UNIVERSITAT ROVIRA i VIRGILI

Role of resident microglia in lymphoid neogenesis after rodent cerebral ischemia

BACHELOR'S THESIS

Degree in Biotechnology

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Table of contents

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Charité Universitätsmedizin Berlin

Charité Universitätsmedizin Berlin is one of the largest university hospitals in Europe. This medical ensemble is distributed in four university campuses - Charité Mitte, Virchow-Klinikum, Benjamin Franklin and Berlin Bunch-, all located in Berlin (Charité - Universitätsmedizin Berlin, 2019).

As a hospital, Charité, founded more than 300 years ago, has a long trajectory and experience and its activity has been expanding to also develop academic and research tasks. Since 2003, Charité has also acted as a joint School of Medicine of Humbold and Freie Universities, both located in Berlin. Charité Universitätsmedizin Berlin offers different undergraduate, masters, and doctoral programs in health sciences (Charité - Universitätsmedizin Berlin, 2019).

The institution is actively involved in biomedical research. Multiple research projects are carried out both in their own facilities and in the different institutions and associated centers. One of them is the Center for Stroke Research Berlin (CSB), founded in 2008 as an integrated center for research and treatment of cerebrovascular accidents. Its mission is to decrease the mortality of the patients, as well as to improve the quality of life of those who survive. The direct contact between patients and the scientific advances developed in the CSB laboratories leads to a faster development of clinical trials and allows to accelerate the implementation of new treatments and therapeutic strategies (Center for Stroke Research Berlin, 2019).

"The role of myeloid cells in autoreactive B cell responses after experimental stroke" is a collaborative project between the Department of Experimental Neurology at Charité Universitätsmedizin Berlin and the CSB. This thesis is based on the results obtained during my internship (January-May 2019) as a part of this research project.

Abstract

In certain inflammatory responses, it has been observed that lymphocytic infiltrates cluster in organized assemblies that can remain in the tissue for a long time. There is emerging evidence that these lymphoid follicles contribute to the maintenance of chronic inflammation through a sustained autoimmune response over time. Lymphoid neogenesis, the process of formation of these compartmentalized aggregates, is thought to be driven by lymphoid chemokines. However, the cell types involved after an acute cerebral ischemia remains unknown, which made us hypothesize about the role or resident microglia.

Cx3cr1.FiDTR was used to deplete microglia at day 11 post-stroke. Mice underwent a middle cerebral artery occlusion during 60 minutes and samples were extracted 14 days after the surgery. Cx3cr1.FiDTR was found to be a consistent mouse model to study microglia functions after stroke. Nonetheless, a longer extent of the depletion would enable better research on long-term effects of this cellular subset.

Immunohistological and expression analyses showed that depletion of resident microglia leads to an inhibition of lymphocytic accumulation in the brain 14 days after stroke. Cx3cr1.FiDTR mice also presented an altered cytokine expression profile after stroke, when compared to our previous data obtained in wild-type and 2D2 mice. We observed lower mRNA levels of the lymphoid chemokines *Cxcl13, Cxcl12,* and *Ccl19*. *Ltβ* and *Cxcr5* genes presented a not significant tendency towards a decreased expression. Regarding to other myeloid populations, microglia depletion did not appear to change the recruitment of infiltrating monocytes and monocyte-derived macrophages.

Therefore, it can be concluded that resident microglia is essentially needed in lymphoid neogenesis and its actively involved in the molecular changes triggered after cerebral ischemia in mice.

Keywords: lymphoid follicles, lymphoid neogenesis, ischemia, microglia, Cx3cr1

1. Introduction

1.1. Chronic inflammation: from protection to pathogenesis

The rapid development of an inflammatory response is a crucial survival mechanism. An effective activation of the immune system allows not only the elimination of the antigen, but it also involves other relevant functions, such as restoration of physiological tissue activity or generation of immunological memory. However, unavoidable damage is also caused at sites of inflammation. For this reason, protective effects of inflammation are highly time-dependent, which underlines the importance of an accurate resolution process (Buckley *et al.*, 2015; Nasef, Mehta and Ferguson, 2017).

Sometimes, inflammatory responses are triggered by unresolved infections, self- or tumor antigens, which prevents the pathogenic origin from being completely eradicated. Consequently, activation of immune system is maintained over time, leading to sever pathological problems that cause the destruction of the affected tissue and, thus, a degeneration of physiological activities (Pitzalis *et al.*, 2014; Nasef, Mehta and Ferguson, 2017).

In the past decades, inflammation has been suggested to be the underlying cause of the chronicity of a myriad of diseases with a wide range different symptoms and etiologies, from rheumatoid arthritis (Buckley *et al.*, 2015; Bombardieri, Lewis and Pitzalis, 2017) to cancer (Korniluk *et al.*, 2017). Thus, controlling the dual role of inflammation has become a major biomedical challenge.

Heterogeneity among diseases and the lack of in-depth knowledge of the initial cause hinders a permanent cure for chronic inflammation. More research on the molecular mechanisms that lead to chronic states is needed, which would enable the development of therapeutic strategies to prevent chronicity and ensure a better outcome for the patients.

1.2. Ectopic lymphoid-like structures in maintenance of chronic inflammation

In certain chronic diseases, it has been observed that lymphocytic infiltrates cluster in organized assemblies that can remain in the inflamed tissue for a long time. Ectopic lymphoid-like structures (ELSs) have been found in many diseases, such as chronic allograft rejection or multiple sclerosis, all of them with chronic inflammation as a common factor (Pitzalis *et al.*, 2014; Buckley *et al.*, 2015).

ELSs, also named tertiary lymphoid organs (TLOs), are composed by a B-cell core surrounded by T cells, with some infiltrated follicular dendritic cells. High endothelial venules are often found around the follicles, enhancing the infiltration of immune cells (Figure 1). Among other functions, ELSs are thought to locally produce antibodies. Therefore, ELSs might be directly involved in the maintenance of inflammatory responses through the enhancement of a sustained autoimmune response over time (Pitzalis *et al.*, 2014; Buckley *et al.*, 2015).

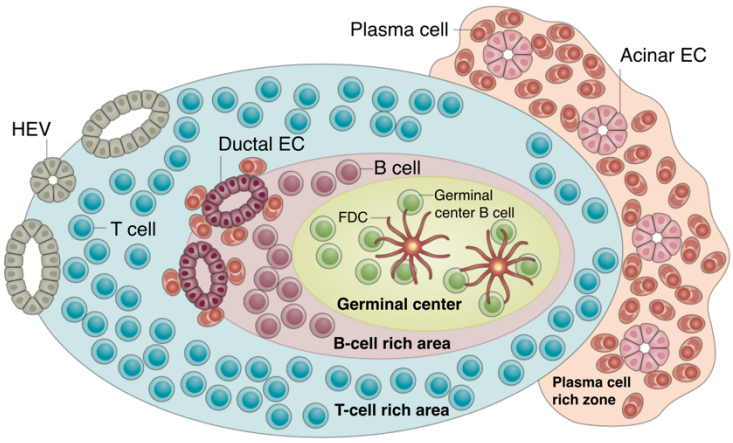

Figure 1. Model of cellular distribution of ectopic lymphoid-like structures. EC, epithelial cell; FDC, follicular dendritic cell; HEV, high endothelial venules (Adapted from Bombardieri, Lewis and Pitzalis, 2017).

However, it must not be forgotten that there are still a lot of uncertainties about ELS and their functions still need to be further investigated. Little is known about the actual incidence of these structures in many diseases and their correlation with the outcome of the patients. there is increasing evidence attributing a key role to homeostatic chemokines for lymphocytic recruitment and compartmentalization, but the cellular types inducing the formation of ELS, known as lymphoid neogenesis, remain unclear.

1.3. Autoimmune B-cell responses and ELSs in ischemic stroke

Cerebral ischemia is one of the lesions in which the presence of ELSs is suggested (Doyle and Buckwalter, 2017; Prinz and Priller, 2017). It is known that inflammatory responses are triggered after stroke, leading to the activation of microglia and the recruitment peripheral immune cells at the lesion site (Chamorro *et al.*, 2012). Recent research provided experimental demonstration that B- and T-lymphocytes remain in the rodent brain for at least 12 weeks in compartmentalized aggregates that forms around 2 weeks after stroke (Doyle *et al.*, 2015).

Neuroinflammation following cerebral ischemia elicits to a complex interaction between central nervous system (CNS) and the immune system. This interplay could contribute to the onset of the cognitive impairment seen after acute ischemia. Indeed, it is estimated that more than 30% of stroke patients will suffer from dementia, which means twice the risk compared to the average population. The molecular mechanisms that cause cognitive decline are currently unknown, but autoimmune B-cell responses might be a potential factor. Detrimental effects of CNS-specific antigens have been proved in other neuroinflammatory disorders, such as spinal cord injury and Alzheimer's disease (Doyle and Buckwalter, 2017).

