The role of PPARγ in myeloid cells in experimental autoimmune encephalomyelitis

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Abbreviations

A. bidest	aqua bidest
APC	antigen presenting cell
APP	amyloid precursor protein
BBB	blood brain barrier
BCP	1-Bromo-3-Chloropropane
BCR	B cell receptor
BSA	bovine serum albumin
β-ME	β-mercaptoethanol
BM-M	bone-marrow macrophages
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked Immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
g	gram
g	gravity
GBSS	Gey's balanced salt solution
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	Human Leukocyte Antigen
Ig	immunoglobulin
IL	interleukin
IFN	interferon
i.p.	intraperitoneal
i.v.	intravenous
LFB	luxol fast blue
LN	lymph node

LPS	lipopolysaccharide
LysM	M-lysozyme
MACS	magnetic-activated cell sorting
MBP	myelin basic protein
M-CSF	macrophage colony-stimulating factor
MFI	mean fluorescence intesity
MHC	Major Histocombatibility Complex
min	minute
mg	milligram
µg	microgram
ml	milliliter
mm	millimeter
mM	millimolar
µm	micrometer
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
NaN_3	sodium azide
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
ng	nanogram
PAMPS	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
PLP	proteolipid protein
PMA	phorbol myristate acetate
Pio	Pioglitazone
pMg	primary microglia
PPARγ	peroxisome proliferator-activated receptor γ
PRR	pattern recognition receptors
RNA	ribonucleic acid
ROS	reactive oxygen species

rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s.c.	subcutaneous
SEM	standard error of the mean
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor

Summary

Multiple sclerosis (MS) is a common chronic inflammatory demyelinating disease of the central nervous system (CNS) affecting more than 1 million people worldwide. It mainly affects young adults and has a great impact on the social economic situation. Although several therapeutic drugs are available for slowing down progression or reducing number of relapses there is a clear need for more efficacious therapies.

The peroxisome proliferator-activated receptor γ (PPARγ), a nuclear transcription factor with anti-inflammatory effects, influences pro-inflammatory responses of several immune cell types during experimental autoimmune encephalomyelitis (EAE), the animal model of MS, which ameliorates the disease course and reduces neuronal damage. Hence, PPARγ seems to be a promising target for therapeutic treatment during EAE and MS.

The aim of this thesis was to investigate the relevance of PPARγ specifically in myeloid cells for control of their inflammatory activity *in vitro* and *in vivo*. To this end, we made use of a conditional knock-out mouse model which exhibits targeted deletion of PPARγ in M-lysozyme (LysM)-expressing cells (LysM-PPARγ^{KO} mice) which comprise myeloid cells such as monocytes, macrophages, CNS-resident microglial cells as well as neutrophilic granulocytes.

We observed significantly enhanced production of pro-inflammatory cytokines, chemokines and neurotoxic mediators by PPARγ-deficient (PPARγ^{KO}) primary microglial cells and macrophages compared to PPARγ^{WT} cells *in vitro*. Importantly, we could demonstrate *in vivo*, that LysM-PPARγ^{KO} mice exhibited an aggravated course of disease during the effector phase of EAE. This aggravation was accompanied by a more pronounced local inflammatory milieu within the CNS as pro-inflammatory cytokines such as tumor necrosis factor (TNF) α, interleucin (IL) -12 and IL-6 as well as chemokines like CCL2 and CCL5 were increasingly expressed in LysM-PPARγ^{KO} mice. Furthermore, this exacerbation was accompanied by an increase of CNS-invading macrophages and T cells as well as an increase in the activation status of PPARγ^{KO} myeloid cells, which show higher levels of Cluster of Differentiation (CD40) and major histocompatibility complexes (MHC) -II expression.

Furthermore, this thesis addressed the question which subgroup of myeloid cells is the main target for PPARγ-mediated regulation during EAE. By combining monocyte transfer and depletion experiments we identified the myeloid cell population that represents the main population regulated by PPARγ during EAE, i.e. Ly6C^{hi} inflammatory monocytes.

Transfer of PPARγ-deficient monocytes into EAE-diseased mice resulted in a more pronounced aggravation of disease than the transfer of wildtype monocytes. In line with this, depletion of inflammatory monocytes during EAE not only ameliorated the disease course of both, LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice but also abrogated the differences observed during the effector phase in LysM-PPARγ^{KO} mice compared to their wildtype littermates.

In summary, these data provide evidence that PPARγ controls the activation status of myeloid cells during autoimmune responses, thereby affecting the extent of CNS inflammation and hence neuronal damage in EAE. Therefore PPARγ in myeloid cells represents a promising therapeutic target for future treatment of CNS-infammation in MS.

Zusammenfassung

Multiple Sklerose ist eine weitverbreitete chronisch-entzündliche, demyelinisierende Erkrankung des Zentralen Nerven Systems (ZNS), an der weltweit mehr als 1 Millionen Menschen erkrankt sind. Sie betrifft überwiegend junge Erwachsene und hat daher eine große Bedeutung für die sozial-wirtschaftliche Lage. Obwohl verschiedene Medikamente verfügbar sind, die zum einen die Progredienz der Erkrankung verlangsamen oder die Anzahl der Schübe verringern, gibt es einen deutlichen Bedarf für wirksamere Therapien.

Der Peroxisom Proliferator-aktivierte Rezeptor γ (PPARγ), ein nukleärer Transkriptionsfaktor mit anti-inflammatorischen Eigenschaften, beeinflusst die Entzündungsreaktionen verschiedener Immunzelltypen während der experimentellen autoimmunen Enzephalomyelitis (EAE), dem Tiermodell der MS. Dies führt zu einer Besserung des Krankheitsverlaufes und mindert neuronale Schäden. Daher scheint PPARγ einen vielversprechender Ansatz für eine therapeutische Behandlung der EAE und der MS zu sein.

Ziel dieser Arbeit war es, die Relevanz von PPARγ speziell in myeloiden Zellen im Hinblick auf deren entzündliche Aktivierung *in vitro* und *in vivo* zu untersuchen. Um dies zu erreichen, wurde ein konditionales Knockout Maus-Modell genutzt, das eine Deletion der PPARγ-Expression ausschließlich in M-Lysozym (LysM) exprimierenden Zellen aufweist, darunter Monozyten, Makrophagen, ZNS-stämmige Mikroglia und neutrophile Granulozyten.

Hierbei wurden *in vitro* in primären Mikrogliazellen und Makrophagen mit fehlerhaft-expremiertem PPARγ ($PPAR\gamma^{KO}$) signifikant erhöhte Produktionen von proinflammatorischen Zytokinen, Chemokinen und neurotoxischen Mediatoren im Vergleich zu Zellen mit funktionalem PPARγ beobachtet. *In vivo* zeigten Mäuse mit einem konditionalen PPARγ-Knockout in myeloiden Zellen (LysM-$PPAR\gamma^{KO}$ Mäuse) einen verschlimmerten Krankheitsverlauf während der Effektorphase der EAE. Diese Verschlechterung wurde begleitet von einem erhöhten inflammatorischen Milieu: die pro-inflammatorischen Zytokine TNFα, IL-12 und IL-6, sowie die Chemokine CCL2 und CCL5 waren erhöht. Zudem wurde die Verschlechterung begleitet von einer erhöhten Anzahl in das ZNS einwandernder Makrophagen und T Zellen sowie eine Zunahme der Aktivierung der $PPAR\gamma^{KO}$ Zellen, die einen Anstieg der CD40- und MHC-II-Expression aufwiesen.

Des Weiteren befasste sich diese Arbeit mit der Fragestellung welche Untergruppe der myeloiden Zellen das Hauptziel für eine PPARγ-vermittelte Regulation während der EAE darstellt. Durch eine Kombination von Experimenten mit Monozyten-Transfer und -Depletion konnte eine bestimmte Population der myeloiden Zellen, die $Ly6C^{hi}$ inflammatorischen Monozyten, identifiziert werden, die die Hauptpopulation der myeloiden Zellen darstellt, die durch PPARγ während der EAE moduliert wird. Der Transfer von PPARγ-defizienten Monozyten in EAE-erkrankte Tiere ergab eine deutlich ausgeprägte Verschlechterung der Erkrankung im Vergleich zum Transfer von Wildtyp-Monozyten. Dementsprechend führte die Depletion inflammatorischer Monozyten während der EAE nicht nur zu einer Verbesserung des Kranheitsverlaufs sowohl in LysM-$PPAR\gamma^{KO}$ Mäusen als auch in LysM-$PPAR\gamma^{WT}$ Mäusen: Zuvor beobachtete Unterschiede während der Effektorphase zwischen LysM-$PPAR\gamma^{KO}$ Mäuse und Wildtyp Kontrolltieren waren aufgehoben.

Zusammenfassend belegen diese Daten, dass PPARγ den Aktivierungsstatus von myeloiden Zellen während der ZNS Autoimmunität kontrolliert. Zudem begrenzt PPARγ in myleoiden Zellen das Ausmaß der ZNS-Entzündung und die nachfolgenden neuronalen Schäden während der EAE. Daher stellt PPARγ in myeloiden Zellen ein vielversprechendes therapeuthisches Ziel für eine zukünftige Behandlung von ZNS-Entzündungen in der MS dar.

1 Introduction

1.1 The immune system

Organisms are ubiquitously exposed to pathogens like bacteria, viruses, fungi, or parasites. These organisms can enter the body through the skin, the respiratory tract, or mucosal epithelium[1].

The immune system is a biological defense mechanism, which protects the organism from those pathogens by identifying and eliminating them. In vertebrates, the immune response is classified into two components:

1) The innate immune response, which is very fast and mediates the first defense in a generic way within the first hours after infection and
2) Whereas the innate immune response is based on conserved receptors, recognizing distinct structures of pathogens and present in all multicellular organisms, only vertebrates possess a more antigen-specific immune response: the adaptive immune system, which specifically recognizes proteins on pathogens[1]. The adaptive immune response occurs later than the innate system. It triggers elimination of pathogens or infected cells in a more specific and a more effective way. Importantly, the adaptive immune response mediates immunological memory, which allows a more rapid immune response at the time of a second infection with the same pathogen[1].

1.1.1 The innate immune system

If microorganisms try to enter the organism through the skin, the respiratory tract, or the mucous membranes, the innate immune response is the first barrier of defense.

Several cells and mechanism, such as natural killer cells, mast cells, eosinophils, and basophils, phagocytic cells like dendritic cells (DCs), macrophages, and neutrophils and the humoral component (e. g. the complement system and serum proteins) compose the innate immune response.

However, only monocytes / macrophages and microglial cells are important cells of the innate immune system in this thesis. Monocytes are the premature macrophages, circulating in the blood stream. Microglia are the resident macrophages of the central

nervous system (CNS)[2]. However, monocytes, macrophages, and microglia are thought to be of myeloid origin and derive from monocytic lineage[3]. Importantly, they recognize molecules specifically associated with pathogens, namely, pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)[1,4]. They can phagocytose these antigens and activate further macrophages by secretion of cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF) α or granulocyte macrophage colony-stimulating factor (GM-CSF). Furthermore, macrophages can release chemokines, attracting immune cells to the side of inflammation. In addition, CCL2, secreted by different tissue cells, e. g. hepatic myofibroblasts[5] or astrocytes in the CNS[6], attracts monocytes, macrophages and microglia.

The importance of the innate immune system is to keep the infection in check for the first 4 to 7 days, until the adaptive immune response begins[1].
During the first days of infection, the major functions of the innate immune system are:

- Identification of pathogens,
- Recruitment of further immune cells to the site of infection by secretion of proteins of the complement system and chemokines [7],
- Activation of the adaptive immune system by cytokines.

1.1.2 The adaptive immune system

Beside tasks, regarding the innate immune system like antigen recognition and immune cell recruitment, macrophages and microglia also belong to the adaptive immune response as they can activate T cells, which are together with B cells the most important cells of the adaptive immune response. Both, T cells and B cells derive from a common lymphatic progenitor cell in the bone marrow. While B cells also mature in the bone marrow, differentiation into a T cell proceeds in the thymus[1]. However, in this thesis, T cells, macrophages and microglial cells play a major role, therefore this part focuses on these cells.
T cells possess a specific T cell receptor (TCR). The specificity of the receptor is not encoded in the genome, but each receptor is distinct due to somatic recombination. Therefore, every T cell carries a unique receptor with a distinct antigen-specificity, which results in a massive repertoire of antigen-recognition by the adaptive immune system[1].

As T cells are not able to recognize “free” antigens, antigen recognition by the TCR requires presentation of antigen-derived peptides bound to molecules of the major histocompatibility complexes (MHC), also known as human leukocyte antigen (HLA) in humans. T cells, classified by expressing cluster of differentiation (CD) 8 on their surface, $CD8^+$ T cells, recognize endogenous peptides, which are presented on MHC class I (MHC-I) molecules on a cell-surface[1].

More prominent for the content of this thesis are $CD4^+$ T cells, which recognize peptides presented on MHC-II molecules: Antigen presenting cells (APCs), e. g. DCs, macrophages, or microglia take up exogenous pathogens by phagocytosis or endocytosis and present peptides in the context of MHC-II on their surface. Beside the recognition of antigens by the MHC–TCR-complex, termed signal 1, full activation of naïve T cells necessitates further signals like CD80 and CD86 (signal 2). An important costimulatory molecule required for the activation of macrophages and microglial cells is CD40, which is recognized by CD40L on the T cell surface.

As a third signal T cell activation requires pro-inflammatory cytokines (signal 3)[8].

Upon activation and expansion, $CD4^+$ T cells, also termed as T helper (T_H) cells, differentiate into different T cell subsets, which are characterized by different cytokine profiles and distinct effector functions[1,9]. Thereby, different cytokine profiles trigger certain T cell subpopulations: For example, presence of interferon (IFN) γ and IL-12 trigger differentiation into T_H1 cells. The main purpose of T_H1 cells is to activate macrophages[10]. During the last decade, a new subset of T_H-cells was described[11-13]: The T_H17 cells, which are induced by transforming growth factor (TGF) β together with IL-6. Beside their importance in clearance of extracellular pathogens like *Klebsiella pneumonia* or *Candida albicans*[14,15], T_H17 cells play an important role in the development and perpetuation of different autoimmune diseases like multiple sclerosis (MS) or colitis[9,11,16,17].

1.2 Self-tolerance and Autoimmunity

The adaptive immune response is a system to specifically defend infection. As mentioned before, based on somatic recombination, various T cells carry their specific TCR on the surface. There is no doubt that T cells recognizing self-antigens develop. Different mechanisms, termed central and peripheral tolerance, overcome the development of autoimmunity: central tolerance take place during the development of T cells in the

thymus. The selection of T cells is controlled by the binding strength of the TCR and the MHC-peptide-complex: If an immature T cell receives a too weak signal, the T cell undergoes apoptosis (positive selection). Within the thymus, self-antigens, derived from the thymus epithelium or the bloodstream, are presented by macrophages and DCs in association with MHC to maturing T cells. If a T cell recognizes a self-antigen by its TCR, apoptosis is induced (negative selection)[18].

Although many auto-reactive T cells and B cells are eliminated by central tolerance, some auto-reactive lymphocytes are present in the periphery. Peripheral tolerance suppresses activation of these T cells by different mechanisms: Self-antigens that are presented in immune privileged areas like the CNS or the eye in a low concentration do not induce activation of auto-reactive T cells (ignorance)[19]. As mentioned before, beside antigen-recognition (signal 1), activation of T cells requires also signal 2 (costimulatory molecules) and 3 (cytokines). If a T cells obtains only signal 1 and signal 2 and 3 is missing, the cell becomes tolerant towards this antigen (anergy)[20].

Breakdown of immunological tolerance results in the development of autoimmunity. Autoimmune disorders can be systemic like rheumatoid arthritis or systemic lupus erythematosus or organ-specific like type I diabetes or MS.

Despite a wide range of analysis, little is known about the development of autoimmune disorders. However, some mechanisms are thought to play a role in progression of autoimmunity: Two major factors for development of autoimmunity are environmental factors and the genetics. Different twin studies revealed a strong involvement of certain genes in the development of autoimmune diseases like type I diabetes[21]. The most prominent protein for genetic disposition to autoimmune disease is the MHC-protein. Several autoimmune diseases have been associated with certain genetic alleles of the human HLA[1].

Furthermore, analysis of different patient populations revealed an important role for environmental factors for the development of autoimmune disorders [23 24].

There are different hypothesis for the development of autoimmune diseases, like infection of APCs that modifies their behavior as antigen-presenting cells, binding of a foreign antigen to self-antigen, resulting in immune reactivity against the self-antigens, or a cross reaction of self-antigens with endogenous antigens (molecular mimicry)[1], like dengue fever inducing lupus[25] or Epstein barr virus, which is associated with MS[26].

Immune responses against self-antigens result in inflammation, and, as a consequence, in severe tissue damage of the particular organ.

1.2.1 Autoimmunity within the central nervous system

The CNS has long been considered to be a non-immunological system[27]. However, studies of systemic inflammation and tissue damage changed this view[28]. MS is one of the most common chronic and inflammatory disorders of the CNS.

1.2.1.1 Multiple Sclerosis

MS is a common neurological autoimmune disease, that typically starts during early adulthood with a high prevalence in Caucasians[29,30]. Its pathology is characterized by inflammation, demyelination, and axonal damage within the CNS[31,32]. Approximately 85% of MS patients start with a relapsing-remitting course, which is characterized by unpredictable recurring relapses, intermediated by a remission, with no signs of disease activity. While the number of clinical relapses decreases over the time, most patients develop progressive neurological deficits that are independent of relapses. In contrast to the relapsing-remitting course, approximately 15% of MS patients do not have acute relapses, but a slowly progressive disease (primary progressive MS)[27,33].

Although the etiology of MS is still unclear, many studies indicate that environmental factors play a role in MS development[34,35]. For example, MS relapses are often associated with viral infections[26], or migration from high- to low-risk areas before adolescence reduces the risk of MS development[34]. One critical factor associated with risk of MS development is a certain gene for the HLA class II, which is associated with MS and plays a role in clinical manifestation as well as influences the disease course of MS[36].

Neuropathologically, MS is characterized by sclerotic lesions within the CNS, which are characterized by demyelination, leading to neurological dysfunction. The current concept about the cascade of pathophysiologic events leading to MS pathology consists of an initial activation of myelin-specific $CD4^+$ T cells in the periphery by professional APCs presenting self-antigens or antigens, which are cross-reactive to self-antigens[37,38]. Especially the T_H1 and T_H17 subsets of $CD4^+$ T cells have been implicated in disease mediation [31,39-41].

