INFLAMMATION AND BEHAVIOR FOLLOWING IRRADIATION-INDUCED INJURY IN THE DEVELOPING BRAIN

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ABSTRACT

Radiotherapy is used in the treatment of pediatric brain tumors and is often associated with debilitating late effects, such as intellectual impairment. Areas in the brain harboring stem cells are particularly sensitive to irradiation (IR) and loss of these cells may contribute to cognitive deficits. It has been demonstrated that IR-induced inflammation negatively affects neural progenitor differentiation. Therefore, it is necessary to investigate the inflammatory mechanism to be able to find potential treatment strategies. One moderate dose of IR to the young rodent brain caused injuries that were detectable after several months, including impaired growth. We have shown that IR to the developing brain induces an acute inflammatory response. An unexpected finding was that microglia died shortly after treatment. The consequences of IR-induced microglia loss can either be that the injury, due to pronounced inflammation, is decreased, or that injury is enhanced due to weakened repair mechanisms. Further investigations are needed to elucidate how the loss of microglia affects the response to IR and brain development.

The third complement component (C3) is a key protein of the complement system which we found to be upregulated after IR. C3 has been shown to be important for neurogenesis, and therefore we wanted to investigate the role of complement activation after IR by using *C3*-deficient mice. Interestingly, the IR-induced injury, measured as tissue loss and decrease of proliferating cells, was not as pronounced in the dentate gyrus of *C3*-deficient mice as in wild type mice. This indicates that manipulation of the complement system could be a fruitful strategy to protect the neurogenic areas from IR-induced injuries.

We have studied functional consequences of IR to the growing brain. We saw that one dose of IR to the young rodent brain caused behavioral changes that were detectable months and even one year after the treatment. Furthermore, non-irradiated animals performed better than irradiated ones in different learning tasks. Importantly, months after IR *C3*-deficient mice made fewer errors in place learning and reversal learning tests than WT mice. These results indicate that the complement system contributes to both morphological and functional IR-induced injury in the young brain.

Keywords: radiotherapy, neuroinflammation, microglia, memory, cognition, hippocampus.
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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Många är omedvetna om de negativa konsekvenser som man kan se hos barn efter att de har fått strålbehandling vid t ex behandling av en hjärntumör. Tyvärr kan dessa biverkningar vara påtagliga och de barn som överlever sin cancer kan få en försämrad livskvalitet på olika sätt, som t ex att de får svårt att lära sig nya saker och att interaktionen med andra människor blir svårare när de blir äldre. Under barndomen och tonåren växer och utvecklas vår hjärna, vilket gör den extra känslig för strålning. Detta beror bland annat på att celler som delar sig är särskilt känsliga för strålning. Tumörceller delar sig ofta och kan därför behandlas med strålning. I våra hjärnor har vi stamceller som delar sig livet ut och dessa är därför också känsliga för strålning. Det har visat sig att när man får strålbehandling så dör dessa stamceller och man tror att det bland annat är på grund av det som barnen får svårt att lära sig nya saker. En del stamceller överlever dock strålbehandlingen, men dessa blir inte nervceller i lika stor utsträckning, som det var tänkt, utan blir istället gliaceller eller stödjeceller. Anledningen till detta tros vara inflammation orsakad av strålningen. Vi ville studera vad som händer mer i detalj, för att sedan kunna använda t ex farmakologiska sätt att förhindra skador på friska celler, utan att minska effekten på maligna celler.

Vi har visat att strålbehandling leder till ett akut inflammatoriskt svar följt av ett oväntat fynd, nämligen att en betydande andel av mikroglia (hjärnans makrofager) dör efter behandlingen och att antalet inte återhämtar sig. En strålningsinducerad förlust av mikroglia var oväntad, och kan antingen innebära att vävnadsskadorna minskar p.g.a. mindre uttalad inflammation, eller att skadan förvärras eftersom reparationsmekanismerna försvagats. Fortsatta studier krävs för att utröna vilket som är fallet.

Vi såg att proteinet C3 ökade efter strålning. C3 har en central roll i komplementsystemet, och används av immunförsvaret för att döda främmande celler, exempelvis bakterier, och inducera inflammation. För att studera betydelsen av C3 efter strålning använde vi möss som på genetisk väg saknar detta protein ($C3^{-/-}$). Vi fann att den strålningsinducerade skadan hos $C3^{-/-}$ -möss, mätt som vävnadsförlust samt minskning av antalet nyfödda celler, inte var lika uttalad i gyrus dentatus i hippocampus. Detta område är viktigt för inlärning och det är där som en stor del av stamcellerna finns. Vi har även studerat beteendeförändringar efter strålning i låg ålder. Vi har då sett att en enda måttlig strålningsdos skapar inlärnings- och beteendeförändringar i vuxen ålder. De möss som strålats och saknade C3 klarade av att lära sig nya saker bättre jämfört med de som hade C3. Detta talar för att manipulering av C3-systemet skulle kunna vara en fruktbar strategi för att skydda stamcellsinnehållande områden i hjärnan mot strålning.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers or manuscripts:

I. Kalm, M., Fukuda, A., Fukuda, H., Öhrfelt, A., Lannering, B., Björk-Eriksson, T., Blennow, K., Márky, I., Blomgren, K.
Transient inflammation in neurogenic regions after irradiation to the developing brain
Radiation Research, (2009) 171, 66-76.

II. Kalm, M., Lannering, B., Björk-Eriksson, T., Blomgren, K.
Irradiation-induced loss of microglia in the young brain
Journal of Neuroimmunology, (2009) 206, 70-75.

III. Karlsson, N.*, Kalm, M.*, Nilsson, M., Björk-Eriksson, T., Blomgren, K.,
Irradiation to the young mouse brain impaired learning and altered the behavior pattern in adulthood and old age
Manuscript
*these authors contributed equally

IV. Kalm, M., Levin, A., Andreasson, U., Björk-Eriksson, T., Pekny, M., Blennow, K., Pekna, M., Blomgren, K.
C3 deficiency protects against impairment of hippocampal growth and learning induced by irradiation to the young brain
Manuscript

TABLE OF CONTENTS

LIST OF ABBREVATIONS

ATM- ataxia teleangiectasia-mutated

C3- complement component 3

CNS- central nervous system

DNA- deoxyribonucleic acid

DG- dentate gyrus

GCL- granular cell layer

GFAP- glial fibrillary acidic protein

Gy- Gray

Iba-1- ionized calcium-binding adapter molecule 1

IL- Interleukin

IR- ionizing radiation

ML- molecular layer

P X- postnatal day X

PCNA- proliferating cell nuclear antigen

SGZ- subgranular zone

SVZ- subventricular zone

TNF-α- tumor necrosis factor-α

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

BACKGROUND

Brain tumors in childhood

Brain tumors represent approximately 30% of all malignancies in children and adolescents in Sweden (Fig. 1). In Sweden there are about 70 new cases each year if all types of CNS tumors are taken into account (Dreifaldt et al., 2004). The locations of the tumors in the brain are different in children compared to adults, e.g. 55% of the tumors are located in the posterior fossa (i.e. by the cerebellum) in children compared with only 10% in adults (Bloom et al., 1990). The goal of modern treatment strategies is improve the chance of survival and also improve the patients' future quality of life.

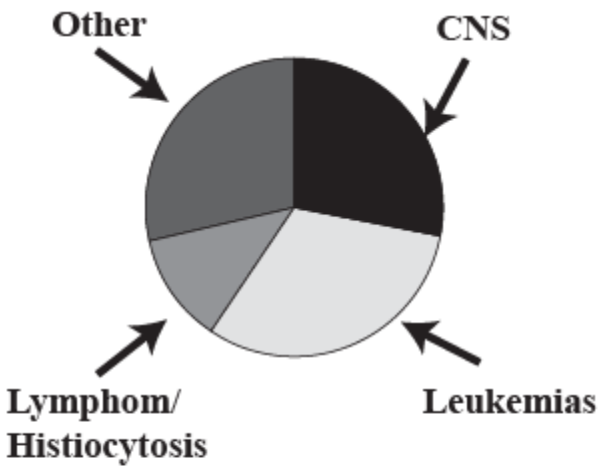

Fig. 1. The distribution of childhood malignancies in Sweden diagnosed 1984-2005 (<15 years of age at the time of diagnosis). The leukemias and CNS tumours constitute roughly 60% of the patients.The picture is modified from the 2007 report from the Swedish childhood cancer registry.

Today, almost 80% of all children with brain tumors survive more than five years, compared with the 1970's when the five-year survival was around 40% (Mariotto et

al., 2009). Improvements in the treatment of brain tumors in childhood have resulted in an enormous increase in long-term survivors. Many of these survivors are now adults living with the consequences of the treatment they received when they were young, the so-called "late effects". For example, brain tumors significantly decrease the likelihood of being employed and, ever getting married or having close friends (Gurney et al., 2009). So, how many long-term survivors suffer from late effects incurred by their treatment? A recent study showed that among brain tumor survivors, 95.7% have late effects (Han et al., 2009). The treatments used today consist of chemotherapy, radiotherapy and surgery. All of them are risk factors for late effects, but radiotherapy has the highest severity when the consequences are graded (Han et al., 2009). Cranial irradiation, young age at diagnosis and female gender are some risk factors for late effects (Fouladi et al., 2005; Lahteenmaki et al., 2007; Han et al., 2009). Radiotherapy significantly increases the need for special education in school due to learning difficulties (Gurney et al., 2009). In addition, 50 to 80% of the children treated with craniospinal radiation for brain tumors experience growth impairment (Duffner, 2004). In general, cognitive decline, growth and puberty impairment after IR are very common late effects (Chin and Maruyama, 1984; Packer et al., 1987; Lannering et al., 1990; Lannering et al., 1995; Habrand and De Crevoisier, 2001; Spiegler et al., 2004; Dietrich et al., 2008).

Radiobiology

Despite the high probability of late effects, radiation is still one of the major treatment strategies used in the treatment of brain tumors. In general, the fundamental principle of radiation is based on the inherent cellular differences in radiosensitivity between normal/healthy tissue and cancer cells. The tumor cells do not have the same capacity

to repair themselves after radiation-induced injury compared to normal cells. To understand this in better detail it is necessary to understand how radiation works and the effects on healthy tissue.

Time scale of effects

One way to get a good overview of the effects radiation has on a biological system is to divide the effects into three phases (Steel, 2002). The first phase is the physical phase which explains the interactions between the charged particle and the tissue (Fig. 2). It takes about 10^{-18}-seconds for a charged particle to cross a DNA molecule. The DNA molecules (or other molecules) then become highly energetic, either by losing an electron (ionization) or moving the electron to a higher energetic state within the molecule (excitation). This then give rise to a cascade of other ionizing events. These events give rise to the second phase, the chemical phase, which means the time when damaged atoms and molecules reacts with other cellular compartments (Fig. 2). It is within this phase where the breaking of chemical bonds and formation of free radicals take place. The free radical reactions are finished in 1 millisecond after radiation exposure. The third and final phase is the biological phase and includes everything that follows the first two phases (Fig. 2). The phase starts with enzyme reactions and repair processes followed by cell death, inflammation and tissue damage. This phase continues for many years and leads to the late effects of radiation treatment.

