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Regulational expression of the CD28-related costimulatory molecule ICOS in Multiple Sclerosis: implications for the role of costimulation in autoimmunity

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1. Introduction

1.1 T cell Mediated Immune Response

T lymphocytes participate in adaptive immune responses. Upon presentation of specific peptides by antigen presenting cells (APC) T cells are activated and expand in a clonal manner and furthermore generate memory cells.

Two major classes of T lymphocytes can be distinguished by their surface markers: CD4+ and CD8+ T cells (Doherty, Zinkernagel et al. 1974). CD4+ T cells are helper T cells and recognize antigens presented by major histocompatibility complex (MHC) class II molecules, whereas CD8+ T cells are mainly cytotoxic cells and respond upon antigen presented by MHC class I molecules.

Among CD4+ T helper cells several cell subsets can be distinguished: T_H1, T_H2 and regulatory T cells (reviewed in Mosmann and Sad 1996). In general, the production of cytokines by T_H1 cells facilitates cell-mediated immunity, including the activation of macrophages and T cell–mediated cytotoxicity; on the other hand, T_H2 cells provide B cell-help and enhance antibody production. CD4+ cells are not determined from the beginning on to differentiate into one of the two distinct subsets. Initially, they leave the thymus as T_H0 cells. The fate to become a T_H1 or a T_H2 CD4+ cell is strongly influenced by environmental conditions during the first antigen contact of the T_H0 cells (reviewed in Romagnani, Parronchi et al. 1997). The cytokine pattern and the way an antigen is presented (MHCI; MHCII) are crucial for subset differentiation.

1.2 The Role of Costimulation in T cell Activation

T cell immune responses are initiated at peripheral lymphoid tissues where immunogenic peptides bound to MHC molecules are presented on the surface of professional APC as antigens. The MHC molecule hereby gets into close contact with the T cell receptor (TCR). This interaction (signal 1) leads to T cell activation by the initiation of a multistep intracellular signalling pathway.

In addition to the signal delivered by the TCR–MHC:peptide interaction, a second signal (signal 2) named costimulation is required for full T cell activation (Bretscher and Cohn 1970). The first signal provides antigen specificity and is necessary but not sufficient to induce complete T cell activation (signal 1). For complete and sustained activation a second costimulatory signal is required (signal 2). This second signal is antigen independent and can be generated by a number of distinct molecular interactions that occur at the APC–T cell interface. The identification of CD28 on T cells interacting with its ligands B7-1 (CD80) and B7-2 (CD86) on APC elucidated the molecular basis of the costimulatory signal. Since then many functional aspects of the second signal have been elucidated (reviewed in Bernard, Lamy et al. 2002).

1.3 The family of B7/CD28 costimulatory molecules

The family of CD28-like costimulatory receptors belong to the immunoglobulin (Ig) superfamily (reviewed in Sharpe and Freeman 2002).

Their ligands belong to the B7-family. B7-family members are expressed on different cell types and are differentially regulated upon activation of APC.

The known members of the CD28 family and their B7 ligands are listed in Table 1.1.

In the following part a brief characterization of the so far known members of B7/CD28 costimulatory molecule family is given with respect expressional regulation and functional properties.

The B7/CD28-CTLA-4 Pathway

CD28 acts as the key molecule delivering the second signal for T cell activation (reviewed in Coyle and Gutierrez-Ramos 2001). It is constitutively expressed on T cells and its ligands are B7-1 and B7-2. B7-2 is the constitutively expressed counterpart of CD28 (Harper, Balzano et al. 1991). The resulting cosignal leads to increased CD28 and B7-1 expression and enables the full activation of the T

cell. One important first step following B7/CD28 interaction is the upregulation of interleukin (IL-) 2 and IL-2 receptor transcription factors (NF-AT, AP-1 and NF-κB) in the T cell that lead to 100 fold stabilization of IL-2 messenger ribonucleic acid (mRNA) (Geginat, Clissi et al. 2000). The activated T cell stimulates itself with IL-2 in an autocrine fashion. Thus CD28 augments and sustains T cell responses initiated by TCR signalling, therefore promoting T cell survival and initiation of clonal expansion and differentiation (Powell, Ragheb et al. 1998). The CD28 signal delivers not only a qualitatively distinct activation signal for T cell activation (see below) but also acts synergistically with the TCR signal: The engagement of CD28 reduces the number of needed TCR for full T cell activation from 8000 to 1500 TCR hits (Iezzi, Karjalainen et al. 1998). In addition, CD28 enforces T cell responses by promoting T cell survival e.g. by induction of the anti-apoptotic gene BCLXL, clonal expansion and differentiation (reviewed in Sharpe and Freeman 2002).

CTLA-4 is a second well-characterized member of the CD28-like receptor family. CTLA-4 surface expression is induced approximately after two days of T cell activation following CD28 interaction (Perkins, Wang et al. 1996). CTLA-4 binds to B7-1 and B7-2 with a much higher affinity than CD28 (Leung, Bradshaw et al. 1995). The engagement of CTLA-4 delivers a negative signal to the activated T cell, opposing the CD28 mediated costimulation: CTLA-4 inhibits IL-2 synthesis, leads to cell cycle arrest and thus counteracts and terminates the T cell response. Therefore, CTLA-4 is considered to be a crucial control of T cell proliferation and for peripheral T cell tolerance.

B7-1 and B7-2, the first members of the B7-family, which have been identified, are expressed on APC (reviewed in Bluestone 1995). B7-2 is constitutively expressed at low levels on professional APC and is upregulated within hours after APC–T cell interaction. In contrast B7-1 is absent on resting APC and is induced on the cell surface 3-4 days after T cell activation (Walunas, Lenschow et al. 1994). It has been suggested therefore that B7-2 is substantial for initial T cell activation, B7-1 being required for sustained T cell activation.

The ICOS/ICOS-Ligand Pathway

Inducible costimulatory signal (ICOS) is the third member of the CD28-family. A more detailed description of the molecule and its ligands follows below (section 1.4).

The PD-1/PDL1/2 Pathway

PD-1 is the fourth member of the CD28-like receptor family (Ishida, Agata et al. 1992). Although it shares some structural homologies with CD28, CTLA-4 and ICOS (like the single extracellular V-like Ig domain) it differs from them in many ways (reviewed in Sharpe and Freeman 2002). Its gene is encoded on the 2q37 locus (instead of CD28, CTLA-4 and ICOS on the 2q33 locus) (Shinohara, Taniwaki et al. 1994). PD-1 is expressed on activated T cells, B cells and approximately 1% of thymocytes (Nishimura, Agata et al. 1996). PD-1 expression is linked to cell activation rather than cell death as the misleading name suggests. PD-1 has been suggested to be a main modulator regulating TCR thresholds of activation (Nishimura, Honjo et al. 2000). It further more inhibits the production of effector cytokines and blocks cell cycle progression. PD-1 is upregulated within 24h after T cell activation.

PDL1 (B7H1) and PDL2 (B7DC) are corresponding ligands for PD-1 (Dong, Zhu et al. 1999; Freeman, Long et al. 2000; Latchman, Wood et al. 2001). PDL1 expression is found on professional APC, but - in contrast to B7-1/-2 – more widely expressed on a variety of tissues (e.g. placenta, lymph nodes, spleen, heart, endothelial, muscle cells and also in various tumor cells (reviewed in Sharpe and Freeman 2002; Wiendl, Mitsdoerffer et al. 2003b&c; Wintterle, Schreiner et al. 2003). PDL2 is expressed on DCs but also on human pancreas, lung and liver. PDL1 precedes PDL2 expression after activation of APC. The expression of PDL1 and PDL2 in both lymphoid and non-lymphoid tissue suggests that the PDL/PDCD1 pathway might be involved in the modulation of immune responses in lymphoid organs as well as in peripheral sites.

B7H3: A new B7-family member

B7H3 is a recently discovered member of the B7-family. It is mainly expressed on immature DC, but is absent on B cells. Its expression is down regulated upon stimulation with LPS (Chapoval, Ni et al. 2001). B7H3 is able to stimulate CD4+ and CD8+ cells and induces in both populations IFN-γ production. B7H3 fusion proteins binds to activated and not to resting T cells what indicates the upregulation of the B7H3 counter-ligand after T cell activation (reviewed in Sharpe and Freeman 2002).

B7H4/BTLA4

B7H4 is expressed on professional APC as well as in non-lymphoid tissues. It controls T cell immunity by the inhibition of T cell proliferation, cytokine production (both the production of T_H1 and T_H2 cytokines) and cell cycle progression (Prasad, Richards et al. 2003; Sica, Choi et al. 2003; Zang, Loke et al. 2003). Therefore, B7H4 may play a role in negative regulation of T cell immunity. B7H4 putatively interacts with BTLA, which is expressed during T cell activation. BTLA-expression remains on T_H1 but not T_H2 cells. BTLA modestly inhibits antigen-induced production of IL-2 (Watanabe, Gavrieli et al. 2003).

APC B7-family members	T cell CD28-like receptors
CD80 ANTIGEN; CD80 Alternative titles; symbols CD28 ANTIGEN LIGAND 1; CD28LG1 B-LYMPHOCYTE ACTIVATION ANTIGEN B7-1; LAB7 B71 ANTIGEN	**ANTIGEN CD28; CD28** Alternative titles; symbols T-CELL ANTIGEN CD28 Tp44
CD86 ANTIGEN; CD86 Alternative titles; symbols CD28 ANTIGEN LIGAND 2; CD28LG2 B-LYMPHOCYTE ACTIVATION ANTIGEN B7-2; LAB7-2; B72 ANTIGEN	**ANTIGEN CD28; CD28** Alternative titles; symbols T-CELL ANTIGEN CD28 Tp44
Programmed Cell Death 1 Ligand 1; PDL1 Alternative titles; symbols B7H1, B7 HOMOLOG 1 PROGRAMMED CELL DEATH 1 LIGAND 1; PDCD1L1; PDCD1 LIGAND 1	**PROGRAMMED CELL DEATH1** Alternative titles; symbols PDCD1; PD1
Programmed Cell Death 1 Ligand 2; PDL2 Alternative titles; symbols PDCD1 LIGAND 2; PDCD1L2 PROGRAMMED DEATH LIGAND 2; PDL2 B7DC	**PROGRAMMED CELL DEATH1** Alternative titles; symbols PDCD1; PD1
B7 HOMOLOG 2 Alternative titles; symbols B7H2 GL50 B7-RELATED PROTEIN 1; B7RP1 AILIM LICOS; ICOS-L	**INDUCIBLE COSTIMULATOR;** Alternative titles; symbols ICOS Activation Inducible Lymphocyte Immunomediatory Molecule;
B7 HOMOLOG 3 Alternative titles; symbols B7H3; B7RP2	not molecularly identified
B7 HOMOLOG 4 Alternative titles; symbols B7H4 B7 Superfamily Member 1; B7S1 B7X	**BTLA** Alternative titles; symbols B- & T- Lymphocyte-Associated

Table 1.1 Names, alternative titles and symbols of the CD28-like receptors and their ligands belonging to the B7-family. The ligand for the newly identified molecule B7H3 remains to be discovered.

1.4 ICOS - ICOSL interactions

1.4.1 Structure and regulational expression of ICOS

Inducible costimulatory signal (ICOS) is the third molecularly described member of the CD28-family. Like CD28 and CTLA-4 it is encoded on the 2q33-34 locus suggesting that all three molecules most likely arose by gene duplication (Hutloff, Dittrich et al. 1999; Mages, Hutloff et al. 2000). Therefore it is not surprising that ICOS resembles CD28/CTLA-4 structure in many ways. The predicted mature ICOS protein is a 199 amino acid type I transmembrane molecule belonging to the Ig-superfamily.

The expression pattern of ICOS differs strikingly to CD28 and CTLA-4. As implicated by its name, ICOS is not constitutively expressed but induced in CD4+ and CD8+ cells upon TCR engagement and following T cell activation (Hutloff, Dittrich et al. 1999; Beier, Hutloff et al. 2000; Mages, Hutloff et al. 2000). Regarding the CD4+ subset, ICOS expression can be induced on naïve T_H0 cells, as well as previously activated T_H1 and T_H2 cells. Cell surface levels of ICOS remain high on T_H2 cells and fall rapidly on T_H1 cells after the initial phase of T cell activation. A constitutive baseline expression can be detected in T_H2 clones and $CD4^+CD44^{hi}CD69^{low}$ cells (memory cells) but not on T_H1 clones. In the course of T cell activation high levels of cell surface expression of ICOS is detected especially in the late stage of activation. Experiments in murine models show no effect of B7-1-blocking and a minor impact of CD28-blocking on ICOS upregulation whereas blockade of B7-2 leads to significant impaired ICOS expression. Immunhistochemical studies showed that $ICOS^+$ cells are mainly distributed in the cortical thymus and the apical light zone of germinal centres in lymph nodes (Mages, Hutloff et al. 2000).

