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MICROGLIA IN THE JUVENILE BRAIN AFTER CRANIAL IRRADIATION

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Microglia in the juvenile brain after cranial irradiation

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To my readers

ABSTRACT

Radiotherapy is used to treat pediatric brain tumors and is often accompanied with debilitating late effects, such as cognitive decline. However, the mechanisms are poorly understood, and consequently there is still no effective treatment. The inflammatory response and microglia activation could change the micro-environment and effect neurogenesis resulting in impairment, and this seems to be one important factor eventually leading to cognitive decline.

Lithium has been used clinically many years for bipolar disorder, recent studies revealed neuroprotective effects of lithium. In the current thesis we show that lithium normalized irradiation induced neural precursor cell death, neurogenesis impairment, inflammation and cognitive decline without obvious side effects. Further studies demonstrated transient microglial activation and long-term microglia loss after irradiation. To further characterize the microglial response after irradiation, our bulk and single cell RNA sequencing data revealed that microglial activation returned towards normal levels 1 week after irradiation, indicating that microglia activation alone did not sustain the chronic inflammation after irradiation in the juvenile hippocampus. So therapeutic interventions aimed at targeting the chronic, detrimental inflammation after cranial radiotherapy may be required prior to or during radiotherapy in a clinical setting.

Microglia are involved in almost all CNS diseases either directly or indirectly. Microglial depletion and repopulation have shown beneficial effects in many animal disease models; however, the signals regulating the microglia repopulation are poorly understood. Here we demonstrate that CX3CR1 could regulate residential microglia repopulation and their competition with peripheral infiltrating monocytes. Hence, CX3CR1-CX3CL1 axis could be manipulated for modulation of repopulation after microglial depletion.

In summary, our studies firstly demonstrate that lithium is a promising novel treatment for radiotherapy-induced intellectual impairment, secondly that there is a narrow time window for microglial therapeutic interventions after radiotherapy, and finally that CX3CR1 is crucial in the regulation of microglial repopulation after depletion. The knowledge generated in this thesis provides the foundation for further research, including clinical trial.

LIST OF SCIENTIFIC PAPERS

- I. **Kai Zhou**, Cuicui Xie, Malin Wickström, Amalia Dolga, Yaodong Zhang, Tao Li, Yiran Xu, Carsten Culmsee, Changlian Zhu[#], Klas Blomgren[#]. Lithium protects hippocampal progenitors, cognitive performance and hypothalamus-pituitary function after irradiation to the juvenile rat brain. *Oncotarget*, 2017, 8(21), 34111-34127
- II. Wei Han, Takashi Umekawa, **Kai Zhou**, Xing-Mei Zhang, Makiko Ohshima, Cecilia A Dominguez, Robert Harris, Changlian Zhu[#], Klas Blomgren[#]. Cranial irradiation induces transient microglia accumulation, followed by long-lasting inflammation and loss of microglia. *Oncotarget*, 2016, 7(50), 82305-82323
- III. Ahmed M Osman, Ying Sun, Terry C Burns, Liqun He, Nigel Kee, Nuria Oliva-Vilarnau, Androniki Alevyzaki, **Kai Zhou**, Lauri Louhivuori, Per Uhlén, Eva Hedlund, Christer Betsholtz, Volker M. Lauschke, Julianna Kele, and Klas Blomgren. Radiation triggers a dynamic sequence of transient microglial alterations in juvenile brain. Revised version resubmitted to *Cell Reports*.
- IV. **Kai Zhou**, Jinming Han, Harald Lund, Shinobu Goto, Volker Lauschke, Nageswara Rao Boggavarapu, Ahmed Osman, Yuyu Wang, Dong liang, Cuicui Xie, Asuka Tachi, Ying Sun, Wei Han, Kristina Gemzell-Danielsson, Christer Betsholtz, Xing-Mei Zhang, Changlian Zhu, Bertrand Joseph, Robert Harris, Klas Blomgren. Repopulation of the microglial niche after genetic depletion is regulated by CX3CL1-CX3CR1 signaling. Manuscript

Shared senior authorship

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

- I. **Kai Zhou**, Martina Boström, Carl Joakim Ek, Tao Li, Cuicui Xie, Yiran Xu, Yanyan Sun, Klas Blomgren, Changlian Zhu. Radiation induces progenitor cell death, microglia activation, and blood-brain barrier damage in the juvenile rat cerebellum. *Scientific Reports*, 2017, 7:46181
- II. Yafeng Wang*, **Kai Zhou***, Tao Li, Yiran Xu, Cuicui Xie, Yanyan Sun, Yaodong Zhang, Juan Rodriguez, Klas Blomgren#, Changlian Zhu#. Inhibition of autophagy prevents irradiation-induced neural stem and progenitor cell death in the juvenile mouse brain. *Cell Death and Disease*, 2017, 8(3):e2694
- III. Yafeng Wang, **Kai Zhou**, Tao Li, Yiran Xu, Cuicui Xie, Yanyan Sun, Juan Rodriguez, Shan Zhang, Juan Song, Xiaoyang, Wang, Klas Blomgren, Changlian Zhu. Selective neural deletion of the *Atg7* gene reduces irradiation-induced cerebellar white matter injury in the juvenile mouse brain by ameliorating oligodendrocyte progenitor cell loss. *Frontiers in Cellular Neuroscience*, 2019, 13:241
- IV. Yiran Xu, Yanyan Sun, **Kai Zhou**, Tao Li, Cuicui Xie, Yaodong Zhang, Juan Rodriguez, Yanling Wu, Min Hu, Linus R Shao, Xiaoyang Wang, Changlian Zhu. Cranial Irradiation induces hypothalamic injury and late-onset metabolic disturbances in juvenile female rats. *Developmental Neuroscience*, 2018, 40(2):120-133

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LIST OF ABBREVIATIONS

BBB	Blood brain barrier
Cereb	Cerebellum
CNS	Central nervous system
CSF1R	Colony-stimulating factor 1
DG	Dentate gyrus
DT	Diphtheria toxin
EGL	External granule layer
FACS	Fluorescence-activated cell sorting
GB	Glioblastoma
GCL	Granular cell layer
GCV	Ganciclovir
GH	Growth hormone
Gy	Gray
h	Hours
Hip	Hippocampus
HSVTK	Thymidine kinase of herpes simplex virus
Iba-1	Ionized calcium-binding adapter molecule 1
i.c.v.	Intra-cerebroventricular
i.p.	Intraperitoneal
IR	Irradiation
KO	Knock out
MB	Medulloblastoma
ML	Molecular layer
P	Postnatal day
RT	Radiotherapy
SGZ	Subgranular zone
SVZ	Subventricular zone
Tam	Tamoxifen
TSH	Thyroid-stimulating hormone
Clo-Lip	Clodronate liposomes

1 Introduction

1.1 Radiotherapy

Brain tumors are the most common pediatric solid tumors and represent 30% of all childhood solid tumors. On average, the annual incidence is 4.6 per 100,000 children, and 81% of them survive more than 5 years (Childhood Cancer Incidence and Survival in Sweden 1984-2010, Childhood Cancer Epidemiology Group).

