Microglia contributes to remyelination in cerebral but not spinal cord ischemia

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Abstract
Inflammation after injury of the central nervous system (CNS) is increasingly viewed as a therapeutic target. However, comparative studies in different CNS compartments are sparse. To date only few studies based on immunohistochemical data and all referring to mechanical injury have directly compared inflammation in different CNS compartments. These studies revealed that inflammation is more pronounced in spinal cord than in brain. Therefore, it is unclear whether concepts and treatments established in the cerebral cortex can be transferred to spinal cord lesions and vice versa or whether immunological treatments must be adapted to different CNS compartments. By use of transcriptomic and flow cytometry analysis of equally sized photothrombotically induced lesions in the cerebral cortex and the spinal cord, we could document an overall comparable inflammatory reaction and repair activity in brain and spinal cord between day 1 and day 7 after ischemia. However, remyelination was increased after cerebral versus spinal cord ischemia which is in line with increased remyelination in gray matter in previous analyses and was accompanied by microglia dominated inflammation opposed to monocytes/macrophages dominated inflammation after spinal cord ischemia. Interestingly remyelination could be reduced by microglia and not hematogenous macrophage depletion. Our results show that despite different cellular composition of the post-ischemic infiltrate the inflammatory response in cerebral cortex and spinal cord are comparable between day 1 and day 7. A striking difference was higher remyelination capacity in the cerebral cortex, which seems to be supported by microglia dominance.

KEYWORDS
brain ischemia, inflammation, photothrombosis, remyelination, spinal cord ischemia, stroke

1 INTRODUCTION

Tissue injury triggers an inflammatory cascade, which is dominated in subacute stages by cells of the innate immune response. Depending on the scope and resolution of the analysis, inflammation is viewed as highly conserved with similar patterns of immune cell activation and cytokine production independent of the tissue involved (Oishi & Manabe, 2018; Seemann et al., 2017) or as individualized reaction...
dependent on the tissue or even the exact localization in a given tissue (Schultze, 2016).

The immune system of the central nervous system (CNS) has traditionally been regarded as an exception being segregated by several barriers from the immune system which is surveilling the organism (Louveau et al., 2015). Recently this view has been challenged by the discovery of a lymphatic system in the murine CNS (Louveau et al., 2018). However, the CNS is at least recognized as being surveilled by a specialized part of the immune system namely the microglia. Given a similar CNS pathology, it is unclear whether inflammation in the CNS can be regarded as a stereotyped reaction or should be viewed as tissue composition dependent and thus different in every CNS compartment. From a clinical perspective, anti-inflammatory treatments to date are not adapted to the CNS compartment, for example, treatment of an MS lesion is similar independent of its localization in the brain or spinal cord (Hart & Bainbridge, 2016).

Only few data exist allowing the direct comparison of the inflammatory response in different CNS compartments. Three studies have revealed a more vigorous inflammatory response after spinal as compared to cerebral traumatic injury based on immunohistochemical and real-time polymerase chain reaction data (Batchelor et al., 2008; Schnell, Fearn, Klassen, et al., 1999; Schnell, Fearn, Schwab, et al., 1999). Furthermore, a recent study compared multiple sclerosis lesions in white and gray matter and revealed increased inflammation in the white matter part just adjacent to the gray matter indicating that the cellular composition of the CNS compartment might have an influence on inflammation (Prins et al., 2015). This finding was corroborated in the cuprizone demyelination model (Buschmann et al., 2012). Moreover it has been shown that phagocytotic activity is greater in white matter versus gray matter (Zrzavy et al., 2018).

As neuroimmunomodulatory treatments enter the clinics and are used in a wide range of indications comprising of course multiple sclerosis but are also in experimental settings extended to for example, ischemic lesions, we were interested to further elucidate in a highly reproducible model whether the inflammatory response differs in different compartments and is tissue composition dependent (e.g., white matter versus gray matter, astrocyte, oligodendrocyte (OL) content). In order to ensure identical pathomechanism of lesion induction with low mortality we undertook the present study in models of photothrombotic cerebral and spinal cord injury, which are non-invasive and highly reproducible in both locations (Gliem et al., 2015; Li et al., 2015). A different inflammatory response should in this case trigger specific therapies in different CNS compartments, but similar inflammatory response in different CNS compartments could lead to development of new strategies in patient treatment independent of CNS compartment.

