CD4+ T cells promote delayed B cell responses in the ischemic brain after experimental stroke

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ABSTRACT

CD4+ T lymphocytes are key mediators of tissue damage after ischemic stroke. However, their infiltration kinetics and interactions with other immune cells in the delayed phase of ischemia remain elusive. We hypothesized that CD4+ T cells facilitate delayed autoimmune B cell responses in the brain, which have been previously linked to post-stroke cognitive impairment (PSCI). Therefore, we treated myelin oligodendrocyte glycoprotein T cell receptor transgenic 2D2 mice of both sexes with anti-CD4 antibody following 60-minute middle cerebral artery occlusion and assessed lymphocyte infiltration for up to 72 days. Anti-CD4-treatment eliminated CD4+ T cells from the circulation and ischemic brain for 28 days and inhibited B cell infiltration into the brain, particularly in animals with large infarcts. Absence of CD4+ T cells did not influence infarct maturation or survival. Once the CD4+ population recovered in the periphery, both CD4+ T and B lymphocytes entered the infarct site forming follicle-like structures. Additionally, we provide further evidence for PSCI that could be attenuated by CD4 depletion. Our findings demonstrate that CD4+ T cells are essential in delayed B cell infiltration into the ischemic brain after stroke. Importantly, lymphocyte infiltration after stroke is a long-lasting process. As CD4 depletion improved cognitive functions in an experimental setup, these findings set the stage to elaborate more specific immune modulating therapies in treating PSCI.

1. Introduction

Ischemic stroke is a leading cause of mortality and long-term disability worldwide (Feigin et al., 2014). Improvements in stroke management have increased survival in developed countries, which in turn led to an increased prevalence of post-stroke cognitive impairment (PSCI) (Mijajlović et al., 2017). Acute ischemic brain damage and neuronal loss contribute to cognitive decline after stroke. However, the patients’ risk of developing cognitive deficits remains elevated and even increases for many years when compared to individuals without stroke history (Pendlebury and Rothwell, 2009). A recent seminal study links PSCI to autoreactive B lymphocyte responses in the brain (Doyle et al., 2015).

In experimental stroke, T and B lymphocytes infiltrate into the CNS in a delayed manner and can be detected for up to 12 weeks (Doyle et al., 2015). This pathway may involve priming in cervical lymph nodes or palatine tonsils and therefore be antigen-specific, as antigen-presenting cells (APCs) co-localize with CNS antigen in these lymphoid organs after experimental and human stroke (Planas et al., 2012; van Zwam et al., 2009).
Myelin oligodendrocyte glycoprotein (MOG) T-cell receptor (TCR) transgenic (2D2) mice where more than 80% of peripheral CD4+ T cells express TCRs recognizing MOG have been used to investigate mechanisms of autoreactive immune responses in models of CNS disorders including stroke. These mice have been used to unravel how stroke-induced immunodepression (SIDS) increases autoreactive CNS antigen-specific T-cell responses in the ischemic brain, a finding which has been demonstrated to be predictive for wild type mice too (Römer et al., 2015). CD4+ T cells from 2D2 mice proliferate and expand in the infarcted brain upon activation by local APCs. When transferred from stroke-experienced 2D2 mice to lymphocyte-deficient mice, CNS antigen-specific CD4+ T cells exacerbated ischemic brain injury and increased neurological deficits when another stroke was induced in the recipients (Jin et al., 2018). Given the interdependence of T and B lymphocyte responses, these antigen-specific T cells may boost B cell autoreactivity after stroke and justify the use of 2D2 mice in investigating antigen-specific lymphocyte infiltration.

Once in the brain, T and B cells aggregate in clusters surrounded by myeloid cells (Doyle et al., 2015). These so-called ectopic lymphoid structures (ELS) have also been observed in chronic tissue inflammation such as in various autoimmune diseases (Corsiero et al., 2012). ELS host formation of germinal centers (GCs), facilitate B cell differentiation and production of antibodies to disease-specific antigens that worsen the outcome (Corsiero et al., 2016). Similar processes might occur after CNS injury as CNS antigen-specific autoantibodies have been described in stroke and were associated with cognitive decline (Becker et al., 2016a). However, little is known about the mechanisms underlying ELS formation in the brain.

In the present study, we aimed to investigate the role of CD4+ T cells in B cell infiltration and ELS formation by depleting peripheral CD4+ T cells after experimental stroke.

2. Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

2.1. Animals and study approval

2D2 mice (Bettelli et al., 2003) (strain name: C57BL/6 Tg (Tcr2D2, Tcrb2D2) Kuch/J; stock number 006912; The Jackson Laboratory; RRID: IMSR_JAX:006912) and C57BL/6J (WT) mice (Janvier Labs, Le Genest-Saint-Ile, France) were used in the study. Mice of both sexes were 10–14 weeks old when entering the study. Mice were housed in the Charité animal facility with a 12 h light/dark cycle (lights on from 6:00 until 18:00) and enriched environment. Mice had ad libitum access to food and water. All animal experiments were conducted in accordance with the ARRIVE guidelines, European Community Council Directives 86/609/EEC and German national laws and approved by local authority (Landesamt für Gesundheit und Soziales, Berlin, Germany).

