The role of the chemokine receptor CXCR3 in mouse models for IL-12-driven CNS-inflammation and Morbus Alzheimer-like neurodegeneration

Kumulative Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, 2012

Angefertigt mit Genehmigung
der Mathematisch-Naturwissenschaftliche Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Mündlichen Prüfung: 18.01.2013
Erscheinungsjahr: 2013

Meinen Eltern

„Wenn man als Werkzeug nur einen Hammer hat, sieht jedes Problem wie ein Nagel aus“

Abraham Maslow (1908-1970)

1. Summary

The chemokine receptor CXCR3 and its corresponding ligands CXCL9, CXCL10 and CXCL11 are well known to be involved in the trafficking and migration of activated $CD4^+$ Th1 T cells, $CD8^+$ T cells and NK cells during inflammation. Because of the high levels of CXCR3 expression on Th1 and NK cells, this chemokine receptor is used as a prototypical marker for these cells. Functional existence of CXCR3 has also been demonstrated on resident cells of the CNS, although the relevance of CXCR3 for CNS immune and non-immune functions is only scarcely defined. The CXCR3 ligands CXCL9, CXCL10 and CXCL11 are induced in a vast variety of inflammatory CNS diseases with a variable degree of immune cell infiltration, but recently these ligands have also been shown to be induced in neurodegenerative diseases without significant infiltration of immune cells. Taken together, the available data argues for a diverse and complex role of CXCR3 in neuroinflammatory diseases, which is beyond simple immune cell attraction. Furthermore, the impact of CXCR3 in neurodegenerative diseases is almost undiscovered.

To further examine the functional role of CXCR3 in CNS disease models, we genetically deleted the CXCR3 receptor in specific CNS disease models. We first focused on the impact of CXCR3 on a highly inflammatory, Th1 cell-mediated immune response in the CNS induced by the CNS-specific production of IL-12 in transgenic mice (GF-IL12 model, Chapter 1). Secondly and in contrast to the first model, we examined the role of CXCR3 signaling in a neurodegenerative disease using transgenic mice co-expressing two human Alzheimer's disease (AD) mutations (APPsw/PS1ΔE9, short APP/PS1 model, Chapter 2) with only minor inflammatory features.

GF-IL12 mice develop ataxia due to severe cerebellar inflammation, but have little overt ocular pathology. In GF-IL12 mice deficient for CXCR3 (termed GF-IL12/CXCR3KO) the incidence of ataxia was drastically reduced, but surprisingly all mice developed cataract and severe inflammatory destruction of the eyes. Histological examination revealed only minimal cerebellar inflammation in the majority of GF-IL12/CXCR3KO mice, but severe retinal disorganization, loss of photoreceptors and lens destruction of the eyes. The number of $CD3^+$, $CD11b^+$ and NK 1.1^+ cells were reduced in the cerebellum, but highly increased in the eyes of GF-IL12/CXCR3KO compared to GF-IL12 mice. In addition, high levels of various transcripts of proinflammatory cytokines were found in the cerebellum of GF-IL12 and the eye of GF-IL12/CXCR3KO mice. These findings demonstrate key, but paradoxical

functions for CXCR3 in IL-12-induced immune pathology in the CNS, promoting inflammation in the brain yet restricting it in the eye. From this experiment we conclude that CXCR3 can have both striking protective and harmful functions in CNS and ocular inflammation and that this effect does not only depend on the trigger as suggested by previous studies but likely also on the micro-milieu of the affected organ.

Early chemokine induction has been described in chronic neurodegenerative diseases such as AD. Descriptive studies in brain tissue from AD patients and the according animal models revealed high levels of the chemokine CXCL10, suggesting an important pathogenetic role of this chemokine and the corresponding receptor CXCR3. To further elucidate the role of CXCR3 in a less inflammatory CNS disease model, we analyzed CXCR3-competent APP/PS1 transgenic mice (APP/PS1) and CXCR3-deficient APP/PS1 transgenic mice (APP/PS1/CXCR3$^{-/-}$) for Aβ-deposition, APP-processing and inflammatory gene transcription. Furthermore, microglial phagocytosis assays were used to analyze the impact of CXCR3 on the microglial phagocytosis of Aβ. We found a strongly reduced plaque burden and Aβ peptide-levels APP/PS1/CXCR3$^{-/-}$ compared to APP/PS1 mice. An alternative morphological activation and diminished accumulation of microglia was detected in APP/PS1/CXCR3$^{-/-}$ mice and after cortical injection of Aβ into CXCR3$^{-/-}$ mice. CXCR3 deficiency led to a reduction of proinflammatory cytokine RNA levels like TNF-α and IL-1β in APP/PS1 brain tissue. *In vitro*, CXCR3$^{-/-}$ and CXCR3 antagonist treated microglia showed enhanced phagocytosis of Aβ. Taken together, we identified CXCR3 as a critical factor modulating the development of the microglial response and thereby the progression of the Alzheimer's like pathology observed in APP/PS1 mice.

The presented studies highlight the potent but also complex functional properties of CXCR3 in both, highly inflammatory and neurodegenerative CNS-disease models. CXCR3 appears to be a novel and promising therapeutic target for AD but our data further underline the functional complexity and unpredictability of this chemokine system in CNS diseases. Until then, therapeutic targeting of CXCR3 has to be proceeded with caution.

2. Zusammenfassung

Im Rahmen von Entzündungsreaktionen sind der Chemokinrezeptor CXCR3 und die CXCR3-Liganden CXCL9, CXCL10 und CXCL11 maßgeblich an der Steuerung und Migration von aktivierten $CD4^+$ Th1 T Zellen, $CD8^+$ T Zellen und NK Zellen beteiligt. CXCR3 ist der typisches Markermolekül für Th1 und NK Zellen. Die funktionelle Expression von CXCR3 ist auch auf Zellen des ZNS nachgewiesen worden, wobei der Einfluss dieses Rezeptorsystems auf immunologische und nicht-immunologische Prozesse im ZNS noch unzureichend geklärt ist. Im ZNS werden die Liganden CXCL9, CXCL10 und CXCL11 in einer Vielzahl von inflammatorischen Erkrankungen mit variabler Immunzellinfiltration induziert. In der Übersicht diverser Studien zu entzündlichen Erkrankungen des ZNS zeigt sich eine komplexe Rolle des CXCR3 Rezeptorsystems, welche jenseits einfacher Zellrekrutierung liegt. Vergleichend dazu ist der Einfluss des Rezeptors auf den Verlauf neurodegenerativer Erkrankungen ohne signifikante Immunzellinfiltration bisher nahezu ungeklärt.

Um die Rolle von CXCR3 besser definieren zu können, wurde der Rezeptor in zwei ZNS Erkrankungsmodellen durch genetischen Knockout deletiert. Der erste Teil der Arbeit fokussiert sich auf eine hochentzündliche, Th1-gesteuerte Immunantwort, die durch die ZNS-spezifische Expression von IL-12 in transgenen Mäusen vermittelt wird (GF-IL12 Modell, *Chapter 1*).

Im Gegensatz zum ersten Modell, wurde im zweiten Modell die Rolle von CXCR3 in einer neurodegenerativen Erkrankung mit entzündlichen Prozessen, jedoch ohne signifikante Immunzellinfiltration analysiert (APP/PS1 Modell, *Chapter 2*). Das hierzu verwendete transgene Mausmodell basiert auf der Koexpression zweier humaner Alzheimer-Mutationen (APPsw/PS1ΔE9, kurz APP/PS1).

GF-IL12 Mäuse entwickeln eine progrediente Ataxie, infolge einer starken zerebellären Entzündungsreaktion, jedoch ohne pathologische Veränderungen des Auges. Bei GF-IL12 Mäusen mit CXCR3-Defizienz (GF-IL12/CXCR3KO) kommt es zu einer drastisch verringerten Inzidenz der Ataxie, aber zu einer frühen Bildung von Katarakten, begleitet von einer starken Entzündungsreaktion, welche bis hin zur Atrophie des Auges führt. Histologische Untersuchungen der GF-IL12/CXCR3KO Mäuse belegen eine zum Großteil sehr milde zerebelläre Entzündung, jedoch die Disorganisation der Zellschichten der Retina konkomitierend mit dem Verlust der Photorezeptor-Zellschicht und der Zerstörung der

Linse. Im Vergleich zu GF-IL12 Mäusen zeigen GF-IL12/CXCR3KO Tiere eine reduzierte Anzahl von $CD3^+$, $CD11b^+$ und NK 1.1^+ Zellen im Zerebellum, jedoch stark erhöhte Populationen dieser Zellen im Auge. Weiterhin wurde die Erhöhung von Zytokin/Chemokin Transkripten im Zerebellum von GF-IL12 und in den Augen von GF-IL12/CXCR3KO Mäusen detektiert. Die vorliegende Arbeit weist auf eine Schlüsselfunktion von CXCR3 in der IL-12 induzierten ZNS-Pathologie hin, welche gewebespezifisch eine Entzündung im Zerebellum fördern, jedoch im Auge unterdrücken kann.

Die Induktion von Chemokinen im frühen Stadium von ZNS-Pathologien ist ein Charakteristikum von chronisch neurodegenerativen Erkrankungen, wie der Alzheimer-Erkrankung (AD). In der Vergangenheit wurde in einer Reihe von deskriptiven Studien die Induktion von CXCL10 im Hirngewebe von AD-Patienten und in AD-Maus-Modellen dokumentiert, jedoch nicht funktionell untersucht.

In der zweiten Studie (*Chapter 2*) wurden APP/PS1 und CXCR3-defiziente APP/PS1 (APP/PS1/$CXCR3^{-/-}$) Mäuse gezüchtet und auf β-Amyloid-(Aβ)-Plaquelast, APP-Prozessierung und Transkription proinflammatorischer Gene untersucht. Weiterhin wurde der Einfluss des CXCR3 Rezeptors auf die Phagozytose von Aβ durch Mikroglia untersucht. Es zeigte sich, dass APP/PS1/$CXCR3^{-/-}$ Mäuse eine stark reduzierte Plaquelast, sowie eine reduzierte Menge an löslichen und unlöslichen Aβ-Peptiden aufweisen. Zudem ließ sich eine differentielle morphologische Aktivierung und verringerte Akkumulation von Mikroglia in APP/PS1/$CXCR3^{-/-}$ Tieren, sowie in $CXCR3^{-/-}$ Mäusen nach intrazerebraler Aβ-Injektion beobachten. Des Weiteren verringert CXCR3-Defizienz im APP/PS1-Model die Genexpression von proinflammatorischen Zytokinen wie TNF-α und IL-1β. *In vitro* kultivierte, primäre $CXCR3^{-/-}$ Mikroglia phagozytieren signifikant mehr fibrilläres Aβ, was sich darüber hinaus auch in WT Mikroglia nach blockieren des CXCR3 Rezeptors beobachten lässt. Zusammenfassend lässt sich sagen, dass CXCR3 eine wichtige Funktion in der Entwicklung der Alzheimer-ähnlichen Krankheit im APP/PS1 Model hat.

Die vorliegenden Modelle für hoch entzündliche und degenerative neurologische Erkrankungen geben Einsicht in potente, aber komplexe Funktionen des CXCR3 Rezeptorsystems. CXCR3 moduliert nicht nur die Verteilung und Aktivierung von infiltrierenden Immunzellen, sondern auch von residenten Immunzellen des ZNS.

3. General introduction

3.1 The brain: an organ with distinctive immunological features

Studies addressing the interaction of the nervous and the immune system have to consider the special properties of the unique immune-modulating milieu of the nervous system. 25 years ago, the idea that the central nervous system (CNS) and the immune system could dynamically interact was considered very unlikely because it was believed that the CNS environment is immunosuppressive. This assumption was based on the observation that tissue grafts are able to survive within the brain for a long period (Geyer et al., 1985; Head and Griffin, 1985).

The immune-modulating milieu differs in between particular regions of the CNS. The CNS is organized into different compartments: the parenchyma, the ventricles containing choroid plexus and cerebrospinal fluid (CSF), and the meninges. Robust proinflammatory T cell responses to grafted tissue and pathogens are often triggered within the non-parenchymal sites of the CNS: the ventricles, the meninges, and the subarachnoid spaces (Murphy and Sturm, 1923; Mason et al., 1986; Matyszak and Perry, 1996; Perry, 1998). The ability of these sites to host a typical peripheral immune response suggests that the ventricular and subarachnoid CSF may function as sites of immune surveillance. Consistent with this, cellular infiltrates that accumulate within the meningeal membranes have been observed to arrange structures resembling secondary lymphoid structures (Serafini et al., 2004), while infiltrates within the parenchyma do not exhibit the features of lymphoid neogenesis (Holman et al., 2011). Recently, it has been shown that $CD4^+$ T cells are restimulated within the subarachnoid space by encountering $MHCII^+$ antigen presenting cells prior to the onset of inflammation in a murine model of autoimmune inflammation of the CNS. This observation further supports the concept of the subarachnoid space and meninges as a site of immunological surveillance (Kivisäkk et al., 2009; Holman et al., 2011).

Apart from these areas, there is a high threshold for initiating leukocyte responses within the CNS parenchyma. Different factors are accountable: firstly, the presence of the blood-brain barrier (BBB) that inhibits or at least controls T cell entry into the CNS, thus impending T cell encounter with CNS antigens; secondly, the absence of a traditional lymphatic system that communicate CNS with regional lymph nodes (CNS can drain antigens by alternate routes such as the physiological CSF circulation into the blood and via some cranial and

spinal nerves roots into the lymph); and third, the inability of microglia, the resident macrophages of the CNS, to present mature antigen to T cells (Romo-González et al., n.d.; Carson et al., 2006; Galea et al., 2007).

During inflammation, different molecules allow leukocytes to interact with endothelial cell barriers and gain access to parenchymal tissues. The sequential process of cellular rolling, adhesion and diapedesis is mediated and guided by the combined expression of chemokines (e.g. CCL19, CCL21, CCL2, CCL4 and CCL5) and chemokine receptors (e.g. CCR1, CCR2 and CCR5) (Cinamon et al., 2001; Adamson et al., 2002; Alon and Feigelson, 2002; Johnston and Butcher, 2002), adhesion molecules (e.g. CAMs, selectins and integrins) (Ley, 1996; Johnston and Butcher, 2002; Ley et al., 2007; Holman et al., 2011) and lipid chemoattractant receptors (e.g. BLT1). Once leukocyte mediated inflammation of the parenchym is established, resident cells of the CNS, like activated microglia and astrocytes can participate in orchestrating the invading immune cells by the production of various factors. The underlying molecular and cellular mechanisms governing inflammation are not completely understood, but one essential group of molecules for both the recruitment and activation of infiltrating and resident immune cells in the CNS are chemokines.

3.2 Introduction to chemokines and chemokine receptors

Chemokines are a family of cytokines with chemoattractive properties. Due to their similarities in molecular structure and their common potential to induce chemotaxis of a variety of cell types, they where categorized separately from other cytokines (Rollins, 1997). The family of chemokines are currently divided into 2 major subclasses, which are named CC- and CXC-chemokines (Zlotnik and Yoshie, 2000; Bacon, 2001). The CXC chemokines are characterized by the interposition of a single amino acid (X) between their first two cysteine residues. This CXC subfamily can be subclassified into two other groups, depending on the presence or absence of the sequence motif glutamic acid– leucine–arginine (ELR) near the N-terminus. In addition, three additional molecules are also regarded as chemokines. These are CX3CL1, with three interventing amino acids between the first cysteines (Bazan et al., 1997), and XCL1 and XCL2 (Stievano et al., 2004), which lack two out of four canonical cysteines. To date, the official nomenclature accounts for almost 50 human chemokines. In parallel to this nomenclature, many chemokines can generally be classified into two functional groups. The first group represents the homeostatic chemokines, which are constitutively expressed and generally involved in lymphoid organ development and

maintenance, as well as immune-surveillance cell trafficking. The second group are inflammation related chemokines, which are induced by stimuli such as pathogens (Luster, 2002; Werling and Jungi, 2003; Pasare and Medzhitov, 2004) or proinflammatory cytokines (e.g. IFN-γ, IL-1 or TNF-α). These chemokines are involved in the mobilization and guidance of effector cells to sites of inflammation (Luster et al., 1985; Aloisi et al., 1992; Cole et al., 1998; Mantovani, 1999).

Secreted chemokines mediate their biological activity through G-protein-coupled cell-surface receptors (GPCRs; Dohlman et al., 1991). These receptors are named according to their subfamily classification and generally share common structural features, which include a polypeptide backbone that loops seven times across the plasma membrane to form three intracellular and three extracellular loops. The human chemokine receptor system at present consists of 19 different GPCRs and represents the most diverse class of cell-surface receptors. The expression of chemokine receptors is heterogeneous and not restricted to hematopoietic cells. Some chemokine receptors are widely expressed, whereas others are restricted to certain specific cells, by specific activation or differentiation states (Luster, 1998). Notably, most chemokine receptors are promiscuous, which means that an individual chemokine receptor can bind several different chemokines, and conversely, individual chemokines can often bind to several different receptors. The chemokine binding to a particular receptor is determined by its receptor affinity, which varies significantly between ligands but does not necessarily translate into functional potency (D'amico et al., 2000).

Successful activation of chemokine receptors is induced by the initial recognition between chemokines and their receptors at exposed loops between the β-strands of the chemokine fold and the chemokine receptor extracellular domains. Secondly, the N-terminal region of the chemokine initiates the activation of the receptor, which is followed by the internalization of the complex regions. G proteins are then activated, driving dissociation of their heterotrimers into α and βγ subunits. Intracellular effectors of signal-transduction include MAPK-associated, CREB-activated and PLC pathways. Downstream, increased Ca^{2+} mobilization results in the activation of proteinkinase C and many other kinases, like serine/threonine- and thyrosine-kinases (Mellado et al., 2001). This activates multiple cellular responses, such as adhesion, polarization and chemotaxis (Asensio and Campbell, 1999; Cardona et al., 2008). Furthermore, chemokine receptor activation and signaling is also controlled by desensitization (Mellado et al., 2001; Vroon et al., 2006). Desensitization implies a multistep process and a plethora of proteins, including G-protein-coupled receptor kinases (GRKs) and

β-arrestins. The process begins with the phosphorylation of the chemokine receptor C-terminal tail by GRKs, which increases the receptor affinity for β-arrestin proteins. The binding of β-arrestins to chemokine receptors prevents any other interaction between the receptor and G proteins. Afterwards, the GRK–β-arrestin complex promotes the internalization of the complete chemokine receptor into vesicular compartments for degradation or recycling (Savarin-Vuaillat and Ransohoff, 2007; Malik and Marchese, 2010; Fox et al., 2011).

Finally, chemokine receptors can also modulate their ligand-induced signaling cascades by receptor dimerization. The current view is that chemokine receptor homo-and heterodimers are constitutively formed and ligand binding stabilizes or reorganizes pre-existing complexes (Salanga et al., 2009; Thelen et al., 2010; Bennett et al., 2011). Importantly, dimerization impacts on the cell's biological response to chemokine exposure. Cross-talk within homomers or heteromers enables regulation of chemokine receptors in response to stimuli other than their own ligands. This process, is known to occur within all types of GPCR dimers (Terrillon and Bouvier, 2004; Bennett et al., 2011).

Besides mediating essential signals, "decoy" receptors, like the Duffy antigen receptor for chemokines (DARC), D6 and CCX-CKR (Haraldsen and Rot, 2006) are able to sequester chemokines without mediating signal transduction. DARC binds with high affinity at least 11 "inflammatory" CXC- and CC-chemokines, D6 binds at least 9 "inflammatory" CC-chemokines and CCX-CKR only the "homeostatic" ones: CCL19 and CCL21, CCL25, as well as CXCL13 (Gosling et al., 2000). These non-signaling receptors can efficiently internalize specific chemokines and thereby control their bioavailability at local level or in the blood stream (Patterson et al., 2002; Lee et al., 2003).

The complexity of the chemokine system and the principal potential to regulate the signaling of the receptors and ligands at different levels explain why researchers are interested in these molecules, but sometimes nominate it as "chemokinese" or "pandora's box"(Rot and von Andrian, 2004; Karin, 2010)

3.3 Chemokines and chemokine receptors in the healthy brain, in CNS inflammation and neurodegenerative disorders

Chemokines are produced during various physiological and pathological processes in the CNS (Asensio and Campbell, 1999; Bacon and Harrison, 2000; Rebenko-Moll et al., 2006).

Because of their involvement in diverse neuroinflammatory diseases, interest in chemokines and chemokine receptors in the CNS has been rapidly increasing over the last decade. As previously mentioned, chemokines can be differentiated into constitutively expressed or inducible chemokines.

In general, constitutively expressed chemokine receptors and chemokine ligands represent nonredundant key regulators of developmental and homoeostatic processes of the CNS. Studies, aimed to clarify the physiological function of chemokines in the brain, have reported that chemokines can influence the neuronal migration in brain development (Bolin et al., 1998; Klein et al., 1999; Lazarini et al., 2000; Zhu et al., 2002; Vilz et al., 2005), cell proliferation (Robinson et al., 1998; Bajetto et al., 2001; Bakhiet et al., 2001; Hatori et al., 2002; Rezaie et al., 2002; Krathwohl and Kaiser, 2004) and the synaptic transmission (Giovannelli et al., 1998; Limatola et al., 2000; Stievano et al., 2004; Bertollini et al., 2006). One prominent chemokine receptor system constitutively expressed in most cells of the brain represents CXCR4/CXCL12 (Jazin et al., 1997; Lavi et al., 1997; Bajetto et al., 1999; McGrath et al., 1999; Tham et al., 2001, 2001; van der Meer et al., 2001; Banisadr et al., 2002, 2003; Tissir et al., 2004). Evidences implicate that CXCR4 signaling is required for the migration of several types of neural cells including neuronal precursor cells from the external germinal layer (Zhu et al., 2002), cortical neuronal progenitors (Lazarini et al., 2000), cerebellar granule neurons (Ma et al., 1998; Klein et al., 1999; Vilz et al., 2005) and dentate gyrus granular neurons (Bagri et al., 2002). In the mature CNS, CXCL12 modulates neurotransmission, neurotoxicity and neuroglial interactions (Li and Ransohoff, 2008). Additional chemokine–receptor networks involving CXCL8/CXCR2 and also CCL2/CCR2, more prominent for its role in brain inflammation and injury, have also nonredundant functions in physiologic processes, including neurogenesis, neuroprotection, and neurotransmission (Meng et al., 1999; Rezaie et al., 2002; Semple et al., 2010). CXCR7 represents another chemokine receptor expressed on embryonic cells during the early development of CNS (Burns et al., 2006). CXCR7 is suspicious to be not directly involved in neurogenesis, but to modulate CXCR4 function (Zou et al., 1998; Sierro et al., 2007; Sánchez-Alcañiz et al., 2011) because of its potential to bind CXCL12, whereas signaling pathways triggered by binding of CXCL12 remain controversial (Burns et al., 2006; Proost et al., 2007; Sierro et al., 2007; Mazzinghi et al., 2008; Wang et al., 2008).

The above described constitutive chemokine receptor systems give a brief impression of their impact on development and homoeostasis of the brain, whereas their specific functional interplay during physiological processes is still not well defined.

In addition to their functional properties under physiological conditions, chemokines are intimately involved in the pathogenesis of disease states of the CNS, in particular in neuroinflammatory diseases (Rot and von Andrian, 2004; Engelhardt and Ransohoff, 2005; Charo and Ransohoff, 2006a; Rebenko-Moll et al., 2006). A plethora of immune and resident CNS cells can produce both chemokines and chemokine receptors during neuroinflammation, thereby modulating the local inflammatory milieu. These cells include granulocytes, lymphocytes, monocytes but also resident neurons, endothelial cells and in particular microglia and astrocytes (Charo and Ransohoff, 2006a; Savarin-Vuaillat and Ransohoff, 2007). Chemokines produced in response to pathogens typically are ligands of CCR1, CCR2, CCR3, CCR5, CXCR2, and CXCR3 (Taub et al., 1993; Dorf et al., 2000; Lee et al., 2002; Ambrosini and Aloisi, 2004; Cartier et al., 2005; de Haas et al., 2007; Farina et al., 2007). Chemokines are involved in both pathogen-driven and autoimmune CNS inflammatory but also in neurodegenerative diseases. In the following section, the role of key inducible chemokine systems during CNS diseases will be dissected.

During acute viral infection of the CNS, astrocytes and microglia are the primary source of chemokines following infection with a wide range of neurotropic viruses, including the JHM strain of mouse hepatitis virus (JHMV), lymphocytic choriomeningitis virus (LCMV), Theiler's murine encephalitis virus (TMEV), herpes simplex virus 1 (HSV1), and human immunodeficiency virus (HIV) (Lane et al., 1998; Aravalli et al., 2005; Christensen et al., 2009; So and Kim, 2009; Lim and Murphy, 2011). Neurons are also capable of secreting chemokines during HIV and West Nile virus (WNV) infection (van Marle et al., 2004; Klein et al., 2005), while endothelial cells express chemokines during simian immunodeficiency virus-induced encephalitis (Sasseville et al., 1996). After viral infection of the CNS, activated and/or virally infected astrocytes, microglia, and endothelial cells secrete chemokines that attract myeloid cells to the CNS. One of the earliest cells to respond to viral infection, neutrophils are recruited into the CNS by virtue of CXCR2 responding to ligands expressed within the CNS (e.g., CXCL1) (Hosking et al., 2009). Beside these cells, monocytes are also attracted into the CNS via the chemokine CCL5 and its receptor CCR5(Glass et al., 2001, 2005; So and Kim, 2009). Neutrophils and monocytes participate in the degradation of the BBB, in part through the release of the matrix metalloproteinase MMP-9, and therefore

ensure successive infiltration of virus-specific lymphocytes into the CNS. During the acute stage of disease, astrocytes, microglia, neurons, and endothelial cells continue to secrete chemokines, serving to attract activated T lymphocytes, NK cells, and monocytes into the CNS. $CD8^+$ and $CD4^+$ T lymphocytes bearing the receptor CXCR3 and/or CCR5 are attracted by the chemokines CXCL10 and CCL5, respectively, and mediate viral control through direct cytolytic activity and/or cytokine secretion (Asensio and Campbell, 1997; Lane et al., 1998; Liu et al., 2001; Trifilo and Lane, 2003; Glass et al., 2004, 2005; Trifilo et al., 2004; Zhang et al., 2008; Hosking and Lane, 2010). CXCL12 may sequester T lymphocytes within the perivascular space and regulate penetration of the parenchyma, thus inhibiting efficient viral clearance (McCandless et al., 2008; Hosking and Lane, 2010).

As outlined a simplified picture has developed that indicates that chemokines and their receptors are intimately involved in generation of effective host responses to viral infections within the CNS, by influencing T lymphocyte, NK cell and monocyte trafficking and activation.

