of MOG-vaccinated DA rats did not bind to either BTN^{exo} or any BTN peptide (Figure 3.2.13 D).

As anticipated from this lack of cross-reactivity, DNA vaccination with pcDNA-BTN/MOG did not enhance the disease induced 4 weeks later by adoptive transfer of 2×10^6 M_{93-109}-selected TCLs (data not presented). Moreover, as described in 3.1.3.4, there was no significant absorption of BTN-specific antibodies in the inflamed CNS of rats with EAE. These observations demonstrate that despite the extensive sequence homology between MOG and

BTN, there is little or no cross-reactivity between the B cell responses to these two proteins in the rat.

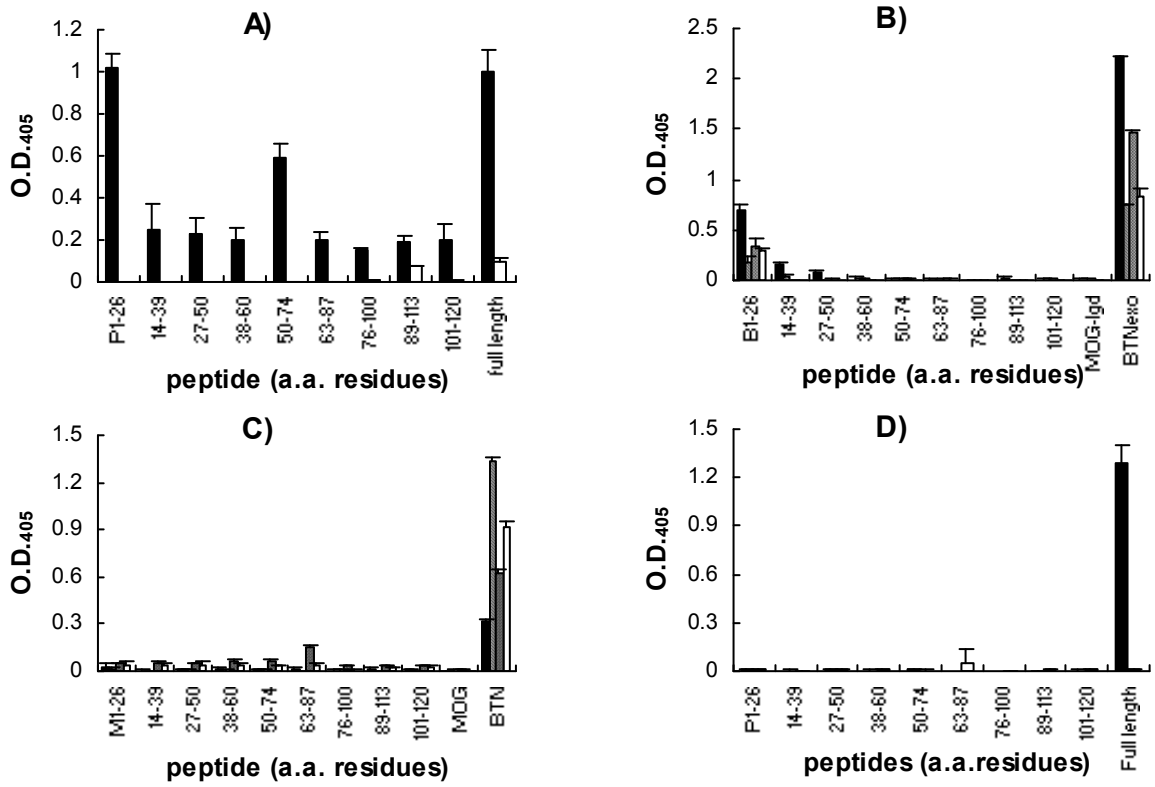


Figure 3.2.13 Specificity of MOG and BTN-specific antibodies in the DA rat

A) Analysis of serum derived 19 d.p.i. with MOG-Igd/IFA. Shown is the mean $O.D._{405}$ and standard deviation of quadruplicate analysis of sera pooled from 3 animals. Black columns: binding to MOG peptides, white columns: binding to BTN peptides. Minimal binding was observed to the BTN peptide 89-113 and BTN^{exo}; **B)** and **C)** antibody response after vaccination with pcDNA-BTN/MOG. **B)** Response to BTN peptides. Note that a linear epitope is recognised in addition to the dominating response to BTN^{exo}; **C)** response to MOG peptides. Shown are mean $O.D._{405}$ and standard deviation of sera derived from 4 rats analysed individually in quadruplicates. No binding was detected to MOG-Igd nor to any linear MOG peptide. **D)** Serum derived from MOG-vaccinated rats also failed to bind to BTN^{exo} or any of the linear BTN peptides (black columns: binding to MOG peptides, white columns: binding to BTN peptides).

3.2.8 Discussion

This study demonstrates that functional modulation of a sub-population of the MOG-specific T cell repertoire is sufficient to modify the clinical course of MOG- induced EAE. The BTN-derived peptide appears to act as an APL for a population of MOG/BTN cross-reactive T cells, skewing their cytokine production towards a less inflammatory phenotype. The demonstration that the environment, in this case the diet, can provide APLs that modify the function of the autoimmune repertoire adds a further level of complexity to the aetiology of complex disorders such as MS and diabetes.

The only other myelin autoantigen for which TCR usage was investigated extensively is MBP (Chluba et al., 1989; Burns et al., 1989; Wucherpfennig et al., 1990). In the Lewis rat, the immunodominant epitope of MBP is MBP_{68-86}, and the T cell response to this peptide is dominated by clones using the TCR Vβ8.2 gene (Chluba et al., 1989; Burns et al., 1989; Matsumoto, 2000). In contrast, the MOG-specific T cell response in the DA rat is far more heterogeneous. Not only are two distinct epitopes recognised, but in each case the T cell response is oligoclonal, as demonstrated by spectratyping. Comparison with the results obtained on epitope specificity and TCR gene usage by human myelin antigen specific TCLs and clones suggests that the MOG-specific T cell response in DA rats more closely resembles the human situation. Despite initial reports that the auto-reactive repertoire was restricted in complexity (Wucherpfennig et al., 1990), detailed studies revealed that for any given myelin antigen, epitope specificity and TCR gene usage are complex in both MS patients and healthy controls. (Ausubel et al., 1997; Pette et al., 1990). Unfortunately, this complexity almost certainly means that immuno-therapies based on the concept that disease can be suppressed by specific elimination of a limited number of autoantigen specific clonal expansions in the T cell repertoire (Vanderbark et al., 1993; Appel et al., 2001) are unlikely to succeed in clinical practise.

The complexity of the T cell response to MOG extends to the cross-reactive response to the BTN peptide 74-90. Although in this case only a single MOG peptide is involved, spectratyping suggest the presence of at least three distinct TCRs that will support the cross-reactive T cell response. However, this must still be confirmed by identifying the TCR α chains associated with these particular TCR β-chains and demonstrating that these TCRs really do support a cross-reactive T cell response. Such studies are the first step towards generating TCR transgenic rats, in which the immune repertoire is skewed in favour of recognition of the cross-reactive peptide epitope. These animals would enable a detailed *in vivo* analysis of the physiological consequences of molecular mimicry between a dietary antigen and self, and the

molecular and cellular mechanisms that regulate the impact of oral tolerance on the autoimmune repertoire.

In DA rats, those T cells recognising both M_{74-90} and BTN_{74-90} appear to be only a minor component of the MOG-reactive repertoire, possibly accounting for less than half of the total response to M_{74-90} induced by immunis ation with MOG-Igd. This estimate is based on two observations. First, the proliferative response of M_{74-90}-selected TCLs to B_{74-90} is approximately 50-80% of the response obtained using M_{74-90} (Figure 3.2.5 A). Second, spectratyping revealed that only a small proportion of the M_{74-90}-and B_{74-90}-selected T cells use the same TCR Vβ elements. Indeed, only three CDR3 sequences were identified that were expanded in both TCLs and none of them were significantly expanded in MOG-Igd-selected TCLs, suggesting that these clones represent a small subpopulation of the MOG-specific T cell repertoire. However, neither of these methods provide a true estimate of the frequency of cross-reactive T cells in the immune repertoire. It is anticipated that the generation of $RT1.B^{av1}$ class II MHC tetramers (Olsson, personal communication) will soon allow this question to be answered directly, as well as providing the means to isolate the cross-reactive T cell population by FACS sorting. This will also facilitate the identification of the TCR α chains.

In this study, HDSA treatment with the BTN peptide reduces, but does not eliminate the pathogenic potential of the cross-reactive T cell response. This observation is in agreement with the spectratyping data, which demonstrate that not all M_{74-90}-selected T cells express the same TCR Vβ rearrangements as the M_{74-90}-selected cells. However, in spite of this heterogeneity, treatment with B_{74-90}, *in vitro* stimulation of M_{74-90}-selected TCLs with this peptide dramatically reduces their ability to induce EAE. Loss of the encephalitogenic/inflammatory potential of the TCLs was associated with a relative increase in the secretion of IL-10, a marker for a Th2 like response in the rat, and conversely with decreased secretion of IFN-γ, a marker of Th1 activity. Stimulation with the BTN peptide therefore induces immune deviation in the M_{74-90}-selected TCLs away from a pro-inflammatory Th1 towards a counter-inflammatory Th0/Th2-like response, suggesting that B_{74-90} acts as an altered peptide ligand (APL).