2.2. Experimental stroke

60 min middle cerebral artery occlusion (MCAo) was performed according to the standard operating procedures of the laboratory (Engel et al., 2011). In brief, anesthesia was induced with 2.5% isoflurane (Forene, Abbott, Wiesbaden Germany) in 1:2 mixtures of O2/N2O and maintained at 1.0%–1.5% isoflurane. A silicon rubber-coated monofilament with a diameter of 0.19 ± 0.01 mm (Doccol, MA, USA) was introduced into the common carotid artery, advanced along the internal carotid artery towards the origin of the MCA, and left there for 60 min. For reperfusion, the inserted filament was withdrawn and the internal carotid artery was ligated permanently under anesthesia. The filament was withdrawn immediately after exposition to the MCA in sham-operated controls. Body temperature was maintained with a heating pad. A drop of 1% Bupivacaine gel was applied to the wound for pain relief. Success of MCAo was verified using the modified Bederson score (Bederson et al., 1986) and MRI infarct volumetry on day 1. Allocation of animals to different operators and sham or MCAo group was randomized. After surgery, animals were allowed to recover in a heated cage before returning to home cages. Following exclusion criteria were applied: Unsuccessful stroke confirmed by MRI assessment, death at the day of operation or death prior to the first antibody injection in depletion experiments.

2.3. T2-weighted magnetic resonance imaging (MRI)

For quantification of ischemic lesion, animals were subjected to T2-weighted MRI as previously described (Hetze et al., 2012). Images were acquired using a 7 T rodent scanner (Pharmscan 70/16, Bruker BioSpin, Ettlingen, Germany) with the Bruker software Paravision 5.1. Acquired images were analyzed semi-automatically with Analyze 10.0 Software (AnalyzeDirect, Inc.; Lenexa, KS, USA). Infarct volumes were expressed as percentage of infarct lesion of edema corrected ipsilateral hemisphere (Gerriets et al., 2004).

2.4. Drug administration

Mice in CD4 depletion and sham group received intraperitoneal injections of 200 µg CD4 depleting antibody (anti-mouse CD4, clone GK1.5, diluted in sterile phosphate-buffered saline (PBS) at 1 mg/ml,) at day 3, 5, 7 and 9 after MCAo. The control group received an isotype control antibody (rat IgG2b isotype control, clone LTF-2) according to the same injection scheme. Randomization of mice to treatment groups was stratified by infarct volumes on day one and has been performed by a group member who did not participate in experiments. Investigators were blinded to treatment groups during data analysis.

2.5. Immunofluorescence staining

PBS-perfused brains were fixed, snap-frozen, cut into 30 µm thick slices on a sliding microtome (LEICA, Wetzlar, Germany) and stored as free-floating sections. Brain tissue was blocked against unspecific binding with 10% normal goat serum (NGS, BIZOZL, Eching, Germany) and 0.3% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Primary and secondary antibodies were diluted in 1% NGS and 0.3% Tween 20 in PBS. Sections were incubated with primary antibodies at 4 °C overnight. Primary antibodies were against B220 (Alexa488 conjugated, 1:200, clone RA3-6B2, BioLegend, San Diego, CA, USA), CD3 (1:200, clone 500A2, BD Bioscience), CD4 (1:200, clone RM-4-5, BD Bioscience),), Laminin 1+2 (1:400, polyclonal, ab7463, Abcam, Cambridge, UK), CD138 (1:200, clone 281-2, BD Bioscience), IgM (coupled with Alexa594, polyclonal, A21044, Life Technologies), IgG (coupled with Oregon Green488, polyclonal, O6380, Life Technologies). After washing with PBS, sections were incubated at room temperature (RT) with secondary antibodies for 2 h on a shaker. Nuclei were counterstained with DAPI (2 µg/ml, Honeywell Fluka, Seelze, Germany). Sections were mounted onto SuperFrost Ultra Plus® slides (R. Langenbrinck, Emmendingen, Germany) with Shandon Immumount (Thermo Scientific, Waltham, MA, USA). The specificity of primary stainings was confirmed by doing control stainings with only secondary antibody. Fluorescent pictures were taken with a confocal microscope (Leica TCS SPE). For representative images maximal projections of 20 µm Z stack scanning with 10 steps were created or single planes of Z stack were chosen. Quantification of infiltrating CD4+ T cells and B cells in 2D2 mice was performed by counting 9 frames (173 µm × 173 µm) per hemisphere using LEICA LAF software. 1 slice around bregma including lesions in both striatum and cortex was chosen to represent each animal.
2.6. Isolation of leukocytes from brain and blood