Elevated levels of ICOS mRNA can be detected already one hour after TCR engagement, followed by surface expression within 12 hours. Protein expression reaches a maximum after 48 hours and declines then slightly (Coyle, Lehar et al. 2000; Mages, Hutloff et al. 2000). Although ICOS expression is supported by CD28 engagement, it is not absolutely CD28

dependent. The fact that ICOS upregulation can be detected under conditions where the TCR is engaged but not CD28 is a strong indicator for the independent costimulatory potency of ICOS.

1.4.2 Functional aspects of ICOS engagement

In contrast to CD28 ICOS blockade has no significant effect on IL-2 production (Yoshinaga, Whoriskey et al. 1999; Riley, Blair et al. 2001). However ICOS expression influences the production of several other cytokines especially in recently activated T cells: Production of IL-4, IL-5, IL-10, IL-13 and IFN-γ is highly ICOS dependent (Hutloff, Dittrich et al. 1999; Beier, Hutloff et al. 2000; Coyle, Lehar et al. 2000). In contrast, CD28 has no significant influence on these cytokines. The cytokine pattern promoted by ICOS engagement reveals a T_H2 milieu and ICOS has therefore been suggested to be crucial for T_H2 cell differentiation and effector function (McAdam, Schweitzer et al. 1998; Kobata, Azuma et al. 2000; Akbari, Freeman et al. 2002). Accordingly T_H2 cell lines in mice express significant higher levels of ICOS than T_H1 cell lines (McAdam, Schweitzer et al. 1998). In conclusion from a considerable number of studies it is suggested that CD28 has an important role in naïve T cell development, for T_H1 differentiation and effector function. Further ICOS plays a crucial role for repeated T cell activation, for T_H2 cell differentiation and effector function.

1.5 Multiple Sclerosis: clinical, epidemiological and immunopathogenetic characteristics

Multiple Sclerosis (MS) is the most common chronic and disabling inflammatory disorders of the central nervous system (CNS). It affects worldwide over 2.5 million individuals (Noseworthy, Lucchinetti et al. 2000; Steinman 2001). The lifetime risk is one in 400 and the disease is the most common cause of neurological disability in young adults. The male: female -ratio is approximately 1:2. Initial features include visual disturbance, paralysis, sensory disturbance and lack of coordination. Single relapses last a few days to weeks followed by periods of remissions (potentially month to years) until the next disease relapse. This type of disease course is termed relapsing-remitting Multiple Sclerosis

(RR-MS) (Lublin and Reingold 1996). However 30% of the patients with RR-MS develop progressive neurological deficits without further remissions called secondary-progressive Multiple Sclerosis (SP-MS). In 10-20% of the patients relapses are absent from the beginning on which is then termed primary-progressive Multiple Sclerosis (PP-MS).

The underlying morphological changes of the disease are the formation of characteristic plaques of demyelination predominately located in the white matter of the CNS - mainly in the cerebellar peduncles, optical nerves, periventricular areas and the spinal cord. Lesions can be detected radiologically by magnetic resonance imaging (MRI) resembling the presence of oedema (T2-hyperintense lesions) or a local breakdown of the blood-brain barrier (gadolinium-enhancing T1 lesions) (Brex, Ciccarelli et al. 2002). High titres of intrathecally synthesized IgG1 and IgG3 antibodies are present in the cerebrospinal fluid (CSF) of MS patients. Altogether an autoimmune disorder is considered being the underlying pathomechanism of the disease (Martin, McFarland et al. 1992). Many studies implicate (1) genetic and (2) environmental /infectious factors of immune dysfunction as key contributors to the onset and disease course of multiple sclerosis, a hypothesis which is not contradictory (Noseworthy, Lucchinetti et al. 2000; Hemmer, Archelos et al. 2002).

Neuropathological studies reveal severe inflammatory infiltrations within the plaques and characteristically a reduced density of myelin fibres often associated with irregular insheathment of axons (Lassmann 2002). The infiltrates contain activated T and B cells, macrophages and activated microglia in which intracytoplasmatic granules of myelin debris are found (Steinman 2001). Within the plaques, elevated levels of the T_H1 cytokines IL-2, IFN-γ and TNF-α and increased expression of the T_H1 chemokine receptors CCR5 and CXCR3 are found (Martinez-Caceres, Espejo et al. 2002). This would be consistent with the presence of increased numbers of autoreactive T cells, which have a T_H1 cytokine spectrum in the peripheral blood of MS patients compared to healthy controls (Merrill 1992; Lucchinetti, Bruck et al. 2001). The formation of a MS plaque is considered to be a multistep process (Lucchinetti,

Bruck et al. 2000). Initially the circulating immune cells have to leave blood vessels to reach the site of inflammation. Thereafter the inflammatory process is initiated by the secretion of certain cytokines attracting macrophages and microglia and generating an optimal milieu for the following immune reaction. The blood brain barrier of the CNS is constituted by endothelial cells connected to each other by tight junctions and a circumferential layer of type IV collagen. Upon an inflammatory signal endothelial cells express adhesion molecules like vascular cell adhesion molecule (VCAM) on their surface where activated T cells can get attached to integrins such as very late antigen-4 (VLA-4)(Steinman 2001). After the lymphocytes have extravasated they make their way through the dense collagen type IV layer by expressing enzymes called matrix metallo proteases (MMPs), which degrade collagen fibrils. Elevated levels of MMPs can be detected in the CSF of MS patients (Kieseier, Clements et al. 1999; Hartung and Kieseier 2000). Lymphocytes then spread into the white matter of the brain and orchestrate a cascade of events finally leading to inflammatory processes. The characteristic features of an immune reaction can be detected within the plaques: Increased levels of TNF-α, IFN-γ, nitric oxygen (NO), monoclonal antibodies (mab) and components of the complement cascade. The result is destruction of the myelin sheath and the phagocytosis of myelin pieces by macrophages and microglia.

1.6 The Role of Costimulation in Multiple Sclerosis Pathogenesis

Autoimmunity is regarded as loss of tolerance of the immune system to self-antigens (Kamradt and Mitchison 2001). MS is viewed as the prototype of an autoimmune disease of the CNS, where T cells specific for myelin antigens are key contributors to the inflammatory demyelination (Steinman 2000; Hemmer, Archelos et al. 2002). One hallmark of T cell mediated autoimmune diseases is the activation of autoreactive and autoagressive T cells. As detailed above, costimulation is the crucial element for delineating T cell fate, e.g. activation versus anergy, modulation of sustained T cell activation (Kobata, Azuma et al. 2000; Chambers 2001). It has therefore been proposed that dysregulation of

costimulatory elements contribute to the initiation, perpetuation or spreading of autoimmunity due to the activation of self-reactive T cells. This assumption was fuelled by the finding that knockout-animals lacking certain costimulatory molecules are resistant to the development of certain autoimmune disorders. B7 costimulation was found to be an important factor for encephalitogenicity as shown by several groups determining the role of B7:CD28/CTLA-4 interaction in the induction and perpetuation of experimental allergic encephalomyelitis (EAE), the animal model for MS (Perrin, Scott et al. 1995; Racke, Scott et al. 1995).

Furthermore the impairment of the balance between T_H1 and T_H2 CD4+ cells seems to be of importance for the pathogenesis of MS (Kobata, Azuma et al. 2000). It is generally thought that MS may result from a dominance of T_H1 cells over T_H2 cells due to impaired lineage distribution. It has to be noted critically that this T_H1-pathogenic/ T_H2-protective paradigm does not fully describe the complexity of the autoreactive immune response in MS, although the concept of a T_H1/ T_H2 dichotomy is widely accepted dogma in the adaptive immunity. The engagement of ICOS mainly leads to the production of the T_H2 cytokines IL-10 and IL-4 but not of the T_H1 proinflammatory cytokines IL-2, IL-12 and IFN-γ. ICOS is considered to deliver a critical costimulatory signal for T_H2 subset polarization and T_H2 responses (McAdam, Schweitzer et al. 1998; Hutloff, Dittrich et al. 1999; Coyle, Lehar et al. 2000). Besides ICOS expressing T_H2 cells, CD4+CD25^{hi} T cells (regulatory T cells) putatively contribute to the maintenance of immunologic self-tolerance. Human CD4+CD25hi T cells, similar to the mouse CD4+CD25+ cells, are anergic to in vitro antigenic stimulation and strongly suppress the proliferation of responder T cells upon coculture. As potentially auto-reactive T cells are present also in the periphery of healthy individuals, mechanisms for inhibiting activation and effector function of activated responder T cells might be crucial for preventing the onset of autoimmune disorders. Thus deficient generation or reduced effector function of CD4+CD25hi T cells may play a role in regulating the autoimmune response in patients with MS. The fact that CD4+CD25hi T cells as well as ICOS expressing

T_H2 cells potentially contribute to the maintenance of immunologic self-tolerance raises the question of a relationship between these T cell subsets.

2. Aim of the Study

The last decades have yielded great insight in cellular and molecular mechanisms contributing to MS. Most findings now strongly suggest a T_H1 cell-mediated autoimmune disorder as the key pathogenic process. As a result of this, inflammatory myelin destruction occurs within the CNS. The fact that the disease is associated with immunological relevant genes and its clinical response to immunosuppression supports the assumption of underlying and driving immunological mechanisms.

Recent years revealed the identification of several costimulatory molecules and their contribution to T cell activation. It becomes increasingly clear that costimulation is crucial for various aspects of adaptive immune responses, e.g. T cell-differentiation, T cell-activation and modulation of the subsequent T cell-response. As a newly identified costimulatory molecule, ICOS was described by Hutloff et al in 1999. Early findings indicated that ICOS drives T-cell responses towards T_H2-responses and augments CD4 T cell responses. The fact that MS is considered to be a T_H1-autoimmune disease therefore challenged the question, whether ICOS regulation, expression and function might be involved in the pathogenesis of MS.

This study aimed to compare MS patients and healthy donors with regard to the expression of ICOS, other costimulatory molecules and related cytokine-production. Using real-time polymerase chain reaction (RT-PCR) and flow cytometry the relative amount of specific mRNA of the respective molecules on peripheral mononuclear cells (under specific stimulatory-conditions and at specific time-points) and of human brain specimen of active MS-plaques and control tissue were determined. Flow cytometry technique was used to analyze isolated mononuclear cells (cultured under certain conditions) from patients with MS and HD. The same technique was used to address the question of a linkage between ICOS-expression as a T_H2-determing molecule and $CD4^+CD25^+$ regulatory T cells, a T cell subpopulation considered important for the regulation of tolerance and autoimmunity.

3. Material and Methods

3.1 Cell Culture

3.1.1 Buffer and Media

Cell culture media:

10% FCS (Fetal Calf Serum Gold)	GIBCO, Invitrogen Corp.
2 mM glutamine	Life Technologies
RPMI 1640 Medium	GIBCO, Invitrogen Corp.

Washing buffer:

Sterile PBS DULBECCO'S	GIBCO, Invitrogen Corp.

3.1.2 Isolation of peripheral blood mononuclear cells

Blood (100 ml) was obtained by venipuncture after informed consent from HD (n=8) and from patients with MS (n=12). The diagnosis of multiple sclerosis was based on the established criteria by Poser et al (Poser, Paty et al. 1983).

Peripheral mononuclear cells (PBMCs) were purified by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway). Blood was diluted 1:1 in PBS and 35 ml of this mixture were layered over 15 ml of ficoll in a 50 ml Falcon tube. In order to form the gradient, tubes were then centrifuged at 2000 rpm without brake for 20 minutes. The PBMC-rich interphase was collected and washed with PBS (1500 rpm, 6min, 4°C). The pellet was resuspended in cell culture medium and the amount of cells was determined with a "Neubauer-Zählkammer".

3.1.3 Myelin peptide and glycoprotein antigens for stimulation of peripheral blood mononuclear cells

Myelin Basic Protein (MBP) (from bovine brain; Sigma-Aldrich Co.)	fc= 10µg/ml
Human recombinant Myelin Basic Protein ($hrMBP_{84-99}$*)	fc= 10µg/ml

Rat recombinant Myelin oligodendrocyte glycoprotein (rrMOG $_{1-125}$)[§] fc= 10µg/ml

Glatirameracetate (standardized, randomized mixture of synthetic polypeptides consisting of L-glutamic acid, L-lysine, L-alanine, and L-tyrosine with a defined molar residue ratio; from TEVA Pharmaceutical Industries, Petah Tiqva, Israel) fc= 50µg/ml

* kindly provided by Dr. Stevanovic, Department of Immunobiology, University of Tübingen

[§] kindly provided by Dr. Weissert, Department of Neurology, University of Tübingen

For stimulation peptides and MOG were diluted in cell culture medium and used in final concentrations (fc) as indicated.