Multiple sclerosis (MS) is the most common autoimmune disorder of the CNS leading to severe inflammation, demyelination and axonal damage of the CNS. The pathogenesis of MS have been widely investigated in descriptive human studies or the experimental autoimmune encephalomyelitis (EAE), an animal model of MS, which recapitulates the basic disease mechanisms (Gold et al., 2000; Lassmann, 2010). Perivascular infiltrates of T cells, B-cells and monocytes/macrophages are a prominent histological feature of MS lesions (Lucchinetti et al., 1998). Many proinflammatory cytokines, including IL-1β (Dinarello, 2009), TNF family members (Ware, 2005) and IL-17 (Kebir et al., 2007) induce chemokines, that might contribute to the recruitment of leukocytes into the vascular cuffs of MS and EAE lesions (Opdenakker and Van Damme, 2011). The chemokines in acute and chronic MS lesions include CCL2, CCL3, CCL4, CCL5 CCL7 and CCL8, localized within the lesion center and suggest a potential role of these chemokines in the inflammatory events (Sørensen et al., 1999; Trebst and Ransohoff, 2001). All receptors, CCR1, CCR2, CCR3, CCR5 and CCR8 were detected on macrophages and activated microglia within chronic active MS lesions, those which also contain the highest levels of ligands for these receptors (Balashov et al., 1999; Simpson et al., 2000a). Furthermore, studies suggests that the expression of CCR5 and CXCR3 by T lymphocytes and CX3CR1 by NK cells is associated with disease activity of MS (Balashov et al., 1999; Infante-Duarte et al., 2005). Finally, there is evidence that both CXCL10 and CXCL9 may play a role in MS pathogenesis (Simpson et al., 2000b). Both have

been detected in actively demyelinating lesions, predominantly expressed by macrophages within the plaques and by reactive astrocytes in the surrounding parenchyma and their cognate receptor, CXCR3, was detected on T cells and astrocytes within the plaques (Balashov et al., 1999; Simpson et al., 2000b). A lot of information has been provided by EAE studies. Using monophasic or relapsing EAE models, functional roles for CXCL1, CXCL10, CCR1, and CCR2 were characterized during the acute phase; CCL2, CCR2, CCL20, and CCR6 were associated with relapses (Ubogu et al., 2006; Savarin-Vuaillat and Ransohoff, 2007).

During Alzheimer's disease (AD) chronic microglia activation in regions associated with Aβ deposition is a prominent feature. Although this neurodegenerative disease exhibits no significant immune cell infiltration of the brain, descriptive studies have demonstrated the presence of chemokines and their receptors in AD tissues. An early induction of different chemokines in AD like CCL2, CXCL10 and CXCL8 in serum, CSF and brain tissue has been described (Galimberti et al., 2003, 2006; Corrêa et al., 2011). CXCR3 was detected on neurons, and its ligand CXCL10, was shown to be increased in astrocytes (Xia et al., 2000). Like CXCR3, CXCR2 was shown to be expressed on neurons, with its expression strongly upregulated in senile plaques (Xia and Hyman, 2002). Furthermore, CCL2 was found in senile plaques and reactive microglia of AD brain tissues (Ishizuka et al., 1997). Additional descriptive studies revealed elevated expression of CCR3 and CCR5 on reactive microglia, associated with amyloid deposits and CCR5 ligand (CCL3 and CCL4) induction in neurons and a subpopulation of reactive astrocytes (Xia et al., 1998).

In summary, it is likely that the production of chemokines plays a role in the recruitment and accumulation of astrocytes and microglia in senile plaques. More recent studies also implicate a relevant impact of chemokine receptors like CCR2, CCR5 and CX3CR1 on the functional polarization of microglia and possibly of astrocytes in different mouse models of AD (Lee et al., 2002, 2009; El Khoury et al., 2007; Fuhrmann et al., 2010; Liu et al., 2010). These receptors are already established to modulate detrimental but also neuroprotective phenotypes of microglia. Related to M1/M2 polarization of peripheral macrophages (Mantovani et al., 2004) there is conclusive data that argue for similar polarization of microglia with distinct functional properties and characteristic chemokine profiles (Durafourt et al., 2012).

However, in neuroinflammatory and neurodegenerative disorders diverse cell types can be induced to produce a wide range of chemokines, which induce chemotaxis, tissue

extravasation and in some instances modulate the functional properties of different resident cells and leukocytes (Sallusto et al., 1998; Tanuma et al., 2006; Farina et al., 2007; Subileau et al., 2009; Fuller et al., 2010; Graeber, 2010). The exact mechanisms and the interplay of the different chemokines molecules and receptors are still not very good defined. Important to keep in mind is the fact that the chemokines are not only active during disease states. Besides their important role in the developing brain, in the mature CNS a lot of physiological responses are regulated by chemokines and their receptors. Because chemokines display pleiotropic functions, blocking one chemokine receptor to treat neurological disease could present unexpected results.

3.4 The function of the CXCR3 chemokine system in neurological disorders and corresponding animal models

CXCR3 is, like all chemokine receptors, a seven–transmembrane GPCR that can be differentially activated by CXCL9, CXCL10 and CXCL11 (Loetscher et al., 1998; Weng et al., 1998; Proost et al., 2001; Colvin et al., 2004, 2006)(Figure 1 A). The CXCR3 ligands can activate the Ras/ERK, Src, and the PI3K/Akt pathway, thereby modulating critical cellular functions, most notably integrin activation, cytoskeletal changes, suppression of angiogenesis and chemotactic migration (Strieter et al., 1995, 2005; Loetscher et al., 1998; Zlotnik and Yoshie, 2000; Bonacchi et al., 2001). Only the Th1 subset of $CD4^+$ cells and NK cells express CXCR3 at high levels (Kim et al., 2001b) and therefore, CXCR3 is established as surface marker of these celltypes. Moreover, studies argue for functional existence of CXCR3 on other cells of the leukocytes lineage and resident cells of the CNS. These cells include monocytes and macrophages (Luster and Leder, 1993; Taub et al., 1993; Luster et al., 1995), microglia (Biber et al., 2002; Rappert et al., 2004; de Jong et al., 2008a), astrocytes (Biber et al., 2002; Flynn, 2003) and neurons (Xia et al., 2000), whereas its biological function on these cells widely remains to be discovered (Figure 1 B).

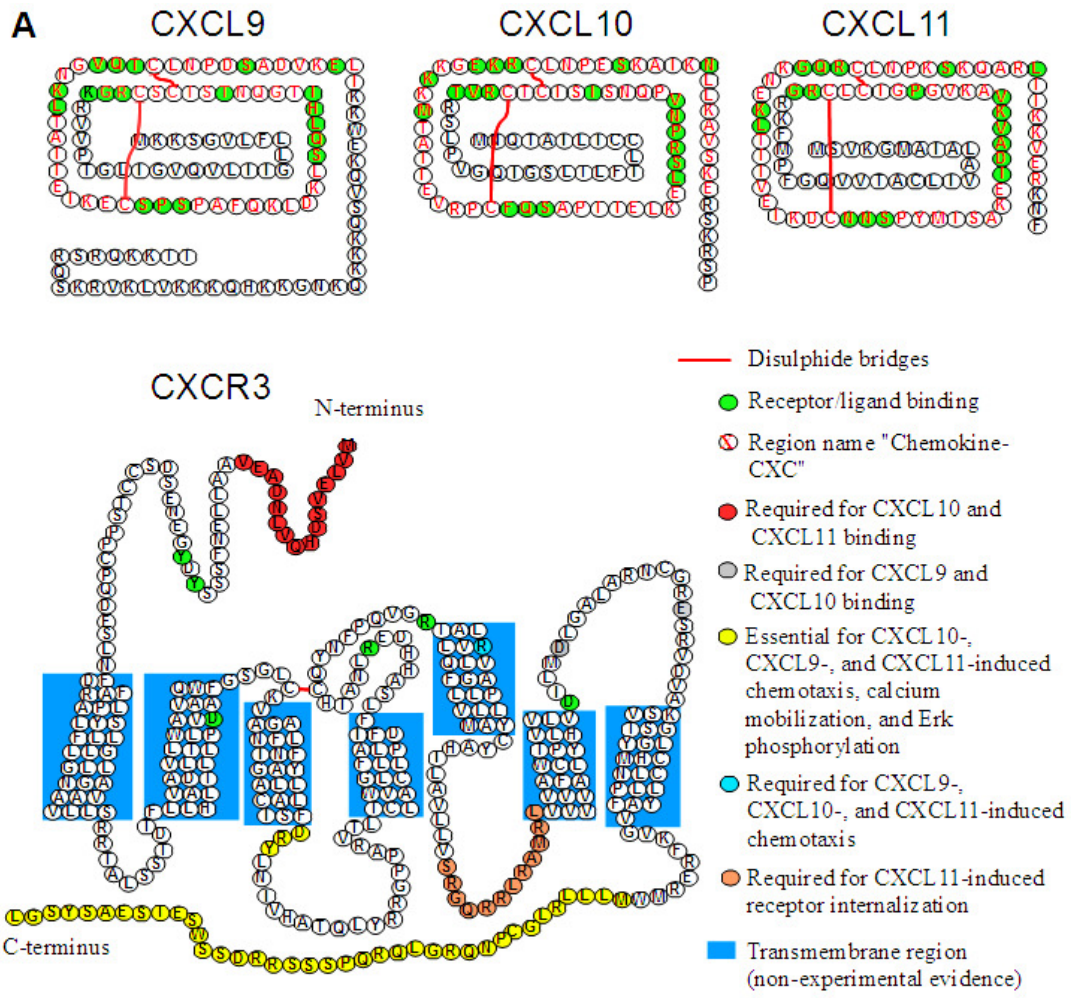
CXCL9, CXCL10 and CXCL11 as the receptor itself are mainly induced and regulated by IFN-γ (Luster et al., 1985; Ferber et al., 1996; Cole et al., 1998), moreover induction of in particular CXCL10 has been detected in response to IFN-β, TNF-α, IL-1β, FasL and TLR ligands (Majumder et al., 1998; Lee et al., 2000; Ghersa et al., 2002; Loos et al., 2006; Choi et al., 2011). IFN-γ is the most potent of these inducers, being capable of inducing the genes for all three chemokines (Luster et al., 1985; Farber, 1990; Farrar and Schreiber, 1993; Cole et al., 1998). The production of IFN-γ itself is largely restricted to activated T cells and NK-cells

(Farrar and Schreiber, 1993). The induction of CXCL9, CXCL10 and CXCL11 occurs almost frequently in humans and rodents during the course of cell-mediated immune responses evoked in a variety of pathologic states including infection, allograft rejection and autoimmunity (Hancock et al., 2000; Simpson et al., 2000b; Garcia-Lopez et al., 2001; Patel et al., 2001; Widney et al., 2005; Hofer et al., 2008; Miu et al., 2008). In this regard it should be noted that C57BL/6 mice have a deletion in the mRNA coding sequence for CXCL11 (NCBI Accession: NT_109320, AK040051.1 and AK050012.1, unpublished observation) and are considered to be functionally deficient for CXCL11.

However, CXCL11 also shows a number of functional differences from CXCL10 and CXCL9, it has significantly higher receptor binding affinity and is a more potent chemoattractant than CXCL10 or CXCL9 (Booth et al., 2004). Moreover, CXCL9, CXCL10 and CXCL11 have also been reported to display differences in the tissue expression patterns during immunoinflammatory responses (Mach et al., 1999; Flier et al., 2001; Goddard et al., 2001; Zhao et al., 2002) hinting at the likelihood that functional specialization exists within this chemokine family (Müller et al., 2010). Studies in mice with gene-targeted disruption of the CXCL9 or CXCL10 genes confirm the non-redundant function of these chemokines in a variety of immunoinflammatory disease states (Kakimi et al., 2001; Menke et al., 2008; Zhai et al., 2008). Even at the level of the same target T cell population the actions of the individual CXCR3 ligands can differ. Thus, while all three chemokines bind and activate CXCR3 (Loetscher et al., 1996, 2001; Cole et al., 1998) this requires different essential intracellular domains of CXCR3 (Xanthou et al., 2003; Colvin et al., 2004, 2006; Dagan-Berger et al., 2006)(Figure 1 A). Consequently, CXCL9, CXCL10 and CXCL11 can differentially regulate the properties of target cells which further increases the individual functional potential of these chemokines.

The complexity of the CXCR3 chemokine subgroup in humans is increased further by the presence of splicing variants of the CXCR3 receptor, which differ in their binding profile for the ligands (Lasagni et al., 2003; Ehlert et al., 2004; Petrai et al., 2008). These splicing variants have not been found in rodents pointing to possible differences in function between humans and rodents. Additional complexity occurs due to the promiscuous interaction of both the CXCR3 receptor and its ligands with other chemokines (e.g. CCL21) and their receptors (e.g. CXCR4) (Soto et al., 1998; Jenh et al., 1999; Van Coillie et al., 1999; Rappert et al., 2002; Dijkstra et al., 2004; Petkovic et al., 2004; de Jong et al., 2008a; Mueller et al., 2008; Müller et al., 2010).

However, initially, *in vitro* chemotaxis assays confirmed that CXCL9, CXCL10 and CXCL11 can promote the chemotaxis of activated T cells and NK cells (Luster and Ravetch, 1987; Farber, 1997). *In vivo*, a T cell recruiting role for CXCR3 was convincingly suggested in rodent cardiac allograft rejection experiments, which focused on CXCR3 and CCR5 and their respective ligands as key mediators of strong alloresponses (Hancock et al., 2000).
Concerning the role of CXCR3 and its ligands in CNS diseases a vast variety of neurological diseases and their corresponding animal models were characterized over the last decades (summarized in Table 2). In the following some specific examples of clinical and experimental neuroimmune diseases are presented to highlight recent concepts and issues on the functional significance of the CXCR3 chemokine system.



B

	CD4+ and CD8+ T cells NK cells, B cells, monocytes	microglia	astrocytes	neurons	oligo-dendrocytes
CXCR3 protein	(murine; Luster and Leder, 1993) (human; Taub et al., 1993) (human; Loetscher et al., 1996, 1998) (human/murine; Farber, 1997) (human; Sallusto et al., 1998) (human; Bonecchi et al., 1998) (human; Qin et al., 1998) (murine; Lu et al., 1999) (human; Yamamoto et al., 2000) (human; Kim et al., 2001a) (murine; Hauser et al., 2002) (human; Thomas et al., 2003) (human; Smit et al., 2003) Reviews: (Liu et al., 2005; Müller et al., 2010; Groom and Luster, 2011)	(human/murine; Biber et al., 2002) (human; Flynn, 2003) (murine; de Jong et al., 2008)	(human/murine; Biber et al., 2002) (human; Flynn, 2003)	(murine; Xia et al., 2000)	(human; Omari et al., 2005)
CXCR3 mediated chemotaxis		(rat/human; Cross and Woodroofe, 1999) (human/murine; Biber et al., 2002) (human; Flynn, 2003)	(human/murine; Biber et al., 2002)	-	-
CXCR3 mediated Ca^{2+} mobilization		(human/murine; Biber et al., 2002)	(human/murine; Biber et al., 2002)	-	-
CXCR3 mediated ERK1/2 pathway activation		-	-	(murine; Xia et al., 2000)	-

Figure 1: Amino acid sequences of human CXCL9, CXCL10, CXCL11 and CXCR3 with highlighted essential sequences for binding and differential activation (*A*). List of studies supply data on (functional) CXCR3 expression on leukocytes and resident cells of the CNS (*B*). The basic information to create hCXCR3 and CXCR3-ligand structure schematics were taken from published studies (Colvin et al., 2004, 2006) and from the NCBI protein database (hCXCL9, Accession: AAH95396.1; hCXCL10, Accession: AAH10954 and hCXCL11, Accession: AAH05292.1).

As introduced above, MS is a chronic demyelinating and neuroinflammatory disorder of the CNS that leads to serious disability in a significant number of affected patients. MS is a heterogeneous disorder of still unknown pathogenesis (Lassmann, 2008; Weiner, 2009). However, in relapsing-remitting MS, current evidence implicates the involvement of a cell-mediated autoimmune process in which $CD4^+$ and $CD8^+$ T cells autoreactive to myelin proteins infiltrate the white matter and co-ordinate an inflammatory response that leads to demyelination and oligodendrocyte loss and concomitant neurodegeneration (Steinman, 1996; Gran et al., 2004; Lassmann and Ransohoff, 2004). As discussed above CXCR3 ligands has key functions in promoting T cell trafficking in cell-mediated immunity. Therefore it is reasonable to believe that these chemokines also would have a crucial role in the pathogenesis of MS. This possibility led to a number of descriptive studies that have explored the relationship of CXCR3 and its ligands in the pathogenesis of MS (Table 1 B, Müller et al., 2010).

Activated T cells, entering the CNS or already have crossed the BBB in MS are shown to be $CXCR3^+$ (Balashov et al., 1999; Simpson et al., 2000b; Kivisäkk et al., 2002). Both CXCL9 and CXCL10 were found to be increased in CSF from MS patients (Sørensen et al., 1999) while CXCL10 was localized to astrocytes present in active but not inactive demyelinating lesions (Balashov et al., 1999). Sorensen et al. examined post mortem tissue from MS patients and found CXCL10 protein in astrocytes around perivascular accumulations of $CXCR3^+$ lymphocytes and in active demyelinating plaques (Sørensen et al., 2002). It was proposed that CXCL10 is important to attract T cells from the CSF into the perivascular space from which they have access to the CNS parenchyma. Available functional data for the role of CXCR3 ligands in EAE is conflicting, the results from the CXCR3-deficient mice reveal that CXCR3 is not required for the recruitment of immune cells to the CNS and signaling through this receptor may actually mediate protection by limiting leukocyte spread and consequent immunemediated tissue injury (Müller et al., 2007, 2010). Furthermore, the idea that CXCR3 signaling does not provide a major stimulus for the recruitment of effector T cells to the CNS in EAE gains indirect support from two additional studies. First, although CXCR3 was found to be a marker for memory $CD4^+$ T cells capable of migrating through the BBB *in vitro*, this receptor was dispensable for the transendothelial cell migration of these cells (Callahan et al., 2004). Second, transgenic mice with astrocyte-targeted production of CXCL10 exhibit only modest CNS accumulation of $CXCR3^+$ T cells that are localized to the

meningeal and ventricular regions-despite the parenchymal production of the chemokine (Boztug et al., 2002).

Although the available functional data for the role of CXCR3 and CXCR3 ligands in EAE is conflicting, the results from the CXCR3-deficient mice demonstrate that CXCR3 is not required for the recruitment of immune cells to the CNS and signaling through this receptor may actually mediate protection by limiting leukocyte spread and consequent immunemediated tissue injury (Müller et al., 2010).

During the pathogenesis of fatal murine cerebral malaria (FMCM), there is, similar to EAE, a cell-specific pattern of expression of CXCL9 and CXCL10. Thus, CXCL9 RNA is found in microglia (Miu et al., 2008), while CXCL10 protein and RNA are localized to neurons (Campanella et al., 2008) and astrocytes (Miu et al., 2008). The majority of mice deficient in CXCR3 were found to be protected from FMCM, and this protection was associated with a reduction in the number of $CD8^+$ T cells in brain vessels. Adoptive transfer of $CD8^+$ T cells from C57BL/6 mice with FMCM abrogated this protection in CXCR3- deficient mice (Miu et al., 2008). These data demonstrate that CXCR3 on $CD8^+$ T cells is required for T cell recruitment into the brain and development of FMCM and suggest that the CXCR3 ligands CXCL9 and CXCL10 play distinct, nonredundant roles in the pathogenesis of this disease (Müller et al., 2010).

Lymphocytic choriomeningitis (LCM) is mediated by anti-viral $CD8^+$ cytotoxic T cells and is characterized by localized breakdown of the BBB that results in convulsive seizures and death. Based on these observations it was proposed that parenchymal infiltration by cytotoxic T cells contributes to the lethality associated with LCM. During LCM, the majority of $CD8^+$ T cells in the CSF express CXCR3 (Christensen et al., 2004, 2006; de Lemos et al., 2005) whereas CXCL9 and CXCL10 protein and RNA transcripts are present in the CNS parenchyma (Hofer et al., 2008). In CXCL10-deficient mice, the numbers of infiltrating T cells were reduced despite increased CXCL9 levels. This finding suggests that CXCL10 is the dominant CXCR3 effector ligand responsible for parenchymal positioning of cytotoxic T cells in the host response to infection with the LCMV-Traub strain. Consequently, the survival rate of CXCL10- and CXCR3-deficient mice are increased following LCMV-Traub infection (Christensen et al., 2004, 2006) this was not found in CXCR3-deficient mice following infection with LCMV-Armstrong strain (Hofer et al., 2008). In contrast to infection with LCMV-Traub, no differences were seen in the number of T cells infiltrating the parenchyma of CXCR3-deficient mice infected with LCMV-Armstrong. The findings

possibly indicate for variable CXCR3 functions, dependent on distinct strain dependent virulence used in these two virus models (Müller et al., 2010).

To study critical aspects of the immunopathology of HSV encephalitis, mouse models of herpes simplex virus CNS infections were established using corneal or intranasal methods of infectious. CXCL9 and CXCL10 are one of the earliest genes upregulated during HSV infection of the CNS (Wickham et al., 2005; Carr and Tomanek, 2006). Studies demonstrate that during the course of HSV encephalitis CXCR3-deficient mice mice have a lower mortality despite higher viral loads. The lower mortality was associated with a reduced CNS infiltration of NK cells (Wickham et al., 2005). This argues for a role of CXCR3 in modulating the immune response towards a more efficient anti-viral response but also towards an immune response that is more harmful to the host. A conflicting finding in CXCL10-deficient mice is the fact that these animals also have a higher HSV virus titer in the CNS during infection but in contrast to CXCR3-deficient mice, have a higher mortality. NK cell infiltration of the CNS was shown to be reduced in both CXCR3- and CXCL10-deficient mice but mobilization of $CD8^+$ T cells was impaired in only CXCL10-deficient mice while $CD8^+$ T cell mobilization was not impaired in CXCR3 / CXCL10 double-deficient mice, these observations arguing against the involvement of an alternative CXCL10-receptor (Wuest and Carr, 2008). Due to inconsistency in the findings it is difficult to draw durable conclusions here. However, it would seem that CXCR3 signaling is involved in the recruitment of NK-cells to the CNS in HSV encephalitis and contributes to the anti-HSV host response since loss of this capacity results in higher levels of virus in the CNS (Müller et al., 2010).

Transmissible spongiform encephalopathies or prion infections of the CNS cause a progressive and ultimately lethal degeneration of neuronal tissue, but the underlying pathomechanisms are still elusive (Prusiner, 1998; Chesebro, 1999; Rezaie and Lantos, 2001; Weissmann, 2004; Riemer et al., 2008). However, a significant increase in trafficking of T cells into prion-infected brain tissue has never been reported. Instead, activation of astrocytes and microglia precedes neuronal death and is a general hallmark of this neurodegenerative protein misfolding disease (Williams et al., 1994, 1997; Riemer et al., 2000; Farina et al., 2007). In mouse models, induction of CXCL9 (Schultz et al., 2004) and CXCL10 (Riemer et al., 2000) is also documented at the early, asymptomatic stages of scrapie infection and is sustained at high levels until the end. Significantly prolonged survival times but accelerated accumulation of prion protein (PrP^{Sc}) was detected in CXCR3-deficient mice compared to

WT controls during scrapie infection (Riemer et al., 2008). These findings were correlated with reduced microglia activation, attenuated induction of proinflammatory genes but an increase in the accumulation of astrocytes in CXCR3-deficient mice. Furthermore, CXCR3 appears not be involved in directing monocyte migration across the BBB in the terminal stage of prion infections (Priller et al., 2006; Riemer et al., 2008). This study argues for a role of CXCR3 in modulating microglia towards a proinflammatory phenotype and possibly enhanced phagocytic function, which ultimately could be reasonable for the upregulation of PrP^{Sc} and enhanced astocytic activation in CXCR3-deficient mice.

Neurodegenerative Alzheimer's disease is also associated with an accumulation of misfolded protein and a correlating activation of microglial and astrocytes. AD brain tissue analysis currently supplies only limited data about the expression pattern of CXCR3 and CXCL10. However, studies argue for $CXCL10^{+}$ periplaque astrocytes and $CXCR3^{+}$ neurons in AD tissue (Xia et al., 2000), whereas nothing is know about their role in the pathogenesis of this disease.

Taken together, the summarized studies outline the early induction of CXCR3 and CXCR3 ligands in a vast variety of neurological disorders. However, the conflicting data from studies in many animal models outline that our knowledge of the CXCR3 chemokine system is still limited.

Table 1: Descriptive studies concerning CXCR3 ligand production in human CNS diseases (Müller et al., 2010)

Disease	Chemokine	Observation	Reference
Bacterial meningitis	CXCL10	CXCL10 is increased in CSF	(Giunti et al., 2003)
Neuroborreliosis	CXCL10, CXCL11	CXCL10 increased in CSF, detection of CXCL11 in CSF	(Lepej et al., 2005)
	CXCL11	CXCL11 in CSF	(Rupprecht et al., 2005)
African Trypanosomiasis	CXCL10	CXCL10 is increased in CSF	(Amin et al., 2009)
Viral meningitis	CXCL9, CXCL10	High levelsof CXCL10 but minimal levels of CXCL9 in CSF	(Lahrtz et al., 1997)
HIV infection	CXCL10	CXCL10 increased in CSF	(Kolb et al., 1999)
	CXCL10	Correlation between CXCL10 level and virus load/white blood cell count in CSF	(Cinque et al., 2005)
HIV-associated dementia	CXCL9	No CXCL9 expression in HIV infected brain tissue	(Wesselingh et al., 1993)
	CXCL10	CXCL10 expressed in astrocytes	(Sanders et al., 1998)
Multiple sclerosis	CXCL9, CXCL10	CXCL9 and CXCL10 increased in CSF; Astrocytic CXCL10 expression	(Sørensen et al., 1999)
	CXCL10	CXCL10 expressed in astro-cytes of active plaques	(Balashov et al., 1999)
	CXCL9, CXCL10	Detection of both CXCL9 and CXCL10 protein in demyelinating lesions	(Simpson et al., 2000b)
	CXCL10	astrocytic CXCL10 ex-pression around peri-vascular $CXCR3^+$ infiltrates	(Sørensen et al., 2002)
	CXCL9, CXCL10, CXCL11	CXCR3 ligands are upregulated under IFN-β treatment	(Cepok et al., 2009)
Optic neuritis	CXCL10	CXCL10 but not CXCL9 or CXCL11	(Sørensen et al., 2004)
Neuro-Behçet	CXCL10	CXCL10 levels in CSF higher than in non-inflammatory controls and MS	(Saruhan-Direskeneli et al., 2003)
Alzheimer's disease	CXCL10	CXCL10 in plaque associated astrocytes	(Xia et al., 2000)

3.5 Interleukin-12: key inducer of cell-mediated immunity

Interleukin-12 (IL-12), a heterodimeric cytokine, consisting of a p35 and p40 subunit, is a key regulator of cellular immunity in both innate and adaptive immune response (Hendrzak and Brunda, 1995; Gately et al., 1998; Trinchieri, 1998). It induces the differentiation of T cells from a Th0 to a Th1 phenotype, which is a prominent immune response in the CNS (Cher and Mosmann, 1987; Trinchieri, 1993). Principal sources of IL-12 are activated macrophages, which secrete the cytokine in response to many microbial stimuli. Many cells appear to synthesize the IL-12p35 subunit, but only the mononuclear phagocytes and dendritic cells produce the p40 component and therefore the biological active cytokine. Secreted IL-12 stimulates the differentiation of $CD4^+$ helper T lymphocytes into IFN-γ producing T cells, which is the key cytokine of Th1 cells. Furthermore IL-12 enhances the cytolytic function of activated NK cells and $CD8^+$ cytolytic T lymphocytes (CTLs). Subsequently, IFN-γ activates macrophages and results in an enhanced capability of killing incorporated microbes.
A transgenic model with an astrocyte targeted expression of IL-12 was established to clarify the impact of IL-12 on CNS inflammation (GF-IL12 mice, (Pagenstecher et al., 2000) Using this model it was demonstrated that astrocyte specific expression (GF-IL12 mice) is a sufficient trigger to induce Th1-type immune response in the CNS (Pagenstecher et al., 2000). The expression of IL-12 in astrocytes causes a spontaneous inflammatory CNS disorder, which is characterized by leukocyte infiltration, tissue destruction, calcification, hypomyelination, and up-regulation of proinflammatory cytokines (TNF-α, IL-1α, IL-1β, and IFN-γ) at the sites of transgene expression. An additional study dissected the impact of infiltrating lymphocytes and IFN-γ as mediators of disease in GF-IL12 mice (Hofer et al., 2004). It was demonstrated that IFN-γ secreting mature lymphocytes are responsible for the disease of GF-IL12 mice using an IFN-γ-deficient model (GF-IL12/IFN-$\gamma^{-/-}$). They found that none of the GF-IL12/IFN-$\gamma^{-/-}$ mice developed spontaneous neurological disorder and histological showed no increased infiltration by mononuclear cells compared with WT mice. These findings clearly demonstrate that in GF-IL12 mice activation and retention of functional lymphocytes in the CNS is crucial for the spontaneous development of disease and that IFN-γ is a pivotal mediator in the pathogenesis of the disorder. Gf.IL12 mice are an excellent model to study Th1 mediated inflammation of the CNS and its consequences.

