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DISSERTATION

Visualization of autoimmune processes in chronic neuroinflammation by means of two-photon laser scanning microscopy.

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Abbreviations

ACSF	artificial cerebrospinal fluid
BDKRB1	bradykinin receptor B1
cGy	centigray
CMTMR	5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)
CNS	central nervous system
ddSNR	depth-dependent signal to noise ratio
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
ECFP	enhanced cyan fluorescent protein
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GM	Goeppert-Mayer
HEK	human embryo kidney
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
IR	infrared
kHz	kilohertz
LB	Lysogeny broth
LFA-1	lymphocyte function antigen 1
MOI	multiplicity of infection
MS	Multiple Sclerosis
NA	numerical aperture
NIR	near infrared
OPO	optical parametric oscillator
PBS	phosphate buffered saline
PMT	photomultiplier tubes
Rag 1	recombination activating gene
tdRFP	tddimer2(12) red fluorescent protein
Ti:Sa	Titanium:Sapphire
TPLSM	two-photon laser scanning microscopy
VLA-4	very late antigen-4

ABSTRACT

Multiple Sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS), characterized by lymphocyte infiltration and inflammation of the CNS leading to dymelination and axonal/neuronal damage. Despite the development of promising treatment strategies in the murine model experimental autoimmune encephalomyelitis (EAE), the detailed therapeutic target mechanisms and the disease underlying cellular and molecular pathways directly in the CNS still remain uncertain, since specific issues regarding immune cell dynamics and the complex neuro-immune crosstalk can not be addressed by conventional experimental approaches.

To overcome these limitations I applied time lapse two-photon laser scanning microscopy (TPLSM) to investigate the cellular migration of various T cell subsets in living brain tissue. First, T cells, isolated from atorvastatin treated mice or after pharmacological activation of the bradykinin receptor B1 revealed a reduced migratory capacity as compared to vehicle treatment. Secondly, cellular dynamics of differentiated effector CD4 T cells are characterized by a predominant vessel alignment in contrast to CD8 T cells, which randomly infiltrate the whole CNS parenchyma. This CD4 T cell compartmentalization was mediated by CXCR4 functioning, whereas the adhesion molecules LFA-1 and the chemokine receptor CCR7 are not involved in this homing process. Obviously, TPLSM allows to visualize cellular dynamics deep in intact tissues and thereby contributes to the clarification of therapeutic but also general pathologic target pathways.

However some pitfalls still remain due to limited excitation wavelengths between 780-1050 nm. The evaluation of long wavelength infrared (IR) excitation by an optical parametric oscillator (OPO) versus near infrared (NIR) excitation by a Titanium:Sapphire (Ti:Sa) laser revealed enhanced penetration depths, an increased depth-dependent spatial resolution and a reduced photobleaching for OPO-excited tdRFP (tddimer2(12) red fluorescent protein) as compared to Ti:Sa-excited EGFP (Enhanced Green Fluorescent Protein) in brain slices, explanted lymph nodes and in the brain of living anesthetized mice. As far as the development of new experimental approaches is concerned, it is further demonstrated that two red fluorescent proteins, i.e. tdRFP and mCherry, can be simultaneously excited and spectrally separated by the OPO-based TPLSM setup. Moreover, using dual Ti:Sa- and OPO-based TPLSM both, cellular dynamics and functional responses, can be visualized during CNS-inflammation. In summary, additionally to the demonstrated advantages regarding image quality new possibilities emerge to elucidate detailed pathomechanisms in the target organ of neuroinflammation by the use of extended excitation wavelengths for TPLSM.

1. Introduction

Multiple Sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS). The initiation of chronic neuroinflammtion in MS is supposed to be mediated by a perivascular accumulation of mononuclear cells preceding the actual infiltration of CNS parenchyma and finally resulting in demyelination and damage of axons and neurons (Compston & Coles, 2008; Trapp *et al.*, 1998). These histopathological hallmarks resemble the findings in the animal model experimental autoimmune encephalomyelitis (EAE), which can be induced in certain rodent strains either by active immunisation with myelin proteins/peptides (active EAE) or by transfer of myelin specific (encephalitogenic) CD4 T helper lymphocytes (passive EAE) (Gold *et al.*, 2006; Wekerle *et al.*, 1986). According to current concepts, which are mainly based on evidence from animal models, myelin specific T cells are activated outside the CNS followed by an upregulation of adhesion molecules and chemokine receptors. Consequently, T cells adhere to and roll along the endothelium via adhesion molecules, such as VLA-4 (Charo & Ransohoff, 2006). Further, the interaction of chemokines with their chemokine receptors induce G-protein-mediated activation of integrins, such as LFA-1, resulting in a firm adhesion and diapedesis through the endothelium (Engelhardt & Ransohoff, 2005). Once in the perivascular space, T cells are reactivated by local antigen presenting cells, such as dendritic cells (Greter *et al.*, 2005) leading to penetration of the CNS parenchyma through *glia* limitants (Bechmann *et al.*, 2007). Proinflammtory cytokines and additional chemotactic factors released by activated T cells and activated microglia lead to further inflammatory cell recruitment and thus enhancement of inflammation finally resulting in demyelination and axonal/neuronal damage (Siffrin *et al.*, 2007).

This complex picture of disease pathogenesis provides various targets for therapeutic interventions. Despite an improved clinical outcome in experimental models, many traditional therapy regimes involving broad immune modulation and suppression, failed to prevent long-term disease progression in the human disease (Killestein & Polman, 2005). Therefore, current research should focus on distinct target mechanisms to develop more specified therapeutic strategies, such as interference with immune cell migration.

