Der Einfluss von immunmodulierender Therapie auf die Glykosylierung von Immunzellen bei Patienten mit schubförmiger multipler Sklerose

The influence of immunomodulating treatment on the glycosylation of immune cells in patients with relapsing-remitting multiple sclerosis

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Content

CNS	central nervous system
CSF	cerebrospinal fluid
DMSO	dimethylsulfoxide C2H6OS
EAE	experimental autoimmune encephalomyelitis
FACS	fluorescence-activated cell sorting
FS	forward scatter
IFN	interferon
IgM, G, A, E	immunoglobulin M, G, A, E
IL	interleukin
HLA	human leukocyte antigen
LSM	lymphocyte separation medium
MAL	maackia amurensis lectin, common name: maackia
MBP	myelin basic protein
MOP	myelin oligodendrocyte protein
MS	multiple sclerosis
nTregs	naturally occurring regulatory T cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PLP	proteolipid protein
PMA	phorbol 12-myristate 13-acetate
RRMS	relapsing-remitting multiple sclerosis
SEM	standard error of the mean
SNA	sambucus nigra lectin, common name: elderberry lectin
SS	sideward scatter
STAT	signal transducer and activator of transcription
Th cells	T helper cells
Tregs	regulatory T cells
UEA	ulex europaeus agglutinin, common name: gorse, furze
VCAM	vascular adhesion molecule
VLA	very late activation antigen

1. Introduction

The immune system is made up of various cell types and proteins that help to clear the body of potential pathogens and keep infections in check. Immunodeficiency and autoimmunity are unfavourable immune disorders that result from the immune response being either too weak or too strong. This study describes phenotypical characteristics of cells of the immune system that are associated with multiple sclerosis and in particular those involved in immunomodulatory treatment. Specifically, changes in the glycan structure on the surface of cells that are associated with MS immunomodulatory treatment (interferon-β and natalizumab) are assessed. This chapter provides an overview of the components of the immune system that are examined in the study.

Multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease of the central nervous system that mainly occurs in young adults. It is characterised by the loss of the myelin sheath around axons, which compromises the conductibility of the affected nerves. The demyelination of axons leaves so-called MS plaques that can often be found in the periventricular white matter, the optic nerves, and the white matter of the spinal cord (Kumar, Cotran et al. 2003). MS plaques often appear as astrocytic scars with relatively well preserved axons (Noseworthy, Lucchinetti et al. 2000). The most common form of MS is relapsing-remitting MS (RRMS) where patients suffer recurring exacerbations of neurologic dysfunction but also experience phases of recovery. Other forms include secondary progressive MS, which is marked by a continuous deterioration of clinical symptoms and evolves from RRMS, and primary progressive MS, which directly begins with a progressive decline in neurologic function (Compston and Coles 2008). Depending on the site of the lesions, patients suffering from MS may present with neurological symptoms such as unilateral optic neuritis, diplopia, Lhermitte's sign (the sensation of a general electric shock caused by flexion of the neck), limb weakness, clumsiness, gait ataxia, and neurogenic bladder and bowel symptoms (Compston and Coles 2008).

The etiopathogenesis of MS has not yet been completely understood. Most immunologists believe that MS is an autoimmune disorder. An increased concordance rate in twins suggests that genetic factors influence the risk of developing MS (Mumford, Wood et al. 1994). Moreover, an association with HLA class II polymorphisms Dw2 and DR2 has long been known (as reviewed by Dyment, Ebers et al. 2004). No single gene was found to be associated with MS and it is instead viewed as a polygenic disorder (Fernald, Yeh et al. 2005). It has been observed that the risk of developing MS was associated with the MS prevalence in the country where people lived at a young age (Elian, Nightingale et al. 1990). Thus, environmental factors, such as an infection with Epstein-Barr virus, are also discussed (Serafini, Rosicarelli et al. 2007).

The primary target of CNS-inflammation in MS is unclear. However, possible candidates for immunogenic brain-antigens are oligopeptides derived from myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte protein (MOP) (Murphy, Travers et al. 2012, p. 636). It is believed that in order to be recognised by T cells these brain-antigens are presented by antigen-presenting cells to naive T cells in places like deep cervical lymph nodes (Hatterer, Davoust et al. 2006). The encounter with the antigen turns T helper cells into activated T memory cells. In addition, an inflammation of the brain set up by a trigger that can still not be pinpointed, increases the permeability of the blood vessels (as reviewed by Frohman, Racke et al. 2006). Consequently, activated T memory cells enter the site of inflammation and are promptly re-activated by local and infiltrating antigen-presenting cells (as reviewed by Fletcher, Lalor et al. 2010; Lucchinetti, Popescu et al. 2011). As a result, the newly activated T cells proliferate and secrete cytokines, which in turn exacerbates the perivascular inflammation and attracts Th1 cells, Th17 cells and myeloid cells via pial vessels (Lucchinetti, Popescu et al. 2011). Subsequently, innate immune cells and plasma cells producing autoreactive antibodies aggravate the inflammation which impinges on oligodendrocytes and eventually results in demyelination of neurites (Lucchinetti, Popescu et al. 2011).

T helper 1 cells

Phenotype, function and differentiation of human Th1 cells

T helper 1 (Th1) cells can be distinguished from other T helper cells by their production of specific cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2) (Mosmann and Coffman 1989).

Under physiological conditions Th1 cells are involved in the defence against intracellular pathogens. They help to keep bacterial infections in check by activating infected macrophages and thus increasing their intracellular microbicidal power (Murphy, Travers et al. 2012, p. 360). In many infections, either Th1 or Th2 cells get the upper hand and become predominant during the course of the inflammatory reaction. Whether Th1 or Th2 cells play a more important role in a given infection is usually dictated by the kind of infection and the cytokines present in the local environment during the early stages of T cell activation (Murphy, Travers et al. 2012, p. 362).

The differentiation of naive CD4 T into Th1 cells requires the exposure to IFN-γ and IL-12 during the initial priming period (Zhu, Yamane et al. 2010). Mature Th1 cells that recognise antigen on target cells increase their IFN-γ production which in turn has a knock-on effect on further Th1 cell differentiation (Murphy, Travers et al. 2012, p. 362).

Research groups have differentiated purified human CD4+ T helper cells into Th1 cells by activating them in the presence of IL-12 and anti-IL-4. Roozendaal and colleagues report that the neutralisation of IL-4 with blocking antibodies leads to the desired differentiation into Th1 cells, even without the addition of IFN-γ (Roozendaal, Vellenga et al. 2001; Heijink, Vellenga et al. 2003).
Another research group has differentiated purified human CD4+ T helper cells into Th1 cells by activating them with anti-CD3 and anti-CD28 in the presence of only IL-12. IL-2 was added as a T cell growth factor (Simhadri, Mariano et al. 2011).

Involvement of human Th1 cells in MS

Inflammation of the brain increases the permeability of blood vessels around the brain and allows Th1 cells to extravasate and exacerbate the inflammation by secreting IFN-γ. As a result, IFN-γ attracts and activates myeloid cells which in turn induce more T cells, B cells, and innate immune cells to travel to the site of inflammation (Murphy, Travers et al. 2012, p. 637).

Both Th17 and Th1 cells play a role in the pathogenesis of multiple sclerosis. The phenotype of Th17 is considered unstable and Th17 cells can turn into Th1 cells (Abromson-Leeman, Bronson et al. 2009). Thus, further research might be necessary to determine whether Th17 or Th1 cells play the more dominant role in MS (Fletcher, Lalor et al. 2010). Mouse studies have shown that either of the two cell types, Th1 or Th17, has the capacity to cause EAE and that, as a consequence, either signature cytokine (IFN-γ or IL-17) can precipitate this kind of inflammation (as reviewed by Fletcher, Lalor et al. 2010).

Th2 cells

Phenotype, function and differentiation of human Th2 cells

Th2 cells are characterised by their secretion of a typical set of cytokines, such as IL-4, IL-5 and IL-10 and IL-13 (Mosmann and Coffman 1989).

By secreting IL-4 and binding to the CD40 molecule on B cells Th2 cells help to initiate the clonal expansion of B cells (Murphy, Travers et al. 2012, pp 391-392).

The most potent cytokine that favours the differentiation of naive T cells into Th2 cells is IL-4. It is secreted from eosinophils, basophils, mast cells, natural killer T cells, and Th2 cells (Mowen and Glimcher 2004). Th2 cells can be obtained in vitro by polarising purified naive human CD4 T cells with IL-4 plus anti-IL-12 (Roozendaal, Vellenga et al. 2001; Heijink, Vellenga et al. 2003).

Involvement of human Th2 cells in MS

In the past, Th2 cells were considered to have immunoregulatory effects. Thus, efforts were made to switch the T cell response from Th1 to Th2. However, these attempts proved unsuccessful and immunomodulatory effects were subsequently associated with regulatory T cells (Tregs) instead (Murphy, Travers et al. 2012, p 619). In fact, Th2 cells have been ascribed to a number of auto-reactive immune responses such as Omenn's syndrome, reduced protection against some intracellular pathogens, chronic graft versus host disease (GVHD), atopic disorders, and some systemic autoimmune diseases (as reviewed by Romagnani 1996).

Th17 cells

Phenotype, functions and differentiation of human Th17 cells

Th17 cells are a subset of CD4+ T helper cells that differ from Th1 and Th2 cells in that they express the IL-23-receptor, transcription factor RORγt and the CC chemokine R6. Furthermore, Th17 cells secrete their namesake cytokine IL-17. They effectively induce the production of IgM, IgG, and IgA, but not IgE in B cells (as reviewed by Romagnani 2008). Th17 cells bring about inflammation by secreting IL-17, which triggers a cascade of inflammatory mediators and eventually culminates in the invasion of innate effector cells, such as neutrophils and macrophages (Mills 2008; Murphy, Travers et al. 2012, p. 434).

The fact that Th17 cells are less susceptible to the immuno-suppressive effects of FoxP3+ regulatory T cells than Th1 and Th2 cells (Annunziato, Cosmi et al. 2007) highlights the detrimental role they play in autoimmunity.

Interestingly, one T helper subset in humans can produce IL-17 as well as IFN-γ (Annunziato, Cosmi et al. 2007; Romagnani 2008; Shi, Cox et al. 2008; Abromson-Leeman, Bronson et al. 2009).

Despite some differing opinions among experts there is the general agreement that IL-23 and IL-1β drive the differentiation of human naive T helper cells towards Th17 cells (Wilson, Boniface et al. 2007). Some authors report that

either IL-1β or IL-23 is able to induce Th17 differentiation alone (Wilson, Boniface et al. 2007). Another study focuses on T memory cells and concludes that bacteria can prompt dendritic cells to produce IL-23 and IL-1 both of which are vital in driving memory T cells towards Th17 development (van Beelen, Zelinkova et al. 2007). While IL-1β and IL-6 can polarise T memory cells to turn into Th17, the differentiation of human naive T cells into Th17 cells requires TGF-β and IL-21 (Yang, Anderson et al. 2008). In sum, IL-1, IL-6, IL-21, IL-23 and TGF-β have all been shown to be involved in human Th17 differentiation in vitro (Wilson, Boniface et al. 2007; Yang, Anderson et al. 2008).

Differentiating human Th17 cells has proven difficult as discrepant combinations of cytokines have been suggested by several groups (as reviewed by Valmori, Raffin et al. 2010). Valmori, Raffin and colleagues report that Th17 originate from FoxP3+ Treg cells and that they can be obtained by differentiating naive Tregs into Th17 in the presence of IL-2 and IL-1β and that adding IL-23 and TGF-β yields an increased number of Th17 cells (Valmori, Raffin et al. 2010).

Involvement of Th17 cells in MS

Th17 cells and their pro-inflammatory effects have been linked to numerous autoimmune disorders. Tissues from systemic lupus erythematosus, rheumatoid arthritis, and psoriasis showed signs of Th17-infiltration (Sallusto and Lanzavecchia 2009). Their pathogenic role in MS and EAE is now widely recognised (Fletcher, Lalor et al. 2010). The number of Th17 cells in the peripheral circulation increases with exacerbation and decreases under interferon therapy (Durelli, Conti et al. 2009). In addition, IL-17 producing cells could be detected in active areas of MS brain lesions besides CD8+ cells and glial cells (Lock, Hermans et al. 2002; Tzartos, Friese et al. 2008; Montes, Zhang et al. 2009). Furthermore, Th17 cells have been shown to travel across the blood brain barrier faster than Th1 cells and impinge on neurons through the secretion of cytolytic granzyme B (Kebir, Kreymborg et al. 2007). It has been postulated that Th17 cells permeabilise the blood brain barrier through IL-17 and IL-22 and thus pave the way for the invasion of other pro-inflammatory CD4+ cells and soluble molecules (Kebir, Kreymborg et al. 2007).

Regulatory T cells

Phenotype and differentiation of regulatory T cells

Regulatory T cells (Tregs) are a heterogeneous group of CD4+CD25+ cells. One of their key features is the ability to detect autoreactive lymphocytes and to suppress them through various mechanisms.

There are two types of regulatory T cells:

One of the types of regulatory T cells are naturally occurring Tregs (nTregs). Recent research suggests that nTregs can be classified into activated suppressor Tregs (FoxP3hiCD45RA-), resting suppressor Tregs (Foxp3loCD45RA+), and non-suppressor Tregs (Foxp3loCD45RA-) according to their function and phenotype (Miyara, Yoshioka et al. 2009).

Natural Tregs develop from moderately auto-reactive naive T lymphocytes after they escape the selection process in the thymus. On encountering self-antigen presented by dendritic cells, however, they do not differentiate into auto-reactive cells that could elicit an immune response. On the contrary, they prevent other self-reactive cells from differentiating into T effector cells or interfere with the function of self-reactive T effector cells. (Murphy, Travers et al. 2012, pp. 364-365)

The other type of Tregs is called adaptive or induced Tregs (iTregs). This type of Tregs evolves in a contact-dependant way from auto-reactive CD4+ cells that recognise self antigen presented by immature dendritic cells.

Secretion of TGF-β by immature dendritic cells in the absence of pro-inflammatory cytokines induces the development of iTregs (Valencia and Lipsky 2007).

Functions of regulatory T cells

There are various mechanisms through which Tregs can control adaptive immune responses:

CTLA-4 on the surface of Tregs competes with naive T cells for the B7 molecule on antigen presenting cells. B7 functions as an important co-

stimulatory signal for naive T cells which is why blocking of B7 by Tregs interrupts T cell activation. Furthermore, Tregs secrete the cytokines IL-10 and TGF-β both of which inhibit T cell proliferation (Murphy, Travers et al. 2012, p. 364-365).

Involvement of human regulatory T cells in MS and interaction with T effector cells

Although there is no difference between the frequency of Tregs in the peripheral blood of healthy individuals and MS-patients, it is the suppressive function of Tregs that is decreased in MS (Viglietta, Baecher-Allan et al. 2004; Feger, Luther et al. 2007; Valencia and Lipsky 2007). Fletcher, Lonergan and colleagues (2009) found that a CD39+ subset of Tregs fails to suppress IL-17 secretion in patients with multiple sclerosis, while the secretion of both IFN-γ and IL-17 was decreased in healthy donors.

B cells

While many studies and experiments focus on the role of T cells in the pathology and prevention of MS, the role of B cells has perhaps been underrated.

Since oligoclonal bands (patterns of immunoglobulin G of which some can only be detected in the CSF but not in the serum of a patient) are a common occurrence in MS patients, the involvement of B cells and plasma cells in the pathogenesis of MS has long been suspected (Frohman, Racke et al. 2006). Even though more recent studies confirm that some of these intrathekal antibodies are specific for brain antigen the exact role of B cells in MS seems to be more complex and remains unclear (Ray, Mann et al. 2011). However, since positive effects of B cell blockage with rituximab (an antibody directed at the CD20 molecule on B cells) have been reported more research could be conducted into the role of B cells in autoimmunity (Naismith, Piccio et al. 2010).

Treatment of relapsing-remitting multiple sclerosis

MS medication can be divided into two categories: Symptom management medication and disease modifying drugs.

An example for symptom management medication is corticosteroids such as methylprednisolone and dexamethasone. Corticosteroids can relieve symptoms and speed up recovery from an acute relapse (Miller, Weinstock-Guttman et al. 2000).

Treatment with disease modifying drugs involves immunemodulators such as interferon-β, natalizumab, glatiramer acetate and Fingolimod. The aim of disease modifying treatment is to delay the accumulation of physical disability and to reduce the frequency of relapses (Compston and Coles 2008).

In this study changes on the surface of cells from RRMS-patients receiving interferon-β or natalizumab are assessed. The following two articles will thus treat those two immunemodulators in greater detail:

Interferon-β

Interferon-β (IFN-β) is a type I interferon (Liu 2005). Type I interferons are pleiotropic cytokines that not only play a role in fighting off viral infections but also act as messengers that mediate a host of cellular functions (van Boxel-Dezaire, Zula et al. 2010).

Together with glatiramer acetate, IFN-β is currently licensed as a first-line treatment in RRMS (de Seze, Borgel et al. 2012). The clinical effects of IFN-β treatment are: reduced MRI lesion activity, reduced brain atrophy, increased time to reach clinically definite MS after the onset of neurological symptoms, decreased relapse rate and reduced risk of sustained disability progression (as reviewed by Kieseier 2011).