3.6 Alzheimer's disease and the AD mouse model APP/PS1

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, with a prevalence of 5% after 65 years of age, the fourth most common cause of death in western industrialized nations, and one of the major contributors to the global burden of disease (WHO, 2000). The disease is clinically characterized by a progressive cognitive impairment often accompanied by psychobehavioural disturbances and language impairment. The two major classical neuropathologic hallmarks of AD are extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) (Lovestone and McLoughlin, 2002; Selkoe, 2004). The development of extracellular senile plaques is associated with hypertrophic astrocytes, activated microglia cells, and various other typical features of inflammatory processes. Etiologically, AD is heterogeneous, most AD cases are sporadic (90%), the early onset familial AD can be caused by mutations in APP, γ-secretases holoenzyme (including PSI, PS2, Nicastrin, Aph-1, and Pen-2) or β-secretases (BACE1) (Francis et al., 2002; De Strooper, 2003). By definition, genes that code for amyloid precursor protein (APP) and the APP processing proteolytic enzymes β- and γ-secretases (Checler, 1995) are good candidates for manipulation; therefore, to date, a multitude of genetically modified mouse strains exist that attempt to dissolve specific parts of the amyloid pathway. Many of the initial attempts to generate transgenic mice using APP with the familial AD mutations did not lead to an AD-specific phenotype. The low expression levels of mutated APP in brain might have been insufficient to trigger AD-like pathogenesis during the short life span of mice (Bornemann and Staufenbiel, 2000). Besides these "Aβ-transgenic" approaches, transgenic mouse models that overexpress human wild-type tau and/or mutated forms of human tau known to be associated with frontotemporal dementia and Parkinson's syndrome have been generated (Lee et al., 2001; Brandt et al., 2005). These models simulate aspects of neurofibrillary pathology, particularly hyperphosphorylation of microtubule-associated protein tau that is believed to be an early event in the pathway that leads from soluble to insoluble and filamentous tau protein (Kenessey and Yen, 1993). The neurofibrillary pathology has often been incriminated as the direct cause of neuronal death. Tangles left in the extracellular space after the death of the neurons that contained them are a direct proof of the neuronal death caused by or at least associated with the NFTs (Braak and Braak, 1991).

However, diverse transgenic strains expressing mutated human genes associated with familial forms of AD offered good models to study the etiology, progression and therapeutic

modulation for AD. A recently developed and available AD model is the APPswe/PS1dE9 model expressing both APPswe and PS1dE9 transgenes. This model was generated by the insertion of the two transgenes of APPswe (mouse/human chimeric APP695 harboring the Swedish mutation (KM594/5N)) and PS1dE9 (exon 9-deleted PS1) driven by a single mouse PrP promoter element at a single locus (Jankowsky et al., 2001). This model displays several pathological characteristics of AD, like progressive accumulation of cerebral amyloid plaques that is accompanied with clustering reactive microglia and astrocytes concentrated around amyloid plaques (Jankowsky et al., 2004; Garcia-Alloza et al., 2006; Ruan et al., 2009). Once activated, microglia and astrocytes release a variety of cytokines, chemokines and free radical oxygen species which might contribute to neuronal dysfunction and death (Sastre et al., 2006; Li et al., 2011). In the APP/PS1 model as well as in brains of AD patients an induction of various of proinflammatory mediators like TNF-α, IL-1β, IL-6, MCP-1, CXCL10, Aβ degrading enzymes, prostanoids, complement proteins, and free radicals have been demonstrated (Akiyama et al., 2000; Hoozemans et al., 2006; Ruan et al., 2009; Malm et al., 2012). The exact function and interplay of the different mediators induced in both AD and AD-like pathology is still inadequately explored. At least microgliosis and astrocytosis associated neuroinflammation in AD is one interesting aspect of this disease, which can critically influence the course of the disease and the APP/PS1 model offers a potential possibility to understand diverse inflammatory signaling pathways modulating innate immunity during this devastating disease (Malm et al., 2012).

3.7 The objective of the presented studies

As discussed above, recent functional studies demonstrated a potent but diverse role of CXCR3 in various experimental disease models. In particular, the role of CXCR3 in neurological diseases is not well defined.

To further clarify the function of CXCR3 in neurological diseases, effects of CXCR3 deficiency was characterized in two very different mouse disease models. The described GF-IL12 transgenic mice was applied to characterize the impact of CXCR3 in IL-12 induced neuroinflammation, a Th1 and NK cell driven immune response. In contrast to this severe neuroinflammatory model, we examined the impact of CXCR3 in a model of neurodegeneration. Therefore, we generated and used CXCR3-deficient APP/PS1 mice, which develop an Alzheimer-like pathology without significant influx of systemic immune cells into the CNS. In both models, the clinical course, histopathological features and the expression of key inflammatory molecules was assessed to determine the role of CXCR3.

The results of both studies were compared to draw conclusions concerning general functional features of CXCR3 in neurological diseases and specific functions in inflammatory or degenerative diseases.

Chapter 1

4. CXCR3 in IL-12-induced cell-mediated immunity in the CNS

Published in "The American Journal of Pathology": Krauthausen M, Carter S, Zimmermann J, Sarris M, Wakefield D, Heneka MT, Campbell IL, Müller M: Opposing roles for CXCR3 signaling in CNS versus ocular inflammation mediated by the astrocyte-targeted production of IL-12: Am J Pathol. 2011 Nov;179(5):2346-59. Epub 2011 Sep 15.
http://dx.doi.org/10.1016/j.ajpath.2011.07.041

4.1 Introduction

Innate and adaptive immune responses play a crucial role in protecting the nervous system from dangerous pathogens. However, this is a two-edged sword as an excessive host immune response can cause more harm than good (Rivest, 2009). The understanding of the pathogenetic mechanisms leading to immune-mediated tissue damage during the course of pathogen driven diseases like bacterial meningitis and herpes encephalitis (Conrady et al., 2010) or autoimmune disease such as multiple sclerosis (Gandhi et al., 2010) is important to develop novel treatment strategies to prevent detrimental effects of the immune response.
Chemokines, chemotactic cytokines, are key molecules in orchestrating innate and adaptive immune responses (Charo and Ransohoff, 2006a). Chemokines are highly upregulated in nearly all neuroinflammatory disorders examined. However, the precise functional properties of these multifunctional and promiscuitive molecules remain somewhat unclear (Cardona et al., 2008). The chemokines CXCL9, CXCL11 and in particular CXCL10 are highly induced in various neuroinflammatory disorders (Müller et al., 2010). They share a common chemokine receptor, which is CXCR3. CXCR3 is mainly found on activated $CD4^+$ and $CD8^+$ T cells but also on NK cells, monocytes and dendritic cells (Cella et al., 1999; Biber et al., 2002; Rappert et al., 2004; Liu et al., 2005). CXCR3 is differentially activated by CXCL9, CXCL10 and CXCL11 (Loetscher et al., 1998; Weng et al., 1998). The ligands and the receptor itself are mainly induced and regulated by IFN-γ (Luster et al., 1985; Ferber et al., 1996; Cole et al., 1998). Only Th1 but not Th2 cells express CXCR3 at high levels (Kim et al., 2001b) and therefore, CXCR3 is a typical marker of Th1 cells (Loetscher et al., 1996). Descriptive studies initially pointed towards a key role of CXCR3 and its ligands in promoting the influx of activated T cells into the CNS (Balashov et al., 1999; Kivisäkk et al., 2002; Sørensen et al., 2002). However, functional studies using gene-deficient mice or

blocking antibodies revealed a much more complex functional profile of this chemokine system (Fife et al., 2001; Narumi et al., 2002; Klein, 2004) which strongly depends on the pathogenesis of the disease model examined. In EAE, CXCR3 surprisingly has a protective effect, which is demonstrated by a more severe and chronic disease in CXCR3-deficient mice (Müller et al., 2007).

However, EAE has a very complex pathogenesis, which is not, as previously assumed, a mainly Th1 driven autoimmune reaction. Different T cell subsets, viz. Th1, Th17 and Tregs have an impact during different stages of the disease and to understand these functions is currently an important issue in neuroimmunology.

To further clarify the functional relevance of CXCR3 in neuroinflammation, we examined the type 1 cell-mediated CNS inflammation in mice with a CNS restricted transgenic expression of the IL-12 gene (Pagenstecher et al., 2000). Interleukin-12 (IL-12) is an important regulator of cellular immunity in both innate and adaptive immune responses. IL-12 activates NK cells, $CD8^+$ and $CD4^+$ T cells in which IL-12 induces differentiation from a Th0 to a Th1 phenotype (Balashov et al., 1999; Kivisäkk et al., 2002). Furthermore it increases the proliferation of T cells and NK cells and stimulates the production of numerous immune effector molecules, in particular IFN-γ. As IFN-γ induces the production of IL-12, the IL-12/IFN-γ cytokine system serves as a positive feedback mechanism, which initiates and maintains immune responses. GF-IL12 mice express the IL-12 gene under the control of an astrocyte (glial fibrillary acidic protein; GFAP) promoter and develop a severe neuroinflammatory response via induction of IFN-γ. In this transgenic mouse model, activated T cells and their production of IFN-γ are critical to the development IL-12-driven CNS-inflammation (Hofer et al., 2004) and the high expression of IFN-γ inducible chemokines like CXCL9 or CXCL10 suggest an additional important role for these molecules. Here we asked whether CXCR3 is a critical molecule for the induction and course of IL-12-induced neuroinflammation. To address this question, we studied the impact of CXCR3 deficiency in transgenic mice with chronic CNS-restricted production of IL-12.

4.2 Material and methods

Animals

CXCR3-deficient (CXCR3KO) mice (originally kindly provided by Drs. Bao Lu and Craig Gerard, Children's Hospital and Harvard Medical School, Boston, MA) have been described previously (Hancock et al., 2000). The mice where backcrossed at least eight generations onto the C57BL/6 strain. CXCR3-deficient mice displayed no clinical and histological abnormalities when compared to wild type (WT) mice.

Transgenic mice expressing both subunits of the IL-12 heterodimer (p35/p40) under the transcriptional control of the astrocyte-specific GFAP promoter (GF-IL12 mice) were described previously (Pagenstecher et al., 2000). To obtain CXCR3-deficient GF-IL12 mice (GF-IL12/CXCR3KO) heterozygous GF-IL12 mice on a C57BL/6 background were successively crossed with CXCR3KO mice. Animals were kept under pathogen free conditions in the *Zentrale Tierexperimentelle Einrichtung (ZTE)* animal facility of the University Hospital Münster and in the Blackburn facility of the University of Sydney (Sydney, Australia). All procedures were approved by the veterinary office of the *Bezirksregierung Münster* (Germany) and of the Sydney Animal Care and Ethics Committee (Australia).

Clinical assessment of mice

The GF-IL12/CXCR3KO, GF-IL12, CXCR3KO and WT mice were clinically evaluated over a period of 36 weeks at least twice a week. The clinical scores for ataxia were assessed for each animal according to a previously established protocol (Quintana et al., 2009). Ataxia signs were scored using a cumulative scale of four points, giving one point to each of these four physical signs: splayed legs, dragging weight on the trunk rather than on the legs, wobbling and falling from side to side. A second score was applied to assess the severity of the ocular phenotype: 1 = monocular cataract; 2 = binocular cataract; 3 = monocular cataract and monocular phthisis bulbi; 4 = binocular phthisis bulbi.

Tissue processing for histology

The tissue for analysis (histology, immunohistochemistry and molecular biology) was obtained from 8 and 24 week old mice of each genotype (WT, CXCR3KO, GF-IL12/CXCR3KO, GF-IL12). Immediately after euthanasia, the brain and eyes were removed and half of the brain (cut along the sagittal midline) and an eye were fixed overnight in PBS-buffered 4% paraformaldehyde at 4 °C, washed in PBS and subsequently embedded in paraffin. Sections (8 μm) were prepared from paraffin-embedded tissue.
For immunohistochemistry on cryosections, tissue was embedded with Tissue Tek® (Sakura Finetek, Staufen, Germany). Sections (8 μm) were prepared and five cerebelli/eyes of each genotype were analyzed.

Routine histology and (fluorescence)-immunohistochemistry

Paraffin-embedded sections were stained with H&E and Luxol fast blue for routine histological analysis and myelin evaluation. For immunohistochemistry, sections were rehydrated in graded ethanol series after deparaffination in xylene and some were pretreated with proteinase K digestion. Slides were then incubated for 1h at room temperature with primary Abs (primary Abs and corresponding protocols for immunohistochemistry are summarized in Table 2). After washing in PBS, a biotinylated secondary Ab (Axxora, Lörrach, Germany; 1/200) and HRP-coupled streptavidin (Axxora; 1/200) was used. The signal was visualized by NovaRED color reagent (Axxora), according to the manufacturer's instructions. Conventional and immunofluorescence-stained sections were examined under a DM4000B bright field and fluorescence microscope (Leica, Wetzlar, Germany). Bright field images and monochrome fluorescent images were acquired using a Leica DFC480 camera and Leica Firecam 1.7.1 software (Leica). The acquired monochrome fluorescence signals were merged using SPOT Advanced 4.5 software (Diagnostic Instruments, Sterling, MI) or "cell^P" imaging software (Olympus Soft Imaging Solutions, Münster, Germany).
To allow clear discrimination of IHC signal and the pigment layer of the eye, and to detect epitopes which are not preserved in paraffin embedded tissue, fluorescent immunohistochemistry on cryo-embedded sections was performed (Table 2). After washing in PBS OD_{594} and OD_{488} fluorescence-conjugated secondary Ab (Invitrogen, Darmstadt, Germany; 1/200) was used to visualize the primary Ab. Sections were mounted and counterstained with DAPI (Sigma-Aldrich, Munich, Germany).

Table 2. Antibody and lectin reagents used for immunohistochemistry

Antibody/Lectin (source)	Specificity	Paraffin Sections (dilution)	Cryo Sections (dilution)
Polyclonal rabbit anti-human CD3 (Dako, Hamburg, Germany)	T cell	1/200	1/200
Biotin-conjugated tomato lectin, *L. esculentum* (Axxora, Lörrach, Germany)	Microglia/ macrophages, endothelial cells	1/50	-
Monoclonal mouse anti -human GFAP (Dako)	Glial fibrillary acidic protein	-	1/200
Polyclonal rabbit anti-Laminin reactive with human and mouse Laminin (Sigma-Aldrich, Munich, Germany)	Basal lamina	-	1/50
Polyclonal rabbit anti-Iba1 reactive with human, mouse and rat Iba1 (Wako Chemicals, Neuss, Germany)	Microglia and macrophages	-	1/500

RNase protection assays

The brain and eyes were collected as described above and snap frozen in liquid nitrogen. Total RNA was isolated using Trizol (Sigma-Aldrich). RNase protection assays were performed as described previously (Ousman and Campbell, 2005). Five μg of total RNA were used for each sample and hybridized with the following probes: *CXCL9, TGF-β, IL1β, IFN-γ, IL-12p40, CCL5* and the *RPL32–4A* gene (Dudov and Perry, 1984) that served as an internal loading control. For autoradiography, Biomax films (Eastman-Kodak, Rochester, NY) were exposed for various periods of time and scanned using a ScanJet 4C (Hewlett-Packard Co., Palo Alto, CA).

Cytokine and chemokine mRNA determination by qRT-PCR

Total RNA (3 μg) was reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR assays were performed using SYBRgreen. The composition of the reaction mixture was as follows: 1 μL of cDNA corresponding to 300 ng of total RNA, 100 nM of each primer, 2×SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) in a total volume of 25 μL. Samples were analyzed simultaneously for GAPDH mRNA as the internal control. The mRNA levels for each target were normalized to mRNA levels of GAPDH and expressed relative to that of nontransgenic C57BL/6J mice. Each sample was assayed in duplicate. Primer sequences used to amplify the GAPDH, IFN-γ, IL-17, CXCL10, TNF-α, VEGF-A, LYVE1 and IL-10 cDNA were: GAPDH sense 5'-TCACCAGGGCTGCCATTTGC-3' and GAPDH anti-sense 5'-GACTCCACGACATACTC AGC-3', IFN-γ sense 5'-CAG CAA CAG CAA GGC GAA A-3' and IFN-γ anti-sense 5'-GCT GGA TTC CGG CAA CAG-3', IL-17 sense 5'-AAG GCA GCA GCG ATC ATC C-3' and IL-17 anti-sense 5'- GGA ACG GTT GAG GTA GTC TGA G-3', CXCL10 sense 5'-GAC GGT CCG CTG TTC T-3' and CXCL10 anti-sense 5'-GCT TCC CTA TGG CCC TCAT T-3', TNF-α sense 5'-ATG AGA AGT TCC CAA ATG GCC-3' and TNF-α anti-sense 5'-ACG TGG GCT ACA GGC TTG TC-3', VEGF-A sense 5'-TTA CTG CTG TAC CTC CAC C-3' and VEGF-A anti-sense 5'-ACA GGA CGG CTT GAA GAT G-3', LYVE1 sense 5'-TCC AAC ACG GGG TAA AAT GT-3' and LYVE1 anti-sense 5'-CCT CCA GCC AAA AGT TCA AA-3' IL-10 sense 5'-TGT CAA ATT CAT TCA TGG CCT-3' and IL-10 anti-sense 5'-ATC GAT TTC TCC CCT GTG AA-3'.

Flow cytometry analyses of cerebellar and ocular leukocytes

Cerebelli and eyes from WT, CXCR3KO, GF-IL12 and GF-IL12/CXCR3KO (n=3) animals were excised and placed into ice-cold PBS buffer solution. The tissue was cut into small pieces and digested for 30 minutes in PBS with collagenase I (0.05 g/ml; Roche Diagnostics, Mannheim, Germany) first and following DNase I (100 μg/ml; Sigma-Aldrich) digestion for 30 min at 37°C in a humidified atmosphere of 5% CO2. The eye homogenates were differentially digested by a combination of collagenase D (1 mg/ml; Roche) and DNase I (100 μg/ml, Sigma-Aldrich) for 30 min. Digestion was stopped with 10% FCS. A pellet was obtained after 10 minutes centrifugation at 340xg. Digested samples were resuspended in

PBS and disrupted/homogenized using needle (0.6x25) and a syringe (5 ml) before passing through a 70 µm cell strainer (BD Biosciences, Heidelberg, Germany). After pelleting homogenates were dissolved in 30% percoll (Amersham Pharmacia Biotech, Braunschweig, Germany). Subsequently, the 30% percoll homogenate mix was layered over 70% Percoll. Leukocytes were collected from the 30%/70% interface after a 800xg centrifugation step for 25 minutes at room temperature. The collected cells were washed in PBS, and blocked with CD16/CD32 (Fc block; BD Biosciences) antibody. Isolated leukocytes were incubated with fluorochrome-conjugated antibodies (eBioscience, Frankfurt/Main, Germany) to detect CD3e (clone 145-2C11, PerCP-Cy5.5), CD4 (clone RM4-5, FITC), CD8a (clone 53-6.7, APC-eFluor 780), CD11b (clone M1/70, APC), CD11c (clone N418, PE-Cy7), CD25 (clone PC61.5, APC), CD45 (clone 30-F11, FITC), Ly6G (clone RB6-8C5, PerCP-Cy5.5), B220 (clone RA3-6B2, APC-eFluor 780) and NK 1/1 (clone PK136, PE-Cy7). Intracellular staining for FoxP3 (PE) was done using the Mouse Regulatory T Cell Staining Kit (w/ PE Foxp3, clone FJK-16s, FITC CD4, APC CD25; eBioscience) according to the manufacturers instructions. After washing, bound Ab was detected using a BD FACSCanto II (BD Biosciences), and the acquired data were analyzed using the flow cytometry software, FlowJo (TreeStar, San Carlos, CA).

Protein lysates and western blot

Tissue was homogenized using a Precellys 24 tissue homogenizer *(*Bertin Technologies*,* Saint-Quentin-en-Yvelines Cedex, France*)* in lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1mM EDTA, 1mM EGTA, 1% NaDOC, 0.1% SDS, 2mM ortho-vanadate, 30 mM NaF, 50 mM Na pyrophosphate, 2 mM PMSF) modified with freshly supplemented protease inhibitor mix (Sigma-Aldrich). Samples were centrifuged at 14000 rpm for 15 min and supernatants were taken. The protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Protein lysates (50 μg) were separated by 10 % SDS-PAGE gel using NuPAGE® MES SDS running buffer (Invitrogen) at 150 V. PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) was used as standard. Proteins were transferred to 0.2 µm nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked for 30 min in TBST containing 5% skim milk. Immunoblotting were performed using anti-phosphorylated (Y693)-STAT4 antibody (Invitrogen), anti-STAT4 (Santa Cruz, Heidelberg, Germany) antibody and antibody CP06 (Oncogene Science, Cambridge, MA) detecting α-tubulin followed by incubation with the

appropriate horseradish-peroxidase conjugated secondary antibodies (Jackson ImmunoResearch, Newmarket, UK). Immunoreactivity was detected by chemiluminescence reaction (Millipore, Schwalbach, Germany) and luminescence intensities were analyzed using Chemidoc XRS imaging system (BioRad, Munich, Germany). With the Quantity One (BioRad) program bands density were determined for each lane and the intensity ratio for the detected proteins were calculated to α-tubulin.

4.3 Results

CXCR3-deficient GF-IL12 mice only rarely develop ataxia but always develop a destructive ocular phenotype

To determine whether the clinical course of IL-12-driven CNS-inflammation was altered in the absence of CXCR3 signaling, the physical status of a cohort of 13 GF-IL12 and 23 GF-IL12/CXCR3KO mice was observed for 36 weeks (Fig. 2 *A*). As described previously (Pagenstecher et al., 2000; Hofer et al., 2004). GF-IL12 mice developed a progressive ataxia at the age of 9-12 weeks. First clinical signs at that time-point were belly dragging and the splaying of legs and resulted in 85% of GF-IL12 mice in an ataxia score of one. At the same time point, only 9% of GF-IL12/CXCR3KO displayed any signs of ataxia. Progression of the ataxia in GF-IL12 mice could be observed resulting in an ataxia score three or four in 92% of the animals at 36 weeks. In contrast, only the GF-IL12/CXCR3KO mice (9%, 2/23), which displayed signs of ataxia at 12 weeks further progressed and developed severe ataxia at 36 weeks (GF-IL12/CXCR3KOsick). The remaining 91% of GF-IL12/CXCR3KO mice developed no signs of ataxia, demonstrating a strongly reduced incidence of the atactic phenotype in GF-IL12/CXCR3KO mice compared to GF-IL12 mice (★★★, $p<0.005$).
Unexpectedly, all GF-IL12/CXCR3KO mice (n=23) developed mono- or bin-ocular cataracts during the first four weeks of life, (ocular score 1-2, Fig. 2 *B*). In contrast, only 15% (2/13) of the GF-IL12 mice developed a very mild opacity of the lens (ocular score 1), which we did not observe in either WT or CXCR3KO controls. After 24 weeks all GF-IL12/CXCR3KO developed a bulbar atrophy (phthisis bulbi) and severe cataracts (ocular score 3-4, Fig. 2 *B*) ultimately leading to blindness in these mice. None of the GF-IL12 mice developed these severe symptoms. The early lens opacity observed in some GF-IL12 animals (6/13) resolved in 66% of these animals and in the remaining animals, the ocular phenotype did not progress any further.

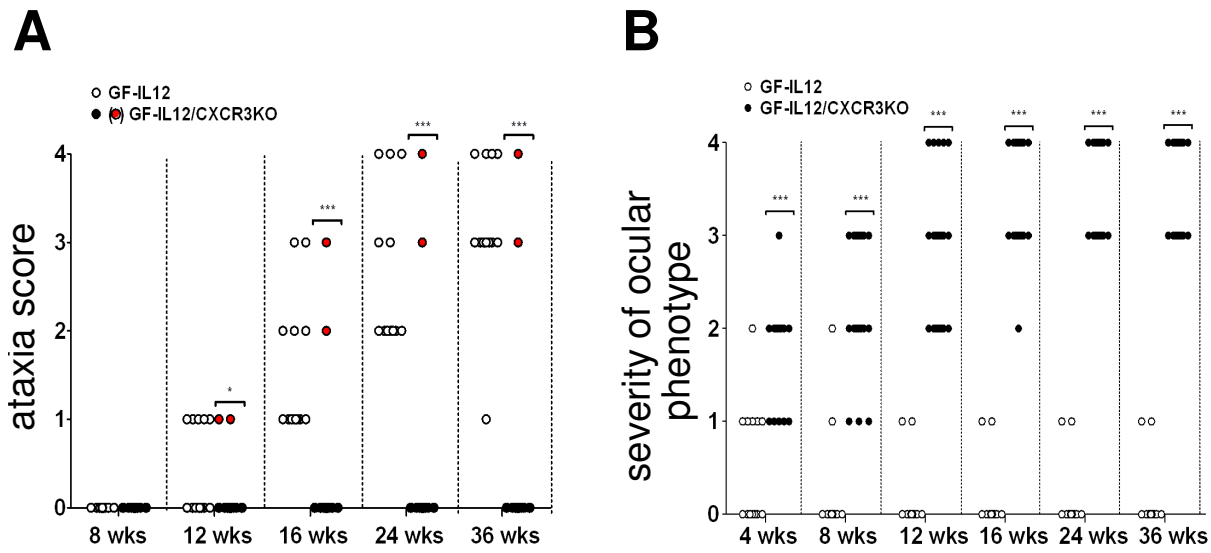


Figure 2: Progression of clinical ataxia (*A*) and the severity of ocular phenotype (*B*) in GF-IL12 (n=13) and GF-IL12/CXCR3KO (n=23) mice over 36 weeks. Ataxic signs were scored using accumulative scale of 4 points, giving 1 point to each of these four physical signs: splayed legs, dragging weight on the trunk rather than on the legs, wobbling and falling from side to side. The ocular score was applied to assess the severity of the ocular phenotype: 1 = monocular cataract; 2 = binocular cataract; 3 = monocular cataract and monocular phthisis bulbi; 4 = binocular phthisis bulbi. While GF-IL12 mice developed severe ataxia over 24 weeks (black dots), most of the GF-IL12/CXCR3KO mice did not display signs of ataxia (circled dots). A few of the GF-IL12/CXCR3KO animals (GF-IL12/CXCR3KO^{sick}; red dots) developed scores of ataxia comparable to GF-IL12 mice until 24 weeks. Surprisingly, during the first four weeks all GF-IL12/CXCR3KO mice developed a progressive disease of the eyes, ultimately leading to binocular bulbar atrophy after 36 weeks. The ocular phenotype of GF-IL12 mice at that age reached a maximum ocular score of one. ⋆, $p < 0.05$; ⋆⋆⋆, $p < 0.005$, mean ± SEM.

