Role of genomic instability in CNS immune cells: Cell autonomous response of microglia-specific Ercc1 knockout

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Abstract

Normal aging and progeroid syndromes have been associated with degenerative changes of Central Nervous System (CNS). Nowadays, increasing evidence suggest that abnormal reactionsof microglia, the immune cells of the brain, contribute to age-related deterioration of the neuronal pathology. However, it is unclear whether the response is due to micro-environmental or intrinsic factors. In this master thesis, adult mice, bearing microglia-specific disruption of ERCC1-XPF damage factor, were examined, in order to delineate its cell autonomous response. According to our data the ERCC1 ablation causes accumulating, unrepaired DNA damage to the microglia, leading to activation of DNA damage response (DDR) dependent on the ATM pathway. Damage sensors were also detected to be present in the cytosol, but their role is not yet defined.

Furthermore, we find change of cell morphology through a transition from resting to activated form, a phenotype connected to both activated and senescent microglia.

Summarizing, our data show the first signs that increased genotoxic damage can alter the functional and morphological properties of microglial cells.

Περίληψη

Η διαδικασία της φυσιολογικής γήρανσης, καθώς και τα σπάνια σύνδρομα πρόωρης γήρανσης, είναι συνδεμένα με ποικίλες εκφυλιστικές αλλοιώσεις του Κεντρικού Νευρικού Συστήματος (ΚΝΣ). Σήμερα, αυξάνεται συνεχώς ο αριθμός ερευνητικών μελετών που υποδεικνύουν τον ρόλο μη φυσιολογικής λειτουργίας της μικρογλοίας, αποκλειστικών κυττάρων ανοσοποιητικού του εγκεφάλου, στην επιδείνωση των ηλικιο-εξαρτώμενων νευρολογικών διαταραχών. Μέχρι πρόσφατα ωστόσο, τα αίτια ενεργοποίησης και η απειλητική για το ΚΝΣ απόκριση της, παρέμενε αδιευκρίνιστο αν οφείλονται στο μικροπεριβάλλον ή την αυτόνομη απόκριση των κυττάρων.

Στην παρούσα μεταπτυχιακή εργασία, εξετάζονται ενήλικα ποντίκια που φέρουν την ειδική απαλοιφή του γονιδίου *Ercc1* – απαραίτητου παράγοντα για την λειτουργία του Μηχανισμού Επιδιόρθωσης Νουκλεοτιδίων (NER - Nucleotide Excision Repair) – αποκλειστικά στα κύτταρα της μικρογλοίας, με σκοπό την διαλεύκανση της αυτόνομης απόκρισης σε περιπτώσεις αυξημένου γενοτοξικού στρες.

Από τα δεδομένα που συλλέχτηκαν προκύπτει ότι απουσία της ERCC1 πρωτείνης, προκαλείται συσσώρευση μη επιδιορθωμένων βλαβών DNA, οδηγώντας σε ενεργοποίηση και ATM-εξαρτώμενη απόκριση (DDR – DNA Damage Response). Πρωτεΐνες που δρουν ως αισθητήρες βλαβών του πυρηνικού DNA, και προωθούν την αναγνώριση από μηχανισμούς επιδιόρθωσης, ανιχνεύτηκαν και στο κυτταρόπλασμα. Ωστόσο ο κυτταροπλασματικός ρόλος αυτών των πρωτεινών δεν αποσαφηνίζεται στα πλαίσια της εργασίας. Περαιτέρω, διεξάχθηκαν πειραμάτα χαρακτηρισμού της «in vivo» μικρογλοιακής μορφολογίας, όπου βρέθηκε η μετάβαση των κυττάρων από τον ομοιοστατικό «resting» φαινότυπο σε ένα προ-ενεργοποιημένο «primed» φαινότυπο, μία κυτταρική κατάσταση που βάσει βιβλιογραφίας έχει συνδεθεί με ανοσολογική ενεργοποίηση και γήρανση.

Συνοψίζοντας, τα αποτελέσματα της εργασίας προσφέρουν τις πρώτες ενδείξεις ότι ο αυξανόμενος φόρτος DNA βλαβών είναι επαρκής για την αλλαγή λειτουργικών και μορφολογικών ιδιοτήτων των μικρογλοιακών κυττάρων.

CHAPTER I:

INTRODUCTION

1.1. Nucleotide Excision Repair Mechanism

DNA is constantly exposed to spontaneous hydrolysis, internal metabolic byproducts (e.g. reactive oxygen species) and environmental toxins. Accumulating damage can cause mutations and chromosomal aberrations, leading to carcinogenesis, or replicative senescence and cell death, promoting aging (Hoeijmakers, J. H., 2009). In order to maintain their genome intact, cells have developed numerous DNA surveillance and repair systems.

Nucleotide excision repair (NER) represents an evolutionarily conserved repair mechanism, that most frequently encounters UV-induced DNA lesions, such as pyrimidine dimer, and helix-distorting damages, like bulky chemical adducts and specific types of oxidative damage (Hoeijmakers, J.H., 2009). The mechanism operates via a “cut and patch” pattern, involving ~30 proteins that act in three steps: lesion recognition, removal of single-strand fragment and resynthesis of the excised strand (Friedberg, E.C*. et al*., 2006).

NER is divided into two sub-pathways, depending on the area where the damage is located: Global Genome Repair (GGR) pathway that targets lesions throughout the genome and Transcription-Coupled Repair (TCR) that removes distorting DNA damage selectively from transcriptionally active genes (Hoeijmakers, J.H., 2009; Bohr, V.A.,*et al*., 1985).

The difference between sub-pathways is the damage recognition step (Hanawalt, P.C.,1994): GG-NER surveys and recognizes distortions by UV-DDB (E3 ubiquitin ligase DDB1-DDB2 heterodimer) and XPC-RAD23-CETN2 complexes (Nishi, R.*et al*., 2005; Masutani, C. et al.,1994) whereas in TC-NER, the initial scanning complex of CSA (ubiquitin ligase) and CSB (DNA-dependent ATPase) proteins, recognize and engage stalled RNA pol ll (Laine, J.P. and Egly, J.M., 2006) and promote further recruitment of the downstream TCR-specific effectors XAB2 (Nakatsu, Y.*et al.,* 2000) p300, HMGN1 (Beiger, Y., *et al.* 2003) and TFIIS (Fousteri, M.*et al.,* 2006) (Figure 1).

The follow through is common for both sub-pathways, including unwinding the DNA helix around a lesion, by TFIIH complex DNA helicases XPB and XPD (Winkler, G.S.*et al*., 2000), stabilization of the ensuing single strand DNA with XPA and RPA proteins (Patrick, S.M. and Turchi, J.J., 2002) and cleavage by the ERCC1-XPF και XPG endonucleases, on the 3’ and 5 ’side of the DNA.

The aforementioned factors lead to the removal of a 25–30 nucleotide fragment and gap filling DNA re-synthesis, that requires further the action of DNA polymerases δ, ε, κ (Ogi, T.*et al*., 2010; Ogi, T. and Lehmann, A.R., 2006), PCNA, RFC, RPA ligase III or I (during S-phase of the cell cycle) (Moser, J.*et al*., 2007).

1.2. NER Syndromes

The significance of NER mechanism can be observed to a heterogeneous group of rare hereditary syndromes: Xeroderma pigmentosum (XP), Cockayne Syndrome (CS), Trichothiodystrophy (TTD) and XFE.

Defects in GGR give rise to cancer-prone XP syndrome (result from mutations in XP(A-G) genes) (DiGiovanna, J.J. and Kraemer, K.H., 2012). Patients have an elevated predisposition for skin cancer while in low rate, they display growth retardation (Lehmann, A.R., 2003) and neurodegeneration (dysmyelination and basal ganglia calcification) (Rapin,I.*et al.,* 2000). Synrdrom DeSanctis-Cacchione (DSC) is the exception to the rule, characterizedby severe neurological abnormalities and dwarfism (Colella, S.*et al.*, 2000).

Instead, defects in TCR generate a heterogeneous set of progeroid syndromes, including the Cockayne syndrome (CS), trichothiodystrophy (TTD) and XFE (Kamileri, I. *et al*., 2012a).

In brief, CS syndrome associates with mutations in TC-NER sub-pathway (CSA or CSB genes) mostly resulting in production of a shorter than normal polypeptide chain (Bertola, D.R. *et al.,* 2006). Patients displaygrowth retardation, photosensitivity, focal cortical dysmyelination, neuron loss or reduction in synaptic conductivity.

TTD and XFE patients are also characterized by developmental and neuronal abnormalities, subcutaneous fat loss and a short lifespan, but not cancer (Garinis,G*. et al*., 2008; Kamileri, I.*et al*., 2012a). Taken together, genetic disorders caused by impaired NER mechanism are frequently associated with premature aging and neuronal degeneration.

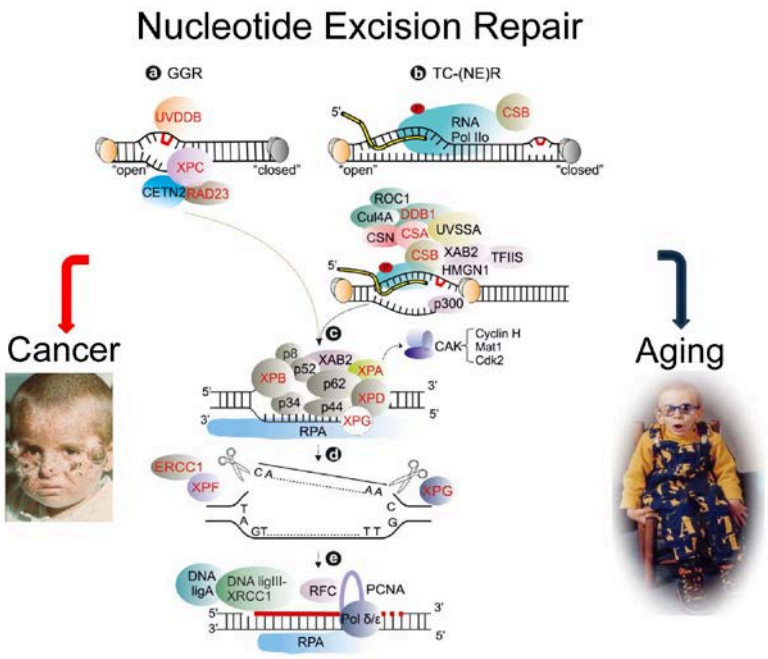


Fig. 1: Nucleotide Excision repair mechanism (NER) is divided into Global Genome Repair (GG-NER) and Transcription Coupled Repair (TC-NER) sub-pathways. Selective defects for GG-NER lead to carcinogenesis and

cause the cancer-prone XP syndrome, whereas defects for TC-NER lead to cellular senescence and potentially cell death, resulting in several human progeroid syndromes.

However, mice models do not fully represent the heterogeneity of patients in NER clinical syndromes.Phenotypic similarities between human and mouse, was mainly observed in double mutants, combining defects in both GG-NER and TC-NER, such as *Csb*$^{-/-}$ *Xpa*$^{-/-}$(Murai, M. *et al.,* 2001), *Xpd*$^{TTD/TTD}$ *Xpa*$^{-/-}$(De Boer, J.*et al.,* 2002), *Xpd*^{XPCS} *Xpa*$^{-/-}$(Niedernhofer, L.J., 2008) and *Xpg*$^{D811stop/D811stop}$ *Xpa*$^{-/-}$ (Shiomi, N. *et al.,* 2005), whereas the only single-gene mutations that approach the patient-like phenotype are *Ercc1*$^{-/-}$ (Niedernhofer *et al.,* 2006), *Xpf*$^{-/-}$ (Tian, M.*et al,* 2004) και*Xpg*$^{-/-}$ (Hiomi, N.*et al*., 2004). Subsequently, there is no perfect correlation between mutations in NER genes and syndromes' phenotypes, implying additional functions for NER components in other DNA repair pathways and/or transcription.

1.3. ERCC1 Role

Ercc1 is detected in the nucleusand together with XPF,forms an endonuclease essential for the "cut and patch" TC and GG-NER pathways (Houtsmuller, A.B.*et al*., 1999; Petit, C.A. and Sancar, 1999). The knockout mice for Ercc1 (Ercc1 KO) die before weaning (<4 weeks old) and display severe premature ageing symptoms including neurodegeneration and ataxia (McWhir, J.J. *et al*., 1993, Weeda, G.I. *et al.,* 1997; Niedernhofer, L. J. *et al*., 2006).

As human NER deficient syndromes are concern,*Ercc1* mutations have not been reported between classical features of XP, however, clinical phenotypes similar to Ercc1 KO mice models have been observed in two cases. The first was found on a patient with a severe XPF mutation, which was suggested to be a consequence of endogenous metabolites suppressing the somatotroph axis (Niedernhofer,L. J. *et al*.., 2006), while in second case, a patient carrying an Ercc1 mutation displayed cerebro-oculo-facio-skeletal syndrome and severe developmental failure (Jaspers,N.G.J. *et al*., 2007).

The explanation offered is that, like for several other proteins of the NER repair mechanism, Ercc1 has functions beyond NER. Nowadays, according to recent evidence is known that, ERCC1 protein is implicated in the repair of interstrand crosslinks (Kuraoka, I.*et al*., 2000), double-strand break repair (Ahmad, A. et al., 2008) and recombination repair (SargentR.G*. el al.,* 1997). Furthermore, the paper of Kamileri, I.*et al*., in 2012 proposed that severe developmental failure of Ercc1$^{-/-}$ mice, may be the consequence of perturbed RNA polymerase II transcription initiation as Ercc1 has been shown to be involved in RNA pol II assembly on promoters.

Close parallels were found recently between DNA damage accumulation in Ercc1 KO and p53 elevated expression in mouse brain (Selfridge, J.*et al*., 2009). As p53 is the principal regulator of DNA Damage Responses (DDR), it has the capability to induce the transcription of key genes involved in DNA repair, cell cycle arrest, cell senescence and apoptosis (Filomeni, G.*et al*., 2015). Furthermore, cells have evolved mechanisms to communicate their compromised state and stimulate the immune system (Gasser, S. and Raulet, D. H., 2006). In cases of chronic DNA damage and persistent DDR activation, cells are driven into senescence, accompanied by Senesce-Associated-Secretory-Phenotype (SASP).The response starts from DNA damage sensors and leads to upregulated expression of immune-stimulatory surface ligands, adhesion molecules and the secretion of pro-inflammatory cytokines.(Rodier, F. *et al*., 2009).This process is likely to show activation of inflammatory pathways and immune responses and consequently, target senescent cells in tissues for removal (Rodier, F. and Campisi, J., 2011).