These activated T cells proliferate and migrate into the CNS across the blood brain barrier (BBB). It has recently been shown that especially during disease initiation, this migration step requires the chemokine receptor CCR6 and its ligand CCL20, which is highly expressed in epithelial cells of the choroid plexus[42].

Within the CNS, T cells recognize myelin peptides in association to the MHC-II-complex on the surface of local APCs, which consist of perivascular macrophages and microglial cells[43]. This local reactivation of auto-reactive T cells and their interaction with local immune cells results in an inflammatory response within the CNS with release of pro-inflammatory cytokines and chemoattractant proteins[29,44-47]. This causes an attraction of further immune cells, such as T and B cells and macrophages, which infiltrate the site of inflammation and contribute to maintenance of inflammation by further release of pro-inflammatory mediators and neurotoxic mediators, ultimately resulting in destruction of the myelin sheath.

Current treatment options for MS therapy consist of IFNβ, glatiramer acetate, and natalizumab. IFNβ reduces relapse-frequency and severity in relapsing remitting MS[48]. Its immunomodulatory activities are broad and encompass reduction of T cell activation and proliferation as well as a reduction in BBB permeability. Glatiramer acetate is a synthetic mixture of amino acids resembling the composition of myelin basic protein (MBP), a major self antigen of the CNS[49]. Treatment of MS patients results in shift from T_H1 cells to T_H2 cells thereby suppressing T cell-mediated inflammatory responses[50]. Another promising therapeutic agent is natalizumab, a monoclonal antibody against very late antigen-4, expressed on activated T cells. Blocking of very late antigen-4 very efficiently inhibits T cell migration through the BBB and hence invasion of auto-reactive T cells into the target organ[51].

1.2.1.2 Experimental autoimmune encephalomyelitis

In order to investigate the mechanism responsible for development of MS, Rivers et al. established a model for MS in 1933[52]: the EAE, which is commonly used for experimental MS research.

EAE is an inflammatory disease of the CNS, which is induced by injection of proteins derived from myelin, like myelin oligodendrocyte protein (MOG), MBP, or proteolipid protein (PLP), into susceptible animals, such as mice, together with Freund's adjuvant as an immunopotentiator. In addition, pertussis toxin is injected, which leads to permeabilization of the BBB.

The disease is characterized by perivascular infiltrates, leading to neurological symptoms like paralysis or ataxia, beginning approximately one week after immunization. The cells, which predominantly initiate the disease, are $CD4^+$ T cells, as transfer of MOG-reactive

$CD4^+$ T cells isolated from EAE-diseased animals results in appearance of disease in healthy recipient mice[53].

It is known, that T cells of T_H1 phenotype mediate CNS inflammation during EAE[54]. However, during the last years several studies identified T_H17 cells to be involved in the induction of CNS-autoimmunity like EAE and MS[39,55,56]. The chemokine CCL20, expressed by epithelial cells of the choroid plexus, and its receptor CCR6, which is predominantly found on T_H17 cells, are involved in T cell migration into the CNS[42,57]. It was shown that T cells migrate through the BBB into the CNS in two steps: A first wave of T_H17 cells migrate into the un-inflamed CNS in a CCR6-dependent manner. In a second wave, T_H17 and T_H1 cells migrate in a CCR6-independent way[42].

Within the CNS, these invading T cells secrete inflammatory mediators, like IFNγ and GM-CSF, which activates microglia and macrophages[47,58] and lead to secretion of further cytokines and chemokines in the inflammed tissue[6,59]. This results in an inflammatory milieu within the CNS, recruiting and activating further immune cells like T cells, macrophages, and microglia into the CNS[60,61]. Furthermore, macrophages and microglia release neurotoxic mediators, like TNFα and nitric oxide (NO), leading to destruction of myelin sheath and astrocytes[45,62-64].

EAE is generally divided into an initial priming phase, that is mediated by auto-reactive T cells followed by the effector phase, where macrophages and microglia get activated by invading T cells, inducing expression of neurotoxic mediators, which leads to demyelination and neurodegeneration[45,62,63].

1.2.1.3 The role of microglia and macrophages for the development of MS and EAE

Microglial cells are resident macrophages of the CNS, scanning the tissue persistently and showing changes in morphology upon activation[2]. They exhibit macrophage-like functions, i.e. phagocytosis, antigen presentation, production of reactive oxygen species (ROS), NO, and pro-inflammatory cytokines[64,65].

MS and EAE have been considered to be mediated by T cells as they crucially amplify CNS autoimmunity[29,66]. As mentioned before, T cells have been proven to initiate the disease. However, effector mechanisms leading to inflammation, demyelination and neuronal damage are thought to be mainly provided by other cells like infiltrating macrophages and microglia[67]. In the last years, these cells have been identified as main effectors of disease progression[47,68] as they both play a crucial role for T cell reactivation

within the CNS[69,70] and directly contribute to demyelination and axonal damage by secretion of pro-inflammatory mediators such as ROS, NO and TNFα[63,64].

1.3 Peroxisome proliferator-activated receptor γ

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear transcription receptor that belongs to the superfamily of nuclear hormone receptors. It is ligand-activated and, upon ligand binding, PPARγ forms a heterodimer with the retinoic X receptor. This complex activates transcription by binding to a specific deoxyribonucleic acid (DNA) element, termed the PPAR response element, in the promoter region of target genes, thus influencing gene transcription[71-73]. In addition, anti-inflammatory effects of PPARγ are mediated by interfering with pro-inflammatory transcription factors, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or the nuclear factor of activated T cells (NFAT)[74,75], which results in modulation of immune responses, cellular proliferation, and differentiation. So far, anti-inflammatory effects of PPARγ on immune responses have been demonstrated for dendritic cells[76-78], lymphocytes [77,79], neuronal cells and astrocytes[80], monocytes / macrophages[72,75,81,82], and microglia[83].
Naturally occurring endogenous agonists for PPARγ are polyunsaturated fatty acids, arachidonic acid metabolites (15-Deoxy-Delta-12,14-prostaglandin J2 (15d-PGJ2))[84], 15-hydroxyeicosatetraenoic acid (15-HETE)[85], or fatty acid-derived components like 9- and 13- hydroxy octadecadienoic acid (HODE)[86]. Beside these endogenous ligands, synthetic ligands like Pioglitazone (Pio) and Rosiglitazone were approved drugs for the therapy of diabetes type II in Germany since 2007 and 1999, respectively. Due to several adverse reactions like an increased risk for bladder cancer, cardiovascular diseases and liver diseases[87], competent authorities suggest to give these medication only with high caution since 2011. However, the anti-inflammatory effect of PPARγ-activation has been proven and can be used for treatment of several inflammatory diseases.

1.3.1 PPARγ and multiple sclerosis

Several EAE-studies suggest that PPARγ is a promising immunomodulatory and therapeutic target for treatment of MS, as systemic activation of PPARγ by application of synthetic or endogenous ligands has been shown to reduce the incidence and severity of

disease, local CNS-inflammation, as well as demyelination and axonal damage during EAE pathology[55,88-90].

Our group has previously shown that activation of PPARγ in DCs decreases $CD4^+$ T cell response *in vitro*. Furthermore, knockout of PPARγ in DCs resulted in an enhanced $CD4^+$ T cell response. In *in vivo* experiments it was shown, that PPARγ-activated DCs, transferred into mice, induce prolonged T cell anergy upon antigen-specific interaction with $CD4^+$ T cells [77].

Furthermore, PPARγ in $CD4^+$ T cells also plays a central role for the development of EAE, as activation of PPARγ selectively interferes with T_H17 differentiation[55] and results in reduced auto-reactive T_H17 responses in the EAE model[89].

Interestingly, Klotz et al. showed reduced levels of PPARγ in peripheral blood mononuclear cells (PBMCs) of MS patients, compared to healthy controls[91]. Moreover, activation of PPARγ in PBMCs derived from MS patients reduced inflammatory responses upon stimulation [91,92], identifying PPARγ in MS patients as promising target for regulation of inflammation.

Therefore, PPARγ seems to be an auspicious target for the reduction of immune responses during MS.

1.4 Importance of the CCL2 / CCR2-axis to reinforce EAE pathology

CCL2 and its receptor CCR2 have been shown to play an important role during CNS autoimmunity, as CCR2-deficient mice are resistant to EAE[93-95] and administration of CCL2-antibodies ameliorates established EAE pathology[96]. Geissmann et al. identified two different monocyte populations in both, humans and mice. In mice they can be distinguished based on their CCR2 expression[97]: The "inflammatory monocytes" are $CCR2^+Ly\text{-}6C^{hi}CX3CR1^{lo}$, and the "resident monocytes" are $CCR2^-Ly\text{-}6C^{lo}CX3CR1^{hi}$.

Inflammatory monocytes play a crucial role for the inflammation within the CNS[98-100] and especially during EAE[99,100]. EAE experiments with CCR2 knockout mice as well as specific cell depletion of $CCR2^+$ peripheral monocytes during EAE showed a reduction of the severity of disease[100]. Upon CNS-inflammation astrocytes have been proven to secrete CCL2, which can attract inflammatory monocytes into the CNS[6].

Interestingly, macrophages can switch from a regulatory phenotype to an inflammatory phenotype and back, upon specific signals[101] and several data sets indicate that PPARγ activation skews monocytes towards a regulatory phenotype in mice and in humans[102-104]. However, the role of PPARγ in myeloid cells on the inflammation is not yet fully understood and needs to be elucidated. Therefore, this thesis focuses on the role of PPARγ in myeloid cells on the inflammation during EAE.

2 Aim of the thesis

MS is the most common neuroinflammatory disease in North America and Europe. Because of its expeditious etiopathology and the severity of disease, it is important to further investigate the disease and develop new therapeutic approaches to alleviate the gravity of disease. A common model for MS research is the animal model EAE.
Several EAE-studies focused on encephalitogenic T cells, which play a crucial role in the pathogenesis of EAE. However, recently different studies identified a prominent role for myeloid cells such as monocytes, macrophages, and microglia for the progression of EAE. Since PPARγ has been demonstrated to modulate pro-inflammatory responses by different myeloid cell populations *in vitro*, this work deals with the question whether PPARγ plays a role in myeloid cells during disease initiation and progression of EAE.

In particular, the following questions were addressed:

1) Does lack of PPARγ in myeloid cells influence the disease course during EAE?

2) Does cell-type specific knock-out of PPARγ alter immune responses of macrophages and microglia *in vitro* and *in vivo*?

3) What is the role of PPARγ in $CCR2^{+}Ly\text{-}6C^{hi}CX3CR1^{lo}$ inflammatory monocytes during the pathogenesis of EAE?

4) Which myeloid cells are the main targets for PPARγ activation *in vivo*?

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

Autoclave		Belimed, Köln
Balances		Sartorius, Göttingen
Beakers (50 ml, 100 ml, 150 ml, 250 ml, 500 ml)		Schott, Mainz
Cell Separator, Auto MACS		Miltenyi Biotec, Bergisch Gladbach
Centrifuges (Multifuge3 S-R, Biofuge fresco, Biofuge pico)		Heraeus, Hanau
Counting Chamber, Neubauer improved		Brand, Wertheim
Disperser, Ultraturrax		IKA Werke, Staufen
ELISA-Reader, SpectraMAX 250		Molecular devices, Ismaning
Flow cytometer, FACS CantoII		BD Biosciences, Heidelberg
Heating block,TheromStat plus		Eppendorf, Hamburg
Incubators, HERAcell		Heraeus, Hanau
Injectionpump WPI SP 2101WZ		WPI, Aston, UK
Magnet stirrer, IKAMAG		IKA Werke, Staufen
Measuring cylinders (50 ml, 100 ml, 250 ml 500ml)		Schott, Mainz
pH-meter		WTW, Weilheim
Pipettes	Pipetboy	IBS, Fernwald
	Pipetman	Gilson, Heidelberg
	Reference	Eppendorf, Hamburg
Preparation Instruments		Labotec, Göttingen
Sieves, steel		University Bonn, Department ″Feinmechanik″
Sonicator, Sonopuls		Bandelin Electronic, Berlin
Spectrophotometer, Nanodrop ND 1000		Thermo Scientific, Wilmington, USA

Thermocycler	Biometra, Göttingen
Thermocycler, real-time PCR, ABI Prism 7900HT	Applied Biosystems, Darmstadt
Threaded bottles (100 ml, 250 ml, 500 ml, 1 l, 2 l)	Schott, Mainz
Ultrapure Water System, NANOpure Diamont	Barnstead Thermolyne, Dubuque, USA
Vortexer	VWR International, Darmstadt
Waterbath, TW8	Julabo, Seelbach
Workbench, sterile, HERAsafe	Heraeus, Hanau

3.1.2 Software

EndNote X3	Thomson ISI Research Soft, USA
FACS Diva V6.1.1	BD Biosciences, Heidelberg
FlowJo V8.8.6	Treestar, Inc., USA
Illustrator 10.0.3	Adobe, USA
Microsoft Office 2008	Microsoft, USA
ND-1000 V3.5.1	NanoDrop Technologies, Wilmigton, USA
Prism 4 for Macintosh	GraphPad Software, USA
SDS 2.2	Applied Biosytems, Darmstadt
SOFTmax pro 2.6.	Molecular devices, Ismaning

3.1.3 Consumables

Cryo vials	VWR International, Darmstadt
FACS tubes, polystyrene, 12 x 75mm	Sarstedt, Nümbrecht
Filter, sterile (0.2 µm, 0.45 µm)	Whatman, Dassel
Injection needles (27G, 26G, 20G)	Braun, Melsungen and BD Bioscience, Heidelberg
Gloves, DermaClean	Ansell, Red Bank, New Jersey, USA
Microtiter plates, 96-well round and flat bottom	Greiner bio-one, Solingen
Microtiter plates, 96-well, transparent	Greiner bio-one, Solingen
Microtiter plates, 384-well, MicroAmp	Applied Biosystems, Darmstadt
Optical adhesive film, MicroAmp	Applied Biosystems, Darmstadt

Parafilm	Pechiney Plastic Packaging, Chicago
Pasteur pipettes (150 mm, 230 mm)	Roth, Karlsuhe
Pertussis toxin, from *Bordetella pertussis*	Sigma-Aldrich, München
Petri dishes, 100 mm, 150 mm	Greiner bio-one, Solingen
Pipette tips, (10 µl, 20 µl, 200 µl, 1000 µl)	Greiner bio-one, Solingen
Pipette tips, nuclease free (10µl, 20 µl, 200 µl, 1000 µl)	Nerbeplus, Winsen / Luhe
Pipettes, glass, (5 ml, 10 ml, 25 ml)	Brand, Wertheim
Pipettes, plastic, (5 ml, 10 ml, 25 ml)	Sarstedt, Nümbrecht
Polyamide tissue, Gaze	Labomedic, Bonn
Polypropylene tubes (15 ml, 50 ml)	Greiner bio-one, Solingen and Sarstedt, Nümbrecht
Reaction tubes (0.2 ml)	Nerbeplus, Winsen / Luhe
Reaction tubes (0.5 ml, 1 ml, 1.5 ml, 2 ml)	Sarstedt, Nümbrecht
Scalpel	Feather, Osaka, Japan
Syringes (1 ml, 2 ml, 5 ml, 10 ml, 20 ml)	BD Bioscience, Heidelberg
Tissue Culture Plates (6-, 12-, 24-, 48-, 96-well)	TPP, St. Louis USA and Sarstedt, Nümbrecht
Tissue culture plates, 100 x 20 mm	Sarstedt, Nümbrecht
Tissue culture flasks, 75 cm^2	Sarstedt, Nümbrecht

3.1.4 Chemicals, reagents and Kits

Bovine serum albumin (BSA)	Roth, Karlsruhe
1-Bromo-3-Chloropropane (BCP)	Sigma-Aldrich, München
Complete Freund's adjuvant (CFA)	Difco, Detroit, USA
Counting beads, CountBright Absolute Counting Beads	Invitrogen, Frankfurt
Collagenase	Roche, Basel, Switzerland
Dimethylsuloxide (DMSO)	Merck, Darmstadt
Ethanol, absolut	Merck, Darmstadt
Ethanol, absolut for nucelase	Aplichem, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, München
Fetal calf serum (FCS)	PAA, Cölbe
Fixation / Permeabilization Kit, BD Cytofix / Cytoperm	BD Bioscience, Heidelberg
Gey's balanced salt solution (GBSS)	Sigma-Aldrich, München

Heparin	Ratiopharm, Ulm
Inhibitors, Monensin and Brefeldin A	eBioscience, Frankfurt
Isopropyl alcohol	Aplichem, Darmstadt
L-Glutamine	Lonza, Köln
Lipopolysaccharide (LPS) from *Echerichia coli* O127:B8	Sigma-Aldrich, München
Medium, DMEM	Invitrogen, Darmstadt
Medium, RPMI 1640	Invitrogen, Darmstadt
Mycobakterium tuberculosis H37 RA, dessicated	Difco, Detroit, USA
non-essential amino acids	PAA, Pasching, Austria
Nuclease-free water	Applied Biosystems, Darmstadt
Paraformaldehyde (PFA)	Fluca, Buchs
Penicillin / Streptomycin	PAA, Pasching, Austria
Pioglitazone (for cell culture)	Alexis Biochemicals, Lörrach
Percoll	GE Healthcare, Freiburg
Phenol-Chloroforme	Roth, Karlsruhe
Phorbol myristate acetate (PMA)	Sigma-Aldrich, München
Phosphat buffered saline (PBS)	Biochrom, Berlin
Phosphat buffered saline (PBS), solution	Sigma-Aldrich, München
Poly-L-lysine hydrobromide	Sigma-Aldrich, München
RNAlater	Qiagen, Hilden
RNeasy-Kit	Qiagen, Hilden
Sodium azide (NaN_3)	Sigma-Aldrich, München
Sodium bicarbonat ($NaHCO_3$)	Sigma-Aldrich, München
SuperScript III	Invitrogen, Frankfurt
TaqMan Gene Expression Master Mix	Applied Biosystems, Darmstadt
TaqMan Power SYBR Green Master Mix	Applied Biosystems, Darmstadt
Tibromoethanol (Avertin)	Sigma-Aldrich, München
Trypane blue (0.4%)	Lonza, Köln
Trysin EDTA	Invitrogen, Frankfurt
TriReagent	Invitrogen, Frankfurt
Triton X-100	Sigma-Aldrich, München
Tween20	AppliChem, Darmstadt

3.1.5 Primer and Probes

For quantitative real-time RT-PCR the following primers were used:

Target	Orientation	Sequence
IFNγ	forward	5′-GAACTGGCAAAAGGATGGTGA-3′
	reverse	5′-TGTGGGTTGTTGACCTCAAAC-3′
IL-1β	forward	5′-GCCCATCCTCTGTGACTCAT-3′
	reverse	5′-AGGCCACAGGTATTTTGTCG-3′
IL-6	forward	5′-AAGTCGGAGGCTTAATTACACATGT-3′
	reverse	5′-CCATTGCACAACTCTTTTCTCATT-3′
TNFα	forward	5′-CATCTTCTCAAAATTCGAGTGACAA-3′
	reverse	5′-TGGGAGTAGACAAGGTACAACCC-3′

Quanti Tect Primer assays for analysis of iNOS and IL-12 were purchased from Qiagen. Examination of the chemokines CCL2, CCL3, CCL5, and CXCL10 as well as internal control of GAPDH were made with FAM-labeled TaqMan Probes.