The mechanism of action of IFN-β is multifactorial and only partly understood. IFN-β reduces the secretion of the inflammatory cytokine IFN-γ by CD4+ cells in RRMS (Skrzipek, Vogelgesang et al. 2011). Since IFN-γ is a signature cytokine of Th1 cells this is in line with the finding that IFN-β also skews T cell differentiation towards the Th2 phenotype (Martin-Saavedra, Gonzalez-Garcia et al. 2008). More recent research shows that, depending on the dosage, IFN-β either increases IL-4 secretion or has no effect on it (Skrzipek, Vogelgesang et al. 2011). This suggests that IFN-β does not compromise Th2 activity. Another anti-inflammatory mechanism of IFN-β is instrumented by an

increased expression of Bcl-XL in nTregs which prevents them from undergoing apoptosis (Martin-Saavedra, Gonzalez-Garcia et al. 2008). Furthermore, IFN-β causes the differential induction of STAT and thus increases the survival of primary human B cells and CD4+ cells. At the same time, IFN-β leads to the apoptosis of monocytes (van Boxel-Dezaire, Zula et al. 2010). Previous observations have suggested the involvement of IFN-β with the adhesion cascade which could hinder activated T helper cells from leaving the blood vessels and setting up an inflammation in the CNS (Calabresi, Tranquill et al. 1997).

Natalizumab

Natalizumab is a humanised monoclonal antibody directed against the α4 subunit of the α4β1 integrin (also called very late activation antigen 4, VLA-4) and α4β7 integrin. Natalizumab is a second-line therapy for patients with so-called treatment-resistant RRMS and with very active disease (Kappos, Bates et al. 2011). It lowers the risk of sustained progression of disability and reduces the rate of clinical relapse as well as the number of lesions detected by gadolinium-enhanced MRI (Polman, O'Connor et al. 2006).

Inflammatory leukocytes use their VLA-4 molecule to dock to VCAM-1 (a vascular adhesion molecule expressed on cerebral endothelial cells) to extravasate out of blood vessels and reach the site of inflammation (Elices, Osborn et al. 1990). The mechanism of action of natalizumab is believed to be mediated through its ability to block the leukocytes' VLA-4 molecule which in turn hinders them from escaping the blood circulation to enter the brain parenchyma (Baron, Madri et al. 1993).

A serious complication of natalizumab treatment is the rare but dangerous reactivation of a latent JC-virus infection (Planas, Jelcic et al. 2011). While the exact connection of JC-virus reactivation and natalizumab treatment is still under discussion, many studies have detailed the changes in the composition of the white blood cells under natalizumab treatment (as reported on page 61).

MS-patients treated with natalizumab show a lower leukocyte count in their cerebrospinal fluid (CSF) which is in keeping with the ability of natalizumab to

hinder leukocytes from crossing the blood brain barrier (Planas, Jelcic et al. 2011). Because the number of CD4+ cells was found to be lower than the number of CD8+ cells in the CSF Planas and colleagues hypothesized that natalizumab has a more potent effect on CD4+ cells than it has on CD8+ cells (Stuve, Marra et al. 2006; Planas, Jelcic et al. 2011).

Glycans on the cell surface

Every cell in a multicellular organism expresses distinct glycans on its surface. Glycans are sugar residues that are attached to transmembrane glycoproteins and other molecules via post-translational modification. Glycosylation is the process of enzymatic addition of these sugars to proteins or peptides, which may change, modify or complete their function (Bertozzi and Rabuka 2009; Varki and Lowe 2009). The glycosylation of peptides within or on the surface of cells can, for example, mediate the specific recognition by other molecules as in the process of cell-cell interactions, cell development and differentiation, the adhesion of cells to certain tissues or the recognition of cells by pathogens and ligands (Varki and Lowe 2009).

Some glycans are composed of sialic acids, which are nine-carbon acidic sugar residues located at the branching point of sugar chains. Their most common form is N-acetylneuraminic acid (also called Neu5Ac, NeuNAc, NeuAc, or NANA) (Varki and Lowe 2009). It is believed that sialic acids stabilise molecules, membranes and enzymes, modify the affinity of receptors and play a role in transmembrane signalling, growth and differentiation (Varki and Schauer 2009).

For the sake of simplicity, in the following text “Neu5Ac/Gcα2,3Galβ1,4Glc(NAc)” is shortened to “α2,3 galactose residues”, “α2,6 galactose residues” stands for “Neu5Acα2,6Gal(NAc)-R”, and the designation “α1,2 fucose residues” is used for “Fucα1-2Gal-R.”

Lectins

Lectins are proteins that recognise and bind to glycans without catalysing or modifying the glycan. They are isolated from animals or plants and thus differ structurally from glycan-specific antibodies (Varki and Lowe 2009). Some

lectins specifically recognise the sialic acid linkage that is embedded in distinct sugar chains, and some other lectins bind specifically to glycans, that are not linked to sialic acid (Varki, Etzler et al. 2009). In our study we used the lectins MAL-II and SNA to detect sialylated α2,3 galactose residues and α2,6 galactose residues, respectively, and UEA-I to mark α1,2 fucose residues.

Aim of the study

While the root cause of MS can still not be exactly pinned down, recent years have seen a rapid expansion of the knowledge of the cell types that accelerate or prevent the mechanism of the disease. In particular, Th1 and Th17 cells (both subsets of CD4+ T helper cells) are now thought to orchestrate the autoreactive immune reaction (Fletcher, Lalor et al. 2010). Understanding the roles of the individual cell types is crucial for tailoring new strategies for the alleviation and prevention of exacerbation in MS.

It has been recently shown that the number of Th17 cells in the circulation of RRMS-patients increases during exacerbation and decreases under interferon beta treatment (Durelli et al, 2009). Further examples that illustrate treatment-related changes in the blood cells of MS patients are lymphocytosis occurring under natalizumab treatment (Polman, O'Connor et al. 2006). Moreover, the suppression of IFN-γ secretion by CD4+ cells following exposure to IFN-β also demonstrates the influence that MS treatment has on the cells of the immune system (Skrzipek, Vogelgesang et al. 2011). Pro-inflammatory autoreactive cells that travel to the site of inflammation dock to certain vascular endothelium molecules to leave the blood vessels. Whether this docking process is influenced by a certain type of glycan on their surface has yet to be established (Sperandio, Gleissner et al. 2009).

The purpose of the study was to document whether MS not only changes the quantitative composition of leukocytes but also whether it has an effect on the glycosylation of surface proteins. To this end, a flow cytometric analysis of the influence of MS and its immunomodulatory treatment on the glycosylation of white blood cells was performed. Cell type specific antibodies and lectins reactive with various different types of glycans (UEA-I, MAL-II, SNA and anti-CD22) were used to mark the cell types and the glycans under study.

Patients, methods and material

Peripheral blood mononuclear cells (PBMC) were taken from four different groups of trial subjects: RRMS patients treated with natalizumab, RRMS patients treated with interferon-β (IFN-β), and RRMS patients who were not receiving any immunomodulatory treatment and healthy volunteers, as control groups.

Patients

The study was carried out at the neurological outpatient department of the University Hospital in Tübingen. The participants included

- 17 patients (10 women and 7 men) with relapsing-remitting multiple sclerosis (RRMS) who were treated with natalizumab (trade name: Tysabri®). Blood from the patients treated with natalizumab was drawn prior to each infusion of natalizumab.

- 8 RRMS patients (6 women and 2 men) treated with interferon-β (trade names: Rebif®, Betaferon®, Avonex®).

- 12 patients (8 women and 4 men) with RRMS who were not receiving any immunomodulatory therapy.

- 14 healthy volunteers (5 women and 9 men).

The patients had been diagnosed with multiple sclerosis according to the McDonald criteria (Polman, Reingold et al. 2005). None of the patients received any corticosteroids during the four weeks before the blood sample was obtained. All participants were informed and provided written consent according to the regulations of the local ethics committee. The project was approved by the Ethical Review Board of the Medical Department of the University of Tübingen on August 4, 2008.

Cell isolation

PBMC (peripheral blood mononuclear cells) were isolated through density centrifugation on lymphocyte separation medium (LSM). 7 to 10 mL of

peripheral blood was drawn from each patient using EDTA-tubes. The blood was diluted with PBS buffer to a final volume of 25 mL and then carefully layered onto 15 mL of lymphocyte separation medium in a 50 mL Falcon™ tube. The separation process was performed by centrifugation for 35 minutes at 20°C at 813x g (the equivalent of 2200 revolutions per minute with a rotor radius of 15 cm). The break of the centrifuge was switched off as a sudden stop of the centrifuge would have shaken up the individual layers in the Falcon™ tube. The PBMC located at the interface between the lymphocyte separation medium and the layer of PBS were carefully harvested into a fresh tube using a disposable pipette. This single cell suspension was washed twice, the first time in 30mL of PBS (20 minutes, 544x g, 20°C) and the second time in 10 mL of PBS (8 minutes, 330x g, 20°C).

Extracellular staining of cells for flow cytometric analysis

After the isolation process the cells were counted and checked for viability using a counting chamber and trypan blue. The cells were then transferred into the wells of a 96-well-plate at 500,000 cells per well. The cells were washed with 150 µL of FACS buffer (PBS 1x, 1% BSA, 0.02% NaN_3).

To prevent unspecific binding of the antibodies Fc-receptors were blocked by adding 50 µL of intravenous immunoglobulin (Ivig, Kiovig 1mg/mL; diluted 1:50) and incubation for 15 minutes at 4°C in darkness.

Without any further washing step, the following antibodies and the lectins were added to the cells and left at 4°C for 30 minutes in darkness: Anti-CD4 and anti-CD8 to mark T cells, anti-CD11c for dendritic cells, anti-CD14 for monocytes, and anti-CD20 for B cells. At the same time, the cells were stained with specific lectins and antibodies to compare their glycosylation patterns: SNA, MAL-II, UEA-I, and anti-CD22.

As some of the antibodies were not labelled with a fluorescent dye but with biotin, the primary antibodies were stained with fluorescently labelled streptavidin after two washing steps with 150 µL of FACS buffer. Finally, the stained cells were washed two more times (to wash away the residual superfluous antibodies that might not have bound to the cells) and

resuspended in fixation buffer (10 mL of PBS and 280 µL of formaldehyde 37%).

T cell stimulation with PMA and ionomycin

To augment the cellular production of IFN-γ, IL-4 and IL-17, 500,000 PBMC were incubated in 2 ng/mL PMA, 0.2 nmol/mL ionomycin, 0.2 nmol/mL monensin and complete RPMI in a 96-well flat bottom assay plate at a final volume of 200 µL at 37°C for 4 hours.

Intracellular cytokine staining

After stimulation with PMA/ionomycin, the cells were washed and stained, first extracellularly (as described on page 22), then intracellularly. Staining cells for intracellular antigens involves stabilising the cell membrane with formaldehyde and at the same time permeabilising the membrane with a detergent called saponin. To this end, cells were incubated in darkness for 60 minutes at 4˚C in 100 µL of fixation/permeabilisation buffer (eBioscience). For the next washing step 200 µL of permeabilisation/wash buffer (eBioscience) were used to maintain the cell membrane's permeability. Antibodies directed at intracellular antigens (anti-IFN-γ, anti-IL-4 and anti-IL-17) were added in the presence of permeabilisation buffer and incubated for 30 minutes at 4˚C in the dark.

Intracellular FoxP3 staining

Intracellular staining of the transcription factor FoxP3 is, in essence, performed the same way as intracellular cytokine staining with the exception that cell stimulation with PMA/ionomycin is not required.

Flow cytometric analysis

Cells were acquired on a CyAn Advanced Digital Processing High-Performance Flow Cytometer and analysed with the Dako Summit software. The PBMC were gated based on forward and sideward scatter. The gate as it is drawn in the top panel of Figure 1 on page 30 includes activated and unactivated lymphocytes, dendritic cells and monocytes. 100,000 events in the lymphocyte gate were acquired for each analysis.

Buffers and solutions

Complete RPMI: RPMI 1640 with HEPES and L-Glutamine, 10% FCS, 0.1% mercaptoethanol, 1% penicillin/streptomycin

10% $CuSO_4$-solution for incubator, solved in demineralised water

Fixation solution: 280 µL of formaldehyde, 10 mL of PBS (phosphate buffered saline)

FACS-buffer: 500 mL PBS, 5 g of BSA (bovine serum albumin), 1 mL NaN_3

Tools and reagents

Tool/reagent	manufacturer
15 mL Tube CELLSTAR®	Greiner bio-one, Frickenhausen
50 mL Tube CELLSTAR®	Greiner bio-one, Frickenhausen
6-Mercaptoethanol, 50mM GIBCO®	Invitrogen, Auckland, NZ
70% Ethanol	
96-well Zellkulturplatten Flat-Bottom, mit Deckel, Falcon®	BectonDickisonLabware, Franklin Lakes (USA)
96-well Zellkulturplatten U-Bottom, mit Deckel, CELLSTAR®	Greiner bio-one, Frickenhausen
Auflichtmikroskop WilovertA	A Hund, Wetzlar
Bovine Serum Albumine (BSA) pH 7,0	PAA Laboratories GmbH, Pasching, Austria
CO2 Water-Jacketed Incubator	Nuaire US Autoflow
CuSO4	SIGMA Chemical Co., St. Louis, USA
CyAn Advanced Digital Processing (ADP) High-Performance Flow Cytometer	Beckman Coulter, Inc., 4300, N. Harbor Blvd. Fullerton, CA 92835, USA
DMSO (Dimethylsulfoxide) C2H6OS, Research Grade	SERVA Electrophoresis GmbH, Heidelberg, Deutschland
EDTA	Merck KGaA, 64271 Darmstadt, Germany
EDTA KE/9mL Monovette®	Sarstedt, Nümbrecht
Elektrische Eppendorf-Pipette „Easypet“	Eppendorf Vertrieb Deutschland
Eppendorf-Cups	
Eppendorf-Pipetten (verschiedene Volumina und Ausführungen)	Eppendorf Vertrieb Deutschland
FACS CyAnTM ADP	BeckmanCoulter GmbH,Krefeld, Deutschland
FACS-Tubes (PP-Tubes 1,3mL 8,55/44MM)	Greiner bio-one, Frickenhausen
Fetal bovine serum – heat inactivated, certified	Cat# 10082-139, Invitrogen, Auckland, NZ
Formaldehyd	Merck Schuchardt OHG, 85662 Hohenbrunn, Germany
FoxP3 Fixation/Permeabilization	Ebioscience, San Diego, CA

Concentrate	
FoxP3 Fixation/Permeabilization Diluent	Ebioscience, San Diego, CA
FoxP3 Permeabilization Buffer (10x)	Ebioscience, San Diego, CA
Gilson-Pipetten, Pipetman (verschiedene Volumina)	Gilson, Inc., Middleton, USA
IMDM (Iscove's Modified Dulbecco's Medium) 12440 (1x) & L-Glutamine, 25mM HEPES GIBCO®	Invitrogen, Auckland, NZ
Interferon-β (betaferon®)	Bayer AG 51368 Leverkusen, Germany
Ionomycin, Calcium Salt (2mM), Streptomyces conglobatus,Calbiochem®	Merck KGaA, Darmstadt, Deutschland
KHCO3	Merck KGaA, 64271 Darmstadt, Germany
Kühl-/Gefrierkombination glassline	Liebherr-Holding GmbH, Biberach
KühlzentrifugeHeraeusMegafuge 1.0 R	Kendro Laboratory Products
LSM 1077 Lymphocyte Separation Medium	PAA Laboratories GmbH, Pasching, Austria
MACS Multi Stand	MiltenyiBiotecInc, Auburn (USA)
MACS-Separation-Collums (LS, MS, LD)	MiltenyiBiotecInc, Auburn (USA)
Monensinsodiumsalt (2mM)	Sigma-Aldrich Chemie GmbH, Steinheim, Deutschland
NaN3 10%	Merck
Neubauer-Zählkammer, improved, Bright Line, 0,100mm Tiefe	Glaswarenfabrik Karl Hecht KG
NH4Cl	SIGMA Chemical Co., St. Louis, USA
Parafilm	Pechiney, Plastic Packaging Menasha (USA)
Pasteuerpipetten, Einweg, 3mL	Carl Roth GmbH, Karlsruhe, Deutschland
PBS: Dulbecco's PBS (1x) without Ca& Mg, sterile	PAA Laboratories GmbH, Pasching, Austria
Penicillin/Streptomycin (10.000 U/mL Penicillin & 10.000 µg/mL Streptomycin) GIBCO®	Invitrogen, Auckland, NZ
Pipettenspitzen, verschiedeneGrößen	Sarstedt, Nümbrecht
PMA (Phorbol-12-myristate-13-acetate) 1mg/mL (Purity 99,07%), Calbiochem®	Merck KGaA, Darmstadt, Deutschland
S1-Biogard-Hood, Modell B60-112	The Baker Co. Inc., Sanford, Maine
Safety-Multifly®-Set	Sarstedt, Nümbrecht
Serologische Pipette costar® verschiedene Volumina	CorningIncorperated, Corning (USA)
Serologische Pipette Falcon® verschiedene Volumina	BectonDickisonLabware, Franklin Lakes (USA)
Spritze, 2mL, BD PlastipakTM	BectonDickison, Madrid (Spain)
Sterilisator, A16/50, BJ 1922, Inh. 14,6ltr	WEBECO, Selmsdorf, Deutschland
Summit (FACS software)	Dako Colorado, inc., 8450 Innovation Drive, Fort Collins, CO 80525, USA
TrypanBlue Solution (0,4%)	SIGMA Chemical Co., St. Louis, USA