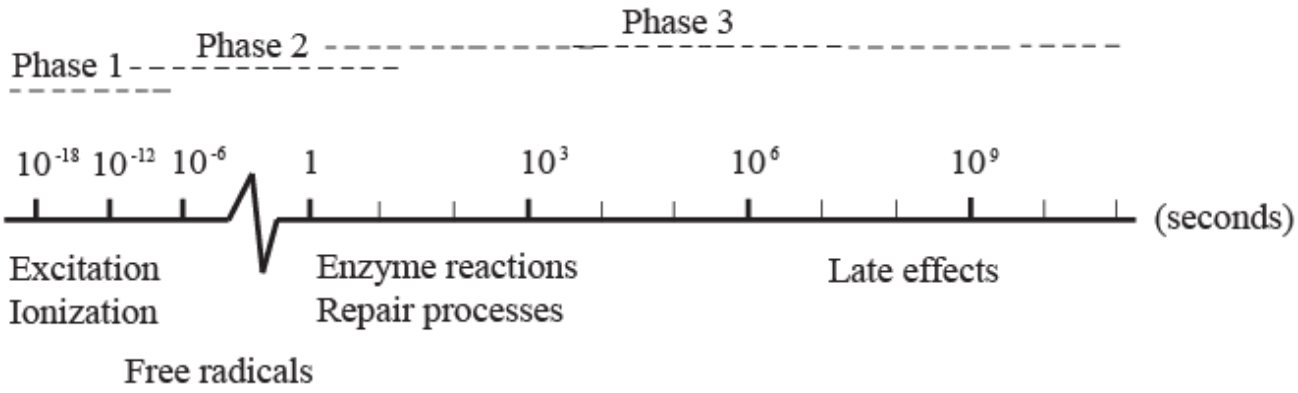

Fig. 2. Time scale after radiation exposure.

DNA strand breaks

The most detrimental outcome of radiation is the biological effects on DNA, which can result in cell death. The DNA damage can be either direct or indirect from the radiation. The direct damage is when parts of the DNA are ionized directly from the radiation. An indirect effect of radiation is the generation of free radicals, which are a major source of DNA damage. Biological systems contain 70-80% water molecules, which easily become ionized and then form free radicals. These radicals can then react with either the DNA molecule or another nearby macromolecule. Another important factor during this process is oxygen. If oxygen is present it has a high affinity for free radicals which induces more double strand breaks in comparison with an anaerobic environment (Distel et al., 2006). The CNS is very susceptible to oxidative stress because it is highly active in oxidative metabolism. The CNS is rich in fatty acids that are easily oxidized and it has a high iron content that can catalyze the generation of reactive oxygen species (ROS). It has also a limited capacity to perform anaerobic glycolysis and low levels of antioxidant defenses (Robbins and Zhao, 2004).

The DNA damage created by radiation can take different forms. The easiest damage to repair is damage to the bases in the nucleotides, but it can also be single strand breaks. When two single strand breaks take place on opposite DNA strands, approximately 10-20 base pair apart, a double strand break occurs (Mahaney et al., 2009). A double strand break is one of the most toxic and mutagenic DNA lesions experienced in the human cell. Therefore a functional repair system for double strand breaks is crucial not only for cell survival but also for correct repair. A faulty repair of the double strand break can result in chromosomal translocations and genomic instability (Mahaney et al., 2009). When double strand breaks occur they activate DNA damage checkpoints to initiate either cell death or cell survival through repair mechanisms.

DNA damage checkpoints

When DNA double strand breaks occur, cell cycle checkpoints become activated. These cell cycle checkpoints monitor the structural integrity of the chromosome before the cell is allowed to continue through crucial cell cycle stages. These checkpoints take place at different time points throughout the cell cycle (Fig. 3). The first and second checkpoints prevent replications of damaged DNA and take place in the G1/S and intra-S phase respectively. The third checkpoint prevents segregation of damaged chromosomes in the G2/M phase (Khanna and Jackson, 2001).

One of the key proteins in checkpoints pathways is the tumor suppressor gene p53, which coordinates DNA repair with cell cycle progression and apoptosis (Fei and El-Deiry, 2003). A simplified way of describing the IR-induced response to DNA damage is to describe it as three phases (Fei and El-Deiry, 2003). The first phase is the initiator phase where sensor proteins recognize the damage, bind to it and activate signaling proteins. In the second phase, the transducer phase, these activated signaling

proteins, e.g. ATM, are capable of phosphorylating target proteins known as effector kinases. The third and final phase is then called the effector phase and the outcome of this phase is either apoptotic cell death or cell cycle arrest coupled with DNA repair and cell survival (Khanna and Jackson, 2001; Fei and El-Deiry, 2003).

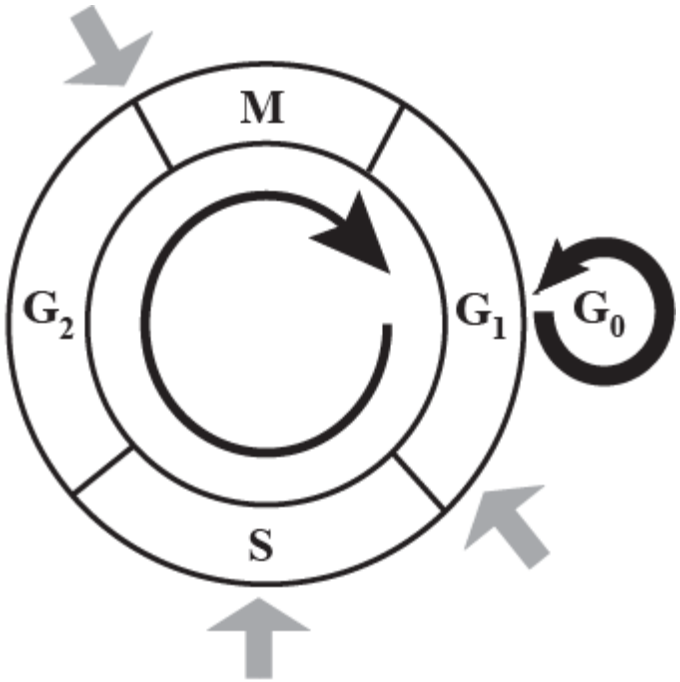

Fig 3. The cell cycle and its checkpoints.

DNA repair

There are several systems that deal with DNA repair in the mammalian cell. For less serious damage, "base excision repair" is used to replace the broken base. "Nucleotide excision repair" is devoted to repairing UV-induced damage by exchanging the nucleotides. And the "mismatch repair" system deals with mismatches occurring during replication (Hoeijmakers, 2001). Two key pathways are activated when a double strand break occur. The first pathway is called HDR (homolog-directed repair)

which is a very accurate repair system, but it requires an undamaged sister chromatid to act as template and it only functions after replication (Mahaney et al., 2009). The second system is called NHEJ (non-homologous end-joining) and this pathway can be active throughout the cell cycle. NHEJ is considered to be the major repair pathway of IR-induced double strand breaks in the DNA (Mahaney et al., 2009).

In general, the NHEJ pathway entails a straightforward ligation of two severed DNA ends in a sequence-independent fashion (Helleday et al., 2007; Mahaney et al., 2009). Due to the fact that an IR-induced injury to the DNA often is very complex and frequently consists of non-locatable end groups and *e.g.* damaged nucleotides, this repair system is very prone to errors (Mahaney et al., 2009). The reason is that the DNA ends then need processing before the joining can proceed, which can lead to loss of nucleotides. Of course, the pathway is much more complex than that, and it requires a machinery of enzymes to repair the DNA. Most of the DNA damage takes just 30 minutes to repair, but the more complex injuries can take up to six hours. Therefore it is important to wait at least 6 to 8 hours before giving a new radiation dose to a patient to avoid severe side-effects (Degerfält et al., 2009).

Malignant cells are generally highly sensitive to radiation because of their genomic instability and loss of growth regulation. As part of the perturbed growth regulation, most tumor cells have lost their ability to undergo apoptosis, which means that they will die through mitotic catastrophe or senescence-like permanent growth arrest upon radiation (Gudkov and Komarova, 2003). Brain neurons are post-mitotic, in a permanent state of growth arrest, and can tolerate relatively high doses of IR, but the recently discovered neurogenic regions, the dentate gyrus (DG) and the subventricular

zone (SVZ), where new neurons are produced throughout life, are highly sensitive to IR, particularly in the developing brain (Fukuda et al., 2005).

Neurogenic regions and IR

There are several reasons for the adverse late effects following brain tumor treatment. One reason that is believed to explain the learning difficulties after IR is that neurogenic areas, the SVZ and the subgranular zone (SGZ) of the DG in the hippocampus, are highly susceptible to IR-induced injury (Fig. 4). This has been demonstrated in rodents (Tada et al., 2000; Fukuda et al., 2004; Raber et al., 2004; Fukuda et al., 2005) and appears to be true also for humans (Monje et al., 2007).

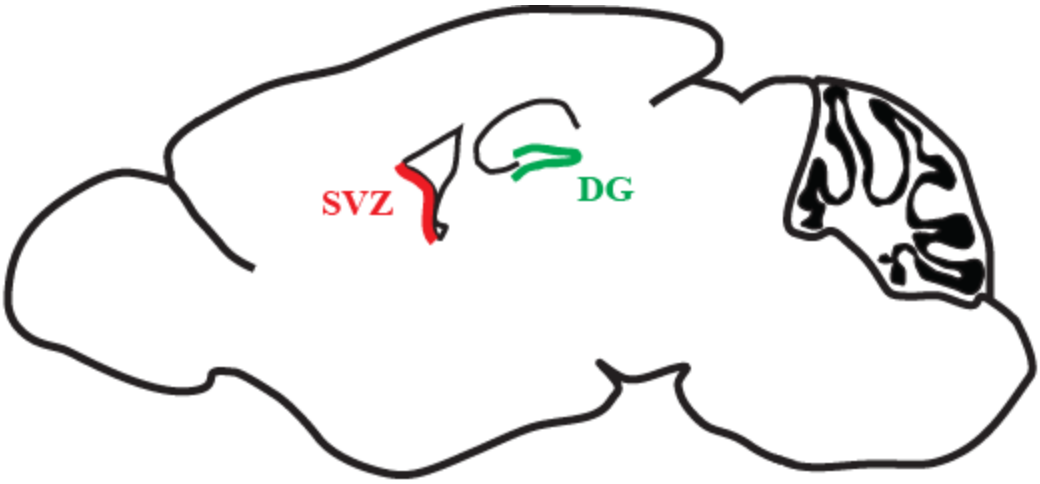

Fig 4. A sagittal overview of the mouse brain. The SVZ and the DG are both neurogenic areas where neural precusor cells reside.

In addition to acute cell loss in the neurogenic areas, IR can influence the survival of stem and progenitor cells in those regions, leading to a limited potential in terms of repopulation or regeneration (Tada et al., 2000). We have previously shown that a

single, moderate dose of IR (8 Gy) to the young rat brain dramatically impaired growth of the DG and the SVZ and that there was no morphological recovery, as judged by the size of the neurogenic regions (Fukuda et al., 2005). Recently, however, it was demonstrated that whereas the initial response to radiation injury was similar in both neurogenic niches, *i.e.* virtually abolished neurogenesis, the long-term effects differed significantly; the dentate gyrus was severely affected long-term, whereas the SVZ appeared to recover with time (Hellstrom et al., 2009). It has been shown that IR causes a change in the microenvironment, making the progenitor cells in the DG shift their proliferative response from neurogenesis to gliogenesis, and this was attributed to an inflammatory response (Monje et al., 2002). Recent studies have suggested that IR, in addition to inducing acute cell death, affects the fate of the precursor cell pool by altering the local microenvironment and the signaling necessary for neurogenesis. IR-induced inflammation was demonstrated to cause progenitors of the SGZ to differentiate into astrocytes instead of neurons (Monje et al., 2002; Monje et al., 2003). Inflammation in the brain can have negative effects on recovery following injury, but some actions appear to be beneficial and essential. Therefore, understanding the principles of neuroinflammation is crucial to fully understand the mechanisms of IR induced injury.