The concept of APL mediated immune deviation in EAE was first investigated by Nicholson et al., 1995, who demonstrated that single amino acid substitutions in an encephalitogenic PLP peptide inhibits EAE in SJL/J mice by inducing immune deviation. Prior to this, different peptide analogues were shown to induce suppression of autoimmune diseases by variable mechanisms, sometimes by blocking TCR-antigen binding without triggering a response, acting as TCR antagonists (De Magistris et al., 1992; Kuchroo et al., 1994; Franco et al., 1994), in other cases by inducting anergy (Sloan-Lancaster et al, 1993) or by partial

activation of T cells (Evavold and Allen, 1991). In other cases, modifications of encephalitogenic peptides induced enhanced responsiveness, and they were therefore termed "superagonist ligands". (Vergelli et al., 1997)

In the current study, stimulation of M_{74-90}-selected TCLs with B_{74-90} increased the synthesis of IL-10 (a marker for enhanced Th2 activity in the rat)(Xiao et al., 1998; Vandebriel et al., 2000) relative to the classical Th1 associated "pro-inflammatory" cytokine IFN-γ *in vitro*. This was paralleled by marked reduction in the pathogenicity of the TCLs *in vivo*. As no increase in TGF-β production was observed in this system, these effects may be attributable to the counter-inflammatory effects of IL-10, which have previously been exploited to suppress EAE. Systemic application of IL-10 was shown to suppress induced EAE (Rott et al., 1994), and mRNA transcripts for IL-10 are reported to be up-regulated in both the CNS and peripheral immune organs during remission (Kennedy et al., 1992; Rott et al., 1994; Diab et al., 1997). IL-10 is also up-regulated after the induction of nasal tolerance (Burkhart et al., 1999), and combined application of IL-10 and MBP-peptide enhances both oral and nasal tolerance in Lewis rats (Slavin et al., 2001). Its importance for disease suppression was further demonstrated by nasal application of IL-10 alone, which prevents EAE in rats in both acute and relapsing remitting disease models (Xiao et al., 1998). In addition, IL-10 seems to be involved in protection mediated by antigen-pulsed DCs (Yang et al., 2000). Last but not least, transgenic mice over-expressing IL-10 were resistant to the induction of EAE (Bettelli et al., 1998; Cua et al., 1999), while IL-10-deficient mice show enhanced clinical EAE (Bettelli et al., 1998). The mode of action of IL-10 in these systems is unclear and its effects are probably pleiotropic.

The confirmation that environmental antigens such as BTN can provide APLs that modulate the functional activity of the self-reactive immune repertoire is important with respect to both our understanding of the aetiology of complex autoimmune disorders such as MS, as well as for the development of APL based immunotherapies. Currently, the degeneracy of the T cell repertoire makes it virtually impossible to design an APL which will have a predictable therapeutic outcome on an established immune response. This was recently demonstrated in MBP-induced EAE in the B10.PL mouse. In this well defined mouse model with a highly restricted TCR usage (8/9 T cell clones had virtually identical TCRs, Acha-Orbea et al.,1988), antagonistic APLs for the dominant T cell receptor were found to induce EAE rather than protect from disease induction (Anderton et al., 1998). Similarly, the first clinical trials of APLs in MS using peptides identified by *in vitro* assays had to be terminated due to various unexpected side effects. In one trial (142 patients, double blind, placebo controlled, three doses tested; Kappos et al., 2000), treatment with the lowest concentration of APL induced immune

deviation associated with decreased MRI activity, but the study had to be stopped after induction of hypersensitivity reactions in 9% of the patients. In contrast, treatment with the same APL in another trial (8 patients, not blinded, no controls, using only the highest dose applied in the Kappos trial; Bielekova et al., 2000) was associated with enhanced Th1-responses together with enhanced MRI and relapse activity. Not surprisingly this trial was also terminated prematurely. However, a different "modified peptide", Copaxone 1 or glatiramer acetate, is an approved treatment for MS. Copaxone 1 is a synthetic random polymer (4.7-11 kDa) consisting of only 4 amino acids (A, K, E and Y) in a defined ratio, which was initially designed as a MBP-analogue to induce EAE (Teitelbaum, 1971). In contrast, however, it reduces disease activity in a significant proportion of MS patients, although its mode of action is still poorly understood. It is possible that this random mix of amino acids results in a variety of agonistic and antagonistic peptides, limited only by the restraints of the amino acid composition, which favour anti-inflammatory Th2-responses (Neuhaus et al., 2001, Farina et al., 2001).

In the current study, the amino acid sequence identity between the encephalitogenic MOG peptide and its BTN peptide analogue was only 53% (9/17 residues). Four of the residues which are conserved between the two peptides (E78, G79, V81 and R84) are vital for the cross-reactive response, while another five residues seem to play a minor role (Figure 3.2.10 A) and none of the modifications enhanced proliferation. In collaboration with Drs K. de Graaf and R. Weissert in Tübingen, we are currently identifying the residues involved in the binding to MHC and those that are critical interaction points with the TCR. This information will allow the design of further $M_{74\text{-}90}/B_{74\text{-}90}$ homologues substituted only at the TCR interaction site, which can then be used to investigate the molecular determinants involved in the APL mediated modulation of cytokine production by the MOG-reactive T cell repertoire.

This study has investigated the influence of xenogenic BTN on the rat MOG-reactive T cell repertoire. However, it should not be forgotten that this repertoire develops in the neonatal animal during the same period that it is exposed to autologous BTN in the mothers milk. It was recognised several years ago that sensitisation with MOG-Igd in the Lewis rat fails to induce a strong proliferative response to the encephalitogenic MOG peptides $M_{1\text{-}20}$ and $M_{35\text{-}55}$, although these are present in the repertoire and can be selected *in vitro*. Could this reflect the presence of a suppressor cell population induced by rat BTN that cross-reacts with MOG? The sequencing of rat BTN provides the basic information necessary for further investigations of this speculation. The availability of synthetic rat BTN peptides will not only permit the analysis of the development of oral tolerance to BTN/MOG in neonates, but also of the mechanisms responsible for the maintenance of tolerance to BTN in the lactating mother.

A surprising outcome of the study was the virtual absence of cross-reactivity between MOG and BTN within the B cell response, as demonstrated by both active immunisation and DNA vaccination. This may be due to the high level of homology in terms of both structure and amino acid sequence that exists between the N-terminal domains of MOG and BTN and an expanding family of BTN-like proteins. This family of proteins contains at least 5 closely related subfamilies (MOG, BTN, BT2, BT3 and BTL II), with up to three members per group (Henry et al., 1997; Stammers et al., 2000), as well as closely related homologues in other species e.g the B-G antigens in birds (Henry et al., 1997). These proteins are also related to the co-stimulatory proteins B7-1 and B7-2 (Linsley et al., 1994) and have been termed the "extended B7 family". All these proteins are encoded in the MHC and the amino acid sequence identity between sub-families ranges from 30-50%, while for any individual subfamily the sequence identity of the N-terminal domain is approximately 95%. The pattern of tissue expression of these proteins is broad, and some are expressed in immune organs. It is conceivable that each member provides a partially overlapping set of tolerogenic signals, which in DA rats combine to effectively eliminate any B cell clones that may recognise epitopes shared by MOG and BTN.

Although the relevance of these findings for MS is still uncertain, molecular mimicry involving BTN may have a significant impact on the composition and function of the MOG-specific T cell repertoire. Whether this leads to tolerance or the induction or a potentially pathogenic, auto-aggressive T cell response will be determined by multiple genetic and environment factors, which are poorly understood.

4 Conclusion and Perspectives

This study investigated the immunobiology of MOG-induced EAE in the DA rat, an animal model, which reproduces the immunopathology of the type II MS lesion (Lucchinetti et al., 2000). A newly established immunisation protocol results in a highly synchronised biphasic form of EAE, which mimics the disease course of secondary progressive MS, albeit in a strongly abbreviated time course (Figure 3.1.1). This study demonstrates that MOG-specific autoantibodies are responsible for initiating clinical relapse and driving disease progression. On the background of mild, sub-clinical inflammatory activity in the CNS, pathogenic antibodies enter the CNS and mediate demyelination, a process that in turn amplifies the local inflammatory response (Figure 3.1.14 A). It should however be noted that lethal clinical relapses may also occur in the absence of a pathogenic antibody response if an inflammatory lesion develops in a region of the CNS that is particularly sensitive to damage, or where it may perturb vital functions, such as the brain stem. Although antibodies have been shown to amplify the severity of ongoing clinical EAE (Schluesener et al., 1987; Linington et al., 1988; Lassmann et al., 1988), firm evidence for a role in driving relapse and disease progression was missing. This study has now established this principal, which in all probability is relevant to our understanding of the pathogenesis of severe, steroid non-responsive relapses in MS patients.

However, this model of EAE is an artificial system, in which the role of antibody is only apparent because of the different kinetics of MOG-specific T and B cell responses. In MS we still have to answer two crucial questions, namely the identity of the autoantigens targeted by the demyelinating antibody response, and the factors that may trigger this response.

