Aus dem Universitätsklinikum Münster
Klinik für Neurologie mit Institut für Translationale Neurologie
-Direktor: Univ.-Prof. Prof. h.c. Dr. med. Heinz Wiendl-

Influence of a dietary supplement with conjugated linoleic acid (CLA) on systemic immune responses in patients with multiple sclerosis

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ZUSAMMENFASSUNG

Teipel, Flavio Jan

Einfluss einer Nahrungsergänzung mit konjugierter Linolsäure (CLA) auf systemische Immunantworten bei Patient*innen mit multipler Sklerose

Der Einfluss von Umweltfaktoren, und speziell der Ernährung, auf die Pathophysiologie der multiplen Sklerose (MS) ist Gegenstand aktueller Forschung. Ergebnisse vorheriger Studien im Kontext verschiedener Autoimmunerkrankungen lassen eine Nahrungsergänzung mit konjugierter Linolsäure (CLA) als einen vielversprechenden Ansatz für die ergänzende Therapie der MS erscheinen. Hier wurde der Einfluss von CLA auf systemische humane Immunantworten genauer untersucht.

Die *in vitro* Behandlung von $CD4^+$ T Zellen gesunder Proband*innen mit CLA resultierte in einer nicht zytotoxisch bedingten reduzierten Ausschüttung pro-inflammatorischer Zytokine sowie einem herabregulierten Zellmetabolismus. In einer explorativen klinischen Pilotstudie wurden 15 Patient*innen mit schubförmiger MS für 24 Wochen mit 2,1 g CLA *per os* täglich behandelt. $CD4^+$ T Zellen in ihrem peripheren Blut zeigten danach ebenfalls einen weniger aktiven Zellmetabolismus im Vergleich zur Voruntersuchung. Die durchflusszytometrische Phänotypisierung peripherer Immunzellen ergab eine Herabregulation verschiedener pro-inflammatorischer Zelltypen und eine reduzierte Ausschüttung pro-inflammatorischer Zytokine durch $CD4^+$ und $CD8^+$ T Zellen. Die Einordnung weiterer Beobachtungen bedarf weiterführender Untersuchungen. Klinisch zeigten sich keine deutlichen Veränderungen und die Nahrungsergänzung mit CLA wurde überwiegend gut vertragen.

Zusammenfassend deutet der überwiegende Teil der Ergebnisse auf anti-inflammatorische Effekte von CLA im Kontext von MS hin. Zukünftige, größere und placebokontrollierte Studien sollten den Wirkmechanismus, die Wirksamkeit und die Verträglichkeit von CLA bei MS weiter untersuchen.

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Erklärung

Ich gebe hiermit die Erklärung ab, dass ich die Dissertation mit dem Titel

Influence of a dietary supplement wit conjugated linoleic acid (CLA) on systemic immune responses in patients with multiple sclerosis

in der

Klinik für Neurologie mit Institut für Translationale Neurologie, Universitätsklinikum Münster

unter der Anleitung von

Univ.-Prof. Dr. med. Luisa Klotz

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Münster, 29.06.2020 Flavio Jan Teipel

To Katharina and Joachim

Contents

Abbreviations

SI units and their standard prefixes are used according to the general conventions. Additionally, the units minute (min), hour (h), day (d) and degree Celsius (°C) are used.

12/15 LOX	12/15 lipoxygenase
AA	antimycin A
ACC1	acetyl-CoA carboxylase 1
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BBB	blood-brain barrier
BG	basal glycolysis
BR	basal respiration
CIS	clinically isolated syndrome
CLA	conjugated linoleic acid
CNS	central nervous system
CSF	cerebrospinal fluid
cT_{reg} cell	colonic T_{reg} cell
ctrl	control
Cyt C	cytochrome C
DMT	disease-modifying therapy
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus (human herpesvirus 4 [HHV-4])
ECAR	extracellular acidification rate
ECD	R Phycoerythrin-Texas Red®-X
ECE®	electrical current exclusion
EDSS	Expanded Disability Status Scale
ELISA	enzyme-linked immunosorbent assay
ETC	electron transport chain
FAO	fatty acid oxidation
FAS	fatty acid synthesis

FCCP	carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescence channel
FOXP3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glycolytic capacity
Gd	gadolinium
GLAT	glatiramer acetate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glycolytic reserve
GWAS	genome-wide association study
HC	healthy control
HLA-DR	human leukocyte antigen – antigen D related
HLA-DRB1	major histocompatibility complex, class II, DR beta 1 (gene)
HODE	13-hydroxyoctadecadienoic acid
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IFNβ	interferon beta
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
LAC	Leukocyte Activation Cocktail
Lin	Lineage Cocktail
LLOQ	lower limit of quantitation
MACS®	magnetic-activated cell sorting
MCAM	melanoma cell adhesion molecule
MHC	major histocompatibility complex
MR	maximal respiration
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
mTOR	mechanistic target of rapamycin

NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
NMR	non-mitochondrial respiration
OCR	oxygen consumption rate
OSE	opticospinal EAE
OXPHOS	oxidative phosphorylation
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohemagglutinin-L
PMA	phorbol 12-myristate 13-acetate
PPARγ	peroxisome proliferator-activated receptor γ
PPMS	primary progressive multiple sclerosis
PSA	polysaccharide A
pT_{reg} cell	peripherally derived T_{reg} cell
PUFA	polyunsaturated fatty acid
Q	coenzyme Q = ubiquinone
RCT	randomized controlled trial
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute, a cell culture medium
RRMS	relapsing-remitting multiple sclerosis
SAE	serious adverse event
SCFA	short-chain fatty acid
SD	standard deviation
SEM	standard error of the mean
shRNA	small hairpin RNA
SPMS	secondary progressive multiple sclerosis
SRC	spare respiratory capacity
SSc	systemic sclerosis
stim	stimulation
T_c17 cell	type 17 cytotoxic T cell
TCA cycle	tricarboxylic acid cycle = citric acid cycle (CAC)

T_{EMRA} cell	T effector memory RA cell
T_H1 cell	type 1 T helper cell
T_H17 cell	type 17 T helper cell
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumor necrosis factor alpha
T_R1 cell	type 1 regulatory T cell
T_{reg} cell	regulatory T cell
t-SNE	t-distributed stochastic neighbor embedding
tT_{reg} cell	thymus derived T_{reg} cell
ULOQ	upper limit of quantitation
viSNE	visual stochastic network embedding
xMAP®	multiple-analyte profiling beads

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic, immune-mediated disease of the central nervous system (CNS) in which demyelination plays a key pathophysiological role. It affects roughly 2.3 million patients worldwide (2013). Causing severe symptoms and leading to profound disability, MS is a heavy burden for afflicted individuals and a major challenge for society. Up to now, no curing therapy is available (30, 71, 152, 173).

1.1.1 Epidemiology

The incidence and prevalence of MS vary notably comparing different regions and populations worldwide. In general, Western Europe and North America face a comparatively high MS prevalence. Although prevalence values from numerous studies differ largely even within these regions, most lie between 50 and 200 per 100,000. In contrast, MS prevalence estimates are remarkably lower for example in the Middle East and Africa (< 50 per 100,000) and especially in Asia (< 20 per 100,000) (30, 91).

Highly varying MS incidence values across the world can be explained by genetic differences between the populations and by a differing exposition to environmental influences such as cigarette smoke, infectious agents or dietary habits (12, 20, 51). Both these groups of influencing factors have been investigated in detail and an introduction in the current state of knowledge is given in the section on MS pathogenesis (section 1.1.3). However, further research on how MS risk and development are influenced by environmental factors, which are generally known to have a greater influence on the immune system than genetic factors (29), is of outstanding importance.

MS affects more women than men with reported sex ratios averaging approximately 2.7 : 1 (female : male) in Central Europe (89). The disease sets in at a mean age of approximately 30 years. However, initial MS diagnoses occur also, but rarely, in children and elderly patients (40, 51).

1.1.2 Clinic

MS exhibits a highly variable clinical phenotype. Numerous neurologic dysfunctions can be caused by distinct demyelinating MS lesions in brain and spinal cord, according to the respective

location of the defect within the CNS. Furthermore, the clinical course develops differently between different patients (52, 92).

1.1.2.1 Symptoms

Despite the large variety of MS symptoms, some signs occur particularly often in MS patients: Sensory disturbances like hypo-/ hyperesthesia, dysesthesia, paresthesia or allodynia are reported by most of the patients during the disease course. Also, optic neuritis occurs frequently in MS patients. It presents with pain upon eye movement, visual field loss (e.g. central scotoma) and a relative afferent pupillary defect. Motoric and eye movement disturbances as well as fatigue and an affection of sexual and vegetative functions are further commonly observed symptoms of MS (14, 92).

Upon first presentation, the most reported symptoms relate to the sensory, visual and motoric systems. Both mono- and polysymptomatic forms of disease onset are observed (52, 92).

Some clinical signs are quite characteristic for MS, for example the Lhermitte sign which denotes a transient sensation described as an electric shock running down the spine after flexion of the neck and Uhthoff's phenomenon, a worsening of MS symptoms upon body temperature increase. However, none of these signs is pathognomonic for the disease (92, 175).

1.1.2.2 Clinical course

MS can proceed in several different disease forms which feature different grades of disease activity and disease progression. Disease activity refers to the occurrence of clinical relapses or of new or enlarging MS lesions. Relapses are characterized by acutely or subacutely arising neurological dysfunctions not accompanied by infection or fever. In contrast, disease progression describes the continuous development and accumulation of lasting disability (109).

Relapsing-remitting MS (RRMS) is the most common disease form upon initial MS diagnosis (85-90% of all cases). It is characterized by acute and distinct relapses followed by at least partial resolution of the impairments. Between the relapses, disease progression is absent. However, the relapses may leave profound disability upon recovery (52).

In most RRMS patients, disease develops to secondary progressive MS (SPMS) later gradually. In SPMS, relapses and minor recoveries occur only sporadically, and the disease progresses independently from relapses. Most lasting disability is accumulated in this phase (52, 109, 193).

Primary progressive MS (PPMS) is the alternate form of disease onset. It is only distinguished from SPMS by the fact that no RRMS preceded the progressive disease phase. Approximately 10-15% of the patients are initially diagnosed with PPMS (52, 109).

Observing a first clinical attack resembling an RRMS relapse but not yet fulfilling the MS diagnostic criteria, a clinically isolated syndrome (CIS) can be diagnosed. A CIS often presents with the most common MS presentation symptoms mentioned above, sensory, visual and motoric impairments (146, 178).

Disease severity and consecutive disability are most commonly measured with the Expanded Disability Status Scale (EDSS). It attributes values between 0 (normal clinical findings) and 10 (death due to MS) to different grades of disability (52).

MS usually progresses slowly although the velocity alters highly interindividually. The median time from disease onset to reaching EDSS 6 (assistance required for walking) is approximately 28 years (180). MS reduces the life expectancy by about 10 years in average (164).

1.1.2.3 Diagnosis

Even if clinical findings alone can suffice for the diagnosis of MS, they should always be supported by results from brain magnetic resonance imaging (MRI) (178). Typical CNS lesions in MS present hyperintense in T2-weighted sequences and sometimes hypointense in native T1 sequences. New, acute lesions enhance gadolinium (Gd)-based contrast media which appear hyperintense on T1 images. This accumulation of Gd in acute lesions is due to a damaged blood-brain barrier (BBB) in these sites. Chronic lesions, by contrast, do not enhance Gd-based contrast media (96, 160).

The commonly employed McDonald diagnostic criteria for MS, last revised in 2017, require the demonstration of CNS lesion dissemination in space and either evidence for lesion dissemination in time or the detection of oligoclonal bands specific for the cerebrospinal fluid (CSF) (178).

1.1.3 Current therapy options

Current therapy strategies for RRMS differentiate between disease-modifying therapies (DMTs) and the treatment of acute relapses (42).

First line DMTs include the administration of broadly acting immunomodulatory drugs like interferon beta (IFNβ) preparations or glatiramer acetate. Drugs that are more specifically directed against individual targets in the immune system, e.g. fingolimod or monoclonal antibodies like alemtuzumab or natalizumab, are applied in cases of higher active or therapy refractory disease (42, 73).

Acute RRMS relapses are usually treated with highly dosed glucocorticoids (42).

1.1.4 Pathogenesis

The etiology and pathogenesis of MS are still by far not completely elucidated (173).

Numerous pathophysiological mechanisms have been hypothesized to underlie disease initiation and progression. It is widely accepted that inflammation and neurodegeneration play key roles in the pathology of MS. However, different theories compete, to which degree and in which direction these two major mechanisms are mutually dependent (73).

It is most commonly thought that inflammation triggered by autoreactive lymphocytes drives MS pathophysiology at disease initiation. As the disease progresses, the migration of peripheral immune cells into the CNS wanes and neurodegeneration as well as local inflammatory processes tend to dominate (40, 73).

The inflammatory action seems to be autoimmune, directed against the myelin sheaths covering the axons in the CNS with ensuing demyelination which causes the symptoms. However, no specific antigen has yet been proven to be the target of such an autoimmune response. Moreover, it is unclear how the immune reaction could be primed as the CNS for long has been thought to be an immune-privileged site where immune surveillance is limited what would prevent autoreactive immune cells from encountering their target antigen (40, 73).

Two major hypotheses try to explain how this aberrant immune response nevertheless could be initiated: First, it could be primed in the periphery independently from the CNS and subse-

quently cross the BBB to take effect in brain and spinal cord. The peripheral activation of autoreactive immune cells that have escaped central and peripheral tolerance mechanisms in this case would be due to mechanisms like molecular mimicry, bystander activation or direct cross reactivity (40, 73).

The second hypothesis proposes that a primary CNS defect like a viral infection or primary neurodegeneration leads to local activation of innate immune cells and the sequestration of antigens into the periphery with an ensuing immune response which then re-enters the CNS and causes further damage. However, this theory seems to be less likely. Several aspects, including results from genetic studies and the failure to find an etiologic infectious agent speak against it (73).

In later disease stages, neurodegeneration plays a major role in disease pathology. However, it is debated whether this is rather primary or secondary. In the primary model, the destruction of the myelin sheath by the early disease inflammation would cause the ensuing neuroaxonal damage without a direct attack of the immune system against the neuron. By contrast, in the secondary model, ongoing though rather compartmentalized inflammation would directly harm the axons. Most probably, these two mechanisms act together and are mutually dependent in the late neurodegenerative disease phase (73).

As described above, demyelination is an important characteristic of MS, especially in the early phase (40, 73). However, also remyelination can be observed in MS and is involved in the restoration of tissue integrity and functionality at sites of MS lesions. Remyelination is therefore associated with clinical remission in MS (32, 142). Defects in remyelinating mechanisms are also proposed to contribute to MS pathophysiology (57).

1.1.4.1 Lesional and systemic immune system alterations

Immune cell infiltration, myelin destruction and neuroaxonal degeneration mainly focus on distinct sites in the CNS, called MS lesions or plaques. They can be identified in neuroimaging and their occurrence in different CNS locations is responsible for the variety of possible MS symptoms (40, 160). MS lesions are typically disseminated in both time and space, which means that they emerge temporally independently from each other at different locations (40, 178). For long time, the white matter has been considered the main location for MS lesions. However, rising attention is now being paid also to gray matter lesions (60, 97). Also, MS pathology is not limited

to these plaques, but diffuse pathological alterations are also found in the so-called normal appearing white matter as well as globally spread over the gray matter (97).

MS immune pathology features highly complex multicellular interactions involving both the adaptive and the innate branch of the immune system (73).

Lymphocytes are the key contributors of the adaptive immune system (40). T cells play a key role in the immunopathology of MS and their action is crucial for the initiation of the autoimmune reactions which are responsible for the disease onset (73). B cells are supposed to contribute to MS immunopathology both via antibody secretion and via other mechanisms such as antigen presentation (85).

T cells are the predominating adaptive immune cells in active MS lesions (96). The infiltrating B cell numbers are generally lower (96) and more variable throughout the disease course (40).

Within the T cells, the relative importance of $CD4^+$ T cells in comparison to $CD8^+$ T cells is not entirely elucidated and seems to differ between human MS disease and experimental autoimmune encephalomyelitis (EAE), the most commonly used mouse model of the condition (40, 148). While EAE is predominantly $CD4^+$ T cell-driven, $CD8^+$ T cells overweigh in MS immune cell infiltrates and seem to contribute more to MS pathology than they do to the development of EAE (148).

However, both types of T cells play important roles in EAE and MS (55, 73, 148). Both $CD4^+$ and $CD8^+$ T cells can induce EAE upon adoptive transfer into naïve recipient mice (55, 81) and the considerable involvement of $CD4^+$ T cells in EAE pathogenesis emphasizes their general importance in CNS autoimmunity (148). The most important $CD4^+$ T cell subtypes for the immunopathology of CNS autoimmunity are the $CD4^+$ type 1 (T_H1) and type 17 (T_H17) T helper cells. The plentiful presence and importance of interferon gamma (IFNγ) and macrophages, the key produced cytokine and the main activated cell type of T_H1 $CD4^+$ T cells, respectively, support a major role of T_H1-mediated immunological pathways in MS pathology. Further experimental evidence suggests also a key role of T_H17 $CD4^+$ T cells in the pathogenesis of CNS autoimmunity (73, 90).

According to the current understanding of MS pathogenesis, the dysregulation of the immune-regulatory cell network also plays a crucial role in the development of the disease. In health, these regulatory cell types help maintain immune homeostasis by promoting peripheral tolerance, i.e. they control autoreactive immune cells which already have escaped central tolerance

mechanisms and prevent them from unfolding action. If the peripheral tolerance fails to suppress these autoreactive cells, they can be activated and attack their specific target (40).

Findings from various studies investigating the number and functionality of regulatory T (T_{reg}) cells in MS patients did not reveal entirely consistent results (54) and the exact characteristics of the dysregulations in the immune-regulatory cell network remain elusive. But several analyses demonstrated an impaired suppressive capacity of different peripheral T_{reg} cell subsets in RRMS patients, such as type 1 regulatory T (T_R1) cells which act in an immunoregulatory fashion via secretion of interleukin (IL)-10 (10) and $FOXP3^+$ $CD4^+$ T_{reg} cells (FOXP3 = forkhead box P3) (185, 186). Venken *et al.* also found the number of total peripheral T_{reg} cells to be reduced in RRMS patients (186). A study by Fletcher *et al.* focusing on the subset of peripheral T_{reg} cells which are CD39 positive found these cells to occur in reduced numbers and with impaired function in RRMS (54).

Furthermore, various experimental findings from animal and human studies suggest that also dysregulations concerning regulatory $CD8^+$ T cells and regulatory B cells are implicated in the pathogenesis of MS (40).

Not only a compromised regulatory cell function can contribute to the failure of peripheral tolerance mechanisms. Also, resistance of effector cells to immunoregulatory signals can empower them to escape the control (114, 166).

Within the innate immune system, activated mononuclear phagocytes are the main contributors to MS pathology (73). Their quantity in acute MS lesions is at least 10-20 times higher than the count of lymphocytes (15). They can be differentiated into two subsets: macrophages evolve from monocytes that infiltrate the CNS from the periphery in phases of acute inflammation (73). Microglial cells, in contrast, enter the CNS during embryogenesis after emerging from myeloid progenitor yolk sac cells and can be activated locally (62, 73).

A study by Yamasaki *et al.* in a transgenic animal model of MS suggested that, especially at disease onset, these two cell types have divergent functions: While the monocyte-derived macrophages are highly inflammatory and largely contribute to tissue damage, the locally activated microglia appear comparatively inert with a down-regulated cell metabolism and are involved in debris cleaning (196) what could facilitate tissue recovery (73).

However, these differential functions are not entirely fathomed and in general, within each of the two phagocyte subsets, both inflammatory and immunoregulatory or neuro-damaging and neuroprotective, respectively, phenotypes can be distinguished (40, 73). Macrophages are categorized in a pro-inflammatory M1 and an immunoregulatory M2 type. M1 macrophages emerge from so-called inflammatory circulating monocytes whereas patrolling monocytes develop into M2 macrophages (73). For microglia, different study results suggest an involvement in both CNS tissue destruction and neuroprotection (40, 73).

As disease progresses, the infiltration of peripheral immune cells into the CNS wanes (40). Nevertheless, inflammation still contributes to disease pathology although the exact modality of this remains elusive, as described above (73). However, this inflammatory late disease component, is rather residing inside the CNS than further maintained by immune cell infiltration and therefore is more independent from the peripheral immune system which could explain the diminished effectiveness of peripherally operating immunomodulatory or -suppressive drugs (40, 73). This is also consistent with the observation of meningeal tertiary lymphoid structures in SPMS which are suspected to contribute to further damage (76).

1.1.4.2 Genetic influences

As mentioned above, many investigations provided evidence for the fact that both genetic and environmental factors can confer susceptibility to or protection from MS and numerous complex gene-environmental interactions play a major role in disease formation (40, 73). Although the genetic contribution to the disease etiology itself cannot yet be therapeutically influenced in human MS, its understanding is of high value since the investigation of how genetic variations influence the risk for developing MS could lead to the discovery of pathophysiological pathways which could also be targeted by changing the exposure to environmental factors or by pharmaceutical interventions (40, 162).

Reported concordance rates for MS in monozygotic twins range around 20-25% and are considerably higher than those for dizygotic twins (ca. 5%) (194). This fact as well as the results from large genome-wide association studies (GWAS) (19, 163) strongly suggest a genetic predisposition for MS. The genetic component is estimated to be responsible for around 25-30% of the general risk to develop the disease (40, 73). However, MS is not caused by a single gene defect. Indeed, more than 100 genetic variations have already been linked to the disease which all together explain roughly 28% of the observed recurrence between siblings (19).

A great share of the identified MS risk variants is associated with genes involved in immunological functions what emphasizes the role of the immune system in the disease. Especially in the vicinity of genes linked to the adaptive immune system, GWAS identified important risk variants associated with MS (19, 163). Many MS-linked polymorphisms can be found in specific major histocompatibility complex (MHC) genes with the HLA-DRB1 locus being the one with the largest individual impact on the disease risk (70) which varies more than 10-fold according to the respective allele combination in this locus (131).

Epidemiologic studies investigating potential associations between MS and other autoimmune diseases revealed inconsistent results (65, 86, 130, 151) which may in part be due to a poor study quality in many of these analyses (113). However, there is a substantial overlap between the genetic variants linked to MS and those associated with other autoimmune conditions (19, 163).

Such an overlap, by contrast, can only marginally be observed with primary neurodegenerative diseases what supports the assumption that MS is not a primary neurodegenerative condition (40).

1.1.4.3 Environmental influences

Since environmental factors generally have a large impact on the diverse development of the immune system (29), it is not surprising that numerous of them also contribute to MS susceptibility (40). The exposition to many environmental factors can be influenced and therefore the investigation of their impact on the MS risk promises the development of novel therapeutic strategies for MS (40). Also, the elucidation of their effect mechanisms could promote the understanding of MS pathogenesis and possibly even lead to the development of specific drugs which act over similar pathways.

The best accepted environmental factors conferring susceptibility to MS are Epstein-Barr virus (EBV) infection and smoking (20). Regarding EBV, it has been observed in well-designed epidemiologic studies that Anti-EBNA IgG seropositivity (4) and history of infectious mononucleosis (69) are associated with an increased MS risk. However, the underlying mechanisms are not yet identified (20). Also smoking has been demonstrated to be associated with a higher risk for developing MS. Again, the underlying mechanisms as well as the concrete responsible agent are not clear (68).

Furthermore, many study results suggested the MS risk to vary between regions, nations and ethnic groups. In this context, higher latitude, higher state of socioeconomic development and white ethnic group membership were assumed to be associated with an elevated risk for MS (5, 91, 171). To make reliable statements regarding these associations, however, is difficult since differences in life expectancy and health care access and insufficient diagnostic consistency may lead to differentially collected and therefore in comparison adulterated prevalence and incidence data. Moreover, it is very challenging to deduce concrete causal relationships from epidemiologic observations regarding these potential MS risk factors because latitude, socioeconomic development and ethnicity are highly associated with each other. Furthermore, even a proven causal relationship between one of these factors and an increased MS risk would leave open the question which mediator variables are directly responsible for such an impact and if they are influenceable or not (e.g. a causal association between ethnicity and MS could be due to behavioral or genetic factors) (91). Finally, the previously assumed increased MS risk in regions of higher latitude (5, 91) and in ethnic groups of white skin color (99, 190) is being reconsidered due to more recent study results or even might be currently diminishing.

However, a potentially increased MS risk in regions of higher latitude could be explained by reduced sunlight exposure, probably at least partly mediated through lower vitamin D serum levels (8). Various studies have suggested associations between sunlight exposure, UV radiation exposure and serum vitamin D levels on the one hand and MS disease risk, activity and progression on the other hand (8, 9, 122, 125, 137).

Recently, rising attention in MS research has been paid to another field of environmental influences: various studies suggest that several nutritional factors can protect from or worsen MS. Since dietary habits could easily be modified as a complementary therapeutic approach, and since the elucidation of their impact on MS could improve our general understanding of MS pathophysiology, research in this area is of high interest (156).

Various animal studies provided evidence for the capability of dietary factors to promote or dampen CNS autoimmunity. While high sodium chloride intake was associated with higher inflammatory activity and worsened disease scores, the experiments suggested protective effects of – among others – zinc, caloric restriction and the vitamins A, E and D3 (183). Moreover, variations in the lipid intake were associated with an altered EAE disease course. While administration of long-chain fatty acids (14-18 carbon atoms (77)) was associated with an aggravated EAE

(67), short-chain fatty acids (SCFAs, 2-6 carbon atoms (77)) (67) as well as polyunsaturated fatty acids (PUFAs) (183) seemed to reduce the severity of EAE, measured by clinical scores.

Studies translating these findings into human MS, have suggested for example vitamin D, biotin, caloric restriction, a Mediterranean diet and PUFAs to be protective (156). High sodium chloride intake, in contrast, appeared to act disease-worsening also in MS (47). However, these effects were mostly demonstrated in studies with an observational or insufficiently controlled design, a small study size or other methodological impairments (47, 156) and most interventional studies so far were not able to reproduce these findings (183). Therefore, there is an urgent need for large randomized controlled trials (RCTs) with strict control of the participants' diet which can detect also small group differences to obtain more reliable information about the influence of dietary factors on MS.

However, the dietary supplementation with PUFAs appears as one of the most auspicious interventions in MS. Although the best designed RCTs until now failed to prove protective effects, various other observational and interventional studies suggested such an impact (120, 165). Therefore, further investigations are needed to clarify the potential of specific PUFAs as preventive or therapeutic intervention in the context of MS.

Several mechanisms have been proposed to be responsible for the effects of nutritional factors on CNS autoimmunity. Some of these include an involvement of the gut microbiota (53, 183). Considering that approximately 70-80% of the whole immune system is residing in the gastrointestinal tract (117, 187), it is easily conceivable that immune cells and gut bacteria are in close interaction and that the microbiota composition can influence the immune system's behavior (21).

Various observations in EAE studies (22, 53, 133, 156) and in MS patients (22, 53, 183) suggested effects of the gut microbiota on CNS autoimmunity. Dietary habits and interventions can modify it, conceivably leading to a more disease-protective or -promoting composition what could in part mediate the various effects of nutritional factors on MS. Further investigations on the underlying mechanisms and the identification of potential protective and disease-promoting bacterial species are required since the findings could lead to novel dietary therapy approaches (53).

For example, the effects of the bacterial species *Bacteroides fragilis* on the immune system and CNS autoimmunity have been investigated in detail (21, 133). In mice, the species promotes anti-

inflammatory responses by colonic T_{reg} (cT_{reg}) cells (157) which are crucial for the tolerance towards nutritional and microbial antigens (77). Ochoa-Repáraz *et al.* demonstrated *B. fragilis* to confer protection from EAE (134). Both these effects were largely dependent on and therefore probably mediated by the expression of the capsular antigen polysaccharide A (PSA) (134, 157). Also administered alone as purified substance, PSA is capable to promote T_{reg} cell development and to confer protection from EAE (133). Results from *in vitro* experiments indicate that PSA could also be beneficial in the context of human MS (177).

It has been observed that the gut microbiota composition differs between healthy controls (HCs) and MS patients what further indicates that it could play a role in the pathogenesis of MS. For example, MS patients exhibit decreased shares of bacteria from the *Lactobacillus*, *Parabacteroides* and *Prevotella* genera and increased shares of *Blautia*, *Dorea* and *Methanobrevibacter* bacteria (168, 183). Alterations could even be observed within twin pairs with one untreated MS-affected and one healthy individual (22). However, statistical-methodological impairments and a small sample size limit the reliability of this result. Furthermore, transgenic mice developed more often a spontaneous model of MS after receiving a transplantation of the gut microbiota from the MS twin than after receiving the healthy twin's microbiota (22).

One prominent alteration in the gut microbiota observed in MS patients is the reduction of SCFA-producing bacteria, for example bacteria from the *Lactobacillus*, *Parabacteroides* and *Prevotella* genera (168, 183). SCFAs are suggested to have a beneficial influence on CNS autoimmunity (67, 183), an effect which is supposed to be mediated in part via an induction of cT_{reg} cells (59, 172). Animal model findings suppose that binding of SCFAs to the receptor GPCR43 is crucial for this cT_{reg} cell induction. A knock-out of its encoding gene *Ffar2* abrogated the beneficial effects of SCFAs on cT_{reg} cell numbers, their immunoregulatory function and their expression of FOXP3 and IL-10 *in vivo* (172). However, SCFAs could also exert effects directly in the brain (45). Combined with the fact that the proportion of SFCA-producing bacteria in the gut and caecal SCFA concentrations can be altered by variations of the dietary lipid composition (112, 143), this underlines the possible capacity of dietary interventions as supplementary MS treatment strategies.

Interestingly, experimental findings led to the hypothesis that also the typical oral MS DMTs (fingolimod, dimethyl fumarate and teriflunomide) (158) and vitamin D (110) could be capable to alter the gut microbiota composition and that their effects on MS could in part be mediated by this mechanism.

However, large studies investigating the effects of the microbiota on MS are needed to prove its influences on CNS autoimmunity and to identify specific protective or harmful microbial species. But considering the collected evidence for an influence of the gut microbiota on MS, its alteration seems to be a promising therapy approach. For this, dietary interventions could be a particularly easy and safe method in comparison to other opportunities like the administration of pro- or antibiotics (183).

Apart from the environmental factors influencing the MS risk discussed above, further proposed but less well-established ones include cytomegalovirus infection, obesity and circadian disruption (20, 72, 126, 174).

1.1.4.4 Immune cell metabolism in MS

Currently, rising attention in immunology research is focused on the emerging sector of immunometabolism (136). It has been found that the cell metabolism has an influence on the function of various immune cells (77, 105, 136). These findings raise the question if a therapeutic manipulation of the immune cell metabolism could be able to positively influence the risk for and course of diseases in which the immune system plays a major role. Indeed, the ensuing research revealed a potential of various substances affecting the immune cells' metabolism to support the treatment of several immunity-related diseases (58, 77). Therefore, it is of high interest to further study how an affection of the immune cell metabolism influences the development of MS.

So far, the knowledge about the immune cell metabolism in MS is limited, but rising insight is being gained in the metabolism of immune cells in general and in several disease conditions (58, 105). In contrast to most body cells in adults, which are fully differentiated and do not undergo sudden activation and rapid proliferation, T cells need the potential to swiftly change their cellular functions and to massively proliferate during an immune response. Naïve T cells are rather quiescent, non-proliferative cells which do not produce large amounts of biomolecules and consume little energy. However, upon activation they must rapidly develop to effector T cells, grow and proliferate fast and produce substantial amounts of effector molecules such as cytokines to fight the trigger of the immune reaction (105, 111, 139). After the pathogen is successfully combated, most of the effector T cells undergo apoptosis while some remain as memory T cells in the body for long time to be able to react quickly in case of a recontact with the antigen. In this state, the cells resemble rather the activity state of naïve T cells again. However, they can re-

increase their level of cytokine production and proliferation even faster than naïve T cells (77, 105).

Between these steps in the life of a T cell – naïve, effector and memory state – it undergoes profound changes in its metabolic system to meet its respective cellular demands of each state. Generally, different non-regulatory T cell subtypes can be categorized in two major groups with similar metabolic functions (136).

First, there is a group of long-lived, rather quiescent, non-proliferative cells to which naïve and memory T cells can be assigned. Even though there are also differences between their metabolic systems, many similarities can be observed: These cells have rather low biosynthesis rates and require from their metabolic system mainly the generation of adenosine triphosphate (ATP) and the facilitation of longevity. For these purposes, the cells largely rely on highly efficient ATP production by engaging the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) with the input metabolites derived from glycolysis, fatty acid oxidation (FAO) and the amino acid catabolism (44, 105, 136, 139).

Second, highly inflammatory and rather short-lived effector T cells can be distinguished, e.g. T_H1, T_H17 and effector $CD8^+$ T cells. These cells are characterized by fast cell growth and proliferation and high synthesis rates for biomolecules, such as cytokines and cell organelles. Therefore, they require from their metabolic system not only a sufficient production of ATP, but also of large amounts of intermediate metabolites which can be used as precursor molecules for the production of proteins (e.g. receptors, cytokines, structural proteins), lipids (e.g. for membranes) and DNA components (e.g. ribose). For these purposes, the key catabolic pathway in this group of cells is the so-called aerobic glycolysis, i.e. the metabolization of glucose to lactate in the presence of oxygen. Glucose is thus used as energy and carbon source for the cells. Compared with OXPHOS, aerobic glycolysis is very inefficient in the generation of ATP but can be activated much faster (no need for the generation of new mitochondria) and provides metabolic intermediates for the synthesis of various structural and functional biomolecules. In contrast to the first group of cells, FAO is low in these inflammatory, effector T cells and instead, fatty acid synthesis (FAS) is high to meet the demands for the generation of lipids for cellular growth and proliferation (44, 58, 77, 105, 136).

Besides naïve, effector and memory T cells, T_{reg} cells are another important subset of T cells. They have higher synthesis rates of biomolecules than naïve and memory T cells and can proliferate upon stimulation by appropriate signals (105). Nevertheless, their metabolic signature largely resembles that of naïve and memory T cells, highly employing the TCA cycle and FAO while exhibiting comparatively low levels of glycolysis and FAS (44, 58, 136).

The metabolism in T cells is controlled by various mechanisms. In this context, the mechanistic target of rapamycin (mTOR) pathway plays a major role. Its activation leads to the upregulation of aerobic glycolysis and supports increased cell growth and proliferation rates by upregulating further necessary metabolic pathways. This matches the observation that the mTOR pathway is activated in pro-inflammatory, highly active effector T cells and shows low activity in memory T cells. Its activity is also low in T_{reg} cells (44, 58, 139). Furthermore, the transcription factors MYC and HIF-1a play an important role in the control of the immune cell metabolism as well, having similar effects to the activated mTOR pathway (44, 139).

It is extensively being studied, if the current immunologic activity and function of T cells determines the cellular needs for ATP and metabolic intermediates for biosynthetic processes and therefore induces specific metabolic pathways while downregulating others or if changes in the cellular metabolic activity, induced by external factors, can influence the immunologic function of a T cell. Such external factors could be, for example, the activation of cell surface receptors or nuclear receptors, the availability of substrates or the composition of the gut microbiota. This second option would raise the hope that therapeutic interferences with the cellular metabolic system could be applied to control immunological processes medically (77, 139).

Research findings so far indicate that most probably both mentioned mechanisms act together. For example, evidence suggests that an inhibition of glycolysis and a promotion of FAO stimulate the development of memory and regulatory T cells and impede the generation of effector T cells while an enhanced FAS and glycolysis appear to favor the development of pro-inflammatory immune cells (44, 77, 136).

In several *in vitro* and *in vivo* experiments, it could be demonstrated that therapeutically influencing the metabolic system of immune cells can change their cellular function. For example, this was found in investigations on acetyl-CoA carboxylase 1 (ACC1), a key enzyme of the FAS. T_H17 cell development was impaired and T_{reg} cell development promoted *in vitro* by pharmacological ACC1 inhibition with soraphen A (murine and human) and by genetic ACC1 knockout (only

murine), probably at least partly mediated by a reduction of FAS. Furthermore, in murine *in vivo* experiments, both the pharmacological inhibition and the T cell-specific genetic knockout of ACC1 ameliorated the course or prevented the development of CNS autoimmunity, respectively (23).

Some metabolism-affecting substances have even been studied in human MS. Statins, which are widely prescribed for lowering serum cholesterol levels, blocked T_H1 and T_H17 polarization and promoted T_{reg} cell development *in vitro* (56) and showed protective effects in EAE *in vivo* (198). However, several clinical trials investigating the effect of various statins on MS so far had inconsistent results (145).

Administration of the mTOR inhibitor rapamycin, which is already clinically used under the name sirolimus for the prevention of organ transplant rejections, shortly before induction of EAE resulted in an ameliorated disease course and increased T_{reg} cell numbers *in vivo* (147). The mTOR inhibitors sirolimus (clinical trial NCT00095329) and temsirolimus (clinical trial NCT00228397) have also been tested for safety in MS (58) without final results having been published yet and preliminary efficacy data suggests a protective effect of temsirolimus in MS treatment (84).

