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Role of eosinophils in experimental autoimmune encephalomyelitis

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Summary

Experimental autoimmune encephalomyelitis (EAE) is the rodent model of multiple sclerosis (MS), a chronic autoimmune neuroinflammatory disease that has a devastating impact on various neurological functions of the patients. The hallmarks of both, MS and EAE, are neuroinflammation, demyelination and neuroaxonal degeneration. Various types of lymphoid and myeloid cells were shown to infiltrate the central nervous system and to participate in disease pathology. However, the role of eosinophil granulocytes has been less explored thus far. An early study showed that eosinophils infiltrate into the spinal cord of EAE mice and suggested their role in the disease progression. Recently, it was reported that eosinophils can play a protective role against EAE when mice are treated with an extract from helminths. Furthermore, it was shown that EAE development is not altered in mice deficient for interleukin-5, an important eosinophil pro-survival factor. Taken together, the role of eosinophils in EAE is currently unclear and needs to be investigated in detail.

In the present study, we use the active model of EAE, whereby we immunized the C57BL/6 mouse strain with MOG_{35-55} peptide emulsified in the complete Freund's adjuvant, in order to study a possible contribution of eosinophils to the disease pathology. Using the flow cytometry and RT-qPCR analysis of the spinal cord, we show that eosinophils infiltrate into the tissue in the course of EAE. The infiltration is likely driven by eosinophil chemoattractants, such as eotaxin-1, as the concentration of the latter was increased in the spinal cord during EAE, as shown on mRNA and protein level. Moreover, detailed flow cytometry analysis of spinal cord eosinophils revealed that they show signs of activation, namely an increase in CD11b and decrease in CCR-3 surface expression. Furthermore, we observed signs of degranulation of spinal cord eosinophils in EAE which was measured as a decrease of the side scatter parameter and an upregulation of CD63 surface expression. These data suggest a potential role of eosinophils in the pathology of EAE. In order to elucidate whether eosinophils are important for the disease development, eosinophil-deficient mice were subjected to EAE and the clinical development of the disease was observed. For this purpose, we used two independent models of eosinophil deficiency - ΔdblGATA1 and interleukin-5-depleted mice. ΔdblGATA1 mice are a genetically manipulated mouse strain bearing a deletion in GATA1 promoter that causes a specific depletion of eosinophils. Interestingly, clinical development of EAE was not affected in these mice when compared to their wild-type controls. As a next step, we depleted eosinophils by injecting wild-type mice with an antibody against the eosinophil pro-survival factor interleukin-5 in order to reduce eosinophil numbers in the effector phase of EAE. In accordance with the result from the experiment with ΔdblGATA1 mice, EAE progression was not altered in the eosinophil-depleted mice when compared to mice that were injected with an isotype control antibody. Further, we analyzed the neuroinflammation and demyelination in the spinal cord of

ΔdblGATA1 mice subjected to EAE. Specifically, the infiltration of inflammatory cell populations, including CD4 and CD8 T cells, neutrophils and macrophages, was assessed by flow cytometry. In agreement with the unchanged clinical EAE development, inflammatory cell infiltration was not affected in ΔdblGATA1 mice. Furthermore, we analyzed expression of pro-inflammatory cytokines in the spinal cord of ΔdblGATA1 mice subjected to EAE in order to better characterize the inflammatory status. No significant changes were detected further confirming that eosinophils do not contribute to neuroinflammation in EAE. Finally, we assessed the demyelination in the spinal cord of ΔdblGATA1 EAE mice using luxol fast blue staining to detect myelin. In accordance with the unaffected clinical development and inflammatory status, we did not observe any difference in the spinal cord demyelination in ΔdblGATA1 mice when compared to their wild-type littermates.

Taken together, although eosinophils infiltrate into the spinal cord of EAE mice and are activated and degranulate therein, they are dispensable for EAE development.

Zusammenfassung

Experimentelle Autoimmunencephalomyelitis (EAE) ist das Nagetiermodell für Multiple Sklerose (MS), eine chronische, neuroinflammatorische Autoimmunerkrankung, welche drastische Auswirkungen auf zahlreiche neurologische Funktionen des Patienten hat. Charakteristisch für beide, MS und EAE, sind Neuroinflammation, Demyelinisierung und neuroaxonale Degeneration. Es wurde gezeigt, dass verschiedene Typen lymphoider und myeloider Zellen das zentrale Nervensystem infiltrieren und zur Pathologie der Krankheit beitragen. Die Rolle von eosinophilen Granulocyten dabei wurde bis jetzt jedoch noch nicht genau untersucht. Eine frühe Studie zeigte, dass Eosinophile das Rückenmark von EAE-Mäusen infiltrieren und legt nahe, dass sie eine Rolle beim Fortschreiten der Krankheit spielen. Kürzlich wurde berichtet, dass Eosinophile eine schützende Rolle gegen EAE spielen, wenn Mäuse mit einem Helminthextrakt behandelt wurden. Außerdem wurde gezeigt, dass sich der Verlauf von EAE in Interleukin-5 – ein wichtiger Überlebensfaktor für Eosinophile -defizienten Mäusen nicht verändert. Insgesamt, ist die Rolle von Eosinophilen in EAE derzeit unklar und bedarf detaillierter Untersuchung.

In der vorliegenden Studie nutzen wir das aktive EAE-Modell, wobei wir Mäuse des C57Bl/6 Stammes mit MOG_{35-55} Peptid emulgiert in Komplettmedium Freund's Adjuvant injizieren, um die mögliche Beteiligung von Eosinophilen am pathologischen Verlauf von EAE zu erforschen. Mit Hilfe von Durchflusszytometrie und RT-qPCR Analyse des Rückenmarks zeigen wir, dass im Verlauf von EAE Eosinophile in das Gewebe infiltrieren. Die Infiltration wird wahrscheinlich durch chemische Lockstoffe von Eosinophilen, wie Eotaxin-1, angetrieben, da dessen Konzentration im Rückenmark auf mRNA- und Proteinebene während der EAE erhöht war. Weiterhin legte detaillierte Durchflusszytometrieanalyse von Eosinophilen des Rückenmarks dar, dass diese Zeichen von Aktivierung aufweisen, namentlich ein Anstieg in CD11b und eine Abnahme von CCR-3 Oberflächenexpression. Außerdem beobachteten wir Anzeichen von Degranulation der Rückenmarkseosinophilen bei EAE, was über eine Abnahme des Seitwärtsstreulichtparameters und einer Hochregulierung von CD63 Oberflächenexpression bestimmt wurde. Diese Daten legen eine potentielle Rolle von Eosinophilen in der Pathologie von EAE nahe. Um festzustellen, ob Eosinophile wichtig für das Fortschreiten der Krankheit sind, wurde EAE in Eosinophil-defizienten Mäusen induziert und die klinische Entwicklung der Krankheit aufgezeichnet. Dafür wurden zwei unabhängige Modelle der Eosinophildefizienz herangezogen - ΔdblGATA1 und Interleukin-5-dezimierte Mäuse. ΔdblGATA1 Mäuse gehören zu einem genetisch manipulierten Mausstamm, welcher eine Deletion innerhalb des GATA1 Promotors aufweist, die eine spezifische Dezimierung von Eosinophilen hervorruft. Interessanterweise war die klinische Entwicklung von EAE im Vergleich zu den Wildtypkontrollen unverändert. Im nächsten Schritt dezimierten wir Eosinophile durch Injektion von Antikörpern gegen den

Eosinophilüberlebensfaktor Interleukin-5 in Wildtypmäusen, um die Anzahl von Eosinophilen in der Effektorphase von EAE zu reduzieren. Im Einklang mit dem Ergebnis vom Experiment mit ΔdblGATA1 Mäusen war das Fortschreiten der EAE in den Eosinophil-dezimierten Mäusen im Vergleich zu den Mäusen, die mit dem Isotypkontrollantikörper injiziert wurden, unverändert. Weiterhin untersuchten wir Neuroinflammation und Demyelinisierung des Rückenmarks von ΔdblGATA1 Mäusen, die dem EAE Modell unterzogen wurden. Insbesondere die Infiltration von inflammatorischen Zellpopulationen, inklusive CD4- und CD8- T-Zellen, Neutrophilen und Makrophagen wurden mit Hilfe von Durchflusszytometrie analysiert. In Übereinstimmung mit der unveränderten klinischen EAE Entwicklung war die inflammatorische Zellinfiltration in ΔdblGATA1 Mäusen nicht beeinträchtigt. Des Weiteren analysierten wir die Expression von proinflammatorischen Zytokinen im Rückenmark von ΔdblGATA1 Mäusen während EAE, um den inflammatorischen Status besser zu charakterisieren. Dabei wurden keine signifikanten Veränderungen entdeckt, was zusätzlich darauf hindeutet, dass Eosinophile nicht zur Neuroinflammation bei EAE beitragen. Schlussendlich, untersuchten wir die Demyelinisierung im Rückenmark von ΔdblGATA1 Mäusen. Im Einklang mit der unbeeinträchtigten klinischen Entwicklung und dem unveränderten inflammatorischen Status, können wir keine Unterschiede in der Demyelinisierung des Rückenmarks von ΔdblGATA1 Mäusen im Vergleich mit Wildtypwurfgeschwistern feststellen.

Zusammenfassend sind Eosinophile nicht entscheidend an der Entwicklung von EAE beteiligt, obwohl sie in das Rückenmark von EAE-Mäusen einwandern, dort aktiviert werden und degranulieren.

Table of contents

List of figures

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
APRIL	a proliferation-inducing ligand
ASIC1	acid-sensing ion channel-1
AUC	area under the curve
BCA	bicinchoninic acid assay
BSA	bovine serum albumine
CCR3	C-C chemokine receptor type 3
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
dNTPs	deoxynucleotides
DTA	diphtheria toxin A
EAE	experimental autoimmune encephalomyelitis
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eMBP	eosinophil major basic protein
EPX	eosinophil peroxidase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GATA1	GATA-binding factor 1
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon γ
IgA	immunoglobulin A
IL	interleukin
IL2RA	interleukin-2 receptor A
IL7R	interleukin-7 receptor
iNOS	inducible nitric oxide synthase

LFB	luxol fast blue
MACS	magnetic-activated cell sorting
MBP	myelin basic protein
MHCII	major histocompatibility complex II
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MS	multiple sclerosis
NK	natural killer
NMO	neuromylelitis optica
NOD	non-obese diabetic
PBS	phosphate buffered saline
PFA	paraformaldehyde
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI medium	Roswell Park Memorial Institute medium
RPS29	40S ribosomal protein S29
RRMS	relapsing-remitting multiple sclerosis
RT-qPCR	real-time quantitative polymerase chain reaction
SEM	standard error of mean
SPMS	secondary progressive multiple sclerosis
SSC	side scatter
TCR	T-cell receptor
TGFβ	transforming growth factor β
Th1	T helper 1
Th17	T helper 17
TLR2	toll-like receptor 2
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Tregs	regulatory T cells
TRMP4	transient receptor potential melastatin 4
WT	wild-type

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune neuroinflammatory demyelinating disease affecting about 2.5 million people world-wide. The majority of the patients are women and the onset of the disease is typically around 30 years of age. The pathology of the disease is characterized by lesions (also called plaques), which are located in the central nervous system (CNS), and are linked with the infiltration of immune inflammatory cells, including predominantly macrophages, CD8 and CD4 T cells, B cells and plasma cells. These cells trigger CNS inflammation that leads to the activation of resident glial cells, demyelination of neurons and subsequent neuroaxonal damage. As the disease progresses, the impaired nerve conduction in various regions of the brain and spinal cord causes a range of neurological dysfunctions including motor incoordination, weakness, numbness, and sensory and cognitive disturbances that often lead to a severe disability, thereby, substantially worsening the quality of life (Dendrou et al., 2015).

Based on the clinical and magnetic resonance imaging activity and the clinical disease progression, MS is classified into four subtypes. The majority of the MS patients (approximately 85%) suffer from the relapsing-remitting MS (RRMS) characterized by attacks of the neurological dysfunctions (relapses) followed by periods of complete or partial recovery (remissions). RRMS often progresses into the secondary progressive MS (SPMS) distinguished by a gradual worsening of the symptoms. In approximately 10 % of the cases, MS shows the steady worsening phenotype already from the onset of symptoms. This MS subtype is classified as primary progressive MS (PPMS). The fourth subtype of MS is the clinically isolated syndrome (CIS) and represents the first clinical manifestation of MS but has not met the MS criteria of dissemination in time, yet (Lublin et al., 2014).

1.1.1 Risk factors for MS

The exact cause of MS remains unknown. However, both, genetic and environmental risk factors were described to be involved in the disease etiology. Among the genetic risk factors, mostly immune system-related genes were identified. Namely, variants of human leukocyte antigen (HLA) class II were suggested to have the largest effect on MS susceptibility, possibly directing CD4 T cell specific immune response against CNS antigens. Moreover, genome-wide association studies revealed several susceptibility genes outside the HLA regions, including predominantly genes involved in T and B lymphocyte activation pathways, e.g. genes for cytokine receptors *IL2RA* and *IL7R*. Polymorphisms in these genes are probably responsible for modifying of the threshold of immune system activation rather that the CNS specificity (Gourraud et al., 2012, Beecham et al., 2013). Besides the genetic risk factors, several environmental factors were shown to play a role in MS development, including infections, vaccinations, other diseases, traumatic events and physical and biochemical agents. Out of all factors, Epstein-Barr virus infection, infectious mononucleosis and smoking are those proven to be associated with MS development (Belbasis et al., 2015).

1.1.2 Immunopathology of MS

It is mostly believed that the primary event of MS induction is an autoimmune T cell-mediated attack directed against antigens in CNS, especially myelin components, which initiates the neurodegenerative processes mentioned above. This hypothesis is called „outside-in“ model of MS. However, there are also findings indicating that the neurodegeneration might be the initiation impulse for MS development. This “inside-out” MS model suggests that the neurodegenerative process generates a tissue damage that activates the primed immune system (Stys et al., 2012).

