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The effect of Natalizumab on functional properties of monocytes in patients with Multiple Sclerosis

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Abbreviations

APC	antigen presenting cell
BBB	blood brain barrier
CD	cluster of differentiation
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
DC	dendritic cell
DGN	Deutsche Gesellschaft für Neurologie
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethyl fumarate
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EBV	Epstein-Barr virus
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FDA	Food and Drug Administration
FMO	fluorescence minus one
GA	glatiramer acetate
HLA	human leukocyte antigen
i.v.	intravenously
IC	internal control
IFN	interferon
IL	interleukin
JCV	John Cunningham virus
LFA-1	lymphocyte function-associated antigen 1
mab	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MFI	mean fluorescent intensity
MMP	matrix metalloproteinases
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	Multiple sclerosis
NEDA	no evidence of disease activity
NTZ	Natalizumab
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PML	progressive multifocal leukoencephalopathy
PPMS	primary progressive multiple sclerosis

RIS	radiologically isolated syndrome
RMS	relapsing multiple sclerosis
RPMI-1640	Roswell park memorial institute-1640
RRMS	relapsing remitting multiple sclerosis
s.c.	subcutaneous
SLAM	signaling lymphocytic activation molecule
SPMS	secondary progressive multiple sclerosis
TGF	transforming growth factor
TLR	toll like receptor
Tmem119	transmembrane protein 119
TNF	tumor necrosis factor
TSPO	translocator protein
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS), affecting more than two million people worldwide (Feigin et al. 2017). The demyelinating disease was first described as an own disease entity in 1868 by Leopold Ordenstein, a scholar of Jean-Martin Charcot (Lehmann et al. 2007; Lehmann et al. 2018). While the etiology of MS remains unknown, a variety of genetic and environmental factors are discussed (Compston and Coles 2002). To this day, MS cannot be cured. However, various drugs for preventing relapses and slowing down progression exist.

1.1.1 Epidemiology, etiology impact

In 2013 the global median prevalence of MS was 33/100,000 (Leray et al. 2016). In 2015 the yearly incidence of MS was approximately 18/100,000 in Germany, a country with a high prevalence (Holstiege et al. 2017). About twice as many women as men are affected by MS (Milo and Kahana 2010). A study on a cohort of Canadian MS patients even indicates that the ratio of women to men increases over time (Orton et al. 2006).

MS is most likely a multifactorial disease. Regarding the environmental factors, it should be noted that the prevalence of MS correlates with geographical latitude (Simpson et al. 2011). For instance, this was shown in a study by Acheson and colleagues (Acheson et al. 1960) for US veterans diagnosed with MS. They observed that higher rates of MS correlate with a low average of total annual hours of sunshine at the birthplace and high degrees north latitude. Migration studies showed that the risk of getting MS for people from high-risk regions declines with migration to low-risk areas, while conversely, the risk seems not to increase with migration to areas of higher risk (Ascherio and Munger 2007b). Moreover, low vitamin D levels are discussed to enhance the risk of MS (Ascherio and Munger 2007a; Ascherio et al. 2010). This might be connected to the geographical latitude and sun exposure, as the generation of vitamin D_3 (cholecalciferol) in the skin is dependent on ultraviolet (UV) radiation. However, vitamin D can also be provided through vitamin supplements or a diet rich in vitamin D. Another environmental factor associated with a higher risk of MS is smoking (Hernan et al. 2001). Furthermore, Epstein-Barr virus (EBV) infection and the resulting infectious mononucleosis are considered to be risk factors for the development of MS (Ascherio et al. 2010).

The familial recurrence rate of MS is about 20%, indicating that genetic factors play a role in the development of MS (Compston and Coles 2008). Various genes such as human leukocyte

antigens (HLAs) and interleukin (IL) 17R are discussed to increase the risk of MS (Leray et al. 2016).

1.1.2 Symptoms, clinical courses

MS is a disease that can present itself with a variety of neurological symptoms, most commonly with an onset at the age of 20 to 50 years (Milo and Kahana 2010). The clinical manifestation depends on the location of demyelinating lesions. Therefore, an assortment of different symptoms can occur during the course of the disease. Typical manifestations are unilateral loss of vision, including scotoma, reduced color vision and visual acuity, and musculoskeletal symptoms, such as weakness or stiffness of the limbs and painful spasms. Autonomic dysfunction, for example bladder dysfunction, constipation, and erectile impotence, can also appear. Moreover, central symptoms like cognitive impairment, affective disorders, and fatigue are experienced by MS patients (Compston and Coles 2008).

In order to assess the degree of clinical impairment of MS patients, the Extended Disability Status Scale (EDSS) was introduced in 1983. It combines the evaluation of different functional systems and ranges from zero (normal neurological exam) to ten (death due to MS) (Kurtzke 1983).

In 1996 a standardized terminology to describe the clinical courses of MS was introduced by the US National Multiple Sclerosis Society. Four main courses were distinguished: Relapsing remitting MS (RRMS), the most common course, is characterized by intermittent exacerbations followed by intervals of complete or partial recovery and a stable course between these exacerbations. The relapsing remitting course can transition into secondary progressive MS (SPMS), being defined as an initial RRMS followed by progression with or without relapses. RRMS and SPMS can be summarized under the term relapsing MS (RMS). Disease progression from the onset of disease with infrequent plateaus and minor improvements is termed primary progressive MS (PPMS) (Lublin and Reingold 1996). Moreover, progressive-relapsing MS (PRMS) was described as category of disease but was later eliminated from the terminology (Lublin et al. 2014).

In 2014, the above-mentioned definitions were revised. In order to classify the MS phenotype more accurately, activity – defined as relapse, new or enlarging T_2 lesion or gadolinium-enhancing lesion – and progression – understood as ongoing inflammatory and neurodegenerative processes – were suggested to be assessed. Therefore, the disease course could for instance be described as follows: *SPMS-active and progressing*, meaning during the assessment period, the patient has experienced both disease activity and gradual disease worsening (Lublin 2014). Moreover, two new terms were introduced: Clinically isolated syndrome (CIS) is the clinical presentation of symptoms while patients do not yet fulfill the criteria for dissemination in time as described below. Incidental magnetic resonance imaging (MRI) findings suggesting inflammatory demyelination without the occurrence of clinical symptoms are defined as radiologically isolated syndrome (RIS). RIS does not count as a course of MS

itself, since clinical evidence is a criterion for the diagnosis of MS, but RIS may increase the likelihood of a subsequent MS diagnosis (Okuda et al. 2011).

1.1.3 Diagnosis

The diagnosis of MS is based on a combined evaluation of clinical, radiological, and laboratory data. To the present day, the most common diagnostic criteria are the McDonald criteria (**Table 1**), which were introduced in 2001 and most recently revised in 2017 (McDonald et al. 2001; Thompson et al. 2018). In order to diagnose MS according to the McDonald criteria, a dissemination in time and space of CNS lesions must be present. Since the latest criteria revision in 2017, the presence of CSF-specific oligoclonal bands (OCBs) is considered a substitute for the fulfillment of dissemination in time in patients with CIS (Thompson et al. 2018).

Table 1: 2017 McDonald criteria (Thompson et al. 2018)

Clinical attacks	Lesions with objective evidence	Additional data needed for diagnosis
≥2	≥2	None
≥2	1 (as well as clear-cut historical evidence)	None
≥2	1	Dissemination in space demonstrated by an additional clinical attack involving a different CNS site or by MRI
1	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI or presence of oligoclonal bands (OCBs)
1	1	Dissemination in space and dissemination in time or demonstration of OCBs
0 (progressive disease from onset)	0	1 year of disability progression independent of clinical relapse + 2 of the following • ≥1 T2-hyperintense lesions characteristic for MS in ≥1 of the following regions: periventricular, cortical/juxtacortical, infratentorial • ≥2 T2-hyperintense in the spinal cord • Presence of OCBs

1.1.4 Pathophysiology and Pathology

The pathology of MS is characterized by destruction and selective loss of myelin sheaths formed by oligodendrocytes (Stadelmann-Nessler and Brück 2015). The existence of myelin sheaths is crucial for the saltatory propagation of the action potential along the axon. The pathophysiology of MS involving demyelination and remyelination, inflammation, loss of oligodendrocytes and astrogliosis, and neuronal and axonal degeneration results in the formation of sclerotic plaques (Compston and Coles 2008). MS lesions can develop at any location within the CNS and can be detected by MRI (Reich et al. 2018). These macroscopic lesions were first described and depicted by Sir Robert Carswell and Jean Cruveilhier after studying the autopsy of a MS patient between 1838 and 1841 (Compston 1988). Today it is known that the histopathological appearance of MS lesions is heterogeneous. Lucchinetti et al. (2000) characterized these demyelinating lesions based on the investigation of a large number of biopsies and autopsies of MS patients. Overall, in this study lesions were identified as areas with a reduced density of myelinated fibers infiltrated by macrophages, activated microglia, and T lymphocytes. Four different patterns of demyelination were defined: Pattern I lesions are associated with an infiltration of T cells and macrophages and are located nearby small veins and venules. The most commonly described pattern II presents itself similar to pattern I regarding location and the dominance of T cells and macrophages. It is characterized by the presence of immunoglobulins (Igs), mainly IgG, and complement C9neo antigen, which are specific to this pattern. Pattern III and IV show signs of oligodendrocyte dystrophy. Characteristic of pattern III is the preferential loss of myelin-associated glycoprotein, while all other patterns show a simultaneous loss of all myelin proteins. Moreover, apoptosis of oligodendrocytes was described in pattern III plaques. Pattern III appeared to be rare in chronic MS but was detected more commonly in biopsies/autopsies of patients with a disease duration of less than two months. Pattern IV is the least common pattern. Its occurrence was only described in patients with a variant of PPMS with pronounced cognitive impairment. It shows similar features to pattern I and II regarding location of plaques but differs from these classical patterns due to an extensive loss of oligodendrocytes. Lucchinetti and colleagues described an intraindividual homogeneity regarding the morphology of the lesions and therefore, suggested that each individual patient had one distinct pattern of demyelination. However, these findings were questioned by a more recent study, which proposed that after a longer disease duration, acute plaques generally appear as pattern II in every patient (Breij et al. 2008).

1.1.5 Pathogenesis of MS

Inflammation and neurodegeneration both play an important role in the formation of the clinical course of MS. Currently four theories on the interdependence of these two processes are being discussed. First, inflammation acts as fundamental pathogenic event, neurodegeneration, namely axonal loss, follows thereafter. Second, neurodegeneration appears initially, causing an inflammatory response. Third, inflammation and neurodegeneration are two

thoroughly independent factors in the pathogenesis of MS. Last, inflammation reveals an intrinsic predisposition for neurodegeneration which results in axonal vulnerability leading to cumulative injury (Compston and Coles 2008). Studies on autopsies and biopsies of MS patients reported a strong correlation between the amount of inflammatory cells and the severity of acute axonal damage (Frischer et al. 2009; Kuhlmann et al. 2002). This correlation could even be demonstrated for autopsies from patients with a progressive disease course. Moreover, in later, pathological inactive stages of progressive disease, a decrease of inflammation was detected which also correlated with a decline of the extent of axonal damage. These findings suggest a connection between inflammation and neurodegeneration (Frischer et al. 2009).

Regarding the provenance of CNS inflammation, two main models are discussed: the outside-in and the inside-out theories. According to the outside-in theory, immune activation originates in the periphery and subsequently is transmitted to the CNS causing inflammation. Autoreactive T cells are thought to be activated via molecular mimicry, cross reactivity or bystander activation (Hemmer et al. 2015). After these cells migrate to lymph nodes a part of the activated T and B cells cross the blood brain barrier (BBB) and thus enter the CNS. The production of inflammatory cytokines such as IL17 and IL22 produced by Th17 cells within the CNS then facilitates the invasion of the CNS for further immune cells such as monocytes and additional lymphocytes by disrupting the BBB (Kebir et al. 2007). Experimental autoimmune encephalomyelitis (EAE), a widely used animal model for MS, bases on the outside-in theory as EAE is commonly induced by subcutaneous (s. c.) injection of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide along with an adjuvant (Lassmann and Bradl 2017). In contrast, the inside-out theory suggests that an initial event inside the CNS activates microglia. This subsequently leads to an expansion of the immune response resulting in a secondary recruitment of peripheral immune cells. This initial event might be the spontaneous death of oligodendrocytes. While hypoxic stress and primary defects of oligodendrocytes, such as mutations, are being discussed as a possible trigger, the cause of oligodendrocyte loss remains unknown (Aboul-Enein et al. 2003; Hemmer et al. 2015). According to this hypothesis, the activation of peripheral immune cells takes place in deep cervical lymph nodes where antigens from within the CNS are drained to. The route of the antigen drainage remains uncertain. On the one hand, the antigens might reach the lymph nodes via dendritic cells (DCs). This is supported by a study indicating that DCs in mice have the ability to leave the brain in association with the rostral migratory stream (Mohammad et al. 2014). On the other hand, it is discussed whether the cerebrospinal fluid (CSF) might carry the antigens outside the CNS (Hemmer et al. 2015).

Although there is an ongoing debate on the sequence of events regarding the pathogenesis, it is widely accepted that the adaptive immune system as well as cells of the innate immune system play a crucial role in the pathogenesis of MS (Reich et al. 2018).

1.1.6 Role of myeloid cells in MS

For a long time, T cells were considered the main effector cells in the pathogenesis of MS (Kaskow and Baecher-Allan 2018). More recently, the involvement of B cells in the pathogenesis of MS was increasingly discussed (Comi et al. 2021). While the role of lymphocytes in MS is widely recognized and researched, the contribution of myeloid cells – including macrophages, dendritic cells (DCs), microglia, and monocytes – is significantly less reviewed.

Monocytes evolve from common myeloid progenitor cells, which are also precursor cells of granulocytes, mast cells, and DCs as well as erythrocytes and platelets (Murphy and Weaver 2018a). Monocytes are then released from the bone marrow into the blood. Three subgroups of monocytes can be distinguished by their expression of the surface markers cluster of differentiation (CD) 14, which is a receptor for lipopolysaccharide (LPS), and CD16, which is a low affinity receptor for IgG. $CD14^{++}CD16^{-}$ monocytes represent the most common subset which is also referred to as classical monocytes. CD16 expressing monocytes can be divided in $CD14^{++}CD16^{+}$ so-called intermediate monocytes and $CD14^{+}CD16^{++}$ non-classical monocytes. Interestingly, it was demonstrated that $CD16^{+}$, but not $CD16^{-}$ classical monocytes, promote the migration of $CD4^{+}$ T cells across an endothelial barrier, which might contribute to the breakdown of the BBB (Waschbisch et al. 2016). A developmental relationship between the above-mentioned subsets with non-classical monocytes as most mature stage is assumed (Ziegler-Heitbrock and Hofer 2013; Ziegler-Heitbrock et al. 2010). Once monocytes infiltrate tissue, they differentiate into macrophages (Hemmer et al. 2015). Macrophages can be divided in two subgroups: On the one hand, the pro-inflammatory M1 phenotype, activated by toll-like receptor (TLR) ligands such as LPS, and on the other hand, M2 macrophages that develop in the presence of IL4 or IL13 and act in an anti-inflammatory way (Murray and Wynn 2011). As their CNS-resident counterpart, microglia, unlike monocytes, do not differentiate from bone marrow hematopoietic stem cells, but derive from yolk-sac progenitor cells during embryogenesis and subsequently self-renew inside the CNS (Li and Barres 2018; Zia et al. 2020). The histological and immunohistochemical distinction of activated microglia and macrophages is often difficult to draw (Yamasaki et al. 2014). Recently, transmembrane protein 119 (Tmem119) was identified as highly expressed microglia-specific cell-surface protein allowing for the differentiation of microglia macrophages (Bennett et al. 2016).