3.1.4 Stimulation of PBMCs

After viability of freshly isolated PBMCs was checked by tryptan blue exclusion, $0{,}5x10^6$ cells were plated in culture media containing peptide or glycoprotein at a final volume of 0,5ml in 24 well flat bottom microtiter plates.

Cells were then maintained at 37°C in a humified atmosphere of 5% CO_2 / 95% air. For further flow cytometry and quantitative gene expression analysis cells were harvested at given time points.

3.1.5 Cryopreservation of human cells

For cryopreservation of human PBMCs, a double-concentrated freezing medium containing 40% culture medium (RPMI 1640), 40% FCS and 20% dimethylsulfoxide (DMSO) was prepared. Aliquots of 500µl of cell suspension with a concentration of $1x10^7$ cells/ml were cooled in cryotubes. After the addition of 500µl of freezing medium the suspension was mixed and tubes were frozen at –80°C for two days. Tubes were then stored in liquid nitrogen.

3.2 Flow cytometry

3.2.1 Buffers and Reagents

FACS Buffer:

0,1% BSA (Bovine Serum Albumin, SIGMA)

0,1% Na-Azide (Sodium-Azide, SIGMA)

in PBS

Primary antibodies:

Molecule	Label	Isotype	Origin	Dilution from stock
IgG1	FITC	IgG	SIGMA	1/100
IgG2a	PE	IgG	COULTER	1/400
TCRab	FITC	IgM	BD Pharmingen	1/3
CD4	FITC	IgG1	BD Pharmingen	1/5
CD8	FITC		Immunotech	1/10
CD4	biotin	IgG1	Immunotech	1/5
ICOS	PE	IgM	Kindly given by R.A. Kroczek*	1/30
ICOS	pure	IgM	Kindly given by R.A. Kroczek*	1/30
CD25	PE		Caltag Labs	1/750
Vd2	FITC		BD Pharmingen	1/100

*R.A. Kroczek, Molecular Immunology, Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany

Conjugates:

Goat anti-Mouse

phycoerythrin (PE) IgG (H + L) F(abV)2 fragment	Dianova
Streptavidin-PerCP	Dianova
Streptavidin-FITC	Dianova

Sera:

Normal goat serum (NGS)	Dianova
Purified mouse IgGs	Sigma
Purified human IgGs	Bayer

3.2.2 Flow cytometry analysis

For membrane staining cultured cells were harvested by extensive washing of 24 well microtiter plates with PBS. Cells were then centrifuged and resuspended in fluorescence-activated cell sorting (FACS) buffer. Freshly isolated PBMC were washed with PBS. After washing, cells were resuspended in FACS buffer and preincubated for 10 minutes on ice with human Ig at 10µg/ml to block surface Fc-receptors. 90µl of cell suspension were placed in FACS tubes cooled on ice. 10µl of primary antibody at the appropriate dilution were added and incubated for 45 minutes on ice and in the dark. Cells were then washed with 3-4 ml of FACS buffer.

After blocking with NGS for 10 minutes on ice, cells were incubated with the secondary antibody for 30 minutes also on ice. Cells were then washed again with 3-4ml of FACS buffer and finally resuspended in 300µl of FACS buffer.

Flow cytometry analysis was performed on a FACSCalibur® (Becton Dickinson) using the company's software CellQuest®.

3.3 Molecular Biology

3.3.1 Total RNA extraction

Stimulated cells were harvested at given time points by extensive washing of 24 well microtiter plates with PBS. Cells were then centrifuged for 5min at 1200rpm, and total RNA extraction was performed by using RNeasy® according to the manufacturer's description (Quiagen, Germany)

3.3.2 cDNA synthesis

Purified RNA was eluted in 21,5 µl DEPC-treated water. For reverse transcription 2 µl of random hexamers (200ng/µl) were added, incubated for 10 min at 70°C and chilled on ice to remove any secondary structures of nucleic acid.

RT-reaction mixture:	reagent	1 x sample
	5 x M-MLV RT-Buffer	10,0µl
	dNTPs (each 10mM)	10,0µl
	RNAse Inhibitor	0,25µl
	M-MLV RT	1,0µl
	DEPC-treated water	5,25µl

26,5µl of the RT-reaction mixture were added to the RNA/random hexamer solution, samples were carefully mixed and incubated for 10 min at room temperature. Samples were then incubated for 50 min at 42°C before reverse transcriptase was denaturized by incubation for 15 min at 70°C. The cDNA-solution was stored at –20°C.

3.3.3 cDNA samples from T_H1 and T_H2 specific T cell lines

cDNA samples from T_H1 and T_H2 specific T cell lines were generated and kindly provided by Dr.med. O. Neuhaus, Department of Neurology, Heinrich-Heine-Universität, Düsseldorf.

3.3.4 cDNA samples from APCs

cDNA samples from APC, namely B cells, monocytes, mature and immature DC from PBMCs of healthy individuals were generated and kindly provided by P. Schnorrer, Department of Neurology, University of Tübingen Medical School.

3.3.5 cDNA samples from cerebral tissue specimen of MS patients

cDNA samples from cerebral tissue specimen of MS patients were kindly provided by B.C. Kieseier, Department of Neurology, Heinrich-Heine-Universität, Düsseldorf.

3.3.6 Conventional PCR for qualitative gene expression

Conventional PCR was performed by the addition of 1µl of the previously synthesized cDNA to 0,2 µl of Hot Star Taq DNA polymerase diluted in a PCR-mixture containing 1µl of forward primer and 1 µl of reverse primer.

PCR-mixture:	reagent	1x sample
	H_2Odd	13,2 µl
	10 x PCR-Buffer	2,0 µl
	dNTPs	1,6 µl
	Forward-Primer (5µM)	1,0 µl
	Reverse-Primer (5µM)	1,0 µl
	Taq-Polymerase	0,2 µl

The thermocycler parameters for PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. PCR products were afterwards visualized by agarose gel electrophoresis (see below).

3.3.7 Oligonucleotide primers used for RT-PCR analysis

Primers were designed to bind to span exon/exon-boundaries within one gene to avoid false positive results arising from amplification of contaminating genomic DNA. Criteria for the selection of a specific single stranded primer included melting temperature (T_M), optimal length (18-22 bases), Guanin/Cytosin-content (G/C content: 20-80%) and a short product length (100-150 base pairs) to enhance efficiency of PCR amplification. The problem of non-specific priming was minimized by selecting primers with only one or two G/Cs within the last five nucleotides at the 3' end.

HUSAR Genius software package (http://genius.embnet.dkfz-heidelberg.de, DKFZ, Heidelberg, Germany) was used to design primers for amplification and they were ordered at BioChip Technologies GmbH, Freiburg, Germany. To avoid unspecific interactions with other human genes, nucleotide query sequences of the newly designed primers were compared against a human nucleotide sequence database by using the HUSAR software BlastN. Additionally, specificity was tested by using Hot Star Taq DNA polymerase and a 1:1 mixture of cDNA from PBMCs.

Lists of primers used for quantitative gene expression measurement and for qualitative gene expression (conventional PCR) are given in Table 3.1 (B7 molecules), Table 3.2 (chemokine receptors) and Table3.3 (cytokines).

Gene	Oligonucleotide				Amplicon
	name	sequence 5'→3'	length	T_M [°C]	length [bp]
CD80 Taq	CD80 Taq F 120-140	AGTTAGAAGGGGAAATGTCGC	20	60	111
	CD80 Taq R 209-230	TCAGGGTAAGACTCCACTTCTG	21	59	
CD80 conventional	CD80 Konv F	ACTCGCATCTACTGGCAAAAGGA	23	55	553
	CD80 Konv R	ATGGGAGCAGGTTATCAGGAAAA	23	55	
CD86 Taq	CD86 Taq F 509-530	ATTCTGAACTGTCAGTGCTTGC	22	60	125
	CD86 Taq R 633-612	CTTCTTAGGTTCTGGGTAACCG	22	59	
CD86 conventional	CD86 Konv F	TGGTGCTGCTCCTCTGAAGATTC	23	55	300
	CD86 Konv R	ATCATTCCTGTGGGCTTTTTGTG	23	55	
ICOS Taq	ICOS Taq F 886-905	GCAACCAGCTTTGGAGAAAG	19	60	153
	ICOS Taq R 1040-1020	TGCTTTGCAGATTCAGTACCC	20	60	
ICOS-L conventional	ICOS-L Konv F	CCGCGGCCCAAGTTCT	16	57	
	ICOS-L Konv R	GCCTCATTCCAGGATCACAG	20	59	
GL50 Taq	GL50 Taq I F 819	CTTGTGGTCGTGGCGGTG	18	60	131
	GL50 Taq R (VL162)	TCACGAGAGCAGAAGGAGCAGGTTCC	26	58	
B7H2 I Taq	B7H2 F 1198 F	GATCCTGGAATGAGGCCCTTT	21	60	114
	B7H2 R 1311	CCATCACCAGTCCCTTCCTGT	21	60	
PDCD1 Taq	PDCD1 F 715	TGCCTGTGTTCTCTGTGGAC	20	60	129
	PDCD1 R 836	TCCGCTAGGAAAGACAATGG	20	60	
B7H1 = PDL1 Taq	B7H1 = PDL1 F 389	TCAATGCCCCATACAACAAA	20	60	119
	B7H1 = PDL1 R 508	TGCTTGTCCAGATGACTTCG	22	60	
PDL2 Taq	PDL2 F	GTACATAATAGAGCATGGCAGCA	23	57	101
	PDL2 R	CCACCTTTTGCAAACTGGCTGT	22	60	
B7H3 Taq	B7H3 F 720	GCCTATGACATTCCCCCCAG	20	60	104
	B7H3 R 823	TGATCTTTCTCCAGCACACGAA	22	59	

Table 3.1: List of oligonucleotides for B7 family members

Gene	Oligonucleotide				Amplicon
	name	sequence 5'→3'	length	T_M [°C]	length [bp]
CCR5 (Th1)	CCR5 F	TGCTACTCGGGAATCATAAAAACT	24	53	280
	CCR5 R	TTCTGAACTTCTCCCCGACAAA	22	56	
18SrRNA	18SrRNA for	CGGCTACCACATCCAAGGA	19		
	18SrRNA rev	GCTGGAATTACCGCGGCT	18		

Table 3.2: Chemokine receptor oligonucleotides and 18SrRNA for housekeeping gene normalization

Gene	Oligonucleotide				Amplicon
	name	sequence 5'→3'	length	T_M [°C]	length [bp]
IFN-γ	IFN-γ-for	TTCAGCTCTGCATCGTTTTG	20	60	153
	IFN-γ-rev	CTTTCCAATTCTTCAAAATGCC	22	60	
IL-4	IL-4-for	CTTTGAACAGCCTCACAGAGC	21	60	127
	IL-4-rev	AACTGCCGGAGCACAGTC	18	60	
IL-10	IL-10-for	GTTTTACCTGGAGGAGGTGATG;	22	60	151
	IL-10-rev	GGCCTTGCTCTTGTTTTCAC	20	60	
CD4	CD4 for 257->275	TTCTGGGAAATCAGGGCTC	19	60	102
	CD4 rev 358->340	TCAGGGGGAAGTTTCCTTG	19	60	

Table 3.: List of oligonucleotides for cytokines and CD4

3.3.8 Relative quantification of gene expression by RT-PCR analysis

The amount of mRNA expression in PBMCs cultured under different conditions was quantified by the use of a two-step RT-PCR. Previously synthesized cDNA served as template and its amplification was achieved by using Applied Biosystems SYBR Green PCR Master Mix. Analysis was performed in the ABI

PRISM 7700 SDS from Perkin Elmer under the following thermal cycler conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Binding of the SYBR GREEN dye to double-stranded DNA results in fluorescence thus allowing the quantification of the DNA since fluorescence is directly proportional to the amount of the generated PCR product. The cycle number (C_T), at which a threshold of fluorescence intensity is reached, was elucidated. The threshold was chosen to be in the exponential phase of the PCR, which makes the C_T values very reproducible and for subsequent relative quantitation the comparative C_T method was used (PE-Applied Biosystems, User Bulletin #2, pages 11-15, 11/12/1997). PCR assays were performed in duplicates and reported as the average. Values were normalized to an endogenous housekeeping gene control (18 sRNA) to account for the variability in initial concentrations of total RNA and the conversion efficiency of the RT-reaction. Results were expressed relative to the level found in PBMCs cultured in medium only. As ICOS-, IL-4-, IL-8-are mainly expressed in CD4+-cells values for this molecules were furthermore normalized to CD4-expression since PBMC-subsets were not isolated prior to culture. Product specificity was confirmed by agarose gel electrophoresis and dissociation curve analysis.

3.3.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA fragments of variable size. TAE-buffer with EtBr (100ng/ml) was used as electrophoresis and gel buffer. Samples were mixed 4:1 (v/v) with the sample buffer and separated at 65mA. DNA bands were visualized with UV light (254nm). 100bp ladder (GIBCO) was used as marker for size.