MOG is the only myelin protein known to initiate a demyelinating antibody response in EAE, and MOG-induced EAE has provided a valuable tool to identify the role of pathogenic autoantibodies in immune mediated demyelination. However, there is a major discrepancy between the proportion of MS patients with pathogenic MOG-specific antibodies in their circulation (5%; Haase et al., 2000) and the frequency of patients with pathological changes suggestive of antibody-mediated pathomechanisms (>50%; Lucchinetti et al., 2000). This discrepancy may in part be accounted for by the absorption of the pathogenic antibodies into the CNS, which will lead to a dramatic reduction of the antibody titre in the periphery, as demonstrated in section 3.1.3.4 of this study. On the other hand, it is unlikely that MOG is the only target autoantigen, which is exposed on the myelin surface and can therefore initiate a demyelinating autoantibody response. The identification of potential targets is a prerequisite to

develop diagnostic kits to identify those patients with pathogenic autoantibody responses and then provide an appropriate therapy such as plasma exchange, or immuno-absorption.

As demonstrated in this study, DNA vaccination using a plasmid encoding a myelin antigen is one approach to generate high titre autoantibody responses directed against the native protein. The pathogenicity of this antibody response can then be assayed in the same animal by inducing EAE. This method circumvents problems such as purity, yield and denaturation, all of which complicate any study using antigens isolated from the CNS or generated using recombinant technologies. Coupling this approach to a proteomics based analysis of the myelin membrane and reverse genomics to identify candidate gene products provides the means to map out those protein antigens that can be targeted by a demyelinating autoantibody response. The feasibility of this concept is currently being tested in the rat using PLP and MAG as myelin components that may in certain circumstances provoke a pathogenic autoantibody response.

Such an analysis will, however, not detect pathogenic antibody responses to glycolipid antigens, which are major target autoantigens in a number of diseases affecting the peripheral nervous system such as Guillain Barré syndrome (GBS). In GBS a pathogenic antibody response to gangliosides appears to be triggered by infections with particular serotypes of *Campylobacter jejuni* (Fredman, 1998; Willison and O´Hanlon, 1999). In the majority of patients these antibody responses are an acute phenomenon and disappear as the patients recover (Hahn, 1998). It is conceivable that a similar mechanism is responsible for the initiation of severe relapses in some MS patients, if an infection triggers a cross-reactive antibody response to a surface glycolipid epitope. This would induce an episode of acute CNS demyelination that would not be immediately responsive to immunosuppressive therapy, as tissue damage and amplification of the local inflammatory response would be driven by the pre-existing antibody response. Analysis of the autoantibody responses in MS should therefore be extended to examine lipid as well as protein autoantigens. Such studies should also not be restricted to myelin, but also address the question of responses to other structures such as the axon and oligodendrocyte progenitor cells.

Such autoantibody responses are however only conditionally pathogenic, in other words their pathogenic potential is only expressed if they can enter the CNS across the blood brain barrier (BBB)(Litzenburger et al., 1998; Bourquin et al., 2000). In EAE the inflammatory insult to the CNS is responsible for the disruption of BBB function and the entry of antibody into the nervous system. MS is characterised by repeated episodes of CNS inflammation but what initiates and maintains this response is unclear. The observation, that DA rats develop a similar, although eventually self-limiting response in the CNS after immunisation with MOG-peptide in

CFA provides a model to investigate the immuno-regulatory deficit(s) responsible for chronic CNS inflammation. The disease model is very reproducible with >90% of animals relapsing after peptide immunisation as opposed to <40% after immunisation with MBP in IFA (Lorentzen et al., 1995). This will make it feasible to use genetic methods, such as disease induction in congenic and intra-MHC congenic rat strains and whole genome screens in F2 backcrosses, to identify genetic loci responsible for this defect. The understanding of the mechanisms involved may help to identify new targets for therapeutic strategies concerning chronic inflammatory diseases like MS and rheumatoid arthritis (RA).

The second part of this thesis investigated a very different aspect of the autoimmune response to MOG, the consequences of immunological cross-reactivity with BTN, a major component of the milk fat globule membrane. The demonstration of cross-reactive T cell responses between MOG and a dietary antigen opens a new perspective for the aetiology of MS, since former investigations of environmental influences were concentrated on molecular mimicry with microbial peptides (Bray et al., 1983; Wucherpfennig et al., 1995; Challoner et al., 1995; Gautam et al., 1998; Ufret-Vicency et al., 1998; Burgoon et al., 1999). Epidemiological studies identified a link between milk consumption and other dietary factors and MS (Butcher, 1986; Malosse et al., 1992; Lauer, 1997), but the identity of the mechanistic basis in the immune system was unknown. It would be naïve to imagine that milk in the diet would per se induce an auto-aggressive response to MOG and thereby trigger MS. Indeed, disease induction is now thought to involve the chance interactions of several environmental factors on a susceptible genotype. In the case of the cross-reactive pair of antigens BTN/MOG, BTN in the diet would normally induce oral tolerance to the cross-reactive epitope, but this may be broken either by early post-natal exposure to bovine milk products (Miller et al., 1994), gastro-intestinal infections (Hornquist and Lycke, 1993; Weiner 1997) or a combination of both (as discussed in Introduction 3.2). However, would this combination of effects be sufficient to induce an inflammatory autoimmune mediated response in the CNS?

Any prediction would at this time be premature. Analysis in the DA rat revealed that the cross-reactive repertoire in this model is complex and involves multiple clonal expansions. Moreover, the sequence of the BTN peptide is not identical to the corresponding MOG sequence and the cross-reactive BTN peptide acts as an APL. In view of the degeneracy of TCR - peptide/MHC recognition, the BTN peptide may initiate a range of responses ranging from superagonistic (Vergelli et al., 1997) to antagonistic (DeMagistris et al., 1992) in the different T cell clones. The identification of TCR β-chains used by the cross-reactive T cells is a first step towards generating a transgenic animal model. This may allow us to examine the

immunopathological consequences of cross-reactivity involving a dietary antigen and the impact of manipulating the gastro-intestinal flora on the immune response.

Whether or not this is relevant for the aetiology of MS is uncertain. Certainly T cell cross-reactivity between the two proteins is very limited and as yet was only demonstrated in the context of the *RT1*^{av1} rat MHC haplotype (Stefferl et al., 2000). No cross-reactive T cell response was detected in LEW and BN rats (Stefferl et al., 2000), as well as in SJL/J, C57/BL6, DBA.1 and CBA.1 mice (Schubart and Wissing, unpublished results). In addition, despite the presence of regions with a high level of sequence identity between the two proteins it was not possible to induce a significant cross-reactive antibody response in the DA rat (section 3.2.7). Why is this?

In the course of this study it became apparent that BTN is a member of a family of structurally related gene products that are termed the BTN-, or extended B7- gene family. The N-terminal IgV-like domain of all these proteins exhibits a high degree of amino acid sequence identity with both MOG and BTN (Henry et al., 1999; Stammers et al., 2000; Rhodes et al., 2001; see discussion of chapter 3.2), and members of this family are expressed in a variety of organs. It is possible that during B and T cell maturation in the bone marrow and thymus, cross-reactive peptides derived from these proteins will eliminate many clones that would otherwise cross-react with MOG reactive T and B cells, and only those cells which escape this network of tolerogenic stimuli enter the periphery.

As the sequences of the BTN-gene family members in the rat are currently unknown (with the exception of MOG), the identification of the extracellular domains of rat BTN provides the first opportunity to test this hypothesis. The oral consumption of milk during suckling should induce rat-BTN-specific suppressor T cells, characterised by low proliferative responsiveness and the secretion of IL-10 and TGF-β (Weiner et al., 1994). The identification and characterisation (epitope specificity) of these suppressor T cell responses in the different rat strains (LEW, BN and DA) may provide an explanation why MOG-specific T cell responses are only poorly encephalitogenic in Lewis rats and might help to elucidate the mechanisms of the development of tolerance in the newborn animals.

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6 Appendices

6.1 MOG-Igd-induced EAE as a model system to investigate novel treatment strategies for MS

MOG-Igd/IFA-induced EAE in the DA rat is a disease model, which closely mimics multiple sclerosis (Storch et al., 1998), reproducing the complex histopathological changes seen in a large subset of MS patients (Lucchinetti et al., 2000; Lassmann et al., 2001). The pathology of this subset of patients is characterised by sharply demarcated demyelinating lesions, the deposition of antibody and C9 at the edge of active lesions as well as $CD4^+$ and $CD8^+$ T cell infiltrates, suggesting an important role of both antibodies and T cells in the pathogenesis of these diseases (Lucchineti et al., 1996, 2000; Lassmann et al., 2001). Other features, which are similar in both diseases, are myelin vesiculation as well as the presence of macrophages containing myelin debris, Ig and complement and ultimately partial remyelination resulting in shadow plaques (Storch et al., 1998; Genain et al., 1999; Raine et al., 1999; Prineas and Graham, 1981). In addition, lesions are also characterised by the activation of microglia, the formation of gliotic scars and irreversible axonal loss leading to persistent neurological deficits (Ferguson et al., 1997; Trapp et al., 1998; Kornek et al., 2000).

Currently, therapeutic strategies for MS target the immunological component of the disease and while beneficial, fail to prevent disease progression in many patients (Noseworthy, 1999 and 2000). There is therefore a vital need to further explore the immunological, as well as the neurological basis of MS and develop strategies that target other pathogenic pathways. One potential target are microglia, which upon activation can not only act as APCs to potentiate the local inflammatory response (Aloisi et al., 2001 A and B), but may also mediate neuro-toxic responses via secretion of pro-inflammatory cytokines (Boje and Arora, 1992; Streit, 1996, Hohlfeld, 1996).