According to the “outside-in” model (Figure 1), there are myelin-specific T lymphocytes that accidentally escaped the negative selection in the thymus. In healthy individuals, these

cells are controlled by mechanisms of peripheral tolerance so that their activation is prevented. However, when these mechanisms fail, either because of non-functional regulatory T cells or due to the resistance of the autoreactive T cells to the action of the regulatory T cells, the autoreactive T cells are activated and differentiate into effector T cell subsets, most importantly cytotoxic CD8 T cells and CD4 helper T cells of Th1 or Th17 subtype. The activation can be mediated through molecular mimicry, newly formed autoantigens, bystander activation or antigens released from CNS. The activated effector T cells then activate other immune cell types, like B lymphocytes and monocytes, and infiltrate into CNS, crossing the blood-brain barrier, where they induce the chronic neuroinflammation (Dendrou et al., 2015).

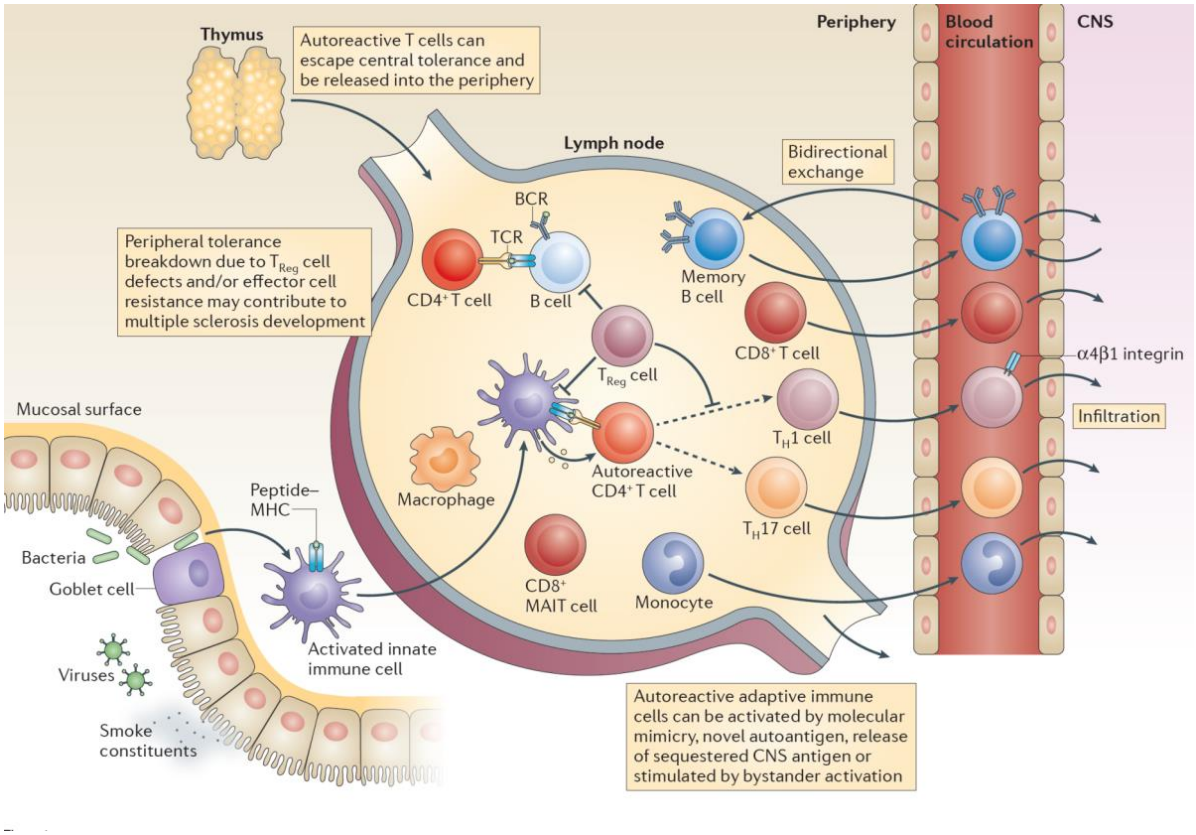

Figure 1 Initiation of MS (from Dendrou et al., 2015)

A scheme presenting the initiation events of MS according to the "outside-in" model.

A wide range of immune cells from both, lymphoid and myeloid lineage were shown to infiltrate into the brain and spinal cord in the course of MS. Among the CD4 T helper cell subsets, Th1 and Th17 lymphocytes are the main populations implicated in the disease

pathology. The typical Th1 cell-produced cytokines, interferon γ (IFNγ) and tumor necrosis factor (TNF), were shown to influence the disease development significantly, however, their exact role remains controversial. CD4 T cells isolated from MS patients express higher levels of IFNγ in comparison to healthy donors (Crawford et al., 2004). In accordance, a treatment of SPMS patients with an IFNγ-blocking antibody ameliorates the disease progression (Skurkovich et al., 2001) and an administration of recombinant IFNγ led to an exacerbation of the disease in RRMS patients (Panitch et al., 1987). Similarly, IFNγ overexpression by oligodendrocytes prevented disease remission in EAE, the mouse model of MS (Renno et al., 1998). On the other hand, studies on mice lacking IFNγ or its receptor showed that IFNγ is dispensable for EAE development (Ferber et al., 1996, Willenborg et al., 1996). Moreover, treatment of EAE mice with antibodies blocking IFNγ enhanced the disease progression (Billiau et al., 1988, Duong et al., 1992). Similarly, ambiguous results were reported about a role of TNF in MS. A blockage of TNF with a soluble receptor reduced disease severity in EAE (Korner et al., 1995, Korner et al., 1997a, Glabinski et al., 2004, Selmaj et al., 1995), indicating a possible use in MS treatment. However, a similar treatment of MS patients led to disease exacerbations (1999). Contradictory data were obtained when TNF knock-out mice were subjected to EAE since both, delayed onset (Sean Riminton et al., 1998, Korner et al., 1997b, Kassiotis et al., 1999) and disease exacerbation (Liu et al., 1998) were observed in these mice. This discrepancy might be caused by opposing actions of TNF receptors since TNF receptor 1 signaling seems to have a pro-inflammatory effect while TNF receptor 2 acts in an anti-inflammatory way (Eugster et al., 1999).

Furthermore, Th17 cells were implicated in the pathology of MS and EAE. In MS, numbers of Th17 cells in cerebrospinal fluid (CSF) were shown to be increased in patients with the disease relapse in comparison to patients in remission (Brucklacher-Waldert et al., 2009). Moreover, interleukin-17 (IL-17), the product of Th17 cells, was suggested to disrupt the blood-brain barrier, thereby promoting the immune cell infiltration (Kebir et al., 2007). Additionally, Th17 cells were shown to infiltrate into CNS in the course of EAE, which appeared to be necessary for the disease development (Reboldi et al., 2009). Studies using

IL-17 and IL-17 receptor knock-out mice revealed the importance of this cytokine in EAE pathology (Gonzalez-Garcia et al., 2009, Komiyama et al., 2006). However, treatment of RRMS patients with an antibody against a subunit of interleukin-23 (IL-23), a Th17 differentiation factor, failed to ameliorate disease development (Segal et al., 2008).

In addition to CD4 T subsets, CD8 T cells were shown to contribute to the pathology of MS and EAE significantly. Clonally expanded CD8 T cells were found in brain lesions of MS patients and showed even lower heterogeneity that CD4 T cells (Babbe et al., 2000, Skulina et al., 2004). Moreover, clonally expanded CD8 T cells are also present in CSF and blood of MS patients and persist there for more than 5 years (Skulina et al., 2004). CD8 T cells are also well known to infiltrate CNS of mice subjected to EAE and are important for disease development (Camara et al., 2013, Luo et al., 2014). Moreover, the adoptive transfer of myelin-specific CD8 T cells induces EAE which is even more severe than EAE induced by the direct immunization with myelin antigen (Sun et al., 2001, Huseby et al., 2001).

In contrast to the T cell subsets discussed above, having mainly pro-inflammatory roles, regulatory T cells (Tregs) were suggested to protect against MS. Importantly, there is a decreased proportion of Tregs in blood of patients with RRMS when compared to healthy individuals (Venken et al., 2008), and the frequency of Tregs is increased in blood of patients being in the remission (Dalla Libera et al., 2011, Peelen et al., 2011). Moreover, an impaired function of Tregs in MS patients was reported (Fletcher et al., 2009, Viglietta et al., 2004, Haas et al., 2005). EAE studies confirmed the protective role of Tregs since they accumulate in CNS of mice in the recovery phase of EAE and their depletion inhibits the recovery (McGeachy et al., 2005, O'Connor et al., 2007).

Opposing roles in MS pathology were reported for B cells, another lymphocyte subset. On one hand, B cell deficiency, induced either by genetic manipulation or antibody-mediated depletion (anti-CD20 injection before EAE induction), led to an inhibition of disease recovery or to its overall exacerbation, respectively (Wolf et al., 1996, Matsushita et al., 2008), suggesting a regulatory role of B cells in EAE. As a mechanism, the production of interleukin-10 (IL-10) (Fillatreau et al., 2002) and interleukin-35 (IL-35) (Shen et al., 2014), and induction

of Tregs (Weber et al., 2010) were suggested. On the other hand, B cells were shown to promote the disease development since B cell depletion induced by anti-CD20 injection after the disease onset was able to ameliorate EAE development (Matsushita et al., 2008, Weber et al., 2010). Studies by Matsushita and Weber suggest that B cells promote EAE when acting as the antigen-presenting cells. Moreover, B cell-derived interleukin-6 (IL-6) seems to contribute to the pathogenic role of B cells both, in EAE and MS (Barr et al., 2012). Indeed, B cell depletion appeared to be a promising therapy of MS since monoclonal antibodies against CD20 passed phase II and III of clinical trials for treatment of RRMS and PPMS, respectively (Hauser et al., 2008, Montalban et al., 2017).

Besides the above discussed lymphoid cells, myeloid cells were also implicated in the pathology of MS. Neutrophils, although present in relatively low numbers in MS lesions, seem to contribute to the disease development. Numbers of blood neutrophils are increased in MS patients and they exhibit a primed phenotype (Naegele et al., 2012, Ziaber et al., 1998, Guarnieri et al., 1985, Hertwig et al., 2016). Furthermore, neutrophils accumulate in CSF of MS patients and their numbers correlate with increased levels of IL-17A that is implicated in the neutrophil chemoattraction (Kostic et al., 2014). Another example of a neutrophil-attracting chemokine increased in CSF of MS patients is interleukin-8 (IL-8) (Ishizu et al., 2005). Moreover, expression of granulocyte colony-stimulating factor (G-CSF), the neutrophil activating cytokine, is upregulated in MS lesions (Lock et al., 2002) and its administration to MS patients can worsen the disease (Openshaw et al., 2000). The important role of G-CSF was confirmed in EAE model. Mice deficient for G-CSF receptor showed reduced neutrophil infiltration into the spinal cord and an ameliorated disease progression (Rumble et al., 2015). Other EAE studies demonstrated that neutrophils might contribute especially to the initiation of the disease when stimulating maturation of antigen presenting cells (Steinbach et al., 2013) and facilitating the blood-brain barrier breakdown (Carlson et al., 2008, Christy et al., 2013).

Other myeloid populations playing a crucial role in MS pathology are macrophages and microglia, the macrophage-like glial cells. Although sharing many features, which also makes

it difficult to distinguish them in MS studies, macrophages and microglia are of a different origin. While CNS macrophages differentiate from bone marrow-derived monocytes, primitive yolk sac-derived macrophages populate CNS during the embryonic development and give rise to microglia (Ginhoux et al., 2010). Both of these cell types were shown to promote MS and EAE progression. Microglia are activated already before the disease onset and immune cell infiltration, proliferate and express molecules necessary for antigen presentation (Ponomarev et al., 2005). Later, monocytes infiltrate into CNS, differentiate to macrophages and promote disease progression (Ajami et al., 2011). Several studies revealed that inhibition of macrophages and microglia ameliorates EAE development (Bhasin et al., 2007, Wu et al., 2013, Ponomarev et al., 2011, Goldmann et al., 2013). However, microglia were also shown to clear the myelin debris, thereby attenuating the neuroinflammation (Yamasaki et al., 2014), and to produce neurotrophic factors like insulin-like growth factor 1, thereby promoting tissue repair (Wlodarczyk et al., 2015). These contradictory reports about the contribution of macrophage/microglia cells to MS could be explained by the existence of two distinct subtypes characterized by pro- (neurotoxic) and anti-inflammatory (neuroprotective) properties that are called M1 and M2 cells, respectively (Bogie et al., 2014). Indeed, there are studies revealing that M1 cells promote EAE development. On the other hand, M2 cells perform neuroprotective functions (Liu et al., 2013, Mikita et al., 2011, Moreno et al., 2014).

1.1.3 Neuroaxonal damage in MS

There are several ways how the CNS-infiltrated immune cells cause the neuroaxonal damage. First, T cells directly induce apoptosis in neuronal cells by expressing ligands of death receptors, e.g. Fas ligand (Giuliani et al., 2003) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Aktas et al., 2005), or by secretion of perforin (Nitsch et al., 2004). Further, activated macrophages and microglia produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) that cause mitochondrial dysfunction followed by energy deficiency in neuronal cells and these changes lead to the focal axonal degeneration

(Nikic et al., 2011, Fischer et al., 2012). Indeed, impaired mitochondrial function, shown as complex IV inactivation, was observed in the demyelinated axons within MS lesions (Mahad et al., 2009). Demyelination leads to upregulation and redistribution of ion channels, including transient receptor potential melastatin 4 (TRMP4), acid-sensing ion channel-1 (ASIC1), Nav1.2, Nav1.6 and Na+/Ca2+ exchanger, which in turn causes increased intracellular concentration of calcium and subsequent neuronal death (Craner et al., 2004, Friese et al., 2007, Schattling et al., 2012). Further, excessive glutamate levels were proposed to play a role in the neuroaxonal damage in MS when increased glutamate concentrations were observed in the acute MS lesions in comparison to the normally appearing white matter (Srinivasan et al., 2005). Indeed, EAE studies revealed that activated immune cells produce glutamate that exerts its excitotoxic effect on neurons and oligodendrocytes (Pitt et al., 2000). This effect is probably mediated by an increase of intracellular calcium levels to a toxic concentration (Nitsch et al., 2004). Furthermore, increased glutamate sensitivity of neurons can be induced by a proinflammatory cytokine IFNγ that forms a calcium-permeable complex with a subunit of glutamate receptor AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic) (Mizuno et al., 2008). Taken together, these pathological changes lead to neuroaxonal damage and subsequent neuronal deficits as discussed above.