2 | RESULTS

2.1 | Comparable lesion size in brain and spinal cord after ischemia

We used photothrombotically induced ischemia in mice to compare inflammation in subacute stages in brain versus spinal cord as lesions in this model are highly reproducible and do not require major manipulation of the tissue (Figure 1a). Duration of cold light exposure was optimized in order to achieve comparable lesion extent at both sites and to avoid excessive mortality in the spinal cord model. Cresyl violet staining showed stronger cellular accumulation at lesion site at day 14 after ischemia in spinal cord versus brain (Figure 1b). Furthermore, comparison of lesion diameter indicated a significantly lower size at day 14 compared to day 1 after ischemia in brain (Figure 1c) and non-significant results in spinal cord (Figure 1d).

2.2 | Similar inflammation in brain and spinal cord after ischemia

To determine the inflammatory pattern induced by ischemia in different tissues of the CNS, we performed a microarray analysis in brain and spinal cord at day 1, 3 and 7 after ischemia and compared data to control.

Principal component analysis of the 22,206 genes clearly segregated brain (cPT) and spinal cord ischemia from their control groups, indicating differentially regulated genes in all groups (Figure 2a). Under control conditions we could detect a higher level of typical gray matter markers like dopamine, dendrin and neurogranin. On the other hand in spinal cord we found a higher expression of myelin markers (Mbp, Mog and Mobp) and astroglial markers (S100, Gfap and Aqp4) confirming the different composition of both CNS compartments. In order to eliminate these preexisting differences, we made a second analysis comparing only the regulated genes between control condition and at day 1, 3 and 7 after ischemia between photothrombosis in the brain cortex and the spinal cord. Heat map analysis of inflammation markers did not reveal qualitative differences in regulation, with slightly stronger expression in spinal cord versus brain after ischemia in time course experiments (Figure 2b). Furthermore, RT-PCR analysis of inflammatory genes TNFα, iNOS and Cd32 showed similar regulation pattern by comparing both tissues brain and spinal cord after ischemia, indicating same inflammatory response in the two different tissues of the CNS (Figure 2c). Additionally, a comparison of repair (Figure S1(a)) and phagocytotic marker (Figure S1(b)) did not reveal qualitative differences. The phagocytotic activity was more pronounced in spinal cord as determined by histological (Figure S1(c),(d)) and RT-PCR (Figure S1(e)) analysis of the phagocytotic marker Cdh68. Even a comparison by ingenuity pathway analysis software showed no qualitative difference in regulation of canonical pathways between brain and spinal cord after ischemia. There was only a difference in level of activity with higher pathway activity in spinal cord versus brain 1 (Figure S1(f)), 3 (Figure S1(g)) and 7 (Figure S2d) days after ischemia as shown by comparison of Top 10 canonical pathways.

2.3 | Differential infiltration of hematogenic and resident cells after brain and spinal cord ischemia

Next, we analyzed which cell population is responsible for inflammatory response in CNS after ischemia by comparison of unique
microglia and monocytes (Butovsky et al., 2014; Gautier et al., 2012) cell marker of our microarray analysis. We could reveal a difference between both tissues with stronger microglia marker expression in brain and stronger monocytes marker expression in spinal cord (Figure 3a). We confirmed these results by further RT-PCR analysis of Arg1 (arginase 1) and Fcgr4 (Fc receptor IgG low affinity IV), that are only expressed by hematogenous MO/MP. The analysis showed lower expression in brain compared to spinal cord after ischemia (Figure 3b). Additionally, a RT-PCR analysis of specific microglia marker P2ry12 (purinergic receptor P2Y, G-protein coupled, 12) and Slc2a5 (solute carrier family 2 member 5) showed stronger P2ry12 and Slc2a5 expression in brain than in spinal cord. Surprisingly both markers were downregulated (P2ry12 (0.6-fold in brain versus 0.2-fold in spinal cord) and Slc2a5 (0.2-fold in spinal cord)) at day 1 after ischemia with stronger downregulation in spinal cord versus brain indicating that microglia loss after lesion induction was better compensated by brain tissue or/and that differentiation of P2ry12<sup>+</sup> homeostatic microglia into inflammatory/repair microglia subsets is more pronounced in spinal cord (Figure 3c).

