Aus dem Institut für Neuroimmunologie
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DISSERTATION

Modulation of human antigen-specific T cell response – therapeutic implications for multiple sclerosis

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No scientist is admired for failing in the attempt to solve problems that lie beyond his competence. The most he can hope for is the kindly contempt earned by the Utopian politician. If politics is the art of the possible, research is surely the art of the soluble. Both are immensely practical-minded affairs.

P B Medawar, **The Art of the Soluble** (1967) Nobel Prize Laureate in Physiology or Medicine, 1960 for the discovery of acquired immunological tolerance (Medawar, 1999).

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SUMMARY

Multiple sclerosis (MS) is a heterogeneous disease of the central nervous system whose pathological mechanisms are far from completely understood. The current hypothesis is that pro-inflammatory T cells are orchestrating the pathogenesis of this condition. It is considered that a dysregulation in T cell control to be involved, with an imbalance in apoptosis-regulating molecules possibly playing a role. In fact, therapeutic strategies aim to reduce T cell activation, proliferation and cytokine production or to promote T cell elimination. The focus of this thesis was to identify the role of regulatory molecules for T cell survival in the immune pathogenesis of MS, and to investigate antiproliferative or apoptosis-promoting effects on T cells by potential therapeutic molecules.

A limitation in the apoptotic regulation of autoreactive T cells in the periphery and in the CNS may contribute to the pathophysiology of MS. As key regulators of apoptosis, members of the Bcl-2 family were investigated in both MS patients and controls. These factors were examined in relation to the susceptibility of T cells, from both groups, towards activation-induced cell death (AICD). To mimic the in vivo elimination of antigen-reactive T cells, an in vitro model of AICD involving repetitive T cell receptor mediated stimulation was utilized. In fact, polyclonal T cells from MS patients showed a decreased susceptibility to undergo AICD as shown by both caspase activity (p=0.013) and DNA fragmentation (p=0.0071) assays. Furthermore, Bcl-X_L protein levels, as measured by immunoblotting, were increased in the peripheral immune cells of MS patients (p=0.014). An inverse correlation observed between Bcl-X_L levels and susceptibility of T cells to undergo AICD is in line with previous data on the significance of this anti-apoptotic protein in T cell resistance. Since this molecule has already been shown to aggravate the outcome of experimental autoimmune encephalitis, the animal model for MS, the observation of elevated Bcl-X_L levels in patients offers perspectives towards therapeutic manipulation in MS.

Apart from promoting apoptotic elimination, current therapeutic strategies aim at inhibiting activation and further proliferation of potentially harmful T cells. Based on clinical experience with rather non-selective therapies that promote T cell elimination, a therapeutic goal is to identify newer immunomodulatory substances with better selectivity in order to maximize the therapy's benefit to risk ratio. Thus, two different substances, both interfering with cell cycle regulation, were investigated. The *first* candidate was the recently discovered member of the TNF/NGF family of death ligands, TNF-related apoptosis inducing ligand (TRAIL) since it has been reported to have immunoregulatory functions and since human antigen-specific T cells were shown to be resistant towards apoptosis induction by this ligand. The *second* candidate drug with potential in MS therapy is atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme

(HMG-CoA) reductase inhibitor and lipid-lowering drug, already indicated for anomalies in lipid metabolism.

In order to prove the hypothesis that these substances interfere with T cell receptor signaling, human antigen-specific T cell lines from both MS patients and controls, characterized with regards to T helper differentiation and peptide specificity, were employed. Exogenous treatment of TRAIL resulted in an inhibition in proliferation, albeit to varying degrees (6.2% - 63.8% inhibition). Atorvastatin also inhibited proliferation of antigen-specific T cell lines in a dose-dependent manner. Both compounds induced hypoproliferation independently of antigen presentation, as shown by their ability to block T cell proliferation in response to direct T cell receptor engagement, thus indicating a direct influence on T cell function. The growth inhibition by TRAIL was associated with a downregulation of the cell cycle regulator CDK4, indicative of an inhibition of cell cycle progression at the G1/S transition. Incubating T cells with atorvastatin also induced a downregulation of CDK4 expression, which was accompanied by an upregulation of $p27^{Kip1}$ expression. The atorvastatin-mediated inhibition in proliferation and cell cycle progression could be reversed by mevalonate, an intermediate product of the HMG-CoA reductase pathway, suggesting a direct involvement of atorvastatin in this pathway, necessary for the isoprenylation of small GTPase proteins of the Rho family.

Utilizing a thapsigargin model of calcium influx to activate the same calcium-release activated calcium (CRAC) channels as T cell receptor-stimulation by antigen, an inhibition in calcium influx could be observed on pre-incubating T cells with TRAIL. Co-incubating with human recombinant TRAIL receptor 2 fusion protein, a competitive antagonist for TRAIL, reversed this inhibition. A direct influence on calcium influx is indicative of an influence of TRAIL on the activation status of human T cells. Therefore, TRAIL directly inhibits activation of these cells via blockade of calcium influx. However, no impact of atorvastatin on early T cell activation was observed, since calcium influx was unaffected.

While TRAIL-mediated interference with T cell activation and further cell cycle progression is still in the pre-clinical phase, statins, which have also been shown here to interfere with the T cell cycle, are already employed in the clinic for other ailments. In fact, clinical trials are currently being undertaken with this group of drugs for MS. Further studies on detailed mechanisms of antiproliferative substances effective in MS will allow the development of highly selective immunomodulatory agents with increased beneficial profile as MS therapy.

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1 INTRODUCTION

Multiple sclerosis (MS) is the most common disease of the central nervous system (CNS) that causes prolonged and severe disability in young adults in Europe and North America. Over a century and a half ago Charcot, Carswell, Cruveilhier, and others described the clinical and pathological characteristics of this enigmatic neurological disease (Carswell, 1838; Cruvellhier, 1842; Charcot, 1868), defining it as 'la sclerose en plaques' (Charcot, 1868). As an enigmatic, relapsing, and often eventually progressive disorder of the CNS, multiple sclerosis continues to challenge investigators to understand its pathogenesis and come up with new therapies to prevent its progression (rev. Noseworthy et al., 2000).

Although MS has a variable prognosis, fifty percent of patients need help with walking within 15 years after the onset of disease (rev. Noseworthy et al., 2000). Recent progress has occurred in understanding the cause, the genetic components, and the pathologic process of multiple sclerosis. Advanced magnetic resonance imaging (MRI) and spectroscopy has also recently allowed clinicians to follow the pathological progression of the disease and monitor the response to treatment (Miller et al., 1998).

The pathological hallmark of chronic MS is the demyelinated plaque, lesions commonly occurring in the optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord white matter, and often surrounding one or several medium-sized vessels. MS is characterized by multifocal infiltration of autoreactive T lymphocytes from the systemic immune system across the blood-brain barrier (BBB). Infiltrating T cells orchestrate an inflammatory response. This response leads to demyelination (rev. Martin et al., 1992; rev. Martino and Hartung, 1999) and, according to recent knowledge, damage of neurons (Peterson et al., 2001) and their axons (Bitsch et al., 2000), which can already occur early during disease and lesion formation (Trapp et al., 1998; Kuhlmann et al., 2002).

MS is associated with genes relevant to the immune response, especially genes of the 'Human Leukocyte Antigen' region (rev. Martin et al., 1992; Zipp et al., 1995; Zipp et al., 1998a). These association studies, together with observations of T cell infiltration in the brain parenchyma as well as a clinical response of MS patients to immunosuppressive and immunomodulatory therapy, designate MS as an autoimmune disease of the CNS. However, the strongest evidence stems from studies on the animal model of MS, experimental autoimmune encephalomyelitis (EAE), which show that immunization of animals with candidate CNS antigens or by transferring activated encephalitogenic T cells that are specific for such proteins leads to damage of CNS tissue and, subsequently, to neurological deficits (rev. Wekerle et al., 1994).

A multi-step model for the initiation of T cell-mediated autoimmune inflammatory disease of the CNS is assumed (Fig. 1). This involves the peripheral activation of T cells specific for myelin antigens and T helper (Th) 1-type differentiation (rev. Martin et al., 2001). Activated antigen-specific T cells that have survived and have not undergone elimination by regulatory mechanisms transmigrate the BBB where they respond to CNS antigens in situ. Adhesion molecules, cytokines, chemokines, leukocytic enzymes, cerebrovascular endothelium, and the parenchymal cells also contribute to this process (Cannella et al., 1991; rev. Karpus and Ransohoff, 1998). Once in the CNS, myelin-specific T cells encounter their target autoantigen on antigen presenting cells (APC), presumably microglia and astrocytes, and, on further stimulation, secrete cytokines, such as apoptosis-mediating TNF and CD95L, which contribute to the local effector mechanisms (rev.Hartung, 1993; rev. Zipp et al., 1999).

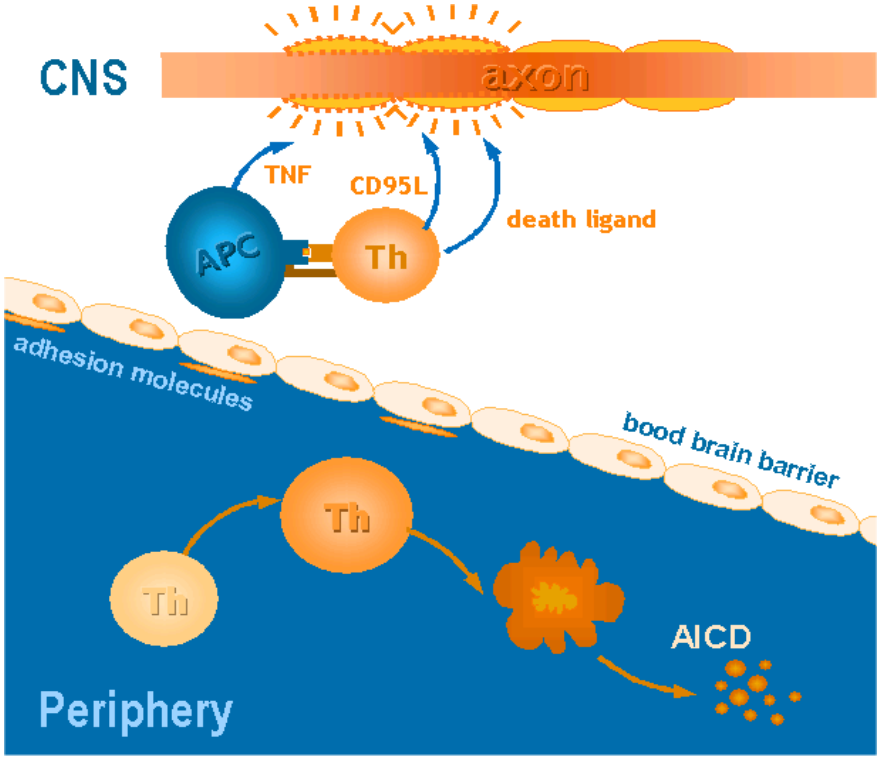

Figure 1 **The pathophysiology of multiple sclerosis** Autoantigen-specific T cells that escape AICD are capable of transmigrating the blood-brain barrier. In the CNS, these myelin-specific T cells encounter their specific target autoantigen and are induced to secrete cytokines, such as TNF or CD95L to cause the tissue damage (adapted from (rev. Zipp et al., 1999)).

Both elimination of T cells in the periphery and damage to CNS tissue such as myelin, oligodendrocytes, and neurons by immune cells are central pathogenic mechanisms of MS. Apoptosis seems to play a central role in both of these processes (rev. Gold et al., 1997; rev. Zipp et al., 1999). While elimination of T cells by apoptosis in the periphery is diminished, CNS damage by apoptosis is an occurring unwanted feature. To develop selective therapies, one has to understand the pathophysiologic progression in detail. The aim is to interfere with T cell activation and proliferation and to promote T cell elimination.

1.1 T CELLS: CENTRAL ROLE IN MULTIPLE SCLEROSIS

The pathogenesis of chronic inflammatory autoimmune disease such as MS, diabetes and rheumatoid arthritis seems to be initiated by $CD4^+$ T helper (Th) cells (rev. Lafaille, 1998). In MS, T cells specific for myelin antigens are thought to initiate (rev. Hohlfeld et al., 1995) and, in cooperation with other immune cells, perpetuate the pathogenic processes (rev. Noseworthy et al., 2000; rev. Zipp, 2000).

1.1.1 T HELPER CELL DIFFERENTIATION

In 1986 Mosmann and colleagues reported that $CD4^+$ T cell clones could be classified (based upon the cytokines they secrete) into two groups which they named **Th1** and **Th2** (Mosmann et al., 1986). The two main subsets, nowadays designated as Th1-like and Th2-like cells, are characterized by the pattern of cytokines secreted upon stimulation, by distinct activation (Munoz et al., 1990), by differential expression of chemokine receptors (Sallusto et al., 1998) and by different sensitivities to undergo AICD (Ramsdell et al., 1994; Varadhachary et al., 1997; Zhang et al., 1997). While Th2-like cells (producing IL-4, IL-5 and IL-13) play a role in the pathogenesis of allergic diseases, it is known that Th1-like cells (producing functionally opposite cytokines which are proinflammatory in nature: IFN-γ, IL-2, TNF-α) are involved in chronic inflammatory reactions (rev. Abbas et al., 1996).

The role of the different Th cell populations on autoimmune diseases has received considerable attention over the past several years (rev. Lafaille, 1998). Th1 cells have been implicated in the pathogenesis of MS and Th1/Th2 balance potentially affects prognosis (rev. Olsson, 1995). Th1 cells stimulate macrophages and can directly destroy or mediate injury of target cells. In fact, the main cytokines produced by Th1-like cells (TNF-α, TNF-β, IFN-γ) are all present in the MS plaque. Interferon-γ and TNF-α can upregulate MHC class II and adhesion molecules, allowing T cells to interact with the endothelium at the BBB and with glia (antigen-presenting cells) such as microglia and astrocytes in the parenchyma (rev. Zipp, 2000).

Therefore, MS is considered to be a Th1-mediated autoimmune condition and treatment with glatiramer acetate (GA), one of the currently available immunomodulatory therapies for MS, induces a Th1 to Th2 immune deviation both in vivo (Miller et al., 1998) as well as in vitro (Neuhaus et al., 2000). Apart from an induction of Th2-type regulatory T cells, another proposed mechanism of action of GA, is a competition with myelin basic protein (MBP) at the MHC and T cell antigen receptor (TCR) level (Fridkis-Hareli et al., 1994).

Nevertheless, Th1 and Th2 cells represent only extremes of a polarized spectrum and Th subdivision is far more complex. In fact, a further subset of immunoregulatory CD4 cells,

Th3, producing transforming growth factor-beta (TGF-β), has also been defined. This cytokine is increasingly being recognized in immunoregulation and tolerance and its production correlates with protection and/or recovery from autoimmune diseases. While encephalitogenic T cells producing Th1 cytokines can transfer disease in EAE (Racke et al., 1994), spontaneous recovery from EAE correlates with a switch to TGF-β and Th2 cytokines (Khoury et al., 1992). An upregulation of TGF-β has also been detected in CNS conditions with a traumatic or inflammatory etiology and has been implicated in the induction of a prominent astrocytic reaction (Logan et al., 1994), which mediates the structural reorganization of tissues and scar formation (rev. McCartney-Francis et al., 1998).

1.1.2 T CELL APOPTOSIS

In order to cross the blood-brain barrier and to mediate their effector damage-function in the CNS, autoreactive T cells need to escape regulatory mechanisms of the immune system. An ongoing immunological means of controlling autoreactive T cells is *tolerance* (immunologic unresponsiveness), a process that begins centrally during T cell maturation in the thymus and continues throughout the cell's life in the periphery by a network of regulated restraints. In both central and peripheral tolerance, *apoptosis* is a common way of eliminating potentially harmful T cells.

The term apoptosis was coined in 1972 by Kerr et al. (rev. Kerr et al., 1972). It is derived from the Greek word απατοσισ, meaning falling leaves. On the contrary to necrosis, apoptosis results in condensation of the nucleus and cytoplasm, release of cytochrome *c* from mitochondria into cytosol, condensation of chromatin and cleavage into regular fragments, shrinking/blebbing of plasma membrane and formation of apoptotic bodies (rev. Wyllie et al., 1980). The rapid clearance of apoptotic cells makes it difficult to observe the phenomenon in vivo (rev. Manfredi et al., 2002).

Apoptosis is considered a physiologic process and a major form of cell death that is used to remove excess, damaged or infected cells throughout life (rev. Bratton and Cohen, 2001). It is therefore important in normal cell development, occurring during embryonic development as well as in the maintenance of tissue homeostasis. Loss of control of the apoptotic program contributes to many diseases, including accumulation of unwanted cells through insufficient apoptosis (e.g. lack of elimination of autoreactive cells as in autoimmune disease) and cell loss due to excessive apoptosis (e.g. neurodegeneration, stroke and heart failure). Since it describes a process in which a cell actively participates in its own destruction, it had been earlier termed ‘programmed cell death’ (Lockshin, 1969).

Ligation of plasma-membrane death receptors and nuclear DNA damage have long been recognized as initial triggers of apoptosis that induce mitochondrial membrane permeabilization and/or the direct activation of cysteine aspartyl-specific proteases (caspases). Principally, two alternative apoptosis pathways (Fig. 2) exist: one is mediated by death receptors on the cell surface — the 'extrinsic pathway'; the other is mediated by mitochondria — 'intrinsic pathway' (rev. Igney and Krammer, 2002). In both pathways, caspases are activated to cleave cellular substrates, leading to the biochemical and morphological changes characteristic of apoptosis (rev. Bratton and Cohen, 2001).

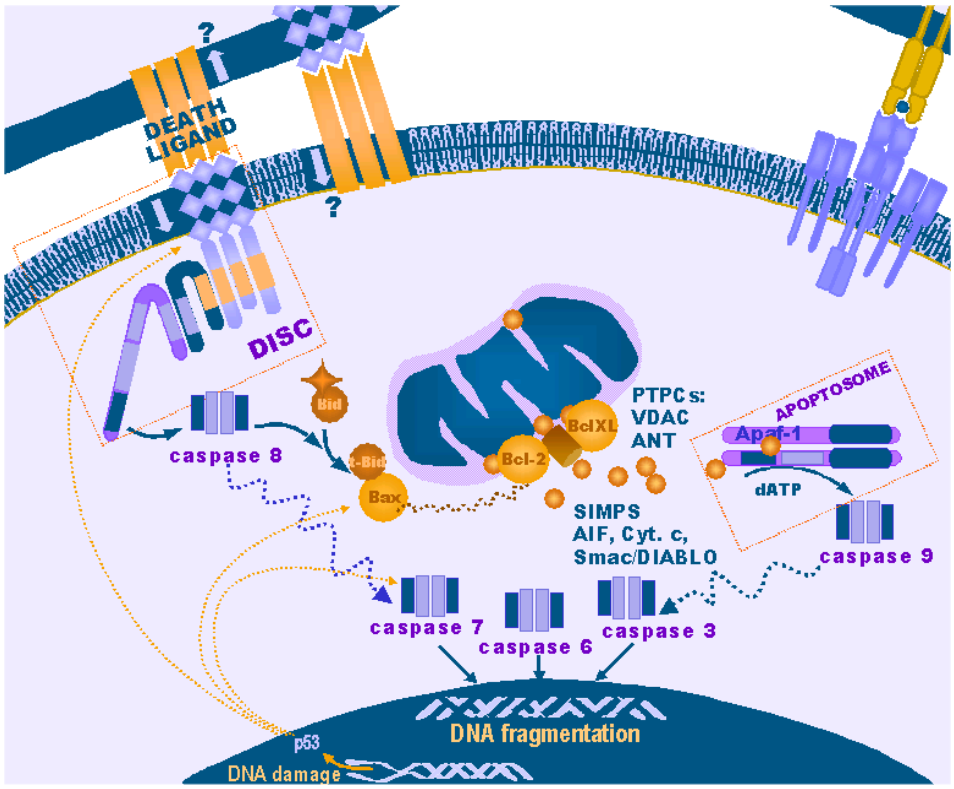

Figure 2 **Apoptotic signaling cascades** Two alternative apoptosis pathways exist: one — referred to as the extrinsic pathway — is mediated by death receptors on the cell surface, the other — referred to as the intrinsic pathway — is mediated by the mitochondria (adapted from (rev. Bratton and Cohen, 2001; rev. Igney and Krammer, 2002)).

Intrinsic Pathway of apoptosis: Bcl-2 family of apoptosis regulators

The cell intrinsic pathway triggers apoptosis chiefly in response to DNA damage, defective cell cycle, hypoxia and loss of survival factors among many types of severe cell stresses. This pathway involves activation of the pro-apoptotic arm of the **Bcl-2 superfamily**. Members of the Bcl-2 family, pro-apoptotic (Bid, Bax, Bak, PUMA, Noxa) and anti-apoptotic (Bcl-2, Bcl-X_L), are key regulators of the cell suicide program critical for normal development and maintenance of tissue homeostasis (rev. Adams and Cory, 1998; rev. Green, 2000; rev. Hunt and Evan, 2001). This pathway is also induced following death receptor engagement and acts as amplifier to the extrinsic pathway (rev. Igney and Krammer, 2002). Pro-apoptotic Bid, cleaved by active 'initiator' caspase-8, translocates to the mitochondria and

stimulates the insertion or oligomerization of Bax or Bak in the outer membrane or agents acting on the PTPC of the mitochondria (Fig. 2). Membrane permeabilization, which is controlled by anti-apoptotic Bcl-2 molecules, such as Bcl-2 or Bcl-X_L, then causes the release of apoptogenic factors, SIMPs, such as cytochrome *c* (Li et al., 1998), AIF and SMAC/DIABLO (Du et al., 2000; Verhagen et al., 2000) into the cytosol. Cytochrome *c* binds the adaptor APAF1, forming an 'apoptosome' and, in the presence of dATP, activates the apoptosis-initiating protease caspase-9. In turn, caspase-9 activates 'executioner' proteases caspase-3, -6 and -7. SMAC/DIABLO promotes apoptosis by binding to inhibitor of apoptosis (IAP) proteins, preventing them from attenuating caspase activation (Du et al., 2000; Verhagen et al., 2000).

Engagement of the cell intrinsic pathway results in the rapid induction of nuclear p53 (Wu and Lozano, 1994) as well as localization of p53 protein to mitochondria in vivo (Marchenko et al., 2000). The transcriptional activity of p53, induced through post-translational mechanisms, is important for its pro-apoptotic function (Fig. 2). Apart from inhibiting expression of Bcl-2, p53 can induce pro-apoptotic members of the Bcl-2 family such as Bax (Miyashita and Reed, 1995), Noxa (Oda et al., 2000) and PUMA (Yu et al., 2001), all of which can translocate from the cytosol to the outer mitochondrial membrane to induce mitochondrial membrane permeabilization. Moreover, mitochondrial anti-apoptotic regulators like Bcl-2 and Bcl-X_L specifically block stress-induced mitochondrial p53 localization and apoptosis but not nuclear p53 induction and cell cycle arrest. p53 also induces some death receptors (Fig. 2), such as CD95 (Muller et al., 1998) and TRAIL-R2 (DR5) (Wu et al., 1997).

Death receptor upregulation increases cellular sensitivity to death receptor ligands. In some cell types, death receptor engagement alone, without use of the cell intrinsic pathway suffices for commitment to apoptotic death. In other cell types, commitment to apoptosis requires amplification of the death receptor signal by the above-described cell intrinsic pathway (Scaffidi et al., 1999). It is therefore of consequence that both the intrinsic and the extrinsic apoptosis signaling pathways communicate with each other.

Extrinsic Pathway of apoptosis: Death ligands and receptors of the TNF superfamily

The cell extrinsic pathway is becoming recognized as an important path used by T cells to trigger apoptosis. This pathway triggers apoptosis in response to engagement of death receptors by their ligands. Death receptors and their ligands are members of the tumor-necrosis factor (TNF) receptor/ligand superfamily. The ligand conferring its name to this group TNF is the prototype member of the ligand superfamily (rev. Locksley et al., 2001).