The presence of macrophages and activated microglia was described in all designated patterns of MS lesion (Lucchinetti et al. 2000). Myeloid cells were found in large numbers in active lesions and could also be detected in plaques of patients with SPMS (Lassmann et al. 2001; Prineas John W. et al. 2001). Their predominance in MS lesions as well as their ability to function both as effector- and as antigen presenting cells (APCs) suggests that they play a crucial role in the pathogenesis of MS (Mishra and Yong 2016). With regards to EAE, it was shown that shortly after immunization with myelin peptide, pro-inflammatory monocytes were released from the bone marrow into the circulation, where they first accumulate before

entering the CNS. Moreover, when mobilization of pro-inflammatory monocytes was augmented, a clinical exacerbation of EAE resulted (King et al. 2009). Conversely, the depletion of monocytes/macrophages reduces disease worsening or even prevents the clinical manifestation of EAE (Brosnan et al. 1981; Huitinga et al. 1990; Moreno et al. 2016). Experiments depleting only peripheral blood-borne macrophages indicate that monocyte-derived macrophages are necessary for a proper activation of CNS resident microglia in EAE (Benveniste 1997).

Myeloid cells are essential for the pathogenesis of MS since they act as APCs and, therefore, activate and polarize T cells (Mishra and Yong 2016). One study showed that in coculture, myeloid cells promote the differentiation of T cells into pathogenic Th17 cells under Th17-polarizing conditions (Yi et al. 2012). A further study indicated that Th1 cells are incapable to execute the encephalitogenic Th1 effector program if recruitment of macrophages to the CNS is compromised (Huang et al. 2001).

In addition to their role as APCs, myeloid cells have the capacity to produce cytokines, chemokines, free radicals, and other mediators of tissue injury (Benveniste 1997; Mishra and Yong 2016). Tumor necrosis factor (TNF) α is one of the pro-inflammatory-cytokines produced by both infiltrating macrophages and CNS-resident microglia in EAE (Renno et al. 1995). Elevated TNFα production by myeloid cells is assumed to lead to a higher capacity of these cells to polarize T cells towards pathogenic Th1 and Th17 cells (Weber et al. 2010). Furthermore, TNFα is thought to be associated with demyelination through oligodendrocyte cytotoxicity (Zajicek et al. 1992). Peripheral blood monocytes/macrophages of patients with active MS were reported to produce significantly higher amounts of TNFα and IL6 upon stimulation compared to normal controls (Imamura et al. 1993). In accordance with that, MS patients were found to have a higher number of IL6 secreting monocytes in the peripheral blood compared to healthy controls. Moreover, the same study revealed higher levels of monocytes secreting IL12, an important cytokine for the encephalitogenic Th1 differentiation of T cells, in MS patients compared to controls. Controversially, this study did not show a difference between the groups regarding the number of TNFα secreting monocytes (Kouwenhoven et al. 2001).

Furthermore, macrophages and microglia both express matrix metalloproteinases (MMP), which facilitate the crossing of leukocytes across the BBB (Bar-Or et al. 2003; Benveniste 1997). In addition to that, MMPs are discussed to disrupt the BBB and take part in demyelination (Cuzner and Opdenakker 1999; Rosenberg 2002). Monocytes of MS patients showed higher levels of two MMPs (namely MMP-2 and MMP-14) compared to monocytes of healthy individuals (Bar-Or et al. 2003).

Regarding relapse-independent disease progression, lately, evidence suggesting a possible contribution of myeloid cells to this continuous process was gathered. In patients with progressive MS, a correlation of microglial/macrophage activation and disease disability and prognosis was detected in a study using positron emission tomography (PET) to measure

the mitochondrial translocator protein (TSPO). This correlation could not be detected in RMS patients (Giannetti et al. 2014). Moreover, monocytes and microglia were detected in gray matter lesions of patients with progressive MS (Giannetti et al. 2014; Prineas J. W. and Parratt 2021).

In contrast to their role as disease promoting cells, regulatory and regeneratory functions of myeloid cells are discussed. Due to their phagocytic abilities, bloodborne macrophages can remove myelin debris and pro-inflammatory by-products in MS lesions (Reich et al. 2018). Their regulatory capacity is among others characterized by the production of anti-inflammatory cytokines such as transforming growth factor (TGF) β1 and IL10 (Kiefer et al. 2001). Studies suggest that activated macrophages can exhibit a mixture of both pro- and anti-inflammatory phenotypes in MS lesions (reviewed in (Mishra and Yong 2016)). A switch from pro-inflammatory M1 to regulatory M2 phenotype was described to coincide with the beginning of remyelination. Microglia and peripherally derived macrophages even seem to play a role in the process of remyelination as they are suggested to enhance oligodendrocyte differentiation (Miron et al. 2013).

1.1.7 Overview on MS therapies

To date, there is no known cure for MS. Therefore, the goal of treatment is the relief of symptoms and the reduction of relapse frequency and relapse-independent progression. Regarding therapy, three categories can be distinguished: treatment of acute relapses, symptomatic treatment, and disease modifying therapeutics.

1.1.7.1 Treatment of acute relapses

A relapse is defined as a monophasic exacerbation in the absence of fever with a duration of at least 24 hours. It depicts a focal or multifocal inflammatory demyelination in the CNS and can be followed by full or partial recovery (Thompson et al. 2018). The standard therapy for these deteriorations is high-dosed methylprednisolone, which is a glucocorticosteroid (Miller et al. 2000). Commonly it is administered intravenous (i.v.), but evidence suggests that an oral administration leads to a comparable clinical benefit (Alam et al. 1993). A daily dose of 500-1,000 mg for 3-5 days is recommended by the Deutsche Gesellschaft für Neurologie (DGN) (DGN 2021). For patients not benefitting from treatment with glucocorticoids, plasma exchange may be considered (Weinshenker et al. 1999).

1.1.7.2 Symptomatic treatment

Since the symptoms of MS are very diverse and can include severe impairments and disabilities, comprehensive therapy has to be multimodal and interdisciplinary (Compston and Coles 2008; Soelberg Sorensen et al. 2019). For instance, spasticity is a disabling symptom that is experienced by approximately 90% of all MS patients. Drugs like baclofen or botulinum toxin have positive effects on spasticity. Moreover, physiotherapy is reported to reduce spasticity (de Sa et al. 2011). Another complex of symptoms that is accompanied by strong

impairment are affective disorders like depression. Patients with depression, however, might benefit from cognitive behavioral therapy (Walker and Gonzalez 2007).

1.1.7.3 Disease modifying therapy

Interferon (IFN) β and glatiramer acetate (GA) were introduced as first disease modifying drugs for MS in the 1990s (Wingerchuk 2006; Ziemssen et al. 2001). To date, various disease modifying drugs with different modes of action are approved for the treatment of RMS. The goal of the treatment is the reach of a disease-free status, also known as no evidence of disease activity (NEDA). It is defined as the absence of relapses, absence of disability worsening and no detection of new or enlarging T2 lesions or T1 gadolinium-enhancing lesions in the MRI (Rotstein DL et al. 2015).

Disease modifying therapeutics can be divided into four main groups based on their mode of action: immune modulation, inhibition of cell replication, cell depletion, and inhibition of migration into the CNS (Brück et al. 2013). IFNβ preparations, GA, and dimethyl fumarate (DMF) act via diverse immunomodulatory effects (Brück et al. 2013; Wingerchuk 2006). Teriflunomide, cladribine, and mitoxantrone target the replication of lymphocytes (Brück et al. 2013; Faissner and Gold 2019). Although highly effective regarding the prevention of relapses, mitoxantrone is only recommended as second line therapeutic due to its high toxicity (Brück et al. 2013). The mode of action of different monoclonal antibodies (mabs) is the depletion of immune cells. While the anti-CD20 antibodies ocrelizumab and ofatumumab selectively deplete B cells and a small population of $CD20^+$ T cells, alemtuzumab binds to CD52 that is expressed on various leukocytes, including T cells, B cells, and monocytes (Coles et al. 2006; Frisch et al. 2021). Fingolimod, Siponimod, and Natalizumab (NTZ) are drugs that inhibit trafficking of immune cells. Fingolimod and Siponimod restrain lymphocytes from exiting lymph nodes, while NTZ prevents leukocytes from crossing the BBB (Brück et al. 2013).

Currently, the only drugs approved for the treatment of CIS are IFNβ and GA (Förster et al. 2019). Regarding PPMS, Ocrelizumab is the only disease modifying drug approved for this disease course to date (Frampton 2017). According to the DGN guideline, IFNβ preparations, GA, DMF, and teriflunomide are suitable drugs for the initial treatment of RRMS patients with low disease activity. If disease activity persists under this treatment, a therapy escalation is recommended. Regarding patients suffering from more severe course of disease with high disease activity, more efficient drugs should already be taken into consideration for initial treatment (DGN 2021). However, when making therapeutic decisions, patient-related factors, including medical history, course of disease, personal preferences, and pregnancy planning, always have to be taken into account (Rotstein D and Montalban 2019).

1.2 Natalizumab

NTZ is a highly effective medication regarding the reduction of relapses and was the first mab to be approved for MS therapy (Shirani and Stüve 2017). It was first introduced to the market in 2004, only 12 years after its target α4β1 integrin (also known as very late antigen-4 (VLA-4)) was discovered to be for the development of CNS inflammation in 1992 (Yednock et al. 1992). Despite promising data from pivotal studies, it was withdrawn from the market only a few months after its approval by the Food and Drug Administration (FDA) due to an unanticipated severe adverse event: progressive multifocal leukoencephalopathy (PML). However, after the development of a guideline for the surveillance of the PML risk, it was reintroduced and became an important drug in the management of RRMS (Rudick R et al. 2013).

1.2.1 Mode of action

Cell adhesion is crucial for leukocyte trafficking across tissue barriers such as the BBB. NTZ binds to α4β1 and α4β7 integrins. Therefore, it blocks the adhesion of α4β1 and α4β7 to the endothelial receptor vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1), respectively (Luster et al. 2005). The blockage of the interaction of α4β1 integrin with its endothelial receptor therefore leads to an inhibition of the migration of lymphocytes as well as monocytes across the BBB (Niino et al. 2006; Shirani and Stüve 2017). In 1990, α4β1 and VCAM-1 were first described to constitute a ligand-receptor-pair. This pair was suggested to be of importance for the recruitment of mononuclear leukocytes to sites of inflammation (Elices et al. 1990). Yednock and colleagues (1992) observed that lymphocytes and monocytes bind selectively to inflamed brain vessels using an in vitro adhesion assay on sections of EAE CNS tissue. Furthermore, they showed that this binding is hindered by anti- α4β1 antibodies. On the basis of these promising in vitro data, an in vivo experiment was conducted to determine the effect of this antibody on the clinical manifestation of EAE. Intriguingly, paralysis was prevented by anti- α4β1 antibodies in 75% of the animals. In addition to its important effect on leukocyte migration, NTZ is discussed to modulate ongoing inflammation inside the CNS since it inhibits the binding of α4β1 integrin to osteopontin and fibronectin (Bayless et al. 1998; Chabas et al. 2001; Davis et al. 1990).

1.2.2 Clinical trials

In 2006 the results of two important phase 3 trials, that were conducted simultaneously, were published: the AFFIRM trial and the SENTINEL trial (Polman et al. 2006; Rudick RA et al. 2006). The stunning results of these trials led to the first approval of NTZ by the FDA (Shirani and Stüve 2017). The placebo-controlled AFFIRM study assessed the i.v. administration of 300 mg NTZ every four weeks. The primary end points were the relapse rate after one year and the rate of sustained disability progression after two years. Sustained

disability progression was defined as an increase in the EDSS score of at least 1.0 from the baseline, or of at least 1.5 if the baseline score was 0, that remained for at least 12 weeks. After one year, the NTZ treatment led to a 68% decrease of the relapse rate. The analysis of the cumulative probability of progression at two years showed a relative risk reduction of 42% (Polman et al. 2006). In the SENTINEL trial, 1171 patients either received IFNβ1a in combination with 300 mg NTZ or IFNβ1a with placebo every four weeks for up to 116 weeks. The primary end points were defined similar to the AFFIRM study. The study met both its primary end points. After one year, in the IFNβ1a+NTZ group, the annual relapse rate was reduced by 54% compared to the IFNβ1a monotherapy. The sustained disability progression was reduced by 24% in the group receiving the combination therapy. Moreover, the combination therapy significantly reduced the number of new or enlarging lesions in the T_2-weighted MRI and the mean number of gadolinium-enhancing lesions after two years (Rudick RA et al. 2006). In 2021, the results of a dose- and frequency-blinded, prospective randomized study (REFINE trial) on patients diagnosed with RRMS were published. Interestingly, regarding efficacy and safety, s.c. application of 300 mg NTZ every four weeks was comparable to i.v. injection (Trojano et al. 2021).

1.2.3 PML and other adverse events

In a randomized, placebo-controlled phase 1 study designed to evaluate the drug's safety, NTZ was reported to be overall well tolerated and safe. Three of the 21 patients receiving NTZ reported at least one of the following mild adverse events: urticaria, nausea, headache, and shakiness (Sheremata et al. 1999). In the SENTINEL trial, the proportion of patients experiencing serious adverse events was comparable in both groups. Relapse of MS was the most frequently reported serious adverse event. Regarding infection rates, no difference was detected between the two groups (Rudick RA et al. 2006). The AFFIRM trial showed a significantly higher rate of fatigue and allergic reaction in the NTZ group compared to the placebo group (Polman et al. 2006).

PML is an opportunistic infection of the CNS caused by John Cunningham virus (JCV). It is a rare disease strongly associated with immunosuppression and mainly affects patients with AIDS (Berger and Khalili 2011). According to the seroprevalence, a high proportion of the world's population acquired a JCV infection. Presumably, PML occurs when the infection is reactivated (Stüve et al. 2007). In 2005, two case reports on patients developing PML under NTZ treatment were published. Both patients received NTZ and IFNβ1a as a part of the SENTINEL trial. One patient developed the first PML approximately one year after study entry. NTZ treatment was discontinued after the 28th dose (Langer-Gould et al. 2005). A second patient was diagnosed with PML after the end of the study. She was enrolled in an open-label extension study and therefore received additional seven infusions of NTZ, adding up to a total of 37 doses. The patient died from PML approximately one month after the last NTZ infusion (Rudick RA et al. 2006). Furthermore, one patient, who received NTZ due to Crohn's disease, developed PML (Van Assche et al. 2005). Based on these cases NTZ was

voluntarily withdrawn from the market three months after the first approval by the FDA. In 2006, NTZ was reapproved by the FDA for the treatment of highly active MS. However, it was only allowed to be administered as part of a restricted distribution program that was designed to diminish the risk of PML (Avasarala 2015). Three risk factors for PML under NTZ treatment were determined: positive status of anti-JCV antibodies, prior use of immunosuppressants and long duration of NTZ treatment (Bloomgren et al. 2012). According to the guideline of the DGN, the status of anti-JCV antibodies has to be determined before initiating the treatment and must be evaluated regularly during therapy. After seroconversion, the patient should be switched to another drug within a defined time frame, depending on the antibody-titer (DGN 2021).