Considering these experimental findings, it seems to be a promising approach to influence the immune cell metabolism therapeutically to treat immune system-related diseases.

The fact that the immune cell metabolism seems to be able to affect the immune cell function raises the question how such an effect could be mediated. One mediator can be seen in the action of so-called moonlighting proteins – proteins that can exert multiple functions in different cellular systems. For example, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds – while not being enzymatically active – the messenger RNAs (mRNAs) for the pro-inflammatory cytokines IFNγ and IL-2, thus inhibiting their translation. If, however, GAPDH is recruited to exert its enzymatic function by an upregulation of the glycolysis as seen in effector T cells, it can no longer bind these mRNAs which hence are beginning to be translated. Therefore, an upregulation of the glycolysis directly causes the production of the pro-inflammatory cytokines IFNγ and IL-2. Similar mechanisms have been described for a variety of metabolic enzymes (44, 105).

To sum up, rising evidence suggests that the cell metabolism in immune cells is not only crucial for the generation of ATP and metabolic intermediates for biosynthetic purposes but is also

closely interlinked with and directly controls elementary immune cell functions like the differentiation to certain immune cell subsets (pro- vs. anti-inflammatory) and the production of cytokines (105, 136).

1.2 Conjugated linoleic acid

As described above, PUFAs are a group of substances which were suggested to have protective effects on CNS autoimmunity in various EAE experiments (165, 183) and some clinical MS studies (120). Further experiments are needed to identify the differential effects of individual PUFAs in the context of CNS autoimmunity. Their results could lead to the development of more specific and potentially more effective dietary interventions in MS. One promising candidate as a PUFA with beneficial effects on MS is conjugated linoleic acid (CLA) (79, 188).

CLA occurs naturally in dairy products and meat and increasing experimental evidence suggests it to have positive health effects in several disease conditions, including inflammatory disorders. For instance, the administration of CLA seems to confer protection from or to ameliorate the course of inflammation-driven colorectal carcinoma, asthma and inflammatory bowel disease (IBD) (188).

Even in the field of CNS autoimmunity CLA already showed beneficial effects: Both *in vivo* studies in a spontaneous mouse model of MS and *in vitro* studies with MS patients' immune cells, which are further described in section 1.2.3.3, revealed protective properties of the substance (79).

Therefore, it is of high interest to further examine the effects of CLA on the immune system in general and on human MS in particular.

1.2.1 Chemical aspects

The term CLA denotes a set of structural and spatial isomers of linoleic acid. They are all PUFAs with an 18-atom carbon chain and two C-C double bonds. In contrast to linoleic acid, the double bonds in CLA are conjugated, i.e. exactly one single bond is lying in between. Varying in the location and the *cis* / *trans* configuration of the double bonds, 28 CLA isomers have been identified that occur naturally. Within these, two constitute together almost 100% of the CLA consumed with the diet: While approximately 90% of the CLA taken in naturally is the *cis*-9, *trans*-11 isomer, the *trans*-10, *cis*-12 isomer makes up roughly 10%. Fig. 1.1 shows the structural formulas of these two isomers (188).

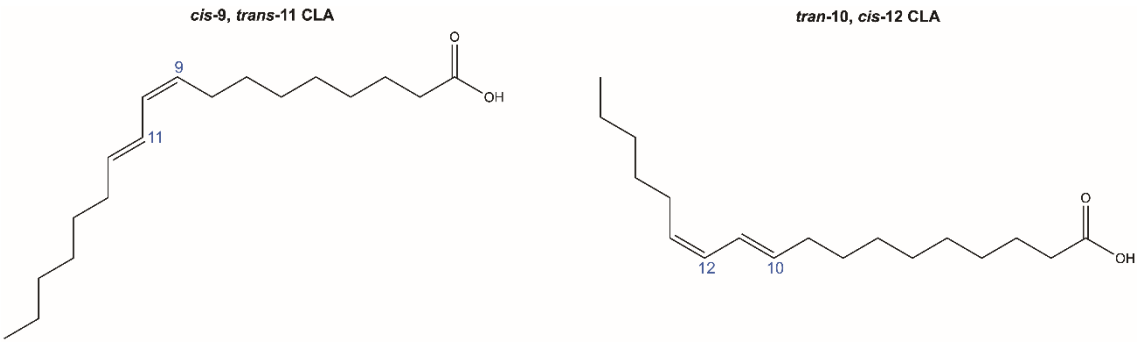

Fig. 1.1 Structural formulas of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA
Graphic based on "Fig. 1. Structure of linoleic acid, *cis*-9, *trans*-11 CLA isomer and *trans*-10, *cis*-12 CLA isomer." in (188).

The health effects seem to differ between the different CLA isomers and synergistic effects of different isomers have been described. However, most suggested favorable effects are attributed to the two main isomers (188).

1.2.2 Occurrence in food items and natural intake

Being generated in different pathways especially, but not exclusively, in ruminant animals, CLA is part of the average daily nutrition (188). Different foodstuffs contain highly variable amounts of CLA with up to more than 8 mg CLA / g fat in some dairy and meat products which generally have the greatest CLA contents (34, 188). Also, the respective proportions of the different CLA isomers vary largely. However, the *cis*-9, *trans*-11 isomer predominates in the CLA occurring naturally in food (34).

The consequent natural CLA intake is depending on nutritional habits. An American study employing 93 healthy individuals estimated the average nutritional CLA intake to be approximately 212 or 151 mg/d for men and women, respectively. For data acquisition, 3-day food duplicates had been analyzed chemically (154).

1.2.3 Effects in the context of inflammatory diseases

Beneficial properties in the context of several disease conditions have been suggested for CLA based on results of a range of animal model experiments and some human trials. Besides anti-carcinogenic and anti-allergic properties, the anti-inflammatory capabilities of CLA are in the focus of current research (79, 188).

1.2.3.1 *Effects on the immune system*

The documented anti-inflammatory effects of CLA seem to be elicited by modulating both the innate and the adaptive immune responses (16, 79, 188). For example, in the innate immune system, CLA appears to reduce the inflammatory activity of macrophages and to influence their development from monocytes (188).

Experimental evidence suggests that in the adaptive immune system, CLA, *inter alia*, can promote the development of regulatory lymphocytes, regulate the production of many cytokines such as the pro-inflammatory tumor necrosis factor alpha (TNFα), IFNγ and IL-17A (16, 79), and modulate the production of immunoglobulins in a class-specific manner (188). For example, CLA appears to be able to dampen allergic reactions by downregulating the expression of IgE while maintaining or even enhancing humoral responses against pathogens by promoting the production of IgG, IgM and IgA class immunoglobulins (188).

1.2.3.2 *Effects on inflammatory diseases*

Since inflammatory processes play a key role in a wide range of diseases, beneficial anti-inflammatory effects of CLA could possibly be exploited for the treatment of many health problems. In this line, the action of CLA has been investigated in animal models and clinical trials for several diseases associated with inflammatory processes. Favorable effects could be observed in many of the animal models and some of the human trials (16, 17, 79, 188).

For example, Moloney *et al.* suggested CLA to reduce obesity-induced insulin resistance, probably due to its anti-inflammatory properties, based on findings from cell culture and murine *in vivo* experiments (123). Also, in the field of asthmatic respiratory inflammation, CLA is suggested to exert beneficial effects (188). Finally, CLA ameliorated chronic inflammation in different animal models of IBD (17, 188) as well as the human disease in a small clinical trial (16) and appeared to protect from inflammation-driven colorectal cancer (18, 46).

In many studies it could be demonstrated that the different CLA isomers can exert differential effects (24, 188). However, although this might vary depending on the treated disease, a mixture of the two main isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA appeared to most efficiently exert anti-inflammatory effects in several studies (17, 79). Results from a murine IBD model *in vivo* experiment suggested that a very early exposition to CLA via placenta and milk in

addition to the oral administration from weaning until disease induction augments the positive effects of the substance (17).

1.2.3.3 Effects in the context of CNS autoimmunity

Hucke, Hartwig *et al.* investigated the effects of CLA on CNS autoimmunity in opticospinal EAE (OSE) mice which are genetically modified so that they develop CNS autoimmunity spontaneously, and with human immune cells from HCs and RRMS patients (79).

Their so far unpublished data revealed a delayed disease onset and an ameliorated disease course in OSE mice supplemented with CLA from conception on. The development of the clinical disease score over a period of 100 d past birth is illustrated in Fig. 1.2 (79).

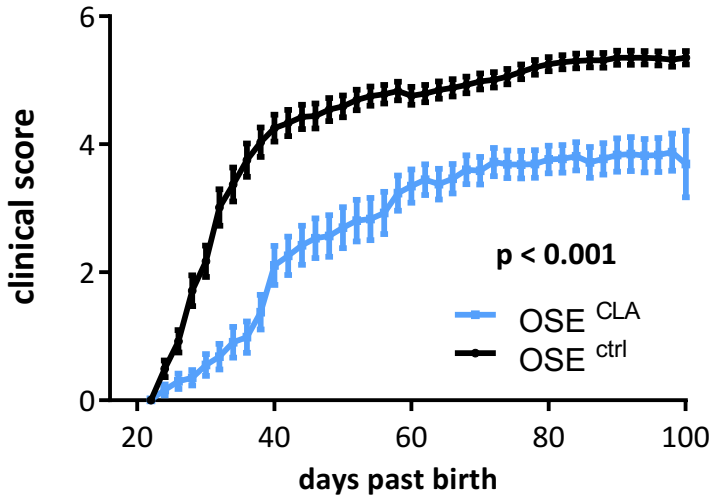

Fig. 1.2 Effect of a dietary CLA supplementation on CNS autoimmunity in OSE mice measured by clinical score
Clinical disease score in the first 100 d past birth of OSE mice supplemented or not with CLA from conception on. The graph shows pooled data from two independent experiments. *n* (OSE^{ctrl}) = 83, *n* (OSE^{CLA}) = 40. The data points represent mean scores across the cohorts ± SEM. p-value calculated with linear mixed model. Unpublished data by Hucke, Hartwig *et al.* (79).

These findings were in line with reduced frequencies of pro-inflammatory cytokine producing peripheral $CD4^+$ T cells (IFNγ and IL-17A, illustrated in Fig. 1.3) and CNS-located $CD4^+$ T cells (IFNγ) and with increased shares of small intestinal and splenic T_{reg} cells in CLA-supplemented OSE mice (79).

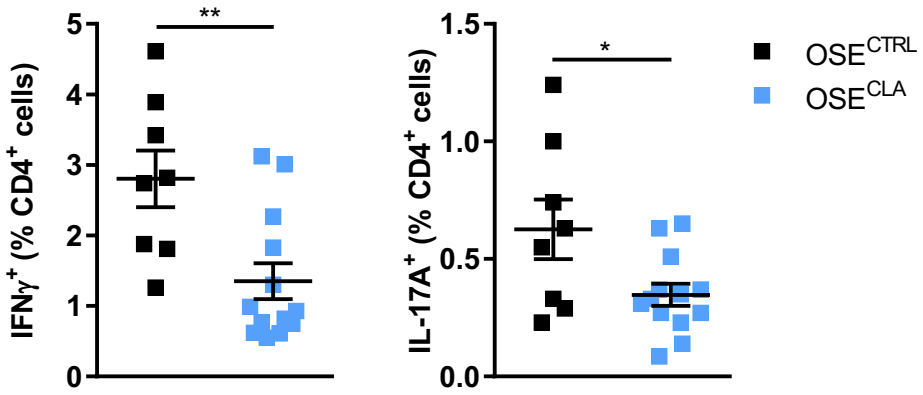

Fig. 1.3 Effect of a dietary CLA supplementation on the number of cytokine producing CD4⁺ T cells in OSE mice
Share of IFNγ and IL-17A producing CD4+ T cells, respectively, as a percentage of all CD4+ T cells, investigated in inguinal lymph nodes and compared between OSE mice supplemented with CLA or not from conception on. *n* (OSE^{ctrl}) = 8, *n* (OSE^{CLA}) = 13. Each data point represents one individual's value. The lines indicate the respective cohort's mean value ± SEM. Results from unpaired t-test: * $p < 0.05$ ** $p < 0.01$. Unpublished data by Hucke, Hartwig *et al.* (79).

In the *in vitro* experiments, Hucke, Hartwig *et al.* investigated the effects of a CLA *in vitro* treatment on human peripheral blood $CD4^+$ T cells derived from HCs and RRMS patients. Upon treatment, they exhibited a reduced proliferation and pro-inflammatory cytokine production. The polarization of naïve $CD4^+$ T cells towards a T_H1 or T_H17 phenotype was restricted by the CLA treatment. Even the pro-inflammatory cytokine production of existing restimulated peripheral blood effector $CD4^+$ T cells could be dampened by the exposure to CLA (Fig. 1.4). This latter effect was also demonstrated with effector $CD4^+$ T cells from MS patients' CSF. A relevant cytotoxicity of CLA on HC peripheral blood $CD4^+$ T cells was excluded (79).

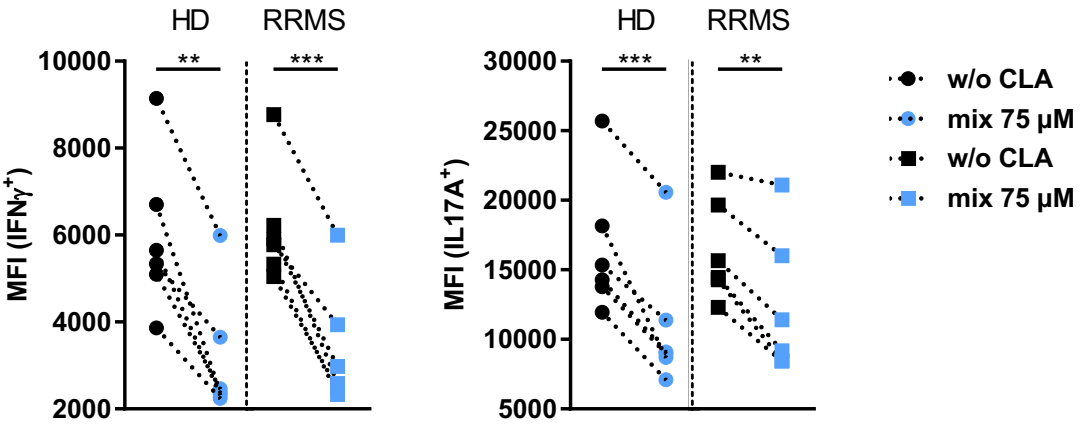

Fig. 1.4 Effect of CLA *in vitro* on stimulated peripheral blood CD4⁺ T cells' cytokine production capacity
Mean fluorescence intensity of cytokine producing CD4⁺ T cell populations for IFNγ and IL-17A, respectively, measured by flow cytometry. The cells were derived from the peripheral blood of healthy donors (HD) or RRMS patients (RRMS) and treated or not with 75 µM CLA during 3 h preincubation and 3 h LAC restimulation. n(HD) = 6, n(RRMS) = 6. Each pair of data points with connection line represents one individual's values. Results from paired t-test: ** $p < 0.01$ *** $p < 0.001$. Unpublished data by Hucke, Hartwig *et al.* (79).

1.2.4 Discussed modes of action

The effect mechanisms underlying the anti-inflammatory properties of CLA are not entirely fathomed. However, several possible modes of action have been hypothesized based on experimental findings (188). Fig. 1.5 gives an overview over the most important discussed mechanisms.

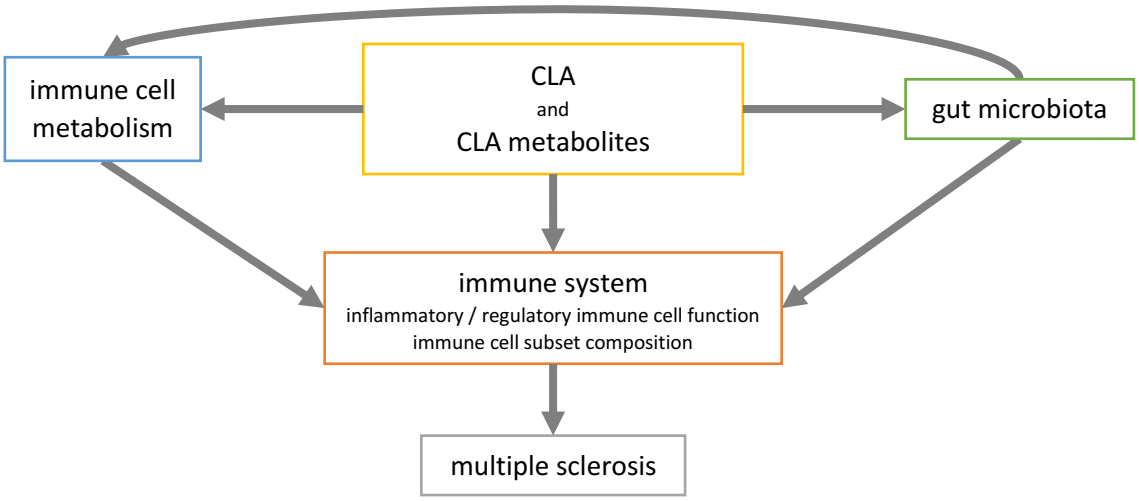

Fig. 1.5 Overview over possible mechanisms underlying the anti-inflammatory effects of CLA

1.2.4.1 CLA influencing the immune system directly

First, CLA could act beneficial in the context of autoimmune diseases by directly affecting the immune cell function. Such effects could be exerted by the activation of receptors for fatty acids. Both cell surface and nuclear receptors are identified which can be activated by different fatty acids (77, 188).

Regarding the effects of CLA on the immune system, the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is of outstanding interest. It has many target genes which mainly play a role in the regulation of the metabolic and the immune system. Many experimental results suggested that its activation is responsible for at least part of the beneficial effects of CLA (188).

PPARγ is expressed in many immune cells. Its activation evokes a shift in the cell's immunologic function towards an anti-inflammatory phenotype, observable in cytokine expression and gene transcription (188). PPARγ can be activated by binding of endogenous (e.g. 13-hydroxyoctadecadienoic acid, HODE) or exogenous (e.g. pioglitazone) ligands (90). The activation of PPARγ suppresses the polarization to T_H17 cells in human and murine cells (90) while its capability to promote the development of T_{reg} cells is discussed controversially (90, 128, 192, 197). In contrast, PPARγ deficiency in murine cells contributes to the polarization towards a T_H17 phenotype (90). Since T_H17 cells play a crucial role in MS pathology, these findings match with the observation that a treatment with pioglitazone exerts beneficial effects in EAE models (49, 90) while a $CD4^+$ T cell-specific PPARγ knock-out leads to an exacerbated EAE course (90).

Among other PUFAs, also CLA is known as a strong activator of PPARγ (188). Upon CLA treatment of mice, the colonic transcription of genes regulated by PPARγ was altered in a fashion indicating PPARγ activation. Furthermore, a colonic tissue-specific PPARγ knockout abrogated the protective effects that had been demonstrated for CLA in an IBD mouse model (17). These findings suggest an involvement of PPARγ in the mediation of the beneficial effects of CLA on autoimmunity.

1.2.4.2 CLA influencing the immune cell metabolism

Second, CLA could exert its beneficial effects by manipulating the immune cells' metabolic system. As described in section 1.1.4.4, it is closely interlinked with the cells' immunologic function

and alterations in the predominantly active metabolic pathways can lead to an altered immunologic phenotype of the cell (44, 77). It has been described *in vitro* and *in vivo* that various triggers can change metabolic processes in immune cells and in this way modify their immunologic functions (23, 44, 77, 139).

These triggers can be categorized in several groups. First, triggers like statins and rapamycin influence the immune cell metabolism by inhibiting certain metabolic enzymes (statins) or interfering with regulatory pathways (rapamycin) (58). However, such an effect is not described for CLA.

Second, the availability of distinct types of metabolic substrates can influence the immune cell metabolism and thereby their function (77). In this line, the PUFA CLA could act as a metabolic substrate for immune cells and unfold its action also this way. However, this could probably explain only rather short-timed effects.

Third, the activation of receptors can elicit changes in the cells' metabolic system. Among these, the before mentioned nuclear receptor PPARγ plays an important role. As described above, CLA is among its strongest activators and its target genes include not only immunologic but also metabolic ones (188). Its activation has an impact on the glucose and lipid metabolism (83) and could therefore change the immunological cell phenotype in this way as well (44, 136).

Finally, the gut microbiota composition can likely influence the metabolic system in intestinal immune cells (23, 139) and since CLA can alter this composition (33), this could be a mode of action for the beneficial CLA effects as well. This is further discussed in the following section 1.2.4.3.

1.2.4.3 CLA influencing the gut microbiota

Third, CLA could exert its anti-inflammatory effects via modulating the gut microbiota composition (33). Its alteration is suggested to be able to influence immune cell functions and the immune cell subset composition directly or via influencing the immune cell metabolism (21, 23, 139).

So far, only few data is available regarding the specific effects of a dietary CLA supplementation on the gut microbiota composition (33). However, studies have suggested that CLA indeed is

capable to act as a prebiotic promoting the expansion of certain bacterial species in the intestinal system (33, 112). Among these promoted bacterial species are, according to a study by Chaplin *et al.*, bacteria from the *Bacteroides / Prevotella* genera and the *A. muciniphila* species (33).

The impact, that changes in the gut microbiota have on the immune system function directly, are summarized in section 1.1.4.3. In the context of CLA the effects evoked by members of the *Bacteroides* genus are of special interest since the number of intestinal *Bacteroides* bacteria is influenced by dietary CLA consumption as described above (21, 33, 133). *B. fragilis* from the *Bacteroides* genus supports the anti-inflammatory activity of cT_{reg} cells and expresses PSA, which has an impact on the risk for CNS autoimmunity as demonstrated in animal studies (133, 134, 157).

Also, the effects that SCFAs have on autoimmunity, which are described in section 1.1.4.3, are relevant in the context of CLA since CLA administration has been shown to increase the concentration of SCFAs in the intestinal lumen by promoting microbial fermentation (67, 112, 183).

Indirect effects of an altered gut microbiota composition on the immune cell function could be mediated by influencing the immune cell metabolism. For example, as mentioned in section 1.1.4.4, the bacterial product soraphen A can inhibit the FAS and thereby shift the balance between regulatory and pro-inflammatory T cells towards the regulatory side and has beneficial effects on the course of an MS mouse model (23, 139).

Considering the impact of alterations in the gut microbiota composition on the immune system function, future research is crucial to reveal the exact influences that dietary CLA exerts on the human gut microbiota (21, 53).

1.2.4.4 CLA metabolites as key molecules

Finally, also enzymatically generated metabolites of CLA could be responsible for the beneficial effects of a dietary CLA supplement. For example, results from experiments with murine T cells raised the hypothesis that the enzyme 12/15 lipoxygenase (12/15 LOX) is essential for the effects of CLA. These effects were diminished in cells lacking the enzyme due to genetic modification (79). 12/15 LOX catalyzes reactions generating eicosanoids out of CLA which might act immune-regulatory in a PPARγ-dependent fashion, e.g. HODE. These metabolites could at least in part be responsible for the beneficial CLA effects (79, 90). However, substantial inter-species variations

in the functionality of 12/15 LOX require caution when concluding from animal study results to the human disease (43).

1.3 Aim of the thesis

Considering the numerous beneficial effects CLA is suggested to have based on results from animal experiments and clinical trials (79, 188), aim of this work was to further elucidate the properties of CLA in the context of human MS disease.

In *in vitro* experiments, the effect of a direct CLA exposure on the cytokine production capacity of HC $CD4^+$ T cells was examined employing two different methods. Furthermore, its impact on the cell metabolism of HC $CD4^+$ T cells was studied in an Agilent Seahorse Mito Stress Test.

The major part of this work was an exploratory clinical study employing 15 patients whose diet was supplemented with 2,100 mg CLA daily for a total of 24 weeks. Baseline demographical and clinical data was collected. Clinical study endpoints were adherence to the study medication, EDSS development, relapse frequency and adverse events. Furthermore, the patients' natural fat and CLA intake were measured with food diaries.

Blood samples collected during the treatment were analyzed to gain an insight into the effects of an intervention with CLA *in vivo* on human MS. With in-depth functional immune phenotyping, potential changes in the composition of the different peripheral blood immune cell subsets were investigated. Peripheral blood $CD4^+$ T cells' cytokine production capacity was examined using flow cytometry and the Luminex® technique comparing samples generated before, during (only flow cytometry) and after the treatment. Changes in the metabolic activity of $CD4^+$ T cells during the CLA treatment were studied employing an Agilent Seahorse Mito Stress Test.

Besides that, stool samples were collected before and after the treatment to study the effects of CLA on the gut microbiota in MS affected individuals. The analysis of these samples, however, is not part of the present work.

2 Material

Apart from the standard laboratory equipment and agents the devices and substances listed in the following tables were used for the experiments.

2.1 Devices

Device	Manufacturer
Bio-Plex® MAGPIX™ Multiplex Reader	Bio-Rad Laboratories, Inc., Hercules, CA, USA
CASY® TT Cell Counter and Analyzer	OLS OMNI Life Science GmbH & Co. KG, Bremen, Germany
counting chamber Neubauer Improved bright-line	Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim / Rhön, Germany
Gallios Flow Cytometer (for *in vitro* experiments)	Beckman Coulter Inc., Brea, CA, USA
Navios Flow Cytometer (for *in vivo* experiments)	Beckman Coulter Inc., Brea, CA, USA
OctoMACS™ Separator	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Seahorse XFe96 Analyzer	Agilent Technologies, Inc., Santa Clara, CA, USA

2.2 Disposables

Product	Manufacturer
MACS® MS columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

2.3 Study medication

Product	Manufacturer
CLA 70plus-Trofocell™ - 1000 mg soft gelatin capsules - ≥ 70% CLA[1] - ≥ 34% *cis*-9, *trans*-11 CLA - ≥ 34% *trans*-10, *cis*-12 CLA - ≤ 3% linoleic acid (*cis*-9, *cis*-12 C18:2) - free of preservatives	TrofoCell Research and Trade GmbH, Hamburg, Germany

2.4 Constituents of media and buffers

Medium	Manufacturer
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Inc., St. Louis, MO, USA
fetal calf serum (FCS)	Sigma-Aldrich, Inc., St. Louis, MO, USA
PBMC cryopreservation medium: CTL-Cryo™ ABC Media kit	Cellular Technology Limited (CTL), Cleveland, OH, USA
RPMI-1640 medium	Sigma-Aldrich, Inc., St. Louis, MO, USA
XF Base Medium Minimal DMEM	Agilent Technologies, Inc., Santa Clara, CA, USA
X-VIVO 15™ medium	Lonza Group AG, Basel, Switzerland

[1] CLA for the study medication was synthesized out of natural safflower oil. The concentration of CLA in the synthesis product depends on the concentration of linoleic acid in the starting material which can vary. However, the product is guaranteed to contain at least 70% CLA. For this work, the administered study medication was considered to contain 70% (700 mg) CLA per capsule with a 1 : 1 ratio of the isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA.

2.5 Composition of media and buffers

Medium	Contents
ELISA stop solution	- 189.4 ml ddH_2O - 10.6 ml H_2SO_4
FACS buffer	- PBS (1X) - 2% (v/v) FCS - 2 mM EDTA
FCS-supplemented RPMI	- RPMI-1640 medium - 10% (v/v) FCS
MACS® buffer	- PBS (1X) - 1% (v/v) FCS - 2 mM EDTA
Mito Stress medium	- XF Base Medium Minimal DMEM - 10 mM glucose - 1 mM pyruvate - 2 mM glutamine - NaOH 0.1 M until pH = 7.4

2.6 Chemicals and reagents

Chemical	Manufacturer
antimycin A	Sigma-Aldrich, Inc., St. Louis, MO, USA
CLA for *in vitro* treatment	Sigma-Aldrich, Inc., St. Louis, MO, USA
FCCP (carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone)	Sigma-Aldrich, Inc., St. Louis, MO, USA
ionomycin	Cayman Chemical Company, Ann Arbor, MI, USA
Leukocyte Activation Cocktail, with BD GolgiPlug™ (LAC)	BD Pharmingen™, BD Biosciences, Franklin Lakes, NJ, USA
Lymphoprep™	STEMCELL Technologies Inc., Vancouver, Canada
MACS® CD4 MicroBeads, human	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
oligomycin	Sigma-Aldrich, Inc., St. Louis, MO, USA
phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Inc., St. Louis, MO, USA
phytohemagglutinin-L (PHA)	Roche Diagnostics GmbH, Mannheim, Germany
propidium iodide	ImmunoChemistry Technologies, LLC, Bloomington, MN, USA
rotenone	Sigma-Aldrich, Inc., St. Louis, MO, USA
trypan blue	Sigma-Aldrich, Inc., St. Louis, MO, USA

2.7 Assay kits

Kit	Manufacturer
Human IFN gamma ELISA Ready-SET-Go! Kit	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
Human TNF alpha ELISA Ready-SET-Go! Kit	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
ProcartaPlex Mix&Match Human 10-plex (PPX-10-MXAAACA)	Thermo Fisher Scientific Inc., Waltham, MA, USA
Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human ProcartaPlex™ Panel (EPX180-12165-901)	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA

2.8 Antibodies for cell activation

Antigen	Antibody	Clone	Manufacturer
CD3	purified, Ultra-LEAF™	Okt3	BioLegend, San Diego, CA, USA
CD28	purified	28.2	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA

2.9 Antibodies for flow cytometry

FL	Antigen	Fluorochrome	Clone	Manufacturer
FL01	CD1c/BDCA-1	FITC	AD5-8E7	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
FL01	CD25	FITC	7G7B6	Ancell Corporation, Bayport, MN, USA
FL01	CD45RA	FITC	ALB11	Beckman Coulter Inc., Brea, CA, USA
FL01	CD45RO	FITC	UCHL1	BioLegend, San Diego, CA, USA
FL01	CD80	FITC	2D10	BioLegend, San Diego, CA, USA
FL01	CD161	FITC	DX12	BD Biosciences, Franklin Lakes, NJ, USA
FL01	CD183/CXCR3	FITC	G025H7	BioLegend, San Diego, CA, USA
FL01	CD206	FITC	15-2	BioLegend, San Diego, CA, USA
FL01	Granzyme K [GrK]	FITC	GM6C3	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
FL01	Helios	FITC	22F6	BioLegend, San Diego, CA, USA
FL01	IFNγ	FITC	4S.B3	BioLegend, San Diego, CA, USA
FL01	IgD	FITC	IA6-2	BioLegend, San Diego, CA, USA
FL01	Lin-3 (CD3/14/19/20)	FITC	SK7 (CD3) SJ25C1 (CD19) L27 (CD20) MϕP9 (CD14)	BD Biosciences, Franklin Lakes, NJ, USA
FL01	mIgG1	FITC	MOPC-21	BD Biosciences, Franklin Lakes, NJ, USA
FL01	mIgG2a	FITC	G155-178	BD Biosciences, Franklin Lakes, NJ, USA
FL01	TNFα	FITC	MAb11	BioLegend, San Diego, CA, USA
FL02	CD27	PE	1A4CD27	Beckman Coulter Inc., Brea, CA, USA
FL02	CD57	PE	HCD57	BioLegend, San Diego, CA, USA
FL02	CD69	PE	TP1.55.3	Beckman Coulter Inc., Brea, CA, USA
FL02	CD80	PE	L307.4	BD Biosciences, Franklin Lakes, NJ, USA
FL02	CD195/CCR5	PE	J418F1	BioLegend, San Diego, CA, USA
FL02	CD200R	PE	OX-108	BioLegend, San Diego, CA, USA
FL02	CD215/IL-15Ra	PE	eBioJM7A4	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL02	FOXP3	PE	PCH101	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL02	Granzyme B [GrB]	PE	REA226	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

FL02	IL-17A	PE	eBio64CAP17	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL02	IL-22	PE	22URTI	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL02	IL-23R	PE	218213	R&D Systems, Inc., Bio-Techne Corporation, Minneapolis, MN, USA
FL02	mIgG1	PE	MOPC-21	BD Biosciences, Franklin Lakes, NJ, USA
FL02	mIgG2b	PE	27-35 (RUO)	BD Biosciences, Franklin Lakes, NJ, USA
FL02	rIgG2b	PE	Clone A95-1 (RUO)	BD Biosciences, Franklin Lakes, NJ, USA
FL03	CD3	ECD	UCHT1	Beckman Coulter Inc., Brea, CA, USA
FL03	CD4	ECD	SFCI12T4D11	Beckman Coulter Inc., Brea, CA, USA
FL03	CD8	ECD	SFCI21Thy2D 3	Beckman Coulter Inc., Brea, CA, USA
FL03	CD16	ECD	3G8	Beckman Coulter Inc., Brea, CA, USA
FL03	CD24	ECD	ALB9	Beckman Coulter Inc., Brea, CA, USA
FL03	CD31	PE-Dazzle594 [Dazzle]	WM59	BioLegend, San Diego, CA, USA
FL03	CD56	ECD	N901	Beckman Coulter Inc., Brea, CA, USA
FL03	HLA-DR	ECD	Immu-357	Beckman Coulter Inc., Brea, CA, USA
FL04	CD3	PC5.5	UCHT1	Beckman Coulter Inc., Brea, CA, USA
FL04	CD8	PC5.5	SFCI21THy2D 3 (T8)	Beckman Coulter Inc., Brea, CA, USA
FL04	CD38	PC5.5	HIT2	BioLegend, San Diego, CA, USA
FL04	CD45RO	PerCP/Cy5.5	UCHL1	BioLegend, San Diego, CA, USA
FL04	CD56	PC5.5	N901 (HLDA6)	Beckman Coulter Inc., Brea, CA, USA
FL04	CD68	PerCP/Cy5.5	Y1/82A	BioLegend, San Diego, CA, USA
FL04	CD86	PC5.5	HA5.2B7	Beckman Coulter Inc., Brea, CA, USA
FL04	CD152/CTLA-4	PerCP-e-Fluor710 [PeF710]	14D3	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL04	CD163	PerCP/Cy5.5	GHI/61	BioLegend, San Diego, CA, USA
FL04	CD194/CCR4	PC5.5	L291H4	BioLegend, San Diego, CA, USA
FL04	CD335/NKp46	PerCP/Cy5.5	9E2	BioLegend, San Diego, CA, USA
FL04	mIgG2b	PC5.5	MPC11	BioLegend, San Diego, CA, USA
FL05	CD1c/BDCA-1	PE/Cy7	L161	BioLegend, San Diego, CA, USA
FL05	CD25/IL2RA	PC7	B1.49.9	Beckman Coulter Inc., Brea, CA, USA
FL05	CD27	PC7	1A4CD27	Beckman Coulter Inc., Brea, CA, USA
FL05	CD56	PC7	N901 (HLDA6)	Beckman Coulter Inc., Brea, CA, USA
FL05	CD123	PC7	6H6	BioLegend, San Diego, CA, USA
FL05	CD196/CCR6	PE/PC7 [PC7]	11A9	BD Biosciences, Franklin Lakes, NJ, USA
FL05	CX3CR1	PECy7	2A9-1	BioLegend, San Diego, CA, USA
FL05	Granzyme A [GrA]	PECy7	CB9	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL06	CD3	APC	Hit3a	BioLegend, San Diego, CA, USA
FL06	CD4	APC	13B8.2	Beckman Coulter Inc., Brea, CA, USA

FL06	CD19	APC	HIB19	BioLegend, San Diego, CA, USA
FL06	CD23	APC	EBVCS-5	BioLegend, San Diego, CA, USA
FL06	CD40	APC	5C3	BioLegend, San Diego, CA, USA
FL06	CD56	APC	AF12-7H3	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
FL06	CD117/cKit	APC	104D2	BioLegend, San Diego, CA, USA
FL06	CD124/IL-4R	APC	G077F6	BioLegend, San Diego, CA, USA
FL06	CD226/DNAM-1	APC	11A8	BioLegend, San Diego, CA, USA
FL06	Granzyme M [GrM]	eFluor660 [eF660]	4B2G4	eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL06	mIgG1	APC	MOPC-21	BD Biosciences, Franklin Lakes, NJ, USA
FL06	mIgG2a	APC	G155-178	BD Biosciences, Franklin Lakes, NJ, USA
FL07	CD8	APC-A700 [A700]	B9.11	Beckman Coulter Inc., Brea, CA, USA
FL07	CD11c	Alexa Fluor 700 [A700]	Bu15	BioLegend, San Diego, CA, USA
FL07	CD14	Alexa Fluor 700 [A700]	M5E2	BioLegend, San Diego, CA, USA
FL07	CD19	APC-A700 [A700]	J3-119	Beckman Coulter Inc., Brea, CA, USA
FL07	CD45RO	Alexa Fluor 700 [A700]	UCHL1	BioLegend, San Diego, CA, USA
FL07	CD127	APC-A700 [A700]	R34.34	Beckman Coulter Inc., Brea, CA, USA
FL08	CD3	APC-A750 [A750]	UCHT1	Beckman Coulter Inc., Brea, CA, USA
FL08	CD16	APC-A750 [A750]	3G8	Beckman Coulter Inc., Brea, CA, USA
FL08	CD20	APC-Fire750	2H7	BioLegend, San Diego, CA, USA
FL08	CD56	APC-A750 [A750]	N901	Beckman Coulter Inc., Brea, CA, USA
FL08	CD62-L	APC-A750 [A750]	Dreg-56	Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA
FL08	CD141/BDCA-3	APC-Vio770	AD5-14H12	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
FL08	CD294/CRTH2	APC/Cy7	BM16	BioLegend, San Diego, CA, USA
FL09	CD8	PacB	B9.11	Beckman Coulter Inc., Brea, CA, USA
FL09	CD16	PacB	3G8	Beckman Coulter Inc., Brea, CA, USA
FL09	CD21	VioBlue	HB5	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
FL09	CD39	BV421	A1	BioLegend, San Diego, CA, USA
FL09	CD45RO	BV421	UCHL1	BioLegend, San Diego, CA, USA
FL09	CD192/CCR2	BV421	K036C2	BioLegend, San Diego, CA, USA
FL09	CD303/BDCA-2	VioBlue	AC144	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
FL09	GM-CSF	BV421	BVD2-21C11	BD Biosciences, Franklin Lakes, NJ, USA
FL09	IL-4	V-450	8D4-8	BD Biosciences, Franklin Lakes, NJ, USA
FL09	mIgG1	BV421	MOPC-21	BioLegend, San Diego, CA, USA
FL09	Perforin	VioBlue	delta G9	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

FL10	CD4	BV510	OKT4	BioLegend, San Diego, CA, USA
FL10	CD14	BV510	M5E2	BioLegend, San Diego, CA, USA
FL10	CD127	BV510	A019D5	BioLegend, San Diego, CA, USA
FL10	CD146	BV510	P1H12	BD Biosciences, Franklin Lakes, NJ, USA
FL10	HLA-DR	BV510	L243	BioLegend, San Diego, CA, USA
FL10	IgM	BV510	MHM-88	BioLegend, San Diego, CA, USA

Table 2.1 Information about the antibodies employed in flow cytometry
Fluorescence channel, antigen, conjugated fluorochrome, clone and manufacturer for each antibody are listed. Abbreviations used for antigens and fluorochromes in section 3.3.4 are indicated in brackets. FL = fluorescence channel.