Most TNFR-superfamily members function as transmembrane signal transducers that respond to ligand binding and comprise a subfamily characterized by an intracellular domain — the death domain (orange box, Fig. 2). Decoy receptors are closely related to the death receptors but lack a functional death domain. When death ligands bind to their respective death receptors, intracellular adaptor protein FADD (Fas-associated death domain protein) gets attracted to the receptors via death domains.

This complex, death-inducing signaling complex (DISC), recruits the inactive proforms of certain members of the caspase protease family — caspase-8 (FLICE) and caspase-10 (Kischkel et al., 1995). At the DISC, these procaspases are cleaved and function as active 'initiator' caspases (Sprick et al., 2000; Kischkel et al., 2001). A regulator of this pathway is FLIP (FLICE-inhibitory protein), a molecule structurally similar to caspase-8 but lacks a functional catalytic domain and therefore interferes with apoptosis signaled through death receptors (Thome et al., 1997). Although FLIP is expressed early during T cell activation, it disappears when T cells become susceptible to CD95L-mediated apoptosis (Irmler et al., 1997). As already described, although some cells known as type I cells, contain sufficient amounts of active caspase-8 to initiate apoptosis directly, some cells, type II cells, contain too small an amount and employ the intrinsic pathway, using mitochondria as 'amplifiers' of the apoptotic signal (Scaffidi et al., 1999).

Following a scan in the human genome database for sequences with homology to TNF, identification of expressed sequence tags led to the cloning of a novel TNF-superfamily member (Wiley et al., 1995; Pitti et al., 1996). Due to its protein sequence homology to CD95L (APO1L) and TNF, the newly discovered protein was named 'APO2L' for APO2 ligand (Pitti et al., 1996) or **TRAIL** for TNF-related apoptosis-inducing ligand (Wiley et al., 1995), respectively. Similar to CD95L and TNF-alpha, TRAIL was shown to rapidly induce apoptosis in susceptible cells upon trimerization of its receptors and subsequent activation of the caspase cascade leading to fragmentation of DNA (Wiley et al., 1995; Pitti et al., 1996). TRAIL can interact with five different receptors. Of these, only TRAIL receptor 1 (TR1), also referred to as DR4 (Pan et al., 1997) and TR2 or DR 5 (Sheridan et al., 1997; Walczak et al., 1997) are capable of transmitting a death signal. Transmembrane TR3 (DcR1, TRID) contains no death domain and TR4 (DcR 2, TRUNDD) a truncated one. They have been suggested to act as decoy receptors by binding TRAIL without transmitting a death signal, thereby inhibiting apoptosis (Pan et al., 1997; Sheridan et al., 1997; Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b). TRAIL and its receptors were shown to be constitutively expressed by a variety of cell types including human (auto)antigen-specific T cells (Wendling et al., 2000).

Involvement of apoptotic mechanisms in MS

There is already growing evidence for the involvement of apoptosis in the pathogenesis of MS. Particularly, a Janus-faced function: while failing to control potentially dangerous autoreactive T cells, apoptosis contributes to the CNS tissue damage. Infiltrating T cells, escaping control mechanisms, are thought to mediate their effect via death receptor-ligand interactions. The induction of T cell death, especially via death receptor-mediated apoptosis, reduces the possibility of invading T cells and thus autoimmune-mediated tissue damage: a regulatory process which is effective during recovery (Schmied et al., 1993) and treatment of EAE (Critchfield et al., 1994).

An impairement of CD95-dependent T cell elimination, both in the CNS (Ciusani et al., 1998) as well as in the periphery (Zipp et al., 1998b; Zipp et al., 1998c; Macchi et al., 1999; Zang et al., 1999) has been reported in MS. Factors which protect against apoptosis, such as soluble CD95 (Zipp et al., 1998c) or FLIP (Semra et al., 2001) are increased in peripheral immune cells of MS patients, indicating an overall reduction of T cell apoptosis in MS patients (rev. Zipp et al., 1999). On the other hand, an upregulation of disease-promoting apoptosis-inducing ligands of the TNF superfamily such as TNFα, CD95L and TRAIL in MS might indicate counterregulatory mechanisms or the involvement of apoptosis in T cell effector mechanisms in the CNS (Zipp et al., 1995; Hermans et al., 1997; Huang et al., 2000; Tejada-Simon et al., 2001).

In T cells, the CD95/CD95L system plays a major role in the induction and regulation of AICD, a signal-induced programmed cell death initiated at the TCR (rev. Kabelitz et al., 1993). An involvement of CD95L-CD95 interaction in AICD is derived from studies on mice carrying CD95 (lpr) or CD95L (gld) mutations (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). These animals spontaneously develop a multi-organ autoimmune disease, with symptoms that are similar to SLE, due to a defect in AICD of mature T cells (Russell et al., 1993; Russell and Wang, 1993). CD95L-CD95 interactions therefore regulate immune selection and peripheral tolerance (Russell, 1995; rev. Kabelitz and Janssen, 1997). CD95L binds to CD95 expressed on the same or on neighboring cells, triggering CD95-dependent apoptosis (Singer and Abbas, 1994; Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995) in either an autocrine “suicide” or paracrine “fratricide” manner (Mariani et al., 1996). In MS, an increase in MBP-specific T cell frequency, only in the presence of CD95 ligand-blocking antibody in vitro, suggests that a significant proportion of MBP-reactive T cells, although sensitive to CD95L-mediated apoptosis, are not deleted in vivo (Zang et al., 1999), possibly due to an impairment of AICD. Although the role of the CD95/CD95L system in the induction of T cell apoptosis is unambiguous, it is evident that

other molecules are equally important (Peter et al., 1995). Other members of the TNF superfamily, including TNFα (Zheng et al., 1995) and TRAIL (Martinez-Lorenzo et al., 1998), have also been implicated in AICD. Additionally, the induction of AICD is influenced by other cytokines such as IL-2. This T cell growth factor has been implicated in the priming of mature T cells for AICD (Lenardo, 1991) by driving cells into the S phase of the cell cycle, where they are sensitive to TCR-triggered AICD (Boehme and Lenardo, 1993). Additionally, susceptibility of T cells towards CD95-mediated AICD is associated with subsiding levels of Bcl-X_L (Boise et al., 1995; Broome et al., 1995; Peter et al., 1997).

Concerning the damage mechanisms within the CNS, members of the TNF superfamily have also been reported to be involved in the T-cell mediated effector mechanisms. An earlier report which has been supported by a more recent study, describes a role for the TNF system in oligodendroglial cell loss in MS (Selmaj et al., 1991; Akassoglou et al., 1998). An involvement of the CD95 system in MS stems from the observation of an enhanced CD95/CD95 ligand expression in brain lesions of MS patients (Dowling et al., 1996). TRAIL induces massive cell death of brain cells, including neurons, astrocytes, and oligodendrocytes (Nitsch et al., 2000). Additionally, the presence of TRAIL receptors but absence of the death-inducing ligand on these parenchymal cells (Dorr et al., 2002a), makes them potentially susceptible to attack by TRAIL-expressing T cells (Dorr et al., 2002b). These findings indicate a potential role for the TRAIL receptor–TRAIL system as an effector mechanism in neuroinflammation such as MS. On the other side of the BBB, studies on animal models of autoimmune diseases have reported an influence of TRAIL on T cell growth and effector function. Systemic neutralization by TRAIL receptor 2 was demonstrated to exacerbate collagen-induced arthritis (Song et al., 2000) and experimental autoimmune encephalomyelitis (Hilliard et al., 2001). This suggests a dual role for TRAIL: regulation in the immune system and toxicity at sites of inflammation.

In EAE, data also exist for the role of both the CD95/CD95 ligand (Sabelko et al., 1997; Waldner et al., 1997) and the TNF receptor/TNF system (Akassoglou et al., 1998) in tissue damage. While lpr and gld mice are protected from active EAE (Sabelko et al., 1997; Waldner et al., 1997), gld mice with passive EAE, induced by transfer of autoreactive T cells, developed prolonged clinical signs when immunized with wildtype T cells (Sabelko-Downes et al., 1999). Thus, in the passive EAE model, inhibiting the CD95L results in reduced disease remission. Although this indicates a role of CD95-mediated apoptosis in the regulation of T cells in EAE, it is still unclear under which conditions T cells are rendered susceptible to apoptosis (Klas et al., 1993; Peter et al., 1997; Zipp et al., 1997).

1.1.3 T CELL ACTIVATION

While naïve T cells cannot readily penetrate the BBB, activated autoantigen-specific T cells that have survived elimination transmigrate into the CNS and get reactivated on further autoantigen presentation (Merrill and Benveniste, 1996).

In T cells, activation is initiated by **signal 1**, which occurs at the T cell antigen receptor (TCR) (Fig. 3). This comprises of a ligand-binding subunit (α and β chains) and a signaling subunit (CD3ε, γ and δ chains and TCRζ chain). The physiologic ligand for the TCR is antigen presented by MHC expressed on APCs such as dendritic cells, macrophages or B cells. Although signals generated by the TCR determine the specificity of the T cell response to antigen, costimulatory receptors, such as CD28, contribute to **signal 2**, which is important for the realization of the TCR response. In fact this dual signaling is necessary for the prevention of anergy, a state of unresponsiveness which develops in the absence of CD28 costimulation (rev. Nel and Slaughter, 2002). The coordinated activation of T cells by antigen leads to clonal expansion, differentiation, cytotoxic killing, or induction of their own programmed cell death.

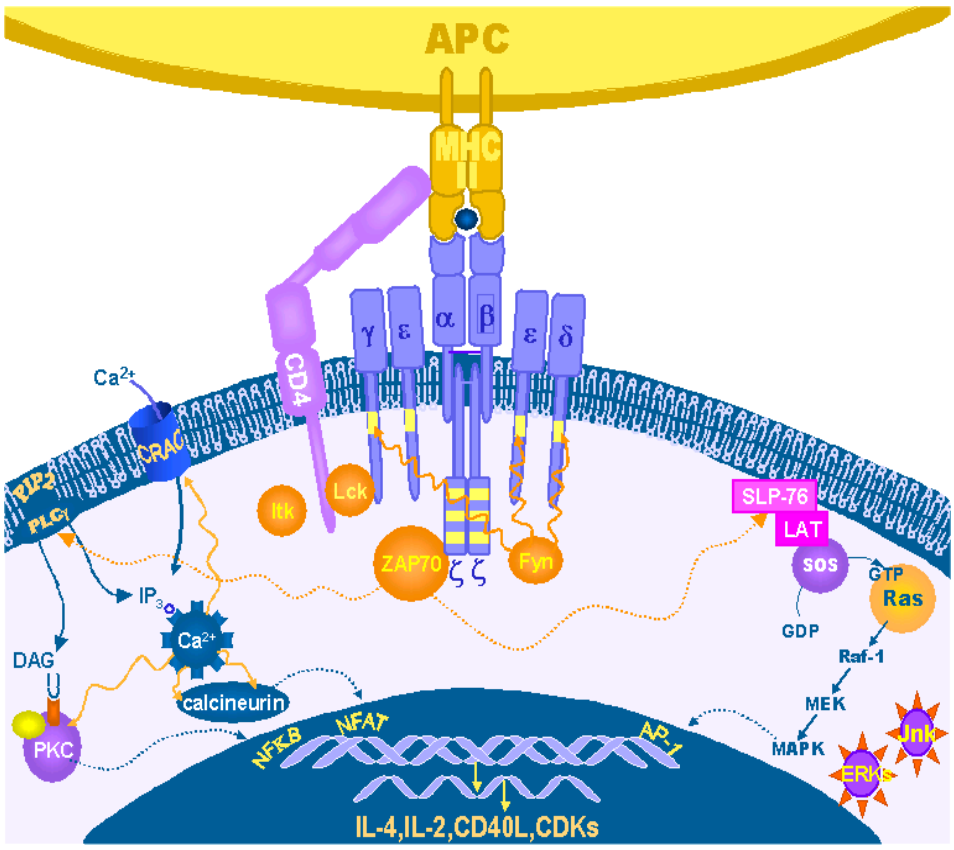

Figure 3
Signaling cascades in T cell activation
The initial steps of a T cell response involve protein tyrosine kinase activation following MHC-Ag-TCR binding and a subsequent activation of downstream signaling pathways including intracellular free calcium increase and MAPK activation, all necessary for T cell activation and further proliferation. (adapted from (rev. Nel, 2002)).

TCR signaling commences with an early wave of protein tyrosine kinase activation, which is mediated by the Src kinases Lck and Fyn, the 70-kd ζ-associated protein (ZAP70) kinase,

and members of the Tec kinase family such as Itk. This early wave of protein tyrosine phosphorylation leads to two main paths: an initiation in inositolphospholipid (IP) turnover resulting in activation of downstream signaling pathways including intracellular free calcium increases (left arm of Fig. 3) and Ras–mitogen-activated protein kinase (MAPK) activation (right arm of Fig. 3). Both arms of this signaling cascade activate transcription factors, such as activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB), ultimately leading to the expression of genes that control cellular proliferation, differentiation, anergy, or apoptosis (rev. Nel, 2002).

Intracellular calcium

Calcium is one of the many ubiquitous secondary messengers that regulate innumerable cellular responses. Elucidation of the role of calcium as an intracellular messenger began over 100 years ago with early observations by Ringer (Ringer S, 1883; rev. Barritt, 1992). It has since been considered that calcium is responsible for certain cell responses in both electrically-excitable and non-electrically-excitable cells. "Ja, Kalzium …das ist alles" is a famous statement by the Nobel Prize laureate Otto Loewi (1873-1961).

As shown in the left-arm of the signal cascade in Fig. 3, calcium is released from endoplasmic reticulum storage sites following activation by inositol-1,4,5-trisphosphate (IP_3) cleaved from phosphatidyl inositol-4,5 biphosphate (PIP_2) by tyrosine phosphorylated PLC-γ (rev. Berridge et al., 1998). A rise in $[Ca^{2+}]_i$ following T cell activation is necessary for most of the physiological functions of T cells including proliferation and cytokine production. Following TCR engagement, mobilization of Ca^{2+} from intracellular stores and depletion of these stores triggers prolonged Ca^{2+} influx through store-operated Ca^{2+} (SOC or CRAC, calcium release-activated calcium) channels in the plasma membrane. The elevation in $[Ca^{2+}]_i$ produced is required for T cell activation (Zweifach and Lewis, 1993) and a lasting rise greater than 200nM is required to induce transcriptional activation in the nucleus such as IL-2 synthesis (Negulescu et al., 1994).

The spatiotemporal characteristics of $[Ca^{2+}]_i$ signaling (transient, sustained, or oscillatory) are important in determining which genes are activated. For instance, sustained $[Ca^{2+}]_i$ elevation is critical for the activation of calcineurin, a calcium-calmodulin–dependent serine phosphatase, to dephosphorylate NFAT (rev. Baksh and Burakoff, 2000) which leads to its nuclear translocation and binding to IL-2 promoter (rev. Rao et al., 1997). In fact, a lasting rise greater than 200 nM is required to induce IL-2 synthesis (Negulescu et al., 1994) and substances which reduce $[Ca^{2+}]_I$ to lower levels than this, such as CD95-stimulation (Lepple-Wienhues et al., 1999), might play a role in anergy induction.

Cell cycle regulation

T cell proliferation is tightly controlled by a large number of positive regulators such as cyclins and cyclin dependent kinases (CDKs), and negative regulators such as CDK inhibitors (Nagasawa et al., 1997; Appleman et al., 2000). These regulate progression of T cells from the G_0 to $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$ phases of the cell cycle. The right arm of the signal cascade (Fig. 3) involves activation of downstream MAPKs pathways, which switch on another set of transcription factors such as AP-1, factors also involved in T cell proliferation and cell cycle progression possibly via the influence of Jun/Fos complexes on cell cycle–activating protein. Cells entering the G1 phase after TCR engagement are characterized by an upregulation of cyclin D and CDK4/6 (Modiano et al., 1994; Kwon et al., 1997). Apart from their known function in cell cycle progression, cell cycle regulators have also been shown to control mechanisms implicated in T cell tolerance, such as anergy (rev. Balomenos and Martinez, 2000). In fact $p27^{Kip1}$ has been found to be responsible for the blockade of clonal expansion of anergic T cells (Boussiotis et al., 2000).

The decision between activation and anergy upon TCR occupancy is generally considered to be dependent on the balance between positive and negative signals in T cells, with costimulatory pathways tipping this balance from anergy to activation (rev. Kamradt and Mitchison, 2001).

These intracellular pathways of signal transduction, initiated by the binding of extracellular ligands to their specific receptors, represent an obvious target for pharmacological intervention. In many instances at least some of the intracellular signaling proteins are specifically linked to the ligand–receptor system so that relatively selective inhibition should be possible not only at the level of the receptor–ligand interaction but also at the level of intracellular signalling.

1.2 AIMS OF THIS THESIS

Pro-inflammatory T cells, which are not properly controlled by regulatory mechanisms such as activation-induced cell death (AICD), are assumed to orchestrate the pathogenesis of MS.

The primary focus of this thesis was:

i. to identify the role of regulatory molecules for T cell survival in the pathogenesis of MS and
ii. to investigate the antiproliferative or apoptosis-promoting effects on T cells by potential therapeutic targets and the underlying mechanisms involved.

I. To address the first question, i.e. to investigate regulatory molecules that might be responsible for the lack of T cell control in MS, the Bcl-2 family of apoptotic regulators was studied. For this purpose a group of MS patients and healthy controls were selected and the expression of three main members of the Bcl-2 family were investigated. In relation to this, the susceptibility of T cells from both groups towards AICD was examined. For this, an in vitro method of AICD involving repetitive T cell receptor mediated stimulation was employed.

II. To address the second question, i.e. to study new therapeutic strategies capable of reducing T cell activation/proliferation and promoting elimination, investigations on TRAIL, a novel member of the TNF/NGF family of death ligands, and atorvastatin, a drug belonging to the HMG-CoA reductase inhibitors, were undertaken. Human antigen-specific T cell lines, characterized with regards to T helper differentiation and peptide specificity, were employed to prove the hypothesis that these two molecules influence T cell signaling. To dissect the underlying mechanisms involved in the TRAIL-mediated and atorvastatin-mediated immunomodulation of antigen-specific T cell response, the interference of both molecules on T cell activation and cell cycle regulation was investigated. For this, calcium influx and the expression of cell cycle regulators, respectively, were monitored following incubation with either molecule. To determine whether the underlying mechanisms involved in the atorvastatin-induced growth inhibition were mediated via an inhibition of the HMG-CoA reductase pathway, an intermediate product of this pathway, mevalonate, was coincubated with atorvastatin.

2 MATERIALS

Cell Culturing

Media: **AB Medium** (ABM). RPMI supplemented with 10mM HEPES buffer (Gibco Invitrogen Corp., Paisley, Scotland), 2mM L-glutamine (Gibco Invitrogen Corp.), 100U/ml penicillin and 100µg/ml streptomycin (Biochrom AG, Berlin, Germany), 10µg/ml ciprofloxacin (Ciprobay® 100, Bayer, Germany) and 5% pooled human AB serum (obtained from clotted blood from normal human AB donors, Institute of Transfusion Medicine, Charite, Berlin).

FBS Medium (FBM). RPMI supplemented with 10mM HEPES buffer, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% heat-inactivated FBS serum (Gibco Invitrogen Corp.)

Freezing Medium (FM). RPMI supplemented with 10mM HEPES buffer (Gibco Invitrogen Corp.), 2mM L-glutamine (Gibco Invitrogen Corp.), 20% heat-inactivated FBS serum (Gibco Invitrogen Corp.) and freshly added ice-cold 10% DMSO (99.9%; Sigma, Steinheim, Germany).

All media stored at 2-8°C

Density gradient: **Lymphoprep™**: diatrizoate and polysaccharide (Nycomed Pharma AS, Oslo, Norway), stored at RT

Dye exclusion: **0.4% Trypan Blue**: prepared in 0.81% NaCl and 0.06% KPO4, dibasic (Sigma Cell Culture, Irvine, UK), stored at RT

IL-2: recombinant human IL-2 **Teceleukin**, Tecin™ (Hoffmann-La Roche Inc., Nutley, NJ, USA) provided by Dr. C.W. Reynolds, National Cancer Institute, Frederick Cancer Research and Development Center, MD, USA, stored for short-term at 2-8°C, otherwise aliquoted at -20°C

[methyl-^{3}H]thymidine: specific activity 185GBq/mmol, 5.0Ci/mmol; 777MBq/mg, 21mCi/mg (stock: 37MBq/ml, 1.0mCi/ml) (Amersham, Braunschweig, Germany), stored at 2-8°C

Antigens: **Myelin Basic Protein** (MBP) from autopsied brains, supplied from Dept. of Clinical Cell- and Neurobiology, Institute of Anatomy, Charite, Berlin; **Birch pollen** (Bet) Betula verrucosa (Allergon, Pharmacia & Upjohn, Sweden); **Tetanus toxoid** (TT): vaccine concentrate (Chiron Behring, Marburg, Germany), all stored at -20°C

Stimuli/Costimuli: **PHA** lectin from *Phaseolus vulgaris* (Sigma, Steinheim, Germany); **PMA** tumor promoter and activator of PKC (Sigma); **ionomycin** calcium ionophore (Sigma); goat anti-SF21-derived recombinant human soluble CD28 (rhsCD28) purified (R&D Systems, MN, USA), all stored at -20°C. **OKT3** (Orthoclone®, Ortho Biotech, Janssen-Cilag, Neuss, Germany), stored at 2-8°C

Flow cytometric and fluorometric measurements

Antibodies: Intracellular staining: FITC-conjugated mouse anti-human interferon **IFN-γ-FITC IgG_1** (PharMingen, Heidelberg, Germany) and **FITC-labeled mouse IgG_1 isotype** control (Sigma). PE-conjugated mouse anti-human **IL-4-PE IgG_1** (PharMingen) and **PE-labeled mouse IgG_1 isotype control** (Becton Dickinson, Heidelberg, Germany), all used at conc of 2µg/ml and stored at 2-8°C

Buffers: 1x **FACS wash buffer**: 0.5% BSA, 0.05% NaN_3 in PBS

1x **FACS permeabilization buffer**: 0.1% saponin, 0.5% BSA, 0.1% NaN_3 in PBS

20x **DNA fragmentation buffer**: 0.1% sodium citrate, 0.1% Triton X-100 in H_20)

1x **Annexin binding buffer**: 10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$

1x **Caspase 3 lysis buffer**: 60mM NaCl, 5mM Tris-HCl, 2.5mM EDTA, 0.25% NP40

all stored at 2-8°C

Intracellular calcium measurement

Fluorescent probes: acetoxymethyl (AM) esters of ratiometric calcium indicators: **FURA-2AM,** $C_{44}H_{47}N_3O_{24}$ (Molecular Probes, Eugene, OR, USA) and **INDO-1/AM,** $C_{47}H_{51}N_3O_{22}$ (Molecular Probes), both stored at -20°C

Reagents: **Thapsigargin** endoplasmic reticular Ca^{2+}-ATPase inhibitor $C_{34}H_{50}O_{12}$ (Calbiochem, San Diego, CA, USA), **EGTA** calcium chelator (Sigma, Steinheim, Germany), **$CaCl_2$** (Sigma), all stored at -20°C

Western Blotting

Protein extraction: 1x **Lysis buffer** (0.15M NaCl, 0.01M Tris-HCl, 0.005M EDTA, 1% Triton X-100), stored at 2-8°C, freshly added with **protease inhibitors**: 2µg/ml aprotinin (Sigma, Steinheim, Germany), 100µg/ml PMSF (Sigma) and 200µM sodium orthovanadate (Sigma), all stored at -20°C

Protein quantification: **BCA Protein Assay Kit** (Pierce, Illinois, USA) contains: BCA Reagent A (Na_2CO_3, $NaHCO_3$, BCA detection reagent, sodium tartrate in 0.1N NaOH), BCA Reagent B (4% $CuSO_4$•$5H_2O$) and BSA concentrate (2mg/ml in a 0.9% aqueous NaCl solution containing NaN_3) for standard curve, stored at 2-8°C

Electrophoresis: 2x **Loading buffer** (50mM Tris-HCl (pH 6.8), 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and full-range **Rainbow molecular weight marker** recombinant protein (Amersham LifeScience, Uppsala, Sweden), both stored at -20°C. 10x **Electrophoresis buffer** (0.25M Tris pH 8.3, 1.92M glycine, 1% SDS), stored at RT. **30% Acrylamide/Bis Solution 29:1 ratio** (Bio-

Rad, California, USA)**;** TEMED (GibcoBRL, Karlsruhe, Germany); APS (GibcoBRL); SDS (Sigma, Steinheim, Germany), stored at 2-8°C

Blotting/Blocking: S&S Protran® BA **nitrocellulose transfer membranes** pore size 0.45µm (Schleicher & Schuell, Dassel/Relliehausen, Germany), stored at RT

1x **Blot Buffer** (2.5mM TrisOH, 11mM glycine, 20% methanol), freshly prepared

1x **Blocking buffer** (0.01M Tris-HCl (pH 7.5), 0.15M NaCl, 0.1% Tween 20, 5% skimmed milk powder, 2% BSA, 0.1% NaN_3), stored at -20°C

1x **Washing buffer** (0.05% Tween 20 in PBS), stored at RT

Antibodies: **Primary antibodies**: monoclonal mouse anti-Bcl-X recognizing **Bcl-X_L** (long) protein (BD PharMingen, California, USA) used at 5µg/ml, monoclonal mouse anti-**Bcl-2** (DAKO, California, USA) at 3.6µg/ml, polyclonal rabbit anti-**Bax** (DAKO) at 13µg/ml, monoclonal mouse anti-**$p27^{Kip1}$** (Santa Cruz, California, USA) at 2µg/ml, polyclonal rabbit anti-**CDK4** (Santa Cruz) at 1µg/ml and monoclonal anti-**β-actin** (Sigma-Aldrich, Steinheim, Germany) at 24ng/ml, stored at 2-8°C

Secondary antibodies: anti-mouse, rabbit or goat coupled to horse radish peroxidase (DAKO, California, USA), stored at 2-8°C

Protein Detection: **Hyperfilm ECL** double-coated detection film (Amersham Life Science, Uppsala, Sweden), **ECL Plus™ chemiluminescent** detection reagents (Amersham LifeScience) contains acridinium ester intermediates react with peroxide under slight alkaline conditions to produce a chemiluminescence with max emission at λ 430nm, both stored at 2-8°C

Pharmacological Reagents

TRAIL: human recombinant form of soluble TRAIL employed together with an enhancer antibody for multimerization (Alexis Corporation, Lausen, Switzerland) and **rhTRAIL-R2:Fc fusion protein**: human recombinant protein consisting of the extracellular domain of TRAIL receptor 2 (DR5) fused to the Fc portion of human IgG_1 (Alexis Corporation), both stored at -20°C

Atorvastatin: atorvastatin calcium powder (supplied by Pfizer GmbH, Karlsruhe, Germany) insoluble in aqueous solutions of pH 4 and below, slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile, slightly soluble in ethanol, and freely soluble in methanol), stored at 2-8°C

Mevalonate: 1N NaOH-activated L-mevalonic acid lactone (Sigma, Steinheim, Germany) was neutralized with 1N HCl to pH 7.2, diluted with distilled water, and filter-sterilized, stored at -20°C

3 METHODS

3.1 CELL CULTURE

Cell preparation and handling was always performed under a laminar flow hood, HERASafe HS15 or HSP12 (Kendro Laboratory Products, Hanau) and carried out under strict aseptic conditions. All cultures and assays were done in culture medium (CM) such as ABM or FBM at 37°C in a 5% CO_2 atmosphere and 95% humidity.