Neuroinflammation

Neuroinflammation is a concept that was first introduced in the mid 90's. During the last decade, awareness of the importance of neuroinflammation has increased and also the knowledge of this very complex phenomenon. Earlier, it was believed that the brain was a privileged site, devoid of inflammation. Today we know that this is not the case. The brain differs from other organs in the body in response to inflammation. The

immune response can be divided into the innate (non-specific) and the adaptive (antibody-mediated) immune system. However, as the brain is relatively isolated from immunosurveillance under normal conditions, this limits the adaptive response (Francis et al., 2003). The most important reason for this difference is the blood-brain barrier, reduced expression of adhesion molecules and an immunosuppressive environment creating an immunological barrier to the brain (Schwartz et al., 1999). This is demonstrated by comparing the recruitment of leukocytes to the site of injury, a very rapid process in other organs, but a much slower and more modest response in the brain (Lucas et al., 2006). It has also been shown that neurons, astrocytes and microglia suppress infiltration of peripheral immunocompetent lymphocytes by expressing tumor necrosis factor-related death ligands that mediate apoptosis of infiltrating cells (Flugel et al., 2000; Griffiths et al., 2009). A contributing factor to this could be that the brain harbors microglia (the immune effector cells of the brain), which respond to injury very rapidly.

Microglia

One major factor of neuroinflammation is the activation of microglial cells. Little is known about their role in the healthy brain and their immediate reactions to brain damage. It is believed that during development, microglia enter the brain and play an important role during maturation of the brain, *e.g.* by removing debris from cells that are dying (Dalmau et al., 2003). It has not yet been resolved how the microglia population is maintained during homeostasis and injury, but they do comprise 5-20 % of all glial cells in the brain (Tambuyzer et al., 2009). Assuming that there are 10 times more glia than neurons in the CNS, it means that there are as many microglia cells as there are neurons. The density of microglia varies between the different regions in the brain, but in general there are more microglia in the gray matter

(Lawson et al., 1990). One important thing that is known is that microglial cells are highly active in their presumed resting state, continually remodeling their processes, in apparent surveillance of the extracellular milieu (Nimmerjahn et al., 2005). Therefore it is nowadays more correct to say that in terms of injury, microglia progress from a surveillant state to an effector microglia instead of saying that they become activated.

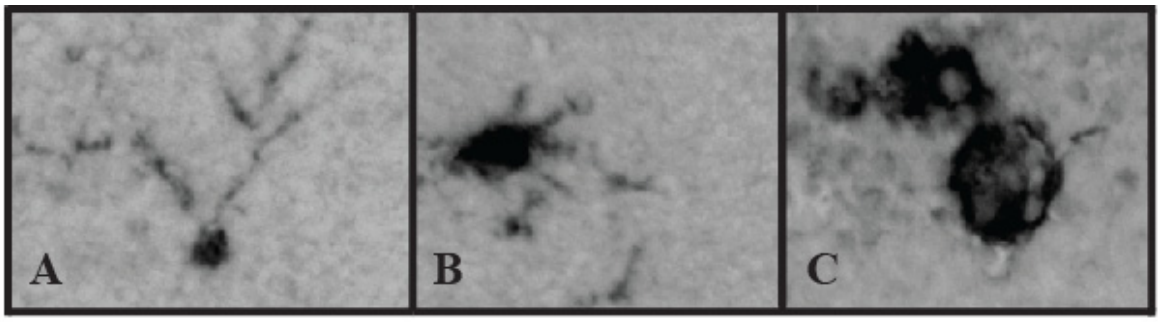

Fig 5. A resident, "resting" microglia is shown in the left microphotograph (A). In the middle an activated microglia is shown which can be identified by the shorter and thicker processes (B). In the right microphotograph (C) two amoeboid microglia that are fully capable of phagocytosing are shown.

In terms of injury, microglia are able to respond quickly. The morphological change is an important characteristic seen after activation (Fig. 5). When microglia are initially activated, they first take on a "bushy" appearance with shorter and thicker processes, followed by an amoeboid phenotype that is fully capable of phagocytosing (Tambuyzer et al., 2009). Microglia can become activated by disruption of the blood brain barrier, fluxes in extracellular glutamate, extracellular ATP from damaged cells, and disrupted tonic inhibition via CX3CR1 (Ransohoff and Perry, 2009). Release of pro-inflammatory cytokines is an early sign of activated microglia and already 15 minutes after injury, the upstream pro-inflammatory cytokine IL-1β is released from microglia (Herx et al., 2000). This leads to activation of surrounding glial cells,

recruitment of blood-borne leukocytes, cell adhesion, migration, differentiation, replication, and cell death (Harry and Kraft, 2008).

Cytokines and chemokines

The acute effects occur within the first hours, and as mentioned above, the upstream cytokine IL-1β is released just 15 minutes after irradiation. We have shown that the cytokine and chemokine peak is around 6 hours after a single dose of irradiation to the developing brain (Kalm et al., 2009b). The first cytokines that are released are the pro-inflammatory cytokines, e.g. IL-1 and TNF-α. These small proteins are usually secreted by microglia (Tambuyzer et al., 2009). Neurons, astrocytes and oligodendrocytes can also produce inflammatory mediators, and cytokine receptors are expressed constitutively all over the brain (Lucas et al., 2006). The pro-inflammatory cytokines initiate expression of chemokines and adhesion molecules necessary to recruit leukocytes (Lee and Benveniste, 1999). Chemokines are small peptides that are secreted during neuroinflammation and they play a crucial role in migration and intracellular communication during neuroinflammation (Tambuyzer et al., 2009). Chemokines also play a key role in leukocyte trafficking into the CNS (Ubogu et al., 2006). The most up-regulated marker we have investigated after IR is the chemokine CCL2 (also called MCP-1) which is expressed both by astrocytes and microglia (Kalm et al., 2009b). CCL2 can also influence the neural precursor cells to migrate towards the injury (Belmadani et al., 2006). Both cytokines and chemokines belong to the innate (nonspecific) immune system.

Complement system

The complement system is an important part of the innate immune system. Within the CNS the complement proteins are produced by neurons, oligodendrocytes, microglia

and astrocytes (Gasque et al., 2000). The system includes over 40 proteins, with receptors and regulators, and the complement component 3 (C3) being a central part of the system (Fig. 6). The system can be activated through three alternative pathways; classical, alternative and lectin. A fourth pathway has recently been discovered that involves the terminal cascade when lacking C3 (Huber-Lang et al., 2006). The pathway that activates the system depends on the stimuli. The classical pathway becomes activated by interaction with immune complexes or nonimmune activators, e.g. C-reactive protein. The lectin pathway becomes activated by carbohydrates found on pathogens. The alternative pathway becomes activated without the presence of an immune complex. Activation of that pathway is performed by deposition of C3 on target cells (Francis et al., 2003). When the complement system becomes activated, a cascade of protein cleavage, including the key proteins C3 and C5, takes place (Alexander et al., 2008). This process initiates the assembly of a membrane attack complex (MAC) that can result in cellular death or activation, recruitment of inflammatory cells and induction of inflammation. Since the complement system is so powerful, it is under strict control to prevent self-injury. The complement system is, however, also involved in other processes than inflammation. A recent finding shows that unwanted synapses are tagged by complement for elimination (Stevens et al., 2007). In addition, it has been demonstrated that progenitor cells and immature neurons are directly affected by the complement system via receptors for C3a and C5a, such that complement activation was shown to promote both basal and ischemia-induced neurogenesis (Rahpeymai et al., 2006).

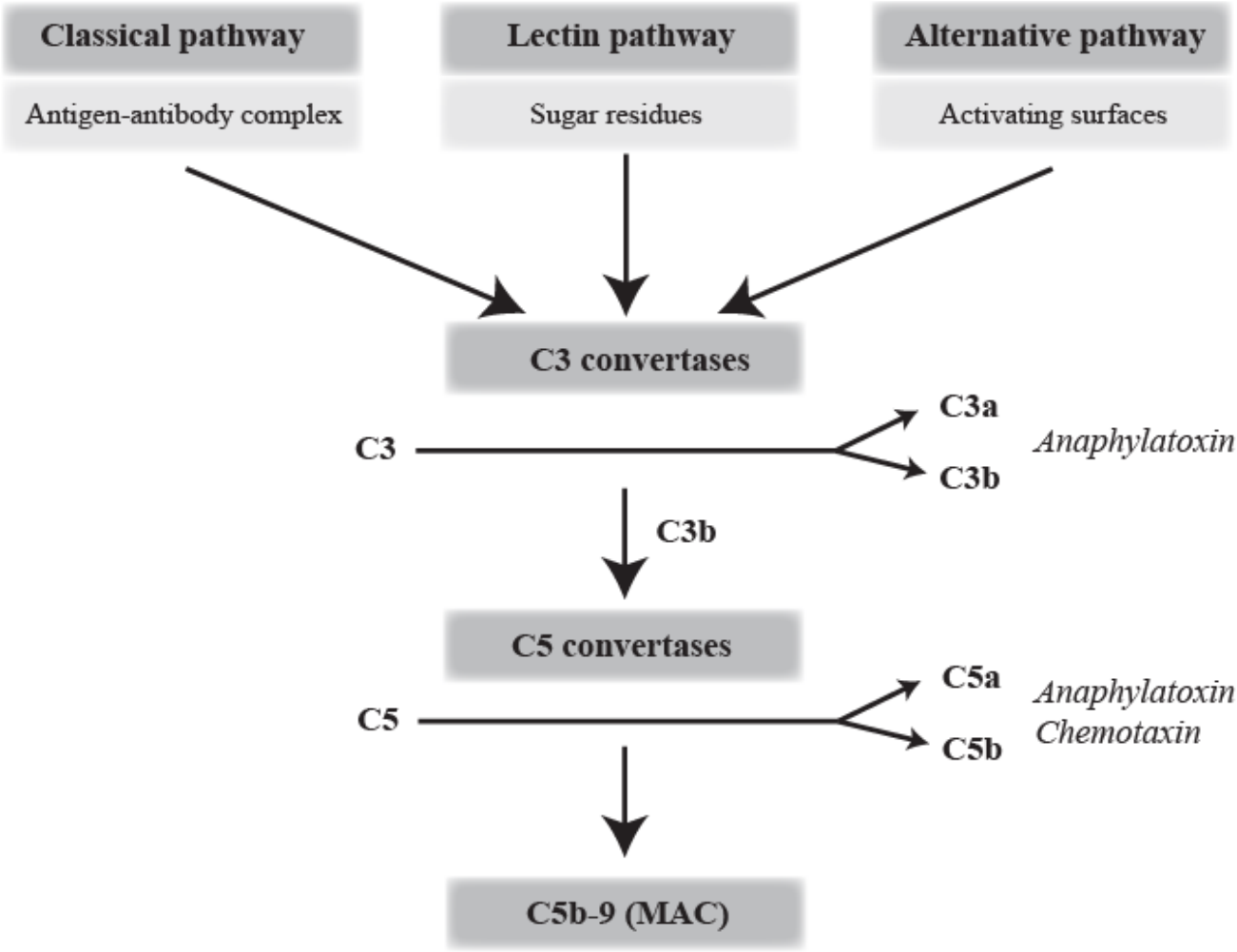

Fig 6. A schematic overview of the three complement activation pathways.