Mice were deeply anesthetized with an intraperitoneal injection of ketamine 10% (150 mg/kg, CP-Pharma, Burgdorf, Germany) and Xylazine (15 mg/kg, Xylavet, CP-Pharma) diluted with 0.9% NaCl and transcardially perfused with PBS. Whole brains without cerebellum were dissected and kept in RPMI 1640 medium (Biochrom, Berlin, Germany) on ice, supplemented with 10% FCS (Biochrom), 50U/ml penicillin, 50 µg/ml streptomycin (Biochrom), 2 mM L-αalanine-glutamic acid (Biochrom). Hemispheres were separated and a single cell suspension was prepared by pressing the tissue through a 70 µm cell strainer (Corning Science, Mexico S.A. de C.V.) into RPMI 1640 medium. After 10 min centrifugation at 250g, the cell pellet was re-suspended in 35% Percoll (GE Healthcare, Buckinghamshire, UK). The 35% Percoll solution was carefully layered on top of 4 ml 70% stock isotonc Percoll and centrifuged at RT for 30 min at 1159g without acceleration and break. Mononuclear cells were collected from the 35%/70% interface. The peripheral blood for was collected either by submandibular bleeding from living animals (baseline and any intermediate time points) or from vena cava in deeply anesthetized mice (for endpoint). Blood was collected in lithium heparin coated tubes (Sarstedt AG, Nümbrecht, Germany). 50 µl of blood were subjected to erythrocyte lysis (BD Pharm Lyse, BD Biosciences, San Jose, CA, USA).

2.7. Flow cytometry

Blood or brain leukocytes were stained with primary, fluorochrome-conjugated antibodies diluted in PBS + 0.5% BSA + 2 mM EDTA for 20 min at 4 °C in the dark. The following fluorescent anti-mouse monoclonal antibodies were used for brain leukocytes: CD11b (ECD, clone M1/70), CD138 (BV605, clone 2B11-2), CD19 (BV785, clone 6D5), CD25 (BV711, clone FC61), CD3e (Pacific Blue, clone 17A2), CD4 (APC-eF780, clone RM4-5), CD44 (PE-Cy7, clone IM7), CD45 (Alexa706, clone 30-F11), CD8 (PE-Cy5, clone 53-6.7), GL7 (Alexa488, clone GL7), IgD (BV510, clone 11-26c2a), TCR Vβ11 (PE, clone RR3-15). The following fluorescent anti-mouse monoclonal antibodies were used for blood leukocytes: CD11b (PE-Cy7, clone M1/70), CD19 (PerCP-Cy5.5, clone 1D3), CD3 (PE, clone 17A2), CD4 (FITC, clone RM4-5), CD8 (Alexa647, clone 53-6.7), Ly6C (BV421, clone HK1.4), Ly6G (APC-Cy7, clone 1A8). Pacific Orange staining was used for discrimination between living and dead cells acquired from blood samples only. Leukocytes were phenotyped using LSRII SORP and FACSCanto II flow cytometers (BD, Franklin Lakes, NJ, USA) and FlowJo (BD) vers.10.0 software. Gating strategies are provided in Fig. 1.

2.8. mRNA expression analysis in brain by qRT-PCR

Total RNA was isolated from ipsilateral and contralateral hemispheres separately by phenol–chloroform extraction, digested with DNaseI (Roche) RNase-free DNase, Promega Corporation, Madison, WI, USA) to remove genomic DNA and reverse-transcribed to cDNA using the M-MLV reverse transcriptase (Promega) with random hexamers (Roche, Basel, Switzerland) according to the protocol provided by the manufacturer. The cDNA was then quantified using a LightCycler 480 (Roche) and the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) according to the manufacturer’s guidelines. Target gene expression was normalized to Reep5 as housekeeping gene. The relative expression of the target gene in ipsilateral to contralateral hemisphere expression. Primers are specified in Table 1.

2.9. Y-maze

Spatial working memory performance of mice was assessed by the Y-maze test (Maurice et al., 1994). This test is based on the natural tendency of mice to explore novel environments more than familiar ones. Spontaneous exploration behavior in 8 min session was recorded at baseline (day −1) at day −1 and 2 weeks and 7 weeks after MCAo. Arm entries were quantified to determine cognitive functions where a decline in arm entries at subsequent time-points was interpreted as functional memory. The y-maze consisted of three wooden, black-painted arms positioned at 120° to each other (arm dimensions: 40 cm long, 10.5 cm high and 3 cm wide). The test was performed in a testing room with diffused dim light (35 lux).

2.10. Statistical analysis

Data are presented as scattered dot plot with mean ± standard deviation or box plot (25–75 percentile) with whiskers (5–95 percentile) using Prism 7.0 software (GraphPad, San Diego, CA, USA). Where data were not normally distributed, Mann-Whitney U test was performed for comparison of 2 groups and paired data were analyzed using the Wilcoxon test. Where applicable, data were log-transformed to attain normal distribution. Normal distribution was verified by Kolgomorov-Smirnov test. The effectiveness of CD4 depletion in the peripheral blood was analyzed by 2-way ANOVA and Sidak’s multiple comparison. Comparison between 2 groups was made with paired or unpaired T-test. Correlation was analyzed by Spearman r. Survival curves were analyzed with the log-rank (Mantel-Cox) test. Power analysis was performed using G*Power software (Faul et al., 2007) where an effect size of 0.5 with α = 0.05 and 1−β = 0.8 was determined based on previous comparable experiments. Statistical analysis was performed using Prism 7.0 software (GraphPad, San Diego, CA, USA), where tests were two-sided and p < 0.05 was considered statistically significant.