Less severe cerebellar histopathology correlates with the attenuated clinical phenotype of CXCR3-deficient GF-IL12 mice

To correlate the clinical differences observed in GF-IL12 versus GF-IL12/CXCR3KO mice with histopathological findings, we used routine histology and immunohistochemistry to examine WT, CXCR3KO, GF-IL12, non-atactic GF-IL12/CXCR3KO and atactic GF-IL12/CXCR3KOsick mice at the age of 24 weeks. Non of the WT or CXCR3KO animals showed any histopathological changes in routine histology. However, extensive tissue destruction, calcifications and mononuclear cell accumulation in the parenchyma and meninges was observed in the brain of GF-IL12 mice and was consistent with previous reports (Pagenstecher et al., 2000; Hofer et al., 2004). Furthermore, widespread demyelination and spongiosis predominantly of the cerebellar white matter was observed (Fig. 3; GF-IL12, LFB)(Pagenstecher et al., 2000; Hofer et al., 2004) In contrast, non-atactic GF-IL12/CXCR3KO mice had only minimal histological alterations without demyelination and calcifications (Fig. 3; GF-IL12/CXCR3KO, LFB). Only the few GF-IL12/CXCR3KOsick mice with signs of ataxia (9% of all GF-IL12/CXCR3KO mice) displayed perivascular cell clustering and demyelination, without calcifications. However, these pathological changes did not reach the level of severity observed in CXCR3-competent GF-IL12 mice (Fig. 3; GF-IL12, LFB). To further characterize the histopathological differences, we examined accumulation and distribution of T cells (CD3) and microglia/macrophages (lectin) by immunohistochemistry. In CXCR3-competent GF-IL12 mice, T cells were found in a perivascular position and throughout the parenchyma (Fig. 3; GF-IL12, CD3). Activated microglia /macrophages were found in the parenchyma and correlated well with tissue destruction and demyelination after 24 weeks (Fig. 3; GF-IL12, Lectin). In contrast at this timepoint 91% of GF-IL12/CXCR3KO mice had only slightly increased numbers of $CD3^+$ T cells, which were most often localized in a parenchymal perivascular position and not in the subarachnoidal space (Fig. 3; GF-IL12/CXCR3KO, CD3). T cells accompanying activated microglia/ macrophages were drastically reduced in non-atactic CXCR3-deficient GF-IL12 mice (Fig. 3; GF-IL12/CXCR3KO, Lectin). Only clinically affected GF-IL12/CXCR3KOsick mice showed perivascular infiltrates consisting of T cells surrounded by activated microglia/macrophages localized to the white matter of the cerebellum (Fig. 3; GF-IL12/CXCR3KO^{sick}, CD3, Lectin). These findings demonstrate that the less severe phenotype in GF-IL12/CXCR3KO mice is associated with a marked decrease in cerebellar inflammation.

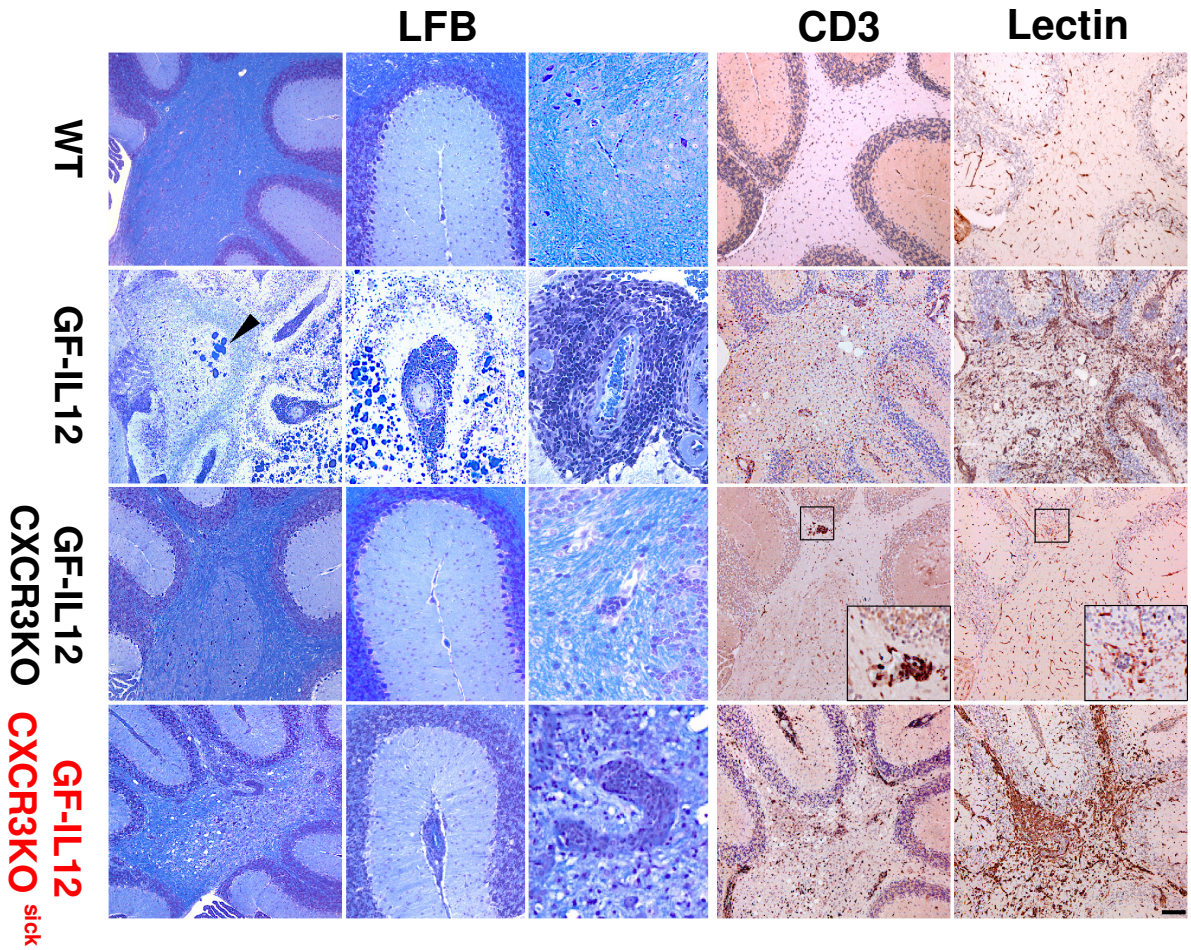


Figure 3: Histological alterations in the cerebellum of GF-IL12 and GF-IL12/CXCR3KO mice at 24 weeks of age. Colocalization of LFB routine histology, T cells (CD3) and microglia/macrophages (tomato lectin). Extensive cell loss in the granule layer (GF-IL12, LFB), white matter calcifications (GF-IL12, LFB, arrowhead) and demyelination was detectable in all GF-IL12 animals. In contrast, most GF-IL12/CXCR3KO mice had non or only minimal histopathological alterations (GF-IL12/CXCR3KO, LFB). A few GF-IL12/CXCR3KO mice with moderate signs of ataxia displayed histopathological alterations (GF-IL12/CXCR3KO^{sick}, LFB), which did not reach the level of tissue disruption in GF-IL12 mice. Meningeal and perivascular (GF-IL12, GF-IL12/CXCR3KO, GF-IL12/CXCR3KO^{sick}, LFB) infiltrates were drastically reduced in GF-IL12/CXCR3KO mice compared to GF-IL12 mice. Widespread distribution and perivascular accumulation of T cells (GF-IL12, GF-IL12/CXCR3KO^{sick}, CD3) are accompanied by diffuse microglia/macrophage activation in the white matter (GF-IL12, GF-IL12/CXCR3KO^{sick}, Lectin) of GF-IL12 mice. Most GF-IL12/CXCR3KO displayed only minor T cell infiltration and less activated microglia/macrophages (GF-IL12/CXCR3KO, CD3). Original magnifications, 20 x. Bar, 250 μm (overview of cerebellum) and 100 μm (meningeal/vascular focus).

The cerebellar cytokine profile correlates with the histopathology and the clinical outcome in GF-IL12 and GF-IL12/CXCR3KO mice.

We wanted to determine if the different cerebral disease phenotypes in GF-IL12/CXCR3KO and GF-IL12 were linked with a specific cytokine RNA pattern. Therefore, the mRNA transcripts of key inflammatory cytokine and chemokine genes in the cerebellum of GF-IL12 vs. GF-IL12/CXCR3KO mice were determined by RNase protection assay (Fig. 4 *A*).
In the cerebellum of WT and CXCR3KO mice, only TGF-β could be detected unequivocally. Consistent with the clinical ataxia and the described histopathological features, induction of all examined cytokine and chemokine genes was prominent in GF-IL12 mice at 24 weeks (Fig. 4 *A*; GF-IL12). In GF-IL12/CXCR3KO mice with a clinical score of 0-1 only and without prominent histological damage, only low levels of CXCL9, IL-1, CXCL10 and CCL5 could be detected. In the GF-IL12/CXCR3KOsick mice we observed a similar pattern to GF-IL12 mice with an induction of CXCL9, IL-1, CXCL10 and CCL5. However, the RNA level for IFN-γ as well for CXCL9 and CXCL10 were considerably lower. As IFN-γ is a key cytokine in this model, we additionally measured RNA levels of IFN-γ by quantitative PCR and found significantly higher levels in GF-IL12 mice (**, $p=0.0015$, 4397 fold increase ± 804) compared to GF-IL12/CXCR3KO mice (782 fold increase ± 227) (Fig. 4 *B*). Within the GF-IL12/CXCR3KO group, the animals with clinical symptoms (GF-IL12/CXCR3KOsick, red dots) did have the highest levels of IFN-γ RNA. To evaluate a possible induction of Th17 subset effector cells in parallel to the predominant Th1 driven immune response observed in this model, we examined the level of IL-17 transcripts in both GF-IL12 and GF-IL12/CXCR3KO cerebelli (Fig. 4 *B*). The IL-17 transcript levels in GF-IL12, GF-IL12/CXCR3KO and GF-IL12/CXCR3KOsick mice were unaltered compared to WT-animals. In summary, the RNA levels of various inflammatory cytokines correlated well with the clinical and histopathological observations. The IFN-γ RNA level was markedly reduced, even in GF-IL12/CXCR3KOsick mice with clinical symptoms.

CXCR3 and CCR5 mRNA expression in GF-IL12-mediated cerebellar inflammation

CCL5 and CCR5 are, like CXCR3 and its ligands, involved in the trafficking of type 1 immune cells (Loetscher et al., 1996; Schrum et al., 1996; Sallusto et al., 1998). The RPA results revealed a prominent upregulation of CCL5 in clinically affected GF-IL12/CXCR3KOsick but not in unaffected GF-IL12/CXCR3KO mice. To determine if CCL5 might compensate for the lack of CXCR3, we further examined and compared cerebellar CXCR3 and CCR5 RNA levels by qRT-PCR in GF-IL12, unaffected GF-IL12/CXCR3KO and clinically affected GF-IL12/CXCR3KOsick mice. The mRNA levels for each transcript were normalized to the mRNA levels of GAPDH and correlated with the level of control mice. We found increased levels of CXCR3 (10.67 fold increase ± 2.460) and CCR5 (7.733 fold increase ± 1.330) transcripts suggesting a functional role of both $CXCR3^+$ and $CCR5^+$ cells. Significantly lower levels of CCR5 transcripts were found in unaffected GF-IL12/CXCR3KO animals (**, $p<0.01$, 2.5 fold increase ± 0.8). In clinically affected GF-IL12/CXCR3KOsick mice, we found CCR5 RNA at a level (8.6 fold increase ± 1.3) comparable to the findings in GF-IL12 mice (Fig. 4 *B*). The high level of CCL5 and CCR5 in GF-IL12/CXCR3KOsick mice but not in clinically unaffected GF-IL12/CXCR3KO mice could argue for a compensation of the CXCR3 deficiency by CCR5 in GF-IL12/CXCR3KOsick mice.

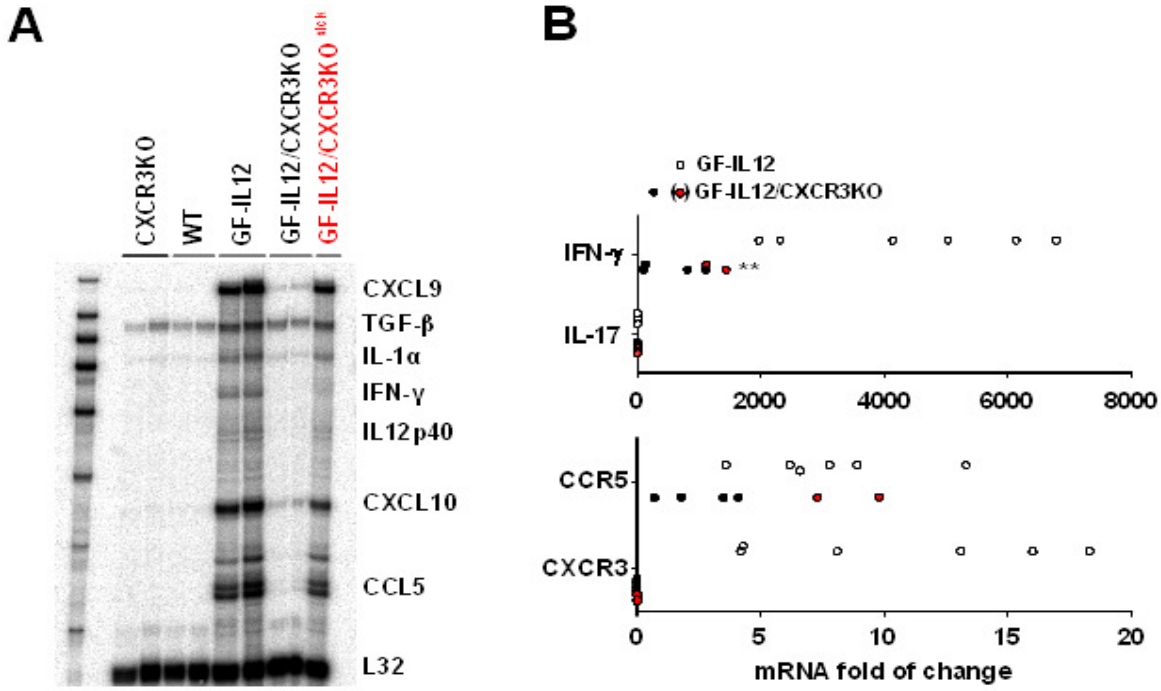


Figure 4: The mRNA expression pattern of selected cytokine and chemokines in the cerebellum of GF-IL12, GF-IL12/CXCR3KO and controls detected by RPA (*A*) Total RNA was isolated from the cerebellum of mice and 5 μg was used for analysis by RPA. The level of L32 was used as loading control. An overall increase of proinflammatory cytokine and chemokine transcripts was found in GF-IL12 and GF-IL12/CXCR3KO^{sick} mice with the exception of a low IFN-γ level in GF-IL12/CXCR3KO^{sick} mice. QRT-PCR was performed to further determine the level of IFN-γ mRNA in the cerebellum of aged GF-IL12 (n=6) and GF-IL12/CXCR3KO (n=6) mice (*B*). Compared with GF-IL12, the GF-IL12/CXCR3KO mice had significantly lower levels of IFN-γ RNA transcripts at 24 weeks of age. Further RNA analysis revealed high levels of cerebellar CXCR3 and CCR5 transcripts in GF-IL12 animals after 24 weeks (*B*). In contrast only the diseased GF-IL12/CXCR3KO^{sick} mice (red dots) revealed elevated levels of CCR5 receptor transcripts in the cerebellum. For statistical significance, ** $p < 0.01$, mean ± SEM.

Cerebellar leukocyte recruitment in GF-IL12/CXCR3KO mice is reduced early and associated with lower levels of IFN-γ mRNA

To further examine the clinical and histopathological differences observed at a late timepoint, we performed flow cytofluorometric analysis from cerebellar cell suspensions at the age of eight weeks when both GF-IL12 versus GF-IL12/CXCR3KO mice did not show any clinical symptoms (Fig. 5 *A*). At that timepoint, the total number of CD45$^+$ leukocytes were already increased in GF-IL12 mice compared with GF-IL12/CXCR3KO animals (243250 cells ± 62268 cells in GF-IL12 vs. 102067 cells ± 20972 cells in GF-IL12/CXCR3KO) (Fig. 5 *A*). However, the relative proportion of T cell subsets (CD4$^+$ vs. CD8$^+$ T cells) remained similar between the two strains of transgenic mice (66.4 ± 13.3% vs. 21.7 ± 0.4% in GF-IL12 and 59.1 ± 9.2 vs. 29.2 ± 6.5% in GF-IL12/CXCR3KO). Further characterization of the CD45$^+$ cells revealed an increase of CD45$^+$/NK-1.1$^+$ cells in GF-IL12 compared to GF-

IL12/CXCR3KO mice (15.2 ± 4.9% in GF-IL12 vs. 6.7 ± 4.0% in GF-IL12/CXCR3KO) resulting in a more than 5-fold increase in the absolute cell counts in GF-IL12 cerebellum. An approximate 4-fold increase in $CD45^+/CD11b^+$ macrophages isolated from GF-IL12 transgenic animals could be observed over that found in GF-IL12/CXCR3KO mice, but with similar frequency of $CD45^+/CD11b^+$ cells (21.8 ± 12.5% in GF-IL12 vs. 13.4 ± 2.5% in GF-IL12/CXCR3KO). Furthermore, qRT-PCR revealed significantly higher IFN-γ transcript level in the cerebellum of GF-IL12 mice than in GF-IL12/CXCR3KO animals (Fig. 5 *B*). In summary, cellular infiltrates and IFN-γ RNA level are increased early in GF-IL12 mice compared with GF-IL12/CXCR3KO animals, arguing for an early role of CXCR3 in cerebellar leukocyte accumulation and IFN-γ induction in the GF-IL12 mice.

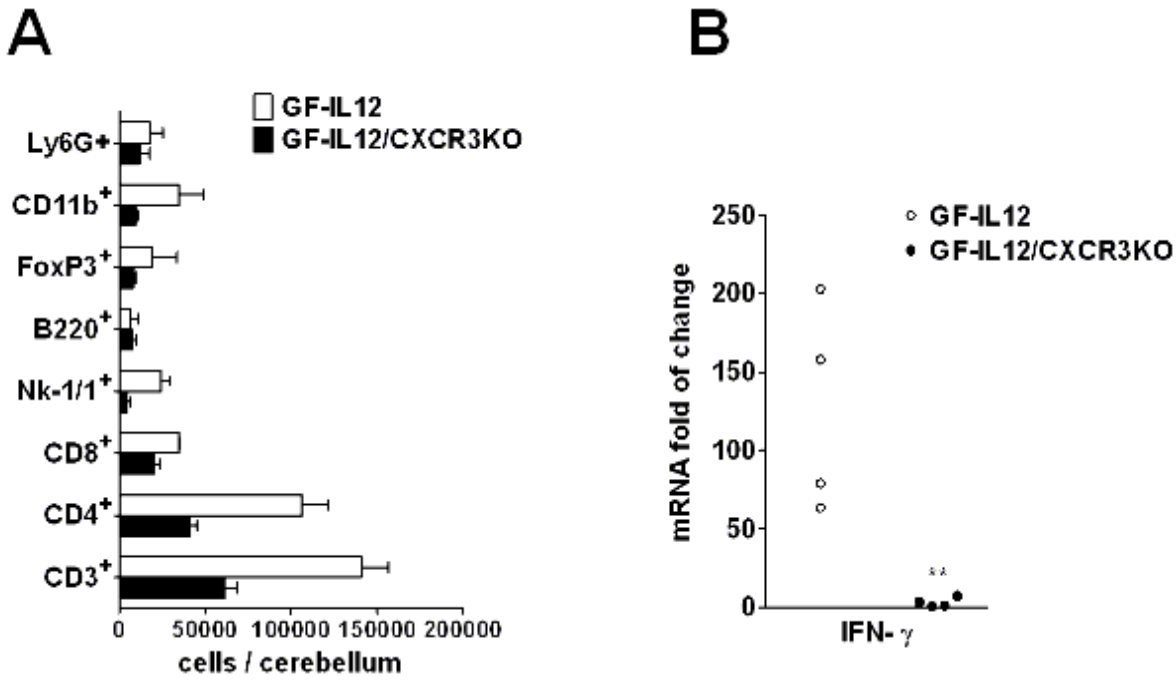


Figure 5: Flow cytometric analysis of leukocyte subsets in the cerebellum of GF-IL12 and GF-IL12/CXCR3KO mice at the age of 8 weeks (*A*). Cerebellar tissue leukocytes were isolated and analyzed as described in the *Materials and Methods*. A strong decrease of $CD4^+$, Nk $1/1^+$ and $CD45^+$ / $CD11b^+$ cells was found in GF-IL12/CXCR3KO mice compared with GF-IL12 animals. Low level of IFN-γ transcripts (qRT-PCR detection) at this early time point correlated with FACS analysis findings of diminished counts of NK- and T cells found in cerebellar tissue of GF-IL12/CXCR3KO compared to GF-IL12 animals (*B*).

CXCR3KO mice developed a progressive and destructive inflammation of the eyes, ultimately leading to bulbar atrophy and blindness.

Macroscopic examination of the eyes of GF-IL12/CXCR3KO mice revealed a severe ocular atrophy (phthisis bulbi) at 24 weeks (Fig. 6 *M*). None of the GF-IL12 mice developed this ocular phenotype (Fig. 6 *I*), eyes from GF-IL12 mice were indistinguishable to WT or CXCR3KO mice at that time-point (Fig. 6 *A, E*).

To further examine the histopathology of the eye phenotype, we performed routine histological staining at 4, 8 and 24 weeks of age and immunohistochemistry at 8 weeks. The eyes of all genotypes were examined (each n=6). Routine H&E staining at 4 weeks revealed that non of the genotypes displayed any pathological alterations, which ruled out developmental abnormalities (Fig. 6 *B, F, J, N*). At the age of 8 weeks, lens damage and changes in the retinal architecture with injury or elimination of photoreceptors inner and outer segments was found in GF-IL12/CXCR3KO mice (Fig. 6 *O*). At 24 weeks, GF-IL12/CXCR3KO mice displayed a severe retinopathy, a disrupted pigment epithelium and destruction of the lens architecture (Fig. 6 *P*). In contrast, GF-IL12 animals did not display any major histopathological alterations of the eye at 8 or 24 weeks (Fig. 6 *K, L*), neither did the WT or CXCR3 control mice (Fig. 6 *C, D, G, H*).

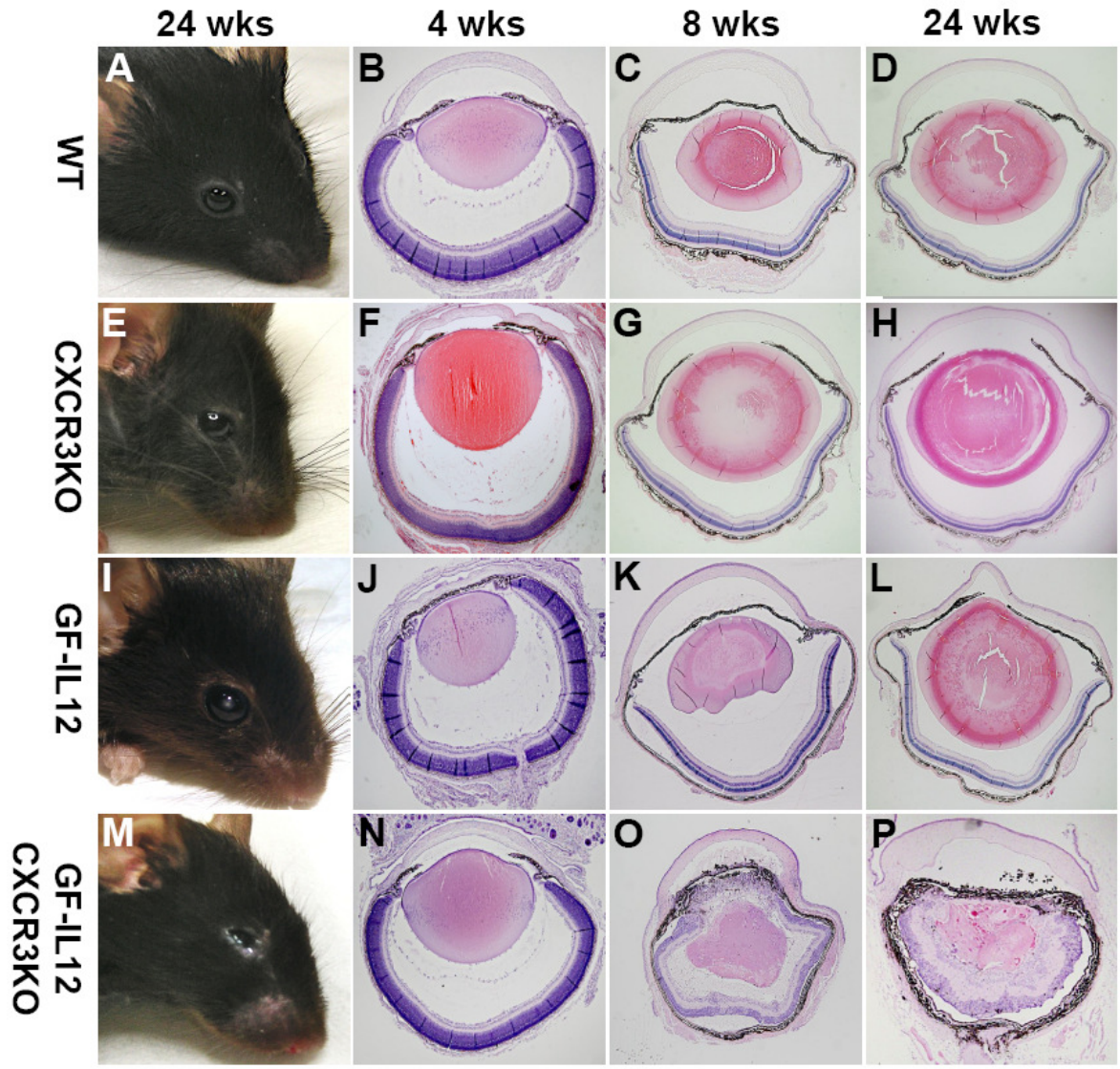


Figure 6: Progressive postnatal ocular inflammation of GF-IL12/CXCR3KO ultimately leading to bulbar atrophy and destruction. Macroscopic ocular phenotype from WT, CXCR3KO, GF-IL12 and GF-IL12/CXCR3KO mice at the age 24 weeks (*A, E, I, M*) and. H&E stained ocular sections at the age of 4, 8, and 24 weeks (*B-D, F-H, J-L, N-P*). Sections of the eyes at the age of 4 weeks appeared normal (*B, F, J, N*) in all genotypes, thereby ruling out developmental abnormalities in the mutant strains. At 8 weeks of age disorganization and loss of photoreceptors in the retina of CXCR3-deficient GF-IL12 mice and with cellular infiltrates in the peripheral cornea, iris, and ciliary body and pigment loaded cells in the anterior chamber (*O*). After 24 weeks of age the whole structure of the retinal layers and the lens were disrupted or destroyed in the eyes of GF-IL12/CXCR3KO mice (*P*). WT, CXCR3KO and GF-IL12 mouse eye/retina without alterations observable in the overview of all ages (*B-D, F-H, J-L*).