3.1.1 DENSITY GRADIENT CENTRIFUGATION

Mononuclear cells were isolated from whole blood using density gradient centrifugation according to the method by Boyum (Boyum, 1968). The **principle** of this method is based on the fact that different cell types differ in their density. Density gradients are used to separate mononuclear cells (low density) from erythrocytes/granulocytes (high density) and to separate live cells (low density) from dead ones (high density).

Blood was withdrawn from both healthy controls as well as patients suffering from multiple sclerosis using EDTA Monovette® blood tubes and infusion set. After diluting with PBS in a ratio of blood:PBS of 3:2, the blood suspension was carefully layered on top of Lymphoprep™, an iso-osmotic density barrier (ρ = 1.078g/ml at 20°C). Importantly, the density gradient and blood had the same temperature, room temperature (RT). Lymphoprep™ contains the impermeant ion diatrizoate and a polysaccharide, which causes the erythrocytes to aggregate. Following centrifugation at 700g, at RT for 40min, living mononuclear cells and thrombocytes form a layer on the surface of the gradient (as a misty white interface) whereas granulocytes and erythrocytes sediment lower down in the tube. The interface was carefully collected with a pipette, transferred to another tube and centrifuged for 15min at 500g at RT. Cells were washed twice (once with PBS, once with medium) with centrifugation steps of 10min 250g RT, counted using Trypan blue exclusion staining (see Cell Viability Assay below) and resuspended in either culture or freezing medium, depending on whether cells were to be placed in culture or frozen at -80°C for 24h for subsequent storage in liquid nitrogen.

3.1.2 CELL VIABILITY ASSAY

The viability of cells was determined by the dye exclusion test. This test is based on the **principle** that certain dyes such as propidium iodide and trypan blue are not able to pass the intact membranes. Therefore, one can distinguish between viable cells and dead cells:

living cells remain unstained whereas dead cells, permeable to these dyes, are eventually stained.

Cell suspension was mixed 1:1 with Trypan blue. An aliquot of this mixture (c. 10µl) was transferred to an improved Neubauer-hemacytometer covered with a coverslip. Unstained (viable) cells were counted using a binocular microscope. The total number of viable cells was calculated as follows: Total living cells = n x df x V x 10^4 (n is the no. of cells counted in one field of the hemacytometer, df is 2 the dilution factor, V is the volume in which cells are suspended)

3.1.3 GENERATION AND MAINTENANCE OF ANTIGEN-SPECIFIC TCLs

Human antigen-specific $CD4^+$ T cell lines (TCLs) specific for myelin basic protein (MBP), tetanus toxoid (TT), or birch pollen (Bet) were generated, as previously described (Zipp et al., 1997), from both healthy individuals and from patients suffering from multiple sclerosis. MBP was isolated from human brain (see Methods 3.5.1 Protein Purification), birch pollen was purified from a crude extract of Betula verrucosa (Allergon, Pharmacia & Upjohn, Sweden) and tetanus toxoid was purchased as a vaccine concentrate (Chiron Behring, Marburg, Germany).

3.1.3.1 PRIMARY CULTURES AND SPLIT-WELL TECHNIQUE

Antigen-specific $CD4^+$ TCLs were established using a modified "split-well" protocol (Fig. 4). Two hundred thousand peripheral blood mononuclear cells (PBMC) in 200µl AB medium (Materials) in the presence of 20µg/ml myelin basic protein (MBP), 8µg/ml birch pollen extract (Bet) or 4µg/ml tetanus toxoid (TT) were seeded in 96-well round bottom microtiter plates. After 7 days, 20IU/ml recombinant human interleukin-2 (IL-2) (Tecin™, teceleukin, Hoffmann-La Roche Inc., Nutley, NJ) were added to the cultures. Seven days thereafter, 100µL of the 14 day primary cultures were taken from each well of the original master plate and split into 2 wells on a new split plate preseeded with 50µl antigen presenting cells (3000rad irradiated autologous 1 x 10^5 PBMC) in the presence or absence of antigen. The split plates served for the analysis of antigen specificity by a proliferation assay (see below). In parallel, the rest of the 14-day primary cultures (100µl) from the wells of the original master plate were added to a new daughter plate preseeded with 100µl antigen presenting cells preincubated with antigen (as for split plates). Proliferation plates consisting of T cells with and without relevant antigen were incubated for 72h following antigen stimulation. Wells in the daughter plate that showed stimulation index of > 2 (indication of antigen

specificity) in the corresponding wells of split plates used for the proliferation assay were cultured further and expanded.

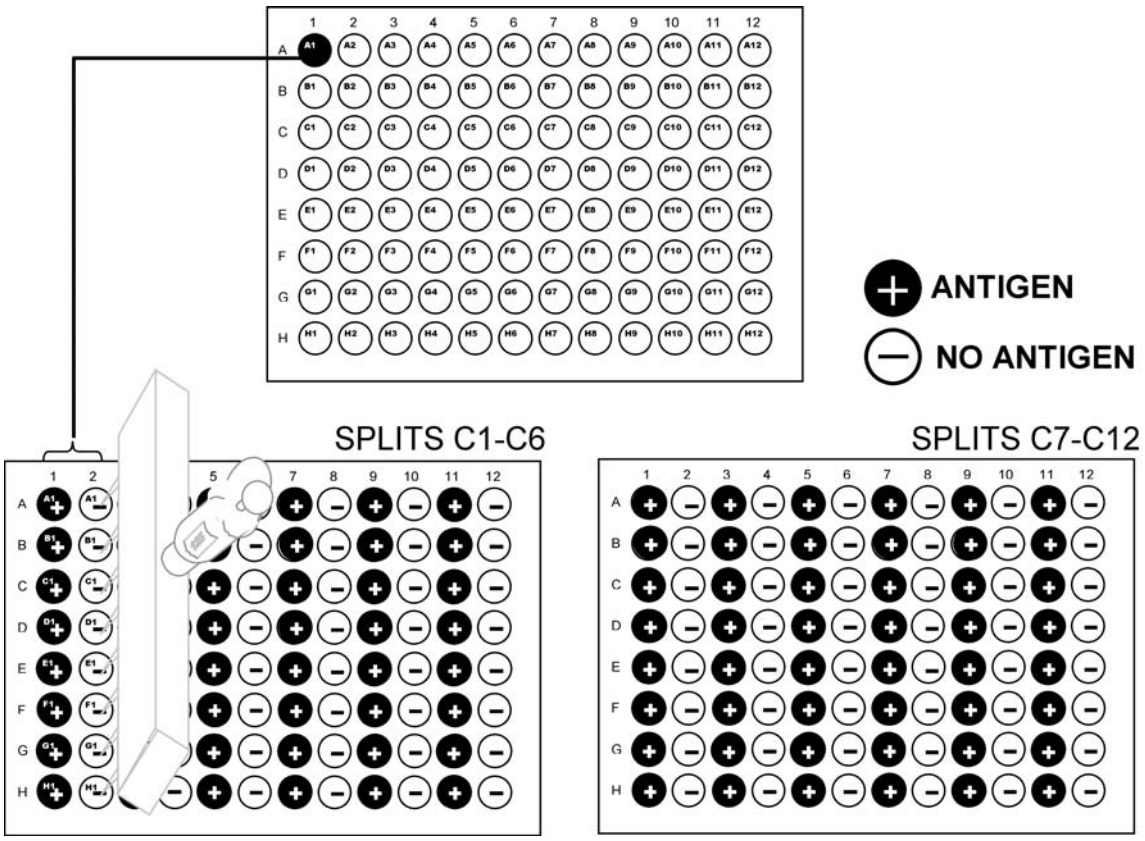

Figure 4 **Split-well approach**
A master plate contains 14-day primary cultures with specific antigen in 200µl AB medium per well. From each original well 100µL were taken and split into 2 wells on a new split plate preseeded with 50µl antigen presenting cells (irradiated autologous PBMC) in the presence or absence of antigen. The split plates served for proliferation assays. In parallel, 100µl antigen presenting cells preincubated with antigen were added to each well of the master plate. Wells that show stimulation index of > 2 in the proliferation assay were further cultured and expanded.

3.1.3.2 PROLIFERATION ASSAYS

Specificity of the different antigen-specific T cell lines was tested by a standard proliferation assay via ^{3}H-thymidine incorporation. Proliferation plates consisting of T cells with and without relevant antigen were incubated for 72h following antigen stimulation. ^{3}H-thymidine (Amersham, Braunschweig, Germany) was then added at a dose of 0.5µCi to each well. After 18h, incorporation of radioactivity was measured in counts per minutes (cpm) with a Microbeta β counter (Wallac ADL, Freiburg, Germany). In some cases, results of the ^{3}H-thymidine uptake are expressed as stimulation index (SI). SI = cpm obtained from stimulated wells/cpm obtained from control unstimulated wells.

3.2 FLOW CYTOMETRIC TECHNIQUES

Flow cytometry has been used to identify cell populations expressing a given antigen, to measure the production of intracellular molecules, in particular cytokines, and to identify different DNA populations. Antigens or cytokines were stained by antibodies coupled to a specific fluorescent dye and analyzed using a FACSCalibur® flow cytometer (Becton Dickinson and Co., Mountain View, CA) equipped with CELLQuest™ software (BD Biosciences).

The **principle** of flow cytometry is based on light scatter and fluorescence to analyze particles or cells in suspension while they flow in a fluid stream one by one through a laser ray (488nm, 200mW). The scattered and fluorescent light produced by cells passing through the illuminated capillary is collected by a system of lenses, mirrors, filters and photodetectors that convert the photon pulses into electronic signals. Further electronic and computational processing results in a graphic display and statistical analysis of the measured parameters. This technology provides quantitative, multiple analysis on single cells. The FACSCalibur is equipped with a 480nm Argon Laser and a 630nm Diode Laser and is able to measure and analyze up to six different parameters:

P1 is Forward scatter (FSC) and is proportional to the cell size.

P2 is Sideward scatter (SSC) and is proportional to the cell granularity.

P3 is Fluorescence 1 and is usually proportional to dye intensity of fluorescein isothiocynate (FITC) - absorption maximum at 492nm and an emission maximum at 520-530nm.

P4 is Fluorescence 2 and is usually proportional to dye intensity of phycoerythrin (PE) - absorption maximum at 488nm and an emission maximum at 570-576nm.

P5 is Fluorescence 3 and is usually proportional to dye intensity of propidium iodide (PI) absorption max. 495nm and emission max. 639nm and PerCP (peridinin chlorophyll A protein).

P6 is Fluorescence 4 and is usually proportional to dye intensity of Cy5 and allophycocyanine, absorption max. 625-650nm and emission max. 660-670nm.

3.2.1 STAINING OF SURFACE ANTIGENS

Sample preparation and staining of antigen were performed in blocking wash buffer (Materials). Cells (10^5-10^6) were harvested, washed in a centrifugation step at 300g and resuspended in the appropriate volume of buffer. To avoid unspecific binding of antibodies to low affinity Fc receptors (expressed on many cell types), cells were incubated before staining in FACS wash buffer with 10% pooled human serum (IVIg) for 10min at 4°C. Cells were then washed once and incubated with antibody. Antibody solutions were prepared

separately in FACS wash buffer, at a final concentration ranging between 0.5 and 7.5µg/ml, 50µl of this master solution was then added to each sample, in order to ensure that each sample received the same concentration of a given antibody.
After incubation with the primary antibody coupled to fluorescent dye, cells were washed twice and pellet was resuspended in 500µl FACS wash buffer. Surface expression was then analysed with a FACSCalibur. Data were analyzed using the program CELLQuest.

3.2.2 STAINING OF INTRACELLULAR CYTOKINES

T cells were stimulated in vitro using the polyclonal activators phorbol ester (PMA) and ionomycin. Before stimulation, cells were harvested, washed twice, counted and resuspended at $2x10^6$ cells/ml. PMA and ionomycin were added to the culture at 100ng/ml and 1µg/ml final concentrations, respectively. Cells were then incubated with the activators for 5-6h. In order to block intracellular transport processes, 5µg/ml Brefeldin A was added to the culture for the last 2h of incubation. Addition of this protein transport inhibitor during cell activation, assures accumulation of the specific cytokines within the cell.
Following incubation, cells were harvested, washed once with PBS and fixed by 20min incubation with 2% paraformaldehyde at a concentration of $1\text{-}2x10^6$ cells/ml. Fixation of activated cells allows manipulation of the cell membrane without destroying its structure. Fixed cells can be stored at 4°C for weeks before performing the intracellular staining.
After fixation, cells were washed twice with FACS permeabilization buffer (Materials). Cell membranes were permeabilized with the detergent saponin (Sigma) to facilitate the passage of antibodies through the membrane and staining of intracellular molecules. Permeabilized cells were stained for intracytoplasmatic interferon (IFN)-γ and IL-4 by incubating with FITC-conjugated mouse anti-human IFN-γ and PE-conjugated mouse anti-human IL-4 and FITC-/PE-labeled rat IgG1 isotype control antibodies diluted in 0.1% saponin permeabilization solution. After 30min in the dark and at 4°C, cells were washed twice with FACS permeabilization buffer and finally in FACS wash buffer (Materials) to allow membrane closure. Analysis was performed with the FACSCalibur and 1×10^4 events were acquired.

3.2.3 DNA FRAGMENTATION ASSAY

Degree of DNA fragmentation as a measure of late apoptotic cell death was analyzed as previously described by staining DNA with propidium iodide (Nicoletti et al., 1991). The **principle** of this method is based on the observation that fragmented DNA from apoptotic cells shows diminished propidium iodide (PI) staining than G_0/G_1 population of normal

diploid cells, identified as a distinct hypo-diploid cell population in flow cytometric histograms.

After 24h incubation in 96-well U-bottomed plates with apoptotic stimulus, $2x10^5$ cells were lyzed and nuclei stained by incubating with a hypotonic fluorochrome solution (50µg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100) for 3h at 4°C. The extent of apoptotic nuclei undergoing DNA fragmentation was analyzed by measuring the magnitude of the hypodiploid DNA peak with the FACSCalibur. Results were expressed as percentage of apoptotic populations from total events and DNA fragmentation indexes calculated by dividing the percentage of hypodiploid nuclei from cells incubated with apoptotic stimulus by the percentage of hypodiploid nuclei obtained from controls (without stimulus).

3.2.4 STAINING OF APOPTOTIC CELLS: ANNEXIN V ASSAY

Annexin V–FITC was used to quantitatively determine the percentage of cells undergoing apoptosis as previously described (Vermes et al., 1995). The **principle** of this method relies on the property of cells to lose membrane asymmetry during the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a 35 kDa Ca^{2+}-dependent phospholipid-binding protein that binds to PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas as the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V–FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V–FITC and PI either are in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V–FITC and PI are alive and not undergoing measurable apoptosis. After incubation with apoptotic stimulus in 96-well flat-bottomed plates, cells were washed twice with PBS and stained with Annexin-V for 15min at RT in the dark. Following this incubation 100µl binding buffer was added and transferred to FACS tubes. PI (0.5µg/ml) was added directly before measurement with the FACSCalibur and 1 x 10^4 events were acquired.

3.3 CASPASE 3 ACTIVITY ASSAY

For the analysis of aspargine-glutamine-valine-asparagine-7-amido-4-methylcumarine (DEVD)-amc-cleaving caspase activity, a previously described protocol (Wendling et al., 2000) was used. Briefly, 10^5 polyclonal T cells were plated in 96-well flat bottom microtitre plates, with or without second stimulus. Six hours following induction of apoptosis, T cells

were incubated for 10min in lysis buffer and cytosolic extracts were thereafter incubated for 30min with 20μM of the fluorogenic substrate Ac-DEVD-AMC (Bachem, Heidelberg, Germany). The level of fluorescence measured at 360nm excitation and 480nm emission wavelengths using a CytoFluor 2400 cytofluorimeter (Millipore Corp., Eschborn, Germany) is an indication of caspase 3-like activity. Results are expressed as fluorescence indexes calculated by dividing values obtained from wells treated with second stimulus by values from control wells (untreated with second stimulus).

3.4 INTRACELLULAR CALCIUM MEASUREMENTS

Antigenic stimulation of T cells triggers intracellular calcium release and the consequent opening of calcium release-activated calcium channels (CRAC) that in turn generate the prolonged elevation of intracellular calcium ($[Ca^{2+}]_i$) required for T cell activation (Zweifach and Lewis, 1993). Two methods have been employed to measure $[Ca^{2+}]_i$, namely a spectro-fluorometric method and a flow cytometric method. Since thapsigargin (Tg) has been shown to activate the same CRAC as TCR-stimulation by antigen (Aussel et al., 1996), a Tg model of calcium influx to bypass TCR signals upstream of the endoplasmic calcium store was used for direct monitoring of the influence of external agents on CRAC. Tg blocks the Ca-ATPase pumps of calcium stores and this model was used in both methods of $[Ca^{2+}]_i$ measurements.

Cells, 5 x 10^6/ml, were loaded with either 2μM of the acetoxymethyl ester precursor of FURA-2, FURA-2/AM (for fluorescence spectrophotometer method) or 5μM Indo-1/AM (for flow cytometry method) for 30min at 37°C in a shaking water bath. Unloaded dye was removed by centrifugation, and cells were resuspended in calcium-free PBS. During measurements, cells loaded with either ratiometric fluorescence dyes were incubated with 0.5mM EGTA for 5 minutes to bind any extracellular calcium. After incubation, 0.25μM Tg was added to block the calcium from going in against a concentration gradient and therefore allowing calcium to leak out by diffusion. Once all the calcium had leaked out of the intracellular stores and CRAC channels were activated, 1.2mM calcium was added to the cell suspension.

3.4.1 FLUORESCENCE SPECTROPHOTOMETRY

A Hitachi F4500 fluorescence spectrophotometer coupled to a PC with F-4500 Intracellular Cation Measurement System© software was used. The spectrophotometer is equipped with 2 excitation monochromators and a dual mirror chopping mechanism to permit rapid alternating (30Hz) excitation of FURA-2/AM at 2 wavelengths (340nm and 380nm). The

instrument's parameters were set at 0.1-second response level, 700V PM voltage, 10nm ex bandpass, 10nm em bandpass and 300s scan time. The processing parameters were set according to Grynkiewicz's formula and the apparent dissociation constant (K_D) set at 224nm. Measurements were carried out in the dark. 1mL of cell suspension was incubated with EGTA or calcium (depending on the type of experiment) for 5min within siliconised cuvettes (cuvette compartment is equipped with continuous stirring). After incubation, selecting 'Run Sample' from the 'Next Sample' window initialized the fluorescence trace. The 'Add Marker' was used to record the time point at which external agents were administered to the cell suspension. The fluorescence trace for the 2 wavelengths was followed on the monitor. Measurements were terminated by addition of 20µL 10% Triton X-100 to lyse cells (maximal fluorescence) and 20µL $MnCl_2$ to quench all fluorescence (minimal fluorescence). The results obtained (intracellular calcium concentration expressed in nM) were calculated using the standard equation: $[Ca^{2+}]_i = K_D * (R - R_{min})/(R_{max} - R)$ as described (Grynkiewicz et al., 1985) using 224nm as K_D for Ca^{2+} and FURA-2.

3.4.2 FLOW CYTOMETRY

The flow cytometric method used was adapted from a method from Griffioen et al. (Griffioen et al., 1989). The analyses were performed on a flow-activated cell sorter (BD FACS LSR, Becton Dickinson) with up to six fluorescence channels, fitted with three lasers (Ar^+ 488nm, HeNe 630nm, HeCd 325nm) and designed for Ca^{2+} flux measurements. The FL4 (510/20) and FL5 (380LP) fluorescence channels were used to measure free Indo-1 and the complex Ca^{2+}-Indo-1 concentrations, respectively. Calculation of the ratio of these 2 fluorescence wavelengths allows the evaluation of changes in cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$ independently of the cell size and the intracellular Indo-1 concentration. The flow rate was set to 300 events/s and the mean ratio of 2000 cells were noted every 20s. Typical measurements involved 10^6 cells in 1ml of calcium-free buffer. Approximately 80s after measurement was acquired (baseline), 5µM thapsigargin (Tg) (Calbiochem) was added to fully deplete intracellular Ca^{2+} stores. Following a 5min-incubation with Tg, 1.2mM calcium was added to the cell suspension to monitor the extent of Ca^{2+} influx.