To analyze PPARγ-deletion in cells, following primers were used:

Target	Orientation	Sequence
PPARγ	forward	5′-TATCACTGGAGATCTCCGCCAACAGC-3′
	reverse	5′-GTCACGTTCTGACAGGACTGTGTGAC-3′

3.1.6 Buffers and solutions

ACK-Lysis Buffer

ACK-Lysis Buffer for lysing of red blood cells, 16.58 g NH_4CL, 2 g $KHCO_3$, and 74.4 mg Na_2EDTA were dissolved in 2000 ml H_2O and adjusted to pH 7.2-7.4. ACK-Lysis Buffer was stored at room temperature.

Fetal calf serum (FCS)

FCS was heat-inactivated at 56°C for 30 min and stored at –20°C at 50 ml aliquot.

0.5 mM EDTA

For a solution of 0.5 mM, 186.1 g EDTA and 20 g NaOH were dissolved in 1000 ml ultra-pure water and pH was adjusted to 7.8 - 8.0. EDTA was stored at room temperature after autoclavation.

Fluorescence-activated cell sorting (FACS)-buffer

For flow cytometric analysis, cells were stained, washed, and stored in FACS-buffer, which is 1 x PBS with 1% (v/v) FCS and 0.1% (v/v) NaN_3. FACS-buffer was stored at +4°C.

Brain perfusion buffer

Before isolation of brain and spinal cord, mice were perfused with GBSS with 2% heparin.

4% (w/v) PFA solution

8 g PFA was dissolved in 200 ml 1 x OBS by gradual heating. The pH was adjusted to 7.4 and aliquots were stored at –20°C.

Phosphat buffered saline (PBS)

1 x PBS was adjusted to pH 7.4, aliquoted à 500 ml, autoclaved, and stored at +4°C.

Buffers for Enzyme-linked Immunosorbent assay (ELISA)

Coating buffer (1 M $NaHCO_3$)

0.84 g $NaHCO_3$ were dissolved in 100 ml A. bidest. The pH was adjusted to 8.2.

Blocking buffer (1 % BSA)

0.1 g BSA was dissolved in 100 ml PBS by agitation.

Washing buffer (0.05 % Tween)

100 µl Tween20 were added to 2 l PBS.

3.1.7 Media

Medium for bone marrow macrophages (BM-M)

DMEM (high glucose)
10% (v/v) FCS
10^5 U Penicillin / 0.1mg/ml Streptomycin
50µM β-Mercaptoethanol (β-ME)
2mM L-Glutamin
30% (v/v) supernatants from M-CSF transduced L929 cell-line

Medium for primary microglia (pMg)

DMEM (high glucose)
10% (v/v) FCS
1% (v/v) non-essential amino acids
10^5 U Penicillin / 0.1mg/ml Streptomycin
50µM β-ME
2mM L-Glutamin

Media for T cells

RPMI 1640
10% (v/v) FCS
10^5 U Penicillin / 0.1mg/ml Streptomycin
50µM β-ME
2mM L-Glutamin

3.1.8 Peptides and Proteins

MOG_{35-55}	Biotrend, Köln
MOG_{35-55}	Pineda, Berlin
recombinant mouse IL-12	R&D Systems, Minneapolis, USA
recombinant mouse IFN γ	R&D Systems, Minneapolis, USA

3.1.9 Antibodies

Fluorochrome conjugated antibodies for flow cytometry

The following antibodys were used for flow cytometric analysis of extracellular surface molecules or for intracellular cyokine staining. Antibodies, labeled with Phycoerythrin (PE), Pacific blue, Allophycocyanin (APC), Fluoresceinisothiocyanat (FITC), Biotin, or tandem conjugate Peridinin Chlorophyll Protein with a cyanin dye (PerCP-Cy5.5), were used at previously determined concentrations.

Antigen	Isotype	Clone	Conjugate	Company
CD4	Rat IgG2b, κ	GK 1.5	PE	eBioscience
			Pacific blue	eBioscience
CD8a	Rat IgG2a, κ	53-6.7	PE	BD Bioscience
CD11b	Rat IgG2b, κ	M1/70	APC	eBioscience
CD115	Rat IgG2a, κ	AFS 98	Biotin	eBioscience
CD40	Rat IgG2a, κ	1C10	PE	eBioscience
CD45	Rat IgG2b, κ	30-F11	Pacific blue	eBioscience
			FITC	eBioscience
IFNγ	Rat IgG1, κ	MG1.2	PE	eBioscience
IL-17A	Rat IgG2a, κ	eBio17B7	APC	eBioscience
			PE	eBioscience
Ly-6C	Rat IgG2c	HK1.4	PerCP-Cy5.5	eBioscience
Ly-6G	Rat IgG2a, κ	1A8	APC	BD Bioscience
			FITC	BD Bioscience
MHC-II	Mouse IgG2a, κ	AF6-120.1	PE	BD Bioscience
TNFα	Rat IgG1, κ	MP6-XT22	PE	eBioscience

Blocking Fc fragments to avoid unspecific signals, Fc Receptor blocker 2.4G2 was used.

Antibodies for ELISA

Primary and secondary antibodies for TNFα ELISA as well as the ELISA-Kit CCL2-ELISA Ready-SET-Go were purchased from eBioscience.

Antibodies for immunohistochemistry

Immunohistochemical analysis were kindly performed by the laboratory of Prof. M. Prinz, Freiburg. Following antibodies were used:

Antigen	Clone	Company
amyloid precursor protein (APP)	polyclonal goat	Chemicon, Temecula, USA
CD3	CD3-12	BD Pharmingen, Germany
MAC-3	M3/84	BD Biosciences, Heidelberg

3.1.10 Antibody-coated beads for Magnetic Cell Separation

For magnetic-activated cell sorting (MACS) following antibody-coated beads from Miltenyi Biotec, Bergisch Gladbach were used:
anti-CD4, anti-CD-8, anti-CD11b, anti-CD11c, anti-CD19, and anti-Biotin.

3.1.11 Mouse strains

All mice were bred and maintained under specific pathogen-free conditions at the "Haus für Experimentelle Therapie" or at the "Instituts of Molecular Medicine and Experimetal Immunology" at the University Hospital Bonn.

Strain	Description
C57BL/6	Mice bear MHC-I haplotype H-2^b
PPAR$\gamma^{fl/fl}$	Mice with two *loxP* sequences flanking exon 1 and 2 of the PPARγ gene.[105]
LysMCre	Mice, expressing a Cre-recombinase under the control of the lysosyme M promotor.[106]
NestinCre	Mice, expressing a Cre-recombinase under the control of the nestin promotor and enhancer.[107]

All mice were with C57BL/6 background.
Crossing LysMCre or NestinCre mice with PPAR$\gamma^{fl/fl}$ mice results in specific ablation of PPARγ in cells of myeloid origin (LysM-PPARγ^{KO} mice) (e. g. microglia, monocytes,

macrophages and neutrophil granulocytes) or neuronal and glia cell precursors (Nestin-PPARγ^{KO} mice) (e. g. astrocytes, neurons and oligodendrocytes).
LysM-PPARγ^{KO} and Nestin-PPARγ^{KO} mice were born at the expected Mendelian ratio, were viable and fertile and developed normally.
For Experiments, mice aged between 6 and 13 weeks were used.

3.2 Methods

3.2.1 Cell culture

All cells were cultured in an incubator with a temperature of 37°C, a relative humidity of 90%, and 5% CO_2.

3.2.1.1 Production of M-CSF for BM-M differentiation

1.5 x 10^6 M-CSF transduced L929 cell were cultured in 30 ml BM-M medium for 3-4 days in 150 mm cell culture dishes. Then, supernatants were centrifuged at 1500 rpm for 10 min and afterwards filtered with 0.2 μm cellulose acetate membrane bottle top filters for sterilization. Supernatants were stored at -20°C.

3.2.1.2 Isolation and culture of primary murine cells

Preparation of BM-Ms
Bone marrow was isolated from tibiae and femur. Therefore, mice were sacrificed, hind limps were cleaned with 70% (v/v) ethanol and tissue was relieved from tibiae and femur. Under sterile conditions, bones were flushed with 10 ml sterile PBS to isolate bone marrow. To get rid of small pieces of bone, cell suspension was filtered through a nylon sieve and afterwards centrifuged by 1500 rpm for 10 min. Cells from one mouse were split on three 10 cm Petri dishes in 10 ml BM-M medium, and cultured at 37 °C and 5 % CO_2. Medium was chanced after four days. On day 7, cells were detached with 5 mM EDTA for 10 min at 37 °C.

Preparation of mixed glial cultures

Mixed glial cultures were prepared from postnatal mice on day 3-5. Therefore, mice were sacrificed and cleaned with 70% (v/v) ethanol. The cerebrum was isolated by carefully open the cranium. Then, meninges were removed with a tweezers. Then, the cerebrum was mechanically dissociated by use of a 5 ml pipette. Cell suspension was incubated for 5 min, and to get rid of non dissociated particles, the supernatant was collected and centrifuged at 1500 rpm for 5 min. Then, supernatant was discharged and cells were seeded into 0.01% Poly-L-lysine pre-covered 75 cm^2 cell-culture flasks. Medium was refreshed every week.

Isolation of pMg

Two weeks after preparation, pMg were harvested from mixed glial cultures by tapping the flasks vigorously. Afterwards, medium was refreshed. Cells were centrifuges at 1500 rpm for 10 min and seeded into 48-well plates for stimulation or for coculture.
Cells from mixed glial cultures could be harvested again after a minimum of one week.

Isolation of astrocytes

Astrocytes were isolated from mixed glial cultures. Therefore, microglial cells were harvested as indicated above. By microscope, culture was checked for microglial cells. If the number of microglial cells compared to astrocytic cells is less than 5%, astrocytes were isolated from 75 cm^2 cell-culture flasks by incubation with 10 ml Trypsin EDTA for 10 min at 37°C. Trypsin EDTA was removed by centrifugation at 1500 rpm for 10 min.

Isolation of T cells

Mice were sacrificed by asphyxiation, cleaned with 70% (v/v) ethanol and spleens were isolated. Organs were dissociated by punching through a steel sieve. Afterwards, cells were centrifuged at 15000 rpm for 10 min. To gain a single-cell suspension, cells were filtered through a nylon sieve and centrifuged at 15000 rpm for 10 min. Then, cells were incubated with 20 µl CD4 MACS-beads (per spleen) in 500µl T cell medium (per spleen) for 15 min at 4 °C. Then, non-bonded beads were washed by centrifugation at 15000 rpm for 10 min. $CD4^+$ T cells were separated from other cells, by immuno magnetic cell separation using auto-MACS. To get rid of the MACS-buffer, cells were washed, counted and 1 x 10^6 cells were seeded into a 48 well-plate in 1 ml T cell medium. To activate T cells, wells were pre-coatet with 4 µg/ml αCD3 and 4 µg/ml αCD28 in PBS for 1h at 37°C.

After three days, cells were harvested washed with medium, counted and cocultured with macrophages or microglia.

Isolation of peritoneal cells

Mice were sacrificed by asphyxiation and the body surface was cleaned with 70% (v/v) ethanol. Then, skin was removed from peritoneum and 8 ml ice cold PBS was injected into the abdomen with a 20 G needle and reabsorbed.

3.2.1.3 *In vitro* stimulation of myeloid cells

For *in vitro* stimulation, pMg were harvested and seeded into a 48 or 96 well-plate. Indicated concentrations of LPS and IFNγ were added for depicted hours. Then, supernatants were collected for quantification of NO by Griess assay and ROS by addition of H_2DCFA. For intracellular quantification of TNFα by FACS or mRNA isolation for quantification of IL-6 and IL-12 mRNA levels by quantitative real-time RT-PCR, cells were harvested by incubation of 5mM EDTA for 10 min at 37°C.

BM-Ms were stimulated in 48 or 96 well-plates with indicated concentrations of LPS and IFNγ for depicted hours. Supernatants were collected for NO determination by Griess assay and analysis of TNFα by Sandwich ELISA. For quantification of IL-6, IL-12 and CCL2, cells were harvested, mRNA was isolated and levels were analysed by quantitative real-time RT-PCR.

3.2.1.4 Coculture experiments

For coculture of astrocytes with BM-Ms or pMg, 8 x 10^5 astrocytes were cocultured with 8 x 10^5 BM-Ms or pMg in 1 ml pMg medium in a 24 well-plate with or without 1 μg/ml LPS and 10 ng/ml IFNγ. If indicated, BM-M or pMg were pre-stimulated with 1 μg/ml LPS and 10 ng/ml IFNγ for 1 h. Afterwards, stimuli were washed out by centrifugation and cells were added to astrocytes.

3.2.1.5 Determination of cell number by Neubauer counting chamber

The cell suspension was diluted in 0.04 % trypane blue to discriminate between live and

dead cells. 10 µl of this suspension was applied to a Neubauer counting chamber and living cells were counted in all four large squares. The cell count was calculated using the following formula:
Cell number / µl = counted viable cells / 4 (squares) x dilution x 10^4

3.2.2 EAE experiments

For EAE experiments, 6-8 week old female mice were immunized. Disease progress was analyzed daily.

3.2.2.1 Immunization of EAE

For active EAE immunization, MOG-CFA-mix was prepared by sonificating 50 µg MOG_{35-55} peptide and CFA including mycobacteria (ratio 1:1). 100 µl MOG-CFA-mix were injected into the base of tail. As the fluid is very viscous, this was done with a 20 G needle. On day 0 and day 2 after immunization, 200 ng PTX (in 200 µl) were injected i.p..

3.2.2.2 Adoptive transfer EAE

For adoptive transfer EAE, T cells were primed in $PPAR\gamma^{fl/fl}$ donor mice, isolated and injected into LysM-$PPAR\gamma^{WT}$ or LysM-$PPAR\gamma^{KO}$ recipients. Therefore, donors were immunized with 50 µg MOG_{35-55} peptide and CFA s.c. in the left flank (nearby the spleen). Donors did not receive PTX injections. After 10 days, spleens were isolated. Under sterile conditions, spleens were dissociated by a steel sieve. To get a single-cell-suspension, cells were pass through a nylon filter. Then, cells were centrifuged, and mononuclear cells were counted. 20 ng/ ml MOG_{35-55} peptide was added to 2 x 10^7 cells and seeded into a 6 well-plate at a volume of 4 ml T cell medium.
After 3 days, cells were harvested and washed twice with PBS. Afterwards, cells were resuspended in PBS and counted in a Neubauer counting chamber. 1.6 x 10^7 cells were injected i.p. into recipient mice. On days 0 and 2 after cell injection, mice obtained 200 ng PTX (in 200 µl) by i.p. injection.

3.2.2.3 Analysis of disease progression

The clinical assessment of diseased mice was performed daily using the following scale:

- 0: no paralysis
- 1: limp tail
- 2: ataxia or unilateral hind limb paresis
- 3: severe unilateral or weak bilateral hind limb paresis
- 4: severe bilateral hind limb paresis
- 5: complete bilateral hind limb plegia
- 6: complete bilateral hind limb plegia and partial fore limb paresis
- 7: severe tetraparesis / plegia
- 8: moribund / dead animals.

3.2.2.4 Isolation of CNS-mononuclear cells from EAE-diseased mice

At indicated timepoints, mice were sacrificed by i.p. injection of a lethal dose of the anesthesia Tribromoethanol (Avertin). Then, aorta was dissected and bloodstream was perfused with 50 ml brain perfusion buffer. Afterwards, brain and spinal cord were carefully isolated. Under sterile conditions, organs were fragmented and incubated in 10 ml GBSS + 0.5% (v/v) collagenase at 37°C for 30 min. Then, cells were dissociated through a steel sieve and washed with PBS + 1% (v/v) FCS. Mononuclear cells were isolated from suspension by a percoll gradient. Therefore, cells were suspended in 30% percoll (in PBS) and underlayed with 37 % and 70 % percoll. Gradient was centrifuged at 2000 rpm for 30 min at 4°C (without brake). Both interphases were taken of and cells were washed twice with PBS + 1% (v/v) FCS (1500 rpm; 10 min). For flow cytometric analysis of macrophages and microglia, cells were resuspended in FACS buffer. For examination of T cell activation, cells were resuspended in medium.

3.2.2.5 Isolation of T cells from draining lymph nodes from EAE-diseased mice

Mice were sacrificed and inguinal lymph nodes (LN) were isolated. Under sterile conditions, LN were dissected and washed twice with PBS + 1% (v/v) FCS.

3.2.2.6 Analysis of T cell activation ex vivo

For intracellular analysis of IFNγ and IL-17-production by FACS, T cells were isolated from brain and spinal cord or draining LN of EAE-diseased mice. 4×10^5 cells were seeded into a 96 well-plate (round bottom) for 4 h. For restimulation of T cells, 5 ng/ ml PMA and 200 ng/ ml Ionomycin were added. In order to accumulate cytokines, monensin and brefeldin A (inhibiting cytokine secretion; 1 µl per ml medium) were also added.

3.2.2.7 Immunohistochemistry

The laboratory of Prof. M. Prinz, Freiburg, kindly performed immunohistochemical analysis of the CNS.

For histological staining of the CNS, mice were anesthetized with a lethal dose of Tribromoethanol (Avertin) on day 35 after EAE immunization. The CNS was perfused with 25 ml brain perfusion buffer and afterwards fixed by perfusion with 25 ml 4 % PFA. Then, brain and spinal cord were carefully isolated and stored in 4 % PFA. Immunohistochemistry of the spinal cord was kindly performed by Dr. Ildiko Dunay and Prof. Dr. Marco Prinz (Department of Neuropathology, University of Freiburg, Germany) as described[108]. Degree of demyelination was assessed by staining myelin with luxol fast blue (LFB) followed by incubation in Schiff reagent, staining demyelinated areas in pink. The percentage of demyelinated areas related to total area was quantified using Cell Analysis Software.

For detection of axonal damage, deposits of APP were counted after staining sections for APP. To detect CNS-resident microglia and infiltrating macrophages and T cells, immunohistochemical staining of MAC-3 and CD3 was performed. Five mice per group and three sections per animal were analyzed for quantification of demyelination, APP deposits and immune cell infiltration, which are indicated as numbers per total CNS area per mm^2.