Hippocampal function

A region that is highly affected by IR is the hippocampus, more specifically the dentate gyrus. In this thesis, most of the focus is on the dentate gyrus, due to the hypotesis that it is involved in the memory-related late effects seen after IR (Fig. 7). The establishment and maintenance of memories take place in the cortex, but the hippocampus and the parahippocampal regions are critical in supporting the

persistence and organization of memories (Eichenbaum et al., 1996). In the 1950's it was shown that whenever the hippocampus was damaged bilaterally during surgery, declarative memory was severely impaired (Scoville and Milner, 1957). The declarative memory is our capacity to recall everyday facts and events.

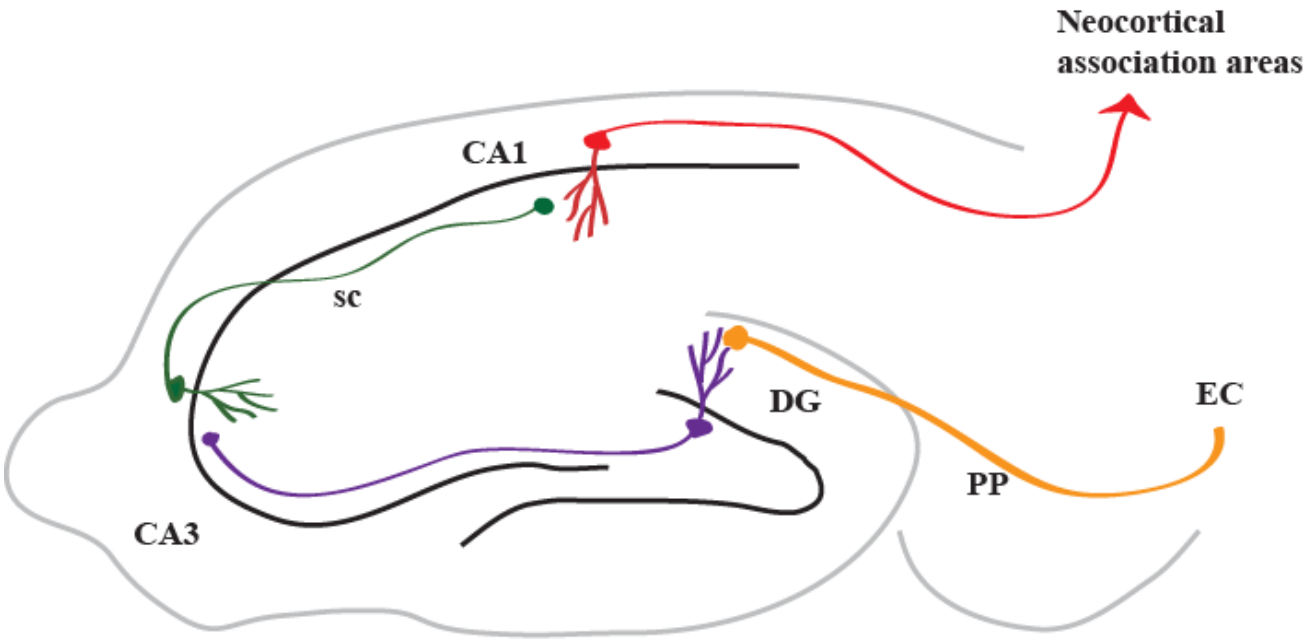

Fig. 7. An overview of the dentate gyrus circuitry. The granule cells of the dentate gyrus (DG) recieve information from the entorhinal cortex (EC) via the perforant pathway (PP). The granule neurons' axons project to CA3 neurons, where the signal is relayed through Schaffer collaterals (sc) which in turn project to the CA1 region. The signal is then relayed back to the neocortical association areas for long-term storage.

In the adult dentate gyrus, newborn neurons migrate from the SGZ into the GCL and integrate into the existing circuitry and receive functional input (Zhao et al., 2008). The survival of newborn cells is regulated through diverse mechanisms and many of them die within four weeks after birth. Once they mature, the granule cells receive excitatory glutamatergic input mainly from the entorhinal cortex and GABAergic

input from interneurons within the dentate gyrus (Laplagne et al., 2007; Zhao et al., 2008). It has also been shown that dentate granule cells born in early postnatal and adult mice acquire a remarkably similar afferent connectivity and firing behavior, suggesting that they constitute a homogeneous functional population (Laplagne et al., 2006). It has also been shown that there is a correlation between hippocampal neurogenesis and spatial memory tasks, particularly the acquisition of new information (Kempermann and Gage, 2002). Nevertheless it is still unclear whether or not hippocampal neurogenesis is a major fundamental factor for the changes seen in cognition (Leuner et al., 2006).

GENERAL AIM

The aim of this project was to investigate how inflammation influences the injury and repair after irradiation to the developing brain. It is necessary to investigate the inflammatory mechanisms to be able to find potential treatment strategies to prevent late effects following radiotherapy.

Specific aims

I. To characterize the inflammatory mechanisms after irradiation to the young rodent brain.

II. To characterize the inflammatory response on a cellular level by investigating the effects on microglia after irradiation.

III. To investigate if the IntelliCage platform is suitable to detect irradiation-induced injuries.

IV. To investigate if modulation of the complement system could alter the outcome after irradiation.

MATERIAL AND METHODS

Animals

Rats (Papers I and II)

Wistar rat pups of both sexes, obtained from B&K Universal, Solna, Sweden, were used for the studies made in paper I and II. The animals were kept in a 12-h light/dark schedule (lights on at 07:00 h) with food and water available *ad libitum,* which means that they were able to drink and eat when they wanted to. All animal experimentation was approved by the Gothenburg committee of the Swedish Animal Welfare Agency (204-2001, 184-2003 and 79-2004).

Mice (Papers III and IV)

In paper III, C57BL/6 litters with male mice were used (Charles River Laboratories, Sulzfeld, Germany). In paper IV $C3^{-/-}$ mice of both sexes were produced as described earlier (Pekna et al., 1998). Wild type (WT) C57BL/6 mice of the same substrain as the mutant mice served as controls, ordered from the same source as the $C3^{-/-}$ mice (Charles River Laboratories, Sulzfeld, Germany). All mice in this study were bred in-house. The animals were kept on a 12-hour light cycle where food and water was provided *ad libitum.* After weaning, the animals were kept in groups of up to 10, separated depending on genotype and gender. Before weaning, all mice were anesthetized with isoflurane and injected subcutaneously with microtransponders (DATAMARS, PetLink, Youngstown, USA) to be able to identify individual animals in the IntelliCages. All animal experiments where approved by the local committee of the Swedish Animal Welfare Agency (46/2007, 30/2008)

Comments: In this thesis, mice and rats were used as model organisms to better understand what happens after IR-induced injury in the human brain. We have tried to extrapolate the timing of brain development from the rodent models to human, but there is still a major difference between rodents and human which one needs to keep in mind when interpreting the results.

In our earlier studies rats were used due to their ease of handling, but because we wanted to use knockout models and also examine the behavior using the IntelliCages, we switched to mice. The IntelliCage platform has until recently only been available for mice. The age in most of the studies was postnatal day 9 (P9) and P10 which corresponds to a newborn human in brain development (Dobbing and Sands, 1979). In paper III, we changed the ages at the time of irradiation in our experiments to P14, which would approximately correspond more to a 3-year-old than a new born (Quinn, 2005). Considering that radiotherapy to children younger than 3-4 years of age is very unusual, we wanted in paper III to move towards a more clinically relevant age. Nevertheless, the biological phenomena studied are equally relevant, and comparing the developmental brain age of rodents and humans is complicated. When for example, comparing human and rodent hippocampal development, the majority of dentate gyrus granule cells, which begin to develop by 13 weeks, are born during the third trimester and the first year of life in humans (Seress et al., 2001). In rodents, granule cells of the dentate gyrus began to develop only 2-3 days before birth, as a consequence 85% of the dentate granule cells are formed postnatally and 10% of the populations forms after P18 (Bayer, 1980), meaning after we performed the IR. Importantly, the granule cell production continues throughout the life, also in the adult human brain (Eriksson et al., 1998).

Irradiation model

For IR, a linear accelerator (Varian Clinac 600 CD; Radiation Oncology Systems LLC, San Diego, CA, USA) with 4 MV nominal photon energy was used. In papers I and II, P9 or P21 pups of both sexes were used. In paper III, P14 male mice were used. In paper IV, P10 mice were used. All animals were anaesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg tribromoethanol (Sigma, Stockholm, Sweden) to immobilize them during the IR procedure. The animals were placed in prone position (head to gantry) on a styrofoam bed. The radiation field size depended on if one or both hemispheres were irradiated (1 × 2 or 2 × 2 cm). To obtain an even IR dose throughout the underlying tissue, the head was covered with a 1 cm tissue equivalent material. The dose variation within the target volume was estimated to be ±5 %. The entire procedure was completed within 10 min. After IR, the pups were kept on a warm bed (36°C) not to decrease in body temperature (which would influence the IR response itself), then returned to their biological dams until sacrificed. Sham-irradiated control animals were anaesthetized but not subjected to IR. A single absorbed dose of 8 Gy was administered. This dose is equivalent to approximately 18 Gy when delivered in repeated 2 Gy fractions, according to the linear-quadratic formula (Fowler, 1989) and an α/β-ratio of 3 for late effects in the normal brain tissue. The α/β-ratio is used to quantify the fractionation sensitivity of tissues. The 8 Gy dose represents a clinically relevant dose, equivalent to the total dose used in treatment protocols for prophylactic cranial irradiation in selected cases of childhood acute lymphatic leukemia (18 Gy). The doses used for pediatric brain tumors are often higher, up to 55 Gy.

Comments: Regarding the irradiation procedure, it would have been optimal to use fractionated radiation to be able to mimic the clinical setting as much as possible. That was not possible in these studies due to practical reasons. The animals had to be transported to the hospital from the animal facility and after irradiation, to be returned to the animal facility. This is stressful for the mice/rats and there is also a risk of bringing back microorganisms to the animal facility. In addition, tribromoethanol was used to sedate the animals. This compound irritates the tissue and should therefore only be used once.

Gene chip expression analysis

To investigate the genetic profile after IR, we used gene chip expression analysis according to the Affymetrix platform. When doing this it is very important to preserve the RNA. We did that by removing the forebrains and rapidly freezing them on powdered dry ice. Total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The purity of the samples was determined by 260/280 spectrophotometry and degradation estimated by agarose gel electrophoresis. For genetic profiling, we used Affymetrix GeneChips (MG-U74Av2). Array expression analysis was performed according to the Affymetrix expression analysis technical manual at the Swegene facility in Lund, Sweden.