1.4. Microglia – Immune defense of the brain

Microglia are characterized as the tissue resident macrophages of the Central Nervous System (CNS) and are the only immune cells in the healthy brain parenchyma. This cell type account for 10% (Chew,L.J. *et al*., 2006, Lawson,L.J.*et al.*, 1990; Banati, R.*et al*., 2003) to 20% (Imai, F. et al., 1997; Soulet, D. andRivest, S., 2008) of the total population in the CNS and has a negative bias between grey and white matter. As the main cells in brain immune surveillance, they are intensively involved in all forms of neuropathology and have the ability to phagocytose and become Antigen Presenting Cells (APC) for naive T cells (Fedoroff, S. *et al.,* 1997). Furthermore, insults to the CNS provoke a complex and multi-stage morphological and functional microglial activation, the distinct phenotypes of which described for the first time by Pio Del Rio Hortega, in 1932.

1.4.1. Origins of microglia

The origin of microglia has been debated for many decades. Controversies centered on whether all glial cells derive from a common neuroectodermal ancestor, or microglia

originate from a mesodermal ancestor as macrophages and other hematopoietic cells (Lawson,L.J*. et al.,* 1990; Perry, V.H.*et al*., 1985; Prinz, M. and Mildner, A., 2011).
Recent studies have delineate the microglial origin, with delicate fate-mapping techniques, targeting Runx1 transcription factor (Ginhoux, F.*etal*., 2010) and Cx3 Chemokine Receptor 1 (Cx3CR1; also known as the fractalkine receptor) (Goldmann, T.*et al*., 2013; Parkhurst,C.N.*et al*., 2013). These elegant evidence helped to define kinetics and distinguish that microglia derived from a myeloid population identified in the yolk sac (Ginhoux, F. *et al*., 2010; Kierdorf, K.D.*et al*., 2013; Schulz, C.*et al*., 2012).

In more detail, the first wave of myeloipoiesis, called primitive haematopoiesis occurs at embryonic day 7.5 (E7.5)–E8.0. At this "developmental window", stem cells develop in blood islands of the yolk sac and produce erythromyeloid progenitors (EMPs). EMPs gradually disappear and an immature population of F4/80+CX3CR1− (A1) and F4/80+CX3CR1+ (A2) arise. Later, mature A2 cells obtain distinct genetic features, differentiated from A1, and express the myeloid cell markers F4/80 and fractalkine receptor. (Prinz, M., and Priller, J. ,2014; Hashimoto, D.*et al*., 2011). Microglia are found to locate on the brain surface and populate the CNS parenchyma at E8.5, about the time that neurulation has been completed (Kierdorf, K.D.*et al*., 2013; Ginhoux, F*. et al.,* 2010; Alliot, F*. et al.,* 1999).
The second wave of myelopoiesis, named definitive haematopoiesis is taken over by founders emerged at E10.5 at the aorta–gonad–mesonephros (AGM) region and at E12.5 at fetal liver. Subsequently, these monocytes are engrafted, throughout the circulation, in all tissues with the exception of brain, which is already disconnected by blood–brain barrier (BBB) (Prinz, M., and Priller, J., 2014).

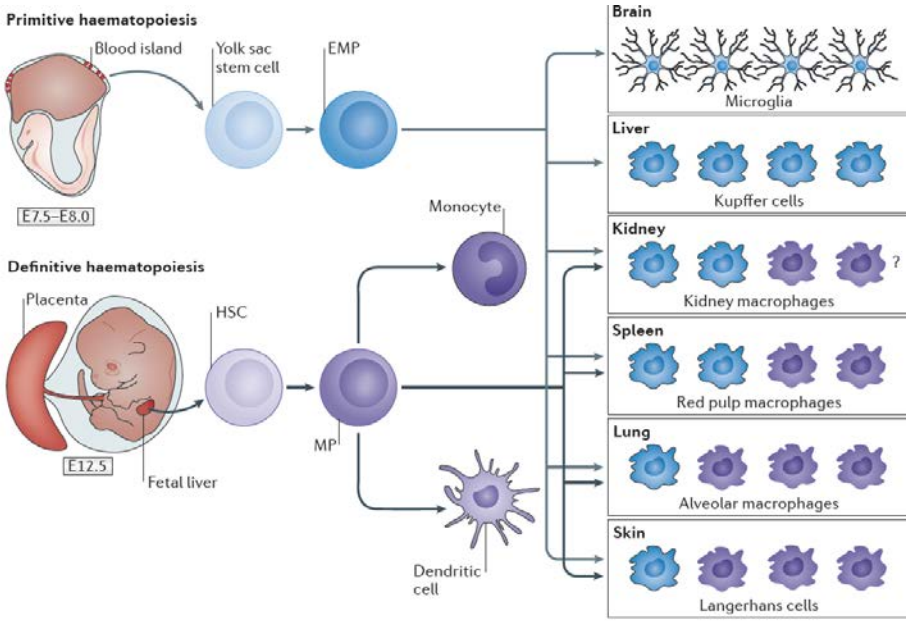


Fig. 2:.Several sources of myelopoiesis conntribute to the development of resident macrophages in different tissues. (Prinz, M., and Priller, J., 2014)

Over perinatal stages, the fountains of ameboid microglia appear(Kettenmann, H.*et al.,* 2011), in distinct positions (particularly, in the supraventricular corpus callosum) (Ling, E.A. and Wong, W.C., 1993; Hurley, S.D.*et al*., 1999) and increase the number of microglial cells by 20-folds from P0 to P11 (Alliot, F.*et al.,* 1999). Until postnatal day 14, microglial progenitor cells migrate from the corpus callosum into cerebral cortex and forebrain, where they lose monocytic properties and differentiate in a branched, ramified morphological phenotype, known as the resting microglia (Kettenmann, H. and Ransom, B.R., 2012; Rio-Hortega, P.,1932). The ameboid cells almost vanish by the late postnatal period, while they are only observed at specific locations of CNS during development (Giulian, D. and Baker, T.J., 1986) and at sites of inflammation in adult organism (Brierley, J.B. and Brown, A.W., 1982; Rio-Hortega, P., 1932).

1.4.2. Physiological Role

Microglia is responsible for homeostasis and defense in the CNS, as BBB prevents peripheral immune responses. In homeostatic conditions they have a ramified morphology exhibiting a small size soma with various long thin protrusions and a decreased proliferative capacity (Priller*,* J.*et al.,* 2001a). Each cell covers a non-overlapping territory and surveys the CNS parenchyma with highly motile ATP-dependent processes (Davalos, D.*et al.*, 2005; Raivich, G., 2005). Communication is necessary to homeostasis, and is achieved through contact with a variety of membrane receptors or through secreted mediators, sach as purines, neurotransmitters and growth factors. (Bessis, A*. et al*., 2007 ; Pocock,J. M. and Kettenmann, H. 2007; Aloisi, F., 2001).The above, allow microglia to "talk to each other" or communicate with astrocytes, neurons, T-cells, and myeloid progenitor cells.

During development microglia participate in maturation of synaptic connections (Tremblay, M.È.*et al*., 2010; Zhan, Y.*et al*., 2014) by phagocytosis (Paolicelli, R.C.*et al*., 2011) and afterwards in adult life support neuron survival through secretion of growth factors and anti-inflammatory cytokines (e.g. Transforming Growth Factor beta (TGF-β)) (Colton, C.A., 2009). Furthermore, in healthy adult organisms, their regular role is wiring of the brain and scavenging of debris and apoptotic cell material by forming phagosomes at the end of their protrusions (Peri, F. and Nüsslein-Volhard, C., 2008), whereas they lack their macrophage-like properties (Oehmichen, 1983) and do not present antigens to CD4 positive T cells. (Havenith, C.E*. et al*., 1998; Aloisi, F. *et al.*, 1999).

To highlight the relevance of microglia and associated diseases to neurons, we should mention the indispensable microglia role to modulate adult neurogenesis (Walton, N.M.*et al*., 2006). The persistent neurogenesis takes place in the subgranular zone of the hippocampal dentate gyrus. In homeostatic conditions, increased activated microglia is related to elevated neurogenesis, formation of long term memories and increase in spatial learning abilities (Battista, D,,*et al.* 2006). A small subset of neural progenitor arising from dentate gyrus survive to become integrated into neuronal circuits (Gould, E. and McEwen, B.S., 1993).

1.4.3. Pathological conditions

To maintain balance, microglia screen relentlessly the environment, they get activated and regulate morphological and functional changes in response to a multitude of potential scenarios:

1) Bacteria, Parasites and microbial products (Johnston, R.B., 1981).
2) Tumor cells (Hussain, S.F.*et al.*, 2006)
3) Cytokines, chemokines and growth factors
4) Complement factors, T–cell response and antibodies
5) Abnormal endogenous proteins (Colton, C.A.*et al.*, 2009; Ford, A.L.*et al*., 1995)
6) Activating stimuli such as Interferon/γ (IFN/γ).

In case of injury, the presence of extracellular calcium waves and release of the adenosine triphosphate, stimulate microglial processes to rapidly move towards the lesion site (Chemotactic migration). In order to respond to stressors, microglia activate, undergo phenotypic alterations and transform into amoeboid cells. (Davalos, D. et al., 2005; Nimmerjahn, A*. et al*., 2005; Schwartz, M*. et al*., 2006). The morphological changes have been described by six stages (Jonas R.A. et al., 2012) and depend to the grade of the insult and the location of the cell. Briefly, cell soma increase in size, and cellular protrusions thicken, decrease and orient rapidly toward the focal site of damage, where microglia lose all processes and regain full macrophage activities (Stage 6A) (Jonas, R.A.*et a*l., 2012; Davalos, D. *et al*., 2005). Phagocytic microglia is also able to present antigens (MHCII positive cells) and control adaptive immune responses (Kettenmann, H., 2007)

In normal conditions, microglia are not replaced by circulating macrophages or myeloid progenitors from the blood (Ginhoux, F.M*. et al.,* 2010; Schulz, C.*et al.,* 2012) and secure cell expansion in a context-dependent manner (Prinz, M., and Priller, J., 2014). However, under inflammatory conditions; the vascularization of the CNS is the basis for microglial distribution

and the recruitment of monocytes and adaptive immunity cells from the circulation (Eglitis, M. A. and Mezey, E., 1997; Flugel, A. et al., 2001; Ladeby, R. et al., 2005). Previous reports support that brain endothelium has a role in the penetration of perivascular cells into CNS parenchyma by expressing adhesion molecules ICAM-1 (Dalmau, I. et al. 1997) and -2 (Rezaie, P. et al. 1997) as homing receptors.

Once activated, microglia functions reprogram, and produce multiple pro-inflammatory, excitatory and neurotoxic factors (Schilling, M.*et al*., 2003; Town, T. *et al*., 2005).

In general, microglia exacerbate in the injured or diseased CNS and:

1. Produce pro-inflammatory & pro-apoptotic cytokines and chemokines, such as tumor necrosis factor alpha (TNF-a), IL-6, IL-1B, and IL-1ra (IL-1 receptor antagonist) IL-10, IL-12 and p40.
2. Transform into efficient APC Cells, positive for major histocompatibility complex (MHC) II (Kreutzberg, G.W., 1996).
3. Produce Neurotoxic/cytotoxic molecules: Nitric oxide (NO), reactive oxygen and nitrogen species (ROS RNS) (Paakkari, I. and Lindsberg, P., 1995)
4. Upregulate a number of surface molecules (e.g. Mac2*)* (Raj, D.D.A. *et al*., 2014).
5. Upregulate lysosomal markers and elevate lipid droplets inclusion, indicating enhanced phagocytosis.

It should be pointed that in the last years several studies have dealt with NO potential detrimental effect for brain, in order to examine the severity of adult microglia responses. NO is synthesized from NADPH, by NO synthases (NOS1-3), has an antimicrobial activity and can potentially harm neurons by increasing oxidative stress and activating cell death pathways. Furthermore, NO can regulate *invitro* adaptive immunity by inhibition of T-cell proliferation (Albina, .J.E.*et al*., 1991) and innate immunity by shaping the transcription of macrophages, NK cells to pro-inflammatory responses (Diefenbach, A*, et al*., 1999; Ehrt, S.*et al.,* 2001). Recent evidence obtained by Brannan C.A. and Roberts M.R. in 2004suggest that adult primary microglia, challenged by IFN-γ or LPS, produce low NO compared to neonatal microglia, or other tissue resident and peripheral macrophages. This observation, suggests that adult brain can control oxidative stress more efficiently than other tissues, modulating immune processes, and securing CNS from serious damage. However, the brain resident macrophages continue to upregulate a number of surface molecules and cytokines, such as high levels of TNF-α, IL-1β, and IL-6.

To conclude, the process of microglia migration to the damage site and the proliferation in acute injury and chronic neuronal diseases is called reactive microgliosis; is rare and tightly regulated (Fujita, S. and Kitamura, T., 1975; Kettenemann, H.*et al*., 2011; Ladeby, R.*et al*., 2005). In addition to proliferative resident population, the composition of the increased

immune population consist from a mix of migratory microglia from unchallenged areas, and a small contribution of circulating monocytes (Ajami, B.*et al*., 2007).
Following the clearance phase of the damaged site, microglial cells also provide reparative functions and stimulate astrogliosis for and tissue reformation (wound healing procedure) (Schilling, M.*et al*., 2009; Zhang, D.*et al*., 2010). Afterwards, activated phagocytic cells are believed to adapt again their ramified quiescent form (Ajami, B*. et al*., 2011).

1.5. Neurodegeneration and associated pathological conditions

Neurodegeneration is an umbrella term indicating inappropriate pro-inflammatory activity. If microglia does not shift from activated to resting form when inflammatory response ends or if they become inappropriate activated for extended periods, chronic responses will contribute to tissue damage. This is the case for many neurodegenerative pathologies like Alzheimer, Huntington and Parkinson (Schulte, T.*et al*., 2002; Griffin, W.S., 2006).Additionally, resting microglia have the role of synaptic stripper between the pre- and postsynaptic elements (Blinzinger, K. and Kreutzberg, G., 1968). However, extensive and incorrect wiring of synapses is able to cause autism spectrum disorders (ASDs) and psychiatric diseases, like depression and schizophrenia (Palop, J.J. and Mucke, L., 2010; Penzes, P. *et al*., 2011).Cell-type specific mutations for myeloid cells also point to two known syndromes: (a) The Rett syndrome (RTT) through altered expression of the methyl-CpG-binding protein 2 (MECP2) have proved to changethe phagocytic state of microglia (Derecki, N.C.*et al*., 2012). (b) The Obsessive Compulsive Disorder (OCD) through a loss of function mutation in the transcription factor gene, Hoxb8 (Chen, S.K*. et al.*, 2010).