**FIGURE 1** (a) Schematic protocol of cerebral(cPT) and spinal cord photothrombosis (scPT). (b) Representative images of cresyl violet staining in brain and spinal cord at day 1 to 14 after injury. (c) Brain and (d) spinal cord lesion diameter quantification. Data show mean ± SEM (n = 3–6), *p < .05 (one-way ANOVA with Tukey’s multiple comparison test). Scale bar = 500 μm
Furthermore, the use of bone marrow chimeric mice (Cd45.1), generated via total body irradiation and subsequent transplantation of donor bone marrow with a distinct marker profile (Cd45.2), allowed us to differentiate resident microglia cells (MG, Cd45.1+) from hematogenous monocytes and macrophages (MO/MP, Cd45.2+) in a FACS analysis (Figure 3d). In addition to microglia and hematogenous MO/MP we analyzed neutrophils and Cd11b− cells in a time course experiment in brain (Figure 3e) and in spinal cord (Figure 3f).
Legend on next page.
In accordance with our microarray analysis of unique cell markers we found increased infiltration of microglia in brain compared to spinal cord with greatest difference at day 7 after ischemia with 89.8 ± 0.48 × 10^3 cells per mg tissue in brain versus 20.5 ± 3.16 × 10^3 cells per mg tissue in spinal cord (Figure 3g). Furthermore, infiltration of hematogenous MO/MP was lower in brain than in spinal cord. As in microglia recruitment the greatest difference was also at day 7 after ischemia with 13.85 ± 1.06 × 10^3 cells per mg tissue in brain versus 148.2 ± 34.69 × 10^3 cells per mg tissue in spinal cord (Figure 3h). Similar to hematogenous MO/MP recruitment we could detect stronger neutrophils recruitment in spinal cord than in brain after ischemia. Greatest difference was found at day 7 after ischemia with 46.09 ± 20.06 × 10^3 cells per mg tissue in spinal cord and only 1.43 ± 0.14 × 10^3 cells per mg tissue in brain. Recruitment of Cd11b^+ cells was not significantly different between brain and spinal cord after ischemia.

### 2.4 Differential myelin compensating mechanism in CNS

As microglia dominated the inflammatory cell population after cerebral photothrombosis but not after spinal cord photoinjury and recent papers imply a role of microglia in myelogenesis and OL precursor maintenance (Hagemeyer et al., 2017) we analyzed genes associated with myelination (Fernandez-Castaneda et al., 2020). We found an upregulation of myelin marker expression in brain and a down-regulation in spinal cord between day 1 and 7 after ischemia (Figure 4a). This was paralleled by the recruitment of Sox10 positive OLs accumulating at the border zone of the infarcted tissue expressing Bcas1 as an indicator for active remyelination (Figure 4b). In a time course experiment the number of Bcas1/Sox10 double positive cells reached its peak at day 7 (Figure 4c).

### 2.5 Inhibition of remyelination by inducing microglia depletion

To test whether microglia predominance and enhanced remyelination in the cerebral cortex are linked, we performed depletion experiments in mice by using pexidartinib (PLX3397), a Csf1r inhibitor to deplete microglia (Szalay et al., 2016) and clodronate liposomes (CloLip), which specifically deplete hematogenous MO/MP after phagocytosis. We fed our mice for 3 weeks with PLX3397 to develop a sufficient depletion of microglia or we injected CloLip at day 1 and 2 after ischemia to achieve a sufficient depletion of hematogenous MO/MP and afterwards performed 3 days after ischemia a reverse transcription polymerase chain reaction (RT-PCR) and 7 days after ischemia histological staining to analyze myelination marker expression (Figure 5a).

Microglia was depleted by over 70% in PLX3397 treated mice (Figure 5b). Successful depletion of hematogenous MO/MP was already proved in our previous work (Gliem et al., 2012). In following RT-PCR analyses we could detect a significant downregulation of myelination marker Olig2, Myrf, and Nkx2.2 3 days after ischemia in brains of microglia depleted mice indicating that microglia depletion interferes with upregulation of myelination markers in brain after ischemia. Furthermore, we also detected a significant downregulation of myelination inhibitor marker Hes1 and Id4 indicating some autocrine regulation (Figure 5c). Next, we were interested in the role of hematogenous MO/MP in brain after ischemia. We observed opposing results with significant upregulation of myelination marker Sox10 and Myrf, indicating that hematogenous MO/MP do not play a main role in upregulation of myelination in brain after ischemia but could play an inhibitory role. Therefore, we also analyzed expression of myelin inhibitors after depletion of hematogenous MO/MP in brain after ischemia but we could not detect any significant differences concerning control and hematogenous MO/MP depleted brains (Figure 5d).