One promising therapy, which has already entered a clinical phase II trial (Paul *et al.*, 2008) and has been shown to reduce EAE severity (Aktas *et al.*, 2003), is the treatment with the 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitor atorvastatin. Although anti-inflammatory as well as immuneregulatory effects have been suggested to be involved in prevention and suppression of disease progression (Waiczies *et al.*, 2005; Youssef

et al., 2002) the detailed target mechanisms are not well understood. Since the reduced EAE severity correlated well with a decreased immune cell infiltration of the CNS (Aktas *et al.*, 2003), the impact of atorvastatin on T cell migratory capacity should be elucidated in more detail and might provide an additional explanation for the observed treatment effects.

Another encouraging specific pharmacological intervention in disease progression consists in modulation of the renin-angiotensin and the opposing kallikrein-kinin systems, which have shown unexpected changes in inflammatory MS lesions (Han *et al.*, 2008; Lock *et al.*, 2002), suggesting that pharmacological modulation of these pathways might influence disease progression. Indeed, activation of the bradykinin receptor B1 (BDKRB1) with its agonist R838 (Sar-[D-Phe]des-Arg^{9}-bradykinin) resulted in decreased clinical symptoms of EAE whereas treatment with its antagonist R715 (Ac-Lys-[D-ßNal^{7}, Ile^{8}]des-Arg^{9}-bradykinin) led to increased disease severity, consistent with observations in *BDKRB1 -/-* EAE affected mice, which revealed increased clinical deficits and increased immune cell infiltration as compared to wildtype controls (Schulze-Topphoff *et al.*, 2009). These observations bring along the question, whether BDKRB1 is involved in modulation of CNS-inflammation by affecting migration of encephalitogenic T cells.

As outlined above, the interactions between chemokines and their receptors display a central requirement for immune cell entry into CNS as well as local homing to immune relevant sites in the CNS. In this context the CXCL12-CXCR4 pathway has been shown to be critically involved in modulation of disease pathogenesis, since pharmacological blockade of CXCR4 led to enhanced EAE severity (McCandless *et al.*, 2006). However its role in local trafficking within the CNS and the contribution of different T cell subpopulations needs further investigation.

Although the treatment strategies, described above, have been shown to improve clinical parameters, the underlying cellular and molecular mechanisms have to be elucidated to get a deeper understanding of pathogenetically and therefore therapeutically significant pathways in chronic neuroinflammation. To this end, new experimental approaches are required to get a detailed view on dynamic processes in the target organ, i.e. the inflamed brain. As far as the complex neuro-immune crosstalk is concerned two-photon laser scanning microscopy (TPLSM) has become the method of choice for intravital imaging studies (Niesner *et al.*, 2008), since it counteracts the severe disadvantages of conventional (one-photon excitation) microscopy techniques (Centonze & White, 1998; Germain *et al.*, 2006; Rocheleau & Piston, 2003). The simultaneous absorption of two photons twice the wavelength employed in one-photon excitation microscopy is confined to a focal spot, providing three-dimensional

sectioning without absorption and, thus, without photobleaching and phototoxicity above and below the focal plane (Zipfel *et al.*, 2003b). Additionally, long wavelength excitation light is less scattered, leading to more than 5 fold larger tissue penetration (Helmchen & Denk, 2005; Rocheleau & Piston, 2003).

However, there are still limitations of standard Titanium:Sapphire (Ti:Sa) laser-based TPLSM setups, i.e. restricted imaging depths in long-term imaging experiments depending on tissue constitution (Germain *et al.*, 2006), high non-linear photobleaching of chromophores at the focal plane (Eggeling *et al.*, 2005; Hopt & Neher, 2001; Petrasek & Schwille, 2008), dramatic depth- and tissue-dependent deterioration of spatial resolution (Niesner *et al.*, 2007) and a restricted range of applicable fluorescent proteins, such as ECFP (enhanced cyan fluorescent protein), EGFP (enhanced green fluorescent protein) and EYFP (enhanced yellow fluorescent protein), excluding the whole range of state-of the art red fluorescent proteins, e.g. tdRFP (tddimer2(12) red fluorescent protein) and mCherry (Campbell *et al.*, 2002; Shaner *et al.*, 2004). Since most of these limitations are mainly caused by scattering of the excitation photons in tissue, the question arises, whether extending the excitation wavelength to the infrared (beyond 1050 nm) by the use of an optical parametric oscillator (OPO) (Rimke *et al.*, 2009) could be a solution to these limitations and thereby improve intravital imaging capacities. Moreover, the simultaneous use of OPO- and Ti:Sa- based excitation would offer new opportunities, regarding the visualization of functional responses of CNS cells to autoimmune inflammation in addition to cellular dynamics, since more than two fluorophores and fluorophores with extremely different excitation spectra might be excited simultaneously.

2. Aims

Referring to the elucidation of pathological mechanisms and target pathways of therapeutic interventions in chronic neuroinflammation, the aim of the first part of this PhD thesis was to investigate the migratory capacities and movement behavior of various T cell subsets in living brain tissue. For this purpose time lapse TPLSM was applied on a brain slice T cell co-culture model to visualize and characterize movement pattern of celltracker Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) labeled T cells.

First, the migratory capacity of T cells, isolated from atorvastatin treated EAE affected mice was investigated and compared to T cells of vehicle treated control mice.

Secondly, the impact of pharmacological blockade and activation of the bradykinin receptor B1 on T cell motility was evaluated in living brain tissue, quantified and compared to that of vehicle treated T cells.

Thirdly, cellular dynamics of differentiated effector CD4 T cells as compared to CD8 T cells were described and quantified. To dissect the underlying mechanisms of T cell homing in living brain tissue, the involvement of adhesion molecules, such as LFA-1 and chemokine receptors, such as CCR7 and CXCR4 has been investigated.