1.6. Microglia in Aging

Aging has been shown to cause harmful phenotypic and functional alterations in microglial cells (Conde, J. R. and Streit, W. J., 2006), and it is well-known that gradually can lead to progressive neurodegeneration and cell death. Having been exposed to a variety of triggers, for extended period of time, aged microglia are less sensitive or unable to respond to a stimuli. Older mice and human microglia are not universally senescent, instead they have shown extensively distributed dystrophic cells (characterized by hypertrophy and retraction and thickening of processes), nearby resting microglia (Streit, W. J.*et al.,* 2004; Wasserman,

J. K.*et al*., 2008). As a conclusion, abnormal function in senescence is distributed to only a part of aged–resident immune population, which displays dystrophic morphology and the ability to release greater amounts of IL-6 and TNF-a (Njie, E.G. *et al*., 2012; Streit, W. J. and Graeber, M. B., 1993).

Furthermore, what makes aged microglia characterization challenging is that we cannot exclude the possibility of a challenged phenotype due to peripheral infections. So far, the most effective way to discriminate senescence versus activation phenotype is to determine whether microglia can recover to quiescent form after an inflammatory response (Luo, X. G. *et al*., 2010).

1.7. Fractalkine receptor andquiescent microglial surface markers

As we need to deal withthe Cx3CR1 (or Fraktalkine Receptor) promoter-driven Ercc1 conditional KO, it is important to understand how Cx3CR1 works to maintain the organism's homeostasis.

Fraktalkine, also known as CX3C ligand1 (CX3CL1) is a transmembrane glycoprotein, that can potentially undergo proteolytic processing (by members of ADAM family) and releases a soluble chemokine. Neurons express the ligand Cx3CL1, whereas its receptor, Cx3CR1, is uniquely expressed on microglia. Both proteins are constitutively present in the CNS, however, during brain development and damage, neurons start emitting the Cx3CL1 attractor in high concentrations (Streit, W.J.*et al*., 1999; De Jong E. K. *et al*., 2005, Tarozzo, G. *et al*., 2003). Elevated levels of Fractalkineinfluence the ability of microglia to extend their protrusions and migrate in response to ATP (Harrison, J.K.*et al*., 1998). Nowadays, Cx3CR1 transgenic mice models have been studied and are remarkably useful for characterization of microglial physiology in homeostatic and pathological conditions (Davalos, D.*et al*., 2005 ; Cardona, A.E.*et al.,* 2006; Nimmerjahn, A. et *al., 2005*)

Having a common hematopoietic progenitor, microglia share many characteristics with blood derived macrophages. Typically, the exclusion of other brain macrophages in cell cultures derived from microglia isolation techniques (described in 1.1.8) is confirmed by co-staining with CD11b and CD45 antibodies (Sedgwick, J.D*. et al.*, 1991). Cd11b is expressed exclusively in the surface of myeloid cells, regulating adhesion and migration during inflammatory responses, while CD45 is expressed low onsurveillant microglia (Ford, A.L. *et al.* 1995) and high on BM macrophages(Kim, S.U. and De Vellis, J. 2005). In the list below, quiescent microglia markers are sorted based on Immuno-histochemical data of previousstudies.

Markers Of Surveillant Microglia	Level Of Expression	Indicative References
Cx3CR1	High	Nimmerjahn, A.*et al.,* 2005
CD11b (Beta2-Integrin)	High	Giulian and Baker, 1986
F4/80 (known as EMR1)	High	Prinz, M. and Mildner, A., 2011
Iba1 (Ionized calcium binding adaptor molecule1)	High	Prinz, M. and Mildner, A., 2011
CD45	Low to intermediate	Kim S.U. and de Vellis J., 2005
P2Y12, purine receptor	High	Haynes S.E.*et al*., 2006
Lysozyme M	Undetectable to intermediate	Guillemin, G. J. *et al*. 2004
NSE (neuron specific enolase)	Undetectable to intermediate	Guillemin, G. J.*et al*. 2004
Calprotectin	Undetectable to intermediate	Guillemin, G. J.*et al.* 2004
CD14	Undetectable to intermediate	Guillemin, G. J.*et al*. 2004

Table 1.8.1.: List of markers to identify microglia in ramified state.

1.8. Microglia Isolation Procedure and Culturing: critical aspects

The rebirth of microglia in the 1980s, also signaled the beginning of microglia culture cells. Giulian, D., and Baker, T. J. achieved for the first time in 1986 to isolate 95% pure primary microglia from the cortex of neonatal rodents, through a process of adhesion and shaking of glial cells. Nowadays, microglial cell lines and primary microglia isolated from embryonic (Gingras, M.*et al*., 2007) or neonatal animals (Giulian, D., and Baker, T. J., 1986; Floden A. M. and Combs CK., 2007) are widely used as *in vitro* models. However, these cells have not experienced the CNS *in vivo* milieu and exhibit different behavior than mature microglia (Sierra, A. et al., 2007).
So far, adult primary cultures are the appropriate in vitro model for neurodegenerative syndromes, where aging is important**.**

Different protocols are available in order to isolate post-natal (young to aged mice) microglia *ex vivo* and have been used to isolate from intact CNS structures (brain and spinal cord) or from various separate CNS regions such as cerebellum, hippocampus and spinal cord. (Carson, M.J.*et al*., 1998; De Haas, A.H. *et al.*, 2007; Frank, M.G. *et al*., 2006; Hickman, S.E.*et al*., 2008; Ponomarev, E.D.*et a*l., 2005). Methods include a step to dissociate tissue

into single cell population, either by an enzymatic digestion (e.g. enzymatic cocktail (Cardona, A.E. et al., 2006)) or by mechanic dissociation procedure (Havenith et al., 1998).
Moving on, for the isolation of microglia from single cell suspension, two methods are widely used: trapping microglia using antibodies to cell specific antigens (Hickman, S.E.*et al*., 2008; Tham, C.S.*et al*., 2003), and separating microglia using discontinuous density centrifugation (De Haas, A.H. *et al*., 2007; Frank, M.G. *et al*., 2006;Cardona A.E. et al., 2006)). In the second case, the cells that interest us are isolated from myelinated cells and also from more non-myeloid elements of CNS. (Ford, A.L.*et al*., 1995), due to their isopycnic position.Comparison of efficacy between the two techniques, showed that density centrifugation does not include the risk of artificial cellular reactions caused by cross-linking, but isolated cells have an elevated contamination of non–microglia glia debris.

Characterization of brain microglial function requires isolation of enriched microglia from other CNS populations. If microglia purity is low and the number of cells is satisfying for further treatments, microglia can be enriched further by an additional step of activated fluorescence cell-sorting (FACs) or by immunomagnetic cell sorting (Ford, A.L. et al., 1995; Guillemin, G. et al., 1997; De Groot C.J.et al., 2000; Frank, M.G. et al., 2006; Cardona, A.E. et al., 2006; De Haas, A.H. et al., 2007).
At this point, we should mention that enriched ameboid microglia differed from peritoneal or tissue-associated macrophages by the capability to proliferate in vitro. Peritoneal phagocytes show limited survival after the DIV 5, whereas ameboid microglia do survive well and proliferate rapidly(Moussaud, S., and Draheim, H. J., 2010).

Consequently, given the rise of the number of isolated cells, further enhancement of the percentage of microglia in a mix glial culture can be achieved by culturing microglia following these steps:

1. In-frequentmedium changes: Opposed to microglia, astrocytes have a more vivid metabolism and demonstrate a progressive reduction in nutrients in the culture medium. As a result, astroglial cells cannot survive more than a few days without medium changes (Hao, C.et al. 1991).
2. Coating: Higher purity obtained in the absence of any substrata, on plastic or glass surfaces. Contrarily, laminin coats favor neuron and astroglial growth due to their adhesive properties, while they inhibit microglial growth (Milner, R. and Campbell, I.L., 2002; Wong,L.F. et al., 2006).
3. Culture Medium: Microglial cells are less abundant in DMEM than in DMEM:F12 (unpublished observations) (Saura,J., 2007).

Furthermore, the age of the animal and the CNS regions do not affect the proportion between microglia and other cell types or glial activation state. Newborns have higher yields in both glia populations (Saura,J., 2007).

CHAPTER II:

AIM OF THE THESIS

Based on previous studies in our lab, that have focused to tissue-specific disruption of Ercc1 and its role in age-dependent degeneration, at the present project we explore the effects of microglia-specific Ercc1knockout and the potential disorders of CNS.

An important reference point for our work is Karakasilioti, I. et al paper from our lab, in which was used a floxed Ercc1 allele with an adipose-specific Cre recombinase transgene (aP2-Cre). Data indicated that high levels of genotoxic stress cause persistent DDR, secretion of pro-inflammatory signals and elevated macrophage infiltration in adipose tissue. Consequently, Ercc1 self-perpetuating pro-inflammatory cycles led to chronic gradual tissue degeneration.
In this masterthesis, an effort was undertaken to study the role of unrepaired DNA damage in *Ercc1* defective immune cells of the CNS and delineate their capacity to induce brain inflammation through autonomous responses. In order to examine our hypothesis, a Cre transgene was driven to microglia under the control of Cx3CR1 gene promoter.
In a (in other respects) physiological tissue environment, are these cells capable of disrupting the homeostasis?

Since the aim of experiments was to find the causalrelationshipbetweenageand accumulative DDR responses, the experiments conducted in adult Cx3CR1 KO and control animals, 3 ½ to 8month-old. In order to access function and morphology of microglia, new techniques were needed to develop. More specifically, microglia isolation and culture were developed for *in vitro* observation, while the *in vivo* effects were observed in brain cryo-sections. Immune-cytochemistry (ICC) or immune-histochemistry (IHC) tools were used as evaluation approaches.
In an attempt to detect enhanced susceptibility to genotoxic stress in isolated primary adult Cx3CR1 KO cells, I examined the presence and levels of proteins that either mark DNA damage sites (γH2Ax/ FANCI) or are implicated in DDR Signaling (ATM modulator staining).

Furthermore, I studied the effects of persistent damage and the possibility of perturbed neuron-glia interaction according to phenotypic features of microglia on brain cryo-sections.

If there is a link, we expect to occur a “switching” in morphology from ramified to a primed and gradually activated state (as described in 1.1.4).
Finally, microglial cells isolated from control mice were treated with MMC, in order to find differences and similarities between persistent and acute DDR responses.
Experimental results of the aforementioned techniques may give rise to complementary data and create an initial profile for Cx3CR1 Cre; Flox/ - mice.

CHAPTER III:

MATERIALS AND METHODS

Theexperimentshave beenconductedin the Laboratory of Functional Genomics and Aging, at the Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology - Hellas (IMBB-FORTH).

Materials and Methods

3.1 Materials

3.1.1 Drugs and Chemicals

Material	Company
40 μm Cell-Strainer	Falcon
Acetone	MERCK
Anti-Freeze Slides	O. Kindler Gmbh
Avertin	
Bovine Serum Albumin (BSA)	Applichem
Collagenase A	Sigma
Dako-Pen	Kisker Biotech Gmbh
Dulbecco's Modified Eagle Medium (DMEM)	Sigma
Fetal Bovine serum (FBS)	Biochrom
Formaldehyde	MERCK
Gentomycin	
Glycerol	MERCK
Isopentane	
Optimal Cutting Temperature compound (OCT)	Tissue-Tek
Paraformaldehyde (PFA)	
Penicillin-Streptomycin-Glutamine (PSG)	Sigma
Percoll	Sigma
Trition X-100	MERCK
Phosphate Buffered Saline (PBS)	Home-Made
Sucrose	

Table 3.1: List of drugs and chemicals.

3.1.2 Antibodies

Antibody	Company
a-GFP	
ATM p1981 (Phospho ATM)	Merck Millipore
DAPI	THERMO
Ercc1	Santa Cruz
F4 / 80	BioLegend
FANCI	Santa Cruz
H2A.x S139 (γH2A.x)	Merck Millipore
HMGB1	BioLegend
ICAM-1	Developmental Studies Hybridoma Bank (DSHB)
LC3β	Santa-Cruz
Mac1	DSHB
PECAM 1	DSHB

Table 3.2: List of antibodies

3.1.3 Cells

Cells	
Microglia Cells	Isolated from 5 ½ το 8 month-oldmice

Table 3.3: Primary cells

3.1.4 Devices

Devices	Company
Centrifuge5810 R Perfusion Pump	Eppendorf
Confocal Laser Scanning Microscope Leica TCS SP8	Leica
CryostatCM1860	Leica
Phase-Contrast Microscope	Olympus

Table 3.4: List of devices

3.1.5 Software

Software	Company
Adobe Photoshop CC	Adobe Systems
Excel	Microsoft
Fiji (Image J)	http://imagej.net/Fiji

Table 3.5: List of software

3.2 Methods

3.2.1 Microglia Preparations

Animals

The mice were housed in the Animal House Facility at IMBB, in a controlled 12-hour light/dark cycle, provided with food and water.

- Heterozygous *Ercc1*mice,on a C57BL/6 genetic background, were crossed with knock-in mice containing floxed/flanked alleles of the *Ercc1* gene, on a FVB/n genetic background (Doig, J. et al., 2006).
- Progeny of the cross, with a wild type and a floxed *Ercc1* allele, were chosen as control.
- Progeny of the cross, bearing a knocked out and a floxed allele were further crossed with transgenic mice expressing Cre recombinase under the fractalkine receptor or Lysozyme2 promoter, on a C57BL/6 background. As result experimental animals were acquired on a mixed background.
- Using the same principles, mice expressing Rosa26-YFP$^{st/st}$, on a C57BL/6 background, were used either as control or were outbred with Cx3CR1-Cre mice, to obtain YFP expression in brain monocytes. Rosa26-YFP$^{st/st}$are knock-in mice, that a floxed STOP cassette is inserted between the promoter and coding sequence of Yellow fluorescent protein (YFP).
- The presence of transgenes and the excision of floxed *Ercc1* allele were confirmed by a genomic PCR assay.

Experiments for microglia Isolation were performed on adult mice, 5½ to 8 months old, which were euthanized by cervical dislocation. On the contrary, the 3½ to 6 months of age, used for tissue cryosections, were anesthesized deeply with Avertin (about 250 mg/kg body weight) were transcardially perfused with 1X PBSand fixed with 4% paraformaldehyde suspended in 1X PBS. The brain was dissected afterwards.

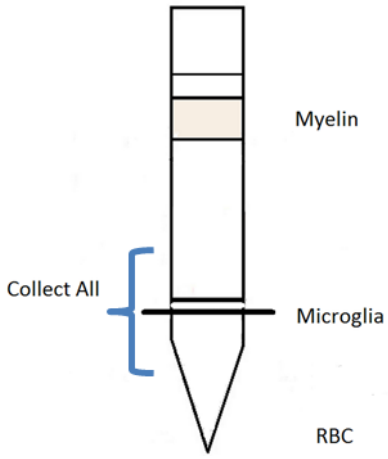
Percoll Solutions

Stock Percoll was diluted 1: 10 with 10X PBS to create 100% isotonic solution. 1X PBS was added then to isotonic Percoll to prepare working concentrations considered as 35% and 75% Percoll.Solutions were used at room temperature (RT), otherwise, cells had the tendency to clump and separation was less efficient.