Samples of cerebrospinal fluid of stroke patients present brain-specific antibodies, proving an intrathecal synthesis (Doyle *et al.*, 2015; Doyle and Buckwalter, 2017), which could take place in ELSs. This suggests that the inflammatory response following ischemia can become chronic due to autoimmune reactions. Therefore, ELSs might be the main mechanism by which detrimental long-term consequences occur.

1.4. Resident microglia in ischemic stroke

Microglia has been proved responsible of beneficial and detrimental effects after stroke, which vary depending on the severity and stage of ischemic lesion, age or surrounding molecular components. The reason why microglia exerts ambivalent functions might be explained by its polarization in different phenotypes after activation, action driven by cytokines (Ma *et al.*, 2017).

There are two main phenotypes known to be associated with activated microglia, and both of them have been detected in ischemic brain. The M1 phenotype enhances the inflammatory response, through an increase in the levels of ROS, nitric oxide, proteases, and pro-inflammatory cytokine production. In contrast, M2 microglia has a role in suppressing inflammation and enhancing the survival of neurons and brain tissue regeneration (Benakis *et al.*, 2015; Ma *et al.*, 2017).

As a response to stroke, rodent microglia acquire a M2 phenotype as soon as one day after the ischemic event with a peak at day 7, whereas the M1 phenotype is not observed until day 3 in mouse models (Ma *et al.*, 2017). Interestingly, the highest levels of M1 phenotype, associated with the detrimental functions of microglia, are detected at day 14 after ischemia (Ma *et al.*, 2017), which correlates with ELSs formation (Pitzalis *et al.*, 2014).

This knowledge supports the hypothesis that microglia could be the main source of cytokines in lymphoid neogenesis. Moreover, neurological impairments have also been associated with microglia dysfunction in rodents (Ma *et al.*, 2017). Therefore, microglia might actively contribute to the pathological chronic inflammation observed in ischemic patients, causing an increasing risk to develop neurodegenerative processes, such as dementia.

1.5. Genetic approaches to deplete microglia: Cx3cr1$^{Cre/ERT2}$-FiDTR mice

Research on individual function of the different subsets of CNS myeloid cells has been challenging for a long time. Common molecular markers and similar morphology kept impeding the differently target infiltrating monocytes-derived macrophages and resident microglia. Both cellular types are the two most common myeloid cells after an acute stroke.

Advances on genetic strategies first allowed ablation of $CD4^+$ T cells (Buch *et al.*, 2005) and dendritic cells (Okuyama *et al.*, 2010) in mice using Cre-loxP recombination in combination with diphtheria toxin receptor (DTR). This novel mouse model proved that lineage-specific depletion was feasible in the CNS. This strategy has been recently used to deplete microglia cells targeting C-X3-C Motif Chemokine Receptor 1 (CX3CR1), expressed in resident microglia and infiltrating inflammatory monocytes (Bruttger *et al.*, 2015).

Considering the current genetic tools, Cx3cr1$^{Cre/ERT2}$-FiDTR mice transgenic mice was found to be the most promising mouse model to evaluate the microglia functions in homeostatic and pathological states. This animal model provides a novel approach to deplete resident microglia using Cre-recombinase technology. It was proved that after three days of treatment, 80% of depletion is achieved with this molecular strategy. However, resident microglia is able to re-populate the affected area within 7 days (Bruttger *et al.*, 2015).

The vector Cx3cr1-CreERT2 uses *Cx3cr1* gene expression to selectively produce Cre-ERT2 fusion proteinin myeloid cells. Cre enzyme is fused to the estrogen receptor ERT2. In order to function, Cre-recombinase needs to be located in the nucleus. The fusion with ERT2 prevents Cre-recombinase to get into the nucleus without tamoxifen, which binds to ERT2. Hence, only in the presence of tamoxifen, Cre-recombinase can enter the nucleus and perform the recombination. This recombination will eliminate the STOP codon from the ROSA26 locus and thus allow the expression of DTR. Therefore, *Cre* would only be expressed in myeloid cells of mice previously treated with tamoxifen (Figure 2a). Mice were heterozygous for Cx3cr1-CreERT2 to avoid interference with homeostatic functions of CX3CR1 (Bruttger *et al.*, 2015).

Simultaneously, R26-iDTR vector induces expression of a monkey diphtheria toxin receptor (Hbegf) upon Cre-mediated excision of a STOP cassette. The vector is inserted into ROSA26 locus, which is known to be constitutively expressed in all cellular subsets. However, diphtheria toxin receptor would only be found on the surface of cells where Cre has been previously expressed, which are myeloid cells (Figure 2b). Mice were homozygous for R26-iDTR insert to ensure greater efficacy of diphtheria toxin (DT) effects (Bruttger *et al.*, 2015).

In order to selectively deplete microglia, we took advantage of the different turnover rates of myeloid cell populations. Infiltrating monocytes have a shorter lifespan than resident microglia. Thus, 4 weeks after tamoxifen treatment, only microglia cells will remain sensitive to DT (Figure 2c) (Bruttger *et al.*, 2015).

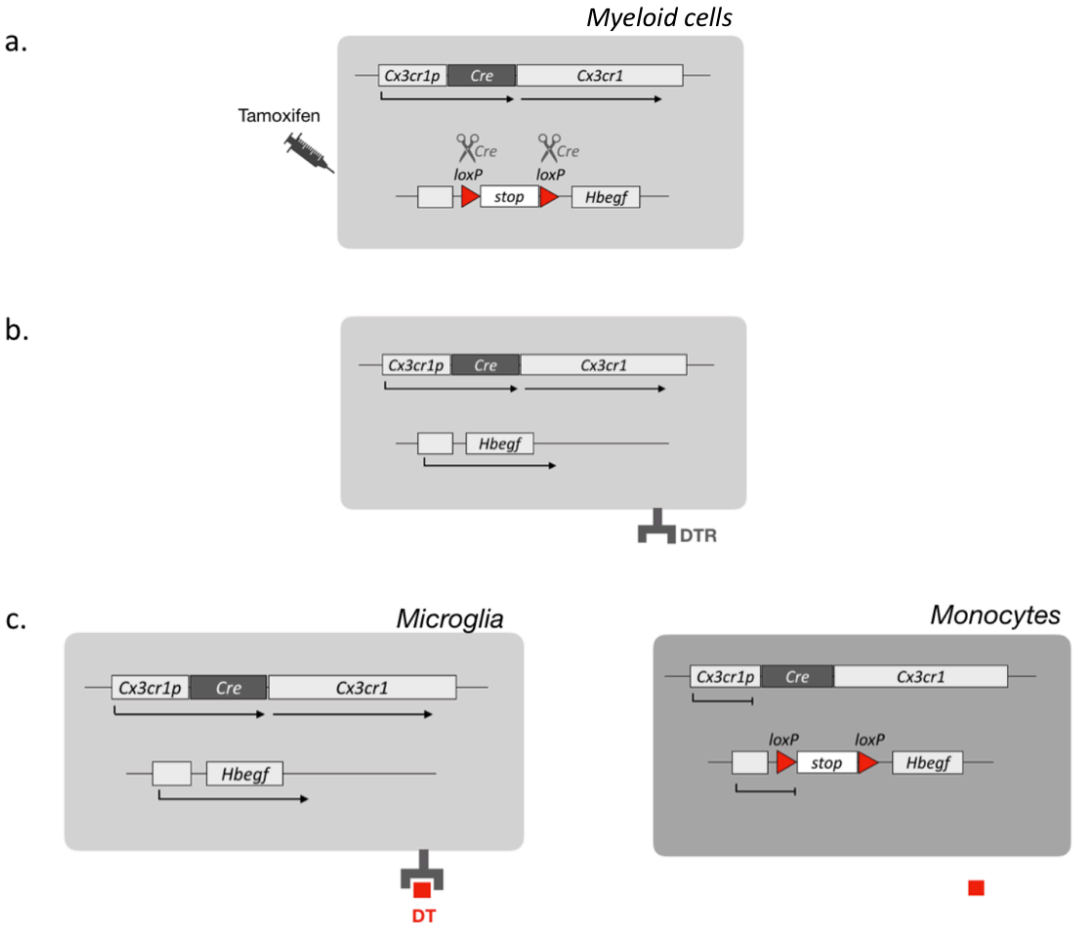

Figure 2. Molecular microglia depletion mechanism in Cx3cr1$^{Cre/ERT2}$-FiDTR mice. Schematic representation of the vectors can be seen in the picture, only parts which were relevant to the explanation have been represented. **a**. Tamoxifen treatment lead to the expression of Cre-recombinase in myeloid cells. **b**. DTR is expressed on myeloid cells after excision of loxP-flanked stop cassette. **c.** After a certain time from tamoxifen treatment, DTR-expressing monocytes will be replaced and only microglia cells, which have a longer lifespan, will be depleted by diphtheria toxin. Hbegf, Heparin binding EGF-like growth factor which encodes for diphtheria toxin receptor; DTR, diphtheria toxin receptor; DT, diphtheria toxin (Schematic representation designed from the information in Buch et al., 2005; Okuyama et al., 2010 and Bruttger et al., 2015.)