Literature searches identified the second-generation tetracycline minocycline as a substance, which not only has anti-inflammatory properties, but also inhibits microglial activation in models of focal and global ischemia (Yränheikki et al., 1998; Yränheikki et al., 1999) and in a mixed spinal cord culture system exposed to excitotoxins (Tikka et al., 2001). Its anti-inflammatory properties include the reduced production of matrix metalloproteinases (MMPs), TNF-α and inducible nitric oxide synthase (iNOS), factors intimately involved in the pathophysiology of MS and EAE. Minocycline treatment of a transgenic mouse model for Huntington's disease delayed disease progression, an effect associated with inhibition of

caspases 1 and 3 and iNOS by microglia (Chen et al., 2000). In addition to these pleiotropic effects on both immune and central nervous system, another advantage of minocycline is that it is an established therapy against rheumatoid arthritis and acne, with minor side effects. These pleiotrophic actions make minocycline an interesting candidate to inhibit disease progression in multiple sclerosis.

In a collaborative study with Drs. I. Duncan and N. Popovic, University of Madison, Wisconsin, U.S.A., we therefore investigated the effects of minocycline on both actively induced MOG-Igd-EAE and EAE induced by adoptive transfer of committed MOG-specific Th1-effector cell lines.

6.1.1 Minocycline delays and reduces the severity of MOG-Igd-induced EAE

Daily treatment of DA rats with 45 mg/kg minocycline, beginning 1 d.p.i. significantly reduced disease activity after active immunisation with 100 µg MOG-Igd in IFA (Figure 6.1 A). Minocycline not only delayed disease onset by 48-72 hours, but also reduced disease severity. All animals then recovered from the first phase of EAE and were completely healthy at 20 d.p.i., at which time the PBS treated controls had developed a severe relapse and had to be sacrificed in accordance with the local animal care regulations. However, the therapeutic effect of minocycline was unable to influence the ultimate outcome of this clinically severe model of EAE, as the treated animals also relapsed 2 to 4 days later.

In view of these encouraging observations, the effect of minocycline treatment was then investigated in a clinically less aggressive, relapsing remitting disease model induced by immunisation with 10 µg MOG-Igd in CFA (performed at the University of Wisconsin, Madison, U.S.A by Dr. N. Popovic). The clinical course of this disease model more closely resembles that seen in most cases of MS than the relatively acute disease induced by 100 µg MOG-Igd and is characterised by focal demyelinating lesions and varying degrees of axonal loss. Daily treatment with minocycline delayed disease onset and dramatically reduced disease severity for at least 30 d.p.i. (Figure 6.1 B). This protective effect could be observed even if treatment was delayed until the animals developed the first clinical symptoms of EAE, in particular when the dose of minocycline given on the first two days of disease was doubled (Figure 6.1 B, Popovic, 2001).

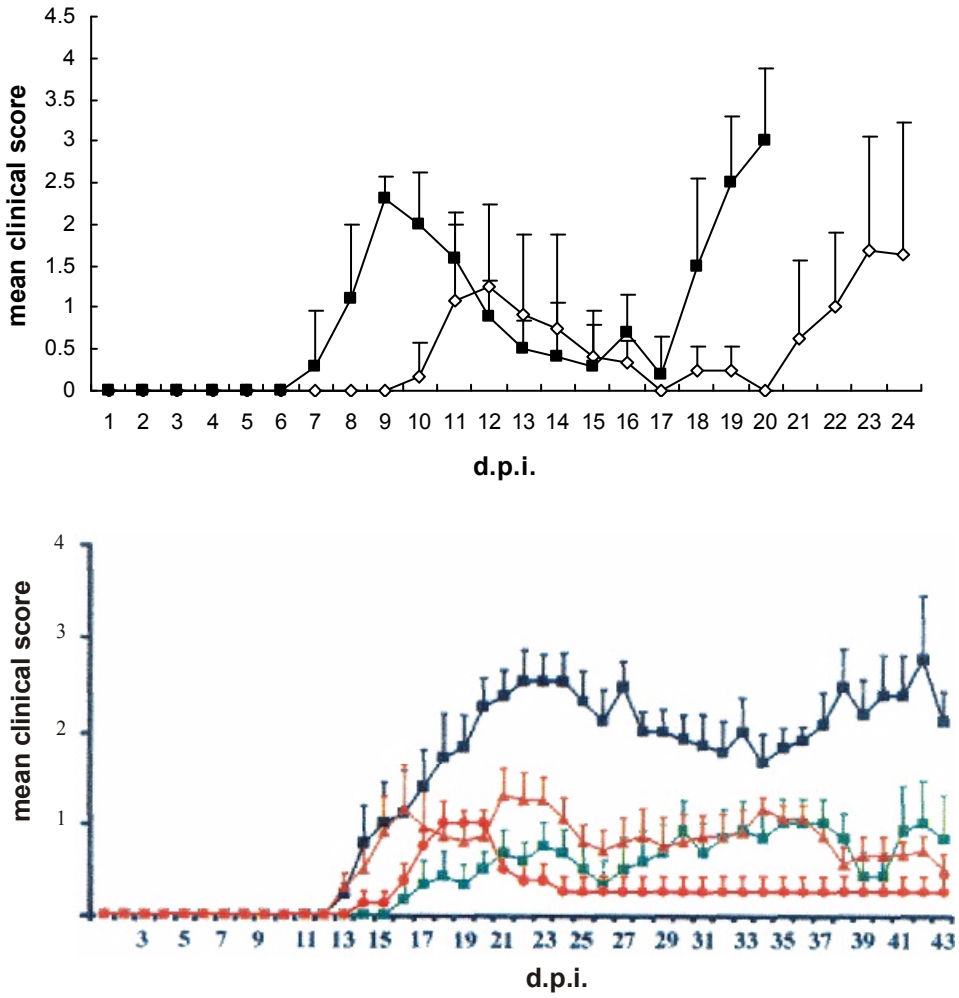

Figure 6.1 Daily treatment with minocycline reduces disease activity after immunisation with MOG-Igd
A) Mean clinical score and standard deviations after immunisation with 100 µg MOG-Igd/IFA. Animals were treated daily beginning 1 d.p.i.. **Black squares**: PBS treated controls (n=5); **white diamonds**: minocycline treated, (n=6; n=4 after 20 d.p.i.). **B)** Mean clinical score and SEM after immunisation with 10 µg MOG-Igd/CFA. **Blue squares**: PBS-treated controls; **green squares**: minocycline treated from 1 d.p.i.; **orange triangles**: minocycline treated from disease onset; **red diamonds**: double dose from disease onset. Figure B) provided by Dr. N Popovic, U.S.A.

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6.1.2 Immunomodulatory effects of minocycline

Previous studies have attributed the beneficial effects of minocycline in the treatment of rheumatoid arthritis to immuno-suppression (Alarcon, 1998; Greenwald, 1994). In our study, prophylactic treatment with minocycline strongly influenced the induction phase of MOG-Igd-induced EAE. In addition, the rapidity of its effects was revealed, when treatment was started after disease onset in the low dose paradigm. However, its mode of action was unclear. In order to determine, whether the protective effects of minocycline were due to the direct modulation of

the MOG-Igd-specific immune response, we analysed its *in vitro* effect on MOG-specific T cell lines as well as the *in vivo* effect on the developing immune response to MOG-Igd.

6.1.2.1 In spite of several *in vitro* effects, minocycline has no effect on the pathogenicity of committed MOG-specific T cells

6.1.2.1.1 Minocycline enhances T cell proliferation

Serial dilutions of minocycline were added to antigen specific T cell restimulation assays of MOG-or MOG-peptide-specific T cells in the presence of irradiated thymus cells and antigen. In contrast to published observations (Kloppenburg et al., 1995; Sewell et al., 1996), proliferation was not reduced, but enhanced in a dose dependent fashion. At concentrations of 50-250 µM, minocycline resulted in a 50 to 70% increase in proliferation, but at higher doses was toxic (Figure 6.2). Interestingly, although the absolute proliferation remained very low, minocycline also strongly enhanced proliferation in the absence of antigen, but did not significantly enhance proliferation in response to the T cell mitogen Concanavalin A (Con A).

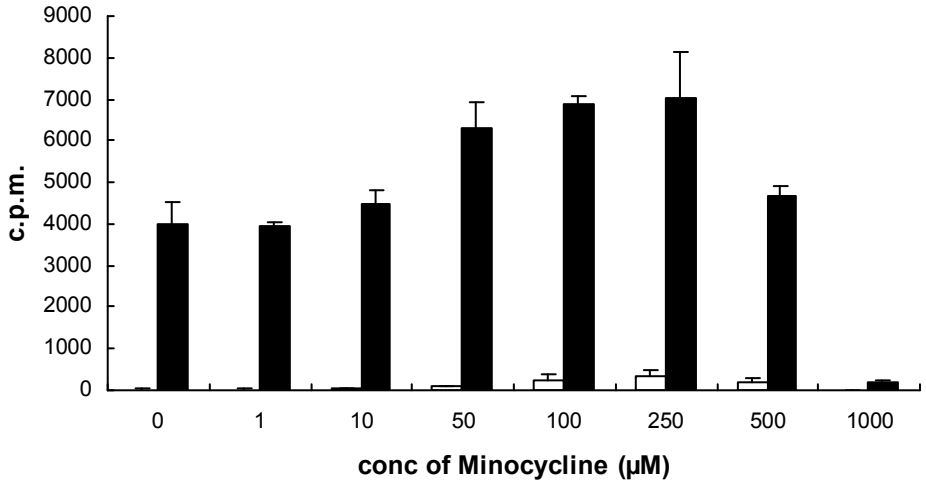

Figure 6.2: Minocycline enhances the proliferative response *in vitro*

MOG-specific T cells were restimulated for 72 hours in the presence of irradiated thymocytes and 20 µg/ml antigen **(black columns)** or in the absence of antigen **(white columns)** and increasing doses of minocycline. Note that minocycline induces increased proliferation both in the presence and absence of antigen. High doses are toxic

6.1.2.1.2 Minocycline modifies the expression of activation markers on T cell blasts

FACS analysis of T cell blasts restimulated in the presence or absence of 250 µM minocycline and antigen demonstrated that these changes in proliferation were accompanied by alterations in the surface expression of several cell surface markers. While the expression of CD4 and MHC class I was unaltered (Figure 6.3 E; C), the presence of minocycline led to a reduced expression of the activation markers OX40 and IL2-R (Figure 6.3 D; F). In a subset of

the cells, there was also a reduction of CD3 and the αβ−TCR on the cell surface (Figure 6.3 B; G).