1.2 Experimental autoimmune encephalomyelitis

EAE is an animal model of MS that helps us to investigate various aspects of MS pathology, including neuroinflammation and demyelination (Rangachari and Kuchroo, 2013). It was discovered accidentally as a side effect of rabies vaccination at the end of the 19th century. Louis Pasteur and his colleagues injected dogs with spinal cord homogenates isolated from rabbits infected with attenuated rabies virus. Apart from the protection against the active virus, however, they often observed neurological complications in the injected animals, including paralysis. Later, the true EAE, used as the animal model of MS, was

developed immunizing rodents, rabbits and monkeys with CNS antigens emulsified in complete Freund's adjuvant (CFA) (Behan and Chaudhuri, 2014).

EAE covers a wide range of models that differ in animal species, strain and the way of induction. In mice, EAE can be induced by active immunization or passively by an adoptive transfer of myelin-specific T cells.

1.2.1 Active EAE

Active EAE in mice is induced by subcutaneous immunization with myelin peptides, typically myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG), emulsified in CFA, that activates myelin-specific T cells and finally leads to neuroinflammation and demyelination. Mice subjected to EAE exhibit ascending paralysis starting with tail limpness and progressing to paralysis of hind and front legs. The course of EAE differs according to the mouse strain and the encephalitogenic peptide used (Figure 2). When SJL/J mice are immunized with a peptide derived from PLP ($PLP_{139-151}$), the majority of them develops EAE with a relapsing-remitting course resembling the relapsing-remitting MS in humans (Figure 2A). A similar course of EAE is induced when C57BL/6J (B6) mice are immunized with a low dose of MOG_{35-55} peptide. On the other hand, mice develop a monophasic form of EAE with a partial remission when immunization with a high dose of MOG_{35-55} peptide is used on C57BL/6J background (Figure 2B). When non-obese diabetic (NOD) mice are immunized with the same MOG fragment, they firstly exhibit a relapsing-remitting disease with mild severity followed by a secondary worsening that resembles the secondary progressive MS in humans (Figure 2C). Currently, the immunization of C57BL/6J mice with MOG_{35-55} peptide is the most frequently utilized EAE model since genetic modifications are introduced relatively easily in this mouse strain, thereby, enabling to study the role of various genes and cell populations in this disease (Rangachari and Kuchroo, 2013).

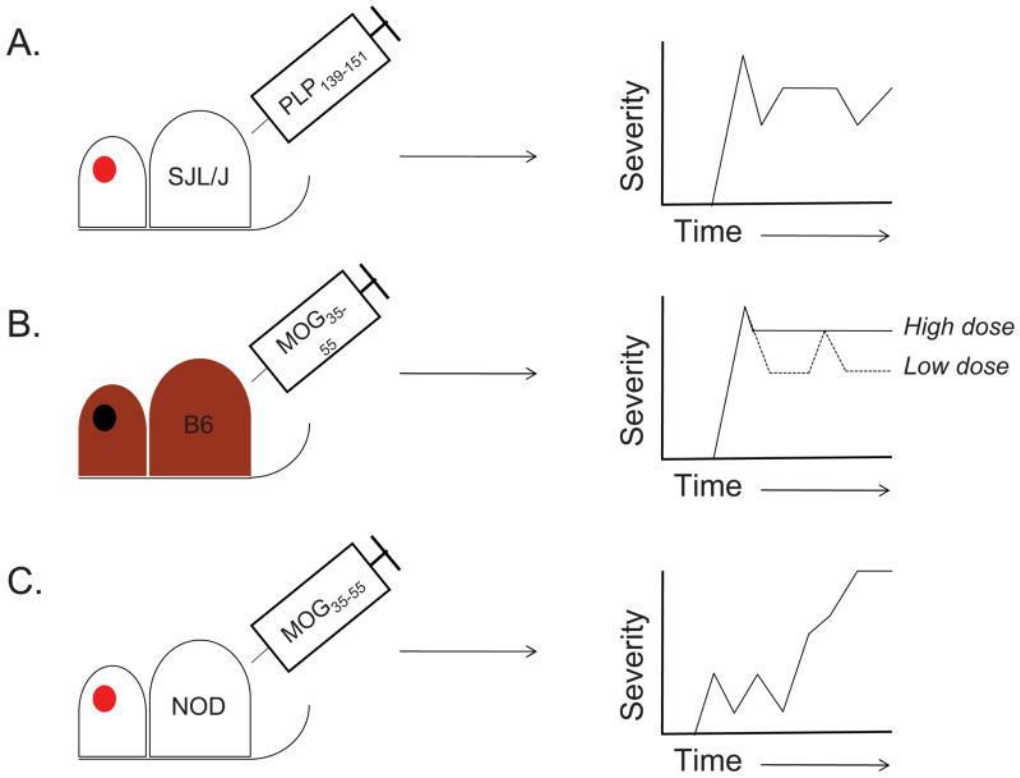

Figure 2 EAE models in mice (adapted from Rangachari and Kuchroo, 2013)

A scheme of the commonest models of active EAE, showing the mouse strain and myelin peptide used and the subsequent disease course.

1.2.2 Passive EAE

Besides the direct immunization with myelin peptides, EAE can be also induced passively, i.e. by the adoptive transfer of myelin-specific T cells. This alternative method modeling the effector phase of the disease enables to avoid possible side effects of CFA injection and better mimics the real pathology of MS that is believed to start spontaneously with T cell attack. Originally, the myelin peptide-immunized animals were used as a source of the T cells, which were transferred to recipients after *in vitro* restimulation with a specific antigen (Naparstek et al., 1983). However, this method produced a clonally heterogenic T cell population. Later, transgenic mouse strains with T cells bearing T-cell receptor (TCR) specific for myelin peptides were generated, thereby, enabling to transfer T cells with homogeneous TCR. Additionally, these transgenic mice develop EAE spontaneously with

various incidences, depending on genetic background and TCR specificity, that further increases the possibilities for studying MS pathology (Rangachari and Kuchroo, 2013, Ben-Nun et al., 2014).

1.3 Eosinophils

Eosinophils are a subtype of granulocytes, named according to their specific staining with an acidophilic dye eosin. They differentiate from hematopoietic progenitors in the bone marrow upon exposure to granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and mainly interleukin-5 (IL-5) and are released to the blood circulation as fully differentiated, mature cells. They are attracted to target tissues typically following IL-5 cytokine or chemokines of the eotaxin family. At steady state, they are predominantly present in the gastrointestinal tract and to a lesser extent in thymus, adipose tissue, lung, mammary glands and uterus where they contribute to the maintenance of tissue homeostasis. Regarding pathological conditions, eosinophils were described to play a crucial role as proinflammatory effector cells in allergic asthma, and other eosinophilic diseases including eosinophilic esophagitis, eosinophilic myopathy and hypereosinophilic syndrome. Furthermore, they significantly contribute to the immunity against infections, most importantly against helminths, but also against bacteria and viruses (Rosenberg et al., 2013).

1.3.1 Immunomodulatory functions of eosinophils

Besides the roles described above, eosinophils also modulate immune reactions affecting processes of both, innate and adaptive immunity, and usually promoting the Th2-polarized immune response (Long et al., 2016). In specific conditions, eosinophils can even work as antigen-presenting cells (Figure 3A). In a murine model of allergic lung inflammation, eosinophils were shown to upregulate the expression of MHCII and costimulatory molecules CD80 and CD86 upon antigen challenge. They migrate to the draining lymph nodes where they present a processed antigen to T cells and activate them while producing interleukin-4

(IL-4) which promotes Th2 polarization and M2 macrophage polarization (Shi et al., 2000, Wang et al., 2007a). Also in humans, eosinophils can express MHCII and costimulatory molecules upon activation and are able to present antigen to T cells *in vitro* (Weller et al., 1993, Lucey et al., 1989, Celestin et al., 2001). Eosinophils support the antigen presentation also indirectly by activating dendritic cells (Figure 3E). Namely, eosinophil-derived eosinophil peroxidase (EPX), one of the eosinophil granule proteins, activates dendritic cells and triggers their migration into a draining lymph node (Chu et al., 2014a). Furthermore, eosinophil-derived neurotoxin (EDN), another eosinophil granule protein, works as a dendritic cell chemoattractant and activates them through toll-like receptor 2 (TLR2) engagement while enhancing Th2-polarized immune response (Yang et al., 2003, Yang et al., 2008). In contrary, eosinophils were proposed to inhibit antigen-specific responses by production of transforming growth factor β (TGFβ) (Nakagome et al., 2007). Moreover, eosinophils increase levels of active TGFβ by production of matrix metalloprotease 9 that converts latent TGFβ to the active form (Chu et al., 2014b).

Another way how eosinophils promote Th2 polarization of the immune response is production of indoleamine 2,3-dioxygenase (IDO) (Figure 3B). IDO is an enzyme generating kynurenines that induce cell death of IFNγ-producing Th1 cells but not Th2 cells (Odemuyiwa et al., 2004). Furthermore, eosinophils produce interleukin-25 (IL-25), thereby enhancing function of Th2 memory cells (Figure 3C) (Wang et al., 2007b). Finally, eosinophils were shown to suppress Th17 cells in small intestine by production of interleukin-1 receptor antagonist (Sugawara et al., 2016).

Except of modulating T cell responses, eosinophils affect also other immune cells. In bone marrow and gut, eosinophils produce factors like a proliferation-inducing ligand (APRIL) and interleukin-6 (IL-6), thus being crucial for plasma cell survival (Chu et al., 2011, Chu and Berek, 2012, Chu et al., 2014b). Moreover, eosinophils were shown to be important for immunoglobulin A (IgA) class switching and generation of CD103+ regulatory T cells (Tregs) and CD103+ dendritic cells in the gut (Chu et al., 2014b).

Finally, released eosinophil major basic protein (eMBP) induces superoxide generation in neutrophils (Haskell et al., 1995) and triggers histamine release from basophils and mast cells (Figure 3D) (Piliponsky et al., 2003, O'Donnell et al., 1983).

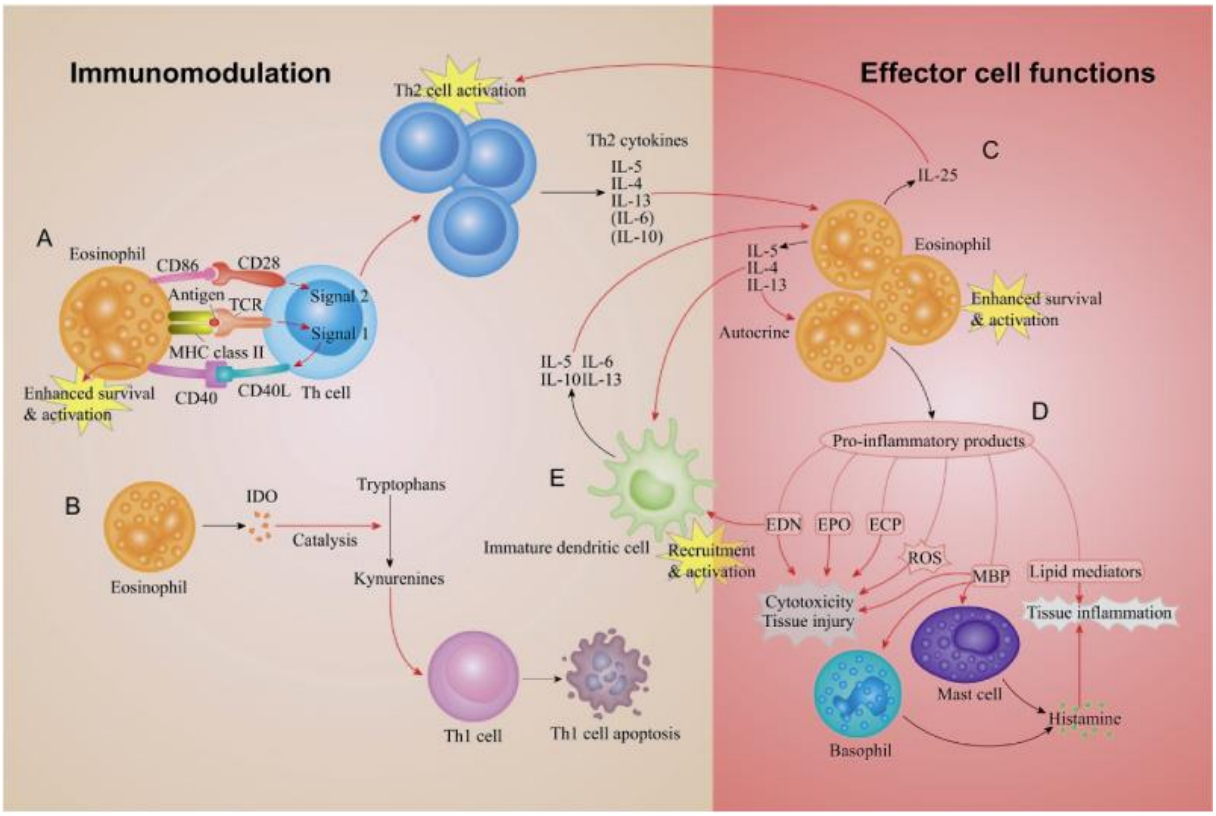

Figure 3 Immunomodulatory and effector functions of eosinophils (adopted from Long et al., 2016)

A scheme describing interactions of eosinophils with other immune cells and effector functions of eosinophils.

1.3.2 Eosinophil-derived granule proteins

As mentioned above, eosinophils also work as effector cells, predominantly using degranulation in order to perform their functions. In numerous granules, they store a variety of preformed cytokines, chemokines, growth factors, enzymes and lipid mediators, prepared for a rapid degranulation after receiving a signal. Moreover, secondary granules of eosinophils contain cationic eosinophil-specific proteins, namely eMBP, EDN, ECP and EPX that are cytotoxic to pathogens but also damage the host tissues (Figure 3D) (Rosenberg et al., 2013). Highly basic eMBP was shown to disrupt lipid bilayers which is proposed to be the

mechanism of cytotoxicity (Abu-Ghazaleh et al., 1992). Further, in the context of asthma, eMBP induces bronchoconstriction and airway hyperactivity (Gundel et al., 1991). EDN has less cationic character, however, possesses RNase activity, which is necessary for its anti-viral function (Domachowske et al., 1998b). Due to the RNase activity, EDN also exerts neurotoxic effect which is manifested as the Gordon phenomenon when EDN is injected intrathecally (Sorrentino et al., 1992). It is of note that EDN is not present in mice (Acharya and Ackerman, 2014). ECP is another cationic granule protein from RNase family (Acharya and Ackerman, 2014); however, the dependence of its cytotoxicity on the RNase activity is controversial (Rosenberg, 1995, Domachowske et al., 1998a). ECP was also shown to elicit symptoms of the Gordon phenomenon when injected intraventricularly (Fredens et al., 1982). EPX is the most abundant eosinophil granule protein and possesses peroxidase activity (Acharya and Ackerman, 2014). Interestingly, reactive oxygen species generated by EPX are released mainly extracellularly, unlike ROS produced by neutrophils, promoting tissue damage (Lacy et al., 2003).