Comments: To be able to handle the analysis of the 15 923 probe sets investigated we used GeneSifter™ Analysis Edition, which is a data analysis software. It helped us to analyze the microarray data in a "user friendly" manner. In this study we chose to investigate only genes linked to the immune response, even though several other genes were affected by the IR. An important issue when investigating RNA is that it doesn't

necessarily correlate with the protein expression, therefore it is recommended to follow up the most interesting genes also on the protein level, e.g. by using immunohistochemistry or ELISA.

Immunoassay

To investigate the IR-induced response on the protein level we used two different immunoassays. To avoid protein degradation before analysis we dissected out the brains quickly and froze them in isopentane and dry ice, followed by storage at -80ºC. The tissue was further homogenized by sonication in a buffer with PBS containing protease inhibitor cocktail, EDTA and Triton-X-100. To minimize the risk of aggregation when running the immunoassay the tissue was diluted in 5 volumes of buffer. In paper I we used the Luminex platform and in paper IV we used the Meso Scale technology. Both methods are multiplex analysis.

Luminex (paper I)

Before running the analysis, the total protein concentration was measured according to Whitaker & Granum (Whitaker and Granum, 1980), adapted for microplates. Crude cytosolic fractions were assayed for the following rat cytokines/chemokines: GMCSF, IL-1α, CCL2, IL-4, IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-5, IFNγ, IL-18, GRO/KC and TNFα, using a multiplex bead-based immunoassay kit (#RCYTO-80K-PMX, Linco, St Charles, Missouri, USA) essentially according to the protocol of the manufacturer. Samples were analyzed using the Bio-Plex™ Protein Array System and the related Bio-Plex Manager™ (Bio-Rad, Sundbyberg, Sweden).

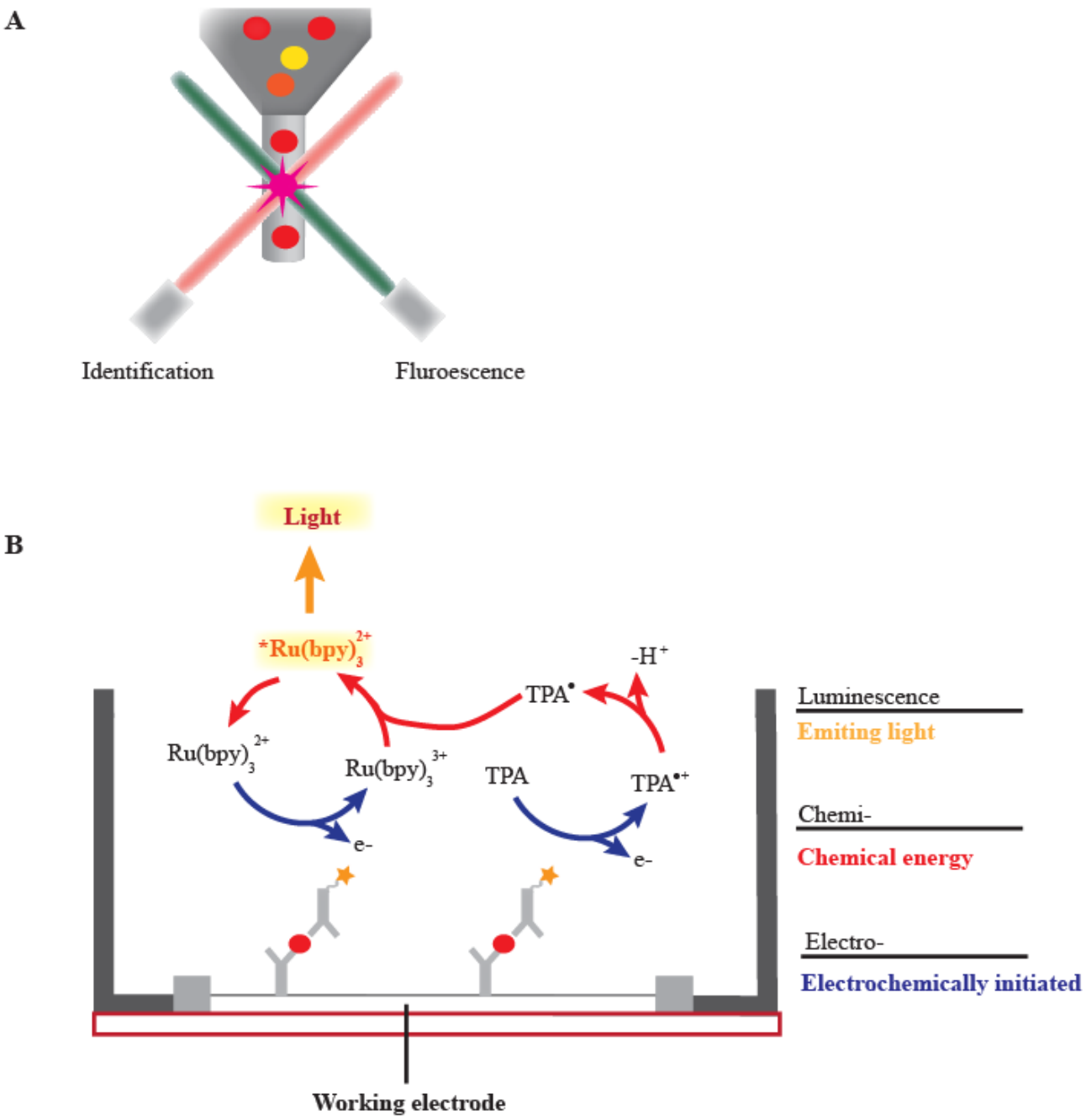

Fig. 8. The flow cytometry based system is showed in A. It has two lasers one for detection of the identification signal and the other for detection of the fluorescence signal. In B the Meso Scale system is illustrated. The SULFO-TAG ($Ru(bpy)_3^{2+}$) emits light upon electric stimulation.

Meso Scale (paper IV)

In this study, total protein concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Crude cytosolic fractions were assayed for the following mouse proteins: KC, CCL2, VEGF, IL-1β, IL-6, GM-CSF and TNF-α using a custom-designed 7-plex assay (Meso Scale Discovery, Gaithersburg, Maryland). The assay was performed according to the protocol from the manufacturer using a Sector® Imager 6000 instrument (Meso Scale Discovery, Gaithersburg, Maryland) and the Discovery Workbench® software (Meso Scale Discovery, Gaithersburg, Maryland) for reading and quantification, respectively.

Comments: Luminex and Meso Scale are based on two different principles (Fig. 8). When using Luminex the antibody of interest is attached to a bead that emits an identification signal. Since it is possible to design beads with up to 100 unique signatures it is theoretically possible to test 100 analytes within a single reaction volume. The system is based on flow cytometry with a single bead passing the detection chamber at any time. The detection chamber has 2 lasers; one detecting the identification signal from the bead and the other detects the signal from fluorescence associated with the analyte. The Meso Scale system is based on a different technology. The antibodies of interest are attached to the bottom of the plate (divided into different spots), where they are attached to an electrode surface. The analyte attaches to the capture antibody (on the electrode surface). After that, a labeled detection antibody is attached to the analyte. The detection is made by electrochemiluminescence, i.e. light is emitted upon electrical stimulation from the bottom of the plate via activation of a SULFO-TAG™ that is in the reaction solution. In one well it is possible to have several spots detecting up to 10 analytes (1 per spot). If one wants to test more than 10 analytes simultaneously, Luminex could be preferably. Otherwise, the systems are

quite similar and available kits on the market could be the reason to choose one system and not the other.

Immunohistochemistry

Tissue preparation

Animals were deeply anaesthetized with sodium pentobarbital (60 mg/ml, 2 ml/100 g body weight i.p.) and transcardially perfusion-fixed with Histofix (EMD Chemicals Inc., an Affiliate of Merck KGaA, Darmstadt, Germany) or 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed and immersion-fixed in the same solution at 4°C for 24 h. In papers I and II the brains were cut coronally into two pieces, one of which contained the striatum, and the other containing the hippocampus. After dehydration, with a graded series of ethanol and xylene, followed by embedment in paraffin, the brains were cut into 5 µm coronal sections and mounted on glass slides. This means that the paraffin has replaced the water in the tissue. In papers III and IV the left and right hemispheres were separated and put in 0.1 M phosphate-buffer with 30% sucrose, pH 7, for storage. The right hemisphere was cut in 25 µm sagittal sections on a sliding microtome. The sections were collected and stored in a cryoprotection solution containing 25% glycerine and 25% ethylene glycol in 0.1 M phosphate buffer.

Comments: In papers I and II we used paraffin sectioning whereas in the other papers free floating methodology was used. The two methods are very different and this has to be considered before starting an experiment. If the tissue is very sensitive due to, for example, lesions and could therefore fall apart, paraffin-embedding is preferable. Using free floating sectioning makes it possible to cut thicker sections compared to

paraffin sectioning. This means that it is possible to stain several cell layers within a section. To be able to do that it is also of importance to know what antibodies you want to use in your experiment. Some antibodies have problems to penetrate the tissue, and for that reason paraffin sections are more suitable.

Staining

In these studies we have used immunohistochemistry for single, double or triple labeling. In papers I and II we used paraffin sections and in paper IV free floating sections. Before the staining of paraffin sections can start, it is necessary to burn (melt) the sections to the slides so they don't fall of during the staining procedure. This step is not needed for free floating since the sections are kept in wells during the staining. When using paraffin sections it is usually beneficial to perform antigen retrieval, which we do by boiling the sections in citric acid buffer. Similar steps could be necessary for some antibodies that have problems penetrating the tissue when using free floating. For both paraffin and free floating blocking nonspecific binding before incubating with the primary antibody is performed. Incubating with the primary antibody is often made over night so the antibody has enough time to find its antigen. All primary antibodies used in these studies are presented in table 1. The following day all unbound antibody is washed of and the appropriate, fluorophore-conjugated or biotinylated secondary antibody is added. When performing double labeling, fluorophore-conjugated secondary antibodies are used to make it possible to detect two or more different antibodies at different wavelengths. When immunofluorescence is used, it is also common to stain for the nuclei. In these studies we have used To-Pro3 (for the confocal) and DAPI to visualize the chromatin. Visualization when staining with a biotinylated secondary antibody was in these studies performed using

an avidin-biotin-peroxidase solution. Stainings were then developed with 3,3-diaminobenzidine.

Primary antibody	Dilution	Company
goat anti MCP-1 (CCL2)	1: 50	Santa Cruz Biotechnology
rabbit anti S-100β	1: 100	Swant®
mouse anti NeuN	1: 200	Chemicon
rabbit anti-Iba1	1: 2000	WAKO Pure Chemical Industries
rabbit anti CCR2	1: 100	Abcam
mouse anti GFAP	1: 500	Chemicon International
mouse anti rat ED-1	1: 2000	Chemicon International
goat anti Iba-1	1: 500	Abcam
rabbit anti-cleaved caspase 3	1: 50	Cell Signaling Technology
goat anti-synapsin 1a/b	1: 250	Santa Cruz Biotechnology
rabbit anti-phospho-histone H3	1: 100	Upstate
goat anti- Neuro D	1: 100	Santa Cruz Biotechnology
rabbit anti-S100β	1: 5000	Dako

Table 1. List of primary antibodies.