Microglia adult mouse brain - Percoll Isolation

Thisprotocol was adapted and reproducedmostly from the earlier publicationNjie, E.G.*et al*., 2012.It can be easily modified to add various digesting enzymes, so that the yield of cells rises (Njie, E.G. et al., 2012; Cardona, A.E. et al., 2006).
The procedures were followed under aseptic conditions. All reagents were filtered (0.2 μm) to prevent contamination and each brain was processed individually to prevent cross-contamination.

Briefly, brains were removed from the skull and placed in 50 ml tubes with standard ice-cold medium (DMEM 10% FBS, 1% PSG), followed by two same medium washes. Tissue was placed in 1x PBS and minced into <2 mm pieces. Cell suspension was packed by short spin centrifugation and the pellet was resuspended in collagenase mix*.
Two commonly used procedures: enzymatic digestion and mechanical dissociationwere used to isolate CNS microglia until the acquirement of single cell suspension. More specifically, the pieces of tissue wereincubated in collagenase solution for 40-45 min at 37^{o}C and were gently triturated every 5 minutes. Collagenase activity was halted by adding 10 ml medium to the resultant homogenate.
Cells were suspended in ice-cold medium, centrifuged 5 min at 280g, 18^{o}C twice and the supernatant was cautiously discarded. Cell suspension was further homogenized using a syringe (21G needle) and filtered through a sterile pre-moistened 40 μm cell strainerto separate the clumped cells, meninges and tissue fragments from the larger pieces.Follows resuspension in ice-cold medium and a 5-min centrifugation step.
The supernatant was discarded and the pellet of an individual brain was resuspended in 2.5 - 3 ml of 75% isotonic Percoll (high Percoll) into a 15mL polystyrene tube. On top of high Percoll 5 ml of 35% isotonic Percoll (low Percoll) was layered, followed by 1 ml of 1x PBS. The gradient was then centrifuged at 800*g* for 40 minutes, 4°C, low acceleration and no brake.



Samples should be processed immediately after centrifugation to avoid a lesser yield. A thinband of desirable cells was captured between low and high Percoll layers.Two ml of interphase (microglia +lymphocytes) were collected and diluted with 1x PBS more than three times, in order to lessen the contact of cells with Percoll.The diluted homogenate was vigorously agitated and finally was spun at 1000 *g* for 10 minutes at 4°C, full acceleration and brake. A final Washing-Centrifugation Step is recommended in 500 μl medium volume, 800g, 7 min at 4C andtransferred into 1.5-mL Eppendorf tubes.

The pellets of Isolated cells were suspended in DMEM that included 10% FBS 1 %PSG (100 units/ml) and Gentomycin (0.1mg/ml) and were placed on un-coated glass coverslips in a 24-well plate.

*Collagenase, is a frequently used enzyme for cell dissociation. Stock was diluted in 1X PBS in order to acquire Working concentration 1 mg/ml, 3 ml solution per one brain.

* *Cells were kept at 15–18^{o}C during the whole gradient centrifugation. After this step, and during washes cells were kept at 4^{o}C, since mononuclear macrophages are highly susceptible to autolysis.

Culture Preparation

In order to compare the functional responses of adult brains, microglial cells were seeded in a 24-well tissue culture plate (Costar) at a density of >50.000 cells per well.Cultures were incubated at 37°C with 5% CO2 and the medium was changed every three days, to remove cell debris, releasedinflammatory or apoptotic factors and avoid a higher apoptotic rate.

MMC Treatment

At 10thday*in vitro*, microglia were stimulated by replacing the original culture media with Mitomycin (MMC)(0.4 mg/ml) diluted in standard medium.After 4 hours of incubation, at 37°C with 5% CO2, cells were washed and fixed for immunostaining.

Adherent cell immunocytochemistry

Cells were rinsed three times, fixed in 4% Formaldehyde solution in 1X PBS for 15 min and washed three times (rotating, 10 min, 45 rot/min). Mixed cultures were then permeabilised and non-specific binding sites were blocked using a solution containing 1% BSA, 3% FBS and 0,5% Triton-X in 1X PBSfor 45 min and processed for immunofluorescence of listed antibodies (4.1.1., Table 4.2). After three washing steps with PBS, cells were incubated with secondary antibodies, rinsedagain three times and mounted in 80% Glycerol.

3.2.2. Brain Preparation

Mice were perfused through the ascending aorta with 15 ml of Saline, clearing the intravascular compartment of blood cells, followed by 35 ml 4% paraformaldehyde suspended in 1X PBS, pH 7.3 at a rate of 5 ml/min, using a peristaltic pump. Brains were rapidly removed and post-fixed in 4% paraformaldehyde, exceeding five times the brain volume, overnight at 4^{o}C. In the following day, brains were placed in 30% sucrose, used as a cryoprotectant, until they sank at 4^{o}C.Then, blocked tissues were embedded in OCT medium and a freezing cryostat was used for sectioning at 10-12 microns. Coronal sections of the brains were then placed on anti-freeze slides and stored at -20^{o}C.

Brain cryosections immunochemistry

Tissue Sections were removed from -20^{o}C and were defined using Dako-Pen. They were incubated at RT for 5 minutes. The samples were further post-fixed in Acetone, for 10 min, at -20^{o}C. Three washing steps followed with 1X PBS, for 6min, at RT and blocking in a solution of 1% BSA, 3% FBS and 0.5% Triton-X in 1X PBS at RT, for 45-60 minutes. Then, the primary antibody solution was placed overnight at 4^{o}C. Samples were immunostained with the corresponding fluorescent-labeled antibodies for 2 hours, at RT. A separate 10-minute incubation was carried out in DAPI and the slides were coverslipped with 80% Glycerol. Three 6 min washes with a solution of 0.2% Triton in 1X PBS were performed between the incubations.

3.2.3. Statistical data analysis

Samples were visualized with a Confocal Laser Scanning Microscope Leica TCS SP8 and analysis was performed with 20x, 40x or 63x lens. The files were saved in the format of LIF. Stack confocal images were performed at 0.5-μm intervals for microglial cells and 1-μm intervals for sections. Consecutive Z-stack Images were converted to a maximum intensity projection image using Image J software.

Quantitative analysis was performed for everyMac1-positive cell, for/toboth cell culture and brain sections. For brain sections morphometric analysis was manually performed for microglial cells, by measuring maximum cell soma area (μm^2), thus determining the variation between the knocked out for Ercc1 gene and control mice.
For microglial cultures stained cells were quantified to determine the percent contribution to the total cells of an image. The values from at least 50 cells per condition of experiment were combined and averaged to estimate the contribution of each DNA Damage Factor within a particular culture. Phospho-ATM and FANCI antibody intensities were measured and quantified in respect to microglial surface area value. Statistical analysis was carried out using ImageJ and Microsoft Excel for Windows.

CHAPTER IV:

RESULTS

4.1. Microglia isolation protocol from adult mouse brain

In order to achieve the goals of the project, it was necessary to develop new protocols and techniques for our lab. One of these tools,that were optimised during the first months of thesis, was the ex vivo microglia isolationprocedure fromadultmice brains.After a series of tests, we came up with the discontinuousPercollgradients of arecent paper byNjie, E.G.etal., published in 2012,as it resulted to the purest celllayer, separatedfrommostofthemyelinanddebris.

Isolation experiments were performed to non-perfusedmice, utilizing the mild effect of CollagenaseA and mechanicaldissociation.

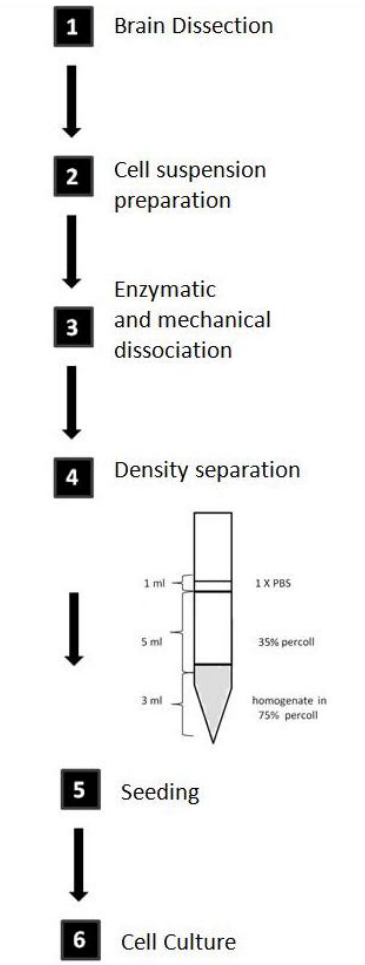


Fig.3: Overview of microglia isolation steps using a discontinuous gradient and density centrifugation. Microglia is enriched in the interphase between low and high Percoll, while myelin and unwanted brain matter are trapped in an isopynic position between 35% Percoll concentration and 1X PBS.After Density Centrifugation, the majority of Red Blood Cells (RBC) drops below the discontinuous gradient.

Density separation yielded on average3*10^5 cells per whole brainbut unfortunately the population was mixed, as opposed to 90% pure microglial culture of protocols,described in literature. Our observation data suggests a significant loss in the total cell count,possibly attributed to cell death occurring between 1 and 2 DaysIn Vitro (DIV) (Pino P.A. and Cardona A.E., 2011). The first days of culture, cells had the tendency to formaggregates that displayed a tense spinning property till they were established on the coverslips. Futhermore,the preference of other cell type-populations for un-coated surfaces and the high density of cells in the centre of coverslipcould have limited the adherence of microglia (Devarajan, G. et al., 2014).At 3rd DIV, cell debris and few alive cells in the supernatant were discarded by medium change, loweringsignificantly the number of remaining adherent cells per well. However, microgliaarecells with ahigh mitotic potential,underproperconditions (Moussaud, S., and Draheim, H. J., 2010; Jones-Villeneuve, E.M. et al., 1982) and thrive in mixed glia cultures, whereas astrocytes and other cell types are unable to survive due to the lack of proper growth factors (Devarajan, G. et al., 2014;Saura, J., 2007). Subsequently, production of new cells on the surface of the established primary layer was observed, suggesting a constantly rising proliferation. At 10th DIV, the mixed glial culture had formed tight feeder-layers in high-density areas, that in addition to FBS may be vital for survival and proliferation of microglia (Moussaud, S., and Draheim, H. J., 2010).
According to literature (Devarajan, G. et al., 2014) and elevated data of Mac1 positive immunostained population (described below), DIVs 9 to 10 seem to be the appropriate time-point to proceed to immunostaining.

Briefly, after day 10, when coverslips were removed from the 24-well-plate, the remaining cells continuedthe intensive cell divisions. At DIV 14, the daughter cells were established in empty space of the well and started to differentiate(Murabe, Y., and Sano Y., 1982). AtDIV 23, most of the cells have developed a distinct morphology simulated microglia, accordingto phenotype of cell lines (Devarajan, G. et al., 2014) and published data of adultprimary microglia cultures.Cells then continued to divide and expand rapidly until day 27, when they were passaged and subcultured to a 60 mmPetri. The total retention andobservation of microglia reached 37 days. Afterwards, cells were discarded.

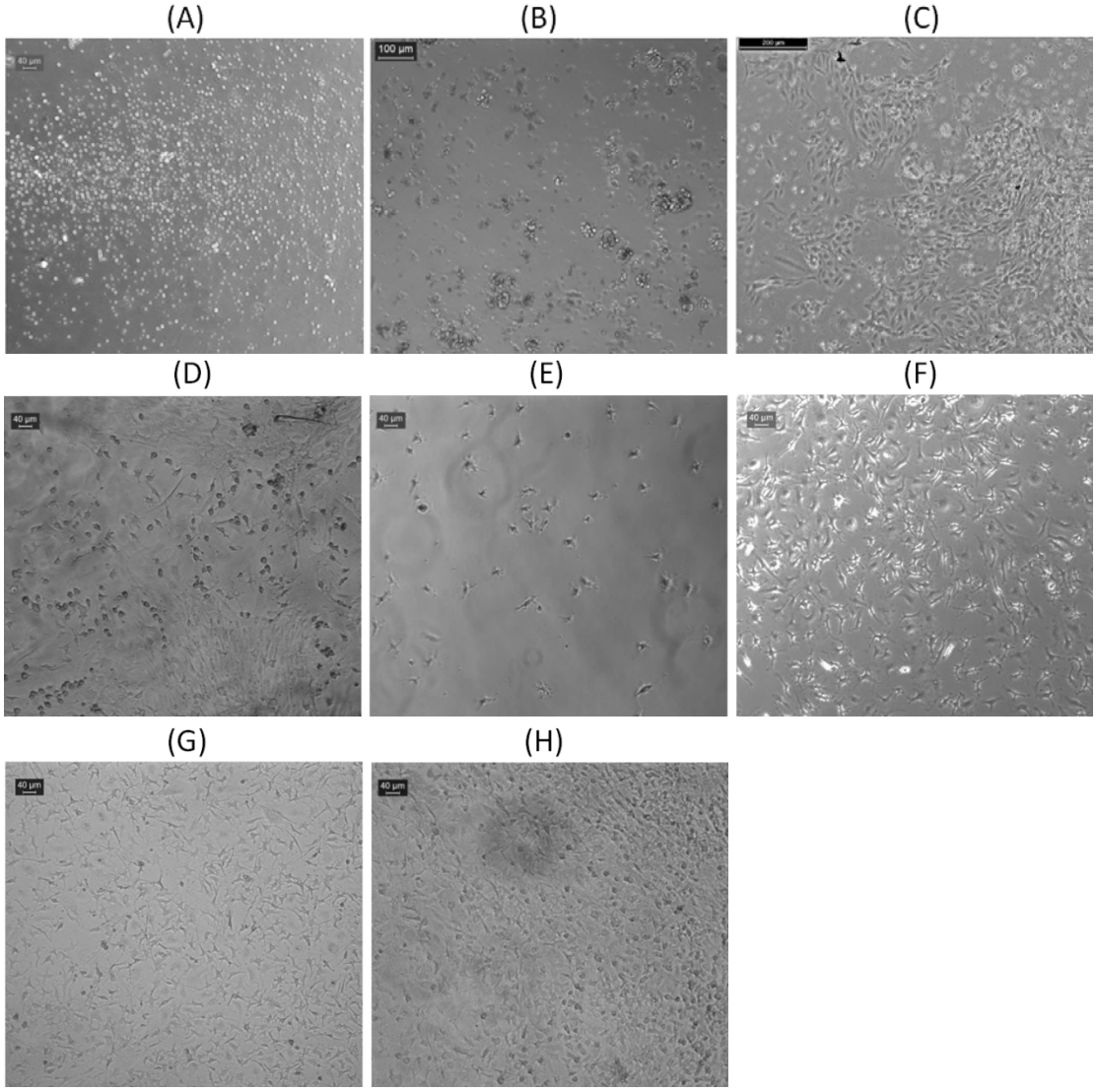


Fig.4: Morphology and proliferation of the cultured microglial cells in different days (A) DIV 0: Isolated cells were seeded in 24-well plate, with glass coverslips. (B) DIV 1: Cellaggregates, in attempt to attach to the coverslip. (C) DIV 4: The cells have been attached and morphologically differentiated in medium with 10% FBS. Dead cells, debris and aggregates had already been removed at DIV 3. (D) DIV 12: Representative image of cell-divisions (E) DIV 14: Round daughter cells in search of a free surface for establishment. (F) DIV 23: Newly established cells developed typical microglia characteristics. (G,H) DIV 27: Rapid expansion and cell divisions in different areas of well. Microglial cells were observed using phase-contrast Olympus microscope. Magnification:x10.