1.2.4 Discontinuation of NTZ treatment

At some point, patients may need to discontinue NTZ therapy due to a high PML risk or for other reasons. Cases of a rebound of disease activity and clinical deterioration have been described after NTZ treatment cessation (Vidal-Jordana et al. 2015). The pathological analysis of CNS tissue from a patient who experienced severe deterioration after NTZ withdrawal revealed drastic inflammation and a high number of demyelinating and highly inflammatory lesions (Larochelle et al. 2017). Different therapy regimens are discussed as follow-up treatment, for instance starting fingolimod or GA after withdrawal. However, further large-scale long-term studies are needed to assess potential follow-up therapeutics (Clerico et al. 2017).

1.3 Aims of the study

NTZ is a well-established disease-modifying drug for the treatment of RRMS. The mab is characterized by high efficiency regarding the prevention of focal inflammation and relapses (Lanzillo et al. 2012; Polman et al. 2006). It is widely acknowledged that NTZ leads to alterations regarding immune cells in the peripheral blood. The rebound of disease activity after NTZ cessation (described in 1.2.4) renders a close analysis of the impact of NTZ on encephalitogenic and pathological cells critical. Therefore, this study aims to gain a deeper understanding of the effect of NTZ on peripheral immune cells.

Leukocytosis is a well-known effect of NTZ on the peripheral blood (Bridel et al. 2015). Moreover, various studies described a disproportionate increase in B cells after initiation of NTZ treatment (Krumbholz et al. 2008; Planas et al. 2012). However, even though monocytes play a crucial role in the pathogenesis of MS, the effect of NTZ on the population remains largely unknown. Thus, this study aims to provide a close analysis of the immune cell composition of NTZ-treated patients, focusing on monocytes and their subpopulations. The measurement was performed using flow cytometry.

Recently, a study was published revealing increased activation and pro-inflammatory differentiation of peripheral B cells upon NTZ treatment (Traub et al. 2019). Since findings suggest that myeloid cells do not only play an important role in the pathogenesis of MS in general but are also partially responsible for disease progression, the aim of this work is to investigate whether NTZ affects the functional properties of monocytes. Therefore, the expression of the activation markers CD69 and CD150 on the surface of monocytes was assessed. Moreover, to evaluate the monocytic capacity to present antigens, the expression MHCII and costimulatory molecules involved in antigen presentation and cell interaction (CD40, CD80, and CD86) on monocytes were measured. In order to monitor the effect of NTZ on the broad monocytic functions, in a last next step, the cytokine production of monocytes was assessed.

2 Materials and Methods

2.1 Patients

The analyzed blood was collected at the University Medical Center Göttingen between 2014 and 2019 (approval number #3/4/14). All patients were diagnosed with RRMS. The diagnosis was based on the McDonald criteria. 18 NTZ-treated MS patients (MS + NTZ 1) were compared to a group of 17 MS control patients (MS Ctrl). The NTZ-treated group received i.v. infusions of NTZ every four weeks for a mean duration of 3.4 years ranging from 0.3 to 9 years. The patients of the MS control group did not receive any immune modulatory treatment for at least six months and did not receive corticosteroids within the last eight weeks before blood withdrawal. Additionally, five MS patients were analyzed longitudinally (MS + NTZ 2) before and after the initiation of NTZ treatment. One healthy individual's blood was used as an internal control (IC) for the conduction of the experiment. Further demographic and disease related information is provided in **Table 2**.

Table 2: Demographic and disease characteristics of the patients

	MS + NTZ 1	**MS Ctrl**	**MS + NTZ 2**
Number of patients	18	17	5
Age [y] (median)	32.0 (23.4-64.0)	36.5 (22.3-54.6)	24.5 (18.8-40.8)
Female sex [%]	61.1	83.4	60
EDSS score (median)	3 (1-7)	2 (0-4)	2 (1.5-7.5)
Disease duration [y] (median)	9.3 (1-14)	3 (0-21)	0.3 (0-9)
NTZ since [y] (median)	3.4 (0.3-9)	-	1.9 (0.6-2.1)

EDSS Expanded Disability Status Scale, *y* years, *NTZ* Natalizumab, *Ctrl* control

2.2 Materials

Table 3: Human monoclonal antibodies for flow cytometry

Antigen	**Fluorochrome**	**Clone**	**Dilution**	**Manufacturing company**
CD4	APC	RPA-T4	1:100	BD Biosciences
CD8	PE	HIT-8a	1:100	BioLegend

Antigen	Fluorochrome	Clone	Dilution	Manufacturing company
CD14	BV421	M5E2	1:100	BD Biosciences
CD14	BV421	MφP9	1:100	BD Biosciences
CD14	FITC	M5E2	1:100	BD Biosciences
CD14	PECF594	MφP9	1:100	BD Biosciences
CD16	BV510	3G8	1:100	BD Biosciences
CD19	PerCp-Cy5.5	HIB19	1:100	BioLegend
CD40	PE	5C3	1:100	BioLegend
CD56	FITC	B159	1:100	BD Biosciences
CD69	FITC	FN50	1:100	BioLegend
CD80	PerCp-Cy5.5	2D10	1:100	BioLegend
CD86	BV421	2331 (FUN-1)	1:100	BD Biosciences
CD150	BV421	A12	1:100	BD Biosciences
MHCII	APC	Tü36	1:100	BioLegend
IL6	FITC	MQ2-13A5	1:100	BD Biosciences
IL10	PECF594	JES3-19F1	1:100	BD Biosciences
TNFα	AF700	Mab11	1:100	BD Biosciences
Dead/Live	ZombieAqua™		1:50	BioLegend
Dead/Live	ZombieNIR™		1:100	BioLegend

CD cluster of differentiation, *MHC* major histocompatibility complex, *IL* interleukin, *TNF* tumor necrosis factor

Table 4: ELISA Kits

Cytokine	Kit	Manufacturing company
IL6	ELISA MAX™ Standard Set Human IL-6	BioLegend
IL10	ELISA MAX™ Standard Set Human IL-10	BioLegend
TNFα	ELISA MAX™ Standard Set Human TNF-α	BioLegend

ELISA enzyme-linked immunosorbent assay

Table 5: Reagents, proteins, and inhibitors

Product	Manufacturing company
BD FACS Clean™	BD Biosciences
BD FACS Flow™	BD Biosciences
BD FACS Rinse™	BD Biosciences
Biocoll separation solution	Biochrom
BSA (bovine serum albumin)	SERVA Electrophoresis GmbH
Cytofix/Cytoperm™	BD Biosciences
DMEM (Dulbecco's Modified Eagle's medium)	Sigma Aldrich
DMSO (Dimethyl-sulfoxide)	Sigma Aldrich
Fc-block™	BioLegend
FCS (fetal calf serum)	Sigma Aldrich
GolgiPlug™	BD Bioscienes
H_2SO_4 (sulfuric acid) 97%	Merck Millipore
Recombinant Mouse IFNγ	BioLegend
L-glutamin	Life Technologies
LPS	Sigma Aldrich
$NaCO_3$ (sodium carbonate)	Merck Millipore
$NaHCO_3$ (sodium hydrogen carbonate)	Merck Millipore
PBS (phosphate buffered salt solution)	Sigma Aldrich
Pen/Strep	Life Technologies
Perm/Wash™ buffer, 10x	BD Biosciences
PFA (para-formaldehyde)	Merck Millipore
RPMI-1640 (Roswell park memorial institute-1640)	Sigma Aldrich
Sodium pyruvate 100mM	Sigma Aldrich
TMB (tetramethylbenzidine)	eBioscience
TrypanBlue	Sigma Aldrich
Tween	Merck Millipore
β-mercaptoethanol	Sigma Aldrich

Table 6: Solutions, buffers, and cell culture media

Solution	Composition
Complete medium	500 ml RPMI-1640, 50 ml FCS, 5 ml L-glutamine, 5 ml sodium pyruvate, 5 ml Pen/Strep, 0.5 ml β-mercaptoethanol
Cryo-medium	60% DMEM, 20% FCS, 20% DMSO
ELISA blocking buffer	1.8 l distilled water, 200 ml PBS 10x, 20 g BSA
ELISA coating buffer	1 l distilled water, 8.4 g $NaHCO_3$, 3.5 g $NaCO_3$
ELISA stop solution	1 M H_2SO_4 solution
ELISA wash buffer	1.8 l distilled water, 200 ml PBS 10x, 1 ml Tween
FACS buffer	2% FCS in PBS

BSA bovine serum albumin, *DMSO* dimethyl sulfoxide, *ELISA* enzyme-linked immunosorbent assay, *FCS* fetal calf serum, *PBS* phosphate-buffered salt solution, *RPMI-1640* Roswell Park Memorial Institute-1640

Table 7: Consumables

Product	Manufacturing company
96-well plates, flat bottom	Sarstedt
96-well plates, round bottom	Sarstedt
FACS tubes 5 ml	Sarstedt
Mirco tubes (0.1 ml, 0.5 ml, 1 ml, 1.5 ml)	Eppendorf
Multistep pipettes 1 ml	Eppendorf
Nunc™ 96-well ELISA plates	ThermoScientific
Pipettes (10 µl, 200 µl, 100 µl)	Sarstedt
Pipettes (5 ml, 10 ml, 25 ml)	Sarstedt

ELISA enzyme-linked immunosorbent assay, *FACS* fluorescence-activated cell sorting

Table 8: Technical devices

Device	Manufacturing company
BBD 6220 cell incubator	ThermoScientific
Centrifuge 5415R	Eppendorf
Centrifuge 5810R	Eppendorf
CKX41 light microscope	Olympus

Device	Manufacturing company
FACS LSRFortessa™	BD Biosciences
IMARK™ microplate reader	Bio-Rad
Neubauer chamber	Superior Marienfeld
SAFE 2020 clean bench	ThermoScientific

Table 9: Software

Software	Company
Microplate Manager 6™	Bio-Rad
FACSDiva™ 8.02	BD Biosciences
FlowJo™ 10.6	Tree Star Inc.
GraphPad Prism™ 6.01	GraphPad software Inc.
Excel 16.29.1	Microsoft Office 2016

2.3 Methods

2.3.1 Preparation of peripheral blood mononuclear cells (PBMCs)

The peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. To this end, the patients' blood was collected in Ethylenediaminetetraacetic acid (EDTA) tubes. EDTA blood was diluted 1:4 with PBS. 50 ml tubes were preloaded with 15 ml Biocoll™ separation solution. Afterwards, the blood PBS dilution was carefully applied on top of the Biocoll™ separation solution creating two layers. The tubes were then centrifuged for 35 minutes (min) at 1500 revolutions per minute (rpm) with very slow acceleration and without brake. Due to the density gradient, the PBMC layer appeared between the separation solution and the blood plasma and could easily been collected and suspended in a new tube. Next, the cells were washed twice using DMEM. For each washing cycle, a centrifugation for 10 min at 1250 rpm and 4 °C was used. After the second washing cycle, the sediment was resuspended in 5 ml of DMEM. The cells were counted in a Neubauer counting chamber using Trypan Blue (prediluted 1:10 in PBS). The following formula was used:

*Total cell count = counted cells / 4 * 10000 * dilution factor * volume of suspension [ml]*

After counting the cells, 30 ml DMEM was added and the PBMCs were washed again. Subsequently, the sediment was resuspended and diluted with DMEM to a concentration of

$4*10^6$ cells/ml. For the freezing, each cryotube was filled with 500 µl cell solution and 500 µl cryo-medium containing 60% DMEM, 20% FCS, and 20% DMSO. The cryotubes were stored at -80 °C, the first 48 h in propanol freezing boxes for a slow freezing process.

2.3.2 Fluorescence Activated Cell Sorting (FACS) panels

Four different FACS panels were designed to analyze the PBMCs: one to examine the immune cell composition, two for measuring the expression of activation markers and costimulatory molecules on monocytes, and one to assess the monocytic cytokine production. The panels were established and optimized on PBMCs from healthy donors before using the patients' PBMCs. The established staining panels are provided in **Table 10** and **Table 11**.

Table 10: FACS panels 1

Cell distribution	**Activation/Antigen presentation 1**
CD56 – FITC	CD69 – FITC
CD19 – PerCp-Cy5.5	CD86 – BV421
CD14 – BV421	CD16 – BV510
CD16 – BV510	CD14 – PECF594
CD8 – PE	MHCII – APC
CD4 – APC	ZombieNIR™
ZombieNIR™	

CD cluster of differentiation, *MHC* major histocompatibility complex

Table 11: FACS panels 2

Activation/Antigen presentation 2	**Cytokines**
CD14 – FITC	CD14 – BV421
CD80 – PerCp-Cy5.5	IL6 – FITC
CD150 – BV421	IL10 – PECF594
CD16 – BV510	TNFα – AF700
CD40 – PE	ZombieAqua™
ZombieNIR™	

CD cluster of differentiation, *IL* interleukin, *TNF* tumor necrosis factor

2.3.3 Unfreezing, seeding, and stimulation

For unfreezing of the cryopreserved PBMCs, tubes filled with 40 ml of complete medium were put into 37 °C water quench for 10 min. Next, 1 ml of the warm medium was added to the cryotubes containing frozen PBMCs to accelerate the unfreezing. Since the DMSO in the cryo-medium is toxic for unfrozen cells, they were immediately filled into 40 ml medium to dilute the cryo-medium. The maximum number of cryotubes per 40 ml complete medium was three. After thawing the cells, they were washed two times using a standard centrifugation (1250 rpm, 4 °C, 10 min). After the second washing procedure, the PBMCs were resuspended in 1 ml complete medium. The cells were counted as described above (2.3.1) and then diluted with complete medium to a concentration of $5*10^6$ cells/ml. For the panels 2-4, 500,000 cells (100µl of the solution) were seeded in each well of a flat bottom 96-well plate. For the cell distribution staining (Panel 1), the cell solution was diluted to a concentration of $2*10^6$ cells/ml. Each well of a U-bottom 96-well plate was filled with a total of 200,000 cells (100 µl of the diluted solution).

Panels 2-4 were incubated both with and without the stimulation agents at 37 °C and 5% CO_2 for 20 h. LPS was used to stimulate the expression of activation markers and costimulatory molecules on monocytes. For the stimulation of the monocytic cytokine production, a combination of LPS and IFNγ was used. Different concentrations of LPS and IFNγ were tested on PBMCs of healthy donors. These tests revealed an optimum stimulation of 400 pg/ml LPS for the extracellular staining (Panels 2 and 3) and an optimum stimulation of 100 pg/ml LPS and 10 ng/ml IFNγ for the intracellular staining. An overview of the stimulation regimens is presented in **Table 12**.