3.4 Statistical analysis

If not indicated otherwise, all data are representative of experiments performed at least three times with similar results. Significance was assessed by two-sided t-test.

4. Results

4.1 T lymphocyte subset analysis of ICOS expression

ICOS costimulation presumably affects the differentiation of T_H1/T_H2 cells after primary activation and modulates the immune response of effector/memory T cells. MS patients may have regulatory disturbances in both phases of the immune response. In order to characterize ICOS expression and its regulation in MS, the baseline expression and inducibility of ICOS in healthy control individuals and in MS patients was quantified in freshly isolated PBMC. Furthermore the question of a potential expression of ICOS in $CD4^+/CD25^+$-regulatory T cells was addressed.

4.1.1 Comparison of constitutive cell surface expression and upregulation kinetics of ICOS on $CD4^+$-and $CD8^+$-T cells in MS patients and healthy individuals

ICOS upregulation after stimulation with immobilized anti-CD3 mAb (OKT3) was quantified at given time points in CD4 and CD8 T lymphocyte subsets. Cell surface expression of ICOS peaked within 48 to 72 h after stimulation. Comparing 5 MS patients (five patients with RR-MS: four female, one male, EDSS 0 - 4.5; 3 months to 7 years of disease duration) with 10 age and sex matched HD, this study revealed that constitutive expression of ICOS on CD4 T cells varied between 0.1% and 42.3% on CD4 cells and between 0.1% and 10.1% on CD8 T cells. No significant differences were observed between both groups (two-sided t-test, $p>0.05$; CD4 cells: baseline mean HD 5.5±3.32 S.E.M., MS 7.73±5.62 S.E.M.; CD8 cells: baseline mean HD 1.38±0.85 S.E.M., MS 3.16±1.8 S.E.M.) (Figure 4.1). Upon anti-CD3 stimulation, up to 66.4% of CD4 T cells (range 2.5% to 66.4%, mean HD 35.75±9.78 S.E.M., mean MS 30.51±6.2 S.E.M.) and up to 52.5% of CD8 T cells (range 1.8% to 52.5%, mean HD 16.39±5.54 S.E.M., mean MS 21.02±5.32 S.E.M.) stained ICOS-positive. Again, inducibility did not differ between MS and HD (two-sided t-test, $p>0.05$; Figure 4.1). Taken together, ICOS expression and inducible upregulation on

CD4 and CD8 T cells showed no differences between healthy donors and patients with MS.

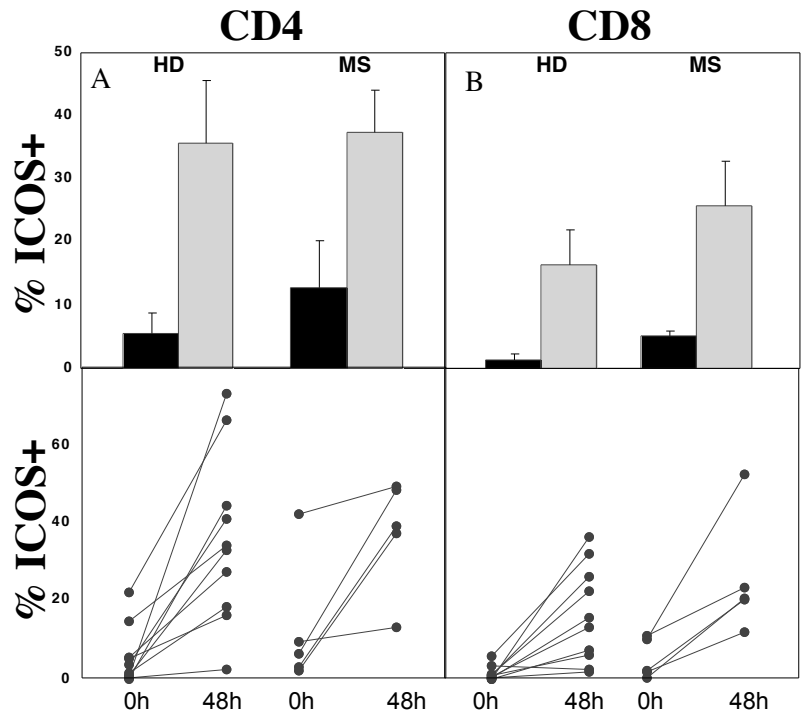

Figure 4.1: Comparison of cell surface ICOS expression and upregulation kinetics.

Percentage of ICOS expressing $CD4^+$- and $CD8^+$-T lymphocytes quantified by the use of flow cytometry. Expression was measured in patients with multiple sclerosis (MS; n=5) and compared to healthy donors (HD; n=10) before and after stimulation with anti-CD3: black bars (0h) and grey bars (48h) in upper panels represent the mean frequency ± SEM of ICOS expression. The lower panels indicate the percentage of ICOS surface expressing $CD4^+$- and $CD8^+$-T lymphocytes in individual patients with MS (n=5) and HD (n=10).

4.1.2 Analysis of constitutive ICOS expression on $CD4^+/CD25^{++}$-regulatory T cells

This study aimed to address the question whether $CD4^+/CD25^+$-regulatory T cells express ICOS constitutively on their cell surface.

Freshly isolated PBMCs from 3 healthy donors were analyzed by 3-color fluorescence for the simultaneous expression of CD4, CD25 and ICOS in the flow cytometer. Figure 4.2 shows one representative experiment.

Among the $CD4^+$cells 18% expressed constitutively CD25 whereas ICOS is expressed in 10% of the cells. Coexpression of CD25 and ICOS was observed in only 4% of the $CD4^+$cells.

Thus, ICOS is not exclusively expressed in $CD4^+CD25^+$cells, and thus it is not a marker for this regulatory subset.

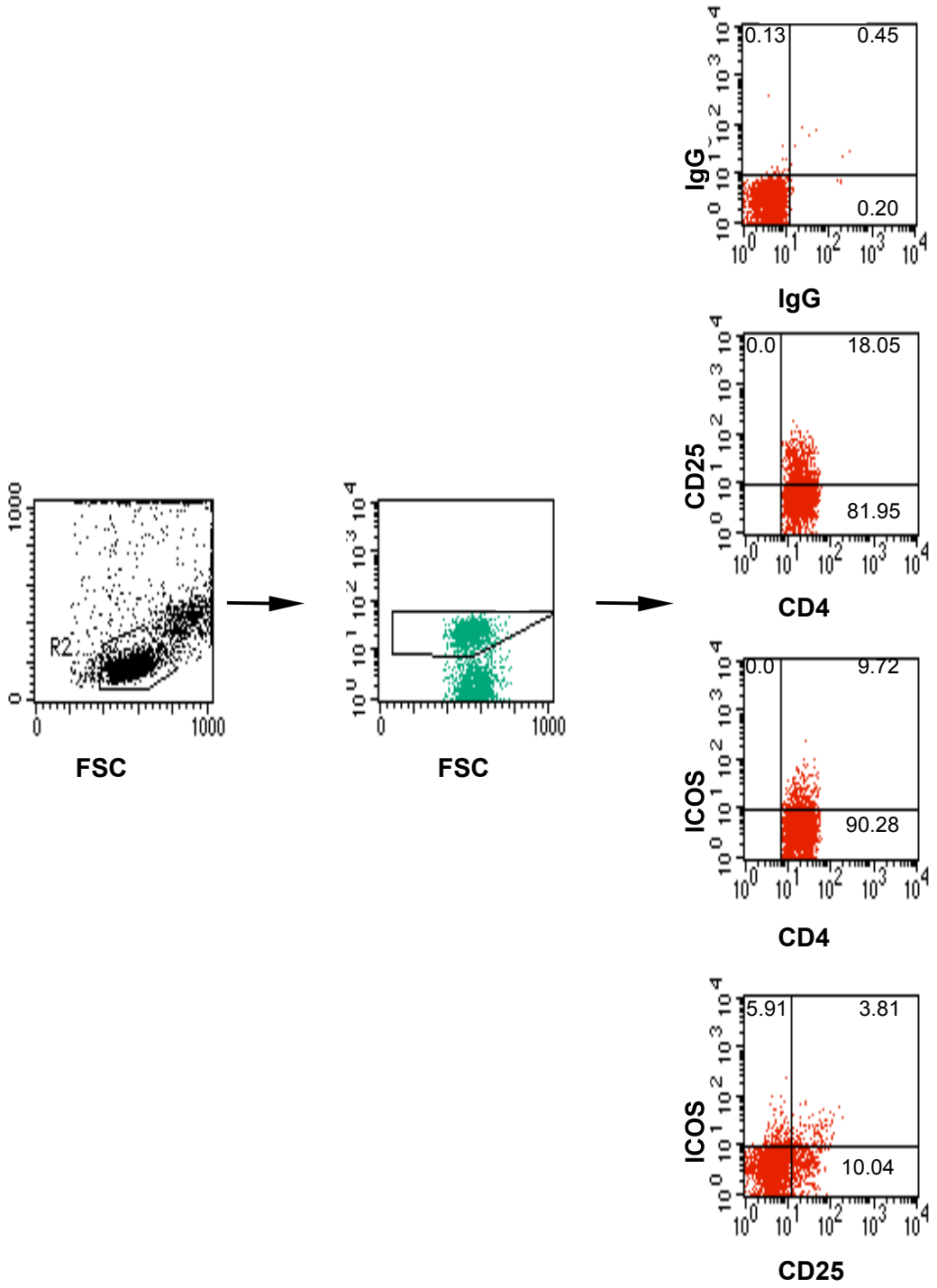

Figure 4.2: ICOS is expressed by some, but not all $CD4^+CD25^+$ regulatory T cells

PBMC were analyzed by 3-color fluorescence technique. Following electronic gating of mononuclear cells on lymphocytes (left), CD4 high positive cells were gated for further double fluorescence staining with CD25 and ICOS. A dot blot analysis of one healthy donor, representative for a series of four experiments performed in a patient with MS and in healthy donors (n = 3) is shown.

4.2 Gene expression analysis of ICOS in T_H1 and T_H2 specific T cell lines

ICOS engagement is considered to deliver a critical costimulatory signal for T-lineage attribution and T_H2 polarization as well as the promotion of T_H2 responses (McAdam, Schweitzer et al. 1998; Coyle, Lehar et al. 2000). The question whether ICOS is mainly expressed by the T_H2 subset was addressed in antigen specific T cell lines. T_H1-specific T cell lines are characterized by the expression of Chemokine Receptor 5 (CCR5) whereas T_H2-specific T cell lines do not express CCR5. By the use of conventional RT-PCR the gene expression of ICOS and CCR5 was assessed in two T_H1- and two T_H2-specific T cell lines. (Figure 4.3). Gene expression of ICOS was restricted to T_H2 specific cell lines and did not occur in T_H1-specific T cell lines, whereas CCR5 expression is restricted to T_H1-specific T cell lines.

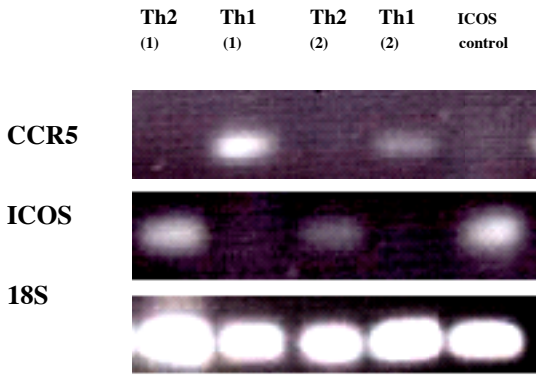

Figure 4.3: Expression of human ICOS and CCR5 in independent T_H1 and T_H2 specific T cell lines. Agarose gel electrophoresis to visualize RT-PCR products of human ICOS and CCR5 (a marker for T_H1-lineage). Expression of the housekeeping gene 18SrRNA served as a control. Plasmid-cDNA encoding for ICOS (ICOS control) served as a positive control.

4.3 Gene expression profile of costimulatory molecules and cytokines in patients with Multiple Sclerosis and healthy individuals

Expression of costimulatory molecules and cytokines might differ in patients with autoimmune disorders and healthy individuals thus contributing potentially to the activation of autoreactive T lymphocytes.

This study aimed at comparing gene expression profiles of the costimulatory molecule ICOS and T_H1- and T_H2-cytokines in stimulated T cells from peripheral blood by QRT-PCR analysis. In addition, gene expression profiles of a wider range of costimulatory molecules and cytokines were measured in cDNA samples of human brain specimen from patients with MS.