Although TPLSM has proved to be an indispensable tool to visualize cellular dynamics deep in intact tissue environments, some pitfalls still remain, i.e. limited imaging depths, depth-dependent deterioration of spatial resolution and a sparse range of applicable fluorophores. However, these parameters are particularly relevant for intravital imaging approaches. In order to improve intravital TPLSM imaging capacities, the aim of the second part of this PhD thesis was to investigate the impact of extended two-photon excitation wavelengths beyond 1050 nm on image quality and on the development of new experimental approaches.

First, the effect of long wavelength infrared (IR) excitation by OPO versus near infrared (NIR) excitation by Ti:Sa was evaluated by comparing penetration depth, spatial resolution and photobleaching characteristics of Ti:Sa-excited EGFP and OPO-excited tdRFP in the brain slice T cell co-culture model, lymph nodes and in the brain of living anesthetized mice.

Secondly, it was tested, whether tdRFP-expressing and lentivirally transduced mCherry-expressing T cells can be simultaneously excited and spectrally separated by the OPO-based TPLSM setup.

Thirdly, the feasibility of dual Ti:Sa- and OPO-based TPLSM was tested for visualization of both cellular dynamics and functional responses during CNS-inflammation by the use of mice, which genetically encode a FRET based calcium sensor in neurons and tdRFP in immune cells.

3. Material and Methods

Mice and Cell culture (publ. 1-4)

The animal experiments were conducted according to current guidelines and regulations, and were approved by the appropriate state committees for animal welfare (LAGeSo, Landesamt für Gesundheit und Soziales, G00029/08, T0271/08). Provenience and cross breeding of individual mouse strains, isolation of T cells and culture/differentiation conditions for individual T cell lines and HEK 293FT cells are described in detail in the publications, referring to in this PhD thesis.

Brain slice T cell co-culture (publ. 1-4)

Brains of C57BL/6 p10-p12 pups were removed immediately and put into 4°C cold aerated (carbogen, 95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 3 mM KCl, 1.6 mM $CaCl_2$, 1.8 mM $MgSO_4$ and 10 mM glucose, adjusted to pH 7.35. 400-µm-thick brain slices were cut with a Vibratome (VT 1200 S, Leica, Heidelberg). For visualization of CNS vessels (publ. 1-3) pups were lethally anesthetized and intracardially perfused with 2% green fluorescent FITC–dextrane in PBS prior to brain slice preparation. Hippocampal slices were isolated, allowed to recover for at least 45 min at room temperature prior to transfer to a heated and with aerated ACSF perfused Luigs & Neumann slice chamber (37°C). T cells, if not expressing EGFP or tdRFP (publ. 4), were stained with celltracker Orange CMTMR and pipetted upon the slice and allowed to invade the slice for about 30 - 60 min before image acquisition.

Two-photon laser scanning microscopy (publ. 1-3)

T cells and vessels were visualized by a two-photon system SP2 (Leica, Heidelberg) equipped with an upright microscope fitted with a 20x water-immersion objective (NA 0.5). Fluorescent dyes were excited simultaneously by a mode-locked Ti:Sa laser (Tsunami, Spectra-Physics, USA) at 840 nm. Fluorescence of CMTMR and FITC was collected using two external non-descanned detectors. Xyz stacks were typically collected over a period of 2-3 h (z-plane distance typically 1.8 µm).

Dual NIR/IR Excitation Setup for TPLSM (publ. 4)

Experiments were performed using a specialized two-photon laser scanning microscope (LaVision BioTec, Bielefeld), which allows for dual NIR (700 – 1020 nm) and IR (1050 – 1600 nm) excitation, i.e. pulsed NIR radiation is generated by an automatically tunable Ti:Sa laser (Mai Tai HP, Spectra Physics, USA), 10% of which is coupled into a scan head (TriMScope, LaVision Biotec, Bielefeld). 90% of Ti:Sa laser power is coupled into a synchronously pumped OPO (APE, Berlin). The generated OPO beam first passes a system of

spectral filters, is entering the scan head and overlapping the Ti:Sa beam. The colocalized beams are coupled into an upright microscope (BX-51WI, Olympus, Hamburg) towards the objective lens (20×, NA 0.95, Olympus, Hamburg). Fluorescence is collected by the same objective lens and directed to a spectrally resolving detection unit containing the respective dichroic mirrors, interference filters and up to three non-descanned photomultiplier tubes (PMT) (H7422-40, Hamamatsu, Japan) for spectral separation of either EGFP and tdRFP or mCherry and tdRFP or Citrine, Cerulean and tdRFP. Applied average laser powers (2 – 17 mW), were measured in the sample using a wavelength-calibrated power meter 818-IR/CM / 842-PE (Newport Spectra-Physics, Darmstadt).

Fluorescent protein purification and measurement of two-photon absorption cross-sections (publ. 4)

Coding sequences for EGFP and tdRFP were amplified by PCR from genomic DNA isolated from EGFP- and tdRFP-expressing splenocytes of *β-actin-EGFP* (Okabe *et al.*, 1997) and *Rosa26 tdRFP ("ΔNeo-flip")* (Luche *et al.*, 2007) C57BL/6 mice and subcloned into the 6x histidine-encoding $pRSET_B$ vector. Plasmids were amplified in *E.coli XL-10 Gold* competent bacteria. *E.coli Ros2-DE3* were transformed by heat shock, amplified and induced for protein expression in carbencillin and chloramphenicol containing LB and autoinduction media. For protein purification bacteria were pelleted and lysed by mechanical cell lysis. Proteins were purified from supernatants of lysats with a cobalt Talon resin followed by gel filtration and dialysis into 20 mM Tris- HCl/150 mM NaCl. Expertise and equipment for protein expression and purification were kindly provided by K. Fälber and O. Daumke (MDC, Berlin). Two-photon cross-sections of EGFP at 850 nm and tdRFP at 1050 nm were measured as described (Xu & Webb, 1996).