Table 12: Stimulation regimes

FACS Panel	Stimulation Regime
Cell distribution	No stimulation
Activation/Antigen presentation 1	400 pg/ml LPS
Activation/Antigen presentation 2	400 pg/ml LPS
Cytokine production	100 pg/ml LPS + 10 ng/ml IFNγ

The supernatant of cells stimulated with 400 pg/ml LPS was collected and frozen in a 96-well plate at -20 °C until ELISA was performed.

2.3.4 Dead/live staining

For excluding dead cells from the analysis, a dead/live staining was performed. The Zombie dye™ is an amine-reactive fluorescent dye that binds to proteins. It is non-permeant to living cells and therefore only binds membrane proteins. However, the dye can enter cells with

compromised membranes and can bind to all proteins inside these cells. Accordingly, dead cells appear brighter than living cells.

After centrifugation (1250 rpm, 4 °C, 7 min), the supernatant of the cells was removed. As described above, the supernatant of cells stimulated with 400 pg/ml LPS was stored at -20 °C. Afterwards the cells were washed (1250 rpm, 4 °C, 7 min) with 200 µl PBS. Next, 30 µl of the diluted Zombie dye™ were added in each well except for the non-Zombie single stains and the unstained well. The cells were resuspended with the dye. The dye was then incubated for 10 min at room temperature in the dark. Subsequently, the cells were washed using 200 µl FACS buffer.

2.3.5 Surface staining

To inhibit unspecific binding of the staining antibodies to F_c-receptors on the cell surface, a F_c-block™ was used. After incubating the Zombie dye for 10 min, 30 µl of the diluted (1:50) Fc-receptor-block were added to all wells. After a 10-minute incubation on ice in the dark, the cells were washed using FACS-buffer (1250 rpm for 7 min 4 °C). Subsequently, the staining antibodies were added in a 1:100 dilution and the cells were resuspended. The antibodies were incubated for 15 min on ice in the dark and then were washed off twice using FACS-buffer (1250 rpm, 4 °C, 7 min). The cells were then resuspended in 100 µl FACS-buffer and fixated using 100 µl PFA resulting in a final concentration of 2%.

2.3.6 Intracellular staining

To accumulate the synthesized cytokines inside the Golgi apparatus, 20 µl GolgiPlug™ (1:20 prediluted) was added to the cells used for the cytokine-staining. The GolgiPlug™ was applied after 20 h of stimulation with 100 pg/ml LPS and 10 ng/ml IFNγ. Following a 3 h incubation at 37 °C and 5% CO_2, the GolgiPlug™ and supernatant were removed, and the extracellular staining was performed as described in above (2.3.4 and 2.3.5). Following the incubation of the extracellular staining antibodies, the cells were stained intracellularly. To this end, the PBMCs were washed once with FACS-buffer (1250 rpm, 4 °C, 7 min) and were subsequently fixated and permeabilized using Cytofix/Cytoperm™. The permeabilization solution was incubated for 30 min at room temperature in the dark. Afterwards, the cells were washed using the washing solution (Perm/Wash™). Next, 30 µl of the intracellular staining antibodies (prediluted 1:100 in Perm/Wash™) were added. The staining antibodies were incubated for 2 h in the dark on ice. Following the incubation, the cells were washed twice using Perm/Wash™ and once using FACS-buffer (1250 rpm, 4 °C, 7 min). The PBMCs were then resuspended and fixated as described above.

2.3.7 Flow cytometry

Flow cytometry was used to analyze the fluorescence labeled cells. The measurement was performed using a LSR II by BD Bioscience flow cytometer and the BD FACSDiva v 8.02

software. The compensation was manually optimized for every individual experiment. The analysis of the obtained data was carried out using the FlowJo 10.6 software.

2.3.8 Gating strategy

For the identification of living cells among all recorded signals, the pre-gating was performed as depicted in **Figure 1A**. Monocytes were identified as CD14$^+$ cells. The expression of surface markers on monocytes as well as the cytokines stained in the intracellular staining were gated according to a fluorescence minus one (FMO) control (**Figure 1B**).

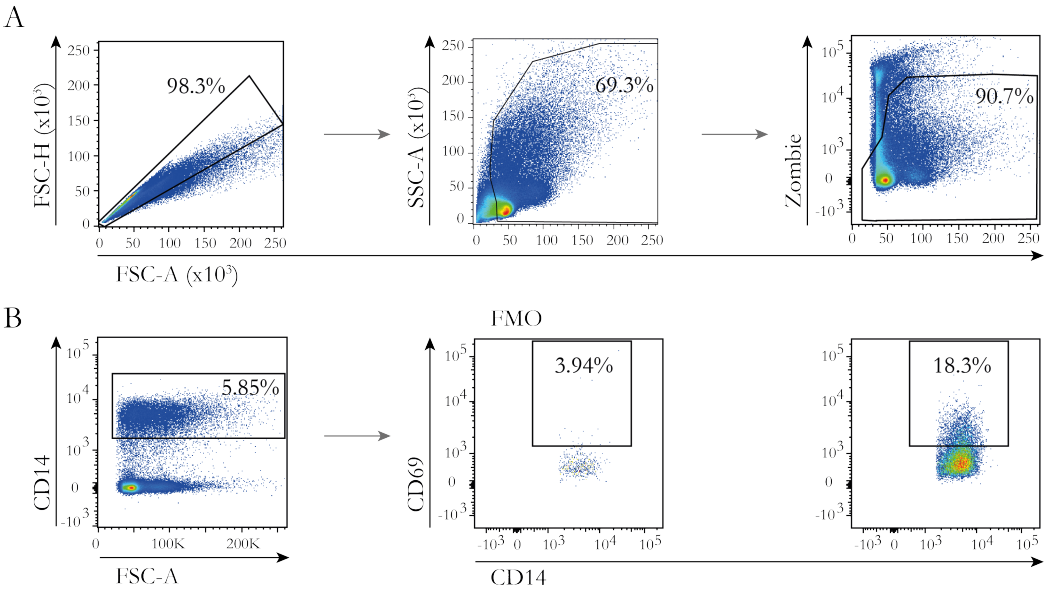

Figure 1: Exemplary pre-gating and gating strategy.

(**A**) Within all recorded events, singlets were determined. Subsequently apoptotic cells were eliminated by size exclusion. Living cells were determined using dead/live staining. (**B**) Monocytes were determined as CD14$^+$ cells. Expression of surface markers on CD14$^+$ cells was gated according to Fluorescence minus one (FMO) controls.

2.3.9 Detection of cytokines using ELISA

In order to assess the effect of the NTZ treatment on the cytokine production, the cytokine concentration in the supernatants was additionally measured by ELISA. The measurements were performed using ELISA MAX™ Standard Set Human IL-6, IL-10, and TNFα. All standards were run as duplicates. The samples were run either as duplicates, or, if enough donor cells were available, as triplicates. The capture antibody was diluted 1:200 with coating-buffer. After 100 µl of the dilution was added to each well, the 96-well plates were sealed and incubated overnight at 4 °C. The day after, the plates were washed thrice with wash buffer before 200 µl blocking buffer was added to each well. The sealed plates were subsequently incubated for 1 h at room temperature while shaking on a plate shaker. During the incubation, the samples were diluted, and standard dilutions were prepared as described in the manufacturer's instruction (see biolegend.com for instructions). The samples for the IL6 ELISA

were diluted 1:200, whereas the samples for the IL10 ELISA were diluted 1:20 and the samples for the TNFα ELISA were diluted 1:5. For all dilutions, blocking buffer was used. After the plates were blocked, it was washed three times with wash buffer. Then, 30 µl of the standard solutions and the samples were added to the wells. For the measurement of IL6 and TNFα, the samples and standard solutions were incubated for 2 h at room temperature on a plate shaker. For the assessment of the IL10 production, the standard solutions and the samples were incubated overnight at 4 °C. After the incubation, the plates were washed three times with wash buffer and 100 µl of the detection antibody solution (prediluted 1:200 with blocking buffer) was added to each well. The plates were sealed and incubated for 2 h at room temperature while shaking. To remove the excess detection antibody, the plates were washed three times with wash buffer. Afterwards, 100 µl 1:100 diluted Avidin.HRP solution was added to each well and the sealed plate was incubated at room temperature on the plate shaker for 30 min in the dark. After the plates were washed four times with wash buffer, 100µl TMB substrate solution were added to each well. The TMB was incubated at room temperature while shaking in the dark for 10-20 min until a color change could be seen. Finally, the reaction was stopped by adding 100 µl stop solution to each well. The absorption was measured at 450 nm with 540 nm wavelength correction using an iMark™ microplate reader.

2.3.10 Statistical analysis

The statistical analysis was conducted using the GraphPad Prism 6.01 software. The data was tested for Gauss distribution using the D'Agostino-Pearson normality test. Regarding the horizontal study, nonparametric data were analyzed using the Mann-Whitney U test, while for nonparametric data the unpaired t-test was applied. Longitudinal data was analyzed using the Wilcoxon signed-rank test. Values are indicated as median. To rule out co-founders, linear regression was applied. The significance level was set at 0.05. A value of $p < 0.05$ is indicated by one asterisk. Two, three, and four asterisks express significances of $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

3 Results

3.1 Cell distribution

NTZ was recently reported to have an impact on frequency and activation status of peripheral B cells. Therefore, it is of further interest to examine effect on the composition peripheral leukocytes. To examine the distribution of immune cell types, their frequency within the PBMC pool was measured directly after unfreezing of PBMCs.

3.1.1 NTZ treatment increases frequency of CD19$^+$ B cells

To determine the impact of NTZ treatment on the frequency of peripheral blood B cells, the proportion of CD19$^+$ cells within all living PBMCs was measured. The NTZ-treated group showed significantly increased B cell frequencies compared to the MS control group (**Figure 2A**). Regarding the longitudinal examination, a trend towards increased B cell frequencies upon NTZ treatment was observed (**Figure 2B**).

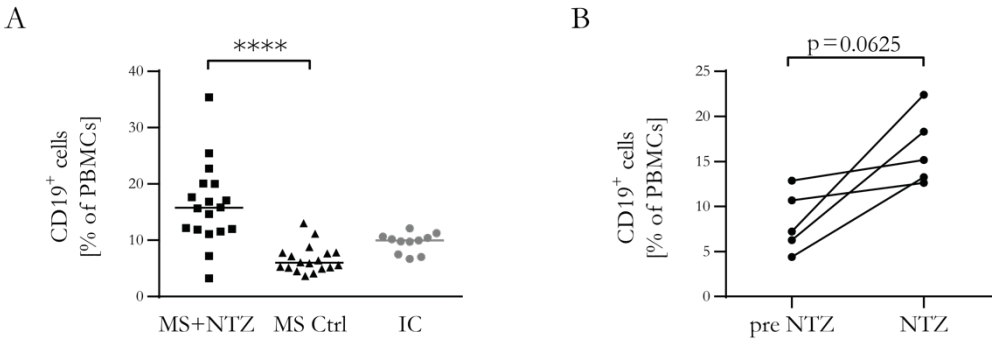

Figure 2: B cell frequency in human blood is increased upon NTZ treatment.

PBMCs were isolated and CD19 expression was measured using flow cytometry. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (**A**) Frequency of CD19$^+$ cells of all living PBMCs is shown for every patient, either receiving NTZ (MS+NTZ; $n = 18$) or untreated (MS Ctrl; $n = 17$). Mann-Whitney U test; data given as median. (**B**) Frequency of CD19$^+$ cells of all living PBMCs is shown for 5 patients before (pre NTZ) and after (NTZ) initiation of NTZ treatment (median treatment duration 1.9 y (0.6-2.1 y)). Wilcoxon matched-pairs single rank test.

3.1.2 NTZ leads to a decreased frequency of CD14$^+$ monocytes, while the absolute number of monocytes remains unchanged

To detect a possible impact of NTZ on peripheral blood monocytes, their frequency within the PBMC pool was measured. The cross-sectional analysis revealed diminished CD14$^+$ monocyte frequencies upon NTZ treatment (**Figure 3A**). In order to determine whether the frequency change of monocytes results from the relative increase of B cells, absolute monocyte numbers as cells per µl blood were determined. For this analysis data from the central lab of the University Medical Center Göttingen was used. Directly after blood withdrawal, the percentage of monocytes within leukocytes in EDTA blood was determined by the

central lab for each patient. These percentages were multiplied with the count of leucocytes that also were determined by the central lab. No decreased monocyte count in the NTZ-treated group compared to untreated MS controls was observed. In contrast, this evaluation even revealed a trend towards an increased number of monocytes ($p = 0.0841$) (**Figure 3B**). The longitudinal analysis showed no significant difference of monocyte frequencies before and after initiation of NTZ treatment (**Figure 3C**).

Since the focus of this study is on monocytes, the $CD14^+CD16^+$ subgroup and the $CD14^+CD16^-$ monocyte subgroup were analyzed separately (exemplary gating shown in **Figure 3D**). This assessment showed a trend towards a reduction of the relative proportion of $CD14^+CD16^+$ cells within the PBMCs upon NTZ treatment ($p = 0.0776$) (**Figure 3E**), while the frequency of $CD14^+CD16^-$ monocytes was significantly reduced in the NTZ-treated group compared to untreated MS controls (**Figure 3F**). When analyzed longitudinally, no significant difference was seen between the samples taken before and after treatment initiation regarding both subgroups (**Figure 3G, H**).