Brain radiotherapies include whole-brain radiotherapy and local radiotherapy [1]. Different conformal strategies have been developed and used in clinical practice in order to reduce damage to the surrounding tissues; however, cognitive decline still occurs [2]. Moreover, whole-brain and spinal cord irradiation is still commonly used in all cases of medulloblastoma (MB) and in the prevention of cranial metastases [3, 4], and it is also used for the treatment of selected cases of leukemia and lymphoma, while whole body irradiation (IR) can be used prior to hematopoietic stem cell transplantation [5]. Generally speaking, radiotherapy combined with surgery and/or chemotherapy can be viewed as standard therapy for malignant (high grade) brain tumors [6].

It is important to note that children may experience relatively more profound impairments after radiotherapy than adults [7, 8]. Radiotherapy can induce cognitive decline later in life, such as memory, attention and executive function deficits [7, 9-11]. The increased number of survivors due to the improvement of treatments has revealed the side effects of IR, drawing more and more concerns about the quality of life for survivors. Neuro-psychological outcomes depend on different factors including radiation dose (number of fractions and dose per fraction), gender, irradiation volume (whole brain irradiation or focal irradiation), age at the time of treatment, and the period of time after treatment [7, 8, 12]. Irradiation can cause damage in different regions of the brain including the cortex, white matter and hippocampus. Specifically, damage to the hippocampus after IR is responsible for learning and memory disabilities, while frontal cortex injury after IR is responsible for executive disability [1, 13]. Loss of white matter induced by IR is also associated with cognitive decline [14, 15]. These potential side effects as well as central nervous system (CNS) damage, are probably due to inhibition of neurogenesis, neuro-inflammatory responses, apoptosis and oxidative stress induced by IR [16-20]. Moreover, IR-induced neurogenic micro-environment alternations, such as microglial activation or inflammatory responses, is a critical factor changing the cell fate of neural precursors and eventually decreasing neurogenesis [21].

1.2 IR-induced neuroinflammation in the juvenile brain

Neuroinflammation is one of the most important mechanisms contributing to or even causing brain injuries in CNS disorders such as multiple sclerosis [22], brain trauma [23], hypoxia-ischemia [24], neurodegeneration disorders [25] and irradiation brain injury [26].

Inflammation can usually be divided in two phases: acute and chronic inflammation. Some have proposed that chronic brain injuries can be treated by blocking the transition from acute to chronic inflammation [25]. Chronic inflammation might also influence the brain long after IR, causing micro vessel damage in the hippocampus [27].

Microglia are local macrophages in the CNS, and they are the first line of defense after brain injury [28]. Under physiological conditions microglia are vital for brain development and work by phagocytosing dead cells or pruning spines. However, microglia activation is always followed by a release of cytokines and chemokines, which changes the CNS microenvironment affecting neurogenesis and neuron maturation [21, 29]. Furthermore, active astrocytes can release cytokines and chemokines and contribute to the inflammatory response [30].

1.2.1 Inflammatory response in the juvenile hippocampus after IR

Neurons and glia can be regenerated throughout life in two neurogenic areas, the subgranular zone (SGZ) of hippocampus and the subventricular zone (SVZ) of the lateral ventricles [31, 32]. Although these processes are conserved throughout life, they are more profound in children. The neurogenesis in the hippocampus is critical for memory formation and cognitive functions. The inflammatory response after IR in the hippocampus is summarized in Table 1.

Microglia activation and cell loss after IR

We have previously shown that the number of both total and active microglia in the SGZ were significantly higher 6 and 24 h after a 6 Gy single dose of IR in postnatal day 11 rats [33]. Consistent results were shown in another study where mice were subjected to IR at postnatal day 10 [34]. IR reduced microglial numbers as early as one week after IR in the SGZ, while total numbers of microglia did not change significantly in the granule cell layer (GCL) of rats subjected to IR at postnatal day 9 [35]. Other studies also found that IR increased microglial numbers in the SGZ during the acute phase, but decreased the number of microglia during the chronic phase, presumably due to microglial death [36, 37].

Astrocytosis after IR

Only two studies have been published referring to the astrocyte response after IR in the young brain hippocampus. In the molecular layer of the hippocampus, astrocyte density (as judged by GFAP positive cell numbers) increased by 12% after IR, even at a long-term time point [38]. Another study found astrocyte hypertrophy rather than hyperplasia 7 days after IR in postnatal day 9 rats [39].

Cytokine and chemokine release after IR

Our study demonstrated that the cytokine interleukin 1 alpha (IL-1 α), and the chemokines CCL2 and GRO/KO significantly increased in the SGZ of the hippocampus 6 h after IR in

postnatal day 11 rats, but returned to normal levels 24 h after irradiation [33]. Long lasting chronic inflammation (IL-6 and CCL2) was detected in both juvenile mice (postnatal day 10) and adult mice after IR [37]. In support of this, another mouse study showed that both CCL2 and IL-1 β were increased 6h after IR, while IL-6 decreased 1 week after IR in postnatal day 9 mice [36]. In a study using microsphere-based xMAPTM technology to quantify cytokine protein expression in the neurogenic regions, CCL2 and IL- α levels were significantly increased 6h after IR on postnatal day 9 rats. They found that the increased CCL2 was mainly produced in astrocytes using immunohistochemistry [39].