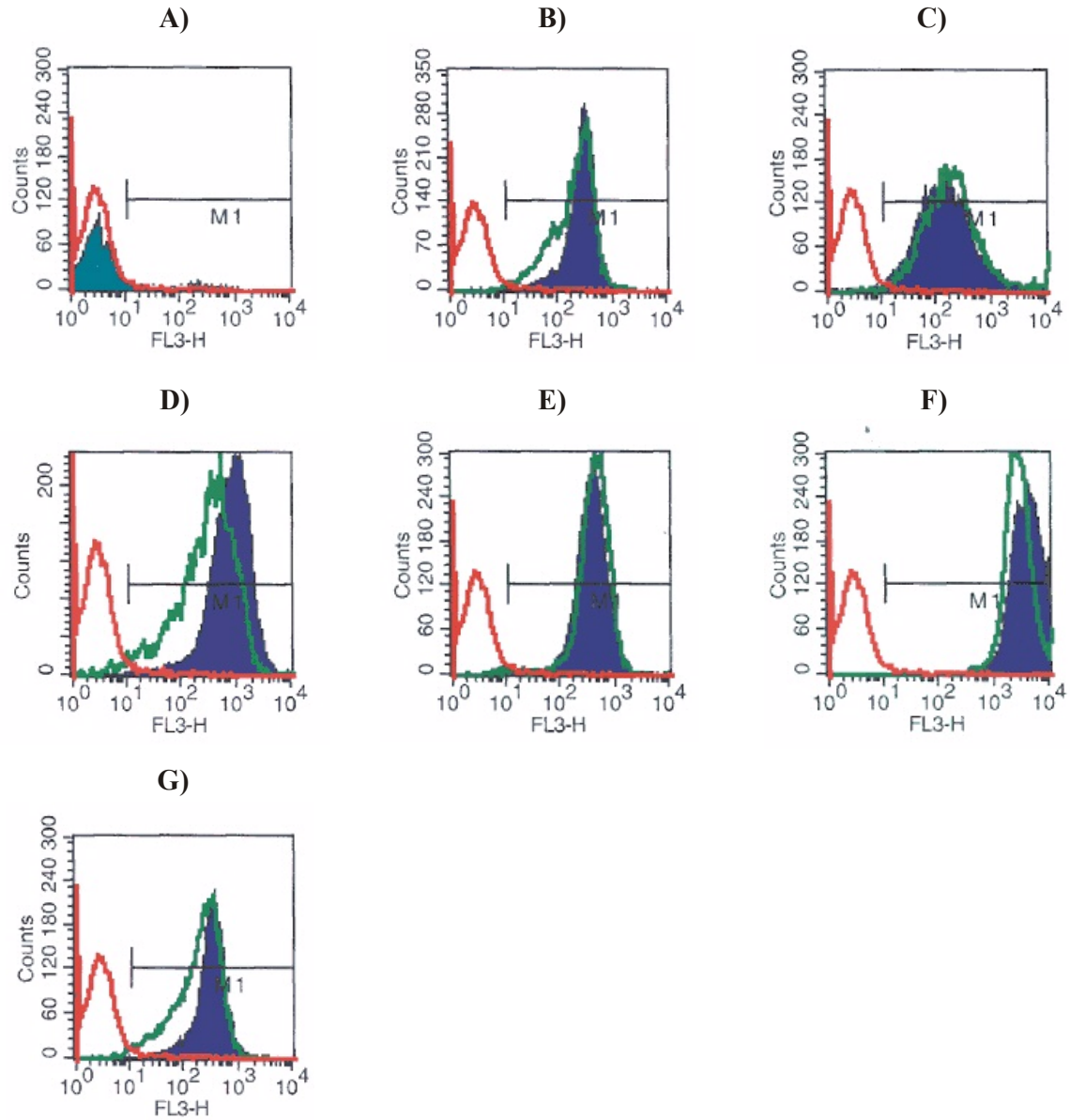

Figure 6.3 Influence of minocycline on the expression of T cell surface markers

MOG-specific T cells were stimulated for three days in the presence of MOG, irradiated thymocytes and with or without 250 µM minocycline. After this, T cell blasts were stained for the expression of the following cell surface markers: **A)** Negative control; **B**) CD3; **C)** MHC class I; **D)** OX40; **E)** CD4; **F)** IL-2 receptor and **G)** α/β T cell receptor. **Blue:** Cells restimulated in the absence of minocycline; **green overlay**: Cells restimulated in the presence of minocycline; **red overlay**: Negative control

6.1.2.1.3 Minocycline does not influence the pathogenicity of MOG-specific T cells

The influence of minocycline on the pathogenic potential of MOG-specific T cells was first studied by adoptive transfer of T cell blasts, which had been restimulated in the presence or absence of 250 µM minocycline, into naïve syngeneic recipients. We could not observe any significant effect of *in vitro* treatment with minocycline on the pathogenicity of the T cells. This indicates that in spite of its effects on the expression of various activation markers, minocycline was incapable of modulating the pathogenic potential of a pre-existing encephalitogenic Th1- T cell response *in vitro* (Figure 6.4, red and orange).

However, since minocycline may act directly in the CNS by suppressing microglial activation and MMP synthesis in the target organ, we also investigated the influence of *in vivo* treatment with minocycline on EAE induced by adoptive transfer of encephalitogenic Th1 T cells. Naïve syngeneic recipients were treated daily with 45 mg/kg minocycline or PBS beginning either 1 d.p.t. or 6 days prior to the transfer of MOG specific T cell blasts (Figure 6.4; blue and green). Once again we could demonstrate no effect on the ability of the transferred T cells to induce disease, suggesting that minocycline was inefficient to suppress a committed encephalitogenic Th1- response *in vivo*.

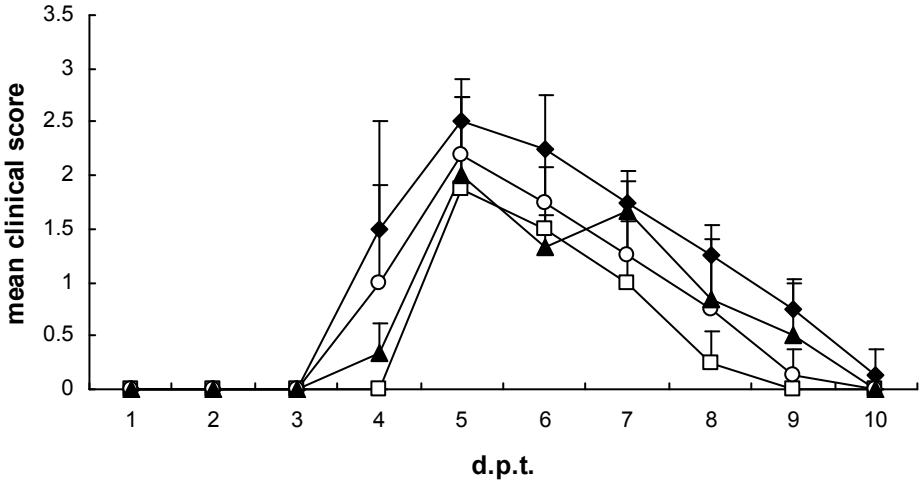

Figure 6.4 Minocyline does not significantly influence the clinical course of EAE induced by T cell transfer
Shown is the mean clinical course and standard deviation after the transfer of 1.7 x10^6 freshly restimulated MOG-specific T cell blasts. **White squares**: Control rats treated daily with PBS from 7 days before transfer, cells untreated (n=4); **black diamonds**: Rats treated daily with minocycline from 7 days before transfer, cells untreated (n=4). Note: this increase in disease activity was not observed consistently in different experiments; **black triangles:** T cells restimulated in the presence of 250 µM minocycline, animals not treated (n=3); **white circles**: Cells untreated, rats untreated (n=3)

6.1.2.2 Minocycline induces immune deviation in the periphery

The above observations demonstrated that minocycline does not influence EAE induced by a committed Th1 effector T cell population. This suggests that its suppressive effect on EAE induced by active immunization with MOG-Igd may be due to a modulation of the developing immune responses. DA rats were therefore immunised with 100 µg MOG-Igd in IFA and treated daily as described above. The peripheral immune response to MOG was investigated 10 d.p.i., at the peak of clinical disease in the PBS treated controls.