3.2.2.8 Monocyte transfer experiments

Inflammatory monocytes were isolated from the bone marrow of LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice, respectively, using a biotinylated antibody for CD115 and biotin-MACS-beads. Then, 1×10^7 CD115$^+$ PPARγ^{KO} or PPARγ^{WT} monocytes were injected i.v.

into EAE diseased mice. Transfer of monocytes was performed on days 12, 14, 16, and 19 after immunization. To get enough cells, monocytes were isolated from 15 LysM-PPARγ^{KO} and 15 LysM-PPARγ^{WT} mice at each time point.

3.2.2.9 Monocyte depletion during EAE

During EAE, depletion of CCR2^{+} inflammatory monocytes in LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice was performed using the anti-CCR2 antibody MC-21. 200 µl with 20 µg of the antibody were injected i.p. into diseased mice every 24 h on days 13-17 after EAE-immunzation. Depletion of the cells was controlled in the blood by FACS-analysis 8 h after the first injection. For negative control, isotype control of the antibody was injected into control mice.

3.2.3 Magnetic-activated cell sorting (MACS)

Prior to the isolation of cells by MACS-beads separation, spleens for isolation of DCs were perfused with 5 ml GBSS +0.5% (v/v) collagenase and incubated for 20 min at 37°C.
For isolation of macrophages, DCs, T cells, or B cells from the spleen by magnetic cell separation, spleens were minced by using a steel sieve. Afterwards, cell suspension was centrifuged for 10 min at 1500 rpm at 4°C and pellet was re-suspended in 500µl of ice-cold MACS-buffer. Then, antibody coated MACS-beads were added (15µl per spleen for DC separation and 25µl per spleen for separation of macrophages, T cells or B cells). After 15 minutes of incubation at 4°C, 50 ml of ice-cold MACS-buffer were added and the cell suspension was centrifuged at 1500 rpm for 10 min at 4°C. Afterwards, cell suspension was filtered through a nylon sieve and cell separation was performed with the autoMACS™ separator using program 'POSSEL'.

3.2.4 Flow Cytometry

For all flow cytometry examinations, cells were stained in 96 well-plate (round bottom) and measurements were performed with FACS Canto II from BD Bioscience was used.
Antibodies for surface marker or intracellular staining were diluted in buffer as follows:

Antigen	Conjugate	Dilution
CD4	PE	1:100
	Pacific blue	1:200
CD8a	PE	1:100
CD11b	APC	1:100
CD40	PE	1:100
CD45	Pacific blue	1:200
	FITC	1:100
IFNγ	PE	1:100
IL-17A	APC	1:100
	PE	1:100
Ly-6C	PerCP-Cy5.5	1:300
Ly-6G	APC	1:300
	FITC	1:100
MHC-II	PE	1:100
TNFα	PE	1:100

3.2.4.1 Surface marker staining

For surface marker staining, cells were washed with FACS buffer. Cells were resuspended in FACS buffer containing 2.4G2 FcBlock and the appropriate diluted antibody. Cells were incubated for 15 min on ice and protected from light. Then, 100 ml FACS buffer were added and cells were centrifuged for 2 min at 1600 rpm to wash away unconjugated antibodies.

3.2.4.2 Intracellular staining of cytokines

Intracellular cytokine staining was performed by using BD Cytofix/Cytoperm fixation and permeabilisation solutions. In order to accumulate cytokines, monensin and brefeldin A (inhibiting cytokine secretion; 1 µl per ml medium) were added to cells 4 h prior intracellular staining of cytokines. Then, after surface markers staining cells were resuspended and fixed in 100 µl of BD Cytofix/Cytoperm fixation solution for 20 min on ice and protected from light. Cells were washed twice with 100 µl BD Cytofix/Cytoperm

permeabilisation solution (diluted 1:10 in A. bidest) (1600 rpm, 5 min, 4°C). Then, staining was done with 50 µl BD Cytofix/Cytoperm permeabilisation solution containing 2.4G2 FcBlock and the appropriate diluted antibody for 30 min on ice and cells were protected from light. Afterwards, cells were washed twice with 100 µl BD Cytofix/Cytoperm permeabilisation solution (diluted 1:10 in A. bidest) (1600 rpm, 5 min, 4°C) and cells were resuspended in FACS buffer for analysis.

3.2.4.3 Determination of absolute cell number by flow cytometry

Prior to FACS analysis, CountBright Absolute Counting Beads micropsheres were added to cells. Therefore, microspheres were vortexed and approx. 2000 beads were added right before FACS measurement. During measurement, cells as well as beads were counted by the system. Afterwards, absolute cell number per vial was calculated by following formula:

Total cell number = (counted cells / counted beads) x 2000

3.2.4.4 FACS-sorting

For quantitative real-time RT-PCR of microglia and macrophages in the CNS of EAE diseased LysM-PPARγ^{WT} or LysM-PPARγ^{KO} mice, cells were isolated from the CNS at day 21 after immunization as indicated at 3.2.2.4 (Isolation of CNS cells from EAE-diseased mice). Then, surface markers CD11b and CD45 were stained with fluorescent labeled antibodies for 10 min at 4°C and CD11b^{+}CD45^{+} cells were sorted using a BD FACS DiVa.

3.2.5 Evaluation of mRNA-levels

3.2.5.1 Isolation of mRNA from cells suspension

mRNA was isolated from pMg or BM-Ms with RNeasy Mini Kit:
Cells were washed with PBS and resuspended in 350 µl RLT buffer with 10 µl / ml β-ME. Cells were homogenized by passing five times through a 20 G needle using a 1 ml syringe. Then, 350 µl 70% ethanol was added to cells and lysate was applied to RNeasy spin

columns and centrifuged at 11000 rpm for 15 sec. The flow-trough was discarded. Then RNA was washed with 700 µl of the buffer RW1 (11000 rpm, 15 sec), 500 µl RPE buffer (1100 rpm, 15 sec), and 500 µl RPE buffer (1100 rpm, 2 min). Afterwards, columns were dried by centrifugation (13000 rpm, 1 min). Then, 30 µl nuclease-free water was incubated on columns for 1 min and for eluation of RNA, columns were centrifuged at 11000 rpm for 1 min. The concentration of m-RNA was evaluated by Nanodrop and stored at -80°C.

3.2.5.2 Isolation of mRNA from CNS-tissue

For mRNA isolation from CNS-derived tissue, a part from the cervical spinal cord was dissected (approx. 50-100 mg) and stored in RNAlater at -20°C until isolation with TriReagent. Then, 1 ml TriReagent was added to tissue and suspension was made using an ultra-turrax. After incubation for 5 min at RT, suspension was centrifuged (13000 rpm, 5 min, 4°C). Supernatants were transferred into a new tube, incubated with 100 µl BCP for 10 min at RT and centrifuged (13000 rpm, 15 min, 4°C). Aqueous phase was transferred into a new tube, mixed with 500 µl isopropyl alcohol and incubated for 10 min at RT. mRNA was centrifuged (13000 rpm, 8 min 4°C), supernatants were discarded and pellet was washed twice with 75% cold ethanol (-20°C) (10000 rpm, 5 min, 4°C). After drying the pellet was resuspended in 50 µl DEPC-treated water. mRNA-concentration was evaluated by Nanodrop and stored at -80°C.

3.2.5.3 Reverse transcription

mRNA was transcribed into cDNA by reverse transcriptase using SuperScript III. Therefore, 8 µl of the mRNA, containing 1000 ng, was mixed with 10 µl 2 x RT buffer and 2 µl RT Enzyme Mix. Then, mixture was incubated for 10 min at RT and PCR program was proceeded by Thermocycler at 50°C for 30 min and afterwards 85°C for 5 min. Then 1 µl of the *E. coli* RNase H was added into the tubes followed by an incubation at 37°C for 20 min.

3.2.5.4 Quantitative real-time RT-PCR

Real-time RT-PCR was performed in an Abi Prism 7900 HT thermocycler. Therefore the PCR reaction mixture was prepared with PowerSYBR Green PCR Master Mix (for primers) or TaqMan Gene Expression Master Mix (for FAM-labeled TaqMan probes) in triplicates.

PCR reaction mix component	TaqMan Gene Expression	Power SYBR Green
Taq Man Gene Expression Assay	0,5 µl	-
SYBR Green primers (forw and rev)	-	0,5 µl (each)
Taq Man Gene Expression Master Mix	5 µl	-
Power SYBR Green Master Mix	-	5 µl
cDNA template	1 µl	1 µl
Nuclease free water	3,5 µl	3 µl

Amplification of cDNA was carried out for 40 cycles. Quantification of the relative data was examined using the $\Delta\Delta C_T$ method, normalizing data to GAPDH according to manufacturer's protocol (described in the User Bulletin 2 of the Abi Prism®).

3.2.6 Measurement of cytokine concentration by Sandwich-ELISA

For cytokine-detection in supernatants, 96-well ELISA plates were coated with 50 µl of the capture antibody diluted in coating buffer at 4°C over night.

Then, plates were washed five times with washing buffer and plate was incubated with 100 µl blocking buffer to overcome unspecific binding for 30 min at room temperature (RT). Afterwards, plates were washed five times with washing buffer and 50 µl of the samples were added to the wells and incubated over night at 4°C. For quantitative analysis, a titration of the particular cytokine was added among the 96 well-plate as well as a negative control.

Afterwards, plates were washed five times with washing buffer and 50 µl of the secondary, biotin-labelled antibody (detection antibody) diluted in blocking buffer was added to the plate for 2 h at RT. After washing the plate five times with washing buffer, 50 µl of the

horseradish peroxidase streptavidin conjugate (diluted 1:1000 in PBS) was added for 30 min at 4°C. Plates were washed five times and 50 µl TMB substrate was added into the wells and the optical density (OD) of the samples were analyzed by ELISA reader at a wavelength of 650 nm. When the OD of the highest concentrated positive control was 1, by adding 25 µl of the stop solution to the substrate immediately reaction was stopped and plates were analyzed at o wavelength of 450 nm. The concentration of cytokine in the samples was calculated by the computer program SOFTmax pro and indicated as pg/ml, ng/ml, or µg/ml.

Cytokine	Dilution of 1st antibody	Dilution of 2nd antibody
TNFα	1:500	1:500
CCL2	1:250	1:250

3.2.7 Determination of ROS

To analyze the production of ROS by pMg, cells were seeded into a 96-well cell-culture plate. When cells attached, medium was discarded and cells were incubated with 100 µl of the H_2DCFA solution (20 mM in PBS) at 37°C for 5 min. Afterwards, emission was excited with 485 nm and determined at 530 nm in a pre-heated (37°C) ELISA reader. Cells incubated without the indicator H_2DCFA served as a negative control.

3.2.8 Griess assay for nitrite determination

Determination of nitrite was done with Griess assay purchased from Molecular probes.
For analysis, equal volumes of component A and B were mixed freshly (20 µl per sample). Then, 20 µl of this mixture (Griess reagent) and 130 µl deionised water were added into a 96-well ELISA microtiter plate. 150 µl of the sample to be analyzed or different concentrations of the sodium nitrite solution (component 3) were incubated with griess reagent in the wells at RT. For negative control 150 µl of the deionise water was used. After 30 min, reaction was analyzed by ELISA reader at a wavelength of 548 nm.

3.2.9 Determination of PPARγ-ablation by PCR

PPARγ-ablation was analyzed in cells derived from LysM-PPARγ^{KO} or LysM-PPARγ^{WT} mice. The following cells were isolated by MACS from spleens using indicated MACS-beads:

Cells	MACS-beads
Macrophages	CD11b
DCs	CD11c
B cells	CD19
$CD4^+$ T cells	CD4
$CD8^+$ T cells	CD8

Peritoneal macrophages were isolated from the peritoneum as indicated in section 3.2.1.2. For isolation of peritoneal neutrophilic cells, 2 ml thioglycollate was injected i.p. 3 days prior to isolation.
Then, mRNA was isolated with RNeasy Mini Kit and transcribed into cDNA using Superscript III. 10 µl of the transcribed cDNA was used for PCR with following components purchased from invitrogen:

280 µl H2O
50 µl 10 x buffer
20 µl 50mM MgCl2
20 µl dNTPs
10 µl primer forward
10 µl primer reverse
10 µl TaqPolymerase

PCR program was performed using following steps:

94°C 5 min
94°C 30 sec
60°C 45 sec } 39 cycles
72°C 45 sec
72°C 10 min
4°C

The analysis of PCR products was validated by agarose gel electrophoresis using a 2% agarose gel.

3.2.10 Statistics

Results are depicted as mean +/- standard error of the mean (SEM). Statistic significances were calculated by unpaired two-tailed Student's t test. p values of 0.01 to 0.05 were considered as significant (*), p values of 0.001 to 0.01 as highly significant (**), and p values of < 0.001 as extremely significant (***).
The number of independent experiments are indicated in the legend.

4 Results

4.1 Role of PPARγ in myeloid cells during CNS-inflammation

The importance of CNS-invading auto-reactive $CD4^+$ T cells, primed in the periphery and initiating EAE, has been sufficiently documented[27,30,109-112]. However, recent data indicate that the effector mechanisms leading to progression of inflammation and demyelination within the CNS are mediated by other cell types, especially infiltrating macrophages and resident microglia[113,114]. Activation of PPARγ in myeloid cells has been proven to impair expression of pro-inflammatory mediators in these cells[72,75,115]. Therefore, the question arose if PPARγ in microglia and macrophages might be a promising regulator during inflammatory diseases like EAE.

4.1.1 PPARγ in myeloid cells controls the effector phase of EAE

This thesis adressed the role of the anti-inflammatory transcription factor PPARγ in myeloid cells during CNS inflammation. To this end, mice with a conditional knock-out of PPARγ in LysM expressing cells, encompassing monocytes / macrophages, microglia, granulocytes, and partially in $CD11c^+$ splenic DCs[106] (LysM-$PPAR\gamma^{KO}$ mice) were utilized. PPARγ cDNA analyzed by real-time RT-PCR in various cell populations showed a truncated 300bp fragment of PPARγ cDNA in cells expressing LysM. In contrast, non-myeloid cells showed full-length 700bp wildtype PPARγ cDNA (Figure 1a). As shown in Figure 1a, LysM-$PPAR\gamma^{KO}$ mice lack PPARγ in microglia, monocytes / macrophages, and neutrophils, whereas PPARγ-expression was only partially affected in $CD11c^+$ splenic cells, but not in T cells and B cells.

To analyze the role of PPARγ in myeloid cells during CNS-inflammation, EAE, the animal model of MS, was used. Therefore, active MOG-EAE was induced in LysM-$PPAR\gamma^{KO}$ mice and their wildtype littermates by immunization with MOG_{35-55} peptide in complete Freund's adjuvant (CFA) and Pertussis toxin, which was injected together with MOG_{35-55}-CFA and in addition on day two after immunization. Disease progress was monitored daily. (EAE-experiment was performed with Stephanie Hucke.)

Importantly, during the effector phase of EAE the course of disease was significantly aggravated in LysM-PPARγ^{KO} mice compared to their wildtype littermates (LysM-PPARγ^{WT}) beginning at day 15 after immunization (Figure 1b). In contrast, alterations in course of disease were not observed during the T cell-dependent induction phase (days 1 to 14) (Figure 1b).

To address the question whether the aggravated disease course in LysM-PPARγ^{KO} mice was accompanied by enhanced inflammation and tissue damage, spinal cords of either LysM-PPARγ^{KO} mice or wildtype littermates were histopathologically analyzed during the effector phase of EAE on day 35 after immunization. As shown in Figure 1c, both demyelination and axonal damage were increased in the spinal cord of LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} controls. Additionally, infiltration of T cells and macrophages was significantly increased in LysM-PPARγ^{KO} mice compared to the wildtype littermates at that point of time (Figure 1d).

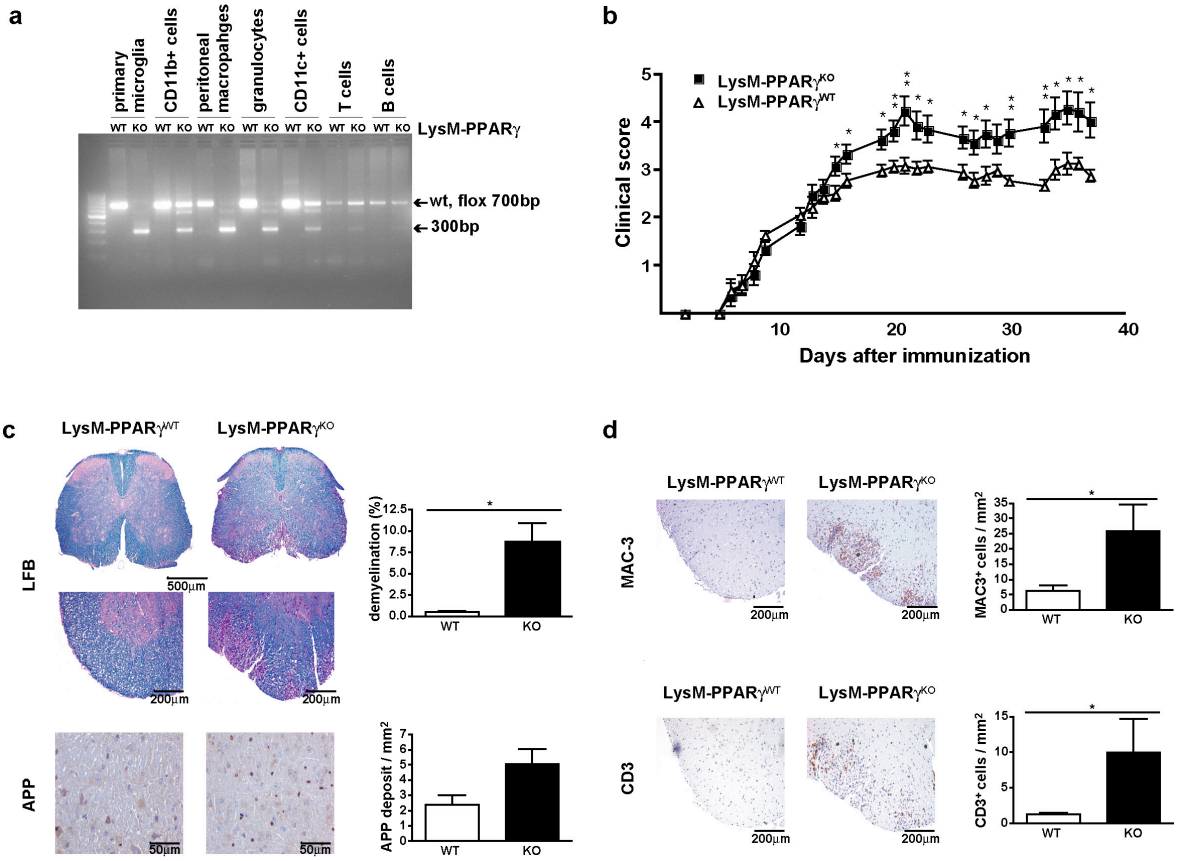

Figure 1 PPARγ in myeloid cells controls the effector phase of EAE (Experiments performed with Stephanie Hucke)

(a) PPARγ ablation in myeloid cells: RT-PCR analysis of mRNA isolated from primary microglia, CD11b^{+} splenic cells, peritoneal macrophages, neutrophil granulocytes, CD11c^{+} splenic cells, T cells and B cells derived from LysM-PPARγ^{KO} (KO) mice or their wildtype (WT) littermates.