Hip	Age	Dose (Gy)	6h after IR	24h after IR	1w after IR	4w after IR	1 year after IR	
Mice	P10	6	MG number (SGZ) Active MG (SGZ)					Wang, et al., 2017
Mice	P10	8	MG number (GCL & SGZ)	MG number (GCL & SGZ)	MG number (GCL & SGZ)	MG number (GCL & SGZ)		Han, et al., 2017
Mice	P10	8	Active MG (SGZ)	Active MG (SGZ)	Active MG (SGZ)			Han, et al., 2017
Mice	P10	8	MG number (DG) IL-6, CCL-2 (Hip)	MG number (DG) IL-6, CCL-2 (Hip)	MG number (DG) IL-6, CCL-2 (Hip)	MG number (DG) IL-6, CCL-2 (Hip)		Han, et al., 2017
Mice	P14	8					Astrocyte Number (ML)	Kalm, et al., 2013
Rats	P11	6	MG number (SGZ) Active MG (SGZ) IL-1 α , CCL-2, GRO/KC, IL-1 β (hip)	MG number (SGZ) Active MG (SGZ) IL-1 α , CCL-2, GRO/KC (hip)				Zhou, et al., 2017
Rats	P9	8	MG number (DG)					Sabel, et al., 2017
Rats	P9	6	CCL-2, IL-1 β (hip)		IL-6 (Hip)	MG number (GCL & ML) Active MG (GCL & ML)		Blomstrand, et al., 2014
Rats	P9	8	MG number (Hip) Active MG (Hip)		MG number (Hip)			Kalm, et al., 2009
Rats	P21	8			MG number (Hip)			Kalm, et al., 2009
Rats	P9	8	CCL-2, IL-1 α (hip)		Astrocyte hypertrophy			Kalm, et al., 2009

Table 1. Inflammatory responses in the hippocampus.

p = postnatal, Dose = IR dose, Gy = Gray, h = hours, SGZ = sub granular zone, GCL= granular cell layer, DG = dentate gyrus, hip=hippocampus, ML= molecular layer. Red means the values increased. Black means no change, blue means the values decreased. Decreases or increases only refer to the protein level.

1.2.2 Inflammatory response in the juvenile subventricular zone (SVZ) after IR

The SVZ is one of the two regions where neurogenesis was found to take place throughout the life span. The new born neurons in the SVZ migrate to the olfactory bulb and mature into local interneurons [40]. As in the SGZ of the hippocampus, the SVZ is highly vulnerable to IR due to the continuously dividing cells.

Changes in microglial number after IR

Seven days after 8 Gy IR on postnatal day 9 rats, the microglial density significantly decreased in SVZ [35]. Another study using both postnatal day 9 and day 21 rats subjected to 8 Gy IR found that microglial numbers increased in both groups 6h after IR; however, 7 days after IR microglial number decreased only in postnatal day 9 rats [41].

Cytokine and Chemokine release after IR

CCL2 expression increased significantly in the SVZ 6 h after IR in postnatal day 9 rats, due to activation of astrocytes and microglia [39]. No data are available for other cytokines and chemokines in the SVZ after IR.

1.2.3 Inflammatory response in the corpus callosum after IR

The development of white matter consists three phases: myelination of axons during the first 3 months following birth, followed by the thickening of the myelin sheaths and the maturation at 48 months. During the first phase of development, the white matter is highly vulnerable to injury after IR [7].

Microglial response after IR

Eight Gy IR results in decreased microglial numbers in the corpus callosum in postnatal day 9 rats, but not in 21-day-old rats [41]. Another study showed that male but not female mice showed increased microglial numbers in the corpus callosum 4 months after IR in postnatal day 14 mice [42].

1.2.4 Inflammatory response in the cerebellum after IR

The cerebellum was thought to be responsible for motor function only; however, an increasing number of new studies are hinting at important roles for the cerebellum in cognitive development and cognitive functions [43-49]. The data are summarized in Table 2.

Microglial response after IR

Many proliferating cells still existed in EGL of the cerebellum when mice were subjected to IR [50], Therefore, Similar results that the number of microglia increased both 6 and 24 h after IR, were found in EGL of cerebellum as in the SGZ and SVZ [34, 51];

Cytokine and chemokine release after IR

Our previous research showed that the blood brain barrier in this region is more vulnerable to IR compare to other brain regions, and that the relative lack of growth (“loss of volume”) after IR was bigger in the cerebellum than the other regions, revealing that the postnatal growth continues long after birth in this region [26]. Therefore, the inflammatory response after IR in the cerebellum may be stronger than in other brain regions. Pro-inflammatory cytokines like IL-1 α , IL-1 β and CCL2 were increased at both 6 and 24 h after IR, while the anti-inflammatory cytokines IL-6, IL-18, CX3CL1 or GRO/KC, GM-CSF and VEGF only increased 24 h after IR [51]. As mentioned before, the EGL still had many proliferating cells when the mice were subjected to IR, which explains the extensive amount of cell death that accompanied the severe inflammation.

Cereb					
	Age	Dose (Gy)	6h after IR	24h after IR	
Mice	P10	6	MG number (EGL)	MG number (EGL)	Wang, et al., 2017
Rats	P11	6	MG number (EGL) IL-1 α , IL-1 β , CCL-2 IL-18, IL-6, GRO/KC GM-CSF, VEGF (Cereb)		Zhou, et al., 2017

Table 2. Inflammatory responses in cerebellum

Cereb = Cerebellum, P = postnatal, Gy=Gray, EGL = external granule cell layer

Red means that the values increased. Black means no change.

1.2.5 Inflammatory responses in other brain areas after IR

Increased IL-1 β expression was detected in the hypothalamus after IR in postnatal day 18 rats, influencing hypothalamic-pituitary-adrenal axis regulation [52], and perhaps explaining the changes in pituitary hormone levels found in another study [33]. In the cortex, CCL2, GRO/KC, IL-1 α , IL-18, TNF- α , IL-2 and IL-12p70 expression was up-regulated 6 h after IR in postnatal day 9 rats [39].