4.3.1 Gene expression profile of ICOS and the cytokines IL-4, IL-10 and IFN-γ in patients with MS and HD

Freshly isolated PBMC from patients with MS (n=12) and HD (n=8) were cultured with peptides of proteins found in the myelin sheath for 36 h. Peptides used for stimulation were MBP whole protein, MBP_{84-99} (immunodominant domain) and the extracellular domain of the glycoprotein MOG_{1-125}. Further stimulatory conditions were glatirameracetat (GA) and one condition without peptide stimulation (media only), which served as negative control. Cells were harvested after 36 h and RNA was extracted. QRT-PCR was performed to quantify the gene expression profile of ICOS, IL-4, IL-10 and IFN-γ. The expression profiles of 18SRNA and CD4 served as references in post-PCR normalization calculations.

Antigen specific stimulation resulted in 1.1 to 5.8 fold higher ICOS expression in patients with MS and in 1.8 to 3.8 fold higher ICOS expression in HD compared to PBMC cultured with media only. No significant differences were observed

between both groups for any antigens used (two-sided t-test, p>0.05; MBP protein: MS 1.47±0.55 S.E.M., HD 3.36±1.24 S.E.M.; MBP_{84-99}: MS 1.12±0.23 S.E.M., HD 1.80±0.46 S.E.M.; MOG: MS 5.82±4.27 S.E.M., HD 3.80±1.23 S.E.M.; GA: MS 1.63±0.44 S.E.M., HD 1.93±0.59 S.E.M.). Thus T cells in MS patients don't differ from controls in their ability to upregulate ICOS under antigenic challenge under the condition of primary proliferation (Figure 4.4).

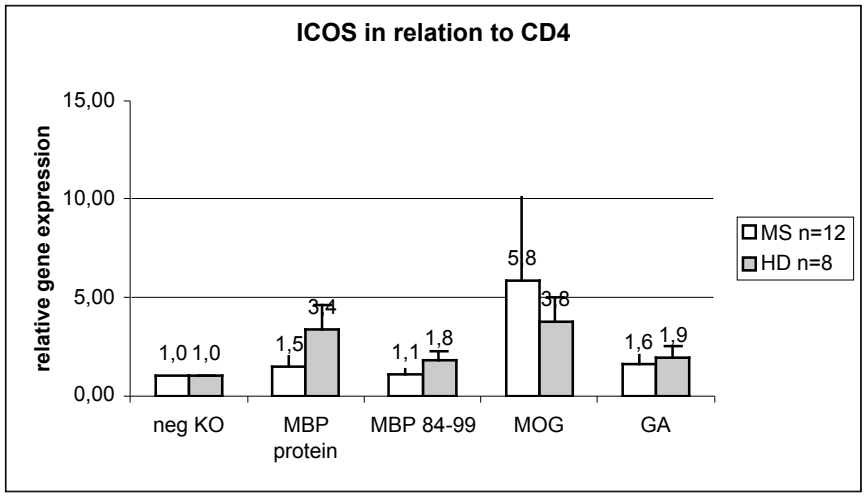

Figure 4.4: Comparison of ICOS mRNA expression in peripheral mononuclear cells (PBMCs) from patients with MS and HD. Freshly isolated PBMCs from patients with MS (n=12) and HD (n=8) were cultured in the presence of myelin peptides (MBP whole protein, MBP_{84-99} immunodominant domain, MOG_{1-125} immunodominant domain), Glatirameracetate (GA) or without peptide stimulation (neg KO) for 36 h under primary proliferation conditions. mRNA was quantified by QRT-PCR analysis. Results are shown after housekeeping gene (18SrRNA) normalization and in relation to CD4-expression and unstimulated PBMCs (neg KO). Bars represent the mean ± S.E.M ICOS mRNA production. in patients with MS (white) and HD (grey).

In the same approach and under the same stimulatory conditions the gene expression profile of the T_H2 cytokines IL-4 and IL-10 and the T_H1 cytokine IFN-γ was measured. Significant (two-sided t-test, $p<0.05$) higher mRNA production of the T_H2 cytokine IL-4 in HD compared to patients with MS was detected under stimulation with MBP_{84-99}, whereas the mRNA-production of the T_H2 cytokine IL-10 is significant (two-sided t-test, $p<0.05$) higher in PBMCs of MS patients cultured with the extracellular domain of the glycoprotein MOG. Otherwise no differences of cytokine production in patients with MS and HD could be detected. Results are shown in Table 4.1 and Figure 4.5.

		MBP protein	**MBP_{84-99}**	**MOG**	**GA**
IL-10	MS	2.57±0.66	1.36±0.56	10.89±3.13	1.70±0.71
	HD	2.69±0.52	1.28±0.27	5.19±1.65	1.33±0.23
IL-4	MS	1.50±0.62	0.74±0.20 **	6.94±5.65	1.38±0.40
	HD	2.75±0.85	1.45±0.33 **	3.30±1.32	2.00±0.80
IFN-g	MS	3.25±1.91	2.87±1.80	16.22±10.18	8.21±5.86
	HD	7.24±5.02	1.96±0.67	24.01±15.27	11.22±5.02

Table 4.1: **Comparison of the mRNA expression of the cytokines IL-10, IL-4 and IFN-γ in peripheral mononuclear cells (PBMCs) from patients with MS and HD.** Freshly isolated PBMCs from patients with MS (n=12) and HD (n=8) were cultured in the presence of myelin peptides (MBP whole protein, MBP_{84-99} immunodominant domain, MOG_{1-125} immunodominant domain), Glatirameracetate (GA) or without peptide stimulation (neg KO) for 36 h under primary proliferation conditions. mRNA was quantified by QRT-PCR analysis. Numbers are given after housekeeping gene (18SrRNA) normalization and in relation to CD4-expression and unstimulated PBMCs (neg KO) and represent the mean ± S.E.M mRNA production of duplicates; **significant differences (two-sided t-test, $p<0.05$)

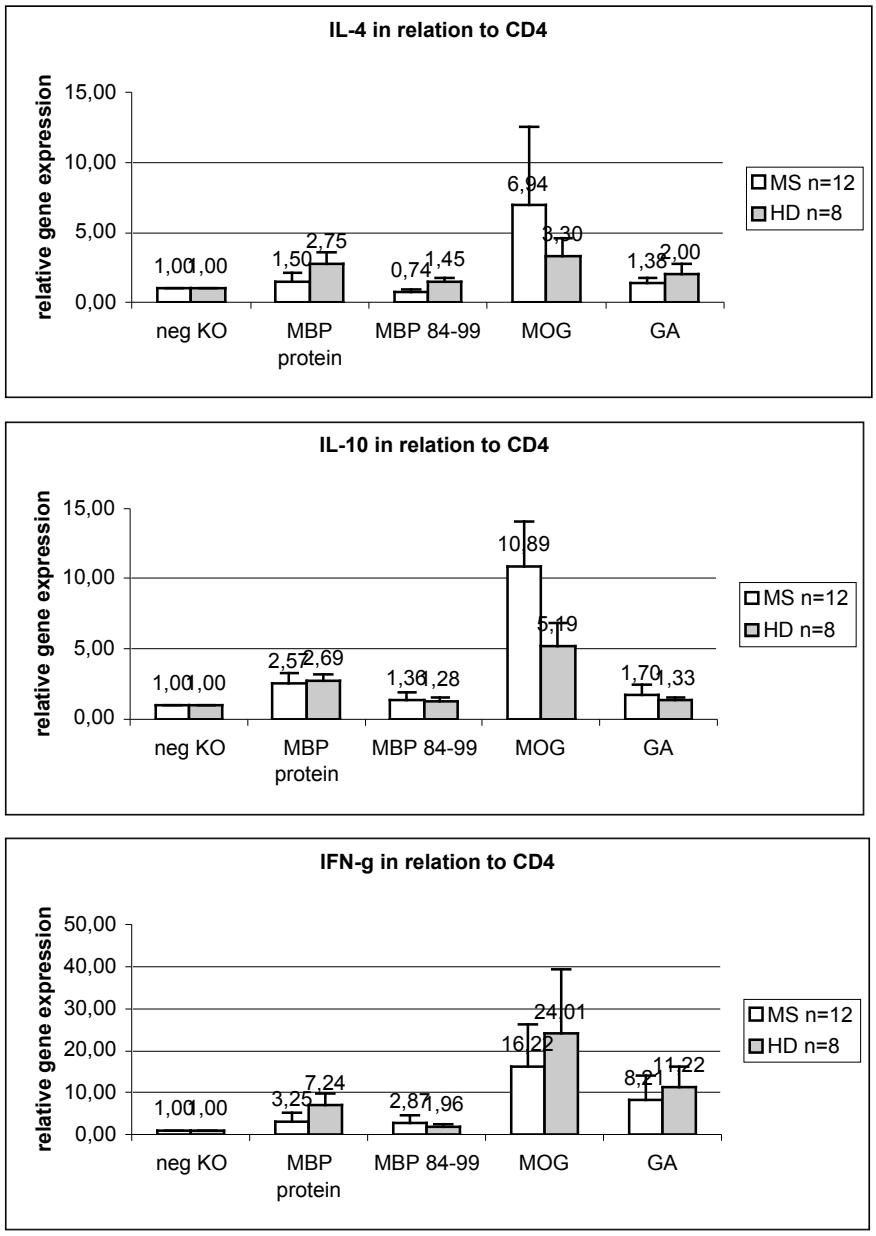

Figure 4.5 Comparison of expression of IL-4, IL-10 and IFN-γ in PBMC from patients with MS and HD. Freshly isolated PBMCs from patients with MS (n=12) and HD (n=8) were cultured in the presence of myelin peptides (MBP whole protein, MBP_{84-99} immunodominant domain, MOG_{1-125} immunodominant domain), Glatirameracetate (GA) and without peptide stimulation (neg KO) for 36 h under primary proliferation conditions. mRNA was quantified by QRT-PCR analysis. Results are shown after housekeeping gene (18SrRNA) normalization and in relation to CD4-expression and unstimulated PBMCs (neg KO). Bars represent the mean ± S.E.M. cytokine mRNA production of in patients with MS (white) and HD (grey).

4.3.2 Assessment of the QRT-PCR approach to detect changes in the gene expression profile in low frequencies of autoreactive T cells

The applicability of QRT-PCR to detect changes in the gene expression profile of low frequencies of antigen-stimulated T cells in a population of non-activated T cells has so far not been determined. Therefore titration experiments were performed to determine the detection threshold of antigen-stimulated T cells. PBMC stimulated with a solid-phase bound anti-CD3 antibody OKT3 for 36 h served as positive control since almost all T cells are activated under this condition. T cells cultured with medium alone served as negative control. Dilution of cDNA of these two samples was performed. Figure 4.6 shows the result of cDNA titration experiments. Aberrations in gene expression were used to calculate the frequencies of detectable cells.

The evaluated frequencies in which changes in the level of gene expression were detectable are 10^{-2} for ICOS (dilution 1:100), 10^{-3} for IL-4 and IL-10 (dilution 1:1000) and 10^{-5} for IFN-γ (dilution 1:10 000). These findings implicate that the ability to detect changes in the gene expression profile of “a single T cell” specific for myelin antigen varied strongly among the assessed molecules, being IFN-γ the best marker for proliferation and activation.