Using immunofluorescence staining of sagittal horizontal sections of eyes we further characterized the histopathological features of the ocular destruction in GF-IL12/CXCR3KO mice at 8 weeks of age. We found high counts of $CD3^+$ T cells throughout the eye (204 ± 23 /section of the eye; n=5 sections) but in particular an accumulation in the ganglion cell layer of the retina (Fig. 7 *M*). At this stage T cell accumulation was closely associated with $Iba1^+$ microglia/macrophages infiltration of the retinal ganglion cell layer, widely found in proximity to the retinal pigment epithelium (Fig. 7 *O*) and anterior chamber (data not shown). GF-IL12 mice had only minor ocular $CD3^+$ T cell accumulation (Fig. 7 *I*; <10 ± 6 /section of the eye; n=8 sections). At 8 weeks we found $Iba1^+$ microglia/macrophages in the GF-IL12 ganglion cell layer and in between the inner and outer nuclear layer of retina (Fig. 7 *K*). No $Iba1^+$ cells were observable in the intact layer where one can find the inner and outer segments of rod and cone photoreceptors. Staining of the vasculature with laminin revealed an increased amount of vessels in the inner plexiform and the granule layers of GF-IL12/CXCR3KO mice but not GF-IL12 mice (Fig. 7 *L, P*). In addition, Mueller cells, the retinal astrocytes, were found highly activated in terms of morphology and GFAP levels in GF-IL12/CXCR3KO mice (Fig. 7 *N*). Non of the examined WT and CXCR3KO eyes showed any histopathological features in respect to CD3 (Fig. 7 *A, E*), GFAP (Fig. 7 *B, F*), Iba1 (Fig. 7 *C, G*) or Laminin (Fig. 7 *D, H*) immunohistochemistry.

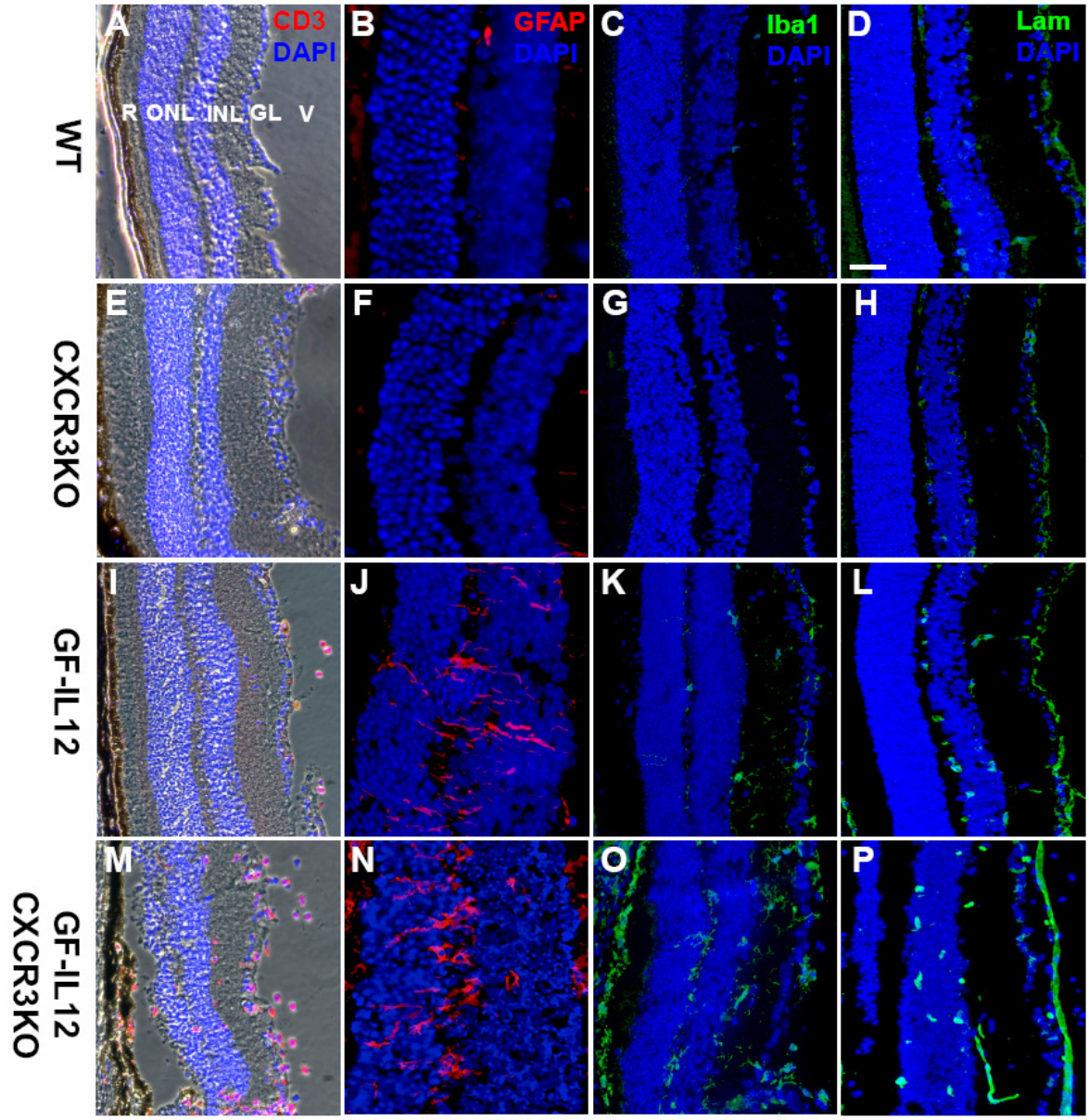


Figure 7: Combined immunofluorescence-staining for T cells (CD3) /phase contrast microscopy (*A, E, I, M*) and single immunofluorescence staining of the retina of examined genotypes (*B-D, F-H, J-L, N-P)*; GFAP to detect Müller cells; Iba-1 to detect microglia/macrophages; Laminin to detect blood-vessels on serial sections. GF-IL12/CXCR3KO but not GF-IL12 mice at 8 weeks revealed massive $CD3^+$ T cell accumulation and destruction of the retinal architecture (V, vitreous; GC, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; R, rod & cones outer segments) with loss of the photoreceptors inner and outer segments (*I, M*). In contrast to only minor changes in the eyes from GF-IL12 mice, pathological neovascularization in the inner plexiform layer and the granule layer (*L, P*; Lam) was observed in the retina of GF-IL12/CXCR3KO mice. Retinal microglia/macrophages were detectable in GF-IL12 and GF-IL12/CXCR3KO, but Iba1 positive cells within the photoreceptor layer were only detected in the GF-IL12/CXCR3KO genotype (*K, O*; Iba-1). In addition, Müller cells in GF-IL12/CXCR3KO mice were highly activated in terms of morphology and GFAP-protein level (*J, N*; GFAP). Nuclear counterstaining with DAPI (blue signal). Bar, 50 μm (*A, C- E, G-I, K-M, O, P*), 25 μm (*B, F, J, N)*.

Increase of specific leukocyte subsets in the eyes of GF-IL12/CXCR3KO

To characterize the composition and numbers of immune cells infiltrating the eyes of GF-IL12/CXCR3KO and GF-IL12 animals at eight weeks, flow cytometric analysis was performed (Fig. 8*A*). Corresponding with the histological findings of high numbers of $CD3^+$ cells in the eyes of GF-IL12/CXCR3KO mice, an increase in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was observed by FACS compared to the GF-IL12 genotype (5299 ± 93 $CD4^+$ T cells and 1606 ± 319 $CD8^+$ T cells in GF-IL12/CXCR3KO vs. 1224 ± 232 $CD4^+$ T cells and 257 ± 176 $CD8^+$ T cells in GF-IL12). Furthermore, the number and relative proportion of $Ly6G^+$ neutrophil granulocytes was found to be markedly elevated in GF-IL12/CXR3KO compared to the GF-IL12 eyes (4.6 ± 0.3% in GF-IL12 vs. 20.7 ± 3.2 in GF-IL12/CXCR3KO). However, the percentage of $CD4^+$ (79.0 ± 15.0% in GF-IL12 vs. 74.5 ± 1.3% in GF-IL12/CXCR3KO), $CD8^+$ (16.6 ± 11.3% in GF-IL12 vs. 22.6 ± 4.5%), $NK\text{-}1.1^+$ (6.6 ± 3.0 in GF-IL12 vs. 9.2 ± 0.8 in GF-IL12/CXCR3KO) and $CD11b^+$ (13.6 ± 0.0% in GF-IL12 vs. 14.2 ± 4.7% in GF-Il12/CXCR3KO) cells remained similar between the two strains.

These findings further demonstrate the inflammatory response in the ocular phenotype observed in GFIL12/CXCR3KO mice. When comparing the cerebellar leukocyte subsets (Fig. 5*A*) with the ocular leukocyte subsets (Fig. 8*A*) of individual mice, we could not detect any relation between the amount of specific inflammatory cell population in these organs (data not shown).

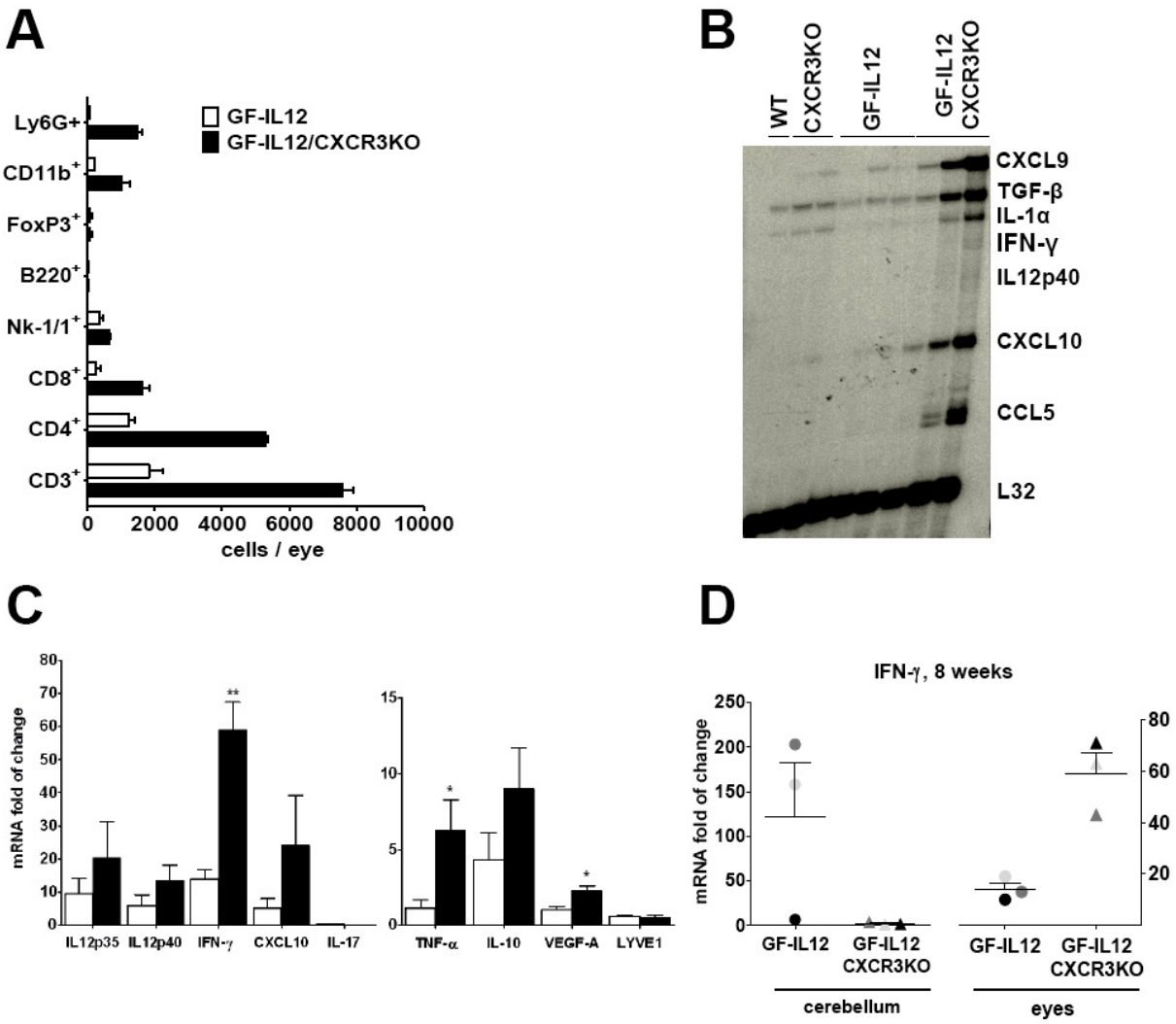


Figure 8: Ocular leukocyte infiltration is related to the induction of various inflammatory gene transcripts in GF-IL12/CXCR3KO mice. Markedly increased accumulation of various leukocyte subsets in the eyes of GF-IL12/CXCRKO mice at the age of 8 weeks (*A*). Ocular leukocytes were isolated and analyzed by cytometry as described in Materials and Methods. Ocular cell counts of CD4+, CD8+, CD45+ CD11b+, CD45+ Ly6G+, CD4+ FoxP3+, and CD19+ B220+ cell populations in GF-IL12 and GF-IL12/CXCR3KO mice. Increased levels of cytokine and chemokine gene transcripts in the eyes of GF-IL12/CXCR3KO compared with GF-IL12 mice (*B*). Real-time quantitative PCR was performed to determine the ocular gene expression level of various proinflammatory transcripts in GF-IL12 and GF-IL12/CXCR3KO mice (*C*). IFN-γ transcript analysis in the eyes and cerebelli of individual animals (*D*, each symbol represents eyes or cerebelli of one analyzed animal). For statistical significance, ⋆P <0.05; ⋆⋆P<0.01, mean±SEM.

Induction of inflammation-related genes in the eyes of GF-IL12/CXCR3KO mice

To examine the RNA levels of key inflammatory cytokines in the eye of GF-IL12 vs. GF-IL12/CXCR3KO mice, we performed RNase protection assay with total RNA from all analyzed genotypes (Fig. 8 *B*). Correlating with the histopathologic changes and inflammatory cells accumulated in the eye at 8 weeks, we found highly increased RNA levels of all examined cytokines, including IFN-γ, CXCL9, CXCL10 and CCL5 (Fig. 8 *B*).

To further characterize the ocular inflammatory response in *GF-IL12/CXCR3KO mice,* we used qRT-PCR to confirm the RPA results and to compare RNA levels from selected key cytokines, chemokines (IL-12p40/35, IFN-γ, CXCL10, IL-17, TNF-α and IL-10) or angiogenesis and lymphangiogenesis markers (VEGF-A and LYVE1) from the eyes of 8 week old animals (Fig. 8 *C*). Fold increase was calculated with respect to expression of the respective target in the CXCR3KO and WT mice. No significant differences were detected between CXCR3KO and WT mice.

We found significantly higher IFN-γ mRNA levels in the eyes GF-IL12/CXCR3KO (⋆⋆, $p<0.01$; 59.0 ± 8.3) vs. GF-IL12 (14.0 ± 2.7) mice (Fig. 8 *C*). However, analysis of the cerebellum and eyes of individual mice at 8 weeks revealed no correlation between cerebellar and ocular IFN-γ mRNA levels (Fig. 8 *D*). The IFN-γ-inducible chemokine CXCL10 mRNA was induced in eyes of GF-IL12/CXCR3KO (24.2 ± 14.9) vs. GF-IL12 (5.0 ± 3.1) mice. However, similar to the cerebellum (see above) no significant induction of IL-17 mRNA was found in the eyes of any genotypes. Next, the eyes mRNA level for the proinflammatory cytokine TNF-α, was found significantly increased in GF-IL12/CXCR3KO mice (⋆, $p<0.05$; 6.3 ± 2.0) vs. GF-IL12 (1.1 ± 0.6) mice. Because of the extensive vascularization observed in the eyes of GF-IL12/CXCR3KO mice, we examined the expression of the vascular endothelial growth factor (VEGF) gene. VEGF induces microvascular permeability and plays a central role in both angiogenesis and vasculogenesis (Malecaze et al., 1994). VEGF-A RNA was found at significantly higher levels in the eye of GF-IL12/CXCR3KO (⋆, $p<0.05$; 2.3 ± 0.3) vs. GF-IL12 (1.0 ± 0.2) ($n=3$, mean ± SEM; Fig. 8 *C*) mice. To further evaluate whether increased vessel formation was also accompanied by an increase in lymphangiogenesis in the eye, we examined LYVE1 transcripts as a lymphatic endothelial cell marker but did not find any significant difference between GF-IL12/CXCR3KO and GF-IL12 mice. In summary, the expression of inflammatory genes is highly upregulated in the eyes of GF-IL12/CXCR3KO mice compared to GF-IL12 mice,

which further provides evidence for an inflammatory driven pathogenesis leading to the severe destruction of eyes in GF-IL12/CXCR3KO mice.

STAT4 phosphorylation is reduced in the cerebellum, but increased in the eyes of GF-IL12/CXCR3KO mice and correlates with IL12rb1 mRNA quantification.

To evaluate possible differences in the cerebellar and ocular tissue response to IL-12, we examined the RNA levels of IL12rb1 by quantitative PCR and STAT4 phosphorylation using immunoblot detection (Fig. 9 *A-F*). Corresponding with the described inflammatory response, we observed higher RNA levels of IL12rb1 in cerebelli of GF-IL12 mice compared with GF-IL12/CXCR3KO mice. Conversely, ocular IL12rb1 was higher in GF-IL12/CXCR3KO mice compared with GF-IL12 mice (Fig. 9 *C,F*).

Densitometric analysis of immunoblots (Fig. 9 *A, B, D*, and *E*) revealed significantly higher protein levels of total and phosphorylated STAT4 protein levels in the cerebelli of GF-IL12 mice versus GF-IL12/CXCR3KO mice (total STAT4, 1.65 ± 0.08 versus 0.96 ± 0.06; phosphorylated STAT4, 0.81 ± 0.07 versus 0.27 ± 0.02; Fig. 9 *A* and *B*). The opposite was observed in the examined eyes: lower levels of total and phosphorylated STAT4 were found in the eyes of GF-IL12 mice compared with GFIL12/CXCR3KO mice (total STAT4, 1.94 ± 0.14 versus 2.76 ± 0.06; phosphorylated STAT4, 0.91 ± 0.09 versus 1.97 ± 0.02; Fig. 9 *D* and *E*).

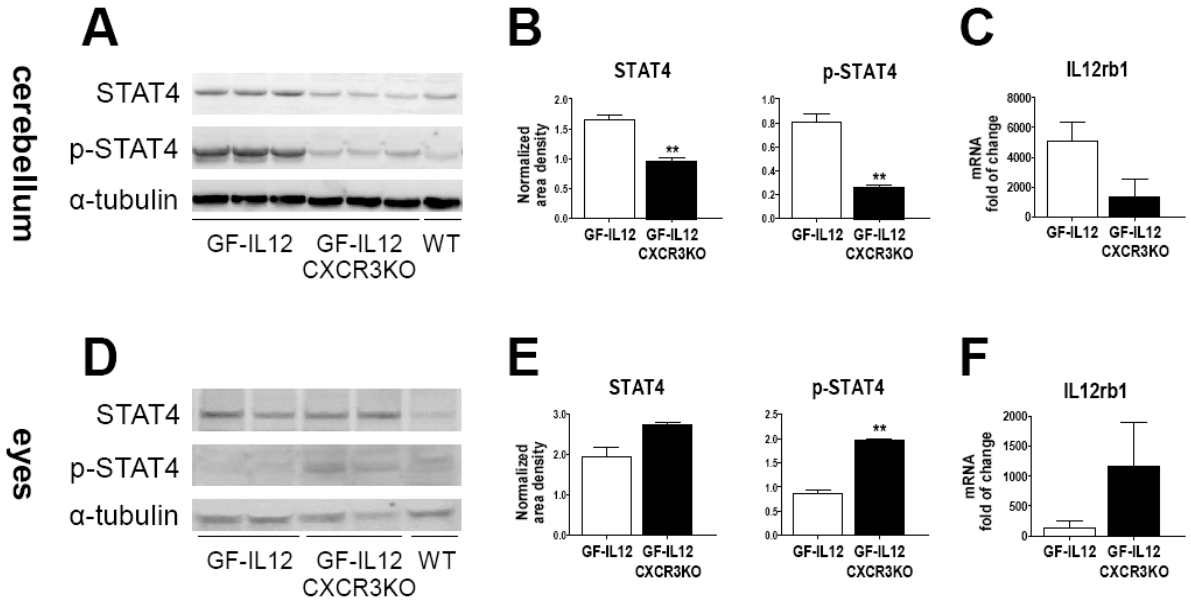


Figure 9: Reduced total STAT4 protein level, STAT4-phosphorylation and IL12rb1 transcripts in GF-IL12/CXCR3KO cerebelli, but increased levels of STAT4, phosphorylated STAT4 and IL12rb1 transcripts in GF-IL12/CXCR3KO eyes. Immunoblot (*A, D*) and densitometric analysis (*B, E*) of the cerebellar and ocular STAT4 and phospho-STAT4 level in GF-IL12 and GF-IL12/CXCR3KO mice was performed as described in Material and Methods section. For statistical significance **, $p < 0.01$, mean ± SEM.

4.4 Discussion

CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 are implicated in the pathogenesis of many neuroinflammatory diseases (Sørensen et al., 2002; Lepej et al., 2005; Rupprecht et al., 2005). However, functional studies examining the role of CXCR3 and its ligands in neuroinflammatory disease models have led to conflicting results without clearly defining the role of the CXCR3 chemokine system in inflammatory CNS diseases (Tsunoda et al., 2004; Müller et al., 2010). In particular, studies in EAE did not reveal the expected disease-promoting effect of CXCR3 but rather protective and disease limiting functions (Liu et al., 2006; Müller et al., 2007). Here, we examined the role of CXCR3 in a less complex type 1 cell-mediated model of spontaneous CNS-inflammation in transgenic mice with astrocyte-targeted production of IL-12. Within the CNS of these mice with CXCR3-deficiency, we observed a markedly attenuated inflammatory response, which corresponded with the well characterized impact of the CXCR3 chemokine system on the migration and attraction of type 1 immune cells (Gao et al., 2003; Xie et al., 2003). However and unexpectedly, these same animals a developed very severe inflammatory disease of the eyes, demonstrating that the effect of CXCR3 in neuroinflammation is not only dependent on the underlying cause but also is strongly influenced by the site of the inflammation.

Neuroinflammation in GF-IL12 transgenic mice is well characterized. Astrocytic IL-12 activates $CD4^+$ and $CD8^+$ T cells and NK cells which produce proinflammatory cytokines including IFN-γ and the CXCR3 ligands CXCL9 and CXCL10 (Gately et al., 1998). An IFN-γ-dependent immune response further increases the local accumulation of activated immune cells and mediates the tissue damage of the CNS (Komatsu et al., 1996; Wenner et al., 1996; Binder and Griffin, 2001; Chesler and Reiss, 2002). Phenotypically these GF-IL12 transgenic mice develop a severe cerebellitis and correspondingly a progressive ataxia (Pagenstecher et al., 2000). However, the majority of GF-IL12/CXCR3KO mice did not develop a clinical phenotype and had only minor histopathological alterations of the cerebellum. This finding argues for a strong disease-promoting function of the CXCR3 chemokine system in IL-12 induced neuroinflammation in the GFAP-IL12 model and is in contrast to previous findings in EAE, where CXCR3 has a disease limiting function-independent of the recruitment of effector T cells to the CNS (Liu et al., 2006; Müller et al., 2007).

The gross reduction of leukocytes in the parenchyma and subarachnoid space of GF-IL12/CXCR3KO mice provides evidence for a functional role of CXCR3 in mediating the

leukocyte accumulation in the CNS as a result of IL-12 overproduction. However, CXCR3-deficiency did not completely prevent cerebellar inflammation in all GF-IL12/CXCR3KO mice. A low percentage of GF-IL12/CXCR3KO mice did develop clinical signs of ataxia and histopathological alterations, which still were less prominent than observed in CXCR3 competent GF-IL12 mice. As CCL5 and CCR5 are highly upregulated in GF-IL12/CXCR3KO^{sick} mice, this chemokine system may possibly have compensated for the CXCR3 deficiency in GF-IL12/CXCR3KO mice that develop clinical signs.

To define early differences between GF-IL12 and GF-IL12/CXCR3KO mice in the inflammatory response, we examined animals of each genotype at the age of eight weeks, where clinical signs were not yet observable. FACS analysis revealed substantial differences in the amount and composition of immune cells in cerebelli from GF-IL12 and GF-IL12/CXCR3KO mice at that early time-point. We did not only observe a difference in T cell subsets but also a marked reduction of CD11b$^+$ microglia / macrophages and NK cells in GF-IL12/CXCR3KO mice. This finding demonstrates that CXCR3 is not only involved in the accumulation of T cells in this model, but also, directly or indirectly, in the accumulation of macrophages and NK cells at an early time point. The observation that CXCR3 and its ligands modulate the CNS accumulation of a variety of immune cells, and not only T cells, is further supported by previous observations in transgenic mice with chronic astrocyte-targeted production of the CXCR3 ligand CXCL10. These mice develop subarachnoidal cellular infiltrates consisting not only of T cells but also of macrophages and neutrophils (Boztug et al., 2002).

Surprisingly, all GF-IL12/CXCR3KO mice developed a severe and progressive ocular phenotype with severe chorioretinitis. In GF-IL12 mice, only mild lens opacities were observed in 50% of the animals. As judged from histology, prominent T cell and neutrophil accumulation in the eyes of GF-IL12/CXCR3KO resulted in a severe disruption of the internal structures of the eye and the loss of the photoreceptor outer segments. FACS analysis revealed a substantial accumulation of both CD4$^+$ and CD8$^+$ T cells, in particular CD4$^+$ T cell, NK cells, CD11b$^+$ and neutrophils. Although the total number of T cells was drastically increased, we did not detect an increase of FoxP3$^+$ regulatory T cells, suggesting a role for CXCR3 in directing and accumulating regulatory T cells into the eye. Proinflammatory cytokines like IFN-γ, TNF-α and VEGF were highly upregulated in the eyes of GF-IL12/CXCR3KO mice, which further underlines the inflammatory nature of the ocular phenotype. A similar ocular pathology was observed in a previous study examining a

transgenic mouse line using the rhodopsin promoter to direct the expression of IFN-γ to photoreceptor cells (rhoγ mice) (Geiger and Sarvetnick, 1996). This assumption is supported by the increased levels of phosphorylated STAT4 in the eyes of GF-IL12/CXCR3KO mice. It is therefore likely that the ocular pathology is driven by the production IFN-γ from IL-12 activated T cells or NK cells. Because GF-IL12 animals do not develop a severe ocular phenotype and have only slightly increased levels of IFN-γ, it is likely that CXCR3 is, indirectly or directly, restricting the activation of immune cells by IL-12, which in turn prevents the inflammatory cascade ultimately leading to the accumulation of leukocytes and the ocular destruction observed in GF-IL12/CXCR3KO mice.

This finding is contrary to other experimental studies, which suggest that CXCR3 increases the production of IFN-γ by T cells in the CNS (Christensen et al., 2004; Liu et al., 2006). An approach to explain the different impact of CXCR3 deficiency for the brain and the eye could be differences in the local immune milieu. It is known that the eye has an immune-suppressive milieu in which factors like high constitutional levels of TGF-β and the induction of high levels of IL-10 during an immune response are able to prevent a potentially harmful Th1 response (D'Orazio and Niederkorn, 1998). Although the brain also displays some properties of a so called immune privileged micro-milieu, milieu-differences between brain and eye could contribute to the different functional properties of CXCR3 leading to enhanced ocular but attenuated cerebellar inflammation in our model (Galea et al., 2007). Future in-vitro studies examining the functional properties of $CXCR3^+$ immune cells in the presence or absence of a specific cytokine milieu including TGF-β and IL-10 could further help to clarify our observations.

The ocular inflammation in GF-IL12/CXCR3KO mice was accompanied by increased vessel formation in the retina. The finding of neovascularization in the retina points toward an angiostatic role of CXCR3 and its ligands in ocular inflammation, which has been described for CXCR3 in many other models of inflammation (Petrai et al., 2008). Retinal neovascularization is not a feature commonly observed in animal models of ocular inflammation (Geiger and Sarvetnick, 1996). We therefore suggest that the observed neovascularization is likely linked to the CXCR3 deficiency in our model.