3.5 PROTEIN PURIFICATION

Some of the antigens utilized to establish human antigen-specific T cell lines were prepared in the lab. These included myelin basic protein (MBP) and birch pollen (Bet). MBP was isolated from human brain and birch pollen was purified from a crude extract of Betula verrucosa (Allergon, Pharmacia & Upjohn, Sweden)

3.5.1 EXTRACTION OF MBP FROM HUMAN BRAIN

Human myelin basic protein (MBP) was purified as described in established protocols (Eylar et al., 1974). Briefly, brain sections obtained from autopsies were homogenized with ice-cold methanol to a turbid suspension and extracted with ice-cold chloroform. Using a separating funnel, the lower clear lipid-phase was discarded, whereas the upper dirty water-phase was preserved and washed with acetone using a Büchner porcelain funnel fitted with a Whatman 41 filter paper. The dried brain mass was resuspended in water, adjusted to pH 2 and the suspension was left to extract overnight at 4°C. On the following day, the extract was ultracentrifuged at 5000g for 30min at 4°C and the supernatant was adjusted to pH 5.5 and mixed for 1h at 4°C for protein precipitation. Following a further centrifugation step, crude MBP was obtained from the supernatant. For precipitation of MBP, 50% saturated ammonium sulphate was added to the supernatant, mixed for 20min at room temperature, adjusted to pH 6 and left stirring overnight at 4°C. On the next day, solution was centrifuged at 500g for 30min at 4°C and pellet was carefully resuspended in 10% acidified acetone. For protein coagulation, solution was left standing for a maximum of 1h and then centrifuged at 585g for 20min at 4°C without lid. The pellet, containing MBP, was resuspended in water and dialyzed against water overnight at 4°C. The dialyzed solution was then centrifuged at 585g for 20min at 4°C and the supernatant was frozen as a thin-layered coat at the base of a large round-bottomed flask for lyophilization. The lyophilized powder was eventually reconstituted in PBS, sterile filtered through a 0.22µm filter and protein was quantified using the BCA assay (3.6.1). Final step protein was run on an SDS-polyacrylamide gel and stained with Coomasie Blue to confirm purity of MBP.

3.5.2 EXTRACTION AND PURIFICATION OF BIRCH POLLEN (BETV1)

Birch pollen was purified from a crude extract of Betula verrucosa (Allergon, Pharmacia & Upjohn, Sweden) according to established protocols (Wiedermann et al., 1998). Allergen extracts were prepared using phosphate-buffered saline (PBS, pH 7.8) as extraction medium. After stirring the pollen overnight in PBS (c. 100mg/ml) at 4°C, the medium was centrifuged at 4000g for 60min at 4°C. The supernatant was collected, filtered (cellulose

acetate, 0.45µm) and dialyzed (Membra-Cel™ Dialysis Membranes, MWCO 7000; Ø 22mm, Serva) against PBS for 24h. The protein content of the allergen extracts was determined using the BCA assay (3.6.1). Final step protein was run on an SDS-polyacrylamide gel and stained with Coomasie Blue to confirm purity of birch pollen.

3.6 PROTEIN DETECTION BY WESTERN BLOTTING

Western blotting, a conservative semi-quantitative method for the detection of protein is useful for the identification and quantification of specific proteins in complex mixtures of protein. However, it often requires a subjective interpretation of results. For this reason, representative blots have also been included in the Results section. The **principle** of this method is based on a three-step approach for identifying protein: size resolution by gel electrophoresis, transfer of separated proteins to a membrane, and specific identification by labeled antibodies.

3.6.1 PREPARATION OF TOTAL PROTEIN FROM CELL LYSATE

Cell pellets (minimum of $3x10^6$ cells) were resuspended in lysis buffer (Materials) with a Hamilton syringe and incubated for a minimum of 15min on ice with occassional vortexing in between. Cell lysates were centrifuged for 15min at 4°C in a Eppindorf microfuge at 6000g. The supernatant was carefully aspirated and transferred to a new microfuge tube and protein amounts were determined by the bicinchoninic acid (BCA) method (Pierce, Illinois, USA).

Using the BSA concentrate (Materials) different dilutions for a standard curve was prepared (62.5, 125, 250, 500 and 1000µg/ml BSA). Once protein samples and standards were pipetted in a 96-well U-bottom plate, a working BCA reagent solution was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B (Materials) and 150µl was added to all samples and standards. Plates were incubated for 30min at 37°C and after cooling read with a spectrophotometer (MRX® microplate reader, Dynex Technologies) at 562nm and measured with Revelation™ (Dynex Technologies) program.

3.6.2 SDS-PAGE AND IMMUNOBLOTTING

For SDS-polyacrylamide gel electrophoresis (PAGE), 10-15% polyacrylamide resolving gels were prepared (1 part TEMED, 25 parts freshly prepared 10% ammonium persulphate (APS), 25 parts 10% SDS, 625 parts 1.5MTris (pH 8.8) and 1250 parts 30% acrylamide mix

(Materials) and 575 parts water) and 5% polyacrylamide stacking gels layered on top of them. Quantified protein samples were denatured by boiling, cooled on ice and resolved on the precast gels. Electrophoretic chambers were attached to an electric power supply and a voltage of 8 to 15 V/cm was applied to the gel. Transfer of protein from gel to blots was done using the wet method. Nitrocellulose membranes, stacking filter paper and resolved gel were soaked in transfer buffer and stacked on top of each other such that membrane, gel and filters were aligned exactly on top of each other (air bubbles squeezed out) with gel on cathode side and the electrical leads connected to a power supply. A current of 0.65mA/cm^2 gel was applied for a period of 1½-2 hours. After blotting, membranes were blocked overnight at 4°C or 2h at RT in blocking buffer (Materials). Membranes were incubated with specific monoclonal primary antibodies (Materials) for 1h at room temperature or overnight at 4°C depending on the specific antibody. In most cases, antibodies were diluted in a 1:40 dilution of the blocking buffer to prevent unspecific binding. Following a series of washing steps, membranes were incubated for <1h with 1.25µg/ml secondary antibody coupled to horse radish peroxidase (Materials). Specific bands were detected using the ECL-plus system (Materials). The membranes were exposed to film in the dark for a time period, ranging from a few seconds to 1h, depending on the antibodies used and developed using a Kodak X-OMAT film-developing machine. Bands were densitometrically quantified using TINA Version 2.09g. All immunoblots were sequentially incubated with anti-β-actin as control, and specific signals adjusted in relation to the expression of this housekeeping gene. Blotting and exposure times were kept constant throughout for each molecule under investigation. However, in the case of semi-quantitative purposes such as in the Bcl-2 study on MS patients, both patient and healthy control samples were loaded and transferred on each immunoblot in order to compensate for minor gel-to-gel variations.

3.7 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Using peqGOLD purification kit (peqLab, Erlangen, Germany), total RNA was isolated from PBMC pellets stored at -80°C. Any contaminating genomic DNA was removed by Dnase I digestion (Boehringer Mannheim, Mannheim, Germany). cDNA was synthesized from 1µg of RNA, using a first-strand cDNA synthesis kit (Pharmacia Biotech, Freiburg, Germany). The amplification profiles for the primer pairs were as follows: Bcl-X_L 30 cycles, 45s/95°C, 45s/65°C, 60s/72°C, primer sequences ACAAGGAGATGCAGGTATTGGT (nucleotides 601-622) and GAGTGGATGGTCAGTGTCTGGT (nucleotides 836-857), glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (G3PDH) 26 cycles, 45s/95°C, 45s/54°C,

60s/72°C, primer sequences GTCAACGGATTTGGTCGTATT (nucleotides 82-102) and AGTCTTCTGGGTGGCAGTGAT (nucleotides 601-621). The PCR fragments were separated with 2% agarose gels and visualized by ethidium bromide. For all genes, PCR protocols were standardized such that the cycle number ensured that PCR amplification was in its exponential phase (Schmidt et al., 1998). A water control was included in each amplification reaction to rule out the possibility of cross-contamination between reaction tubes. For quantification of Bcl-X_L, optical density (OD) was measured with BioDocII (Biometra, Göttingen, Germany) documentation system. Specific signals were adjusted in relation to the expression of the housekeeping gene G3PDH, and expressed as arbitrary OD index.

3.8 STATISTICS

Statistical analyses were performed with SPSS 10.0 software for Windows (SPSS, Chicago, USA). Data is typically represented as mean ± SEM (standard error of mean). For group comparisons, the non-parametric Mann-Whitney U test was used. Degree of correlation was analyzed with the non-parametric Spearman Bivariate Correlation Coefficient test. p values < 0.05 were regarded significant.

4 RESULTS

4.1 ANALYSIS OF THE BCL-2 FAMILY MEMBERS

The expression of the apoptosis-regulating Bcl-2 family members (Bcl-2, Bcl-X_L and Bax) was analyzed in MS patients and compared to healthy individuals. An increased protein expression of anti-apoptotic Bcl-X_L (Fig. 5), but no alteration in anti-apoptotic Bcl-2 or pro-apoptotic Bax (Fig. 7), was observed in patients.

Twenty-three patients (13 females, 10 males) with clinically definite MS (Poser et al., 1983) and 29 healthy individuals (15 females, 14 males) were included. Approval from the local ethics committee and informed consent from each patient was obtained for this study. Fourteen MS patients participating in this study presented with a relapsing-remitting course of the disease (RRMS), 4 patients were diagnosed as secondary chronic progressive (SPMS) and 5 patients presented with primary chronic progressive MS (PPMS) (Table 1).

Table 1 **Clinical features of MS patient groups**

Clinical group	Total no. (females)	Age (years)
Multiple sclerosis	23 (13)	40.0 ± 11.8
RRMS[a]	14 (10)	33.8 ± 8.2
PPMS[a]	5 (1)	53.8 ± 5.6
SPMS[a]	4 (2)	42.8 ± 11.3
Healthy controls	29 (15)	33.6 ± 12.2

[a] RR = relapsing-remitting; PP = primary progressive; SP = secondary progressive.

[b] Values expressed as mean (SD)

Sixteen out of the 23 patients did not receive any immunomodulatory treatment; five patients were on interferon-beta (IFN-β) and two patients on other therapies, namely mitoxantrone and azathioprine (Table 2).

4.1.1 Bcl-X_L but not Bcl-2 or Bax protein upregulation in MS

Bcl-2, Bcl-X_L and Bax protein expression was investigated in resting peripheral mononuclear cells of patients with MS and healthy controls using western blotting. The immunoblots were sequentially incubated with β-actin as control.

Bcl-X_L bands, detected at 26 kDa, were more intense in MS patients than healthy controls (Fig. 5A). Bcl-X_L levels were calculated in relation to β-actin, in three independent

experiments. A significant difference in the expression of Bcl-X_L was observed between the 23 MS patients and 29 healthy controls (p=0.014) but no correlation was observed between the levels of Bcl-X_L and the type of MS (Fig. 5B).

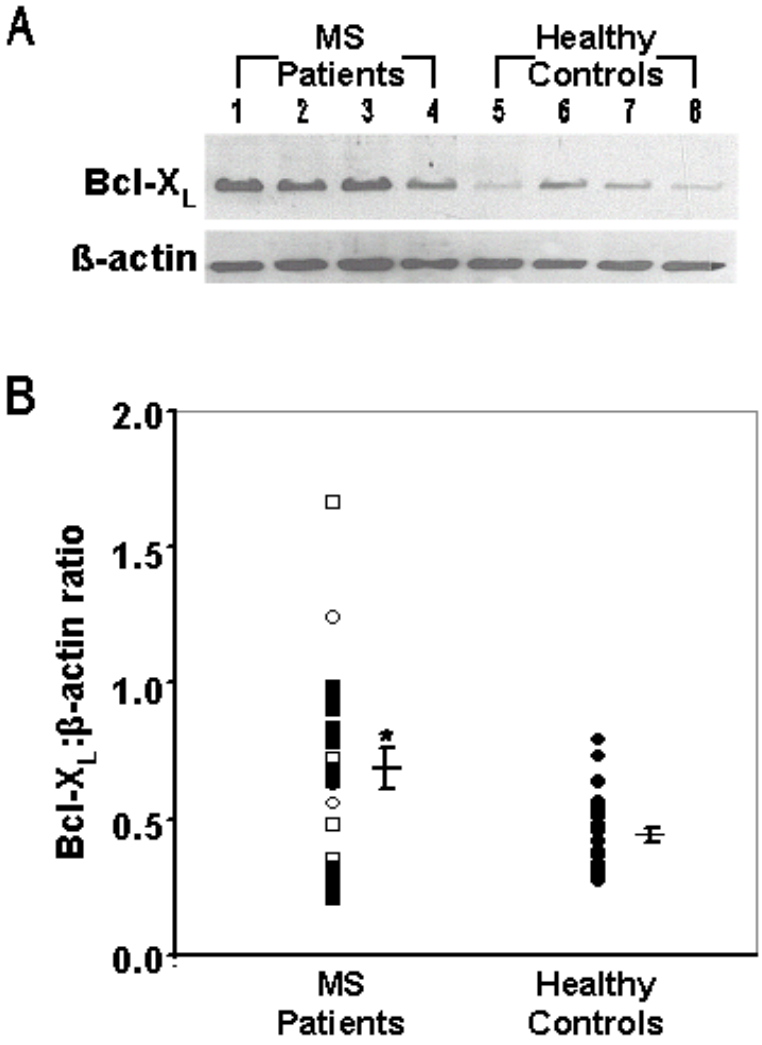

Figure 5 **Elevated Bcl-X_L protein expression in MS patients**

(A) A representative western blot shows that specific Bcl-X_L bands, detected at 26 kDa, are more intense in MS patient samples (Lane 1-4) compared to healthy controls (Lane 5-8) and in relation to β-actin reference protein, detected on the same blot.

(B) Each point represents the mean Bcl-X_L level of 3 independent experiments. Bcl-X_L levels were calculated from the density ratio of Bcl-X_L:β-actin specific signals on the same blot. MS patients (O PPMS; □ SPMS; ■ RRMS) express significantly higher levels of Bcl-X_L protein than normal healthy controls (●) (p=0.013). Indicated are mean ± standard errors of mean (SEM) for both, MS patients (0.69 ± 0.07) and controls (0.44 ± 0.03).

Table 2 **Bcl-X_L expression of MS patients on different treatment regimen**

Clinical groups	Bcl-X_L protein levels [a]
Multiple sclerosis	0.69 ± 0.07 [c]
Untreated	0.70 ± 0.08 [d]
IFN-β [b]	0.70 ± 0.29
Other treatment (MT, AZ) [b]	0.59 ± 0.34
Healthy controls	0.44 ± 0.03

[a] expressed as an Bcl-X_L:β-actin index, [b] IFN-β = interferon-beta; MT = mitoxantrone; AZ = azathioprine

[c] p = 0.013 compared to levels in healthy controls, [d] p = 0.006 compared to levels in healthy controls

Interestingly, the subgroup of untreated MS patients revealed a larger difference in the Bcl-X_L protein levels compared to healthy controls (p=0.006) (Table 2). Patients treated with azathioprine (AZ) and mitoxantrone (MT) expressed lower Bcl-X_L levels than untreated patients or patients treated with IFN-β, although this finding was not statistically significant (p>0.05) (Table 2).

To determine whether the increased Bcl-X_L protein expression in MS patients was based on a regulation of gene expression, messenger RNA levels by RT-PCR were investigated in both, MS patients as well as healthy controls.

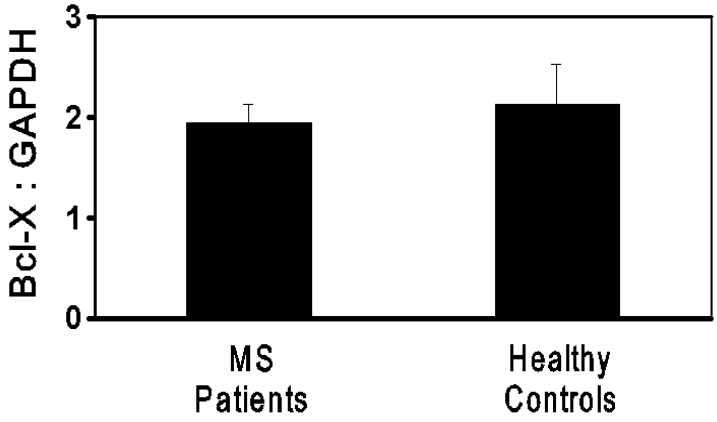

Figure 6 **No alterations in Bcl-X_L mRNA expression**
Specific Bcl-X_L signals were adjusted in relation to the expression of the housekeeping gene, GAPDH, in three independent experiments and expressed as arbitrary OD indices (see Methods).

On the contrary to protein expression, no alteration in Bcl-X_L mRNA expression (Fig. 6) was observed between MS patients and healthy controls. Specific Bcl-X_L signals were adjusted in relation to the expression of the housekeeping gene, G3PDH, in three independent experiments and expressed as arbitrary OD indexes. A mean OD index ± SEM revealed no difference between MS patients (1.56 ± 0.22) and healthy controls (1.88 ± 0.48) (p>0.1).

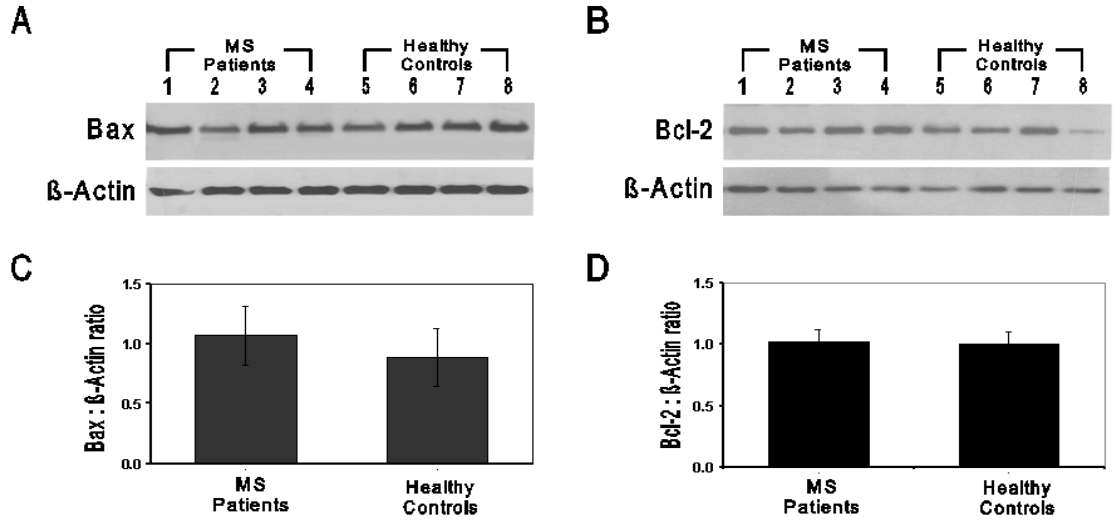

Figure 7 **No alterations in Bax and Bcl-2 protein expression**

Figure 7 (cont.) Representative western blots show no apparent differences in the expression of (A) pro-apoptotic protein Bax, detected at 24 kDa, and (B) anti-apoptotic protein Bcl-2, detected at 30 kDa, between patients (Lane 1-4) and healthy controls (Lane 5-8). (C,D) Bax and Bcl-2 protein levels were calculated from the density ratio of Bax:β-actin specific signals and Bcl-2:β-actin specific signals, respectively. The data represent the mean ± SEM of (C) Bax/β-actin and (D) Bcl-2/β-actin expression in immune cells of all MS patients and healthy controls. Samples from each individual donor were analyzed in 3 independent instances and a mean was calculated.

Unlike Bcl-X_L, no apparent differences in the expression of both, anti-apoptotic protein Bcl-2, detected at 30 kDa, and pro-apoptotic protein Bax, detected at 24 kDa, were seen between patients and controls (Fig. 7A,B). Bcl-2 and Bax basal levels were calculated in relation to β-actin, in three independent experiments, and no statistically significant differences were observed between MS patients and healthy controls (Fig. 7C,D).

4.1.2 Increased resistance of immune cells from MS patients to undergo AICD

The present study made use of a modified model of in vitro AICD (Klas et al., 1993) by stimulating day 0 peripheral mononuclear cells *ex vivo* with 1µg/mL of PHA and eventually inducing apoptosis in polyclonal day 6 T cells with 10µg/mL PHA. Mononuclear cells were thawed and resuspended at 10^6/ml in FBM (Materials). Cell viability was determined by trypan blue dye exclusion assay (Methods).

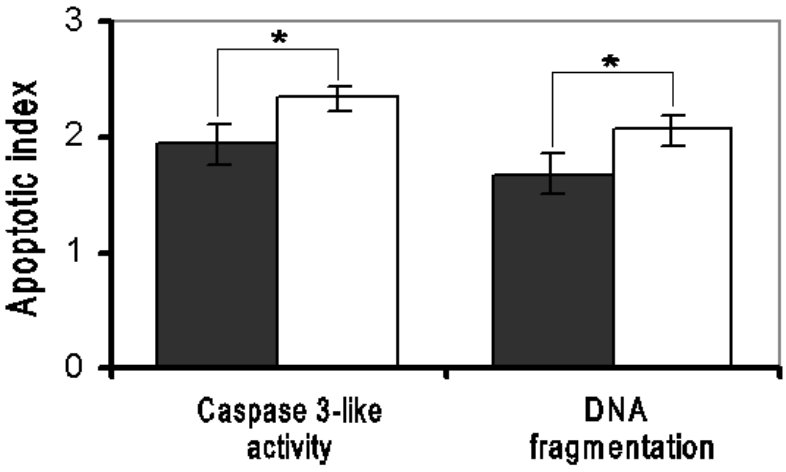

Figure 8 **Decreased susceptibility of T cells towards AICD in MS**
Susceptibility of activated polyclonal T cells towards apoptosis was analyzed with caspase 3-like activity and DNA fragmentation assays. MS patients (closed bars) show a significant decrease in apoptotic cell death when compared to healthy controls (open bars) with respect to caspase 3-like activity (p=0.013) and DNA fragmentation (p=0.0071). Data represent the mean apoptotic index ± SEM. Mean absolute values for *caspase 3-like activity*: 237 ± 11.3 fluorescence units for unstimulated controls and 499.4 ± 26.4 fluorescence units for stimulated cells. Mean absolute values for *DNA fragmentation*: 19.7% ± 0.13 cells with hypodiploid DNA for unstimulated controls and 35.22% ± 0.18 cells with hypodiploid DNA for stimulated cells. Both methods for AICD quantification correlated significantly with each other (R=0.740, p=0.01).

Following 24h stimulation, T cell blasts (day 1 cells) were washed twice with PBS, split in two wells, and thereafter cultured for a further 5 days in complete medium supplemented

with 25U/ml IL-2 (Klas et al., 1993). Apoptosis of polyclonal (day 6) T cells was induced with a second stimulus of 10µg/ml PHA. The extent of AICD was determined by analyzing the activity of downstream caspases, specifically DEVD-amc-cleaving caspase activity, and the level of DNA fragmentation. Following kinetic studies, the incubation time of day 6 T cells with PHA was established at 6h for DEVD-amc-cleaving caspase activity assays and at 24h for DNA fragmentation assays. T cells from MS patients showed a significantly lower susceptibility towards AICD than T cells from controls, as shown by both, DEVD-amc-cleaving caspase activity (p=0.013) and DNA fragmentation (p=0.0071) assays (Fig. 8).

4.1.3 Inverse correlation between Bcl-X_L levels and AICD

Using the Spearman Bivariate Correlation Coefficient test, and taking the whole population of patients and controls into account, a significant inverse correlation was observed between Bcl-X_L levels in peripheral immune cells and the susceptibility of these cells to undergo DNA fragmentation (R=-0.406, p=0.016) (Fig. 9). The latter finding implicates a role for Bcl-X_L in the protection of T cells against AICD. No correlation could be extracted between the degree of apoptosis sensitivity and the clinical course of MS.

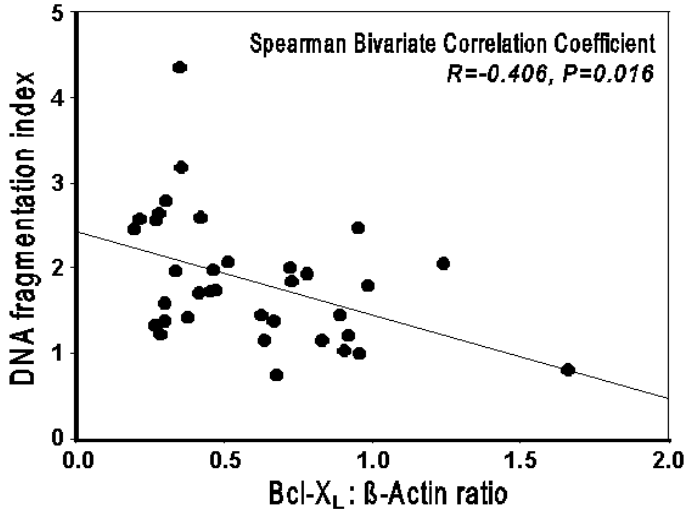

Figure 9 **Bcl-X_L levels inversely correlate with T cell AICD**
Increasing Bcl-X_L levels in peripheral immune cells accompany a reduced susceptibility of T cells towards AICD as shown by the significant inverse correlation obtained between DNA fragmentation indices and Bcl-X_L protein levels (R=-0.406, p=0.016).