Antibodies and lectins

Antibody/ lectin	clone	fluores-cence	concen-tration[1]	Cat#	manufacturer
Anti-CD25	BC96	FITC	12,5µg/mL	302603	Biolegend
Anti-CD11c	B-ly6	apc	1.25 µg/mL	559877	BD Bioscience
Anti-CD14	61D3	apc	1.25 µg/mL	17-0149-71	eBioscience
Anti-CD20	L27	PerCP	1/50	345794	BD Bioscience
Anti-CD4	RPA-T4	APC	0.25 µg/mL	17-0049-42	eBioscience
Anti-CD69	FN50	FITC	25µg/mL	310904	Biolegend
Anti-CD8	RPA-T8	APC	1/100	555369	BD-Biosciences
Anti-FoxP3	PCH101	PE	2.5 µg/mL	12-4776-42	Ebioscience
Anti-IFN-γ	4SB3	PE	2.5 µg/mL	12-7319-41	Ebioscience
Anti-IL-17	BL168	PE	1/100	512305	Biolegend
Anti-IL4	8D4-8	PE	2.5 µg/mL	12-7049-41	Ebioscience
anti-CD22	HIB22	FITC	1/75	F-5546	sigma
Ivig			10mg/mL		Kiovig
MAL-II		biotin.	10 µg/mL	B-1265	Vector
Normal rat-Serum				24-5555-94	Ebioscience
SNA		biotin.	6.66 µg/mL	B-1305	Vector
Strept-avidin		Pacific-Blue	10 µg/mL	S11222	Invitrogen
UEA-I	BAL-22012	biotin.	4.44 µg/mL	B-1065	Vector

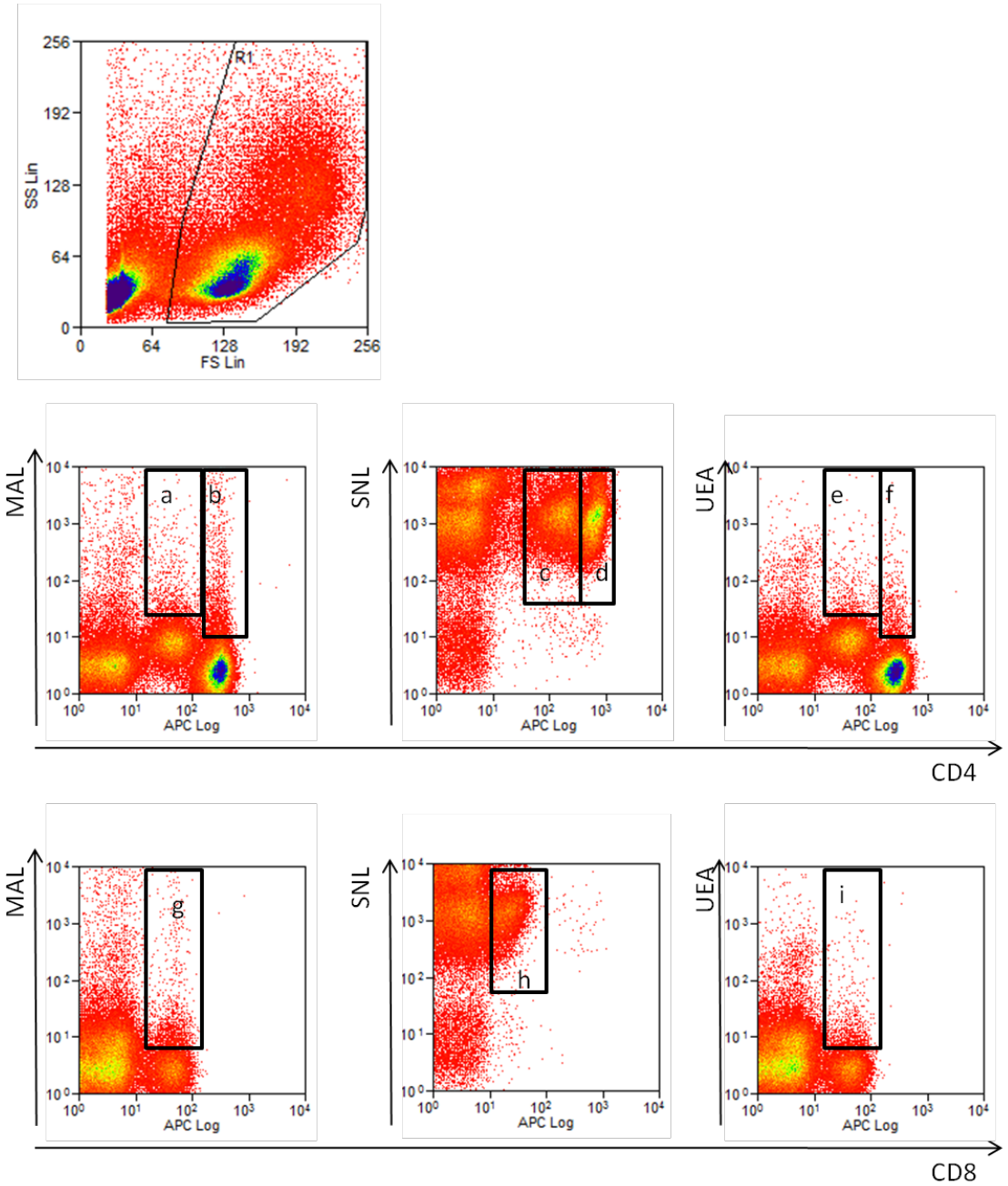
1 *Concentration* here indicates the concentration at which the antibody was used. The dilution at which the antibody from the vial was diluted is only given in cases where the manufacturer withheld the antibody concentration of the original vial.

Statistical analysis

The statistical analysis of the data was performed with IBM® SPSS® Statistics version 19. The non-parametric Mann-Whitney-U-test was used to detect statistical significance between two different groups. A p-value of <0.05 was considered significant.

Results

FACS analysis of PBMC



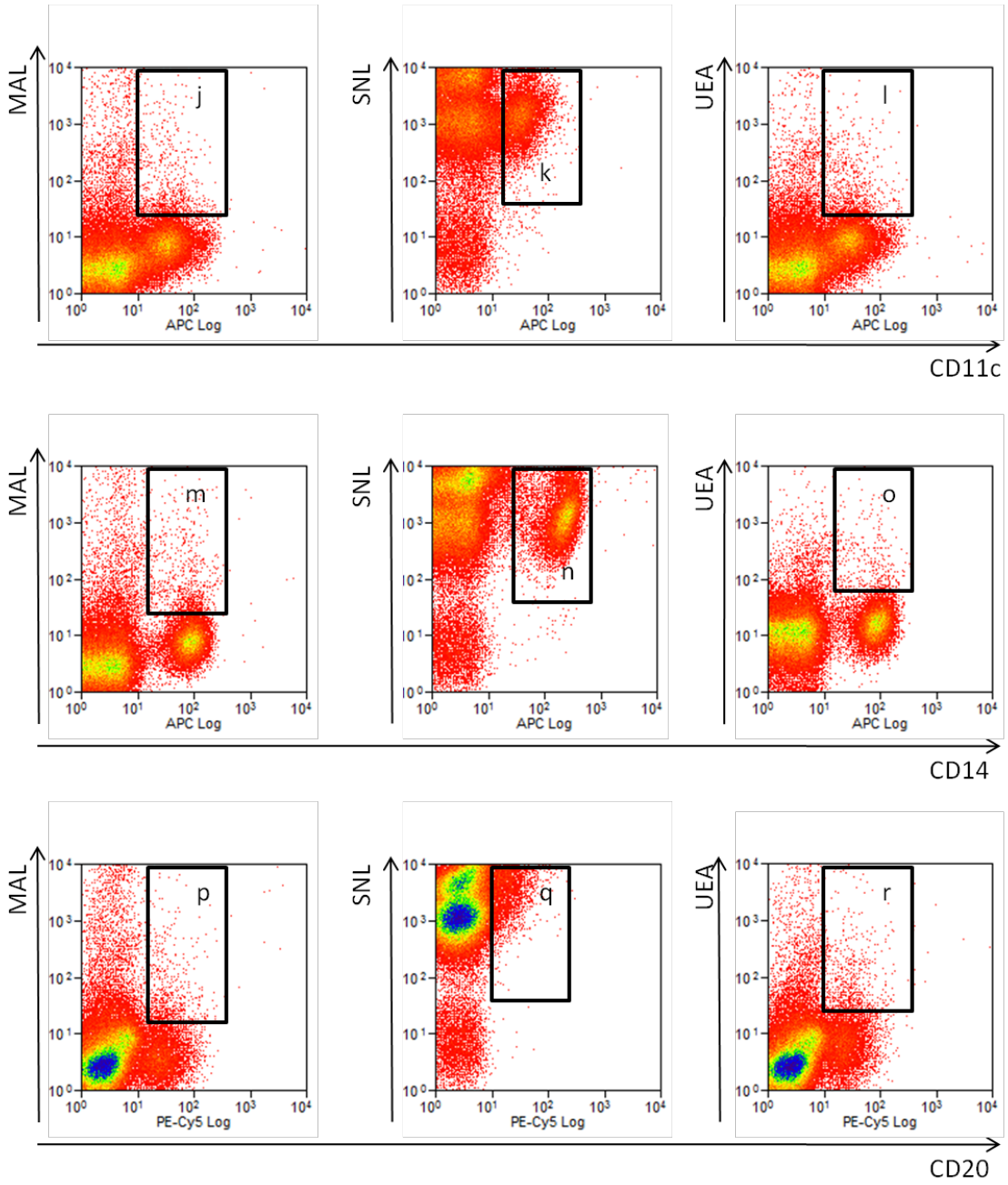


Figure 1 *FACS analysis of human peripheral blood cells stained with fluorescent antibodies directed against CD4, CD8, CD11c, CD14 and CD20. At the same time the cells were also stained with MAL-II, SNA and UEA-I that bind to α2,3-galactose residues, α2,6-sialylic acid and α1,2-linked fucose residues respectively. The graph at the top shows PBMC in the forward and sideward scatter. The gate was drawn around all PBMC to include lymphocytes (CD4+, CD8+ and CD20+), dendritic cells (CD11c+), and monocytes (CD14+). The analysis of CD4+ cells shows the existence of a CD4low and a CD4high population, both of which were analysed for their respective sialylation or fucosylation (boxes a-f). Boxes g-i show the sialylated and fucosylated fraction of CD8+ cells, boxes j-l show the*

sialylated/fucosylated fraction of CD11c+ cells, boxes m-o show the sialylation/fucosylation of CD14+ cells and boxes p-r show the sialylation/fucosylation of CD20+ cells.

Distribution of leukocyte subsets under the influence of natalizumab and interferon-β treatment

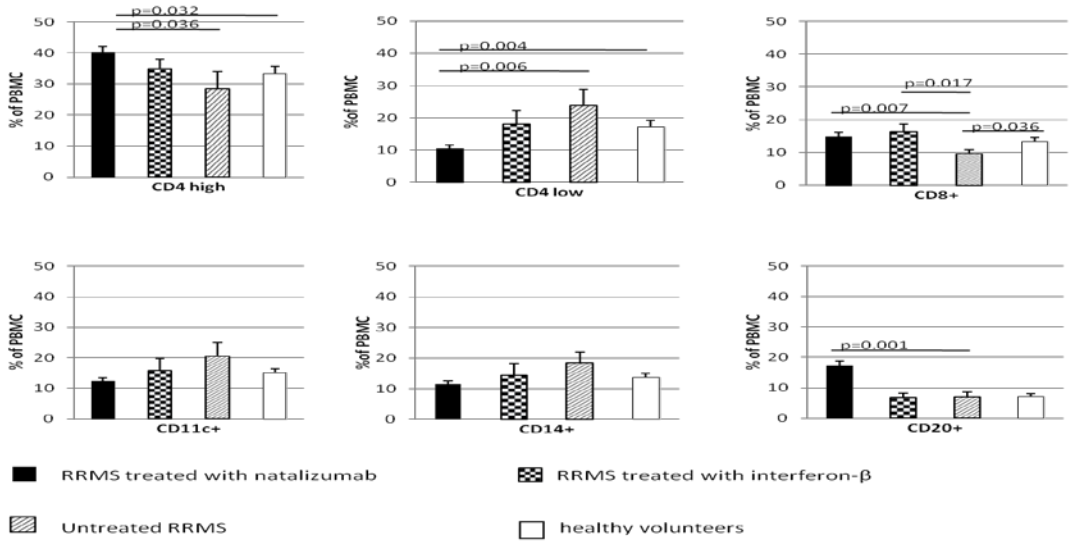


Figure 2 *shows the percentages of various leukocyte subsets within the PBMC population in RRMS patients receiving natalizumab, RRMS patients treated with interferon-β, untreated RRMS patients and healthy controls. The bars indicate the means in % while the whiskers represent the standard error of the mean (SEM). The horizontal lines with the p-value on top indicate statistical significance between the bars that they connect.*

Natalizumab treatment was associated with a rise in relative numbers of CD4high cells, cytotoxic T cells (CD8+) and B cells (CD20+). Quite the contrary, the percentage of CD4low cells was decreased under natalizumab. IFN-β treatment was associated with an increased portion of cytotoxic T cells (CD8+). No significant differences were noted among dendritic cells (CD11c+) and monocytes (CD14+).

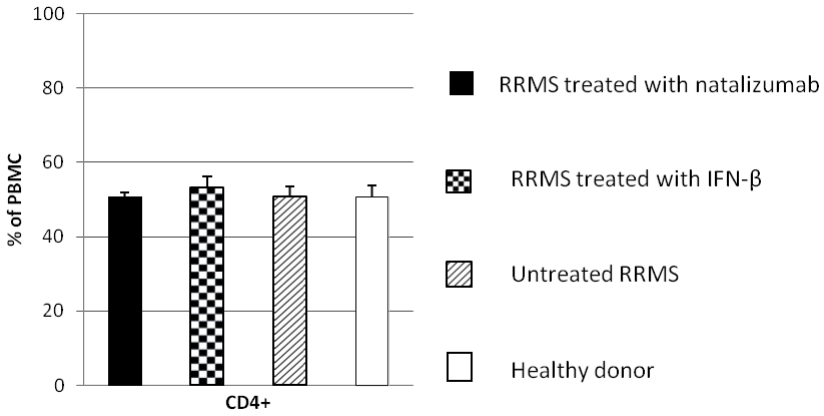


Figure 3 *shows the portions of all CD4+ cells in RRMS patients receiving natalizumab and IFN-β, and in control groups. The portion of CD4+ cells did not differ significantly between groups.*

Table 1 *shows the distribution of various leukocyte subsets in percent of all PBMC (peripheral blood mononuclear cells). The numbers are displayed as means ± SEM. * n=12, ** n=18*

	untreated RRMS	natalizumab	IFN-β	healthy control
n	8	17	8	14
CD4high	28.3 ± 5.5	40.4 ± 1.6	35.0 ± 3.1	33.3 ± 2.3
CD4low	23.9 ± 4.9	10.5 ± 1.1	18.1 ± 4.1	17.2 ± 2.0
CD4+	50.9 ± 2.7*	50.7 ± 1.2**	53.1 ± 3.1	50.6 ± 3.2
CD8+	9.6 ± 1.3	14.9 ± 1.2	16.4 ± 2.4	13.4 ± 1.2
CD11c+	20.5 ± 4.5	12.5 ± 1.1	15.8 ± 4.0	15.0 ± 1.4
CD14+	18.4 ± 3.6	11.5 ± 1.1	14.4 ± 3.9	13.6 ± 1.2
CD20+	7.2 ± 1.5	17.3 ± 1.6	6.8 ± 1.4	7.1 ± 1.0

Natalizumab treatment was associated with a larger percentage of unactivated T helper cells (CD4high) cells, but with a smaller percentage of activated CD4 T cells (CD4low)

RRMS-patients treated with natalizumab had a larger percentage of non-activated CD4high cells and a smaller percentage of activated CD4low cells.

The fraction of non-activated T cells (CD4high cells) was increased by 42% in RRMS-patients receiving natalizumab compared to RRMS-patients under no immunomodulatory treatment (p=0.036). Compared to healthy controls, the

fraction of non-activated T cells (CD4high cells) was increased by 21% in RRMS-patients receiving natalizumab treatment (p=0.032). Conversely, the percentage of activated CD4+ cells (CD4low cells) in RRMS patients under natalizumab treatment was by 56% smaller than in untreated RRMS-patients (p=0.006), and by 39% smaller than in healthy controls (p=0.004) (Figure 2 and Table 1).

The percentage of B cells was increased in natalizumab-treated RRMS patients

With an increase of 140% the percentage of B cells (CD20+) more than doubled under natalizumab therapy compared to RRMS without treatment (p=0.001) (Figure 2 and Table 1).

Natalizumab- and IFN-β-treatment were both associated with an increase in the percentage of cytotoxic T cells in RRMS patients

The percentage of cytotoxic T cells (CD8+ cells) was increased by 54% under natalizumab-treatment (p=0.007) and by 70% under interferon-β-treatment (p=0.017) compared to RRMS patients under no immunomodulatory therapy. Besides, healthy volunteers also showed greater numbers of CD8+ cells (39 % higher, p=0.036) than untreated RRMS patients (Figure 2 and Table 1).