2. Hypothesis and objectives

Our main hypothesis was that resident microglia is essentially needed for ELSs development in acute stroke in mice. Consequently, depletion of microglia would hamper lymphocytic infiltration and/or follicle organization. For this reason, the aim of this project was to characterize the involvement of microglia in the development of lymphoid follicles after a cerebral ischemia in mice.

Therefore, our particular objectives were:

- To confirm whether Cx3cr1$^{Cre/ERT2}$-FiDTR mouse model is a valid rodent model for the study of resident microglia.
- To determine whether lymphocytes infiltrate into the ischemic tissue and form ELSs without the contribution of resident microglia using the mouse model Cx3cr1$^{Cre/ERT2}$-FiDTR after an experimental transient acute cerebral ischemia.
- To define expression changes on relevant genes related with ELSs formation after microglia depletion.

3. Materials and methods

3.1. Animal model: Cx3cr1.FiDTR mice

To evaluate the role resident microglia in the formation of ELS, nine 10- to 14-weeks-old male Cx3cr1$^{Cre/ERT2}$-FiDTR transgenic mice (henceforth referred as Cx3cr1.FiDTR) on a C57Bl/6J genetic background were used. Possible tamoxifen or DT unexpected effects were considered. Thus, two mice of the nine initial mice had a wild-type genotype for Cx3Cr1-CreERT2 or ROSA26-iDTR vectors and were used as controls (tamoxifen and DT were applied likewise). In addition, ten WT were used as a second control. Experimental groups are detailed in Table 1. All mice were bred and housed at Charité institution under specific-pathogen-free conditions.

Prior to the experiments, mice followed a one-week acclimatization. They were group housed, fed with standard chow diet, no restriction to water and kept under a 12 hours dark/light cycle. Conditions were maintained during following experiments. Once used to the laboratory, Cx3cr1.FiDTR mice were treated with tamoxifen to trigger DTR expression in myeloid cells (Figure 3). After four weeks, all mice (including WT mice) underwent middle cerebral artery occlusion (MCAo) surgery for 60 minutes to induce brain ischemia. One day after stroke, magnetic resonance imaging was performed to measure brain injury. At days 11, 12 and 13 post-stroke, DT was injected to selectively deplete microglia on Cx3cr1.FiDTR mice. On day 14, samples were extracted and immunohistochemical and gene expression analyses, were carried out as explained in the following sections.

Table 1. *Experimental groups and number of animals in each group.*

	Histopathology	*Expression analyses*	
		Pax5, Cd3e, Lta, Ltb, Cxcl13, Cxcr5, Cxcl12, and Ccl19 genes	*Ccr2, Ly6c, and Iba-1 genes*
Control	▪ 5 WT mice	▪ 5 WT mice ▪ 2 control Cx3cr1.FiDTR mice	▪ 2 control Cx3cr1.FiDTR mice
Test sample	▪ 3 Microglia-depleted Cx3cr1.FiDTR mice	▪ 4 Microglia-depleted Cx3cr1.FiDTR mice	▪ 4 Microglia-depleted Cx3cr1.FiDTR mice

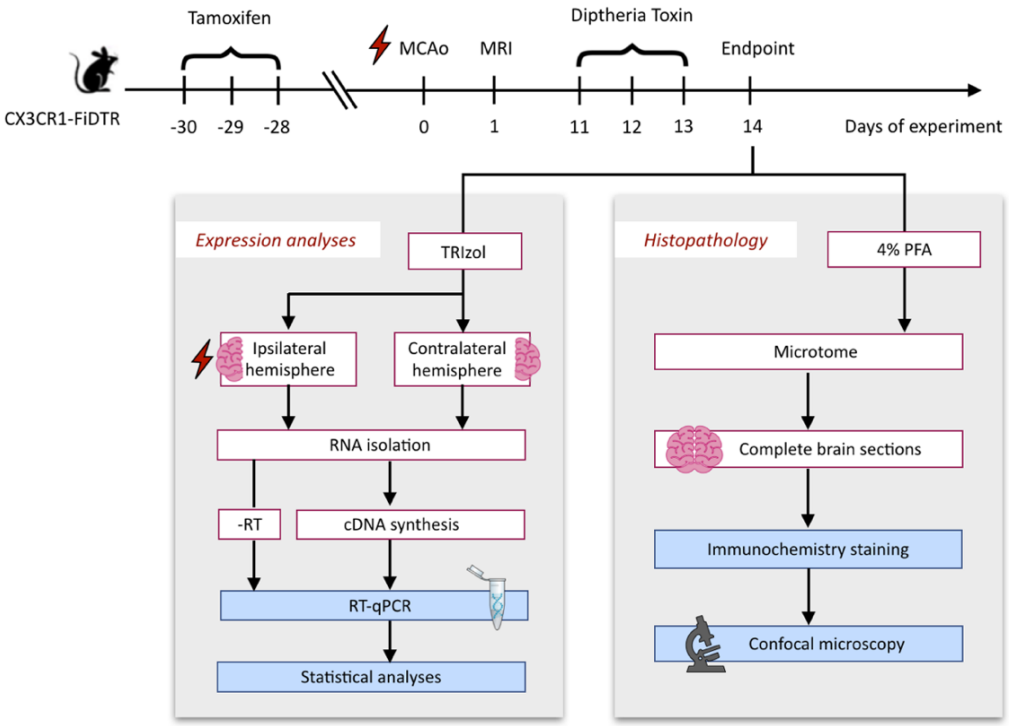

Figure 3. Schematic representation of the experimental design to evaluate the role of microglia in acute ischemia in Cx3cr1.FiDTR mice. In blue, the experiments I personally performed. A more detailed explanation for each step is written below.

3.2. Brain ischemia

Experimental focal brain ischemia was induced by transient occlusion of the left middle cerebral artery (MCA). As a result, stroke was triggered in the left, further referred to as ipsilateral, hemisphere. Mice were first anesthetized with Isoflurane in 70:30 N_2O/O_2. A nylon-coated (8/0) monofilament (Doccol Corp, Redlands, CA, USA) was introduced into the common carotid artery through a midline neck incision and advanced until blockage of blood flow through the MCA. Recirculation was established after 60 mins by withdrawing the filament. Animals recovered in heated cages and body temperature was maintained at 36.5+/-0.5 °C during surgery. Mice without stroke or that did not survive to experimental ischemia were excluded. Surgery was carried out in compliance to standard operating procedures of the Department of Experimental Neurology, Charité Berlin (Dirnagl *et al.*, 2012) and approved by the Landesamt für Gesundheit und Soziales, Berlin.

3.3. Assessment of infarct volume

Magnetic resonance imaging was performed one day after stroke in order to quantify the generated lesion (PharmaScan 7.0 T, 16 cm, Bruker, Ettlingen, Germany). The analysis was carried out under the anesthetic effects of 1.5% Isoflurane in 70:30 mixture of N_2O/O_2. During MRI analysis, breath and body temperature were monitored to minimize animal discomfort and ensure anesthetic depth.

Infarct volumes were determined using the software Analyze 10.0 (AnalyzeDirect, Overland Park, KS, USA) based on the total volume of the brain. Edema caused by stroke leads to a strong horizontal displacement of midline structures on MRI. Therefore, the space-occupying effect of the edema was considered to correct the infarct size, according to the following formula (Gerriets *et al.*, 2004):

$$\mathrm{HLVe}\ (\%) = \frac{\mathrm{HVc} - (\mathrm{HVi} - \mathrm{LVu})}{\mathrm{HVc}} \cdot 100$$

HLVe = Edema-corrected hemispheric Lesion Volume
LVe = Lesion Volume (edema corrected)
LVu = Lesion Volume (uncorrected)
HVc = Hemispheric Volume contralateral
HVi = Hemispheric Volume ipsilateral

3.4. Post-stroke care

Animal health was controlled on a daily basis by body weight, body temperature and behavioral indicators. Body posture, opening of the eyes, grooming, mobility and environment exploration were scored and if animals did not meet a minimum standard, they were sacrificed to avoid suffering. Mice were fed twice daily with syringe when a weight loss of 15% was noticed. In addition, they received soaked food on the cage floor after surgery.

3.5. Sample extraction

14 days after surgery, mice were anesthetized with a mixture of ketamine hydrochloride (150 mg/kg) and xylazine (15mg/kg). After transcardial perfusion with PBS, brains were extracted and followed different procedures depending on the post-mortem analysis. For expression analysis, the cerebellum was removed and the two hemispheres separated. Brain samples were kept in TRIzol (Thermo Fisher Scientific, US). For immunohistochemical staining, brains

were fixed overnight in 4% PFA. Then, samples were transferred to 30% sucrose in PBS. After 2 days, they were frozen in methylbutane placed on dry ice.