In this study, the expression of the apoptosis-regulating Bcl-2 family members was investigated and, in relation, the T cell susceptibility towards AICD in MS patients compared to healthy individuals. An increased protein expression of anti-apoptotic Bcl-X_L, but no alteration in anti-apoptotic Bcl-2 or pro-apoptotic Bax, was observed in MS patients. Overexpression of Bcl-X_L was correlated with a decreased susceptibility of polyclonal T cells from MS patients to undergo AICD.

4.2 CHARACTERIZATION OF HUMAN ANTIGEN-SPECIFIC T CELL LINES

A panel of human antigen-specific $CD4^+$ T cell lines (TCLs) specific for myelin basic protein (MBP), tetanus toxoid (TT), or birch pollen (Bet) from both, healthy individuals and patients suffering from multiple sclerosis (Table 3), were generated as previously described. TCLs were characterized as T helper 1 (Th1), T helper 2 (Th2) or T helper 0 (Th0)-like cells according to their intracellular cytokine profile (Table 4).

4.2.1 Donors for human T cell lines

MS patients and control individuals donated fresh blood at start of the primary MBP, Bet or TT culture and 2 weeks later for the split-well. For the restimulations, frozen autologous PBMCs serving as APCs were thawed each week and preincubated with antigen prior to addition to the TCLs. The clinical status of the MS donors is listed in Table 3 and the reactivity of allergic individuals to birch pollen, measured by RAST method (Pharmacia CAP – System, Uppsala, Sweden) was analyzed.

Table 3 **Donors for human antigen-specific TCLs**

MS Patient	Clinical group	Gender	Age
BA	RRMS	F	29
PE	SPMS	M	47
BL	RRMS	F	43
GU	RRMS	F	31
KI	RRMS	M	30
CH	RRMS	F	21
BÜ	RRMS	F	32
Control	**Birch pollen allergy**	**Gender**	**Age**
AV	NA	M	NA
MA	NA	F	30
FZ	0.89 kU/l IgE	F	39
MB	5.52 kU/l IgE	M	31
EG	8.89 kU/l IgE	F	34
OW	NA	M	26
SE	NA	M	24
SR	NA	F	28

Most of the patients donating blood for the TCLs were suffering from the relapsing-form of MS (RRMS). Control individuals donating for the birch pollen specific TCLs suffer from one or more allergies, including birch pollen (IgE conc in serum indicated in kU/l); § NA = not available

4.2.2 Characterization of T helper phenotype

The T helper differentiation was based on the ratio of IFN-γ and IL-4 production. Predominant IFN-γ staining by flow cytometry determined a Th1-like cell line, double staining a Th0 cell line, and IL-4 staining a Th2-like cell line (Fig. 10). The classification of each antigen-specific TCL, both MS patient and healthy control-derived, is listed in Table 4.

Table 4 **Human antigen-specific T cell lines**

Control TCL[a]	Antigen Specificity	Th-status
AV4	MBP	Th2
MA1	MBP	Th0
MA14	MBP	Th0
FZ2	MBP	Th1
FZ3	MBP	Th0
FN8	MBP	Th1
MB2	Bet	Th0
MB7	Bet	Th2
MB8	Bet	Th2
MB10	Bet	Th0
MB12	Bet	Th2
EG1	Bet	Th2
EG3	Bet	Th2
EG4	Bet	Th2
OW4	Bet	Th0
OW8	TT	Th0
SE4	TT	Th0
SE5	TT	Th0
SE13	TT	Th0
SR6	TT	Th0
SR12	TT	Th2
FZ4	TT	Th0
MS Patient TCL[a]	**Antigen Specificity**	**Th-status**
BA1	MBP	Th1
BA4	MBP	Th1
DE1	MBP	Th1
DE4	MBP	Th2
PE4	MBP	Th0
BL4	MBP	Th1
GU3	MBP	Th0

MS Patient TCL[a]	Antigen Specificity	Th-status
KI2	MBP	Th1
ES10	MBP	Th1
CH2	MBP	Th0
CH3	MBP	Th2
BÜ1	Bet	Th2
BÜ2	Bet	Th2

[a] Each TCL is denoted with the initials of the donor (MS patient or control) and the indicated antigen specificity (MBP = myelin basic protein, Bet = birch pollen, TT= tetanus toxoid)
All T cell lines exhibited a minimum SI of three at all times indicating acceptable antigen specificity

As shown in Table 4 most TCLs specific to MBP are Th1-like with only a few showing a Th2 shift (AV4, DE4 and CH3) and allergic TCLs, specific to birch pollen allergen are Th2-like. Tetanus-toxoid specific T cells lines typically stain for both IFN-γ and IL-4 cytokines, denoting them as Th0-like.

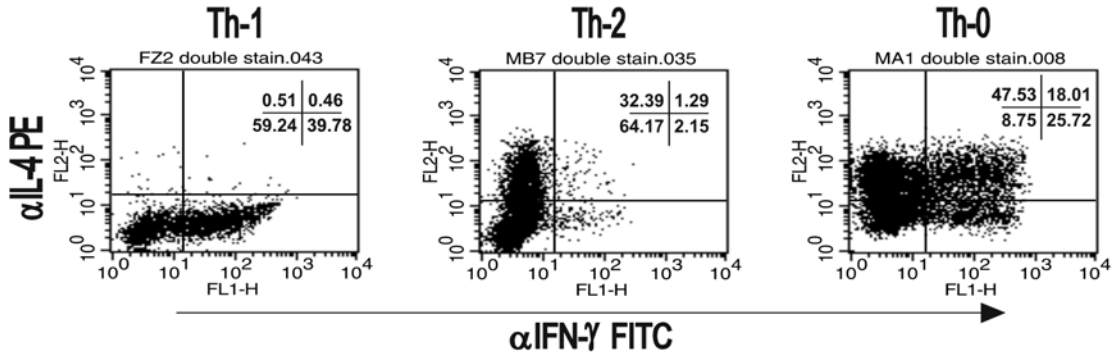

Figure 10 **Typical Th1, Th2 and Th0-like cell lines**
FZ2 is a typical Th1, MB7 a typical Th2 and MA1 a typical Th0-like cell line. Resting TCLs (Day 7-8) were stimulated with 0.1µg/ml PMA and 1µg/ml ionomycin in the presence of 5µg/ml brefeldin A. After 5h, cells were harvested, fixed and stained for intracytoplasmic interferon (IFN)-γ and IL-4 by incubating with fluorescein (FITC)- conjugated rat anti-human IFN-γ (XMG1.2, PharMingen), phycoerythrin (PE)-conjugated rat anti-mouse IL-4 (11B11, PharMingen) and FITC-/PE-labeled rat IgG1 isotype control antibodies (R3-34, PharMingen). Analysis was performed with a FACSCalibur® flow cytometer (Becton Dickinson and Co., Mountain View, CA) equipped with CELLQuest software, and 1 x 10^4 events acquired.

4.2.3 MBP epitope mapping of human antigen-specific T cells

T cell reactivity to major encephalitogenic myelin antigens has been studied extensively in Caucasian patients with MS (Meinl et al., 1993; Correale et al., 1995; Wallstrom et al., 1998). The peptide-binding motif of the *HLA-DR2* (*DRA*0101-DRB1*1501*) gene product has been determined in the MBP peptide recognized by T cell clones derived from Caucasian patients with MS (Wucherpfennig et al., 1994).

To identify the epitopes recognized by the MBP-specific TCLs utilized in this study, the reactivity of the TCLs against MBP antigen peptides was screened. Sixteen 19-mer peptides (Table 5) spanning the whole MBP molecule, displaced by 10 aa and overlapping by 9 aa, were synthesized using standard F-moc chemistry on an Abimed AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany). Mass spectroscopy was performed on a matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer (Laser BenchTopII; Applied Biosystems). The purity of the products was characterized by analytical high-pressure liquid chromatography. All peptides were stored at 10mg/mL in DMSO and aliquots of 1mg/ml in medium.

Table 5 **Amino acid sequences of synthetic peptides for ^{170}MBP**

Peptide	Amino acid sequence
$MBP^{1–19}$	ASQKRPSQRHGSKYLATAS
$MBP^{11–29}$	GSKYLATASTMDHARHGFL
$MBP^{21–39}$	MDHARHGFLPRHRDTGILD
$MBP^{31–49}$	RHRDTGILDSLGRFFGGDR
$MBP^{41–59}$	IGRFFGGDRGAPKRGSGKD
$MBP^{51–69}$	APKRGSGKDSHHAARTTHY
$MBP^{61–79}$	HHAARTTHYGSLPQKSHGR
$MBP^{71–89}$	SLPQKSHGRTQDENPVVHF
$MBP^{81–99}$	QDENPVVHFFKNIVTPRTP
$MBP^{91–109}$	KNIVTPRTPPPSQGKGRGL
$MBP^{101–119}$	PSQGKGRGLSLSRFSWGAE
$MBP^{111–129}$	LSRFSWGAEGQRPGFGYGG
$MBP^{121–139}$	QRPGFGYGGRASDYKSAHK
$MBP^{131–149}$	ASDYKSAHKGLKGVDAQGT
$MBP^{141–159}$	LKGVDAQGTLSKIFKLGGR
$MBP^{151–170}$	SKIFKLGGRDSRSGSPMARR

Resting T cell lines (Day 7 following last restimulation) were cultured in the absence or presence of MBP, to determine the level of specificity of the cell line, and in parallel with 5µM of each of the above listed overlapping MBP peptides. Reactivity towards peptide was analyzed 96h after incubation with whole antigen/peptides by proliferation assays utilizing [^{3}H]–thymidine uptake. Peptide specificities from eight TCLs (5 from healthy controls: MA1, MA14, FN8, FZ2, LS7 and 3 from MS patients: CH3, KI2, GU3) are shown in Fig. 11. Data are expressed as mean stimulation index (as compared to unstimulated controls) ± SEM.

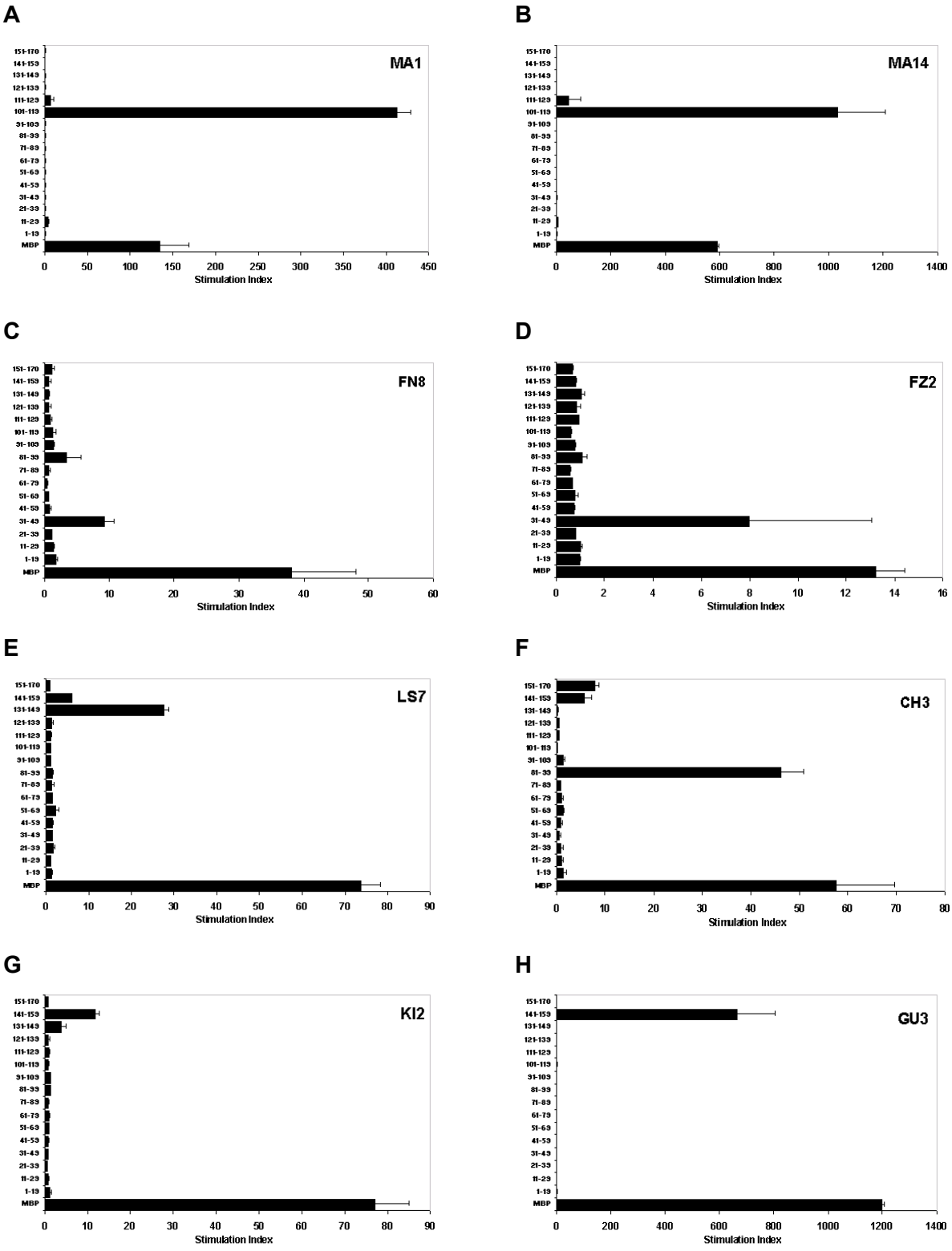

Figure 11 **Peptide specificity of MBP-autoreactive TCLs**
T cell lines from healthy controls (A-E) and MS patients (F-H) were cultured in the absence or presence of different MBP peptides for 96h. Peptide specificity was analyzed by proliferation assessment utilizing [^{3}H]–thymidine uptake in triplicates. Results are expressed as mean stimulation index (as compared to unstimulated controls) ± SEM.

As shown in Fig. 11, TCLs from 2 MS patients (KI2, GU3) showed reactivity to $MBP^{141–159}$ epitope, whereas TCLs from a third patient (CH3) showed reactivity to $MBP^{81–99}$ epitope. TCLs MA1 and MA14 (both from the same healthy control, MA) showed reactivity to $MBP^{101–119}$ peptide and TCLS FZ2 and FN8 (both from the same healthy control, FZ) showed reactivity to $MBP^{31–49}$ peptide. This is in line with original work on the MBP-specific T cell repertoire where the $MBP^{29–48}$ domain was mostly recognized by control-derived TCLs and the $MBP^{80–105}$ domain frequently by MS patient-derived TCLs (Meinl et al., 1993).

The TCR of the MBP-specific $CD4^+$ TCLs recognizes MBP antigen in the context of MHC class II on APC. In principle, there are two types of MHC molecules: class I, which includes human leukocyte antigens (HLA)-A, -B and -C, and class II, which includes HLADR, -DP and -DQ. The latter antigens take up peptide, which is recognized by $CD4^+$ T cells. Donors for the MBP-specific TCLs were tissue typed for the HLA class II antigens. Genomic DNA was prepared from PBMC of each individual by the salting-out method (Miller et al., 1988). Typing for the polymorphism of HLA class II-loci was performed by polymerase chain reaction amplification with sequence specific primers, as described previously (Olerup and Zetterquist, 1992). The DR, DQ and DP type of most of the donors for the TCLs is presented in Table 6 according to established nomenclature (Schreuder et al., 1999).

Table 6 **HLA Typing of MS patients and controls**

Patient	**DR**		**DQ**	**DP**
BL (F)	B1*0808/1515	B5*pos(51)	B1*0404/0606	B1*0401/1301
CH (F)	B1*1515/1616	B5*pos(51)	B1*0505/0606	-
KI (M)	B1*0101/0404	B4*pos(53)	B1*0303/0505	-
Control	**DR**		**DQ**	**DP**
LS (F)	B1*0404	B4*pos(53)	B1*0303	-
MA (F)	B1*09012(9)/1313	B3*pos(52), B4*pos(53)	B1*0606/0303	-
FZ (F)	B1*1313/1515	B3*pos, B4*neg, B5*pos	B1*0606	-

4.3 ROLE OF TRAIL IN HUMAN ANTIGEN-SPECIFIC T CELL LINES

Although expressing death inducing receptors TRAIL receptor 1 and 2, human T cells are not killed by soluble leucine-zipper TRAIL in vitro (Wendling et al., 2000). However, studies on animal models of autoimmune diseases suggested an influence of TRAIL on T cell growth and effector function. Systemic neutralization by TRAIL receptor 2 was demonstrated to exacerbate collagen-induced arthritis (Song et al., 2000) and EAE (Hilliard et al., 2001). Therefore, immunoregulatory effects of TRAIL other than clonal deletion by induction of apoptosis were investigated in the human immune system using characterized antigen-specific human T cell lines. For this, a human recombinant form of soluble TRAIL was employed together with an enhancer antibody for multimerization (Materials)

4.3.1 TRAIL inhibits proliferation of human antigen-specific T cells

As outlined in Table 7, TRAIL inhibited the antigen-induced proliferation of 22 characterized T cell lines, in eight of them (highlighted in gray) by more than 40%. The inhibitory effect was independent of the antigen-specificity and Th1/Th2 differentiation of these T cells. Furthermore, no differences in the proliferative response of the T cell lines derived from MS patients or healthy individuals were observed. TRAIL at concentrations ranging from 30 to 300ng/ml did not affect proliferation of the T cell lines cultured with APC in the absence of the nominal antigen (data not shown).

Table 7 **TRAIL inhibits proliferation of human antigen-specific T cell lines**

TCL	Donor	Antigen Specificity	TH-status	% Inhibition$_{Max}$*	TRAIL†
BA1	MS	MBP	Th1	32.1	300
BA4	MS	MBP	Th1	46.5	100
BL4	MS	MBP	Th1	63.8	300
GU3	MS	MBP	Th0	47,9	100
KI2	MS	MBP	Th1	39.1	300
ES10	MS	MBP	Th1	6.2	100
BU2	MS	MBP	Th2	38.6	300
CH2	MS	MBP	Th0	54.1	300
AV4	control	MBP	Th2	53.8	300
MA1	control	MBP	Th0	19.8	300
MA3	control	MBP	NA§	16.85	300
MA7	control	MBP	NA§	9.3	300
MA14	control	MBP	Th0	27.8	100

TCL	Donor	Antigen Specificity	TH-status	% Inhibition$_{Max}$*	TRAIL†
FZ2	control	MBP	Th1	47.3	300
MB2	control	Bet	Th0	19.9	100
MB7	control	Bet	Th2	49.3	300
MB8	control	Bet	Th2	47.0	300
OW8	control	TT	Th0	20.5	30
SE4	control	TT	Th0	18.2	100
SE5	control	TT	Th0	15.7	30
SE13	control	TT	Th0	17.6	300
FZ4	control	TT	Th0	36.8	100

* % Inhibition = (cpm in the presence of TRAIL/cpm in the absence of TRAIL) x 100; max % inhibition from TRAIL concentrations used; † Lowest TRAIL conc in ng/ml at which max inhibition is observed, § NA = not available

Proliferation was assessed by [^{3}H]–thymidine uptake and measured as counts per minute (cpm). All T cell lines showed a stimulation index (SI) >3 and the effect of TRAIL on antigen-stimulated proliferation was investigated in each particular cell line >3 times. Highlighted in gray are TCLs, whose proliferation is inhibited by more than 40%

4.3.2 TRAIL-induced hypoproliferation is independent of Ag presentation

To investigate whether the TRAIL-induced hypoproliferation is due to interference with antigen-processing or -presentation, 12 representative T cell lines were stimulated with plate-bound anti-CD3 (1µg/ml) and soluble anti-CD28 (2.5µg/ml) antibodies.

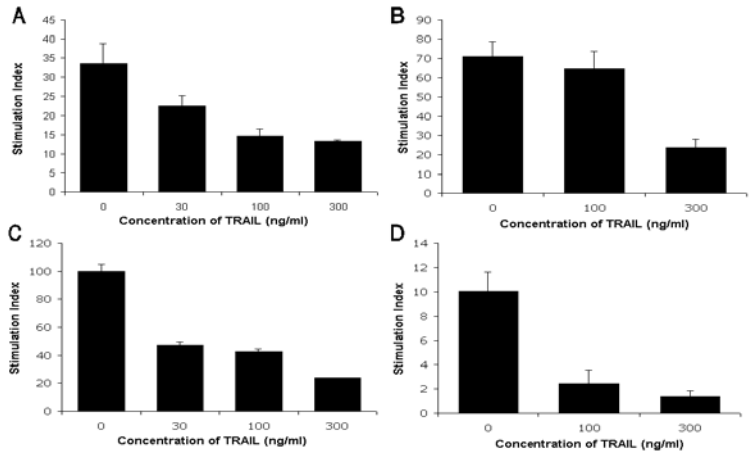

Figure 12 **TRAIL inhibits proliferation of TCLs independently of APC**
T cell lines were cultured in the absence or presence of different TRAIL concentrations for 96h. Proliferation was assessed by [^{3}H]–thymidine uptake in triplicates. Results from 2 representative TCLs, stimulated once with specific antigen and once by direct triggering of TCR are expressed as mean stimulation index (as compared to unstimulated controls) ± SEM. (A) MBP-specific T cell line FZ2 antigen-stimulated (background 198cpm), (B) MBP-specific TCL FZ2 anti-CD3/CD28-stimulated (background 227cpm), (C) Bet-specific TCL MB7 antigen-stimulated (background 121cpm), (D) Bet-specific TCL MB7 anti-CD3/CD28-stimulated (background 276cpm).

As demonstrated in Fig. 12, TRAIL substantially inhibited T cell proliferation, also in the absence of APC, indicating that this cytokine influences T cell function directly rather than via APC-mediated signals.

4.3.3 TRAIL dose-dependently decreases Ca^{2+} influx

Antigenic stimulation of T cells triggers intracellular calcium release and the consequent opening of calcium release-activated calcium channels (CRAC) that in turn generate the prolonged elevation of cytosolic calcium ($[Ca^{2+}]_i$) required for T cell activation (Zweifach and Lewis, 1993). A lasting rise greater than 200nM is required to induce IL-2 synthesis (Negulescu et al., 1994). Since thapsigargin (Tg) has been shown to activate the same CRAC as TCR-stimulation by antigen (Aussel et al., 1996), a Tg model of calcium influx was used to bypass TCR signals upstream of the endoplasmic calcium store and to directly monitor the influence of TRAIL on CRAC. As shown in Fig. 13A, addition of external calcium to Tg-treated cells causes a peak in intracellular calcium, and incubation with TRAIL causes a downregulation of this calcium entry into the cell. The blockade of calcium influx was further accompanied by an inhibition of proliferation (Fig. 13B) indicating that TRAIL negatively regulates human T cell calcium channels.