Sialylation and fucosylation of various immune cells

FACS analysis of MAL binding to leukocyte subsets in natalizumab- and IFN-β-treated patients

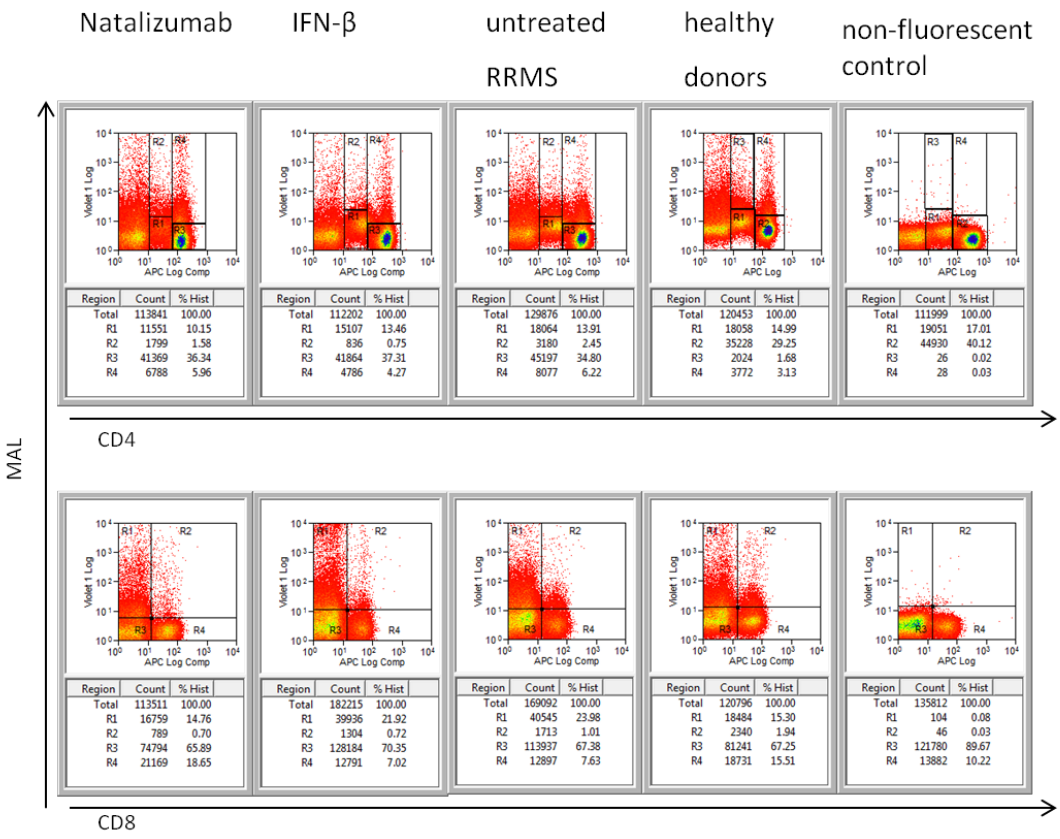


Figure 4 *(This figure also includes the diagram on the next page) shows a FACS analysis of lymphocytes, dendritic cells and monocytes and their binding to MAL. Cells obtained from natalizumab-treated and IFN-β-treated RRMS patients, from untreated RRMS-patients and from healthy controls are compared. IFN-β treatment was associated with a lower binding affinity of MAL to unactivated T cells (CD4high).*

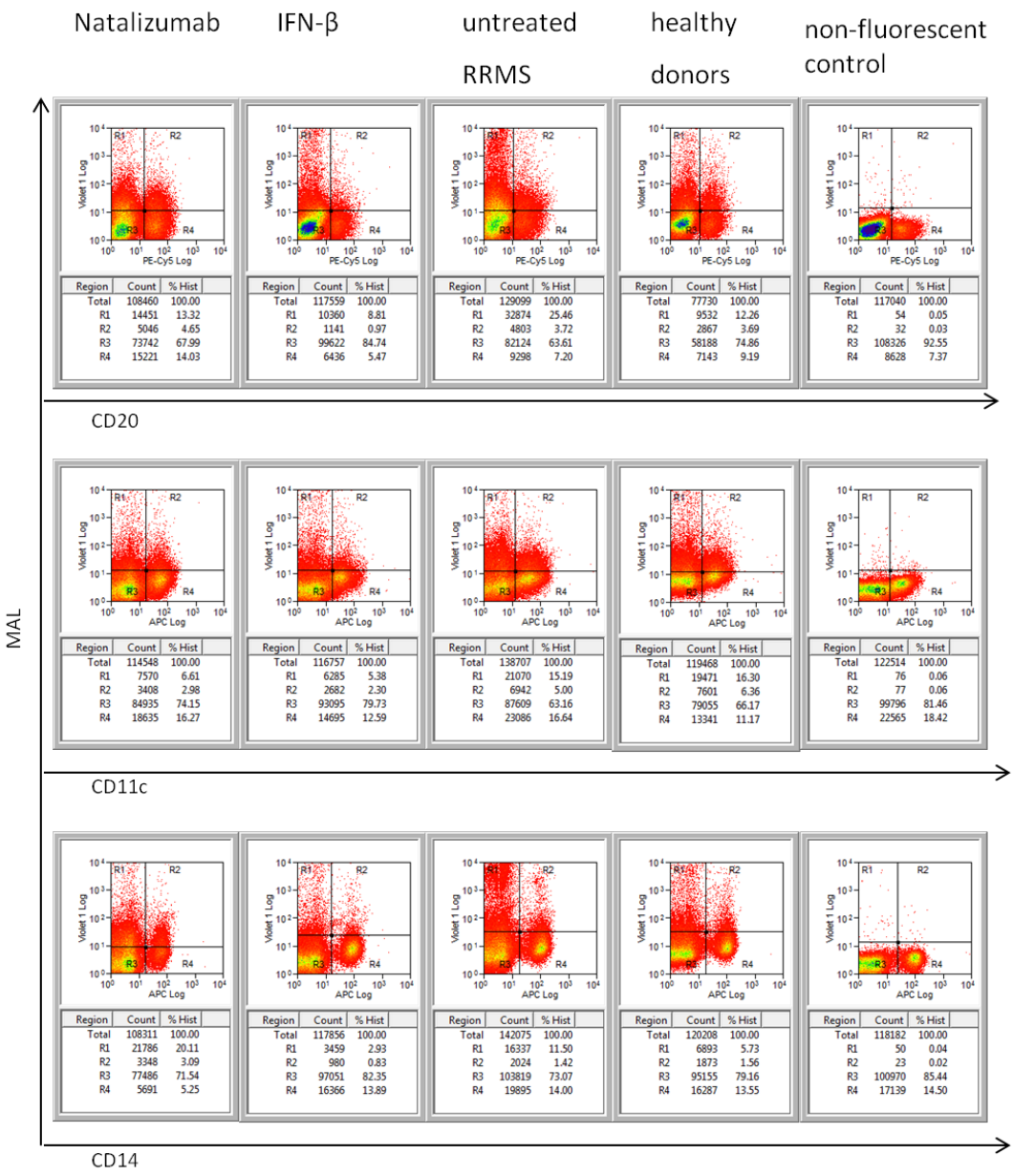

Natalizumab
IFN-β
untreated RRMS
healthy donors
non-fluorescent control
MAL
CD20
CD11c
CD14
R1
R2
R3
R4
Violet 1 Log
PE-Cy5 Log
APC Log
Region | Count | % Hist
Total 108460 100.00
R1 14451 13.32
R2 5046 4.65
R3 73742 67.99
R4 15221 14.03
Total 117559 100.00
R1 10360 8.81
R2 1141 0.97
R3 99622 84.74
R4 6436 5.47
Total 129099 100.00
R1 32874 25.46
R2 4803 3.72
R3 82124 63.61
R4 9298 7.20
Total 77730 100.00
R1 9532 12.26
R2 2867 3.69
R3 58188 74.86
R4 7143 9.19
Total 117040 100.00
R1 54 0.05
R2 32 0.03
R3 108326 92.55
R4 8628 7.37
Total 114548 100.00
R1 7570 6.61
R2 3408 2.98
R3 84935 74.15
R4 18635 16.27
Total 116757 100.00
R1 6285 5.38
R2 2682 2.30
R3 93095 79.73
R4 14695 12.59
Total 138707 100.00
R1 21070 15.19
R2 6942 5.00
R3 87609 63.16
R4 23086 16.64
Total 119468 100.00
R1 19471 16.30
R2 7601 6.36
R3 79055 66.17
R4 13341 11.17
Total 122514 100.00
R1 76 0.06
R2 77 0.06
R3 99796 81.46
R4 22565 18.42
Total 108311 100.00
R1 21786 20.11
R2 3348 3.09
R3 77486 71.54
R4 5691 5.25
Total 117856 100.00
R1 3459 2.93
R2 980 0.83
R3 97051 82.35
R4 16366 13.89
Total 142075 100.00
R1 16337 11.50
R2 2024 1.42
R3 103819 73.07
R4 19895 14.00
Total 120208 100.00
R1 6893 5.73
R2 1873 1.56
R3 95155 79.16
R4 16287 13.55
Total 118182 100.00
R1 50 0.04
R2 23 0.02
R3 100970 85.44
R4 17139 14.50

Table 2 *shows the percentage ± SEM of cells expressing α2,3 sialylated galactose residues within each leukocyte subset (CD4low, CD4high, CD8+, CD20+, CD11c+ and CD14+ cells) from RRMS-patients treated with natalizumab, RRMS-patients treated with IFN-β, untreated RRMS-patients and healthy donors, respectively . The number of cases "n" is given underneath each cell type.*

	Natalizumab	IFN-β	untreated RRMS	healthy donors
CD4low	7.0 ± 0.9	4.4 ± 0.8	9.0 ± 3.4	7.1 ± 1.2
n	17	8	8	14
CD4high	7.3 ± 0.7	3.4 ± 1.3	17.8 ± 7.7	6.0 ± 0.8
n	17	8	8	14
CD8+	8.7 ± 1.0	9.0 ± 1.7	13.8 ± 5.9	8.4 ± 1.3
n	15	8	8	14
CD20+	10.4 ± 1.5	8.2 ± 1.7	27.3 ± 8.1	16.9 ± 2.7
n	16	6	6	13
CD11c+	8.7 ± 1.0	7.3 ± 2.0	16.4 ± 6.5	13.7 ± 5.4
n	16	8	8	14
CD14+	9.5 ± 1.4	11.4 ± 4.6	18.1 ± 7.1	12.0 ± 2.2
n	16	8	8	14

Figure 5 *shows the percentages of various MAL+ leukocytes. The horizontal lines indicate statistical significance. Note the small portion of CD4highMAL+ cells and CD20+MAL+ cells under natalizumab and IFN-β treatment.*

INF-β treatment was associated with a lower amount of α2,3-galactose sialylation on non-activated CD4high cells

IFN-β treatment was associated with a decrease in α2,3-galactose sialylation of non-activated CD4high cells. Specifically, the proportion of unactivated T helper cells that bind to MAL was 81% lower in IFN-β-treated patients than in untreated RRMS patients (p=0.019).

Natalizumab treatment was associated with a lower amount of α2,3-galactose sialylation on B cells

Double staining with anti-CD20 and the lectin MAL showed that compared to healthy controls the portion of CD20+ cells that bound to MAL was 51% lower in RRMS patients under IFN-β treatment (p=0.065) and 39% lower in RRMS

patients under natalizumab (p=0.034). The percentage of MAL+ B cells in natalizumab- and IFN-β-treated patients was also smaller than in untreated patients, although the p-values did not show significant differences (p=0.133 between natalizumab-treated patients and untreated patients and p=0.107 between IFN-β-treated patients and untreated patients) (Figure 5 and Table 2 on page 37).

Percentages of SNA+ leukocyte subsets in natalizumab- and IFN-β-treated RRMS patients

Table 3 *The results are presented as percentage ± SEM of cells expressing α2,6-sialylic acid within each leukocyte subset (CD4low, CD4high, CD8+, CD20+, CD11c+ and CD14+ cells) from RRMS-patients treated with natalizumab, RRMS-patients treated with IFN-β, untreated RRMS-patients and healthy donors, respectively. The number of cases "n" is given underneath each cell type.*

	Natalizumab	**IFN-β**	**untreated RRMS**	**healthy donors**
CD4low	99.6 ± 0 .2	98.9 ± 0.6	97.7 ± 0.9	99.0 ± 0.4
n	17	8	10	13
CD4high	99.6 ± 0.2	98.5 ± 1.1	98.2 ± 0.7	99.5 ± 0.14
n	17	8	11	14
CD8+	99.6 ± 0.2	96.3 ± 1.3	98.9 ± 0.4	90.6 ± 7.4
n	16	7	11	11
CD20+	99.4 ± 0.2	98.3 ± 0.4	99.4 ± 0.3	99.4 ± 0.1
n	17	6	10	13
CD11c+	99.3 ± 0.2	98.0 ± 1.2	99.2 ± 0.4	99.0 ± 0.2
n	16	7	11	13
CD14+	99.4 ± 0.3	96.6 ± 0.9	99.0 ± 0.3	98.9 ± 0.3
n	16	8	11	13

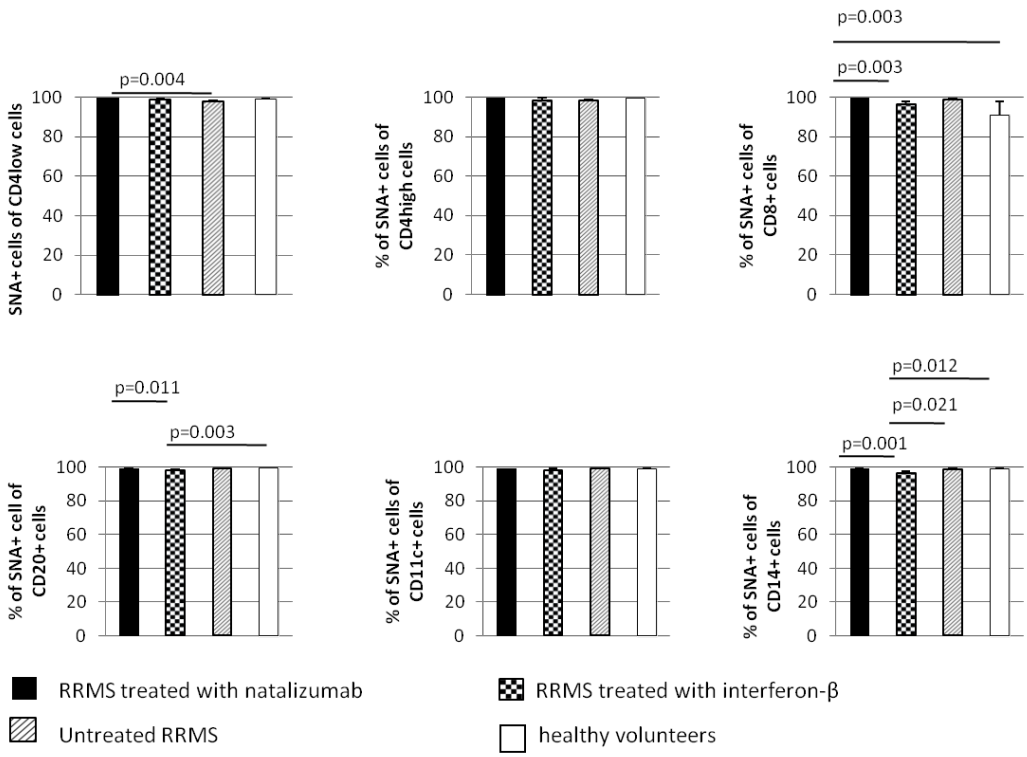


Figure 6 *Percentage of SNA+ cells of the respective leukocyte subset. The horizontal lines connect bars that show significant differences. The bar charts reflect that SNA bound abundantly to all sorts of immune cells within our scope from all groups of trial subjects.*

All leukocytes expressed plentiful amounts of α2,6-linked galactose

All the leukocyte subsets within our scope expressed α2,6-sialylic acid abundantly. The presence of RRMS and the type of therapy had no or only a minor influence on the binding capacity of those cells (Figure 6 and Table 3).

Percentages of UEA+ leukocyte subsets in natalizumab- and IFN-β-treated patients

Table 4 *shows the percentage ± SEM of cells expressing α1,2-linked fucose residues within each leukocyte subset (CD4low, CD4high, CD8+, CD20+,*

CD11c+ and CD14+ cells) from RRMS-patients treated with natalizumab, RRMS-patients treated with IFN-β, untreated RRMS-patients and healthy donors, respectively. The number of cases "n" is given underneath each cell type

	Natalizumab	**IFN-β**	**untreated RRMS**	**healthy donors**
CD4low	5.9 ± 0.9	5.8 ± 2.1	5.3 ± 1.4	5.0 ± 0.9
n	17	8	8	14
CD4high	3.8 ± 0.3	4.8 ± 1.6	4.0 ± 1.4	4.4 ± 0.5
n	17	8	8	14
CD8+	4.8 ± 0.5	4.4 ± 1.0	12.1 ± 7.0	4.6 ± 0.5
n	15	7	8	12
CD20+	12.8 ± 2.5	12.5 ± 5.6	44.3 ± 9.9	27.5 ± 6.2
n	16	6	6	13
CD22+	24.2 ± 2	51.3 ± 9.4	33.9 ± 5.6	53.7 ± 6.4
n	14	4	9	11
CD11c+	7.9 ± 0.8	8.6 ± 2.8	16.7 ± 6.8	12.4 ± 3.8
n	15	8	7	14
CD14+	8.4 ± 1.3	7.9 ± 3.6	14.1 ± 6.2	6.9 ± 0.7
n	16	8	8	14

Figure 7 *Percentage of UEA+ cells of the respective leukocyte subset. The horizontal lines connect bars that differ significantly from each other. Natalizumab and IFN-β treatment were associated with a lower binding capacity for UEA in B cells (CD20+ cells).*

B cells expressed a smaller amount of α1,2-linked fucose in response to natalizumab and interferon-β treatment

UEA-I was used to asses the expression of α1,2-linked fucose residues. Among the various leukocyte subsets only B cells seemed to change their bindig capacity of UEA-I in response to natalizumab- and IFN-β treatment: Both IFN-β and natalizumab treatment were associated with a lower expression of α1,2-linked fucose residues on CD20+ cells.