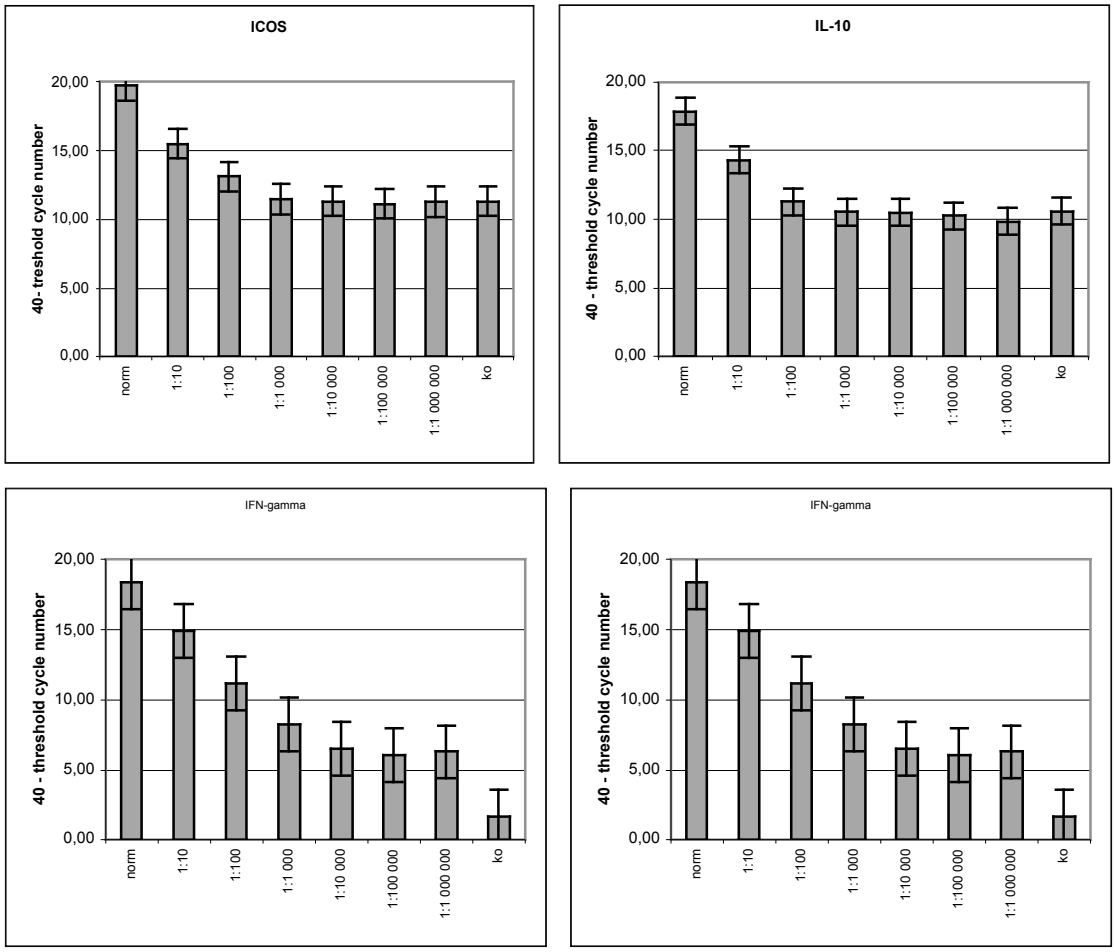

Figure 4.6 Titration study of cDNA from activated T cells by solid phase bound anti-CD3 antibodies against cDNA from unstimulated T cells. Hereby the ability of quantitative real-time RT-PCR to detect changes in the gene expression profile of one single activated T cell in a given number of non-activated T cells was to be determined. By subtracting threshold cycle numbers from the value 40, high values on the y-axis indicate high amounts of gene expression. cDNA from stimulated PBMCs was diluted 1:50 in pure water. This concentration served as positive control (norm). cDNA (diluted 1:50 in pure water) of unstimulated PBMCs served as negative control (ko). The evaluated frequencies where changes in the level of gene expression were detectable were 10^{-2} for ICOS (dilution 1:100), 10^{-3} for IL-4 and IL-10 (dilution 1:1000) and 10^{-5} for IFN-g (dilution 1:10 000).

4.3.3 Gene expression profile of ICOS, IL-4 and IFN-γ in brain tissue specimens from a patient with Multiple Sclerosis

Myelin reactive T-cells migrate into the CNS where they reencounter their antigen and orchestrate myelin damage (Steinman 1993; Steinman 1996). In this course the T_H1 cytokines IL-2, IL-12, TNF-α and IFN-γ create a pro-inflammatory cytokine milieu, which recruits other immune cells and thus perpetrates the local immune response.

Gene expression of ICOS, IL-4 and IFN-γ were quantified in CNS tissue specimens from different brain regions in a MS patient

Human brain tissue specimens were classified by means of histopathology as “normal appearing white matter” (NAWM 1 & 2) or “control”. Normal appearing white matter is defined as cerebral white matter tissue specimen containing myelinated axons that already show histopathologically inflammatory processes. Control tissue specimens were obtained from post-mortem biopsies of individuals without pathological cerebral processes. RNA was extracted from these regions and cDNA synthesis was performed. Unfortunately plaque material was not available. By the use of QRT-PCR analysis the gene expression profiles were measured and results are shown in Figure 4.7. Interestingly the results reveal upregulated gene expression for ICOS (3528 fold), IL-4 (119 fold) and IFN-γ (9 fold) in one of the samples classified as NAWM1 in contrast to moderate to low expression in NAWM2 (ICOS: 3,3 fold; IL-4: 1,4 fold; IFN-γ: 0,5 fold) when compared to control.

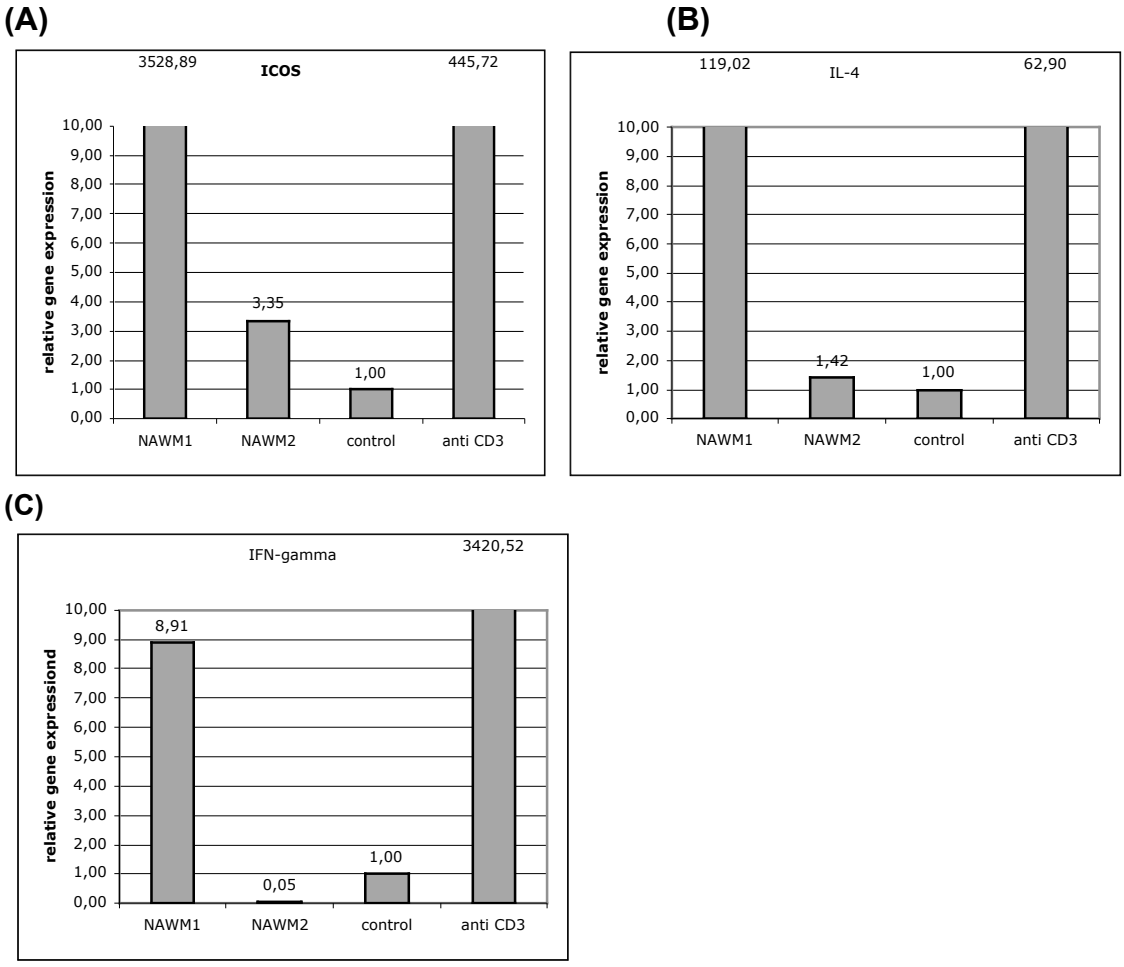

Figure 4.7: Comparison of gene expression profile of the costimulatory molecule ICOS (A) and the cytokines IL-4 (B) and IFN-γ (C) in central nervous system (CNS) tissue samples from a patient with Multiple Sclerosis. Prior to mRNA quantification by real-time RT-PCR analysis cDNA samples originating from cerebral tissue specimen and were classified by means of histopathology as “normal appearing white matter” (NAWM 1 &2) or “control” tissue. In normal appearing white matter myelinated axons as well as clues for inflammatory processes are found. Peripheral mononuclear cells (PBMCs) stimulated for 36h with solid phase bound anti CD3 antibody served as positive control. Results are shown after 18SrNA normalization and in relation to the sample “control”.

4.4 Gene expression profile analysis of the extended B7-family ligands in professional APC and human brain specimens

The gene expression profile of the extended B7-family ligands in CNS tissue samples from a patient with MS was assessed by means of QRT-PCR. Activated T cells and different kinds of APC served as positive control.

4.4.1 Gene expression analysis of B7-family costimulatory molecules in professional APC

Oligonucleotides of the so far known members of the B7/CD28-family were designed and tested by the use of QRT-PCR analysis in cDNA samples of different kinds of APC namely mature and immature dendritic cells, B-cells and monocytes. Results are shown in Figure 4.8 and resemble earlier described gene expression profiles (see introduction).

PDL1 and PDL2 bind to the same ligand, PD1. Compared to unstimulated PBMC both molecules are expressed in moderate levels in B cells (PDL1: 54 fold; PDL2: 108 fold) and monocytes (PDL1: 415,9 fold; PDL2: 225,2 fold). Higher mRNA levels are found in immature DC (PDL1: 1820,4 fold; PDL2: 1273,9 fold), but it is in mature DC where highly increased mRNA levels of both molecules (PDL1: 6539,7 fold; PDL2: 3929,2 fold) are found.

B7H2 and GL50 are splice variants of LICOS. Compared to unstimulated PBMC low mRNA levels of B7H2 and GL50 are found in B cells (B7H2: 31,7 fold, GL50: 3.4 fold) and monocytes (B7H2: 16,5 fold; Gl50: 15,7 fold), whereas gene expression was upregulated in immature DCs (B7H2: 196 fold, Gl50: 65 fold) and mature (B7H2: 115,0 fold, Gl50: 44,8 fold). Interestingly higher expression levels of both molecules were found in immature DC than in mature DC.

B7H3 is a recently identified member of the B7 family and up to date the ligand has not been identified. Compared to unstimulated PBMC the mRNA expression moderately elevated levels were found in B cells (39,4 fold) and

monocytes (623,8 fold) whereas the levels detected in mature (42347,8 fold) and immature (50886,7 fold) DC are very high.

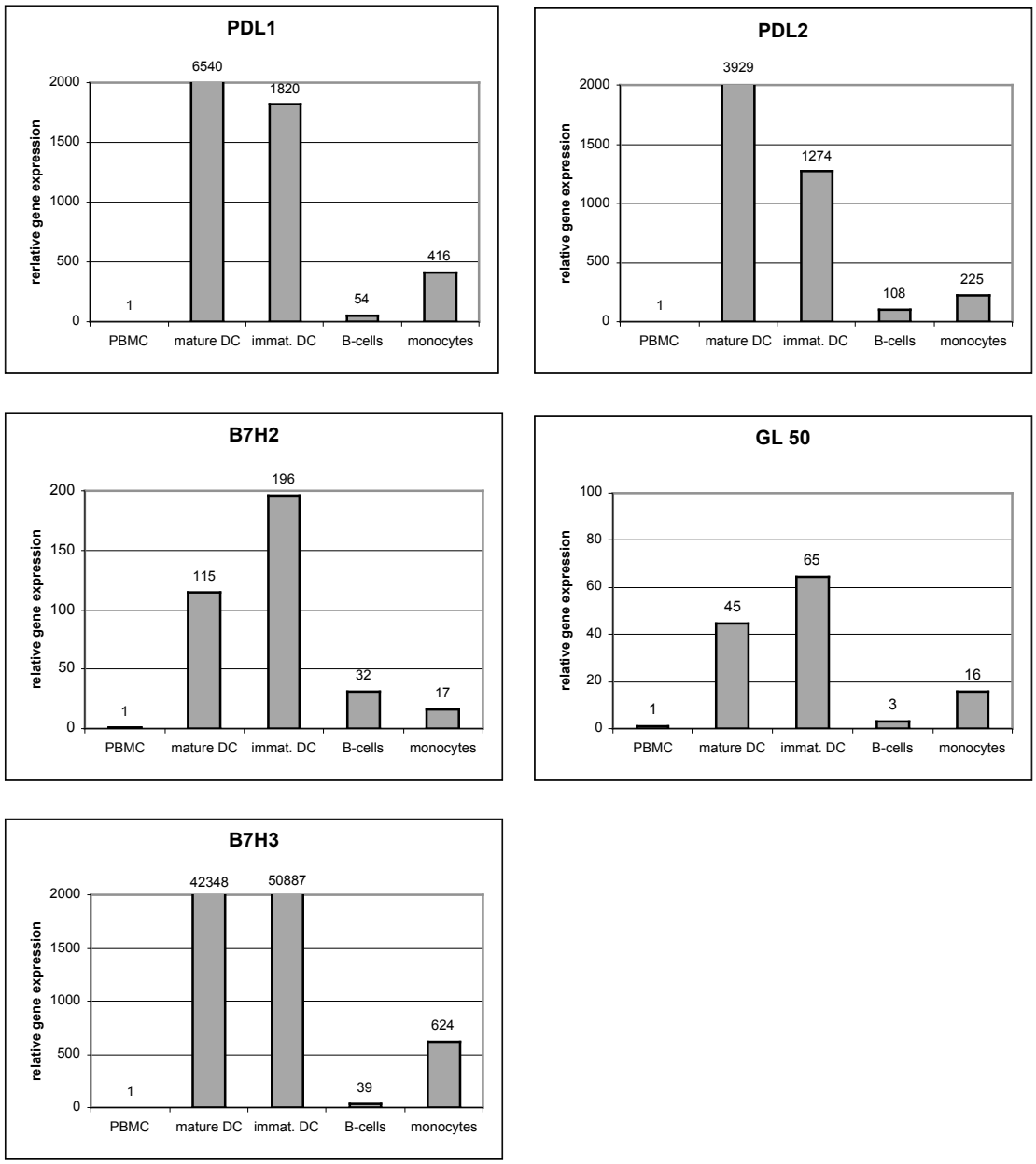

Figure 4.8: Gene expression profiles of the B7-family members PDL-1, PDL-2, B7H2, GI50 and B7H3 in APC from peripheral blood. mRNA gene expression levels were compared in peripheral mononuclear cells (PBMC), immature and mature monocyte-derived (48h LPS) dendritic cells (DC), B cells and monocytes from one healthy donor. Results are shown after housekeeping gene normalization with 18SrRNA and in relation to PBMCs.