To verify the identity of cells and determine the percentage of microglia in mixed culture, on day 10, cells were stained with Macrophage-1 antigen (Mac-1) membrane marker, which recognizes CD11b receptor of myeloid cells. During visual observation of cell culture with confocal laser-scanning microscopy, microglia exhibited smaller and denser nuclei morphology, while astrocytes and other cell types had larger and paler nuclei, as mentioned byMoore K. L. and Barr M. L., 1953 (Fig.5).

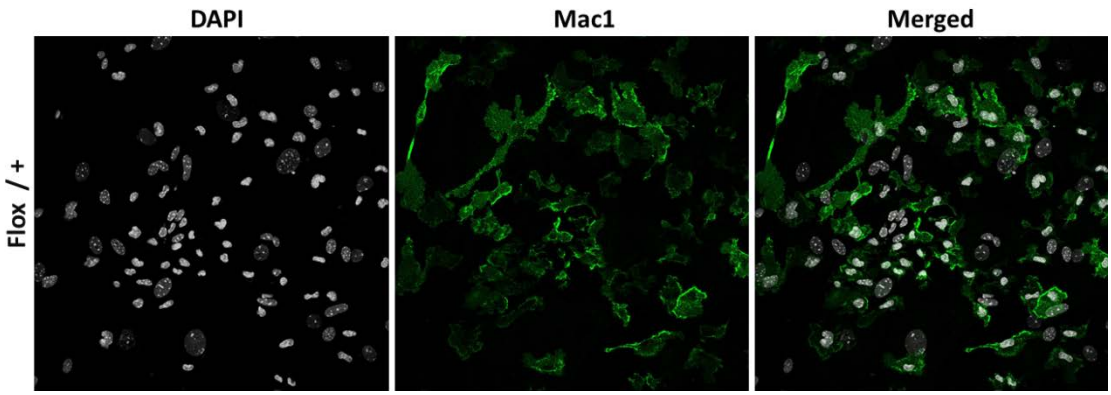


Fig.5: Representative Confocal z-stack projections showing a microglial culture, on day 10 (white:DAPI, green: Mac-1). Microglial cells were observed using Confocal Laser-Scanning Leica Microscope. Magnification:x40, Zoom: 0.75. Data are representative of more than 3 experimental repeats.

Quantitive analysis at DIV 10 showed that the number of positive Mac1 cells was more than 40% of the isolated population (Fig.5). Microglia were immunostained and were also found positive for monocyte marker F4/80 (Data Not Shown).

Furthermore, we observed that cell density per area addresses changes in the microglial phenotype. A more ramified state was acquired at high-density areas, possibly due to close proximity to other glia, whereas a more activated rounded and flattened morphology was observed at the low-populated edges of coverslip. For each experiment described below, Mac1 was used as a microglial marker.

4.2. Generation of microglia-specific Ercc1 knock-out

To determine the frequency of *Ercc1* deletion in microglia, weused two different mice models, in which Cre Recombinaseallowed gene deletion or expression under the Fractalkine Receptor promoter. The experiments were performed for (a) Cx3CR1-Cre; Flox/- Ercc1 heterozygous mice, quantifying the ablation of Ercc1 and (b) Rosa26-YFP reporter mice, that allowed to quantify the expression of YFP protein (genotypes of mice are extensively described in Materials and Methods 3.2.1).Each experiment was conducted to its Flox/+ or Rosa26-YFP$^{st/st}$respective control.

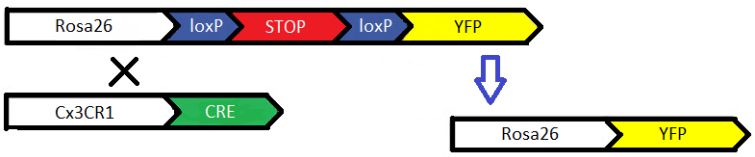


Fig.6: Graphic representation for the knock-in construct of Rosa26-YFP$^{st/st}$ mice. The Rosa26 ubiquitous promoter drives a flanked, by loxP sites, stop sequence, upstream of YFP locus. In such a way, Cre-Lox System is used to fluorescently label tissue resident macrophages.

Cells were co-stained with Mac1 and Ercc1 antibodies in the first case (Fig.7A and Fig.8) and with Mac1 and a-GFPantibodies, in order to enhance visualization, in the second case (Fig.7B).

The results of the percentage calculation for Mac1 and YFP positive cells were supportive, as in the first model only 0.7% showed a co-expression of Mac-1 and Ercc1, while in the second model 98.86% co-expressed Mac1 and YFP. According to data, Cx3cr1 KO has a 99 % Efficiency of Cre-Mediated Recombination (Fig.7C,D).

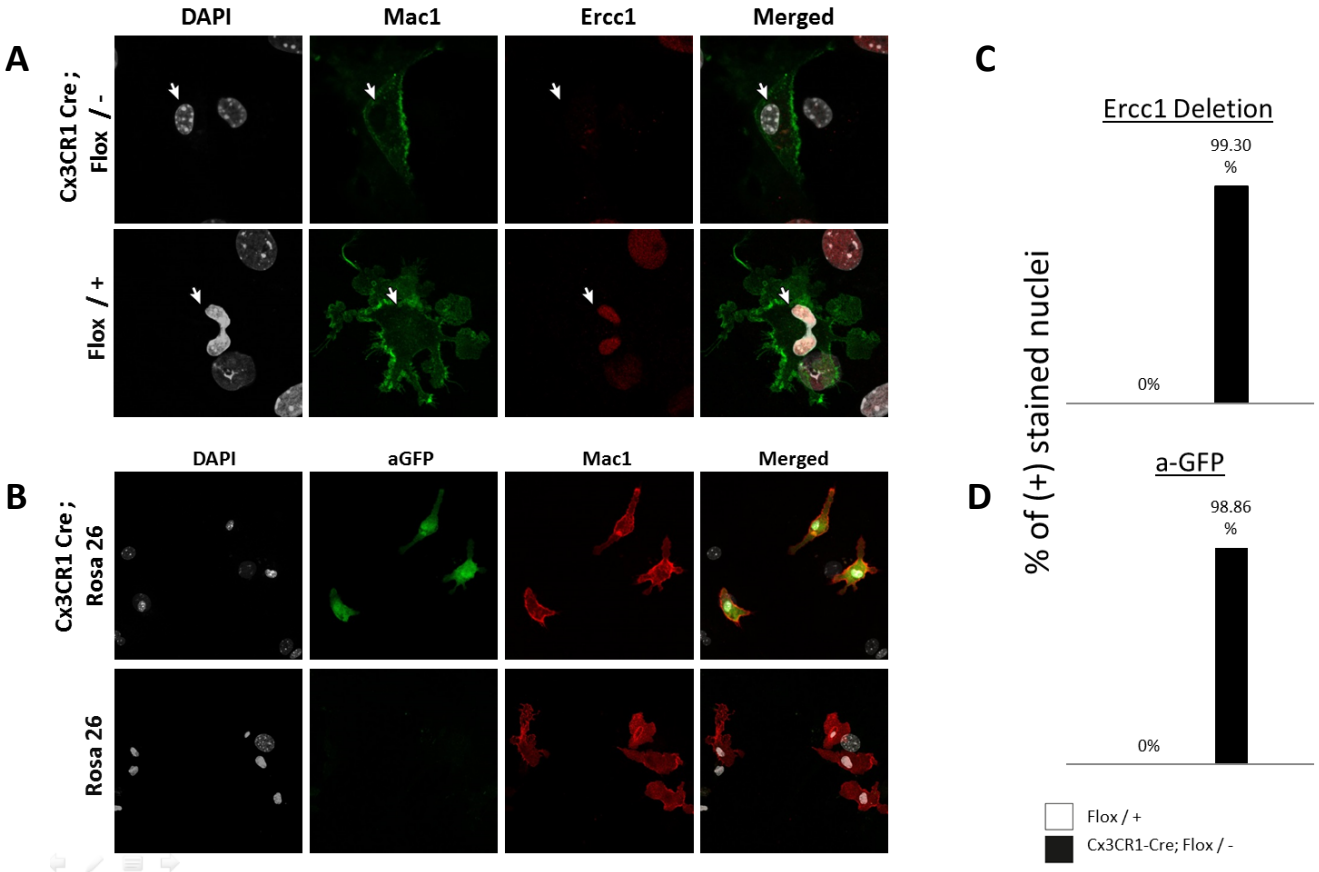


Fig.7: Confirmation of microglia-specific Ercc1 ablation by two different mice models (A,B) Representative confocal images of microglial cells, at DIV 9 for (A) Cx3cr1-Cre;Flox/- and Flox/+ mice. (B) Cx3cr1-Cre; Rosa26-YFP$^{st/st}$ and Rosa26-YFP$^{st/st}$ (white: DAPI; green: Mac1; red: Ercc1). (C,D) Quantitative analysis for (C) Ercc1 deletionof Cx3cr1-Cre;Flox/- and Flox/+ microglia (Cx3CR1 KO: 142 cells/ 3 coverslips, CONTROL: 165 cells/ 3 coverslips).(*D*)YFP protein expression of Cx3cr1-Cre; Rosa26-YFP$^{st/st}$ and Rosa26-YFP$^{st/st}$ microglia (Cx3CR1 KO: 88 cells/ 1 coverslips, CONTROL: 362 cells/ 4 coverslips). Magnification x63, Zoom : 2.5. Data is representative of three experimental repeats for Ercc1 and one experimental repeat for YFP.

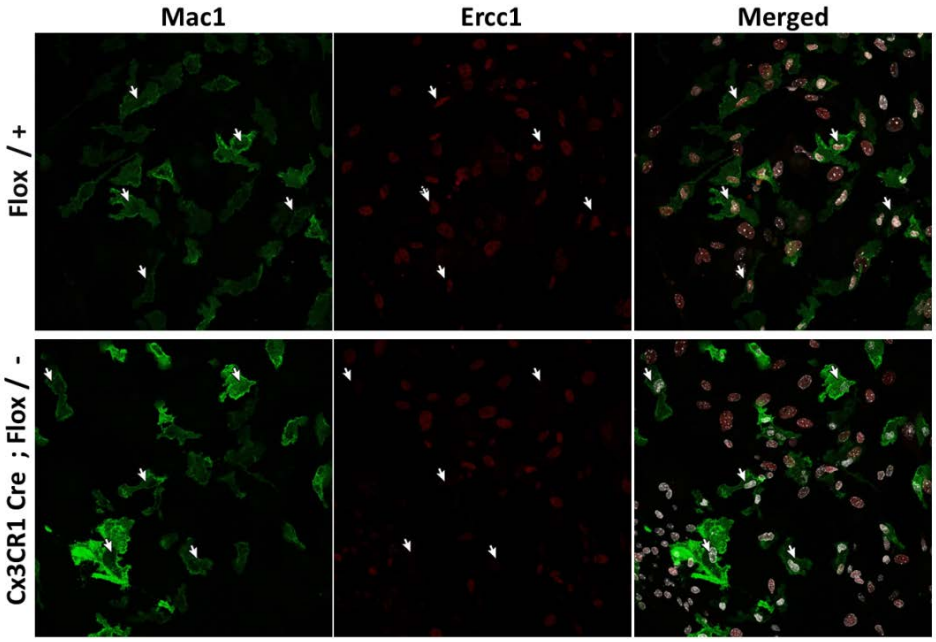


Fig.8: Representative confocal Images showing Ercc1 deletion in mixed population for Cx3CR1 KO and Control, on day 10. (white: DAPI; green: Mac1; red: Ercc1). Magnification x40, Zoom : 0.75.

4.3. Accumulation of unrepaired DNA damage cause autonomous stress responses

In order to examine the functionality of the Cx3CR1 KO model, resting assure the tissue resident macrophage-specific inactivation of the *Ercc1* gene, we tried to detect the activation of specific protein factors involved in DNA Damage. Hence, Cx3CR1 KO and Control cell cultures were immune-stained with:

- Phosphorylated histone H2Ax (γH2Ax), which marks the sites of DNA breaks.
- Phosphorylated Ataxia telangiectasia mutated (pATM), the active form of a serine/threonine protein kinase, recruited to DNA double-strand breaks.
- FANCI involved in the repair of DNA Interstrand Crosslinks (ICLs).
- High-mobility group protein B1 (HMGB1) (Davalos, D. et al., 2013), expressed in elevated levels by senescent phenotypes, that is identified as a Damage-associated molecular pattern (DAMP). The DAMPS molecules are secreted by stressed cells undergoing necrosis, initiate and perpetuate immune responses.