The portion of CD20+UEA+ cells decreased by 53% under natalizumab treatment compared to healthy controls (p=0.029) and by 71% compared to untreated RRMS patients (p=0.025).

Similarly, the percentage of UEA+CD20+ B cells in IFN-β-treated patients was 72% smaller than in RRMS patients without immunomodulatory therapy (p=0.016) (Table 4 and Figure 7 on pages 40 and 41).

The α1,2-fucosylation of CD22+ cells roughly corresponded to the α1,2-fucosylation of CD20+ cells. A notable exception to this was that IFN-β treatment did not seem to decrease the UEA binding capacity of CD22+ cells (Figure 7 on page 41).

CD20+ cells and CD11c+ cells express CD22 on their surface

Table 5 *shows the percentage ± SEM of CD20+ cells and CD11c+ cells expressing CD22 on their surface. "n" is given underneath each cell type to designate the number of cases.*

	Natalizumab	IFN-β	untreated RRMS	healthy donors
CD20+	88.5 ± 1.6	91.6 ± 1.4	89.6 ± 2.3	94 ± 0.7
n	10	5	9	13
CD11c+	1.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
n	15	8	10	14

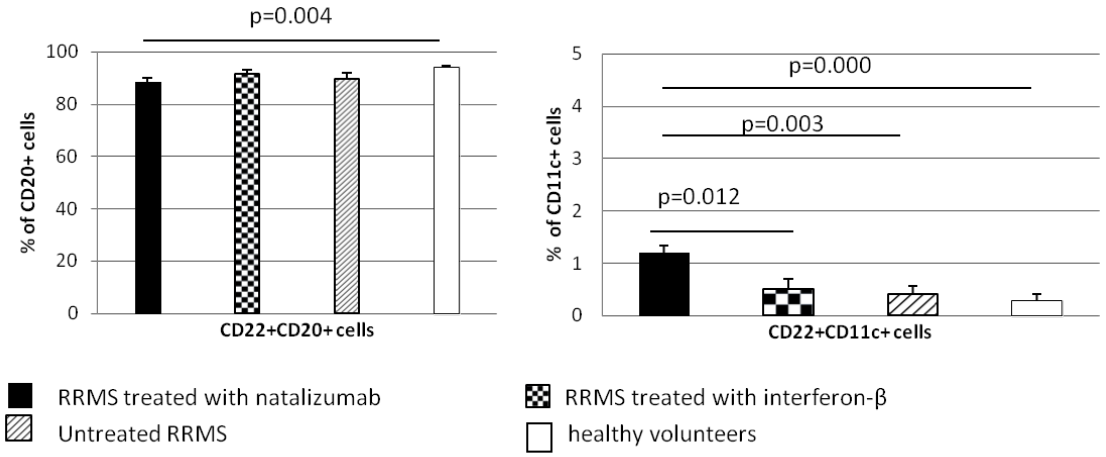


Figure 8 *Anti-CD22 bound to B cells (CD20+ cells) and to dendritic cells (CD11c+ cells).*

The aim of this FACS analysis was to document which cell types express the surface molecule CD22. It turned out that anti-CD22 bound to CD20+ cells and (to a much lesser extent) to CD11c+ cells (Figure 8) but not to the other cell types under study (data not shown).

Distribution of CD4 subsets

Distribution of Th1 cells, Th2 cells, Th17 cells and regulatory T cells in healthy volunteers

Figure 9 *displays the frequencies of T cell subsets (Th1, Th2, Th17 and regulatory T cells) in percent of CD4+ cells in healthy donors, measured by FACS analysis*

The percentage of Th1 cells was larger in natalizumab-treated patients than in healthy volunteers

Table 6 *shows the percentage ± SEM of CD4+ cells expressing IFN-γ, IL-4 and IL-17 in natalizumab-treated RRMS patients and healthy controls. The expression of the transcription factor FoxP3 was assessed in IFN-β-treated RRMS patients and in treatment-naive RRMS patients in addition. The number of cases "n" is given underneath each row.*

	natalizumab	healthy control
IFN-γ+ of CD4+	25.3 ± 3.5	13 ± 3.5
n	7	10
IL-4+ of CD4+	6.1 ± 1	5.1 ± 1.1
n	7	10
IL-17+ of CD4+	1.9 ± 0.3	1.2 ± 0.2
n	7	5

	natalizumab	IFN-β	untreated	healthy c.
FoxP3+ of CD4	4.9 ± 0.8	5.8 ± 1.5	3.5 ± 0.8	3.2 ± 0.7
n	9	4	5	6

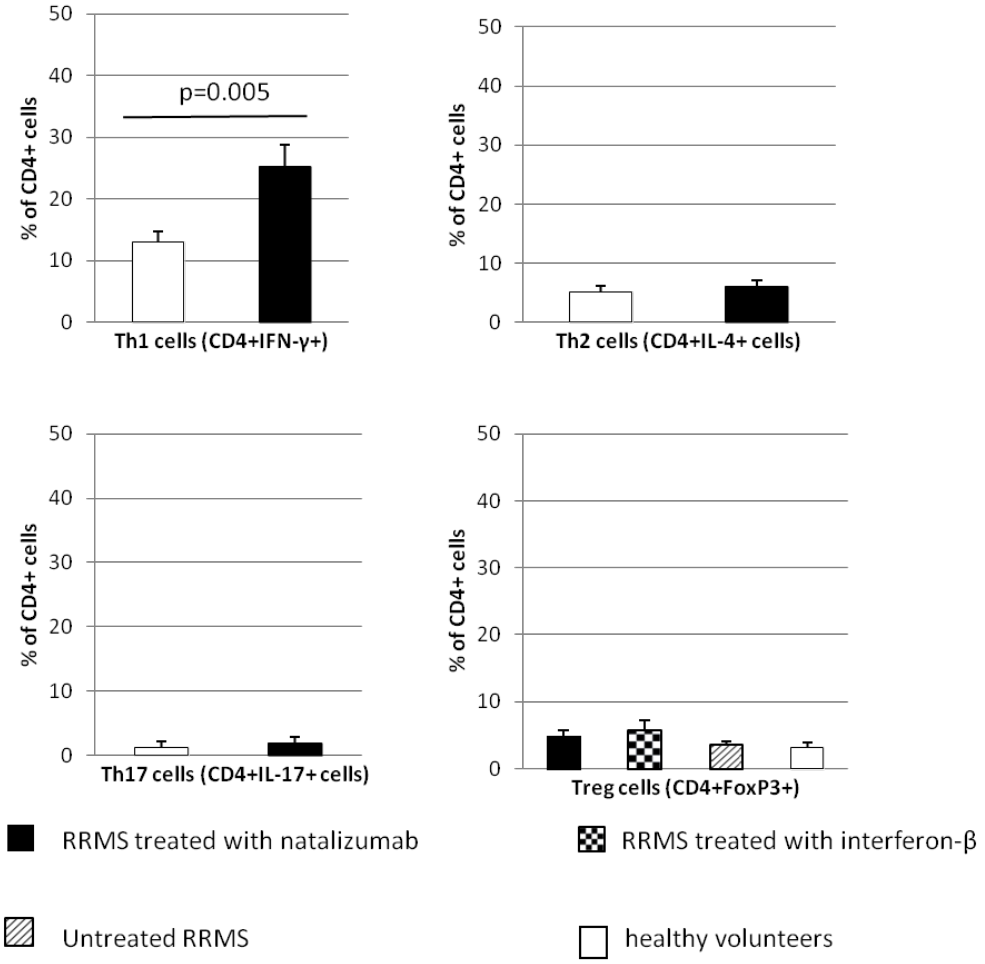


Figure 10 *shows the percentage of the particular T helper subset among all CD4+ cells, comparing healthy volunteers to natalizumab-treated patients.*

PBMC were stained for CD4 and intracellular, subset-specific markers. The aim was to assess possible differences between natalizumab-treated patients and healthy volunteers. The portion of Th1 cells (IFN-γ secreting CD4+ cells) under natalizumab was 94% larger than healthy controls (p=0.005) (Figure 10).

Sialylation and fucosylation of CD4+ Th1, Th2, Th17 and regulatory T cells in healthy volunteers

Th2 cells had the highest binding capacity to MAL

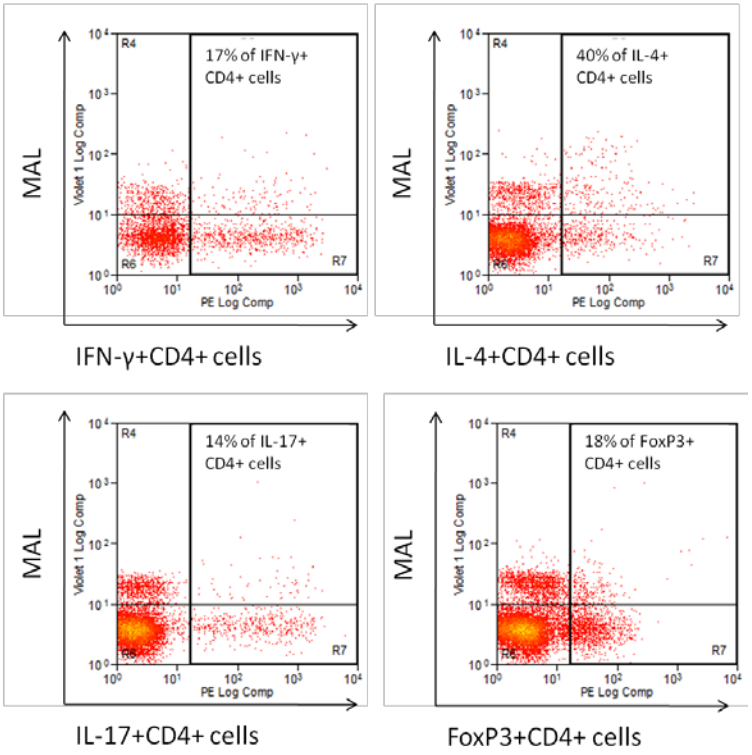


Figure 11 *FACS analysis of MAL binding to Th1, Th2, Th17 cells and regulatory T cells*

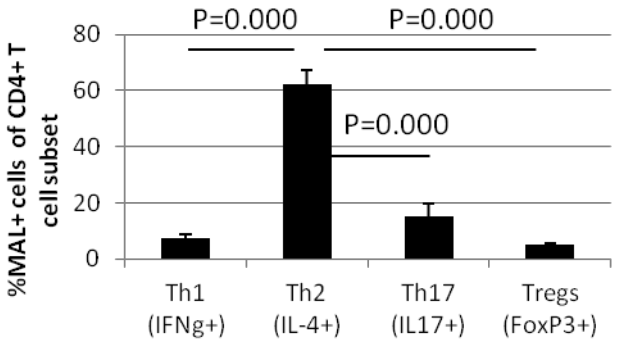


Figure 12 *While 62% of Th2 cells were MAL+, other T cell subsets expressed significantly fewer binding sites for MAL*

Table 7 *displays the percentage ± SEM of CD4+ T cell subsets (Th1, Th2, Th17 and regulatory T cells) expressing binding sites for MAL*

	Th1 (IFN-γ+)	Th2 (IL-4+)	Th17 (IL-17+)	Tregs (FoxP3+)
% MAL+	7.6 ± 1.2	62.1 ± 5.0	15.3 ± 4.3	4.9 ± 0.7
n	10	10	9	10

MAL was used as a marker to assess the α2,3-sialylation on T cell subsets. It turned out that significantly more Th2 cells expressed binding sites for MAL, than did Th1 cells (p=0.000), Th17 cells (p=0.000) and Tregs (p=0.000).

UEA binding to Th2 cells was higher than in other T helper subsets

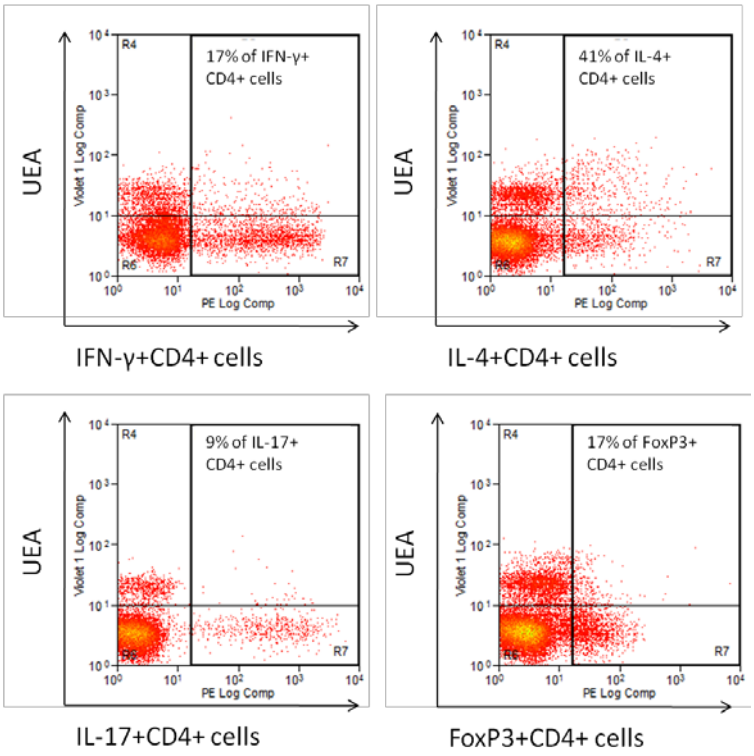


Figure 13 *Flow cytometry analysis of ulex europaeus lectin (UEA) sialylation of Th1, Th2, Th17 and regulatory T cells*.

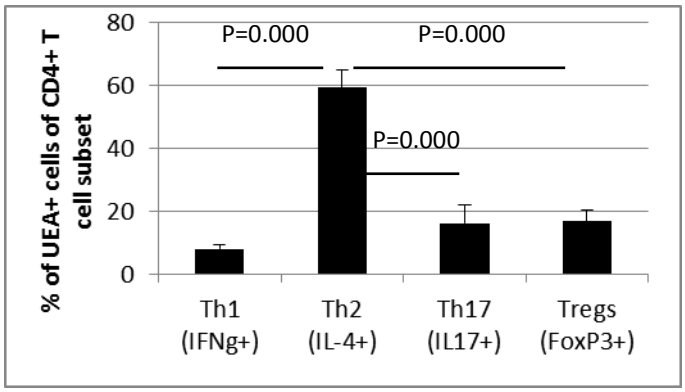


Figure 14 *shows the percentage of T cell subsets that show reactivity to UEA as measured by FACS analysis. Horizontal bars designate statistical significance*

Table 8 *displays the percentage ± SEM of CD4+ T cell subsets (Th1, Th2, Th17 and regulatory T cells) expressing binding sites for UEA*

	Th1 (IFN-γ+)	**Th2 (IL-4+)**	**Th17 (IL-17+)**	**Tregs (FoxP3+)**
% UEA+	8.0 ± 1.4	59.4 ± 5.4	16.2 ± 5.7	16.9 ± 3.5
n	10	10	9	10

PBMC were stained for CD4 and markers specific for T helper subsets. UEA was used to assess their α1,2-fucosylation.

Similarly to the MAL+ T cell subsets, there were more UEA+Th2 cells than UEA+Th1 cells (p=0.000), UEA+Th17 cells (p=0.001) and Tregs (p=0.000). Also, the difference between Th1 and Tregs is p=0.04.

All CD4 T cell subsets showed a high affinity to SNA

Table 9 *displays the percentage ± SEM of CD4+ T cell subsets (Th1, Th2, Th17 and regulatory T cells) expressing binding sites for UEA*

	Th1 (IFN-γ+)	**Th2 (IL-4+)**	**Th17 (IL-17+)**	**Tregs (FoxP3+)**
% SNA+	95.9 ± 2.3	97.2 ± 1.5	87.7 ± 7.1	88.3 ± 5
n	10	9	6	6

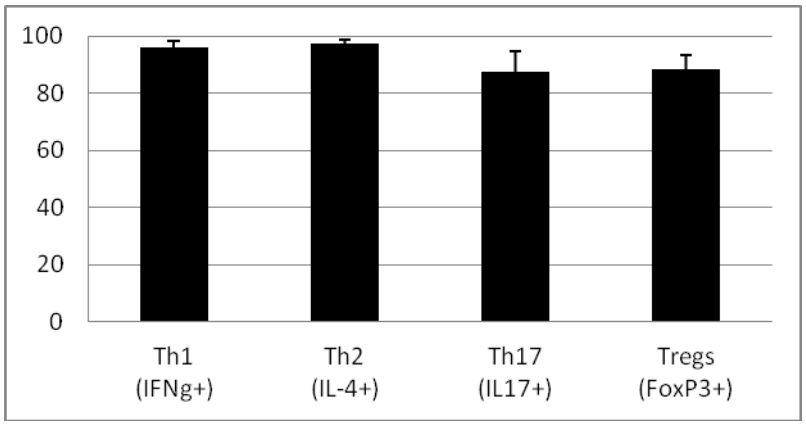


Figure 15 *shows the percentage of T cell subsets that show reactivity to SNA as measured by FACS analysis*

The assessment of α2,6-sialylation of CD4+ T cell subsets showed that SNA bound abundantly to all T cell subsets (Figure 15). This phenomenon could also be observed in other types of leukocytes (Figure 6 on page 39).