Expression of Cre-recombinase in myeloid cells results in a specific ablation of PPARγ cDNA (300 bp fragment), in contrast to wildtype cDNA (700 bp). **(b)** Mean clinical score of EAE-diseased LysM-PPARγ^{WT} (n=12) or LysM-PPARγ^{KO} (n=13) mice. Active EAE was induced by immunization with MOG_{35-55} and disease was monitored daily. Mean clinical score ± SEM of two representatives out of five independent experiments is shown. **(c+d)** Histopathological analysis (left) and quantification (right) of **(c)** structural damage: demyelination (luxol fast blue (LFB)), axonal damage (amyloid precursor protein (APP) (n=5 per group), **(d)** and CNS-infiltration by macrophages / microglia (MAC-3) and T cells (CD3) (n=5).
Data are shown as mean ± SEM. * $p<0.05$; ** $p<0.01$.

These results show that lack of PPARγ in myeloid cells leads to an aggravated disease course during the effector phase of EAE, accompanied by enhanced immune cell infiltration and tissue damage.

To further analyze the inflammatory milieu within the CNS during the effector phase, mRNA levels of several pro-inflammatory mediators were evaluated by quantitative real-time RT-PCR.

Compared to non-diseased mice, mRNA levels of pro-inflammatory cytokines like IL-1β, IL-12, TNFα, and IFNγ were increased in diseased LysM-PPARγ^{WT} mice, and were further augmented in LysM-PPARγ^{KO} EAE mice (Figure 2a). Additionally, mRNA levels of iNOS were significantly augmented in LysM-PPARγ^{KO} mice. Moreover, as shown in Figure 2b, mRNA levels of the chemokines CCL2, CCL3, CCL5, and CXCL10, were three- to seven-fold higher in LysM-PPARγ^{KO} mice compared to wildtype littermates.

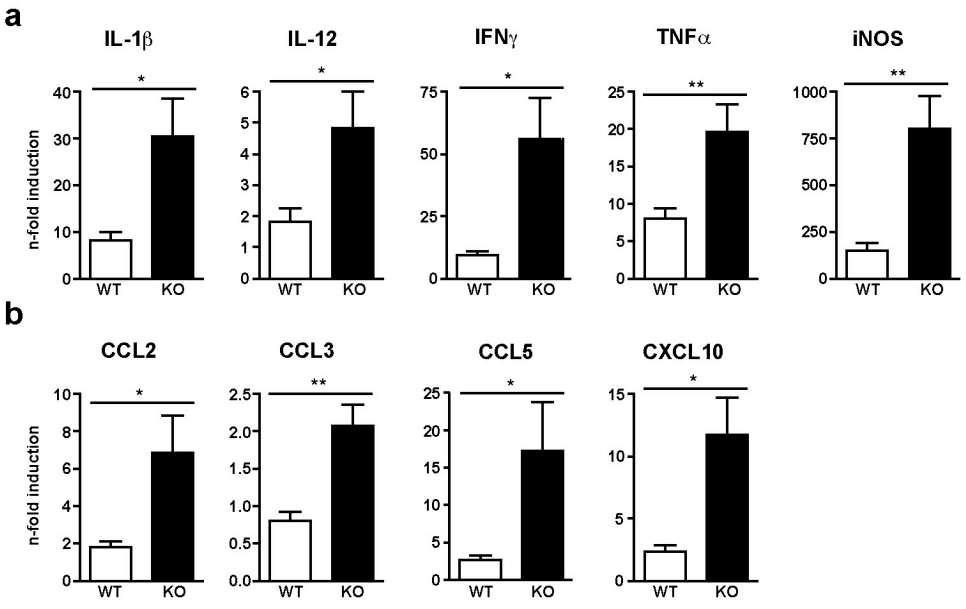

Figure 2 PPARγ in myeloid cells controls the inflammatory milieu within the CNS during the effector phase of EAE

(a-b) mRNA levels of **(a)** inflammatory cytokines, iNOS**, (b)** and chemokines in cervical spinal cord of LysM-PPARγ^{KO} (KO) mice or LysM-PPARγ^{WT} (WT) EAE mice (n=10 per group) quantified by quantitative real-time RT-PCR. mRNA was isolated from tissue on day 21 after immunization. Data are shown as n-fold induction compared to healthy controls (n=3) and were normalized to endogenous GAPDH expression.
Data expressed as mean ±SEM. * $p<0.05$; ** $p<0.01$.

To analyze whether PPARγ modulates the activation status of myeloid cells during EAE *per se*, mRNA levels of IL-1β in microglia and macrophages isolated from the CNS of EAE-diseased LysM-PPARγ^{WT} or LysM-PPARγ^{KO} mice were examined.
Although the alteration did not reach statistical significance, enhanced levels of IL-1β mRNA were observed in macrophages and microglia from LysM-PPARγ^{KO} mice compared to wildtype littermates (Figure 3).

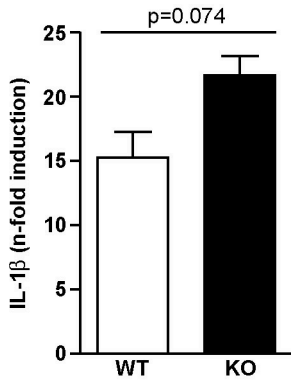

Figure 3 PPARγ controls IL-1β-expression in myeloid cells during the effector phase of EAE
mRNA-level of IL-1β in macrophages and microglia from LysM-PPARγ^{WT} (WT) (n=4) or LysM-PPARγ^{KO} (KO) (n=3) EAE mice. Cells were isolated by fluorescence-activated cell sorting, based on their CD11b^{+}CD45^{+} expression. Levels were quantified by quantitative real-time RT-PCR, normalized to endogenous GAPDH-expression and shown as n-fold induction compared to unstimulated BM-Ms.

In summary, lack of PPARγ in myeloid cells resulted in an aggravated disease course during the effector phase of EAE, accompanied by an increased release of inflammatory and chemoattractant mediators in the CNS. Thus, these data identify PPARγ in myeloid cells as a crucial regulator for CNS inflammation during EAE pathology.

4.1.2 PPARγ controls immunological activation in myeloid cells during the effector phase of EAE

So far, the *in vivo* findings revealed that PPARγ in myeloid cells effects demyelination, axonal damage, and inflammation during the effector phase of EAE.

As T cells play an important role in EAE-pathology[109,110] and expression of CD40 and MHC-II molecules on the surface of microglia and macrophages are important for interacting with encephalitogenic T cells during EAE[116-118], the question arose, if PPARγ in myeloid cells might also influence the interaction with auto-reactive encephalitogenic T cells, which then might result in altered T cell-activation. CNS mononuclear cells were isolated from LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice on day 21 after EAE immunization and the expression of CD40 and MHC-II on macrophages and microglia was analyzed by flow cytometry.

Both cell-types are CD11b^{+}, but can be distinguished by their different expression of the surface-molecules CD45 and Ly-6C: Macrophages are characterized by high levels of CD45 and Ly-6C (CD45^{hi}Ly-6C^{+}), whereas microglia are characterized by a low to intermediate expression of CD45 and Ly-6C (CD45^{lo}Ly-6C^{lo}) (Figure 4a).

As shown in Figure 4b, both, infiltrating macrophages and microglia derived from LysM-PPARγ^{KO} mice, showed significantly increased levels of the surface marker CD40 compared to their wildtype control on day 21 after EAE immunization (Figure 4c). Further, increased levels of MHC-II surface expression on macrophages and microglia within the CNS of LysM-PPARγ^{KO} mice compared to their wildtype control were observed (Figure 4d). In addition, the percentage of MHC-II-expressing macrophages and microglia was enhanced in LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} mice (Figure 4e). Moreover, flow cytometric analysis revealed a two-fold increase in absolute cell numbers of both, macrophages and microglia, in LysM-PPARγ^{KO} compared to wildtype littermates at that time point (Figure 4f).

In conclusion, the expression of CD40 and MHC-II, molecules that are important for interaction with auto-reactive encephalitogenic T cells, was increased in LysM-PPARγ^{KO} EAE mice during the effector phase of EAE compared to wildtype littermates. This indicates that PPARγ modulates the activation status in microglia and macrophages during the effector phase of EAE.

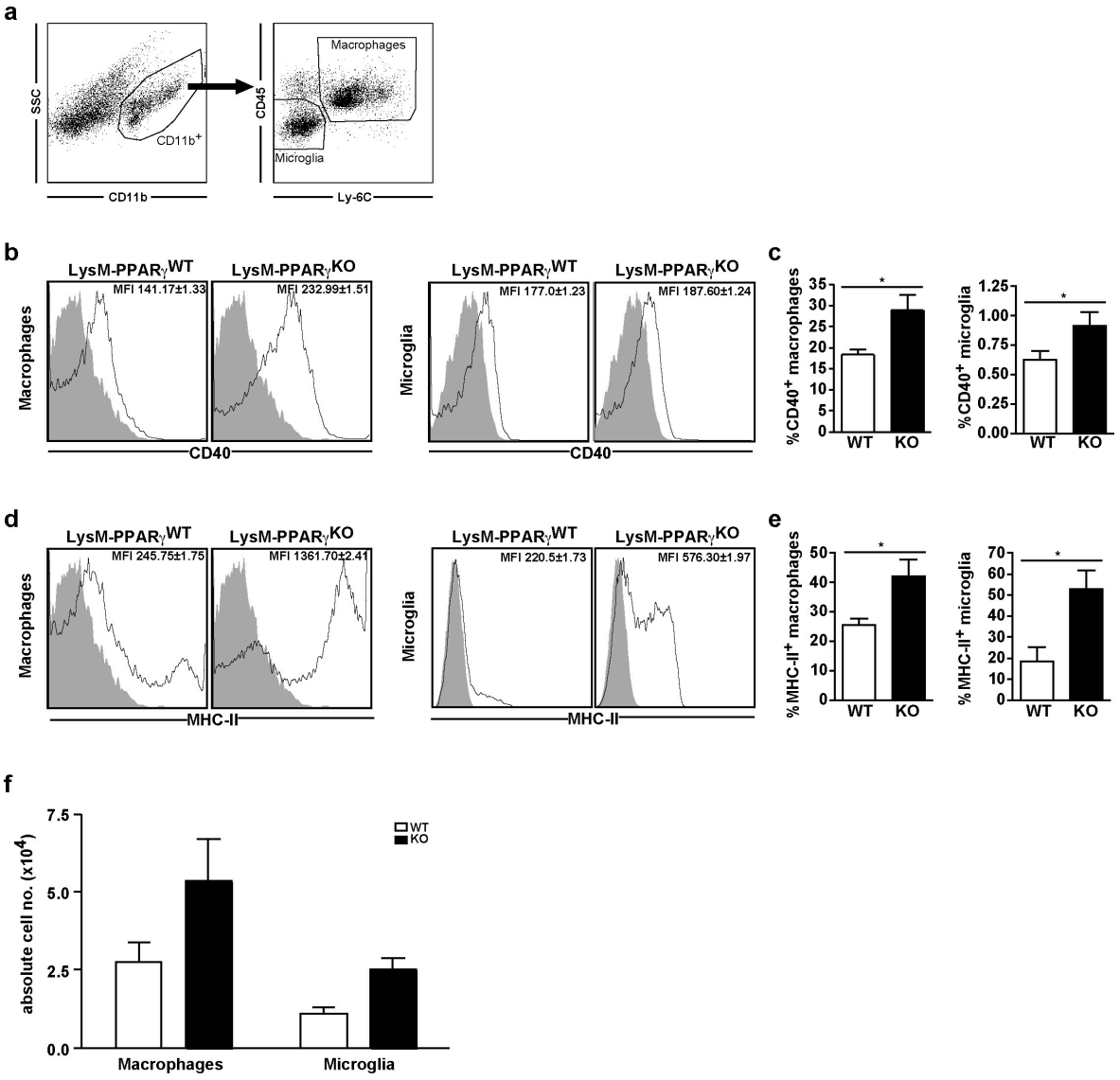

Figure 4 PPARγ controls the immunological activation in myeloid cells during the effector phase of EAE

Analysis of macrophages and microglial cells within the CNS during the effector phase of EAE. CNS-mononuclear cells were isolated from brain and spinal cord of LysM-PPARγ^{WT} (WT) or LysM-PPARγ^{KO} (KO) EAE mice on day 21 after immunization. **(a)** Classification of CD11b^{+} macrophages and microglial cells based on the expression of CD45 and Ly-6C. **(b+d)** Expression of surface molecules **(b)** CD40 (n=12) and **(d)** MHC-II (n=9) was analyzed by flow cytometry. Histograms depict unstained control (tinted) and CD40- or MHC-II-specific antibody (line). Mean fluorescence intensity (MFI) ± SEM is indicated in graphs. **(c+e)** Percentage of CD40- **(c)** (n=12) or MHC-II- **(e)** (n=9) positive macrophages or microglial cells within the CNS. Graphs depict mean percentage ± SEM. **(f)** Absolute cell numbers of macrophages and microglia on day 21 after EAE-immunization. Cells were quantified by flow cytometry using fluorescently labeled beads and indicated as mean ± SEM; n=5 per group. * $p<0.05$.

4.1.3 PPARγ in myeloid cells does not affect T cell priming during the induction phase of EAE

It is known, that activation of PPARγ in APCs impairs T cell priming[77,119]. Hence, it is arguable, whether the observed aggravated disease course during the effector phase of EAE in LysM-PPARγ^{KO} mice compared to wildtype controls (Figure 1) might be a consequence of PPARγ-ablation in peripheral macrophages affecting initial T cell priming in the periphery.

To investigate a potential influence of PPARγ in peripheral myeloid cells (e.g. macrophages) on T cell priming during development of EAE, MOG-specific CD4$^+$ T cell responses were characterized at different time points.

To this end, T cells from the draining lymph nodes or the CNS from either LysM-PPARγ^{KO} EAE mice or wildtype control mice were isolated and MOG-specific IFNγ- and IL-17A-responses were assessed on days 7 or 14 (induction phase) and on days 21 and 27 (effector phase).

However, an increase in MOG-specific IFNγ-production was not observed in the draining lymph nodes from LysM-PPARγ^{KO} mice (Figure 5a). Furthermore, on days 7 and 14, the percentage of IL-17A-producing T cells was not significantly different in the draining lymph nodes. Moreover, no differences in the percentages of IFNγ- or IL-17A-producing CD4$^+$ T cells were found in the CNS of LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} mice on day 14 or 27 after immunization, respectively (Figure 5b). Likewise, CD8$^+$ T cell responses were not altered on day 27 after immunization (Figure 5c).

To further exclude an effect of PPARγ on the immunological activation in peripheral APCs like macrophages and therefore on T cell priming during the induction phase of EAE, MOG-activated T cells from immunized wildtype mice, were adoptively transferred into either wildtype recipients or LysM-PPARγ^{KO} mice.

Importantly, the course of disease was significantly aggravated during the effector phase in LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} mice during adoptive transfer EAE, although this difference was not as pronounced as during active MOG-EAE (Figure 5d). This difference was observed in three independent experiments.

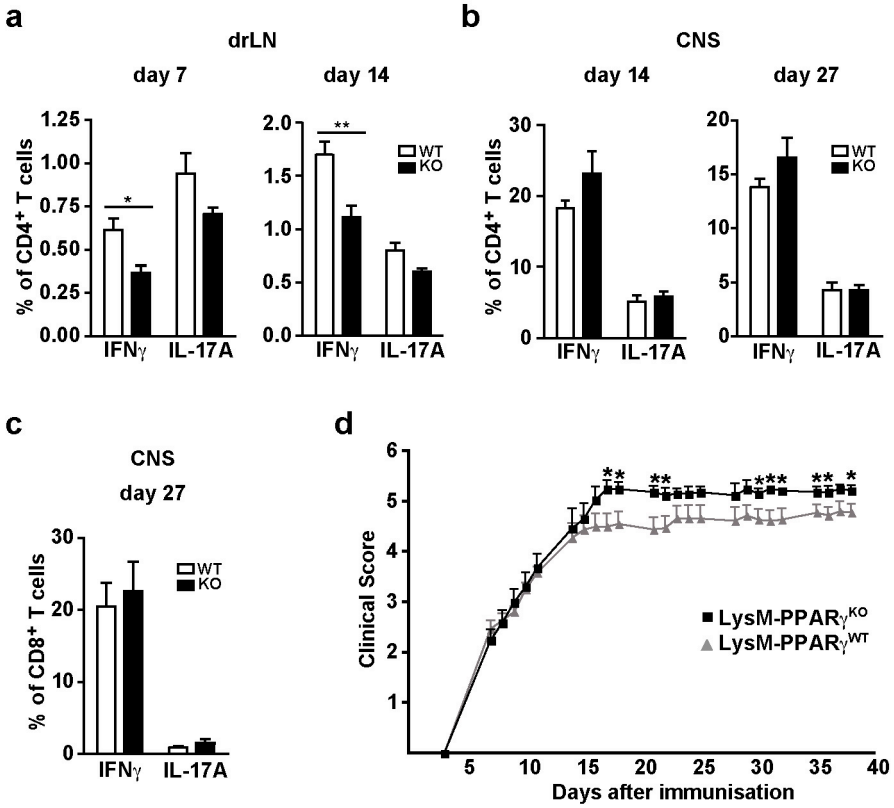

Figure 5 PPARγ in myeloid cells does not influence activation of T cell during EAE
(a-c) Analysis of T cells from draining lymph nodes or CNS from LysM-PPARγ^{WT} (WT) or LysM-PPARγ^{KO} (KO) EAE mice. At indicated time points, mononuclear cells were isolated from **(a)** draining lymph nodes (drLN) or **(b+c)** the CNS, and **(a+b)** CD4$^+$ or **(c)** CD8$^+$ T cells were analyzed for IFNγ- or IL-17A-expression by flow cytometry (n=5 per timepoint). Graphs depict the mean percentage ± SEM. **(d)** Mean clinical score of adoptive transfer EAE: MOG_{35-55}-reactive T cells were primed in wildtype mice, restimulated *in vitro* and transferred into either LysM-PPARγ^{WT} (n=6) or LysM-PPARγ^{KO} (n=7) mice.
Graph shows mean clinical score ± SEM. * $p<0.05$; ** $p<0.01$.