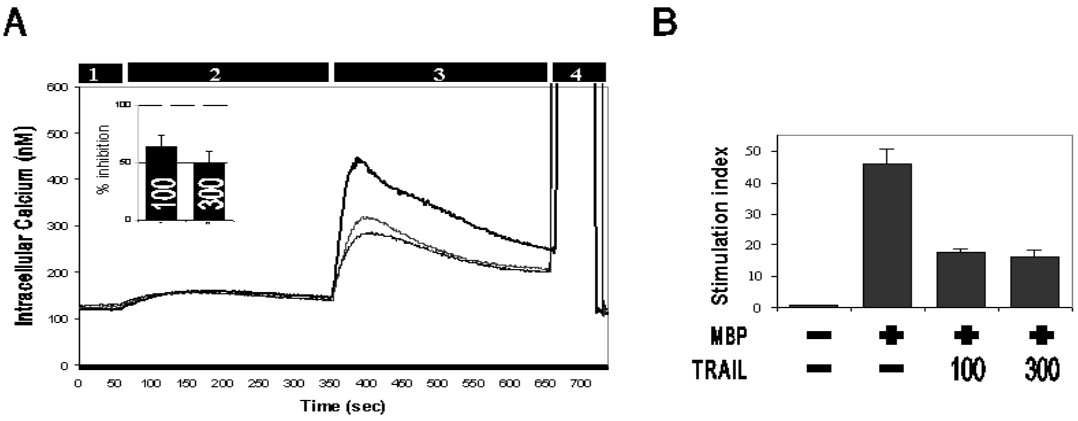

Figure 13 **TRAIL induces a dose-dependent decrease in Ca^{2+} influx**
(A) This panel shows the $[Ca^{2+}]_i$ measured by fluorescence spectrophotometry of an MBP-specific T cell line (GU3) and is representative of 6 independent experiments. Cells were incubated for 1h at 37°C in the absence (bold trace) or presence of 100ng/ml (thinner trace) and 300ng/ml (thinnest trace) TRAIL and enhancer. FURA-2/AM-loaded resting T cell lines were incubated with 0.5mM EGTA for 5min, during the last minute the calcium trace was started ①. To activate CRAC channels cells were treated with 2µM thapsigargin for 5min ②. Ca^{2+} entry was seen upon addition of 1.2mM-extracellular Ca^{2+} ③. Measurements were terminated by Triton-X (maximal calcium) and 3mM $MnCl_2$ fluorescence quencher (minimal calcium) ④ (see Methods) (B) The TRAIL-induced decrease in Ca^{2+} influx was followed by an inhibition of proliferation. During the same time point, GU3 showed hypoproliferation with TRAIL (65% inhibition with 300ng/ml) as analyzed by [^{3}H]-thymidine uptake.

TRAIL reduced Ca^{2+} influx to a minimum, just within the lower concentration limit (200nM) required for IL-2 production and consequent proliferation.

Additionally, co-incubation of TRAIL with 4µg/ml of a human recombinant protein consisting of the extracellular domain of TRAIL receptor 2 (DR5) fused to the Fc portion of human IgG_1 (rhTRAIL-R2:Fc fusion protein; Alexis) antagonized the inhibitory effect of TRAIL (Fig. 14). Thus, this further confirms that interaction of TRAIL with its receptors negatively regulates human T cell calcium channels resulting in reduced T cell activation.

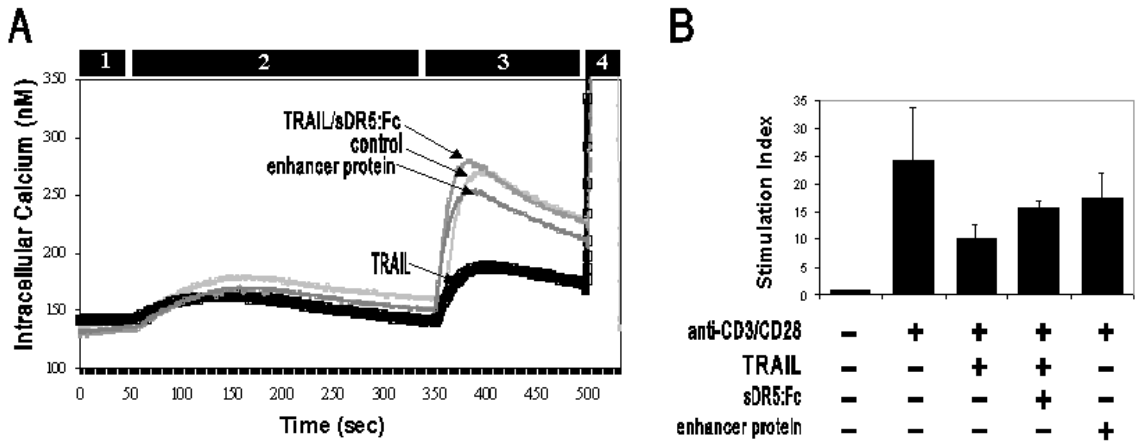

Figure 14 **TRAIL-induced inhibition in Ca^{2+} influx is reversed by sDR5:Fc**
Bet-specific T cell line (MB8) was incubated with the enhancer alone (2µg/ml) as negative control, TRAIL co-incubated with sDR5:Fc (4µg/ml) or TRAIL alone. Addition of the TRAIL R2 fusion protein antagonized the inhibitory effect of TRAIL on calcium influx (A) and subsequent proliferation (B).

4.3.4 TRAIL inhibits G1/S transition

T cell proliferation upon TCR engagement is controlled by a large number of positive regulators such as cyclins and cyclin dependent kinases (CDK), and negative regulators such as CDK inhibitors (Nagasawa et al., 1997; Appleman et al., 2000). Since DNA synthesis and [^{3}H]-thymidine incorporation occur during the S phase of the cell cycle, TRAIL could block cell cycle progression during the G1 to S phase transition. CDK4 allows transit through the G1 phase of the cell. Therefore, the influence of TRAIL on expression of this kinase was examined. Furthermore, the expression of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, was investigated since it has recently been found to be responsible for the blockade of clonal expansion of anergic T cells (Boussiotis et al., 2000; Jackson et al., 2001). The expression levels of CDK4 and the kinase inhibitor $p27^{Kip1}$ were measured following both antigenic stimulation and anti-CD3/CD28 stimulation in the presence or absence of TRAIL in 7 T cell lines. Fig. 15 depicts data from a representative T cell line showing an upregulation of CDK4 upon T cell stimulation as expected (Nagasawa et al., 1997;

Appleman et al., 2000) and a downregulation upon incubation of TRAIL with either of both stimuli, MBP (Fig. 15A) and anti-CD3/CD28 (Fig. 15B). This indicates an inhibition of cell cycle progression at the G1/S transition level. However, $p27^{Kip1}$ expression was unaltered in the presence of TRAIL, which therefore excludes induction of clonal anergy by TRAIL as an underlying mechanism of the observed inhibitory properties on T cell activation.

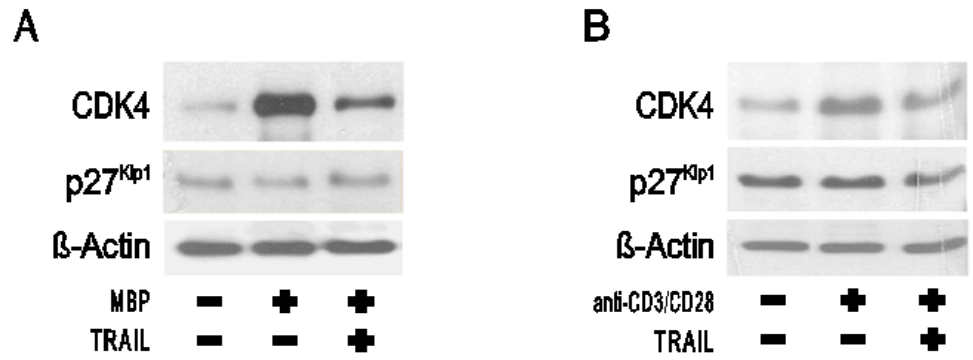

Figure 15 **TRAIL downregulates expression of CDK4**
The MBP-specific T cell line (LS4) was restimulated with either (A) antigen (MBP) for 72h or (B) anti-CD3/anti-CD28 stimulus for 24h in the presence (+) or absence (-) of 100ng/ml of TRAIL and enhancer. Lysates were prepared and equal amounts of protein were analyzed by 12% SDS-PAGE. Blots were sequentially incubated with CDK4, $p27^{Kip1}$, and β-actin antibodies.

Once again, co-incubation of TRAIL with 4µg/ml of the human recombinant TRAIL receptor 2 (rhTRAIL-R2:Fc) fusion protein antagonized the inhibitory effect of TRAIL (Fig. 16) confirming once more a requirement for an interaction between TRAIL and its receptor to regulate human T cell cycle progression.

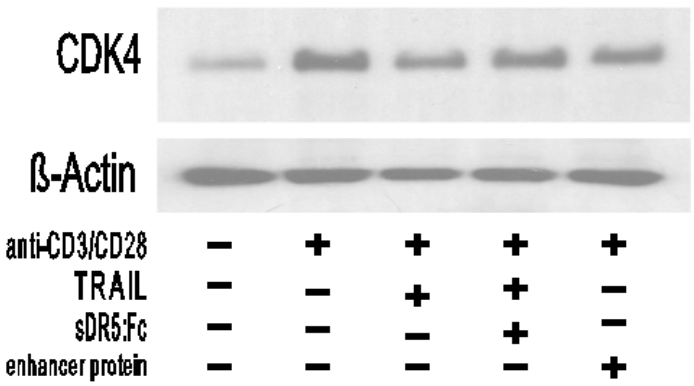

Figure 16 **Influence of TRAIL on cell cycle regulation is reversed by sDR5:Fc**
The CDK4 downregulatory effect of TRAIL could be reversed by co-incubation with sDR5:Fc (4µg/ml), whereas the enhancer antibody (2µg/ml) which served as irrelevant control protein did not affect the regulation of CDK4.

4.4 MECHANISM OF ACTION OF ATORVASTATIN

Atorvastatin is capable of treating and preventing relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells (Youssef et al., 2002; Aktas et al., 2003). Along with several other statins, atorvastatin has been long-approved through several large-scale intervention trials for the treatment of hypercholesterolemia (rev. Maron et al., 2000)
Statins are known to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Fig. 17), thereby inhibiting isoprenoid synthesis and subsequent isoprenylation of signaling molecules such as Ras, Rho and Rac from the Ras superfamily.

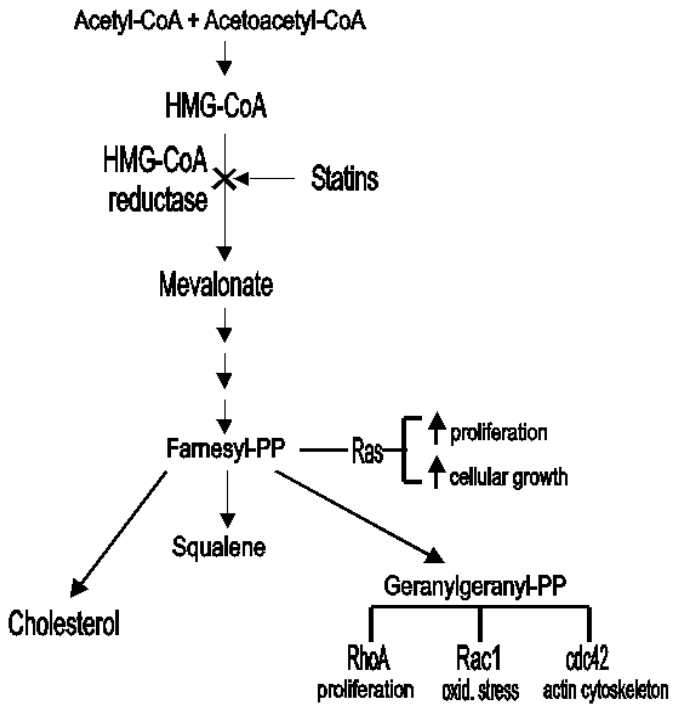

Figure 17 **Cholesterol biosynthesis pathway**
Inhibition of HMG-CoA reductase by statins decreases the synthesis of isoprenoids and cholesterol. PP indicates pyrophosphate.

In order to understand via which mechanisms atorvastatin is inducing its protective and therapeutic effects on EAE, the immunoregulatory effects of this statin were investigated in the human immune system using characterized antigen-specific human T cell lines. Since pure atorvastatin (provided by Pfizer) is not soluble in PBS, the stock was dissolved in 2% DMSO (100µM stock in 2% DMSO). The carrier was used as vehicle control in the same dilution as atorvastatin. Atorvastatin inhibited not only antigen-specific responses, but also decreased T cell proliferation mediated by direct TCR engagement independently of MHC class II and LFA-1. Inhibition of proliferation was not due to apoptosis induction, but linked to a negative regulation on cell cycle progression. However, early T cell activation was unaffected, as reflected by unaltered calcium fluxes. Thus, these results provide evidence for a beneficial role of statins in the treatment of autoimmune attack on the CNS.

4.4.1 Atorvastatin inhibits proliferation of human antigen-specific T cells

To investigate whether atorvastatin interferes with antigen-specific proliferation, antigen-specific TCLs were incubated with varying doses (1-25µM) of statin for 96h. Proliferation was assessed by measuring the extent of ^{3}[H]-thymidine uptake. The doses of 1-25µM atorvastatin used in these assays are comparable to the levels measured in human plasma (rev. Lea and McTavish, 1997; Stern et al., 2000).

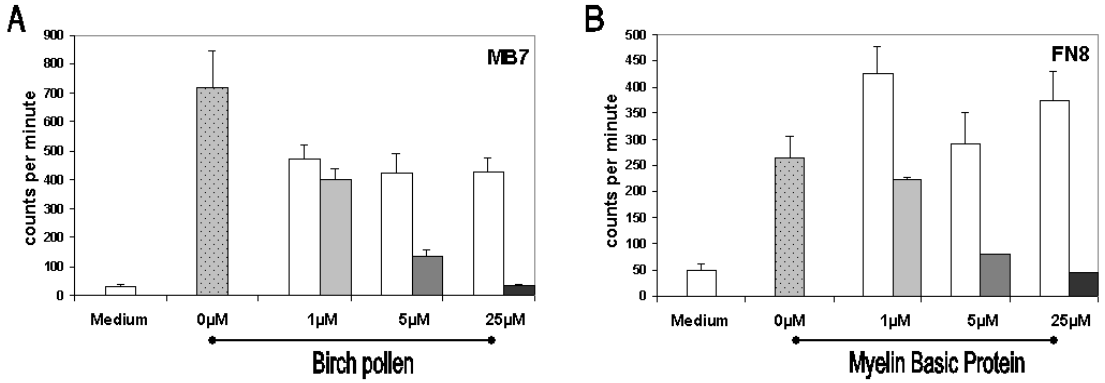

Figure 18 **Atorvastatin inhibits T cell proliferation in a dose-dependent manner**
Bet-specific TCL MB7 (A) and MBP-specific TCL FN8 (B) were stimulated with or without antigen presented by irradiated autologous APC in the absence or presence of different concentrations of atorvastatin (filled bars) or vehicle alone (open bars), and following 96h incubation ^{3}[H]-thymidine uptake, as a measure of proliferation, was assessed.

As indicated in Fig. 18, antigen-specific proliferation of both, the Bet-specific TCL MB7 (Fig. 18A) and the MBP-specific TCL FN8 (Fig. 18B), was suppressed by atorvastatin in a dose-dependent manner. Interestingly, blockade of proliferation was also observed when atorvastatin was added to proliferating T cells, 24h or 48h after stimulation (Fig. 19).

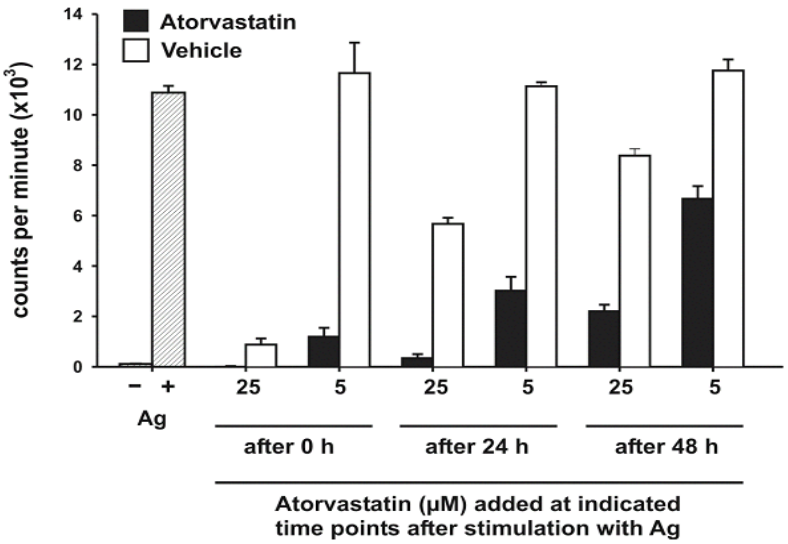

Figure 19 **Atorvastatin inhibits proliferation of already activated T cells**

The representative human Bet-specific T cell line MB7 was stimulated with (+) or without (-) antigen presented by irradiated autologous APC. Atorvastatin (filled bars) or vehicle (open bars) was added immediately (0h), after 24h or 48h antigen presentation.

This points to further

pathways of immunomodulatory function in addition to those involving a reduced MHC class II upregulation by inhibition of the inducible promoter IV of the transactivator CIITA (Kwak et al., 2000), and the blockade of LFA-1/ICAM-1 interactions (Weitz-Schmidt et al., 2001) by statins.

4.4.2 Atorvastain inhibits proliferation independently of Ag presentation

To determine whether the inhibition of T cell proliferation to be via direct T cell receptor engagement, TCLs were stimulated independent of Ag presentation, by anti-CD3/CD28.
As shown in Fig. 20, ^{3}[H]-thymidine uptake of the cell lines, FN8, was also markedly suppressed by atorvastatin when stimulated with anti-CD3/CD28. Thus, in an environment lacking APC, expressing MHC class II and ICAM-1, atorvastatin is nonetheless capable of inhibiting proliferation.

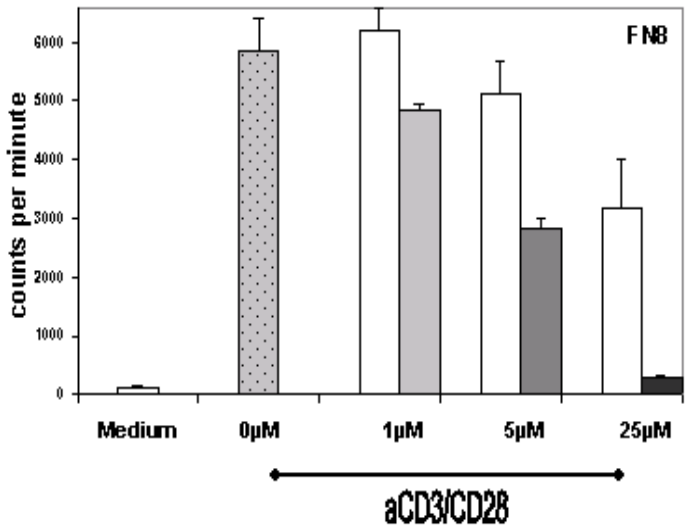

Figure 20 **Atorvastatin inhibits proliferation independently of antigen presentation**
The representative human MBP-specific TCL FN8 was stimulated with or without 1µg/ml coated anti-CD3 and 2.5µg/ml soluble CD28 in the absence or presence of different concentrations of atorvastatin (filled bars in grayscale) or vehicle alone (open bars), and following 72h incubation ^{3}[H]-thymidine uptake, as a measure of proliferation, was assessed.

4.4.3 No role of atorvastatin in early T cell activation

In T cells a rise in $[Ca^{2+}]_i$ is one of the first events occurring following stimulation. In this study, the thapsigargin model of calcium influx was used once again to directly monitor the influence of atorvastatin on CRAC (s. Sections 1.1.1, 3.4 and Results 4.3.3). For this experiment a flow cytometric method was used, adapted from Griffioen et al. (Griffioen et al., 1989). The analyses were performed on a flow-activated cell sorter (BD FACS LSR, Becton Dickinson), designed for Ca^{2+} flux measurements.

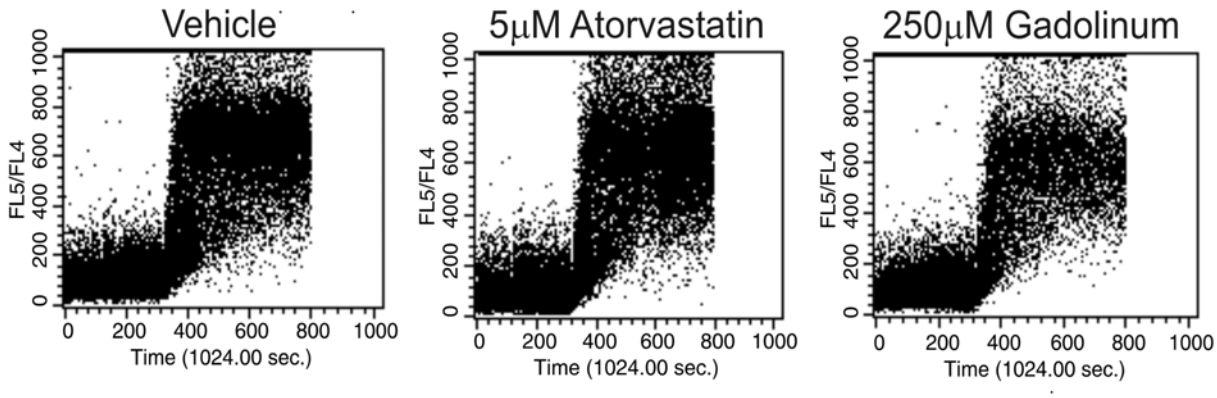

Figure 21 **Intracellular calcium measurement using flowcytometry**
The representative Bet-specific T cell line EG8 is demonstrated as an example for the measurement of Ca^{2+} influx by flow cytometry. Cells were incubated for 1h in the absence (vehicle) or presence of atorvastatin and Ca^{2+} influx was monitored, using a thapsigargin model of CRAC activation. Gadolinum, a Ca^{2+} entry blocker, was used as positive control.

As shown in Fig. 21-22 atorvastatin did not mediate any influence on calcium influx therefore indicating no impact on early T cell activation.

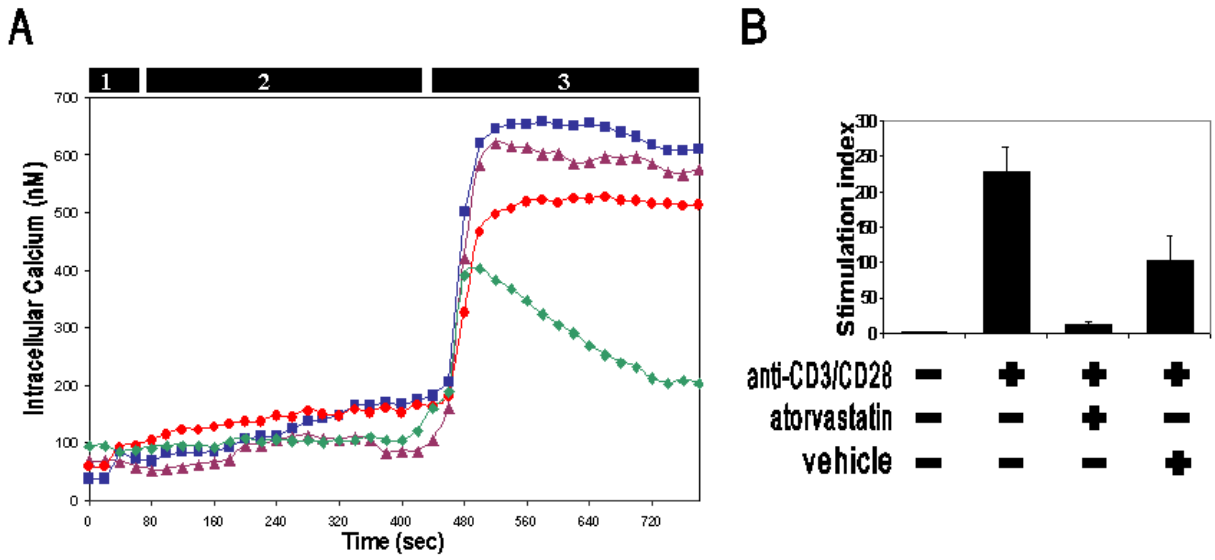

Figure 22 **Atorvastatin does not influence Ca^{2+} influx**
(A) Quantification of the intracellular calcium levels shows no effect of atorvastatin on calcium influx. Cells were incubated for 1h at 37°C in the absence (■) or presence of 5µM atorvastatin (▲) and corresponding vehicle control (●). 250µM Gadolinum (◆) was used as positive control. Indo-1/AM-loaded resting T cell lines were incubated at 3-4 x 10^6 cells/ml with 0.5mM EGTA for 5min, during the last minute of which the calcium trace measurement was started ①. To activate CRAC by depletion of intracellular stores, cells were treated with 2µM thapsigargin for 5-6min ② and Ca^{2+} entry was observed upon addition of 1.2mM-extracellular Ca^{2+} ③. (B) During the same time point, EG8 showed hypoproliferation with atorvastatin as analyzed by [^{3}H]-thymidine uptake.