Additionally, we performed immunohistochemical analysis of myelinating OLs marker Bcas1 and the oligodendroglial lineage marker Sox10 in non-treated, microglia depleted and hematogenous MO/MP depleted mice at day 7 after ischemia. We observed that PLX3397 led to downregulation of Bcas1/Sox10 double positive cells in the infarct border zone (Figure 5e) whereas CloLip left cell numbers unchanged (Figure 5f) indicating that microglia but not hematogenous MO/MP supported remyelination after cortical ischemia.

In conclusion our data demonstrate that similar lesion mechanisms lead to a distinct cellular recruitment but comparable responses on a transcriptomic level in different CNS compartments in case of inflammation, repair and phagocytosis. Interestingly there was a difference in myelination, which could be linked with microglia predominance and deserves further studies.

### 3 DISCUSSION

The inflammatory response after injury is viewed as a promising target for reparative and neuroregenerative therapies not only in...
inflammatory but also in ischemically lesioned tissues. Consequently, clinical studies with immunomodulatory agents after stroke are underway. Whether the immunomodulatory therapy not just after stroke but also in other CNS pathologies should be adjusted to the localization of the lesion and the cellular composition of the surrounding tissue is unclear.

We used the photothrombotic ischemia model to induce ischemic lesions in brain and spinal cord and could achieve similar lesion diameter by optimizing duration of cold light exposure. We performed a direct comparison of inflammation in subacute stages in the cerebral cortex and spinal cord as both tissues differ concerning the cellular composition and amount of gray and white matter.

![Heat map and images showing myelination marker expression and Bcas1 and Sox10 staining in murine brain at day 7 after ischemia.](image)

**FIGURE 4** (a) Heat map of myelination marker in brain versus spinal cord sorted by correlation with i data sets from Fernández-Castañeda et al. and ii further known myelination marker. The scaled expression value is shown in a blue-red color scheme with red indicating higher expression, and blue lower expression (n = 4). (b) Representative images of Bcas1 (red) and Sox10 (green) staining in murine brain at day 7 after ischemia. Inserts show enlargement of double-positive cells indicated by white box. Scale bar = 50 μm. (c) Analysis of the total cell number of Bcas1 and Sox10 double positive cells in mice after ischemia over time (n = 6–8; one-way ANOVA with Tukey’s multiple comparison test). Data show mean ± SEM, **p < .01, ***p < .001, ****p < .0001
Figure 5: Legend on next page.
By FACS analysis of bone marrow chimeric animals, we could distinguish between hematogenously invaded MO/MP and resident microglia. In this regard our study is the first study, which is able to dissect the contribution of hematogenous MO/MP from microglia in a comparative analysis between spinal cord and cerebral cortical lesion. While hematogenous MO/MP were the dominant cell population after spinal cord ischemia with a peak at day 7, microglia dominated after cerebral ischemia and increased until day 14. Interestingly, the microglia population after spinal cord ischemia decreased until day 7 and then slowly recovered. Our data collected after cerebral ischemia are in accordance with data reported by Gelderblom et al. (2009) and our own previously published data (Gliem et al., 2012). Data after spinal cord ischemia are well in line with existing data from spinal cord injury (Jin et al., 2012) and with existing data from traumatic brain injury (Mawhinney et al., 2012), which in a synopsis show also a predominance of hematogenous MO/MP over microglia after spinal cord injury.