Comments: Visualization of different antigens using immunohistochemistry is a great method to use to better understand, for example, the response after injury. It is of great importance to know what you are supposed to see and to know the properties of the proteins you want to visualize, so that you can decide if the staining finally obtained looks like what you should expect. Some antibodies are not as specific as one would wish, yielding unspecific binding. Therefore, optimizing the protocol before performing a big experiment saves both time and money. In these studies we always

used commercially available antibodies, but negative controls and optimization was performed.

Cell counting

Cell counting was performed on both paraffin and free floating sections. In paper II Iba-1-positive cells with a clearly identifiable, DAPI-stained nucleus were counted in the entire hippocampus and the SVZ in the acute phase (6 hours after IR). In the subacute phase, 7 days after IR, Iba-1-positive cells were counted in the entire hippocampus, SVZ, corpus callosum and cortex. In the cortex, a stereological fractionator was used, which means that evenly randomized boxes within the cortex was counted and not the entire area. Cells double-positive for Iba-1 and cleaved caspase-3 were identified in the entire hippocampus. Positive cells were separated into groups with a single DAPI-stained nucleus or several DAPI-stained pieces of chromatin. To investigate if the developmental level of the brain would influence the microglial reaction, both developing (P9) and virtually fully matured (P21) brains were irradiated and evaluated 7 days later. These quantifications were made on one section from each brain. In paper IV, cells were quantified in the dentate gyrus (DG), including the granule cell layer (GCL), the hilus and the molecular layer (ML), using stereological principles. Neuro D-positive cells, though, were counted only in the GCL, using the fractionator function. The volumes of all three areas were measured using DAPI-stained sections in order to minimize the shrinkage by the staining procedures. Every 10^{th} section was counted throughout the whole DG.

Comments: In paper II we investigated one section from each brain, which means there is a risk such that if the areas of interest have few positive cells a potential difference between groups could be lost due to a high variance within the groups.

Also, measuring the volume is not possible since the sections are so thin (5 µm) and just one section is measured in these studies. It is possible to measure the volume if several paraffin sections are used according to stereological principles. In paper IV we used free floating sections and applied stereological principles which give more accurate information than when the quantification is made on paraffin sections. After IR one important factor to investigate is the volume due to the effect IR has on growth and also cell death. Using stereological principles and measuring every 10^{th} section throughout the brain gives stable values, both when counting cells and measuring the volume.

IntelliCage®

In paper III and IV the IntelliCage platform was used to study the mice in a home cage-like environment. The IntelliCage platform has previously been described elsewhere (Galsworthy et al., 2005; Knapska et al., 2006; Onishchenko et al., 2007). Briefly, each IntelliCage contains four learning and conditioning corners. Each conditioning corner is equipped with an antenna that registers the implanted microtransponders when the mice enter the corner. In addition, each corner contains two water bottles that can be sequestered using doors. Sensors in each corner register nose pokes and drinking from the water bottles. Several IntelliCages can be connected to a computer that registers events in the cages. The software can be programmed to act on specific events in the cages, for instance, when the animals perform a nose poke (approaching the door with their nose), the computer can open the door to allow the animal access to the water bottles. In each corner it is also possible to activate the air valve for administration of punishment (air puffs) or to turn on up to 6 different LED lights in different colors (red, blue, green, yellow) in response to an event.

The animals in these studies were house in the IntelliCages in groups of up to 10 animals per cage even though it is possible to house up to 12 animals per cage according to the manufacturer. The experiments always started with an introduction period (4-6 days) where the mice were allowed to acclimatize to the cage and the new way of gaining water through performing nose pokes. The introduction period was followed by a corner training period, where each animal was randomized to one corner; this corner was programmed as the correct corner while all the three other corners were programmed as incorrect. The mice were only allowed to drink from the water bottles in a correct corner, where a nose poke would give them access to the water bottles. In incorrect corners, the doors to the water bottles remained closed after nose pokes. After 5 days, the animals were randomized to a new corner. In two separate experiments with adult animals, the air valve was activated when the mice made a nose poke in a non-allocated corner or the green LED light was turned on when the mice entered a correct corner.

Comments: The IntelliCage platform is a fairly new way of investigating learning and behavior in the animals' home cage environment, i.e. without disturbing the animals by handling or stressing them by changing their environment when testing them, as in the case in e.g. Water Maze or open field. The system is built with advanced technology which puts some demands on the users when handling the IntelliCages. We have tried different set-ups to investigate the IR-induced injury. For example, when using punishment-based learning using air-puffs, unacceptable numbers of animals had to be excluded from the experiment due to them not making any visits (hence were not able to drink). Instead we have used set-ups where the animals did not become afraid of visiting the corners.

The data from the IntelliCages was analyzed using the IntelliCage software (IntelliCage Plus, 2.4, NewBehavior AG, Zurich, Switzerland) together with Microsoft Excel 2007 and SPSS 15 (SPSS Inc, Chicago, USA). Only the active period (dark period) was analyzed and visits lasting longer than 180 seconds were excluded from the analysis. This was performed to remove visits where the mice entered the corners to sleep and accidently did "nose pokes" with their backs. In paper III, all visits that did not lead to a nose poke were also excluded. The corner is a small area so only one mouse is able to be there at a time, even though we have occasionally seen two mice in one corner. Therefore, it is quite important to wait until mice are large enough before using the IntelliCage. Animals that performed 6 or less on two of the three measured parameters (visits, nose pokes and licks) per day were excluded from the experiment since we could not be sure that they understood how the corners worked or if the chip was not registered. It is impossible for the mouse to open the door in a corner if the chip is not registered, so it is necessary to keep track of their visit/ nose poke/ lick frequency.

Open field

In paper III we tested the mice in an open field paradigm. The animals were 21 and 65 weeks old, respectively, when they were introduced to an unfamiliar open field arena for 50 minutes. This method has previously been described by Nilsson et al. (2006). Briefly, four arenas were simultaneously videotaped from above with one single CCD monochrome video camera. The camera was connected to an S-VHS videocassette recorder. The arenas were made of black Plexiglas (*l*, *w*, *h*: 46 × 33 × 35 cm), rubbed with sandpaper and indirectly illuminated to avoid reflexes and shadows. The floors of

the arenas were covered with grey gravel that had earlier been exposed to other mice (Nilsson et al., 2006). The videotapes were after completion of the experiment analyzed using the video-tracking software Ethovision 3.1 (Noldus Information Technologies b.v.). The program gives, for each sampling occasion, the position of the mouse, together with the animal's body area seen from the overhead camera; the animals' body areas are measured in pixels. The analysis results in a track record, which describes the animals' behavioral pattern during the observation period (Nilsson et al., 2006).

Comments: C57BL/6 mice were used in this study. These animals have brown/black fur, hence some information can be lost when the mice are really close to the black plexiglas walls. Still, open field is a very good and widely accepted method to investigate how active the mice are when introduced to an unfamiliar place. One example that can be studied is their anxiety versus fear behavior by looking where in the arena the mouse is located during the analyzed period. If the mouse displays fear-related traits it spends more time along the walls and in the corners whereas if it is anxious it spends more time in the center of the arena. In this study we analyzed the whole arena as one zone and divided the activity into 10-minute intervals. Our results clearly show that the mice explore the arena more and are more curious in the beginning, and that their curiosity decreases over time.

Statistical analysis

In paper I the Gene chip analysis was performed on log-transformed, normalized data using GeneSifter software (VizX Labs, Seattle) with Benjamini and Hochberg correction and a threshold of 1.2. In papers I, II and IV, immunoassay data and the cell

counts were analyzed using unpaired Student's *t*-tests or ANOVA with a Tukey HSD post hoc test, using SPSS 15.0 (SPSS, Chicago, IL, USA). The body weights and lick preference data in paper III were also analyzed using Student's *t*-test. The open field data in paper III were analyzed using a Mann-Whitney test. In paper IV the IntelliCage data were analyzed using Kruskal-Wallis H followed by a Mann-Whitney test. The data were considered statistically significant if $p<0.025$, since two comparisons were made on the same data set (a Bonferroni correction). In paper III the IntelliCage data were analyzed using Generalized Estimating Equations (GEE). This method was used to estimate the average response of the populations for the different parameters measured in the IntelliCage. For integer values a Poisson-model was used and for ratios a binominal model was used. Differences in all tests mentioned above were considered statistically significant at $p< 0.05$.

Comments: Both Student's *t*-test and ANOVA are parametric tests which means in general that they require normal distributions. This is something that can be assumed when counting for example the numbers of cells in a particular area. One-way ANOVA was used when we compared several independent groups with each other and therefore the Student's *t*-test was not applicable. As mentioned, those methods are parametric tests and, generally speaking, parametric methods make more assumptions than non-parametric methods. Therefore, when analyzing behavior it is preferable to not assume that the data have a normal distribution, making the test more robust. Mann-Whitney test and GEE are such examples.

RESULTS AND DISCUSSION

The inflammatory mechanisms after irradiation

Areas in the brain harboring stem cells are particularly sensitive to IR and loss of these cells may contribute to cognitive deficits. It has been demonstrated that IR-induced inflammation negatively affects neural progenitor differentiation (Monje et al., 2003). Therefore it is necessary to investigate the inflammatory mechanism to be able to find potential treatment strategies. We have shown that IR to the developing brain induces an acute inflammatory response.

Genetic profile - acute phase

During the acute period of inflammation (6 h post-IR), genes involved in the acute-phase response were highly activated, *e.g. stat3* (Levy and Lee, 2002). Other genes that showed an increased expression and are known to be involved in homing and migration of lymphocytes to sites of injury, e.g. *CD-44* and *Icam-1* (Brennan et al., 1999; Greenwood et al., 2002). After irradiation, several pathways related to the immune response were also affected, e.g. the Toll-like receptor signaling pathway that has chemotactic properties, *C3* and *PLAU* in the complement and coagulation cascades were both upregulated 6 hours after irradiation.

In more detail, *CCL2* was one of the most significantly up-regulated genes 6 h post-IR. Other inflammation-related genes that were up-regulated 6 h post-IR include *CCL3* and *CCL4*, both involved in Th1 macrophage activation, and *CXCL10*, involved in the T cell response (Mackay, 2001). *Tnfrsf1a*, a TNF receptor, was strongly up-regulated in the acute phase after irradiation. This receptor is involved in various

aspects of the innate immunity, such as regulating the defense by synchronizing the inflammatory process and increasing the acute-phase proteins (Wallach et al., 1999). *C/EBP β, Stat3* and *Fibronectin1* are all linked to the acute-phase response and were also up-regulated 6 hours after IR but had returned to basal levels at the 7 day time-point. *Egr-1*, which is suggested to be involved in macrophage differentiation and affect the transcription of *CD44* and *ICAM-1*, was up-regulated 6 h post-IR (McMahon and Monroe, 1996). *Egr1* also induces the expression of *PDGF-C*, which was increased 6 hours after IR. *PDGF-C* is suggested to be involved in the inflammatory process as well (Reigstad et al., 2005). In addition to *CXCL10* and *CCL3*, mentioned above, *CD14*, *c-fos* and *Fosl1* were also up-regulated 6 hours after IR, and they are all associated with the toll-like receptor signaling pathway, involved in the innate immune system and chemotaxis. *C-fos* is also often used as an activity marker of neuro-immune interaction (Sako et al., 2005). It has been shown that IR causes a decrease in *C-fos* levels when investigating the levels at a late time point (Achanta et al., 2007). Interestingly, *C-fos* has also been shown to participate in the molecular mechanisms of hippocampal learning and memory, and could hence be involved in the learning deficit seen at later time points (Guzowski, 2002). In summary this indicates that IR causes an inflammation in the acute phase.