4.4.4 Impact of atorvastatin on cell cycle regulation

Statins block the synthesis of isoprenoid intermediates essential for the isoprenylation and function of intracellular signaling molecules such as Ras and Rho (rev. Liao, 2002). Geranylgeranylated Rho, for example, has been reported to be essential for the degradation of $p27^{Kip1}$ (Hirai et al., 1997) and therefore Rho's inhibition by statins could result in the upregulation of this inhibitor of cyclin dependent kinases, thereby restricting the progression of T cells from the G1 to S Phase in T cells. For this reason the influence of atorvastatin on the expression of this negative regulator of cell cycle was investigated. In addition, CDK4 expression was analyzed. The expression levels of CDK4 and the kinase inhibitor $p27^{Kip1}$ were measured following 24h anti-CD3/CD28 stimulation in the presence or absence of atorvastatin.

Growth inhibition by atorvastatin was associated with an inhibition in the regulation of cell cycle progression (Fig. 23) as shown by a downregulation of the positive cell cycle regulator CDK4 and upregulation of the negative cell cycle regulator $p27^{Kip1}$. This finding, together with a direct effect of atorvastatin on T cell function suggests an inhibition of T cell cycle progression at the late part of the G1 phase.

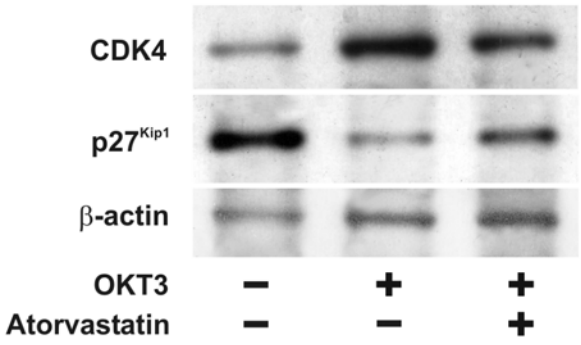

Figure 23 **Atorvastatin modulates expression of cell cycle regulators**
The representative human Bet-specific T cell line EG8 was stimulated by anti-CD3/CD28 in the presence or absence of atorvastatin, and the expression of CDK4 and the CDK inhibitor $p27^{Kip1}$ was assessed after 24h by immunoblotting. During the same time point, EG8 showed hypoproliferation with atorvastatin as analyzed by [^{3}H]-thymidine uptake (see Fig. 22).

4.4.5 Reversibility of atorvastatin-induced effects by L-mevalonate

Since atorvastatin can confer its immunomodulatory effects both via (Kwak et al., 2000) and independently of (Weitz-Schmidt et al., 2001) HMG-CoA reductase inhibition, the role of the HMG-CoA pathway (Fig. 17) in the observed T cell targeted antiproliferative effect was

investigated by employing mevalonate. By employing this product of HMG-CoA reduction the reversibility of the atorvastatin-induced effects could be examined.

Mevalonate was used at a concentration of 200µM. L-mevalonic acid lactone (Sigma) was activated by 1N NaOH. The resulting solution was neutralized with 1N HCl to pH 7.2, diluted with distilled water, and filter-sterilized.

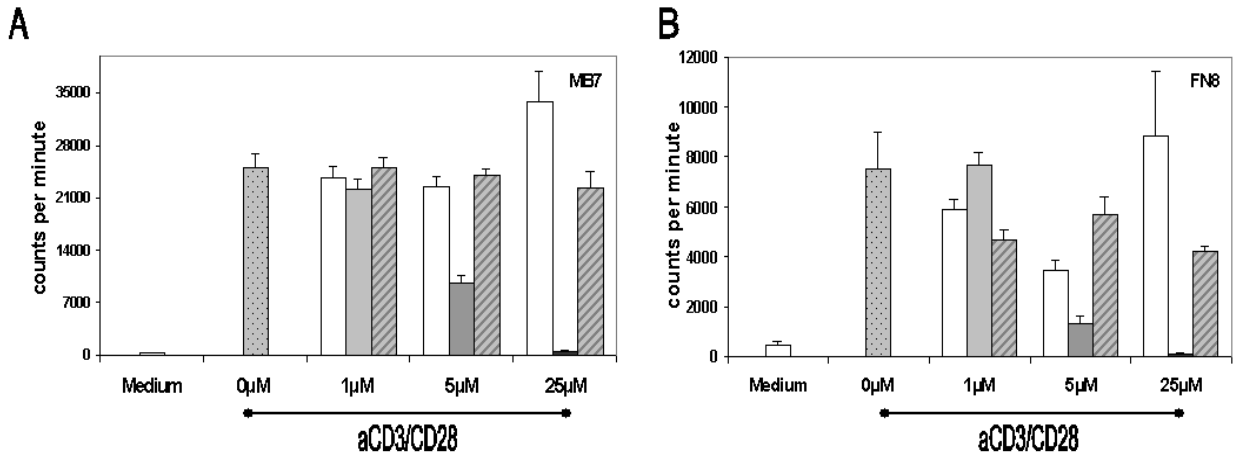

Figure 24 **HMG-CoA-reductase-dependent T cell hypoproliferation by atorvastatin**
The Bet-specific MB7 (A) and MBP-specific FN8 (B) TCLs were stimulated with anti-CD3/CD28 together with increasing doses of atorvastatin and corresponding vehicle dilutions (open bars) and the presence (hatched bars) or absence (grey-scale filled bars) of 200µM L-mevalonate.

As shown in Fig. 24, the inhibitory effects of atorvastatin on ^{3}[H]-thymidine uptake and therefore T cell proliferation could be reversed by L-mevalonate, providing direct evidence that the immunomodulatory effects of atorvastatin are mediated by inhibition of HMG-CoA reductase. Additionally the influence of atorvastatin on cell cycle regulation could also be inverted by addition of mevalonate as shown by the return of p27 to normal levels (Fig. 25). This indicates that the cell cycle arrest brought about by statins could be reversed and could therefore be mediated via HMG-CoA reductase mechanisms.

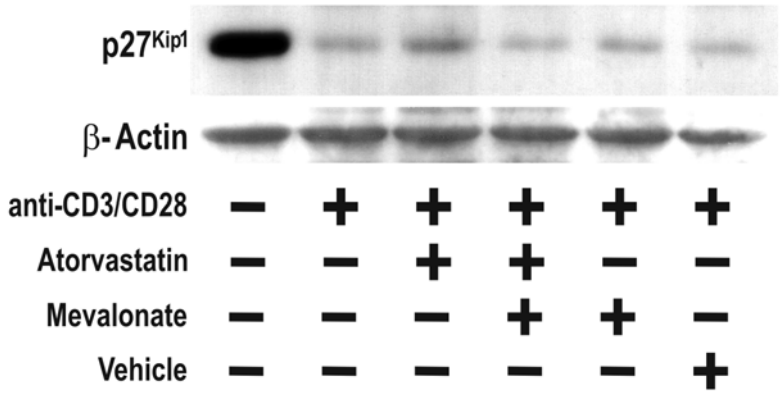

Figure 25 **Role of HMG-CoA-reductase in p27^{Kip1} regulation**
The same cell line, MB7, demonstrated a reversibility of the atorvastatin-induced upregulation of the CDK inhibitor p27^{Kip1} by 200µM mevalonate. Resting cells were co-treated with mevalonate and atorvastatin throughout the 24h anti-CD3/CD28 stimulus.

5 DISCUSSION

The pathological mechanisms involved in the autoimmune disease of the CNS, multiple sclerosis (MS), are far from completely understood. The current hypothesis is that autoreactive T cells, which are not controlled by apoptotic mechanisms such as activation-induced cell death (AICD), are orchestrating the pathogenesis of this condition. In this thesis, a reduction in the susceptibility of T cells from MS patients to undergo AICD was reported. A dysregulation in T cell control might be associated with an imbalance in apoptotic molecules. In fact, this study implicates an increase in Bcl-X_L levels as potential mechanism for the observed resistance towards AICD in MS patients. According to the current hypothesis, autoreactive T cells, specific towards myelin antigen, transmigrate the blood-brain barrier into the CNS. After further presentation of autoantigen by glial cells, these pro-inflammatory T cells get reactivated and instigate the inflammatory and destructive process observed in MS. Thus, it became clear that T cell activation, proliferation and elimination are major players in the pathogenesis and therefore potential targets for therapeutic strategies in MS. The focus of this thesis was to identify the role of regulatory molecules for T cell survival in the immune pathogenesis of MS, and to investigate antiproliferative or apoptosis-promoting effects on T cells by potential therapeutic targets. In this dissertation the 2 candidate substances TNF-related apoptosis inducing ligand (TRAIL) and atorvastatin were investigated and were both shown to interfere with cell cycle progression in antigen-specific T cell lines.

IDENTIFICATION OF THE ROLE OF T CELL APOPTOSIS-REGULATING MOLECULES

A limitation in the apoptotic regulation of autoreactive T cells in the periphery and in the CNS may contribute to the pathophysiology of MS. One of the aims of this thesis was to identify the role of apoptosis regulating molecules associated with T cell elimination in MS. Members of the Bcl-2 family are renowned regulators of apoptosis, critical for normal development and maintenance of T cell homeostasis (rev. Adams and Cory, 1998). In particular, Bcl-X_L has a predominant role in T cell growth and death (Boise et al., 1995; Broome et al., 1995; Peter et al., 1997). On investigating the protein expression of the three main members of the Bcl-2 family (Bcl-X_L, Bcl-2, and Bax), anti-apoptotic member Bcl-X_L was reported to be increased in MS patients (Fig. 5), whereas expression of both, anti-apoptotic Bcl-2 as well as pro-apoptotic Bax were unaltered (Fig. 7). In contrast to Bcl-2, Bcl-X_L was reported to be upregulated upon T cell receptor-mediated activation,

determining resistance of T cells towards CD95-mediated apoptosis. In fact transformed T cells could be rendered resistant to apoptosis by transfection with Bcl-X_L (Boise et al., 1995) and susceptibility towards apoptosis was accompanied by a downregulation of Bcl-X_L levels (Peter et al., 1997). In mice, transgenic expression of Bcl-X_L increased T cell antigen-specific proliferation (Issazadeh et al., 2000) and enhanced T cell survival in vitro when these cells were left unstimulated or stimulated without anti-CD28 (Wells et al., 1999). In fact, blockade of T cell clearance by overexpressing Bcl-X_L led to earlier onset and more severe expression of EAE, as well as a reduction in remission (Issazadeh et al., 2000).

Since Bcl-2 expression was also reported to be comparable in T cells of MS patients and neurologic controls by others (Semra et al., 2001) but was shown to correlate with remyelination in MS plaques, specifically oligodendrocytes (Kuhlmann et al., 1999), this survival molecule might play a more important role in the apoptosis modulation of cells primarily localized within the CNS.

Although the mechanism of action of Bcl-2 family members remains to be fully clarified, these factors have been reported to regulate cell survival. They accomplish this function by either promoting or suppressing apoptotic pathways initiated at the mitochondria (Memon et al., 1995; Strasser et al., 1995; Erhardt and Cooper, 1996), where Bcl-X_L and the other members of the Bcl-2 family are predominantly located (Hsu et al., 1997). The mitochondrion is one checkpoint in intrinsic apoptotic pathways that activates its own initiator and effector caspases via compartmentalization of cytochrome *c* (rev. Vander Heiden and Thompson, 1999). It has been suggested that peripheral T cells might be independent of mitochondrial functions following CD95 signaling (Scaffidi et al., 1998). However, growing evidence shows that the extrinsic and intrinsic pathways of caspase activation are tightly interconnected (Li et al., 1998). Overexpression of Bcl-X_L was correlated with a decreased susceptibility of polyclonal T cells from MS patients to undergo AICD (Fig. 9). AICD is an apoptotic deletional mechanism, involving the CD95 system, which is supposed to regulate peripheral T cell tolerance. In the in vivo situation, AICD occurs when previously primed T cells are repeatedly activated (rev. Van Parijs and Abbas, 1996; rev. Janssen et al., 2000). An in vitro model of AICD was used in this study, a model which via repetitive mitogenic stimulation attempts to exemplify the susceptibility or resistance of antigen-reactive T cells towards apoptosis in vivo. AICD data showed an impairment of T cells from MS patients to undergo apoptosis (Fig. 8). An inverse correlation of Bcl-X_L levels with susceptibility of T cells to undergo AICD (Fig. 9) is in line with previous data on the significance of this anti-apoptotic protein in T cell resistance (Peter et al., 1997).

The observation of elevated Bcl-X_L levels in MS patients together with an involvement of this molecule in the earlier induction and reduced remission of EAE (Issazadeh et al., 2000) offer perspectives towards manipulation of this apoptosis-regulating molecule as a means of therapy in MS. Antisense oligonucleotide therapies directed against members of the Bcl-2 family are in fact currently under investigation in studies on cancer cell lines (Leech et al., 2000; Olie et al., 2002) as well as in the treatment of lymphoma patients (Webb et al., 1997; Waters et al., 2000).

MODULATION OF T CELL RESPONSE AS A THERAPEUTIC STRATEGY

The treatment of autoimmune diseases is still in its infancy: glucocorticoids and other immunosuppressants remain the mainstay therapies. Three types of medications are currently approved for the treatment of MS. These include various formulations of IFN-β; glatiramer acetate (GA), a random copolymer of four amino acids; and mitoxantrone, a drug previously approved for use in cancer. The most encouraging results have been obtained with the Type I interferon, IFN-β (rev. Chofflon, 2000). **IFN-β** has been shown to reduce relapse rates, slow the progression of disability and substantially reduce the accumulation of new MRI lesions in patients with relapsing-remitting (RR) MS (The IFNB Multiple Sclerosis Study Group, 1993; Johnson et al., 1995; Jacobs et al., 1996; Jacobs et al., 2000; Comi et al., 2001). IFN-β has been suggested to mediate its effect by inhibiting T cell activation (Rudick et al., 1993) and by interfering with lymphocyte migration into the brain (Stuve et al., 1996). Type-I interferons have also been reported to augment AICD of T cells, not only in healthy controls (Kaser et al., 1999b) but also in patients suffering from MS (Kaser et al., 1999a). On the other hand, **GA**, which was actually developed due to its ability to suppress EAE in various forms in rodents and primates, alters the cytokine production by autoimmune T cells and competes with MBP at the MHC and TCR level (Milo and Panitch, 1995). Since 1993, IFN-β1b and IFN-β1a, along with synthetic GA have been implemented as immunomodulatory agents for the treatment of MS in Europe and the US (rev. Galetta et al., 2002). However, the heterogeneity of autoimmune diseases such as MS challenges investigations for the discovery of new immune interventions, which are more effective than the present pleiotropic medications available. In fact current therapies are only moderately effective and reduce disease exacerbations by only 30% (Johnson et al., 1995; The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1995). Based on clinical experience with rather non-selective therapies, the goal is to identify newer target-specific substances with better selectivity to maximize the benefit to risk ratio.

Here, two different substances, both interfering with cell cycle regulation, were investigated. The *first candidate* was the recently discovered member of the TNF/NGF family of death ligands, TNF-related apoptosis inducing ligand (TRAIL) and the *second candidate* was atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase inhibitor and lipid-lowering drug already indicated for anomalies in lipid metabolism.

T cell modulation by TNF-related apoptosis-inducing ligand (TRAIL)

Death ligand TRAIL was originally thought to have the capacity to induce T cell elimination due to its high-affinity binding properties to death-inducing receptors (Truneh et al., 2000). However a selectivity of TRAIL in its killing has been shown. Although T cells express the death-inducing TRAIL receptors 1 and 2, they were shown to be resistant to apoptosis by soluble leucine-zipper TRAIL in vitro (Wendling et al., 2000). Nonetheless, studies on *animal* models of autoimmune diseases suggested an influence of TRAIL on T cell growth and effector function since systemic neutralization by TRAIL receptor 2 was demonstrated to exacerbate collagen-induced arthritis (Song et al., 2000) and experimental autoimmune encephalomyelitis (Hilliard et al., 2001). In this study, immunoregulatory effects of TRAIL, other than apoptosis, were investigated in *human* untransformed antigen-specific human T cell lines, which were previously shown to be immune to TRAIL-induced apoptosis. The results obtained from this study show that TRAIL is capable of inhibiting T cell activation, subsequent cell cycle progression, and cytokine production in human antigen-specific T cells. Exogenous treatment of TRAIL to antigen-specific TCLs resulted in an inhibition in proliferation, albeit to varying degrees and irrespective of T helper differentiation or donor (Table 7). Whereas marked dose-dependent inhibition was apparent in one third of the T cell lines, the other T cell lines exhibited only slight effects. Both foreign (TT/Bet-specific) and autoreactive (MBP-specific) T cell lines and T cells derived from patients with multiple sclerosis as well as healthy individuals were inhibited. The reduction of T cell proliferation was also independent of antigen specificity. Since the inhibitory effect on T cell proliferation by exogenously applied TRAIL was shown to be dose-dependent, lower TRAIL concentrations in supernatants of activated T cells, which are comparable to serum levels of healthy volunteers (data not shown), might be ineffective in modulating T cell growth. However, this does not exclude a possible impact of soluble TRAIL on T cell activation and growth in inflammatory situations. Of note, an upregulation of TRAIL in peripheral immune cells of MS patients could be explained as a secondary compensatory mechanism that downregulates the inflammatory response (Huang et al., 2000).

An independency on antigen presentation was concluded since the inhibitory effect was also observed in anti-CD3/CD28-stimulated T cells (Fig. 12). Thus, TRAIL directly influences T cell function. Concerning the mechanisms of the observed apoptosis-independent properties of death ligand TRAIL, an interference with cell cycle regulation was revealed. Cell cycle regulators such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors are important for the coordination of T cell proliferation. Cells entering the G1 phase after TCR engagement are characterized by an upregulation of cyclin D and CDK4/6 (Modiano et al., 1994; Kwon et al., 1997). Investigating CDK4 expression in antigen-specific T cells, in parallel to the TRAIL-induced hypoproliferation, showed a downregulation of this positive cell cycle regulator with TRAIL (Fig. 15), indicating an inhibition of cell cycle progression at the G1/S transition. Apart from their known function in cell cycle progression, cell cycle regulators have also been shown to control mechanisms implicated in T cell tolerance, such as anergy (rev. Balomenos and Martinez, 2000). The cycling inhibitor $p27^{Kip1}$ contributes to the association and activation of cyclin D with their complementary CDK and was recently demonstrated to be important for the induction and maintenance of T cell anergy (Boussiotis et al., 2000; Jackson et al., 2001). No evidence for an involvement of TRAIL in peripheral T cell tolerance was found since $p27^{Kip1}$ expression was unaltered (Fig. 15) and IL-2 production upon secondary antigen challenge remained the same (data not shown).

Calcium influx is crucial to lymphocyte activation, including cytokine generation and cell proliferation (rev. Qian and Weiss, 1997) and the inhibition of calcium-dependent signaling pathways was shown to completely suppress T cell activation (rev. Lewis and Cahalan, 1995). To identify whether the inhibitory effect of TRAIL could be related to alterations in calcium signaling, a thapsigargin-model for monitoring calcium influx was utilized. Following TRAIL incubation, an inhibition of calcium entry through calcium-release activated channels (CRAC) was observed in human T cells. Inhibition of CRAC channels has also been described for other TNF-superfamily members. Applying the same model utilized here for evoking calcium entry, TNF-α was reported to inhibit store-operated calcium influx in a rat thyroid cell line (Tornquist et al., 1999) and CD95-stimulation was reported to inhibit activation of calcium channels and subsequent IL-2 synthesis in apoptosis-resistant Jurkat T cells (Lepple-Wienhues et al., 1999). The latter observation indicates that the CD95 system might play a role in anergy induction prior to or in the absence of apoptosis. TRAIL, however, reduced calcium influx to a level, just within the lower concentration limit required for subsequent IL-2 production and proliferation (Negulescu et al., 1994), and thus showed no anergy-inducing properties in the T cell lines investigated.

The present observations of apoptosis-independent immunomodulatory properties of TRAIL in vitro, implicate TRAIL in the regulation of inflammatory conditions such as those involved in the autoimmune disease MS. However, TRAIL seems to play an additional role in T cell effector-functions within the CNS. TRAIL has been shown to induce massive cell death of brain cells, including neurons, astrocytes, and oligodendrocytes (Nitsch et al., 2000). Therefore, untransformed human brain tissue, which lacks TRAIL but expresses apoptosis-mediating TRAIL receptors on oligodendrocytes and neurons, is potentially susceptible to TRAIL-mediated apoptosis (Dorr et al., 2002a). Since T cells upregulate TRAIL upon activation (Wendling et al., 2000), the scenario might be that T cells, which invade the brain, might induce cell death of the parenchymal cells via TRAIL /TRAIL receptor interaction. In fact activated T cells could induce TRAIL-mediated glioma cell death (Dorr et al., 2002b). Additionally cell-to-cell contact was a prerequisite for this TRAIL-mediated brain cell apoptosis, indicating an involvement of surface-expression of this ligand in the cytotoxicity observed in the CNS pathology. Therefore, the roles of surface-expressed and soluble TRAIL need to be dissected.

As presented here, soluble TRAIL inhibited T cell activation and cell cycle progression in the present in vitro study. This indicates that unlike the membrane-bound form, high amounts of soluble TRAIL are rather involved in systemic immunomodulation. Additionally, the suppression of calcium-dependent lymphocyte activation might represent a primary mechanism responsible for the immunomodulatory properties of TNF/NGF superfamily members. These molecules are known to be critically involved in the regulation of immune responses and are currently being targeted for therapeutic modulation in autoimmune and malignant diseases (Rau, 2002).

T cell modulation by Atorvastatin

Another potential therapeutic candidate analyzed here was atorvastatin, from the statin group of drugs. Statins, also referred to as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, were originally indicated for the sole treatment of lipid anomalies. These effects are attributed to alteration in cholesterol metabolism (end product of HMG-CoA pathway) and reduction in low-density lipoprotein (LDL) formation (rev. Maron et al., 2000). However, in 1995 a new mode of action for statins was discovered since increased survival in cardiac transplant recipients following pravastatin therapy was reported to be independent of its cholesterol-lowering effects (Kobashigawa et al., 1995). This observation prompted subsequent in vitro studies, which demonstrated that statins interfered with production of several important proinflammatory mediators (Pahan et al., 1997; Youssef et

al., 2002). Lovastatin, like atorvastatin an HMG-CoA reductase inhibitor, suppressed production of inducible nitric oxide synthase (iNOS) and secretion of TNFα by IFN-γ-activated astrocytes and microglia (Pahan et al., 1997). iNOS and TNFα may play important roles in the inflammatory process of MS (Steinman, 2001). In fact lovastatin blocked the development of acute inflammation during EAE by inhibiting iNOS, TNFα and IFN-γ expression in the CNS (Stanislaus et al., 2001) but most probably also by inhibiting LFA-1 (Weitz-Schmidt et al., 2001) and IFN-γ-induced MHC class II expression (Kwak et al., 2000). Kwak et al. demonstrated that statins inhibit the IFN-γ-induced expression of MHC class II on most APC, including B cells and macrophages, by suppressing the inducible promoter IV of the transactivator CIITA (Kwak et al., 2000). A recent study showed that certain statins inhibit the LFA-1-dependent stimulation of T cells and that a lovastatin-based LFA-1 inhibitor reduces infiltration of neutrophils in the murine thioglycollate-induced peritonitis model (Weitz-Schmidt et al., 2001). Overall, these data indicate the potential of cholesterol-reducing agents, such as atorvastatin, in MS therapy (Bradbury, 2002).