Analysis of the draining lymph nodes and spleen revealed that minocycline treatment resulted in a slight enhancement of MOG-Igd-specific proliferation in both tissues (Figure 6.5 A for lymph node cells), which was paralleled by marked changes in cytokine production. Strikingly, the synthesis of the Th2-associated cytokine IL-10 was increased, whilst synthesis of the proinflammatory cytokines TNF-α and IFN-γ were either unaffected or slightly reduced (Figure 6.5 B). Analysis of MOG-specific antibody isotypes as a surrogate marker for Th1- and Th2- like T cell responses confirmed that these changes in the cytokine milieu resulted in immune deviation away from a disease-associated Th1-like response in favour of a counter-inflammatory Th2-like response. The production of the Th1-associated isotypes IgG2A, IgG2B and IgG2C was significantly decreased, while the titre of the Th2-associated isotype IgG1 was enhanced, resulting in a reduction of the IgG2b/IgG1 ratio from 0.80 to 0.17 when assayed at a serum dilution of 1/300 (Figure A-5C). In other EAE paradigms, similar shifts from a Th1 to Th2-like responses are associated with decreased disease activity (Karin et al., 1994; Kuchroo et al., 1995), suggesting that this effect on the antigen-specific immune response will contribute to the protection afforded by minocycline.

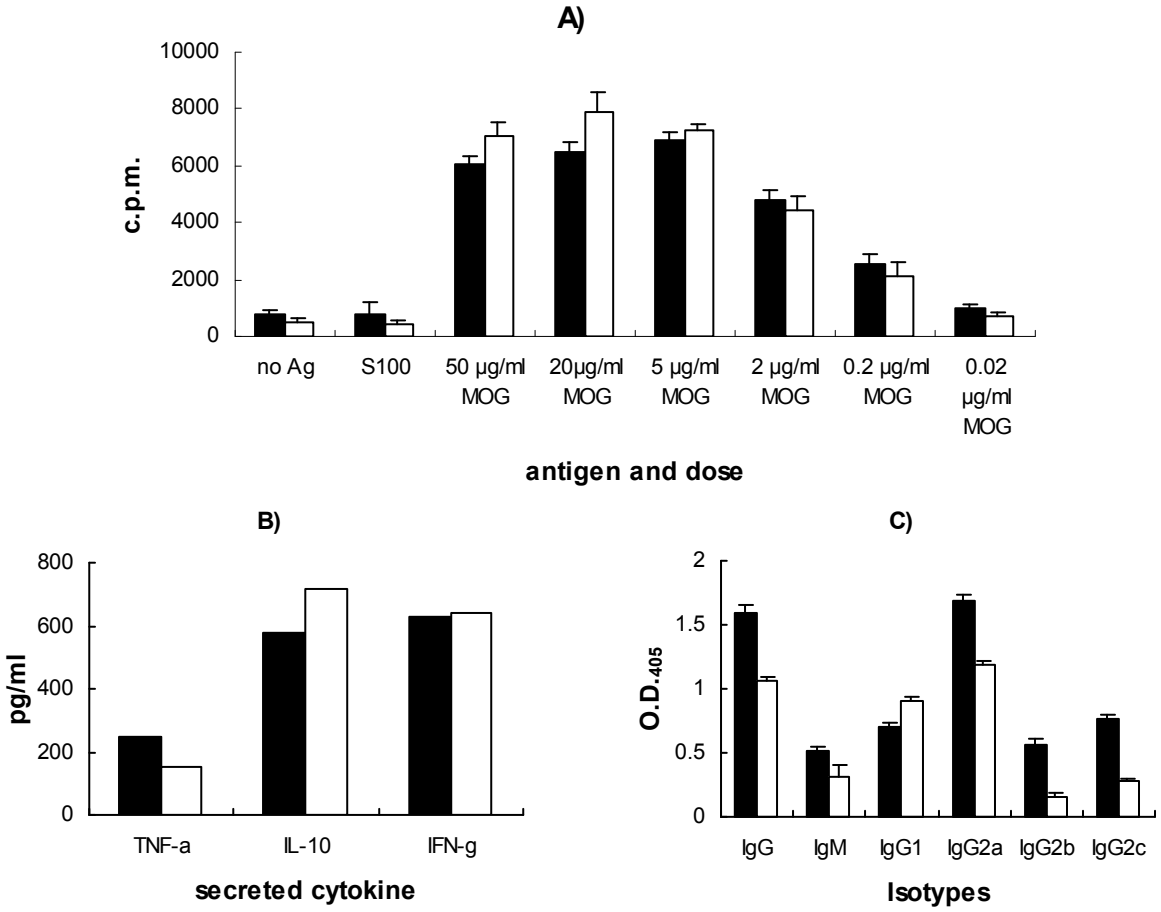

Figure 6.5 *In vivo* treatment with minocycline modulates the peripheral immune response 10 d.p.i. with 100 µg MOG/IFA

A) Shown are mean c.p.m. and standard deviations of a primary stimulation assay of lymph node cells, analysed in quadruplicate; **B) c**ytokines secreted by these primary lymph node cultures stimulated with 20 µg MOG (in the absence of minocycline *in vitro*); **C)** isotypes of MOG-Igd-specific antibodies. Sera were diluted 1/900 (IgG) or 1/300 (others). Shown is the mean $O.D._{405}$ and standard deviation of quadruplicate analyses. DA rats were treated daily beginning 1 d.p.i. with PBS **(black)** or minocycline **(white).** Tissues/Sera were pooled from 3 animals.

6.1.2.3 Does minocycline affect the microglial response?

In order to determine, whether the treatment with minocycline induced any direct effects on microglia, CNS tissue sections were examined for inflammation, microglial activation and MMP-2 expression (performed by B. Goetz in Madison, Wisconsin, U.S.A.).

In both MOG-Igd EAE paradigms, the therapeutic effect of minocycline was paralleled by a strong reduction of inflammation and demyelination (Figure 6.6). Since microglial activation is a response to CNS inflammation, the reduction in inflammation will be associated with a concomitant loss of microglial activation. It was therefore extremely difficult to judge whether the lack of microglial activation was due to a direct effect of minocycline on the microglia, or simply to the lower level of inflammatory activity in the CNS. However, the analysis of CNS tissue in the areas of focal, low grade inflammation suggests that minocycline

induces a reduction of microglial activation (as indicated by microglial morphology and MHC class II-expression) as well as reduced MMP-2 expression (Dr. I. Duncan, personal communication). Analysis for these markers in the CNS derived from minocycline treated rats with T cell mediated disease may be more informative and is currently in progress.

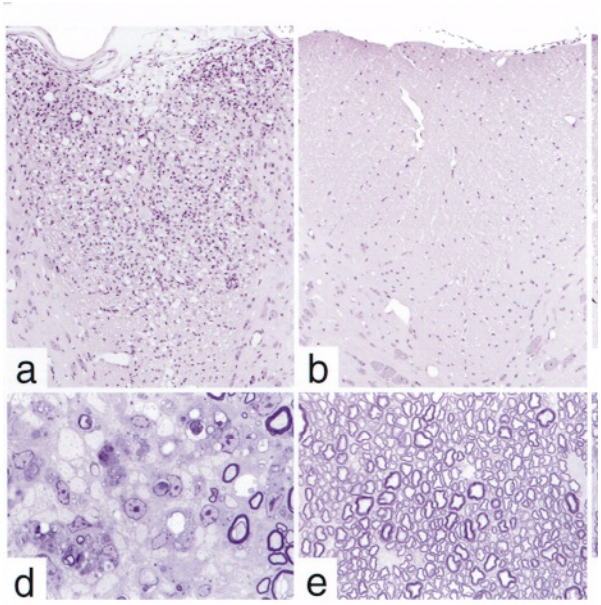

Figure 6.6 Treatment with minocycline reduces inflammation and demyelination in the CNS

DA rats were immunised with with 10 µg MOG/CFA and treated daily with 45 mg/kg minocycline. **a)** and **d)** PBS-treated control rats; **b)** and **e)** minocycline treated rats; **a)** and **b)** stained for inflammatory infiltrates (hematoxylin eosin), **d)** and **e)** stained for myelin (toluidine blue).

6.1.3 Discussion

In this collaborative study, we employed relapsing remitting EAE induced by MOG-Igd to investigate the therapeutic effects of minocycline, a second-generation tetracycline. This drug was chosen, because it not only has anti-inflammatory properties (Alarcon, 1998; Greenwald, 1994), but also suppresses microglial activation (Yränheikki et al., 1998; Yränheikki et al., 1999; Tikka et al., 2001). We demonstrate that minocycline suppresses the developing inflammatory response in the CNS and will even inhibit further activation of the inflammatory cascade in the CNS in established disease. However, at present its mode of action is uncertain.

Previous studies attributed the beneficial effects of minocycline in the treatment of rheumatoid arthritis to global immuno-suppression (Alarcon, 1998; Greenwald, 1994), but the current study indicates that the action of minocycline on the developing immune response is more complex. Continuous treatment with minocycline directly affects the cytokine milieu in the draining lymph nodes, enhancing IL-10 synthesis, reducing TNF-α production, but with a minimal effect on the synthesis of IFN-γ. This functional shift towards a "counter-inflammatory" Th2 response was confirmed by analysis of immunoglobulin isotype usage. In the context of Th1 T cell mediated autoimmune diseases, Th1 and Th2 T cell responses are seen as being mutually antagonistic by virtue of the reciprocal-regulatory effects of Th1 (IFN-γ, IL-12) and Th-2 (IL-4, IL-5, IL-10) associated cytokines (Liblau et al., 1995). Shifting the balance of the immune response in favour of Th2 like responses is associated with suppression of disease activity in EAE in immuno-competent animals (Khoruts et al., 1995) and will contribute significantly to the overall efficacy of minocycline in the current study. In addition, IL-10 might also have a direct effect (see discussion of chapter 3.2). The induction of immune deviation by minocycline is a novel observation that helps to clarify its mode of action in both rheumatoid arthritis and EAE. One the other hand, this study clearly demonstrates that minocycline is incapable of inhibiting EAE mediated by committed encephalitogenic Th1 TCLs.