4.4.2 Gene expression profile of the B7-family in CNS tissue specimens from patients with MS and on activated T cells

In order to quantitate mRNA levels of the B7-family members in brain tissue specimens from patients with MS QRT-PCR was performed. Samples were classified by means of histopathology as “normal appearing white matter” (NAWM 1 and 2; see 4.3.3 for more detailed description of NAWM) or “control”. Results are shown in Figure 4.9.

B7-1 and B7-2 are the corresponding ligands to CD28 and CTLA-4. The gene expression pattern of these two B7-family members varies considerably in the assessed samples: Compared to one control brain specimen, B7-2 expression is upregulated modestly in the samples NAWM2 (3,5 fold) whereas its gene expression level is abundantly high in NAWM 1 (3385,1 fold). In contrast to this B7-1 mRNA expression is modestly upregulated in the sample termed NAWM2 (2,2 fold) whereas its expression is low in NAWM1 (0,6 fold). Although levels of mRNA expression of other costimulatory molecules were also upregulated in the cDNA sample termed “normal appearing white matter 2” (NAWM2) the value assessed for B7-2 mRNA expression was by far the highest.

PDL1 and PDL2 both belong to the B7-family of costimulatory molecules and bind to one ligand, PD1. Their gene expression pattern is more or less similar in the measured cDNA samples: Low levels are expressed in NAWM2 (PDL1: 0,5 fold; PDL2: 1,3 fold) whereas in the sample named NAWM1 abundant levels of mRNA expression were detected for both molecules (PDL1: 260,5 fold; PDL2: 146,5 fold) compared to control.

B7H2 is one splice variant of ICOS ligand. Compared to control gene expression pattern shows low levels in NAWM2 (0,3 fold) whereas in NAWM1 high levels (249 fold) of B7H2 mRNA expression were detected.

B7H3 shows an expression pattern similar to that of the other costimulatory molecules with abundantly high levels of mRNA expression in the sample termed NAWM1 (141,5 fold) and negligible levels NAWM2 (0,5 fold).

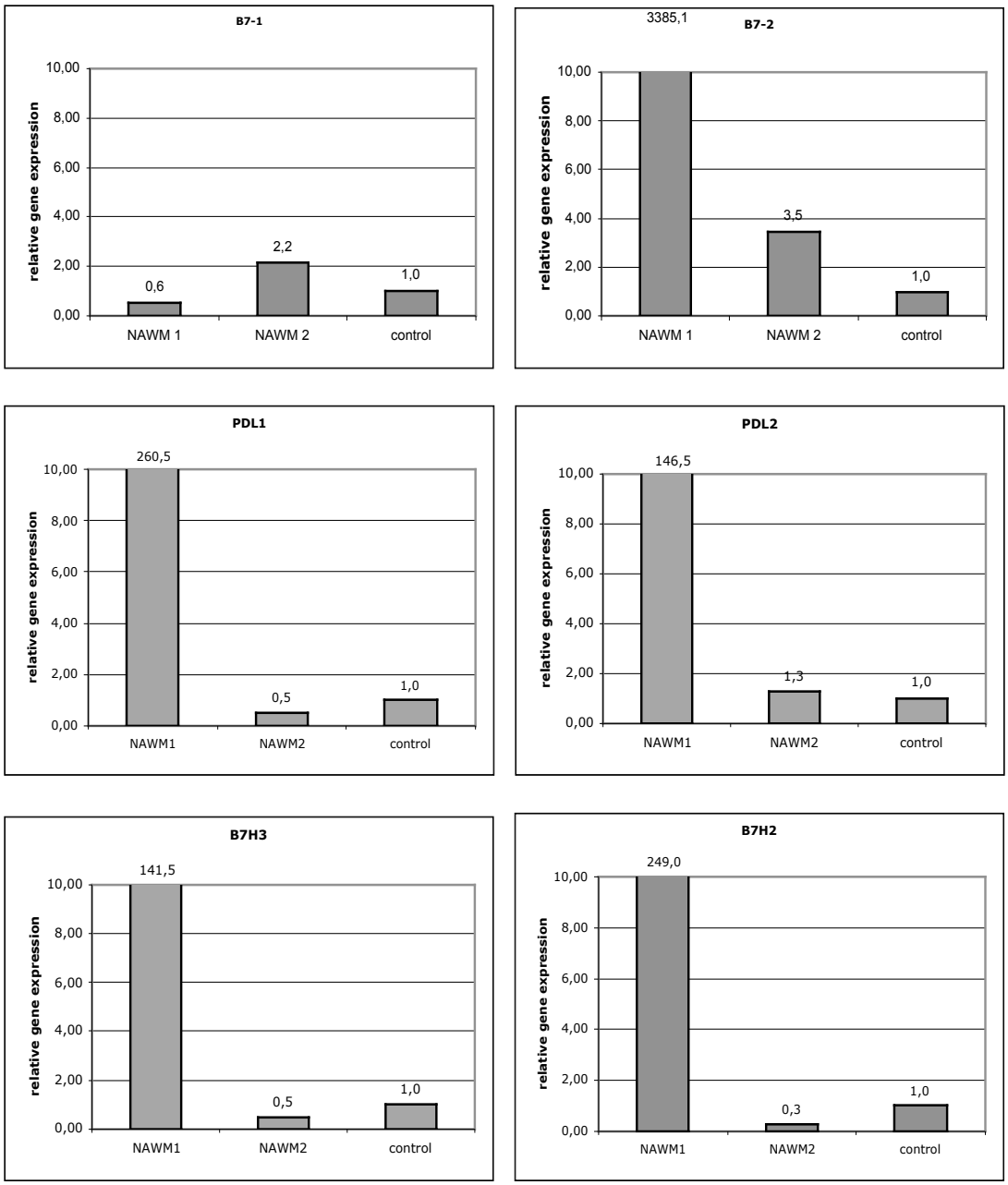

Figure 4.9: Gene expression profile of B7-family members B7.1, B7.2, PDL1, PDL2, B7H2 and B7H3 in central nervous system (CNS) tissue samples from a patient with Multiple Sclerosis and on activated T cells. Prior to mRNA quantification by real-time RT-PCR analysis cDNA samples were classified by means of histopathology as ”normal appearing white matter” (NAWM 1&2) or ”control”. Normal appearing white matter is defined as brain tissue consisting of myelinated axons surrounded by inflammatory processes. Results are shown after housekeeping gene normalization with 18SrNA and in relation to the sample control.

5. Discussion

5.1 Characterization of ICOS in MS patients and healthy controls

The initial characterization of ICOS suggested that ICOS might function as a T_H2-specific costimulatory molecule (Hutloff, Dittrich et al. 1999). ICOS ligation was found to upregulate mainly IL-4 and IL-10 cytokine production by T cells. ICOS $^{-/-}$ mice exhibited a disturbed balance between T_H1/ T_H2 T cells and profound defects in T_H2-induced humoral responses as well as in the production of most T_H2 cytokines (McAdam, Schweitzer et al. 1998; Dong, Juedes et al. 2001; Tafuri, Shahinian et al. 2001). In accordance with this hypothesis, results in this study show that human antigen-specific T_H2 TCL express higher amounts of ICOS than T_H1 TCL thus suggesting a critical role for ICOS in the T_H1/T_H2 development and T cell subset distribution (Fig. 4.3). However, the paradigm of ICOS being a selective T_H2-specific costimulatory molecule has recently been challenged by the observation of its involvement also in T_H1 memory/effector responses (Gonzalo J-A 2001; Ozkaynak, Gao et al. 2001; Rottman, Smith et al. 2001; Sperling and Bluestone 2001; Sporici, Beswick et al. 2001; Tesciuba, Subudhi et al. 2001). In analogy to the observations in ICOS deficient mice, ICOS neutralization in the induction phase of EAE exacerbated the disease course as a consequence of a dysbalanced T_H1/T_H2 helper cell development (Rottman, Smith et al. 2001). However, blockade in the effector phase had a favourable influence on the outcome of EAE (Rottman, Smith et al. 2001; Sporici, Beswick et al. 2001) thus proposing ICOS as an activation-induced cell surface molecule modulating the effector or memory T cell responses of autoreactive T cells (Sperling 2001).

MS is regarded as a paradigm of a predominantly CD4 T_H1-cell mediated autoimmune disease that may result from regulatory disturbances both in the differentiation of T_H1/T_H2 cells after primary activation and in the effector/memory T cell population (Martin, McFarland et al. 1992; Hemmer, Archelos et al. 2002). Results of this study provide information on the regulation of ICOS expression on peripheral blood T cells in patients with MS compared to

healthy donors. ICOS is rapidly induced on CD4 and CD8 T cells after antigen unspecific T cell activation (Fig. 4.1) (Wiendl et al. 2003a). Consistent with this is the finding of this study with respect to ICOS gene expression after antigen-specific stimulation that didn't show differences in MS patients and healthy controls (Fig. 4.4). Since no differences in the regulation of T-cell responses between MS and healthy controls were observed, one can assume that dysfunctions in this costimulatory pathway are not critical for the disturbances in the T_H1/ T_H2 development that may underlie the potentially autoreactive T-cell repertoire in MS. Consistent with this is the finding that the production of IL-4 and IL-10 upon antigen specific stimulation of PBMCs differed in only one cell-culture condition and that these differences of higher T_H2 cytokine mRNA expression were observed in MS patients in the opposite way as with the HD group. These facts are not sufficient to draw any conclusions concerning their role in the pathogenesis of MS. This is underlined by recent findings that ICOS is capable to enhance the production of T_H1 and T_H2 cytokines in effector/memory CD4 T cells and promotes both, T_H1 and T_H2 cell differentiation (Beier, Hutloff et al. 2000; McAdam, Greenwald et al. 2001. Facing these observations the fact that ICOS costimulation is sufficient to enhance TH1 and TH2 cytokine production, both in the absence and the presence of B7/CD28 costimulation is of special importance (Wiendl et al 2003a; Ozkaynak, Gao et al. 2001). Many non-lymphoid tissues are capable to act as non-professional APC by expressing MHCII molecules. Thus, in autoimmune processes generated or perpetuated in non-lymphoid tissues lacking B7.1/2, the ICOSL/ICOS second signal may be of critical importance, as for example recently demonstrated in the case of inflammatory myopathies (Wiendl, Mitsdoerffer et al. 2003c). In MS, the local (re)activation of autoreactive T cells in the CNS by MHC class II-antigen expressing target cells is considered as an important step triggering the autodestructive immune response against myelin structures in situ (Martin, McFarland et al. 1992; Hemmer, Archelos et al. 2002). Further, this issue may be of relevance with regard to the characteristics and the putative prerequisites of (re)activation of myelin-reactive T cells in MS. First, B7.1/ CD28 costimulation seems to be less important for the reactivation

of myelin-reactive T cells in patients than in healthy controls (Lovett-Racke, Trotter et al. 1998; Scholz, Patton et al. 1998). Second, a population of CD4+CD28_ myelin-reactive T cells has recently been defined in MS (Markovic-Plese, Cortese et al. 2001) that may easily be (re)activated peripherally or within the CNS in the absence of B7.1/2. Concerning the implications of further studies for ICOSL/ICOS in the immunopathogenesis of MS it has to be noted that the in vivo expression of ICOSL/ICOS in CNS tissue has not been studied to date. According to the observations in the murine system, ICOSL seems to be constitutively expressed within the CNS (Rottman, Smith et al. 2001). Clarification of its involvement in MS pathogenesis could be achieved by a detailed description of ICOSL/ICOS expression patterns on CNS and inflammatory cells including its characterization in active and chronic MS-plaques.