In summary, Th2 cells (IL-4 secreting CD4+ cells) showed a marked affinity to MAL-II and UEA-I that was unparalleled by any other T cell subset under study.

Discussion

It has been observed that in the course of MS some cell types expand or decrease in number while some other cell types change or lose their function and characteristics. For example, the number of circulating Th17 cells increases during an exacerbation of MS, and decreases under interferon treatment (Durelli, Conti et al. 2009). An example for cells that alter their properties in the course of an autoimmune disease is regulatory T cells. Regulatory T cells occur in the same frequency in MS patients as they do in healthy controls, but their immunosuppressive function is impaired in MS patients (Feger, Luther et al. 2007). Our motivation was to find out if the treatment of RRMS with natalizumab and IFN-β are associated with altered sialylation/fucosylation patterns of immune cells. Further experiments could then show if the changes in sialylation and fucosylation go along with functional changes in these cells.

Our data show that natalizumab and IFN-β treatment were associated with both changes in sialylation/fucosylation and frequency of certain cell types.

Cell types

CD4+ cells

CD4low and CD4high cells

As Figure 1 on page 30 shows FACS analysis revealed two distinct populations of CD4 cells among the PBMC: One that expresses CD4 at a high level and one that downregulates its CD4 antigen. This brought up the question whether the activation state of CD4+ cells influences the way they up- or downregulate their CD4-antigen. To obtain a sample of activated cells human PBMC were stimulated with PMA and ionomycin. Double staining for CD69 (an activation marker) and CD4 showed that a considerable portion of activated CD69+CD4+ cells in the lymphocyte gate sends out a weaker CD4 signal than unstimulated cells (Figure 16). Consequently, it can be inferred that CD4high cells are non-activated CD4+ cells and CD4low cells are activated CD4+ cells.

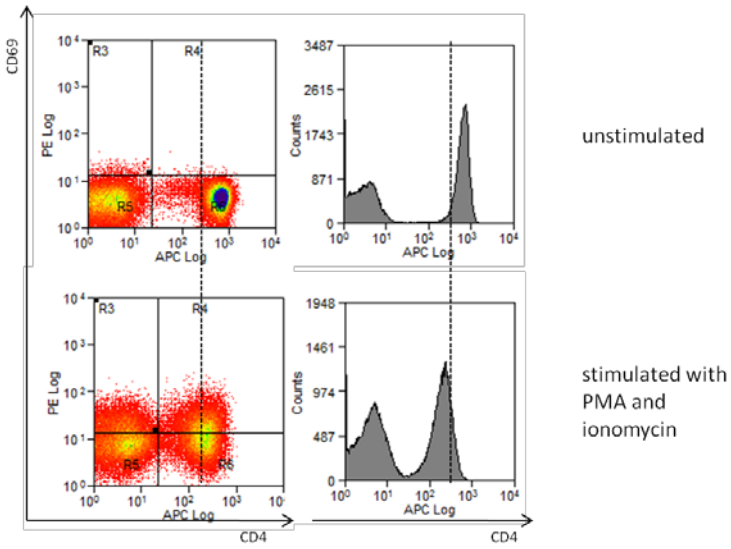


Figure 16 *shows unstimulated CD4 T cells that express CD4 at a high level (upper panels) and stimulated cells expressing CD4 at a lower level. The broken lines illustrate the difference in brightness of CD4 expression between the stimulated and unstimulated lymphocytes. PBMC from the lymphocyte gate were used for analysis.*

Th1 cells

FACS analysis of PBMC obtained from healthy donors revealed a frequency of Th1 cells of approximately 13% of CD4+ cells. This corresponds to the numbers we obtained from literature, where Chi, Xu and colleagues (2010) report that approximately 16% of the CD4+ cells in patients with neurological disease other than demylinating diseases are Th1 cells.

In our study we saw a larger percentage of CD4+IFN-γ+ cells in natalizumab-treated RRMS patients which points to a higher Th1 frequency in that group (Figure 10). Similarly, a higher IFN-γ-production in RRMS patients in proportion to disease activity has been reported (Frisullo, Nociti et al. 2008).

Th17 cells

IL-17 showed a dim signal in FACS analysis (Figure 13), which other authors also report (Chi, Xu et al. 2010). In our study, the frequency of CD4+IL-17+ Th17 cells among CD4+ cells was 1.2 ± 0.2% (Figure 10, Table 6), which is close to what we found in literature: 1.02 ± 0.37% (Chi, Xu et al. 2010).

Regulatory T cells

According to our analysis CD4+FoxP3+ regulatory T cells make up 3.2-5.8% of peripheral CD4+ cells in humans (Table 6 on page 44), which is close to the 5-10% that have been reported in literature (as reviewed by Takahashi, Kuniyasu et al. 1998). The frequencies of regulatory T cells in our study do not vary significantly between groups (Figure 10). Therefore, our data are in line with previous findings which report that MS is not associated with an altered portion of peripheral regulatory T cells (Feger, Luther et al. 2007).

CD20+ cells (B cells)

The percentage of B cells in natalizumab-treated patients more than doubled the percentage of B cells in the untreated RRMS control group (Table 1, Figure 2). This phenomenon will be discussed on page 61.

CD8+ cells (cytotoxic T cells)

The portion of CD8+ cells was increased 1.5-fold in RRMS patients treated with natalizumab compared to untreated RRMS patients (Table 1, Figure 2). This has also been observed by Putzki and Baranwald (2010) who report a 1.7-fold increase of CD8+ cells under natalizumab treatment.

CD14+ cells (monocytes)

Although observations to the contrary have also been made (Polman, O'Connor et al. 2006), natalizumab therapy seems to decrease the number of monocytes in RRMS-patients (Skarica, Eckstein et al. 2011). Our data show an insignificant trend toward this direction (Figure 2 on page 31).

Sialylation and fucosylation

α2,3-linked galactose residues detected with MAL-II

Maackia amurensis lectin II (MAL-II) is a hemoagglutinin and therefore it is sometimes also called Maackia amurensis hemagglutinin (MAH), depending on which vendor it is bought from (Geisler and Jarvis, 2011). It serves as a

detection tool for α2,3-linked sialic acids, in particular Siaα2-3Galβ1-3(±Siaα2-6)GalNAc (Geisler and Jarvis, 2011; Wang and Cummings 1988).

The exact role of α2,3-linked sialic acids on the surface of immune cells still needs to be clarified. It was shown that the molecule responsible for L-selectin mediated leukocyte interaction (PSGL-1, P-selectin glycoprotein ligand-1) is dependent on the α2-3-sialyltransferase (ST3Gal IV) (Sperandio, Frommhold et al. 2006). However, the actual α2,3-sialylation carried out by α2-3-sialyltransferase (ST3Gal-IV) is not needed for the capture and rolling of leukocytes, as was shown in an experiment involving high endothelial cells of Peyer's patch (Sperandio, Frommhold et al. 2006). Thus, it is still not entirely clear whether α2,3-linked sialic residues on leukocytes play a role in chemokine-mediated cell adhesion and extravasation (Sperandio, Gleissner et al. 2009).

We found that IFN-β had similar effects on the expression of α2,3-linked sialic acid on both B cells and unstimulated T cells (CD4high cells): In our study IFN-β treatment of RRMS-patients was associated with a decreased expression of α2,3-linked sialic acid on unstimulated T cells and a trend toward that a lower portion of α2,3 sialylated B cell (Figure 5 on page 37). By way of contrast, natalizumab treatment did not seem to have any significant effect on the α2,3 sialylation of most kinds of immune cells within our scope. In B cells, there was a slightly smaller portion of α2,3 sialylated B cells under natalizumab treatment, but only when compared to healthy controls.

Furthermore, a clear increase in α2,3-linked sialic acid expression was noted in the Th2 cells of healthy donors, compared to other T helper subsets (see page 46). The fact that the focus of the pathogenesis of MS lies on Th1 and Th17 cells rather than on Th2 cells makes it difficult to draw a conclusion concerning the α2,3-sialylation of Th2 cells and autoimmune diseases. To evaluate the role of α2,3-sialylation on T helper cells more research is necessary.

α2,6-linked galactose detected with SNA

SNA (sambucus nigra) is a lectin isolated from elderberry. It is highly specific for α2,6-linked galactose, which makes it an eligible tool to detect these distinct sugar chains on the cell surface (Shibuya, Goldstein et al. 1987).

The current data show that all the immune cells under study were highly reactive with SNA (as shown on page 39). To rule out the possibility of unspecific binding because of too high concentrations of SNA (in spite of the use of intravenous immunoglobulin as an Fc-receptor block) we performed titration studies for SNA (Figure 17). It turned out that lower concentrations of SNA led to a dimmer signal in FACS analysis but did not decrease the percentage of cells that were reactive with SNA. Following this pilot experiment SNA was used at a concentration of 6.66 µg/mL for assessing the α2,6-sialylation of PBMC.

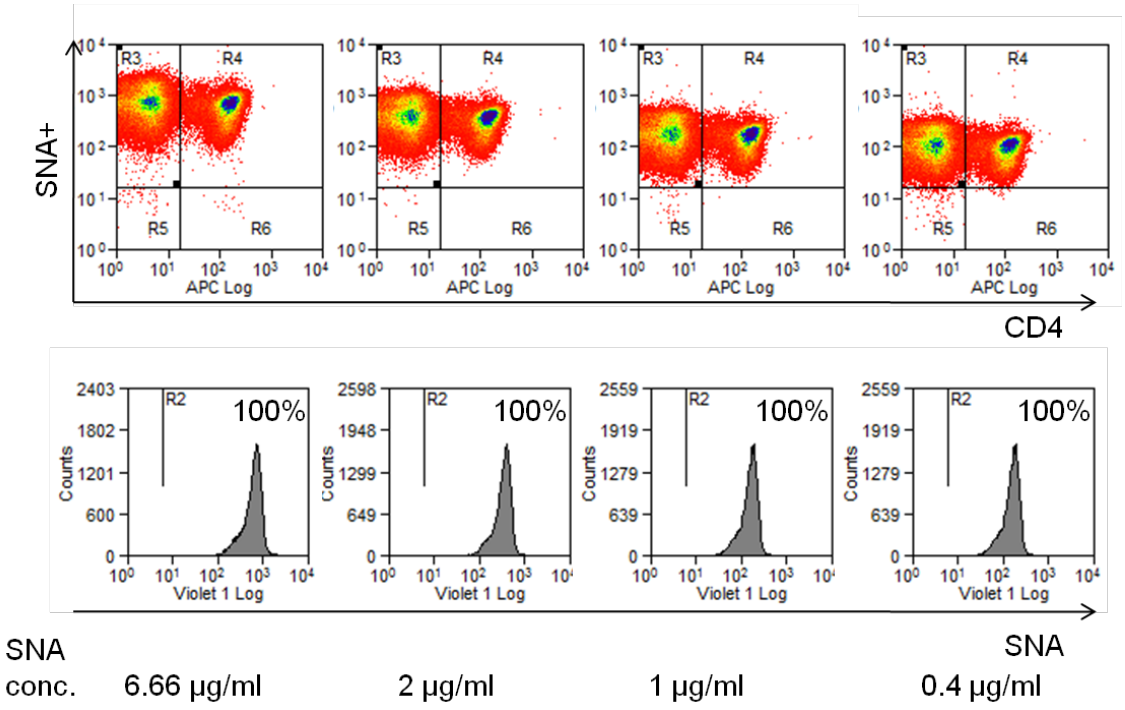


Figure 17 *Titration studies of SNA to rule out unspecific binding of SNA to CD4+ and CD4- lymphocytes. The top panels show the SNA-binding CD4- and CD4+ lymphocytes with the respective SNA-concentrations indicated at the bottom. The bottom panels show the SNA-signal of CD4+ lymphocytes. With smaller concentrations of SNA the brightness of the SNA-signal is reduced. Nevertheless, no change in the percentage of SNA+ cells could be observed.*

It has been suggested that several lectins including SNA are toxic to cells (Batisse, Marquet et al. 2004; Franz, Frey et al. 2006). However, Malagolini, Chiricolo and colleagues (2009) conducted FACS analysis studies and dismissed the allegedly toxic effect of SNA as insignificant. Even so, we often noticed a drop in cell count after we stained PBMC with fluorescent SNA. This phenomen was not observed after incubation with MAL or UEA instead. Consequently, we sought to find out if the presence of SNA really affected our cells more than the exposure to other lectins. FACS analysis is a viable method to determine the size and granularity of cells. Our rationale was that if cells shrink as they undergo apoptosis, smaller forward scatter values in FACS analysis would point to the typical apoptosis-dependent cell shrinkage (Franz, Frey et al. 2006).

Our PBMC did in fact show an altered FACS profile after exposure to SNA for 30 minutes (Figure 18). The population of dead cells (in the bottom left hand corner of the FACS diagram) expanded to greater numbers on both the forward and the sideward scatter scale and their percentage more than doubled (data not shown). In contrast to the “dead cell population”, the lymphocyte population that had been stained with SNA did not show any decrease in its forward scatter value but stayed in the same position. The border between “dead cells” and lymphocytes seemed to be less demarcated in the lower bottom corner of the FACS diagram on the left. This implies that SNA exposure results in a larger percentage of dead cells in the PBMC population but does not change the size and granularity of those cells that could withstand the cytotoxic effects of SNA.

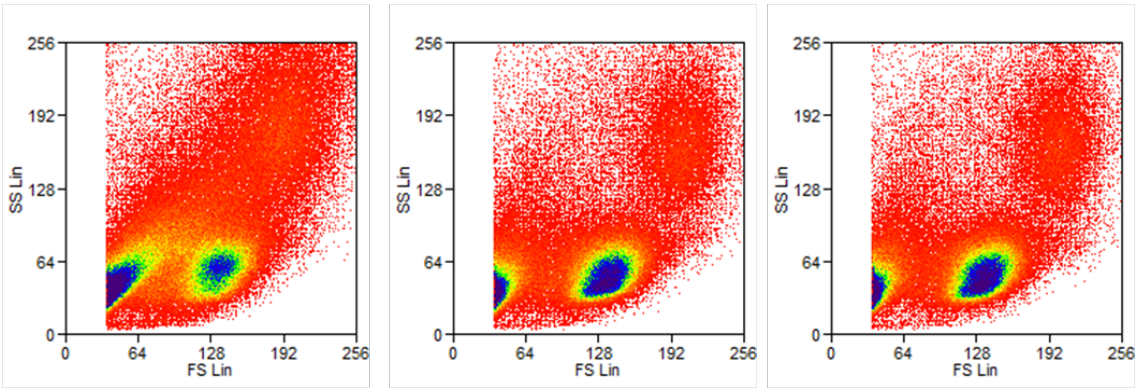


Figure 18 *FACS analysis of size (forward scatter, FS) and granularity (sideward scatter, SS) of PBMC exposed to SNA for half an hour (left) and MAL (centre) and UEA (right) for comparison. Note the expansion of the population of dead cells (in the bottom left hand corner; see arrow) following incubation with SNA.*

It has been shown that several cell types, including human neutrophils, react strongly with SNA when they undergo apoptosis (Malagolini, Chiricolo et al. 2009). This finding is in line with the results of our experiment, in which dead PBMC showed strong reactivity with SNA (Figure 19). On the other hand, Batisse, Marquet et al. (2004) confirmed that apoptotic cells down-regulate their binding sites for both MAL and SNA. It should be noted, however, that Batisse et al. performed their experiment with a myelomonocytic cell line derived from a patient who had histiocytic lymphoma, whereas we focused on PBMC from patients with RRMS and healthy donors. In contrast to what Batisse et al. report, in our experiment SNA bound to lymphocytes to the same extent as it bound to dead PBMC (Figure 19). We cannot rule out the possibility that there were a number of dead or apoptosing lymphocytes in the lymphocyte gate because we did not use a marker for apoptosis, such as annexin-V.

In our experiment the reactivity of PBMC with MAL and UEA is clearly weaker than the reactivity with SNA. “Dead cells” from the lower left hand corner of the FACS diagram and lymphocytes show a high affinity for SNA alike (Figure 19 and Figure 20).