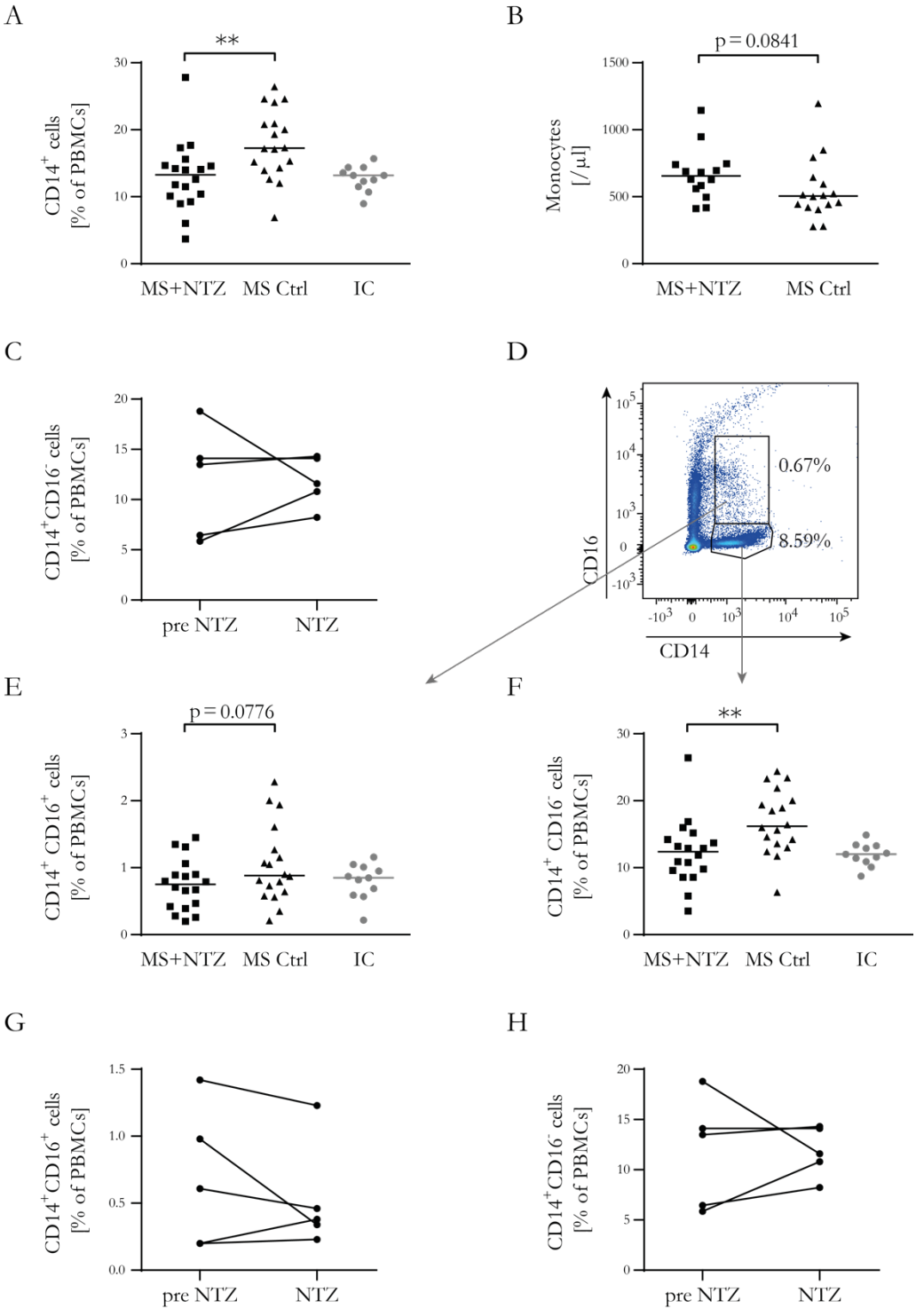

Figure 3: Frequency of monocytes is reduced upon NTZ treatment, while the absolute number of monocytes remains unchanged.

The CD14 and CD16 expression on isolated PBMCs was measured using flow cytometry. (**A**) Median frequency of $CD14^+$ within PBMCs is shown for every patient, either receiving NTZ (MS+NTZ; n = 18) or being untreated (MS Ctrl; n = 17). (**B**) Absolute monocyte counts per µl blood were measured in routine clinical laboratory; data given as median. (**C**) Frequency of $CD14^+$ cells of all living PBMCs is shown for 5 patients before (pre NTZ) and after (NTZ) initiation of NTZ treatment (median treatment duration 1.9 y (0.6-2.1 y)). (**D**) Exemplary subdivision of the monocyte subgroups. (**E, F**) Median frequency of $CD14^+CD16^+$ (**E**) and $CD14^+CD16^-$ (**F**) cells within PBMCs. (**G, H**) Frequency of $CD14^+CD16^+$ (**G**) and $CD14^+CD16^-$ (**H**) cells

within PBMCs before and after initiation of NTZ-treatment. * $p < 0.05$; ** $p < 0.01$; (**A**, **B**, **F**) Mann-Whitney U test; (**E**) unpaired t-test; (**C**, **G**, **H**) Wilcoxon matched-pairs single rank test.

3.1.3 NTZ treatment decreases CD4$^+$ T cell frequency, while it does not change frequency of CD8$^+$ T cells

To determine a potential effect of NTZ on T cells, CD4$^+$ T helper cells and CD8$^+$ cytotoxic T cells were analyzed separately. The cross-sectional evaluation revealed a significantly decreased frequency of CD4$^+$ T cells in NTZ-treated patients compared to MS control patients (**Figure 4A**). In contrast, the relative proportion of CD8$^+$ T cells within the PBMC pool did not change (**Figure 4C**). Regarding the longitudinal analysis, no significant difference between the NTZ-treated and the control group regarding both T helper cells and cytotoxic T cells could be detected (**Figure 4B, D**).

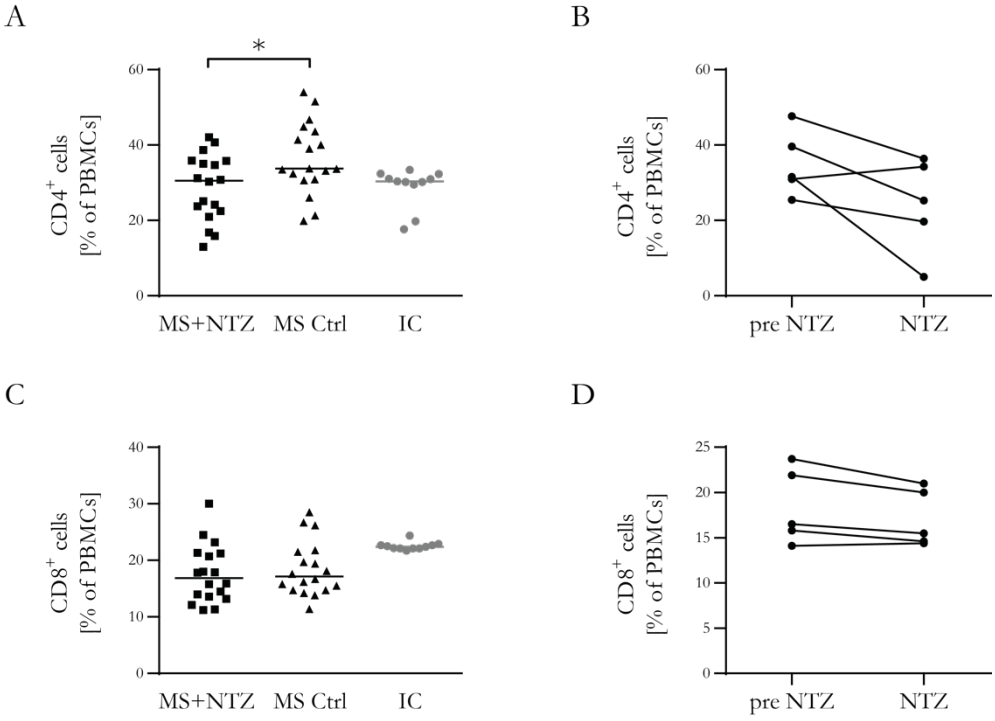

Figure 4: Frequency of CD4$^+$ T cells decreases under NTZ treatment, while CD8$^+$ T cell frequency does not change.

PBMCs were isolated and CD4 and CD8 expression was analyzed using flow cytometry. * $p < 0.05$. (**A**, **C**) Median frequency of CD4$^+$ (**A**) and CD8$^+$ (**C**) cells within living PBMCs is shown for every patient, either receiving NTZ (MS+NTZ; $n = 18$) or being untreated (MS Ctrl; $n = 17$). Unpaired t-test; data given as median. (**B**, **D**) Longitudinal analysis of the frequency of CD4$^+$ (**B**) and CD8$^+$ (**D**) cells within PBMCs is depicted for 5 patients before (pre NTZ) and after (NTZ) initiation of NTZ treatment (median treatment duration 1.9 y (0.6-2.1 y)). Wilcoxon matched-pairs single rank test.

3.1.4 NTZ has no impact on frequency of natural killer cells

Next, the impact of NTZ treatment on the frequency of natural killer cells was examined. To address this, the relative proportion of $CD56^+$ cells within the pool of living PBMCs was measured. In the cross-sectional study, no difference between the NTZ-treated and untreated blood samples could be detected (**Figure 5A**). The longitudinal analysis also revealed no change regarding $CD56^+$ cell frequency upon NTZ treatment (**Figure 5B**).

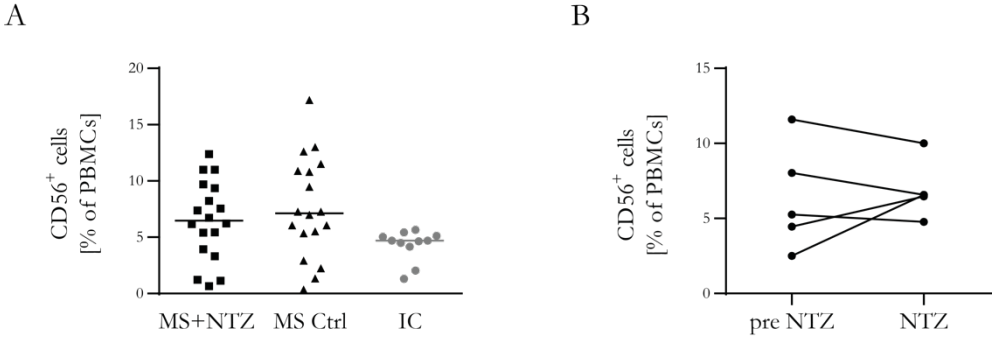

Figure 5: NK cell frequency does not alter upon NTZ treatment.

Frequency of $CD56^+$ PBMCs was analyzed using flow cytometry. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (**A**) Median frequency of $CD56^+$ cells of all living PBMCs is depicted for every patient, either receiving NTZ (MS+NTZ; n = 18) or being untreated (MS Ctrl; n = 17). Unpaired t-test. (**B**) Frequency of $CD56^+$ cells of all living PBMCs is shown for 5 patients before (pre NTZ) and after (NTZ) initiation of NTZ treatment (median treatment duration 1.9 y (0.6-2.1 y)). Wilcoxon matched-pairs single rank test.

3.2 NTZ increases the activation of peripheral blood monocytes

The major purpose of this study was to investigate, whether NTZ treatment is associated with an alteration of the activation status of peripheral blood monocytes. Therefore, the expression of activation markers on $CD14^+$ monocytes was analyzed using flow cytometry. As depicted in **Figure 6A**, when stimulated via TLR stimulation with LPS, the expression of the activation marker CD69 on peripheral blood monocytes increased significantly upon NTZ treatment. This difference could not be observed in the absence of LPS. Furthermore, NTZ treatment significantly upregulated the expression of the activation marker CD150 upon stimulation with LPS. In the absence, of LPS only a small percentage of monocytes expressed CD150 and no alteration upon NTZ treatment could be detected (**Figure 6B**).

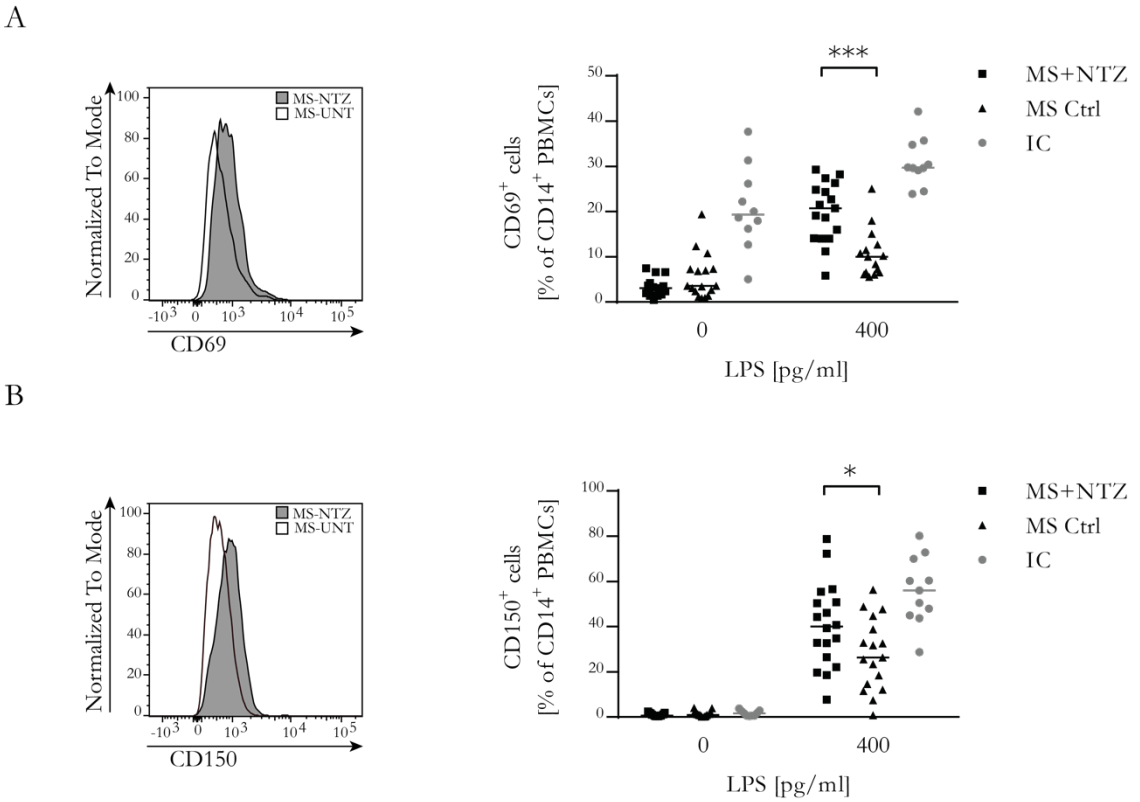

Figure 6: NTZ treatment increases the frequency of CD69$^+$ and CD150$^+$ monocytes in the blood of MS patients.

PBMCs were isolated from NTZ-treated MS patients (MS + NTZ; n = 18; squares) or untreated MS controls (MS Ctrl; n = 17) and subsequently incubated unstimulated or stimulated using 400 pg/ml. Surface markers were stained with respective antibodies and cells were analyzed using flow cytometry. Representative histograms are shown. Median frequency of CD69$^+$ (**A**) and CD150$^+$ (**B**) monocytes. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; (**A**) Mann-Whitney U test (**B**) unpaired t-test.

These robust changes in the cross-sectional analysis failed to be reflected in the few longitudinal samples available. Flow cytometry analysis did not indicate a significant alteration of expression of both CD69 (**Figure 7A**) and CD150 (**Figure 7B**) between NTZ-treated and untreated blood samples.