3.6. Immunohistochemical staining

Immunofluorescence staining was performed on 30µm-thick brain sections at bregma level. Sections were first blocked with 10% normal serum in tris-buffered saline with 0.3% Tween-20 (TBS-T). Normal goat serum or normal donkey serum (Biozol Diagnostica Vertrieb GmbH, Eching, Germany) was used according to secondary antibodies. To detect the main cellular lymphocytes, resident microglia and infiltrating macrophages, samples were incubated overnight with the primary antibodies detailed in Table 2. Appropriate secondary antibodies, diluted 1:500 in TBS-T, were applied for 2 hours. 1% serum in TBS-T was used for antibody incubations. Additionally, nuclei were counterstained with DAPI. For CXCL13 staining, a previous antigen retrieval protocol was performed consisting on incubating brain sections at 95ºC for 30 min with 10mM citrate buffer.

Brain sections were protected from light during and after staining procedure. After fixation on glass slides using mounting medium Immumount (Sigma-Aldrich, Oakville, ON, Canada), samples were imaged by confocal microscopy (Leica SP8 microscope, Leica Microsystems, Wetzlar, Germany). Images acquired with LAS software (Leica Microsystems) were only further processed for presentation purposes. A minimum of two brain samples were stained of each animal.

Table 2. Antibodies used in immunohistochemistry analysis of Cx3cr1.FiDTR mice brain sections. 'Dilution' refers to primary antibody concentrations in TBS-T with 1% serum. Secondary antibodies were all 1:500 concentrated. Antibodies were provided by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Target protein	*Target cell*	*Primary antibody*	*Dilution*	*Secondary antibody*
B220 (CD45R)	B-lymphocytes	Rat anti-mouse B220-A488	1:200	Goat anti-rat A488 Donkey anti-rat A488
CD45	Lymphocytes	Hamster anti-mouse CD45	1:200	Goat anti-hamster A546
Iba-1	Infiltrating macrophages and resident microglia	Rabbit anti-mouse Iba-1	1:500	Goat anti-rabbit A635 Donkey anti-rabbit A647
CXCL13	Unknown	Goat anti-mouse Cxcl13	1:50	Donkey anti-goat A546 Rabbit anti-goat biotin + Streptavidin A633

3.7. RT-qPCR

Phenol-chloroform extraction of total RNA was performed on brain tissue lysed in TRIzol reagent (Invitrogen). Upon completion of DNase digestion to avoid genomic DNA contamination, a final phenolic isolation of RNA was carried out. RNA concentration and purity were measured using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was then reverse transcribed into cDNA using a mixture of random primers (Roche, Switzerland) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, USA). One sample without M-MLV RT (named as -RT in Figure 3) was used as a control.

qPCR analysis was performed on LightCycler® 480 (Roche, Switzerland) by SYBR Green dye detection, following a standardized protocol. The commercial mix LightCycler® 480 SYBR Green I Master (Roche) was added to cDNA samples and the corresponding primers according to the manufacturer's instructions. Primers were designed with Primer BLAST software (NCBI) to bridge the exon-intron boundaries within the gene of interest to avoid genomic amplification. Sequences and annealing temperatures are described in Table 3. Housekeeping gene used for normalization was the receptor expression-enhancing protein 5 *(Reep5).* Melting curve analyses were carried out on completion of 42 amplification cycles to ensure product specificity. All reactions were performed in duplicates.

Table 3. Primers used for expression analysis by RT-qPCR. 'F' refers to forward primer, while 'R' stands for reverse primer.

Target gene	*Primer sequences*	*Efficiency*	*Annealing temp. (ºC)*	*Measurement temp. (ºC)*
Ltα	F: GGACCCCGCACAGCAGGTTCTC R: GCGGAGAAGCGGACACCAGA	1,97	64	80
Ltβ	F: ACCTCAATAGGCGCTTGGATG R: CGACGTGGCAGTAGAGGTAA	1,98	59	82
Cxcl13	F: TACGCCCCCTGGGAATGGCT R: AGTGGCTTCAGGCAGCTCTTCT	1,85	67	78
Cxcr5	F: CCCACTAACCCTGGACATGGGCTC R: AGTGTGCCGGTGCCTCTCCA	1,89	67	78
Ccl19	F: CTGCTGGTTCTCTGGACCTTC R: GCGGAAGGCTTTCACGATGT	1,90	67	80
Cxcl12	F: TGGACGCCAAGGTCGTCGCC R: GAACCGGCAGGGGCATCGGT	1,82	67	78
Pax5	F: CCGACTCCTCGGACCATCAGGACA R: GGGCCTGACACCTTGATGGGCA	1,86	67	80
Cd3e	F: ATGCGGTGGAACACTTTCTGGGGC R: TGTTCTCGGCATCGTCCTGGCAA	1,90	67	80

Ly6c	F: CTGACAGAACTTGCCACTGTGCCTG R: AGCAATGCAGAATCCATCAGAGGC	1,91	58	76
Ccr2	F: AAGGAGCCATACCTGTAAATGCC R: TGCCGTGGATGAACTGAGGTA	1,87	58	76
Iba-1	F: GGCTGGAGGGGATCAACAAGCAAT R: CGGAGCCACTGGACACCTCTC	1,88	60	82
Reep5	F: CTGATAGGTTTCGGATACCCAG R: GACTCGTGCTTGAGGAAGATAG	1,9	64	80

3.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Prism Software, Inc.). Expression values were determined by a variation of the $2^{(-\Delta\Delta CT)}$ method, corrected by the real efficiency for the housekeeping and the genes of interest, previously measured. Differences among groups were analyzed using two-way ANOVA statistical test. In the graphs, genetic expression is shown as mean ± SD.

4. Results

4.1. Previous experiments: overexpression of cytokines after stroke

Our research group has been focused on the study of the cytokine interplay after stroke. During previous studies (prior to my internship), it was reported that acute ischemia triggers the overexpression of some relevant cytokines and other genes related with ELSs development in WT (Figure 4) and 2D2[I] mice (unpublished work, not shown).

These findings, accompanied with the fact that organized lymphoid follicles were found in these mouse models 14 post-stroke, suggested that these cytokines could be involved in ELSs formation and lymphocytic recruitment after stroke. However, it did not give any evidence about which are the cellular components that orchestrate these changes, which made us hypothesize about the role of resident microglia.

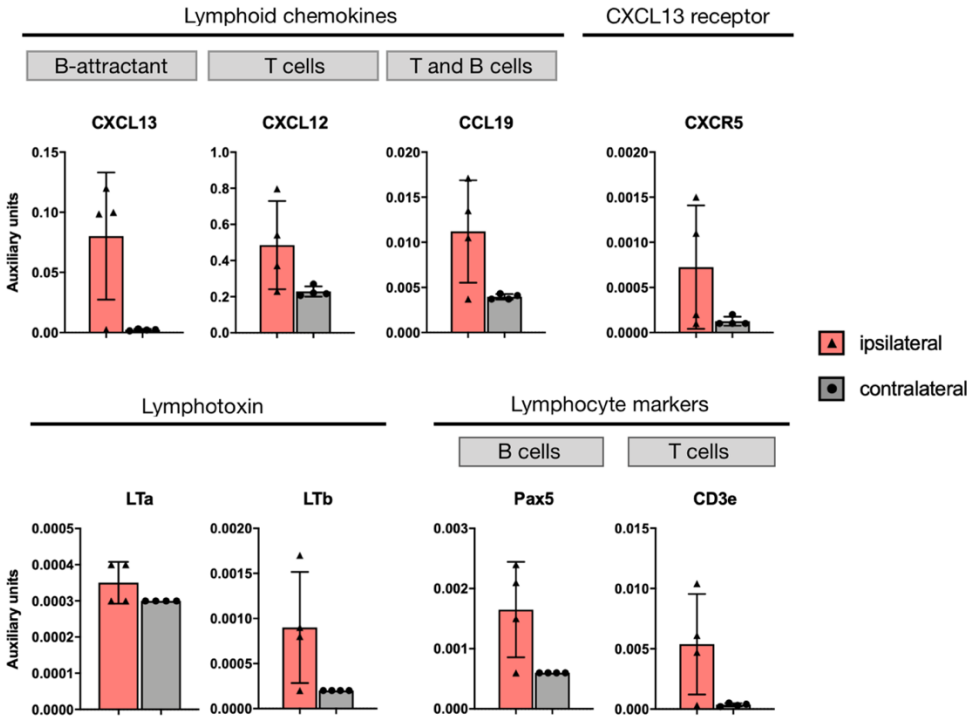

Figure 4. Expression changes 14 days after stroke in WT mice. Cerebral ischemia was induced in the ipsilateral hemisphere. Results are shown as mean ± SD (unpublished data). Molecular functions are also indicated (Aloisi and Pujol-Borrell, 2006; Neyt *et al.*, 2012; Pitzalis *et al.*, 2014). CXCL13, C-X-C motif ligand 13; CCL12, C-X-C motif ligand 12, CCL19, C-C motif ligand 19; CXCR5, C-X-C chemokine receptor type 5; LTa, lymphotoxin-alpha; LTb, lymphotoxin-beta; Pax5, paired box protein Pax-5; CD3e, cluster of differentiation 3 epsilon chain.