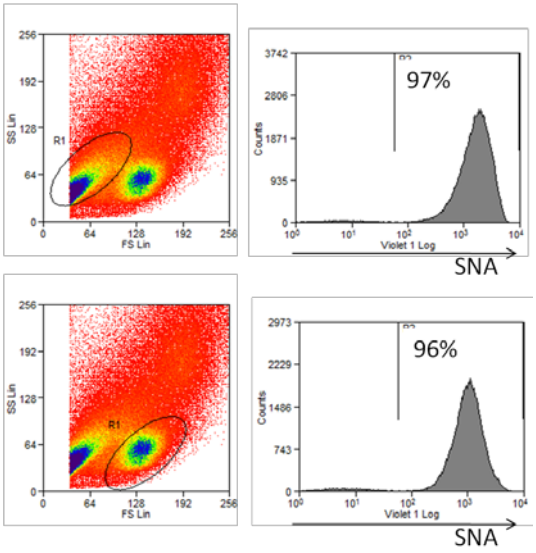


Figure 19 *This FACS analysis shows that almost all dead PBMC (upper panel) and almost all lymphocytes (lower panel) express plenty of binding sites for SNA.*

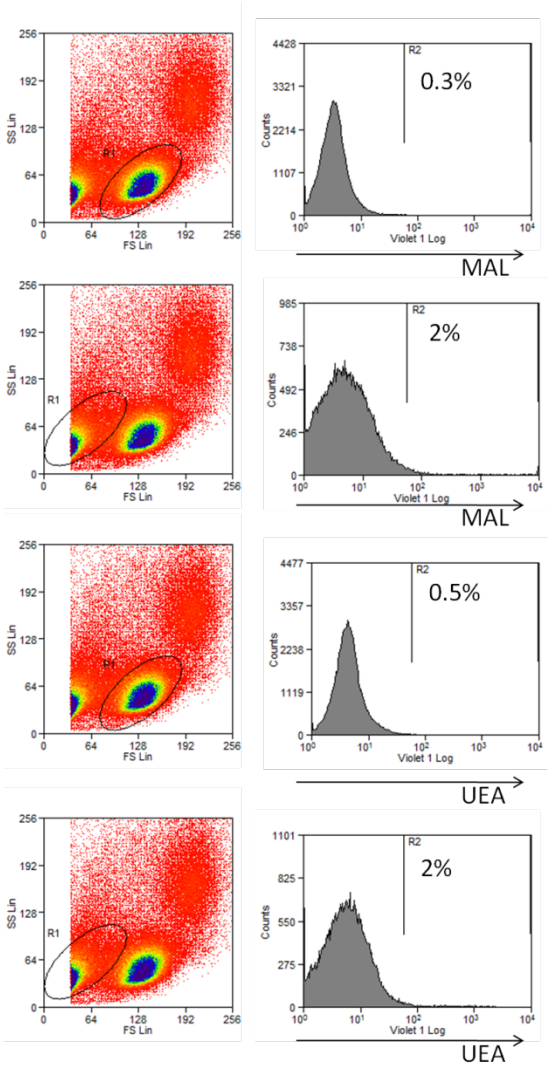


Figure 20 *MAL and UEA show poorer reactivity with lymphocytes than does SNA. MAL and UEA show some affinity to dead cells (second and fourth panels) but not as much as SNA does.*

To answer the question whether the putative cytotoxic effects of SNA (Batisse, Marquet et al. 2004) account for the unusual configuration of SNA-exposed PBMC in the FS SS diagram we compared our SNA-concentration to the results published by another research group. Batisse et al. (2004) demonstrate the dose-dependent cytotoxic effect of SNA on lymphocytes by assessing the percentage of apoptotic and necrotic cells with the help of an annexin V/PI assay. Their results point to a clear dose dependent cytotoxicity of SNA with 50 µg/mL resulting in an apopotosis rate of 12%, and 10 µg/mL of SNA leading to 5% apoptotic cells after 45 minutes (Batisse, Marquet et al. 2004). The SNA-concentration that we used for our experiments, namely 6.66 µg/mL, is close to the concentration that showed only very small numbers of apoptotic and

necrotic cells in the experiment performed by Batisse, Marquet et al. (2004): In their experiment an SNA-concentration of 5 µg/mL lead to only 3% apoptotic cells and 1% necrotic cells (Batisse, Marquet et al. 2004).

To find out whether our SNA-concentration of 6.66 µg/mL has a deleterious impact on PBMC we compared the FS SS diagrams of PBMC incubated for 30 minutes with decreasing concentrations of SNA (Figure 21). Again, PBMC exposed to 6.66 µg/mL of SNA for 30 minutes showed a more marked fraction of dead cells in the FACS diagram than cells incubated with smaller concentrations of SNA. However, the overall percentage of dead cells showed no correlation with the amount of SNA that had been applied.

It can therefore be concluded that the SNA-concentration of 6.66 µg/mL might be somewhat toxic to PBMC but not to an extent that would skew our results significantly.

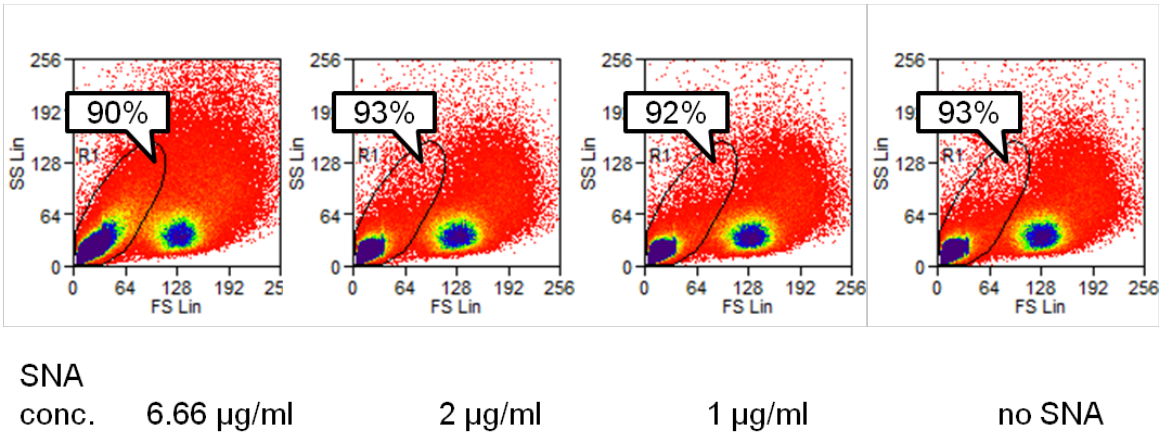


Figure 21 *Forward-sideward-scatter analysis of PBMC incubated for 30 minutes with varying concentrations of SNA. Note the extended dead-cell-fraction in the left panel that extends to the left and seems to be associated with the SNA-concentration. Comparison of the percentages of dead cells of the whole diagram, however, provides no evidence that a concentration of 6.66 µg/mL has a significantly greater cytotoxic effect than smaller concentrations.*

α1,2-linked fucose residues detected with the help of UEA-I

UEA-I (Ulex europaeus agglutinin I; also known as gorse or furze) is a lectin that binds specifically to α1,2-linked fucose residues (Sughii, Kabat et al. 1982).

Fascinatingly, in our study the α1,2 fucosylation of immune cells was similar to their α2,3 sialylation (Figure 5 on p. 37 and Figure 7 on p. 41): The expression of both types of sugar residues (α1,2-linked fucose and α2,3-linked sialic acids) was decreased on B cells from RRMS patients receiving natalizumab treatment. However, the α1,2 fucosylation of CD4 T cells was not subject to any changes. What stands out in the assessment of these data is that Th2 cells were highly reactive with UEA-I which points to plenty of α1,2-linked fucose residues on their surface. This parallels the binding behaviour of MAL to Th2 cells (see Figure 12, page 46).

Because CD22 is an antigen expressed mainly by B cells (see below, Nitschke 2009) we expected the frequency of UEA+CD22+ cells to be similar to UEA+CD20+ cells. Similarly to the findings in CD20+ cells, UEA binding to CD22+ cells seems to decrease under natalizumab (Figure 7, page 41).

CD22

CD22 belongs to the family of siglecs which are members of the immunoglobulin super family that bind to sialic acids (a quality that they share with lectins). CD22 is a cell surface receptor that occurs in the membrane of B cells (Nitschke 2009) and to a lesser extent in the membrane of dendritic cells (Reineks, Osei et al. 2009). An important feature of CD22 is its highly specific binding site for α2,6-sialic acids whose ligation can inhibit intracellular signalling by suppressing Ca++ influx. Mouse models showed that CD22 is a negative regulator of B cell activation (Varki and Crocker 2009). It is thus believed that whenever an antigen-presenting cell that co-expresses α2,6 sialic acids encounters a B cell the immune response of that B cell will be reduced; it has been suggested that CD22 might therefore prevent B cells from overstimulation, or that, conversely, an impaired function of the CD22 molecule on B cells might favour autoimmunity (reviewed by Poe and Tedder 2012).

Considering the possibly immunosuppressive effects of CD22 expression, we sought to examine whether B cells obtained from RRMS patients express fewer CD22 molecules than healthy controls. We found that B cells from natalizumab-treated patients show a somewhat weaker CD22-expression compared to healthy controls ($p=0.004$, Figure 8 and Table 5, page 43).

However, we were unable to detect a significant difference in CD22-expression between untreated RRMS patients and healthy controls. It should be noted, however, that an impaired function of the CD22 molecule may go unnoticed as long as only the presence and not the function of this molecule is analysed. Further tests that examine the signalling cascade in B cells from MS patients might shed more light on the role of the CD22 molecule in MS.

Since CD22 has been primarily seen as a marker for B cells (as reviewed by Nitschke 2009) only few authors, including Reineks, Osei et al, 2009, have reported that dendritic cells (CD11c+ cells) express CD22. Our data confirm those findings in that in our study not only B cells but also dendritic cells (CD11c+ cells) showed reactivity with anti-CD22 (Figure 8 and Table 5, page 43).

Effect of immunomodulatory therapies

Effects of natalizumab on the leukocyte composition

T cells

As we pointed out on page 50, we believe that CD4high cells are unactivated T cells and CD4low cells are activated T cells. Since we analysed CD4high and CD4low cells separately we had the chance to compare the numbers of activated CD4low cells, CD4high cells and CD4+ cells in general. The exact changes in the composition of peripheral blood cells under natalizumab treatment are still the subject of some debate, which is reflected by contradictory findings. While Skarica, Eckstein and colleagues (2011) could not detect an increase in CD4+ cells under natalizumab treatment other researchers report an increase in the numbers of lymphocytes, monocytes, eosinophils, and basophils in patients treated with natalizumab (Polman, O'Connor et al. 2006; Putzki, Baranwal et al. 2010). It has been suggested that this could be down to an increased release of all hematopoietic progenitor cells (Planas, Jelcic et al. 2011).

Our results only partly support the expectation of a larger percentage of lymphocytes related to natalizumab treatment. In particular, in our study the

percentage of CD4high T cells was larger under natalizumab, whereas the portion of CD4low cells was smaller (see Figure 2 on page 31). In fact, the frequencies of CD4high and CD4low cells taken together even cancelled each other out so that the portion of CD4+ cells did not vary significantly from group to group (Figure 3, page 32). Contrary to expectation, our results suggest that natalizumab does not influence the percentage of CD4 T cells on the whole. Nonetheless, our numbers of CD4high cells are in keeping with what can be found about CD4 T cells in literature.

One of the putative effects of natalizumab is to hinder CD4 T cells from leaving the blood vessels and entering the brain parenchyma (Baron, Madri et al. 1993). If this effect were reflected in the relative numbers of peripheral T cells and T cell subsets, we would expect greater numbers of CD4 T cells in natalizumab-treated patients than in treatment-naive RRMS patients. While the distribution of CD4+ cells failed to back this assumption, CD4high cells occurred in greater frequencies in natalizumab-treated patients than in untreated RRMS-patients (Figure 2, page 31).

In our opinion, CD4high cells are unactivated CD4 T cells (see page 50). It is the activated cells, however, that leave blood vessels to engage in inflammation. Hence, it is unclear, whether the effect of natalizumab, that stops cells from extravasating and traps them in the blood vessels, can account for our finding of a larger portion of CD4high cells in the blood of MS patients.

B cells

In our study the percentage of B cells in natalizumab-treated patients more than doubled (Table 1, Figure 2, page 31) which parallels reports of natalizumab-associated lymphocytosis (Polman, O'Connor et al. 2006; Krumbholz, Meinl et al. 2008; Skarica, Eckstein et al. 2011). Interestingly, a rise in the number of B cells under natalizumab led Planas, Jelcic and their colleagues to the hypothesis that an increased B cell mobilisation provides the JC virus with ideal breeding grounds (Planas, Jelcic et al. 2011).

Monocytes

Although observations to the contrary have also been made (Polman, O'Connor et al. 2006), our data show an insignificant trend toward a decreased portion of monocytes under natalizumab (Figure 2). Similarly, a drop in relative numbers of monocytes under natalizumab has also been reported (Skarica, Eckstein et al. 2011).

Cytotoxic T cells

The effect of natalizumab on cytotoxic T cells (CD8+ cells) has still not been fully understood. Some researchers were unable to detect an increase in the number of cytotoxic T cells under natalizumab (Skarica, Eckstein et al. 2011), while other research groups found that CD8+ cells increased 1.7-fold under natalizumab therapy (Putzki, Baranwal et al. 2010). The latter figure is close to what we could find, namely a 1.5 fold increase in the percentage of CD8+ cells under natalizumab (Table 1, Figure 2, page 31).

Effects of interferon-β on the composition of leukocytes

Van Boxel-Dezaire, Zula and their colleagues (2010) report a number of effects on the composition of leukocytes in response to interferon-β, most of which we were unable to reproduce in our study.

For example, van Boxel-Dezaire and Zula found an IFN-β-related increase in the survival of T cells and B cells (van Boxel-Dezaire, Zula et al. 2010), which we could not find. In our study, neither the percentage of B cells in IFN-β-treated patients (Figure 2), nor the percentage of T cells (all CD4+ cells, Figure 3) differed considerably from the ones in control groups.

Furthermore, van Boxel-Dezaire, Zula and colleagues (2010) report a higher apoptosis rate in monocytes when exposed to IFN-β. We can only partly support this finding as our study shows an insignificant trend towards a smaller portion of monocytes under IFN-β-treatment ($p=0.225$ compared to untreated MS patients, Figure 2, page 31).

A possible explanation for why our results did not completely match van Boxel-Dezaire and Zula's findings is that they ran an in vitro experiment in which they

exposed freshly drawn blood to IFN-β in culture-dishes. Unfortunately, they did not state the exact concentration of IFN-β at which they stimulated the blood cells. However, in different experiments carried out by the same group other kinds of interferon were added to the cells at concentrations between 3×10^3 IU/µl and 10×10^3 IU/µl (van Boxel-Dezaire, Zula et al. 2010). It is difficult to say how this corresponds to the serum levels of INF-β in the patients that participated in our study. The half life ($T_{1/2}$) of IFN-β is approximately 8 hours (Lahoz, Kauffman et al. 2009) and thus serum levels in patients who get an IFN-β-injection every other day may be not as steady as in an in vitro experiment.

Subsets of T cells (Th1 and Th17, Th2 and Treg) play a role in the pathogenesis of MS or its prevention, respectively. Therefore, it is interesting to examine whether IFN-β has an effect on T helper cell subsets.

For example, Skrzipek, Vogelgesang and colleagues (2011) activated CD4+ cells obtained from treatment-naive RRMS patients with anti-CD3/CD28 in the presence of IFN-β1b. They report a reduced IFN-γ secretion by CD4+ under the influence of IFN-β1b (Skrzipek, Vogelgesang et al. 2011). Their finding is interesting in light of an earlier report suggesting that IFN-β favours the differentiation of naive CD4 T cells into Th2 cells rather than into Th1 cells (Martin-Saavedra, Gonzalez-Garcia et al. 2008; Skrzipek, Vogelgesang et al. 2011).

One way to further analyse the influence of IFN-β on the differentiation and surface glycosylation of T helper subsets would be to purify naive CD4 T cells and differentiate them into T helper subsets over at least 10 days. This method would involve a repeated activation of these naive CD4 T cells with anti-CD3/CD28 in the presence or absence of IFN-β and appropriate cytokines. The percentage of the respective T helper subset population and their surface glycosylation could then be determined using flow cytometry (personal correspondence with PD Dr. Ghoreschi and PD Dr. Bischof, both UKT Tübingen).

Conclusions

In this study we showed that IFN-β treatment was associated with changes in glycan patterns not only on CD4 T cells but also on B cells. Specifically, IFN-β treatment was associated with lower α2,3-sialylation on CD4high cells and a trend toward this direction on B cells. Furthermore, α1,2-fucosylation on B cells was also markedly reduced under the influence of IFN-β treatment. As regards the frequency of whole cell populations, IFN-β treatment was associated with an increase in the portion of cytotoxic T cells.

Interestingly, some of the changes noted in the presence of IFN-β treatment were also seen in trial subjects treated with natalizumab: Both α2,3-sialylation and α1,2-fucosylation were reduced on B cells, suggesting an impact of natalizumab treatment on the sialylation of B cells.

In addition, we documented changes in the relative frequencies of various different types of immune cells that might well be related to the influence of natalizumab treatment:

The portion of CD4high cells was larger while the portion of CD4low cells was smaller under natalizumab treatment. Furthermore, the percentages of cytotoxic T cells and B cells were increased. We confirm previous reports that document CD22 expression on B cells and dendritic cells. Furthermore, we found a lower CD22 expression on B cells in natalizumab-treated RRMS patients. The assessment of the sialylation of T cell subsets showed plenty of binding sites for UEA-I and MAL-II on Th2 cells, pointing to an increased α1,2-fucosylation and α2,3-sialylation compared to the other T cell subsets. SNA bound abundantly to all cell types under study.