3. Results

3.1. 2D2 mice suffer from greater infarcts and increased lymphocyte infiltration compared to wild-type mice

To focus on CNS antigen-specific lymphocyte infiltration, we used myelin oligodendrocyte glycoprotein (MOG) T cell receptor (TCR) transgenic (2D2) mice, which are prone to autoreactive immune responses due to their enriched CNS antigen-specific T cell repertoire (Betteli et al., 2003). Lymphocyte CNS infiltration and outcome at day 14 after MCAo were compared between 2D2 and C57Bl6 (WT) mice. Immunofluorescence staining of selected brain slices and flow cytometry analysis of leukocytes isolated from homogenized brain tissue revealed about 20-fold increased CNS infiltration of CD4⁺ T cells and B cells into the ipsilateral hemisphere of 2D2 mice compared to WT animals (Fig. 2A-B). No lymphocyte infiltration was observed in the contralateral hemispheres of MCAo mice or in brains of sham-operated control mice (data not shown). Additionally, 2D2 mice showed larger infarct volumes (Fig. 2C) and higher mortality compared to WT mice (Fig. 2D). These findings indicate that 2D2 mice serve as a useful model to investigate CD4⁺ T cell-dependent CNS antigen-specific immune cell responses after experimental stroke.

3.2. Anti-CD4 treatment eliminates peripheral CD4⁺ T cells and prevents their early infiltration into the ischemic brain

In order to investigate the role of CD4⁺ T cells in delayed but not immediate early immune responses after stroke, we depleted circulating CD4⁺ T cells by repeated injections of monoclonal anti-CD4 antibody once every other day between day three and day nine after stroke onset (Fig. 3A). Leukocyte infiltration into the ischemic brain strongly depends on infarct size (Supplementary Fig. 1A). Therefore, mice were assigned to treatment groups by their infarct volume on day 1 after surgery (Supplementary Fig. 1B). Seven days after MCAo, CD4⁺ T cells were eliminated from the periphery and did not recover until day 28 (Fig. 3B). Numbers of blood CD8⁺ T cells, CD19⁺ cells and myeloid...
cells were not affected by peripheral CD4 depletion (Fig. 3C), while infiltration of CD4+ T cells into the ischemic brain at day 14 after MCAo was effectively prevented in anti-CD4 antibody-treated compared to isotype-treated control mice (Fig. 3D).

3.3. Anti-CD4 treatment reduces microglial loss and myeloid cell infiltration 14 days after MCAo

To test whether CD4 depletion influenced myeloid cells in the delayed phase of cerebral ischemia, we quantified resident microglia and infiltrating myeloid cells (IMC) by flow cytometry 14 days after MCAo in 2D2 mice. Microglia and IMC can be distinguished by high CD45 expression in IMC and low CD45 expression in microglia. It was shown previously that microglia upregulate CD45 upon activation (Stein et al., 2007). However, when inducing MCAo in a transgenic microglia reporter mouse, we found that CD45 expression levels are sufficient to distinguish microglia and IMC by flow cytometry 14 days after stroke (Supplementary Fig. 2). The trend towards microglial loss in the ipsilateral compared to the contralateral hemisphere in isotype-treated mice was prevented by CD4 depletion as microglia numbers were significantly higher in the ipsilateral hemisphere of CD4-depleted mice compared to isotype-treated mice (Fig. 4A). Microglia numbers in the ipsilateral hemisphere were lower with increasing lesion size in both treatment groups, without reaching statistical significance in isotype-
treated mice (Fig. 4B). However, there was considerable myeloid cell infiltration in a subpopulation of isotype-treated mice (Fig. 4C). IMC numbers positively correlated with infarct lesion size in isotype-treated animals without reaching statistical significance. In contrast, no correlation between infarct size and myeloid cell infiltration was observed in CD4-depleted mice, and even in animals with large infarcts only low numbers of IMC were found (Fig. 4D). Infiltration of myeloid cells into the ipsilateral hemisphere increased with decreasing microglia numbers in isotype-treated mice, but not in CD4-depleted animals (Fig. 4E).

Expression of specific markers for microglial activation (Cst7, Csf1, Ctsd, Apoe, Lpl, Ctsl) as well as microglial homeostasis (P2ry13, Hexb, Cx3cr1, Tmem119) were upregulated in the ischemic hemisphere compared to the contralateral hemisphere in both treatment groups equally (Fig. 4F).