In conclusion, these data corroborate the hypothesis that PPARγ in myeloid cells regulates local CNS-inflammation during the effector phase of EAE, but does not significantly affect T cell priming or differentiation during the initial priming phase.

4.1.4 PPARγ-ablation in astrocytes, neurons and oligodendrocytes does not affect disease score during EAE

So far, these data reveal an influence of PPARγ in myeloid cells on CNS inflammation during the effector phase of EAE. As other CNS resident cells like astrocytes, neurons and oligodendrocytes also play a role in CNS inflammation[6,120], an effect of PPARγ on these CNS resident cells was examined during CNS inflammation.

To this end, mice with a conditional knock-out of PPARγ in CNS resident cells were employed (Nestin-PPARγ^{KO}).

As shown in Figure 6a, astrocytes derived from Nestin-PPARγ^{KO} mice, but not microglia, exhibited PPARγ-ablation (Figure 6a), characterized by a truncated 300bp fragment of PPARγ cDNA, in contrast to the full-length 700bp wildtype cDNA.

To analyze the role of PPARγ in astrocytes and neuronal cells during CNS-inflammation, Nestin-PPARγ^{KO} mice and wildtype littermates were immunized with MOG_{35-55} peptide and the course of EAE was determined.

Upon MOG-immunization, differences in disease score were not observed in Nestin-PPARγ^{KO} mice compared to wildtype control littermates (Figure 6b), indicating that PPARγ is not a potent regulator in CNS resident astrocytes and neuronal cells during CNS inflammation.

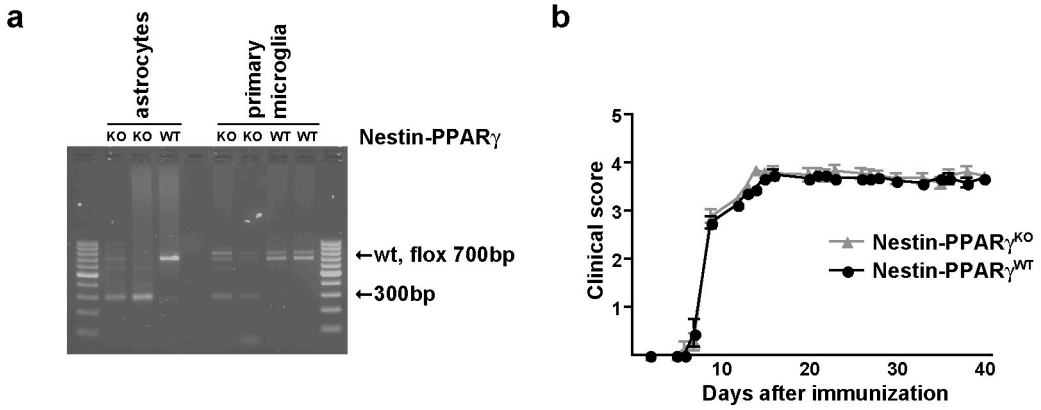

Figure 6 PPARγ ablation in CNS resident cells does not affect disease score during EAE
Nestin-Cre mice were crossed with PPARγ$^{fl/fl}$ mice, resulting in specific PPARγ ablation in CNS resident cells. **(a)** RT-PCR analysis of mRNA from primary astrocytes and primary microglia. Expression of Cre-recombinase in myeloid cells results in a specific ablation of PPARγ cDNA (300 bp fragment), in contrast to wildtype cDNA (700 bp). **(b)** Mean clinical score of EAE-diseased Nestin-PPARγ^{WT} (n=9) or Nestin-PPARγ^{KO} (n=6) mice. MOG_{35-55}-EAE was induced by active immunization and mice were clinically monitored daily. Figure depicts mean clinical score ± SEM of one representative experiments out of four.

4.1.5 PPARγ controls activation of myeloid cells *in vitro*

So far, the *in vivo* findings revealed an influence of PPARγ in myeloid cells on local CNS-inflammation during the course of EAE (Figure 1 and 2). To further characterize the effect of PPARγ on the innate immune response in myeloid cells *in vitro*, levels of cytokines,

chemokines, and neurotoxic mediators were analyzed in bone marrow-derived macrophages (BM-Ms) and primary microglia (pMg) derived from either LysM-PPARγ^{KO} or LysM-PPARγ^{WT} mice.

Upon stimulation, PPARγ^{KO} BM-Ms exhibited a significant 1.5-fold increase in nitrite-release compared to wildtype BM-Ms. In addition, levels of TNFα were increased 1.5-fold in the supernatants of stimulated BM-Ms derived from PPARγ^{KO} mice compared to PPARγ^{WT} BM-Ms. Moreover, analysis of mRNA levels of inflammatory cytokines like IL-6 and IL-12 revealed an 1.5- and 2-fold increase, respectively, in PPARγ^{KO} macrophages compared to wildtype macrophages. Furthermore, CCL2 levels were 3-fold higher in PPARγ^{KO} BM-Ms compared to wildtype macrophages (Figure 7a).

Increased levels of neurotoxic mediators and inflammatory cytokines were also observed in primary microglial cells (pMg) isolated from neonatal LysM-PPARγ^{KO} and wildtype littermate pups: Upon stimulation with LPS and IFNγ, significantly increased levels of nitrite in the supernatants of PPARγ^{KO} pMg compared to wildtype pMg were detected. Additionally, a significant increase in ROS was determined in PPARγ^{KO} pMg compared to wildtype pMg as well as an elevation in the percentage of TNFα^{+} pMg. mRNA-levels of the inflammatory cytokines IL-6 and IL-12 were 2-fold higher in PPARγ^{KO} pMg compared to wildtype pMg (Figure 7b).

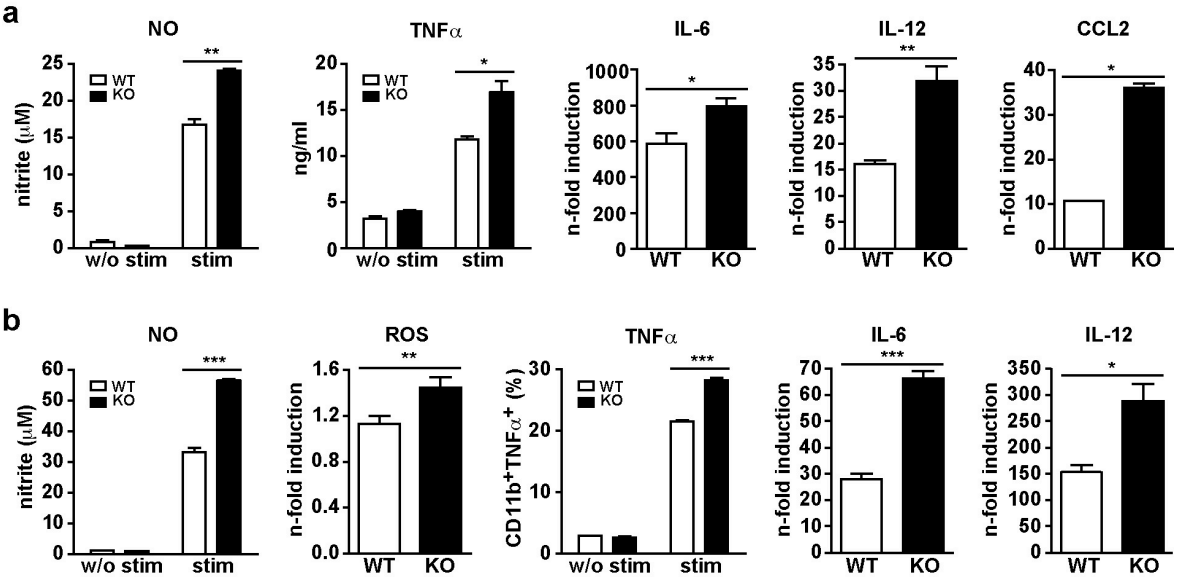

Figure 7 PPARγ regulates innate immune responses in myeloid cells

(a) BM-Ms from PPARγ^{KO} or PPARγ^{WT} mice were stimulated (stim) for determination of NO-production by Griess assay (100 ng/ml LPS and 10 ng/ml IFNγ, 48 h) and TNFα-release by ELISA

(100 ng/ml LPS, 24h). Control cells were not stimulated (w/o stim). mRNA-levels of IL-6, IL-12, and CCL2 were quantified by real-time RT-PCR after stimulation (1 μg/ml LPS and 5 pg/ml IFNγ, 6 h). Data were normalized to endogenous GAPDH expression and shown as n-fold induction compared to unstimulated control. **(b)** PPARγ^{KO} or PPARγ^{WT} pMg were stimulated for quantification of NO- (1 μg/ml LPS and 10 ng/ml IFNγ, 48 h), or ROS-production (10 μg/ml LPS, 24h). Induction of ROS is expressed as fold chance in relative light units compared to unstimulated control. Percentage of TNFα^{+} pMg was intracellularly analyzed by flow cytometry after stimulation (1 μg/ml LPS and 5 pg/ml IFNγ, 4 h) in the presence of monensin and brefeldin A. Stimulation of pMg for mRNA-detection and -quantification was determined as indicated for BM-Ms (7a).
Data are expressed as mean ± SEM (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

In conclusion, PPARγ-ablated macrophages and microglial cells showed augmented innate immune responses upon activation *in vitro*.

4.1.6 PPARγ controls astrocytic CCL2-production in myeloid cells

A number of studies indicate that the CCL2 / CCR2-axis plays a crucial role for the pathogenesis of multiple sclerosis and EAE[60,100]. CCR2 and its ligand CCL2 are important for emigration of inflammatory monocytes, bearing CCR2 on their surface, from the bone marrow and immigration into the inflamed tissue among a CCL2-gradient.

So far, data of this thesis reveal an involvement of PPARγ in myeloid cells in modulation of local CCL2-production within the CNS (Figure 2b) during the effector phase of EAE. As astrocytes are known to be the main producer of CCL2 within the CNS[6,121], we hypothesized that activated myeloid cells might trigger astrocytic CCL2-release during EAE and that this interaction might be influenced by PPARγ in myeloid cells.

To evaluate this assumption *in vitro*, wildtype astrocytes were activated with LPS and IFNγ or cocultured with preactivated PPARγ^{KO} or PPARγ^{WT} BM-Ms or pMg. CCL2-release was examined by ELISA after 24h.

Upon stimulation with LPS and IFNγ, CCL2 production in astrocytes was 7-fold higher. Furthermore, CCL2-release was significantly 2-fold higher in macrophages (Figure 8a) and microglia (Figure 8b). Interestingly, coculture of astrocytes with macrophages (Figure 8a) or microglia (Figure 8b), that were prestimulated for 1h, increased CCL2-production compared to unstimulated astrocytes. Importantly, this macrophage- or microglia-mediated activation was regulated by PPARγ, as PPARγ-deficiency in myeloid cells resulted in enhanced CCL2-release into the supernatant.

In contrast, T cells, activated for 72h with or without T_H17-inducing conditions did not produce relevant amounts of CCL2 (Figure 8c).

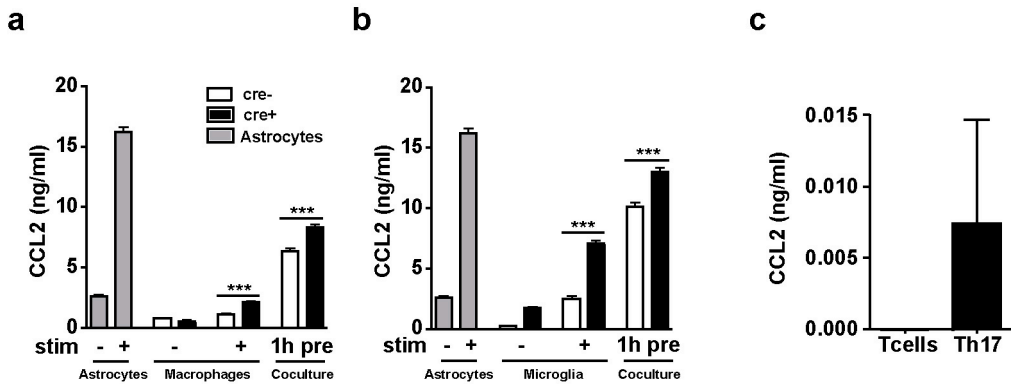

Figure 8 CCL2-release by astrocytes cocultured with PPARγ^{KO} or PPARγ^{WT} macrophages or microglia

(a-b) CCL2-production of astrocytes, **(a)** BM-Ms or **(b)** pMg or coculture: wildtype astrocytes were cocultured with PPARγ^{KO} or PPARγ^{WT} **(a)** BM-Ms or **(b)** pMg at a ratio of 1:1. Cells were stimulated with 1μg/ml LPS and 10ng/ml IFNγ. Supernatants were collected after 48 h and CCL2-production was determined by ELISA. **(c)** Splenic T cells were isolated from a C57BL/6 mouse by immunomagnetic cell separation using CD4-MACS beads, activated by plate-bound αCD3 and αCD28 antibody for 72 h, with or without T_H17-inducing conditions. CCL2 release was determined in the supernatant by ELISA.
Data are expressed as mean ± SEM (n=5). *** $p<0.001$

In conclusion, these results showed that myeloid cells can activate astrocytes producing high levels of CCL2: the chemokine that attracts CCR2$^+$ inflammatory monocytes.

4.1.7 Inflammatory monocytes are the main myeloid cell population controlled by PPARγ during EAE

Several reports indicate a prominent role for a CCR2-bearing subpopulation of myeloid cells, the 'inflammatory monocytes'[97], for the progression of EAE[98,100]. This cell population can be discriminated from resident monocytes: inflammatory monocytes are CCR2$^+$Ly-6C^{hi}CD62L$^+$CX3CR1^{lo}, and the resident monocytes are CCR2$^-$Ly-6C^{lo}CD62L$^-$CX3CR1^{hi} [97].

As the preceding data show an important role of PPARγ in myeloid cells during the effector phase of EAE, the influence of PPARγ in inflammatory monocytes during EAE was further analyzed.

To directly assess the role of PPARγ in inflammatory monocytes during the effector phase of EAE, CD115$^+$ monocytes were isolated from healthy LysM-PPARγ^{KO} or LysM-PPARγ^{WT} mice and injected into MOG-immunized C57BL/6 EAE mice during the effector phase of EAE. Disease score was monitored daily and, on day 10 after the first monocyte transfer, CD40-expression on the surface of macrophages and microglia was analyzed.

Although not significant due to small animal numbers, administration of 1 x 10^7 PPARγ^{WT} inflammatory monocytes per mouse resulted in a slightly augmented disease course during ongoing CNS-inflammation which was visible already from day one after transfer (Figure 9a). In mice that received PPARγ^{KO} inflammatory monocytes, the course of disease was even more aggravated in comparison to mice that received wildtype inflammatory monocytes.

Moreover, analysis of the CD40-expression on CNS-derived macrophages and microglia on day ten after the first transfer revealed a slight increase in the percentage of CD40-expressing cells in mice that received PPARγ^{WT} monocytes. This amount was even more increased in mice that received PPARγ^{KO} monocytes (Figure 9b). These findings indicate that CD40 is upregulated on microglial cells and macrophages during CNS-inflammation in a PPARγ-dependent way.

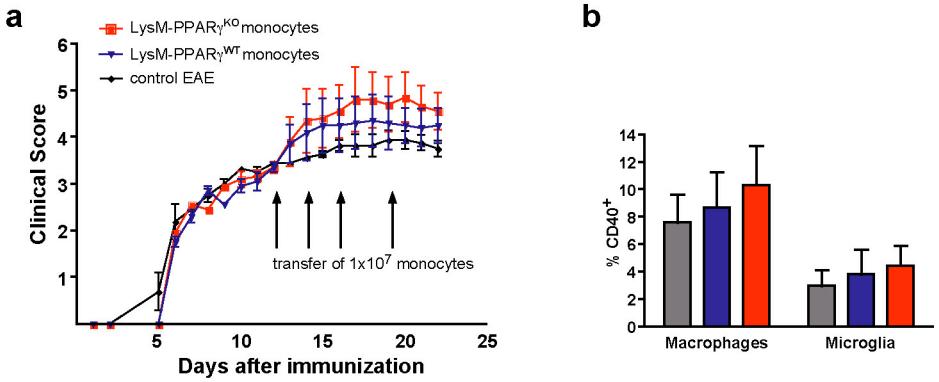

Figure 9 PPARγ in moncytes limits monocyte-mediated severity of disease during EAE
(a) Mean clinical score of EAE-diseased C57BL/6 mice. On days 12, 14, 16 and 19 after active EAE immunization, 1 x 10^7 CD115$^+$ monocytes derived from bone-marrow of healthy LysM-PPARγ^{KO} or LysM-PPARγ^{WT} mice were injected i. v. into EAE mice. Control mice did not receive monocytes. Graph depicts mean clinical score ± SEM, n=5 per group. **(b)** 10 days after the first monocyte transfer, CNS macrophages and microglia were isolated and analyzed for CD40 expression. Graph depicts the mean percentage of CD40$^+$ macrophages or microglia ± SEM (n=5 per group).

It is known that depletion of inflammatory monocytes results in an ameliorated course of disease[98,100]. As we observed that transfer of PPARγ^{KO} inflammatory monocytes aggravates EAE (Figure 9a), we hypothesized that PPARγ plays a crucial role for CNS-inflammation during EAE especially in this myeloid cell population. Therefore, we performed depletion of this population to address the question, if diminution of inflammatory monocytes in LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice may abolish the observed differences during the effector phase of EAE.

To this end, we used an αCCR2 antibody (MC-21), which selectively depletes $CCR2^{+}Ly\text{-}6C^{hi}$, but not $CCR2^{-}Ly\text{-}6C^{lo}$ monocytes *in vivo*[122]. (EAE-experiment and monocyte depletion was performed with Stephanie Hucke.)

LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice were immunized with MOG_{35-55} in CFA and 20μg MC-21 or the appropriate isotype control were injected daily on days 13 to 17 after MOG-immunization.

Application of the MC-21 antibody resulted in approximately 85 % depletion of inflammatory monocyes in the periphery of LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice, compared to isotype controls (Figure 10a).

As expected, depletion of inflammatory monocytes resulted in amelioration of the disease course from day one after the first MC-21 injection in both, LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice (Figure 10b). Importantly, depletion of inflammatory monocytes completely abolished the differences that were observed between isotype treated LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice (Figure 10b). This observation indicates inflammatory monocytes represent the most important population of myeloid cells regulated by PPARγ during EAE.