Genetic profile - subacute phase

During the subacute phase (7 days after IR), genes linked to tissue damage and glial scar formation, e.g. *CDKN1A*, *GFAP*, and *CTGF,* were all activated (Hertel et al., 2000; Ring et al., 2003; Pekny and Pekna, 2004). At this stage genes associated to cell activation were changed. *FcγRIIB*, for example, which has an inhibitory effect on B-cell activation, was upregulated (Ravetch and Bolland, 2001). On the other hand *Tnfsf13*, which has been suggested to induce proliferation of both B and T cells, was

also upregulated (Yu et al., 2000). *ARGDIB,* that is suggested to be involved in lymphocyte activation pathways, was also upregulated during the subacute phase (Scherle et al., 1993). This follows the same line as *Tj6*, which was downregulated at this time point, and is suggested to have an inhibitory effect on lymphocytes (Lee et al., 1990). The levels of both *Anxa1* and *Serping 1* were increased, indicating that there could be an inhibition of the leukocyte adhesion and egression from the blood vessels to the extravascular tissue taking place at this time point as well (Perretti and Gavins, 2003; Cai et al., 2005). This could mean that there had been a disruption of the blood-brain barrier, which is an important feature after radiation, and is believed to be important in both early and late radiation toxicities (Rubin et al., 1994). Some of the genes that were changed have contradictive functions, but nevertheless there is still an irradiation-induced response to injury on the mRNA level 7 days after irradiation.

Protein profile

In the young rat brain there is an acute response 2-12 hours after IR, where acute cell death in the neurogenic region coincides with a transient production of pro-inflammatory chemokines and cytokines (Fukuda et al., 2004; Kalm et al., 2009b). We have shown that CCL2 (MCP-1 or Scya2) was the most strongly up-regulated of the cyto-/chemokines on the protein level in both mice and rats and it was up-regulated more than two-fold also on the mRNA level in rats. CCL2 was recently demonstrated to regulate the migration of neural progenitors to sites of neuroinflammation (Belmadani et al., 2006; Yang et al., 2007). Neural progenitors migrated towards sites of inflammation, but very little migration was observed in CCL2-deficient brain tissue and, conversely, neural progenitors lacking the CCL2 receptor CCR2 exhibited little migration (Belmadani et al., 2006). This indicates that CCL2, in addition to its role in neuroinflammation, is crucial for inflammation-related

neural progenitor migration, and thus constitutes an attractive target for anti-inflammatory strategies.

Another chemokine that also was upregulated after IR was GRO/KC (CXCL1) which displayed a two- to three-fold increase on the protein level in rats but was not significantly up-regulated on the mRNA level. In mice there was a trend towards upregulation in the wild types, and it the C3-deficient mice there was a 50% increase. This is a good example that mice and rats differ in their response to IR, which is important to keep in mind when interpreting the data and before moving to the next step, human patients.

From the IL-1 family, the IL-1α protein was increased more than 70-fold in the hippocampus and more than 13-fold in the cortex in the rats, but none of the interleukins or their receptors were significantly up-regulated on the transcriptional level, neither 6 hours, nor 7 days after IR. Surprisingly, IL-1α was not upregulated in the wild type mice but the other member of the IL-1 family, IL-1β, was upregulated in the *C3*-deficient mice. IL-1β can be detected as early as 15 minutes after cortical injury (Herx et al., 2000), so it possible that 6 h after IR was too late to detect IL-1β expression in the wild type mice brains. IL-1 receptors are expressed constitutively throughout the CNS, but inflammatory mediators may have dual roles, with detrimental acute effects but beneficial effects in long-term repair and recovery (Lucas et al., 2006). The major source of IL-1β in the injured brain is microglia (Eriksson et al., 1999), and microglia appear to respond differently to IR in the adult and the immature brain (Monje et al., 2003; Hellström et al., 2009; Kalm et al., 2009a).

The response of microglia after irradiation

Activated microglia are mainly scavenger cells with the capacity to destroy invading microorganisms, remove potentially deleterious debris, promote tissue repair by secreting growth factors and thus facilitate the return to tissue homeostasis, but they may also be transformed into potentially cytotoxic cells (Kreutzberg, 1996). Cell death after IR occurred only in neurogenic regions (Fukuda et al., 2004; Fukuda et al., 2005) but IR-induced inflammation, as judged by the expression of cytokines and chemokines, was not restricted to injured areas (Kalm et al., 2009b). In areas of injury, microglia activation and clearance of cellular debris is expected to occur. We saw that 6 hours after IR in the P9 rat pups, the microglia morphology was altered throughout the brain. The morphology was drastically changed particularly in the areas where cell death occurred (SVZ and GCL). In those areas most of the microglia showed this altered morphology, including swelling and fragmented nuclei. In both the hippocampus and SVZ the numbers of Iba-1-positive microglia (all microglia) were significantly increased during this acute phase after IR. This phenomenon, with an increase in microglia after IR is comparable to other injury models (Garden and Moller, 2006).

In the subacute phase 7 days after IR, the numbers of Iba-1-positive microglia had decreased significantly compared with control rats in the hippocampus, the SVZ, the cortex and the corpus callosum. Hence, this loss of microglia after IR was not detected only in the neurogenic regions, where cell death occurred, but also in the cortex and corpus callosum, where no overt injury could be detected (Fukuda et al., 2004). In the subacute phase, when cell death is completed (Fukuda et al., 2004) and inflammation, as judged by cytokine and chemokine levels, has subsided (Kalm et al., 2009b),

microglia with altered morphology could no longer be detected. Inspired by the altered morphology in the acute phase, the unchanged numbers of activated (Iba-1/ED-1 double-positive) microglia in irradiated brains but seemingly paradoxical decrease of total (Iba-1-positive) numbers of microglia, we hypothesized that a substantial number of microglia undergo IR-induced cell death. That was also the case. When investigating if microglia die after IR by using markers for DNA damage (TUNEL) and activated caspase-3, we could indeed show that they die. The nuclear morphology, TUNEL and active caspase-3 stainings indicated that microglia were injured and dying (Fig. 9). This occurred both in the neurogenic regions, where large numbers of precursor cells died, and in other brain regions like the cortex, where no obvious cellular injury could be detected. The IR-induced loss of microglia can perhaps best be understood in light of the finding that even resting microglia are highly active (Nimmerjahn et al., 2005) and that microglia are proliferating during brain development (Dalmau et al., 2003). According to Dalmau et al. microglia proliferation, as judged by lectin/PCNA double labeling, peaks at P9. This indicates that if the brain is irradiated during maximal microglia proliferation, there will be subsequent and significant loss of these cells. Another possible explanation is that it has been shown that microglia that become activated undergo apoptosis as a self-regulatory mechanism to inhibit chronic inflammation which can cause tissue damage (Liu et al., 2001; Yang et al., 2002). Nevertheless, it still remains to elucidate whether this loss of microglia is beneficial (due to reduced production of pro-inflammatory factors) or detrimental (due to reduced scavenging and tissue repair) for the tissue.

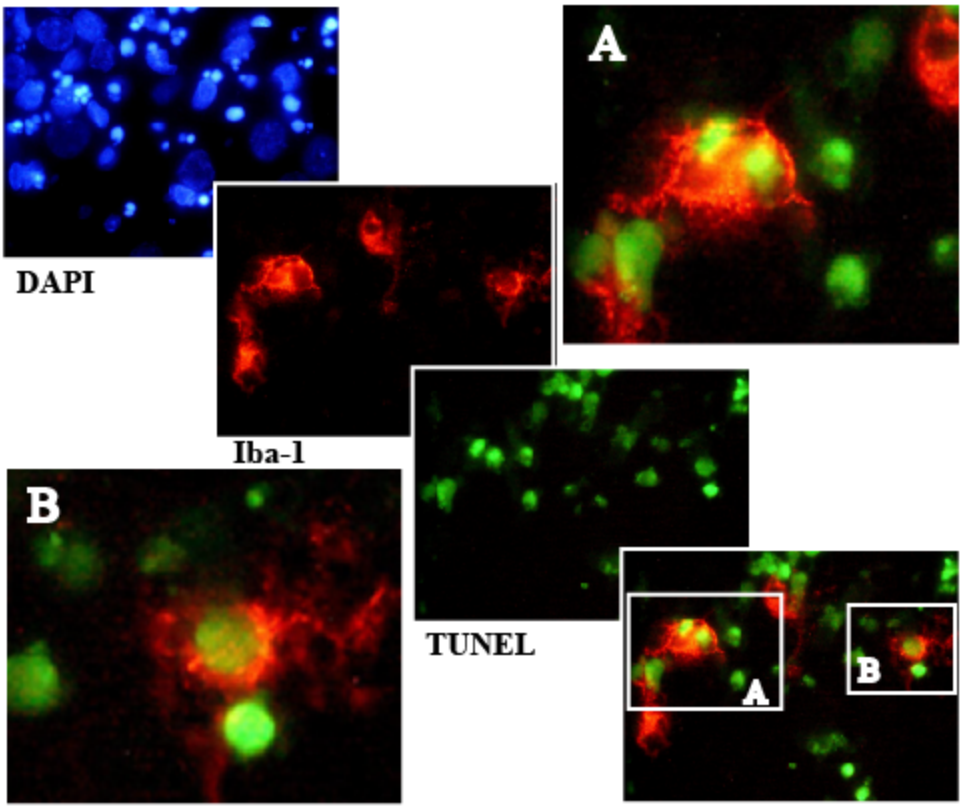

Fig. 9. Microphotographs showing representative fluorescent triple staining with DAPI (chromatin, blue), Iba-1 (microglia, red), and TUNEL (green). A shows a shrunken, non-phagocytosing microglial cell with a single, TUNEL-positive condensed nucleus, presumably undergoing apoptosis. B shows a microglial cell containing multiple, condensed, TUNEL-positive chromatin fragments that presumably have been phagocytosed (Kalm et al. 2009a).

Modulation of the immune response

As mentioned earlier, it has been shown that IR causes a change in the microenvironment, making the progenitor cells in the DG shift from neurogenesis to gliogenesis, and this was attributed to an inflammatory response (Monje et al., 2002). In paper I, we observed that the *C3* gene was highly up-regulated 6 hours after IR (Kalm et al., 2009b). C3 has a central position in the complement system and genetic

ablation of C3 prevents activation of the complement system by any of the three activation pathways (Pekna et a., 1998). In addition, it has earlier been demonstrated that progenitor cells and immature neurons express receptors for C3a and C5a and that C3 promotes both basal and ischemia-induced neurogenesis (Rahpeymai et al., 2006). Therefore, we investigated the effects of IR on the developing brain in *C3*$^{-/-}$ mice. We assessed the morphology and inflammatory response in the DG of the hippocampus, a neurogenic area important for learning and memory.