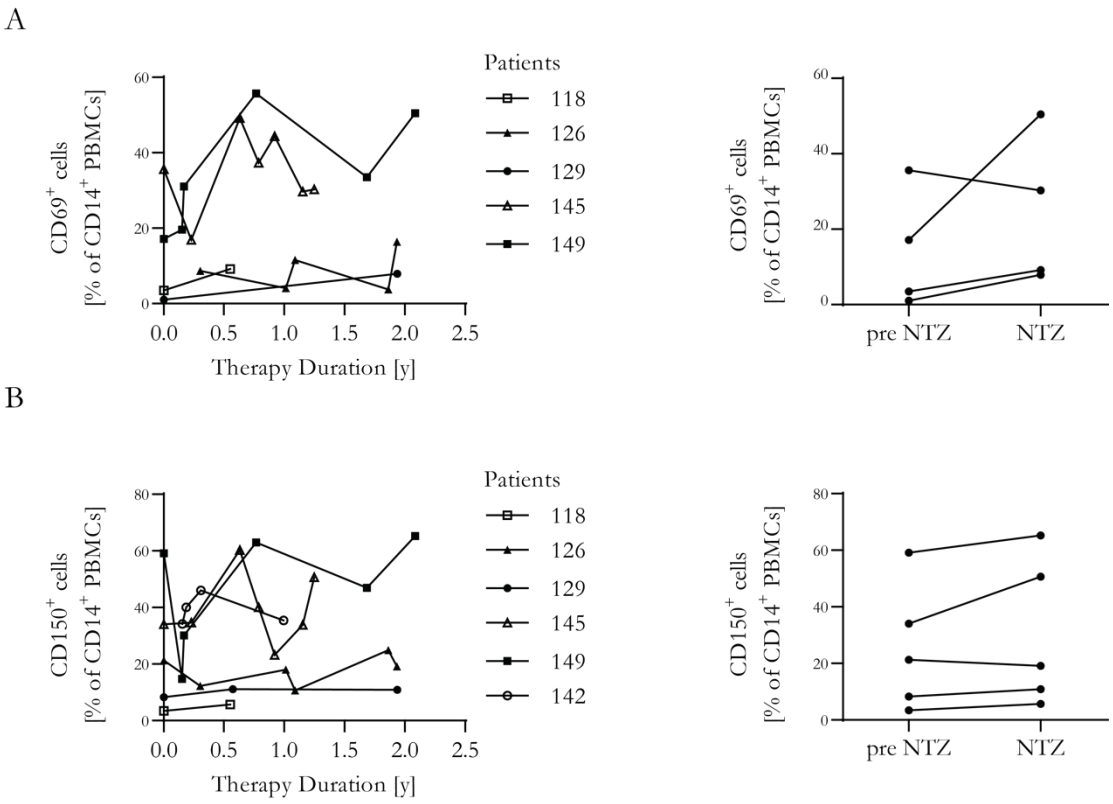

Figure 7: Expression of CD69 and CD150 on peripheral blood monocytes prior and after initiation of continuous treatment with NTZ.

PBMCs were isolated from MS patients before (pre NTZ) and after (NTZ) initiation of treatment (**A**: median treatment duration 1.6 years (0.6-2.1 y); **B**: median treatment duration 1.9 y (0.6-2.1 y)) with NTZ (n = 4-5). PBMCs were stimulated with lipopolysaccharide (LPS) and subsequently analyzed by flow cytometry using cell surface staining. The expression of the activation markers CD69 (**A**) and CD150 (**B**) on monocytes ($CD14^+$) was determined longitudinally. Data is presented as frequency; Wilcoxon matched-pairs single rank test.

3.3 NTZ does not alter the expression of molecules involved in antigen presentation on peripheral blood monocytes

Next, it was aimed to assess, whether the enhanced activation of peripheral blood monocytes was associated with a change in the functional status of these APCs. Many different surface markers are involved in antigen presentation. The level of MHCII expression was evaluated, since it plays a key role in antigen presentation. For the analysis of costimulatory molecules expressed on monocytes, CD40, CD80, and CD86 were selected. Regarding the cross-sectional examination, flow cytometry revealed a trend towards a higher expression of MHCII in the NTZ-treated group compared to the MS control group in the LPS stimulated samples ($p = 0.0793$) (**Figure 8A**). As depicted in **Figure 8B,** the expression of CD40 did not alter upon NTZ treatment. Regarding the expression of both CD80 (**Figure 8C**) and CD86 (**Figure 8D**), the assessment also revealed no significant difference between NTZ-treated and untreated blood samples.

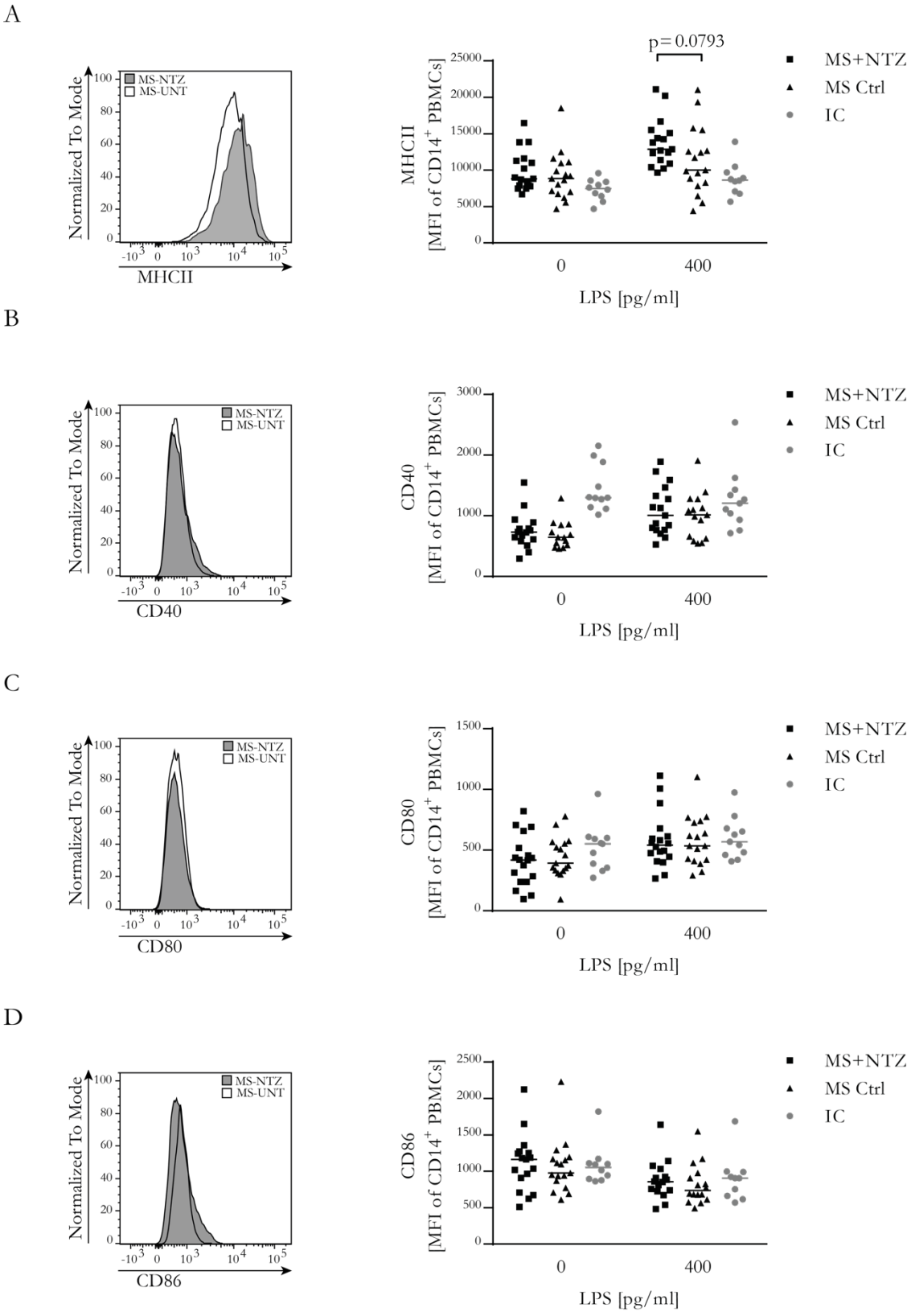

Figure 8: Expression of MHCII, CD40, CD80, and CD86 on peripheral blood monocytes is not altered due to NTZ treatment.

PBMCs were isolated from NTZ-treated MS patients (MS + NTZ; *n* = 18) or untreated MS controls (MS Ctrl; *n* = 17). After incubation with and without 400 pg/ml LPS, the expression of the respective surface markers on CD14+ cells was analyzed using flow cytometry. Representative histograms are shown. MHCII (**A**), CD40

(**B**), CD80 (**C**), and CD86 (**D**) on monocytes is shown as median of mean fluorescent intensity (MFI). (**A**, **B**, **C**) Unpaired t-test; (**D**) Mann-Whitney U test.

Next, the expression of the above-described molecules involved in antigen presentation were measured longitudinally over a period of 0.6-2.1 years. The trend towards a higher expression of MHCII in the NTZ-treated samples detected in the cross-sectional analysis was not reflected in the longitudinal examination (**Figure 9A**). However, the measurement revealed a trend towards an increased expression of CD40 on peripheral blood monocytes upon NTZ treatment ($p = 0.0625$) (**Figure 9B**). The expression of CD80 and CD86 did not alter significantly after the initiation of NTZ treatment, as shown in **Figure 9C** and **D**.

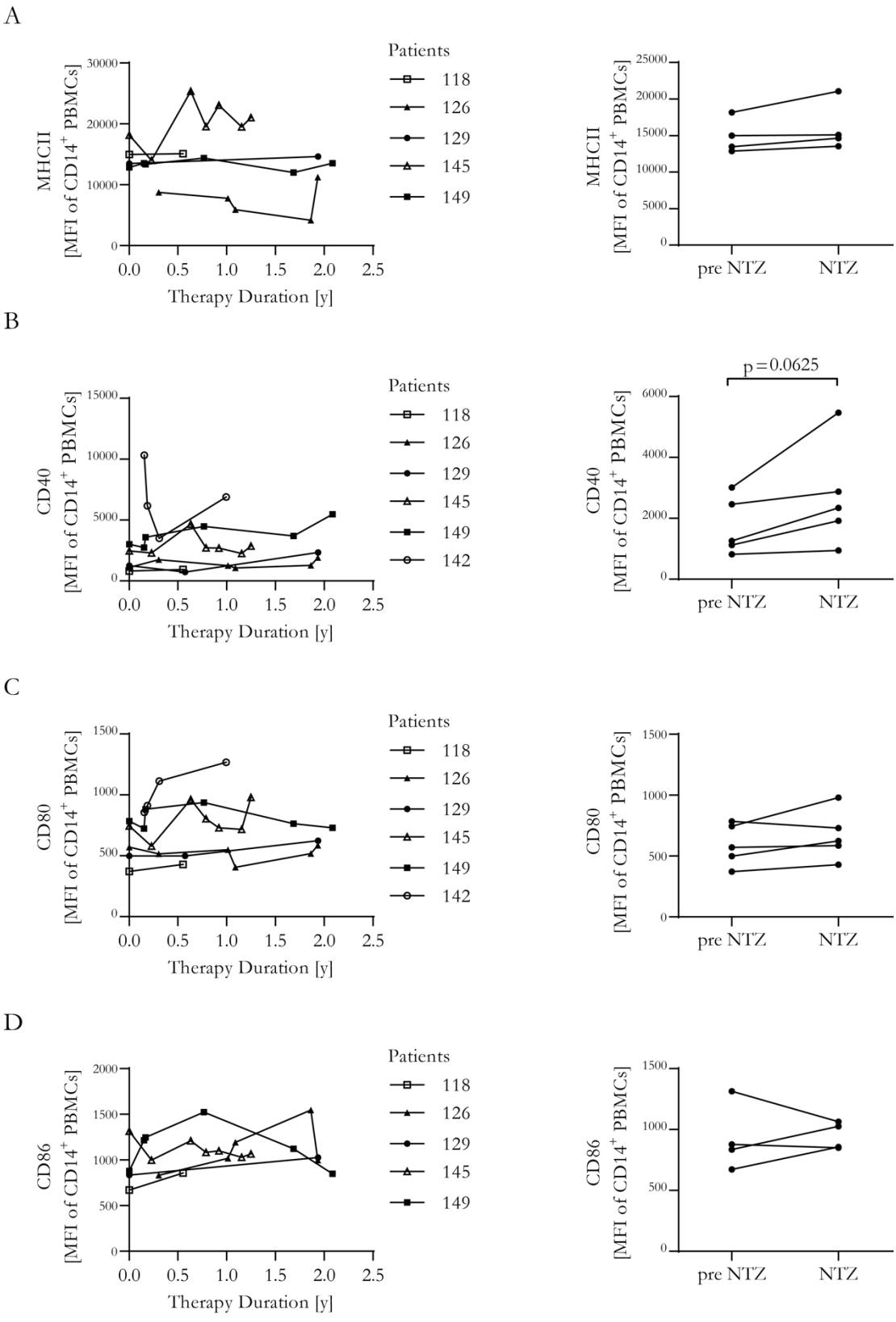

Figure 9: Expression of MHCII, CD40, CD80, and CD86 on peripheral blood monocytes prior and after initiation of continuous treatment with NTZ.

Isolated PBMCs from MS patients before and after initiation of treatment (**A**, **D**: median treatment duration 1.6 years (0.6-2.1 y); **B**, **C**: median treatment duration 1.9 y (0.6-2.1 y)) with natalizumab (NTZ; n = 4-5) were stimulated with 400 pg/ml LPS. Thereafter, the cell surface was stained with the respective antibodies and an

analysis using flow cytometry was conducted. The expression of the following costimulatory molecules on ($CD14^+$ monocytes was measured: MHCII (**A**), CD40 (**D**), CD80 (**E**), and CD86 (**F**). Data is presented as MFI; Wilcoxon matched-pairs single rank test.

3.4 Cytokine Production

In order to characterize the effect of NTZ on the functional properties of peripheral monocytes more precisely, the levels of cytokine production by monocytes were analyzed. To stimulate the cytokine production of monocytes, the PBMCs were incubated with a combination of 100 pg/ml LPS and 10 ng/ml IFNγ. First, the cytokine production was measured by staining the cells intracellularly and using flow cytometry.

As depicted in (**Figure 10A**) no significant difference in production of pro-inflammatory TNFα by peripheral blood monocytes between the NTZ-treated and the untreated group could be detected neither with nor without simulation. The longitudinal examination of stimulated PBMCs as well revealed no change after treatment initiation regarding the production of TNFα when compared to the sample collected before treatment was initiated (**Figure 10B**).

Regarding the cross-sectional analysis, flow cytometry showed no difference of IL6 production between the NTZ-treated and untreated samples both when stimulated and not (**Figure 10C**). When analyzing this parameter longitudinally, no alteration was detected upon NTZ treatment (**Figure 10D**).

Moreover, the production of the anti-inflammatory cytokine IL10 by peripheral blood monocytes was assessed. The cross-sectional and the longitudinal examination both revealed no statistically significant difference regarding the production of IL10, as depicted in **Figure 10E** and **F**.