Agarose films (publ. 4)

A 2% aqueous suspension of agarose was boiled, mixed in a volume ratio of 7:3 with a 0.002% suspension of small (200 nm) red (580/605) and yellow-green (505/515) fluorescent polystyrene beads, pipetted onto a glass slide and cooled down to room temperature.

Lymph node preparation (publ. 4)

tdRFP- and EGFP-expressing T cells were injected intravenously into *Rag 1 -/-* transgenic C57BL/6 mice. 4 weeks later mice were sacrificed and popliteal lymph nodes were isolated, embedded in anti-fade non fluorescent media kept in a specialized spacer on a glass slide and covered by a transparent film prior to image acquisition.

Generation of mCherry-encoding lentivirus and T cell transduction (publ. 4)

cDNA encoding mCherry (Shaner *et al.*, 2004) sequence (RY. Tsien, San Diego) was subcloned into the lentiviral expression vector pFUGW replacing EGFP sequence and generating pFUmChW lentiviral expression vector. Viral particles were generated by transient Lipofectamin cotransfection of HEK 293FT cells with pFUmChW, pdelta8.9 packaging vector and pVSVG envelope vector using vector ratios as described (Lois *et al.*, 2002). pFUGW, pdelta8.9 and pVSVG vector constructs were kindly provided by S. Haesler / C.Scharff (MPI for Molecular Genetics, Berlin). 72 h after transfection viral supernatants were collected, filtered through 0.45 µm filter and concentrated by ultracentrifugation for 1.5 h at 25,000 rpm in a Beckmann SW40 rotor followed by resupsension in HBSS and determination of viral titers. 2×10^6 *in vitro* cultured and differentiated *2d2* TCR transgenic T cells were supplemented with 2-10 $\times 10^6$ viral particles (MOI (multiplicity of infection) of 1-5) on day 5-7 after (re)stimulation and cultured for at least 3 days in cell culture medium prior to use in TPLSM experiments.

Bone marrow chimera and active EAE (publ. 4)

Recipient *CerTN L15* transgenic C57BL/6 (Heim *et al.*, 2007) mice were sublethally irradiated with 1100 cGy and reconstituted with 12-20 $\times 10^6$ tdRFP-expressing, CD90 T cell depleted bone marrow cells isolated from C57BL/6 *Rosa26 tdRFP ("ΔNeo-flip")* (Luche *et al.*, 2007). After engraftment for 8 weeks active EAE was performed as described in publ. 4 (Siffrin *et al.*, 2009). Intravital TPLSM was performed on day 15 after immunization, i.e. at the peak of disease.

Preparation of imaging field for intravial imaging (publ. 4)

Mice were anesthetized using 1.5% isoflurane in oxygen / nitrous oxide (2:1) with a facemask, tracheotomized and continuously respirated. After transferring to a custom-built operation and microscopy table and fixation in a hanging position, the preparation of the imaging field was performed according to adapted protocols for cortical imaging (Gobel & Helmchen, 2007). For imaging of the brain stem, this region was exposed by carefully removing musculature above dorsal neck area and removing dura mater between first cervical vertebra and occipital skull bone. Head was inclined for access to deeper brain stem regions and the brain stem superfused with isotonic Ringer solution. A sterile agarose patch (0.5% in 0.9% NaCl solution) was installed on the now exposed brain surface to reduce heart beat and breathing artefacts. During surgery and microscopy body temperature and anesthesia depth were controlled.

Data analysis (publ. 1-4)

Images were post-processed using acquisition software Imspector (LaVision Biotec). For quantification of fluorescence intensities ImageJ software (Wayne Rasband, NIH) was used. 3D presentation and quantitative cell tracking analysis were performed with Volocity (Improvision, Germany). Statistical analysis and graphical presentation was done with SPSS (SPSS, Germany), GraphPad Prism 4 (GraphPad Software, USA) and Origin (OriginLab).

4. Results

In-vivo atorvastatin treatment inhibits T cell infiltration into CNS tissue

C57BL/6 mice, treated with either atorvastatin or vehicle, were sacrificed 18 h following the last treatment. Isolated T cells of both groups were polyclonally activated for 3 days, then labeled with CMTMR and subjected to acute hippocampal slices from 10 day old C57BL/6 mice for analysis of migratory capacity in living CNS tissue with time lapse TPLSM. Whereas cells of vehicle-treated mice infiltrated brain tissue instantly and were moving around with high motility, T cells from atrovastatin-treated mice were barely able to migrate into deeper brain tissue regions and remained almost stationary, leading to mean track velocities of 0.014 µm/s for statin-treatment as compared to 0.006 µm/s for vehicle treatment.

Modulation of BDKRB1 signalling influences CD4 Th17 cell migration in brain tissue

CMTMR labelled *in vitro* differentiated ovalbumin (OVA)-specific CD4 Th17 cells were incubated either with BDKRB1 agonist R838 or antagonist R715 or PBS for 4 h before coincubation with hippocampal brain slices. Time lapse TPLSM revealed a decreased infiltration of CNS tissue and a decreased motility upon BDKRB1-activation with R838 as compared to BDKRB1-blockade and PBS-treatment, i.e mean track velocity was reduced by 15% and the average number of infiltrated T cells per minute and per defined volume was reduced by 70% for R838 treatment as compared to PBS control.