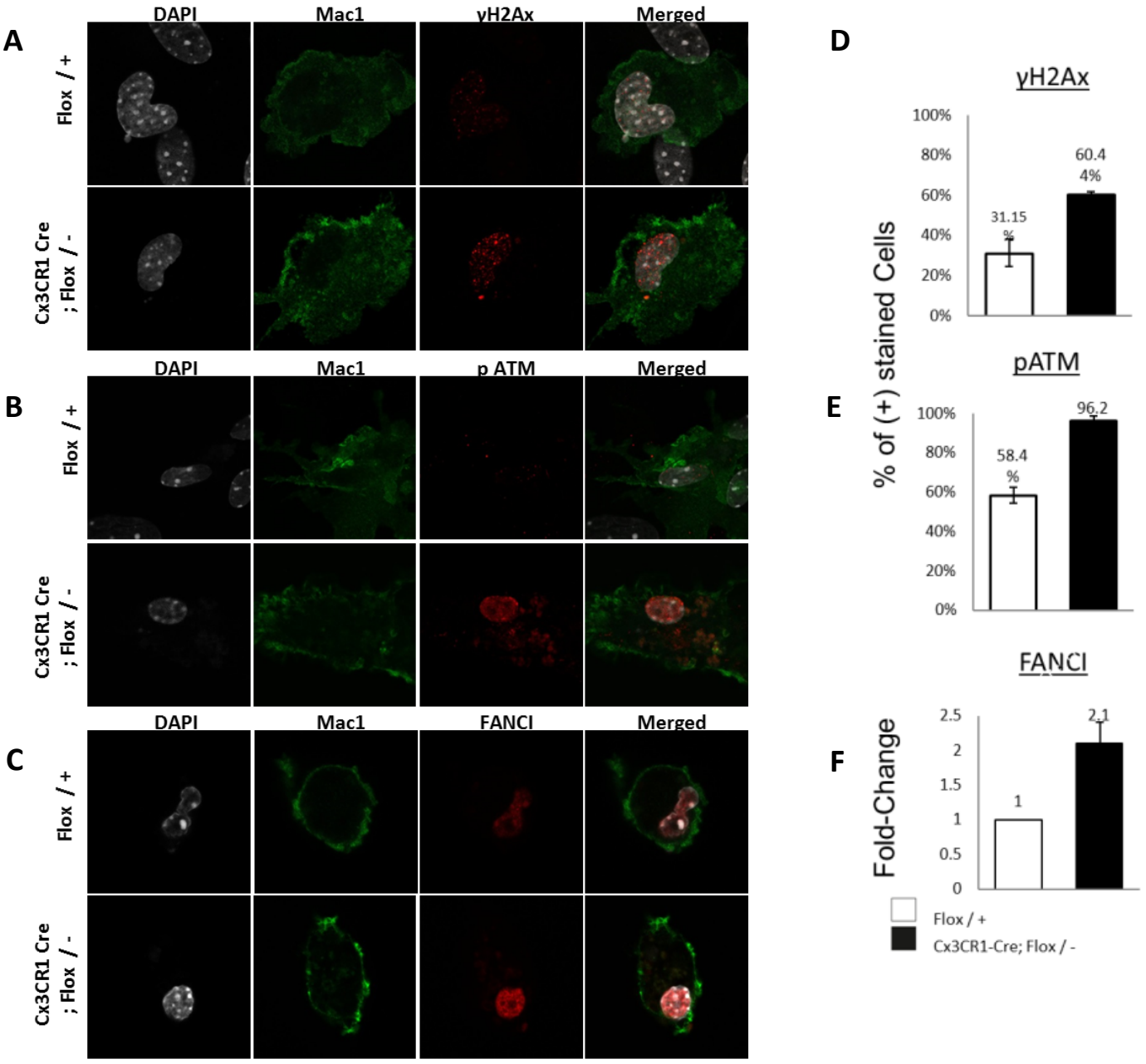


Fig.9: Ercc1 deletion triggers upregulated expression of proteins that participate on DNA Damage Response (DDR) in primary microglia. (A-C) Confocal projections for Mac1 positive cells from control and Cx3CR1 KO mice stained on days 9 and 10, for (A) γH2Ax, (B) pATM and (C) FANCI (white :DAPI; green:Mac1; red: γH2Ax/ pATM/ FANCI).(D-F) Quantitative analysis for (D) γH2Ax microglia(Cx3CR1 KO: 152 cells/ 2 coverslips, control: 305 cells/ 4 coverslips) (E) pATM (Cx3CR1 KO: 71 cells/ 2 coverslips, control: 150cells/ 3 coverslips) and (F) FANCI (Cx3CR1 KO: 207 cells/2 coverslips, control: 171 cells/ 2 coverslips). pATM representative image depicts nuclear boosted expression and diffused and in aggregates cytoplasmic detection of protein. Magnification x63, Zoom: 2.5. Data is representative of two biological replicates.

The quantification showed that on day 10, 60% (±1.5% SD) of primary Cx3CR1 KO microglial cells was γH2Ax positive, while control percentage did not exceed 31 % (±6-7% SD) (Fig.9D). Same results were obtained for pATM, since Mac1 positive cells reached 96% (±2.5%) to 58% (±4% SD) for Cx3CR1 KO and Control respectively (Fig.9E).

Additionally, the entire Cx3CR1 KO and control population of microglia, seems to express FANCI and HMGB1 in the nucleus. However, quantification for intensity of FANCI fluorescence showed a 2-fold (±0.3 SD) increase at Cx3CR1-Cre;Flox/- microglia (Fig.9F), whereas HMGB1 protein levels were not affected (Data not shown).

Furthermore, signal from γH2Ax and pATM proteins is detectable in the nucleus, but also shuttles to the cytoplasm. To determine the role of cytosolicstaining, and whether it reflects a cellular response to accumulating DNA damage, microglia patterns divided into two categories: nuclear and cytoplasmic staining (Fig.10).

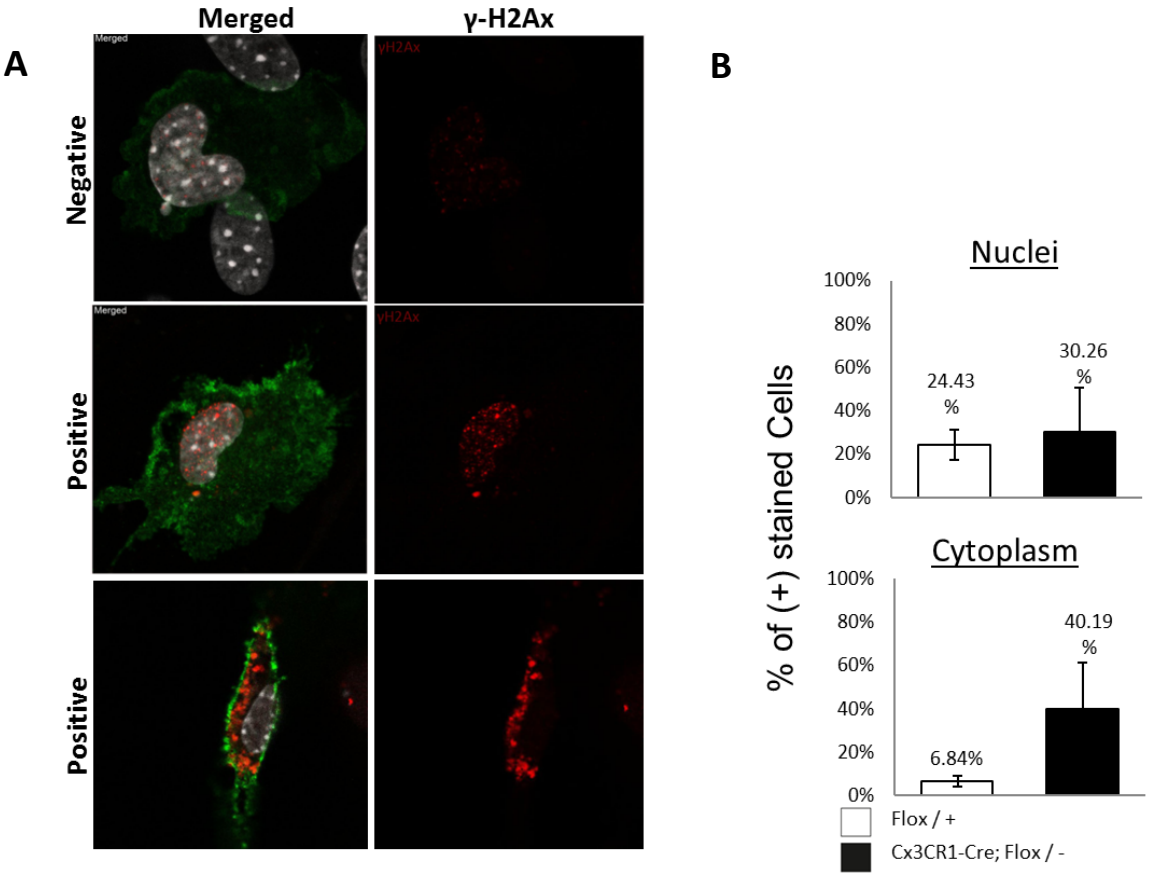


Fig.10: Gamma-H2Ax nuclear and cytoplasmic induced responses of Cx3CR1 KO (A) Representative confocal *z*-stack of microglial cells for negative, nuclear-positive and cytoplasmic-positive γH2Ax staining. (white: DAPI; green: Mac1; red: γH2Ax). (B)Nuclear andcytoplasmic quantitative analysis of control and CX3CR1 KOmicroglia. Magnification x40, Zoom: 6. Data is representative of two experimental repeats.

Gamma-H2Ax was expressed in the nucleus at 24% (±7% SD), and it was rarely found in the cytosol, 6.8%, of Flox/+ (Control) microglia (±2% SD). On the contrary, the phosphorylated H2Ax histone was found in the cytosol of Cx3CR1 KO in a 40% rate (± 20% SD- 2 repeats)(Fig.10B). Pleomorphic (both nuclear and cytoplasmic) positive cells were also found to be present in the cell culture.

Furthermore, γH2Ax histone was present in the cytoplasm only in the form of aggregates and showed co-localization with membrane marker Mac1. It is noteworthy that,Mac1 negative cells, whose nuclei morphology resembles astrocytes, showed equally or higher elevated levels of nuclearγH2Ax, than microglia.

The quantification of pATM expression turned quite challenging because three different distribution patterns were found: nuclear staining, diffused (Dotted) cytoplasmic staining and formatted aggregates cytoplasmic staining. Microglial cells were considered positive for each case if they were above a defined threshold (Fig.11A).

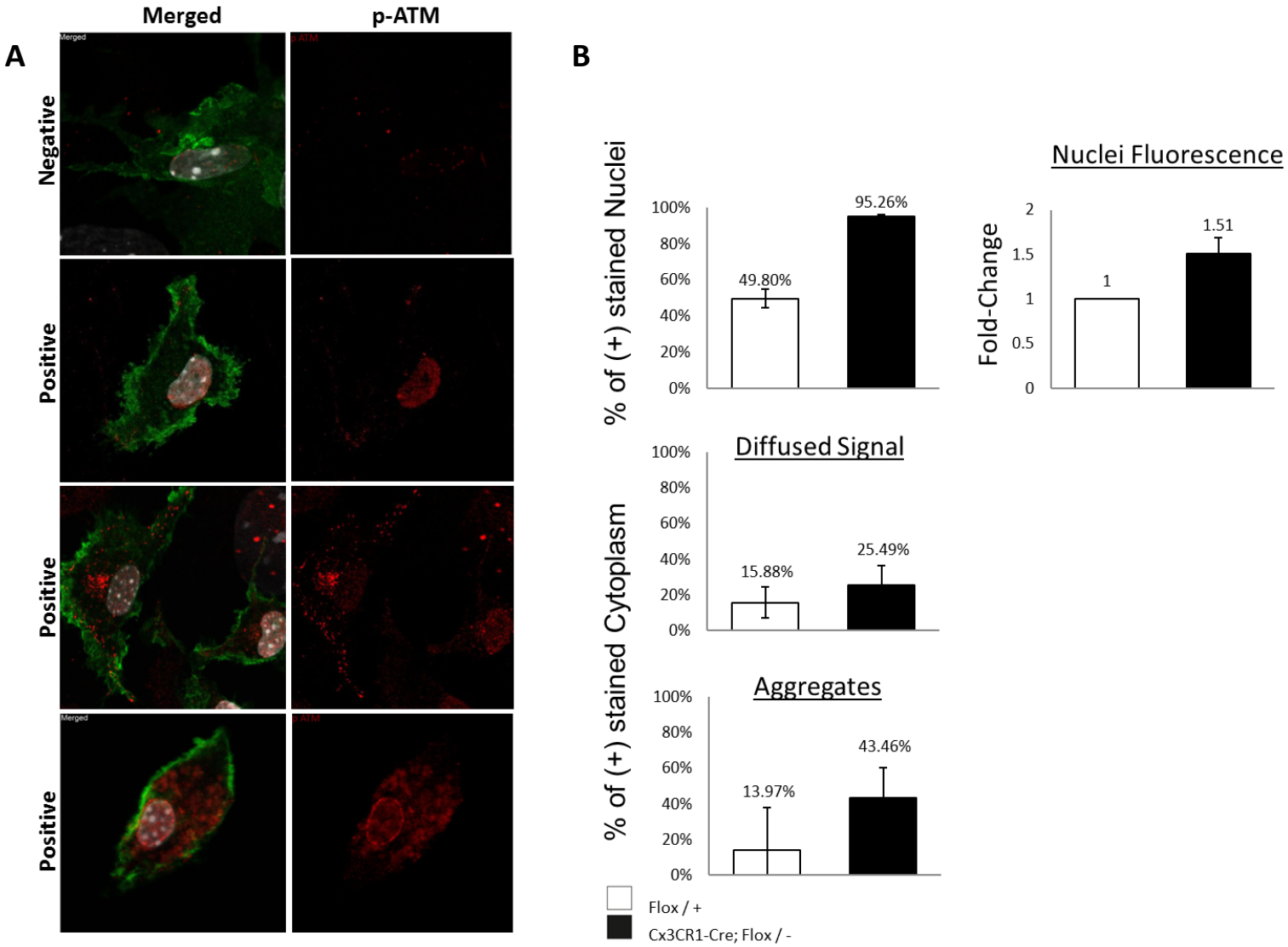


Fig.11: pATM nuclear and cytoplasmic induced responses of Cx3CR1 KO (A) Representative confocal pictures of microglial cells for negative, nuclear-positive, diffused cytoplasmic-positive and aggregate-shape cytoplasmic-positive pATM staining. (white: DAPI; green: Mac1; red: pATM). **(B)** Nuclear andcytoplasmic quantitative analysis of control and CX3CR1 KOmicroglia. Magnification:x40, Zoom: 6. Data is representative of two experimental repeats for Cx3CR1 KO and three experimental repeats for Control.

First category results, exhibited 95% (±1% SD) of Cx3CR1 KO, versus 50% (±5% SD) of control positive microglia, and also showed a 1.5-Fold increase in nuclear fluorescence

intensity (Fig.11B).Moreover, in some cells we observed an antibody ring around the nucleus, possibly meaning that an elevated population of the protein was forced to move to the cytoplasm.Cytoplasmic Quantification found pATM diffused and bound to aggregates in percentages of only 16% and 14% at control. On the contrary, Ercc1 KO for tissue-resident macrophages displayed 25.5 % and 43.5 % respectively (Fig.11B).