Furthermore, we could document a greater neutrophil invasion in the spinal cord and a peak of neutrophil accumulation at day 3 after both, cortical and spinal ischemia. The larger magnitude of the neutrophil accumulation has also been reported in comparative analyses between traumatic brain injury and spinal cord injury (Schnell, Fearn, Klassen, et al., 1999). Neutrophils are predominantly associated with exacerbation of ischemic brain damage and deleterious effects on repair and regeneration (Herz et al., 2015), which would imply a higher degree of destruction in the spinal cord. Interestingly experimental autoimmune encephalomyelitis, a multiple sclerosis model, is also showing stronger neutrophils recruitment in spinal cord than brain, but spinal cord injury showed much less dependence on neutrophil recruitment (Simmons et al., 2014). The mechanism of this regulation is to date unclear.

Although cell recruitment was different between both tissues, and growing evidence underlines the fundamental differences between different cell types for example, between monocytes and microglia (Butovsky et al., 2014; Ginhoux et al., 2010), ischemia in both tissues triggers an inflammatory response that was paralleled from its very beginning on day 1 until day 7 by upregulation of reparative markers. Using standard analysis software, prespecified canonical pathway analyses and published transcriptome signatures for inflammatory markers. Regulation of most genes was slightly increased in spinal cord specimen, like phagocytotic markers, which may be due to increased clearance of myelin debris in the spinal cord, but the overall pattern was comparable. Our data are well in line with data from spinal cord injury (Kigerl et al., 2009; Shechter et al., 2009) compared to data from traumatic brain injury (Walker et al., 2012), showing a simultaneous evolution of an inflammatory and reparative response until day 7. In both injury models the inflammatory signature persists whereas the reparative signature is reduced beyond day 7, leaving an inflammatory surrounding which is suboptimal for regeneration and repair.

Adversely, a prespecified myelination panel previously established by Fernandez-Castaneda et al. (2020) showed a higher expression of most of these markers after cortical cerebral ischemia. Correspondingly, remyelination in multiple sclerosis is faster and consistently more extensive in gray matter lesions than in white matter lesions (Albert et al., 2007). This contrasts to a higher density of remyelinating OLs in white matter versus gray matter, while the density of non-myelinating oligodendrocyte precursor cells (OPCs) is much higher in gray matter lesions versus white matter lesions (Strijbis et al., 2017). A possible explanation might be that gray matter OPCs are less mature, proliferate more and differentiate slower than white matter OPCs, and therefore gray matter OPCs are less sensitive to IFNγ mediated inhibition of OPC differentiation (Lentferink et al., 2018), indicating their stronger remyelinating ability by being less sensitive to inhibiting signal cascades.

Furthermore, recent papers link myelinogenesis to postnatal microglia (Włodarczyk et al., 2017) and could document a role of microglia for OL progenitor maintenance (Hagemeyer et al., 2017). Therefore, we performed depletion experiments by using pexidartinib to deplete microglia and clodronate liposomes to deplete MO/MP to attribute this remyelinating process to one of dominating cell populations in brain ischemia. We could show that microglia depletion led to downregulation of myelination marker Olig2, Myrf and Nkx2.2, while MO/MP depletion did not influence these markers. Furthermore, microglia depletion led to a reduction of Bcas1/Sox10 (Fard et al., 2017) double positive cells, affirming a role for microglia in remyelination after ischemia.

Remyelination of hematogenous MO/MP on the other hand did not reduce remyelination after cerebral ischemia but on the other hand on RNA level even seemed to increase remyelination capacity. A possible explanation might be the role of hematogenous MO/MP in building
up the astroglial and fibrotic scar (Gliem et al., 2012; Gliem et al., 2015), which both interfere with remyelination.

Taken together our study provides evidence for the first time that the inflammatory transcriptome is similar in different CNS compartments if the lesion mechanism is comparable, even though the composition of recruited cells is different. Additionally, we show for the first time that the remyelination process is depending on microglia activity. Consequently, regulation of microglia activity could be a promising therapeutic approach to enhance regeneration. Further studies are necessary to research the mechanisms behind this interaction.

4 | MATERIALS AND METHODS

4.1 | Animals

All animal experiments were approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Approval Nr. 84-02.04.2014.A450) and carried out in accordance with international guidelines on handling laboratory animals. We used male wild-type (WT) C57BL/6J mice (Janvier, Le Genest Saint Isle, France), mice expressing the allotypic marker CD45.1 (WT [CD45.1]) (B6.SJL-PtpcrPepcb/J) and CCR2 knock-out (CCR2−/−) (B6.129S4-Ccr2tm1Ifc/J) mice (all knock-out and transgenic mice were from Jackson Laboratory, Bar Harbor, ME). At the beginning of experiments mice weighted 20–25 g, were 6–12 weeks of age and were used in groups of three to six mice. They were anesthetized for induction with 5% isoflurane and maintained in 1.5%–2% isoflurane in 67% N₂O and 33% O₂ using a vaporizer. Body temperature was maintained at 37°C using a servo-controlled heating pad.