To further investigate the extent of inflammation within the CNS of MC-21-treated EAE mice, mRNA-levels of inflammatory mediators were analyzed by quantitative real-time RT-PCR. As expected, within the isotype control group, mRNA levels of the inflammatory cytokines IL-1β, IL-12, and IFNγ as well as iNOS were increased in the brain tissue of LysM-PPARγ^{KO} EAE mice compared to wildtype littermates (Figure 10c), which is in line with previous results (Figure 2a+b). Importantly and in accordance to the literature, mRNA-levels of inflammatory cytokines were decreased in brains of mice treated with MC-21. Furthermore, differences in pro-inflammatory mediators between LysM-PPARγ^{KO} and wildtype EAE mice (Figure 2a) were completely abrogated upon treatment with MC-21. Similar results were found for mRNA levels of chemoattractant mediators like CCL2, CCL3, CCL5, and CXCL10 (Figure 10c).

Furthermore, the activation status of macrophages and microglia within the CNS in MC-21-treated EAE mice was analyzed during the effector phase of EAE.

As shown in Figure 10d, alterations in CD40 or MHC-II-expression were not observed between MC-21-treated and isotype control (histograms), indicating that MC-21-treatment does not affect myeloid cell activation status on a single cell level. However, the percentage of MHC-II- and CD40-expressing macrophages was reduced in the MC-21 treated mice compared to the isotype control-treated mice.

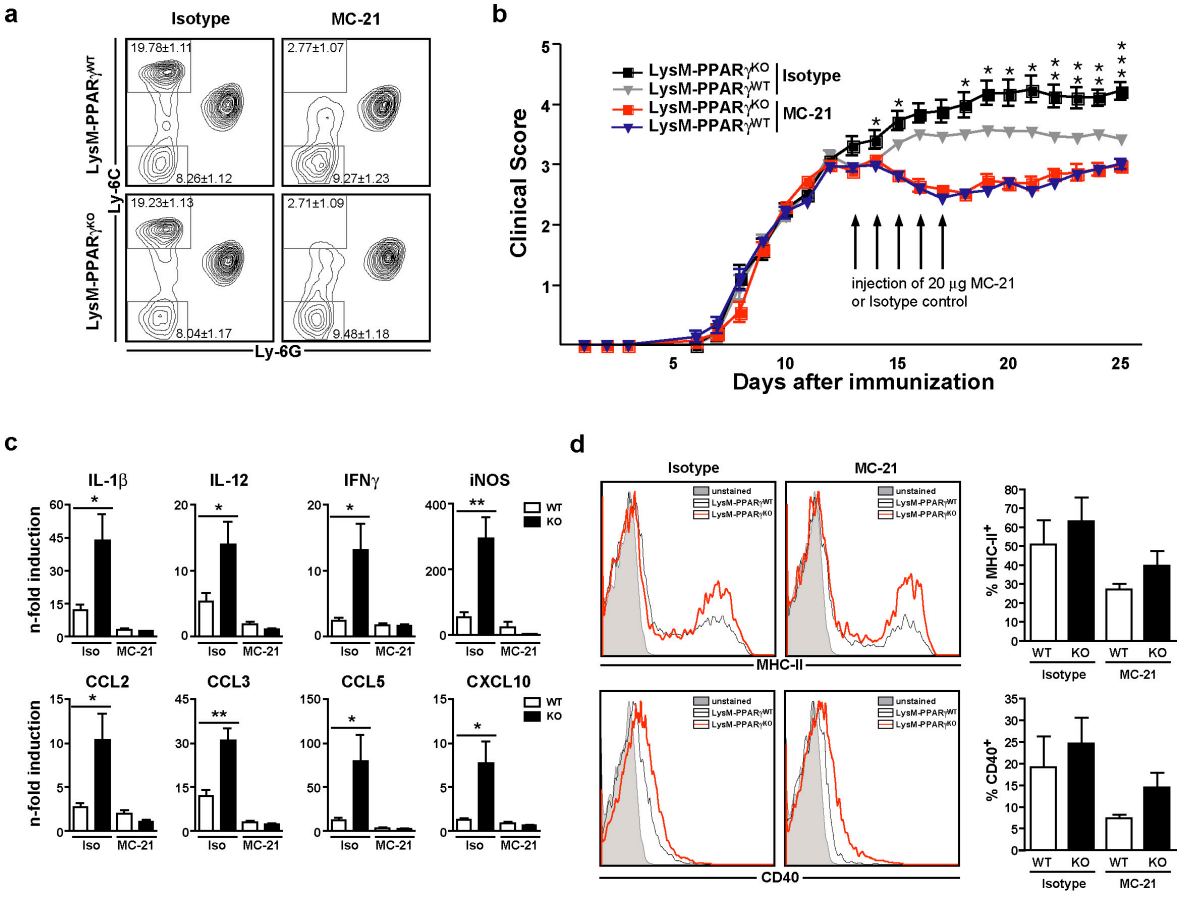

Figure 10 Depletion of inflammatory monocytes ameliorated disease course during EAE and diminished differences between LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice (Experiments performed with Stephanie Hucke)

(a-d) Depletion experiment of inflammatory monocytes in LysM-PPARγ^{KO} or LysM-PPARγ^{WT} mice by the αCCR2 antibody (MC-21) during EAE. **(a)** 8 h after first injection of MC-21 or isotype control, depletion was monitored in the blood. Living cells were gated on CD11b, numbers in dotplots indicate the percentage of Ly-6C^{hi}Ly-6G^{-} cells ± SEM (population in upper gate) and Ly-6C^{lo}Ly-6G^{-} cells ± SEM (population in lower gate). **(b)** Mean clinical score ± SEM of LysM-PPARγ^{KO} or LysM-PPARγ^{WT} EAE mice of two independent experiments is shown. EAE was induced by active immunization and inflammatory monocytes were depleted by repetitive injection of 20 µg MC-21 or isotype control at indicated timepoints (arrows). Graph depicts LysM-PPARγ^{KO} EAE mice administered MC-21 (red squares; n=20) or isotype control (black squares; n=15) and LysM-PPARγ^{WT} EAE mice that received MC-21 (blue triangles; n=20) or isotype control (grey triangles; n=12). **(c)** On day 21 after immunization, expression of inflammatory mediators and chemokines was determined by quantitative real-time RT-PCR (n=5, MC-21; n=10, isotype control). Data were normalized to endogenous GAPDH and are expressed as n-fold induction compared to healthy LysM-PPARγ^{WT} and LysM-PPARγ^{KO} mice, respectively. Data are displayed as mean ± SEM. **(d)** Expression of MHC-II and CD40 was analyzed in CNS-macrophages by flow cytometry on day 21 after immunization (n=3, isotype control; n=5 MC-21).
Graphs show mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

These data clearly show that transfer of PPARγ-ablated inflammatory monocytes during the effector phase aggravates EAE (Figure 9a), whereas depletion of this cell population

results in an ameliorated course of disease, which is comparably in LysM-PPARγ^{KO} and wildtype mice (Figure 10b).

Together, the observation that depletion of inflammatory monocytes completely abolished the differences in the course of disease observed between LysM-PPARγ^{KO} mice and their wildtype littermates identify inflammatory monocytes as the main target for PPARγ-activation within the population of myeloid cells.

5 Discussion

The importance of auto-reactive encephalitogenic T cells for disease initiation in MS and EAE has been analyzed extensively[27,111,112]. However, several publications recently focused on myeloid cells like peripheral macrophages and resident microglial cells involved in CNS inflammation during the development of EAE: For example, microglial cells are important for development and disease progression of EAE by activation of encephalitogenic T cells[118,123] and secretion of neurotoxic mediators like NO, ROS, and TNFα, which directly contribute to tissue damage within the CNS[64]. More recently, peripheral inflammatory monocytes have been reported to contribute to disease progression during EAE[98,100].

Importantly, activation of PPARγ in monocytes and macrophages augmented release of pro-inflammatory and neurotoxic mediators like IL-1β, IL-6, TNFα, and iNOS[75,72].

Based on these findings, the major interest of this thesis was to analyze the role of PPARγ in myeloid cells on the inflammatory and neurotoxic immune responses and its effect on the development of EAE.

Various inflammatory mediators produced by microglial cells and macrophages have been linked to activation and recruitment of disease-promoting cells during EAE and MS, respectively[6,45,56,120]. To address the role of PPARγ in myeloid cells during EAE, alterations of these different parameters due to PPARγ ablation were analyzed in this work. *In vitro* analysis demonstrated that PPARγ controls the production of TNFα, IL-6, NO, ROS, and IL-12 in bone-marrow-derived macrophages and primary microglial cells (Figure 7a+b). IL-12 and IL-6 are cytokines known to be involved in the polarization of T_H1 cells and T_H17 cells. These two important T cell subsets are involved in the onset and maintenance of EAE and MS[16,41]. Therefore, a suppression of IL-6 and IL-12 production in macrophages and microglial cells by PPARγ might control activation and differentiation of these T cell subsets during the development of EAE. This hypothesis is supported by the observation that LysM-PPARγ^{KO} mice showed a significant increase in the quantity of T cells within the CNS compared to LysM-PPARγ^{WT} mice (Figure 1d). Although the percentage of activated $CD4^+$ T cells in the draining lymph nodes and the CNS did not show an increase of IFNγ- or IL-17A-producing T cells in the LysM-PPARγ^{KO} mice compared to wildtype littermates (Figure 5a+b), the absolute numbers of T cells within the CNS were increased in LysM-PPARγ^{KO} mice compared to wildtype controls (Figure 1d).

TNFα and IL-6 refer to activation of macrophages and microglial cells, and TNFα as well as NO have been implicated to directly contribute to MS pathology[63]: TNFα induces NO-synthase[124,125], which leads to cell death of oligodendrocytes[126]. As oligodendrocytes synthesize and maintain the myelin sheath, destruction of these cells results in enhanced demyelination and impaired remyelination during ongoing CNS autoimmunity[62-64]. As PPARγ controls release of these neurotoxic mediators in both, macrophages (Figure 7a) and microglia (Figure 7b) and mice lacking PPARγ in myeloid cells showed a significantly higher demyelination during EAE (Figure 1c), a prominent role of PPARγ in these cells can be assumed for the reduction of demyelination and neuronal damage.

The surface molecules MHC-II and CD40 are both known to be important for the development and perpetuation of disease. Presentation of self-antigens presented by myeloid cells in the context of MHC-II to encephalitogenic, auto-reactive T cells, is a crucial step for T cell reactivation and initiation of disease as inhibition of MHC-II-expression ameliorates the clinical course of EAE[127]. Furthermore, lack of CD40-expression within the CNS decreases the efficacy of T cell priming[118]. These data identify PPARγ in macrophages and microglia as a prominent regulator for the expression of these molecules, as CD40- as well as MHC-II-expression were increased in PPARγ-ablated microglia and CNS-derived macrophages compared to myeloid cells derived from wildtype mice (Figure 4b-e).

We therefore suggest that PPARγ in myeloid cells does not only control pro-inflammatory responses involved in innate immune activation and organ damage, but also controls the capacity of these cells to locally reactivate invading encephalitogenic T cells within the CNS.

Taken together, PPARγ in myeloid cells is a potent regulator for innate and adaptive immune responses important for the development and perpetuation of EAE and MS: On the one hand, production of neurotoxic mediators like TNFα and ROS as well as iNOS is controlled by PPARγ, thus, leading to demyelination and neuronal damage (Figure 11a). On the other hand, PPARγ in myeloid cells regulates several components regarding signal 1, 2, and 3 which are required for T cell priming and activation: MHC-II (signal 1), CD40 (signal 2), and IL-6, IL-12 (signal 3) were all controlled by PPARγ in myeloid cells (Figure 11b). Therefore, PPARγ in macrophages and microglial cells is a promising target for innate and adaptive immune responses promoting the development and perpetuation of EAE.

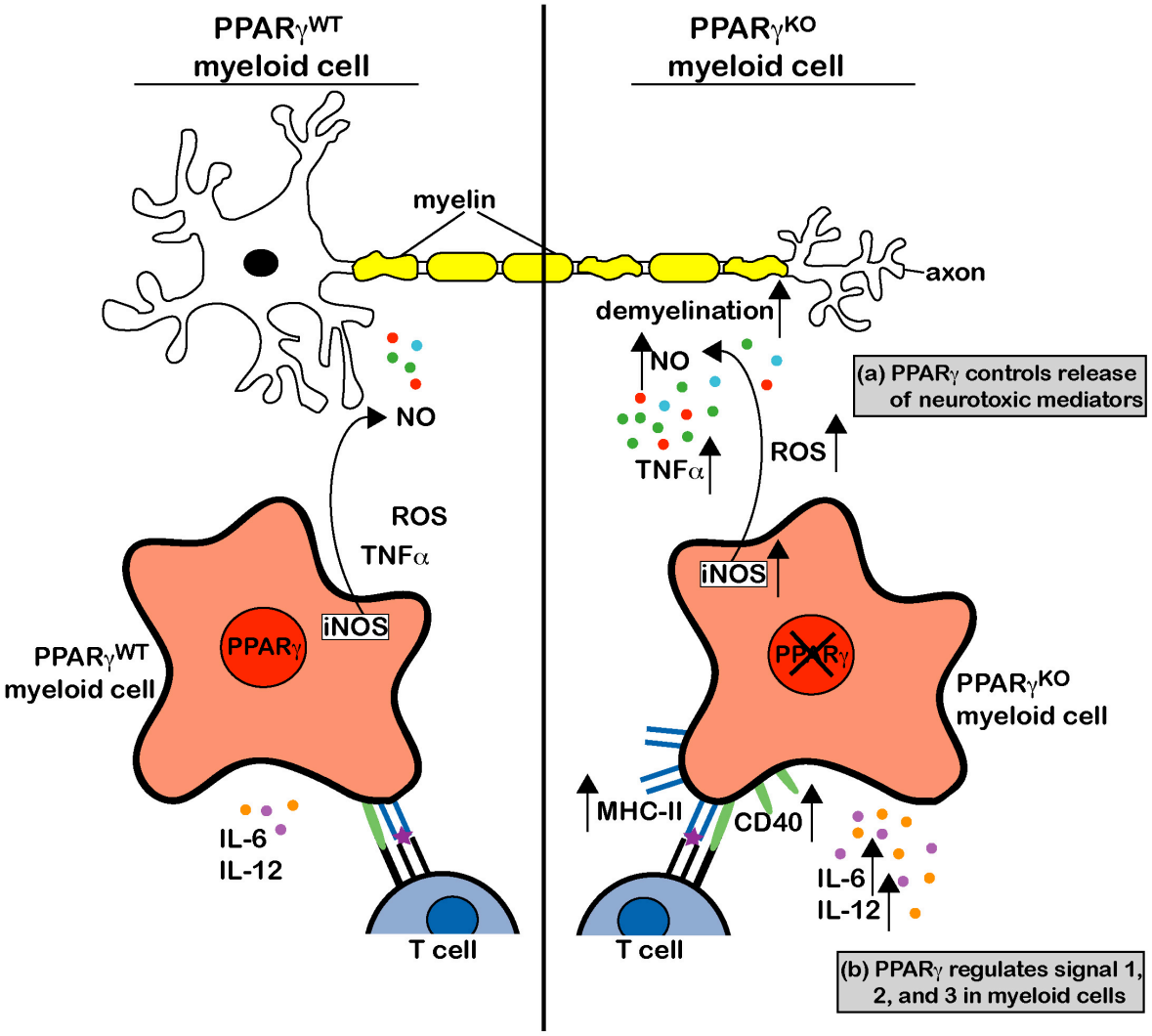

Figure 11 PPARγ in macrophages and microglia is involved in the regulation of innate and adaptive immune responses promoting EAE-development and perpetuation of disease

(a) Expression of neurotoxic mediators by myeloid cells is controlled by PPARγ **(b)** Upregulation of signal 1 (MHC-II), signal 2 (CD40) and signal 3 (IL-6, IL-12) is regulated by PPARγ.

Also *in vivo* we could demonstrate that PPARγ in myeloid cells controls the production of different pro-inflammatory cytokines, as well as neurotoxic mediators within the CNS (Figure 2a) affecting demyelination, neuronal damage (Figure 1 c) and the disruption of the BBB[128], leading to an aggravated disease course in LysM-PPARγ^{KO} mice compared to wildtype littermates (Figure 1b). Together, these data hence provide evidence that regulation of these parameters by PPARγ in myeloid cells might influence the development and perpetuation of disease during EAE and MS. Beside cytokines and pro-inflammatory mediators implicated in disease progression and direct organ damage, also levels of several chemokines like CCL2, CCL3, and CXCL10 were controlled by PPARγ in myeloid cells

during the effector phase of EAE (Figure 2b). These chemokines specifically target cells of the immune system and recruit monocytes, macrophages, microglia, and T cells to the site of inflammation during EAE. In line with this, histological analysis of the spinal cord of EAE-diseased mice showed that PPARγ-ablation in myeloid cells resulted in an increased infiltration of T cells and macrophages during the effector phase of EAE (Figure 2b and Figure 1d), which in turn results in recruitment and infiltration of further immune cells reflected by increased numbers of T cells and macrophages.

In conclusion, during EAE PPARγ in myeloid cells regulates the production of pro-inflammatory mediators activating immune cells and neurotoxic mediators, leading to neuronal and axonal damage, and the release of chemokines resulting in further recruitment of immune cells thus perpetuating local inflammation. These observations underline the hypothesis that PPARγ in myeloid cells plays a prominent role for disease progression during the effector phase of EAE.

Importantly, differences between LysM-PPARγ^{KO} mice and LysM-PPARγ^{WT} mice were observed exclusively during the effector phase of EAE, which suggests that PPARγ in myeloid cells is a dispensable factor during the initial T cell-dependent phase of disease (Figure 1b). Besides their already discussed inflammatory and neurotoxic effects, myeloid cells may also contribute to T cell priming. Therefore the question arose, whether the aggravated course of disease during the effector phase in LysM-PPARγ^{KO} mice compared to wildtype littermates is due to an increased T cell priming in the periphery during the initial priming phase.

As differences in T cell priming were not observed in LysM-PPARγ^{KO} mice compared to wildtype littermates both in the periphery and within the CNS during EAE (Figure 5a-c), it can be concluded that initial T cell activation in the periphery by antigen-presenting cells was not modulated by PPARγ. Moreover, upon adoptive transfer of primed wildtype CD4^{+} T cells into LysM-PPARγ^{KO} mice still revealed a significant difference in disease severity, thus demonstrating that these observed differences are not due to T cell priming (Figure 5d).