2.10 Software

Software	Publisher
Wave Desktop (for Seahorse data analysis)	Agilent Technologies, Inc., Santa Clara, CA, USA
GraphPad Prism 6 and 7	Graphpad Software, Inc., La Jolla, CA, USA
cyt (embedding viSNE algorithm)	Dana Pe'er lab, Columbia University, New York City, NY, USA
MATLAB	The MathWorks, Inc., Natick, MA, USA
FlowJo	FlowJo, LLC, Ashland, OR, USA
ProcartaPlex Analyst 1.0	Thermo Fisher Scientific Inc., Waltham, MA, USA
Kaluza Analysis Software 1.5a	Beckman Coulter Inc., Brea, CA, USA

3 Methods

3.1 Biological sample acquisition

In the following, the origin of the biological material used in the experiments presented in this thesis is described: On the one hand, blood and stool samples were acquired during a clinical study with MS patients, on the other hand, blood samples from HCs were collected for several *in vitro* experiments.

3.1.1 Clinical study

Between April and June 2016, 15 patients with diagnosed RRMS were included in the clinical study in the "Department of Neurology with Institute of Translational Neurology" at the University Hospital Münster. All participants signed informed consent to the planned procedures and the study was approved by the responsible ethics committee (*Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster*; registration number 2016-053-f-S, approved on 05.04.2016).

3.1.1.1 Inclusion and exclusion criteria

Table 3.1 describes the inclusion and exclusion criteria for the clinical study.

Inclusion criteria
age between 18 and 55 years
diagnosed RRMS (according to revised McDonald criteria 2010)[2]
stable immunomodulatory therapy with IFNβ preparation or glatiramer acetate since ≥ 3 months
Exclusion criteria
MS relapse within the previous 3 months
presence of another autoimmune disease
adherence to a specific diet
intake of other dietary supplements
presence of a gastrointestinal disease with malabsorption
intake of systemic glucocorticoids, immune suppressive drugs or cholestyramine
severe liver or kidney disease
status post organ transplantation
pregnancy and lactation
limited or lacking capacity for consent

Table 3.1 Inclusion and exclusion criteria for the clinical study

3.1.1.2 Study design

After their inclusion, the participants were asked to take 2,100 mg CLA (3 capsules CLA 70plus-Trofocell™, 1 : 1 ratio of the isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, refer to section 2.3 for more details) daily *per os* for a total of 24 weeks.

Prior to the treatment start, blood and stool samples were acquired. The blood samples were processed immediately isolating PMBCs which then were deep-frozen and stored in a liquid nitrogen tank at -180 °C. The stool samples were deep-frozen at -80 °C until the further analysis which is not part of this work.

[2] since study patients were recruited in 2016, the 2010 revised McDonald criteria were applied and not the 2017 revised criteria.

The patients were asked to record the intake of the study medication in a diary. Additionally, the emptied capsule packages were collected and the amount of taken capsules documented. Out of this data, the patients' adherence to the study medication was calculated in percent.

Further blood samples were acquired and processed to isolate peripheral blood mononuclear cells (PBMCs) after 8, 16 and 24 treatment weeks. After the 24 weeks treatment, also another stool sample was collected and deep-frozen.

During their treatment time the patients were asked to record all food and drink items they consumed within seven consecutive days with the dietary protocol "Freiburger Ernährung-sprotokoll" (132). The food questionnaires were analyzed to get an impression of the patient's natural, alimentary fat and CLA intake.

Fig. 3.1 summarizes the study's general structure.

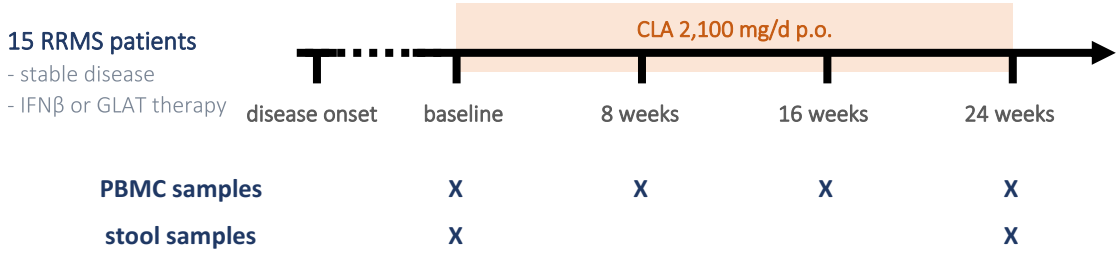

Fig. 3.1 Scheme of the clinical study setup
The diagram illustrates the general structure of the clinical study which is further explained in the text. GLAT = glatiramer acetate.

3.1.1.3 Analysis of baseline demographical and clinical data

At the baseline timepoint, relevant demographical and clinical data has been collected by approbated physicians. Age, sex, EDSS score (52), time since first MS symptoms, time since MS diagnosis and the current immunomodulatory treatment were analyzed to get an overview over the study cohort.

The EDSS score (52) was assessed another time after the study period and changes in the score were evaluated.

All MS relapses and adverse events occurring during the study period were documented and their incidence was determined.

3.1.1.4 Analysis of natural fat and CLA intake

CLA is a PUFA and occurs naturally in several foodstuffs, especially dairy products and meat (188). To investigate how much fat in general and how much CLA the study participants ingested besides the study medication as part of their alimentation, the collected food questionnaires (*n* = 14) were analyzed.

To calculate the natural total fat intake, for most of the relevant food items in the alimentary diaries (i.e. those with a not neglectable fat concentration) the best corresponding entry was chosen in the Swiss Food Composition Database, Version 5.3, published by the Federal Food Safety and Veterinary Office FSVO, Switzerland (48). It lists the average total fat contents in a wide range of food items. If there was more than one entry corresponding equally well to a food questionnaire item, the average of all corresponding entries has been used for the further calculations.

Using the value for the average total fat content and the recorded consumed amount within one week of each product, the average daily alimentary fat intake was determined for each patient.

To calculate also the natural CLA intake, for most food items with a not neglectable CLA concentration the best corresponding entries were selected in a second database, a publication by Chin *et al.* (34). The publication lists the result of the measurement of the CLA content in several food items in mg CLA / g fat. Out of the information from the two named data sources the food items' CLA content in mg CLA / 100 g food was calculated.

With this value and the recorded consumption data, the average daily alimentary CLA intake was calculated for each patient as well.

Mean and standard deviation (SD) were determined for the natural fat and CLA intake out of the cohort's values.

3.1.2 **HC samples**

For further *in vitro* experiments, blood samples were obtained from several voluntary, informed and consenting HCs. PBMCs were isolated out of the blood samples immediately to be then deep-frozen and stored in liquid nitrogen at -180 °C until the experiment.

3.2 Cell culture

3.2.1 PBMC isolation

All experiments except the gut microbiota analysis (which was part of the clinical trial but not of the present work) were performed using PBMCs. They were isolated out of venous blood anti-coagulated with EDTA.

Under sterile conditions, at room temperature, the blood was diluted with phosphate buffered saline (PBS) and then pipetted gently over a phase of Lymphoprep™ density gradient medium without mixing the two phases. During a following centrifugation step with minimal acceleration and break forces the erythrocytes and polymorphonuclear blood cells sunk to the bottom under the Lymphoprep™ medium while the PBMCs built an interphase between the Lymphoprep™ and the topmost phase of plasma. The PBMC phase was carefully extracted and the cells washed with PBS once. After resuspension in PBS the PBMC concentration was determined and the isolated number of cells calculated. The PBS was removed.

For cryopreservation, the PBMCs were resuspended in the necessary volume of CTL-C solution to reach a concentration of $2x10^7$ cells/ml. An equal volume of prepared CTL-A + B solution was then added slowly, reaching the target concentration of 10^7 cells/ml.

The cell solution was distributed into Cryo vials, deep-frozen for at least 48 h (maximum: one week) at -80 °C in a Nalgene® cryofreezing container (Mr. Frosty) to ensure a slow and steady cool down pace and then transferred into a liquid nitrogen tank at ca. -180 °C.

To achieve an enhanced comparability between the different experiments, all of them were performed with thawed and not with freshly isolated PBC samples and all used samples were stored for a minimum of two weeks at -180 °C prior to the experiment.

3.2.2 PBMC thawing

For use in the experiments the PBMC samples were rapidly thawed in a 37 °C water bath. The cell suspension was then immediately diluted with warm cell culture medium (X-Vivo 15™ medium, PBS or FCS-supplemented RPMI, depending on the experiment). After centrifugation the supernatant was discarded, and the cells resuspended in the adequate medium or buffer for the further processing.

3.2.3 Determination of cell concentrations

Two methods were employed to determine cell concentrations, manual cell counting in a hemocytometer and automatic cell counting by a CASY® Cell Counter.

3.2.3.1 Manual cell counting

For manual cell concentration determination, the cells were resuspended in a distinct volume and a sample of this suspension stained with trypan blue. Doing so, the sample was diluted in a known ratio. Using a Neubauer Improved hemocytometer the number of vital cells in a chamber of known volume was counted and the concentration and cell count in the original cell suspension calculated considering the total suspension volume, the dilution ratio, the chamber volume, and the cell count.

3.2.3.2 Automatic cell counting

To determine cell concentrations automatically, a sample of the cell suspension with a distinct volume was drawn and diluted in CASYton buffer. The cell concentration in the diluted sample was quantified in a CASY® Cell Counter measuring the electrical current exclusion (ECE®) by vital cells. The cell concentration in the original suspension was calculated by the CASY® Cell Counter considering the dilution ratio and the measured concentration in the diluted sample. The total cell count was then calculated by multiplying the concentration with the original suspension's volume.

3.2.4 Adjustment of cell concentrations

If a specific number of cells was to be used in an experiment, one of the two following methods was applied.

If the volume in which the requested number of cells was suspended was of no relevance (e.g. since the following procedure was a wash step), the volume containing the needed number of cells was calculated and drawn from the original cell suspension.

If, however, the volume containing the necessary cell number had to be specific (e.g. for seeding the cells in a cell culture) either an adequate volume of the same medium or buffer to reach the

target concentration was added to the cell suspension or the sample was centrifugated, the supernatant decanted and the cells resuspended in an appropriate volume of buffer or medium to reach the target concentration.

3.2.5 CD4$^+$ T cell isolation

In the experiments in which only CD4$^+$ T cells were analyzed those were isolated out of PBMC samples using positive selecting magnetic-activated cell sorting (MACS®).

Performing this method, the target cells are labeled with MACS® MicroBeads, magnetic nanoparticles conjugated with specific antibodies for cell surface markers expressed only on the cells to be isolated. While a strong magnetic field retains the labeled cells, all other particles can be washed out. After removing the magnetic field, the target cells can be eluted (see (118) for basic methodological approach).

After centrifugation and decanting of the supernatant, the PBMCs were resuspended in MACS® buffer and incubated with MACS® CD4 MicroBeads at 4 °C for at least 10 min. After a wash step they were resuspended in MACS® buffer and added to the prepared MACS® column on the magnetic MACS® separator. The column was washed three times to remove all unlabeled cells. After taking the column off the separator, the labelled CD4$^+$ T cells were eluted with MACS® buffer and further processed.

3.2.6 Cell activation

To not only observe the analyzed cells' basal state but to also measure their reactions upon confrontation with stimulating factors, they were activated with different methods in several experiments.

3.2.6.1 PHA activation

In all experiments analyzing the cells' cytokine production capabilities as well as in the cytotoxicity analysis for CLA the respective cells were exposed to 5 µg/ml phytohemagglutinin-L (PHA) in the cell culture medium for 72 h. In all these experiments, however, negative, not activated controls for all treatment groups and biological replicates were examined as well.

3.2.6.2 LAC activation

Cell activation with LAC was employed only in the context of the flow cytometry measurements and is described in section 3.3.4.5.

3.2.6.3 PMA / ionomycin activation

In the experiments analyzing the cell metabolism of *in vivo* treated samples, a short-time activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin was applied. Since this activation was part of the Agilent Seahorse XF assay itself, it is further described in the correspondent section 3.3.5.

3.2.6.4 anti-CD3 and anti-CD28 activation

In contrast, prior to the analysis of the metabolism of *in vitro* treated $CD4^+$ T cells, those were activated during their treatment time with antibodies against the surface proteins CD3 and CD28. This activation method is further described in the context of the methods employed for the metabolism analyses in section 3.3.5.

3.2.7 ***in vitro* CLA Treatment**

For *in vitro* CLA treatment the two isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, constituting together almost 100% of the naturally consumed CLA (188), were added to the cell culture media. The indicated concentrations (25 µM, 50 µM or 75 µM) in this work always refer to the concentration of each of these two isomers in the cell culture medium. The cells were exposed to CLA *in vitro* for 72 h (cytotoxicity assay, cytokine production analyses) or 24 h (metabolism experiments). An untreated negative control group was employed in every experiment with an *in vitro* CLA treatment.

3.3 Analytical methods

3.3.1 **Enzyme-linked immunosorbent assay (ELISA)**

Direct sandwich ELISA was performed to measure the concentrations of cytokines in cell culture supernatants.

The method is based on specific capture and detection antibodies binding different epitopes of the target cytokine. While the capture antibody is coated to the bottom of a 96-well microtiter plate and therefore retains the bound substance in the well during the following wash steps, the detection antibody is biotinylated. Therefore, it binds horseradish peroxidase (HRP)-conjugated streptavidin. The HRP catalyzes a reaction of its substrate TMB with a color development which can be quantified by measuring the spectral absorbance at 450 nm wavelength. The absorbance is then compared between the samples and a set of serially diluted standards with known cytokine concentration to achieve absolute concentration values as a result (see (129) and (103) for basic methodological approach).

Ready-SET-Go!™ ELISA kits were used to measure the concentration of TNFα and IFNγ in cell culture supernatants.

One day prior to the assay the 96-well microtiter plates were coated with the respective coating antibody and incubated overnight.

On the day of the assay, all plates were washed and then blocked for at least 1 h.

After another washing step the samples, as well as the serially diluted standards and blanks only containing cell culture medium, were added to the wells on the TNFα plates. After 2 h incubation time the cell culture supernatants were transferred gently to the before washed IFNγ plates on which also the respective standard series and blanks were pipetted. The IFNγ plates were incubated for 2 h.

After their respective sample incubation step, the plates were washed, the adequate detection antibody was added, and the plates were incubated for 1 h, followed by another wash step. After adding Avidin-HRP the plates were incubated for another 30 min.

The plates were washed and then incubated with the TMB substrate. The proceeding reaction was stopped after a visually judged time by adding ELISA stop solution. The absorbance was then measured at 450 nm.

Analyzing the results, the cytokine concentrations in the samples was extrapolated via a standard curve calculated out of the absorbance values of the serially diluted standards and blanks.

3.3.2 Luminex® assay

The Luminex® xMAP® technology was used to measure the concentration of several cytokines in a single reaction volume of cell culture supernatant simultaneously.

Similar to an ELISA, the technology is based on the binding of specific capture and detection antibodies to the analyte. However, the capture antibodies are not coated to the well bottom, but to fluorescence-labelled magnetic microspheres (beads). For each analyte one specific type of beads is employed, having a distinct color and being coated with only the respective capture antibody. After binding the analyte to the capture antibody, biotinylated detection antibodies are added which then first bind to the analyte as well, and then also to the further added Streptavidin-PE (see (75) for basic methodological approach).

The samples are then analyzed with a MAGPIX® instrument which is based on quantitative fluorescence microscopy. While a magnetic field holds the beads in a monolayer, LED inside the instrument excite the classification (bead dyes) and reporter (PE) fluorescent molecules. With a CCD imager the emitted fluorescence signals from the classification and the reporter fluorochromes are quantified as mean fluorescence intensity for each bead (see (75) for basic methodological approach).

With this information, and by measuring a standard series with all analyzed cytokines and blanks as well, via standard curves, the concentration of all analytes in the samples can be calculated (see (75) for basic methodological approach).

According to the manufacturer's instructions, the experiments were performed using a handheld magnetic plate washer which retains the magnetic microspheres in the wells during the wash steps. All incubation steps were performed in darkness, the plates were sealed before and gently agitated during the incubation period.

First, the magnetic beads were added to the wells and washed with wash buffer. Next, the cell culture supernatant samples as well as the prepared standard series and blanks (cell culture medium only) were added to the wells. The plates were incubated for 2 h.

After two wash steps, the detection antibody mixture was added to the wells and incubated for 30 min. The plates were then washed twice again before adding streptavidin-PE, which was incubated for 30 min.

Two more wash steps were performed. Then, reading buffer was added to the wells and after additional 5 min incubation time, the plates were read on the MAGPIX® instrument.

Two Luminex® assays were performed. In the first one, analyzing *in vitro* treated samples, the concentration of 18 cytokines (IFNγ, TNFα, GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27) was measured with a "Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human ProcartaPlex™ Panel".

The second one analyzed samples from before and after an *in vivo* CLA treatment. Therefore, the concentrations of ten different cytokines (IFNγ, TNFα, GM-CSF, IL-1β, IL-2, IL-4, IL-10, IL-17A, IL-21, IL-22) were determined with a "ProcartaPlex Mix&Match Human 10-plex" kit.

In the second experiment, cytokine concentration values had to be excluded from further analysis if the cytokine production profile clearly indicated technical problems during the experiment.

3.3.3 Propidium iodide (PI) staining

Cell staining with PI and analysis by flow cytometry were performed to measure the portion of living and dead cells.

PI is a fluorochrome intercalating with DNA. Lacking the ability to cross the cell membrane of living cells, PI can only be detected in dead cells characterized by a damaged membrane. By analysis with flow cytometry, this property can be used to measure the amount of living and dead cells in a sample (see (41) for underlying methodological principles).

Therefore, the cells were washed with MACS® buffer and incubated with the PI staining solution (1.25 µg/ml) for 10 min on ice. The staining process was then stopped by adding MACS® buffer which diluted the PI in the reaction volume. The samples were then measured in a flow cytometer. During the analysis a cut-off value for the fluorescence intensity was determined and cells exhibiting a higher fluorescence intensity were considered dead.

3.3.4 Flow cytometry

Ten color flow cytometry analyses were performed to gain a profound insight into the immune cell subset composition in the examined blood samples.

Investigation subjects were PBMC samples from all 15 study patients collected before and after 8, 16 and 24 weeks of CLA *in vivo* treatment.

All 60 samples were analyzed using a total of 14 different staining panels to assess different branches of the immune system. For four of these panels, all samples were additionally stained with a respective staining panel replacing some of the antibodies by their respective isotype control antibodies to enable a more accurate identification of specific immune cell subsets in the ensuing data analysis.

The exact procedure in the staining process differed slightly depending on the staining panel considering the different targets of the staining antibodies and their different requirements for optimal binding.

However, for all staining panels, deep-frozen PBMCs were thawed and their concentration was determined. Depending on the panel, 2 x 10^5 or 5 x 10^5 cells were withdrawn (cell number specified in Table 3.2 - Table 3.6) and further processed as described below.

Besides the stained cell numbers, Table 3.2 - Table 3.6 indicate the used antibodies for each panel sorted by the fluorescence channel (FL01 - FL10) in which their fluorescence could be best detected as well as the volume of each antibody concentrate added to FACS buffer or permeabilization working solution per 1 ml working staining solution.

3.3.4.1 Surface staining at 4 °C

For the staining panels in Table 3.2, the PBMCs were stained with 100 µl antibody staining mixture for 30 min at 4 °C. After washing with FACS buffer, the stained cells were analyzed by flow cytometry, diluted in FACS buffer.

Panel PBMC count	**FL01**	**FL02**	**FL03**	**FL04**	**FL05**	**FL06**	**FL07**	**FL08**	**FL09**	**FL10**
Basic $2x10^5$	10 µl **CD1c**-FITC	5 µl **CD69**-PE	5 µl **HLA-DR**-ECD	5 µl **CD3**-PC5.5	5 µl **CD56**-PC7	5 µl **CD4**-APC	5 µl **CD19**-A700	5 µl **CD16**-A750	5 µl **CD8**-PacB	10 µl **CD14**-BV510
T dev $2x10^5$	10 µl **CD45RO**-FITC	10 µl **CD27**-PE	10 µl **CD3**-ECD	10 µl **CD8**-PC5.5	5 µl **CD25**-PC7	5 µl **CD56**-APC	10 µl **CD127**-A700	5 µl **CD62L**-A750	5 µl **CD39**-BV421	10 µl **CD4**-BV510
CD8 $2x10^5$	5 µl **CD45RA**-FITC	20 µl **CD57**-PE	5 µl **CD31**-Dazzle	5 µl **CD56**-PC5.5	5 µl **CD27**-PC7	10 µl **CD226**-APC	5 µl **CD45RO**-A700	5 µl **CD3**-A750	5 µl **CD8**-PacB	10 µl **CD4**-BV510
B cell $5x10^5$	10 µl **anti-IgD**-FITC	10 µl **CD80**-PE	10 µl **CD24**-ECD	5 µl **CD38**-PC5.5	5 µl **CD27**-PC7	5 µl **CD23**-APC	5 µl **CD19**-A700	10 µl **CD20**-APC-Fire750	5 µl **CD21**-VioBlue	5 µl **IgM**-BV510
DC $5x10^5$	10 µl **CD25**-FITC	10 µl **IL-15Ra**-PE	5 µl **HLA-DR**-ECD	10 µl **CD86**-PC5.5	5 µl **CD1c**-PE/Cy7	**LinX**-APC = 5 µl **CD3**-APC + 5 µl **CD19**-APC + 5 µl **CD56**-APC	10 µl **CD11c**-A700	5 µl **CD141**-APC-Vio770	10 µl **CD303**-VioBlue	10 µl **CD14**-BV510
DC iso $5x10^5$	10 µl **mIgG2a**-FITC	10 µl **mIgG2b**-PE	5 µl **HLA-DR**-ECD	10 µl **mIgG2b** -PC5.5	5 µl **CD1c**-PE/Cy7	**LinX**-APC = 5 µl **CD3**-APC + 5 µl **CD19**-APC + 5 µl **CD56**-APC	10 µl **CD11c**-A700	5 µl **CD141**-APC-Vio770	10 µl **CD303**-VioBlue	10 µl **CD14**-BV510
ILC $5x10^5$	10 µl **Lin-3**-FITC		10 µl **CD56**-ECD	20 µl **NKp46**-PerCP/Cy5.5	10 µl **CD123**-PC7	10 µl **CD117**-APC	10 µl **CD11c**-A700	10 µl **CRTH2**-APC/Cy7	10 µl **CD16**-PacB	20 µl **CD127**-BV510

Table 3.2 Employed PMBC cell counts and antibodies in the 4 °C surface staining panels
The table indicates the number of PBMCs and the volume of antibody concentrate (per 1 ml working staining solution) used in each staining panel. The staining solution was based of FACS buffer. T dev = T development. iso = isotype antibody panel. FL = fluorescence channel.

3.3.4.2 Surface staining at 37 °C

The procedure was very similar for the staining panels in Table 3.3. After staining with 100 µl antibody staining mixture for 30 min at 37 °C, the PBMCs also were washed with FACS buffer and analyzed in the flow cytometer, diluted in FACS buffer.

Panel PBMC count	**FL01**	**FL02**	**FL03**	**FL04**	**FL05**	**FL06**	**FL07**	**FL08**	**FL09**	**FL10**
T_H1 $2x10^5$	5 µl **CD183**-FITC	10 µl **CD195**-PE	5 µl **CD8**-ECD	5 µl **CD194**-PC5.5	5 µl **CD196**-PC7	5 µl **CD56**-APC	10 µl **CD14**-A700	5 µl **CD3**-A750	10 µl **CD45RO**-BV421	10 µl **CD4**-BV510
T_H17 $2x10^5$	10 µl **CD161**-FITC	20 µl **IL-23R**-PE	10 µl **CD56**-ECD	5 µl **CD194**-PC5.5	5 µl **CD196**-PC7	10 µl **CD4**-APC	5 µl **CD8**-A700	5 µl **CD3**-A750	10 µl **CD45RO**-BV421	10 µl **CD146**-BV510
T_H17 iso $2x10^5$	10 µl **CD161**-FITC	20 µl **mIgG2b**-PE	10 µl **CD56**-ECD	5 µl **CD194**-PC5.5	5 µl **CD196**-PC7	10 µl **CD4**-APC	5 µl **CD8**-A700	5 µl **CD3**-A750	10 µl **CD45RO**-BV421	10 µl **CD146**-BV510
Mono 2 $5x10^5$	20 µl **CD206**-FITC	10 µl **CD200R**-PE	2 µl **CD16**-ECD	20 µl **CD163**-PerCP/Cy5.5	3.3 µl **CX3CR1**-PECy7	10 µl **CD124**-APC	10 µl **CD14**-A700	5 µl **CD56**-A750	3.3 µl **CD39**-BV421	10 µl **HLA-DR**-BV510
Mono 2 iso $5x10^5$	20 µl **mIgG1**-FITC	10 µl **mIgG1**-PE	2 µl **CD16**-ECD	20 µl **CD163**-PerCP/Cy5.5	3.3 µl **CX3CR1**-PECy7	10 µl **mIgG2a** -APC	10 µl **CD14**-A700	5 µl **CD56**-A750	3.3 µl **mIgG1**-BV421	10 µl **HLA-DR**-BV510

Table 3.3 Employed PMBC cell counts and antibodies in the 37 °C surface staining panels
The table indicates the number of PBMCS and the volume of antibody concentrate (per 1 ml working staining solution) used in each staining panel. The staining solution was based of FACS buffer. Mono = monocyte. iso = isotype antibody panel. FL = fluorescence channel.

3.3.4.3 Combined surface and intracellular staining at 4 °C

With the panels in Table 3.4 both surface and intracellular markers were stained.

First, the surface staining was performed. Therefore, the PBMCS were incubated for 30 min at 4 °C with 100 µl of the FACS buffer based surface staining solution which contained only the antibodies indicated in the white cells of Table 3.4.

After washing them with FACS buffer, the cells were incubated for 20 min at 4 °C in permeabilization / fixation working solution. Next, after washing with permeabilization working solution, the cells were stained with 100 µl permeabilization working solution based staining mixture containing only the antibodies for intracellular staining, named in the gray cells of Table 3.4, for 30 min at 4 °C.

Then, after washing with FACS buffer, the cells were resuspended in FACS buffer and analyzed with the flow cytometer.

Panel PBMC count	**FL01**	**FL02**	**FL03**	**FL04**	**FL05**	**FL06**	**FL07**	**FL08**	**FL09**	**FL10**
T_{reg} $5x10^5$	25 µl **Helios**-FITC	10 µl **FOXP3**-PE	5 µl **CD4**-ECD	20 µl **CTLA-4**-PeF710	10 µl **CD25**-PC7	10 µl **CD56**-APC	5 µl **CD8**-A700	5 µl **CD3**-A750	10 µl **CD45RO**-BV421	5 µl **CD127**-BV510
Granzymes $5x10^5$	20 µl **GrK**-FITC	5 µl **GrB**-PE	20 µl **CD56**-ECD	5 µl **CD45RO**-PerCP/Cy5.5	5 µl **GrA**-PECy7	10 µl **GrM**-eF660	5 µl **CD8**-A700	5 µl **CD3**-A750	10 µl **Perforin**-VioBlue	10 µl **CD4**-BV510

Table 3.4 Employed PMBC cell counts and antibodies in the 4 °C combined surface and intracellular staining panels
The table indicates the number of PBMCS and the volume of antibody concentrate (per 1 ml working staining solution) used in each staining panel. Two staining solutions were prepared for each panel: The surface staining solution was based on FACS buffer and contained the dyed antibodies in the white table cells. The intracellular staining solution was based on permeabilization working solution and contained the antibodies indicated in the gray table cells. FL = fluorescence channel.

3.3.4.4 Combined surface and intracellular staining at 37 °C / 4 °C

Similarly, the staining for the panels indicated in Table 3.5 was conducted. After staining with 100 µl surface staining solution for 30 min at 37 °C, the cells were washed with FACS buffer and then incubated with permeabilization / fixation working solution for 20 min at 4 °C. After the following washing with permeabilization working solution, 100 µl of the intracellular staining mixture were added and the cells incubated for 30 min at 4 °C. Thereafter, the cells were washed with and then resuspended in FACS buffer for analysis by flow cytometry.

Panel PBMC count	**FL01**	**FL02**	**FL03**	**FL04**	**FL05**	**FL06**	**FL07**	**FL08**	**FL09**	**FL10**
Mono 1 $5x10^5$	20 µl **CD80**-FITC	10 µl **CD195**-PE	2 µl **CD16**-ECD	5 µl **CD68**-PerCP/Cy5.5		10 µl **CD40**-APC	10 µl **CD14**-A700	5 µl **CD56**-A750	3.3 µl **CD192**-BV421	10 µl **HLA-DR**-BV510
Mono 1 iso $5x10^5$	20 µl **mIgG1**-FITC	10 µl **rIgG2b** -PE	2 µl **CD16**-ECD	5 µl **mIgG2b**-PC5.5		10 µl **mIgG1**-APC	10 µl **CD14**-A700	5 µl **CD56**-A750	3.3 µl **CD192**-BV421	10 µl **HLA-DR**-BV510

Table 3.5 Employed PMBC cell counts and antibodies in the 37 °C / 4 °C combined surface and intracellular staining panels
The table indicates the number of PBMCS and the volume of antibody concentrate (per 1 ml working staining solution) used in each staining panel. Two staining solutions were prepared for each panel: The surface staining solution was based on FACS buffer and contained the dyed antibodies in the white table cells. The intracellular staining solution was based on permeabilization working solution and contained the antibodies indicated in the gray table cells. Mono = monocyte. iso = isotype antibody panel. FL = fluorescence channel.

3.3.4.5 Combined surface and intracellular staining at 4 °C after LAC stimulation

Finally, to analyze the cytokine production capacity of the immune cells, they were also measured after activation with LAC with a combined surface marker and intracellular staining.

For this, the cells were, after thawing, incubated overnight at 37 °C in a 5% CO_2 atmosphere in 100 µl X-Vivo 15™ medium. The medium was then changed into 100 µl X-Vivo 15™ medium supplemented with 1 µl LAC and the cells incubated another 4 h under the same conditions.

For the surface staining, the PBMCS were then incubated with 100 µl surface staining solution for 30 min at 4 °C. The staining mixture was prepared as described in Table 3.6. After a wash step with FACS buffer, the cells were then resuspended in permeabilization / fixation working solution and incubated for 20 min at 4 °C. Next, they were washed with permeabilization working solution and stained by incubating with 100 µl intracellular staining mixture for 30 min at 4 °C. Table 3.6 indicates the composition of the intracellular staining solution.

After washing with FACS buffer, the cells were analyzed by flow cytometry, resuspended in FACS buffer.

Panel PBMC count	**FL01**	**FL02**	**FL03**	**FL04**	**FL05**	**FL06**	**FL07**	**FL08**	**FL09**	**FL10**
T_H stim 1 $5x10^5$	25 µl **IFNγ**-FITC	25 µl **IL-17A**-PE	5 µl **CD4**-ECD	5 µl **CD45RO**- PerCP/Cy5.5		10 µl **CD56**- APC	5 µl **CD8**- A700	5 µl **CD3**- A750	5 µl **GM-CSF**- BV421	10 µl **CD146**- BV510
T_H stim 2 $5x10^5$	10 µl **TNFα**- FITC	25 µl **IL-22**-PE	5 µl **CD4**-ECD	5 µl **CD45RO**- PerCP/Cy5.5		10 µl **CD56**- APC	5 µl **CD8**- A700	5 µl **CD3**- A750	5 µl **IL-4**- V-450	10 µl **CD146**- BV510

Table 3.6 Employed PMBC cell counts and antibodies in the 4 °C combined surface and intracellular staining panels after LAC stimulation

The table indicates the number of PBMCS and the volume of antibody concentrate (per 1 ml working staining solution) used in each staining panel. Two staining solutions were prepared for each panel: The surface staining solution was based on FACS buffer and contained the dyed antibodies in the white table cells. The intracellular staining solution was based on permeabilization working solution and contained the antibodies indicated in the gray table cells. stim = stimulation. FL = fluorescence channel.

3.3.4.6 Data analysis

The resulting flow cytometry raw data was analyzed with two methods independently from each other.

First, using the Kaluza Analysis software, after appropriate compensation, classical flow cytometry gating strategies were applied to the datasets manually to identify and quantify distinct known and described immune cell populations. Table 3.7 lists the defining marker combinations applied to identify the immune cell subsets which are the subject of the y-scatter plots in section 4.2.4. Out of the resulting numbers of cells their percentage in superordinate subsets could be calculated. The changes of these portions are illustrated in the y-scatter plots in section 4.2.4.

Immune cell subset	Defining marker combination
Fig. 4.9	
$CD4^+$ memory cells	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$
$CD4^+$ memory cells $CD146^+$ (T_H17 cells)	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ $CD146^+$
$CD4^+$	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$
$CD4^+$ memory cells $CD194^+$ $CD196^+$ $CD161^+$ $CD146^+$ (T_H17 cells)	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ $CD194^+$ $CD196^+$ $CD161^+$ $CD146^+$
$CD4^+$ memory cells IL-$23R^+$	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ IL-$23R^+$
Fig. 4.11	
$CD4^+$	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$
$CD4^+$ IL-17A	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ IL-$17A^+$
$CD4^+$ IL-22	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ IL-22^+
$CD4^+$ memory cells	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$
$CD4^+$ memory cells IL-17A	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ IL-$17A^+$
$CD4^+$ memory cells IL-22	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ IL-22^+
Fig. 4.13	
$CD8^+$	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$
$CD8^+$ $CD226^+$	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD226^+$
$CD8^+$ central memory cells	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD27^+$ $CD45RA^-$
$CD8^+$ central memory cells $CD226^+$	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD27^+$ $CD45RA^-$ $CD226^+$
$CD8^+$ T_{EMRA}	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD27^-$ $CD45RA^+$
$CD8^+$ T_{EMRA} $CD226^+$	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD27^-$ $CD45RA^+$ $CD226^+$
Fig. 4.15	
$CD8^+$	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$
$CD8^+$ IL-17A	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ IL-$17A^+$
$CD8^+$ IL-22	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ IL-22^+
$CD8^+$ memory cells	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD45RO^+$
$CD8^+$ memory cells IL-17A	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD45RO^+$ IL-$17A^+$
$CD8^+$ memory cells IL-22	$CD3^+$ $CD56^-$ $CD4^-$ $CD8^+$ $CD45RO^+$ IL-22^+
Fig. 4.16	
B cells	$CD19^+$ $CD20^+$
naïve ($CD27^-$) B cells	$CD19^+$ $CD20^+$ $CD27^-$
memory ($CD27^+$) B cells	$CD19^+$ $CD20^+$ $CD27^+$

Fig. 4.18	
$CD4^+$	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$
T_{reg} cells	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ $CD127^{low}$ $CD25^+$
pT_{reg} cells	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ $CD127^{low}$ $CD25^+$ $FoxP3^+$ $HELIOS^-$
tT_{reg} cells	$CD3^+$ $CD56^-$ $CD4^+$ $CD8^-$ $CD45RO^+$ $CD127^{low}$ $CD25^+$ $FoxP3^+$ $HELIOS^+$

Table 3.7 Defining marker combinations applied to identify the immune cell subsets which are the subject of the y-scatter plots in section 4.2.4

Data points from the functional stimulation panels employing cytokine staining after LAC stimulation were excluded from this analysis if the measured values clearly indicated that the cell activation had not been successful. In these cases, the reduced number of study subjects is indicated in the figure legends of the related graphs in section 4.2.4.