1.3.3 Mouse models of eosinophil deficiency

In order to study a role of eosinophils in various biological processes, several mouse models of eosinophils deficiency were developed, using either genetic modifications or injection of antibodies reducing eosinophil numbers.

As the first genetically manipulated eosinophil-deficient animal, ΔdblGATA1 mouse was generated. GATA-binding factor 1 (GATA1) is a transcription factor necessary for the development of erythroid, megakaryocytic and eosinophilic cells. However, in ΔdblGATA1 mice, only high-affinity GATA-binding site in GATA1 promoter is removed, enabling selective depletion of the eosinophil lineage (Yu et al., 2002). Later, another eosinophil-deficient mouse strain, called PHIL or EPO-DTA, was created. In PHIL mouse, diphtheria toxin A chain (DTA) is expressed under control of a promoter of the eosinophil-specific EPX which

leads to impaired protein synthesis in eosinophils and thereby to their depletion (Lee et al., 2004).

As an alternative approach to genetic manipulation, injections of various antibodies were shown to induce eosinophil deficiency, bringing a possibility to deplete eosinophils inducibly. Firstly, eosinophils can be depleted by an injection of an antibody against IL-5, which is the main eosinophil differentiation, prosurvival, migration and activation factor. Thus, its blocking significantly reduces eosinophil numbers *in vivo* (Garlisi et al., 1999). Further, it is possible to deplete eosinophils by administration of antibodies against Siglec-F which is specifically expressed on eosinophils and its cross-linking causes their apoptosis (Song et al., 2009). Finally, injection of antibodies against (C-C chemokine receptor type 3) CCR3 receptor can also lead to eosinophil depletion without affecting other cell populations (Grimaldi et al., 1999).

1.4 Eosinophils in MS and EAE

There is very limited knowledge about a role of eosinophils in MS and EAE. A microarray study of MS patients revealed an increased expression of ECP, the eosinophil granule protein, in the active but not inactive lesions in the brain, suggesting the presence of eosinophils (Lock et al., 2002). The brain infiltration of eosinophils was described in patients suffering from a rare rapidly progressive Marburg's variant of MS (Elenein et al., 2011, Suzuki et al., 2013). However, nothing is known about their function in the disease pathology.

In EAE, eosinophil infiltration into the spinal cord was firstly described by Gladue and colleagues. Moreover, they showed that blocking of the infiltration by administration of a leukotriene B4 antagonist ameliorated EAE development (Gladue et al., 1996). Later, the same group described eosinophil infiltration also into the optic nerve early in the course of EAE (Milici et al., 1998). Recently, Finlay and colleagues showed that eosinophilia, induced by administration of helminth products, partially protects from EAE development and this effect is dependent on IL-33 and IL-5. Furthermore, eosinophils infiltrated brains of EAE mice

when treated with the helminth products but not control EAE animals (Finlay et al., 2016). Finally, there is a study reporting that mice lacking IL-5, the eosinophil pro-survival factor, develop EAE with severity comparable to their wild-type (WT) controls (Weir et al., 2003).

Furthermore, there are reports suggesting neurotoxic effects of eosinophil products. As mentioned above, EDN and ECP eosinophil granule proteins were described to elicit symptoms of the Gordon phenomenon when injected into CNS (Fredens et al., 1982, Sorrentino et al., 1992). ECP was shown to have neurotoxic effect on cerebellar granule cells and astrocytes, inducing their apoptosis *in vitro*, and this mechanism was suggested to play a role in pathology of hypereosinophilic syndrome that affects also CNS besides other organs (Gleich and Leiferman, 2009, Navarro et al., 2010). Further, a role of eosinophil products was reported in a neuroinflammatory disease related to MS, neuromylelitis optica (NMO). In a mouse model of NMO, Zhang and colleagues showed that eosinophil degranulation causes demyelination in the spinal cord by mechanisms of antibody- and complement-dependent cell-mediated cytotoxity (Zhang and Verkman, 2013). Taken together, these data would rather implicate a negative role of eosinophils in MS and EAE.

1.5 Aim of the study

As described in the previous chapter, there are controversial data about a potential role of eosinophils in the pathology of MS and EAE. On one hand, there are reports suggesting an involvement of eosinophils in MS, since they are present in the brain in at least a subtype of MS. On the other hand, there are contradictory data showing both, positive and negative role in EAE development; and also a study suggesting no role of eosinophils in EAE. Thus, we attempted to solve this controversy here. For this purpose, we first induced EAE in WT mice and analyzed the course of eosinophil infiltration into the spinal cord. Subsequently, we assessed EAE development in two independent models of eosinophil deficiency with emphasis on the clinical development and neuroinflammation and demyelination in the spinal cord.

2 Material and Methods

2.1 Buffers and media

- MACS buffer for myelin removal – PBS + 0.5 % BSA
- MACS buffer for Pan T cell kit – PBS + 0.5 % BSA + 2 mM EDTA
- FACS buffer – PBS + 0.5 % BSA + 0.1 % NaN_3
- Complete RPMI 1640 – RPMI 1640 + 10 % FBS + 1% PenStrep

2.2 Mice

Wild-type C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). ΔdblGATA1 mice and littermate WT control on C57BL/6 background were also used. ΔdblGATA1 mice were kindly provided by Prof. Avery August (Cornell University College of Veterinary Medicine, NY, USA) who backcrossed them from the original ΔdblGATA1 mouse strain generated on BALB/C background (Yu et al., 2002). Mice were kept in the barrier facility of the Medizinisch-Theoretisches Zentrum, Dresden, Germany. Animal experiments were approved by the Landesdirektion Sachsen, Germany.

2.3 EAE induction and scoring

Active EAE was induced as previously described (Choi et al., 2015). Eight- to twelve-weeks old female mice were shaved at the tail base and subcutaneously injected with 200 µg MOG_{35-55} (myelin oligodendrocyte glycoprotein 35-55; peptides&elephants GmbH, Potsdam, Germany) emulsified in incomplete Freund's adjuvant (Sigma, St Louis, MO, USA) containing 500 µg inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA) and followed by intraperitoneal injection of 400 ng pertussis toxin (Merck, Darmstadt, Germany). The pertussis toxin injection was repeated two days later.

Mice were monitored daily and clinical score was determined by a blinded observer as follows: 0 = no clinical sign; 1 = limp tail; 2 = mild hind limb weakness; 3 = complete unilateral hind limb paralysis or severe weakness of both hind limbs; 4 = complete bilateral hind limb paralysis; 5 = four limb paralysis; 6 = death. In the case of mouse death, score 6 was recorded for the day of death and excluded from the rest of the scoring experiment. When mice started to show EAE symptoms corresponding to score 2, dry food and Solid drink gels (Triple A Trading, Tiel, Netherlands), substituting access to drinking water, were placed inside the cage.

2.4 Isolation of leukocytes from spinal cord

The spine was excised from euthanized mice and the whole spinal cord was collected in PBS (after mouse anesthesia with ketamine-xylazine mixture and systemic perfusion through the left ventricle in order to prevent the contamination of the spinal cord infiltrate with circulating cells). A single-cell suspension was prepared using a 100µm cell strainer and myelin was removed using MACS technology in order to obtain leukocyte-enriched spinal cord fraction. The spinal cord suspension was incubated with Myelin Removal Beads II (Miltenyi Biotec, Bergisch Gladbach, Germany), that are antibodies against myelin conjugated to magnetic beads, and passed through a column placed in the magnetic field. Thus, myelin containing cells and debris were captured in the column and the leukocyte-enriched fraction was collected and used for FACS analysis or sorting.

2.5 Flow cytometry analysis and cell sorting

The leukocyte-enriched spinal cord fraction was isolated as described above, pre-incubated with anti-mouse CD16/32 (Fc block; BD Biosciences, Heidelberg, Germany) and stained in FACS buffer for 45 min with the following antibodies: anti-CD45-PE (clone 30-F11), anti-CD11b-Alexa Fluor 488 (clone M1/70) and anti-Siglec-F-Alexa Fluor 647 (clone E50-2440) from BD Biosciences (Heidelberg, Germany); anti-CCR3-PE-Vio770 (clone REA

122), anti-CD4-APC (clone GK1.5), anti-CD8-PerCP (clone 53-6.7) from Miltenyi Biotec (Bergisch Gladbach, Germany); anti-CD63-APC/Cy7 (clone NVG-2), anti-Ly6G-PerCP/Cy5.5 (clone 1A8) from BioLegend (San Diego, CA, USA); anti-F4/80-PE/Cy7 (clone BM8) from eBioscience (Frankfurt, Germany). Viability staining was performed using Hoechst 33258 (Thermo Fisher Scientific, Waltham, MA, USA).

BD FACSCanto II and BD FACSAria III instruments from BD Biosciences (Heidelberg, Germany) were used for flow cytometry analysis and cell sorting, respectively. Flow cytometry data were analyzed with FlowJo software (FlowJo, LLC, Ashland, OR, USA).

2.5.1 Cell counting for flow cytometry analysis

In order to determine the cell number of different cell populations infiltrating the spinal cord, the total cellularity of the leukocyte-enriched spinal cord fraction was measured. For this purpose, a part of the cell suspension was separated before FACS staining and diluted in PBS containing Hoechst 33258. Cell number was measured using MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and the total cell number of live (Hoechst 33258-negative) singlet cells was calculated according to the dilutions that have been made. The obtained total cell number was than multiplied by percentages of cell populations (out of live singlet cells), determined by flow cytometry, and the resulting cell number was normalized to 100 mg of spinal cord.

2.5.2 Intracellular staining for IFNγ

In order to measure the activation of T cells in the recall response assay (chapter 2.8), production of IFNγ was measured after intracellular staining with FACS. At the end of the recall response assay, Protein Transport Inhibitor Cocktail (eBioscience, Frankfurt, Germany) was added into the co-culture of dendritic and T cells for 3 hours in order to inhibit the

secretory pathway. Thus, IFNγ was captured inside the cells and was detectable with intracellular staining. Afterwards, cells were collected using PBS supplied with 2mM EDTA, centrifuged and stained for surface markers with anti-CD4-FITC and anti-CD8-PerCP antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, cells were washed with PBS and stained with Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA, USA) for 15 min in room temperature in order to distinguish dead cells during FACS analysis. Cells were washed with PBS supplied with 3%FBS and permeabilized using FOXP3 Staining Buffer Set (eBioscience, Frankfurt, Germany) for at least 40 minutes. Then, they were washed with Permeabilization buffer from the above mentioned buffer set and stained with anti-IFNγ-APC antibody (eBioscience, Frankfurt, Germany) for 30 minutes. Afterwards, cells were washed with Permeabilization buffer and analyzed by flow cytometry.

2.6 Molecular biology methods

In order to determine the mRNA expression of specific genes in the spinal cord tissue or sorted cells, RNA was isolated and transcribed to cDNA. Expression levels were measured in the real-time quantitative polymerase chain reaction (RT-qPCR) which allows for simultaneous amplification and quantification of cDNA in the gene-specific manner.

2.6.1 RNA isolation

From the spinal cord tissue, RNA was isolated using a modified Chomczynski and Sacchi protocol (Chomczynski and Sacchi, 1987). Briefly, a piece of tissue was homogenized in 500 µl of TRI Reagent (MRC, Cincinnati, OH, USA) using Precellys 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) and centrifuged at 13000 g, 10 min, 4 °C to remove a fat layer. Afterwards, the homogenate was transferred into a clean tube and 100 µl of chloroform (Roth, Karlsruhe, Germany) was added. Samples were mixed, incubated for 3 min in room temperature and centrifuged at 13000 rpm, 10 min, 4 °C. The

liquid separated into three phases: the lower one containing proteins and lipids, the middle one containing DNA and the upper one containing RNA. The upper phase was collected into a new tube and mixed with 200 µl of isopropanol (VWR Chemicals, East Grinstead, UK). After 10 min incubation at room temperature, samples were centrifuged at 13000 rpm, for 10 min, 4 °C so that a pellet of precipitated RNA was formed. The pellet was washed with 70% ethanol and dried shortly. Afterwards, it was resuspended in water and incubated at 55 °C for 10 min to facilitate the RNA dissolution. The concentration of the isolated RNA was measured with the Synergy HT microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany).

From sorted cells, RNA was isolated using RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Manufacturer's instructions were followed.

2.6.2 Reverse transcription

1 µg of RNA and all RNA isolated from the spinal cord tissue and sorted cells, respectively, was transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) and Mastercycler gradient cycler (Eppendorf, Wesseling-Berzdorf, Germany). The following protocol was used:

- 5 min at 25ºC
- 30 min at 42ºC
- 5 min at 85ºC

2.6.3 Real-time quantitative PCR

RT-qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), containing DNA polymerase, dNTPs and a fluorescent nucleic acid dye. Besides the SsoFast EvaGreen Supermix, the reaction mixture included cDNA diluted in

water and gene-specific primers at a final concentration 400 nM. The primers used are listed in the following table.