We conclude that both natalizumab and IFN-β seem to influence the composition of the white blood cell count and the sialylation/fucosylation of various immune cells. To further clarify the impact of certain types of sialylation on immune cells, functional tests and experiments based on the data that we generated are necessary. In particular, proliferation assays with α1,2-fucosylated and α2,3-sialylated Th2 cells or the influence of IFN-β on the

cytokine production of T helper subsets might reveal further insight into the field.

Summary

It is believed that the inflammation of the CNS in MS is driven by Th1 and Th17 cells and that it is then maintained by other cell types of the immune system such as B cells or plasma cells. Different cell types, such as regulatory T cells, are thought to fight off inflammation and thus have a beneficial effect on the course of the disease. There is also the hypothesis that ligation of certain surface molecules, such as CD22, influences the reactivity of immune cells through intracellular signalling. To determine the frequency and the glycan pattern of the respective cell types, immune cells obtained from natalizumab- and IFN-β-treated RRMS patients and controls were stained with cell type specific antibodies and glycan specific lectins (MAL-II, UEA-I, SNA and anti-CD22) and analysed flow cytometrically. Our findings parallel previous reports insofar as we saw an increase in the portion of B cells and cytotoxic T cells in natalizumab-treated patients. A hitherto undescribed phenomenon that occurred in natalizumab-treated patients was a reduced expression of CD22, α1,2-fucosylation and α2,3-sialylation on B cells. IFN-β treatment was associated with an increase in the percentage of cytotoxic T cells as well as with a reduced α2,3-sialylation of CD4high cells. In addition, we found that Th2 cells obtained from healthy donors expressed markedly larger amounts of α1,2-fucosylation and α2,3-sialylation than any other T cell subset. The portion of regulatory T cells was neither altered by MS itself nor by its immunomodulatory treatment.

Zusammenfassung

Es wird angenommen, dass die Entzündung des ZNS bei MS von Th1 und Th17 Zellen angetrieben wird und durch andere Arten von Immunzellen, wie B-Zellen oder Plasmazellen aufrechterhalten wird. Außerdem wird vermutet, dass regulatorische T-Zellen die Entzündungsreaktion unterdrücken und somit eine positive Wirkung auf den Verlauf der Erkrankung haben. Es besteht außerdem die Hypothese, dass die Ligation bestimmter Oberflächenmoleküle, wie CD22,

die Reaktivität von Immunzellen durch intrazelluläre Signalisierung beeinflussen. Um die Häufigkeit und das Glycanmuster der jeweiligen Zelltypen zu bestimmen, wurden Immunzellen mit zelltyp-spezifischen Antikörpern und glycan-spezifischen Lektinen (MAL-II, UEA-I, SNA-und Anti-CD22) gefärbt und durchflusszytometrisch untersucht. Die Zellen wurden von RRMS-Patienten, die mit Natalizumab und IFN-β behandelt wurden, und von Kontrollgruppen gewonnen. Ein Teil unsere Ergebnisse (die Zunahme der relativen Anzahl von B-Zellen und zytotoxischen T-Zellen in Natalizumab-behandelten Patienten) entspricht früheren Befunden. Ein bisher unbeschriebenes Phänomen, das wir in der Gruppe der mit Natalizumab behandelten Patienten fanden, ist eine verminderte Expression von CD22, α1,2-Fucosylierung und α2,3-Sialylierung auf B-Zellen. Die Behandlung mit IFN-β war sowohl mit einem erhöhten Anteil zytotoxischer T-Zellen als auch mit einer reduzierten α2,3-Sialylierung von CD4high Zellen assoziiert. Zusätzlich fanden wir, dass die α1,2-Fucosylierung und α2,3-Sialylierung auf der Oberfläche von Th2-Zellen von gesunden Spendern deutlich höher im Vergleich zu anderen T-Zell-Untergruppen war. Der Anteil von regulatorischen T-Zellen wurde weder durch MS selbst noch durch ihre immunmodulatorische Behandlung verändert.

References

Abromson-Leeman, S., R. T. Bronson, et al. (2009). "Encephalitogenic T cells that stably express both T-bet and ROR gamma t consistently produce IFNgamma but have a spectrum of IL-17 profiles." J Neuroimmunol 215(1-2): 10-24.

Annunziato, F., L. Cosmi, et al. (2007). "Phenotypic and functional features of human Th17 cells." J Exp Med 204(8): 1849-1861.

Baron, J. L., J. A. Madri, et al. (1993). "Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma." J Exp Med 177(1): 57-68.

Batisse, C., J. Marquet, et al. (2004). "Lectin-based three-color flow cytometric approach for studying cell surface glycosylation changes that occur during apoptosis." Cytometry A 62(2): 81-88.

Bertozzi, C. and D. Rabuka (2009). Structural Basis of Glycan Diversity. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. . Cold Spring Harbor (NY), Cold Spring Harbor Laboratory Press: Chapter 2.

Calabresi, P. A., L. R. Tranquill, et al. (1997). "Increases in soluble VCAM-1 correlate with a decrease in MRI lesions in multiple sclerosis treated with interferon beta-1b." Ann Neurol 41(5): 669-674.

Chi, L. J., W. H. Xu, et al. (2010). "Distribution of Th17 cells and Th1 cells in peripheral blood and cerebrospinal fluid in chronic inflammatory demyelinating polyradiculoneuropathy." J Peripher Nerv Syst 15(4): 345-356.

Compston, A. and A. Coles (2008). "Multiple sclerosis." Lancet 372(9648): 1502-1517.

de Seze, J., F. Borgel, et al. (2012). "Patient perceptions of multiple sclerosis and its treatment." Patient Prefer Adherence 6: 263-273.

Durelli, L., L. Conti, et al. (2009). "T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta." Ann Neurol 65(5): 499-509.

Dyment, D. A., G. C. Ebers, et al. (2004). "Genetics of multiple sclerosis." Lancet Neurol 3(2): 104-110.

Elian, M., S. Nightingale, et al. (1990). "Multiple sclerosis among United Kingdom-born children of immigrants from the Indian subcontinent, Africa and the West Indies." J Neurol Neurosurg Psychiatry 53(10): 906-911.

Elices, M. J., L. Osborn, et al. (1990). "VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site." Cell 60(4): 577-584.

Feger, U., C. Luther, et al. (2007). "Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients." Clin Exp Immunol 147(3): 412-418.

Fernald, G. H., R. F. Yeh, et al. (2005). "Mapping gene activity in complex disorders: Integration of expression and genomic scans for multiple sclerosis." J Neuroimmunol 167(1-2): 157-169.

Fletcher, J. M., S. J. Lalor, et al. (2010). "T cells in multiple sclerosis and experimental autoimmune encephalomyelitis." Clin Exp Immunol 162(1): 1-11.

Fletcher, J. M., R. Lonergan, et al. (2009). "CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis." J Immunol 183(11): 7602-7610.
Franz, S., B. Frey, et al. (2006). "Lectins detect changes of the glycosylation status of plasma membrane constituents during late apoptosis." Cytometry A 69(4): 230-239.
Frisullo, G., V. Nociti, et al. (2008). "IL17 and IFNgamma production by peripheral blood mononuclear cells from clinically isolated syndrome to secondary progressive multiple sclerosis." Cytokine 44(1): 22-25.
Frohman, E. M., M. K. Racke, et al. (2006). "Multiple sclerosis--the plaque and its pathogenesis." N Engl J Med 354(9): 942-955.
Geisler, C. and D. L. Jarvis Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities, Glycobiology. 2011 Aug;21(8):988-93.
Hatterer, E., N. Davoust, et al. (2006). "How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes." Blood 107(2): 806-812.
Heijink, I. H., E. Vellenga, et al. (2003). "Polarized Th1 and Th2 cells are less responsive to negative feedback by receptors coupled to the AC/cAMP system compared to freshly isolated T cells." Br J Pharmacol 138(8): 1441-1450.
Kappos, L., D. Bates, et al. (2011). "Natalizumab treatment for multiple sclerosis: updated recommendations for patient selection and monitoring." Lancet Neurol 10(8): 745-758.
Kazazi, F., J. M. Mathijs, et al. (1989). "Variations in CD4 expression by human monocytes and macrophages and their relationships to infection with the human immunodeficiency virus." J Gen Virol 70(Pt 10): 2661-2672.
Kebir, H., K. Kreymborg, et al. (2007). "Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation." Nat Med 13(10): 1173-1175.
Kieseier, B. C. (2011). "The mechanism of action of interferon-beta in relapsing multiple sclerosis." CNS Drugs 25(6): 491-502.
Krumbholz, M., I. Meinl, et al. (2008). "Natalizumab disproportionately increases circulating pre-B and B cells in multiple sclerosis." Neurology 71(17): 1350-1354.
Kumar, V., R. S. Cotran, et al. (2003). Robbins basic pathology. Philadelphia, PA, Saunders.
Lahoz, M., M. A. Kauffman, et al. (2009). "Pharmacokinetics and pharmacodynamics of interferon beta 1a in Cebus apella." J Med Primatol 38(3): 187-191.
Liu, Y. J. (2005). "IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors." Annu Rev Immunol 23: 275-306.
Lock, C., G. Hermans, et al. (2002). "Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis." Nature Medicine 8(5): 500-508.
Lucchinetti, C. F., B. F. Popescu, et al. (2011). "Inflammatory cortical demyelination in early multiple sclerosis." N Engl J Med 365(23): 2188-2197.

Malagolini, N., M. Chiricolo, et al. (2009). "Exposure of alpha2,6-sialylated lactosaminic chains marks apoptotic and necrotic death in different cell types." Glycobiology 19(2): 172-181.
Martin-Saavedra, F. M., C. Gonzalez-Garcia, et al. (2008). "Beta interferon restricts the inflammatory potential of CD4+ cells through the boost of the Th2 phenotype, the inhibition of Th17 response and the prevalence of naturally occurring T regulatory cells." Mol Immunol 45(15): 4008-4019.
Miller, D. M., B. Weinstock-Guttman, et al. (2000). "A meta-analysis of methylprednisolone in recovery from multiple sclerosis exacerbations." Mult Scler 6(4): 267-273.
Mills, K. H. (2008). "Induction, function and regulation of IL-17-producing T cells." Eur J Immunol 38(10): 2636-2649.
Miyara, M., Y. Yoshioka, et al. (2009). "Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor." Immunity 30(6): 899-911.
Montes, M., X. Zhang, et al. (2009). "Oligoclonal myelin-reactive T-cell infiltrates derived from multiple sclerosis lesions are enriched in Th17 cells." Clin Immunol 130(2): 133-144.
Mosmann, T. R. and R. L. Coffman (1989). "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties." Annu Rev Immunol 7: 145-173.
Mowen, K. A. and L. H. Glimcher (2004). "Signaling pathways in Th2 development." Immunol Rev 202: 203-222.
Mumford, C. J., N. W. Wood, et al. (1994). "The British Isles survey of multiple sclerosis in twins." Neurology 44(1): 11-15.
Murphy, K., P. Travers, et al. (2012). Janeway's immunobiology. New York, Garland Science.
Naismith, R. T., L. Piccio, et al. (2010). "Rituximab add-on therapy for breakthrough relapsing multiple sclerosis: a 52-week phase II trial." Neurology 74(23): 1860-1867.
Nitschke, L. (2009). "CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions." Immunological Reviews 230(1): 128-143.
Noseworthy, J. H., C. Lucchinetti, et al. (2000). "Multiple sclerosis." N Engl J Med 343(13): 938-952.
Planas, R., I. Jelcic, et al. (2011). "Natalizumab Treatment Perturbs Memory- and Marginal Zone-like B-cell Homing in Secondary Lymphoid Organs in Multiple Sclerosis." Eur J Immunol.
Poe, J. C. and T. F. Tedder (2012). "CD22 and Siglec-G in B cell function and tolerance." Trends Immunol 33(8): 413-420.
Polman, C. H., P. W. O'Connor, et al. (2006). "A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." N Engl J Med 354(9): 899-910.
Polman, C. H., S. C. Reingold, et al. (2005). "Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria"." Ann Neurol 58(6): 840-846.
Putzki, N., M. K. Baranwal, et al. (2010). "Effects of natalizumab on circulating B cells, T regulatory cells and natural killer cells." Eur Neurol 63(5): 311-317.

Ray, A., M. K. Mann, et al. (2011). "A case for regulatory B cells in controlling the severity of autoimmune-mediated inflammation in experimental autoimmune encephalomyelitis and multiple sclerosis." J Neuroimmunol 230(1-2): 1-9.
Reineks, E. Z., E. S. Osei, et al. (2009). "CD22 expression on blastic plasmacytoid dendritic cell neoplasms and reactivity of anti-CD22 antibodies to peripheral blood dendritic cells." Cytometry B Clin Cytom 76(4): 237-248.
Romagnani, S. (1996). "Th1 and Th2 in human diseases." Clin Immunol Immunopathol 80(3 Pt 1): 225-235.
Romagnani, S. (2008). "Human Th17 cells." Arthritis Res Ther 10(2): 18.
Roozendaal, R., E. Vellenga, et al. (2001). "Resistance of activated human Th2 cells to NO-induced apoptosis is mediated by gamma-glutamyltranspeptidase." Int Immunol 13(4): 519-528.
Sallusto, F. and A. Lanzavecchia (2009). "Human Th17 cells in infection and autoimmunity." Microbes Infect 11(5): 620-624.
Serafini, B., B. Rosicarelli, et al. (2007). "Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain." J Exp Med 204(12): 2899-2912.
Shi, G., C. A. Cox, et al. (2008). "Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells." J Immunol 181(10): 7205-7213.
Shibuya, N., I. J. Goldstein, et al. (1987). "The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence." J Biol Chem 262(4): 1596-1601.
Simhadri, V. R., J. L. Mariano, et al. (2011). "Differential expression of CD300a/c on human TH1 and TH17 cells." BMC Immunol 12: 62.
Skarica, M., C. Eckstein, et al. (2011). "Novel mechanisms of immune modulation of natalizumab in multiple sclerosis patients." J Neuroimmunol 235(1-2): 70-76.
Skrzipek, S., A. Vogelgesang, et al. (2011). "Differential effects of interferon-ss1b on cytokine patterns of CD4+ and CD8+ T cells derived from RRMS and PPMS patients." Mult Scler.
Sperandio, M., D. Frommhold, et al. (2006). "Alpha 2,3-sialyltransferase-IV is essential for L-selectin ligand function in inflammation." Eur J Immunol 36(12): 3207-3215.
Sperandio, M., C. A. Gleissner, et al. (2009). "Glycosylation in immune cell trafficking." Immunol Rev 230(1): 97-113.
Stuve, O., C. M. Marra, et al. (2006). "Altered CD4+/CD8+ T-cell ratios in cerebrospinal fluid of natalizumab-treated patients with multiple sclerosis." Arch Neurol 63(10): 1383-1387.
Sughii, S., E. A. Kabat, et al. (1982). "Further immunochemical studies on the combining sites of Lotus tetragonolobus and Ulex europaeus I and II lectins." Carbohydr Res 99(1): 99-101.
Takahashi, T., Y. Kuniyasu, et al. (1998). "Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state." Int Immunol 10(12): 1969-1980.

Tzartos, J. S., M. A. Friese, et al. (2008). "Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis." Am J Pathol 172(1): 146-155.
Valencia, X. and P. E. Lipsky (2007). "CD4+CD25+FoxP3+ regulatory T cells in autoimmune diseases." Nat Clin Pract Rheumatol 3(11): 619-626.
Valmori, D., C. Raffin, et al. (2010). "Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors." Proc Natl Acad Sci U S A 107(45): 19402-19407.
van Beelen, A. J., Z. Zelinkova, et al. (2007). "Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells." Immunity 27(4): 660-669.
van Boxel-Dezaire, A. H., J. A. Zula, et al. (2010). "Major differences in the responses of primary human leukocyte subsets to IFN-beta." J Immunol 185(10): 5888-5899.
Varki and Crocker (2009). I-type Lectins. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY), Cold Spring Harbor Laboratory Press: Chapter 32.
Varki, A., M. Etzler, et al. (2009). Discovery and Classification of Glycan-Binding Proteins. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY), Cold Spring Harbor Laboratory Press: Chapter 26.
Varki, A. and J. Lowe (2009). Biological Roles of Glycans. In: Essentials of glycobiology. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press: Chapter 6.
Varki, A. and R. Schauer (2009). Sialic Acids. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY), Cold Spring Harbor Laboratory Press: Chapter 14.
Viglietta, V., C. Baecher-Allan, et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med 199(7): 971-979.
Wang, W. C. and R. D. Cummings (1988). "The immobilized leukoagglutinin from the seeds of Maackia amurensis binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked alpha-2,3 to penultimate galactose residues." J Biol Chem 263(10): 4576-4585.
Wilson, N. J., K. Boniface, et al. (2007). "Development, cytokine profile and function of human interleukin 17-producing helper T cells." Nat Immunol 8(9): 950-957.
Yang, L., D. E. Anderson, et al. (2008). "IL-21 and TGF-beta are required for differentiation of human T(H)17 cells." Nature 454(7202): 350-352.
Zhu, J., H. Yamane, et al. (2010). "Differentiation of effector CD4 T cell populations (*)." Annu Rev Immunol 28: 445-489.

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