Indeed, atorvastatin inhibits the inflammation observed in the animal model of MS in both a preventive as well as therapeutic manner (Youssef et al., 2002; Aktas et al., 2003). Having demonstrated the inhibitory effects of atorvastatin on *murine* cells in vivo and in vitro (Aktas et al., 2003), the influence of this statin on the proliferation of *human* antigen-specific TCLs was investigated. As indicated in Fig. 18, antigen-specific proliferation of both a birch pollen (Bet)-specific TCL (MB7) as well as an MBP-specific TCL (FN8) was suppressed by atorvastatin in a dose-dependent manner. Since inhibition of proliferation was also observed when atorvastatin was added to proliferating T cells, 24h or 48h after antigen presentation (Fig. 19) an MHC class II independent pathway was thought to be involved. To confirm this, the ability of atorvastatin to block T cell proliferation in response to direct T cell receptor engagement, independently of antigen-presentation, was tested. As shown in Fig. 20 proliferation of the same TCLs following stimulation with anti-CD3/CD28 was markedly suppressed by atorvastatin. Thus, in an environment lacking APC, which express MHC class II and ICAM-1, atorvastatin is nonetheless capable of inhibiting proliferation. Therefore, a reduced MHC class II induction (Kwak et al., 2000) and blockade of LFA-1/ICAM-1 interactions (Weitz-Schmidt et al., 2001), required for the transmigration of mononuclear cells into the CNS (Stanislaus et al., 2001) is not sufficient to explain the antiproliferative effect of atorvastatin in human anti-CD3/CD28-stimulated T cells (Fig. 20).
No impact of atorvastatin on early T cell activation was observed, since calcium influx was unaffected (Fig. 21-22). This suggests that the left arm of the T cell signaling cascade initiated by protein tyrosine kinases (PTKs) as depicted in Fig. 3 to be unaffected by statins.

An influence of statins on cell cycle regulation has previously been reported in rat mesangial cells (Danesh et al., 2002) and in aortic cells by upregulation of $p27^{Kip1}$ (Weiss et al., 1999) via Ras and/or Rho, a family of GTPase proteins from the Ras superfamily. Ras promotes cell cycle progression via activation of the mitogen-activated protein kinase pathway (rev. Hughes, 1995) whereas Rho causes cellular proliferation possibly through destabilizing $p27^{Kip1}$ protein (Hengst and Reed, 1996), responsible for the blockade of clonal expansion of anergic T cells (Boussiotis et al., 2000). GTPase proteins function as GDP/GTP-regulated switches that cycle between an active GTP-bound state and an inactive GDP-bound state. They accumulate in the vicinity of the T cell membrane following TCR ligation and phosphorylation of guanine nucleotide exchange factor (GEFs; such as Grb2 and SOS) by upstream protein tyrosine kinases (PTKs) (Nel et al., 1995). Figure 26 shows a schematic representation of how specific Ras signaling pathways link with the regulation of cell cycle progression (rev. Pruitt and Der, 2001).

Apart from necessitating activation by GEFs to switch to an active GTP-bound state, GTPases require posttranslational modification, specifically isoprenylation, in order to fulfill their function. Protein isoprenylation permits the covalent attachment, subcellular localization, and intracellular trafficking of membrane-associated proteins (rev. Liao, 2002). This process is mediated by isoprenoid intermediates of the HMG-CoA cholesterol biosynthetic pathway, such as geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) (see Fig. 17). Farnesylation of Ras by FPP recruits serine-threonine kinase Raf-1 to the membrane (Fig. 3) and results in the activation of the MAPK/ERK signaling pathways (rev. Rincon et al., 2000) and geranylgeranylation of Rho by GGPP has been reported to be essential for the degradation of $p27^{Kip1}$ (Hirai et al., 1997) (Fig. 26). Therefore, the isoprenylation of GTPase molecules is essential for the activation of downstream signaling pathways involved in cell cycle progression. Indeed, MAPK signaling following farnesylation of Ras is important for the induction of AP-1 transcription factors, consisting of dimeric proteins such as the Jun and Fos sub-family. c-Jun was reported to be necessary for the expression of cyclin D1 (a positive regulator of cell cycle, specifically G1/S progression), which associates with cyclin dependent kinases (CDK). Regulators of the cell cycle such as CDK4 are important for the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein (Fig. 26), which otherwise binds to E2F, recruiting histone deacetylases to the promoters of E2F-responsive genes and repressing their transcription (Wisdom and Verma, 1993).

Therefore, by blocking HMG-CoA reductase and mevalonate synthesis, statins prevent the synthesis of important isoprenoid intermediates of the cholesterol biosynthetic pathway,

important for the isoprenylation of Ras and Rho, ultimately important for cell cycle progression. The possible interference at the T cell cycle level by atorvastatin was investigated in this study. The growth inhibition by atorvastatin was associated with a downregulation of CDK4 and an increased expression of $p27^{Kip1}$ (Fig. 23), thus revealing an inhibition of cell cycle progression by atorvastatin at the late part of the G1 phase.

The proposed atorvastatin mechanism of action on APC-independent T cell cycle progression and proliferation, suggests a direct influence of statins on HMG-CoA reductase. To confirm this hypothesis, T cells were cotreated with an intermediate product of HMG-CoA reductase, mevalonate, along with the statin. In fact, this analysis revealed a reversibility of the statin-induced hypoproliferation (Fig. 24) as well as cell cycle arrest (Fig. 25), following co-administration with mevalonate. Therefore, one may deduce that atorvastatin is rather involved in an inhibition of isoprenylation of Rho or Ras (by FPP and GGPP), necessary for MAPK signaling and the destabilization of the CDK inhibitor $p27^{Kip1}$.

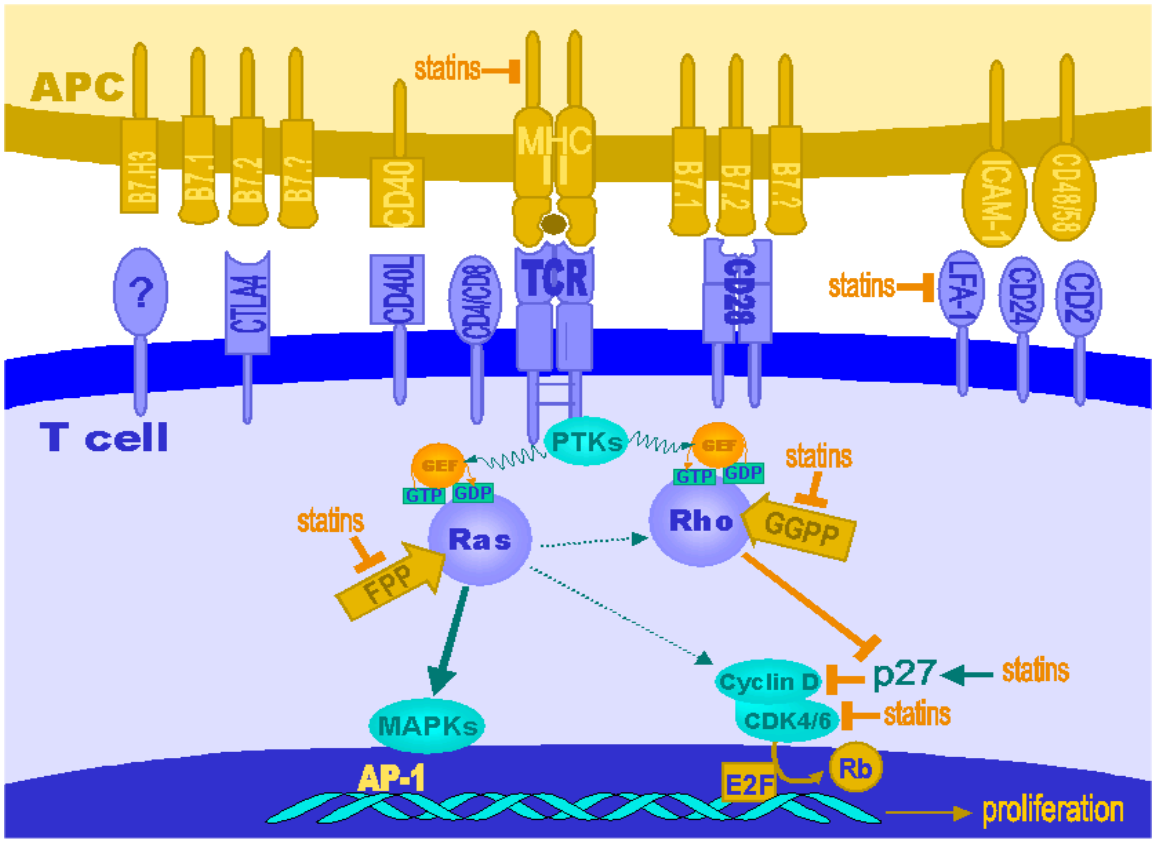

Figure 26 **Proposed mechanisms of action of statins in immunomodulation**
Statins inhibit IFN-γ-induced expression of MHC class II on APC and the LFA-1-dependent stimulation of T cells. This study extends a role for statins in T cell–cycle regulation. By inhibiting the HMG-CoA reductase pathway and therefore isoprenoid synthesis, statins prevent the isoprenylation of Ras and Rho (required for cell-cyle regulation) by farnesylpyrophosphate FPP and geranylgeranyl-pyrophosphate GGPP (adapted from (rev. Pruitt and Der, 2001)).

Since atorvastatin mediated an upregulation of $p27^{Kip1}$, an involvement of statins in the induction of anergy as a mechanism of peripheral T cell tolerance is feasible. Future studies should aim at investigating the involvement of atorvastatin in anergizing T cells, possibly by monitoring IL-2 production and proliferation following preincubation with statin and reencounter with antigen. The unresponsive state observed in some models of T cell anergy may also be the result of CTLA-4 receptor upregulation, which antagonizes the effects of CD28 (Perez et al., 1997). Therefore, such prospective studies would determine whether the HMG-CoA-reductase mediated statin-induced cell cycle arrest to be mediated via an influence on costimulatory signals.

CONCLUSIONS AND PERSPECTIVES

Most therapies, both those currently employed in the clinic and those under preclinical investigation, induce their immunomodulatory effects, either by promoting apoptotic elimination of potentially harmful T cells or by inhibiting activation and further proliferation of these cells. Although this is the mode of action of currently available medications, these drugs are pleiotropic in their actions and have a low success rate in reducing relapses. New treatment approaches are still under preclinical investigation or undergoing clinical trials and include monoclonal antibodies to immune system receptors, cytokines, and chemokines or the application of drugs previously approved and already employed in other diseases. Concerning the latter therapeutic approach and in view of the data described here on atorvastatin, clinical trials, including a simvastatin multicenter phase II study, are currently being undertaken for the safety evaluation of statins in MS (National Multiple Sclerosis Society Advisory Committee, 2002, Internet Communication). Additionally, the observation of an elevation in Bcl-X_L levels in patients which correlates with a decreased susceptibility of their T cells to undergo apoptosis also offers perspectives towards therapeutic manipulation in MS. Antisense oligonucleotide therapies directed against members of the Bcl-2 family are for example already under investigation in the treatment of lymphoma patients (Webb et al., 1997; Waters et al., 2000). Additionally, a large number of pre-clinical studies on other potential therapeutic strategies targeting T cell responses have been reported. Such studies include the selective phosphodiesterase type 4 inhibitor rolipram (Sommer et al., 1997), the tetracycline minocycline (Nessler et al., 2002; Popovic et al., 2002) and the thiazolidinedione peroxisome proliferator-activated receptor agonist pioglitazone (Feinstein et al., 2002). All these candidates have been shown to reduce and/or protect against the clinical signs of EAE. A prerequisite for finding innovative treatment strategies for MS is to identify mechanisms influenced upon inhibiting T cell function and thereafter targeting the

identified molecules within the complex signaling machinery with highly selective agents. Novel molecules targeting specific intracellular molecules with high selectivity would enhance the efficacy to risk ratio associated with therapy. The reported interference with cell cycle regulation as underlying mechanism for TRAIL and atorvastatin-mediated modulation of human T cell responses has therapeutic implications. This is further supported by data on the influence of both agents on T cell growth and effector function in the EAE animal model (Hilliard et al., 2001; Youssef et al., 2002; Aktas et al., 2003). While the TRAIL-mediated interference with T cell activation and further cell cycle progression is still in the pre-clinical phase, statins, which have also been shown here to interfere with the T cell cycle, are already employed in the clinic for other ailments.

The overall goal is to identify new mechanisms involved in the immunomodulatory role of effective therapeutic agents on T cells, thus enabling the successive development of highly selective pharmacological intervention in MS.

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[*]equally contributed

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ZUSAMMENFASSUNG

Multiple Sklerose (MS) ist eine heterogene Krankheit des Zentralnervensystems, deren pathologische Mechanismen noch nicht vollständig aufgeklärt sind. Die gegenwärtige Hypothese ist, daß pro-inflammatorische T-Zellen entscheidend an der Pathogenese der MS beteiligt sind. Man geht davon aus, daß eine Fehlregulation der T-Zell-Kontrolle, möglicherweise bedingt durch ein Ungleichgewicht an Apoptose-regulierenden Molekülen, dabei eine Rolle spielt. Tatsächlich zielen therapeutische Strategien darauf ab, T-Zell-Aktivierung, Proliferation und Produktion von Zytokinen zu verringern, oder T-Zell-Eliminierung zu fördern. Diese Arbeit sollte zum einen die Bedeutung regulatorischer Faktoren klären, die für das überleben der T-Zellen von MS-Patienten verantwortlich sind. Zum anderen sollten die antiproliferative oder Apoptose-fördende Wirkung potentiell therapeutisch wirksamer Moleküle untersucht werden.

Eine eingeschränkte Regulation der autoreaktiven T-Zellen durch Apoptose in der Peripherie und im ZNS trägt möglicherweise zur Pathophysiologie der MS bei. Als Schlüsselfaktoren der Regulation von Apoptose wurden Mitglieder der Bcl-2-Familie in MS-Patienten und Probanden untersucht. Diese Faktoren wurden in Relation zu der Suszeptibilität der T-Zellen gegenüber aktivierungsinduziertem Zelltod (sog. Activation-induced cell death oder AICD) überprüft. Um die in-vivo-Elimination der Antigen-reaktiven T-Zellen nachzuahmen, wurde ein in-vitro-Modell des AICD mit repetitiver T-Zell-Stimulation verwendet. Tatsächlich zeigten polyklonale T-Zellen von MS-Patienten eine verringerte Suszeptibilität für AICD, nachgewiesen sowohl durch verminderte Caspaseaktivtät ($p=0.013$) als auch durch DNA-Fragmentierung ($p=0.0071$). Weiter wurden höhere Spiegel des Proteins Bcl-X_L in den Immunzellen von MS-Patienten mit Immunoblotting gemessen ($p=0.014$). Eine inverse Korrelation zwischen der Expression an Bcl-X_L und der Empfindlichkeit der T-Zellen gegenüber AICD steht in Übereinstimmung mit vorhergehenden Daten bezüglich der Bedeutung dieses Proteins für die Apoptose-Resistenz von T-Zellen. Es wurde bereits gezeigt, daß dieses Molekül die Ausprägung der experimentell-autoimmun Enzephalomyelitis, des Tiermodells der MS, verstärkt. Zusammen mit den erhöhten Bcl-X_L-Werten bei MS-Patienten, ergeben sich nun Perspektiven für einen therapeutischen Ansatz.

Abgesehen von dem Konzept die apoptotische Eliminierung von T-Zellen zu unterstützen, streben gegenwärtige therapeutische Strategien an, die Aktivierung und

weitere Proliferation der schädlichen T-Zellen zu hemmen. Basierend auf klinischer Erfahrung mit eher unselektiven Therapien, ist es ein therapeutisches Ziel, neue immunomodulatorische Substanzen mit besserer Selektivität zu finden, um das Nutzen/Risiko-Verhältnis zu maximieren. Aus diesem Grund wurden zwei unterschiedliche Substanzen untersucht die beide den Zellzyklus beeinflussen. Als *erster* Kandidat wurde der kürzlich entdeckte Todesligand TRAIL (engl.: TNF-related apoptosis inducing ligand) aus der TNF/NGF-Familie untersucht, da diesem bereits T-Zell-regulatorische Funktionen zugeschrieben worden waren, humane Antigen-spezifische T-Zellen jedoch resistent gegenüber TRAIL-induzierter Apoptose sind. Der *zweite* Kandidat mit potenziell therapeutischer Wirkung bei MS ist Atorvastatin, ein HMG-CoA-Reduktase-Hemmer, der bereits als Lipidsenker bei Patienten eingesetzt wird.

Um die Hypothese zu überprüfen, daß diese Substanzen T-Zell-Rezeptor-Signale beeinflussen können, wurden humane Antigen-spezifische T-Zell-Linien von MS-Patienten und gesunden Probanden eingesetzt. Diese wurden hinsichtlich T-Helfer-Phänotyp und Peptid-Spezifität charakterisiert. Eine Behandlung mit TRAIL führte zur Hemmung der Proliferation in unterschiedlichem Ausmaß (6.2% - 63.8%). Atorvastatin hemmte in Abhängigkeit von der Dosis ebenso die Proliferation Antigen-spezifischer T-Zellen. Beide Substanzen wirkten antiproliferativ unabhängig von der Antigenpräsentation, aufgrund ihrer Fähigkeit, die Proliferation in Abwesenheit von professionellen Antigen-präsentierenden Zellen zu vermindern. Diese Eigenschaft weißt auf einen direkten Einfluß auf die T-Zell-Funktion hin. Die TRAIL-induzierte Hypoproliferation war assoziiert mit einer Herunterregulation der Zyklin-abhängigen Kinase CDK4 (engl.: cyclin dependent kinase 4), einem Schlüsselenzym für die nach T-Zell-Rezeptor-Stimulation einsetzende Transition von der G1- zur S-Phase des Zellzyklus. Inkubation mit Atorvastatin induzierte ebenso eine Verminderung von CDK4, begleitet von einer Erhöhung von $p27^{Kip1}$. Die Atorvastatin-vermittelte Proliferations- und Zellzyklus-Blockade konnte durch Mevalonat rückgängig gemacht werden. Mevalonat ist ein Zwischenprodukt des HMG-CoA-Reduktaseweges. Atorvastatin scheint demnach einen direkten Einfluß auf diese Enzymkaskade zu haben, der wichtig für die Isoprenylierung von GTPase-Proteinen der Rho-Familie ist.

T-Zell-Rezeptor-Stimulation führt zur Freisetzung von Kalzium aus intrazellulären Speichern und nachfolgend zur Öffnung transmembranöser Kalzium-Kanäle (sog. calcium release-activated calcium oder CRAC-Kanäle), die eine für die T-Zellaktivierung

notwendige und anhaltende Erhöhung der intrazellulären Kalzium-Konzentration hervorruft. Nach Behandlung mit TRAIL wurde eine konzentrationsabhängige Inhibition des Einstroms extrazellulärer Kalzium-Ionen durch die CRAC-Kanäle beobachtet. Dies wurde mit löslichem TRAIL-Rezeptor-Fusionsprotein, einem TRAIL-Antagonisten, rückgängig gemacht. Die Blockade von Kalzium-abhängigen Aktivierungssignalen stellt damit möglicherweise einen primären immunregulatorischen Mechanismus für diese Todesliganden dar. Jedoch wurde keine Auswirkung von Atorvastatin auf die T-Zellaktivierung beobachtet, da der Einstrom von extrazellulärem Kalzium nicht beeinflußt wurde.

Während Studien zum TRAIL-vermittelten Einfluß auf die T-Zell-Aktivierung und dem Zellzyklus erst in der präklinischen Phase sind, werden Statine, die ebenfalls den Zellzyklus beeinflussen, bereits in der Therapie anderer Erkrankungen angewand. Darüber hinaus werden derzeit bereits klinische Studien mit Statinen zur MS-Therapie durchgeführt. Weitere Untersuchungen zu den detaillierten Mechanismen antiproliferativer Substanzen mit potenziellem therapeutischen Effekt in der MS ermöglichen die Entwicklung von selektiveren immunomodulatorischen Therapien mit höherem therapeutischen Nutzen für MS-Patienten.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt zu haben.

Bei der Verfassung der Dissertation wurden keine anderen als die im Text aufgeführten Hilfsmittel benutzt.

Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze einen entsprechenden Doktorgrad nicht.

Berlin, den

Sonia Waiczies

ABBREVIATIONS

Aa	Amino acid
Ab	Antibody
Ag	Antigen
AICD	Activation-induced cell death
AIF	Apoptosis-inducing factor
AP-1	Activator protein 1
APAF-1	Apoptotic protease activating factor-1
APC	Antigen-presenting cell
APS	Ammonium persulphate
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
Bet	*Betula verrucosa* (birch pollen)
BSA	Bovine Serum Albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
Caspase	Cysteine aspartyl-specific protease
CD	Cluster of Designation
CDK	Cyclin dependent kinase
CIITA	MHC class II transactivator
CNS	Central nervous system
CRAC	Calcium release-activated calcium
CTLA-4	Cytotoxic T-lymphocyte antigen
DAG	1,2-Diacyl glycerol
DEVD	aspargine-glutamine-valine-asparagine-7-amido-4-methylcumarine
DIABLO	Direct IAP binding protein with low pI
DMSO	Dimethyl sulphoxide
DR	Death receptor
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced Chemiluminescence System
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N'N'-tetraacetic acid
ER	Endoplasmic reticulum
Erk	Extra-cellular signal regulated kinase
et al.	et alii (and others)
FADD	Fas-associated death domain protein
FACS	Fluorescence activated cell sorter
FBS	Fetal Bovine Serum
Fc	Constant fragment of immunoglobulin molecule
FITC	Fluorescein-isothiocyanate
FLICE	Fas-associated death domain–like IL-1b–converting enzyme
FPP	Farnesylpyrophosphate
FSC	Forward scatter
FURA-2/AM	1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'- amino-5'- methylphenoxy)-ethane-N,N,N'N'-tetraacetic acid pentaacetoxymethyl ester
GAP	GTPase activating protein
GDP	Guanosine-5`-diphosphate

GEF	Guanine nucleotide exchange factors
GGPP	Geranylgeranylpyrophosphate
Grb2	Growth factor receptor–bound protein
GTP	Guanosine-5`-triphosphate
h	Hours
HLA	Human Leukocyte Antigen
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Horse Radish Peroxidase
IAP	Inhibitor of Apoptosis Protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IgG	Immunoglobulin G
IκB	Inhibitory κB protein
IκK	IκB kinase
IL	Interleukin
INDO-1/AM	1H-Indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2- oxoethyl]amino]-3-[2-[2-[bis[2- [(acetyloxy)methoxy]-2-oxoetyl]amino]-5- methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
Itk	Inducible T cell kinase
JNK	N-terminal c-Jun kinase
LAT	Linker for activated T cells
LFA-1	Lymphocyte function–associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Kinase
MAPKK	Mitogen Activated Kinase Kinase
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
min	Minutes
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
n	Number of experiments
NaN_3	Sodium azide
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NGF	Neural Growth Factor
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PeSt	Penicillin-Streptomycin
PHA	Phytohemagglutinin
PI_3K	Phosphatidylinositol 3'-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein Kinase C
PLC	Phospholipase C

PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PP	Pyrophosphate
PPMS	Primary progressive multiple sclerosis
PTKs	Protein Tyrosine Kinases
PTPC	Permeability transition pore complex
rad	Unit of radiation
Rb	Retinoblastoma
rev.	Review
RPMI 1640	Roswell Park Memorial Institute 1640
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SDS	Sodium dodecylsulphate
s	seconds or soluble
SEM	Standard Error of Mean
SIMP	Soluble intermembrane protein
SLE	Systemic lupus erythematosus
SLP-76	SH2 domain–containing leukocyte protein of 76 kd
SMAC	Second mitochondria-derived activator of caspase
SOS	Sons of Sevenless
SPMS	Secondary progressive multiple sclerosis
Src	Sarcoma
SSC	Sideward scatter
TBS	Tris Buffer Saline
TCL	T cell line
TCR	T cell antigen receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
Tg	Thapsigargin
TNF-α	Tumour Necrosis Factor -α
TR	TRAIL receptor
TRAIL	TNF-related apoptosis-inducing ligand
TT	Tetanus toxoid
Tween 20	Polyoxyethylene-sorbitan monolaurate
Tyr	Tyrosine

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