RPS29 fwd	GAGCCGACTCGTTCCTTTCT
RPS29 rev	TTCAAGGTCGCTTAGTCCAACTTA
18S fwd	GTTCCGACCATAAACGATGCC
18S rev	TGGTGGTGCCCTTCCGTCAAT
eMBP fwd	GGAGCGTCTGCTCTTCATCT
eMBP rev	CCCCTGGAGGACACTCTTCT
EPX fwd	CTGCTTAGCTGTAGTGGGGG
EPX rev	ACGTTGCATGATGCTTTTCTGT
ECP fwd	AAGCAGACAGGGAAACATGGGT
ECP rev	TGTTCTCCTCCGACTGGTGAT
Eotaxin-1 fwd	TGCTCACGGTCACTTCCTTC
Eotaxin-1 rev	CTTGAAGACTATGGCTTTCAGGGTG
Eotaxin-2 fwd	AGGGGTCATCTTCATCACCAAG
Eotaxin-2 rev	AAGCAGCCTGGTAAAGCGT
IFNγ fwd	CTGGAGGAACTGGCAAAAGG
IFNγ rev	CTGGACCTGTGGGTTGTTGA
IL-17A fwd	CGCAAAAGTGAGCTCCAGA
IL-17A rev	TGAGCTTCCCAGATCACAG
IL-6 fwd	CCTTCCTACCCCAATTTCCAAT
IL-6 rev	AACGCACTAGGTTTGCCGAGTA
TNF fwd	AACCACCAAGTGGAGGAGCA
TNF rev	GGGTGAGGAGCACGTAGTCG
iNOS fwd	ACCTTGTTCAGCTACGCCTT
iNOS rev	CATTCCCAAATGTGCTTGTC
IL-1β fwd	ATCCCAAGCAATACCCAAAG
IL-1β rev	GTGCTGATGTACCAGTTGGG

The qPCR reaction was carried out in a CFX384 Real-Time cycler (BioRad). First, DNA polymerase is heat-activated. Afterwards, temperature in the cycler is changed so that cycles of DNA denaturation, primer annealing and DNA extension are repeated. The product amplification is quantified in the real time as the nucleic acid dye binds to the newly formed double-stranded DNA and becomes fluorescent. The following protocol was used:

1. 95 °C … 30 s
2. 95 °C … 5 s
3. 60 °C … 5 s

Go to step 2. 45-times

4. 70 to 95 °C increase (melting curve)

The relative gene expression was calculated by the ΔΔCT method (Livak and Schmittgen, 2001) and normalized to the expression of RPS29 and 18S housekeeping genes in the spinal cord and sorted cell samples, respectively.

2.7 Eotaxin-1 ELISA

After systemic perfusion of mice, spinal cords were collected in PBS supplied with 1x protease inhibitor cocktail cOmplete Mini (Roche Diagnostics, Mannheim, Germany). Samples were homogenized with a T10 basic Ultra-Turrax homogenizer (IKA, Staufen, Germany), centrifuged at 17000 g, for 30 min, at 4°C, and supernatants were collected for analysis. The concentration of eotaxin-1 protein was measured with Mouse CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Concentration of eotaxin-1 was normalized to the total protein amount determined by bicinchoninic acid assay (BCA) method (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA).

2.8 Recall response assay

In order to test the recall response of T cells from eosinophil-deficient and –sufficient mice, ΔdblGATA1 and WT mice were immunized with MOG_{35-55} peptide emulsified in CFA as described in the chapter about EAE induction (chapter 2.3). At day 7 after the immunization, T cells from these mice were isolated and restimulated with MOG_{35-55}-pulsed dendritic cells differentiated from the bone marrow of untreated WT mice. Activation of T cells was measured 24 hours later as IFNγ production using intracellular staining and flow cytometry analysis (chapter 2.5.2).

2.8.1 Differentiation of dendritic cells from bone marrow

WT mice were sacrificed and femurs and tibias were collected. Bone marrow was flushed out from the bones with PBS, passed through a 40 µm strainer and centrifuged at 400 g, for 5 min, at 4 °C. Cells were seeded in complete RPMI 1640 medium supplied with 20 ng/ml of GM-CSF (Prepro Tech, Hamburg, Germany) in suspension culture dishes. Medium was changed 3 days later. On day 7, floating cells, representing mainly dendritic cells, were harvested and used for the recall response assay.

2.8.2 T cell isolation

∆dblGATA1 and WT mice were sacrificed 7 days after the immunization with MOG_{35-55} peptide. Draining lymph nodes were collected in MACS buffer and a single cell suspension was prepared using a 40 µm strainer. Cells were centrifuged at 400 g, for 5 min, at 4 °C and T cells were isolated with Pan T Cell Isolation Kit II, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany). This kit utilizes the MACS technology. At first, cell suspension is stained with a cocktail of biotin-conjugated antibodies against markers of several cell populations except of T cells. Then, anti-biotin antibodies conjugated to magnetic beads are co-incubated. Finally, the stained cell suspension is applied to a column placed in magnetic field where non-T cell types are captured and the flow-through, containing only T cells, is collected. T cells isolated in this way were centrifuged at 400 g, for 5 min, at 4 °C, resuspended in complete RPMI 1640 medium and used for the recall response assay.

2.8.3 Recall response assay

Dendritic cells (20000/ well) were seeded in complete RPMI 1640 medium and incubated for 30 minutes. Afterwards they were loaded with MOG_{35-55} peptide at a final

concentration of 10 µg/ml for 4 hours. T cells were added (180000 cells/ well), co-cultured overnight and subsequently stained intracellularly for IFNγ (chapter 2.5.2).

2.9 Luxol Fast Blue staining

In order to analyze demyelination in the spinal cord of mice subjected to EAE, we used Luxol Fast Blue (LFB) MBSN stain (Sigma, St Louis, MO, USA) that binds to myelin.

After systemic perfusion, a part of the spinal cord was dissected and incubated in 4% paraformaldehyde (PFA) for 1 hour at 4 °C. After washings, the tissue was transferred into 30% sucrose (Sigma, St Louis, MO, USA) and let to sink overnight. It was embedded in OCT Tissue Tek (Sakura, Staufen, Germany), frozen in -80 °C and cut to 12µm sections using CryoStar NX50 cryostat (Thermo Fisher Scientific, Waltham, MA, USA).

Sections were dried for 30 minutes in room temperature and OCT was washed away with PBS. Then, sections were fixed in methanol for 5 minutes, defatted in 1:1 ethanol/chloroform mixture for 4 hours and hydrated in 95% ethanol for 5 minutes. Sections were incubated in luxol fast blue solution, composed of 0.1% luxol fast blue and 0.5% glacial acetic acid dissolved in 95% ethanol, at 58 °C overnight. The excess of the stain was then rinsed with 95% ethanol and distilled water for 1 minute each. The staining was differentiated in 0.05% lithium carbonate for 15 seconds followed by 70% ethanol for another 15 seconds. Slides were washed in distilled water and observed under a microscope. The differentiation steps were repeated until the white and grey matters were sharply defined. Then, sections were washed with 95% ethanol (1 minute), 100% ethanol (2x5 minutes) and xylene (2x5 minutes). Slides were mounted in VectaMount Permanent Mounting Medium (Vector Laboratories, Peterborough, UK).

Imaging was performed with an inverted microscope Apotome (Carl Zeiss, Jena, Germany) where mosaic pictures were collected. 4-6 sections from each mouse were analyzed using Fiji software. The amount of myelin was measured as integrated density after

automatic thresholding divided by area of section. Values were normalized to WT that was set as 100 %.

2.10 Statistical analysis

For all experiments, Mann Whitney test was used to assess the statistical significance of results. P values ≤0.05 were considered statistically significant (* P≤0.05, ** P≤0.01, *** P≤0.001). Data are presented as mean ± standard error of the mean (SEM). GraphPad Prism 6 software was used to calculate the statistics.

3 Results

3.1 Eosinophils infiltrate into the spinal cord in the course of EAE

In order to study neuroinflammatory processes, that are hallmark of MS pathology, EAE mouse model is widely used. To study a possible role of eosinophils in this disease, we chose the active EAE that is induced in C57BL/6 mice by immunization with a high dose of the peptide derived from MOG protein (MOG_{35-55} peptide) (Choi et al., 2015). This immunization protocol induces a monophasic course of the disease with the pre-onset phase around day 7, the onset phase around day 10, and the peak phase around day 17 after the immunization followed by a partial remission of symptoms.

EAE was induced in WT mice and the infiltration of eosinophils into the spinal cord was assessed by FACS. In order to identify eosinophils, Siglec-F was used as a well-established eosinophil marker (Voehringer et al., 2007, Griseri et al., 2015). Furthermore, CD45, CD11b and high side scatter (SSC) were used to better recognize eosinophils by FACS (Griseri et al., 2015). Thus, eosinophils were determined as CD45+, SSC high, CD11b+ and Siglec-F+ cells. The gating strategy is show in Figure 4A. The eosinophil infiltration into the spinal cord was analyzed in the pre-onset (D7), onset (D10) and peak (D17) phase of EAE, and non-immunized (healthy) mice were used as a control. There was a significant increase of eosinophil numbers in all three EAE time points compared to healthy mice (Figure 4B). The eosinophil infiltration was accompanied by an expected increase of total cell numbers which was also significant in all three phases of EAE (Figure 4C). Furthermore, the eosinophil infiltration was confirmed by RT-qPCR analysis of the expression of eosinophil granule proteins, namely eMBP and ECP, which are commonly used as eosinophil-specific markers (Rosenberg et al., 2013). The expression of ECP and eMBP in the spinal cord was significantly upregulated in the peak phase of EAE (Figure 4D, E). Moreover, mRNA levels of ECP were increased already in the onset phase of EAE (Figure 4D). Taken together, these

data indicate that eosinophils infiltrate into the spinal cord during EAE. Thus, we were interested to understand the mechanism of eosinophil chemoattraction into the spinal cord.

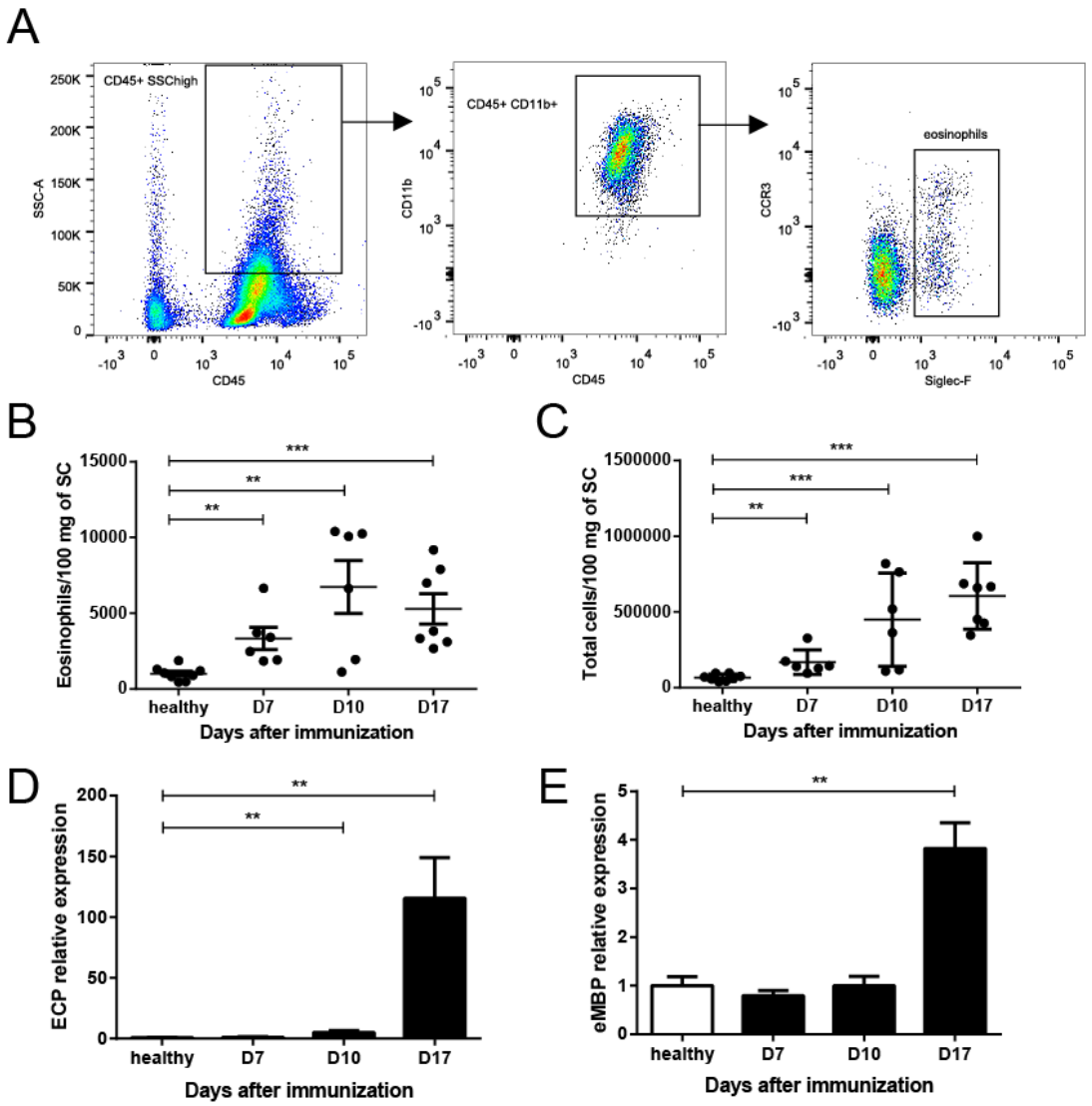

Figure 4 Eosinophils infiltrate into the spinal cord in the course of EAE

Wild-type female mice were immunized with MOG_{35-55} in CFA in order to induce EAE and leukocyte infiltrates in the spinal cord were analyzed at different time points. Non-immunized mice were used as controls. (A) Using FACS analysis, eosinophils infiltrated into the spinal cord were identified as CD45+ SSChigh CD11b+ Siglec-F+ cells out of live cells, determined as Hoechst-negative cells, and singlets. Representative graphs of the gating strategy are shown. Numbers of (B) eosinophils and (C) total cells infiltrated in the spinal cord of healthy or EAE mice 7, 10 and 17 days after immunization (n = 6-8/group). Relative mRNA expression of (D) eMBP and (E) ECP in the spinal cord of healthy or EAE mice 7, 10 and 17 days after immunization (n = 6-7/group). The expression was normalized to RPS29 housekeeping gene and the average expression in controls was set as 1. The P values

refer to comparison of control and EAE mice at different time points. ** P≤0.01, *** P≤0.001. Data are presented as mean ± SEM. eMBP (major basic protein), ECP (eosinophil cationic protein)

3.1.1 Eotaxin-1 is a possible chemokine attracting eosinophils to the spinal cord in the course of EAE

Eotaxins are typical eosinophil chemoattractants, driving CCR3 positive eosinophils to target tissues under homeostatic or inflamed conditions. In context of inflammation, eotaxin-1 and -2 are necessary for the eosinophil infiltration to lung of mice subjected to a murine model of asthma (Pope et al., 2005). To identify a possible mechanism of eosinophil recruitment in our model we analysed eotaxins in the spinal cord during EAE. As eotaxin-3 is a pseudogene in mice (Pease and Williams, 2013), we analyzed mRNA levels of eotaxin-1 and -2 in the spinal cord of EAE and control mice. Expression of eotaxin-1, but not eotaxin-2, was significantly increased in the onset and peak phase of the disease (Figure 5A, B). There was a trend for eotaxin-2 to be increased at the onset of EAE (Figure 5B). In order to confirm the eotaxin-1 upregulation at the protein level, we used ELISA to measure eotaxin-1 in homogenates of spinal cords isolated from EAE and control mice. Since mRNA expression of eotaxin-1 was predominantly upregulated in the onset phase of EAE (D10) we chose this time point to analyze its protein levels. Concentration of eotaxin-1 was significantly increased at this time point, as compared to the spinal cords from control mice (Figure 5C). These data suggest that eotaxin-1 is a possible chemoattractant for spinal cord-infiltrating eosinophils in the course of EAE. However, this mechanism requires further confirmation, for instance by using an eotaxin-1 blocking agent or eotaxin-1 receptor knock-out mice.