Table 1

| Gene   | F:                                      | R:                                      |
|--------|-----------------------------------------|-----------------------------------------|
| Pax5   | CCGACTCTCGAGCCATGCGACAGCA              | GGGCTGAGACAGATGAGGGCA                   |
| Cst7   | AGTCCTAGCTTCAGCAAGAGCC                 | ATATAGAGTCGCCCTCAAGGCAG                |
| Csf1   | CTCTAGGCGGCGCATGTC                    | GCTCTGCTCATTCCACTTG                    |
| Ctsd   | ACTAGCTGCTGTCGAGCTAC                | CCGAGGCGGACTGAGGCAGGTC                 |
| Apoe   | GCTGAGCTGCTGAGCTGAGGTGTC              | TCTGGTCTGAGGTAGGTC                     |
| Lpl    | CTCGAGGCTGCGGCTCCTTTCC             | TCTGGTCTGAGGTAGGTC                     |
| Cx3Cr1 | GCTGAGACTGCGGTGTAAGGTCGTG             | GCTGACTGCTGAGGTAGGTC                   |
| Tmem119| CACCCAGAGCTGTTCCATAGC                  | GGTCTTCCGGGTGGTGGACT                   |

Fig. 2. Enhanced immune responses in 2D2 mice after MCAo. A) Representative immunostaining of CD4+ T cells that infiltrate the ischemic brain through the vascular basement membrane (laminin) in 2D2 mice and WT mice 14 days after MCAo. Scale bar = 50 µm. B) Flow cytometry quantification of CD4+ T cells and CD19+ cells in ipsilateral hemispheres of 2D2 (n = 3) and WT (n = 5) mice 14 days after MCAo (Mann-Whitney U Test). C) Infarct volumes measured by T2-weighted MRI on day 1 after MCAo in 2D2 (n = 31) and WT (n = 24) mice. D) Long-term survival after 60 min MCAo in 2D2 and WT mice (Log-Rank (Mantel-Cox) Test). Mice were sacrificed after 49 days (surviving/total mice used in the study: WT n = 16/17; 2D2 n = 15/21) or 72 days (WT n = 17/19; 2D2 n = 11/16).
3.4. Anti-CD4 treatment reduces brain infiltration of B cells 14 days after MCAo

Infiltrating B cells were not randomly distributed across the affected hemisphere at 14 days after MCAo but clustered in few areas of the infarct core reminiscent of ELS, while CD4 T cells scattered more widely in the surrounding area (Fig. 5A). ELS formation was observed in both isotype-treated and CD4-depleted mice, but B cell infiltration was strongly reduced after CD4 depletion at 14 days after MCAo (Fig. 5B-C).

We confirmed these findings by measurement of the B cell marker Pax5 in the ipsilateral hemisphere using qRT-PCR (Fig. 5D). Flow cytometry analysis also showed a significant reduction in CD19⁺ B cells and CD138⁺ plasmablasts/plasma cells (PC) after CD4 depletion compared to isotype-treatment (Fig. 5E). Only in isotype-treated but not CD4-depleted mice, infiltration of CD19⁺ cells into the brain positively correlated with infarct volumes measured on day 1 (Fig. 5F).
3.5. CD4+ T and B cells infiltrate the ischemic brain as soon as peripheral CD4+ T cell population recovers

Since peripheral CD4+ T cell recovery in CD4-depleted animals started about 28 days after MCAo and administration of CD4-depleting antibody between day 3 and 9 almost completely prevented ELS formation at day 14, we next wondered whether the delayed CD4 depletion had a long-lasting inhibitory effect on lymphocyte infiltration into the brain. In line with our hypothesis that T cells facilitate B cell infiltration, at day 49 and 72 after MCAo in both isotype- and anti-CD4-treated mice, we found high numbers of B cells within the ischemic hemisphere. In addition, we observed equal numbers of CD4+ T cells in both treatment groups (Fig. 6A) and a strong correlation with B cell infiltration after 49 days (Fig. 6B). Interestingly, lymphoid follicles were spacious and highly organized in both treatment groups at day 49 and 72, which may indicate an active adaptive immune response within the ischemic brain even more than 4 weeks after stroke onset (Fig. 6C). In fact, flow cytometry analysis of brain B cell subsets at day 49 and 72 demonstrated the presence of antibody-producing PC and GC-like B cells that express a distinct cell surface marker composition (GL7+IgD−) upon antigen-recognition indicating local B cell differentiation within ELS (Fig. 6D). This finding is supported by an immunostaining depicting IgM+ and IgG+ PC in proximity to ELS in both CD4-depleted and isotype-treated mice at day 72 after stroke (Fig. 6E). Representative images display unspecific staining in green (IgG) or red (IgM), which may be due to autoreactive antibodies produced by the respective PC subset binding to surrounding tissue.
A

B

C

D

E

F

(caption on next page)
3.6. Anti-CD4 treatment has no effect on infarct maturation or long-term survival after ischemic stroke but alleviates cognitive decline

To assess effects of anti-CD4 treatment on long-term outcome we determined infarct maturation by MRI over 10 weeks after stroke onset. Both treatment groups showed a significant decrease in infarct sizes from day 1 to day 14 after stroke and further infarct maturation was similar (Fig. 7A). In addition, we observed no differences in survival (Fig. 7B), weight loss, or body temperature (Supplementary Fig. 1C-D) between CD4-depleted and isotype-treated mice. As a previous study reported a link between infiltrating B cells and CNS antigen-specific autoantibody responses to cognitive impairment after stroke (Doyle et al., 2015), we next investigated whether CD4 depletion and the resulting reduction of brain B cell infiltration would alleviate cognitive decline after stroke. Using the number of arm entries in the Y-maze test to assess learning and memory abilities, we observed that sham-operated mice had progressively decreasing entries 2 weeks and 7 weeks after stroke when compared to baseline. While after 2 weeks all groups behaved similarly, after 7 weeks arm entries rose back to baseline level in isotype-treated mice with a significant difference to sham-operated mice. CD4-depleted mice had arm entry numbers in between sham-operated and isotype-treated animals at week 7 suggesting potential differences in cognitive function between CD4-depleted and isotype-treated animals in the long-term course after stroke (Fig. 7C). However, the effect was not statistically different between both groups at this time-point.