Vessel-associated CD4 T cell movement is actively promoted by CXCR4

In vitro differentiated and CMTMR labelled OVA-specific CD4 and CD8 T cells were co-incubated with acute hippocampal brain slices, in which the vasculature had been highlighted by injection of FITC-dextrane. After an initial invasion period the majority of CD4 T cells assembled around medium-sized CNS vessels and constantly moved along the outer vessel wall. In contrast, CD8 T cells showed highly dynamic trafficking through the whole CNS parenchyma without the predominant vessel restricted alignment as observed for CD4 T cells. To objectify these observations quantitatively, vector-vessel angles for both T cell subsets have been calculated. Whereas CD8 T cells exhibited a characteristic even distribution of individual angles with a mean angle of 44.96° ± 2.37°, an increased number of CD4 T cells revealed significantly smaller vector-vessel angles with a mean angle of 32.36° ± 2.56°.

To check for the involvement of adhesion molecules and chemokine receptors in lymphocyte CNS trafficking, polyclonally stimulated CD4 cells derived from *LFA1*$^{-/-}$ C57BL/6 and from *CCR7-/-* C57BL/6 mice were investigated. To interfere with CXCR4 function, OVA-specific CD4 cells were treated with the small molecule antagonist AMD3100 for 3 h before experimentation. Analysis of motility pattern and vector-vessel angle revealed a significant

change in the vessel-associated motility of AMD3100-treated CD4 T cells, which with a mean vector angle of 42.36 ± 1.99° almost equalled the random-like motility observed for CD8 T cells, whereas LFA-1 and CCR7 deficiency had no impact on vessel association, since mean angles amounted to 32.92° ± 2.31° and 34.95° ± 1.81, respectively.

Improvement of intravital imaging capacities by expanding two-photon excitation to the infrared

First, benchmarking experiments, including the determination of two-photon excitation spectra and two-photon absorption cross-sections were performed, to allow for comparative studies regarding the optical performance, i.e. depth-dependent spatial resolution, maximal penetration depth and photobleaching rates of OPO- versus Ti:Sa-based excitation of tdRFP and EGFP, respectively. According to the experimentally derived excitation spectra experiments on tdRFP were performed at 1110 nm, whereas experiments on EGFP were carried out both at 850 nm, a typical wavelength of Ti:Sa considering the fluorescence spectrum of the crystal, and at 920 nm, the excitation maximum. Since all spectra were corrected for peak laser photon flux and for cellular autofluorescence, they represent relative active two-photon absorption cross-sections spectra. Absolute values of the two-photon absorption cross-sections were derived from measurements on purified fluorescent proteins and amounted to 23.0 ± 1 GM at λ_{exc} = 850 nm for EGFP and 20.2 ± 0.8 GM at λ_{exc} = 1110 nm for tdRFP.

The spatial resolution was determined by the dimensions of the effective point spread function of a punctiform object with dimensions below the resolution limit. For this purpose the local 3D fluorescence signal of red fluorescent (580/605) and yellow-green fluorescent (505/515) microspheres with analogous excitation and emission spectra to tdRFP and EGFP was collected in scattering tissue of hippocampal brain slices. The depth-dependent deterioration of spatial resolution at 1110 nm was significantly lower (laterally 1.5× and axially 1.4×) than that at 920 nm (laterally 2.2× and axially 1.8×), which was slightly lower than that at 850 nm (laterally 2.6× and axially 2.1×).

The maximal penetration depth is defined as the penetration depth, in which the decreasing fluorescence signal reaches the level of the background noise, i.e. the depth-dependent signal to noise ratio (ddSNR) becomes 1. Typical ddSNR curves for excitation of EGFP- and tdRFP-expressing T cells in hippocampal brain slices resulted in maximal imaging depths of 160 µm for EGFP excitation at 920 nm and 150 µm at 850 nm, whereas an improvement of 40% is attained by tdRFP excitation at 1110 nm, i.e. the maximal imaging depth is 210 µm. The maximal penetration depth in lymph nodes of *Rag 1 -/-* mice, reconstituted with EGFP- and

tdRFP-expressing T cells, typically amounted to 130 µm for EGFP at 850 nm and to 140 µm at 920 nm, whereas excitation of tdRFP at 1110 nm resulted in 190 µm imaging depth. ddSNR decay measurements under intravital conditions in the brain stem of mice expressing EGFP or tdRFP in neurons resulted in maximal imaging depths of 160 µm at 850 nm and 174 µm at 920 nm for EGFP excitation as compared to 276 µm for tdRFP excitation at 1110 nm. Similarly, maximal penetration depths in the cortex amounted to 282 µm at 850 nm (EGFP), 292 µm at 920 nm (EGFP) and 508 µm at 1110 nm (tdRFP), demonstrating an improvement of 80%.

Photobleaching rates (k_{bl}) were determined in co-cultures of hippocampal slices with EGFP- and tdRFP-expressing T cells and intravitally in the brain stem of *EGFP Thy1-21* (Feng *et al.*, 2000) mice and *tdRFP CNP* mice. Since the slope of the double logarithmic dependence k_{bl} on the peak photon flux was 2.62 ± 0.13 for EGFP excited at 850 nm and 2.93 ± 0.14 for tdRFP excited at 1110 nm, the typical non-linear dependence of the photobleaching rate on the peak photon flux of the excitation laser was confirmed. However, the photobleaching rate of EGFP (λ_{exc} = 850 nm) is approximately 2 and 6 times higher than that of tdRFP (λ_{exc} = 1110 nm) in brain slices and in intravital imaging of the brain stem, respectively.

To check, whether exclusively red fluorescent proteins can be used for multicolor two-photon analysis, experiments were performed on mixtures of mCherry- and tdRFP-expressing T cells. mCherry expression was induced by lentiviral gene transduction, since a corresponding transgenic model is lacking so far. Using various excitation wavelengths near the excitation maxima of both fluorescent proteins, the optimal wavelength for simultaneous excitation and spectral separation of mCherry and tdRFP-expressing T cells was determined to be 1170 nm.