However, the rapidity of the response, when the therapy was started at disease onset suggests, that minocycline might not simply act by inducing immune deviation. Previous studies indicate that minocycline also suppresses microglial activation. It is, unfortunately, very difficult to test this by histological analysis, as in EAE inflammation and microglial activation are tightly inter-related. Invading encephalitogenic Th1 T cells, which recognise their target antigen in the CNS, secrete IFN-γ and other inflammatory and chemotactic mediators. These factors activate microglia (Shrikant and Beneviste, 1996), which then in turn act as local antigen presenting cells to augment the T-cell mediated inflammatory response (Aloisi et al., 2001), and are thought to play a crucial role in tissue destruction through the production of

proinflammatory cytokines. In the absence of a strong inflammatory response, it is therefore difficult to decide whether the suppression of microglia is responsible for the lack of inflammation, or whether the lack of inflammation, possibly caused by immune deviation, is responsible for the lack of microglial activation. However, we are currently examining tissues of minocycline-treated animals with clinical EAE induced by adoptive transfer of MOG-specific T cells to address this question.

Our data demonstrates that in MOG-Igd-induced EAE, T cell recruitment into the CNS is markedly reduced by treatment with minocycline. This may be in part mediated directly by the counter-inflammatory effects of the enhanced Th-2 like response induced by minocycline or by direct actions on microglia, but is also likely to involve other factors. These may include the down-regulation of metalloproteinase (MMP) activity by minocycline (Greenwald et al., 1992), as MMPs are crucially involved at many stages of the immuno-pathogenesis of EAE (Liedtke et al., 1998; Kieseier et al., 1999). We observed that MMP-2 expression is increased in areas of inflammation in sham-treated rats with EAE. In contrast, this was not seen in animals treated with minocycline, even in areas with evidence of a local T cell infiltrate (I. Duncan, personal communication). Intriguingly, alpha-4 integrin-mediated induction of MMP-2 expression plays a crucial role in the pathogenesis of EAE (Graesser et al, 2000), degrading the sub-endothelial basement membrane and facilitating T cell/macrophage entry into the CNS. Disruption of the coordinate activation of MMP-2 may also influence the subsequent expression of other MMPs, such as MMP-9, which also plays a vital role in EAE and MS (Yong et al., 1998)

Experimental data from models of rheumatoid arthritis support the view that inhibition of MMP expression is at least in part responsible for the clinical efficacy of minocycline (Greenwald et al., 1992). However, once again, it remains to be determined whether the lack of MMP-2-expression after treatment with minocycline might not simply be due to the absence of an inflammatory response in the CNS.

Although not investigated directly in the current study, we speculate that in addition to blocking or reducing inflammation and immune mediated damage *per se*, minocycline treatment may also be neuroprotective and reduce axonal damage directly or through inhibition of microglia. Minocycline prevents the death of cultured neurons exposed to glutamate or kainic acid (Tikka et al., 2001 A/B). Recently, glutamate antagonists have been shown to reduce disease in EAE models and to be neuroprotective (Pitt et al., 2000; Smith et al., 2000). Stimulation of glutamate receptors is thought to cause microglial proliferation with subsequent glutamate release which may not only result in neuronal loss, but also in oligodendrocyte death in both EAE and MS (Matute et al., 2001).

As a safe drug commonly used for extended periods in clinical practice to treat infections and in patients with rheumatoid arthritis, minocycline would thus seem to be an excellent candidate for a clinical trial in MS. While its efficacy might be greatest if given prior to the onset of clinical signs, for example at a time when a triggering episode such as a viral infection occurs, our data suggest that it could be given at relapse onset. Its intermittent use may have advantages over the β-interferon drugs, which need to be given for life; but long-term treatment with minocycline is also possible (as is currently the case in the treatment of rheumatoid arthritis and acne (Goulden et al., 1996) and could extend its use into patients with other forms of MS. It is not yet proven that long-term therapy with β-interferon will result in axonal protection and the possibility that minocycline may also be a useful neuroprotective drug suggests that minocycline could become a therapy of choice in MS. Importantly, minocycline would be a much less expensive therapy than those currently approved for the treatment of MS. In addition, minocycline could be used in other demyelinating disorders such as adrenoleucodystrophy (Powers and Moser, 1998) and the Guillain-Barré syndrome (Griffin et al., 1990), where T cell recruitment and inflammation also play a key role in their pathogenesis.

6.2 Chemicals and Biochemicals

Acetic acid	Merck, Darmstadt
Acrylamid/Bis, Rotiphorese 30	Roth, Karlsruhe
Agar	Fluka, Seelze
Agarose	Biozym, Hess. Oldendorf
L-Aspargine	GibcoBRL, Paisley, Scotland
Ammonium chloride	Merck, Darmstadt
Ammoniumperoxodisulfate	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
β–Mercaptoethanol	Merck-Schuchardt, Hohenbrunn
Boric acid	Merck, Darmstadt
Brome phenol blue	Sigma, Deisenhofen
BSA	Roth, Karlsruhe
Butanol	Riedl-deHaën, Seelze
Calciumchloride	Merck, Darmstadt
Citric acid-Monohydrate	Merck, Darmstadt
Concanavalin A	Sigma, Deisenhofen
Coomassie Brilliant Blue	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
Diethanolamine	Sigma, Deisenhofen
Diethylpyrocarbonat (DEPC)	Sigma, Deisenhofen
Dimethylsulfoxide (DMSO)	Sigma, Deisenhofen
DTT (Dithiothreitol)	Gibco BRL, Paisley, Schottland
dNTPs	Boehringer, Mannheim
Dulbecco´s Modification of Eagles Medium (DMEM)	Gibco BRL, Paisley, Schottland
EDTA	Merck, Darmstadt
Ethanol	Riedl-deHaën, Seelze
Ether	Hoechst, Frankfurt
Ethidiumbromide	Sigma, Deisenhofen
Fetal calf serum	Gibco, Paisley/Schottland
Glucose	Merck, Darmstadt
L-Glutamine	Gibco, Paisley/Schottland
Glycerol	Merck, Darmstadt
Glycin	Riedl-deHaën, Seelze
HEPES	Sigma,Steinheim
Horse serum	Gibco BRL, Paisley, Schottland Hydrochloric
acid (37%)	Riedl-deHaën, Seelze

Hydrogen peroxide	Aldrich-Chemie, Steinheim
Imidazole	Merck, Darmstadt
Isopropanol	Riedl-deHaën, Seelze
Isopropyl-b-D-Thiogalaktosid (IPTG)	bts, Leon-Rot
Kanamycine	Sigma, Deisenhofen
Lowry Protein Assay Kit	Sigma Diagnostics, USA
Lysozyme	Sigma, Steinheim
Magnesiumchloride x $6H_2O$	Merck, Darmstadt
Magnesiumsulfate	Merck, Darmstadt
Manganese chloride	Merck, Darmstadt
MEM Non-essentiel amino acids	Gibco, Paisley/Schottland
Methanol	Riedl-deHaën, Seelze
Milk powder	Frema Reform
Nickel chloride	Sigma, Deisenhofen
p-nitrophenyl phosphate	Sigma, Deisenhofen
Oligo-dT-Primer	Gibco BRL, Paisley, Schottland
Orthophenyldiamin (OPD)	Sigma, Deisenhofen
Ovalbumine	Roth, Karlsruhe
Paraformaldehyde	Merck, Darmstadt
Peptone from casein	Merck, Darmstadt
Penicillin/Streptomycin	Gibco BRL, Paisley, Schottland
Phenol	Appligene, Heidelberg
Phenol red	Sigma, Deisenhofen
Pfu-DNA polymerase	Stratagene
Polyoxyethylensorbitan Monolaurat (Tween 20)	Sigma, Deisenhofen
Potassium acetate	Merck, Darmstadt
Potassium bicarbonate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Mono-Potassiumphosphat	Merck, Darmstadt
Rubidium chloride	Sigma, Deisenhofen
Sodium acetate	Merck, Darmstadt
Sodium azide	Merck, Darmstadt
Sodium bicarbonate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium hydrogencarbonate	Merck, Darmstadt
Sodiumhydroxide	Merck, Darmstadt
Disodium phosphate	Merck, Darmstadt

Sodiumlaurylsulfate (SDS)	Roth, Karlsruhe
Sodiumphosphate	Merck, Darmstadt
Sodium pyruvate	Gibco, Paisley/Schottland
Superscript™ RT with buffer	Gibco BRL, Paisley, Schottland
Taq-Polymerase with buffer	Boehringer Mannheim
Tetramethylethylenediamine (TEMED)	Sigma, Deisenhofen
Tris-(hydroxymethyl)-aminomethan	Riedl-deHaën, Seelze
Triton-X-100	Sigma, Deisenhofen
Trizol	Gibco BRL, Paisley, Schottland
Trypanblue	Sigma Cell Culture, UK
Tryptone	Sigma, Deisenhofen
Tuberculin syringes	Poulten & Graf GmbH, Wertheim
Tween 20	Sigma
Urea	Merck, Darmstadt
Xylene cyanol	Sigma, Deisenhofen
Yeast extract	Merck, Darmstadt

Plastics and films

Plastics were obtained from Assistent (Sondheim), Falcon™ (Becton Dickinson, Plymouth/England), Amersham (Braunschweig), Greiner (Flacht), Schleicher & Schuell (Dassel) and Agfa (München).