1.3 Neuroinflammation as a potential target for the side-effects caused by RT

Blocking inflammation using indomethacin, a commonly used anti-inflammatory drug, could rescue neurogenesis in adult rats after IR [53]. Peroxisomal proliferator-activated receptors (PPARs) also showed an anti-inflammatory response after IR [19]. Only the absence of CCL2 was enough to normalize the neurogenesis loss in an adult mouse study [54]. Recently, lithium has also been indicated as a promising and safe pharmacotherapy to protect the brain by reducing cell death and neuroinflammation in the hippocampus of juvenile mice [55]. Our previous data showed that lithium can prevent IR-induced neuroinflammation, including pro-inflammatory cytokines, even with the activation of microglia [33]. Along this line,

autophagy could be another mechanism that contributes to side effects after IR. A mouse study revealed that Atg7 KO mice had decreased levels of IL-1 β , IL-4, IL-6 and CCL2 after IR [34]. Another study tested hypothermia in irradiated rats, but the protective effect was not significant [35]. Inhibiting microglia by using a colony-stimulating factor 1 (CSF1R) receptor blocker such as PLX5622 was found to reduce inflammation (as judged by monocyte accumulation) and normalize the cognitive dysfunction after three fractions of 3.3 Gy whole-brain irradiation in adult mice [56], although this study neglected the effects of microglial depletion on normal brain development and behavior itself [57]. Also, peroxisome proliferator/activated receptors were tested in the IR mouse model as an anti-inflammatory strategy, and the cognitive outcomes were improved and inflammation responses were reduced [58].

1.4 Microglial depletion and repopulation

1.4.1 Microglial depletion

Microglial depletion methods

Different ways of depleting microglia have been developed recently and are mainly classified into three categories: genetic, conditional genetic and pharmacological depletion [59]. In genetic depletion in mice, the microglia usually have a mutation in the genes that are critical for microglia survival and the mice carrying these mutations usually die before adulthood [60, 61]; thus, genetic depletion is not widely used for microglial depletion and repopulation.

The conditional genetic microglial depletion models include the use of CD11b-HSVTK, CX3CR1^{creER}:iDTR and CX3CR1^{creER}Rosa26^{DTA} mouse strain. In a CD11b-HSVTK mouse strain with ganciclovir (GCV) injection, GCV can be monophosphorylated by HSVTK, and further transforms into a triphosphate form with cellular toxicity. All CD11b⁺ cells are supposed to be eliminated, leading to high mortality [62]. To avoid targeting cells outside the CNS, the GCV was injected intra-cerebroventricularly (i.c.v.), and the efficacy of microglial depletion could reach 95% after 4 weeks of treatment, but with mortality and BBB damage causing the infiltration of peripheral cells [63]. Later, the CX3CR1^{creER}:iDTR strain was developed. Due to the rapid turnover of peripheral monocytes, microglia can be specifically depleted by injecting Diphtheria toxin (DT) several weeks after tamoxifen (Tam) injections, and the microglial depletion rate can reach > 80%, without peripheral infiltrating monocytes contributing to the repopulation [64, 65]. The CX3CR1^{creER}Rosa26^{DTA} mice strain was generated by crossing CX3CR1^{creER} and Rosa26^{DTA} mouse strains, DT can be produced intracellularly. Specifically, Cre recombinase was released after tamoxifen injections and cut the floxed-STOP cassette of DT. DT starts to express and eventually leads to cell death. The efficacy of microglia depletion can reach > 90% but with peripheral infiltrating monocytes contribute to the repopulation in this approach [66].

Clo-Lip can be phagocytosed by microglia, accumulated free form of clodronate eventually leads to microglia apoptosis [67, 68]. Clo-Lip is either injected intracerebroventricular to deplete microglia in the whole brain [69] or injected intracerebral to deplete microglia in specific brain regions [70, 71]; however, Clo-Lip has been shown damage other brain cells and blood vessels [68]. CSF1R is crucial for microglial survival and development [72]. CSF1R KO mice lack microglia and die before adulthood [73, 74]. Different CSF1R inhibitors including PLX3397, PLX647, Ki20227 and GW2580 have been tested, and PLX3397 eventually shows the most robust microglia depletion [75]. Newly repopulated microglia without contribution from the periphery was noted after microglial depletion by PLX3397 and PLX5622 and PLX3397 can be given orally in chow, which means it can be applied to any strains and species. but PLX3397 is not specific to CSF1R, it also can inhibit other kinases including FLT3, PDGFR and KIT [76, 77]. Later, PLX5622 was developed with a more selective profile for CSF1R over FLT3 and KIT [78].

Microglial depletion in the healthy brain

Microglia depletion may affect other cell types in the brain. Western blot data showed no changes in levels of microtubule-associated protein 2 (MAP2), hexaribonucleotide binding protein-3 (NeuN), or oligodendrocyte transcription factor (Olig2) after microglial depletion; however, astrocyte markers such as GFAP and S100 were significantly increased, while the BBB and cognitive behavior were not affected by either short or long term microglial depletion in adult mice [75]. Another adult of adult mice showed transiently spatial memory impairment after microglial depletion [71]. However, early life microglial depletion at postnatal day 1 and 4 induced social, mood-related and locomotor behavior impairments [69].

Microglial depletion in different diseases

Microglial depletion as a novel tool to study the role of microglia has widely broadened our knowledge of microglia under both physiological and pathological conditions. Microglia play important roles in different CNS diseases, and targeting or depleting microglia has become a promising way to treat different CNS diseases that involve dysfunctional microglia [79]. Microglial depletion shows beneficial outcomes in different disease models including experimental autoimmune encephalomyelitis (EAE), intracerebral hemorrhage, Alzheimer's disease (AD), neuropathic pain, IR-induced memory deficits, detrimental pathological outcomes in amyotrophic lateral sclerosis (ALS), cerebral ischemia, Parkinson's disease (PD) and coronavirus encephalitis [79]. This means that microglia play distinct roles in different diseases and that different therapies targeting microglia should be applied accordingly. Moreover, microglial depletion at different time points could also yield different results. For example, microglial depletion in 5-month-old mice in an AD model showed no alternation in A β formation or maintenance [63]; however, early application of long-term microglial depletion significantly reduced neurotic plaque deposition, accumulation of intraneuronal amyloid and pre-fibrillar oligomers [80].