4. Discussion

Delayed infiltration of B cells into the ischemic brain has been linked to PSCI recently (Doyle et al., 2015). Using antibody-based CD4+ T cell depletion after MCAo in 2D2 mice, we demonstrated that B cell infiltration into the ischemic brain and their differentiation within lymphoid follicle-like structures depends on the presence of CD4+ T cells. Importantly, lymphocyte infiltration into the brain is a process lasting for at least 72 days after stroke onset. Finally, we provide further experimental evidence for a crucial role of lymphocytes in PSCI, which can be targeted by CD4+ T cell depletion.

According to current understanding, early ischemic injury is mediated primarily by myeloid immune cells and innate-like lymphocytes and therefore independent of antigen-specificity (Benakis et al., 2014; Kleinschnitz et al., 2010). However, modulation of the adaptive immunity by inducing CNS-specific tolerance or autoimmunity greatly influences stroke severity. It was shown that adaptive transfer of T cells reactive to the CNS-antigen MOG exacerbates early infarct lesions and stroke outcomes (Ren et al., 2012), whereas induction of immunologic tolerance to MOG prior to experimental stroke reduces the infarct size (Frenkel et al., 2003). Here, we demonstrate that 2D2 mice, in which more than 80% of peripheral CD4+ T cells express functional T cell receptors recognizing MOG, suffer from significantly larger infarcts already 1 day after MCAo when compared to WT. These results corroborate that antigen-specific immune responses by pre-existing memory T cells may boost lesions following cerebral ischemia. However, additional factors such as cerebrovascular anatomy, susceptibility to excitotoxicity and varying cytokine expression are mouse strain-specific and may contribute to the observed difference in infarct volumes (Knauss et al., 2020; Barone et al., 1993; Lamberts et al., 2002; Schauwecker and Steward, 1997). Greater infarct volumes in 2D2 mice were associated with higher mortality compared to WT mice, whereas no differences in infarct maturation or mortality were observed between CD4-depleted and isotype-treated mice. Therefore, we propose that survival and infarct volumes are determined by early rather than late CNS-specific immune responses.

We had previously characterized stroke-induced immunodepression (SIDS), a mechanism mediated by activation of the sympathetic nervous system (SNS) predisposing stroke patients to severe infection (Harms et al., 2008; Prass et al., 2003). It was shown that post-stroke infections favored by SIDS further exacerbate CNS-specific autoreactivity and impede recovery after human and experimental stroke (Becker et al., 2011, 2016b; Hoffmann et al., 2017). Importantly, MBP-specific immune responses worsened stroke patients’ outcome independently of infarct severity or age (Becker et al., 2011). Inhibiting the sympathetic nervous system by administration of propranolol prevented SIDS and post-stroke infections in WT and 2D2 mice similarly (Prass et al., 2003; Römer et al., 2015). In contrast, inhibition of SIDS resulted in enhanced CNS-specific T cell responses after stroke in 2D2 mice (Römer et al., 2015). Stroke-induced immunodepression may therefore promote contrary effects after stroke, protecting stroke patients from autoreactive responses to CNS antigens directly after acute injury while predisposing them to infections which in turn may exacerbate CNS autoreactivity.

Infiltration of activated lymphocytes following stroke has been recognized as a potential therapeutic target. In experimental stroke, combined deficiency of T and B cells as well as selective depletion of CD4+ or CD8+ T cells reduce lesion volumes (Hurn et al., 2007; Liesz et al., 2011). Depletion of regulatory CD4+ T cells (Treg) results in beneficial, deleterious or even no effects, depending on the stroke model and the time point investigated (Liesz et al., 2015; Stubbe et al., 2013). A multicenter preclinical trial demonstrated that antibody treatment directed against CD49d, an integrin-α subunit involved in leukocyte extravasation, reduced infarct volumes and leukocyte infiltration into the ischemic brain tissue after permanent but not transient MCAo (Llovera et al., 2015). Recent phase II clinical trials investigating anti-CD49d-treatment in human stroke patients failed in improving post-stroke long-term outcome (Elkins et al., 2017; Simats et al., 2016), which might be due to different timing of treatment.