Second, and independently from the classical manual gating analysis, unsupervised machine learning technologies (159) were applied to the raw data from the most interesting staining panels ("T development", "CD8", "B cell", "T_H17", "T_{reg}", "T_H stim 1", "T_H stim 2") for an additional, less biased (159) analysis. This work was performed in collaboration with working group colleagues.

Two recently developed and published algorithms were the core of this automated analysis, visual stochastic network embedding (viSNE) and PhenoGraph. viSNE (6) is an application of the t-distributed stochastic neighbor embedding (t-SNE) algorithm (184) optimized for the computation of high dimensional single cell data as for example generated by flow cytometry. It is a non-linear dimensionality reduction algorithm which interprets every measured cell as a data point in high-dimensional space with each dimension representing one measured variable. Its aim is to map all datapoints individually (single cell resolution) onto a two-dimensional plot yet preserving the complex organization of the measured cells in high-dimensional space as far as possible. The similarity of each pair of cells is calculated based on their Euclidian distance in high dimensional space and the 2D graph is created by approximating that distribution of the data points on the graph which best reproduces these pairwise similarities between each couple of data points. Thus, cells which appear similar (i.e. which are close) in high dimensional space are mapped in vicinity of each other on the 2D graph, the so-called viSNE map (6). Further details on the methodology of the viSNE and the underlying t-SNE algorithm can be found in the publications (6) and (184), respectively.

PhenoGraph is a clustering algorithm aiming to identify clusters of cells (i.e. cells with a similar phenotype) in high dimensional space. To this end, it identifies a specified number of nearest neighbors of each cell based on their Euclidean distance in high dimensional space and subsequently maps the cells based on the number of shared nearest neighbors between each pair of cells (100). With the Louvain community detection method (25), individual clusters are then identified (100).

In practice, after appropriate compensation, the data was normalized using an algorithm developed to decrease the sample-to-sample acquisition variability (50). Next, a manual pre-gating step using FlowJo software was applied to the data in order to extract only those cells from the data sets which were specifically meant to be investigated by each staining panel (e.g. in the compensated data from the "B cell" panel, B cells were pre-gated and only their flow cytometry data was exported and subsequently analyzed with viSNE and PhenoGraph). The figure legends of the viSNE maps in section 4.2.4 indicate which cell population was pre-gated prior to the automated analysis for each staining panel, respectively.

Next, all samples were subsampled to avoid the problem of "crowding". If the number of cells analyzed by the viSNE algorithm is too high, data points which are far away from each other in high dimensional space tend to collapse and aggregate resulting in a single large area of the 2D plot without segregation of populations (6). The number of cells which were analyzed by the viSNE and PhenoGraph algorithms after subsampling and merging (see next step) is indicated in the respective figure legends in section 4.2.4.

Then, the subsampled cells from all patients at both analyzed treatment conditions (baseline, 24 weeks CLA treatment) were merged to be then analyzed together. Otherwise, the two algorithms would analyze each sample / condition individually, resulting in multiple viSNE maps and cell cluster structures which could not easily be compared with each other. However, it was kept track of which cell in the merged file originates from which treatment condition.

Next, the viSNE algorithm was run using the interactive visualization tool *cyt* written in MATLAB (6). Clustering with the PhenoGraph algorithm, implemented in MATLAB (100), was applied to identify distinct cell populations.

Then, the merged data was divided in two parts based on the sample origination from before or after the 24 weeks CLA *in vivo* treatment. Thus, two viSNE maps were generated for each analyzed staining panel and the datapoints colored based on their affiliation to the cell populations identified by PhenoGraph. These viSNE maps comparing the baseline and post treatment state are shown in section 4.2.4.

Cell clusters which have been substantially up- or downregulated during the CLA *in vivo* treatment were identified and the fold change of their size is indicated in the figures. Based on the marker expression of the cells in these clusters, these populations were assigned names of known immune cell subsets applying expert knowledge.

3.3.5 Metabolism analysis

$CD4^+$ T cells‘ metabolism was examined using Agilent Seahorse XF technology.

This method allows to analyze the OXPHOS and glycolysis in a cell sample label-free. It is mainly based on the measurement of two parameters: The oxygen consumption rate (OCR) indicates how fast oxygen is consumed and correlates with the activity of OXPHOS in the cells, which is the main metabolic pathway consuming oxygen and the last step in aerobic ATP generation. The extracellular acidification rate (ECAR) quantifies how fast the acidity in the cell culture medium is increased, mainly by the production of lactate in glycolysis (2, 3).

OCR and ECAR can be measured during a so-called Mito Stress Test for two reasons: First, this allows to distinguish between the oxygen consumption by OXPHOS and the non-mitochondrial oxygen consumption. Second, in this way it is possible to not only measure the basal respiration (BR) but also the maximal respiration (MR) and the spare respiratory capacity (SRC) regarding the aerobic metabolic pathways, and not only the basal glycolysis (BG) but also the glycolytic capacity (GC) and the glycolytic reserve (GR) regarding the anaerobic ATP generation (see (2) and (3) for underlying methodological principles).

The theoretic background of the investigation of the OXPHOS in a Mito Stress Test, which is explained hereinafter, is illustrated in Fig. 3.2.

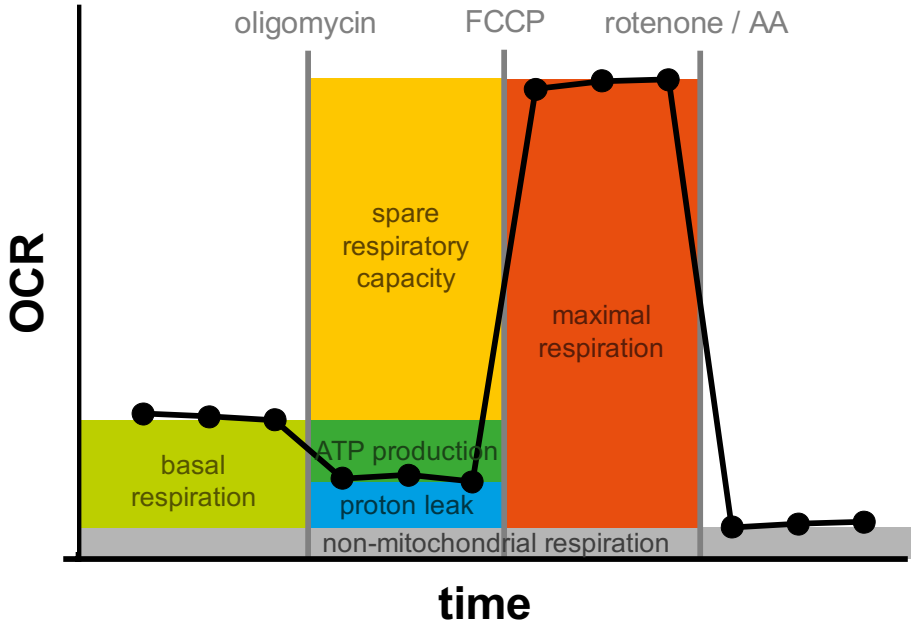

Fig. 3.2 Idealized progression of the OCR during a Mito Stress Test
The graph shows a theoretic OCR curve progression during a Mito Stress Test in which oligomycin, FCCP and rotenone / antimycin A are added as indicated. Each dot represents one measurement (3). The calculation of the parameters for the OXPHOS is visualized in the graph and further explained in Table 3.8. AA = antimycin A. Graphic based on "Figure 1. Agilent Seahorse XF Cell Mito Stress Test profile, showing the key parameters of mitochondrial function" in Agilent Seahorse XF Cell Mito Stress Test Kit User Guide (3).

The Mito Stress Test is performed by adding subsequently oligomycin, then FCCP and then simultaneously rotenone and antimycin A. The targets of these substances in the OXPHOS are illustrated in Fig. 3.3 (3).

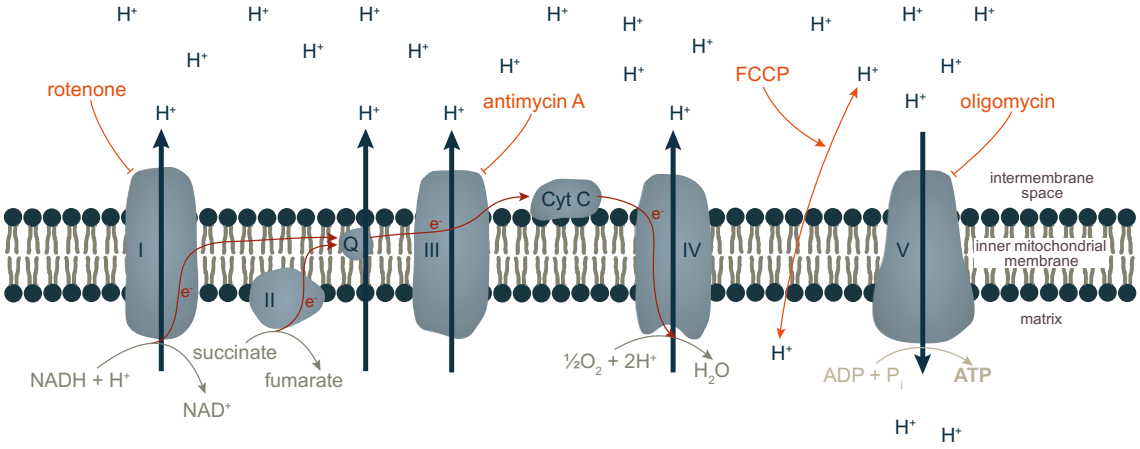

Fig. 3.3 Schematic diagram of the OXPHOS and targets of the Mito Stress Test substances
The complexes of the OXPHOS are situated at the inner mitochondrial membrane. The complexes I to IV, coenzyme Q (ubiquinone) and cytochrome c form the electron transport chain (ETC) which transports electrons from the reducing equivalents generated in the citric acid cycle to oxygen. The oxygen, the accepted electrons and surrounding protons finally react to form water. The energy released by this exergonic electron transport is used by complexes I, III, IV and coenzyme Q to carry protons across the inner mitochondrial membrane into the intermembrane space. This establishes an electrochemical gradient across the inner mitochondrial membrane. From the intermembrane space, the protons flow back following the gradient through complex V which uses this exergonic process to synthesize ATP out of adenosine diphosphate (ADP) and phosphate (27). Oligomycin blocks complex V while rotenone and antimycin A block complexes I and III, respectively. FCCP is an uncoupling protein which allows protons to freely cross the inner mitochondrial membrane aside from the complexes of the OXPHOS (3). Complex I = NADH-coenzyme Q oxidoreductase, complex II = succinate-coenzyme Q oxidoreductase, complex III = coenzyme Q-cytochrome c oxidoreductase, complex IV = cytochrome c oxidase, complex V = ATP synthase, Cyt C = cytochrome C, Q = coenzyme Q = ubiquinone. Graphic based on “Figure 2. Agilent Seahorse XF Cell Mito Stress Test modulators of the ETC“ in Agilent Seahorse XF Cell Mito Stress Test Kit User Guide (3).

Before adding any of these substances, oxygen is consumed by two processes: First, complex IV of the ETC consumes oxygen to enable the transport of protons from the mitochondrial matrix into the intermembrane space by complex I, coenzyme Q, complex III and complex IV. This basal oxygen consumption of complex IV is referred to as the BR. Second, oxygen is consumed by other processes not located in the mitochondria (non-mitochondrial respiration, NMR) (3).

Oligomycin blocks the ATP synthase (complex V of the ETC). As long as the ETC is completely working, the protons in the intermembrane space flow back into the matrix through complex V which uses the electrochemical gradient driving this flow to generate ATP. If complex V is blocked, the protons accumulate in the intermembrane space. This increases the electrochemical gradient against which complex IV must work resulting in a reduction of the complex IV activity and therefore of the OCR (3).

Nevertheless, also after reaching a steady state, oxygen is further consumed, what cannot be attributed to the aerobic ATP production since complex V is blocked. Two mechanisms are responsible for the remaining oxygen consumption: First, some protons can still flow back from

the intermembrane space to the matrix through the inner mitochondrial membrane bypassing complex V. This causes complex IV to not completely stop working and therefore to remain consuming oxygen (OCR attributed to the proton leak). The second mechanism is the NMR described above. The decrease in the OCR after oligomycin addition is attributed to the aerobic ATP production (3).

FCCP is an uncoupling protein. It is a proton channel that is inserted in the inner mitochondrial membrane and lets the protons in the intermembrane space flow back into the matrix bypassing complex V following the electrochemical gradient very fast. The rapid depletion of the proton gradient causes complex IV to work as fast as possible eliciting the highest possible OCR. This resulting oxygen consumption is composed of the MR and the NMR (3, 116).

rotenone and antimycin A block complexes I and III of the ETC, respectively. Hence, no electrons are transported to complex IV which therefore cannot work as well and consumes no oxygen. The remaining oxygen consumption is caused by the NMR only (3).

By measuring the OCR during the Mito Stress Test, the parameters NMR, BR, MR and SRC can be calculated according to the formulas listed in Table 3.8 (1).

Parameter	Calculation
non-mitochondrial respiration (NMR)	lowest OCR after rotenone / antimycin A injection
basal respiration	(last OCR before oligomycin injection) – NMR
maximal respiration	(highest OCR after FCCP injection) – NMR
spare respiratory capacity	(maximal respiration) – (basal respiration)

Table 3.8 Calculation of OXPHOS activity parameters
The activity parameters of OXPHOS were calculated as stated according to the manufacturer's recommendations (1).

Since the analyzer measures the ECAR simultaneously, information about the glycolytic activity of the cells can be generated in the same setup (2). A theoretic ECAR curve progression during a Mito Stress Test and the calculation of the glycolysis parameters are featured in Fig. 3.4 and further explained below (see (2) for underlying methodological principles).

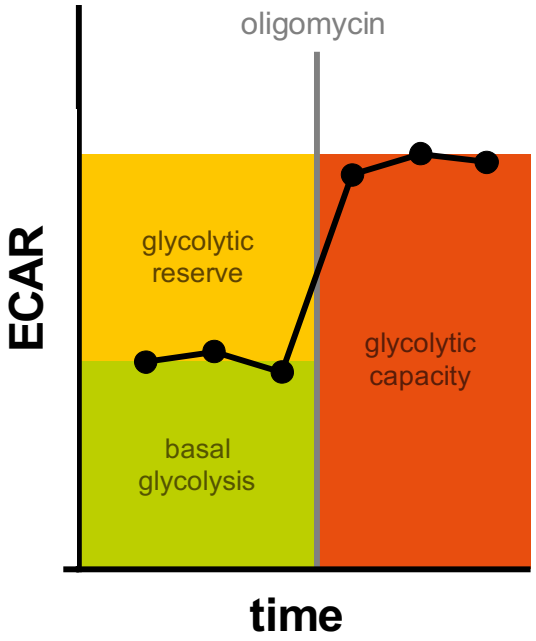

Fig. 3.4 Idealized progression of the ECAR during a Mito Stress Test
The graph shows a theoretic ECAR curve progression during a Mito Stress Test in which oligomycin is added as indicated. Each dot represents one measurement (see (2) for underlying methodological principles). The calculation of the parameters for the glycolysis is visualized in the graph and further explained in Table 3.9. Graphic based on "Figure 1. Agilent Seahorse XF Glycolysis Stress Test profile of the key parameters of glycolytic function. Sequential compound injections measure glycolysis, glycolytic capacity, and allow calculation of glycolytic reserve and nonglycolytic acidification." in Agilent Seahorse XF Glycolysis Stress Test Kit User Guide (2).

Before adding oligomycin, the cells are completely unaffected and the ECAR is considered to represent the BG. Oligomycin inhibits completely the aerobic ATP generation. After its addition, the cells are fully dependent on anaerobic glycolysis for producing ATP. Therefore, the ECAR after oligomycin addition represents the maximal possible glycolytic activity (GC). The difference between BG and GC is referred to as the GR. The formulas used to calculate these parameters are listed in Table 3.9 (see (2) for underlying methodological principles).

Parameter	Calculation
basal glycolysis	mean ECAR in last 3 measurements before oligomycin injection
glycolytic capacity	highest ECAR after oligomycin injection
glycolytic reserve	(glycolytic capacity) – (basal glycolysis)

Table 3.9 Calculation of glycolysis activity parameters
The activity parameters of glycolysis were calculated as stated.

The Mito Stress Test can be performed with unstimulated, but also with stimulated cells to observe the effect of a cell activation on the cells' metabolic behavior.

For this assay, special 96-well cell culture microplates and sensor cartridges compatible with the XFe96 Analyzer were used. The cells were seeded in Mito Stress medium on the cell culture microplates. The solutions for cell stimulation and ETC affection were loaded into the cartridge ports where they stayed until the analyzer injected each solution simultaneously into all wells to achieve maximal comparability between the wells. Between the injections OCR and ECAR were measured several times in distinct intervals.

For this work, in the *in vitro* CLA treatment experiments, $CD4^+$ T cells were isolated out of freshly thawed HC PBMC samples and then stimulated for 24 h in the cell culture while being treated or not with 75 µM CLA simultaneously. The stimulation was performed with purified antibodies against the CD3 and CD28 surface proteins in a concentration of 4 µg/ml each. These *in vitro* treated samples were then not re-stimulated inside the analyzer.

For the *in vivo* CLA treatment experiments, in contrast, $CD4^+$ T cells were isolated out of freshly thawed PBMC samples collected before and after 24 weeks CLA *in vivo* treatment and measured immediately without overnight incubation and stimulation. The measurement was performed both in cells not stimulated *in vitro* at all and in cells short-time stimulated with PMA and ionomycin in the analyzer for 160 min. For the unstimulated samples, Mito Stress medium was injected simultaneously to the PMA / ionomycin injection in the other wells.

Fig. 3.5 illustrates the sequence of injections and measurements in the XFe96 Analyzer. Table 3.10 gives an overview over the applied concentrations of the cell stimulating and the ETC affecting substances. All substances were diluted in Mito Stress medium.

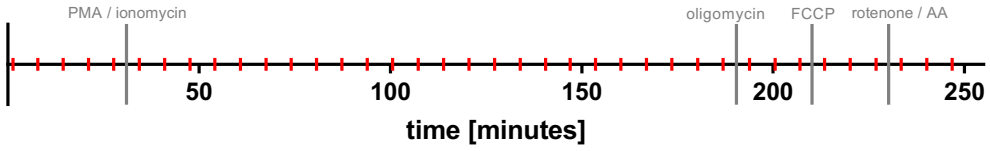

Fig. 3.5 Sequence of injections and measurements in metabolism analysis
The red markers symbolize the measurements between the injections of the cell stimulating and ETC affecting substances. AA = antimycin A.

Substance	Concentration
PMA	50 ng/ml
ionomycin	500 ng/ml
anti-CD3 antibody	4 µg/ml
anti-CD28 antibody	4 µg/ml
oligomycin	2 µM
FCCP	1.5 µM
rotenone	0.1 µM
antimycin A	1 µM

Table 3.10 Concentrations of cell stimulating and ETC affecting substances in metabolism analysis

The *in vivo* samples had to be measured on two 96-well plates on two consecutive days. It was ensured that all samples of one patient (before and after treatment, each restimulated and not restimulated) were measured on the same plate and therefore simultaneously. However, to achieve maximal comparability also between the samples on different plates, the resulting values were standardized. Therefore, on both days one additional HC sample from the same person and the same blood withdrawal was processed parallelly to the study samples and measured concurrently. Its resulting values were used to standardize the study samples' values.

3.4 Statistical methods

The automated flow cytometry data analysis using the viSNE and PhenoGraph algorithms is described in detail in section 3.3.4.6. All other data was graphed and statistically analyzed as described below using the software GraphPad Prism.

3.4.1 Descriptive statistics

The graphed data points represent either one individual's value or the mean value of one individual's technical replicates.

All data is graphed as mean ± SD. For graphs comparing different groups ($n > 1$), mean and SD were calculated out of the individuals' values or out of the mean values of their respective technical replicates. For exemplary graphs comparing data from only one individual ($n = 1$), mean and SD were calculated for the different technical replicates.

3.4.2 Inferential statistics

Except of for the analysis of the data resulting from the Luminex® assay performed after *in vitro* CLA treatment, for all group comparisons, Wilcoxon signed-rank tests were performed. This test was chosen because there was no justification to assume normally distributed data and because this test takes account of the fact that all samples were related since representing different treatment states of the same individual's cells.

To analyze the data from the experiment measuring cytokine concentrations in a Luminex® assay after *in vitro* CLA treatment (results in section 4.1.2.2), paired t-tests were performed. Since the *n* in this explorative experiment was only *n* = 4, a Wilcoxon signed-rank-test is not capable to show significant results, no matter how large and clear group differences are. Although normally distributed data cannot be assumed, the results from a paired t-test can give an exploratory impression of the group differences in this case.

The results of all statistical tests are illustrated in the graphs as * ($p \leq 0.05$), ** ($p \leq 0.01$), ***($p \leq 0.001$) or "n.s." (not significant).

3.4.3 Handling of values < LLOQ or > ULOQ

The assays measuring cytokine concentrations have a lower (LLOQ) and an upper (ULOQ) limit of quantitation. Below or above these values, respectively, the calculated cytokine concentration cannot be considered being accurately measured.

To avoid measuring cytokine concentrations outside the range between LLOQ and ULOQ, samples were measured after a calculated dilution if concentrations higher than the respective ULOQ could be expected.

However, only having at best a rough estimation of the real value before the experiment and – in case of the Luminex® assay – measuring different cytokines with different needs for pre-measurement dilution in a single reaction volume, this procedure could not suffice to completely avoid measuring data < LLOQ or > ULOQ. These data was appropriately handled as described below.

3.4.3.1 Values < LLOQ

For presentation in the graphs, values < LLOQ and mean values of technical replicates of which at least one was < LLOQ were plotted as measured and colored blue. Values lower than zero (which can result from the calculations due to comparing the samples with blanks) were replaced by zero. The LLOQ was delineated in the graphs as a blue line when reasonable.

When measuring a value < LLOQ it can be assumed that the real value is between zero and the LLOQ. Therefore, for the statistical tests, both these most extreme values between which the actual value must lie, were inserted once in the calculation for the p-value. In all cases presented in this work, both calculations led to a result within the same range of p-values (regarding the illustration with *, **, *** or “n.s.”). The respective symbol was inserted in the graph.

For the calculation of mean and SD, however, this method was not applied since this would lead to two values for mean and SD and therefore to confusing graphs. Instead, mean and SD were not calculated for groups containing values < LLOQ.

3.4.3.2 Values > ULOQ

In the graphs, values > ULOQ and mean values of technical replicates of which at least one was > ULOQ were plotted as measured and colored green.

It can be assumed that above the ULOQ the assays rather measure false-low than false-high values. If a false-low measurement would rather contradict the experimental hypothesis (i.e. false-low pro-inflammatory cytokine concentrations before CLA treatment), mean, SD and a p-value were calculated with the measured value. If, however, a false-low result would rather wrongly support the hypothesis (i.e. false-low pro-inflammatory cytokine concentrations after CLA treatment), mean, SD and a p-value were not calculated.

4 Results

Considering the indications for beneficial capabilities of CLA in the context of several autoimmune diseases (188), its disease-ameliorating effects in a spontaneous MS mouse model and its anti-inflammatory influence on healthy controls' and RRMS patients' $CD4^+$ T cells *in vitro* (unpublished data by Hucke, Hartwig *et al.* (79)), aim of this thesis was to further investigate the effects of CLA on the human immune system and human MS disease as well as to find evidence by which mechanisms such effects could be mediated.

4.1 *in vitro* experiments

With *in vitro* experiments conducted in isolated and cultured healthy controls' peripheral blood $CD4^+$ T cells, possible cytotoxic effects of CLA on $CD4^+$ T cells were examined and the capability of CLA to reduce their cytokine production capacity as well as its effects on their metabolic activity were analyzed.

4.1.1 Cytotoxicity investigation

To exclude that potential suppressive effects of CLA on the pro-inflammatory cytokine production capacity or metabolism of $CD4^+$ T cells could be only wrongly suggested by a cytotoxic effect of CLA on these cells, a relevant cytotoxicity of CLA in the concentrations used in the further *in vitro* experiments had to be ruled out. Therefore, the portion of living HC $CD4^+$ T cells after 72 h CLA treatment under PHA stimulation in comparison to a negative control without treatment was measured by performing a PI staining.

Fig. 4.2 illustrates the resulting data: The portion of living stimulated $CD4^+$ T cells is similar in all treatment groups, from the negative control without CLA treatment to the maximum tested concentration of 75 µM CLA (each isomer). Thus, the experiment revealed no relevant cytotoxic effects of CLA.

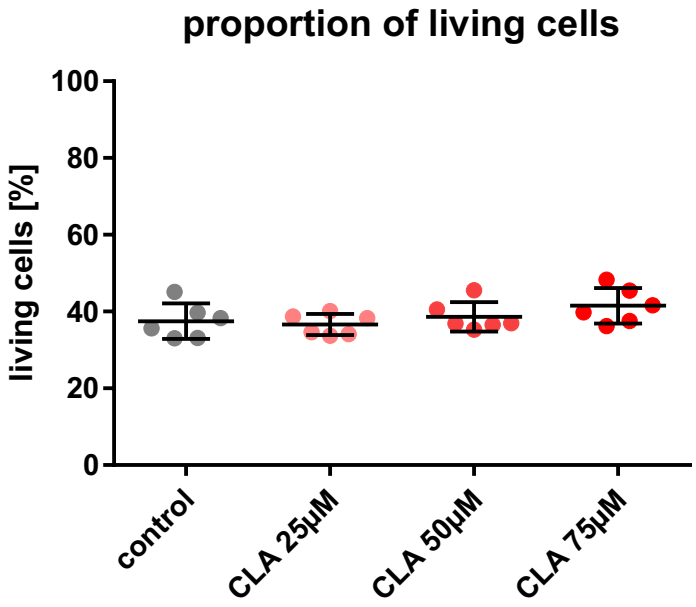

Fig. 4.1 Effect of CLA *in vitro* on activated HC CD4⁺ T cells' survival
Portion of living HC CD4⁺ T cells stimulated with PHA and treated with different concentrations of CLA for 72 h, measured after PI staining. *n* = 6. All data is presented as mean ± SD.

4.1.2 CD4⁺ T cell cytokine production upon activation

4.1.2.1 Quantification in an ELISA assay

By directly measuring the cytokine production with an ELISA assay, the hypothesis was tested that CLA is capable to reduce the pro-inflammatory cytokine production capacity of HC CD4⁺ T cells *in vitro*. After 72 h PHA-activation and CLA treatment or not the concentrations of TNFα and IFNγ produced by the cultured CD4⁺ T cells were measured with an ELISA assay.

The resulting data (Fig. 4.2) shows a profound reduction in the CD4⁺ T cells' production capacity for both investigated cytokines. Moreover, the data illustrates that the effect was dose-dependent: the exposure to increasing concentrations of CLA was associated with a lower cytokine production by the activated CD4⁺ T cells.

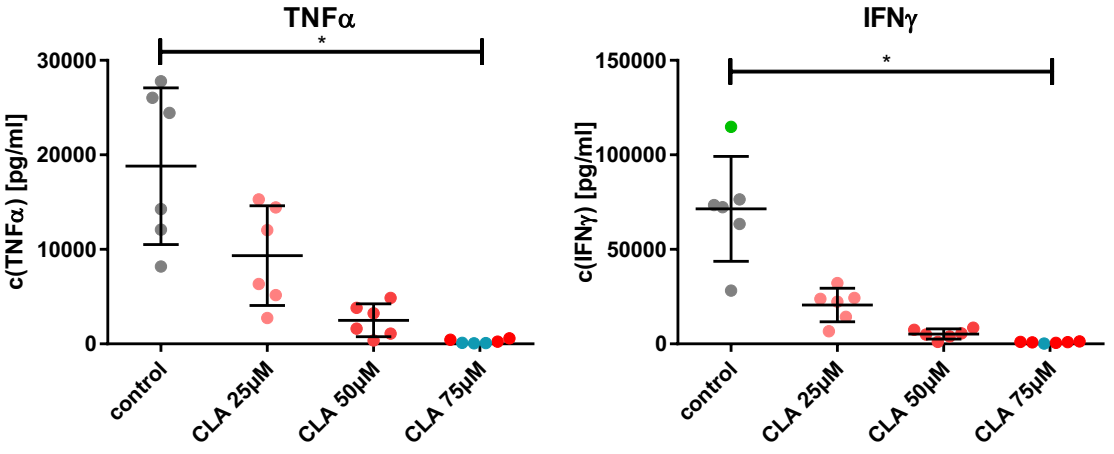

Fig. 4.2 Effect of CLA *in vitro* on activated HC CD4⁺ T cells' cytokine production capacity (ELISA)
Quantity of the respective cytokine produced by HC CD4⁺ T cells stimulated with PHA and treated with different concentrations of CLA for 72 h. *n* = 6. All data is presented as mean ± SD. Results from Wilcoxon test control vs. CLA 75 µM: * p ≤ 0.05. Blue dots represent values < LLOQ, green dots values > ULOQ. The cytokine concentration in all measured unstimulated samples was close to or lower than the LLOQ (1 of 24 could not be measured due to technical issues).

4.1.2.2 Quantification in a Luminex® assay

To reproduce these findings in a different assay and to expand the range of investigated cytokines, the experiment was repeated measuring the cytokine concentration in the cell culture supernatants with a Luminex® assay. Since a CLA concentration of 75 µM showed the largest effects in the ELISA assay but no relevant cytotoxic effects in the cytotoxicity assay, it was employed for this experiment. The supernatants of HC CD4⁺ T cells, again PHA-stimulated and CLA-treated or not for 72 h, were analyzed regarding 18 different cytokine concentrations employing Luminex® technique.

The results for the 15 most interesting cytokines are presented in Fig. 4.3. The cytokine production under PHA activation for all measured cytokines was significantly lower after 72 h *in vitro* CLA treatment in comparison to the untreated control group. This includes a wide range of pro-inflammatory cytokines, such as TNFα, IFNγ, GM-CSF or IL-17A. However, also the production of cytokines showing a rather anti-inflammatory phenotype as IL-4 and IL-10 was lowered during the CLA treatment.

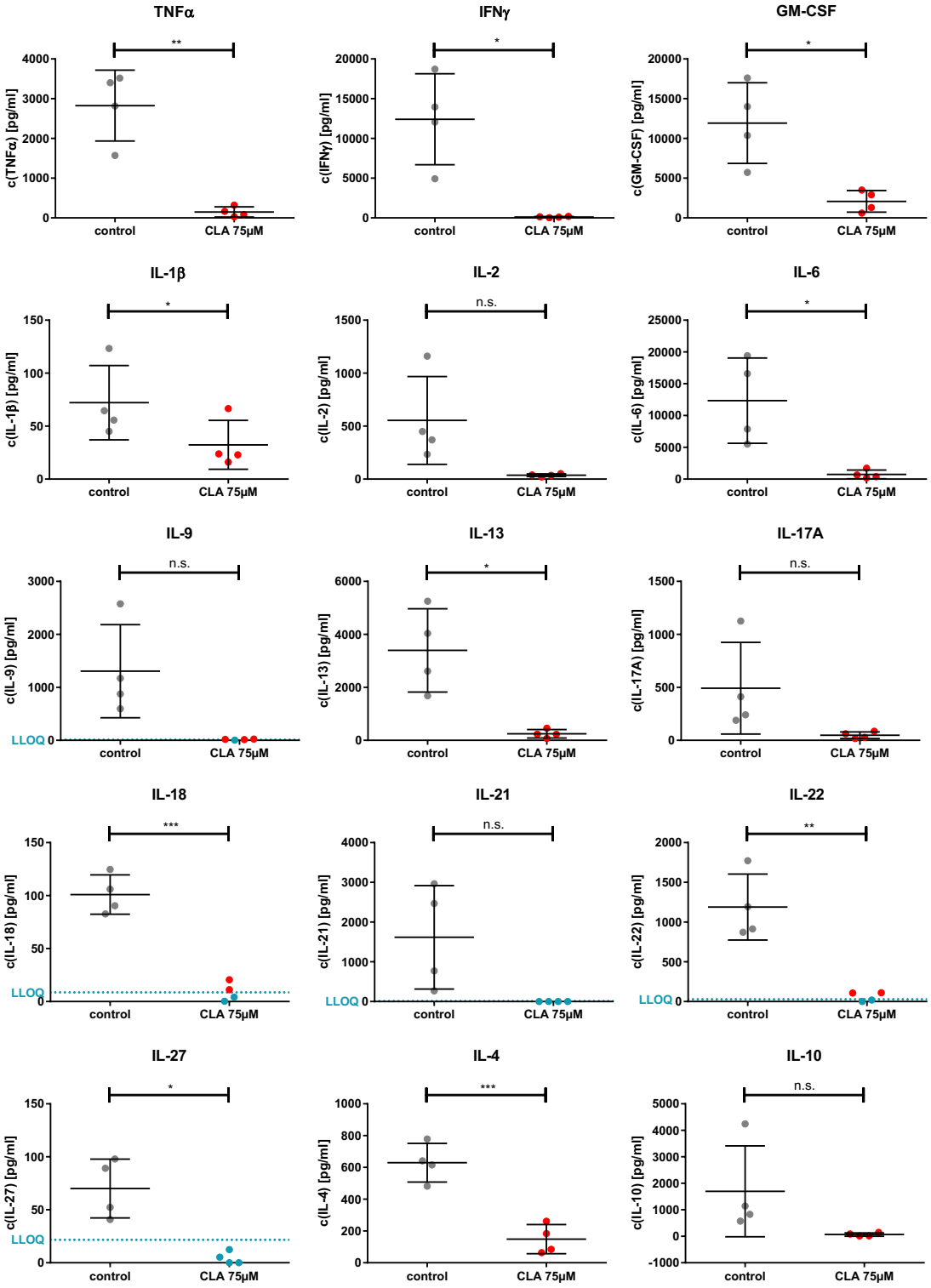

Fig. 4.3 Effect of CLA *in vitro* on activated HC CD4⁺ T cells' cytokine production capacity (Luminex®)
Quantity of the respective cytokine produced by HC CD4⁺ T cells stimulated with PHA and treated or not with 75 µM CLA *in vitro* for 72 h. *n* = 4. All data is presented as mean ± SD. Results from paired t-test: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$ n.s. = not significant. Blue dots represent values < LLOQ, green dots values > ULOQ. The cytokine concentration in all unstimulated samples was < LLOQ.

4.1.3 CD4$^+$ T cell metabolism

As described in the introduction (sections 1.1.4.4 and 1.2.4.2), the metabolic and the immunologic function of immune cells are closely interlinked, and it has been hypothesized that CLA can influence the metabolic activity of immune cells (44, 77). To test the hypothesis that an *in vitro* CLA treatment reprograms the metabolism of immune cells towards a phenotype which is associated with an immunomodulatory cell function, the metabolism of HC CD4$^+$ T cells was analyzed after an *in vitro* CLA treatment. Results from such an experiment could give indications regarding the mechanism of action of CLA.

For this experiment, HC CD4$^+$ T cells were isolated from freshly thawed PBMC samples and incubated overnight (24 h) under stimulation with anti-CD3 and anti-CD28 antibodies and treatment with 75 µM CLA or not. Afterwards, their metabolic activity was measured under Mito Stress conditions employing Agilent Seahorse XF technology.

Analyzing the cells' OCR during the Mito Stress Test, which is representing the activity of the OXPHOS (3), it could be found that the CLA treated CD4$^+$ T cells exposed a significantly lower activity of mitochondrial respiration (Fig. 4.4). BR, MR and SRC were reduced.