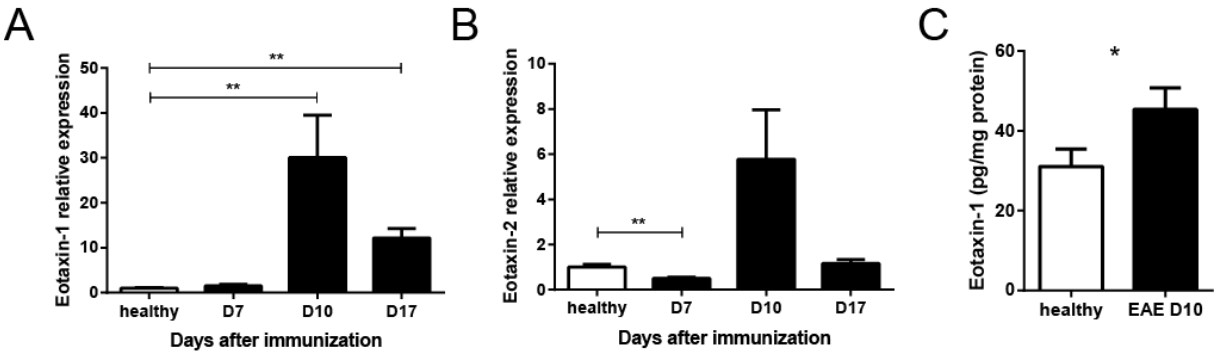

Figure 5 Eotaxin-1 concentration increases in the spinal cord of EAE mice

Wild-type mice were immunized with MOG_{35-55} in CFA in order to induce EAE and eotaxin levels in the spinal cord were analyzed at different time points. Non-immunized mice were used as controls. Relative mRNA expression of (A) eotaxin-1 and (B) eotaxin-2 in the spinal cord of healthy or EAE mice 7, 10 and 17 days after immunization (n = 6-7/group). The expression was normalized to RPS29 housekeeping gene. (C) Protein levels of eotaxin-1 in spinal cord homogenates from healthy or EAE mice were analyzed by ELISA 10 days after immunization. Eotaxin-1 concentration was normalized to the total protein amount (n = 4-5/group). The P values refer to comparison of control and EAE mice at different time points. * P≤0.05, ** P≤0.01. Data are presented as mean ± SEM.

3.2 Spinal cord eosinophils are activated and degranulate during EAE

Since eosinophils accumulate in the spinal cord of EAE mice (Figure 4), we proceeded to the analysis of their activation status. For this purpose, we chose CD11b integrin of which increased surface expression has been reported as a marker of eosinophil activation in a mouse model of colitis (Griseri et al., 2015). Interestingly, its expression by spinal cord eosinophils, measured by FACS, was significantly increased in the onset and peak phase of EAE when compared to spinal cord eosinophils from healthy mice (Figure 6A). The activation of the spinal cord eosinophils was further tested by FACS analysis of CCR3 receptor expression. Decrease of CCR3 expression on eosinophils has been shown as a marker of activation in a mouse model of lung infection (Voehringer et al., 2007). Indeed, surface expression of CCR3 on spinal cord eosinophils gradually decreased in the course of EAE.

(Figure 6B). Since there is the downregulation of CCR3 expression on eosinophils during EAE and we have noticed two distinct populations of eosinophils in the spinal cord of EAE mice differing in the expression of CCR3 (Figure 4A), we questioned whether there is a difference in the expression of eosinophil granule toxins between these two populations which might help us to better understand the activation status of eosinophils in EAE. For this purpose, CCR3-possitive and –negative eosinophils were sorted from the spinal cord of EAE mice in the peak phase of the disease and the expression of the eosinophil granule proteins eMBP, ECP and EPX was analyzed by RT-qPCR. Interestingly, expression of all three proteins was significantly higher in CCR3-negative eosinophils (Figure 6C-E). These results indicate that during the activation of eosinophils (characterized by CCR3 downregulation) they upregulate expression of their granule toxins, which might contribute to the pathology of EAE.

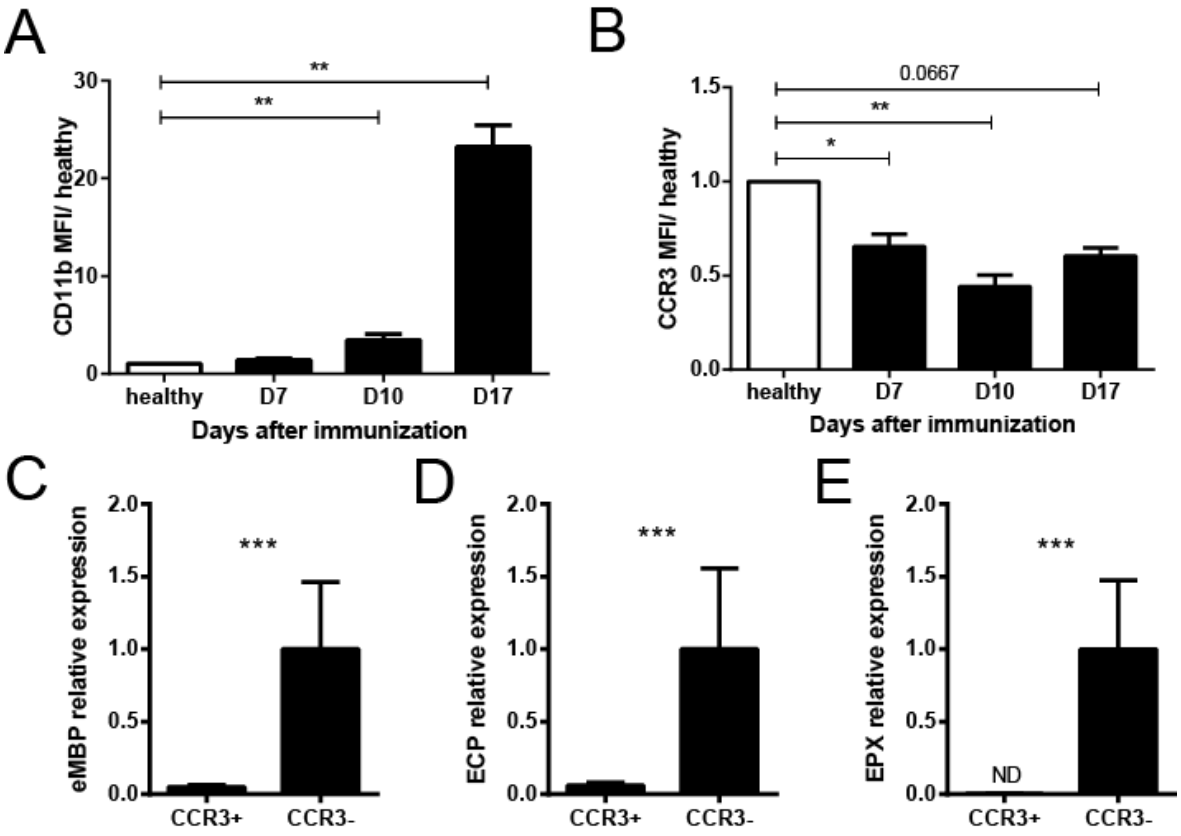

Figure 6 Spinal cord-infiltrating eosinophils are activated in EAE

Wild-type mice were immunized with MOG_{35-55} in CFA in order to induce EAE and leukocyte infiltrates in the spinal cord were analyzed at different time points. Non-immunized mice were used as controls. The expression of (A) CD11b and (B) CCR3 on eosinophils determined as CD45+ SSChigh CD11b+ Siglec-F+ cells by FACS in

healthy or EAE mice 7, 10 and 17 days after immunization. The expression is presented as MFI and normalized to healthy controls (n =4-6/group). CCR3+ and CCR3- eosinophils from the spinal cord of EAE mice at the peak of the disease were sorted by FACS. Relative mRNA expression of (C) eMBP, (D) ECP and (E) EPX was analyzed by qPCR and normalized to 18S expression (n =7-10/group). * P≤0.05, ** P≤0.01, *** P≤0.001. Data are presented as mean ± SEM. MFI (median of fluorescence intensity), eMBP (major basic protein), EPX (eosinophil peroxidase), ECP (eosinophil cationic protein), ND (not detected)

As we found that eosinophils are activated in EAE and that the expression of their granule proteins is upregulated, we were further interested to study whether eosinophils degranulate in the spinal cord of EAE mice. Therefore, we analyzed eosinophil degranulation using FACS by measuring their side scatter parameter which describes the granularity of cells. Thus, SSC decrease corresponds to the loss of granules and, thereby, is a measure of degranulation. Indeed, SSC of spinal cord eosinophils was significantly decreased during all stages of EAE when compared with healthy mice (Figure 7A). In order to confirm the degranulation, we used another marker, tetraspanin CD63. Increased surface expression of CD63 is a marker of degranulation of gut eosinophils in a mouse colitis model (Griseri et al., 2015). In agreement with the decreased SSC, the percentage of CD63-positive eosinophils was significantly increased in the spinal cord of EAE mice in the peak of the disease (Figure 7B). Therefore, our data showed that eosinophils likely degranulate in the spinal cord of EAE mice.

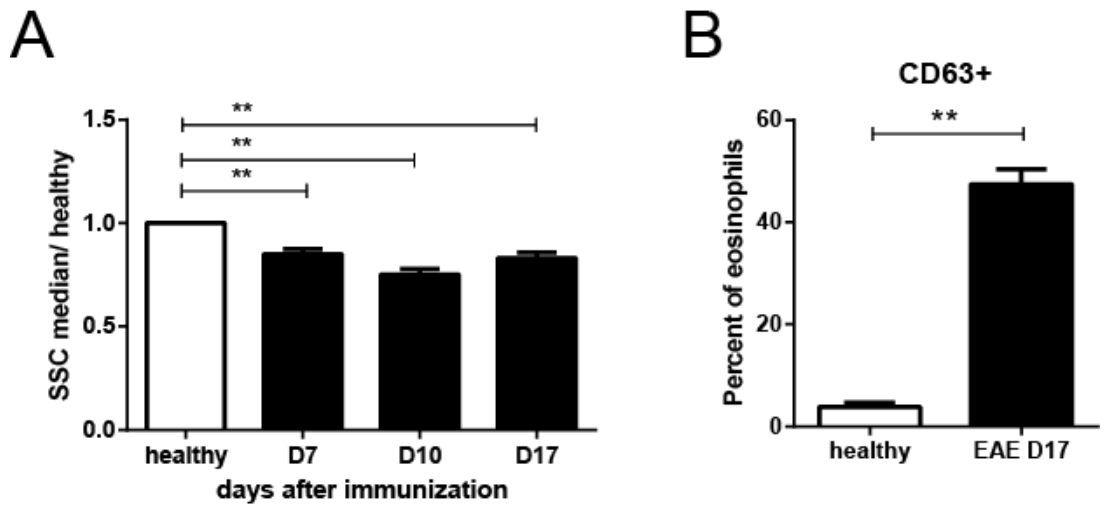

Figure 7 Spinal cord-infiltrating eosinophils degranulate in EAE

Wild-type mice were immunized with MOG_{35-55} in CFA in order to induce EAE and leukocyte infiltrates in the spinal cord were analyzed at different time points. Non-immunized mice were used as controls. (A) SSC median of spinal cord eosinophils from control or EAE mice 7, 10 and 17 days after immunization. Data were normalized to the healthy controls (n =4-6/group). (D) Percentage of spinal cord eosinophils expressing CD63 in control and EAE mice 17 days after immunization determined by FACS (n =4-6/group). ** P≤0.01. Data are presented as mean ± SEM. SSC (side scatter)

3.3 Eosinophils are dispensable for EAE development

Since we demonstrated that eosinophils infiltrate into the spinal cord during EAE (Figure 4) and they are activated and degranulate therein (Figure 6, Figure 7), suggesting a potential role in EAE pathology, we were interested whether they are necessary for disease development. For this purpose, we used two independent models of eosinophil deficiency: ΔdblGATA1 mice (Yu et al., 2002) and anti-IL-5 antibody-mediated eosinophil depletion (Garlisi et al., 1999). At first, ΔdblGATA1 mice and their littermate controls were subjected to EAE using the immunization protocol described above for WT mice, and the clinical development was assessed (for more details regarding the clinical scoring see chapter 2.3). Interestingly, we did not observe any significant changes in EAE severity when comparing ΔdblGATA1 mice with their eosinophil-sufficient controls as shown in an EAE development graph (Figure 8A) and as area under the curve (AUC) (Figure 8B). Second, wild-type mice were injected intraperitoneally with an antibody against IL-5 or its isotype control at day 5 and 8 after the EAE induction in order to deplete eosinophils in the effector phase of EAE when the eosinophil infiltration into the spinal cord was the most prominent (Figure 4). Confirming the result from ΔdblGATA1 mice, there was no difference in EAE severity between eosinophil-depleted and isotype-injected mice as shown again in both, the EAE development graph (Figure 8C) and AUC representation (Figure 8D). Taken together, EAE clinical scoring experiments showed that eosinophils are dispensable for EAE development in two independent models of eosinophil deficiency.