Microglial depletion in the human brain

Early studies from Plexxikon showed that microglial depletion could reduce glioblastoma (GB) burden and spread. PLX3397 significantly reduced GB cell invasion when co-cultured with microglia, and PLX3397 could reach GB and surrounding tissues without causing BBB damage. A clinical phase II study of 37 recurrent GB patients showed that PLX3397 also reduced microglial numbers in human brains, but without improvement of clinical outcomes. Therefore, combined therapies need to be further studied [81].

1.4.2 Microglial niche repopulation

Resident microglia contribute to the repopulation of the microglia niche

Both genetic and pharmacological microglial depletion allow microglia to rapidly repopulate the brain, indicating the inherent property of microglial repopulation independent of the way in which they are depleted [72]. Interestingly, one study showed that microglial niche repopulation is proportional to the microglial depletion rate, in other words the more microglia that are depleted, the faster the new microglia will repopulate. Further, several cycles of microglial depletion will delay the microglia repopulation [82].

The CX3CR1CreER:iDTR microglial depletion model showed that in the absence of IR, microglia repopulated exclusively from the internal microglial pool [65]. Elmore and his colleagues demonstrated that the repopulation of microglia after depletion by CSF1R inhibitor PLX3397 did not stem from the peripheral macrophages. They further found that repopulated microglia were mainly from nestin⁺ microglia precursor cells rather than the surviving microglia [75]. However, this was overturned by a more recent microglia depletion study that used PLX5622, in which the authors demonstrated using parabiosis that repopulated microglia were only from the remaining resident microglia. In fact, newly repopulated microglia were from less than 1% of the surviving microglia. Moreover, the newly formed microglia transiently expressed nestin, and none of the repopulated microglia originated from nestin⁺ non-microglial cells. Another study revealed that repopulated microglia after depletion by PLX5622 are not derived from neuroprogenitor cells (nestin⁺) or the oligodendrocyte precursor cells (NG2⁺ or PDGFra⁺), but were instead from self-renewal of the CX3CR1 lineage without the contribution of peripheral macrophages [72].

One study showed that microglia repopulated from clonal clusters with minimal migratory diffusion by using Microfetti labeled Brainbow mice, and impaired microglia repopulation was found in NF-kb KO mice [72]. In a *Cx3cr1*^{CreER} study, after microglial depletion the remaining microglia that escaped depletion started to proliferate in clusters and then move away to fill the empty microglial niche. Furthermore, the proliferating microglia expressed high levels of IL1R, and IL-1R antagonist impaired the process of microglia repopulation [65].

Single cells analysis of both repopulated and resident microglia showed that newly repopulated microglia expressed higher levels of cell cycle genes (Cdk1 and Mki67), cell survival genes (Bcl2a1a and Bcl2a1d) and microglial migration related genes (Cd36), which

indicated high proliferation and migration of repopulating microglia. The authors further compared the brains before and after microglia depletion and repopulation, the whole brain transcriptome was not influenced, and only three differentially expressed genes, including *Gabra6*, *Cbln3*, *Fat2*, were observed. Furthermore, the gene profile showed no difference after challenging the resident microglia and repopulated microglia with LPS, indicating the similarity of function between newly repopulated microglia and resident microglia [83]. During microglial repopulation the regenerating microglia recapture immature signatures during early repopulation and re-express mature markers during later phase of repopulation. The authors also found that the highly proliferating repopulated microglia also experience active cell death [72]. Another study showed that 2 weeks after microglial repopulation by PLX3397, the repopulated microglia are morphologically indistinguishable from the original resident microglia [75].

Peripheral monocytes repopulate to the microglial niche and become microglia-like cells

After IR and microglial depletion using a *CX3CR1^{CreER}* mice model, BM-derived cells infiltrated the CNS and became microglia-like cells, resembling resident microglia in terms of distribution and morphology 12 weeks after microglial depletion. However, the microglia-like cells are transcriptionally distinct from resident microglia, expressing lower levels of *Hexb*, *P2yr12*, *Olfml3*, *Mertk*, *Pros1*, *Entpd1*, *C1qa* and *Tmem119* [65]. Unlike in other microglial depletion models, peripheral-derived monocytes populated the CNS microglial niche after depletion by administration of tamoxifen in the *Cx3cr1^{CreER/+}R26^{DTA/+}* mice, and the resulting microglia adopted similar phenotypes but remained transcriptionally, epigenetically and functionally distinct. This process is independent of BBB leakage and is regulated by type 1 interferon [66].

2 The aims of the thesis

The overall aim of this thesis is to investigate potential strategies to ameliorate the often debilitating late-appearing side effects after RT to the juvenile brain. Specifically, to investigate the effects of pharmacological lithium treatment, and to explore microglia dynamics after IR or after depletion and during repopulation.

Specific aims:

1. To evaluate the therapeutic and toxic effects of lithium as a potential pharmacological treatment for the side effects after RT in juvenile mice
2. To characterize the short and long term microglial response after IR in juvenile mice
3. To characterize the microglial transcriptome by bulk and single-cell RNA sequencing at different time points after IR in juvenile mice
4. To investigate the contribution the Cx3cl1-Cx3cr1 axis on microglial repopulation after depletion

3 Methods

3.1 Animal models

The CNS develops continually during childhood and adolescence; however, there are no systematic studies comparing the IR-induced brain injury between human and rodents at different ages. In papers 1 and 2 we subjected postnatal day 11 rats and postnatal day 10 mice to IR. The CNS developmental level at postnatal 10 or postnatal 11 in a rodent is approximately equivalent to 2-years-old human or younger [84]. However, radiotherapy is typically avoided in patients younger than 4 years of age, which approximately is equal to postnatal day 21 in rodents [84]. Thus, to be more clinically relevant, we used postnatal day 21 mice in paper 3.

Sex is another factor that may contribute to the susceptibility to side effects after IR. It has been shown that girls are more likely to develop IR-induced cognitive decline than boys [85]. Males were used in paper 1; both sexes were used in paper 2, but without comparison between the two; Females were used in paper 3. Gender differences should be taken into consideration in future IR-induced brain injury studies.

6 Gy and 8 Gy are approximately equivalent to 12 Gy and 16 Gy, respectively, using repeated 2 Gy fractions, according to the linear-quadratic model [86] and an α/β ratio of 3 for late effects in normal brain tissue, representing a moderate radiation dose in clinical settings. From a practical point of view, single dose of 6-8 Gy is often used in rodent models.