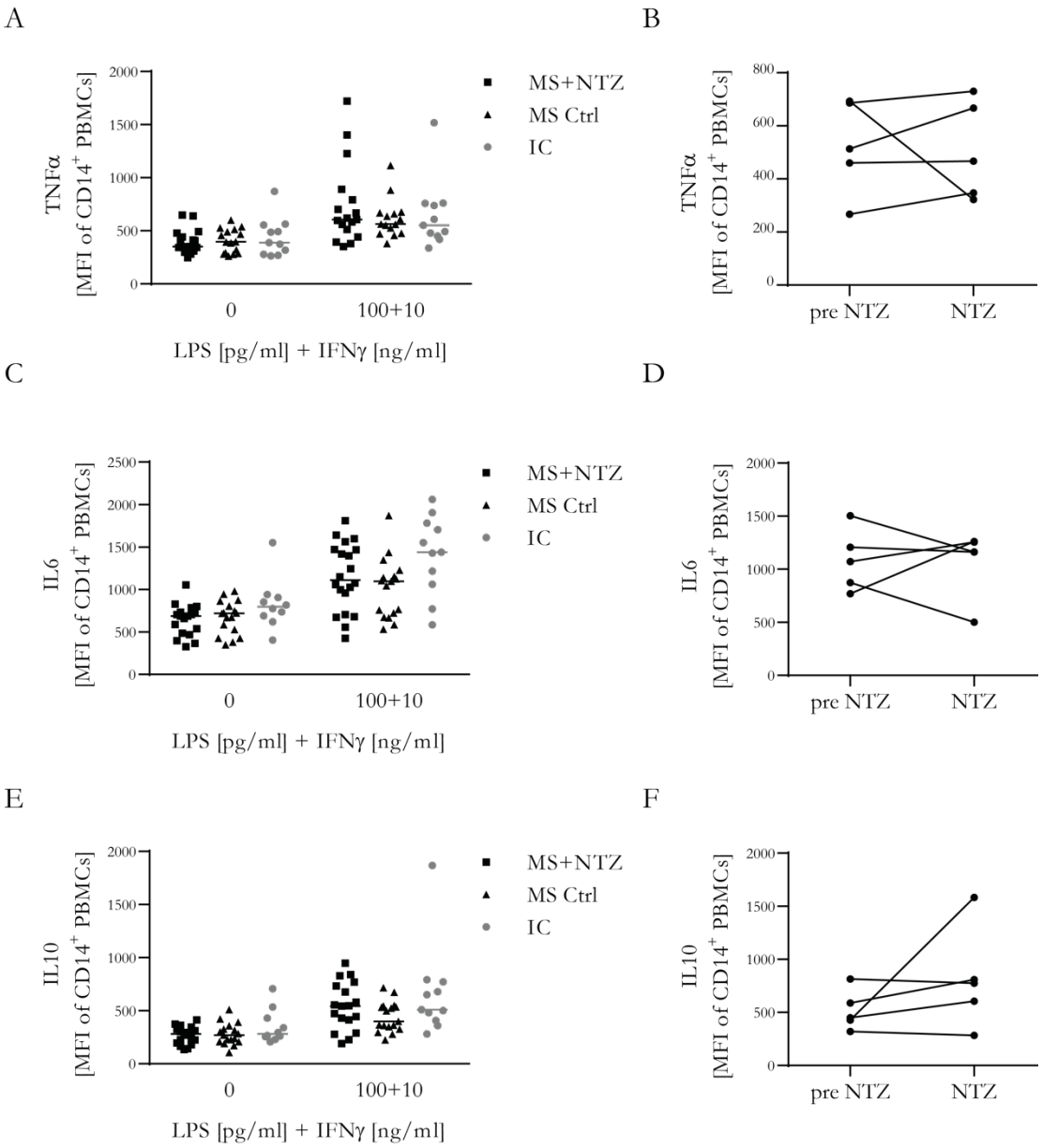

Figure 10: Cross sectional and longitudinal examination of TNFα, IL6, and IL10 production of peripheral blood monocytes.

(**A**, **C**, **E**) PBMCs were isolated from NTZ-treated MS patients (MS + NTZ; *n* = 18) or untreated MS controls (MS Ctrl; *n* = 17) and incubated without stimulation or with a combination of 100 pg/ml LPS and 10 ng/ml IFNγ. After an extracellular staining for CD14, the PBMCs were stained intracellularly for TNFα (**A**), IL6 (**C**), and IL10 (**E**); data are shown as the median of MFI; (**A**, **C**) Mann-Whitney U test; (**E**) unpaired t-test. (**B**, **D**, **F**) Blood samples of 5 MS patients were drawn before (pre NTZ) and after (NTZ) initiation of NTZ treatment (median treatment duration 1.9 y (0.6-2.1 y)). Isolated PBMCs were stimulated and stained as described above. The production of TNFα (**B**), IL6 (**D**), and IL10 (**F**) was measured using flow cytometry. Data is presented as MFI; Wilcoxon matched-pairs single rank test.

In a second step, the cytokine concentration in the supernatant of LPS stimulated PBMCs was analyzed using ELISA. As illustrated in **Figure 11A** the TNFα concentration in the supernatant did not alter significantly upon NTZ treatment. Furthermore, the concentration of IL6 in the supernatant of the NTZ-treated samples did not differ from the concentration

of the untreated samples (**Figure 11B**). Regarding the concentration of anti-inflammatory IL10 in the supernatant, ELISA revealed no difference between the NTZ-treated group and the MS control group (**Figure 11C**).

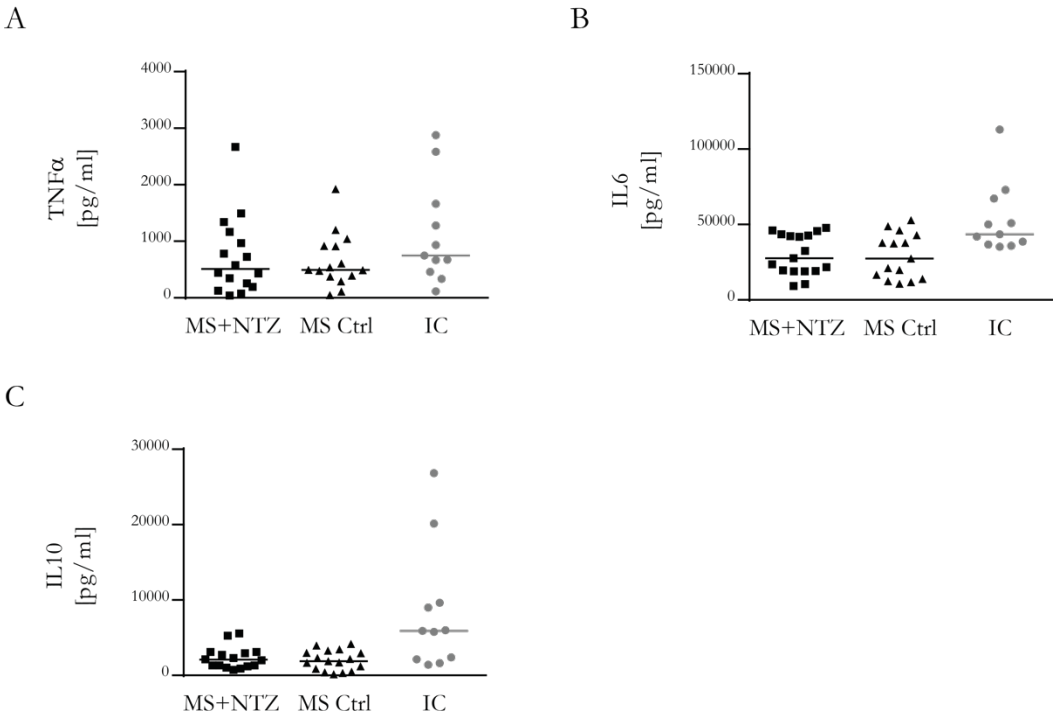

Figure 11: NTZ treatment does not change the concentration of TNFα, IL6, and IL10 concentration in the supernatant.

PBMCs were isolated from NTZ-treated MS patients (MS + NTZ; *n* = 18) or untreated MS controls (MS Ctrl; *n* = 17) stimulated using 100 pg/ml LPS and 10 ng/ml IFNγ. The concentration of TNFα (**A**), IL6 (**B**), and IL10 (**C**) in the supernatant was measured using ELISA. Mann-Whitney U test.

3.5 Epidemiological analysis

Ultimately, it was aimed to exclude an influence of patient- and disease-related characteristics on the NTZ-attributed changes of the expression of activation marker expression on peripheral blood monocytes described in **3.2**. Therefore, age of the patients, duration since the diagnosis of MS was made, and the EDSS score were assessed.

As depicted in **Figure 12A** and **B**, patients' age had no effect on the expression of either CD69 or CD150. Disease duration could be ruled out as a factor influencing the expression of CD69 and CD150 on monocytes likewise (**Figure 12C** and **D**). **Figure 12E** and **F** show, that the severity of the disease assessed as EDSS score did not influence the NTZ-attributed alterations of the expression of CD69 and CD150. To conclude, these findings rule out an age- or disease-related effect on the robust changes of the CD69 and CD150 expression on peripheral blood monocytes detected upon NTZ treatment.

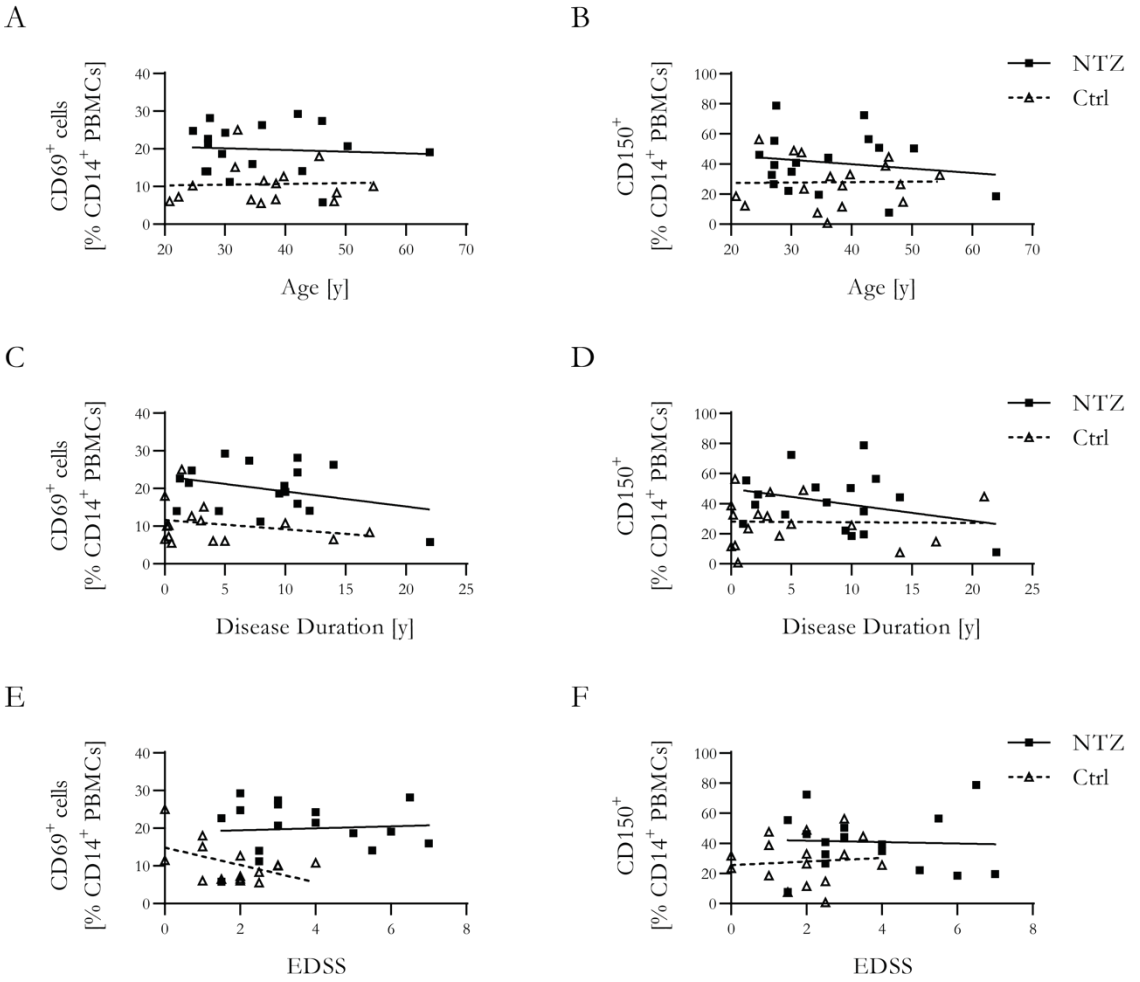

Figure 12: Patients' age, disease duration, and EDSS score have no impact on the frequency of CD69$^+$ and CD150$^+$ monocytes in human blood.

PBMCs were isolated from NTZ-treated MS patients (MS + NTZ; n = 18) or untreated MS controls (MS Ctrl; n = 17) and were stimulated with 400 pg/ml LPS. The frequencies of CD69$^+$ (**A**, **C**, **E**) and CD150$^+$ (**B**, **D**, **F**) cells within the monocytes (CD14$^+$) were correlated to patients' age (**A**-**B**), duration of the disease (**C**-**D**), and EDSS score (**E**-**F**) using linear regression (* $p < 0.05$).

4 Discussion

The target molecule of NTZ, α4 integrin, is not only crucial for the migration of immune cells across the BBB, but also involved in hematopoiesis and immune cell trafficking in general. Hence, NTZ is also suggested to influence these processes. Considering that NTZ was reported to promote activation and pro-inflammatory differentiation of peripheral B cells, it is of interest to closely investigate the effect of NTZ on the amount and functional properties of leukocytes. This study concentrates on monocytes, as they play an important role in the pathogenesis of MS and, just like B cells, function as APCs. Therefore, the composition of immune cells within PBMCs as well as the functional properties of monocytes of MS patients under NTZ treatment was analyzed and compared to an untreated control group.

4.1 Effect of NTZ on immune cell distribution

This study revealed that NTZ has a pronounced effect on the composition of immune cells in the peripheral blood. Upon NTZ treatment, significant changes regarding the frequency of B cells, monocytes, and $CD4^+$ T cells could be detected. It is widely recognized that NTZ has an effect on immune cells in the peripheral blood. A previous study on cynomolgus monkeys demonstrated that NTZ leads to a dose-dependent increased spleen weight and leukocyte count in the peripheral blood (Wehner et al. 2009). Similarly, the treatment of mice with an NTZ analogon resulted in an increase of white blood cell numbers in the peripheral blood (Häusler et al. 2015). Equivalently, an elevated amount of white blood cells has been reported in MS patients upon treatment with NTZ (Bridel et al. 2015; Traub et al. 2019). Moreover, two of the above-mentioned studies detected significant alterations of the composition of immune cell populations, indicating heterogeneous effects of NTZ on distinct leukocyte subsets (Traub et al. 2019; Wehner et al. 2009).

4.1.1 B cells

The present study's results demonstrated that the frequency of B cells within all PBMCs is significantly elevated upon NTZ treatment. Interestingly, various studies have reported an increased proportion of B cells due to NTZ treatment (Krumbholz et al. 2008; Planas et al. 2012; Traub et al. 2019). This is of high clinical importance as lately, evidence has been gathered supporting the crucial role of B cells for the pathogenesis of MS. They do not only act as potent APCs, but also produce pro- and anti-inflammatory cytokines (Frisch et al. 2021). According to studies, B cells of MS patients are chronically activated and present themselves with a more pro-inflammatory cytokine profile compared to B cells of healthy individuals (Bar-Or et al. 2010; Barr et al. 2012).

Different causes for increased B cells in the peripheral blood are being discussed. Studies demonstrated not only an increase of mature B cells, but also a prominent elevation of $CD10^+CD19^+$ pre-B cells in the peripheral blood of NTZ-treated patients (Krumbholz et al.