In conclusion, these data do not support a relevant involvement of PPARγ in macrophages for peripheral T cell priming during disease induction. Instead, it can be assumed that PPARγ in myeloid cells is involved in limiting recruitment of T cells to the inflamed CNS during the effector phase of EAE, as the number of T cells was increased in the CNS of LysM-PPARγ^{KO} mice compared to wildtype littermates at later timepoints (Figure 1d).

In EAE-experiments Natarajan et al. revealed that PPARγ-deficient heterozygous mice developed an exacerbated course of disease with prolonged clinical paralysis compared to their wildtype littermates[129]. Our group has previously demonstrated that a conditional knock-out of PPARγ in $CD4^+$ T cells resulted in a selective increase of T_H17 cells within the CNS[55], which are pivotal for the development of EAE[130]. By augmenting T_H17 differentiation, the PPARγ-ablation in $CD4^+$ T cells resulted in a disease aggravation during the initial priming phase but not the effetor phase during EAE [55]. Interestingly, the data presented in this thesis reveal that PPARγ in myeloid cells regulates the effector phase of EAE only (Figure 1b), indicating that PPARγ exerts distinct immune-regulatory properties in different immune cell populations during different phases of CNS autoimmunity.

Although the contribution of astrocytes to EAE or MS is widely discussed[70,131,132], astrocytes are known to serve as APCs *in vitro* and contribute to release of pro-inflammatory cytokines and chemokines in response to a variety of stimuli[67,133] under the control of PPARγ *in vitro*[134]. However, disease pathology mediated by other CNS-resident cells like astrocytes and neuronal cells was not influenced by PPARγ as reflected by unaltered EAE disease course in Nestin-$PPAR\gamma^{KO}$ mice which lack PPARγ in astrocytes and neuronal cells (Figure 6b).

In summary, these different effects of PPARγ in various cells demonstrate different functions of PPARγ in distinct cell populations during EAE: In $CD4^+$ T cells PPARγ-ablation augmented T_H17 differentiation resulting in an aggravated initial priming phase, whereas PPARγ-ablation in myeloid cells caused a reinforcement of the inflammation and cell recruitment resulting in an aggravation during the effector phase, but PPARγ-ablation in other CNS-resident had cells no effect on the course of disease.

Systemic activation of PPARγ by oral application of a pharmacological PPARγ agonist (e.g. Pio), has been shown to ameliorate the course of disease during EAE[55,88]. In *ex vivo* experiments, our group previously showed that systemic activation of PPARγ by Pio resulted in reduced numbers of T_H17 cells during EAE within the CNS[55]. Moreover, systemic activation of PPARγ by i.p. administration of different PPARγ agonists decreased CNS inflammation and demyelination during the course of EAE[135]. Furthermore, activation of PPARγ by these agonists affected also macrophage activation *in vitro*[135].

Based on these findings, it can be hypothesized that pharmacological activation of PPARγ not only affects T cell activation but might also regulate inflammatory responses in monocytes, macrophages, and microglial cells during EAE. Accordingly, *in vitro* activation of PPARγ in macrophages and microglia showed reduced production of pro-inflammatory cytokines, neurotoxic mediators, chemokines, and surface markers, which are crucial for cell-cell-interaction during the development and perpetuation of EAE.
In light of these promising results from animal studies, Klotz et al. demonstrated a beneficial role of PPARγ in human immune cells: activation of PPARγ *ex vivo* suppressed T_H17 differentiation of $CD4^+$ T cells derived both from healthy individuals and MS patients[55]. Furthermore, *in vitro* activation of PPARγ revealed an anti-inflammatory and anti-proliferative effect in peripheral blood mononuclear cells (PBMCs) from healthy controls but also from MS patients[91] and in these patients a reduced expression of PPARγ was found. Interestingly, both PPARγ expression levels and PPARγ activity could be increased by activation with Pio when given before an acute inflammatory event[91]. Therefore, it can be hypothesized that continuous activation of PPARγ might control disease progression during MS if administration would start prior to an acute relapse.

Based on the observations made in this study, an important question arose: which subgroup of myeloid cells is the main target for PPARγ-mediated regulation during EAE. Based on the literature, the subpopulation of inflammatory monocytes seemed to be the most promising target for PPARγ activation during EAE.
Recruitment of these cells into the CNS has been shown to be a crucial step for the progression and perpetuation of CNS inflammation during EAE[98,100]. Various examinations showed that the chemokine CCL2 is essential for recruitment of inflammatory monocytes in this process[60,98,100]. Within the CNS, this chemokine is mainly produced by astrocytes, attracting inflammatory monocytes into the CNS[6,121].
In this work it was demonstrated that astrocytic CCL2-release is triggered by activated macrophages and microglial cells, and that the extent of astrocytic CCL2 production depends on PPARγ-activity in myeloid cells, as pre-activation of macrophages and microglia elicited CCL2-release in astrocytes, and this inflammation-dependent CCL2-release of astrocytes was significantly higher upon co-culture with PPARγ-deficient macrophages or microglia compared to coculture with wildtype myeloid cells (Figure 8a+b).

Oh et al., showed that human astrocytes produced CCL2 in response to TNFα and IL-1β[136]. Since expression of these cytokines was increased in PPARγ-deficient macrophages and microglia (Figure 3, Figure 7a+b), we hypothesize that enhanced levels of IL-1β and TNFα in the CNS of EAE diseased LysM-PPARγ^{KO} mice might trigger high levels of astrocytic CCL2-release *in vivo*. Importantly, this increase of CCL2 was observed in the CNS in of LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} during the course of EAE (Figure 10c).

Local production of CCL2 within the CNS results in recruitment of CCR2^{+}Ly-6C^{hi}CX3CR1^{lo} inflammatory monocytes, which have recently been shown to be crucial for disease progression during EAE[98,100,118]. By a combination of monocyte transfer experiments and selective antibody-mediated ablation of CCR2 expressing monocytes during EAE, we could provide evidence that these inflammatory monocytes are the main target population of PPARγ during the effector phase of EAE, as transfer of PPARγ^{KO} monocytes resulted in a more aggravated disease course than transfer of PPARγ^{WT} monocytes, whereas ablation of CCR2^{+} monocytes completely abrogated all differences observed during EAE of LysM-PPARγ^{KO} versus LysM-PPARγ^{WT} mice.

Conclusion

The role of PPARγ in regulating the release of neurotoxic and pro-inflammatory mediators by myeloid cells *in vitro*, which are important factors for development and perpetuation of EAE and MS, has been analyzed before[75,72,115]. However, in this thesis we could show for the first time that PPARγ in myeloid cells *in vivo* controls different factors promoting development as well as perpetuation of disease during the effector phase of EAE.

In myeloid cells PPARγ controls the release of pro-inflammatory and neurotoxic mediators such as TNFα, NO, and ROS, causing an inflammatory milieu and neurotoxic damage (Figure 12d). Furthermore, we found that PPARγ in myeloid cells controls activation of macrophages and microglia reflected in the expression of surface molecules (CD40 and MHC-II) and cytokines (IL-6 and IL-12), promoting T cell-activation (Figure 12b). These signals promote development of T_H1 and T_H17 cells, which have been demonstrated to promote EAE and in turn activate myeloid cells[118]. Besides control of immune cell activation and reduction of neuronal damage within the CNS, PPARγ in myeloid cells controls release of IL-1β and TNFα by myeloid cells, and hence reduces astrocytic CCL2-release known to be relevant for further macrophages and – importantly – CCR2^{+} inflammatory monocyte recruitment from the periphery to the site of inflammation

(Figure 12e). Moreover, PPARγ in myeloid cells controlled the release of T cell attracting chemokines during EAE (Figure 12f).

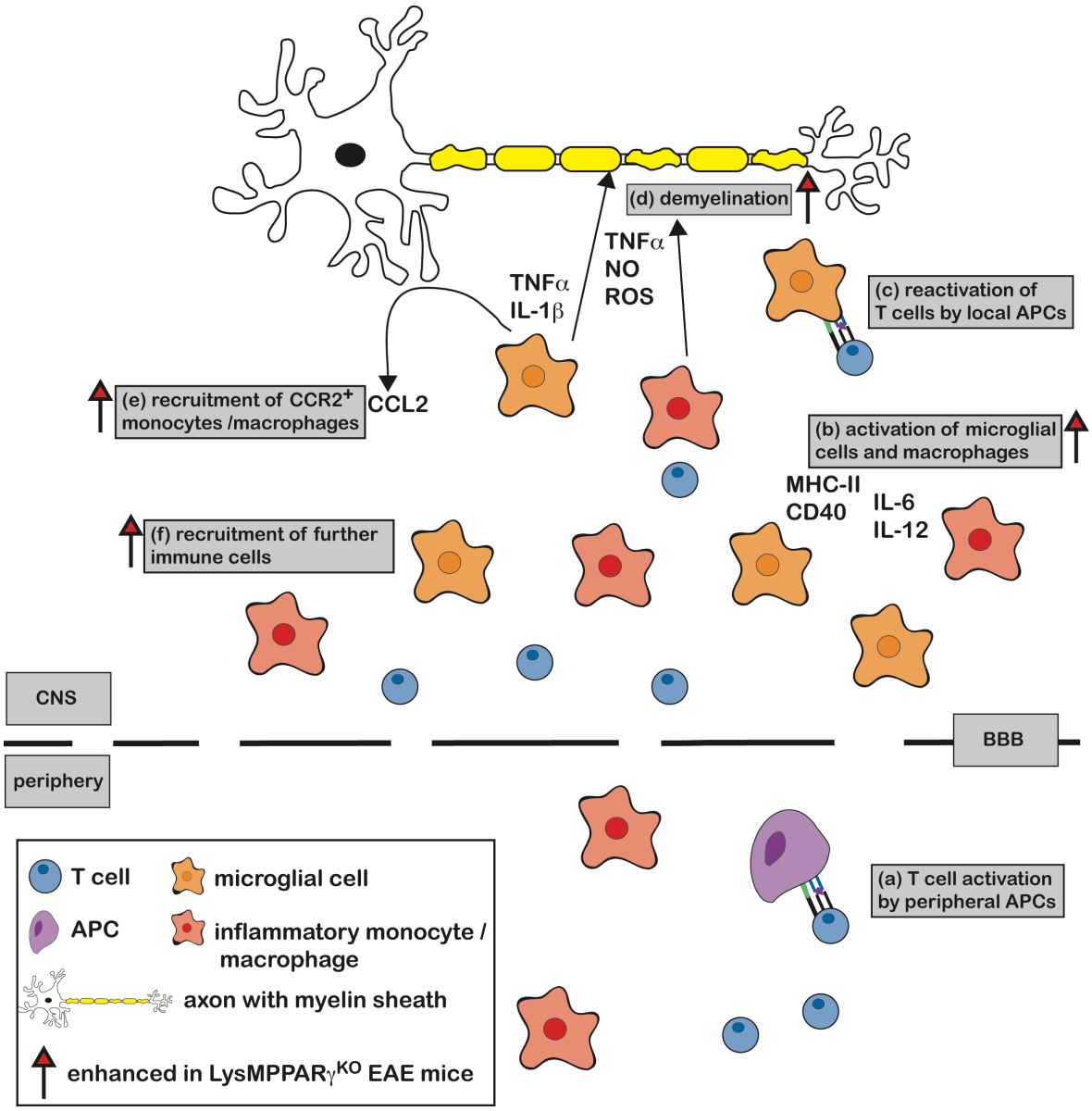

Figure 12 PPARγ in myeloid cells controls different mechanisms during the development and perpetuation of EAE

(a) Priming of auto-reactive cells in the periphery by APCs. **(b)** Upregulation of signal 1 (MHC-II), signal 2 (CD40) and signal 3 (IL-6, IL-12) in myeloid cells is regulated by PPARγ. **(c)** encephalitogenic T cells get reactivated by local APCs like microglia or CNS-invading macrophages. **(d)** Release of neurotoxic mediators resulting in demyelination is controlled by PPARγ in myeloid cells. **(e)** TNFα and IL-1β released by myeloid cells under the control of PPARγ trigger astrocytic CCL2-production, which attracts further $CCL2^{+}$ inflammatory monocytes to the side of inflammation. **(f)** Release of further chemokines recruiting immune cells into the CNS is controlled by PPARγ in myeloid cells. Red arrows indicate parts regulated by PPARγ in myeloid cells during EAE.

Importantly, in this thesis we could show that depletion of inflammatory monocytes in the periphery diminished the differences found in the course of disease between LysM-PPARγ^{KO} mice compared to LysM-PPARγ^{WT} mice, hence identifying this population as crucial target for PPARγ-mediated regulation of inflammation during the effector phase of EAE (Figure 13).

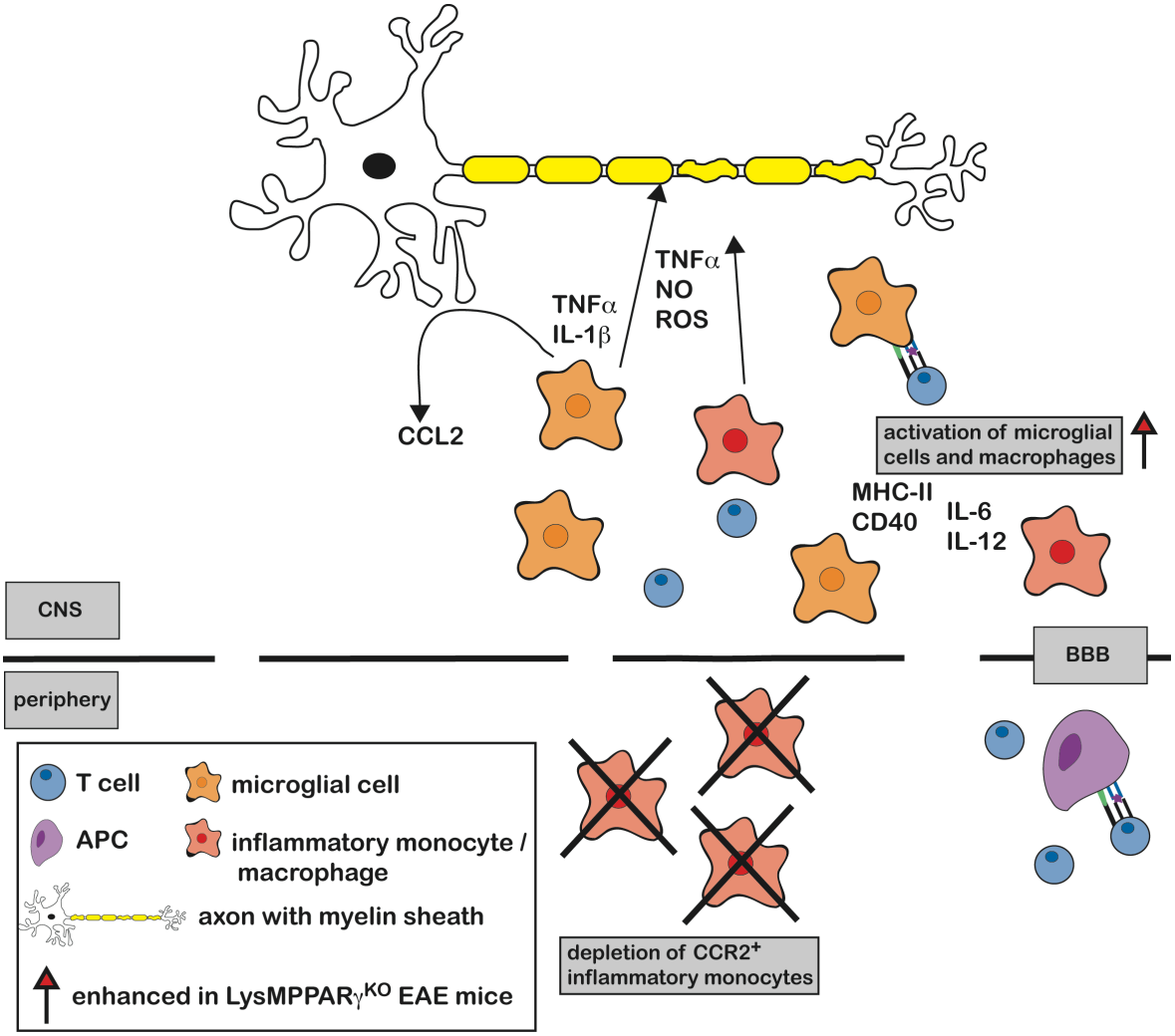

Figure 13 Depletion of CCR2^{+} inflammatory monocytes abolishes differences observed between LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice

Upon depletion of CCR2^{+}Ly-6C^{hi} inflammatory monocytes, differences in the severity of disease between LysM-PPARγ^{KO} and LysM-PPARγ^{WT} mice were abolished. Only activation of CNS-macrophages and microglia on a single cell level were increased in LysM-PPARγ^{KO} mice compared to wildtype littermates.

In conclusion, development of disease during EAE in LysM-PPARγ^{KO} mice in comparison to wildtype mice showed that PPARγ in myeloid cells controls disease during the effector phase of EAE in a complex way. We have shown that not only CNS-resident microglial cells, but also peripheral macrophages and especially inflammatory monocytes play an

important role during the development and perpetuation of disease. Therefore, myeloid cells represent a promising target for PPARγ-activation, which reduces inflammation, cell recruitment into the CNS, and neurotoxicity and thereby ameliorates severity of disease. Importantly, due to the fact that differences were only observed in the effector phase of disease, these data implicate that targeting myeloid cells for treatment of CNS-inflammation, e.g. by pharmacological PPARγ-activation, might still be effective when started after disease manifestation, which more closely reflects the human situation. Therefore, myeloid cells, especially inflammatory monocytes, exhibit a promising target for future therapy of CNS autoimmunity.

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Table of Figures

Auszüge dieser Arbeit waren Bestandteil folgender Publikationen und Kongressbeiträgen:

Publikationen

Licensing of myeloid cells promotes central nervous system autoimmunity and is controlled by peroxisome proliferator-activated receptor γ
Hucke S*, Floßdorf J*, Grützke B, Dunay I, Frenzel K, Jungverdorben J, Linnartz B, Mack M, Peitz M, Brüstle O, Kurts C, Klockgether T, Neumann H, Prinz M, Wiendl H, Knolle P, Klotz L.
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Kongressbeiträge

September 10	Vortrag und Posterpräsentation auf dem 40th Annual Meeting of the German Society for Immunology, Leipzig Titel: Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) ablation in microglia and macrophages augments CNS inflammation during EAE
September 09	Posterpräsentation auf dem 2nd European Congress of Immunology, Berlin Titel: Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) regulates microglial immunogenicity
September 08	Posterpräsentation auf dem Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGFI), Wien Titel: PPARγ controls microglial APC function