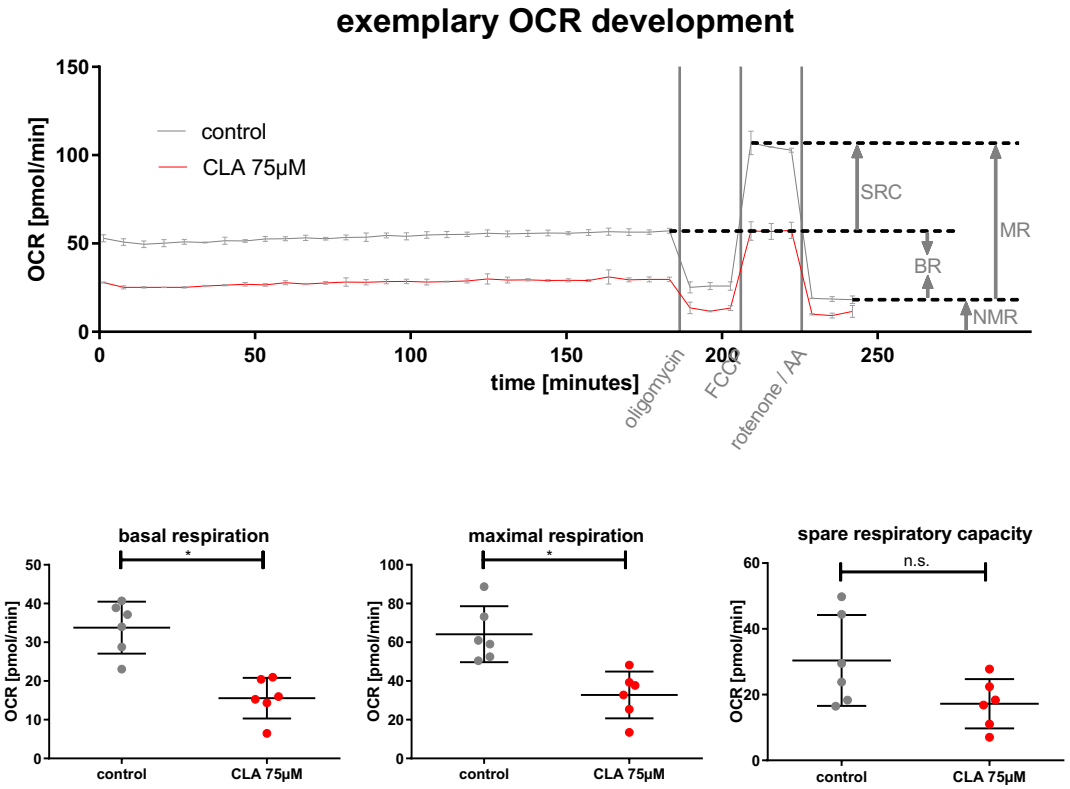

Fig. 4.4 Effect of CLA *in vitro* on activated HC CD4⁺ T cells' OXPHOS
Top. Representative OCR curves during Mito Stress Test of HC CD4⁺ T cells activated with anti-CD3 and anti-CD28 antibodies and treated or not with 75 µM CLA *in vitro* for 24 h. Oligomycin, FCCF and rotenone / antimycin A were added as indicated. The calculations for the parameters NMR, BR, MR and SRC are illustrated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BR, MR and SRC HC CD4⁺ T cells activated with anti-CD3 and anti-CD28 antibodies and treated or not with 75 µM CLA *in vitro* for 24 h. *n* = 6. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$ n.s. = not significant

The ECAR in Agilent Seahorse XF assays represents the activity of lactate-producing glycolysis (2). Its analysis revealed the data presented in Fig. 4.5, illustrating the effect of an *in vitro* CLA treatment on the glycolytic activity of HC CD4⁺ T cells: The BG, the GC and the GR were reduced after the treatment.

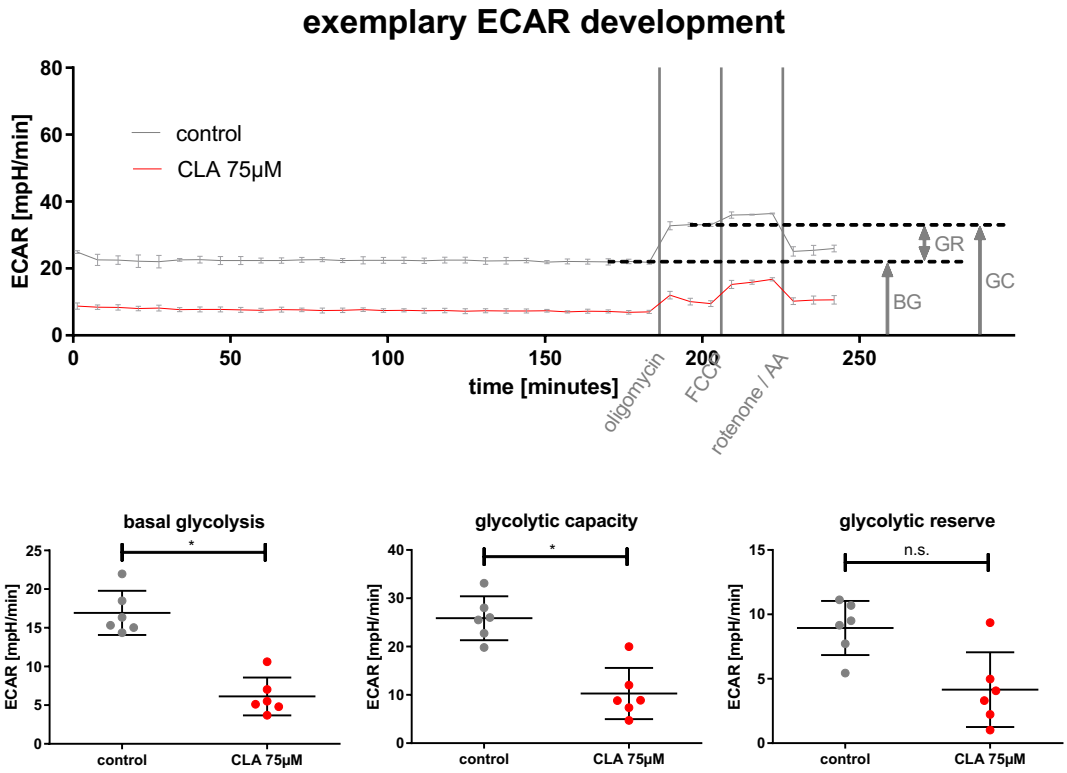

Fig. 4.5 Effect of CLA *in vitro* on activated HC CD4⁺ T cells' glycolytic activity
Top. Representative ECAR curves during Mito Stress Test of HC CD4⁺ T cells activated with anti-CD3 and anti-CD28 antibodies and treated or not with 75 µM CLA *in vitro* for 24 h. Oligomycin, FCCF and rotenone / antimycin A were added as indicated. The calculations for the parameters BG, GC and GR are illustrated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BG, GC and GR of HC CD4⁺ T cells activated with anti-CD3 and anti-CD28 antibodies and treated or not with 75 µM CLA *in vitro* for 24 h. *n* = 6. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$ n.s. = not significant

4.2 Clinical study

Considering the beneficial effects of CLA in the context of autoimmunity suggested by various preclinical and clinical studies previously (16, 17, 79, 188), it was of high interest to examine the effects of CLA in the *in vivo* situation of MS. To gain insight into the function of CLA in MS patients, a clinical study was performed.

4.2.1 Baseline demographical and clinical data

Table 4.1 shows the baseline demographical and clinical data of the study participants. The collective was predominantly female (73,3% women) and aged in average 36.7 years (SD = 10.9 years). The mean baseline EDSS score was 0.8 (SD = 0.9).

age (years)	36.7 (10.9)
women	11 (73.3%)
baseline EDSS score	0.8 (0.9)
time since first MS symptoms (years)	8.5 (9.7)
time since MS diagnosis (years)	6.5 (9.3)
current immunomodulatory treatment	
glatiramer acetate	5 (33.3%)
interferon beta-1a	9 (60%)
peginterferon beta-1a	1 (6.7%)

Table 4.1 Baseline demographical and clinical data in the study cohort
Values are mean (SD) or *n* (%). (*n* = 15)

4.2.2 Natural fat and CLA intake

4.2.2.1 Fat intake

The analysis of the food diaries kept by the study patients revealed that the average natural fat intake was approximately 72 g/d (SD = 23 g/d). Fig. 4.6 illustrates the distribution of the natural fat intake among the study patients.

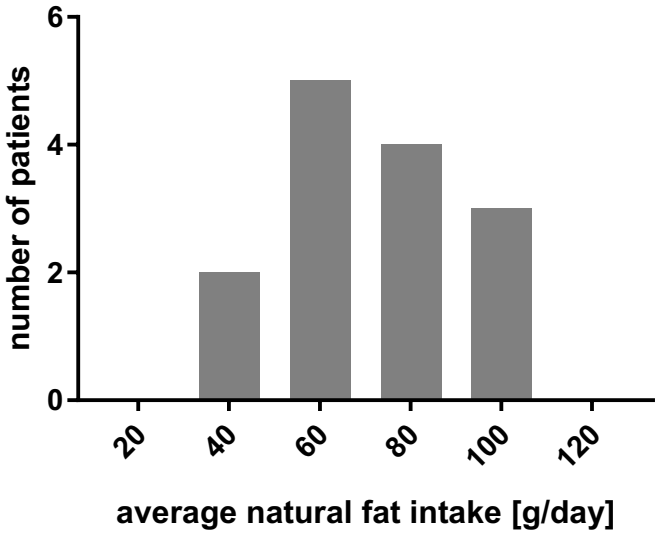

Fig. 4.6 Distribution of natural fat intake among study patients
Histogram of the average daily fat intake by the study patients. *n* = 14.

4.2.2.2 CLA intake

Regarding the natural CLA intake, the food diary analysis showed an average intake of approximately 100 mg/d (SD = 41 mg/d). This Is less than 5% of the study medication the patients received during the clinical trial. Fig. 4.7 shows the distribution of the natural CLA intake within the study cohort and Fig. 4.8 compares the natural CLA intake with the study medication.

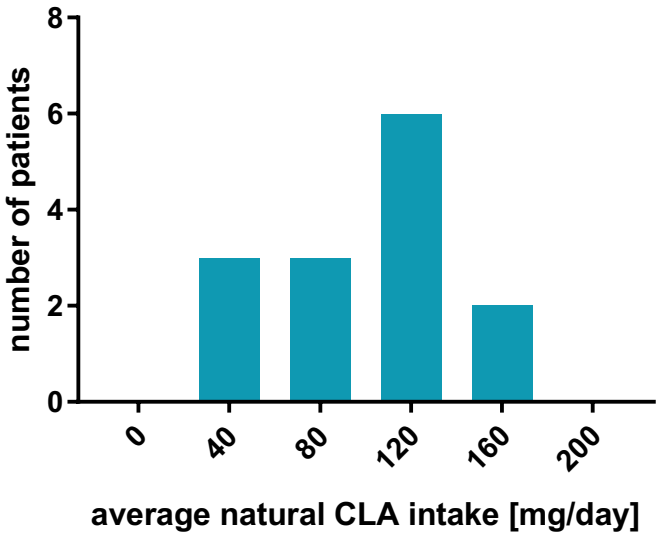

Fig. 4.7 Distribution of natural CLA intake among study patients
Histogram of the average daily CLA intake by the study patients. *n* = 14.

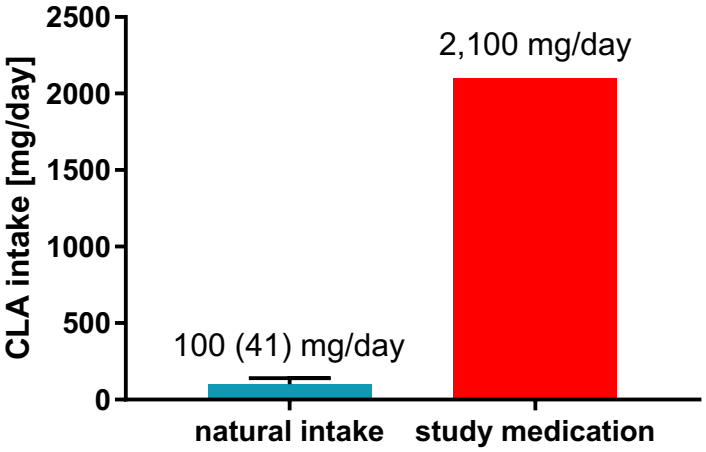

Fig. 4.8 Comparison between natural CLA intake and study medication
Comparison of the average natural CLA intake by the study patients with the study medication. *n* = 14. Data for natural CLA intake is presented as mean ± SD.

4.2.3 Clinical study endpoints

4.2.3.1 Adherence to study medication

The results of the measurement of the patients' adherence to the study medication resulted in the data presented in Table 4.2.

Adherence to study medication	n (%)
≥ 95%	11 (73.3%)
≥ 90%, but < 95%	3 (20%)
≥ 85%, but < 90%	1 (6.7%)

Table 4.2 Adherence to study medication in the study cohort
Values are *n* (%). (*n* = 15)

4.2.3.2 Relapses

The number of relapses sustained by the patients during the clinical study is shown in Table 4.3.

Number of relapses	n (%)
no relapse	14 (93.3%)
one relapse	1 (6.7%)

Table 4.3 Number of relapses sustained by the study patients during the study period
Values are *n* (%). (*n* = 15)

This is equivalent to an average annualized relapse rate of 0.14 (SD = 0.56) relapses per year. In comparison, the average annualizes relapse rate in the last year before study inclusion was 0.27 (SD = 0.46) relapses per year (no relapse in 11 patients, one relapse in 4 patients). The sole patient who suffered a relapse during the study had no relapse in the year prior to the study.

4.2.3.3 EDSS during follow-up

Table 4.4 describes the change in the study patients' EDSS scores over the time of the CLA treatment.

EDSS during follow-up	n (%)	Δ EDSS
EDSS value increase ≥ 0.5 after 24 weeks	2 (13.3%)	1; 1.5
of which relapse-independent increase	2 (13.3%)	1; 1.5
EDSS value decrease ≥ 0.5 after 24 weeks	1 (6.7%)	-0.5
EDSS value stable after 24 weeks	12 (80%)	

Table 4.4 Change of EDSS value within the 24 CLA treatment weeks
Δ EDSS = absolute change of EDSS score over study period in respective patients. Values are *n* (%). (*n* = 15)

4.2.3.4 Paraclinical data

MRI scans were not part of the study protocol in the present exploratory clinical trial. Nevertheless, MRI was obtained from several patients during the study period for other reasons, e.g. as part of the routine periodic clinical and paraclinical evaluation, in the context of other studies conducted parallelly or in case of neurological symptoms (MS relapse).

In total, MRI scans were conducted in 7 of the 15 participants during the study period. Of these, in comparison to the respective previous examination, four patients exhibited a stable paraclinical development while three patients presented with new, enlarging and / or Gd-enhancing lesions.

In this regard it must be noted that the mentioned MRI scans were performed at random time points during the study and compared to images obtained unequal time periods prior to the study inclusion. Thus, it cannot be stated if the neuropathologic processes underlying the paraclinical instability observed in three patients have taken place before or during the study. Therefore, and because of the small study size and lack of a control group, as well as because MRI was obtained only in part of the study subjects, no conclusions about the efficacy of CLA on paraclinical parameters can be drawn from these observations.

4.2.3.5 Correlation of relapses, EDSS during follow-up and paraclinical data

As described above, during the study, one patient suffered a relapse but no EDSS increase (comparing study beginning and ending), two patients a relapse-independent EDSS increase and in three patients MRI scans revealed paraclinical instability (comparing an MRI obtained at a random timepoint during the study with an MRI obtained unequal time periods prior to the study inclusion). These events were partly correlated as follows: One patient suffered a relapse associated with paraclinical instability. In another patient, a relapse-independent EDSS increase and

changes on MRI were observed. One patient exhibited only a relapse-independent EDSS increase (but no MRI was obtained during the study from this patient) and a fourth one only paraclinical instability.

4.2.3.6 Adverse events

The adverse events observed during the study period in the participants are listed with their respective incidence in Table 4.5. One MS relapse requiring an in-patient therapy was the only recorded serious adverse event (SAE).

Adverse events, categorized by organ system	Incidence	Relation to CLA intake
Dermatological		
contact allergy	1	improbable
Gastrointestinal		
abdominal fullness	1	**possible**
abdominal pain	1	**possible**
elevated liver enzymes	1	improbable
enteritis	1	**possible**
nausea	2	**possible**
Infectious		
cold	5	improbable
gastrointestinal	1	improbable
urinary tract	2	improbable
Neurologic		
migraine	1	improbable
MS relapse (SAE)	1	improbable
Ophthalmologic		
reddening and dryness of the eye	1	improbable
Otorhinolaryngological		
allergic rhinitis	1	improbable
dysphagia	1	improbable
sore throat	1	improbable
Other		
influenza vaccination	1	improbable
iron deficiency	1	improbable

Table 4.5 Adverse events and their incidence sustained by the patients during the study period
Values are absolute numbers. (*n* = 15)

4.2.4 In-depth functional immune phenotyping

To gain a broad insight into the effects of CLA on the immune system *in vivo*, changes in the immune cell subset composition were analyzed performing an in-depth functional immune phenotyping by flow cytometry as an exploratory and orientating investigation. The immune cells' cytokine production capacity was measured in this setup as well.

The underlying hypothesis was that pro-inflammatory immune cell subsets and their pro-inflammatory cytokine production capacity are downregulated while immune cells exhibiting a more regulatory phenotype are upregulated during the CLA treatment.

PBMC samples collected before and after 8, 16 and 24 weeks of CLA *in vivo* treatment were analyzed. The resulting data from all staining panels was analyzed with conventional gating strategies identifying distinct known immune cell subsets. The most interesting results from this analysis are shown in the following figures.

Additionally, in collaboration with the working group team, the data from the most promising staining panels ("T development", "CD8", "B cell", "T_H17", "T_{reg}", "T_H stim 1", "T_H stim 2") was also analyzed independently with an automated, less biased method employing the viSNE and PhenoGraph algorithms. The resulting viSNE maps showing cells either from before or after the 24 weeks CLA *in vivo* treatment with the individual cells colored according to their assignment to clusters identified by the PhenoGraph algorithm are presented in the following figures if interesting alterations in the immune cell subset composition could be observed during the treatment period.

In this section, all figures with y-scatter plots present results from the conventional gating analysis and figures with viSNE maps the results from the less biased automated data analysis.

4.2.4.1 $CD4^+$ T cells

Fig. 4.9 illustrates the effect of the CLA *in vivo* treatment on different $CD4^+$ memory T cell subsets. T_H17 cells, defined either as $CD146^+$ $CD4^+$ memory T cells (38, 39) or more strictly as $CD194^+$ $CD196^+$ $CD161^+$ $CD146^+$ $CD4^+$ memory T cells (22, 106) were downregulated during the CLA treatment. This result reached statistical significance for the wider definition of T_H17 cells as $CD146^+$ $CD4^+$ memory T cells. $CD4^+$ memory T cells expressing IL-23R were significantly downregulated as well.

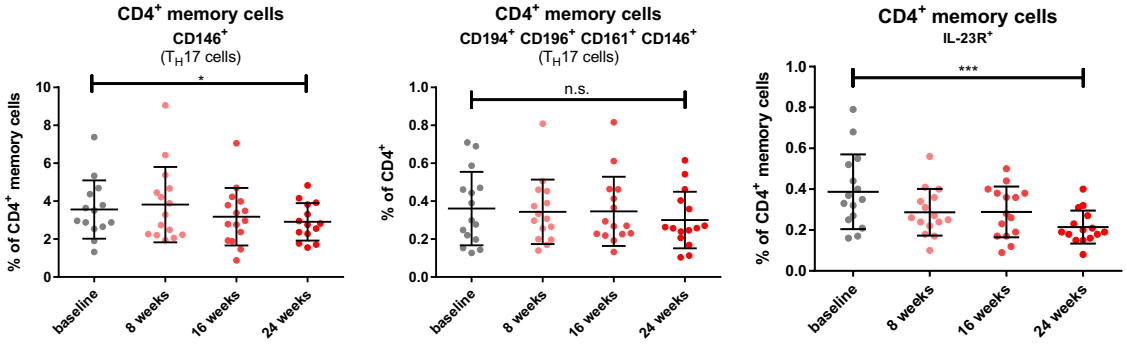

Fig. 4.9 Effect of CLA *in vivo* on the CD4⁺ memory T cell subset composition in MS patients
Share of the respective indicated CD4⁺ memory T cell subset as a percentage of the respective indicated superordinate T cell subset determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment. *n* = 14 (CD4⁺ memory cells CD146⁺ baseline and 24 weeks) or *n* = 15 (all other groups). All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: * p ≤ 0.05 ***p ≤ 0.001 n.s. = not significant.

A similar effect could be observed after applying the viSNE and PhenoGraph algorithms to the data from lymphocytes (pre-gated before automated analysis) activated and stained according to the "T_H stim 1" panel protocol. CD146 positive CD4⁺ T cells were decreased 1.9-fold after the CLA *in vivo* treatment in comparison to before (Fig. 4.10).

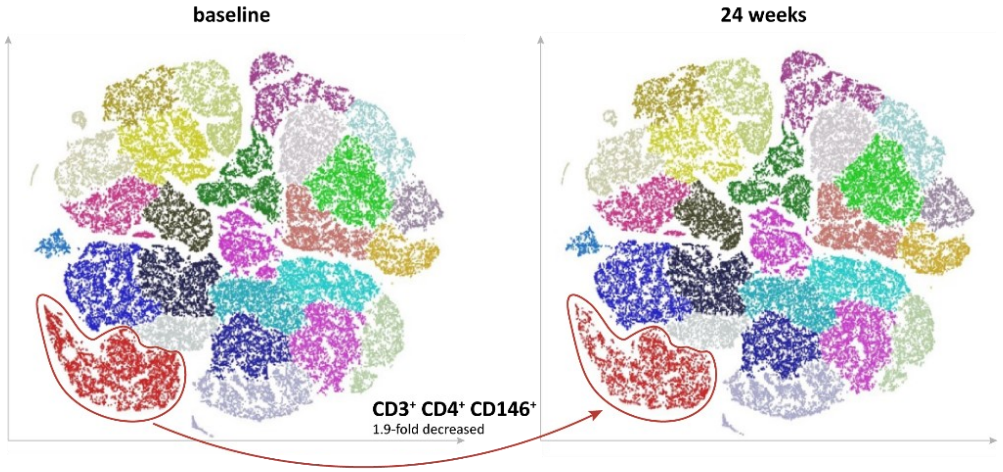

Fig. 4.10 Effect of CLA *in vivo* on lymphocytes in MS patients
viSNE maps of all lymphocytes (pre-gated before automated analysis) before and after 24 weeks CLA *in vivo* treatment, measured by 9 color flow cytometry after 4 h LAC stimulation and staining with the "T_H stim 1" panel. Each dot represents one individual cell. The cells are colored according to their assignment to distinct cell clusters by the PhenoGraph algorithm. Each graph maps a total of 45,000 cells merged from all study participants. The axes are given in arbitrary units. *n* = 15.

Alterations in the cytokine production of CD4⁺ T cells and CD4⁺ memory T cells during the *in vivo* CLA treatment were analyzed and the results are presented in Fig. 4.11. Both the CD4⁺ T cells in

general and the CD4⁺ memory T cells produced significantly less IL-17A and IL-22 upon activation after the CLA treatment.

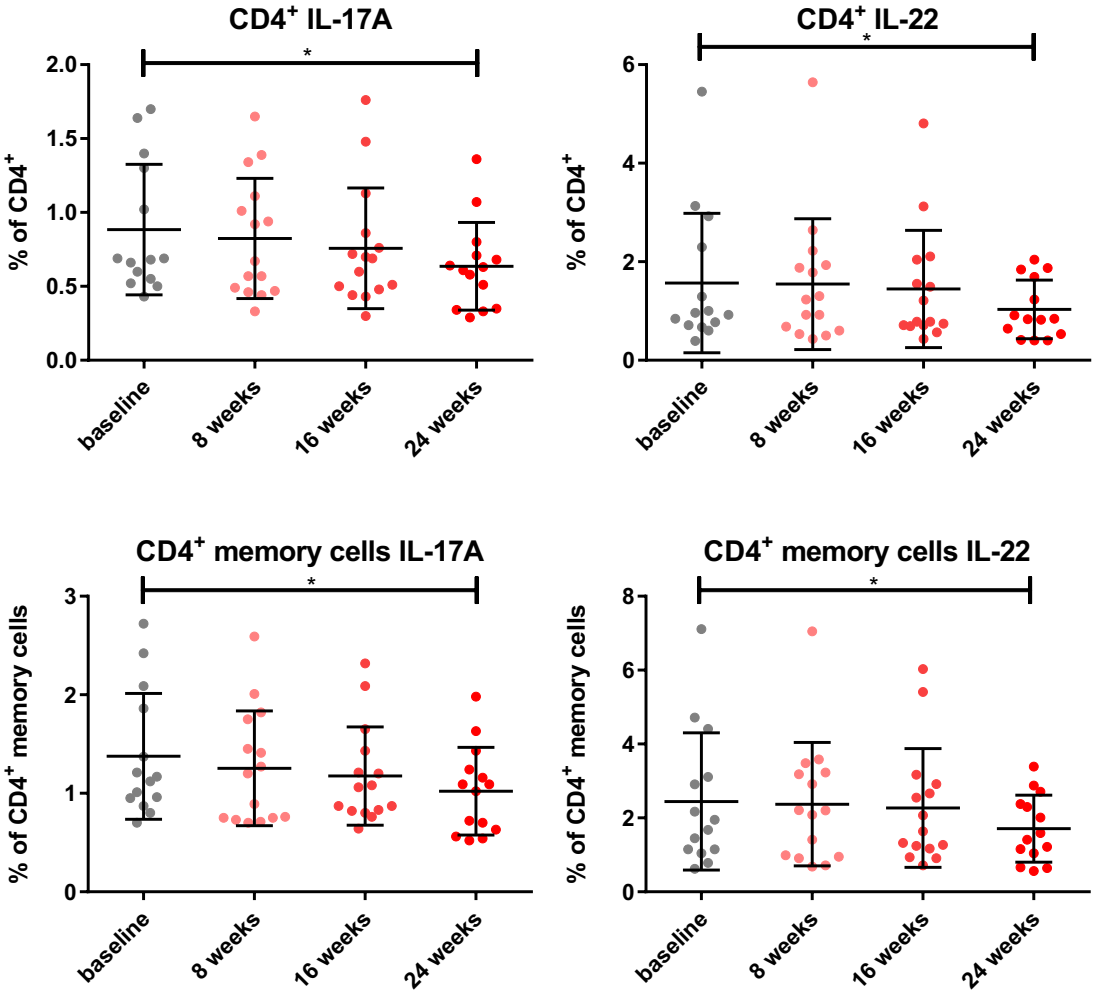

Fig. 4.11 Effect of CLA *in vivo* on CD4⁺ T cell subset cytokine production capacity in MS patients
Share of the cytokine-producing CD4⁺ (memory) T cells as a percentage of all CD4⁺ (memory) T cells determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment, measured after 4 h LAC stimulation. *n* = 14 (baseline and 24 weeks groups) or *n* = 15 (8 and 16 weeks groups). All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: * p ≤ 0.05.

In the automated analysis of all lymphocytes (pre-gated before applying viSNE and PhenoGraph algorithms) after PBMC activation and staining according to the "T_H stim 2" panel protocol, a 1.46-fold decrease of IL-22 producing CD4⁺ CD146⁺ T cells could be detected (Fig. 4.12).

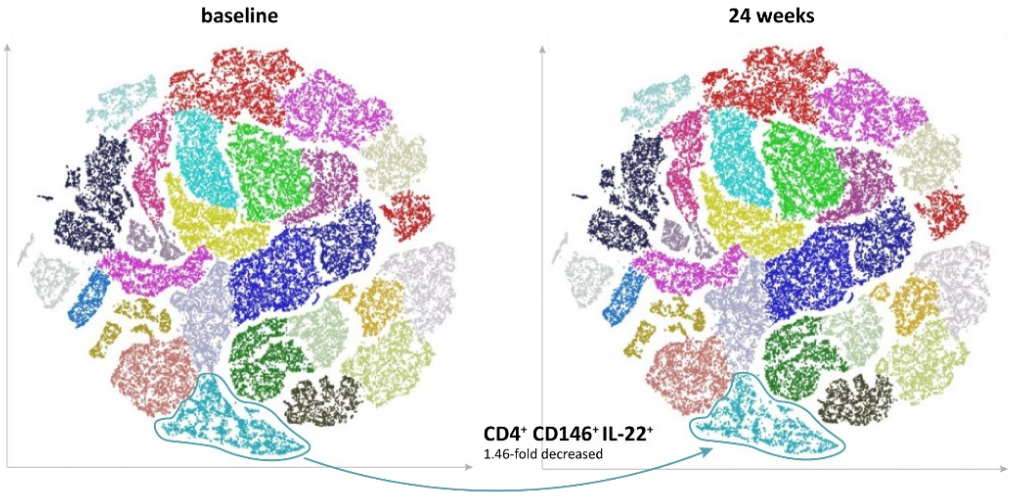

Fig. 4.12 Effect of CLA *in vivo* on lymphocytes in MS patients
viSNE maps of all lymphocytes (pre-gated before automated analysis) before and after 24 weeks CLA *in vivo* treatment, measured by 9 color flow cytometry after 4 h LAC stimulation and staining with the "T_H stim 2" panel. Each dot represents one individual cell. The cells are colored according to their assignment to distinct cell clusters by the PhenoGraph algorithm. Each graph maps a total of 42,000 cells merged from all study participants. The axes are given in arbitrary units. *n* = 15.

4.2.4.2 CD8⁺ T cells

Fig. 4.13 focusses on the expression of CD226 (DNAX accessory molecule-1 [DNAM-1]) on CD8⁺ T cells and subordinate subsets. CD226 was upregulated on CD8⁺ T cells, CD8⁺ central memory T cells and CD8⁺ T effector memory RA (T_{EMRA}) cells during the CLA *in vivo* treatment.

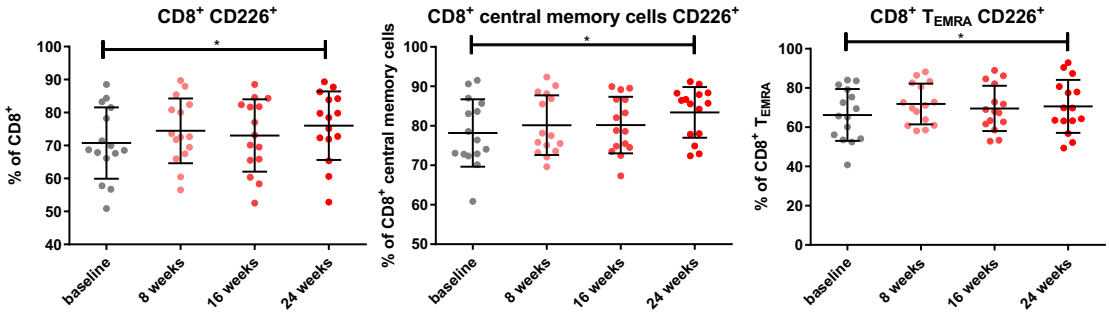

Fig. 4.13 Effect of CLA *in vivo* on expression of CD226 in different CD8⁺ T cell subsets in MS patients
Share of the CD226⁺ fraction in different CD8⁺ T cell subsets expressed in percent determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment. *n* = 15. All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: * p ≤ 0.05.

Similar results were found in the automated analysis of the data from T cells (pre-gated before automated analysis) colored with the "CD8" staining panel. CD8⁺ memory T cells expressing CD226 on their surface were found to be 1.37-fold increased (Fig. 4.14).

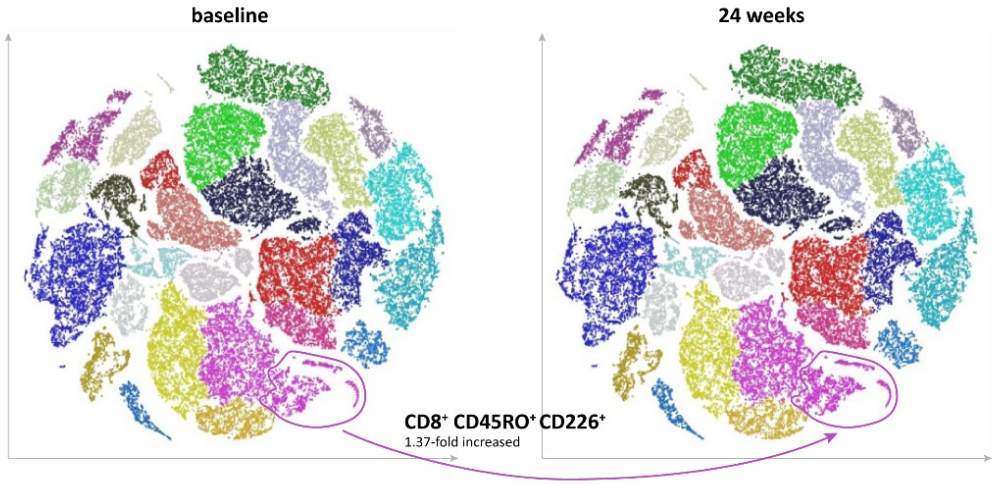

Fig. 4.14 Effect of CLA *in vivo* on T cells in MS patients
viSNE maps of all T cells (pre-gated before automated analysis) before and after 24 weeks CLA *in vivo* treatment, measured by 10 color flow cytometry after staining with the "CD8" panel. Each dot represents one individual cell. The cells are colored according to their assignment to distinct cell clusters by the PhenoGraph algorithm. Each graph maps a total of 84,000 cells merged from all study participants. The axes are given in arbitrary units. *n* = 15.

The effects of the treatment on the production of IL-17A and IL-22 by activated $CD8^+$ T cells in general and by $CD8^+$ memory T cells are shown in Fig. 4.15. The production of both cytokines was decreased in both groups during the treatment.

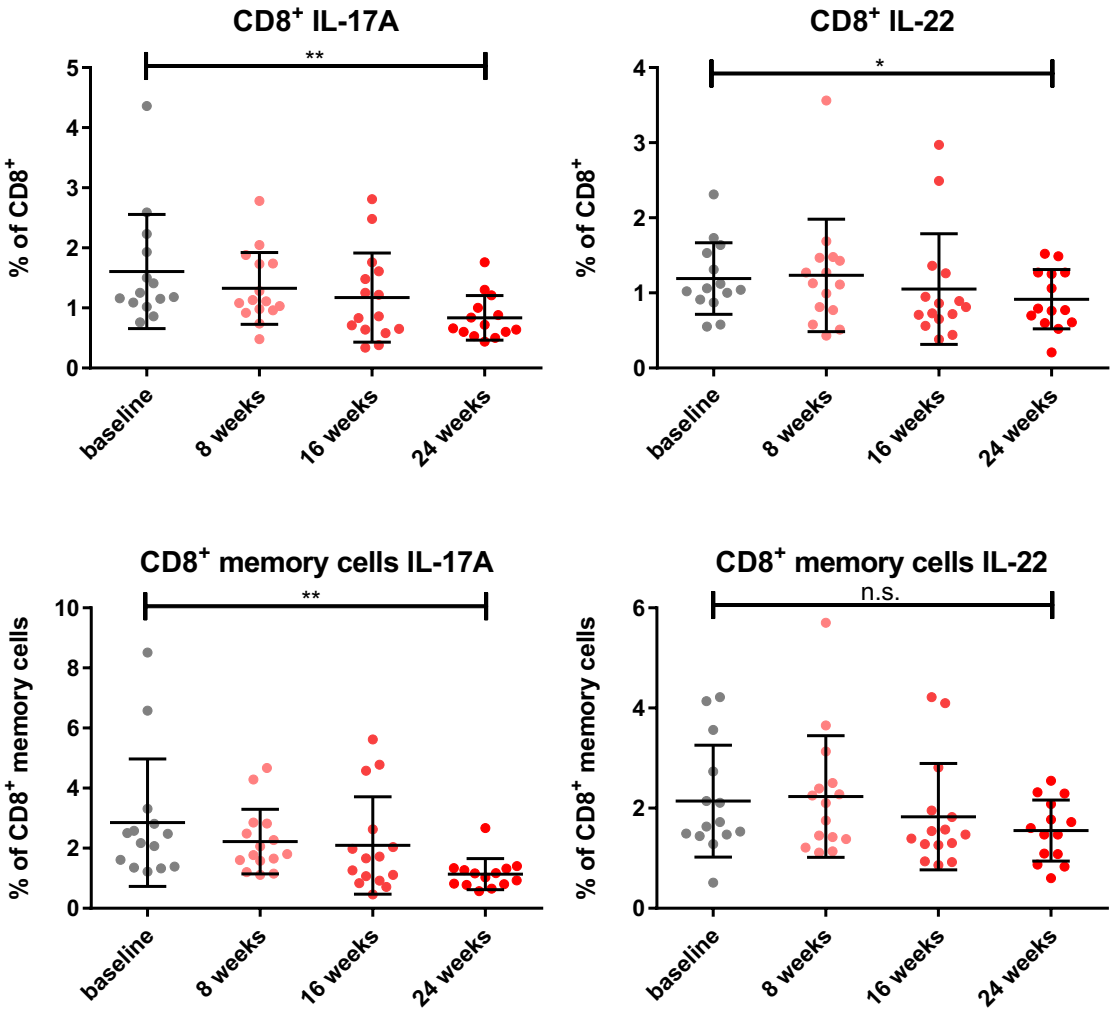

Fig. 4.15 Effect of CLA *in vivo* on CD8⁺ T cell subset cytokine production capacity in MS patients
Share of the cytokine-producing CD8⁺ (memory) T cells as a percentage of all CD8⁺ (memory) T cells determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment, measured after 4 h LAC stimulation. n = 14 (baseline and 24 weeks groups) or n = 15 (8 and 16 weeks groups). All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: * $p \leq 0.05$ ** $p \leq 0.01$ n.s. = not significant.

4.2.4.3 B cells

Next, the B cell subset composition was altered during the treatment as well. Fig. 4.16 shows the upregulation of naïve B cells and the reciprocal downregulation memory B cells, each calculated as percent of total B cells.