4.2 | Induction of cerebral ischemia

Cerebral ischemia was induced by cortical photothrombosis (cPT). A fiber optic bundle coupled to a cold light source (Schott EL 1500, Mainz, Germany) was centered 2 mm posterior and 2.4 mm laterally from bregma. After intraperitoneal injection of 1 mg Rose Bengal (Sigma Aldrich, St. Louis, MO) the brain was illuminated through the intact skull for 15 min. After illumination was stopped, the skin was sealed with the skin adhesive Histoacyrl® and the animal allowed to awake.

4.3 | Spinal cord ischemia

Spinal cord ischemia was induced by photothrombosis with a fiber optic bundle coupled to a cold light source. The spine was exposed between thoracic level 8 and 9 (T8 and 9) and a cold light source was centered between them. After intraperitoneal injection of 1 mg Rose Bengal the spinal cord was illuminated for 5 min to reach a comparable lesion size. After illumination, the skin was sutured and additionally sealed with the skin adhesive Histoacryl® and the animal allowed to awake.

4.4 | Generation of bone marrow (BM) chimeric mice

BM chimeras were generated as described previously (Mildner et al., 2007). Wild-type recipient mice (WT [Cd45.1]) received total body irradiation with 10.5 Gray while the brain was shielded. They were subsequently reconstituted with donor BM cells (WT [Cd45.2] or CCR2−/− [Cd45.2]), that were prepared from hind leg tibias and femurs. Chimerism was confirmed by flow cytometry 7 weeks after irradiation and mice with >80% donor blood cells (Cd11b+Cd45.2+) were used for further experiments.

4.5 | Depletion of hematogenous MO/MP and microglia

For depletion of hematogenous MO/MP in mice, 0.1 ml per 10 g weight of clodronate filled liposome suspension was injected intraperitoneally at days 1 and 2 after stroke. This technique allowed depletion until day 7. PBS-filled liposomes were used in the control groups. For depletion of microglia, mice were fed with 290 mg/kg PLX3397 in standard chow for 21 days to deplete microglia. Control groups were fed with placebo in standard chow.

4.6 | Immunohistochemistry

Mice were euthanized with isoflurane and perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains and spinal cords were carefully removed after the perfusion and fixed in PFA for another 24 h. Tissue samples were then washed in PBS and stored at 4°C until the embedding in paraffin. Paraffin sections were stained with polyclonal goat anti-bovine/human collagen-4 (Meridian Life Science, Saco, ME), polyclonal rabbit anti-mouse GFAP (Sigma, Munich, Germany), and monoclonal rat anti-mouse CD68 (Biorad, Munich, Germany) and analyzed by ImageJ software (NIH, Bethesda, MD).

4.7 | Flow cytometry

Mice were euthanized with isoflurane and perfused with PBS. Brain and spinal cord samples were collected in medium. After homogenization Collagenase/Dispase/DNase I enzyme mix (10 mg/ml) was added and incubated for 30 min. After the incubation, the resulting cell suspension passed through 70 μm cell strainer. The cells were washed and centrifuged at 4°C, 300 g, for 10 min. The supernatant was decanted and the pellet was resuspended in fluorescence activated
cell sorting (FACS) buffer and prepared for FACS analysis. The cell suspensions were incubated with various combinations of antibodies.

All antibodies used were purchased from Mylenyi Biotec (Bergisch Gladbach, Germany), BD Biosciences (Heidelberg, Germany) or Biolegend (San Diego, CA): Cd45, Cd45.1, Cd45.2, Cd11b, F4/80, Ly6g, NK1.1, Ly6c. Data were acquired on a flow cytometer (BD FACSCanto™ II) and analyzed with the FlowJo software (Tree Star, Ashland, OR).