In order to reveal the power of combining both NIR excitation of Ti:Sa with IR excitation of OPO, dual NIR/IR excitation TPLSM was employed for intravital deep tissue imaging of three fluorescent proteins in the brain stem of EAE affected *CerTN L15* mice, previously reconstituted with tdRFP-expressing bone marrow cells, i.e. mice expressing Cerulean (ECFP variant) and Citrine (YFP derivative) as a FRET-pair indicating the cytosolic Ca^{2+}-level in neurons combined with tdRFP expression in immune cells. Intravital three-color 4D-imaging (3D + time) revealed that communication between immune cells and neurons resulted in transient calcium fluctuations and overall increased calcium concentration in neurons.

5. Discussion

Multiple sclerosis represents the prototypic inflammatory autoimmune disorder of the central nervous system and the most common cause of neurological disability in young adults. In spite of a better understanding of the immunopathological processes underlying this disease leading to the establishment of novel treatment options, most of the current neuroimmunological research depends on *in vitro* and *ex vivo* experimental setups, e.g. cell cuture and immunohistochemical experiments, supplying only limited insight into the *in vivo* situation of CNS-inflammation and neglecting the critical aspect of the dynamics, the sequence and functional relevance of immune cell trafficking and communication within the complex cellular network of living brain parenchyma. However, with the advent of TPLSM some of these limitations can be overcome by the visualization of cellular dynamics and interactions deep in intact tissue of living brain slices (Kawakami *et al.*, 2005; Nitsch *et al.*, 2004) but also in various brain regions of living animals (Helmchen *et al.*, 1999).

In this PhD thesis time lapse TPLSM was used for visualization of immune cell trafficking within living CNS tissue to contribute to the clarification of effector mechanisms of two pharmacological interventions, which have been shown to ameliorate and prevent EAE (Schulze-Topphoff *et al.*, 2009; Waiczies *et al.*, 2007). First, *in vivo* treatment with atorvastatin inhibited T cell migration and movement into and within the brain. Secondly, activation of BDKBR1 resulted in reduced T cell motility and infiltrative behavior of CD4 Th17 cells. Since these observations correlated well with an improved clinical outcome and histological representations for both treatment strategies, it can be concluded, that T cell migration into and within the CNS is a central target mechanisms for the development of novel therapies.

However, our understanding of the detailed pathological but also immunregulatory pathways, initiating and controlling chronic neuroinflammtion, is still incomplete, since the role and significance of different immune cell subsets in disease pathogenesis remains uncertain. With regard to these questions time lapse TPLSM was applied to get deeper insights to general routes and rules controlling immune cell trafficking within the CNS. Comparative TPLSM studies on CD4 and CD8 T cell movement in living brain tissue revealed that CD4 T cells compartmentalize to CNS vessels, contrasting CD8 T cell movement, which infiltrate the whole parenchyma. Further, this process depended on CXCR4 functioning, whereas key adhesion molecules and chemokine receptors, such as LFA-1 and CCR7 do not seem to be involved in this homing behavior. These data explain previous observations of an increased lymphocyte infiltration into CNS parenchyma upon CXCR4 blockade in EAE (McCandless *et*

al., 2006). However, the data presented here provide more detailed information, since it is shown, that particularly CD4 T cells home to the perivascular space leading to the assumption that this compartmentalization is an important prerequisite for the following processes involved in inflammation but also immuneregulation, such as interaction with professional antigen presenting cells (Greter *et al.*, 2005).

The next step forward to clarify the functional relevance of these observations consists in the *in vivo* visualization of cellular communications to define cellular targets of these different T cell subsets within the inflamed brain. However, to this end two central requirements have to be met in intravital TPLSM. First, fluorophores must specifically label cells over the whole duration of disease, i.e. genetically expressed compartmentalized fluorescent proteins should be used instead of *ex vivo* staining with synthetic dyes, characterized by leakage and cellular stress due to dimethyl sulfoxide (DMSO) based solvents (Germain *et al.*, 2006). Secondly, multicolour two-photon analysis is required, i.e. the simultaneous excitation of fluorophores with extremely different excitation spectra, such as GFP derivatives combined with red fluorescent proteins, e.g. tdRFP and mCherry (Campbell *et al.*, 2002; Shaner *et al.*, 2004), which cannot be excited with standard Ti:Sa lasers due to limited excitation wavelengths within spectral ranges between 700-1080 nm. Moreover, using conventional Ti:Sa-based TPLSM, one can not completely overcome scattering due to local differences in tissue properties and autofluorescence induced by excitation of intracellular metabolics like the NADH, NADPH and flavoproteins (Niesner *et al.*, 2004; Zipfel *et al.*, 2003a) leading to limited imaging depths and reduced depth-dependent spatial resolution.

Single aspects of these limitations have been addressed, for instance, by using low repetition rate Ti:Sa excitation (400 kHz) imaging depths as large as 1 mm were reached in the cortex (Theer *et al.*, 2003). However, the mean laser power was 1000 mW, i.e. peak power of $1.25 \cdot 10^8$ mW, and verification in long-term imaging experiments is missing. Further technical solutions are based on extension of the excitation wavelength to the infrared by the use of chromium:forsterite lasers (1250 nm – 1350 nm) (Chan *et al.*, 2005) or (linear or non-linear) optical parametric amplifiers ((N)OPA) (Manzoni *et al.*, 2006), but most of these setups still need to be adapted for *in vivo* and intravital application. In this PhD thesis it is shown that limitations in penetration depth, resolution and photobleaching particularly in intravital imaging can be overcome by extending the excitation wavelength range up to 1600 nm using an OPO. Comparing IR excitation by OPO to NIR excitation by Ti:Sa, it was demonstrated that in 70 µm depth in scattering brain tissue a 1.7 fold improvement of both lateral and axial resolution is achieved under 1110 nm excitation as compared to 850 nm excitation. Since in

TPLSM, based on PMT-detection, the spatial resolution is mainly determined by the excitation rather than the emission wavelength, this improvement must be accounted alone to the longer wavelength excitation, which can be explained by the fact, that the mean free path l_s of ballistic excitation photons between two scattering events in tissue approximately scales with λ_{exc}^{-2} (Caccia *et al.*, 2008).