As mentioned for γH2AX, also pATM, HMGB1 and more rarely FANCI were found to have elevated protein aggregates levels in the cytoplasm. The regular co-localization of proteins with Mac1 and low fluorescence DAPI, may suggest a significant difference in phagocytosis, for the Cx3CR1 KO macrophages.

As autophagy has been linked to DNA damage and cellular stress (Park, C. *et al*., 2015), we examined whether in our case autophagy was induced. To do so, we used the Microtubule-associated protein/1B-light chain (LC3β) marker(Fig12A). LC3β is a cytoplasmic protein, which recruits to autophagosomes and expands their membrane through a post-translational modification(phosphatidyl-ethanolamineconjugated (PE)). In normal conditions, low levels of LC3β are detectable in the cytoplasm, while in cellular disorders that induce autophagy, LC3β results in a pattern of autophagosomal vesicles (Tanida, I. et al., 2008).

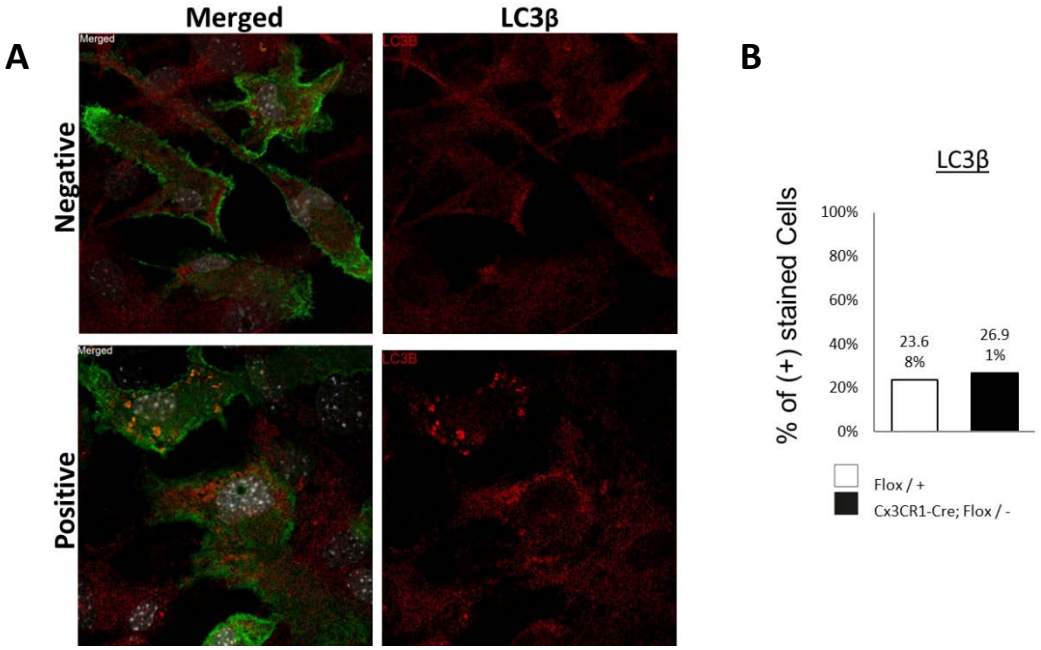


Fig.12: Autophagy levels were not altered for Cx3CR1 KO mouse model (A) Representative confocalimages of microglia LC3β staining for negative and positive conceivable limit microglia. (white: DAPI; green: Mac1; red: LC3β). (B) Nuclear andcytoplasmic quantitative analysis of control and CX3CR1 KOmicroglia. Magnification:x63, Zoom: 1.6. Data is representative of one experimental repeat.

In order to assess the results the LC3βthreshold was the presence of 3-4“foci” per positive cell. The quantification of LC3β staining suggested a rate of 27% positive microglia for Cx3CR1 KO, and 24% positive microglia for control, pointed out that the basal autophagy was

not disrupted. In addition, visual observation displayed a lower LC3β fluorescence for microglia than other cell populations.

4.4. Spontaneous microglia responses upon ICLs induction

So far, we examined the role of persistent DNA damage in the immune-regulatory microglia of Ercc1 KO, in order to suggest a link withinflammatory immune responses.

To further test if our results cope with transient acute DNA Damage cell responses, we used Mitomycin C (MMC), a potent inducer of DNA cross-links. We exposed Flox/+ (Control) microglia to 0.4 mg/ml MMC, for 4 hours. Afterwards, cells were fixed and stained with Mac1 marker to label microglia andγH2Ax, pATM and FANCI antibodies to measure the levels of cell protein expression. The experiment was conducted once and compared to un-treated Flox/+ glia culture.

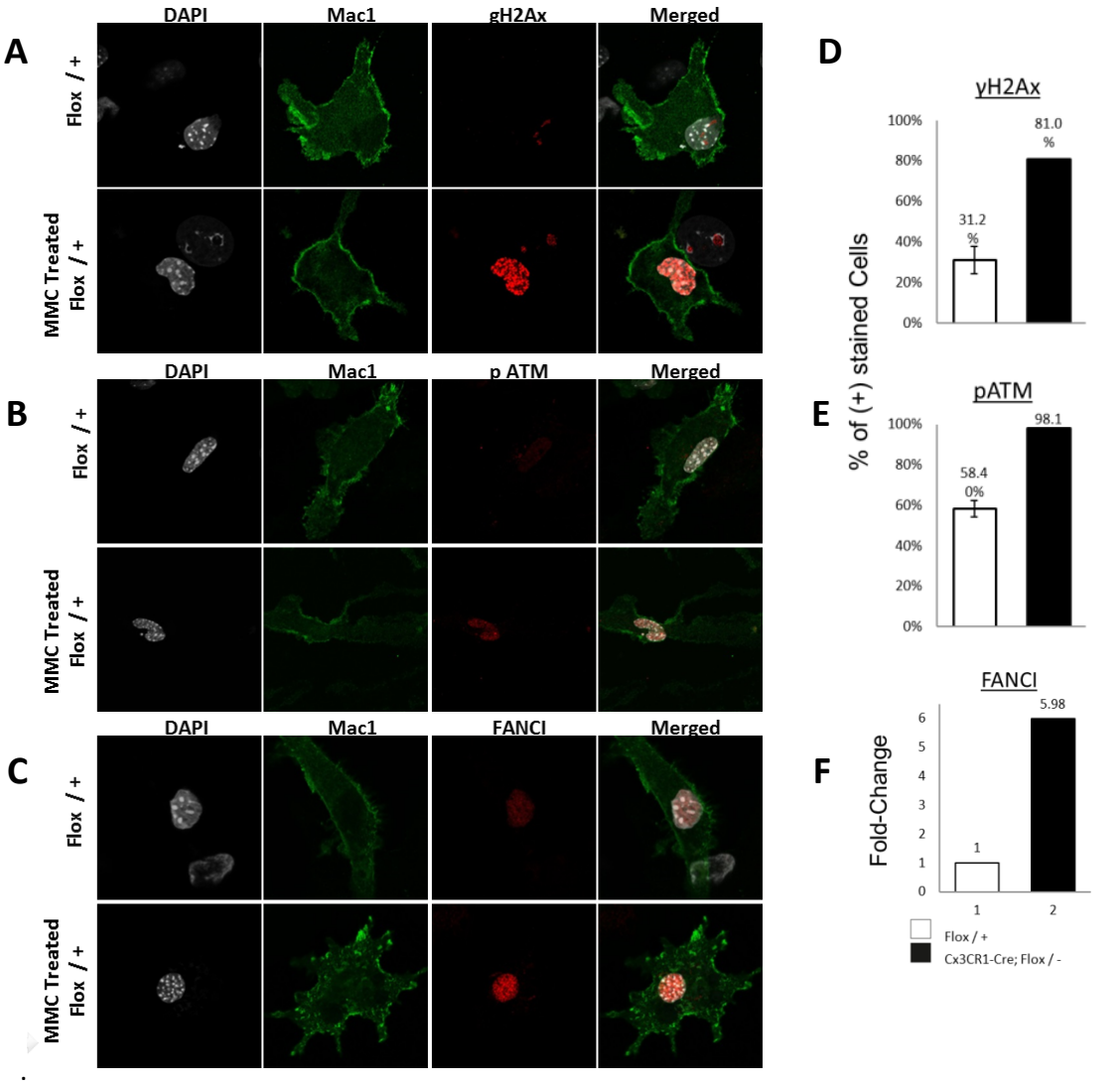


Fig.13: Accumulation of DNA ICLs triggers similar overall responses to Cx3CR1 KO (A-C) Confocal projections for Mac1 positive cells for Flox/+ and MMC-Treated Flox/+ mice stained at DIV 10, for (A) γ-H2Ax, (B) pATM and (C) FANCI. (white: DAPI; green: Mac1; red: γH2Ax/ pATM/ FANCI).(D-F) Quantitative analysis for (D) γH2Ax microglia(Cx3CR1 KO*:* 116 cells/ 1 coverslips, control: 305 cells/ 4 coverslips) (E) pATM (Cx3CR1 KO: 50 cells/ 2 coverslips, control: 150 cells/ 3 coverslips) and (F) FANCI (Cx3CR1 KO: 10 cells/1 coverslips, control: 171 cells/ 3 coverslips). Magnification x63, Zoom : 2.5.

The results showed a dramatic spontaneous upregulation of γH2Ax positive cells for 81% of MMC-Treated microglia, in contrast to only 31% (±6% SD) of Control microglia (Fig.13D). Similar results produced for pATM antibody, reached 98% positive cells in contrast to 58% (±4% SD), for MMC Treated and Control microglia respectively (Fig.13E). Unfortunately, due to technical problems, expression of FANCI was measured for 5-10 cells per condition, showing a 6-fold upregulation (Fig.13F).

We handled MMC treated microglia in the same way as Cx3CR1 KO cells, examining γH2Ax and pATM proteins separately for nuclear and cytoplasmic detection.

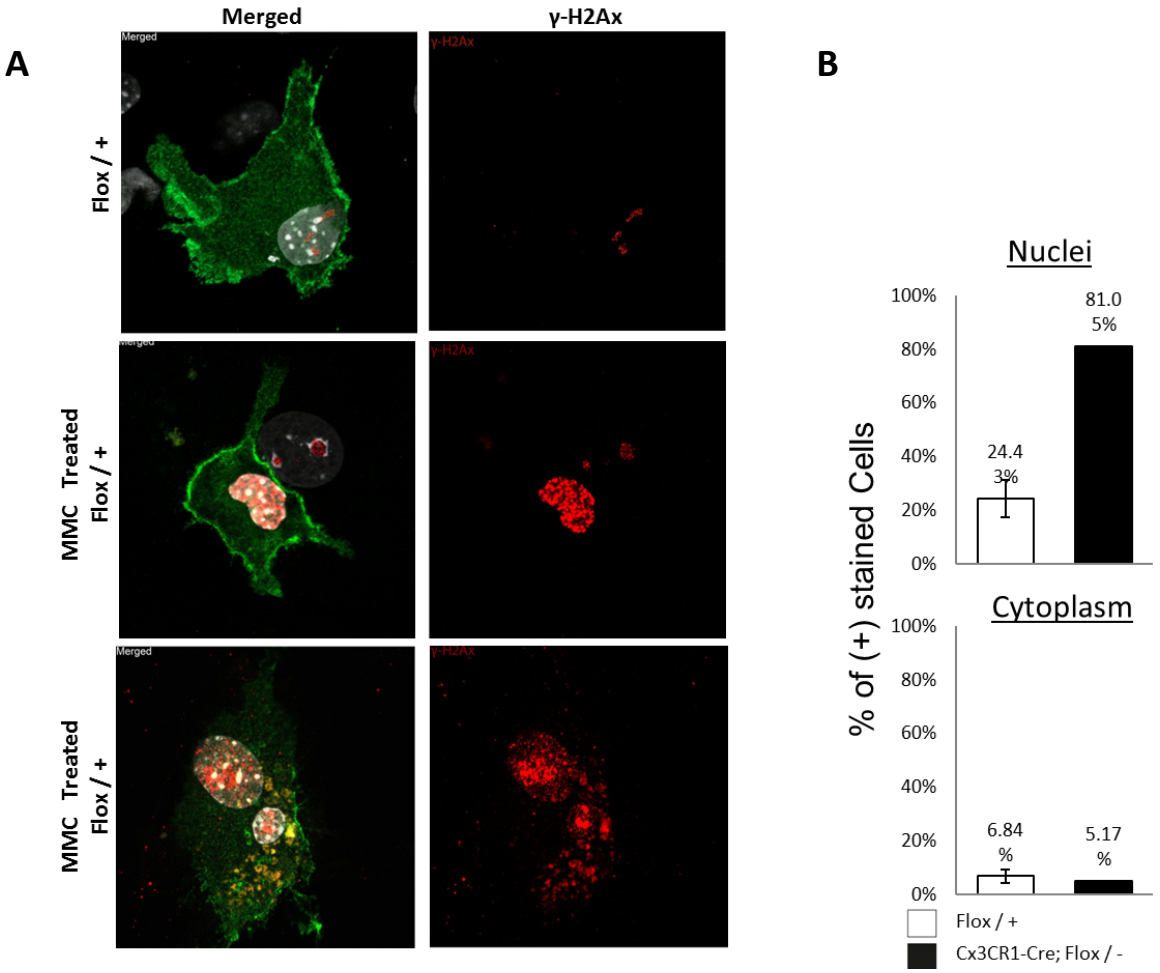


Fig.14: Gamma H2Ax nuclear and cytoplasmic induced responses of MMC Treated Flox/+ microglia (A) Representative confocal images of microglial cells for negative, nuclear-positive and cytoplasmic-positive γH2Ax staining. (white :DAPI; green:Mac1; red: γH2Ax). **(B)** Nuclear andcytoplasmic quantitative analysis of MMC-treated Flox /+ and Flox /+microglia. Magnification x63, Zoom: 2.

After MMC treatment, while the number of positive cells reached to 81% instead of 24% (±7% SD) of un-treated Flox/+, cytoplasmic γH2Ax cells were few and comparable to the microglial number of control (Fig.14B). Moreover, cytoplasmic γH2Ax was found only in cells on the edge of the coverslip. If so, the results are suggesting that the presence of cytoplasmic γH2Ax aggregates may be related with a chronic cell response.