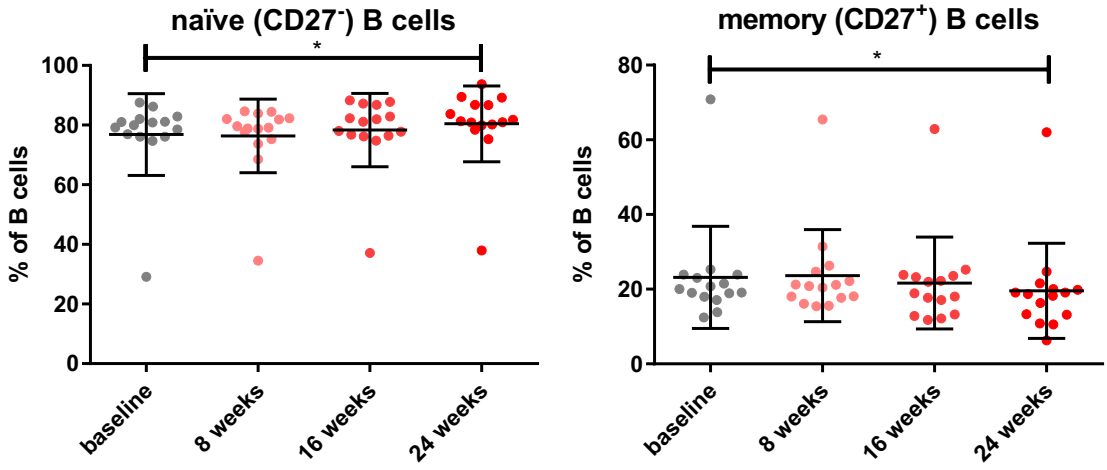

Fig. 4.16 Effect of CLA *in vivo* on B cell subset composition in MS patients
Share of the respective indicated B cell subset as a percentage of all B cells determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment. *n* = 15. All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: * $p \leq 0.05$. The outliers at all timepoints in both graphs are representing one individual.

The automated analysis of the data from B cells (pre-gated before algorithm application) stained with the "B cell" panel also revealed a 1.32-fold upregulation of naïve B cells after the CLA *in vivo* treatment (Fig. 4.17).

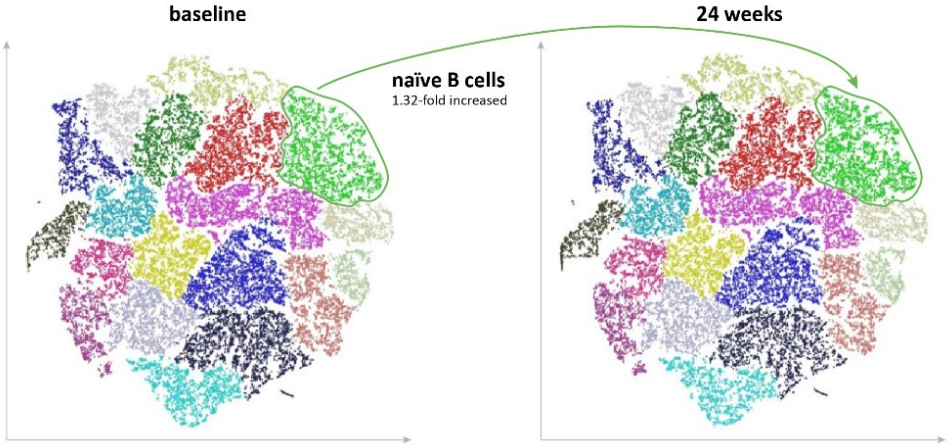

Fig. 4.17 Effect of CLA *in vivo* on B cells in MS patients
viSNE maps of all B cells (pre-gated before automated analysis) before and after 24 weeks CLA *in vivo* treatment, measured by 10 color flow cytometry after staining with the "B cell" panel. Each dot represents one individual cell. The cells are colored according to their assignment to distinct cell clusters by the PhenoGraph algorithm. Each graph maps a total of 56,000 cells merged from all study participants. The axes are given in arbitrary units. *n* = 15.

4.2.4.4 *CD4⁺ T_{reg} cells*

Ultimately, Fig. 4.18 shows the effect of CLA on T_{reg} cells and their major subsets, peripherally (pT_{reg}) and thymus (tT_{reg}) derived T_{reg} cells. While the share of pT_{reg} cells slightly, but statistically significantly decreased both in all T_{reg} cells and in all $CD4^+$ T cells, the share of tT_{reg} cells in all T_{reg} cells slightly increased and the share of tT_{reg} and of total T_{reg} cells in all $CD4^+$ T cells remained more or less constant during the treatment period.

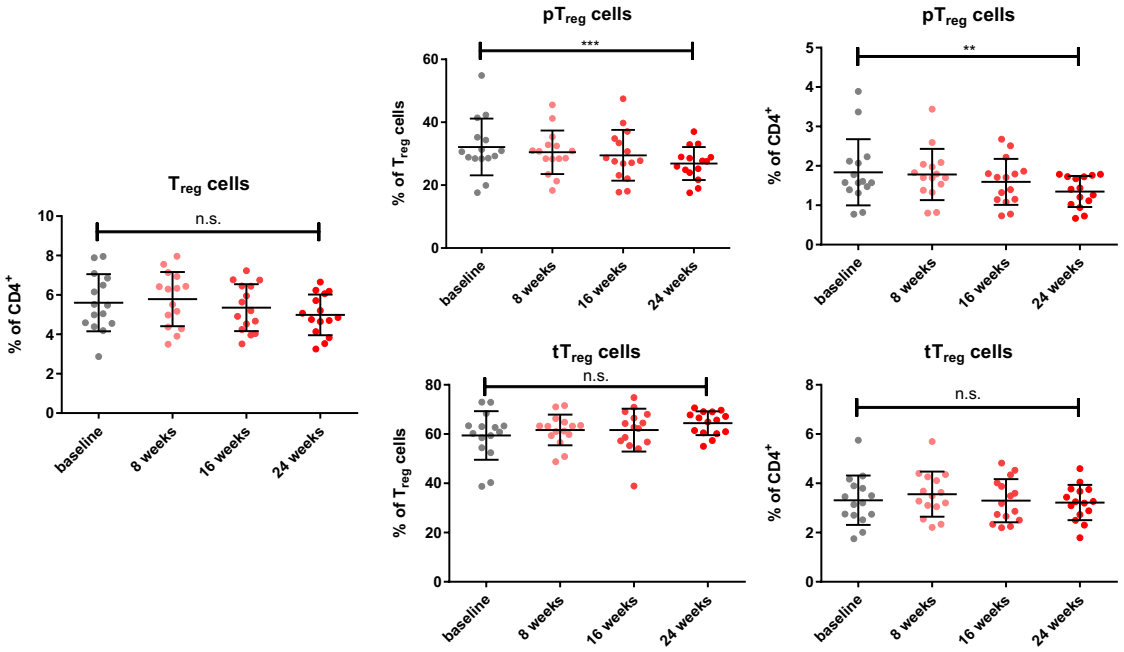

Fig. 4.18 Effect of CLA *in vivo* on T_{reg} cells in MS patients
Share of T_{reg} cells as a percentage of all $CD4^+$ T cells and share of pT_{reg} and tT_{reg} cells as a percentage of all T_{reg} and of all $CD4^+$ T cells determined in a conventional gating analysis and compared before, during and after 24 weeks CLA *in vivo* treatment. *n* = 15. All data is presented as mean ± SD. Results from Wilcoxon test baseline vs. 24 weeks: ** $p \leq 0.01$ ***$p \leq 0.001$ n.s. = not significant.

Also this effect was reproducible in the automated flow cytometry data analysis. After applying the viSNE and the PhenoGraph algorithms to the data from $CD25^+$ $CD4^+$ memory T cells (pre-gated before automated analysis) stained with the "T_{reg}" panel, pT_{reg} cells were found 1.39-fold reduced after the CLA treatment period (Fig. 4.19).

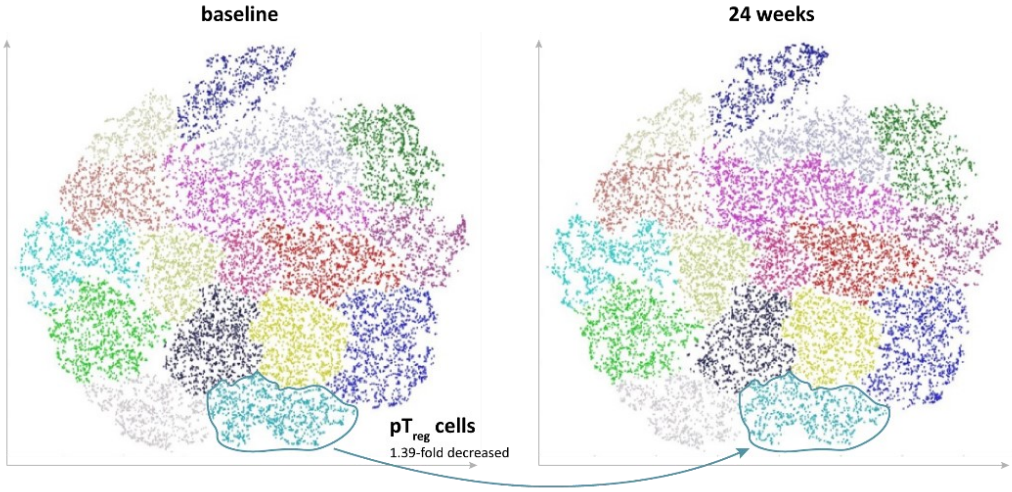

Fig. 4.19 Effect of CLA *in vivo* on CD25⁺ CD4⁺ memory T cells in MS patients
viSNE maps of all CD25⁺ CD4⁺ memory T cells (pre-gated before automated analysis) before and after 24 weeks CLA *in vivo* treatment, measured by 10 color flow cytometry after staining with the "T_{reg}" panel. Each dot represents one individual cell. The cells are colored according to their assignment to distinct cell clusters by the PhenoGraph algorithm. Each graph maps a total of 16,296 cells merged from all study participants. The axes are given in arbitrary units. *n* = 15.

4.2.5 CD4⁺ T cell cytokine production upon activation

The *in vitro* treatment of HC CD4⁺ T cells (measured with ELISA and Luminex® technique – section 4.1.2) and the *in vivo* treatment of RRMS patients' CD4⁺ T cells (measured by flow cytometry – section 4.2.4) with CLA resulted in lower cytokine production capacities of these cells. Next, the hypothesis that an *in vivo* CLA treatment lowers the pro-inflammatory cytokine production capacity of RRMS patients' CD4⁺ T cells was tested with the a Luminex® assay as an additional method, analyzing more cytokines simultaneously than the flow cytometry. Therefore, study patients' CD4⁺ T cells from before and after the 24 weeks CLA treatment were stimulated with PHA for 72 h without *in vitro* exposure to CLA. The cell culture supernatants were analyzed in a Luminex® assay to determine several cytokine concentrations.

The resulting data for the nine most interesting cytokines is presented in Fig. 4.20. No significant changes could be detected between the cytokine production capacity before and after the treatment.

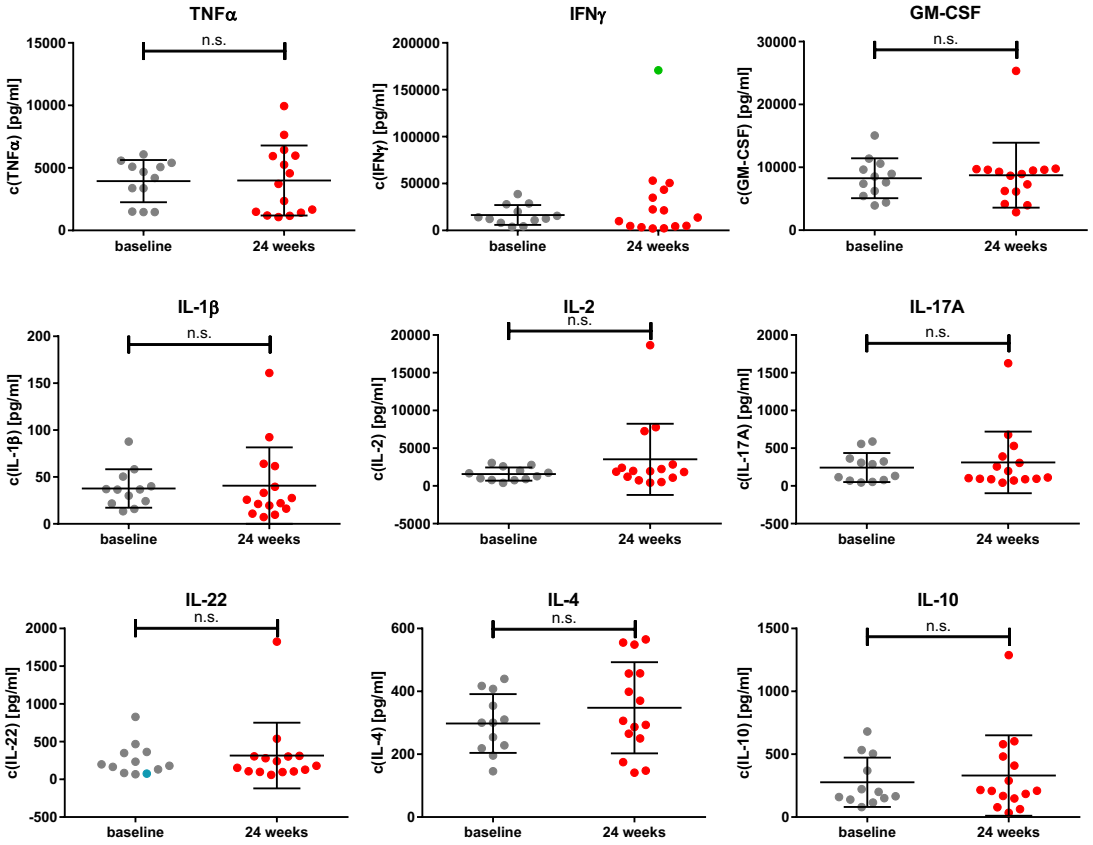

Fig. 4.20 Effect of CLA *in vivo* on MS patients' CD4⁺ T cells' cytokine production capacity
Quantity of the respective cytokine produced by MS patients' CD4⁺ T cells before and after 24 weeks *in vivo* CLA treatment, measured after 72 h stimulation with PHA. baseline: $n = 12$ (due to technical issues), 24 weeks: $n = 15$. All data is presented as mean ± SD. Results from Wilcoxon test: n.s. = not significant. Blue dots represent values < LLOQ, green dots values > ULOQ. The cytokine concentration in all unstimulated samples was < LLOQ.

4.2.6 CD4⁺ T cell metabolism

To reproduce the findings from the corresponding *in vitro* experiment and to gain insight into the effects of an *in vivo* CLA treatment on the immune cell metabolism, the metabolic activity was measured and compared in CD4⁺ T cells collected before and after the CLA treatment under Mito Stress conditions. The underlying hypothesis was that an *in vivo* CLA treatment would change the cells' metabolic activity towards a rather immunomodulatory phenotype. In contrast to the corresponding *in vitro* experiment, since no *in vivo* CLA treatment was necessary, the CD4⁺ T cells were isolated from freshly thawed PBMC samples and their metabolic activity during a Mito Stress Test was measured immediately.

4.2.6.1 *Oxidative phosphorylation (OXPHOS)*

First, the CD4⁺ T cells' metabolic activity was measured without any *in vitro* stimulation. In this case, the analysis of the OXPHOS activity (represented by the OCR (3)) during the Mito Stress Test revealed that the CD4⁺ T cells collected after the treatment exhibited a significantly lower mitochondrial respiration (Fig. 4.21). The cells' BR as well as their MR and SRC were on a lower level than in the cells collected before the treatment.

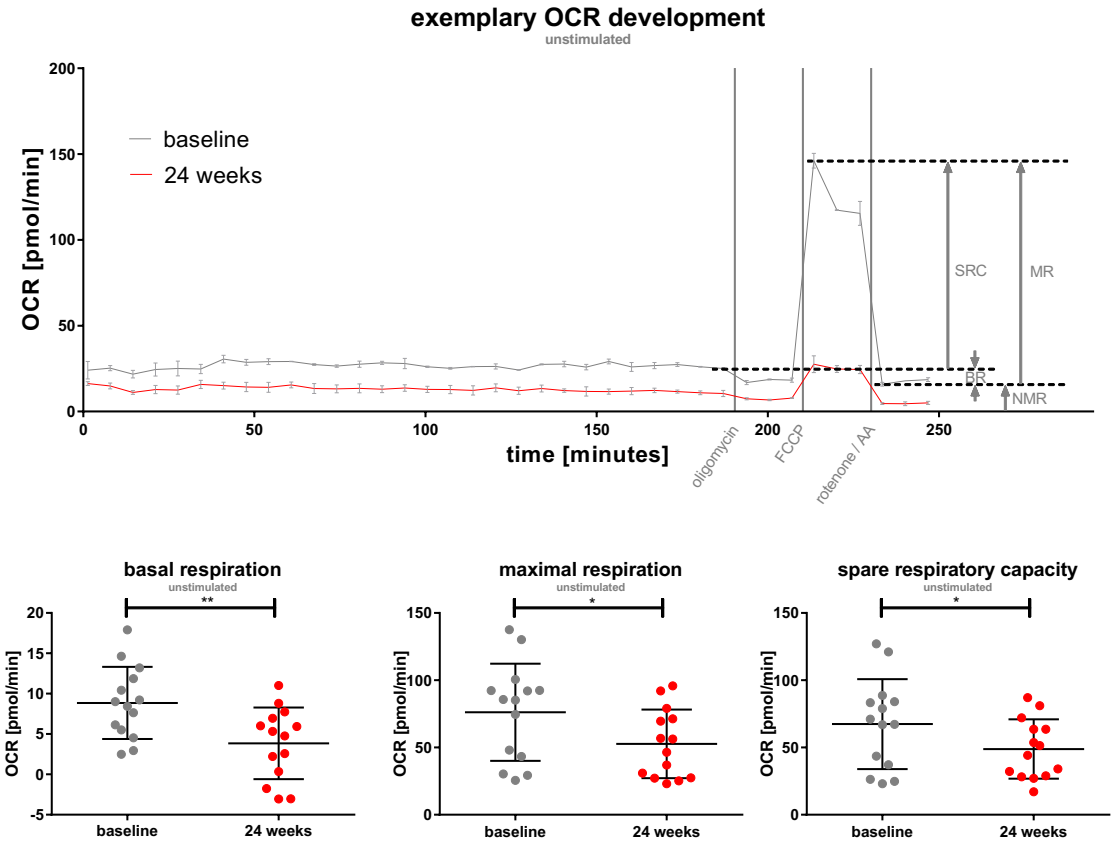

Fig. 4.21 Effect of CLA *in vivo* on MS patients' unstimulated CD4⁺ T cells' OXPHOS
Top. Representative OCR curves of MS patients' CD4⁺ T cells isolated before or after 24 weeks *in vivo* CLA treatment during Mito Stress Test without stimulation. Oligomycin, FCCF and rotenone / antimycin A were added as indicated. The calculations for the parameters NMR, BR, MR and SRC are illustrated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BR, MR and SRC of MS patients' unstimulated CD4⁺ T cells before and after 24 weeks *in vivo* CLA treatment. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: * p ≤ 0.05 ** p ≤ 0.01

In comparison, Fig. 4.22 shows the corresponding results when performing the Mito Stress Test after a short time activation with PMA and ionomycin for 2:40 h. A reduction in the CD4⁺ T cells' activity performing OXPHOS, represented by the basal and MR and the SRC, could be seen here as well.

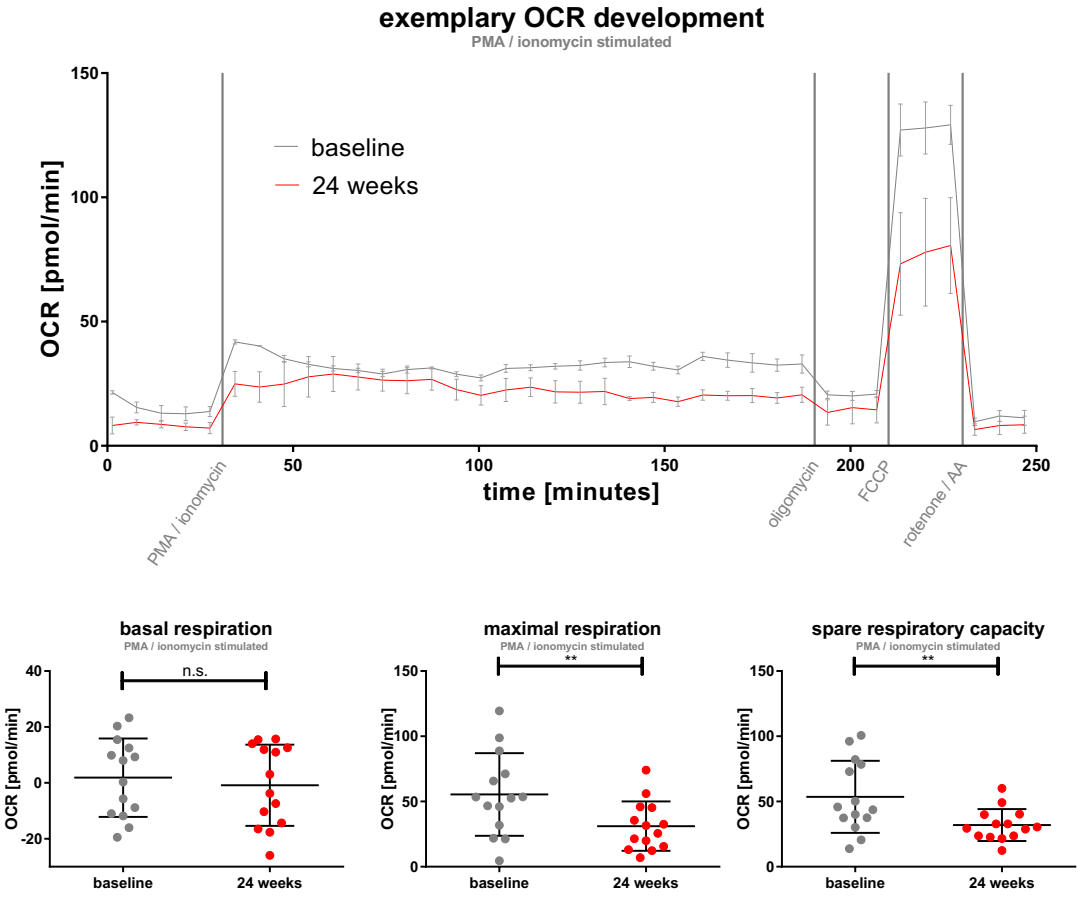

Fig. 4.22 Effect of CLA *in vivo* on MS patients' stimulated CD4⁺ T cells' OXPHOS
Top. Representative OCR curves of MS patients' CD4⁺ T cells isolated before or after 24 weeks *in vivo* CLA treatment during Mito Stress Test with PMA / ionomycin stimulation. PMA / ionomycin, oligomycin, FCCF and rotenone / antimycin A were added as indicated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BR, MR and SRC of MS patients' CD4⁺ T cells collected before and after 24 weeks *in vivo* CLA treatment, measured after PMA / ionomycin stimulation. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: ** $p \leq 0.01$ n.s. = not significant

Comparing the effect of a stimulation with PMA and ionomycin on the MR and the SRC in CD4⁺ T cells collected before versus after the treatment, it could be seen, that the stimulation-induced increase in both parameters was significantly lower after the 24 weeks *in vivo* treatment, expressed by the ratio of the respective values with and without stimulation (Fig. 4.23).

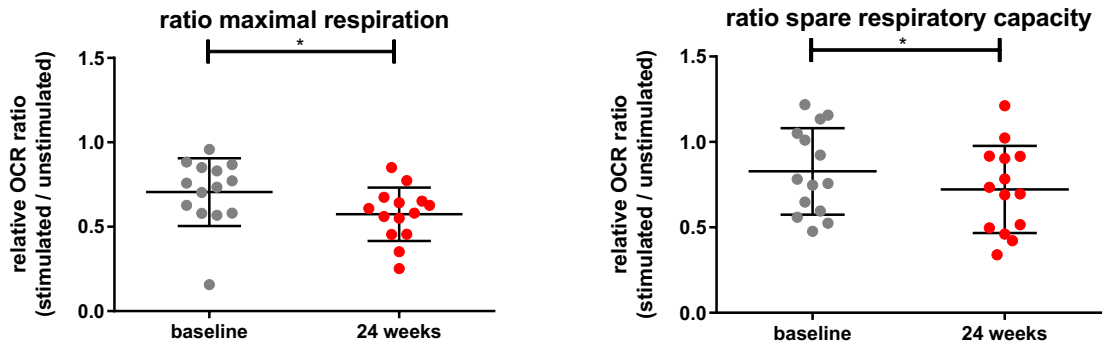

Fig. 4.23 Effect of CLA *in vivo* on MS patients' CD4⁺ T cells' stimulation-induced increase in MR and SRC
Ratio of MR and SRC values measured in samples stimulated or not with PMA / ionomycin (stimulated / unstimulated) calculated for CD4⁺ T cell samples collected before and after 24 weeks *in vivo* CLA treatment. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$

4.2.6.2 Glycolysis

The effect of a 24 weeks *in vivo* CLA treatment on the glycolytic activity of CD4⁺ T cells from MS patients, measured without *in vitro* stimulation, is shown in Fig. 4.24: The BG, the GC and especially the GR were reduced after the treatment.

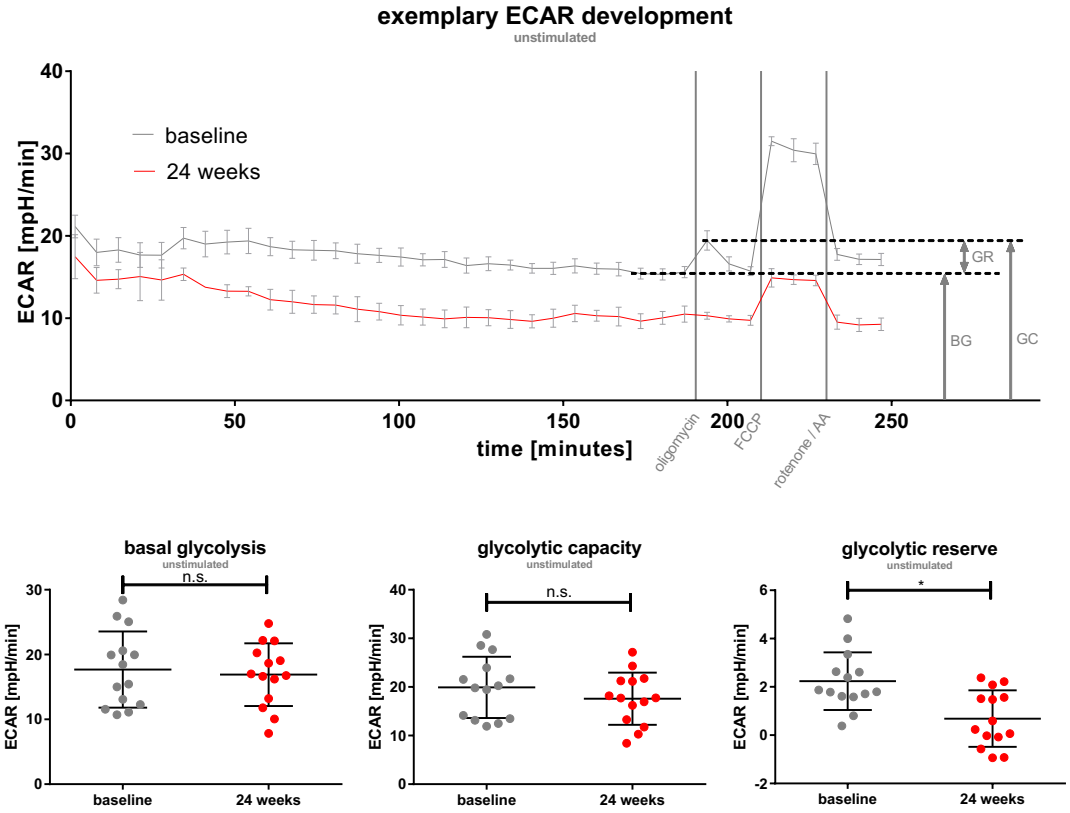

Fig. 4.24 Effect of CLA *in vivo* on MS patients' unstimulated CD4⁺ T cells' glycolytic activity
Top. Representative ECAR curves of MS patients' CD4⁺ T cells isolated before or after 24 weeks *in vivo* CLA treatment during Mito Stress Test without stimulation. Oligomycin, FCCF and rotenone / antimycin A were added as indicated. The calculations for the parameters BG, GC and GR are illustrated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BG, GC and GR of MS patients' unstimulated CD4⁺ T cells before and after 24 weeks *in vivo* CLA treatment. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$ n.s. = not significant

Performing the same experiment after short time activation with PMA and ionomycin for 2:40 h, similar changes could be observed (Fig. 4.25). All three parameters were reduced in the CD4⁺ T cells collected after the treatment.

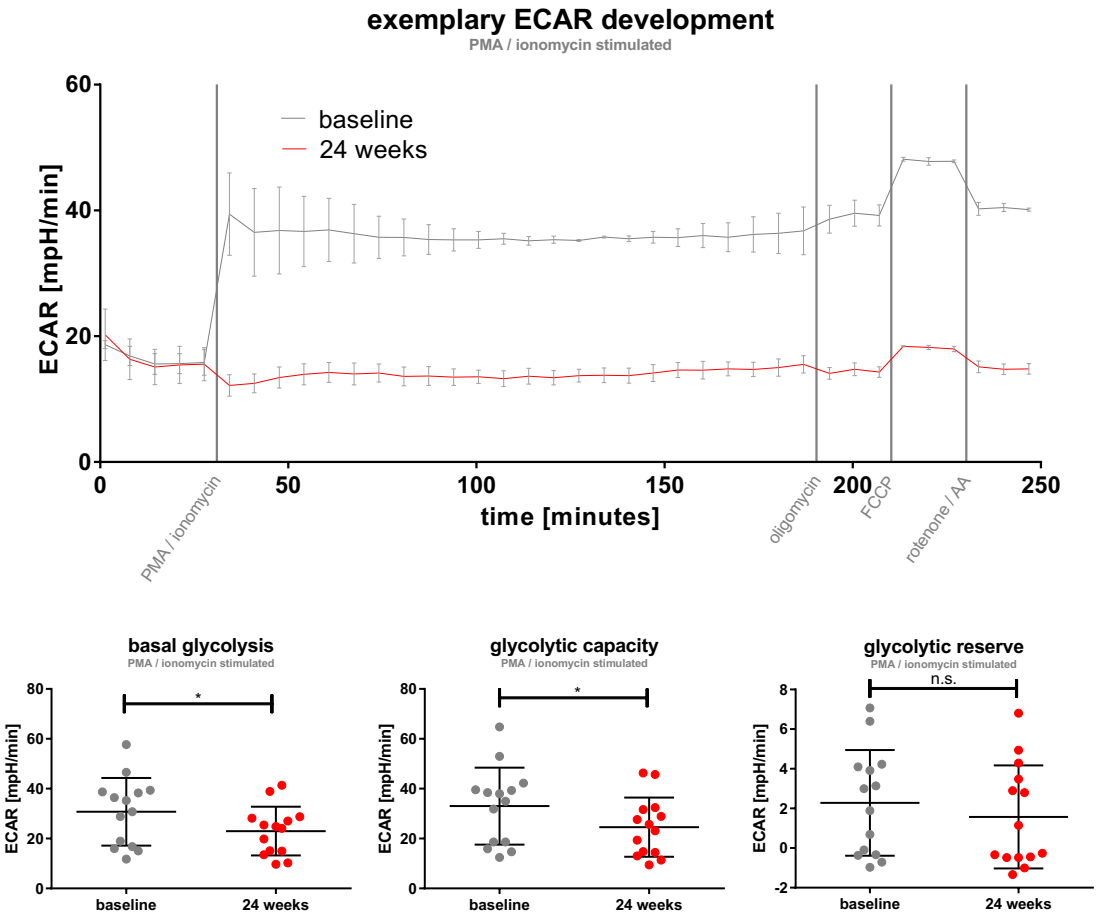

Fig. 4.25 Effect of CLA *in vivo* on MS patients' stimulated CD4⁺ T cells' glycolytic activity
Top. Representative ECAR curves of MS patients' CD4⁺ T cells isolated before or after 24 weeks *in vivo* CLA treatment during Mito Stress Test with PMA / ionomycin stimulation. PMA / ionomycin, oligomycin, FCCF and rotenone / antimycin A were added as indicated. All data points are presented as mean ± SD of the technical replicates. AA = antimycin A. **Button.** BG, GC and GR of MS patients' CD4⁺ T cells collected before and after 24 weeks *in vivo* CLA treatment, measured after PMA / ionomycin stimulation. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$ n.s. = not significant

Again, also a comparison between the stimulation-induced increase of the glycolytic activity before and after the treatment was performed (Fig. 4.26). It could be observed that this increase of the BG and the GC, represented by the ratio of the respective values with and without stimulation, was higher in the baseline samples than in those collected after the treatment.

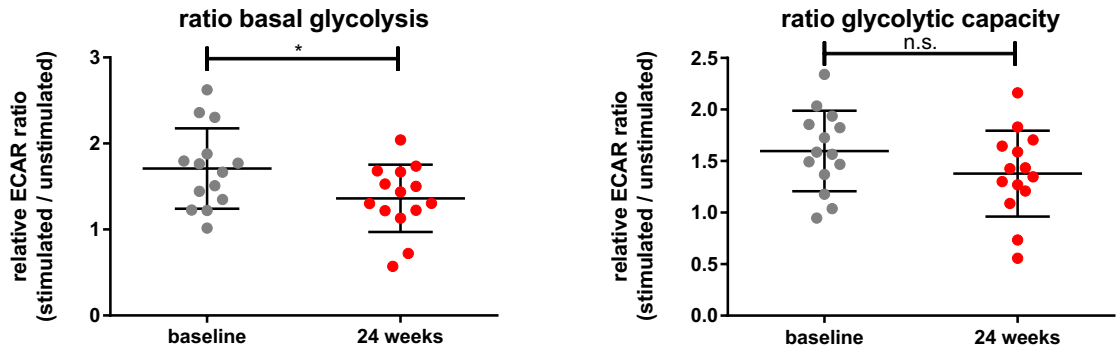

Fig. 4.26 Effect of CLA *in vivo* on MS patients' CD4⁺ T cells' stimulation-induced increase in BG and GC
Ratio of BG and GC values measured in samples stimulated or not with PMA / ionomycin (stimulated / unstimulated) calculated for CD4⁺ T cell samples generated before and after 24 weeks *in vivo* CLA treatment. *n* = 14. All data is presented as mean ± SD. Results from Wilcoxon test: * $p \leq 0.05$ n.s. = not significant

5 Discussion

In current neuroimmunology research, rising attention is being paid to the effects of environmental and especially nutritional factors on CNS autoimmunity. Since the identification of protective nutritional factors could lead to novel treatment approaches for MS by supplementing them with the diet or by targeting their mechanisms of action with new drugs, further research on this complex field of neuroinflammation is urgently needed (40, 156).

CLA was identified as a promising candidate for the complementary treatment of MS. It was demonstrated to exert beneficial effects in the context of various immune-related diseases (16, 17, 188) and – most interestingly – in a spontaneous MS mouse model *in vivo* and in human healthy controls' and RRMS patients' immune cells *in vitro* (79). Moreover, CLA can change the immune cell metabolism (83, 188) and the gut microbiota composition (33), both being factors influencing the immune system function and thereby potentially CNS autoimmunity (21, 23, 44, 77, 133, 139).

Therefore, it was aim of the present work to gain a deeper insight into the effects of CLA on human MS disease. With *in vitro* experiments and an exploratory clinical trial, the impact of a CLA treatment on clinical parameters, the immune cell subset composition, the $CD4^+$ T cell cytokine production and their cell metabolism were studied. Furthermore, the natural CLA intake by the study patients and their adherence to the study medication were measured.

5.1 Clinical data

5.1.1 Baseline demographical and clinical data

In the study, 15 RRMS patients in an early disease phase were included, relapse free and on a stable first-line immunomodulatory medication for at least 3 months prior to the study. The cohort was predominantly female (73,3%) what is in accordance with the share of women in the general Central European MS-affected population (89). In average, the patients were diagnosed with MS 6.5 years before inclusion and had experienced the first symptoms 2 years earlier. The mean EDSS score at the time of inclusion was 0.8. To sum up, the study cohort constituted an average group of RRMS patients in an early disease phase.

5.1.2 Natural fat and CLA intake

Since CLA is also consumed naturally with the diet (188), it was necessary to measure this consume to make valid statements about the effects of dietary supplemented CLA on MS. Only if the amount of additionally consumed CLA would be remarkably higher, potential effects could possibly be attributed to the supplemented CLA. Moreover, it was of interest, how much the natural fat and especially CLA intake vary across the study cohort.