Our data delineate that CXCR3 is important to initiate and maintain an IL-12 driven inflammation in the brain. CXCR3 deficiency drastically reduces the incidence of IL-12-induced inflammation of the cerebellum. However, the protective effect of CXCR3 deficiency in IL-12 driven inflammation is not as complete as previously observed for IFN-γ

deficiency (Hofer et al., 2004). Some GF-IL12 animals did develop cerebral inflammation despite CXCR3 deficiency. This observation suggests that other molecules may compensate for the loss of CXCR3 and mediate the IL-12-induced inflammation even in the absence of CXCR3. Our data suggest that CCL5 and the corresponding receptor CCR5 might be candidates in this role as we found high levels of both CCL5 and CCR5 RNA in affected GF-IL12/CXCR3KO mice.

However, in contrast to the cerebellar effects, CXCR3 deficiency led to a destructive inflammatory phenotype of the eyes, arguing for a protective role of CXCR3 in IL-12-induced ocular inflammation and for micro-millieu dependent functional properties of CXCR3, which remain to be further defined by future studies. Our data support the perspective that CXCR3 can have both striking protective and harmful functions in CNS and ocular inflammation and that this effect does not only depend on the trigger as suggested by previous studies but likely also on the micro-millieu of the affected organ. CXCR3 is a potential therapeutic target but our data further underline the functional complexity of this chemokine system, which has to be better defined in future studies. Until then, our findings caution against the therapeutic targeting of CXCR3 (Müller et al., 2010).

Chapter 2

5. CXCR3 in Alzheimer's-like pathology

Submitted to "The Journal of Clinical Investigation": Krauthausen M, Kummer MP, Zimmermann J, Terwel D, Heneka MT, Müller M: The chemokine receptor CXCR3 is critical for the Alzheimer-like plaque formation in transgenic APP/PS1 mice.

5.1 Introduction

Alzheimer's disease (AD) is a neurodegenerative brain disorder with deposition of beta-amyloid plaques, predominantly in hippocampal and cortical regions (Katzman and Saitoh, 1991; Mattson, 2004).

Periplaque activation of microglia and astrocytes as well as the induction of proinflammatory molecules suggest a pathogenetic role of inflammation in this disease (Heneka et al., 2010; Parpura et al., 2012). Microglia are resident CNS cells with immune modulatory and phagocytic capabilities (Perry and Gordon, 1988; Lawson et al., 1992). Recent studies indicate that the microglial state of activation can determine if these cells have a protective or detrimental functional role in AD (Koenigsknecht-Talboo and Landreth, 2005; Shie et al., 2005; Yamamoto et al., 2005; Town et al., 2008; Krause and Müller, 2010; Reed-Geaghan et al., 2010). Microglia can induce reactive oxygen species, secrete proinflammatory cytokines and additional neurotoxic factors, which contribute to the pathology of AD (Wyss-Coray and Mucke, 2002; Heneka et al., 2010; Parpura et al., 2012). But microglia can also release Aβ degrading enzymes and express scavenger receptors, which can mediate Aβ phagocytosis (Paresce et al., 1996; Weldon et al., 1998; Koenigsknecht and Landreth, 2004). There is compelling evidence that microglial cells can modulate the pathological course of AD, whereas the exact role of microglia in AD remains to be elucidated.

Chemokines are cytokines, which orchestrate the innate and adaptive immune responses and are found to be highly induced in a vast variety of neuroinflammatory disorders (Charo and Ransohoff, 2006b). The non-ELR CXC chemokines CXCL9, CXCL11 and in particular CXCL10 are prominent members of these molecules (Müller et al., 2010). They share the receptor CXCR3 (Loetscher et al., 1996; Weng et al., 1998), that is expressed on T cells and NK cells but also on resident CNS cells (Sallusto and Lanzavecchia, 2000; Biber et al., 2002; Flynn, 2003; Rappert et al., 2004; de Jong et al., 2008b). CXCR3 can be differentially activated

by CXCL9, CXCL10 and CXCL11 (Loetscher et al., 1998; Weng et al., 1998). IFN-γ and TNF-α are major inducers and regulators of both CXCR3 and CXCR3-ligands (Luster et al., 1985; Cole et al., 1998; Carter et al., 2007; Zhang et al., 2010; Choi et al., 2011).

Current studies in experimental AD models have demonstrated that chemokine receptor systems like CCR5 (Lee et al., 2009), CCR2 (El Khoury et al., 2007; Kiyota et al., 2009; Semple et al., 2010) and CX3CR1 (Fuhrmann et al., 2010; Lee et al., 2010; Liu et al., 2010) can modulate the disease course by influencing microglial function, accumulation and clustering (El Khoury et al., 2007; Fuhrmann et al., 2010; Lee et al., 2010; Liu et al., 2010).

Concerning the role of the CXCR3 chemokine system in AD, it has been demonstrated that there is a positive correlation between cerebrospinal fluid CXCL10 levels and cognitive impairment in AD patients (Galimberti et al., 2003, 2006). Moreover, CXCL10 was found to be expressed in astrocytes of AD brains (Xia et al., 2000). Furthermore, CXCL10 was detected in close proximity to Aβ plaques in a corresponding AD mouse model (Duan et al., 2008).

To characterize the role of the CXCR3 chemokine system in the course of this degenerative disease model, we now examined the impact of genetic CXCR3-deficiency in APP/PS1 transgenic mice.

5.2 Material and methods

Animals

CXCR3-deficient (CXCR3$^{-/-}$) mice (originally provided by Drs. Bao Lu and Craig Gerard, Children's Hospital and Harvard Medical School, Boston, MA) have been described previously (Hancock et al., 2000). The mice where backcrossed at least eight generations to the C57BL/6 strain. CXCR3-deficient mice displayed no clinical and histological abnormalities when compared to C57BL/6J (WT) mice.

APP/PS1 double transgenic mice (B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J, The Jackson Laboratory) expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe; APP) and a mutant human presenilin 1 (PS1-dE9; PS1) protein were used (Jankowsky et al., 2001). The animals were hemizygous or littermate control mice and had been backcrossed for at least eight generations onto the C57BL/6 strain. To obtain CXCR3-deficient mice with a transgenic expression of APP/PS1 (APP/PS1/CXCR3$^{-/-}$) heterozygous APP/PS1 mice were successively crossed with CXCR3$^{-/-}$ mice and subsequently intercrossed. Only male mice were studied in order to avoid the possible influence of gender on amyloid plaque formation and inflammation in AD (Candore et al., 2006; Casadesus et al., 2006). Animals were kept under pathogen free conditions and handling was performed on the declaration of Helsinki and approved by local ethical committees.

Immunohistochemistry and thioflavin-S staining

5 and 8 months old mice of each examined genotype (WT, CXCR3$^{-/-}$, APP/PS1, APP/PS1/CXCR3$^{-/-}$) were anesthetized with isoflurane and transcardially perfused with ice cold PBS. Immediately after euthanasia, the brains were removed and half of a brain (cut along the sagittal midline) was fixed over night in PBS-buffered 4% paraformaldehyde at 4 °C, then washed in PBS. The fixed brains were serially sectioned at 40 μm with a vibratome (Leica, Nussloch, Germany). Sections were blocked with 5% BSA in PBST and immunolabeled with antibodies against Iba1, MHC-II, CD11b, CD68, GFAP or Aβ, (Table 3) followed by incubation with Alexa Fluor® 488 or Alexa Fluor® 594-conjugated secondary antibodies (Invitrogen, Darmstadt, Germany). A double staining protocol was used for combined plaque staining by thioflavin-S and microglial immunostaining. For that purpose free floating sections were stained for 10 min with 0.015% Thio-S in 50% ethanol, washed in

50% ethanol and water before entering the immunostaining protocol. Finally, sections were mounted on HistoBond (VWR International, Darmstadt, Germany) slides, dried and coverslipped with Fluorescent mounting medium (Dako) containing 0,1% DAPI (Sigma-Aldrich, Munich, Germany). The percent area occupied by thioflavine-S positive area or Aβ-immunoreactive plaques was used to estimate amyloid load (5 sections of the frontal cortex and hippocampus/ animal). Fluorescence microscopy was done with an Olympus BX61 microscope using identical exposure times. Images were processed and analyzed with Cell ^ P (Olympus Soft Imaging Solutions, Münster, Germany) using identical threshold values. The percent of stained area of the ROIs was generated using analySIS 3.2 (Olympus).

Table 3. Summary of antibodies used for immunohistochemistry

Antibody (source)	Specificity	Vibratome Sections (dilution)
Polyclonal rabbit anti-Iba1 reactive with human, mouse and rat Iba1 (Wako Chemicals, Neuss, Germany)	Microglia and macrophages	1/500
Polyclonal rabbit anti-mouse MHC II (Dianova, Hamburg, Germany)	APC, Dendritc cells	1/500
Monoclonal rat anti-mouse Integrin αM [CD11b], (Millipore, Schwalbach, Germany)	Microglia/ macrophages	1/400
Monoclonal rat anti- mouse CD68, (Serotec, Düsseldorf, Germany)	Microglia/ macrophages	1/1000
Polyclonal rabbit anti -human GFAP (Dako, Hamburg, Germany)	Glial fibrillary acidic protein	1/1000
Polyclonal rabbit antiserum 2964 (Heneka et al., 2005; Wahle et al., 2006) against fibrillar $A\beta_{1-42}$	$A\beta_{1-42}$	1/1000
Monoclonal mouse anti-human $A\beta_{1-16}$ (Jäger et al., 2009)	$A\beta_{1-16}$	1/600

Tissue processing for protein extraction

The tissue for protein analysis was obtained from 5 and 8 months old male mice of each examined genotype (WT, CXCR3$^{-/-}$, APP/PS1, APP/PS1/CXCR3$^{-/-}$). Snap frozen brain hemispheres were homogenized in ice cold modified PBS (1 mM EDTA, 1 mM EGTA, 3 μl/ml protease inhibitor mix, pH 7.4) using an UltraTurax T8 homogenizer (IKA Labortechnik, Staufen, Germany). Homogenates were extracted in RIPA-buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% NaDOC, 0.1% SDS), centrifuged at 20,000 × g for 15 min/4°C and the remaining pellet containing insoluble Aβ was subsequently solubilized in SDS-buffer (2% SDS, 25 mM Tris–HCl, pH 7.4). After pulsed sonication for 15 sec RIPA and SDS fractions protein concentrations were determined using the BCA Protein Assay Kit (Thermo scientific, Schwerte, Germany).

APP processing and amyloid-β detection

Proteins were separated using a 4–12% NuPAGE gel (Invitrogen) with NuPage® MES SDS running buffer (Invitrogen) at 150 V. PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) was used as standard. Proteins were transferred to 0.2 μm nitrocellulose membranes. Membranes were boiled in water for 5 min and blocked for 30 min in TBST containing 5% skim milk. Immunoblotting was performed using antibody CT-15 raised against the C terminus of APP (Martin et al., 1991) (Chemicon, Temecula, CA; 1:2000) and antibody CP06 detecting α-tubulin (1:2000, clone DM1A, Millipore, Schwalbach, Germany) followed by incubation with the appropriate horseradish-peroxidase (HRP) conjugated secondary antibodies. Immunoreactivity was detected by chemiluminescence reaction (Millipore) and luminescence intensities were analyzed using Chemidoc XRS imaging system (Biorad, Munich, Germany). With the Quantity One (BioRad) program, the density of bands was determined for each lane and the intensity ratio for the detected proteins were normalized to α-tubulin. Each gel contained a non-transgenic WT and CXCR3$^{-/-}$ brain extract as controls. HoloAPP signal of APP/PS1 and APP/PS1/CXCR3-/- for each sample was given relative to the endogenous APP of the corresponding control. Graphpad Prism (Graph Pad Software, San Diego, CA) software statistical program was then utilized for statistical analysis.

Aβ ELISA

Aβ peptides levels were quantified by human Aβ1-40 and Aβ1-42 ELISA kits (The Genetics Company, Schlieren, Switzerland) according to the manufacturer's protocol. Both RIPA and SDS fractions were analyzed for Aβ1-40 and Aβ1-42 peptides. Samples were analyzed in duplicates. The results were tabulated as mean±SEM and compared using and statistically compared using an unpaired student's t-test.

Cytokine and mRNA determination by qRT-PCR

Total RNA was isolated and purified from aliquots of homogenized brain samples using Trizol reagent (Sigma-Aldrich). RNA quantity was determined spectrophotometrically using a NanoDrop 1000 (Peqlab, Erlangen, Germany). 3 μg of total RNA was reverse-transcribed into cDNA by using SuperScript™ III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR assays were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) using PowerSYBRgreen (Applied Biosystems). The composition of the reaction mixture was as follows: 1 μL of cDNA corresponding to 40 ng of total RNA, 100 nM of each primer, 2×PowerSYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μL. The used primer sequences are listed in Table 4. Samples were analyzed simultaneously for GAPDH mRNA as the internal control. Each sample was assayed in duplicate, normalized to GAPDH and expressed relative to that of nontransgenic C57BL/6J mice. Data were determined as mRNA fold of change ± SEM.

Table 4. List of oligonucleotide sequences used for qRT-PCR

Gene Name	Forward sequence	Reverse sequence
Gapdh	5'- TCACCAGGGCTGCCATTTGC-3'	5'-GACTCCACGACATACTCAGC-3'
Cd68	5'-ATCCCCACCTGTCTCTCTCA-3'	5'-ACCGCCATGTAGTCCAGGTA-3'
Itgam	5'-GTTTGTTGAAGGCATTTCCC-3'	5'-ATTCGGTGATCCCTTGGATT-3'
Cxcr3	5'-AATGCCACCCATTGCCAGTAC-3'	5'-AGCAGTAGGCCATGACCAGAAG-3'
Cxcl9	5'-GCCATGAAGTCCGCTGTTCT-3'	5'-GGGTTCCTCGAACTCCACACT-3'
Cxcl10	5'-GACGGTCCGCTGCAACTG-3'	5'-GCTTCCCTATGGCCCTCATT-3'
Fasl	5'-TTAAATGGGCCACACTCCTC-3'	5'-ACTCCGTGAGTTCACCAACC-3'
Il1b	5'-GGTCAAAGGTTTGGAAGCAG-3'	5'-TGTGAAATGCCACCTTTTGA-3'
Ifng	5'-CAGCAACAGCAAGGCGAAA-3'	5'-GCTGGATTCCGGCAACAG-3'
Ccl2	5'-TGGCTCAGCCAGATGCAGT-3'	5'-TTGGGATCATCTTGCTGGTG-3'
Ccl5	5'-CAAGTGCTCCAATCTTGCAGTC-3'	5'-TTCTCTGGGTTGGCACACAC-3'
II6	5'-ACCAGAGGAAATTTTCAATAGGC-3'	5'-TGATGCACTTGCAGAAAACA-3'
Tnf	5'-ATGAGAAGTTCCCAAATGGCC-3'	5'-ACGTGGGCTACAGGCTTGTC -3'

Retrieval of primary mouse microglia and astrocytes

Cortical murine microglia was prepared of pups at postnatal day one as previously described (Hanisch et al., 2004). Briefly, meninges of isolated brains were removed mechanically and cells were dissociated by trituration and cultured in high glucose (4.5 mg/ml) DMEM (Gibco BRL, Eggenstein, Germany), supplemented with 10% fetal calf serum (PAN Biotech, Aidenbach, Germany) and 1% penicillin/streptomycin (PAA, Cölbe, Germany) for up to 14 days. Microglia cells were harvested by shake-off. The detached microglia containing medium was collected and the isolated microglia were reseeded and allow to settle for 1h.

Primary astrocyte cultures were obtained from microglial/astrocytic co-culture maintained for a minimum of 7 days to generate a confluent glial culture. Prior to trypsinization, contaminating microglial cells were separated by repeated mechanical agitation and removed by subsequent washing in Hank's Balanced Salt Solution (PAA). Astrocytic monolayers were then dislodged from flasks by trypsinization (0.25% trypsin in HBSS and 1 mM EDTA). Cells were seeded in Ø 9 cm dishes plates (1×10^5 cells/well) and grown for 7 days until

confluent prior to stimulation. Culture purity was determined by double-labeling immunohistochemistry for GFAP (1:1000, Dako) and CD11b (1:500, Serotec) to identify astrocytes (>95%) and microglia (<5%), respectively.

Microglia and astrocytes were stimulated with either unlabeled aggregated 0.7 μM Aβ1-42 (Peptide Speciality Laboratories, Heidelberg, Germany), LPS (100 ng/ml, Sigma-Aldrich, from *Escherichia coli* 0127:B8) or mouse recombinant TNF-α (10 ng/ml, Roche Diagnostics, Mannheim, Germany) in low-serum DMEM (1 % FBS). Three separate cultures were stimulated, such that data represents the mean of three independent experiments. Additional astrocytes were incubated in medium to serve as unstimulated controls. Supernatant was collected after stimulation for 4h, 12h or 20 h and stored at -80°C until analysis, whilst cells were washed in PBS and scraped for collection with 1X RIPA containing protease inhibitor cocktail (Sigma-Aldrich).

Microglial phagocytosis of FAM-labeled Aβ1-42

Primary microglia (PMG) were seeded on cover glasses and incubated with 0,7 μM of fibrillar FAM-labeled Aβ1-42 (FAM-Aβ) (AnaSpec, San Jose, CA). Fibrillar FAM-Aβ was generated using NaOH treated peptide lyophilizate after incubation in acetat buffer to initiate aggregation (Teplow, 2006). Microglia cells were washed with PBST, fixed with 4% PFA and stained with rat polyclonal anti-CD68 (1:500; Serotec) after FAM-Aβ incubation period of one hour. Secondary detection antibody was conjugated to Alexa Fluor® 594 (Invitrogen). Furthermore, a microglial Aβ phagocytosis reader assay was applied as described previously (Fleisher-Berkovich et al., 2010) . Briefly, 5 x 10^4 cells/well were seeded and incubated with 0,7 μM fibrillar FAM-Aβ for 1 h. Afterwards the medium was removed and extracellular FAM-Aβ fluorescence signal was quenched with trypan blue. Fluorescence intensity was measured at 485 nm excitation/535 nm emission using a fluorescence plate reader (Infinite 200M, Tecan, Crailsheim, Germany). To compensate different cell counts, results were normalized with the Hoechst Dye 33342 (Sigma-Aldrich) nuclear stain signal. To evaluate the influence of CXCR3 ligands on microglial phagocytosis, recombinant mouse CXCL9 or CXCL10 (250 ng/ml, R&D systems, Wiesbaden, Germany) was applied 30 min following FAM-Aβ incubation. In additional experiments, a CXCR3 antagonist (30-300 nM, 12o, (Hayes et al., 2008) was added 30 min prior to the phagocytosis experiment to block the chemokine receptor.

Detection of cytokine proteins in culture supernatant

Commercially-available mouse Quantikine Immunoassays (R&D Systems) were used to quantify the production of the chemokines CXCL9 and CXCL10 in culture supernatants. For the detection of TNF-α, the ELISA Ready-Set-Go!Kit (eBioscience, Frankfurt, Germany) were applied. Each sample was measured in duplicate.

Intracerebral injection of fibrillar Aβ

Six- to eight-months old CXCR3$^{-/-}$ (n=5) and WT (n=5) mice were anesthetized with ketamine/xylazine (30 mg/kg/4 mg/kg) and immobilized using a stereotactic device. A 0.5-mm burr hole was drilled in the skull, and 1 μl of fibrillar Aβ1-42 solution was injected intracortically into the right hemisphere (anteroposterior –2.5, lateral 2.0 at 1.0 mm (cortex relative to the bregma) at a rate of 1 μl/min using a 5 μl Hamilton syringe as described previously (Kummer et al., 2011). Control animals received buffer solution into the right hemisphere, accordingly. Mice were sacrificed 48 hours after injection and prepared for histology as previously described. 30 μm thick horizontal brain sections including the needle track were immunostained with the primary antibodies against for Iba1 and IC16 or CD68 and fAβ antiserum 2964 as listed in Table 3. The antibody binding was detected with Alexa dye-conjugated secondary antibodies. Sections were analyzed using a BX61 microscope equipped with a confocal disk scanning unit (Olympus). Image stacks were deconvoluted using Cell ^ P (Olympus). Iba1-positive microglia within multiple 10 μm z-stacks of the needle track were counted and examined for the presence of intralysosomal fAβ1-42. Analysis of microglia phagocytosis of fAβ content within CD68$^+$ microglial lysosoms was performed using NIH ImageJ software. Microglial bodies were identified in *z*-projections from confocal images. For the quantification of intralysosomal fAβ, a region of interest (ROI) was drawn around the CD68$^+$ area and the mean fluorescence intensity values were obtained from the resulting area after subtraction of CD68$^+$ microglia regions. Aβ$^+$ deposit that was not located inside microglia was considered to be in the extracellular space. For the determination of the mean lysosomal perimeter within microglia in proximity to the fAβ injection site ≥587 CD68$^+$ lysosoms in WT and CXCR3$^{-/-}$ were analyzed. The RGB fluorescence intensity profile plot was also obtained using NIH ImageJ. The results were tabulated as mean±SEM and compared using an unpaired *t*-test.

5.3 Results

Decreased Aβ deposition and Aβ level in CXCR3-deficient APP/PS1 mice

The APP/PS1 transgenic model exhibits a progressive increase in plaque burden between the age of four and twelve months (Jankowsky et al., 2001).

We examined APP/PS1 and APP/PS1/CXCR3$^{-/-}$ animals within the early stage of Aβ deposition at 5 months and at the stage of compact and diffuse plaque burden at 8 months, prominent in APP/PS1 mice. We observed a widespread distribution of Aβ plaques stained with ThioS throughout the hippocampus and cerebral cortex of male APP/PS1 mice at 8 months as described previously (Fig. 10 A). In contrast to APP/PS1 mice, APP/PS1/CXCR3$^{-/-}$ mice revealed a strong reduction in Aβ plaque burden in both regions at the age of eight months (Fig. 10 A). Quantification of ThioS^{+} area (%) in cerebral cortex and hippocampus (Fig. 10 B) revealed a highly significant reduction of Aβ plaques in APP/PS1/CXCR3$^{-/-}$ animals compared to APP/PS1 mice at both five (cerebral cortex, APP/PS1: 0.074 ± 0.009% vs. APP/PS1/CXCR3$^{-/-}$: 0.020 ± 0.002%, $p<0.001$; hippocampus, APP/PS1: 0.047 ± 0.008% vs. APP/PS1/CXCR3$^{-/-}$: 0.004 ± 0.001%, $^{###}p< 0.0005$) and eight (cerebral cortex, APP/PS1: 0.589 ± 0.082% vs. APP/PS1/CXCR3$^{-/-}$: 0.047 ± 0.007%, ★★★$p<0.0005$; hippocampus, APP/PS1: 0.634 ± 0.145% vs. APP/PS1/CXCR3$^{-/-}$: 0.044 ± 0.011%, $^{###}p<0.0005$) months of age.

Concerning Aβ protein-levels of the brain, we could also demonstrate a significant lower Aβ1-42 level (Aβ1-40: 15.6 ± 0.2 pg/mg, Aβ1-42: 7.8 ± 0.8 ng/mg) in the insoluble SDS fraction of five months old APP/PS1/CXCR3$^{-/-}$ mice brain when compared to APP/PS1 mice (Aβ1-40: 26.2 ± 6.3 pg/mg, Aβ1-42: 49.4 ± 8.3 ng/mg; $^{##}p<0.001$ for Aβ1-42; Fig. 10 C). Correlating with the strong decrease in ThioS^{+} plaque deposition after eight months, we found a five-fold reduction of Aβ level (Aβ1-40: 32.8 ± 4.7 ng/mg, Aβ1-42: 31.6 ± 1.8 ng/mg) in APP/PS1/CXCR3$^{-/-}$ when compared to APP/PS1 mice (Aβ1-40: 166.0 ± 41.0; Aβ1-42: 113.5 ± 36.2 ng/mg; ★★★$p<0.001$ for Aβ1-40; $^{#}p<0.05$ for Aβ1-42; Fig. 10 D). No significant difference in soluble RIPA fraction could be observed at five months between the two genotypes. However, in eight months old APP/PS1/CXCR3$^{-/-}$ mice the Aβ peptide level (Aβ1-40: 127.8 ± 4.2 pg/mg, Aβ1-42: 59.8 ± 3.5 pg/mg) was significantly reduced compared

to Aβ concentration in APP/PS1 mice brain (Aβ1-40: 85.4 ± 4.2 pg/mg, Aβ1-42: 47.4 ± 2.1 pg/mg; ***$p<0.0001$ for Aβ1-40; $^{\#}p=0.01$ for Aβ1-42; Fig. 10 D).

In summary, CXCR3-deficiency reduces significantly the amyloid plaque deposition and Aβ1-42/Aβ1-40-levels in the APP/PS1 AD mouse model.

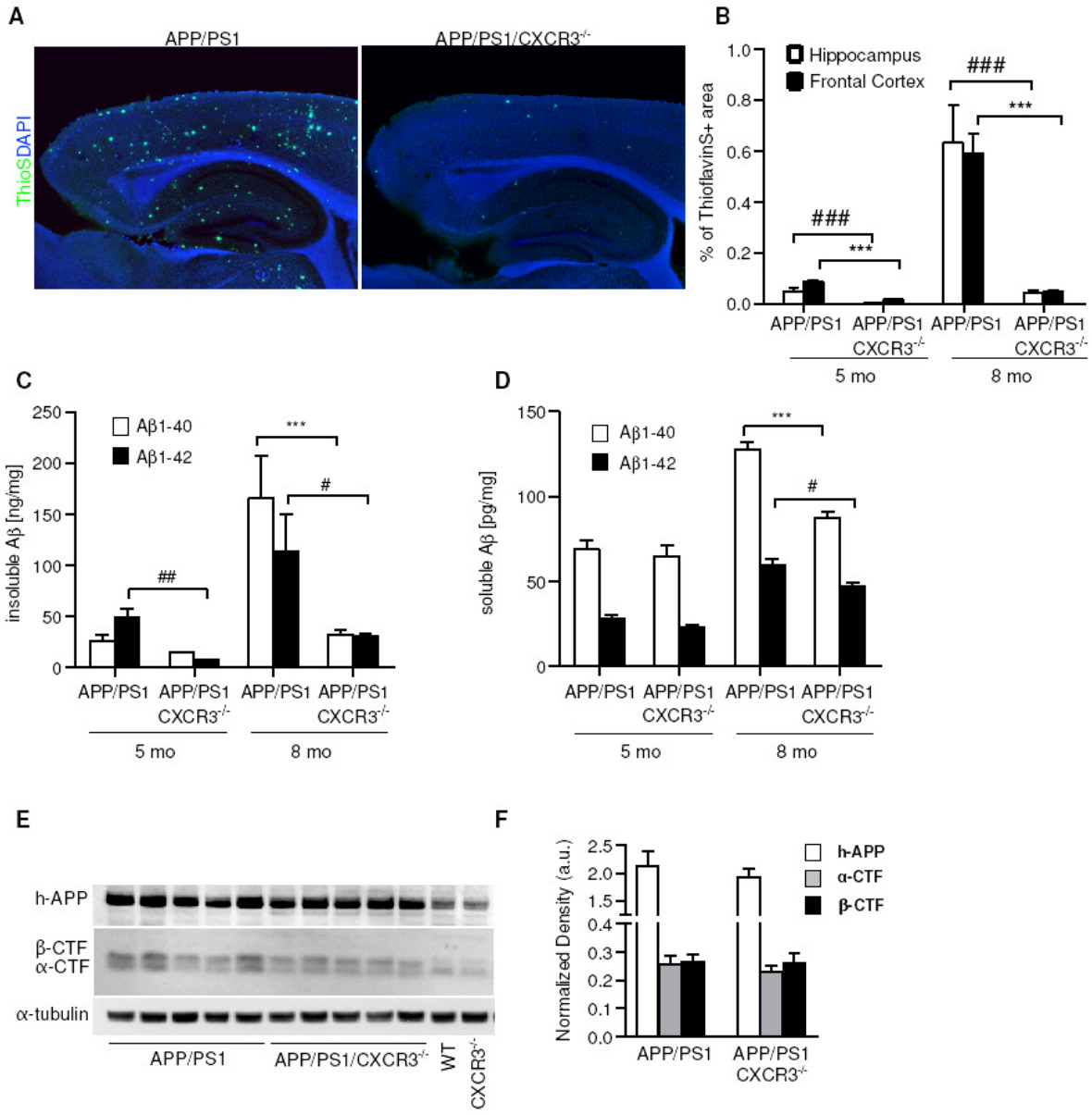


Figure 10: CXCR3 deficiency leads to a strong reduction of Aβ-deposition in APP/PS1 mice. At 8 month sagittal brain sections of APP/PS1 and APP/PS1/CXCR3-/- male mice were stained with Thioflavine S (ThioS) to detect dense-core Aβ deposition (A). Widespread Aβ deposition is visible in hippocampal and cortical regions in APP/PS1 mice, by contrast APP/PS1/CXCR3-/- mice exhibit a strong reduction of Alzheimer's-like plaques in both brain regions (A). Quantification of ThioS+ area in the frontal cortex and hippocampus of all animals demonstrate a significant reduction in Aβ deposition in 5- and 8 month old APP/PS1/CXCR3-/- compared to APP/PS1 mice (B; ###, ***$p < 0.0005$; unpaired t-test; n=5; 16-20 sections, mean±SEM). ELISA measurement of Aβ1-40 and Aβ1-42 peptides at 5- and 8 months documents a significant reduction in the insoluble SDS-fraction of the brain extract compared to APP/PS1 mice (C, #$p < 0.05$, **$p = 0.001$, ***$p < 0.001$, unpaired t-test, n=5-8, mean±SEM). At 5 months no significant differences in the composition of both soluble peptides could be demonstrate between the two genotypes (D, 5 mo), whereas at 8 months the concentration of Aβ1-40 and Aβ1-42 are significantly lower in APP/PS1/CXCR3-/- mice (D, 8 mo, *$p = 0.01$; ***$p < 0.0001$; unpaired t-test; n=5-8, mean±SEM). Immunoblot analysis using a c-terminus detecting anti-holo-APP antibody (CT15) indicates no significant effect of CXCR3 deficiency on APP-processing in APP/PS1 mice at 5 months (E). Densitometric analysis of holo-APP, α-CTFs and β-CTFs levels reveals no significant differences after normalization to β-tubulin (F, unpaired t-test, mean±SEM).