This dependence of the scattering parameter l_s on the excitation wavelength is also the main reason for the increased depth-dependent SNR, demonstrated in this work, i.e. the 40 - 80% increased maximal penetration depth, at 1110 nm excitation of tdRFP as compared to 850 nm or 920 nm excitation of EGFP in brain slice T cell co-cultures, in explanted lymph nodes, and intravitally in the brain stem or cortex of anesthetized mice. Since l_s does not only depend on excitation wavelength but also on tissue constitution, reduced imaging depths in lymph nodes as compared to brain tissue might be explained by shorter l_s of about 20 µm at 800 nm in lymph node due to a high number of nuclei per volume unit as compared to 100-200 µm in brain tissue (Caccia *et al.*, 2008; Oheim *et al.*, 2001). Moreover, in the brain l_s varies with the metabolic state, myelinisation and the density of neural mass (Helmchen & Denk, 2005; Oheim *et al.*, 2001), consistent with observations in this thesis, which indicate larger imaging depths in cortex than in brain stem than in hippocampal brain slices. Since laser pulse width and shape, distance between sample and detection unit and two-photon absorption cross-sections of the investigated fluorescent proteins, were either kept constant or were similar for the comparative studies, it can be assumed that these findings are not subject to artefacts due to differing experimental or molecular parameters.

Almost all intravital imaging studies aim at the visualization of cellular dynamics and interactions requiring the repeated acquisition of fluorescence images of the same region at the sample. Here, most investigators are faced with the central dilemma to reach high penetration depths at high laser powers, implying high photobleaching rates (Patterson & Piston, 2000). Therefore, photobleaching characteristics of EGFP and tdRFP were compared in the brain slice T cell co-culture model and in the brain stem of living mice, revealing a 2 - 6 times higher photobleaching rate of EGFP at both 850 nm and 920 nm as compared to tdRFP at 1110 nm. Since, it can be assumed that mainly the molecular photophysics of EGFP and tdRFP determine this trend, the implicit advantage of OPO IR-excitation over Ti:Sa NIR-excitation is shown, i.e. the possibility to apply the whole range of novel fluorescent proteins with improved properties, e.g. improved photobleaching characteristics.

According to the improved optical performance of OPO-based long wavelength excitation, a step further towards new two-photon applications was to test, whether other red fluorescent

proteins, which cannot be excited by standard Ti:Sa lasers, might be used for multicolor two-photon analysis. To this end, it is shown here that two red fluorescent proteins, i.e. mCherry and tdRFP can be simultaneously excited and spectrally separated using OPO-based TPLSM at an excitation wavelength of 1170 nm.

Referring to the investigation of pathological mechanisms the simultaneous visualization not only of cellular dynamics but also of functional responses requires the application of more than two fluorophores. Here, it is demonstrated that the application of dual Ti:Sa- and OPO-based TPLSM on chimeric mice expressing a Citrine-Cerulean-based FRET pair in neurons (Heim *et al.*, 2007) and tdRFP in immune cells enables to monitor neuronal calcium fluctuations, i.e. neuronal function, in response to immune cell interactions in the CNS of living mice affected by EAE, thereby highlighting an important aspect of the neuro-immune crosstalk during chronic neuroinflammation.

Collectively, two-photon microscopy has become a leading tool in neuroimmunolgical research, since it allows to clarify therapeutic target mechanisms in autoimmune CNS-inflammation, as shown here for pharmacological interventions by HMG-CoA reductase inhibition, by CXCR4 blockade and by BDKBR1 modulation. Moreover, exploiting the full capacity of long (IR) wavelength excitation beyond 1050 nm, as demonstrated in this thesis, combined with the use of lineage specific red fluorescent protein expressing reporter mice (Wan & Flavell, 2005; Yang *et al.*, 2008) and functional fluorescence-based constructs that for example allow to measure enzyme activities, such as caspase-3 (Chiang & Truong, 2005) for the visualization of apoptosis, may further contribute to the development of new experimental approaches to investigate the general pathological processes and their functional relevance, which is indispensable for the development of effective therapies.

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Appendix

Individual contribution to seleceted publications

Publication 1

Waiczies, S.*, I. Bendix*, T. Prozorovski, M. Ratner, I. Nazarenko, C. F. Pfüller, A. U. Brandt, **J. Herz**, S. Brocke, O. Ulrich, F. Zipp. 2007. Geranylgeranylation but not GTP loading determines Rho migratory function in T cells. *J. Immunol*. 179:6024-6032

Individual contribution: 10-20 %

Josephine Herz isolated T cells, did the short-term cultures and characterization of activation status of T cells by FACS analysis. She carried out TPLSM experiments on hippocampal brain slice T cell co-cultures and acquired imaging data. Moreover, she made critical revision of the manuscript.