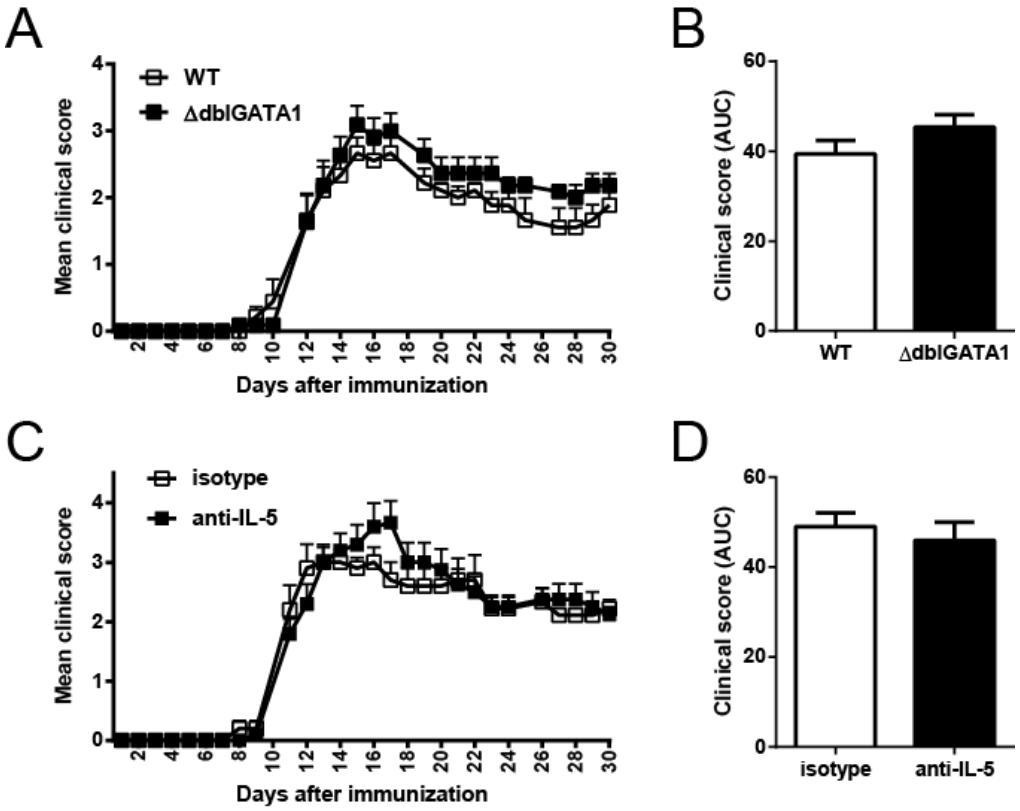

Figure 8 Eosinophils are not necessary for EAE development

Female ΔdblGATA1 mice and their littermate controls were immunized with MOG_{35-55} in CFA in order to induce EAE and the clinical development was scored by a blinded observer (n=9-11/group). (A) Mean clinical score was plotted in the time course and statistical analysis was performed for every day separately. (B) Area under the curve was calculated from the data in (A). Female WT mice were immunized with MOG_{35-55} in CFA in order to induce EAE and intraperitoneally injected with anti-IL-5 antibody or isotype control (10 µg/mouse) at day 5 and 8 after the immunization (n=10/group). The clinical development of EAE was scored by a blinded observer. (C) Mean clinical score was plotted in a time course and statistical analysis was performed for every day separately. (D) Area under the curve was calculated from the data in (C). Data are presented as mean ± SEM. AUC (area under the curve)

3.3.1 No difference in inflammation in the spinal cord of ΔdblGATA1 mice

In order to further assess a potential functional contribution of eosinophils in EAE pathology, we analyzed the infiltration of several inflammatory cell populations into the spinal

cord of eosinophil-deficient and –sufficient mice at the peak of EAE (day 17). For this purpose, ΔdblGATA1 mice were chosen since they are the more specific model of eosinophil deficiency. The spinal cord infiltrating cells were analyzed using FACS. Leukocyte-enriched fraction of the spinal cord homogenate was prepared and two separated FACS stainings for myeloid and lymphoid cells were performed. Eosinophils were identified as CD45+ SSChigh CD11b+ Siglec-F+ Ly6Glow F4/80int cells (Figure 9A), neutrophils as CD45+ CD11b+ F4/80- Ly6G+ cells (Figure 9B), macrophages as CD45+ CD11b+ Ly6G- F4/80+ CD45high cells (Figure 9B), CD4 T cells as CD45+ CD8- CD4+ cells (Figure 9C) and CD8 T cells as CD45+ CD4- CD8+ cells (Figure 9C).

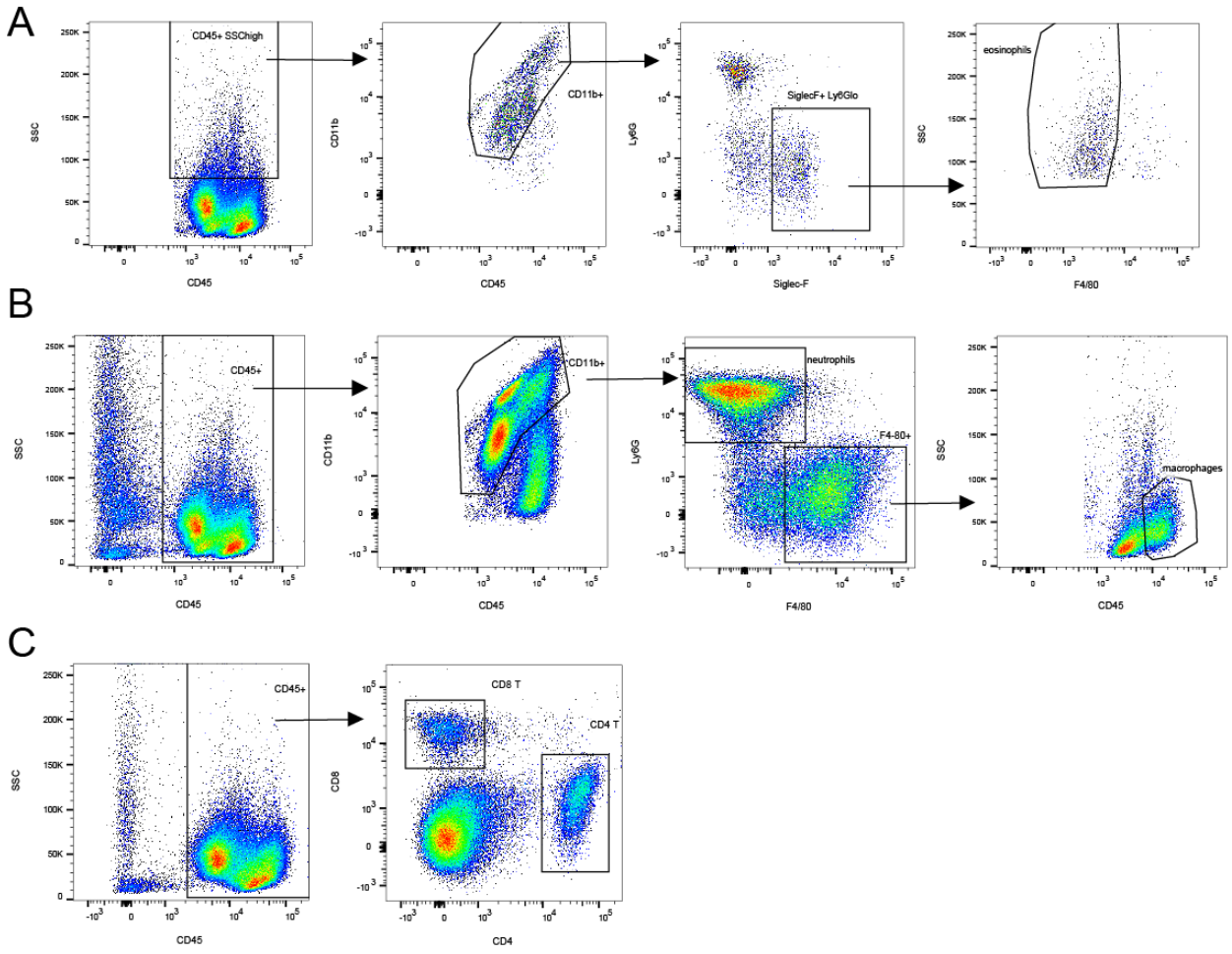

Figure 9 Gating strategy for inflammatory cell FACS analysis

Representative FACS plots show gating strategy for the identification of (A) eosinophils, (B) neutrophils, macrophages and (C) CD4 and CD8 T cells infiltrating the spinal cord of WT control mice subjected to EAE.

We did not observe any significant difference in the numbers of total CD45+ cells (Figure 10A), CD4 T cells (Figure 10B), CD8 T cells (Figure 10C), neutrophils (Figure 10D) and macrophages (Figure 10E) infiltrating the spinal cord of ΔdblGATA1 and WT control mice at the peak of the disease. As expected, there were nearly no eosinophils detected in ΔdblGATA1 mice in contrast to their WT controls (Figure 10F). In accordance with the scoring experiments presented above, these mice had a comparable clinical score (Figure 10G).

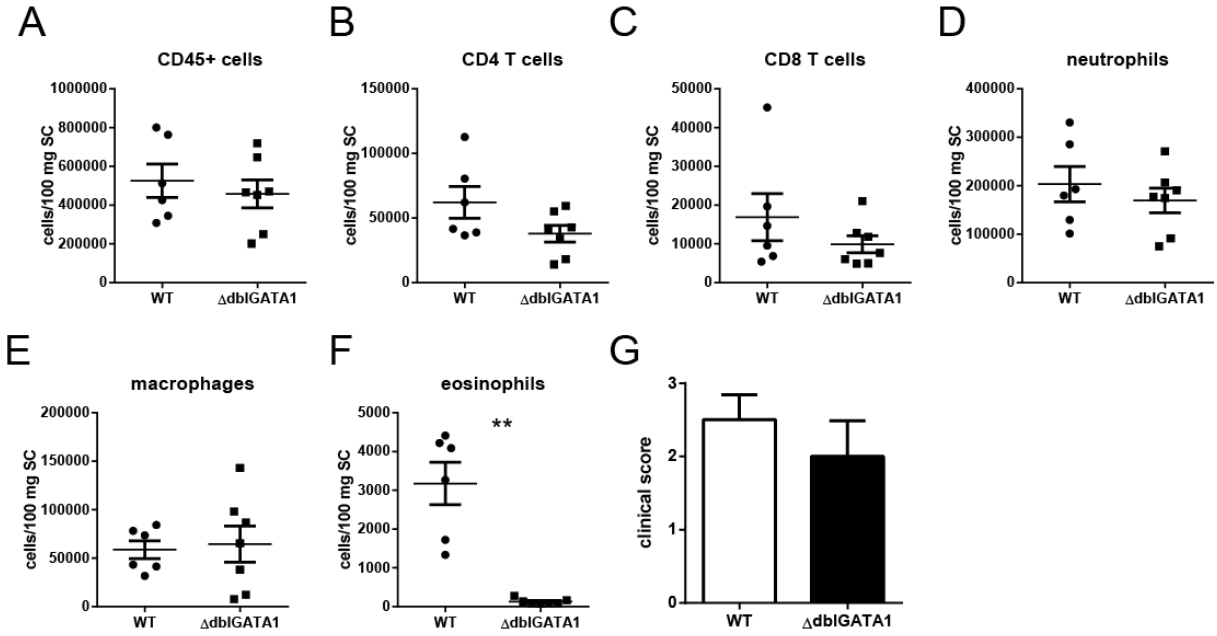

Figure 10 No difference in inflammatory cell infiltration in ΔdblGATA1 mice

Female ΔdblGATA1 mice and their littermate controls were immunized with MOG_{35-55} in CFA in order to induce EAE. Numbers of spinal cord-infiltrated (A) CD45+, (B) CD4 T and (C) CD8 T cells, (D) neutrophils, (E) macrophages and (F) eosinophils were analyzed by FACS at day 17 after EAE induction. (G) Clinical score of the analyzed mice at day 17 after EAE induction (n=6-7/group). ** P≤0.01. Data are presented as mean ± SEM.

In order to further analyze the inflammatory status of the spinal cord in ΔdblGATA1 and WT mice, expression analysis of inflammatory molecules (IFNγ, IL-17A, IL-6, TNF, iNOS, IL-1β) in the spinal cord was performed in the peak phase of EAE. We did not observe any significant difference in any of the assessed molecules (Figure 11), showing that also the cytokine milieu is not changed in ΔdblGATA1 mice. In order to verify eosinophil deficiency, expression of eMBP, one of the eosinophil markers, was also analyzed by RT-qPCR. Indeed,

mRNA levels of eMBP were significantly downregulated in ΔdblGATA1 mice as compared to their littermate controls (Figure 11).

Taken together, these data show that there is no difference in the inflammatory status of the spinal cord when comparing eosinophil-deficient and –sufficient mice subjected to EAE which is in agreement with their identical clinical scores during EAE development (Figure 8).

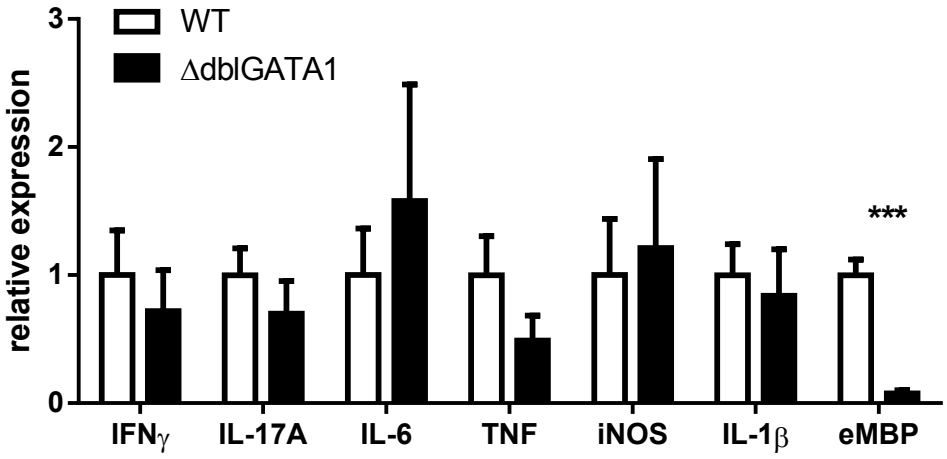

Figure 11 No difference in proinflammatory cytokines in ΔdblGATA1 mice

Female ΔdblGATA1 mice and their littermate controls were immunized with MOG_{35-55} in CFA in order to induce EAE. mRNA levels of IFNγ, IL-17A, IL-6, TNF, iNOS, IL-1β and eMBP in the spinal cord were analyzed using RT-qPCR at day 17 after EAE induction (n=6-7/group). The expression was normalized to RPS29 housekeeping gene. *** P≤0.001. Data are presented as mean ± SEM.