[I] 2D2 mice: transgenic mouse used for research in CNS autoimmunity, which spontaneously develops autoimmune responses through recognition of the brain antigen myelin oligodendrocyte glycoprotein.

4.2. Effect of microglia depletion on ELSs development

Organized lymphoid follicles were observed on brain samples of WT mice 14 days after the stroke event during previous experiments of our research group (Figure 5A). Interestingly, depletion of microglia resulted on the inhibition of ELSs development in Cx3cr1.FiDTR mice at this time point, as shown in Figure 5B. Histological analyses correlated with a lower expression of lymphocytic markers Pax5 (B-lymphocytes) and CD3e (T-lymphocytes) in microglia-deficient mice after stroke (Figure 6), which reached equivalent levels of contralateral hemispheres.

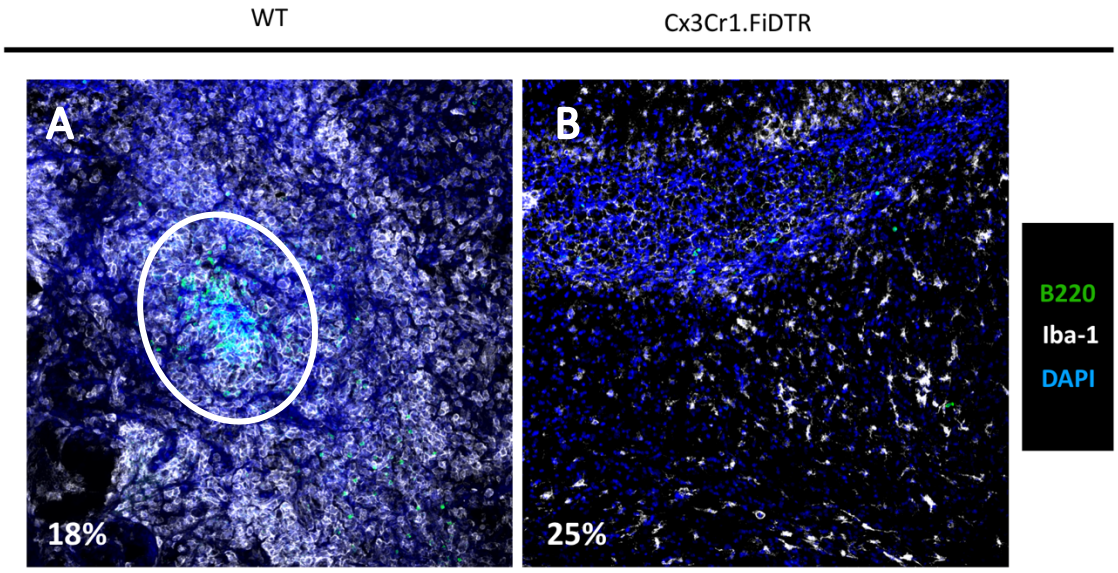

Figure 2. Immunohistochemical staining of brain sections of WT (A) and microglia-depleted Cx3cr1.FiDTR mice (B) 14 days after experimental cerebral ischemia. B220 shows B-cell infiltration, ionized calcium-binding adapter molecule 1 (Iba-1) was used as a marker of resident microglia and infiltrating macrophages and DAPI is a cell marker. Infarct sizes of each animal are indicated as percentages. Lymphoid follicles are marked with a white circle.

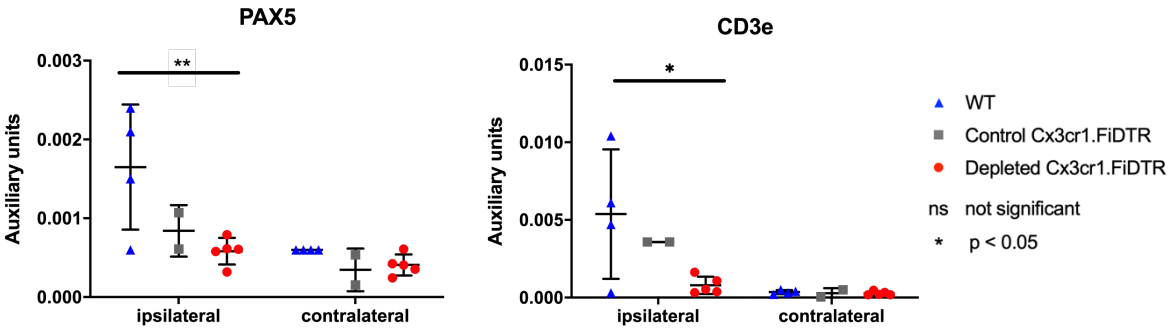

Figure 3. Expression of the lymphocytic markers Pax5 (B cells) and CD3e (T cells) in WT and Cx3cr1.FiDTR mouse models 14 days after experimental cerebral ischemia. Stroke was induced in the ipsilateral hemisphere.

4.3. Molecular pathways affected by resident microglia

Once we confirmed a relevant role of resident microglia in ELSs formation and lymphocytic recruitment, we further investigate the molecular mechanisms orchestrated by this cellular subset. With this aim, expression of relevant genes related with ELSs formation was analyzed by qPCR. Considering previous experiments from our research, we wanted to establish whether the microglia-depletion produces variations in the genes overexpressed after an ischemia in WT and 2D2 mouse models.

Intragroup big differences among ipsilateral samples were attributed to variable infarct sizes (1-26%). Nonetheless, only total expression levels is graphically represented in the following sections due to the lack of a minimum amount of Cx3cr1.FiDTR control samples to accurately determine a mathematical correlation with the infarct volume. For the same reason, WT mice was used to statistically determine expression changes in microglia-depleted mice, instead of control Cx3cr1.FiDTR mice.

4.3.1. Lymphotoxin

As shown in Figure 7, lymphotoxin-alpha was not affected by the suppression of resident microglia. Moreover, when comparing ischemic and contralateral hemispheres of all three animal groups, stroke only triggered minor changes on *Lta* expression. In contrast, a lower expression of *Ltb* in microglia-depleted mice was suggested.

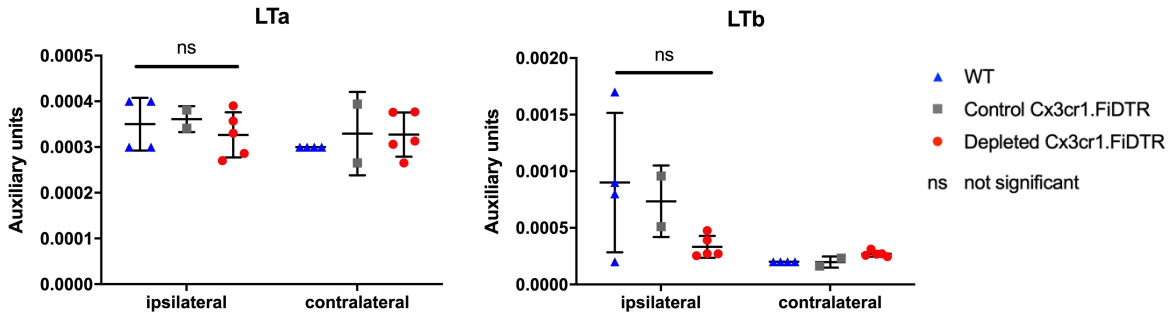

Figure 4. Expression of lymphotoxin-alpha (*Lta*) and lymphotoxin-beta (*Ltb*) genes in WT and Cx3cr1.FiDTR mouse models 14 days after experimental cerebral ischemia. Stroke was induced in the ipsilateral hemisphere.

4.3.2. Lymphoid chemokines

Absence of resident microglia in the brain after stroke greatly decreased levels of the lymphoid chemokines CXCL13, CXCL12 and CCL19 (Figure 8), involved on the recruitment of lymphocytes to the infarct area. The CXCL13 receptor, CXCR5, also showed a clear tendency towards a decreased expression. It is known that CXCR5 is expressed mainly on B-cells. Therefore, even not being significant, the lack of B-lymphocytes in microscopical observations (Figure 5) gave supporting evidence on the diminished levels of *Cxcr5*. In all cases, microglia-depleted ischemic hemispheres showed a comparable expression to contralateral hemispheres.