Paper 1 described the use of Wistar rat pups (Charles River, Germany) to study lithium and IR. Postnatal day 11 rats were anesthetized by an i.p. injection of 50 mg/kg tribromoethanol. The rats were placed in a prone position and the whole brain was irradiated with single dose of 6 Gy. Sham animals were anesthetized but not irradiated.

Paper 2 described the use of C57BL/6 mice (Charles River, Germany), Cx3cr1^{GFP/+}CCR2^{RFP/+} mice generated by crossbreeding Cx3cr1^{GFP/GFP} and CCR2^{RFP/RFP} (Jackson Laboratory, US), and Casp8^{fl/fl} LysMCre^{+/-} or Casp8^{fl/fl} LysMCre^{-/-} mice generated by crossbreeding Caspase-8^{fl/fl} and Casp8^{fl/fl} LysMCre^{+/-}. The mice were anesthetized with isoflurane and irradiated in a cabinet X-ray irradiator to a dose of 8Gy at postnatal day 10 or 90.

Paper 3 described the use of C57BL/6 mice (Charles River, Germany), with the same IR procedure as paper 2

Paper 4 described the use of Cx3cr1^{CreER/+}Rosa26^{DTA/+} mice generated by crossbreeding Cx3cr1^{CreER/CreER} (Jackson Laboratory, US) with Rosa26^{DTA/DTA} (Jackson Laboratory, US) mice and Cx3cr1^{GFP/+} mice generated by crossbreeding Cx3cr1^{GFP/GFP} (Jackson Laboratory, US) and C57BL/6 mice (Charles River, Germany).

3.2 Behavioral evaluation

Paper 1 described the use of behavioral tests, including openfield and object recognition. The mice were allowed to move on a 100 x100 cm open-field arena, with a camera automatically recording the movements of the mice. The speed and distance reflected the mouse activity, while staying in the central zone (30x30cm) reflecting less anxiety. Object recognition was tested the next day, consisting of two trials, where two identical objects were put in the arena. The mice were allowed to freely explore them to get familiar with the objects for 5 minutes. Six hours later, one of the objects was replaced by a new, differently shaped object, and the total time spent exploring the new object and the old object were recorded. The health mice usually spend more time exploring the new object since the mice have an innate preference for novelty.

3.3 Bulk and single cell RNA sequencing

Paper 3 described the use of bulk and single cell RNA sequencing. For the bulk RNA sequencing, the RNA was isolated using a miRNeasy micro kit (Qiagen, 217084). The library was prepared using a QIAseq FX single cell RNA library kit (Qiagen), and then sequence on an Illumina HiSeq 2000. Single-cell sequencing was performed using the 10x Genomics Chromium method and sequenced using an Illumina HiSeq 2500.

Paper 4 described the use of bulk and single cell RNA sequencing. For bulk RNA seq, the cells were sorted into Smart-Seq lysis buffer directly. The library was built by following the Smart-Seq protocols, and then sequenced on an Illumina HiSeq 2000. In the single RNA seq, we re-analyzed the same data that was generated in paper 3.

4 Results and discussion

4.1 Paper 1: Lithium as a promising drug for radiotherapy induced pediatric brain injury

Radiotherapy is an indispensable brain tumor treatment; however, it also causes severe late effects, such as cognitive decline including deficits in memory, executive functions and attention, especially in children [7]. However, as of yet there is no effective treatment for radiotherapy-induced side effects in children. The only strategy that has been clinically tested in children is exercise, which partially restore neurogenesis and behavioral deficits in mice [87] and has positive effects also in human [88, 89]. Lithium has robust neuroprotective roles after radiotherapy [18]; however to be able to move to clinical trials we have to test the long-term effects of lithium on juvenile animals.

We first found that relatively high concentrations of lithium are toxic to tumor cells, but not to the immortalized neural progenitor cells. Then, when we irradiated an IR resistant medulloblastoma cell line, and found that 10 mM of lithium increased the IR sensitivity, without any signs of protective effects on the tumor cells with either 1 or 10mM of lithium. This is consistent with other studies indicating that lithium do not protect tumor cells [90], and acts as a radiosensitizer [91].

As described above, the neurogenic regions, including the SGZ, are particularly susceptible to IR, and the loss of neural precursor cells in the SGZ is associated with cognitive decline. We next found that lithium is able to inhibit and delay cell death in the SGZ and increases neurogenesis following IR. We further assessed a behavior test, novel object recognition; and as we hoped, lithium prevented the IR-induced cognitive deficits. We further found that lithium could prevent IR-induced acute inflammation in the hippocampus, but had no effect on numbers of microglia of SGZ. Moreover, IR can also induce systemic effects through radiation-induced endocrinopathy [5]. Body weight gain was delayed 4 weeks after IR, but lithium failed to rescue the body weight gain loss. It is well-known that patients treated with craniospinal radiotherapy often need replacement therapy with thyroid hormone and growth hormone. One study showed that IR induces deficits of pituitary weight and related hormones, and the deficits are bigger in younger adult rats [92]. Here, we found in the juvenile rat brain, that IR reduced or even abolished pituitary-related hormonal increases when body weight and the concurrent metabolic needs increased, Lithium restored TSH and GH levels after IR and reduced the IR-induced BDNF increase.

Finally, we checked the possible side effects that may arise from administration of lithium. Blood samples were collected at different time points after IR and lithium injections. Creatinine levels in the blood can be used to monitor kidney function, and increased levels may indicate kidney damage. Creatinine increased along with the rapid growth, as expected when the muscle mass increases, but this was not different between lithium- and vehicle-treated mice. In summary, lithium has multiple effects on IR-induced juvenile rat brain

injury, and without any obvious side effects, making it a promising pharmacological treatment for radiotherapy-induced late-appearing side effects.

4.2 Paper 2: Transient microglia accumulation with long lasting loss of microglia after IR

In paper 1, we discussed that lithium can reduce inflammatory response, but not microglial activation. However, microglial response time after IR is poorly understood. IR can induce cognitive decline in both juveniles and adults; however, the mechanisms seem to be different. Here, postnatal day 10 and day 90 mice were irradiated, after which microglial accumulation was more pronounced in juvenile mice than adult mice. Both juvenile and adult mice demonstrated a transient microglial increase, followed by a decrease in microglial numbers over time due to microglia cell death. The increased microglial number in the juvenile brain was at least partly due to proliferation after IR, but no Ki67⁺ microglia were detected at any time after IR in adult mice. The increased microglial numbers of the SGZ may also be a result of migration from the neighboring areas. One transcriptional study of microglia indicated an aging-like microglial profile after IR. The study further demonstrated an inverse correlations between CSF1R and IR [93]. Given that CSF1R is important for microglial survival and development, IR-induced CSF1R impairment may be the reason behind the induction of microglial cell death.