6.3 Common abbreviations

a.a.	Amino acids
ADCC	Antibody-dependent cellular cytotoxicity
AP	Alkaline phosphatase
APC	Antigen presenting Cell
APL	Altered peptide liganc
APS	Ammoniumperoxisulfate
BBB	Blood brain barrier
BN	Brown Norway (inbred rat strain)
Bov	Bovine
BTN	butyrophilin
BTN^{exo}	Extracellular domains of bov. Butyrophilin, derived from insect cells
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius (centigrades)
cDNA	DNA which is complementary to mRNA
CDR	Complementarity determining region
CFA	Complete freunds adjuvans
CNS	Central nervous system
ConA	Concanavalin A
c.p.m.	counts per minute
dATP	2´-Desoxyadenosin-5´-triphosphat
DA	Dark Agouti (inbred rat strain)
DEPC	Diethylpyrocarbonat
DMEM	Dulbecco´s Modified Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	2´-Desoxynukleosid-5´-triphosphate
d.p.i.	days post immunisation
d.p.t.	days post transfer
d.p.v.	days post vaccination
EAE	Experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*
e.g.	For example
EH	Eagles HEPES
ELISA	Enzyme linked immunosorbant assay

et al.	And others
EtBr	Ethidiumbromide
FACS	Fluorescence activated cell sorting
FCS	Fetale calf serum
h	hours
HS	Horse serum
HP	Horseradish peroxidase
IFA	Incomplete freunds adjuvant
IFN	Interferon
Ig	Immunoglobulin
IgV	Variable domain of Ig
IL-4 /10	Interleukin 4/10
IPTG	Isopropyl-β-Thiogalactoside
kb	Kilobases
kDa	Kilodalton
LEW	Lewis (inbred rat strain)
LNCs	Lymph node cells
M	Molar
mM	Millimolar
mAB	monoclonale antibodies
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
min	Minutes
ml	Milliliter
MOBP	Myelin oligodendrocyte basic protein
MOG-Igd	Extracellular immunoglobulin domain of rat MOG
MOG	Myelin oligodendrocyte-glycoprotein
MS	Multiple Sclerosis
µg	Microgramm
µl	Microliter
mRNA	Messenger-RNA
ng	Nanogramm
nm	nanometer
OD_X	Optical density at wave length x
PAGE	Polyacrylamidgelelektrophorese

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pers. comm	Personal communication
PFA	Paraformaldehyde
pg	Picogramm
PLP	Proteolipidprotein
PMN	Polymorphonuclear cells
PNS	Peripheral nervous system
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription-Polymerase chain reaction
s.	See
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBS	Tris buffered saline
TBE	Tris-Borat/EDTA
TCGF	T-cell growth factor
TEMED	Tetramethylendiamin
TGF	Tumor growth factor
Th	T-helper cell
TNF	Tumor Necrosis Faktor
Tris/Cl	Tris-(hydroxymethyl)-aminomethan titred with HCl
U	Unit, (for enzyme activities)
UV	Ultraviolet
V	Volt

6.4 Curriculum Vitae

Anna Schubart, born 25.09.1971 in Frankfurt/Main, Germany

June 1990	Abitur at the Lessing-Gymnasium, Frankfurt/Main (1.7)
1990-1991	Au-pair in Paris, France

University Career

1991-1993	Biology student at the J.W.Goethe-Universität, Frankfurt, Germany
July 1993	Vordiplom (1.0)
1993-1994	Biology student at the University of Edinburgh, Scotland
1994-1997	Biology student at the Ludwig-Maximilian-Universität, Munich
Feb1995	Four weeks Neuroimmunology research training, Dr. M. Bradl, MPI for Psychiatry, Department of Neuroimmunology (Prof. Wekerle)
Sep/Oct 1995	Eight weeks research training in Autoimmunity (rheumatoid arthritis), Dr. A. Davidson, Albert Einstein College of Medicine, New York
1997	Diploma project with Dr C. Linington, MPI for Psychiatry, Department of Neuroimmunology (Prof. Wekerle). Title: "Induction of tolerance to MOG in experimental autoimmune encephalomyelitis (EAE)."
Nov 1997	Awarded Diploma in Biology, grade 1.0 (outstanding)
Since Jan 1998	PhD studies with Dr. C. Linington, MPI for Neurobiology, Department of Neuroimmunology (Prof. Wekerle)

Teaching experience

June 1999	Course tutor, Farmades Neuroimmunology course, Martinsried
1998-2001	Supervision of biochemistry and medical student projects:
	Hannah Pellkofer : "T cell mediated autoaggresion to neuronal paraneoplastic autoantigens."
	Simone Dill : "RNA expression analysis of MOG-reactive T cell lines."
	Silke Wissing : "Baculovirus expression of butyrophilin and analysis of the butyrophilin immune response in different mouse strains."
	Jessica Mittelstädt : "Induction of pathogenic autoantibody responses by DNA vaccination,"

6.5 List of Publications

1. Original Articles

Schwaeble WJ, Stover CM, Schall TJ, Dairaghi DJ, Trinder PKE, Linington C, Iglesias I, **Schubart A**, Lynch NJ, Weihe E and Schäfer MKH (1998)
Neuronal expression of fractalkine in the presence and absence of inflammation
FEBS Letters 439: 203-207

Stefferl A*, **Schubart A***, Storch M*, Amini A, Mather I, Lassmann H and Linington C (2000)
Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis
J Immunol 154: 2859-2865
* These authors contributed equally to this publication

Popovic N, **Schubart A**, Goetz B, Zhang S-C, Linington C and Duncan ID (2001)
Inhibition of autoimmune encephalomylitis by a tetracycline
Ann Neurol in press

Pham-Dinh D, Daubas P, Vizler C, Delarasse C, Dimitri D, Goude K, Della Gaspera B, Bauer J, Litzenburger T, Iglesias A, **Schubart A**, Dierich A, LeMeur M, Roussel G, Nussbaum JL, Dautigny A and Liblau RS (2001)
Myelin/oligodendrocyte glycoprotein (MOG)-deficient mice reveal a critical role for MOG in autoimmue encephalomyelitis
submitted

Schubart A, Höftberger R, Stefferl A, Lassmann H and Linington C
Spontaneous relapse and disease progression in myelin oligodendrocyte glycoprotein experimental autoimmune encephalomyelitis is driven by the autoantibody response
in preparation

Schubart A, Eiglmeier I, Kosin S, Höftberger R, De Graaf K, Weissert R, Lassmann H , Goebels N and Linington C
Butyrophilin provides a naturally occurring source of altered peptide ligand that modulates the encephalitogenic potential of the MOG-specific T cell repertoire
in preparation

2. Review Articles

Iglesias A, Bauer J, Litzenburger T, **Schubart A** and Linington C (2001)
T and B cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis
Glia 36: 220 – 234

3. Book Chapters

Schubart A and Linington C (2001)
Aktuelle tierexperimentelle Aspekte der Multiple-Sklerose-Therapie: Antigen-spezifische Immuntherapien bei Myelin-Oligodendrozyten-Glykoprotein (MOG)-induzierter experimenteller autoimmuner Enzephalomyelitis
In: Zettl UK and Mix E (Hrsg): Multiple Sklerose: Kausalorientierte, symptomatische und rehabilitative Therapie, Springer –Verlag Berlin Heidelberg

4. Oral presentations:

Departmental Seminar in the Institute of Veterinary Medicine, Madison, Wisconsin, U.S.A., group of Dr. Ian Duncan, August 1998
MOG-induced EAE as a model system for multiple sclerosis

Annual meeting of the Deutsche Gesellschaft für Immunologie, Düsseldorf, Dec. 2000
Schubart AS, Guggenmos J, Haase C, Mather I and Linington C (2000)
Molecular mimicry between the myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in EAE and multiple sclerosis
Immunobiology 203:503

VIth International Congress of Neuroimmunology, Sept. 2001
Schubart A, Höftberger R, Lassmann H and Linington C
Antibody dependent mechanisms mediate relapse in MOG-induced EAE
J Neuroimmunol 118: 46 (No 130)

Schubart A, Eiglmeier I, Goebels N and Linington C
The molecular basis of T cell cross-reactivity between the myelin oligodendrocyte glycoprotein and the milk protein butyrophilin
J Neuroimmunol 118: 55 (No 157)

5. Poster Presentations

5th annual congress of the British Society for Immunology 1997

Schubart A, Storch M, Lassmann H and Linington C

Treatment with soluble antigen modulates the clinical course of myelin oligodendrocyte glycoprotein (MOG)-induced EAE

Immunology 92 (Suppl 1): 9 (2.16)

Vth International Congress of Neuroimmunology, Aug. 1998

Schubart A, Storch M, Lassmann H and Linington C (1998)

Treatment with soluble antigen modulates the clinical course of myelin oligodendrocyte glycoprotein (MOG)-induced EAE

J Neuroimmunol 90: 83

Joint annnual meeting 2000 of the german and dutch societies of Immunology, Nov 2000

Schubart AS, Guggenmos J, Haase C, Mather I and Linington C (2000)

Molecular mimicry between the myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in EAE and multiple sclerosis

Immunobiology 203:503