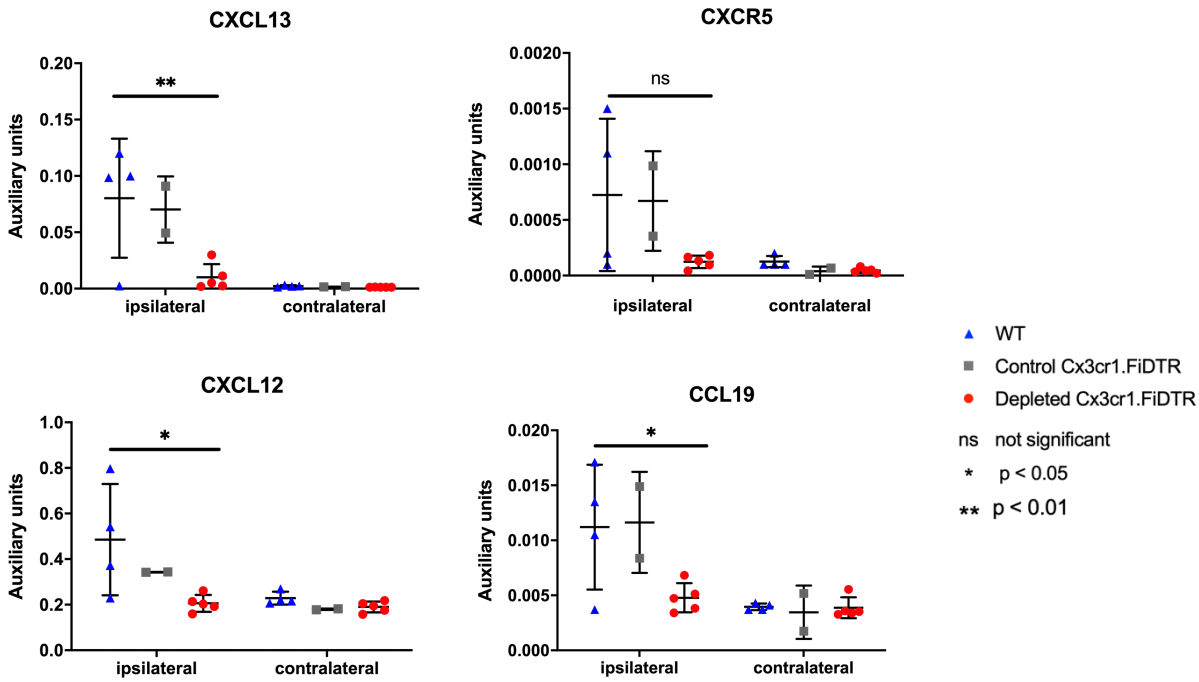

Figure 5. Expression of lymphoid chemokines *CXCL13, CXCL12,* and *CCL19* and receptor CXCR5 in WT and Cx3cr1.FiDTR mouse models 14 days after experimental cerebral ischemia. Stroke was induced in the ipsilateral hemisphere.

4.3.3. Myeloid cells

In addition to previously analyzed gens, we studied the effect of the depletion of resident microglia and other myeloid populations. Since infiltrating monocytes/macrophages and microglia are suggested to have similar functions in neuroinflammation, it could have been possible to observe compensation mechanisms to replace the absent microglia.

Infiltrating inflammatory monocytes, which further differentiate into macrophages, are classified as Ly6C^{high}CCR2^{+}CX3CR1$^{int.}$ cells and, as shown in Figure 9, they do not seem to

develop higher proliferation ratios as a response to the absence of resident microglia cells after an acute stroke. Lymphocyte antigen 6C *(Ly6c)* expression remained equal among group, both when comparing ipsilateral-contralateral hemispheres and control-depleted mice. On the other side, C-C chemokine receptor type 2 (*Ccr2)* presented higher levels after an ischemia, but this variation was attenuated in microglia-depleted mice.

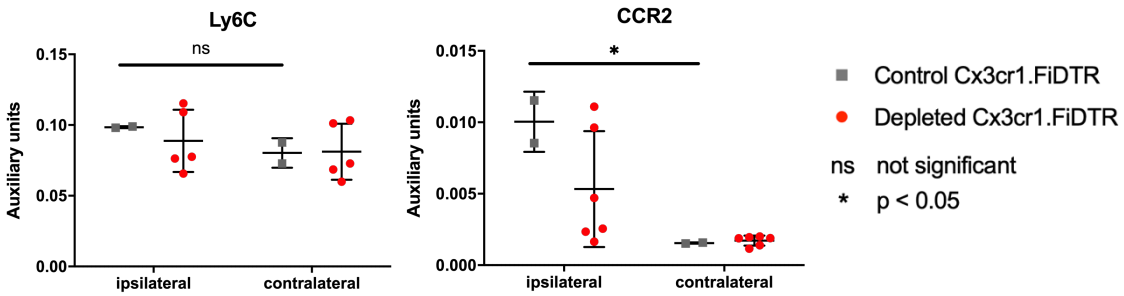

Figure 6. Expression of inflammatory monocytes markers Ly6C and CCR2 in Cx3cr1.FiDTR mice 14 days after experimental cerebral ischemia. Stroke was induced in the ipsilateral hemisphere.

Infiltrating macrophages were analyzed using *Iba-1* expression, which is expressed on macrophages and resident microglia. A clear overexpression after an ischemic stroke in both control and depleted animals was observed (Figure 10). Not significantly lower levels were noticed in microglia-depleted mice, which were probably caused by the lack of microglia contribution to *Iba-1* total expression levels. Moreover, by looking at the histology results (Figure 5) a clear reduction of Iba-1^{+} cells is suggested, although not quantified.

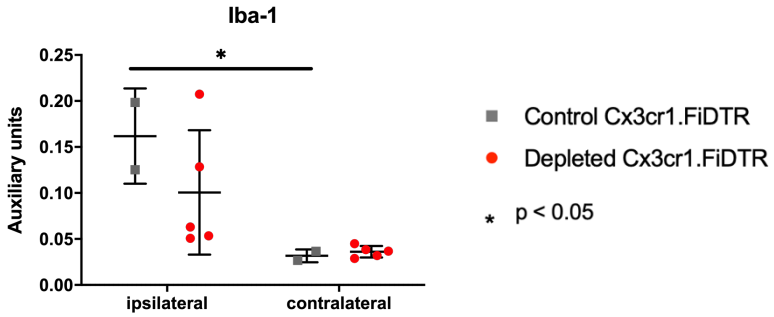

Figure 7. Expression of myeloid marker Iba-1 (infiltrating macrophages and resident microglia) in Cx3cr1.FiDTR mice 14 days after experimental cerebral ischemia. Stroke was induced in the ipsilateral hemisphere.

It is important to note that only a limited interpretation of the results can be done due to the lack of data from WT mice (*Ccr2, Ly6c,* and *Iba-1* expression was not analyzed during previous experiments) and the low number of Cx3cr1.FiDTR control samples. Thus, an evaluation of the Cx3cr1.FiDTR mouse model was not available. However, considering the results obtained as representative of the average animal population, infiltrating monocytes or macrophages were not affected by microglia depletion.

4.4. CXCL13-producing cells

Further immunohistochemical staining was done for CXCL13, the most relevant B-attractant chemokine, in order to conclude which type of cells produce this chemokine. Immunohistochemical staining in WT mice allowed to verify that the cells that produce CXCL13 were located within the ELSs area, meaning there is a local CXCL13 production associated with the lymphoid follicle (Figure 11A). In contrast and confirming gene expression analyses, CXCL13 signal was almost absent on microglia-depleted mice samples (Figure 11B).

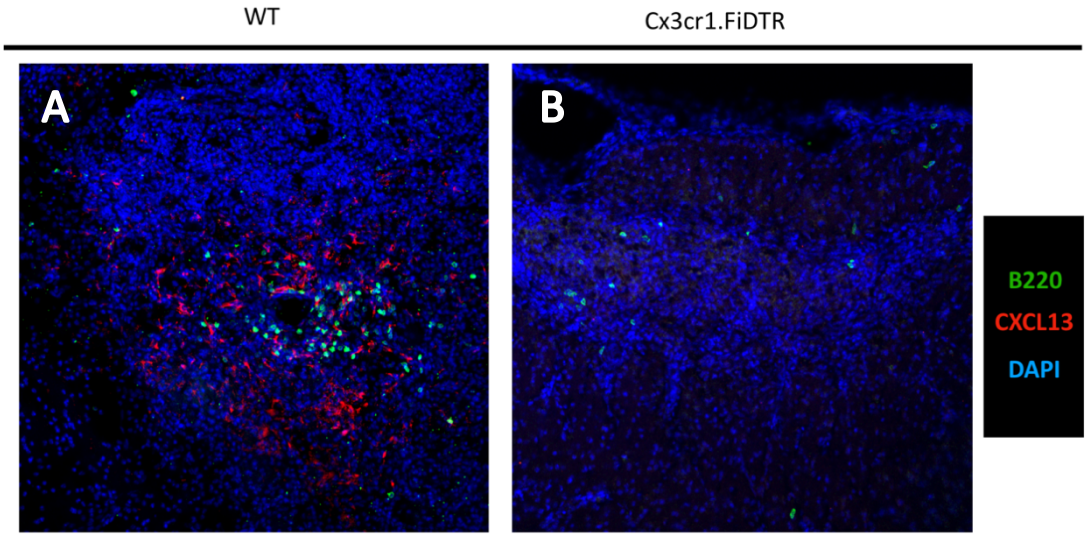

Figure 8. Immunohistochemical staining of brain sections of WT (A) and microglia-depleted Cx3cr1.FiDTR mice (B) 14 days after experimental cerebral ischemia. B220 was used as a B cell marker, which indicates de lymphoid follicle area and DAPI was used as a cell marker.