CXCR3 deficiency does not alter APP processing in APP/PS1/CXCR3$^{-/-}$ mice

To examine the influence of CXCR3 on neuronal APP processing as a cause for a reduced plaque load, we determined the protein level of holo-APP and APP cleavage products β-CTF and α-CTF in WT, CXCR3$^{-/-}$, APP/PS1 and APP/PS1/CXCR3$^{-/-}$ mice by western blot analysis. As expected control WT and CXCR3$^{-/-}$ samples showed a weak band of endogenous APP and weak signal for α- and β-CTF cleavage fragments (Fig. 10 E). We found no significant differences in normalized band intensity of holo-APP between APP/PS1 and APP/PS1/CXCR3$^{-/-}$ animals (APP/PS1: 2.1 ± 0.3 vs. APP/PS1/CXCR3$^{-/-}$: 1.9 ± 0.2 normalized band intensity; Fig 10 F). Furthermore, no significant differences in the levels of β-CTF (APP/PS1: 0.3 ± 0.0 vs. APP/PS1/CXCR3$^{-/-}$: 0.3 ± 0.0 normalized band intensity) and α-CTF (APP/PS1: 0.3 ± 0.0 vs. APP/PS1/CXCR3$^{-/-}$: 0.2 ± 0.0 normalized band intensity; Fig. 10 F) were detectable reflecting a lack of alteration of the APP processing by CXCR3-deficiency.

CXCR3 modulates the in vivo phenotype of microglia and astrocytes in APP/PS1 transgenic mice

To elucidate the impact of CXCR3$^{-/-}$ microglia on the morphological phenotype of microglia in APP/PS1 mouse model, we characterized periplaque accumulation and morphological activation of microglia. Using combined Aβ-plaque staining with ThioS and Iba1 immunofluroescent labeling of microglia, we specifically detected Aβ-plaque associated microglia/macrophages in the brains of APP/PS1 and APP/PS1/CXCR3$^{-/-}$ mice.

The strong periplaque microglial association to Aβ-plaques, documented in APP/PS1 brains (Fig. 11 D) was substantially decreased in CXCR3-deficient APP/PS1 mice (Fig. 11 D). Independent of size or region of the detected plaques, we observed a strongly reduced, almost abrogated clustering microglia around plaques in APP/PS1/CXCR3$^{-/-}$ mice. At eight months of age in APP/PS1 animals MHCII positive cells were found to be in proximity to clusters of Iba1-positve microglia. At that time point only few MHC-II positive microglial cells were detected in APP/PS1/CXCR3$^{-/-}$ male mice. Concurrent with the immunohistochemical staining of activated microglia in APP/PS1 mice, CD11b and CD68 transcript quantification outlined a significant increase in APP/PS1 mice. Both microglial marker in APP/PS./CXCR3$^{-/-}$ mice remained at WT control level (CD11b in APP/PS1: 1.7 ± 0.2 vs. 1.1 ± 0.0 folds

increase in APP/PS1/CXCR3$^{-/-}$; **p<0.01 and CD68 in APP/PS1: 3.8 ± 0.7 vs. 1.2 ± 0.1 folds increase in APP/PS1/CXCR3$^{-/-}$; **p<0.01; Fig. 11 C).

Double staining for GFAP and ThioS demonstrated in APP/PS1 mice that most amyloid plaques were surrounded by astrocytes, which appear activated by morphological criteria (Fig. 11 E). Some scattered activated astrocytes were not associated with amyloid plaques, however, correlating with the substantial reduction of Aβ-plaques, we could also observe a markedly reduction of GFAP immunoreactivity in APP/PS1/CXCR3$^{-/-}$ brain using immunofluorescence and immuno blotting (Fig. 11 E, F).

In summary CXCR3-deficiency strongly reduces microglial activation and clustering in proximity to Aβ-plaques stained by ThioS. Moreover, morphologically activated astrocytes were strongly reduced in APP/PS1/CXCR3$^{-/-}$ compared to APP/PS1 brain.

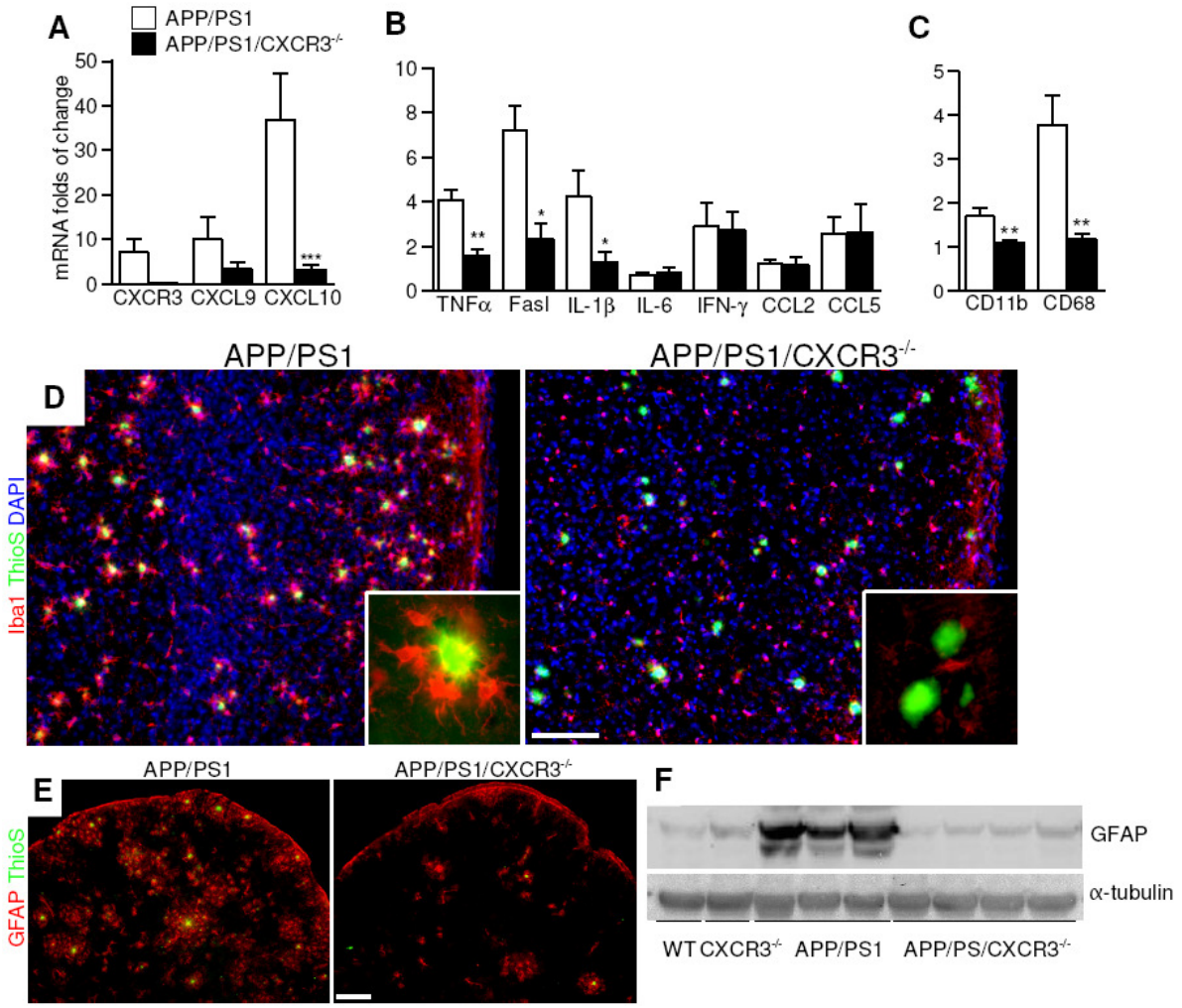


Figure 11: CXCR3 deficiency strongly reduces the level of inflammatory transcripts, plaqueassociated microglial accumulation and the periplaque activation of astrocytes in APP/PS1 mice. Quantitative real-time PCR revealed reduced expression of CXCR3 ligands (A), inflammatory cytokines/chemokines (B) and selective microglial markers (C) in APP/PS1/CXCR3-/- brains. RNA transcripts were normalized to GAPDH and expressed relative to that of age matched WT controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$; unpaired t-test; n=5-8, mean±SEM). Sagittal brain sections of age-matched APP/PS1 and APP/PS1/CXCR3-/- mice were immunostained with the microglial marker Iba1 and densecore Aβ plaques were visualized using Thioflavine S (ThioS, D). A markedly reduction of microglial accumulation around Aβ deposition is visible in APP/PS1/CXCR3-/- compared to the periplaque clustering of Iba1+ microglia in APP/PS1 brain (D, insets). Combined staining for Aβ plaques and an astrocytic marker reports a substantial reduction of GFAP+ astrocytes in APP/PS1/CXCR3-/- mice compared to the strong immunoreactivity of the Aβ plaques ringed astrocytes in APP/PS1 mice (E, scale bar=250 μm). Western blots of RIPA brain extracts probed with antibodies to GFAP revealed reduced GFAP-levels in APP/PS1/CXCR3-/- mice compared to APP/PS1 transcripts in APP/PS1/CXCR3-/- mice (F).

CXCR3 and the corresponding ligands CXCL9 and CXCL10 are induced in APP/PS1 transgenic mice

To correlate the histological findings of altered microglial activation with RNA transcripts for the CXCR3 and its ligands CXCL9 and CXCL10, we performed quantitative PCR analysis from eight months old APP/PS1 and APP/PS1/CXCR3$^{-/-}$ total brain RNA. We first could demonstrate an induction of CXCR3 transcripts in APP/PS1 mice (7.2 ± 3.0 fold increase) relative to WT controls (Fig. 11 A). Furthermore analysis of CXCR3 ligand transcripts revealed elevated levels for CXCL9 (5.8 ± 4.2 fold increase) and in particular for CXCL10 (36.7 ± 10.5 fold increase) in APP/PS1 mice (Fig. 11 A).

Proinflammatory cytokines are attenuated in CXCR3-deficient APP/PS1 mice

In APP/PS1/CXCR3$^{-/-}$ mice we found the levels of proinflammatory TNF-α (APP/PS1: 4.1 ± 0.5 fold increase vs. APP/PS1/CXCR3$^{-/-}$: 1.6 ± 0.3 fold increase; $^{**}p<0.002$), IL-1β (APP/PS1: 4.2 ± 1.2 fold increase vs. APP/PS1/CXCR3$^{-/-}$: 1.3 ± 0.5 fold increase,; $^{*}p<0.03$) and the death receptor pathway associated ligand FasL to be significantly down-regulated (APP/PS1: 7.2 ± 1.1 fold increase vs. APP/PS1/CXCR3$^{-/-}$: 2.3 ± 0.7 increase; $^{*}p<0.02$) compared to APP/PS1 mice (Fig. 11 B).
Moreover, we detected slightly elevated levels of mRNA for CCL2, CCL5, IL-6 and IFN-γ without finding significant differences among APP/PS1 and APP/PS1/CXCR3$^{-/-}$ genotype relative to WT controls. In summary the presented data demonstrate that CXCR3-deficiency reduces the expression of distinct transcripts associated with microglial activation in APP/PS1 mice and suggests a possible alternative inflammatory milieu in APP/PS1/CXCR3-/- mice.

Fibrillar Aβ stimulates microglia to produce TNF-α and CXCL10, while TNF-α but not Aβ induces CXCL10 production by astrocytes.

To determine the cellular source of TNF-α and CXCL10 in response to fibrillar Aβ1-42 (Aβ), primary microglia and astrocytes were stimulated with Aβ and TNF-α. LPS stimulated cultures served as positive controls. We analyzed the supernatants of 4h, 12h and 20h stimulated cultures and detected microglial/astrocytic secreted TNF-α and CXCL10 via ELISA (schematic diagram, Fig. 12 A).
Aβ stimulation of primary microglia led to a significant induction of TNF-α after 12h (105.0 ± 14.0 pg/ml) and 20h (264.0 ± 4.0 pg/ml), compared to unstimulated controls (52.8 ± 20.4 pg/ml after 12h, 180.0 ± 4.0 pg/ml after 20h; $^{**}p < 0.005$, Fig. 12 B).

In contrast, primary astrocytes displayed only a minimal TNF-α production in response to Aβ stimulation at the examined time points (Aβ, 8h: 0.1 ± 0.1 pg/ml vs. control, 8h: 0.4 ± 0.2 pg/ml; Aβ,12h: 3.5 ± 0.1 pg/ml vs. control, 12h: 0.4 ± 0.2 pg/ml; Aβ, 20h: 4.2 ± 0.4 pg/ml vs. control, 20h: 1.9 ± 0.1 pg/ml; Fig. 12 C).

In addition, we determined the microglial synthesis of CXCL10 in response to Aβ. The detected levels of secreted CXCL10 were found significant higher to untreated controls over all 3 time points ($^{\star}p < 0.05$, $^{\star\star}p < 0.005$; Fig. 12 D). Moreover, Aβ induces CXCL10 as strong as LPS after 12h and 24h (LPS, 12h: 433.5 ± 89.5 pg/ml vs. Aβ, 12h: 390.0 ± 84.0 pg/ml; LPS, 24h: 409.5 ± 33.5 pg/ml vs. Aβ, 24h: 491.0 ± 91.0 pg/ml).

Furthermore, astrocytic CXCL10 production after Aβ or TNF-α stimulation were observed. In contrast to the robust induction of TNF-α by Aβ-stimulated microglia, we did not detect a relevant production of CXCL10 by Aβ-stimulated astrocytes. However, when stimulating primary astrocytes with TNF-α, we observed a stronger CXCL10 induction than observed by LPS stimulation (Fig.12 E, LPS, 12h: 2178.0 ± 13.0 pg/ml vs. Aβ, 12h: 2258.0 ± 15.0 pg/ml; LPS, 24h: 2316.0 ± 61.5 pg/ml vs. Aβ, 24h: 2739.0 ± 586.0 pg/ml).

Taken together, primary microglia are potent sources of TNF-α and CXCL10 after Aβ stimulation. Astrocytes are not directly responsive to Aβ, but can significantly contribute to the production of CXCL10 after being stimulated with TNF-α.

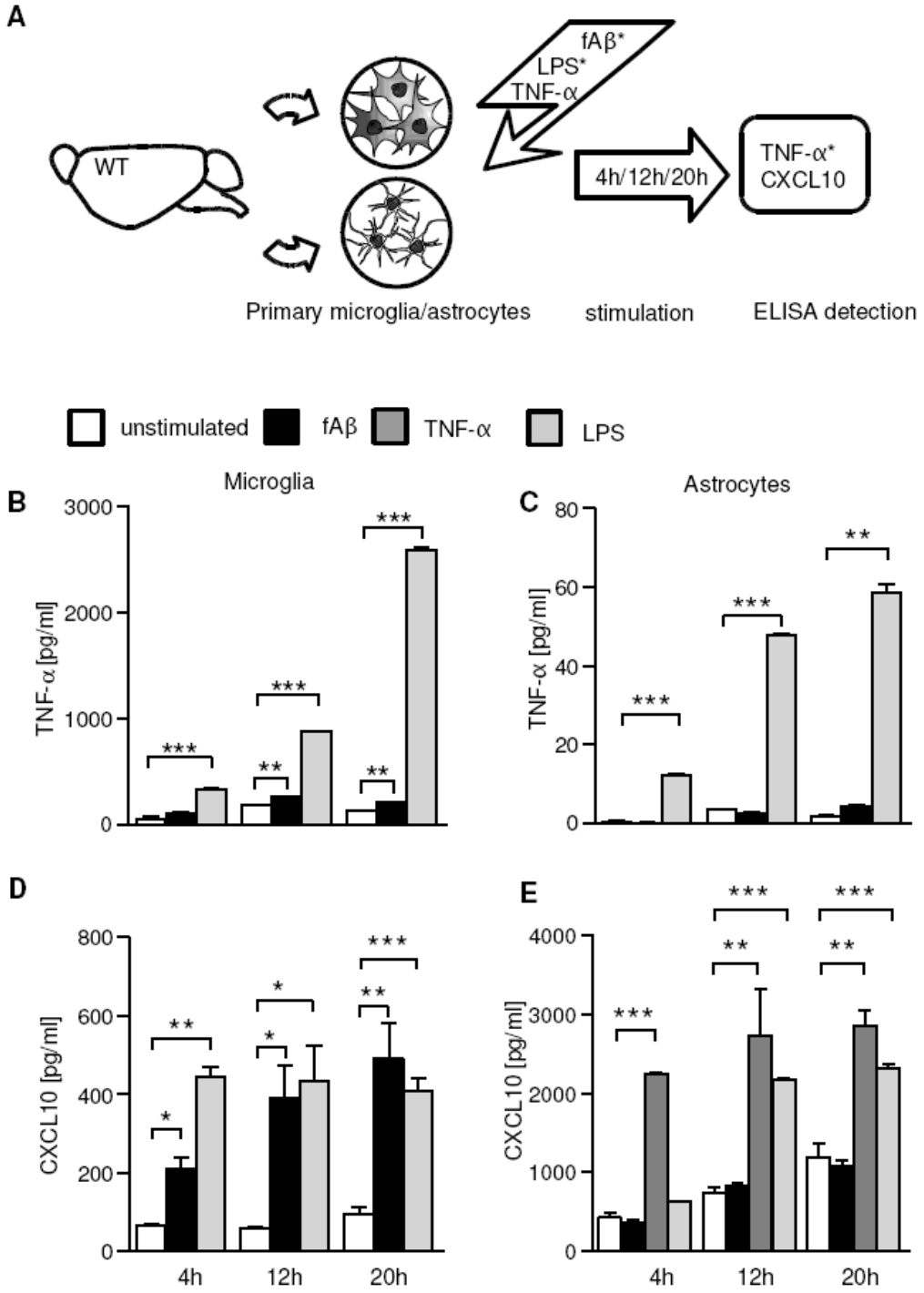


Figure 12: Effect of fibrillar Aβ on the induction of microglial and astrocytic TNF-α and CXCL10 secretion. Primary microglia and astrocytes were prepared from primary co-culture of newborn WT mice as described in Material and Methods. After stimulation with 0,7 μM of fibrillar Aβ or 100 ng/ml LPS (10 ng/ml TNF-α) for 4, 12 and 20h primary culture supernatants were analyzed for the level of secreted TNF-α and CXCL10 using ELISA (schematic diagram, A). A significant induction of TNF-α is demonstrated after 12h of fAβ stimulation of microglia culture (B), whereas no TNF-α induction could be determined in astrocytes after fAβ treatment over all analyzed timepoints (C). Microglial treatment with fAβ induced an equal strong secretion of CXCL10 after 12h and 20h like the LPS stimulus (D). Primary astrocytes did not respond to Aβ with enhanced CXCL10 secretion compared to nontreated controls across the time course. Contrary, administration of TNF-α strongly stimulates astrocytes to produce CXCL10 after 4h on (E, $\star p<0.05$, $\star\star p<0.01$, $\star\star\star p<0.001$, unpaired t-test; 2-3 experiments; mean±SEM).

CXCR3 signaling reduces the Aβ-phagocytosis of FAM-Aβ uptake of primary microglia but increases the production of TNF-α after FAM-Aβ treatment

To examine the impact of CXCR3 on microglial phagocytosis of fAβ, we quantified FAM-Aβ uptake in WT and CXCR3$^{-/-}$ primary microglia. Using quantitative imaging of FAM-Aβ^{+} particles in CD68^{+} microglia cells (Fig. 13 A) we found an over 30% higher capacity of CXCR3$^{-/-}$ microglia (area/cell = 60.7 ± 7.6 μm2) to uptake FAM-Aβ than WT microglia (area/cell = 82.1 ± 7.2 μm2, *$p < 0.05$, Fig. 13 B). To further elucidate if CXCR3 signaling is able to conversely reduce the phagocytic capacity of WT microglia, we pretreated the microglial cells with CXCL9 or CXCL10 and then examined the phagocytosis capacity for FAM-Aβ. Both CXCL9 and CXCL10 pretreatment reduced the phagocytic capacity of WT-microglia significantly (1.03 ± 0.04 of unstimulated control vs. 0.62 ± 0.13 of CXCL9 pretreatment and 0.52 ± 0.08 of CXCL10 pretreatment, normalized phagocytosis ± SEM; Fig. 13 B), whereas no effect was observed in pretreated microglia from CXCR3-deficient mice (1.47 ± 0.07 of unstimulated control vs. 1.39 ± 0.09 of CXCL9 pretreatment and 1.50 ± 0.06 of CXCL10 pretreatment, normalized phagocytosis ± SEM; Fig. 13 B). Preincubation with the CXCR3 antagonist before initiating the phagocytosis assay led to increased fAβ phagocytosis in CXCR3 at the level of CXCR3$^{-/-}$ microglia (Fig. 13 D).

To examine the influence of CXCR3 on the production of proinflammatory cytokines during Aβ-stimulation, we analyzed the culture supernatants from WT and CXCR3$^{-/-}$ microglia after the incubation with FAM-Aβ. The FAM-Aβ incubated WT cells were found to secret a 10-fold higher level of TNF-α (220.4 ± 10.5 pg/ml) than CXCR3-deficient cells (19.1 ± 1.7 pg/ml, ***$p < 0.0001$, Fig. 13 C). In addition, we detected a lower baseline level of TNF-α secretion in control CXCR3$^{-/-}$ (5.9 ± 3.8 pg/ml) compared to control WT cells (46.1 ± 14.4 pg/ml, Fig. 13 C).

Moreover, we found CXCR3 antagonist treated WT microglia to produce significantly lower level of TNF-α after fAβ stimulation (18.8 ± 5.5 pg/ml, 100 nM) than stimulation without the CXCR3 antagonist (32.7 ± 1.1 pg/ml, 100nM, Fig. 13 E).

In summary, CXCR3-deficiency results in an increased phagocytotic capacity of microglia and reduces the expression of TNF-α. Moreover, CXCL9/10-CXCR3 signaling can reduce the phagocytic ability in WT, but not in CXCR3$^{-/-}$ microglia.

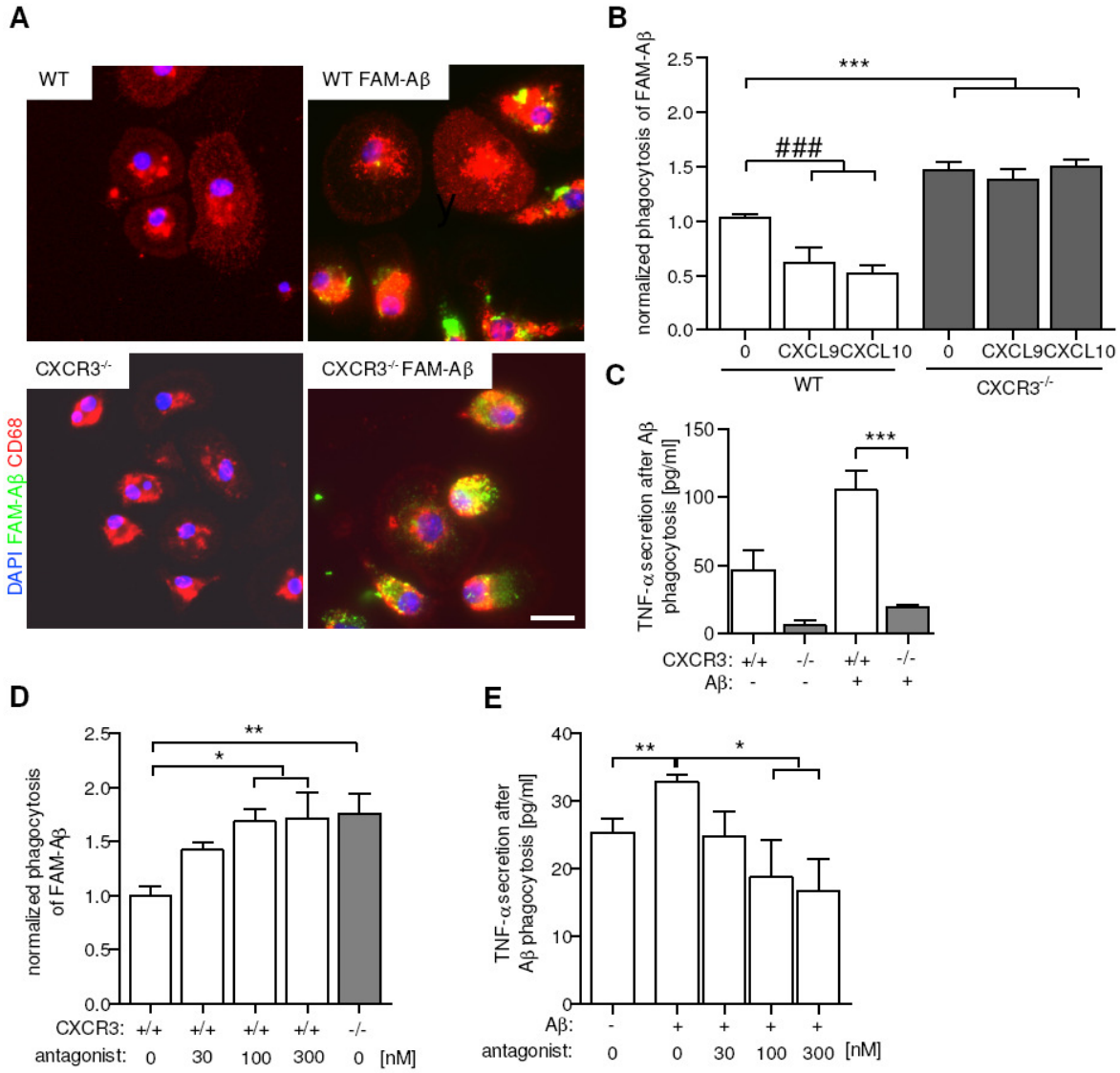


Figure 13: CXCR3 deficiency and CXCR3-antagonism enhances the microglial phagocytosis of FAMAβ and reduces the production of TNF-α in vitro. Primary WT and CXCR3-/- microglia were incubated with or without 0.7 µM FAM-Aβ for 1 h. After washing and fixation, cells were stained for CD68 and visualized using Alexa 594-conjugated secondary antibody (A, Scale bar=20 µm). A phagocytosis reader assay of WT and CXCR3-/- microglia revealed a significant increase of FAM-Aβ uptake in CXCR3-/- compared to WT microglia (B). mCXCL9 and mCXCL10 (each 250 ng/ml) treatment of WT and CXCR3-/- microglia significantly diminished phagocytosis in WT but not in CXCR3-/- cells (B, ###, ***$p<0.0005$, unpaired ttest; 3-5 individual experiments; mean±SEM).Detection of TNF-α concentration in the cell supernatants after phagocytosis reader assay revealed a significant reduction of TNF-α secretion in Aβ stimulated CXCR3-/- compared to WT cells (C, ***$p<0.005$; unpaired t-test; mean±SEM). Functionally blocking of CXCR3 significantly upregulated microglial phagocytosis at an equal level like observed in CXCR3-deficient microglia (D, CXCR3 antagonist ≥ 100nM, **$p<0.05$, *$p<0.02$, Tukey's Multiple Comparison Test; mean±SEM). Analysis of the TNF-α level in CXCR3-antagonist incubated WT microglia exhibit a reduction of TNF-α secretion below WT control level when stimulated with Aβ (E, **$p<0.01$, *$p<0.05$, Tukey's Multiple Comparison Test, mean±SEM).