2008; Saraste et al. 2016). α4β1 integrin is relevant for the attachment of hematopoietic precursor cells in the bone marrow (Lichterfeld et al. 2000; Zohren et al. 2008). Therefore, the blockage of α4β1 integrin causes an accelerated release of lymphoid precursors from the bone marrow (Krumbholz et al. 2008; Planas et al. 2012). Moreover, Planas et al. (2012) suggest, that the higher count of B cells in the peripheral blood under NTZ treatment might be additionally caused by a disturbance of the homing of mature B cells in secondary lymphoid organs due to NTZ. This hypothesis is further supported by their finding of an isolated increase of memory and marginal zone-like B cells which require the interaction of α4β1 integrin and VCAM1 to remain in the splenic marginal zone. In 2014, a study revealing that in the peripheral blood the proportion of $CXCR3^+$ cells within the B cells is significantly increased upon NTZ treatment was published. The chemokine receptor CXCR3 is thought to be relevant for the migratory capacity of B cells. Therefore, this finding leads to the assumption the accumulation of B cells, which without NTZ would have been extravasated into the inflamed CNS, might as well be responsible for the increased number of B cells (Saraste et al. 2016).

4.1.2 Monocytes

This study detected a decreased frequency of $CD14^+$ monocytes within all PBMCs upon NTZ treatment. A reduced relative frequency of monocytes upon NTZ treatment was previously described in other studies (Dallari et al. 2015; Skarica et al. 2011). The present study evaluated, whether this decrease of frequency is caused by a reduced absolute count of monocytes or the disproportionate increase of B cells. Remarkably, this analysis revealed a trend towards an increased absolute number of monocytes. These findings are comparable with results of previous studies which described a moderate increase of monocyte counts upon NTZ treatment (Bridel et al. 2015; Kaufmann et al. 2018; Planas et al. 2012). Monocytes express high levels of VLA-4 (Niino et al. 2006). Therefore, their migration into the CNS is restrained by NTZ treatment, which might result in an accumulation of monocytes in the peripheral blood. However, monocytes might also be capable of entering the CNS independently of VLA-4 as they express not only VLA-4 but also lymphocyte function-associated antigen 1 (LFA-1) (Séguin et al. 2003). This is further supported by the fact that higher percentages of monocytes were detected in the CSF of NTZ-treated patients compared to untreated RRMS patients (Schneider-Hohendorf et al. 2014). Furthermore, a study that showed an increase of lymphoid progenitors in the peripheral blood upon NTZ treatment (see 4.1.1), detected no influence of NTZ on the release of myeloid progenitors to the blood stream (Planas et al. 2012). This finding provides an explanation for the smaller impact of NTZ on the monocyte count when compared to the disproportionate increase of B cells. The potential increase of monocytes upon NTZ therapy demonstrated in this study is highly relevant, since monocytes, as APCs and cytokine producing cells, play a crucial role in the pathogenesis of MS.

In addition to the measurement of the proportion of $CD14^+$ monocytes within all PBMCs, an analysis of the frequency of monocyte subgroups was performed. The frequency of $CD14^{++}CD16^-$ classical monocytes within all PBMCs was significantly reduced upon NTZ treatment and thus behaved like the entirety of the $CD14^+$ monocytes. The frequency of the $CD14^+CD16^+$ population however, only showed a trend towards a reduction under NTZ treatment. The $CD14^{++}CD16^+$ population consisted of both intermediate ($CD14^{++}CD16^+$) and non-classical monocytes ($CD14^+CD16^{++}$). Due to a low count of $CD14^+CD16^+$ cells, the population could not be further distinguished.

Waschbisch et al. (2016) described a reduced frequency of $CD16^+$ cells within all monocytes in blood samples from untreated RRMS patients compared to healthy donors. Interestingly, their study revealed that upon NTZ, the percentage of $CD16^+$ cells within the monocyte population is higher than in untreated MS patients and comparable to the frequency in samples from healthy donors. These data point in the same direction as the results of the present study, which showed that the decrease in total monocytes under NTZ treatment is mainly driven by a reduction of $CD16^-$ monocytes. However, it needs to be considered that in the study by Waschbisch et al. (2016), the untreated group was diagnosed rather recently, while the group receiving NTZ had a longer disease duration. Therefore, an effect of the disease duration on the described changes regarding the monocyte subpopulations under NTZ therapy cannot be ruled out. Yet, the developmental sequence and the plasticity of the monocyte subpopulations are still subject to debate. Therefore, consequences of the described alterations of monocyte subpopulations cannot be predicted.

4.1.3 T cells

For a long period of time, MS was seen as a T cell mediated disease. Although nowadays the role of B cells and myeloid cells is widely acknowledged, the migration of peripherally activated T cells into the CNS is still considered crucial for the pathogenesis. Previous studies demonstrated a significantly increased absolute number of T cells in blood samples from NTZ-treated patients (Krumbholz et al. 2008; Putzki et al. 2010). Remarkably, in the present study, the frequency of $CD4^+$ T helper cells within all PBMCs was significantly reduced upon NTZ treatment. In contrast, the proportion of $CD8^+$ cytotoxic T cells did not change significantly. In accordance with this, Traub et al. (2019) described a reduced frequency of $CD4^+$ T cells and an unchanged frequency of $CD8^+$ T cells within all lymphocytes upon NTZ treatment. However, when calculating the absolute numbers of T cells, an increase of both $CD4^+$ and $CD8^+$ T cells upon NTZ treatment was detected. Furthermore, Putzki et al. (2010) detected a stronger increase in absolute numbers of $CD8^+$ T cells (1.7-fold) compared to $CD4^+$ T cells (1.4-fold) upon NTZ treatment. However, no change of the $CD4^+/CD8^+$ ratio in the peripheral blood was described following the onset of NTZ therapy. Opposing to this, a study detected a gradually declining $CD4^+/CD8^+$ ratio in the peripheral blood of RRMS patients under increasing numbers of NTZ infusions. Yet, the $CD4^+/CD8^+$ ratio in the peripheral blood remained within normal limits (Stüve et al. 2006). Jointly considered, these

findings lead to the assumption that the reduction of the relative frequency of $CD4^+$ T cells described in the present study might be a result of the disproportionate increase of B cells due to NTZ. Furthermore, it suggests a possible alteration in the ratio of $CD4^+/CD8^+$ T cells due to NTZ, presumably caused by different levels of increase or accumulation of the two subpopulations in the peripheral blood.

4.1.4 NK cells

The present study did not detect significant alterations regarding the frequency of NK cells within all PBMCs upon NTZ treatment. This observation is contrary to a study of Skarica et al. (2011) describing a significantly increased proportion of NK cells within all PBMCs after NTZ therapy. Moreover, previous studies have described a significant increase of absolute counts of NK cells after initiation of NTZ treatment (Planas et al. 2012; Putzki et al. 2010). In the present study however, an increase in NK cells cannot be ruled out since no absolute numbers were assessed and an increase in frequency might have been masked by the disproportionate increase in B cells. Interestingly, a study on patients treated with the IL2 receptor-blocking mab daclizumab revealed a correlation between expansion of $CD56^+$ NK cells and treatment response, ascribing them a potential immunoregulatory function (Bielekova et al. 2006). This raises the question, whether the expansion of NK cells in the peripheral blood under NTZ treatment might correspondingly contribute to the beneficial effect of NTZ (Putzki et al. 2010).

4.2 Impact of NTZ on functional properties of monocytes

4.2.1 Activation status of monocytes

In addition to analyzing the composition of peripheral immune cells, functional properties of monocytes upon NTZ treatment were examined. Recent studies indicate that NTZ promotes pro-inflammatory differentiation and activation of T and B cells in the peripheral blood (Benkert et al. 2012; Traub et al. 2019). These findings raise the question, whether the activation status of monocytes is as well influenced by NTZ treatment. In order to determine the activation status of monocytes, the expression of CD69 and CD150 on the surface of $CD14^+$ cells was measured. CD69 is a transmembrane protein and belongs to the C-type lectins. It functions as a signal-transmitting receptor expressed on leukocytes and as marker of monocyte activation (Farina et al. 2004; Murphy and Weaver 2018b; Ziegler et al. 1994)

. CD150, also known as signaling lymphocytic activation molecule (SLAM), is a transmembrane molecule involved in the B cell T cell interaction and is expressed on activated monocytes (Farina et al. 2004; Murphy and Weaver 2018b).

Remarkably, in the present study, both CD69 and CD150 were significantly upregulated on LPS-stimulated monocytes of NTZ-treated samples compared to untreated controls. This

indicates, that NTZ leads to a higher activation status of monocytes and goes in line with the previously described activating effects on B and T cells upon NTZ treatment (Benkert et al. 2012; Traub et al. 2019). However, the study design of the present work does not allow for distinguishing a direct effect of NTZ on monocytes from a possible indirect effect due to activated B cells. Importantly, the epidemiological analysis exhibited that this increase in monocyte activation was not dependent on disease duration, age nor EDSS score.

These findings are of high relevance because an activated peripheral immune system after NTZ therapy could have detrimental effects regarding the MS pathogenesis, as discontinuation of NTZ treatment is associated with rebound disease activity (Vellinga et al. 2008; Vidal-Jordana et al. 2015). Moreover, the observed activation of monocytes could have a negative impact on chronic progression of MS, as evidence suggests that myeloid cells contribute to relapse-independent disease progression (Giannetti et al. 2014; Sucksdorff et al. 2020). Furthermore, a recent clinical trial investigating whether NTZ has beneficial effects in SPMS patients in addition to the prevention of superimposed relapses was unsuccessful, which underlines the described assumption (Kapoor et al. 2018). Additionally, it was suggested that a relatively high proportion of RRMS patients undergoing NTZ therapy at an early stage of their disease course develop confirmed relapse-independent disease progression under NTZ (Graf et al. 2021).

4.2.2 Antigen presentation by monocytes

Monocytes act as APC, which is crucial for the pathogenesis of MS as they activate and polarize peripheral T cells (Mishra and Yong 2016). To assess the antigen presenting function of monocytes under NTZ therapy, the expression of MHCII and the costimulatory molecules CD40, CD80, and CD86 was measured. MHCII is a surface molecule used by APCs for the antigen presentation and crucial for the activation of $CD4^+$ T cells (Murphy and Weaver 2018a). CD40 is a TNF receptor expressed on B cells and myeloid cells and is involved in the interaction of monocytes and T cells (Murphy and Weaver 2018b; Wagner Jr. et al. 1994). CD80 and CD86, also known as B7.1 and B7.2, are costimulatory molecules expressed on myeloid cells. They are important for the activation of $CD4^+$ T cells (Murphy and Weaver 2018b).

The phenotypical characterization of monocytes in the present study revealed a trend towards a higher expression of MHCII on monocytes of NTZ-treated patients compared to untreated controls. However, regarding the expression of CD40, CD80, and CD86, no difference between NTZ-treated and untreated blood samples was detected. These results contrast the study by Traub et al. (2019) finding a significantly higher expression of both MCHII and costimulatory molecules on B cells upon NTZ compared to untreated controls. This suggests that with regards to antigen presentation, NTZ does not affect monocytes to the same extent as B cells.

4.2.3 Cytokine production by monocytes

Finally, it was analyzed whether NTZ affects the cytokine production of peripheral blood monocytes. The pro-inflammatory cytokines IL6 and TNFα as well as the anti-inflammatory cytokine IL10 were evaluated. Both flow cytometry and ELISA detected no difference between NTZ-treated MS patients and untreated control patients regarding the cytokine production of monocytes upon NTZ. In contrast, Traub et al. (2019) described an increased production of the pro-inflammatory cytokines IL6 and TNFα by B cells upon NTZ treatment. Moreover, NTZ is associated with an induction of the expression of pro-inflammatory cytokines IL2, IFNγ, and IL17 by $CD4^+$ T cells (Benkert et al. 2012). It can therefore be assumed, that the cytokine production by monocytes may not be affected by NTZ to the extent as T and B cells. However, regarding the ELISA analysis, it must be considered that the supernatants for the ELISA were collected from LPS and IFNγ stimulated PBMCs. Even though IFNγ augmented LPS stimulation is well-established for the simulation of monocytes and myeloid cells are considered the primary cellular sensory for LPS, cytokine production by other cells like e.g. B cells cannot be ruled out (Alexander and Rietschel 2001; Hayes et al. 1995).

4.3 Outlook

This study reveals, that human blood monocytes become activated upon NTZ treatment, while NTZ does not alter the expression of molecules involved in antigen presentation and the production of both pro- and anti-inflammatory cytokines. Moreover, we were able to confirm that NTZ leads to a disproportionate increase of B cells as described in previous studies (Krumbholz et al. 2008; Planas et al. 2012; Traub et al. 2019). However, the robust alterations regarding the expression of CD69 and CD150 failed to be reflected in the longitudinal analysis. Regarding the longitudinal evaluation of the immune cell composition, the B cell frequency revealed a trend towards an increase in B cells although remaining statistically insignificant. This might have been caused by the low number of longitudinally observed patients. Therefore, it would be of interest, to conduct a longitudinal analysis with a larger sample size.

Remarkably, this study revealed that peripheral blood monocytes become activated upon NTZ treatment. Yet, it remains unclear whether NTZ itself exerts this effect on monocytes or whether they are activated indirectly for exemplarily by NTZ influenced B cells. In order to address this, experiments on purified human monocytes would be valuable to clarify, whether the observed monocytic activation is induced directly or indirectly via other immune cells.

5 Summary

Natalizumab (NTZ) is a monoclonal antibody against very late antigen-4 (VLA-4) used in treating relapsing remitting multiple sclerosis (MS), characterized by its high effectiveness in preventing relapses. Since VLA-4 is a vital adhesion molecule for peripheral immune cells, its blockage inhibits the migration of leukocytes into the central nervous system. Importantly, it is widely acknowledged that NTZ affects peripheral immune cells. Leukocytosis, a disproportional increase in B cells and B cell activation are alterations reported upon NTZ treatment. Although myeloid cells play a crucial role in the pathogenesis of MS, the effect of NTZ on peripheral monocytes is poorly investigated. Therefore, the impact of NTZ on the immune cell composition and functional properties of monocytes was evaluated. Peripheral blood mononuclear cells (PBMCs) of NTZ treated MS patients were analyzed and compared to an untreated MS control group. In addition to fluorescence-activated cell sorting analysis, the supernatant of stimulated PBMCs was analyzed using enzyme-linked immunosorbent assay.

The analysis of the immune cell composition revealed a decreased frequency of monocytes within all PBMCs. In order to assess whether this decrease is caused by the widely acknowledged disproportionate increase in B cells, which was confirmed by this study, the absolute monocyte count was determined. In contrast, this analysis even detected a trend toward an increased absolute number of monocytes ($p = 0.0841$). Furthermore, a decreased frequency of $CD4^+$ T cells was measured, while no alterations regarding the frequency of $CD8^+$ T cells and NK cells were observed. Remarkably, the study revealed an association of NTZ treatment with an enhanced activation status of peripheral monocytes. The expression of two independent activation markers, CD69 and CD150, was significantly upregulated upon NTZ treatment compared to untreated controls. Other properties, however, namely antigen presentation and cytokine production, did not alter significantly upon NTZ treatment. The robust changes in the cell composition and the increased monocytic activation failed to be reflected in the longitudinal analysis, possibly caused by a low number of longitudinally observed patients.

As myeloid cells are assumed to play a prominent role in chronic MS progression, the identified monocytic activation upon NTZ treatment could implicate that NTZ exerts non-desirable effects on MS pathogenesis. Yet it remains uncertain whether NTZ causes the observed activation of monocytes itself or only indirectly, exemplarily through an NTZ-induced activation of B cells.

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