CXCL13-producing cells were found to be negative for Iba-1 maker (Figure 12), which indicates that they can not be characterized as resident microglia nor infiltrating macrophages. CD45 marker proved that neither B- nor T-lymphocytes produced CXCL13 chemokine.

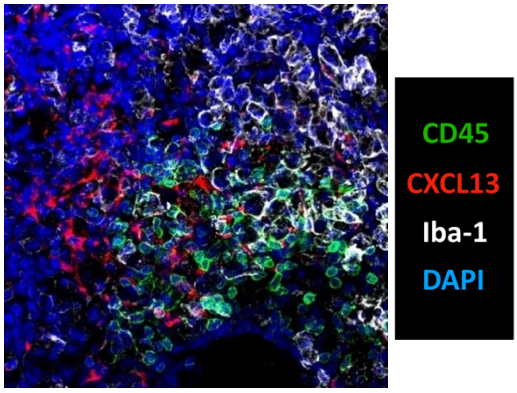

Figure 9. Immunohistochemical staining of brain sections of WT mice 14 days after experimental cerebral ischemia. CD45 was used as a lymphocytic marker, Iba-1 for resident microglia and infiltrating macrophages and DAPI as a cell marker.

5. Discussion

5.1. Evaluation of Cx3cr1.FiDTR mouse model

This genetic approach did not appear to have unspecific alterations on the neuroinflammatory processes triggered after a cerebral ischemia. On the one hand, ipsilateral mRNA expression of the genes of interest in control Cx3cr1.FiDTR and WT mice remained equal in ipsilateral hemispheres, suggesting inflammatory response after stroke was not increased or diminished.

In Cx3cr1.FiDTR mouse model, resident microglia dies by necrosis, which could result in neuroinflammation (Huang *et al.*, 2018). Thus, Cx3cr1.FiDTR could not have not been accurately reflecting the inflammatory response after stroke. However, as said before we did not observe an increased chemokine expression when compared to WT and neither in the contralateral hemispheres, where stroke was not induced. Therefore, it could be concluded that variations seen in microglia-depleted Cx3cr1.FiDTR mice were only caused by the inhibition of microglia functions after stroke and not by the treatment with DT. Nevertheless, in the future, we plan to use a pharmacological approach to deplete resident microglia by colony-stimulating factor 1 receptor (CSF1R) antagonists. This would lead to microglia apoptosis, instead of necrosis, avoiding any possible contribution of the mouse model to neuroinflammation.

Cx3cr1.FiDTR mice appears to be a suitable rodent model to study resident microglia after ischemic stroke. However, it is important to note that the small amount of control samples did only permit to intuit the consistency of Cx3cr1.FiDTR mouse model. A higher number of samples would be needed to conclude whether the data discussed is representative of the total population, but publications from other research groups also gave supporting evidence of the promising applications of this animal model (Bruttger *et al.*, 2015).

Regarding histological observations, it was proved that microglia-depleted Cx3cr1.FiDTR mice presented lower quantities of Iba-1$^+$ cells than WT mice, suggesting a successful depletion. Considering other studies (Bruttger *et al.*, 2015), we did not expect to achieve a complete depletion of resident microglia, however, Iba-1$^+$ cells could also correspond monocyte-derived macrophages, which are known to infiltrate into the ischemic brain (unpublished work of our research group). Iba-1 marker did not allow to accurately distinguish between both myeloid

populations. Consequently, the efficacy of the molecular approach or the percentage of resident microglia that did already repopulated the inflamed area could not be calculated.

As indicated by other research, this genetic approach does only permit a time-limited depletion (Bruttger *et al.*, 2015). This fact did only permit the analysis of resident microglia functions between days 11-14 post-stroke, which hinders the research on the long-term involvement of this cellular type on lymphoid neogenesis. Thus, so far it could not be concluded whether the absence of ELSs seen after microglia depletion is maintained over time. For example, it could be possible that lymphocytic migration is slowed down by microglia depletion and lymphocytes achieve to cluster, forming ELSs at later time points.

Considering the variations observed during this short time frame, Cx3cr1.FiDTR allowed to conclude that the role of microglia cells is essential during initial stages of ELSs development, a novel knowledge which could not be achieved without this mouse model. Thus, Cx3cr1.FiDTR is a very promising animal model to study resident microglia after ischemic stroke. However, a longer duration of the depletion would provide new insights of microglia kinetics and long-term effects.

5.2. Role of resident microglia in ELSs development

Our study revealed the importance of resident microglia in lymphocytic recruitment 14 days after an acute stroke. Previous findings in WT and 2D2 mice from our research group, where organized lymphoid follicles were present at this time point, contrasted with the absence of lymphocytes in the immunohistological analyses of Cx3cr1.FiDTR mice. These observations correlated with low mRNA levels of lymphocytic markers Pax5 and CD3e. Ischemic hemispheres of microglia-depleted samples presented a comparable expression with contralateral hemispheres, which proves the absence of the trigger that causes an overexpression after stroke. Therefore, both expression and histological approaches provided clear evidence that this cellular type plays a key role on ELSs formation after an acute cerebral ischemia.

Research on the molecular mechanisms that underlie microglia function in ELSs formation indicated an alteration of the expression after stroke of lymphoid chemokines, receptors and lymphotoxins. Overexpression formerly seen in WT and 2D2 mice did not occur after the depletion of resident microglia in Cx3cr1.FiDTR mice.

A clear contribution of microglia to the increase of lymphotoxin-beta levels after stroke was proved. Cx3cr1.FiDTR microglia-depleted mice presented the same expression of LTb than the contralateral hemispheres, which were not affected by ischemia. In contrast, lymphotoxin-alpha appears not to be affected by the inhibition of microglia, but its upregulation after stroke was not as significant as previously thought. Therefore, our results suggest that, even LTa could still be a crucial factor on the initiation of ELSs after cerebral ischemia, its function might not be associated with an increased synthesis. It could be possible that, in stroke, LTβ is the limiting component of the $LT\alpha_1\beta_2$ heterodimers. This complex is thought to be one of the initial triggers with lymphoid neogenesis, inducing the expression of lymphoid chemokines CXCL13, CXCL12, and CCL19, among other molecules. In other diseases also characterized by autoimmune responses, such as rheumatoid arthritis (RA) the importance of LTβ to predict ELSs development has already been suggested (Tang *et al.*, 2017).

CXCR5 is mainly expressed on B-lymphocytes, which were proved not to be able to infiltrate into the ischemic tissue without the support of resident microglia. Consequently, CXCL13 receptor, CXCR5, showed a tendency towards a reduced expression in microglia-depleted mice, as it could be expected.

Other factors discussed below could have also hampered the inhibition of the recruitment of B-lymphocytes after stroke, but the most notable effects were observed on CXCL13. The overexpression triggered by stroke was successfully inhibited by the depletion of resident microglia. Considering established knowledge of the B cell attractant properties of this chemokine (Pitzalis *et al.*, 2014), lower *Cxcl13* expression 14 days post-stroke correlated with the lack of B-lymphocytes in histological samples of microglia-depleted mice. CXCL13-producing cells could not be properly characterized, even though proved not to be myeloid cells nor other recruited leukocytes.

Resident microglia was also proved to significantly contribute to the increase of the lymphoid chemokines CXCL12 and CCL19 after stroke. Both cytokines are involved on T-lymphocyte migration to inflamed areas (Pitzalis *et al.*, 2014), which is consistent with the low expression of the T cell marker CD3e found in microglia-depleted mice.

Our group recently provided evidence that the interplay between B- and T-lymphocytes appears to be crucial on ELSs formation after stroke (unpublished data). Depletion of T-lymphocytes appears to avoid infiltration of B-lymphocytes into the brain, indicating a tight correlation between both cellular subsets. These results might also suggest that the lack of T-lymphocytes could be the main cause of the inhibition of B-lymphocyte migration in microglia-depleted mice.

Therefore, considering the above, the most feasible causes of the inhibition of B-lymphocyte recruitment are the low levels CXCL13 and the absence of T cells. Whether both hypotheses are interrelated or contribute independently to the infiltration of B-lymphocytes remains unknown.