Permanent CD4 T cell deficiency and early depletion after stroke onset have reduced infarct volumes consistently in experimental stroke models (Hurn et al., 2007; Kleinschnitz et al., 2013; Liesz et al., 2011). This is most likely due to early detrimental T cell effects within the ischemic brain following stroke (Gu et al., 2012; Kleinschnitz et al., 2010). On the other hand, peripheral T cells play a crucial role in protecting from post-stroke infections (Prass et al., 2003). Our experiments aimed at investigating delayed B cell infiltration and ELS formation. Considering our previous data on a delayed T cell infiltration into the ischemic brain (Stubbe et al., 2013) and in order to avoid interference with the early T cell effects, we established a depletion protocol eliminating CD4+ T cells between day 7 and 28. Using this setup we focused our investigation on delayed lymphocyte infiltration and autoreactivity without interfering with immediate or early immune responses. The observation of a “chronic” lymphocyte infiltration as late as day 49 and 72 after stroke was an unexpected finding of our study. This very important finding needs to be addressed in further...
longitudinal experiments.

Additional effects of the anti-CD4 treatment that are unrelated to CD4+ T cell reduction may influence leukocyte infiltration after stroke, for instance by altering the blood brain barrier. However, the observation that B cells infiltrate the ischemic brain at late time-points, once the CD4+ population recovers in the periphery, indicates that infiltration of CD4+ T cells and B cells are directly linked. It was recently found that interferon-γ (IFNγ) promotes transendothelial lymphocyte migration in the CNS by upregulation of endothelial adhesion molecules (Sonar et al., 2017). We had previously observed enhanced IFNγ production upon ex-vivo antigen stimulation in MOG-specific CD4+ T cells, suggesting that CD4 depletion may have further neuroprotective effects by preventing leukocyte transmigration across the blood brain barrier (Römer et al., 2015).

In accordance with very recently published data (Harris et al., 2020), delayed elimination of CD4+ T cells had no effect on long-term survival or infarct maturation in our experiments. Therefore, CD4+ T cells’ effect on infarct size seems limited to the acute phase of stroke. CD4+ T cell subsets such as T-helper (Th)17, follicular Th (TFH) and Treg exert distinct and partly opposing functions after stroke (Cramer et al., 2019). Global CD4+ T cell depletion approaches may therefore produce distorted results neglecting subset specific effects. For instance, previous findings indicate upregulation of regulatory cytokines such as IL-10 and TGF-β in MOG-specific Tregs isolated from the infarcted brain upon ex-vivo MOG stimulation. However, Tregs only made up less than 1% of peripheral CD4+ T cells in 2D2 mice before and after MCAo probably limiting their effect in stroke pathology (Römer et al., 2015). In addition, CD4- T cell populations such as IL-17-producing γδ T cells may also contribute to ischemic brain injury in a delayed manner and cannot be targeted by CD4 depletion (Shichita et al., 2009). In order to account for subset specific T cell functions in B cell infiltration after stroke, further experiments with more specific depletion or adoptive cell transfer approaches are required.

Delayed B lymphocyte accumulation in ELS in the ischemic brain after experimental stroke was recently linked to PSCI that could be prevented by genetic B cell deficiency or antibody-mediated B cell depletion (Doyle et al., 2015). Here, we demonstrate that CD4+ T cells are essential in B cell infiltration after stroke, further experiments with more specific depletion or adoptive cell transfer approaches are required.
cytokines and chemokines have been shown to promote B cell infiltration and ELS formation during chronic inflammation, including IL-17, lymhotoxin-α1β2 and various members of the CCL and CXCL family (Aloisi and Pujol-Borrell, 2006). CD4+ T cell subsets participate in lymphoid neogenesis during chronic inflammation either directly by secreting cytokines such as IL-17 or indirectly by promoting the expression of lymphoid chemokines in myeloid and stromal cells (Al-Kufaisy et al., 2017; Carlsen et al., 2004; Corsiero et al., 2012; Feige et al., 2014). In addition, we had previously described that CNS-infiltrating MOG-specific T cells produce IFN-γ following stroke (Klehm et al., 2016; Römer et al., 2015). Exposure to IFN-γ induces the expression of adhesion molecules in brain endothelial cells which enable transendothelial lymphocyte migration (Sonar et al., 2017).

The unique cellular composition of the CNS makes comparison between ELS formation in stroke and other autoimmune diseases difficult. Nevertheless, ELS formation in the meninges has been observed in multiple sclerosis (MS) (Serafini et al., 2004). As B cell depleting therapy is highly effective in relapsing-remitting MS, B cells and ELS are suspected to play a fundamental role in the pathogenesis of this disease as well (Naismith et al., 2010).

Here, we demonstrate that CD4+ T cells are essential for B cell infiltration and formation of ELS in the ischemic brain. As previously reported in WT mice (Doyle et al., 2015), we found that B cells aggregated in the ischemic tissue, forming dense clusters with surrounding T cells in 2D2 mice. However, B cell infiltration and ELS formation was earlier and more pronounced in 2D2 mice. Stroke complications such as hemorrhagic transformation (HT) could possibly aggravate lymphocyte infiltration. However, we have not observed HT in the present study suggesting that B and T cell infiltration occurs independently of HT. In fact, our findings in isotype-treated mice suggest that the magnitude of B cell infiltration depends on lesion size. Although CD4+ T cell depletion reduces B cell brain-infiltration to a large extent it did not prevent ELS formation entirely. This finding and further data discussed by Zbesko et al. in this issue indicate that additional CD4+ T cell independent mechanisms are involved in B cell infiltration (Zbesko et al., 2020).