Another possible mechanism by which ICOS may influence the differentiation of T_H1/T_H2 cells is its potential role in regulatory cells such as CD4/CD25$^+$ cells. So far no firm relationship between CD4/CD25+ regulatory cells and ICOS expressing T_H2 cells has been made and one important issue of this study were the investigations in this respect. Although no direct association of ICOS with CD4/CD25 regulatory T cells was found (Fig. 4.2) (Beier, Hutloff et al. 2000), one possibility may still be an impact on regulatory T cell functions, which could also be detrimental. The advantage of ICOS selective blockade of activated T cells may be regulated by the unselective interference with T_H1 and T_H2 cytokines, which may impede anti-inflammatory or beneficial local immune responses.

5.2 Expression of the extended B7 family members on professional APC and in MS tissue specimen

Although precise functional aspects of costimulation are just beginning to be explored it is very likely that engagement of the different costimulatory molecules at distinct time points of T cell differentiation and activation contributes to T cell regulation and orchestrates immune responses.

The results of this study concerning the gene expression profile of B7-molecules on APC confirm previously reported expression patterns of these molecules. As mature DC are the most potent antigen presenting cells it is not surprising that the highest mRNA levels of the B7 costimulatory molecules were found in these cells: PDL1, PDL2, B7H2, GL50 and B7H3 (Figure 4.8). Interestingly mRNA levels of B7 molecules in APC differed substantially with marginal amounts of ICOS ligand mRNA compared to high mRNA levels observed for other B7 family members. As the studies were performed with mRNA from APC at one given time point of their maturation or activation the result reveals only a snapshot in the development and maturation of the cells. Therefore studies addressing the upregulation kinetics of costimulatory molecule on different APC could be proposed.

Analysis of peripheral T cells, which have been previously activated with myelin antigen before assessment of the gene expression profile is an artificial attempt to gain insight into the inflammatory process of plaque formation within the CNS occurring during the course of MS. Therefore cDNA samples from brain tissue from patients with MS are of special interest, as they allow a more direct “view” on the processes of demyelination and inflammation. Interestingly the findings in this study reveal upregulated gene expression of ICOS as well as other B7 molecules and certain cytokines in one human brain specimen classified as “normal appearing white matter” (Figures 4.7 and 4.9). An explanation for this finding might be the fact that the extent of inflammatory processes is difficult to estimate by means of histopathology. Therefore histopathologically comparable regions may be of different immunological activity. Furthermore the assessment of only one cDNA sample is by far not sufficient enough to draw any conclusions and in the future quantification of gene expression in more samples may help to understand the pathological processes underlying MS plaque formation.

5.3 Considerations on the technical approach: Is QRT-PCR an appropriate method to assess differences in the gene expression profile of myelin specific antigen specific T cells in peripheral blood?

The use of QRT-PCR for the approach to detect differences in the gene expression profile between different cell types or cell samples bears several widely accepted advantages: The technique is characterized by a high sensitivity, a low intra-assay variation, no need for post-PCR analysis and the specificity of the products can be monitored easily by analyzing the melting curves. Although gene expression profiles can be detected in very small numbers of cells (Bustin 2000) this study shows that the minimal frequencies of antigen specific T cells to assess gene expression differ substantially for distinct molecules: f= 10^{-2} for ICOS, f= 10^{-3} for IL-4 and IL-10 and f= 10^{-5} for IFN-γ (Figure 4.6). Potential explanations for this observation are unknown (perhaps greatly differing) affinities of the used oligonucleotides to small numbers of target-cDNA or differing amounts of mRNA production under the unphysiological high stimulatory conditions of solid-phase bound anti-CD3 antibodies.

In the past reports of studies concerning the frequency of autoreactive T cells specific for peptides of the myelin sheath in the peripheral blood of patients with MS differed in a great range. For instance frequencies of autoreactive T cells specific for MBP were described in one study to range from 10^{-5} to 10^{-6} whereas other studies report the frequency for MBP specific T cells in a range from 10^{-2} to 10^{-3} (Ota, Matsui et al. 1990; Markovic-Plese, Cortese et al. 2001; Markovic-Plese and McFarland 2001). The use of different peptides (synthetic peptides versus recombinant or native peptides) as well as different technical approaches may contribute to these findings (Koehler, Genain et al. 2002).

Therefore quantitative real-time RT-PCR is an elegant method bearing several advantages as long as frequencies of autoreactive myelin-specific T cells are higher as the elucidated detection threshold, whereas it might be not sensitive enough for lower frequencies As the question concerning the frequency of

autoreactive T cells remains open the approach of QRT-PCR to detect changes in the gene expression profile of the few autoreactive myelin-specific T cells found in peripheral blood is a critical attempt. Further studies could be proposed to address this question more in detail.

6. Summary

Costimulatory signals play a key role in regulating T cell activation and are believed to have decisive influence in the inciting and perpetuating cellular effector mechanisms in autoimmune diseases such as multiple sclerosis (MS). Inducible costimulator protein (ICOS), a recently identified member of the CD28-family, presumably affects the differentiation of Th1/Th2 cells after primary activation and modulates the immune response of effector/memory T cells.

This study examined the constitutive expression as well as upregulated expression after antigen-specific stimulation of ICOS and the cytokines IL-4, IL-10 and IFN-g in healthy donors and patients with MS. Gene-expression of these molecules and numerous B7-costimulatory molecules was also examined in brain tissue specimen from a patient with MS as well as in antigen presenting cells. Additionally ICOS expression in $CD4^+CD25^+$ cells and in Th1 and Th2 specific T cell lines was examined. For this purpose techniques such as flow cytometry and QRT-PCR were used. Furthermore the question whether QRT-PCR is an appropriate approach to detect changes in the gene expression profile in low frequencies of autoreactive T cells was addressed.

Analysis of ICOS gene-expression in Th1 and Th2 specific T cell lines clearly revealed ICOS gene expression being restricted to Th2 specific cells whereas it was absent in Th1 specific cell lines. Comparison of constitutive cell surface expression and upregulational kinetics of ICOS on $CD4^+$- and $CD8^+$-T cells showed no differences in patients with MS and healthy individuals. It was furthermore shown that ICOS is not exclusively expressed in $CD4^+CD25^+$ cells and thus is not a marker for this regulatory subset. Gene expression of ICOS, IL-4, IL-10 and IFN-g in PBMCs after antigen specific stimulation in MS patients and healthy individuals differed for IL-4 and IL-10 after stimulation with MBP_{84-99} and MOG, respectively. IL-4 expression was higher in healthy individuals, whereas greater IL-10 expression was detected in MS patients. However, as these two Th2 cytokines were upregulated in MS patients and healthy individuals in an opposing way, no conclusions with respect to their role and the

role of ICOS in the pathogenesis of MS can be drawn. With regard to the expression of B7-costimulatory molecules in APC it is not surprising that the greatest amounts of gene expression was found in mature DC as these cells are the most potent APC. Some interesting findings could also be made for the gene-expression of ICOS and other costimulatory molecules in tissue specimen from a patient with MS: In one human brain specimen classified as “normal appearing white matter” upregulated gene expression of ICOS, other B7 costimulatory molecules and of inflammatory cytokines could be detected whereas in other specimen also classified as “normal appearing white matter” only moderate levels of these molecules could be found. This noteworthy observation indicates that in CNS of MS patients inflammatory activity is potentially present in histopathologically normal appearing areas. With regard to the applicability of QRT-PCR to detect changes in the gene expression profile of autoreactive T cells it was found that the method is useful as long as frequencies of the cells are higher as the elucidated detection threshold.

7. References

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8. Abbreviations

APC	Antigen presenting cell
CD	Cluster of differentiation
CCR	Chemokine receptors
CNS	Central nervous system
CSF	Cerebro spinal fluid
DC	Dendritic cell
DMSO	Dimethylsulfoxide
EAE	Experimental allergic encephalomyelitis
EDSS	Expanded disability status scale
FcR	Fc-receptor
FCS	Fetal Calf Serum Gold
G/C	Guanin/Cytosin
HD	Healthy donor
HEV	High endothelial venule
ICOS	Inducible Costimulator
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBP	Myelin Basic Protein
MHC	Major histocompatibility complex
MMP	Matrix metallo protease
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis

NAWM	Normal appearing white matter
NGS	Normal goat serum
NO	Nitric oxygen
PBMC	Peripheral mononuclear cell
PBS	Phosphate buffered saline
PP-MS	Primary-progressive Multiple Sclerosis
RR-MS	Relapsing-remitting Multiple Sclerosis
RT-PCR	Reverse transcriptase polymerase chain reaction
S.E.M	Standard error of the mean
SH	Src homology domain
SP-MS	Secondary-progressive Multiple Sclerosis
TCR	T cell receptor
T_H	T helper
TNF-α	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion molecule
VLA-1	Very late antigen

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Diese Doktorarbeit möchte ich meinen Eltern widmen, die mich im Studium immer uneingeschränkt unterstützt haben.

10. Curriculum Vitae

Personal Specifications

born on 09/11/1976 in Eichstätt/Germany

marital status: unmarried

nationality: german

Academic Records

97 - 04 medical education at the Hannover and University of Tübingen Medical School, result of final examination (Staatsexamen, 14.05.2004): high above average (sehr gut)

studies abroad: medical school final year clinical rotation (Praktisches Jahr – PJ) at the University of Zürich/Switzerland (surgery) and the University of Montpellier/France (internal medicine); clinical clerkships in Great Britain, United States and The Netherlands in internal medicine, neurology, neuro-ophthalmology, general medicine and surgery

83 – 96 elementary School and secondary School with emphasis on english, physical sciences and history (Robert-Bosch-Gymnasium, Langenau)

Civil Service

96 - 97 Department of Orthopaedics, University of Ulm Medical School

Career

08/04 start of a residency program at the Department of General Neurology of the University of Tübingen Medical School

Scientific Record

02 - 04 MD Thesis at the Laboratory of Neuroimmunolgy, Department of Neurology University of Tübingen Medical School; Original title: “The costimulatory molecule inducible costimulator (ICOS) in Multiple Sclerosis” and subsequent extracurricular continuation of scientific work in the field of costimulation in Multiple Sclerosis; financially supported from the Department of Neurology University of Tübingen Medical School

2003 attendance at the 4th Course of the European School of Neuroimmunology (ESNI) in September 2003; bursary: “ ESNI Travel Grant for Young Investigators”

Publications

A regulatory polymorphism in the Programmed Death 1 Gene (PD-1) impairs inhibition of T cell activation and is associated with disease progression in Multiple Sclerosis
A. Kroner*, M. Mehling*, B. Hemmer, P. Rieckmann, K.V. Toyka, M. Mäurer*, H. Wiendl* (*equal contribution); submitted

The CD28 related molecule ICOS: T cell modulation in the presence and absence of B7.1/2 and regulational expression in multiple sclerosis;
H. Wiendl, O. Neuhaus, M. Mehling, S. Wintterle, B. Schreiner, M. Mitsdoerffer, W. Wienhold, R. Weissert, J. Wessels, HP. Hartung, M. Weller, E. Tolosa, A. Melms
Journal of Neuroimmunology 140(1-2):177-87

The humoral response in the pathogenesis of gluten ataxia;
H. Wiendl, M. Mehling, J. Dichgans, A. Melms, and K. Bürk.
Neurology 2003; 60: 1397

Abstracts:

A polymorphism in the Programmed Death 1 Gene (PD-1) impairs inhibition of T cell activation and is associated with disease progression in Multiple Sclerosis
M. Mehling, A. Kroner, B. Rosche, B. Hemmer, K.V. Toyka, P. Rieckmann, M. Mäurer, H. Wiendl
Oral presentation at 7th International Congress of the International Society of Neuroimmunology (ISNI), September 2004; abstract listed in: Journal of Neuroimmunology 154(1-2):22 [oral presentation].

Untersuchungen zur humoralen Immunantwort bei sporadischer Ataxie mit Glutensensitivität
H. Wiendl, M. Mehling, C. Globas, J. Dichgans, A. Melms und K. Bürk
Aktuelle Neurologie 2002;29(suppl 2):545 [Abstract].

Inducible Costimulator Protein (ICOS) in Multiple Sclerosis: Preferential Expression on Th2 Cells Reveals a Regulatory Role in CNS-Autoimmunity
H. Wiendl, O. Neuhaus, E. Tolosa, M. Mehling, R. Weissert, HP. Hartung, R. Kroczek, A Melms
Meeting of the International Society of Neuroimmunology (ISNI) September 2001 [Poster].

Extracurricular Activities

languages: English and French (writing, reading and oral)

00/02	active member of the DFA (Deutscher Famulantenaustausch), which is a non-profit organization belonging to the International Medical Student's Associations (IFMSA) that coordinates international exchange programs for medical students
97/99	student delegate of the Hannover Medical School