Finally, absence of resident microglia did not trigger variations on proliferation ratios of other myeloid populations. Markers of infiltrating monocytes and macrophages did not show significant changes, indicating that the apparent functions of these cell types after stroke are independent from microglia signaling. However, the role of infiltrating monocytes and macrophages in ischemic neuroinflammation is still under study. Furthermore, it has to be considered that markers could not accurately reflect cell counts. It is possible that different activation states, such as a lower or higher expression of the cellular markers, might mask changes in cell numbers.

Our results showed similarities between organized follicles formed after an acute cerebral ischemia and those of other diseases in terms of cellular distribution of the different components of the follicles. In addition, the cytokines that appear to have a more important role on ELSs formation are also equivalent. Therefore, advances in other autoimmune diseases with lymphoid neogenesis could indicate possible hypothesis of what is occurring in cerebral ischemia. However, knowledge in other disorders needs to be further verified in stroke models, since disease-specific molecular and cellular processes are possible. Indeed, our results revealed the crucial role of resident microglia, a cellular type which is not present in other tissues where lymphoid neogenesis is also known to occur.

6. Conclusion

Cx3cr1.FiDTR mice resulted to be a promising mouse model to study microglia functions after stroke. Currently, the major drawback of this genetic approach is the short duration of the depletion, which hinders the research on long-term role of resident microglia. Therefore, it has to be considered that our results only shown the effect of a delayed depletion of microglia, starting from d11 post-ischemia.

Immunohistological and expression analyses of Cx3cr1.FiDTR mice showed that absence of resident microglia leads to an inhibition of lymphocyte migration to the ischemic brain 14 days after stroke. Consequently, compartmentalized lymphoid follicles previously observed in WT mice at this time point are not able to develop without the support of microglia cells. Whether ELSs form at a later time point need to be further verified. More research is also needed to verify the detrimental effects of ELS and its correlation with post-stroke cognitive decline. However, abnormal presence of lymphocytes in the lesion area is a strong evidence that stroke enhances the onset of B-cell autoimmune responses in the brain.

Microglia depletion also altered cytokine expression profile observed in WT mice after stroke, which is thought to be the main cause of the inhibition of ELS development. Interestingly, lower mRNA levels of the lymphoid chemokines CXCL13, CXCL12, and CCL19 were observed. Furthermore, *Ltβ* and *Cxcr5* genes presented a tendency towards a decreased expression, even not statistically significant. Regarding to other myeloid populations, depletion did not appear to change the recruitment of infiltrating monocytes and monocyte-derived macrophages.

Therefore, confirming our initial hypothesis, resident microglia has a crucial role in lymphoid neogenesis and its actively involved in the molecular changes triggered after cerebral ischemia.

References

Aloisi, F. and Pujol-Borrell, R. (2006) 'Lymphoid neogenesis in chronic inflammatory diseases', *Nature Reviews Immunology*, 6(3), pp. 205–217. doi: 10.1038/nri1786.

Benakis, C. *et al.* (2015) 'The role of microglia and myeloid immune cells in acute cerebral ischemia', *Frontiers in Cellular Neuroscience*, 8(January), pp. 1–16. doi: 10.3389/fncel.2014.00461.

Bombardieri, M., Lewis, M. and Pitzalis, C. (2017) 'Ectopic lymphoid neogenesis in rheumatic autoimmune diseases', *Nature Reviews Rheumatology*, 13(3), pp. 141–154. doi: 10.1038/nrrheum.2016.217.

Bruttger, J. *et al.* (2015) 'Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System', *Immunity*, 43, pp. 92–106. doi: 10.1016/j.immuni.2015.06.012.

Buch, T. *et al.* (2005) 'A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration', *Nature Methods*, 2(6), pp. 419–426. doi: 10.1038/nmeth762.

Buckley, C. D. *et al.* (2015) 'Stromal Cells in Chronic Inflammation and Tertiary Lymphoid Organ Formation', *Annual Review of Immunology*, 33(1), pp. 715–745. doi: 10.1146/annurev-immunol-032713-120252.

Center for Stroke Research Berlin (2019) *Homepage CSB*. Available at: https://www.schlaganfallcentrum.de/en/ (Accessed: 10 March 2019).

Chamorro, Á. *et al.* (2012) 'The immunology of acute stroke', *Nature Reviews Neurology*. Nature Publishing Group, 8(7), pp. 401–410. doi: 10.1038/nrneurol.2012.98.

Charité - Universitätsmedizin Berlin (2019) *Charité – Universitätsmedizin Berlin*. Available at: https://www.charite.de/en/ (Accessed: 10 March 2019).

Dirnagl, U. and Group members of the MCAO-SOP (2012) 'Standard operating procedures (SOP) in experimental stroke research: SOP for middle cerebral artery occlusion in the mouse', *Nature Precedings*. doi:10.1038/npre.2012.3492.3.

Doyle, K. P. *et al.* (2015) 'B-Lymphocyte-Mediated Delayed Cognitive Impairment following Stroke', *Journal of Neuroscience*, 35(5), pp. 2133–2145. doi: 10.1523/JNEUROSCI.4098-14.2015.

Doyle, K. P. and Buckwalter, M. S. (2017) 'Does B lymphocyte-mediated autoimmunity contribute to post-stroke dementia?', *Brain, Behavior, and Immunity*. Elsevier Inc., 64, pp. 1–8. doi: 10.1016/j.bbi.2016.08.009.

Gerriets, T. *et al.* (2004) 'Noninvasive Quantification of Brain Edema and the Space-Occupying Effect in Rat Stroke Models Using Magnetic Resonance Imaging', *Stroke*, 35(2), pp. 566–571. doi: 10.1161/01.STR.0000113692.38574.57.

Huang, Z. *et al.* (2018) 'Necroptosis in microglia contributes to neuroinflammation and retinal degeneration through TLR4 activation', *Cell Death and Differentiation*. Nature Publishing Group, 25(1), pp. 180–189. doi: 10.1038/cdd.2017.141.

Korniluk, A. *et al.* (2017) 'From inflammation to cancer', *Irish Journal of Medical Science*. Springer London, 186(1), pp. 57–62. doi: 10.1007/s11845-016-1464-0.

Ma, Y. *et al.* (2017) 'The biphasic function of microglia in ischemic stroke', *Progress in Neurobiology*. Elsevier Ltd, 157, pp. 247–272. doi: 10.1016/j.pneurobio.2016.01.005.

Nasef, N. A., Mehta, S. and Ferguson, L. R. (2017) 'Susceptibility to chronic inflammation: an update', *Archives of Toxicology*. Springer Berlin Heidelberg, 91(3), pp. 1131–1141. doi: 10.1007/s00204-016-1914-5.

Neyt, K. *et al.* (2012) 'Tertiary lymphoid organs in infection and autoimmunity', *Trends in Immunology*, 33(6), pp. 297–305. doi: 10.1016/j.it.2012.04.006.

Okuyama, M. *et al.* (2010) 'A novel in vivo inducible dendritic cell ablation model in mice', *Biochemical and Biophysical Research Communications*. Elsevier Inc., 397(3), pp. 559–563. doi: 10.1016/j.bbrc.2010.05.157.

Pitzalis, C. *et al.* (2014) 'Ectopic lymphoid-like structures in infection, cancer and autoimmunity', *Nature Reviews Immunology*, 14, pp. 447–462. doi: 10.1038/nri3700.

Prinz, M. and Priller, J. (2017) 'The role of peripheral immune cells in the CNS in steady state and disease', *Nature Neuroscience*, 20(2), pp. 136–144. doi: 10.1038/nn.4418.

Tang, H. *et al.* (2017) 'Lymphotoxin signalling in tertiary lymphoid structures and immunotherapy', *Cellular and Molecular Immunology*. Nature Publishing Group, 14(10), pp. 809–818. doi: 10.1038/cmi.2017.13.

Self-assessment

Before starting my internship in Charité, I felt insecure about working abroad as a trainee because I didn't know to what extent I could adapt to the academic and formative requirements of a foreign country. However, at the same time, it was also an exciting challenge. After the internship, I have been able to realize that it has been a very enriching and recommendable opportunity to improve my scientific skills, as well as, to gain confidence.

Regarding to the theoretical apprenticeship, I have been able to refresh basic concepts in the field of immunology and I have also acquired new knowledge, methodologies and work routines in neuroscientific research. Neurology is an area for which I have been always curious about and this thesis has helped me to deepen into a subject in which I would like to focus my professional career and further studies. In addition, qPCR and immunohistochemical staining are two scientific techniques widely used in any research field. Gaining practical experience is something I also consider very useful for my future.

Interestingly, working with staff from a medical background and in direct contact with patients was of great added value. It helped me to have a clinical and anatomical perspective of the project, both of them subjects that are not so present in a degree in Biotechnology. It was also made me appreciate the relevance of cross-sectionalism in research groups.

In conclusion, the stay at the Charité institution has provided me with theoretical and practical knowledge that perfectly complement the university degree I have been studying, which has served as a crucial basis to address the internship.