Additionally, we found that CD4 depletion attenuates microglial loss in the ipsilateral hemisphere 14 days after MCAo independently of infarct size. Recruitment of IMC to the ischemic hemisphere was highly variable in isotype-treated mice, but myeloid cell infiltration in CD4-depleted mice was consistently low. IMC could facilitate ELS induction as monocyte-derived macrophages drive lymphoid neogenesis during autoimmunity by CXCL13 production (Carlsen et al., 2004). In experimental stroke, myeloid cell infiltration was considered to be limited to the acute and subacute phase of stroke. However, a second peak of T cells and macrophages in the CNS after experimental stroke was described recently, raising the possibility of interaction between T cells and macrophages contributing to ELS formation (Vindeggaard et al., 2017).

Consistent with previous reports, we observed post-ischemic microglial loss (Otxoa-de-AMezaga et al., 2018) and infiltration of myeloid cells, mainly monocytes/macrophages. Mechanisms linked directly to cerebral hypoxia account for the majority of microglial cell death during early stroke pathology (Eyo et al., 2013; Wang et al., 2017). However, at later time-points autoreactivity could contribute to this process, as immune responses directed against a number of CNS-specific antigens have been described after stroke (Ortega et al., 2015). Accordingly, compared to isotype-treated mice we observed higher numbers of microglia in the ipsilateral hemisphere in mice in which delayed CD4+ T cell responses were prevented by anti-CD4 treatment. This finding may suggest that prolonged autoreactive inflammatory CD4+ T cell responses could increase microglial loss after stroke. Despite the pronounced effect on myeloid cell numbers, CD4 depletion did not affect the expression of microglial homeostasis and activation markers after stroke. CD4+ T cell signals that could mediate microglia cell death are currently unknown and need further investigation.

We speculate that IMC compensate for post-ischemic microglial loss, especially in animals with large infarcts (Mildner et al., 2007). Indeed, we found that numbers of IMC increased with infarct volumes and decreased with the number of resident microglia. Both correlations were completely abrogated by CD4 depletion indicating an essential role for CD4+ T cells in delayed IMC recruitment after stroke. Considering the reduction of both, infiltrating B cells and myeloid cells by CD4 depletion, CD4+ T cells may either be involved in B cell and myeloid cell recruitment independently or promote myeloid cell infiltration, which in turn further facilitate B cell recruitment (Ortega et al., 2015).

Circulating CD4+ T cells were eliminated until day 28 after stroke but recovered after 49 days. Importantly, with the recovery of CD4+ T cells in the periphery, T as well as B lymphocyte infiltration into the brain started also in the depletion group, indicating that the cues for increased lymphocyte trafficking across the blood–brain barrier are present over a long period of time after ischemic brain injury.

Antibodies to neuronal antigens worsen stroke patients’ functional outcome (Becker et al., 2016a). B cell expansion, hypermutation and differentiation to antibody-producing PC occur in the GCs of ELS (Corsiero et al., 2016). The presence of lymphocytes expressing GC B cell and PC markers in brains 49 and 72 days after MCAo suggests that these GC-specific processes leading to autoreactive CNS antigen-specific antibody production also occur in stroke. Conclusively, we observed IgG and IgM producing PC in proximity to ELS. The antigen specificity of these antibodies remains yet to be determined. Unspecific IgM and IgG staining in the infarcted tissue suggest reactivity to multiple target antigens however. We also observed CD138+ IgM+ IgG- PC that could possibly produce IgA as described (Zbesko et al., 2020).

Production of CNS-specific antibodies may contribute to PSCI (Becker et al., 2016a), which could be prevented by antibody-based B cell depletion or primary B cell deficiency in an experimental setup (Doyle et al., 2015). Increased B cell numbers and IgG bound to brain tissue were also observed in post-mortem brain samples analyzed from stroke patients with dementia compared to those without dementia (Doyle et al., 2015). To our knowledge, ELS have not been reported in brain tissue after stroke in humans, yet. This may be due to the fact that ELS in experimental stroke can only be detected in the core area of ischemia. However, tissue samples from patients who have died after a stroke are usually not collected from these areas during autopsy for the brain banks. Interestingly, a recent study using B cell depletion suggests that B cells contribute to post-stroke recovery by promoting neurogenesis (Ortega et al., 2020). In our hands, anti-CD4 treatment reduced B cell infiltration only temporarily. Even though CD4+ T cells and B cells infiltrated the CNS also in the depletion group at later time points, our behavioral data suggest that CD4+ T cell depletion could improve long-term functional outcomes after stroke.

Altogether, our results indicate that B cell infiltration into the CNS after experimental stroke is facilitated by CD4+ T cell-mediated responses, which might include additional responses by microglia and infiltrating peripheral myeloid cells. Furthermore, we demonstrate that post-stroke lymphocyte infiltration is a long-lasting process that could serve as a target to prevent PSCI.

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Appendix A. Supplementary data

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