Whether peripheral monocytes contribute to the microglial pool after IR is not clear. Here, we used a Cx3cr1^{GFP/+}CCR2^{RFP/+} strain, for which in the CNS, GFP was exclusively expressed in microglia and RFP was expressed on peripheral “inflammatory” monocytes, identified as Ly6c^{hi}CCR2⁺Cx3cr1⁻, which are commonly found in inflamed tissues. No RFP⁺ microglia were detected in either juvenile or adult brains, indicating that no peripheral infiltrating monocytes contributed to the microglial pool after IR. Monocytes may however have down-regulated CCR2 after infiltrating into the brain [94], so we used an RFP antibody to amplify the signal; still, no RFP⁺ cells were detected, and no BBB leakage was detected, as judged by the lack of extravascular albumin. These data are consistent with a previous study, which only found infiltrating monocytes in the brain after high-dose IR [95]. Long-lasting expression of CCL2 was detected in both juvenile and adult hippocampus, and CCL2 is known to regulate the BBB and attract infiltrating peripheral monocytes [96]. Together with our data, increased CCL2 alone is not enough for BBB opening and peripheral monocyte infiltration.

Microglial activation and inflammation were also compared between juvenile and adult mice after IR. Both morphological changes and CD68 expression indicated a more pronounced and long-lasting microglial activation in the juvenile brain (until after one week in the juvenile brain, as compared to 24 hours in the adult brain). Long-lasting IL-1 β expression was also detected in both juvenile and adult brains, indicating chronic inflammation. IL-1 β increases BBB opening by targeting astrocytes [97]. It also impairs SGZ neurogenesis [98], which maybe another mechanism leading to IR-induced cognitive decline.

4.3 Paper 3: Dynamic microglia alterations in juvenile brain

In paper 2, we discussed that the increase in microglial numbers during the acute phase in the SGZ was at least partly due to the microglial proliferation in the juvenile brain and may also involve microglial migration from neighboring regions of the dentate gyrus. The SGZ microglia were transiently active until one week after IR in the juvenile brain, and there was no peripheral monocyte contribution to the microglial pool, neither in juvenile, nor in adult brains. Microglia display distinct molecular changes across brain developmental stages, which may influence microglial responses in different pathological CNS conditions [99]. Here we used bulk and single-cell RNA sequencing to unveil dynamic microglial molecular profiles in irradiated juvenile brains.

Consistent with paper 2, histology showed that juvenile microglia react most exuberantly early after IR and return toward baseline after one week. Overall, microglial density decreased in the entire DG, indicating microglial loss after IR. Double staining of microglial markers Iba-1 and active caspase-3 in the acute phase and microglial pouches containing caspase-3⁺ cellular structures were observed, indicating that microglial numbers increased transiently as well as that their activation was associated with engulfment of apoptotic cells in the SGZ. Furthermore, bulk RNA sequencing of the microglia of the hippocampus showed that a majority of upregulated genes were related to lysosomal biogenesis, modulation of cytoskeleton and cell adhesion, which are important for microglial phagocytic activity [100-102]. The vast neural precursor cell death-induced microglial activation and phagocytic activity in the juvenile brain may be the reason for the distinct microglial and inflammatory response in the juvenile brain, different from the adult brain after IR, since in the later there are much fewer dying precursors.

After IR, only the microglia in the SGZ of the hippocampus were active due to the vast amount of cell death. The microglia returned to baseline, morphologically, when the dead cells were cleared. These finding strongly indicate that IR-induced cell death is a key trigger for microglial activation [103]. Since the bulk RNA sequencing includes all the microglia in the hippocampus, and thus cannot distinguish the SGZ microglia from the microglia of other parts of the hippocampus, we next performed single-cell RNA sequencing.

Cx3cr1⁺ microglia were fluorescence-activated cell sorting (FACS) sorted from the hippocampus 6 hours and 1 week after IR for single-cell RNA sequencing. As expected, the one-week IR microglia clustered together with the sham microglia, and only the 6 hours IR microglia clustered separately, which was consistent with our bulk RNA sequencing and histology study. Further clustering analysis revealed that different clusters adopted different profiles after IR, indicating considerable heterogeneity of the microglia in the hippocampus. Moreover, we detected genes associated with both pro- and anti-inflammatory states expressed together in individual microglia. This was consistent with our bulk RNA sequencing data showing transcriptional changes overlapping between pro- and anti-inflammatory genotypes 2, 6 and 24 hours after IR. But bulk sequencing doesn't allow us to determine if the pro- and anti-inflammatory genes were expressed in different cells or the

same cell. This overturns the M1 and M2 classification of microglia [104], which were only validated in vitro, since the in vivo model failed to reveal a distinct M1 or M2 phenotype [105-107]. Velocity analysis showed that one cluster displayed greater transcriptional activity compared to other clusters and expressed high CCL4 and CCL3. Immunohistochemical analysis identified that this cluster of microglia as the phagocytically active SGZ microglia, which stained positive for CCL4.

To further compare the juvenile and adult microglia after IR, and the aging microglia, gene set enrichment analysis (GSEA) was used to compare data from our juvenile microglia after IR with data available from with previously published work on adult microglia after IR or ageing. The results showed that except for the one-week timepoint after IR, which appeared to return to normal, the GSEA further confirmed our histology and bulk RNA sequencing data showing the transient nature of microglial alteration after IR in the juvenile brain. However, the adult microglia after IR showed a high degree of overlap of hallmark gene sets with ageing microglia one month after IR, indicating that they hold an aged microglial phenotype following IR, consistent with previously reported results [93].