The analysis of the food diaries filled out by the study patients revealed an approximate daily fat intake of 72 g with individual values ranging from 35 to 107 g/d. The average fat consume of the general German population was measured in 2008 with a survey asking about the food intake during a period of 4 weeks. The study was conducted in 15,371 individuals representative for the German population. It revealed an average daily fat intake of 73,8 g (women) and 102 g (men), respectively (115). Therefore, the fat intake of the CLA study cohort seems to have been slightly lower but considering the share of women in the study cohort above the general population's average, the difference is marginal. The World Health Organization recommends a fat consume constituting less than 30% of the total calorie intake (195). Depending on the study patients' daily energy demand which has not been surveyed this target could have been reached by the participants. Calculating with an energy density of 9 kcal/g for fat (155), the average calorie intake from fat in the study cohort was 648 kcal/d.

The natural intake of CLA by the study participants was measured with the nutrition diaries to be approximately 100 mg/d with values ranging from 27 to 167 mg/d. Different studies measured the natural CLA intake by the average population. They came to largely varying results which may be due to regional and cultural differences (127). However, the study method plays an important role for the accuracy of the results (154), and different employed methods may be partially responsible for the highly varying results (127). One US-American study by Ritzenthaler *et al.* compared different methods to estimate the dietary CLA intake in a single study cohort. Thereby, dietary records and food-frequency questionnaires underestimated the amount of dietary CLA in comparison to the results from chemical food duplicate analyses which revealed a natural CLA intake of 212 and 151 mg/d by men and women, respectively (154). When comparing the values from the present work with the results from Ritzenthaler *et al.*, the increased share of female study participants must be considered again, as the sex obviously is a major determinant for the dietary CLA intake (154) as it is for the overall fat consume (see above) (115).

Nevertheless, the present work estimates the natural CLA intake by the participants lower than Ritzenthaler *et al.* in their study population with the food duplicate method. This may be due to methodological impairments as the food diary method cannot be considered as accurate as for example a food duplicate analysis and could underestimate the dietary CLA intake (154). This could be explained by, *inter alia*, incomplete dietary recording, wrong estimation of consumed amounts, difficulties when categorizing food items and insufficient knowledge about their exact chemical composition. However, also a lower real CLA intake due to different dietary habits is conceivable. This could represent either statistical coincidence evoked by the selection of participants in the two rather small studies (n = 14 in present work; n = 93 in study by Ritzenthaler *et al.* (154)) or different cultural habits between the US-American and German population.

The natural CLA intake varied up to 6-fold between the individual study patients. However, this fact as well as the natural CLA consumption in general probably did not influence the study results since the interventional CLA supplementation with 2,100 mg/d was by far, at least 10-fold, higher than any individual natural intake value. In average, the natural CLA intake was lower than 5% of the supplementary consume.

To sum up, the fat and CLA intake values surveyed with food intake diaries for this study cannot be considered as optimally reliable. However, the results for the fat consume are approximately in accordance with those of the average German population (115) and those for the study cohort's natural CLA intake lie within the range of estimates made by different methods published previously (127). Considering that the supplementary CLA consume was much higher than the natural one, the reliability of the natural CLA intake calculation might be considered sufficient to state that the natural CLA intake did not substantially influence the study results.

5.1.3 Adherence to study medication

Patients' adherence to the medication is crucial for every medicinal therapeutic intervention (98). To validate the results of the study and to determine the readiness for a daily CLA supplementation, the participants' adherence to the study medication was surveyed with intake diaries and empty package counting. The investigation revealed a remarkable adherence to the study medication of ≥ 95% in 73.3% (11/15) of the participants and ≥ 90% in 93.3% (14/15). One patient (6.7% of the study cohort) showed an adherence of 88.1%. It must be considered that the

adherence may differ in potential patients treated with CLA regularly and long-termed in comparison to the present cohort under study conditions. However, these values promise a potential of CLA as a supplementation with high adherence.

5.1.4 Relapses, EDSS and paraclinical development during follow-up

In the long term, the aim of an MS therapy should be a reduction of the frequency of relapses, of the chronic accumulation of disability (as reflected by the EDSS score) and / or of mortality. The analysis of the study patients' clinical course revealed no major clinical effects of the 24 weeks CLA treatment.

With one out of 15 patients suffering a relapse, the average annualized relapse rate during the 24 weeks study period (0.14 relapses per year) was lower than in the year before study inclusion (0.27 relapses per year; 4 out of 15 patients suffered one relapse each). However, the small study size and the lack of a control group limit the reliability of these results. Especially in view of the lacking control group it must be considered that on the one hand a recent MS relapse might have motivated patients to participate in the study, and that on the other hand a relapse in the last three months prior to the study was an exclusion criterion. In which way these circumstances might have caused a selection bias is unclear, but they certainly limit the comparability of the annualized relapse rates before and during the study. Anyway, due to the study-independent development of diseases like MS a comparison between relapse rates before and during the study can never be as informative as a comparison between the relapse rates during the study in a treated and an untreated group.

Interestingly, the sole patient suffering a relapse during the study reported not to have applied the prescribed DMT (IFNβ-1a, Rebif®) regularly in the months prior to the relapse. However, it cannot be stated if this has directly caused or favored the attack and the adherence to their basic DMT was not systematically measured in the study participants, neither before nor during the study.

The EDSS score remained stable in 12 out of 15 patients, one participant had a reduced and two an increased score after the study compared with the inclusion timepoint. In these two patients with an increased score, the increase was relapse-independent. However, a control group would be necessary to draw conclusions about the effect of CLA on the chronic accumulation of disability from this observation.

As explained in section 4.2.3.4, the data about the paraclinical development of the study participants is very limited. Therefore, this study cannot give information about the effect of CLA on paraclinical processes in MS. However, in a putative RCT investigating the influence of CLA on MS, changes on MRI scans should be included in the study endpoints.

In the experiments investigating the effects of CLA on murine CNS autoimmunity presented in the introduction (see section 1.2.3.3), also an effect on the clinical course could be demonstrated represented by the development of the clinical disease score (unpublished data by Hucke, Hartwig *et al.* (79)). However, several major methodological differences must be considered when comparing these findings with the present human trial. For example, in the mouse model, the beneficial clinical effect was not observed by comparing different timepoints (end of treatment vs. baseline) but by comparing to a control group. In both study groups, the average clinical score increased continuously. Furthermore, OSE develops much more rapidly than human MS and therefore the CLA treatment and the observation period in comparison to the disease development in the murine experiment were much longer than in the present clinical trial. Also, the CLA treatment was started with conception in the murine experiment while it was initiated after disease onset (8.5 years in average) in the human trial. Moreover, the study groups were larger in the animal model experiment (n (OSE^{CLA}) = 40; n (OSE^{ctrl}) = 83) (79).

Difficulties when translating animal model findings about CLA into human disease have been reported in the context of several other autoimmune conditions (188). Nevertheless, for example a small (n = 13) clinical trial by Bassaganya-Riera *et al.* investigating the effects of CLA on human IBD could also reveal an ameliorated clinical disease activity and in improved quality of life after a 12 week CLA treatment. With 6 g CLA daily, a higher dosage was applied to the study participants in this trial compared to the present study (16).

Reliable statements on the clinical efficacy of CLA in MS could only be made in a double-blind randomized placebo-controlled clinical trial with a much higher number of study participants, especially considering the fact that the hypothesized CLA effect would be rather small and therefore more difficult to detect and a potential treatment would have only a complementary character. Results would be even more reliable when prolonging the CLA treatment period and adding MRI scans as additional paraclinical endpoints.

However, the aim of the present pilot study was not more than a first, exploratory investigation of the effects of CLA on human MS and the main read-outs were not the clinical parameters, but

the analysis of the immune cell subset composition, the $CD4^+$ T cell cytokine production and their cell metabolism.

5.1.5 Adverse events

Also, a highly important characteristic of any medicinal treatment is the risk of adverse reactions. Therefore, it was of interest to systematically document adverse events occurring during the CLA supplementation period.

The collected data is presented in section 4.2.3.4. In total, 23 adverse events were recorded, mostly infections and gastrointestinal symptoms / disorders. One SAE occurred, an MS relapse leading to hospitalization of the affected patient. In five of the recorded AEs (abdominal pain, abdominal fullness, enteritis, 2 x nausea) a relation to the CLA supplementation appeared possible.

In other studies investigating CLA in humans, CLA generally appeared to be well-tolerated (16, 188). However, after long-term CLA supplementation, some patients exhibited an elevated insulin resistance and mild gastrointestinal irritations (188).

With the acquired data of the present study, no conclusions about the capacity of CLA to evoke adverse reactions can be drawn. Also other studies on CLA in humans so far were methodologically not suitable to make final statements about adverse reactions to a CLA treatment (188). Only a large-scale double-blind randomized placebo-controlled clinical trial could confirm statements about this topic. However, the findings in the present trial are in line with previously published data about adverse reactions to CLA supplementation and underline CLA as a potential complementary treatment in MS with few side effects.

5.2 In-depth functional immune phenotyping

Considering the various effects on different parts of the immune system attributed to CLA and the influence it seems to have on several immune system-driven diseases, including CNS autoimmunity (16, 79, 188), it was of high interest to analyze the impact of a dietary CLA supplementation on different immune system components *in vivo* in the context of human MS.

A broad overview over this impact was gained by flow cytometry analysis of PBMC samples collected before and after 8, 16 and 24 weeks of dietary CLA supplementation from RRMS patients.

The data was subsequently analyzed both with manual gating strategies (all four treatment timepoints) and in an automated, less biased approach applying a dimensionality reduction and a clustering algorithm to the data (before vs. after treatment). The effect of CLA on both phenotypical (shares of different types of immune cells) and functional (activity of immune cells) parameters were analyzed.

The underlying hypothesis was that the CLA supplementation would reduce the shares of pro-inflammatory immune cell subsets and decrease their pro-inflammatory cytokine production capacity while promoting immune-regulatory cell types.

5.2.1 **$CD4^+$ T cells**

5.2.1.1 T_H17 cells and related immune cell subsets

The conventional gating analysis revealed a downregulation of T_H17 cells, defined either as $CD146^+$ $CD4^+$ memory T cells (statistically significant downregulation) or more strictly as $CD194^+$ $CD196^+$ $CD161^+$ $CD146^+$ $CD4^+$ memory T cells (statistically not significant downregulation). The automated data analysis showed a 1.9-fold reduction of $CD146^+$ $CD4^+$ T cells. The manual gating further revealed a statistically significant downregulation of IL-$23R^+$ memory $CD4^+$ T cells during the CLA treatment.

These observations imply an anti-inflammatory effect of CLA. The concurrent expression of CD194, CD196, CD146 and CD161 (CD194 = CCR4; CD196 = CCR6; CD146 = MCAM) on $CD4^+$ memory T cells is a well-established criterion for the flow cytometric identification of T_H17 cells (22, 106). However, also the downregulation of $CD146^+$ $CD4^+$ memory T cells and of $CD146^+$ $CD4^+$ T cells upon CLA treatment detected in the manual and automated data analysis, respectively, can be interpreted as an effect on T_H17 cells. $CD146^+$ $CD4^+$ T cells both from HCs and from patients with different autoimmune diseases exhibit an effector memory phenotype and partial co-expression of the above mentioned T_H17 cell-associated cell surface markers CD194, CD196 and CD161. Most importantly, $CD146^+$ $CD4^+$ T cells produce IL-17A and show several other characteristics of T_H17 cells, thus resembling a T_H17 phenotype (38). CD146, also called melanoma cell adhesion molecule (MCAM), plays an important role in the migration of T_H17 cells into the CNS (28).

T_H17 cells are a highly pro-inflammatory type of T helper cells characterized by the production of IL-17A (synonym IL-17). Additionally, they secrete a variety of other cytokines including IL-17F

and IL-21. While IL-21 is involved in the signaling to other immune cells and promotes the generation of further T_H17 cells, IL-17A and IL-17F are pro-inflammatory effector cytokines directly causing local inflammation. T_H17 cells play an important role in the control of specific bacterial and fungal pathogens which are not sufficiently combated by T_H1 and T_H2 cells. However, since their cytokine secretion profile enables them to evoke profound tissue inflammation, their action also contributes to the pathogenesis of several autoimmune diseases (93, 189).

One of these diseases is MS. Due to many experimental observations, T_H17 cells are considered a key pathogenic contributor to MS development. Several effector mechanisms and characteristics are underlying the pathogenic potential of this cell type in MS. Among these are their ability to enter the CNS by crossing the BBB and to facilitate the entry for other immune cells, potential neurotoxic and anti-remyelinating properties and the secretion of pro-inflammatory effector cytokines. Many new therapeutic approaches are currently being developed and tested for a possible application in MS treatment which interfere with T_H17 cell differentiation or effector activity (189). Also the $CD146^+$ $CD4^+$ T cells, which seem to be T_H17 cells or at least to strongly resemble their phenotype, as described above, are suggested by experimental findings to be implicated in the pathogenesis of several autoimmune diseases (38).

IL-23R, which is one subunit of the heterodimeric receptor for IL-23, is another marker of T_H17 cells (90, 141). IL-23 signaling is important for their terminal differentiation and survival (7, 93). Several research findings suggested an implication of IL-23A (synonym IL-23p19; one subunit of the heterodimeric cytokine IL-23) and IL-23R in the pathogenesis of MS and various other autoimmune diseases (93, 101). These findings include the resistance of IL-23A knockout mice to EAE (36), elevated IL-23A serum levels in MS patients and the association of variants in the IL-23A and IL-23R genes with MS risk (101). Interestingly, $IL\text{-}23R^+$ $CD4^+$ T cell levels were found to be decreased upon treatment with the first line drug for DMT IFNβ-1a, suggesting that the effect of IFNβ-1a on MS could at least partially be mediated via downregulation of this cell type. However, the very low number of study participants (n = 6) reduces the reliability of this result (42, 94).

Considering this information, the observed downregulation upon CLA treatment of immune cell subsets exhibiting T_H17 phenotypic characteristics suggests an anti-inflammatory effect of CLA in the context of MS. This effect on T_H17 cells could mediate potential beneficial effects of CLA on the risk for or course of MS.

The finding that CLA acts in an anti-inflammatory fashion on T_H17 cells is consistent with previous experimental results. In CD patients, dietary CLA supplementation reduced the IL-17A production by $CD4^+$ T cells (16). Also in OSE mice, the IL-17A production by peripheral $CD4^+$ T cells was lowered upon oral CLA treatment. Furthermore, CLA exposition *in vitro* inhibited the polarization of both murine and human naïve $CD4^+$ T cells towards T_H17 cells. Finally, also the IL-17A production of stimulated peripheral blood effector $CD4^+$ T cells isolated from HCs and RRMS patients was reduced under *in vitro* exposition to CLA (79).

Interestingly, the nuclear receptor PPARγ, which has been described in the introductory section 1.2.4, and which can be activated by CLA (188), has been found to be a key specific inhibitor of T_H17 cell differentiation both in the murine and the human system while not interfering with T_H1 and T_H2 differentiation. In this context, PPARγ activation also reduced the IL-23R expression by human $CD4^+$ T cells. The inhibition of T_H17 cell differentiation by activation of PPARγ could be a mechanisms of action of potential protective effects of CLA in the context of CNS autoimmunity as this has been found to mediate EAE-protective effects of pioglitazone, another activator of PPARγ (90).

5.2.1.2 $CD4^+$ T cell cytokine production

Focusing on the cytokine production of $CD4^+$ T cells, the flow cytometry analysis revealed a statistically significant downregulation of IL-17A and IL-22 producing $CD4^+$ T cells and $CD4^+$ memory T cells (manual gating analysis) and a reduction of IL-22 producing $CD4^+$ $CD146^+$ T cells (automated analysis), respectively, all measured after 4 h LAC activation.

IL-17A and IL-22 are two signature cytokines of the highly pro-inflammatory T_H17 cells which are assumed to play an important role in the pathogenesis of MS as described in the previous section 5.2.1.1. While IL-17A is well-known as a pro-inflammatory cytokine, the exact role of IL-22 is less well-established (93, 189). On the one hand, a genetic knockout of the gene encoding IL-22 has no influence on the risk for or course of EAE what does not support a key pathogenic role of the cytokine in CNS autoimmunity (95). On the other hand, several observations suggest IL-22 to contribute to the pathogenic role of T_H17 cells in the context of CNS autoimmunity. IL-22 promotes the secretion of various antimicrobial molecules by epithelial cells (161), MS patients exhibit elevated IL-22 levels in their peripheral blood and CSF (189) and the cytokine is suggested to contribute to the BBB damage in MS lesions (87).

The observation of a reduced IL-17A and IL-22 production by activated $CD4^+$ T cells upon CLA treatment is in line with previous experimental results. Since these are signature cytokines of T_H17 cells (189), the inhibitory effect of the PPARγ agonists CLA and pioglitazone on T_H17 cell differentiation in both the murine and the human system described in the previous section 5.2.1.1 corresponds to the present findings (79, 90). The IL-17A production by peripheral $CD4^+$ T cells was reduced upon *in vivo* CLA treatment of OSE mice (79) and CD patients (16). Upon *in vitro* CLA treatment, peripheral blood $CD4^+$ T cells from HCs and RRMS patients exhibited a diminished production of IL-17A (79). Regarding IL-22, Klotz *et al.* observed a reduced expression by murine and human $CD4^+$ T cells exposed *in vitro* to the PPARγ agonist pioglitazone (90). Finally, also the observation in the present work that an *in vitro* CLA treatment reduced the IL-17A and IL-22 production capacity of HC $CD4^+$ T cells (4.1.2.2), is in line with the findings from the immune phenotyping of the study participants' PBMC samples.

To sum up, the observed downregulation of the IL-17A and IL-22 production by activated $CD4^+$ T cells, $CD4^+$ memory T cells and $CD4^+$ $CD146^+$ T cells, which can be regarded as T_H17 cells as described in the previous section 5.2.1.1 (38), upon *in vivo* CLA treatment further suggests an anti-inflammatory effect of the dietary supplement mediated by dampening the frequency and / or activity of T_H17 cells.

5.2.2 **$CD8^+$ T cells**

5.2.2.1 CD226 expression on $CD8^+$ T cells

The immune phenotyping revealed an upregulation of the surface marker CD226 (DNAM-1) on $CD8^+$ T cells, $CD8^+$ central memory T cells, $CD8^+$ T_{EMRA} cells (manual gating analysis) and on $CD8^+$ memory T cells (automated analysis) upon 24 weeks CLA *in vivo* treatment.

CD226 is an adhesion and signaling protein expressed by most T cells, natural killer cells, monocytes and some B cell subsets (170).

The observed downregulation of CD226 on different $CD8^+$ T cell subsets upon CLA treatment cannot clearly be interpreted as a protective or detrimental effect of CLA on MS since the role of CD226 in MS and other autoimmune diseases is discussed controversially. GWAS identified the rs763361 C>T (Gly307Ser) genetic variant of the CD226 gene to confer susceptibility to various autoimmune diseases, including MS (61, 66, 149). This risk variant in the CD226 gene results in a decreased CD226 expression suggesting a protective effect of the surface marker in the

context of autoimmune diseases (104). CD226 is suggested to be important for the function of the immune-regulatory cell network (64, 144). The observed downregulation of CD226 on the surface of immune-regulatory cells in MS is hypothesized to lead to their compromised function (64).

EAE experiments were performed to further elucidate the role of CD226 in CNS autoimmunity. However, their results were inconsistent. While in a study by Piédavent *et al.* mice with a genetic knockout of CD226 suffered from an exacerbated disease course (144), Zhang *et al.* described an ameliorated EAE course and an elevated IL-10 production upon CD226 knockout in mice (199).

Also in the human system, some experimental results suggested a pro-inflammatory role of CD226. Lozano *et al.* investigated the role of the protein in $CD4^+$ T cells from HCs. Most IFNγ-producing and all IL-17A-producing T helper cells also expressed CD226. A knockdown of CD226 with small hairpin RNA (shRNA) in $CD4^+$ T cells from HCs resulted in a decreased expression of IFNγ and an elevated expression of IL-4 (108). Moreover, in this study and another one by Zhang *et al.*, antibody-mediated CD226 blockade impaired the proliferation, activation and IFNγ- and IL-17A-production of human HC $CD4^+$ T cells while elevating their IL-10 expression (108, 199).

Less information is available about CD226 expressing $CD8^+$ T cells. A study by Ayano *et al.* investigated their role in systemic sclerosis (SSc), another autoimmune disease. The authors reported that HC $CD226^+$ $CD8^+$ T cells exhibited an elevated expression of IFNγ, IL-13 and IL-4 in comparison to $CD226^-$ $CD8^+$ T cells. They found $CD226^+$ $CD8^+$ T cells, $CD226^+$ $CD8^+$ effector memory T cells and $CD226^{high}$ $CD8^+$ T cells to be upregulated in SSc patients compared to HCs and the amount of $CD226^{high}$ $CD8^+$ T cells to correlate with disease severity. Furthermore, their data suggested that CD226 could be pathogenetically involved in SSc by enhancing the cytotoxicity of $CD8^+$ T cells and elevating the expression of profibrotic IL-13 (11). Taken together, these observations suggest a rather detrimental role of CD226 in autoimmunity. However, the findings regarding IL-13 somewhat contradict the observation in the above-mentioned study by Lozano *et al.* that the production of IL-13 by human HC $CD4^+$ T cells is elevated upon CD226 knockdown (108).

Considering these inconsistent and contradictory observations regarding CD226 positive immune cells, it is difficult to interpret the reported upregulation of CD226 on different $CD8^+$ T cell subsets upon CLA *in vivo* treatment in the present study. The inconsistency in previous study results regarding CD226 may be due to a differing function of the protein in the various analyzed

conditions (EAE, HCs, SSc) and on the different examined immune cells (regulatory cells, $CD4^+$ and $CD8^+$ T cells). Therefore, further research is necessary to elucidate the specific role of $CD226^+$ $CD8^+$ T cells in MS immunopathology and to interpret the results of the present study. Finally, also experimental information about the effect of CLA or other PPARγ agonists on $CD226^+$ in the context of other autoimmune diseases would help to clarify the relevance of the effect observed in this study.

5.2.2.2 $CD8^+$ T cell cytokine production

Similar as observed regarding the $CD4^+$ T cells, also the share of IL17A and IL-22 producing $CD8^+$ T cells and $CD8^+$ memory T cells was reduced upon 24 weeks CLA *in vivo* treatment, measured after 4 h LAC activation and calculated by manual gating analysis.

As described in detail in section 5.2.1.2, IL-17A and IL-22 are well-established as two key cytokines of T_H17 cells which largely contribute to the immunopathology of MS. In this context IL-17A plays a clearly pro-inflammatory role and some research evidence also suggests a pathogenic function of IL-22 in CNS autoimmunity (87, 93, 161, 189).

Various experimental observations also suggested an involvement of IL-17A or IL22 producing $CD8^+$ T cells in the pathogenesis of CNS autoimmunity and other autoimmune disorders. For example, these cell types were found in elevated numbers in lesional skin from patients with psoriasis, a probably autoimmune mediated disease, compared with healthy skin (153). Evidence from research in mice and humans suggests a pathogenic function of type 17 cytotoxic T (T_C17) cells ($CD8^+$ T cells defined by the production of IL-17A) in the development of CNS autoimmunity (78). During relapse, T_C17 cells were upregulated in the peripheral blood of RRMS patients (191). The share of T_C17 cells in all peripheral blood $CD8^+$ T cells was positively correlated with T1 and T2 MRI lesion volumes in patients with active RRMS (176). Huber *et al.* observed that in CIS and early stage MS patients the share of T_C17 cells in the CSF is higher than in the peripheral blood, what was different in control patients suffering from noninfectious headache. Also, the CIS and early stage MS patients had higher T_C17 cell concentrations in their CSF than the control patients (78). A study by Tzartos *et al.* also suggested elevated numbers of T_C17 cells in active MS lesions (181). Finally, findings by Tao *et al.* suggested that the involvement of T_C17 cells in MS immunopathology might even have therapeutic implications, as their study revealed a downregulation of T_C17 cells in the peripheral blood upon IFNβ-1a treatment (176). Evidence supporting a pathogenic role of IL-22^+ $CD8^+$ T cells in MS includes the observations that RRMS

patients have higher shares of IL-22$^+$ cells within all peripheral blood CD8$^+$ T cells than HCs and that in patients with active MS, this share is positively correlated with T2 lesion volume (176).

While various previous studies demonstrated a dampening effect of PPARγ agonists such as CLA on the IL-17A and IL-22 production of CD4$^+$ T cells under different conditions (see section 5.2.1.2) (16, 79, 90), information about their effect on the respective cytokine production by CD8$^+$ T cells is more limited. However, Bassaganya-Riera *et al.* observed a reduced IL-17A production by peripheral blood CD8$^+$ T cells from CD patients upon 12 weeks *in vivo* CLA treatment (16).

In summary, the observed dampening effect of a 24 weeks CLA *in vivo* treatment on the IL-17A and IL-22 production by activated peripheral blood CD8$^+$ T cells from RRMS patients in the present study supports a beneficial effect of the substance in the context of MS and is in line with previous experimental findings.

5.2.3 B cells

Both automated and manual gating analyses demonstrated an upregulation of naïve (CD27$^-$) B cells and the manual gating analysis additionally revealed a downregulation of memory (CD27$^+$) B cells, each calculated as a percentage of all B cells, upon 24 weeks CLA *in vivo* treatment.

CD27$^-$ B cells are termed naïve after maturing from the transitional B cell state. After antigen exposure and germinal center reaction they can differentiate to memory B cells (CD27$^+$), which are rather quiescent cells ready to initiate a faster immune response when re-encountering their specific target antigen (13, 167). The surface marker CD27 is widely used to distinguish between naïve (CD27$^-$) and memory (CD27$^+$) B cells (102, 106, 167).

Especially observations from studies in MS with B cell depleting therapies led to the assumption that particularly memory B cells might play a pathogenic role in MS. After an anti-CD20 treatment, which targets both naïve and memory B cells, the repopulation with naïve B cells tends to occur faster than the generation of new memory B cells. While this prolonged memory B cell depletion seems to correlate with a longer MS disease suppression, the eventual repopulation with memory B cells in other autoimmune diseases treated with anti-CD20 therapies was shown to coincide with increasing disease activity. Furthermore, experimental treatment of MS patients with Atacicept, an agent which predominantly depletes naïve B cells but less affects memory B cells, was shown to exacerbate the disease activity. Taken together, these observations indicate that memory B cells are driving MS pathology more than naïve B cells (13, 102).

Interestingly, many of the approved DMTs influence the peripheral blood B cell subset composition in MS patients (107). Most of them, including glatiramer acetate (80), fingolimod (26, 35, 121) and dimethyl fumarate (37, 124), increase the proportion of naïve B cells while decreasing the share of memory B cells within the total peripheral blood B cell pool (107). In the assumption that memory B cells are more pathogenic in the context of MS than naïve B cells, these effects of the named DMTs could partly explain their efficacy in MS treatment (107).

Considering these observations about the involvement of different B cell subsets in the pathogenesis of MS, the effect of CLA on naïve and memory B cells observed in the present study suggests a beneficial, protective effect of CLA in MS. The observations are in line with the hypothesis that CLA increases the share of rather protective and decreases the proportion of rather harmful immune cell subsets in the peripheral blood of MS patients. Regarding the B cell subset composition, the effects of CLA seem to resemble those that most approved MS DMTs have.

5.2.4 $CD4^+$ T_{reg} cells

The manual gating analysis of the flow cytometry data revealed a downregulation of pT_{reg} cells, calculated as a percentage both of all $CD4^+$ T cells and of total T_{reg} cells, a slight upregulation of the share of tT_{reg} cells in all T_{reg} cells and constant proportions of total T_{reg} cells and tT_{reg} cells in all $CD4^+$ T cells. The downregulation of pT_{reg} cells was also observed in the automated data analysis after pre-gating to $CD25^+$ $CD4^+$ memory T cells.

As described in detail in section 1.1.4.1, T_{reg} cells play a crucial role in the maintenance of peripheral tolerance and help to prevent the development of various autoimmune diseases (40, 169). Although there is conflicting evidence regarding the precise characteristics, the dysregulation of the immune-regulatory cell network has repeatedly been implicated in the pathogenesis of MS (40, 54). Different investigations revealed not entirely consistent information about the number and functionality of T_{reg} cells in MS patients (54). However, most authors proposed T_{reg} cells to be present in reduced numbers and with impaired functionality in RRMS patients, similar as it has been reported also in other autoimmune diseases (31, 82, 185, 186). These observations as well as several other experimental findings strongly suggest T_{reg} cells to play a protective role in the context of CNS autoimmunity (82).

Two subsets of T_{reg} cells can be distinguished. While most T_{reg} cells arise in the thymus (thymus derived T_{reg} cells, tT_{reg} cells), some also develop in the periphery from activated non-regulatory $CD4^+$ T cells under exposure to certain signals (peripherally derived T_{reg} cells, pT_{reg} cells). It is assumed that this development of pT_{reg} cells can be induced by activated tT_{reg} cells (31, 169). Although having been discussed controversially, the transcription factor Helios is currently widely accepted as a good marker for distinguishing tT_{reg} ($Helios^+$) from pT_{reg} ($Helios^-$) $FoxP3^+$ T_{reg} cells (169, 179).

Research is ongoing to elucidate if and how $Helios^+$ and $Helios^-$ T_{reg} cells functionally differ from each other. Findings from several *in vitro* and *in vivo* experiments revealed functional differences between both T_{reg} cell subsets and mostly suggested either an overall equal suppressive capacity of both cell types or a slightly superior regulative function and increased stability of $Helios^+$ (tT_{reg}) cells (169, 179). In the context of CNS autoimmunity, little is known about the differential involvement of pT_{reg} versus tT_{reg} cells, but both cell types have been supposed to be implicated in the pathogenesis of EAE or MS (82, 140).

The influence of glucocorticoids and established MS DMTs on peripheral T_{reg} cell populations is only partly known. For example, in a small trial with 15 RRMS patients, a six months dimethyl fumarate treatment was shown to elevate the share of pT_{reg} cells in all peripheral blood $CD4^+$ T cells (63). While most studies suggested a glucocorticoid treatment to expand the T_{reg} cell population of patients with different autoimmune diseases, data is not entirely consistent and especially in the context of MS, studies investigating the effect of glucocorticoids on T_{reg} cells had contradictory results (31).

Several PPARγ agonists, including pioglitazone (128), eicosapentaenoic acid (182, 197) and α-lipoic acid (192), have been shown to increase the number, proportion or functionality of T_{reg} cells in the peripheral blood, in lymph nodes or in the spleen and to be capable to ameliorate parameters of CNS inflammation. However, also regarding the influence of PPARγ agonists on the promotion of T_{reg} cells, experimental data is not completely consistent. For example, the PPARγ agonist pioglitazone was not able to enhance the cytokine-mediated *in vitro* induction of T_{reg} cells (90).

In several murine *in vivo* experiments, CLA was demonstrated to expand the population of T_{reg} cells at different body sites, probably at least partly mediated by PPARγ activation (46, 79).

The observed downregulation of pT_{reg} cells upon 24 weeks CLA *in vivo* treatment observed in the present trial contradicts the hypothesis that CLA would promote the expansion of immune-regulatory cell types in the peripheral blood of MS patients. Although the precise characteristics of the disturbances in the immune-regulatory cell network in MS pathogenesis are not entirely clear and although there have been conflicting results regarding the numbers of T_{reg} cells in the peripheral blood of MS patients, most research findings suggest T_{reg} cells to play a protective role in MS immunopathology (54, 82). Thus, the downregulation of pT_{reg} cells in this study appears as a detrimental effect of CLA in MS. Furthermore, this finding is not in line with the observations from the previous studies summarized above which predominantly suggested a promotion of T_{reg} cell development by exposure to MS DMTs, PPARγ agonists in general or CLA specifically (46, 63, 79, 128, 192, 197).

However, several considerations must be made when interpreting these results. First, and most importantly, only the share of the pT_{reg} cell subset (in all T_{reg} cells and in all $CD4^+$ T cells) was found decreased after CLA treatment. In contrast, the percentage of tT_{reg} cells (in all T_{reg} cells) was slightly – although not statistically significantly – increased. And the share of total T_{reg} cells (in all $CD4^+$ T cells) fluctuated during the treatment course, overall decreasing only marginally and not statistically significantly. Since pT_{reg} cells anyway represent the minor part of all T_{reg} cells – in this and in previous (169) studies – their downregulation might not be of high relevance, especially since the share of total T_{reg} cells (in all $CD4^+$ T cells) remains relatively constant during the treatment period. The increased share of tT_{reg} cells (in all T_{reg} cells) might even not only be a relative increase due to the reduction of pT_{reg} cells but also represent a true expansion of tT_{reg} cells what would suggest a beneficial effect of CLA on the T_{reg} cell population. This is especially important since the results from several experiments suggested tT_{reg} cells to have a higher immunoregulatory capacity than pT_{reg} cells (169, 179).

Furthermore, it must be noted that numbers or proportions of different T_{reg} cell subsets do not necessarily reflect their collective immunoregulatory capacity. While for non-regulatory $CD4^+$ T cells and $CD8^+$ T cells this study also evaluated functional properties (cytokine staining in flow cytometry [section 4.2.4] and cytokine production measurement with Luminex® technique [section 4.2.5, only for $CD4^+$ T cells]), the investigations regarding T_{reg} cells were limited to a phenotypical characterization. Thus, the reduced proportion of pT_{reg} cells might have been compensated by an increased suppressive function of pT_{reg} or tT_{reg} cells. However, this study provides no evidence for such a phenomenon.

Next, although many studies suggested Treg cell promoting effects of MS DMTs, CLA and other PPARγ agonists (46, 63, 79, 128, 192, 197), not all studies could reproduce these findings. For example, research concerning the effect of glucocorticoids on T_{reg} cells in MS led to contradictory results (31) and the PPARγ agonist pioglitazone could not promote the *in vitro* induction of T_{reg} cells (90). Therefore, the role of T_{reg} cells in MS pathophysiology and the influence that MS therapeutics and PPARγ agonists have on this cell population appear very complex (54) and future research will be necessary to further clarify these interactions. The results from such investigations might also help interpreting the relevance of the observed pT_{reg} cell downregulation in the present study better.

Finally, it could be hypothesized that the decrease of pT_{reg} cells in the peripheral blood is at least partly due to their recruitment into organs of relevance in the pathogenesis of MS. As described in detail in section 1.1.4.3, many observations suggested that processes in the gut involving nutritional factors, the gut microbiota and cT_{reg} cells play a role in MS pathogenesis (21, 53, 133, 134, 157, 183). In the experiments described in section 1.2.3.3, the authors investigated how a dietary CLA supplement influences the disease course and the immune system in OSE mice which develop spontaneous autoimmunity. Regarding T_{reg} cells, they observed that the CLA treated mice had higher shares of T_{reg} cells (in all $CD4^+$ T cells) in the small intestine lamina propria and in the spleen than their littermates fed with a control diet (79). Thus, it appears possible that CLA promotes a recruitment of T_{reg} cells from the peripheral blood into peripheral lymphoid tissues. It is also conceivable, that T_{reg} cells are recruited from the peripheral blood into the CNS and exert beneficial functions there. In these cases, the decrease of pT_{reg} cells in the peripheral blood of the MS patients in the present study could even represent a beneficial therapeutic effect of the dietary CLA supplement. However, it would be difficult to prove such a redistribution of T_{reg} cells from the peripheral blood into peripheral lymphoid tissues or into the CNS in humans since biopsies from the gut, the spleen or the CNS of study participants would be necessary but ethically not justifiable for this purpose.

To sum up, the observed decrease of pT_{reg} cell proportions in the peripheral blood in the present study is contradictory to the experimental hypothesis. But several considerations require a careful interpretation of this result. Several hypotheses could at least partly explain the effects without implicating a detrimental effect of CLA on MS and future research is necessary to interpret the findings properly. This research needs to further clarify (1) the differential role of pT_{reg} and tT_{reg} cells in MS, (2) the influence of other PPARγ agonists and MS DMTs on pT_{reg} and tT_{reg} cells,

(3) the effect of CLA on these T_{reg} cell subsets focusing also on their functionality, (4) if the observations from the present study are reproducible also in a larger patient cohort and (5) if a redistribution of T_{reg} cells from the peripheral blood to organs such as the gut or the CNS could be responsible for the effects observed here.

5.2.5 Methodological limitations

Both analyses of the flow cytometry data have some methodological limitations. One main problem of the manual gating analysis is its subjectivity. The position and size of the applied gates is dependent on the investigator leading to a reduced inter-rater-reproducibility of the results. Also the order of the gating steps influences the results what contributes to the bias introduced by the investigator. Furthermore, the manual analysis can only identify previously described and purposively examined cell subsets, thus potentially missing relevant, but so far never identified or in the applied gating strategy not included cell populations. Finally, manual gating employs hard threshold values for distinguishing positive and negative (and sometimes intermediately expressing) cells regarding one marker. This dichotomous approach neglects a potential continuum of marker expression between different cell subsets (159).

Automated analyses with unsupervised machine learning technologies such as the viSNE and PhenoGraph algorithms overcome these problems by working investigator-independently, by simultaneously including all markers in the analysis, by not being biased towards pre-described cell populations and by not working with dichotomous cell classifications (159). However, also these techniques entail methodological limitations. The 2D viSNE map never can reflect the whole complexity of the underlying data set. The subsampling of data points prior to the viSNE analysis – necessary to avoid the “crowding” problem – potentially impedes or even precludes the identification of changes in small cell populations (6). And the interpretation of the results from the automated analysis in the context of the current literature can be difficult when it shows alterations of completely unknown, never pre-described cell populations.