For FACS analysis the flow cytometer MoFlo™ XDP (Beckman Coulter, Germany) was configured to detect with 488 nm excitation (100 mW) antibody-Ly6g-FITC signal in FL1 (521/28 bandpass filter), anti-Cd11b-PE in FL2 (575/25 bandpass filter), propidiumiodid in FL4 (670/30 bandpass filter), and anti-NK1.1-PE-Cy7 in FL5 (785/62 bandpass filter). Anti-CD45-APC-Cy7 was excited with 633 nm Laser (80 mW) and emit in FL12 (785/62 bandpass filter). Gating strategy was set to exclude debris with FSC/SSC. The exclusion of doublets was done by SSC Width/SSC Height parameters. We include living cells based on propidiumiodid negative population.

4.8 | RNA analysis

For RNA analysis tissue samples were collected directly after the euthanasia and snap frozen. Total RNA was extracted using the TRizol™ reagent (Thermofisher Scientific, Waltham, MA). RNA quality and concentration were quantified by a Nanodrop spectrophotometer (Thermofisher Scientific), and samples were stored at −80°C until analysis. Analysis of total RNA was performed by quantitative real-time PCR as described previously (Schroeter, Kury, & Jander, 2003). Relative gene expression levels were determined according to the AΔΔct method. Sequence of primers are shown in Table S1. Microarray analysis was performed in Genomics & Transcriptomics Labor, at the BMFZ facility in Duesseldorf, Germany. The totalRNA samples used for transcriptome analyses were quantified and quality measured by capillary electrophoresis Bioanalyzer assay (Eukaryote Total RNA Pico, Agilent). Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturer’s protocol (GeneChip® Pico Reagent Kit 703308 Rev. 4; ThermoFisher scientific). Briefly, 10 ng of total RNA were converted to cDNA, amplified to complementary RNA (cRNA) followed by in vitro transcription and biotin labeling of cDNA. After fragmentation labeled cDNA was hybridized to Applied Biosystems™ Clariom™ S Mouse Gene Expression Microarrays for 16 h at 45°C, stained by streptavidin/phycoerythrin conjugate, and scanned as described in the manufacturer’s protocol. Data analyses on Affymetrix CEL files were conducted with GeneSpring GX software (Vers. 12.5; Agilent Technologies). Probes within each probe set were summarized by GeneSprings' ExonRMA16 algorithm after quantile normalization of probe-level signal intensities across all samples to reduce interarray variability (Bolstad, Irizarry, Astrand, & Speed, 2003). Input data preprocessing was concluded by baseline transformation to the median of all samples. After grouping of samples (for biological replicates each) according to their respective experimental condition, a given probe set had to be expressed above background (i.e., fluorescence signal of that probe set was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in three replicates in at least one of three conditions to be further analyzed in pairwise comparisons. Differential gene expression was statistically determined by Moderated t test. The Resulting P values were corrected for multiple testing by Benjamini-Hochberg-correction. A p value of ≤.05 was considered significant. Hierarchical cluster analysis was performed with Euclidian similarity measures and Ward’s linkage. The data was further evaluated with the Ingenuity-Pathway analysis software (Qiagen Inc., Release 2016).

4.9 | Statistical analysis

All values are expressed as means ± SEM. Statistical analyses between groups were performed by unpaired Student’s t test or one-way ANOVA followed by a Bonferroni post hoc test. A value of p < .05 was regarded as statistically significant.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

Goran Pavic designed contributed to the conception and design of the study and executed experiments, gathered and analyzed data, constructed figures and wrote the manuscript. Patrick Petzsch and Karl Köhrer executed microarray experiments, gathered and analyzed data. Robin Jansen and Nicole Rychlik executed experiments, gathered and analyzed data. Katharina Raba executed FACS sorting experiments, gathered and analyzed data. Ioannis Simiantonakis executed irradiation experiments and gathered data. Sven G. Meuth, Hans-Peter Hartung, Peter Göttle and Patrick Küry contributed to the conception and design of the study and writing of the manuscript. Sebastian Jander contributed to the conception and design of the study, analyzed data and contributed to writing of the manuscript. Michael Gliem contributed to the conception and design of the study, designed experiments, constructed figures and wrote the manuscript.

DATA AVAILABILITY STATEMENT

Raw and processed microarray data is registered under NCBI GEO Expression Omnibus with the accession number GSE167274 and can
be accessed using the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167274.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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