4.4 Paper 4: Cx3cr1 regulated microglia repopulation after depletion

Microglia are derived from immature macrophages at E8.0, which develop into A1 (CD45⁺ c-kit^{lo}Cx3cr1⁻) and further mature into A2 (CD4⁺ c-kit⁺Cx3cr1⁺) cell types. The expression of Cx3cr1 in microglia is initiated from the A2 phase at E9.0 and is maintained in microglia in the later phase. Therefore, Cx3cr1-GFP and Cx3cr1^{Cre/ERT2} mice can be used from E9.0 onward to label and target microglia [74, 108]. Cx3cr1 is exclusively expressed by microglia in the CNS, and the Cx3cl1/ Cx3cr1 axis plays a key role in microglia-neuron contact [109, 110]. Moreover Cx3cr1 is widely used to genetically label microglia by inserting EGFP [75, 111], and thus can be easily used for microglial tracing, visualization and sorting [74, 112-115]. Furthermore Cx3cr1^{cre} and Cx3cr1^{Cre/ERT2} are commonly used for studying microglial fate mapping [116, 117] and depletion [57, 118, 119] by modifying the genome of the microglia with floxed specific target genes [120]. Previously publications reviewed the current available approaches to specifically deplete microglia by targeting Cx3cr1 [121, 122], and these methods have important implications for understanding microglia and associated CNS diseases. In the current study, we found that approximately 1% of all microglia escaped genetic labelling, and that these microglia were transcriptionally and functionally different from the modified ones. In microglial depletion based on the expression of Diphtheria toxin in cells carrying the Cre-ERT2, after injection of tamoxifen, the unmodified microglia were not affected, as expected. During repopulation, these unmodified microglia appeared to have an advantage when competing with surviving resident and modified microglia and with infiltrating macrophages. The unmodified cells established their territories in large colonies originating from the small numbers of surviving cells without mixing with the other two cell types, while the latter two established smaller interspersed colonies, mixing with each other.

Further analysis revealed that the wild type microglia that had escaped genetic modification expressed higher levels of Cx3cr1 compared to the Cx3cr1^{Cre/ERT2/YFP} microglia, approximately twice as high, possibly because in the modified cells one allele is replaced with the inserted Cre/ERT2/YFP. We hypothesized that Cx3cr1 may be a critical regulator for microglial repopulation in the CNS and that higher levels of Cx3cr1 entail a competitive advantage in this context, and that Cx3cl1- Cx3cr1 signaling is related to the repopulation advantage observed. This notion is supported by the finding that microglial repopulation after depletion using CSF1 blockade was slower in the retina in Cx3cr1 deficiency and accelerated with exogenous Cx3cl1 administration [123]. Therefore, we depleted microglia in Cx3cr1-deficient mice, and 6 weeks later, the Cx3cr1-deficient brains were fully repopulated. However, the majority of the repopulated microglia were derived from infiltrating macrophages. Meanwhile, in the Cx3cr1^{Cre/ERT2/+} brains, around 50% of the repopulated cells were derived from resident microglia and 50% were derived from infiltrating macrophages, indicating that Cx3cr1 is essential for resident microglia repopulation but not for peripheral macrophage infiltration.

4 Future Perspectives

4.1 IR-induced cognitive decline

Predicting which patients will suffer from cranial RT induced intellectual impairment

Random coefficient models were developed to predict intellectual outcome decline in MB patients after cranial RT [124]; however, different individuals responded differently, and it is still difficult to predict who will develop cognitive impairment and who will not [125]. It is conceivable that biomarkers of neuroinflammation reflective of brain injury, could help predict RT-induced intellectual outcome. We propose that at least some of the markers we identify in the brain tissue also can be detected in the blood, and could serve as such biomarkers.

The mechanisms of IR-induced cognitive decline need to be further investigated

IR can induce neurogenesis impairment, white matter injury, neuroinflammation and vessel damage; however, the exact mechanisms leading to cognitive decline are still unclear, and there are no effective therapeutic interventions [126, 127]. New technologies, such as in vivo imaging, single cell RNA sequencing and mass cytometry should be applied in future research to uncover the mystery of IR-induced brain injury.

4.2 Combined radiotherapy and immunotherapy

Immunotherapy has been successfully used in the treatment of tumors like malignant melanoma and lung cancers [128-136]. However, it is less successful with CNS tumors, since there are no or very few infiltrating immune cells in these tumors, except in the case of high grade GB and brain metastases [137-139]. The complicated immune suppression in the tumor microenvironment of GB makes it hard to treat using one immunotherapy intervention alone [138], and combined immunotherapies may increase the risk of side effects. Radiotherapy could increase T cell infiltration to the tumor tissues, and immunotherapies could further boost the immune response [140-142]. Therefore, RT and immunotherapy seem to be a good combination for brain tumors; however, the interplay between RT and immunotherapy needs to be further investigated, especially in children. The immune system is not fully mature until 12 years of age, so the immune response may be different from adults, and pediatric tumors usually have a low mutational burden compared with tumors in adults. Thus, the check point blockade strategies may be less effective [143]. Further, most preclinical modeling of immune therapeutics has been done with adult mice, thereby hampering the development of pediatric immunotherapy [144, 145]. Therefore, pediatric brain tumor immunotherapies urgently require more research.

4.3 Microglial depletion and repopulation

Microglial depletion has shown beneficial effects in different diseases [79]; however, many questions need to be solved before it can be applied in the clinic. PLX3397 has been used in a clinical trial for GB, depleting microglia in order to reduce the tumor burden and

invasiveness, but it showed no beneficial effects [79]. Studies combining PLX3397 together with RT are still ongoing (<https://clinicaltrials.gov/ct2/show/NCT01790503>). As CSF1R blockade drugs including PLX3397 and PLX5622, not only target microglia, but also all other cells expressing CSF1R, including peripheral monocytes and macrophages, a microglia-specific depletion drug needs to be developed.

Repopulated microglia all come from the surviving microglia after depletion. Both the repopulated microglia and the brain with the newly repopulated microglia will resume the same transcriptomic profile as it had before the depletion [83]. Microglia replacement therapy in disease models has been limited by the transcriptomic similarity before and after depletion; however, microglia replacement by peripheral monocytes or even engineered cells, is still promising. The mechanisms regulating the repopulation of both resident microglia and peripherally derived cells need to be further investigated. Our current paper 4 indicates that CX3CR1 regulates resident microglia and peripheral monocyte repopulation.

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