Intracerebral fAβ injection reveals enhanced microglial phagocytosis and differences in clustering in CXCR3$^{-/-}$ mice compared to wild type controls

To evaluate in a second experimental approach whether loss of CXCR3 signaling leads to an enhanced phagocytosis of fAβ *in vivo*, fibrillar Aβ1-42 was injected into the brains of WT and CXCR3$^{-/-}$ animals (Fig. 14 A). First, quantification of microglia within an 80 μm radius proximal to the fibrillar Aβ1-42 injection site revealed a significant reduction of microglial accumulation 20% in CXCR3$^{-/-}$ animals compared to WT mice (WT Aβ: 18.3 ± 0.9 vs. CXCR3$^{-/-}$ Aβ: 14.0 ± 0.7; Fig. 14 B). In addition, sham injection did not show any significant difference in respect to the total number between WT and CXCR3$^{-/-}$ animals (WT TRIS: 11.6 ± 0.8 vs. CXCR3$^{-/-}$ TRIS: 11.0 ± 0.9; Fig. 14 B). Secondly, we detected a significantly increased amount of intracellular fAβ in CD68^{+} phagolysosomes from CXCR3$^{-/-}$ mice compared with WT mice. (WT Aβ: 50.2 ± 3.1 a. u. vs. CXCR3$^{-/-}$ Aβ: 65.1 ± 3.6 a. u.; Fig. 14 C, D). Notably, the microglial cells of CXCR3$^{-/-}$ animals exhibited a less ramified morphology with retracted extant branches and a more large rounded morphology with large Iba1 negative vacuoles (asterisks, Fig. 14 A). Further, these changes correlate with a significant larger size of CD68^{+} lysosoms in these cells. We measured the lysosomal perimeter of microglia in close proximity to the injected fAβ deposition and found a mean size difference of >30% in CXCR3$^{-/-}$ lysosoms compared with WT lysosoms (WT Aβ: 5.6 ± 0.1μm, n=717 vs. CXCR3$^{-/-}$ Aβ: 7.4 ± 0.2 μm, n=587; Fig. 14 E). Using a RGB fluorescence intensity profile we confirmed the intralysosomal location of fibrillar Aβ (Fig. 14 F).

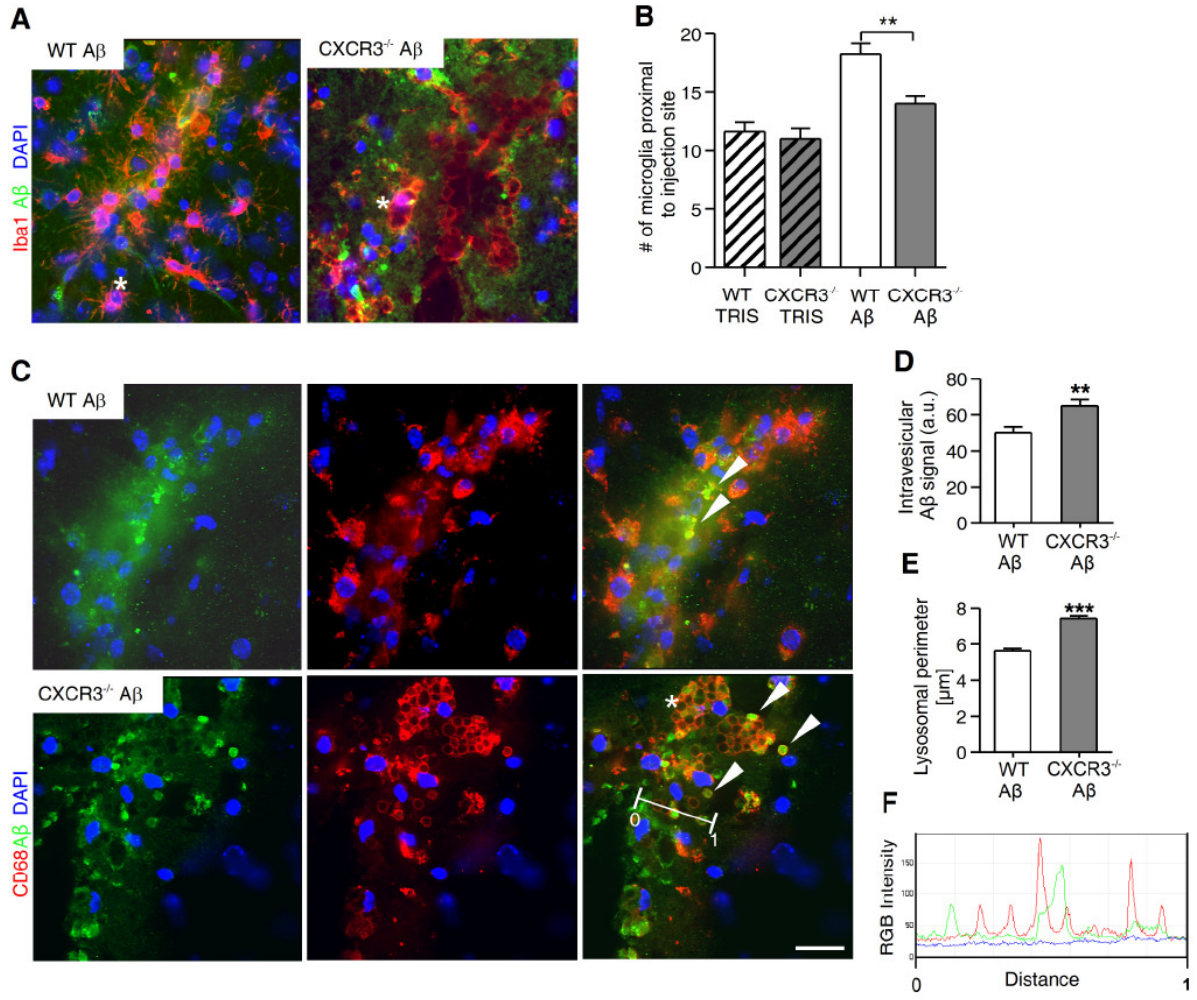


Figure 14: CXCR3-deficient mice show augmented microglial phagolysosomal uptake of fAβ1-42 but reduced microglial accumulation after intracerebral Aβ injection. 6-month-old CXCR3-/- (n=5) and WT (n=5) mice were injected with fibrillar Aβ1-42 (A, C) or with TRIS buffer. Horizontal brain sections (30 μm) containing the needle track were immunostained with antibodies against Iba1 and Aβ (A) or CD68 and Aβ (C) to localize and quantify microglia proximal to the Aβ injection site (B) or intralysosomal Aβ content (D). Quantification of Iba1+ microglia cells within an 80 μm radius of Aβ application center shows a significant higher number in WT mice than in CXCR3-/- (B). Using confocal microscopy we detected large CD68+ lysosoms (red) containing fAβ immunoreactive content (green) in CXCR3-/- rather than in WT (C, arrowheads, scale bar=20 μm). Analysis of microglial CD68+ lysosoms content in CXCR3-/- mice exhibit a significant increase in Aβ fluorescence compared to WT mice (D, **p<0.01, unpaired t-test, mean±SEM). Furthermore, we observed an increase of the mean lysosomal size distribution (expressed as perimeter) within CXCR3-/- microglia compared to WT (E, ***p<0.0001, mean±SEM, ≥580 lysosoms per genotype). Quantification of fluorescence intensity during confocal microscopy for CD68 (red stain and line) and Aβ (green stain and line) are shown next to the corresponding RGB image (C, F). Simplified dimensioning arrow (C, CXCR3-/-, 0-1) indicates area transected in line plot depictions (F). RGB intensity profile localizes peak Aβ (green) intermediate to peak CD68 (red) fluorescence intensity.

5.4 Discussion

The presented study provides insight into the functional role of the CXCR3 chemokine system on the progression of Alzheimer's-like pathology in the APP/PS1 AD-mouse model. The rationale for this experimental study was the recent detection of high levels of CXCL10 in cerebrospinal fluid and brain tissue from AD patients and the corresponding animal models, suggesting a role for this chemokine and its receptor CXCR3 in AD (Xia et al., 2000; Galimberti et al., 2003, 2006). Here we show a strong attenuation of Aβ plaque formation and significantly diminished Aβ-peptides in brain tissue from CXCR3-deficient APP/PS1 mice arguing the first time, for a critical role for CXCR3 in the generation of AD-like pathology in this model.

A growing number of studies implicate that Aβ-deposition in AD and AD-like models is modulated by the function and activation state of microglia and astrocytes (Wyss-Coray et al., 2003; Koistinaho et al., 2004; Farina et al., 2007; Fuller et al., 2010). As previously demonstrated, resident glial cells of the brain functionally express CXCR3, but its impact on AD progression is not clarified (Biber et al., 2002; Flynn, 2003; Rappert et al., 2004; de Jong et al., 2008b).

In APP/PS1 mice deficient for CXCR3, we found a strong modulation of microglial and astrocytic activation both by morphological and molecular criteria. This was independent from the local plaque load and was even observed around large Aβ-plaques in old animals, pointing towards a primary modulation of the glial response in APP/PS1/CXCR3$^{-/-}$ mice and not only less activated glia due to a generally reduced plaque load. Moreover and consistent with the findings in APP/PS1 model, we detected a distinct microglial phenotype and distribution pattern within Aβ injected CXCR3$^{-/-}$ mice. This demonstrates that CXCR3 is able to modulate the microglial state in the presence of Aβ which is in line with recent studies examining the role of CXCR3 in other models of neurodegeneration These studies demonstrated that absence of CXCR3 is associated with an attenuated microglial activation, reduced expression of inflammatory factors, constrained microglial recruitment (Rappert et al., 2004; de Jong et al., 2008b; Riemer et al., 2008; van Weering et al., 2011).

Concerning the mechanisms, which lead to a diminished Aβ load in CXCR3-deficient APP/PS1 mice, we hypothesized that CXCR3-deficient microglia might be more capable in phagocytosing Aβ in the APP/PS1 model, contributing to the observed plaque reduction in APP/PS1/CXCR3$^{-/-}$ mice. Strikingly, we could show that in vitro, CXCR3-deficient microglia

have a higher phagocytic capability for fAβ than WT microglia. Conversely, CXCR3 activation with CXCL9 or CXCL10 reduced the phagocytosis of fAβ by WT microglia but not by CXCR3-deficient microglial cells, providing evidence for a CXCR3-specific effect. In addition, treatment with a CXCR3 specific antagonist elevated phagocytosis in WT microglia. The observation of enhanced microglial fAβ phagocytosis and augmented lysosomal size in $CXCR3^{-/-}$ animals after intracerebral injection further demonstrate the impact of CXCR3 on microglial phagocytosis in our model: CXCR3 decreases the phagocytic capabilities for Aβ and thereby promotes the deposition of Aβ plaques in APP/PS1 mice.

We also detected a strong reduction of proinflammatory transcripts like TNF-α, IL-1β or FasL in CXCR3 deficient APP/PS1 mice. TNF-α and IL-1β are known to be upregulated in senile plaques and dystrophic neurites in AD (Amor et al., 2010) and in an AD mouse model (Ruan et al., 2009). TNF-α is thought to amplify brain inflammation and cognitive impairment in both AD patients and AD models (Ramos et al., 2006; Tobinick et al., 2006; McAlpine et al., 2009; Pardridge, 2010; Frankola et al., 2011). Disregulated apoptosis has been implicated in several neurodegenerative disorders including AD. The key apoptosis regulator FasL but also IL-1β are potentially involved in both neuronal and immune cell apoptosis in AD and AD models (Hofmann and Tschopp, 1995; Felderhoff-Mueser et al., 2000; Ethell and Buhler, 2003; Su et al., 2003; Shaftel et al., 2007; Pinteaux et al., 2009).

Therefore, the reduced levels of TNF-α, IL-1β and FasL molecules likely contribute to the alleviated phenotype in APP/PS1/CXCR3-/- mice.

Our in vitro Aβ stimulation of glial cells and numerous experimental studies (Klegeris et al., 1997; Gregersen et al., 2000; Lue et al., 2001; Hanisch, 2002) identify microglial cells as the main source for TNF-α. We found that CXCR3 signaling drastically increase the TNF-α production of microglia after fAβ-stimulation. This further corroborates that the attenuated disease course in CXCR3 deficiency is mediated by the modulation of microglial function.

We also confirmed the presence and a striking induction of the receptor and ligands of the CXCR3 chemokine system, whereas the induction of other chemokines possibly involved in the pathogenesis of AD, like CCL2 (Ishizuka et al., 1997) or CCL5 (Tripathy et al., 2010) were much less prominent in our model.

Because IFN-γ is a strong stimulator for the induction of CXCR3 ligands (Luster et al., 1985; Ferber et al., 1996; Cole et al., 1998), we examined the IFN-γ gene expression in our model but could not find any relevant differences between APP/PS1 and CXCR3-deficient APP/PS1

mice. Other factors like TNF-α and IL-1β, which are induced in our model can also induce CXCR3 ligands, in particular CXCL10 (Majumder et al., 1998; Choi et al., 2011). To further elucidate the factors, which induce CXCL10 in APP/PS1 mice, we stimulated glial cells in vitro with TNF-α and fAβ. Our data revealed that fAβ is a strong stimulus for both microglia and astrocytes to secrete CXCL10. Besides, TNF-α is also able to induce a CXCL10 secretion of astrocytes. In summary, this data suggests that fAβ is able to directly induce the production of CXCL10 by microglial cells and that proinflammatory cytokines, in particular microglial TNF-α, can further increase the astroglial production of CXCL10.

As neurons are also able to functionally express CXCR3 (Coughlan et al., 2000; Xia et al., 2000; Nelson and Gruol, 2004), these cells could principally contribute to the observed plaque reduction in APP/PS1/CXCR3$^{-/-}$ mice as well. We evaluated the impact of neuronal CXCR3 deficiency on the neuronal expression and processing of APPswe and found no significant differences in total APP and cleavage products (CTFs) levels in APP/PS1 and APP/PS1/CXCR3$^{-/-}$ brain tissue, arguing against a relevant impact of neuronal CXCR3 on the observed phenotypical changes.

In summary, the presented data demonstrates for the first time the functional importance of the CXCR3 chemokine system during the course of AD-like pathology. We conclude that the direct and indirect induction of CXCL10 by fAβ and the subsequent activation of the CXCR3 chemokine system are able to modulate the activation state of glial cells. CXCR3 activation reduces the phagocytotic capability of microglia for fAβ, which ultimately promotes plaque formation in the APP/PS1 model. CXCR3 has a key role in the progression of the AD-like pathology in the APP/PS1 model and should therefore be considered as a potential novel therapeutic target in AD.

6. Conclusion

The presented studies further support the important function of the CXCR3 chemokine system in neuroinflammatory and neurodegenerative disease models.

In the Th1/IFNγ driven GF-IL12 model, CXCR3 displays very diverse functions. On the one hand, CXCR3 strongly promotes the accumulation of tissue damaging immune cells in the cerebellum but on the other hand also prevents ocular inflammation. These findings underline that CXCR3 can have a major impact on the recruitment and activation of immune cells that is very likely milieu-dependent. CXCR3 can cause but also prevent inflammation of specific regions of the CNS.

In contrast to the GF-IL12 model, the secondly applied APP/PS1 model is characterized by a chronic glial activation with induction of cytokines like TNF-α and the absence of infiltrating immune cells. Here we found, that CXCR3 signaling critically controls accumulation, clustering and activation of microglia. Ultimately, CXCR3 enhances the Alzheimer-like pathology in APP/PS1 mice, providing for the first time evidence that this chemokine system is involved, not only in severe neuroinflammation, but also in chronic neurodegeneration.

In both models, CXCR3 not only orchestrates the distribution of resident and infiltrating immune cells, but also changes the local inflammatory milieu by modulating the cytokine patterns. In particular, CXCR3 changes the levels of proinflammatory cytokines like IFN-γ and TNF-α, which further intervenes with the course of the diseases. As demonstrated this effect directly or indirectly is also able to modulate cellular property like microglial phagocytosis.

In conclusion, CXCR3 is a key molecule in neuroinflammatory and neurodegenerative disease models. It modulates not only the distribution and activation of infiltrating cells, but also of resident immune cells. Therefore the development of therapeutic approaches, addressing the function of CXCR3 is a reasonable and an exciting challenge for the future. However, with respect to the unexpected ocular inflammation observed in GF-IL12/CXCR3KO mice, a CXCR3 specific treatment requires caution, as this receptor can mediate both beneficial but also harmful effects during the course of CNS diseases.

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8. Abbreviations

°C	Degree Celsius
µ	Micro (as preset to SI-units, 10^{-6})
Ab	Antibody
AD	Alzheimer's disease
APC	Antigen presenting cell
APC (FACS)	Allophycocyanin
APC-eFluor (FACS)	Allophycocyanin coupled to eFluor®
APP	Amyloid precursor protein
APP/PS1	Transgene containing mutant APPsw and PS1dE9
APS	Ammonium persulfate
AS	Antisense
Aβ	Amyloid beta
B220	B cell isoform of 220 kDa
BBB	Blood brain barrier
BCA	Bicinchoninic acid
bp	Base pair
BSA	Bovine serum albumin
C57BL/6	C57 black 6, inbred strain of laboratory mice
CCL	Chemokine ligand with two near amino-terminal cysteines
CCR	Receptor for CC-chemokines
CD	Cluster of differentiation
cDNA	Complementary DNA
CHO	Chinese hamster ovary, cell line
CNS	Central nervous system
CREB	Cyclic adenosine monophosphate response element-binding
cRNA	RNA derived from cDNA through standard RNA synthesis
CSF	Cerebrospinal fluid
CTL	Cytolytic T lymphocytes
CX_3CL1	Type of chemokine ligand with three near amino-terminal cysteines, separated by one amino acid, represented in this name with an "X",
CX_3CR1	Receptor for CX_3CL1
CXCL	Type of chemokine ligand with two near amino-terminal cysteines, separated by one amino acid, represented in this name with an "X",
CXCR	Receptor for CXC-chemokines
CXCR3KO	CXCR3-deficient
Da	Dalton
DAPI	4'-6-Diamidino-2-phenylindole
DARC	Duffy antigene receptor for chemokines

DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELR	Amino acid motif of glutamic acid-leucine-arginine
Fa.	Company
FACS	Fluorescence-activated cell sorting
FAM-Aβ	Fluorescent (FAM)-labeled β- amyloid 1-42
FasL	Fas ligand
fAβ	Fibrillar Aβ1-42
Fig.	Figure
FITC	Fluorescein isothiocyanate
FLIPR	fluorometric imaging plate reader, Ca flux assay
FMCM	Fatal murine cerebral malaria
FoxP3	Forkhead box P3
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GF-IL12	Transgene containing both subunits of the IL-12 heterodimer (p35/p40) under the transcriptional control of the astrocyte-specific GFAP promoter
GF-IL12/CXCR3KO	CXCR3-deficient GF-IL12 mice
GPCR	G-protein coupled cell-surface receptor
GRK	G-protein coupled receptor kinases
h	Hour(s)
H&E	Haematoxylin and eosin
HBSS	Hank's buffered salt solution
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromotography
HRP	Horseradish peroxidase enzyme
HSV	Herpes simplex virus
Iba1	Ionized calcium binding adaptor molecule 1
IC50	half maximal inhibitory concentration
IFN	Interferon
IHC	Immunohistochemie
IL	Interleukin
IL12rb1	Interleukin 12 receptor, beta 1
ISH	In situ Hybridization
JHMV	JHM strain of mouse hepatitis virus

l	Litre
Lam	Laminin
LCMV	Lymphocytic choriomeningitis
LFB	Luxol fast blue
LPS	Lipopolysaccharide
Ly6G	Granulocyte differentiation antigen 1
LYVE1	Lymphatic vessel endothelial hyaluronan receptor
m	Milli (as preset to SI-units, 10^{-3})
M	Mole
MAPK	Mitogen-activated protein kinases
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
min	Minute(s)
MMP	Matrix metallopeptidase
mRNA	Messenger Ribonucleic Acid
MS	Multiple sclerosis
n	Nano (as preset to SI-units, 10^{-9})
NaCl	Sodium chloride
NaDOC	Sodium deoxycholate
NaF	Sodium fluoride
NK	Natural killer
NP-40	Nonyl phenoxypolyethoxylethanol
NuPAGE	Polyacrylamide gel electrophoresis
ON	Over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Tandem fluorochrome composed of phycoerythrin coupled to the cyanine dye Cy™7
PerCP-Cy5.5	Tandem fluorochrome composed of peridinin chlorophyll protein coupled to the cyanine dye Cy™5.5
PFA	Paraformaldehyde
PGE	Prostaglandin E
PK	Proteinase K
PMG	Primary microglia
PMSF	Phenylmethanesulfonylfluoride
PS1	Presenilin1
PS2	Presenilin2
qRT-PCR	Quantitative real time polymerase chain reaction
RAG2	Recombination activating gene 2
rATP	Ribonucleotide, adenosine triphosphate
rCTP	Ribonucleotide, cytidine triphosphate
rGTP	Ribonucleotide, guanosine triphosphate
RIPA	Radioimmunoprecipitation assay

RLB	Radiolabeling binding assay
RNA	Ribonucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RNasin	Ribonuclease inhibitor
ROI	Region of interest
RPA	RNase protection assay
RPL32-4A	60S ribosomal protein L32
rpm	Rounds per minute
RT	Room temperature
S	Sense
SDF-1	Stromal cell-derived factor-1
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
SEM	Standard error of the mean
SSPE	Buffer solution containing sodium chlorid, sodium hydrogen phosphate and EDTA
STAT4	Signal transducer and activator of transcription 4
StAV488	Streptavidine, Alexa Fluor 488™ conjugated
StAV594	Streptavidine, Alexa Fluor 594™ conjugated
TBE	Buffer solution containing Tris base, boric acid and EDTA
TBST	Tris-buffered saline and Tween 20
TE	Tris-EDTA-buffer
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
TGF-β	Tumor growth factor beta
Th0	T helper 0
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
ThioS	Thioflavin-S
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tris	2-Amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	Transfer RNA
U	Units
V	Volt(s)
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
vs.	versus
w/v	Weight per volume
wks	Weeks
WNV	West nile virus

WT	Wild-type

9. List of figures

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Publikationen

Krauthausen M, Kummer MP, Zimmermann J, Terwel D, Heneka MT, Müller M: The chemokine receptor CXCR3 is critical for the Alzheimer-like plaque formation in transgenic APP/PS1 mice. Manuskript bei "The Journal of Clinical Investigation" eingereicht.

Zimmermann J, **Krauthausen M**, Hofer MJ, Heneka MT, Campbell IL, Müller M: CNS-targeted production of IL-17A induces glial activation, microvascular pathology and mediates synergistic effects in neuroinflammation without inducing severe tissue damage. Manuskript in Vorbereitung.

Krauthausen M, Zimmermann J, Conrad C, Campbell IL, Heneka MT, Müller M: Different effects of IFN-beta on IL-6 and IL-12 driven CNS-inflammation. Manuscript in Vorbereitung.

Krauthausen M, Carter S, Zimmermann J, Sarris M, Wakefield D, Heneka MT, Campbell IL, Müller M: Opposing roles for CXCR3 signaling in CNS versus ocular inflammation mediated by the astrocyte-targeted production of IL-12: Am J Pathol. 2011 Nov;179(5):2346-59. Epub 2011 Sep 15.
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Kongressbeiträge

Poster:

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Vorträge:

M. Krauthausen
Neurowoche 2010, Mannheim, 21.-25.09.10
The chemokine receptor CXCR3 is critical for the Alzheimer-like plaque formation in APP/PS1 mice
Neurol 2010; V333

M. Krauthausen
1. NEUROWIND meeting, Berlin/Motzen, 30.10.-01.11.09
CXCR3-deficieny in APP/PS1 transgenic mice
Report on the 1st scientific meeting of the "Verein zur Förderung des Wissenschaftlichen Nachwuchses in der Neurologie" (NEUROWIND e.V.) held in Motzen, Germany, Oct. 30th - Nov. 1st, 2009. ExpTransl Stroke Med. 2010, 2:7

M. Krauthausen
2. NEUROWIND meeting, Berlin/Motzen, 29.10.-31.10.10
Astrocytic overexpression of CXCL10 and CXCL9 in APP/PS1 transgenic mice
Report on the 2nd scientific meeting of the „Verein zur Förderung des Wissenschaftlichen Nachwuchses in der Neurologie" (NEUROWIND e.V.) held in Motzen, Germany, Oct. 29'th – Oct. 31'st, 2010. Exp Transl Stroke Med. 2011, 3(1):3

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Krauthausen M, Carter S, Zimmermann J, Sarris M, Wakefield D, Heneka MT, Campbell IL, Müller M: Opposing roles for CXCR3 signaling in CNS versus ocular inflammation mediated by the astrocyte-targeted production of IL-12: Am J Pathol. 2011 Nov;179(5):2346-59. Epub 2011 Sep 15 http://dx.doi.org/10.1016/j.ajpath.2011.07.041

Eingereicht bei "The Journal of Clinical investigation": Krauthausen M, Kummer MP, Terwel D, Zimmermann J, Bulic B, Heneka MT, Müller M: The chemokine receptor CXCR3 is critical for the Alzheimer-like plaque formation in transgenic APP/PS1 mice.

Ich habe früher noch keinen Promotionsversuch unternommen.

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Bonn, September 2012

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Marius Krauthausen