5.3 $CD4^+$ T cell cytokine production upon activation

An influence of CLA on the cytokine production by immune cells has been demonstrated in various *in vitro* and *in vivo* experiments (17, 79, 188). The immune phenotyping of the clinical study samples revealed a diminished number of pro-inflammatory cytokine producing immune cells

(see section 4.2.4). Considering these findings, the results from direct measurements of the cytokine production after *in vitro* CLA treatment of HC $CD4^+$ T cells and *in vivo* CLA treatment in the clinical trial are of high interest. The hypothesis behind these experiments was that both an *in vitro* treatment of HC $CD4^+$ T cells and an *in vivo* CLA treatment of MS patients would lead to a reduced pro-inflammatory $CD4^+$ T cell cytokine production capacity.

However, a relevant cytotoxicity of CLA had to be ruled out before since any reduction of cytokine production otherwise could be due to cell death only. A potential cytotoxicity on human HC PHA-activated $CD4^+$ T cells was investigated with a PI staining, the results of which are presented in section 4.1.1. It revealed no relevant cytotoxicity of a 72 h exposure to CLA in the concentrations 25 µM, 50 µM and 75 µM (each isomer) which were employed in the further *in vitro* experiments.

Hucke, Hartwig *et al.* investigated a potential cytotoxic effect of CLA on $CD4^+$ T cells as well. On murine $CD4^+$ T cells activated with purified antibodies against CD3 and CD28, a cytotoxic effect was observed only for 75 µM CLA (each isomer). For any examined lower concentration (25 µM, 50 µM) of the mix and for 25 µM, 50 µM and 75 µM of any of the two isomers alone, no relevant cytotoxicity was found. Importantly, investigating human HC $CD4^+$ T cells, activated with anti-CD3 and anti-CD28 antibodies and treated with 75 µM CLA (each isomer) for 96 h, no relevant cytotoxicity of CLA was observed (79). This is in accordance to the data acquired in the present work and in conclusion, it can be assumed that a reduced cytokine production was not caused by cell death.

Measuring the cytokine production of PHA-activated human HC $CD4^+$ T cells in an ELISA assay it was found that a 72 h CLA treatment potently reduced the production of the pro-inflammatory cytokines TNFα and IFNγ in a dose-dependent fashion. Higher CLA concentrations (25 µM, 50 µM, 75 µM – each isomer, respectively) resulted in a lower TNFα and IFNγ production. These findings are in line with results from various *in vitro* and *in vivo* studies. CLA has been demonstrated to reduce the production of TNFα and IFNγ in several *in vitro* studies (188) as well as in *in vivo* studies conducted in humans (16) and mice (latter regarding TNFα) (17). Notably, Hucke, Hartwig *et al.* observed a reduced IFNγ production by *in vitro* CLA treated LAC-restimulated $CD4^+$ T cells collected from the peripheral blood of HCs and RRMS patients and from the CSF of RRMS patients, measured by flow cytometry. Furthermore, they found diminished numbers of IFNγ

producing $CD4^+$ T cells in the periphery and in the CNS of CLA-treated OSE mice (unpublished data, (79)).

The results from the ELISA experiment could be reproduced in a similar setup investigating the effect of a 72 h CLA treatment on the cytokine production of PHA-activated HC $CD4^+$ T cells, measuring the produced amount of 18 cytokines with the Luminex® technique. Thereby, the production of many pro-inflammatory cytokines (including TNFα, IFNγ, IL-1β, IL-6, IL-17A) was reduced upon CLA exposure. However, also the production of anti-inflammatory IL-4 and IL-10 was reduced.

Regarding the pro-inflammatory cytokines these findings are in accordance to the literature as the above mentioned publications reported similar effects of CLA on the synthesis of IL-6, Il-1β (both (188)) and IL-17A (16, 79) as on the TNFα and IFNγ production.

However, the observed reduction of the anti-inflammatory cytokine production (IL-4 and IL-10) is irritating on the first view. Since no data is available concerning specifically the production of anti-inflammatory cytokines by $CD4^+$ T cells in the above-mentioned studies (16, 17, 79, 188), and barely information about the effects of CLA on their production in literature, a comparison to previous findings is difficult. But since no cytotoxicity of CLA can be made responsible for the reduced cytokine production, the present data suggests that CLA rather generally dampens the cytokine production activity of $CD4^+$ T cells than specifically inhibiting the pro-inflammatory cytokine synthesis in the used experimental setup. However, further experimental research is required to elucidate the level of specificity of the dampening effect of CLA on the $CD4^+$ T cell cytokine production.

The conduction of a Luminex® assay comparing the cytokine production capacity of $CD4^+$ T cells collected before and after 24 weeks *in vivo* CLA treatment of RRMS patients revealed disappointing results. No changes in the production could be detected regarding any of the 10 investigated pro- and anti-inflammatory cytokines. These findings are not in line with those from the *in vitro* experiments, from the immune phenotyping and not with those published before (16, 17, 79, 188).

Basically, two different theories could explain this observation. First, it could be that 24 weeks *in vivo* CLA treatment of MS patients indeed has no effects on their peripheral blood $CD4^+$ T cells' cytokine production capacity in contrast to what the *in vitro* experiments suggested. However,

the findings from other *in vivo* settings in mice (17, 79) and humans (16) and especially the findings from the immune phenotyping in the present work with clear reductions of the numbers of pro-inflammatory cytokine producing $CD4^+$ and $CD8^+$ T cells speak against this possibility.

The second option would be that the experimental setup was not suitable to detect a reduction in the cytokine production capacity which was actually there. Several circumstances could explain such a failure of this experimental setup.

First, the effects of CLA *in vivo* on the cytokine production could be much smaller than the massive reduction observed in the *in vitro* experiments. Two differences between the *in vitro* and the *in vivo* experiment in the present work could be responsible for this. First, the direct exposure of the immune cells to CLA *in vivo* could be reduced compared with the *in vitro* situation. Second, RRMS patients' $CD4^+$ T cells might react differently to the CLA treatment than healthy controls' cells. MS patients' immune cells are generally more activated (40) and could be less susceptible to the immunomodulatory effects of CLA. If – due to one of these reasons – the CLA effect in the *in vivo* situation is much smaller than in the *in vitro* experiments of the present work, the quite strong and rather unphysiological PHA stimulation (88) could have "overshadowed" the CLA effect. It could have activated the cells collected before and after the *in vivo* treatment so strongly that the rather small *in vivo* effect of CLA was no longer detectable with a Luminex® assay. The previously cited literature contradicts the idea that the two above-mentioned circumstances could be responsible for an effect of CLA *in vivo* in MS patients which is negligibly small and therefore uninteresting as a treatment approach. Data from the *in vivo* experiments in mouse models of MS (unpublished data by Hucke, Hartwig *et al.* (79)) and IBD (17) as well as in IBD-affected humans (16) demonstrated that also a CLA exposure *in vivo* can affect the immune cells' cytokine production. Furthermore, the unpublished data by Hucke, Hartwig *et al.* showed that CLA also has profound effects on the cytokine production of generally more highly activated $CD4^+$ T cells from RRMS patients (observed after *in vitro* treatment) (79). However, the named possible mechanisms could cause the CLA effect *in vivo* in MS to be so much smaller than in the HC *in vitro* situation – though still potentially therapeutically relevant – that the PHA stimulation „overshadowed" it and therefore made it unable to be detected in the used analytical setup.

Second, it is conceivable that the dampening effect of CLA *in vivo* on the cytokine production in MS which could clearly be observed in the immune phenotyping in the present study is largely

dependent on the mediation by for example regulatory cells. Those were never separated from the pro-inflammatory cytokine producing $CD4^+$ T cells in the flow cytometry setup but they were separated in the Luminex® experiment for 72 h between thawing and measuring, i.e. during the PHA stimulation period. This could explain the differences in comparison to the findings from the immune phenotyping and to those from other *in vivo* experiments which used methods for measuring the cytokine production which did not require a separation of the $CD4^+$ T cells from regulatory cells before analysis (e.g. flow cytometry in human IBD trial (16) and quantitative real-time reverse-transcriptase polymerase chain reaction (PCR) of total colonic RNA and therefore also of cytokine mRNA in murine IBD trial (17)). However, it could not explain the differences to the findings in the *in vitro* experiments of the present study in which $CD4^+$ T cells were PHA-stimulated and CLA-treated for 72 h alone, in the absence of regulatory cells.

Third, and maybe most importantly, the $CD4^+$ T cells the cytokine production of which was later measured with the Luminex® technique were deep-frozen directly after acquisition from the study participants (as PMBC samples). After thawing they were immediately isolated and then stimulated for 72 h with PHA. During this stimulation period they were not exposed to CLA to only measure the effects of an *in vivo* treatment. Therefore, they could only be subject to any potential direct or indirect beneficial effect of CLA while they were in the patient's blood. A theory explaining the disappointing results in the *in vivo* Luminex® measurement could be that the effect of CLA on the cytokine production by $CD4^+$ T cells is a rather short-term effect and dependent on the continuous presence of CLA. This could explain the different findings compared with the immune phenotyping results where the cells for the cytokine production measurement were analyzed on the day after thawing and therefore after much shorter absence of CLA. Also the differences in comparison with the other *in vivo* experiments using different methods for the cytokine production measurement which do not require such a long incubation of the $CD4^+$ T cells in the absence of CLA (16, 17) could be explained by this theory.

To conclude, it could be demonstrated that CLA is capable to potently reduce the cytokine production capacity of $CD4^+$ T cells by *in vitro* treatment. This affected the production of pro- and anti-inflammatory cytokines, a result which suggests a rather unspecific dampening effect of the $CD4^+$ T cell activity by CLA. Further research must clarify in more detail the effects of CLA on the production of anti-inflammatory cytokines and the potential clinical relevance of this.

During an *in vivo* treatment of RMMS patients, these results could be reproduced by immune phenotyping but not by direct measurement of the produced quantity of cytokines. However, the disappointing latter observation could have been evoked by methodological impairments only. For further measurements in similar conditions, the $CD4^+$ T cells of which the cytokine production capacity shall be measured, should be incubated shorter in absence of CLA and should be stimulated less strongly.

In total, the results regarding the influence of CLA on the $CD4^+$ T cell pro-inflammatory cytokine production capacity promise beneficial capabilities of CLA in the treatment of autoimmune-driven diseases like MS.

The direct measurements of produced cytokines were conducted in the cell culture supernatants of $CD4^+$ T cells because many of the previous data showing beneficial effects of CLA on the immune system was acquired in experiments with this cell type (16, 79) and because $CD4^+$ T cells are assumed to be the main drivers of CNS autoimmunity in EAE (148). However, the relative importance on $CD4^+$ vs. $CD8^+$ T cells seems to differ between murine and human CNS autoimmunity and $CD8^+$ T cells might play a larger role in human MS than they do in EAE (40, 148). Therefore, and because the immune phenotyping also showed reduced numbers of pro-inflammatory cytokine producing $CD8^+$ T cells (see section 4.2.4), a future conduction of the presented or similar direct cytokine production measurements in the cell culture supernatants of $CD8^+$ T cells would be interesting as well. Nevertheless, the presented findings in $CD4^+$ T cells from the *in vitro* experiments and the immune phenotyping are of high interest in the context of human MS since this cell type plays an important role in the pathogenesis of MS, too (73, 90, 148).

5.4 $CD4^+$ T cell metabolism

Rising interest in immunology research is currently being paid to the effects that the immune cell metabolism has on the immune cell function. Interventions influencing the immune cell metabolism appear as promising treatment strategies to control aberrant immune responses (77, 136).

It has been demonstrated that various interventions in the immune cell metabolism can alter their function (44, 77, 136). Some of them showed beneficial effects in mouse models of MS (23, 198) and some have even been tested in clinical trials with MS patients (58, 84, 145). It is conceivable that also potential MS-protective effects of CLA could be mediated at least partly via

affection of the immune cell metabolism. CLA could influence the metabolism for example by binding to specific cell surface or nuclear receptors, of which PPARγ is of outstanding interest, or by altering the gut microbiota composition, favoring the expansion of bacterial strains which produce cell metabolism-affecting substance such as soraphen A (23, 33, 77, 139, 188).

Considering these potential mechanisms of action of CLA, the results of the *in vitro* and *in vivo* experiments analyzing the effects of CLA on the metabolism of healthy controls' and RRMS patients' $CD4^+$ T cells are of high interest. The underlying hypothesis of these analyses was that an *in vitro* or an *in vivo* CLA treatment would shift the metabolic signature of $CD4^+$ T cells towards a less inflammatory, more regulatory phenotype.

In the *in vitro* experiment, HC $CD4^+$ T cells were treated or not for 24 h *in vitro* with CLA under simultaneous stimulation with antibodies directed against CD3 and CD28. Afterwards, their metabolic profile was measured in a Mito Stress assay.

In the *in vivo* experiment, $CD4^+$ T cells were isolated from MS patients' PBMC samples collected before and after 24 weeks of dietary CLA supplementation and their metabolic activity during a Mito Stress Test was compared. The Mito Stress Test was performed both without any previous cell activation and after a short time stimulation with PMA and ionomycin.

The results suggest that CLA indeed influences the metabolism of $CD4^+$ T cells. Both the *in vitro* treatment of activated HC $CD4^+$ T cells and the *in vivo* treatment of RRMS patients' $CD4^+$ T cells, measured with and without previous short-time activation, resulted in a reduced activity of the OXPHOS and glycolysis metabolic systems, reflected by a lowered BR and BG as well as by decreased MR / GC and SRC / GR.

As described in detail in section 1.1.4.4, immune cells exhibit various specific characteristics compared with other body cells (139). In a simplified model, they can be categorized in two major groups according to their cell metabolic profile. Naïve, memory and regulatory T cells largely rely on the highly efficient ATP generation in the TCA cycle and OXPHOS using metabolites generated by glycolysis, FAO and the amino acid catabolism (44, 58, 105, 136, 139). In contrast, highly inflammatory cells like T_H1, T_H17 and effector $CD8^+$ T cells feature high rates of aerobic glycolysis using glucose as energy and carbon source to ensure a rapid upregulation of ATP production and the generation of intermediate metabolites for biosynthetic purposes (44, 105, 136).

Seeing the experimental results only in the light of these two major groups of immune cells, characterized by their contrasting metabolic signatures, the downregulation of both OXPHOS and glycolysis upon CLA treatment could be surprising. However, importantly, upon antigen- or cytokine dependent activation T cells strongly increase their rates of both OXPHOS and glycolysis to meet their rapidly growing demand for ATP and precursor molecules for biosynthesis (105, 139). During the development from a naïve to an effector T cell, the shift in the signature metabolic pathway from OXPHOS to glycolysis may be due to a more emphasized upregulation of glycolysis than of OXPHOS because glycolysis can be increased much faster (no need for the generation of mitochondria) and it is needed not only for the generation of ATP but also for the provision of intermediate metabolites for biosynthesis (44, 136). However, the activation of T cells is accompanied by an upregulation of both these major metabolic pathways (105, 139).

Considering this, the results from the present CLA experiments could indicate that the $CD4^+$ T cells indeed exhibited a less activated, inflammatory metabolic phenotype upon CLA treatment with an overall reduced metabolic activity regarding both glycolysis and OXPHOS. The consistency of this result between the *in vitro* and the *in vivo* setup with healthy controls' and RRMS patients' $CD4^+$ T cells, respectively, underlines the credibility of this observation. CLA might have reduced the metabolic activity of the examined cells which could be a mechanism of action underlying the hypothesized anti-inflammatory effects of CLA.

In the *in vivo* setup, it could be observed that the $CD4^+$ T cells collected after 24 weeks *in vivo* CLA treatment were less strongly activated by the exposition to PMA and ionomycin. The stimulation-induced increase was calculated for the metabolic parameters by dividing the respective value after PMA / ionomycin stimulation by the value measured without previous stimulation. After 24 weeks of *in vivo* CLA treatment, the stimulation-induced increase of the MR, SRC, BG and GC was reduced in comparison to before the treatment.

This suggests that the CLA treatment might not only have reduced the cells' overall metabolic activity but might also have impeded their activation by PMA and ionomycin. The untreated cells may have been more readily and strongly activatable than the CLA treated cells. Translated to the *in vivo* system this indicates that CLA could potentially reduce the responsiveness of $CD4^+$ T cells to aberrant inflammatory stimuli.

However, one observation concerning the stimulation-induced increase of the named parameters is surprising. For the two parameters of glycolysis (BG, GC) the values of almost all individuals both before and after CLA treatment are greater than 1.0 what means that the PMA / ionomycin stimulation resulted in an elevation of the parameters as it was expected since the stimulation was supposed to increase the cells' metabolic activity. In contrast, the values for the stimulation-induced increase of the OXPHOS parameters (MR, SRC) are lower than 1.0 in most individuals at both treatment conditions indicating that the cells were metabolically less active after stimulation.

This finding and that it was only observed for the OXPHOS parameters could be explained by the following considerations. First, as described above, the activation of T cells causes the upregulation of both OXPHOS and glycolysis (105, 139) but glycolysis in general is probably more strongly increased than OXPHOS (44, 136). Second, the PMA / ionomycin stimulation might have been too strong, leading to an "exhaustion" of the cells' metabolic system, thus lowering its responsiveness to the stimuli of the Mito Stress Test. This could have resulted in an apparently less active metabolic system after stimulation as a false result representing only a damage to the cells' metabolic system due to overstimulation. If now this damage was not very severe its effect could have only been visible in the parameters of OXPHOS and not of glycolysis because the potential overstimulation might have damaged the glycolytic system less strongly. This could have been either because it might be generally less susceptible to such a damage or because its in comparison to OXPHOS stronger upregulation in the inflammatory RRMS patients' $CD4^+$ T cells (44, 136) might have made it less susceptible.

However, even if this finding is indeed the consequence of a damaging overstimulation of the cells and the results from the measurements after PMA / ionomycin stimulation thus are not fully reliable, the observations in the completely unstimulated *in vivo* treated cells as well as the findings in the *in vitro* experiments strongly suggest a beneficial, anti-inflammatory effect of CLA on the cell metabolism of $CD4^+$ T cells in MS.

Further research is urgently needed regarding the immune cell metabolism in general and the effects that CLA exerts on it, especially in the context of autoimmune diseases. Gene expression analyses could reveal mechanisms underlying a potential effect of CLA on the immune cell metabolism. Distinct gene expression profiles could give evidence for certain receptors the activa-

tion of which could be key to the mediation of CLA effects. One promising candidate in this context is PPARγ, a nuclear receptor activated by CLA having an impact on cell metabolic functions (188). Apart from a direct mediation by receptors, another discussed mechanism underlying the potential effect of CLA on the immune cell metabolism is the alteration of the gut microbiota increasing or decreasing the share of bacterial strains with effect on the metabolism (23, 33, 139). The finding in this work that also direct exposition to CLA *in vitro* influenced the $CD4^+$ T cell metabolism indicates that a mediation via the gut microbiota would at least not be the sole explanation of such a CLA effect. However, it could also play a role in the *in vivo* situation and further research regarding the effect of CLA on the gut microbiota composition and the effect of certain bacterial strains on the metabolism of immune cells is necessary to elucidate the mechanisms of action of CLA.

5.5 Study limitations

The *in vitro* experiments and the exploratory clinical trial in the present work gave a first insight into the effects CLA exerts on the human immune system in the context of MS. Although some findings did not prove the previously hypothesized effects, most of the results let CLA appear as a promising new complementary therapy approach for RRMS patients.

However, several limitations of the present work must be considered when interpreting the results. It must be noted that the clinical trial can only be considered a first exploratory pilot study and is not capable to prove the presence or absence of any beneficial effect of CLA on MS.

To prove the assumed beneficial properties of CLA with regard to efficacy, a large-scale double-blind randomized placebo-controlled clinical trial would be necessary. The small number of participants in the present study, the lack of a placebo-treated control group and the unblinded design require a cautious interpretation of the clinical results.

Especially considering that MS is a slowly progressing disease (180), the relatively short treatment duration in the present trial is another limitation of the study. Although previous human CLA *in vivo* experiments were also conducted with even shorter treatments (e.g. 12 weeks in the human IBD trial by Bassaganya-Riera *et al.* (16)), a longer supplementation and observation period might reveal more profound effects. Especially the findings concerning the effects of CLA on clinical parameters would be much more reliable.

Furthermore, in a larger future clinical trial, investigations on the optimal dosage of CLA would be reasonable. Therefore, the effects in different treatment groups with distinct CLA dosages could be compared. In previous human *in vivo* experiments investigating the effects of CLA, the applied dosages of CLA varied largely with frequently used dosages ranging between 1.7 g/d and 6 g/d (119, 135, 150). Comparing with these, the dosage applied in the present study (2.1 g/d) was rather low. A higher dosage could potentially evoke stronger effects. However, a higher risk for adverse reactions must be considered in this context.

In the clinical study – as well as in the *in vitro* experiments –, the effects of a 1 : 1 mixture of the two main natural CLA isomers – *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (188) – on the human immune system were investigated. However, it must be considered that various previous studies identified these two main isomers to have differential effects (24, 188). The 1 : 1 mix was chosen for the present work because it had been demonstrated to more efficiently exert beneficial effects than the individual isomers alone in previous investigations (17, 79). Nevertheless, since the precise differences in the properties of individual CLA isomers so far remain elusive (188), further research on this topic is essential.

The readouts are another limitation of the present clinical trial. First, the effects of CLA on components of the immune system were only observed in the peripheral blood. It would be of high interest to examine also the impact CLA *in vivo* has on immune cells residing in the gut and the CNS. In the OSE experiment by Hucke, Hartwig *et al.*, increased numbers of T_{reg} cells in the small intestine lamina propria upon CLA treatment were detected (79). To prove such an effect also in humans would be intriguing. However, ethical reasons would prohibit to perform otherwise unnecessary intestinal biopsies only for this purpose. With *in vitro* experiments conducted in CSF residing immune cells extracted from RRMS patients' CSF samples taken during the clinical routine as well as with the OSE *in vivo* experiment, Hucke, Hartwig *et al.* also demonstrated an anti-inflammatory effect of CLA on CSF-residing immune cells (79). These cells may reflect more directly inflammatory processes taking place in the CNS. However, an observation of the effects of CLA on CSF residing immune cells during a clinical trial again would not be ethically reasonable since the necessary lumbar punctures would be a too invasive intervention for research purposes only. Therefore, the peripheral blood immune cells remain as the only easily accessible part of the immune system in a human clinical study.

The cytokine production and cell metabolism experiments in the present study focused on peripheral blood $CD4^+$ T cells. Especially considering the diverse effects of CLA on different immune cell subsets revealed in the flow cytometry assay, it would be interesting to also analyze the effects of CLA on other peripheral blood immune cell subsets such as $CD8^+$ T cells or B cells in further experimental setups as well. For example, it could be of interest if the findings regarding the metabolism of $CD4^+$ T cells upon CLA treatment can be reproduced in other cell types.

Clinical and paraclinical readouts besides immunological parameters would be important in a future clinical trial. The clinical readouts in the present study can only be considered orientating due to the methodological impairments described in section 5.1.4. But in a larger, placebo-controlled trial, clinical readouts like relapse rates and EDSS development could potentially prove a clinical efficacy of a CLA treatment with a measurable benefit for the patient. Of high importance in such a future study would also be the comparison of paraclinical MRI parameters between the treatment and the control group. Information about the number of Gd-enhancing and of T2 hyperintense lesions can give a direct insight into the degree of inflammation in the CNS and could serve as an additional readout proving potential clinical efficacy of CLA in MS.

Furthermore, in the above-mentioned murine *in vivo* experiments by Bassaganya-Riera *et al.* and Hucke, Hartwig *et al.* on colitis and CNS autoimmunity, respectively, CLA was administered very early in life, i.e. already before birth by feeding to the dams (17, 79). This is a major difference to the present clinical trial where CLA was administered after onset of the disease. An earlier administration could possibly influence earlier phases of immune system development and disease formation and thus be more effective. This hypothesis is supported by the finding that even a CLA feeding beginning after weaning was associated with diminished protective effects in comparison to the administration via placenta and milk by feeding CLA already to the dams in the *in vivo* colitis model (17). To translate these observations into human MS, however, would pose difficulties since a prophylactic interventional trial would not be easy to conduct.

But observational studies like case-control or especially cohort studies could reveal associations between the natural CLA consumption and the risk for MS and therefore give information also about the effects of CLA consumed before the disease onset and of long term CLA exposure. In this context, it must be kept in mind that the natural CLA consumption is quite low in comparison to dosages commonly used in interventional studies (see section 5.1.2 and above in this section, (119, 135, 150, 154)) and potential effects of CLA therefore might be much smaller if CLA is only

consumed with the diet than if it is supplemented. Nevertheless, an investigation of associations between the amount of naturally consumed CLA and the risk for MS would be highly interesting. This is especially true since the natural CLA intake might vary even much more strongly in the general population than suggested by the findings from the food diaries kept by the participants of the present study, because patients following specific diets were excluded from this trial to observe only the effects of the study medication (see Table 3.1). Since the natural occurrence of CLA is highly variable in different foods (34, 188), a great variation concerning the natural CLA consumption could exist between individuals having specific dietary habits (e.g. vegan diet, meat- and dairy product-rich diet etc.) and the effects of these on the risk for MS could be observable in a cohort study. Also, the highly varying data for the dietary CLA consumption published previously (127) might be not only due to methodological differences between the studies but also due to actual differences in the CLA intake between the investigated populations. This further suggests that indeed the natural CLA intake highly varies in the general population and effects of this variation on the MS risk could possibly be detected in large cohort studies.

To sum up, the present clinical trial has several limitations which require a cautious interpretation of the results. However, the study was intended to be the first interventional trial with CLA in MS patients and thus to be a first, exploratory pilot project. Since the immunologic study endpoints were in the focus of interest and revealed interesting anti-inflammatory properties of CLA also in the context of human MS, the chosen experimental setting seems to have been suitable for the aims of this investigation. The results from this work might pave the way for a future larger clinical study with interventional CLA supplementation in RRMS patients.

5.6 Gut microbiota

The *in vitro* experiments in this and in previous works demonstrated that CLA is capable to influence mechanisms and pathways in immune cells directly. For example, it can directly exert effects on immunologic cell functions and on the immune cell metabolism which itself is closely interlinked with the immunologic cell activity (44, 77, 90, 188) (see section 1.2.4).

However, the capacity of CLA to interact directly with immune cells does not exclude that *in vivo* also other, indirect mechanisms play a role in the mediation of CLA effects. As described in section 1.2.4.3, CLA is capable to change the gut microbiota composition (33, 112) and several connections between this composition and immunologic as well as metabolic cell functions have been described previously (21, 23, 139) (see section1.2.4.3).

Therefore, it is of high interest to further investigate the exact effects of CLA on the human gut microbiota and to clarify if these could also be partly responsible for immunomodulatory capabilities of CLA *in vivo*.

To this end, during the clinical trial, patient stool staples were collected before and after the 24 weeks CLA supplementation. In these, the composition of the bacterial microbiota shall be analyzed by sequencing of the bacterial DNA. The analysis of these data is still ongoing in the working group and not part of this work. However, the results might reveal an impact of CLA *in vivo* on the human gut microbiota composition. But like regarding the other readouts of the clinical trial, it must be noted that these data will be only orientating and will not be able to prove the presence or absence of such an impact. However, the results of this analysis might encourage to analyze the gut microbiota composition also in a potential future RCT investigating CLA in MS which then could confirm hypothesized effects.

But even if CLA could be proven to alter the gut microbiota composition in MS patients and to exert beneficial effects on the disease course in an RCT, this would not necessarily mean that the influence on the gut microbiota would be partly responsible for the beneficial effects on the disease course since both effects of CLA could be independent from each other. To prove that alterations in the gut microbiota composition can ameliorate the course of MS, it would be necessary to specifically eradicate or administer single bacterial species or genera which are suspected to be detrimental or beneficial, respectively, in an RCT and observe the effects on the disease course. However, this would be so far impossible (eradication) or ethically not justifiable in humans (administration). Such experiments can only be conducted in mouse models of MS and there reveal interesting new information about the effects of the gut microbiota on CNS autoimmunity.

5.7 Outlook

Further animal, translational and clinical research is needed to investigate the effects of CLA on CNS autoimmunity in more detail. It should be targeted on the elucidation of the mechanisms of action, isomer-specific effects and optimal timing and dosing of a CLA treatment and a large RCT with clinical and radiologic endpoints should be used to prove the clinical efficacy of CLA in MS treatment.

But the results from the present work suggest that the previously demonstrated anti-inflammatory properties of CLA (16, 17, 79, 188) could also be valuable in the treatment of MS. The great advantage of a CLA treatment for MS would be its very low potential for side effects and the possibility to be administrated complementary to the standard medication, thus offering patients substantial benefits without causing major adverse events (16, 188). In addition, patients could profit from further potential health benefits of CLA simultaneously, as for example anti-carcinogenic effects or the enhancement of specific immune responses against viral and bacterial infections, which could be of especially high value in patients with potentially impaired immunity due to immunosuppressive MS treatment (74, 138, 188).

6 Summary

The influence of environmental and especially nutritional factors on the pathophysiology of multiple sclerosis (MS) is in the focus of current research. Results from previous studies in the context of various autoimmune diseases suggest a dietary supplementation with conjugated linoleic acid (CLA) to be a promising approach for the complementary treatment of MS. Here, the influence of CLA on systemic human immune responses was further investigated.

The *in vitro* treatment of healthy controls' $CD4^+$ T cells with CLA resulted in a reduced secretion of pro-inflammatory cytokines and a downregulated cell metabolism not caused by cytotoxicity. In an exploratory clinical pilot trial, 15 patients with relapsing-remitting MS were treated with 2.1 g CLA *per os* daily for 24 weeks. In comparison to the baseline status, $CD4^+$ T cells in their peripheral blood exhibited a less active cell metabolism after the treatment as well. The flow cytometric phenotyping of peripheral immune cells revealed a downregulation of several pro-inflammatory cell types and a reduced secretion of pro-inflammatory cytokines by $CD4^+$ and $CD8^+$ T cells. The interpretation of other observations requires further investigations. Clinically, no major changes occurred and the dietary supplementation with CLA was overall well tolerated.

To sum up, most of the results suggest anti-inflammatory effects of CLA in the context of MS. Future, larger and placebo-controlled trials should further investigate the mechanism of action, efficacy and tolerability of CLA in MS.

Figures

Tables

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Curriculum vitae

Appendix

Approval for the clinical study by the responsible ethics committee (*Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster*; registration number 2016-053-f-S, approved on 05.04.2016) – page 1

Patientenschutz | Forschungsfreiheit

ÄRZTEKAMMER WESTFALEN-LIPPE

WESTFÄLISCHE WILHELMS-UNIVERSITÄT MÜNSTER

EINGEGANGEN
11. April 2016

ETHIK KOMMISSION
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5. April 2016

Unser Aktenzeichen:	**2016-053-f-S** (bitte immer angeben!)
Studiencode:	
Sponsor / Finanzierung:	**Universitätsklinikum-Münster, Klinik für Allgemeine Neurologie, Prof. Dr. med. Heinz Wiendl, Albert-Schweitzer-Campus 1,-Gebäude-A1, 48149 Münster**
Titel des Forschungsvorhabens:	„Untersuchung molekularer und zellulärer Mechanismen neuroimmunologischer Erkrankungen: Implikationen für Wirmechanismus- und Beurteilung von Therapien und Differentialdiagnose entzündlciher Erkankungen des Nervensystems"

Beratung und Bewertung

Sehr geehrter Herr Professor Wiendl,

für das oben genannte Forschungsvorhaben haben Sie mit Schreiben vom 28.01.2016 die Beratung durch die Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster („Ethik-Kommission") beantragt.

Die Ethik-Kommission hat in ihrer Sitzung am 16.02.2016 über Ihren Antrag beraten, über ergänzend vorgelegte Unterlagen in ihrer Sitzung am 18.03.2016 beraten und beschlossen:

Die Ethik-Kommission hat keine grundsätzlichen Bedenken ethischer oder rechtlicher Art gegen die Durchführung des Forschungsvorhabens.

Die vorliegende Einschätzung gilt für das Forschungsvorhaben, wie es sich auf Grundlage der in Anhang 1 genannten Unterlagen darstellt.

Für die Entscheidung der Ethik-Kommission erhebt die Ärztekammer Westfalen-Lippe Gebühren nach Maßgabe ihrer Verwaltungsgebührenordnung. Über die Gebühren erhalten Sie von der Ärztekammer einen gesonderten Bescheid.

Allgemeine Hinweise:

Mit der vorliegenden Stellungnahme berät die Ethik-Kommission die der Ärztekammer Westfalen-Lippe angehörenden Ärztinnen und Ärzte zu den mit dem Forschungsvorhaben verbundenen berufsethischen und berufsrechtlichen Fragen gemäß § 15 Abs. 1 Berufsordnung ÄKWL.

Approval for the clinical study by the responsible ethics committee (*Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster*; registration number 2016-053-f-S, approved on 05.04.2016) – page 2

Die Einschätzung der Kommission ist als ergebnisoffene Beratung für den Antragsteller nicht bindend. Die Ethik-Kommission weist darauf hin, dass unabhängig von der vorliegenden Stellungnahme die medizinische, ethische und rechtliche Verantwortung für die Durchführung des Forschungsvorhabens bei dessen Leiter und bei allen an dem Vorhaben teilnehmenden Ärzten bzw. Forschern verbleibt.

An der Beratung und Beschlussfassung haben die in Anhang 2 aufgeführten Mitglieder der Ethik-Kommission teilgenommen. Es haben keine Mitglieder teilgenommen, die selbst an dem Forschungsvorhaben mitwirken oder deren Interessen davon berührt werden.

Die Ethik-Kommission empfiehlt im Einklang mit der Deklaration von Helsinki nachdrücklich die Registrierung klinischer Studien vor Studienbeginn in einem öffentlich zugänglichen Register, das die von der Weltgesundheitsorganisation (WHO) geforderten Voraussetzungen erfüllt, insbesondere deren Mindestangaben enthält. Ausführliche Informationen zur International Clinical Trials Registry Platform (ICTRP) stehen im Internetangebot der WHO zur Verfügung:
http://www.who.int/ictrp/about/en/
Zu den Kriterien des International Committee of Medical Journal Editors (ICMJE) sei beispielsweise verwiesen auf die Informationen unter:
http://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html
Das WHO Primär-Register für Deutschland ist das Deutsche Register für Klinische Studien (DRKS) in Freiburg. Es erfüllt die Forderungen der Fachzeitschriften:
http://www.drks.de/index.html

Die Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster ist organisiert und arbeitet gemäß den nationalen gesetzlichen Bestimmungen und den GCP-Richtlinien der ICH.

Die Kommission wünscht Ihrem Forschungsvorhaben gutes Gelingen und geht davon aus, dass Sie nach Abschluss des Vorhabens über die Ergebnisse berichten werden.

Mit freundlichen Grüßen

i.V. [signature]

Univ.-Prof. Dr. med. Wolfgang E. Berdel
Stellv. Vorsitzender der Ethik-Kommission

Approval for the clinical study by the responsible ethics committee (*Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster*; registration number 2016-053-f-S, approved on 05.04.2016) – page 3

Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster
unser Az.: 2016-053-f-S
Schreiben vom: 5. April 2016

Anhang 1

Folgende Unterlagen haben bei der Beschlussfassung vorgelegen:

Eingang	Datierung	Anlage
01.02.2016	28.01.2016	Anschreiben_28 02 2016
01.02.2016	28.01.2016	Aufklärung-Einwilligung_molekulare und zelluläre Mechanism
01.02.2016	28.01.2016	Ethik-Antrag_molekulare und zelluläre Mechanismen
01.02.2016	28.01.2016	Klotz_CV
01.02.2016	28.01.2016	Kostenübernahmeerklärung
01.02.2016	28.01.2016	Meuth_CV
01.02.2016	28.01.2016	Wiendl_CV
04.03.2016	29.02.2016	aktualisierter Antrag inkl. Stellungnahme des Antragstellers ohne

S. 3 von 4

Approval for the clinical study by the responsible ethics committee (*Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster*; registration number 2016-053-f-S, approved on 05.04.2016) – page 4

Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster
unser Az.: 2016-053-f-S
Schreiben vom: 5. April 2016

Anhang 2

Folgende Mitglieder der Ethik-Kommission haben an der Beratung und Beschlussfassung in der Sitzung am 18.03.2016 teilgenommen:

Univ.-Prof. Dr. med.
Wolfgang E. **Berdel**
Medizinische Klinik A
Universitätsklinikum Münster

Prof. Dr. med. Dr. phil.
Gerhard **Brodner**
Anästhesie, Intensivmedizin und Schmerztherapie,
Fachklinik Hornheide, Münster

Univ.-Prof. Dr. med.
Frank Ulrich **Müller**
Institut für Pharmakologie und Toxikologie, UKM Münster

Univ.-Prof. Dr.
Wolfgang **Köpcke**
Institut für Biometrie und klinische Forschung, WWU Münster

Präsident des Landgerichts a. D.
Klaus **Schelp**
Münster

Univ.-Prof. Dr. med. dent.
Petra **Scheutzel**
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Dr. med.
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S. 4 von 4