3.3.2 No difference in demyelination in the spinal cord of ΔdblGATA1 mice

Not only neuroinflammation but also demyelination is an important hallmark of EAE pathology and these two aspects are interconnected (Dendrou et al., 2015). Thus, we tested the degree of demyelination in the spinal cord of ΔdblGATA1 and WT mice at the peak of EAE using LFB staining of myelin. In accordance with no change in neuroinflammation and clinical development, we also did not observe any difference in demyelination (Figure 12) further suggesting that eosinophil deficiency does not affect EAE development.

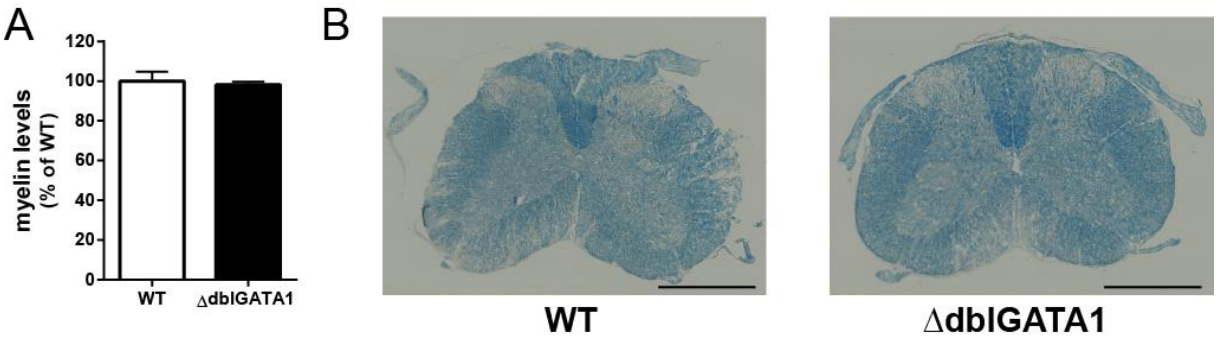

Figure 12 Demyelination in ΔdblGATA1 mice

Female ΔdblGATA1 mice and their littermate controls were immunized with MOG_{35-55} in CFA in order to induce EAE. At day 17 after immunization, 12µm spinal cord cryosections were stained with LFB for myelin levels measurement (n=3-5/group). (A) quantification, (B) representative pictures. Scale bar 500 µm. Data are presented as mean ± SEM.

3.3.3 No difference in recall response in ΔdblGATA1 mice

In order to complete the analysis of a possible impact of eosinophils on EAE development, the initiation phase of EAE was investigated in ΔdblGATA1 mice. These mice and their littermate controls were immunized with MOG_{35-55} peptide in order to induce EAE. They were sacrificed 7 days later and a recall response of T cells isolated from draining lymph nodes to the MOG_{35-55} antigen was analyzed. The isolated T cells were co-cultured with MOG_{35-55}-pulsed dendritic cells differentiated from bone marrow cells isolated from untreated WT mice. In order to measure the activation of T cells, IFNγ production was assessed by intracellular staining followed by FACS analysis. No difference in the activation of CD4 and CD8 T cells was found since the percentage of IFNγ-producing CD4 and CD8 T cells, respectively, was not changed in ΔdblGATA1 mice (Figure 13A, B).These data show that eosinophil deficiency does not affect the initiation phase of EAE.

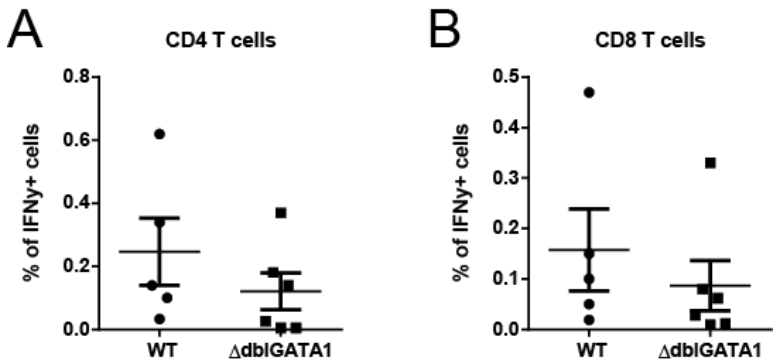

Figure 13 No difference in recall response in ΔdblGATA1 mice

Female ΔdblGATA1 mice and their littermate controls were immunized with MOG_{35-55} in CFA. At day 7, T cells isolated from draining lymph nodes were co-cultured with MOG_{35-55}-pulsed dendritic cells for 24 hours. Surface expression of CD4 and CD8 and intracellular expression of IFNγ were analyzed by FACS (n=6/group). Data are presented as mean ± SEM.

4 Discussion

MS is an autoimmune neuroinflammatory demyelinating disease, afflicting 2.5 million people worldwide, causing a variety of neurological problems often leading to a permanent use of wheel chair. Moreover, the typical onset of the disease is around 30 years of age, thus, the quality of life in the productive age is seriously affected. Unfortunately, the pathology and especially the cause of MS are not well characterized and therefore the successfulness of the treatment is limited. Although, the cause of the disease is controversial, it is mostly believed that the launching event is a dysregulation of immune system, which is followed by infiltration of immune cells into CNS and subsequent neuroinflammation (Dendrou et al., 2015). Many immune cell types were described to be involved in the immunopathology of MS, playing either positive or negative roles. However, there is only very limited information about a possible role of eosinophils in this disease. Thus, we were encouraged to investigate it in order to reveal new mechanisms of MS pathology and, maybe, to contribute to development of a more efficient MS treatment.

In order to study a role of eosinophils in MS, we employed its mouse model EAE that mimics both, the neuroiflammatory and demyelinating aspects of the disease. At first, we assessed whether there is eosinophil infiltration into CNS of EAE-subjected mice. For this purpose, EAE was induced in WT mice and the eosinophil numbers in the spinal cord were analyzed in the course of the disease and compared to healthy controls. In the present study, we showed that eosinophils infiltrate into the spinal cord during EAE and, moreover, that they are activated and degranulate there. Additionally, concentration of eotaxin-1, the eosinophil chemoattractant, was increased in EAE mice, suggesting eotaxin-1-mediated mechanism of eosinophil infiltration. Further, we induced EAE in two independent mouse models of eosinophil deficiency, ΔdblGATA1 and IL-5-depleted mice, and observed the clinical symptoms. Interestingly, we found that eosinophils are dispensable for EAE development in

both models. We chose ΔdblGATA1 mice, as the more specific model, and analyzed these mice in detail with regards to different aspects of EAE pathology. In accordance with the comparable clinical course, we did not observe any difference in the infiltration of various immune cell populations into the spinal cord, or in the proinflammatory molecule expression and demyelination. Furthermore, T cells isolated from ΔdblGATA1 mice behaved similarly in the recall response assay, indicating that the immunization phase was not affected by the eosinophil deficiency that is also in accordance with the results of the scoring experiments.

Recently, Finlay and colleagues reported that eosinophilia induced by an injection of helminth products has a protective effect against EAE development (Finlay et al., 2016). In contrast, we show herein that eosinophil deficiency does not influence EAE development. A possible explanation for this discrepant result is that, in their study, eosinophils acquire a regulatory phenotype due to the helminth products injection, thereby ameliorating EAE. Indeed, *in vitro* differentiated eosinophils from bone marrow without any additional treatment were not able to reduce EAE severity when transferred into EAE mice. In contrast, when they transferred eosinophils from mice treated with interleukin-33 (IL-33), a mediator of the helminth product-induced eosinophilia, development of EAE in recipient mice was impaired (Finlay et al., 2016). In accordance with the hypothesis that eosinophils with different phenotypes might play different roles in the immunity, Mesnil and colleagues showed that lung resident eosinophils have a different character than eosinophils infiltrating the lung after allergic stimulus. They propose that resident eosinophils, but not the infiltrated ones, possess a regulatory phenotype, as shown by transcriptome analysis, and are able to reduce immune response by inhibiting dendritic cell maturation (Mesnil et al., 2016). Thus, it might be that only eosinophils that have been polarized to a specific phenotype (e.g. after a parasitic infection) are capable to affect EAE development.

Another study assessing a role of eosinophils in EAE was performed by Gladue and colleagues. They were the first who reported eosinophil infiltration into the spinal cord of EAE mice. Further, they proposed that a treatment of EAE mice with a leukotriene B4 antagonist reduces the eosinophil infiltration and EAE severity. Moreover, they claimed that the

lymphocyte infiltration remains unaffected by this treatment and, therefore, that the effect is eosinophil specific (Gladue et al., 1996). However, leukotriene B4 is known to have a broad range of proinflammatory effects, including recruitment of immune cells and their activation (Le Bel et al., 2014). Therefore, it is likely that other cell types besides eosinophils were affected by the leukotriene B4 antagonist. Moreover, Gladue et al. induced EAE by the adoptive transfer of encephalogenic T cells into SJL mice (Gladue et al., 1996), which is a model different from ours. Thus, different experimental conditions may also account for different findings between this previous study and ours.

Weir et al. showed that IL-5 deficiency does not affect EAE development, using IL-5 knock-out mice (Weir et al., 2003), which is in agreement with our results, especially with the experiment using IL-5 blockade to deplete eosinophils.

Zhang and colleagues reported that eosinophil degranulation causes demyelination in the spinal cord in a mouse model of NMO (Zhang and Verkman, 2013). However, although we see that eosinophils infiltrate into the spinal cord and likely degranulate therein during EAE, we did not observe any difference in demyelination between eosinophil-deficient and –sufficient mice. A possible explanation for this disagreement is that the mechanisms of antibody- and complement-dependent cell-mediated cytotoxity, described in NMO, do not occur in EAE. Another possible explanation is that the contribution of eosinophil degranulation to the overall demyelination is minor and, thus, the impact is not observable in eosinophil-deficient mice.

There are several reports that eosinophils can support initiation of an antigen-specific immune response, namely antigen presentation, either directly, when acting as antigen presenting cells, or indirectly by promoting dendritic cell maturation and migration. In contrast, there is a study proposing that eosinophils inhibit antigen-induced immune responses by TGFβ-mediated suppression of CD4 T cells (see chapter 1.3.1). In the present thesis, we show that eosinophil deficiency did not affect antigen-specific T cell activation, since we did not observe any difference in antigen-specific *in vitro* restimulation of T cells isolated from MOG-immunized ΔdblGATA1 and control mice. In accordance with our results,

Weir and colleagues also did not see any abnormalities in recall response of T cells isolated from MOG-immunized IL-5-deficient mice (Weir et al., 2003). The discrepancy in reports describing the role eosinophils in the initiation of antigen-specific immune response might result from use of different mouse strains and immunization models. While we and Weir et al. use mice in a C57BL/6 background immunized with MOG in order to induce EAE, the majority of the other studies used mice with BALB/c background subjected to a model of allergic inflammation.

Furthermore, we show a significant upregulation of eotaxin-1, the eosinophil chemoattractant, at the peak of EAE but most substantially at the onset of the disease. Additionally, expression of eotaxin-2, another eosinophil chemoattractant, was increased at the onset of EAE although not significantly. In a mouse model of asthma, it was shown that expression of eotaxin-1 and -2 is necessary for eosinophil recruitment into lung which is mediated by CCR3 receptor expressed on eosinophils (Pope et al., 2005). Thus, we propose that the eotaxin-1/2-CCR3 axis is responsible for eosinophil recruitment also into the spinal cord. In the future, this hypothesis will be tested using CCR-3 knock-out mice subjected to EAE.

In conclusion, we present herein that eosinophils infiltrate into the spinal cord in the course of EAE and they are activated and degranulate there. We suggest that the infiltration is mediated by eotaxins and their receptor CCR3. Finally, we show that eosinophils are dispensable for EAE development in our experimental setting. However, as described in literature, eosinophils can play a protective role against EAE development when they acquire a regulatory phenotype. This is a phenomenon that should be further studied in the context of MS pathogenesis.

5 Literature

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6 Declarations

Appendix 1

Technical University Dresden

Medical Faculty Carl Gustav Carus

Doctorate regulations, 2011/07/24

Declarations for the opening of the doctoral examination procedures

1. I herewith declare, that I prepared this thesis without prohibited assistance of a third party and without making use of aids other than those specified; notions obtained directly or indirectly from other sources are indentified as such.
2. The following persons supported me in material selection and evaluation as well as in preparation of the manuscript:
    - Ales Neuwirth, *Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Dresden, Germany*
    - Jong-Hyung Lim, *Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Dresden, Germany*
    - Georgia Fodelianaki, *Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Dresden, Germany*
    - Triantafyllos Chavakis, *Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Dresden, Germany*
3. No further persons were involved in the generation of this work. Particularly, I did not get help of a commercial doctoral adviser. Third parties did not directly or indirectly receive monetary values for work related to contents of this thesis.
4. This paper has not been previously presented in identical or similar manner to any other German or foreign association board.

5. The contents and data of this thesis have not been published elsewhere yet
6. I declare not to have undertaken any previous unsuccessful doctoral examination procedures.
7. I declare to recognize the doctorate regulations of the Faculty of Medicine of the Dresden University of Technology.
8. I noticed and followed the doctorate citation guidelines of the Faculty of Medicine of the Dresden University of Technology.

Dresden, 2017

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Klara Ruppova

Appendix 2

I herewith declare, that all work in this thesis was performed according to the current legal specifications.

- Approval of the Ethics Committee for clinical studies, epidemiological studies referring to individuals or situations, which concern the Medicinal Devices Act.

  Project's file number of the responsible Ethics Committee:

  Not applicable

- Conformance with regulations of the Protection of Animals Act

  Project's file numbers of the approving authority:

  DD24-5131/207/14 TVV 2014/13

- Conformance with the Genetic Engineering Act

  Reference numbers:

  56-8811.72/1/39 21.11.2002

- Accordance with the regulations of data protection of the Faculty of Medicine of the Dresden University of Technology.

Dresden, 2017

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Klara Ruppova

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