We investigated the injury by measuring the volumes of the three DG subregions, the GCL, the hilus and the ML, 7 days after IR. The DG is known to be affected by irradiation, particularly the proliferating cells in the subgranular zone of the GCL. The lack of growth is caused by a combination of a loss of cells that would have generated new tissue and negative effects on cells that survived the insult. We showed that the wild type mice but not the *C3*$^{-/-}$ mice have a reduced DG volume 7 days after IR. These findings were consistent throughout all three subregions, indicating an essentially different response after IR in the *C3*-deficient mice compared with the wild type mice. Since this was seen not only in the GCL, where the absolute majority of the precursor cells in the DG are located, but also in the hilus and ML, it indicates that the growth inhibition after IR reflects a general tissue reaction not restricted to neurogenic regions, and that this reaction was different in hippocampal tissue devoid of C3. To our knowledge, the IR-induced disruption of growth in the non-neurogenic hilus and ML has not been investigated before. It has been described that anti-inflammatory strategies can ameliorate some of the negative effects seen after IR, which supports our finding (Tikka et al., 2001; Monje et al., 2003; Moore et al., 2004).

On the protein level we saw differences in the chemo/cytokine response after IR when comparing *C3*-deficient mice, wild type mice and rats. When looking at the mice only, comparing the genotypes, there were in particular two inflammatory markers that diverged markedly in the *C3*-deficient mice, namely IL-1β and IL-6. IL-1β and IL-6 are proinflammatory cytokines and they were up-regulated after IR, but only in C3-deficient brains. This appears paradoxical, in light of the detrimental effects on neurogenesis attributed to increased levels of IL-6 after IR of the adult rat hippocampus (Vallieres et al., 2002; Monje et al., 2003; Nakanishi et al., 2007). Recent studies have indicated that the presence of IL-6 after injury could have beneficial, neuroprotective effects in the brain (Penkowa et al., 2000; Yamashita et al., 2005). One study also showed that IL-6 plays an important role in CNS repair by promoting axon regeneration and functional recovery (Hakkoum et al., 2007). While some reports show that IL-6 promotes glial differentiation of neural precursor cells (Monje et al., 2003; Nakanishi et al., 2007), two recent publications demonstrate that IL-6 can promote neuronal differentiation (Johansson et al., 2008; Islam et al., 2009). As mentioned before, IL-1β is an early, upstream cytokine in the signaling cascades of neuroinflammation. IL-1β can be detected as early as 15 minutes after cortical injury (Herx et al., 2000), so it possible that 6 h after IR was too late to detect IL-1β expression in the WT brains in the present study. The increased level of IL-1β after IR in the *C3*$^{-/-}$ brains largely follows that of IL-6, and might represent an apparently opposite reaction, as for IL-6, or might represent a delayed or stronger expression than in WT brains, thereby remaining detectable 6 hours after IR. Furthermore, IL-1β can promote the expression of IL-6 (Basu et al., 2004), so it is possible that they are expressed in sequence. Our present findings indicate that higher expression of IL-1β and IL-6 early after IR correlates with a better outcome, but the underlying mechanisms remain to be investigated. Nevertheless, it remains to be elucidated if the

altered effects observed in C3-deficient hippocampal tissue after IR are related to inflammatory or other mechanisms, or both.

Behavioral outcome after irradiation

The number of survivors after cancer treatment has increased over the last few decades, which creates a greater understanding of the long-term cognitive deficits and decline observed as a major adverse late effect after such treatments. It is crucial to find an unbiased method to detect this decline in preclinical models in order to further increase our understanding of these negative effects. In addition, better behavioral tests will help to evaluate potential therapeutic interventions, i.e., novel drugs or physical activity after irradiation-induced injury to the developing brain but also in other injury paradigms (Naylor et al., 2008). We have shown that one moderate dose of IR to the young rodent brain causes injuries that are detectable after several months. In patients, the irradiation-induced injury to the developing brain is aggravated over time (Mulhern et al., 2004). Some of the behavioral alterations from IR are evident one year after treatment, but due to a greater variance in this group, these differences are difficult to detect. Indeed this indicates individual differences in the long-term recovery of these animals after IR, not unlike the situation for IR treated patients.

We have used two different behavioral methods, the IntelliCage platform and open field, to investigate the IR induced long-term effects. The IntelliCage platform is a suitable tool to investigate the IR-induced memory deficits previously demonstrated (Highfield et al., 1998; Davids et al., 2003). The cognitive alterations after IR have been studied before, but with tests involving extensive handling of the animals, and in non-social environments (Ordy et al., 1968; Nash et al., 1970; Gazzara and Altman,

1981; Rola et al., 2004). Our research group has previously used open field to detect differences in movement pattern months after IR, but with younger mice at the time of IR (Naylor et al., 2008). This suggests that the age used in that study (P9) is developmentally more sensitive to IR damage compared to the age we have used (P14). In the current study we could only detect significant differences in movement pattern between the aged groups (approximately one year after IR) in the open field. The irradiated aged mice did significantly more stops and rearing during the 50 minutes in the open field arena, and they also tended to move a greater distance compared with the controls. This presumably means that they are more curious or hyperactive. It is most likely the latter, as hyperactivity has been demonstrated in another experiment involving irradiation induced injury (Davids et al., 2003).

Learning deficits constitute a major problem for children who survive their brain tumors. Therefore, parameters reflecting memory and learning are relevant and useful to study in a preclinical setting. Learning after IR has previously been studied using other behavioral tests, for instance the Morris Water Maze (Rola et al., 2004). However, it has previously been shown that memory tests are negatively affected by the stress of swimming (Whishaw and Tomie, 1996), suggesting that Morris Water Maze might not be as useful for mice as it is for rats. Therefore, in this study, we wanted to use a model that does not involve extensive handling of the animals and provides a non-stressful environment over a long period of time in a home cage-like environment.

One parameter from the IntelliCage platform, which is a good measurement of learning, is to investigate the number of nose pokes per incorrect visit. When using

wild type C57BL/6 mice that were P14 at the time of IR, we saw that both adult and aged animals displayed IR-induced differences, with irradiated animals performing worse (i.e. making more nose pokes in incorrect corners). This indicates that the irradiated mice do not learn or understand which corner is the correct one; instead they try several times to gain access to the water bottles. To investigate possible functional effects of IR and C3 deficiency, the IntelliCage platform was used 2-3 months after IR. The number of nose pokes (i.e. attempts to open the door) per visit in non-allocated, incorrect corners was used also in this study to measure the animals' ability to learn that they cannot open the door in such a corner, i.e. there was an inverse relation between memory retention and the number of nose pokes in non-allocated corners. There was no difference in number of nose pokes/incorrect visit in non-irradiated wild type and *C3*$^{-/-}$-mice. Both during the learning phase (corner 1) and the reversal learning phase (corner 2), the irradiated *C3*$^{-/-}$-mice made fewer attempts to open the door in non-allocated corners compared with irradiated wild type mice, indicating both better place learning and better reversal learning.

In summary, we saw that months after IR, the irradiated *C3*$^{-/-}$-mice performed better in a place learning task and in a subsequent reversal learning task in the IntelliCages, both of which can be claimed to be, at least partly, hippocampal-dependent (Colgin et al., 2008). Neurogenesis has been demonstrated to be important for hippocampal-dependent learning (Shors et al., 2002; Kempermann et al., 2004). It has also been shown that IR-induced changes in neurogenesis were associated with spatial memory retention deficits determined using the Morris water maze (Rola et al., 2004; Fan et al., 2007). The neural progenitors were not protected in the *C3*-deficient mice, as judged by the number of NeuroD-positive cells, at least not 7 days after IR. It needs to be further investigated if this is persistent over time. Together, these results indicate

that the complement system contributes to IR-induced injury in the young hippocampus. Therefore, it is possible that targeting the complement system is a fruitful strategy to protect the neurogenic areas from IR induced injuries.

GENERAL CONCLUSION

This thesis concludes that IR causes a major acute inflammatory response. This could explain some of the negative consequences seen after IR, especially since it has been demonstrated that IR-induced inflammation negatively affects neural progenitor differentiation.

Specific conclusions to given aims

I. We have shown that IR to the developing brain induces an acute inflammatory response which activates several pathways in the immune system. Most of the inflammation was downregulated 7 days after IR.

II. We showed that a substantial number of microglia die shortly after IR treatment. An IR-induced loss of microglia could either mean that the injury, due to pronounced inflammation, is decreased or that the injury is exacerbated due to weakened repair mechanisms. How this loss of microglia affects both the response to IR and the brain development still remains to be elucidated.

III. We showed that one moderate dose of IR to the young rodent brain causes injuries that are detectable after several months. Furthermore, non-irradiated animals performed better than irradiated mice, both when they were adult and when they were aged, as judged by different learning parameters analyzed using the IntelliCage platform.

IV. Modulating the immune response by deleting the third complement component, as in *C3*-deficient mice, revealed that they were, at least partly, protected from IR-induced injury to the dentate gyrus 7 days after IR, as judged by tissue loss and decrease in proliferating cells. Notably, months after IR the *C3*-deficient mice made fewer errors in place learning and reversal learning tests than wild type mice. These results indicate that the complement system is involved in the IR–induced injury in the developing brain.

CLINICAL PERSPECTIVE AND FUTURE DIRECTIONS

The improvements in the treatment for brain tumors in childhood have resulted in an enormous increase in long-term survivors. Therefore, it is vital to increase the knowledge of the injuries inducing the late effects seen especially after radiotherapy. One important factor is the individual differences seen after radiotherapy. Even though the children receive the same dose, the outcome can be very different. We found that the chemokine CCL2 was highly upregulated after IR, a marker that should be possible to measure in blood. It would be interesting to investigate if CCL2 could be used as a biomarker for IR-induced inflammation and possible injury to the brain tissue in general, thereby providing a means to determine the individual radiosensitivity of patients. CCL2 is a chemokine important for macrophage activation (Mackay, 2001), but it has also been shown to have important effects on migration of the neuronal precursor cells of locations for pathological processes (Belmadani et al., 2006). Further investigations need to be done to evaluate the potential of inhibiting CCL2 expression after IR.

We found that microglia, which are important after injury due to their role in removing potentially deleterious debris, promote tissue repair by secreting growth factors and thus facilitate the return to tissue homeostasis, die after IR. How this influences the repair and healing process after IR we still don't know, but we do know that inflammation is a very important key for this injury. One method that has been used in other injury models is "preconditioning". It has been shown that pretreatment with lipopolysaccharide (LPS; a big molecule from the outer membrane of Gram-negative bacteria) could be protective (Mallard and Hagberg, 2007). This means that at the time

of injury, the immune system is already activated, or that the inflammation has induced other "preconditioned" mechanisms. Preconditioning with LPS would activate microglia before the time of IR, but the question remains if it would be beneficial and protect from the IR induced injury or not.

As mentioned above, the immune system seems to play a fundamental role for the IR-induced injury, especially looking at the outcome from the C3-study. We showed that the growing hippocampi devoid of a functional complement system appear to withstand ionizing radiation better that wild type hippocampi. Also when investigating the behavior, the *C3*-deficient mice performed better in a place learning task and in a subsequent reversal learning task, both of which can be claimed to be, at least partly, hippocampal-dependent (Colgin et al., 2008). In the C3 study the number of neural progenitors, as judged by the number of NeuroD-positive cells, was not different between the genotypes 7 days after IR. Further investigations are needed to elucidate whether this difference persists over time, by determining the phenotype of the newborn cells in the hippocampus. Are the altered effects observed in the $C3^{-/-}$ hippocampal tissue after IR related to inflammatory or other mechanisms, or both?

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