Publication 2

Schulz-Topphoff, U., A. Prat, T. Prozorovski, V. Siffrin, M. Paterka, **J. Herz**, I. Bendix, I. Ifergan, I. Schadock, M. A. Mori, J. Van Horssen, F. Schröter, M. Htwe Han, M. Bader, L. Steinmann, O. Aktas §, F. Zipp §. 2009. Activation of kinin receptor B1 limits encephalitogenic T lymphocyte recruitment to the central nervous system. *Nat. Med*. 15(7):788-93

Individual contribution: 10-20 %

Josephine Herz isolated naïve T cells by MACS followed by in vitro differentiation. She characterized the activation and differentiation status by FACS analysis and performed pharmacological treatment of antigen-specific T cell lineages. She performed TPLSM on hippocampal brain slice T cell co-cultures, acquired, analysed and interpreted the imaging data and did the statistical analysis on these data. She critically revised the manuscript,

Publication 3

Siffrin, V.*, A. U. Brandt*, H. Radbruch*, **J. Herz**, N. Boldakowa, T. Leuenberger, J. Werr, A. Hahner, U. Schulze-Topphoff, R. Nitsch, F. Zipp. 2009. Differential immune cell dynamics in the CNS cause $CD4^+$ T cell compartmentalization. *Brain*. 132:1247-1258

Individual contribution: 30-40 %

Josephine Herz did the isolation, in vitro differentiation and characterization of activation and differentiation status of antigen-specific T cell lineages and of CCR7 -/- and LFA-1 -/- T cells by FACS analysis. She carried out TPLSM on hippocampal brain slice T cell co-cultures,

acquired, analysed and interpreted imaging data of slice experiments and performed statistical analysis on these data. She made critical revision of the manuscript.

Publication 4

Herz, J.*, V. Siffrin*, A. E. Hauser, A. U. Brandt, T. Leuenberger, H. Radbruch, F. Zipp, and R. A. Niesner. 2010. Expanding two-photon intravital microscopy to the infrared by means of OPO. *Biophys. J.* 98(4):715-23

Individual contribution: 70-80 %

Josephine Herz conceived and designed the research. She carried out the benchmarking experiments, i.e. measurements of relative two-photon absorption cross-section spectra, cloning procedures for protein expression and purification of fluorescent proteins for measurement of absolute two-photon absorption cross-sections. She performed isolation and culture of T cells and hippocampal brain slice T cell co-cultures, generated lentiviruses and carried out transduction of T cells. She acquired, analysed and interpreted imaging data of TPLSM measurements on brain slices, lymph nodes and of intravital imaging experiments. She drafted the manuscript, corrected and critically revised the manuscript in response to reviewers comments.

Berlin, 06.01.2010

Josephine Herz Prof. Dr. med. Frauke Zipp

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Eidesstattliche Erklärung

„Ich, Josephine Herz, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertationsschrift mit dem Thema: ‚Visualization of autoimmune processes in chronic neuroinflammation by means of two-photon laser scanning microscopy' selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Berlin, 06.01.2010

Josephine Herz

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

Bibliography *[5-Year Impact Factors (IF)]*

Original articles

Waiczies, S.*, I. Bendix*, T. Prozorovski, M. Ratner, I. Nazarenko, C. F. Pfüller, A. U. Brandt, **J. Herz**, S. Brocke, O. Ulrich, F. Zipp. 2007. Geranylgeranylation but not GTP loading determines Rho migratory function in T cells. *J. Immunol*. 179:6024-6032 *[6,165]*

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Siffrin, V., A. U. Brandt, **J. Herz**, F. Zipp. 2007. New insights into adaptive immunity in chronic neuroinflammation. *Adv. Immunol*. 96: Chapter 1 *[7,078]*

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Congress contributions

PhD symposia

Berlin Brain Days 2006, Berlin, 29.10.06-3.11.06

Herz, J., A. U. Brandt, V. Siffrin, H. Radbruch, N. Boldakowa, N. Asselborn, F. Zipp. The influence of different T cell populations on neural damage in neuroinflammation. (oral presentation)

Neurizons 2007, Göttingen, 30.05.-02-06.07

Herz, J., V. Siffrin, A. U. Brandt, N.Boldakowa, H. Radbruch, H.Salmon, N. Asselborn, F. Zipp. Visualizing autoimmune processes leading to neural damage in a rodent model for Multiple Sclerosis – focusing on the impact of different T cell subpopulations. (poster presentation)

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Herz, J., A. U. Brandt, V. Siffrin, N. Boldakowa, H. Radbruch, H. Salmon, N. Asselborn, F. Zipp. Visualizing autoimmune processes in the CNS using Multi-photon microscopy. (oral presentation)

Berlin Brain Days 2008, Berlin, 11.12.-12.12.08

Herz, J., R. A. Niesner, A. E. Hauser, A. U. Brandt, T. Leuenberger, H. Radbruch, H. J. Fehling, V. Siffrin, F. Zipp. Improvement of intravital two-photon microscopy. (poster presentation)

International Congresses

World Immune Regulation Meeting 2007, Davos, 11.04.-15.04.07

Luenstedt, S., **J. Herz**, C. F. Pfüller, T. Leuenberger, I. Bendix, T. Prozorovski, F. Zipp, S. Waiczies. Modulating the T cell response by HMG-CoA reductase inhibitors: mechanisms for treating neuroinflammation?
(poster presentation)

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Siffrin, V., A. U. Brandt, H. Radbruch, **J. Herz**, N. Boldakowa, T. Leuenberger, J. Werr, A. Hahner, U. Schulze-Topphoff, R. Nitsch, F. Zipp. Differential immune cell dynamicx in the CNS cause CD4 T cell compartmentalization.
(poster presentation)

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Herz, J., V. Siffrin, R.A. Niesner, A. U. Brandt, H. Radbruch, H. J. Fehling, F. Zipp. Using dual two-photon excitation to visualize lymphocytes in compact CNS tissue in vivo and in vitro.

(poster presentation)

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