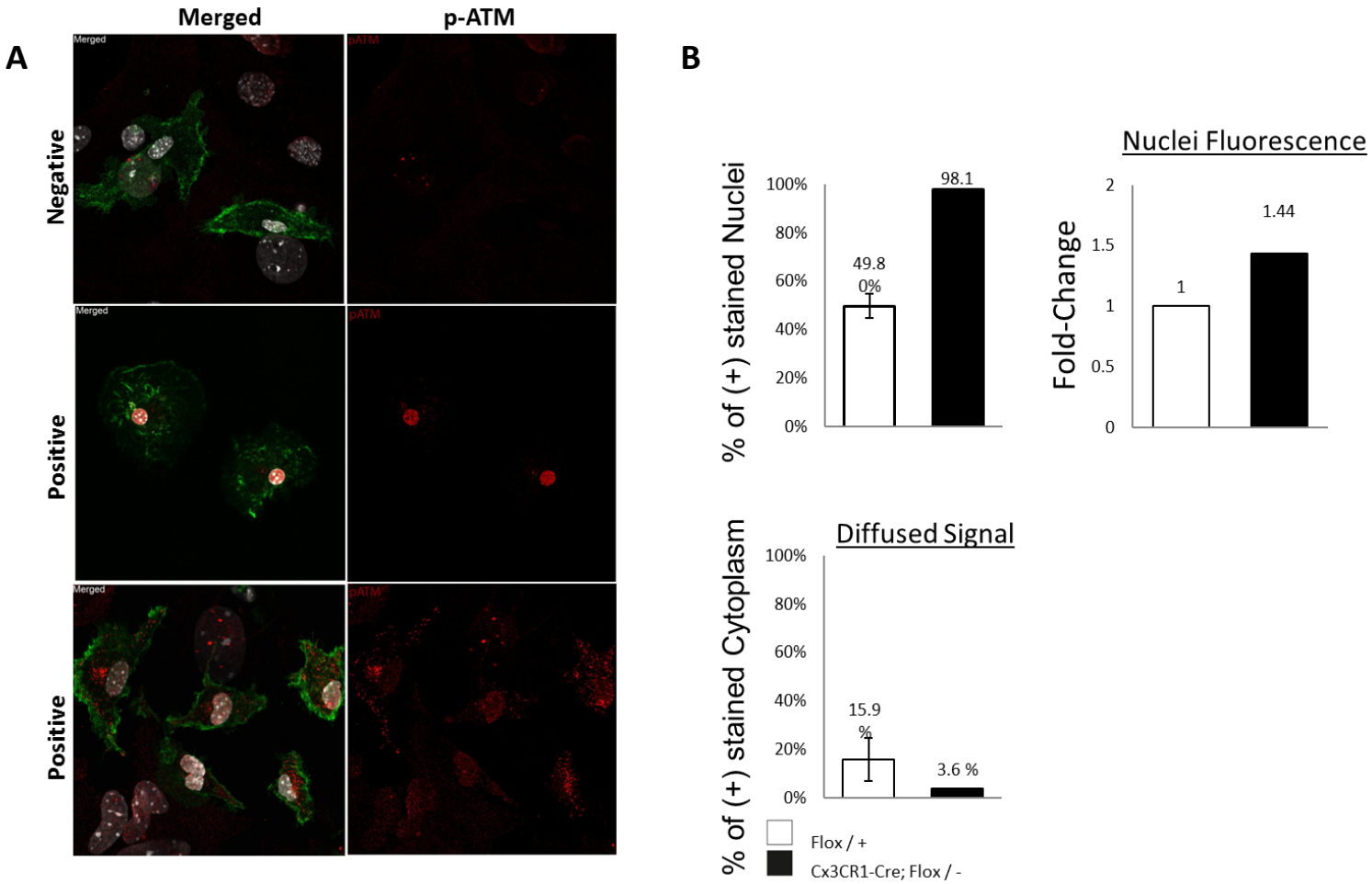


Fig.15: pATM nuclear and cytoplasmic induced responses of MMC Treated Flox/ + (A) Representative confocal images of microglial cells for negative, nuclear-positive, diffused cytoplasmic-positive and aggregate-shape cytoplasmic-positive pATM staining. (white: DAPI; green: Mac1; red: pATM). (B)Nuclear andcytoplasmic quantitative analysis of MMC-treated Flox /+ and un-treated Flox/+microglia. Magnification x63, Zoom: 1.6.

The measurement of phosphorylated-ATM indicated that nuclear detection rose to 98% in the MMC-treated microglia instead of 50% (±5% SD) of the un-treated and had a 1.5-Fold upregulation in nuclear fluorescence intensity. In the cytoplasm, no differences were found between MMC and control (Fig.15B).

Further observation on our MMC-treated glia culture, revealed that microglia were the only cell type that showed elevated levels for pATM and γH2Ax nuclear accumulation, whereas astrocytes displayed no alteration from control. Once again, the results of MMC treated cells are opposed to the results of Cx3CR1 KO, that γH2Ax expression in astrocytes was upregulated.

4.5. Evidence on interplay between DDR and immune regulation mechanisms

The role of NER in microglia has already been examined, by neuron-specific disruption of major NER factors (RajD.D.A. et al., 2014; Jaarsma, D. et al., 2011). In the Ercc1 case, microglia was found in an activated dystrophic state, having regained their ability to phagocytose (Raj, D.D.A. et al., 2014). The aim of our project is to support a functional connection between cell-autonomous accumulative DNA damage and persistent immune responses. In order to do that, we approached the inflammatory phenotype of microglia in our model, *in vivo*. Otherwise, microglial morphology could not be determined, as the isolation procedure torments-activates the cells (Streit W.J. et al., 1999), and the culture conditions do not mimick tissue environment.

To monitor the *invivo* state of microglia, more new tools were optimised during the first months of the thesis and included perfusion technique (collaboration with the lab of Dr Sidiropoulos, Assistant Professorof Physiology), preparation for cryosections and brain-tissue immunofluoresence for membrane markers.

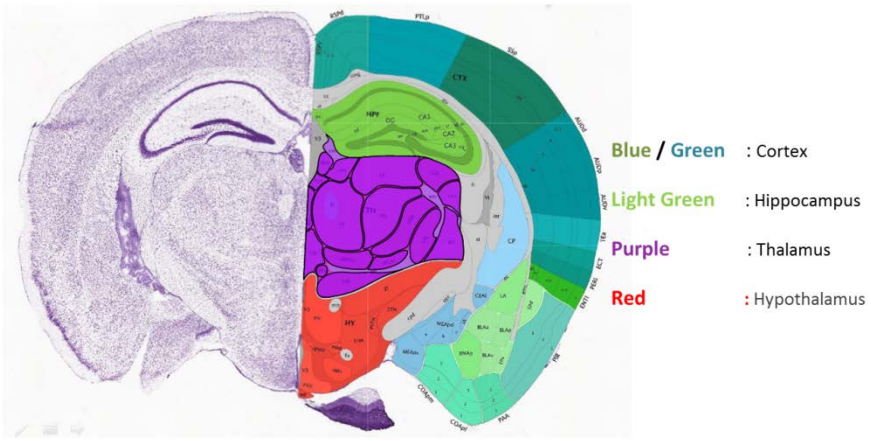


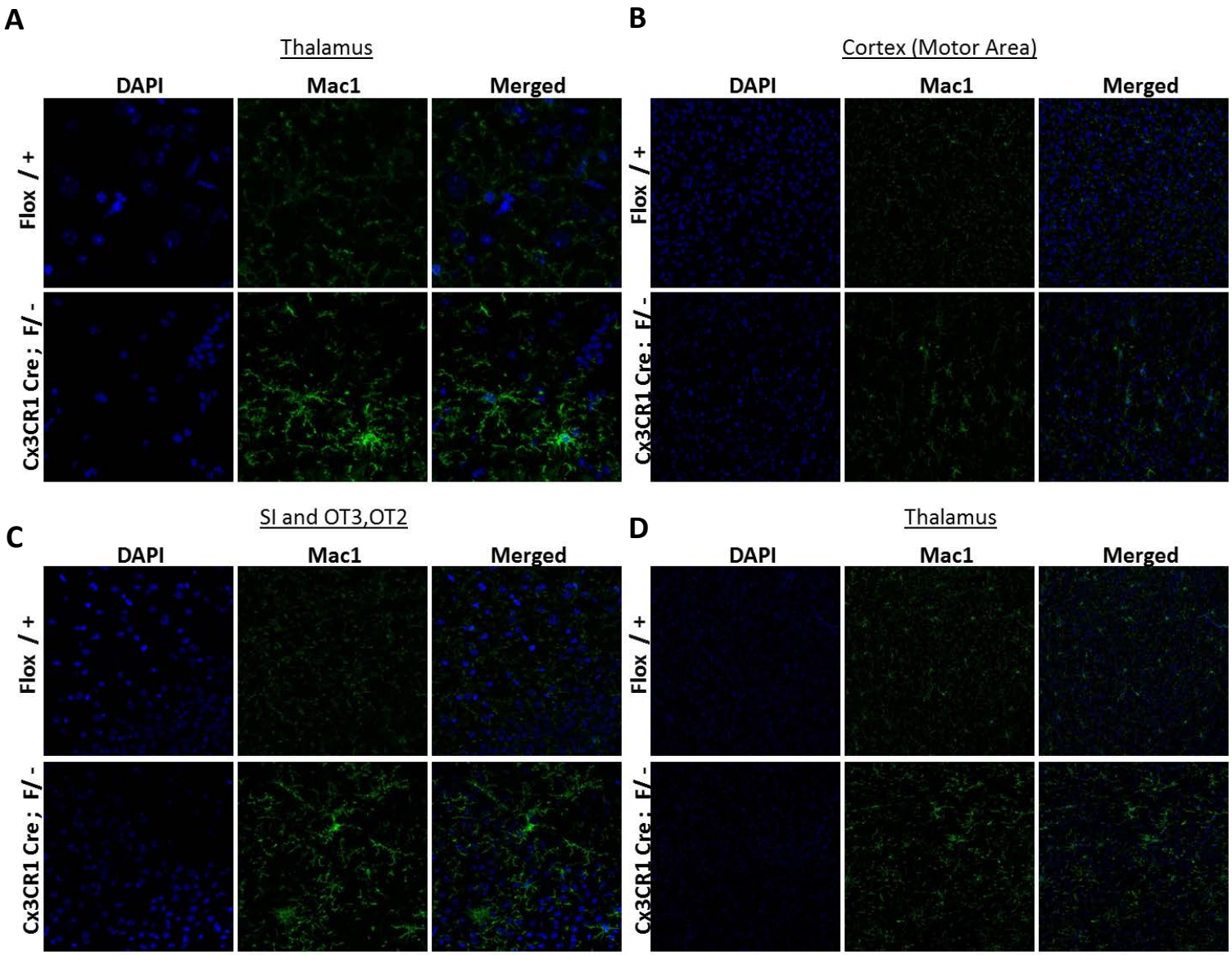
Fig.16: Coronal section from mouse brain interactive Atlas. Different areas examined for variations in microglia morphology are depicted.

In order to obtain a better characterization of the phenotype, we analyzed microglia on tissue sections for five different areas: cortex, hippocampus, thalamus, hypothalamus and striatum (forebrain). To define anatomical position of brain structureswe used the online ALLENBRAINATLAS and the ReferenceAtlases tool (Fig.16).

As expected from preview reviews (Jaarsma et al., 2011),histological organization of CNS was the same, between Cx3CR1 KO and control (3 ½ to 5 month old) mice with no neuro-developmental deficits.

To further characterize Cx3CR1 KO tissue-resident macrophage morphology, adult brain cryosections were stained for the Mac1 marker (preparation procedures described at 3.2.2).

Activated microglial phenotype with enlarged bodies and thick processes appeared in every region examined for the Cx3CR1 KO sample. On the other hand, microglia from Control cryosections had several processes and a small soma, resembling ramified state (Fig.17).



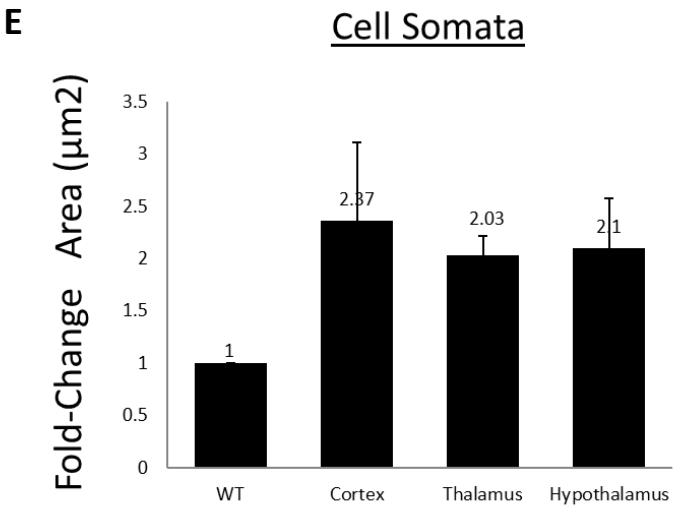


Fig.17: Cx3CR1 KO mice show microglia activation in different brain areas. (A-D) Representative superimposed confocal images (1 µm z-stacks of 8 images) for microglia morphologyof 3 ½ to 5 month-old Cx3CR1 KO and control mice. Images are zoomed out in incremental steps, in order to show that a large number of activated microglia is present in Cx3CR1 KO model. (blue: DAPI; green: Mac1) (E) Quantitative analysis of microglial cell volume for cortex, thalamus and hypothalamus brain areas (Cortex:140 cells Thalamus:70 cells Hypothalamus: 137 cells/3 slides/ 3 mice) Data is representative of three experimental repeats.. (A) Magnification:x63, Zoom: 2.5 (B) Magnification:x63, Zoom: 0.75 (C) Magnification:x40, Zoom: 0.75 (D) Magnification:x20, Zoom: 0.75

According to previous studies, the regional and the micro-environmental alterations determine microglial phenotype (Neumann, H., 2001). Reports based on immune histochemical data, suggested spatial heterogeneity of rested and activated microglia, upon damage (Schwartz, M. et al., 2006; Vilhardt, F. 2005). Hence, we expected differences in microglial morphology among the five examined areas under both normal and pathological conditions, and therefore each region was separately analyzed (Fig. 17A-D).

The Morphometric quantification of the Cellsoma volume depicteda 2-fold increase in Cx3Cr1 KO slides for the regions of cortex, thalamus and hypothalamus. Hippocampus and striatum have not been quantified, but visual observation suggested similar phenotypic differences between control and Cx3CR1 KO (Fig.17E).
Consistent with our results, Raj A.D.D. et. al., 2014 showed the same morphometric results for experiments performed at 4-month-old Ercc1 mutant animals (Ercc1Δ/-).

As mentioned in the introduction (1.4.3.), circulating macrophages have a site of entry in the CNS, so that they can be recruited in emergency cases. To test whether in our model, endothelium is the key and contributes to macrophage infiltration into the brain; we stained slides forIntercellular Adhesion Molecule 1 (ICAM1), whose expression is upregulated in inflammatory conditions (Rodier, F. *etal*., 2009; Dalmau, I. et al. 1997).

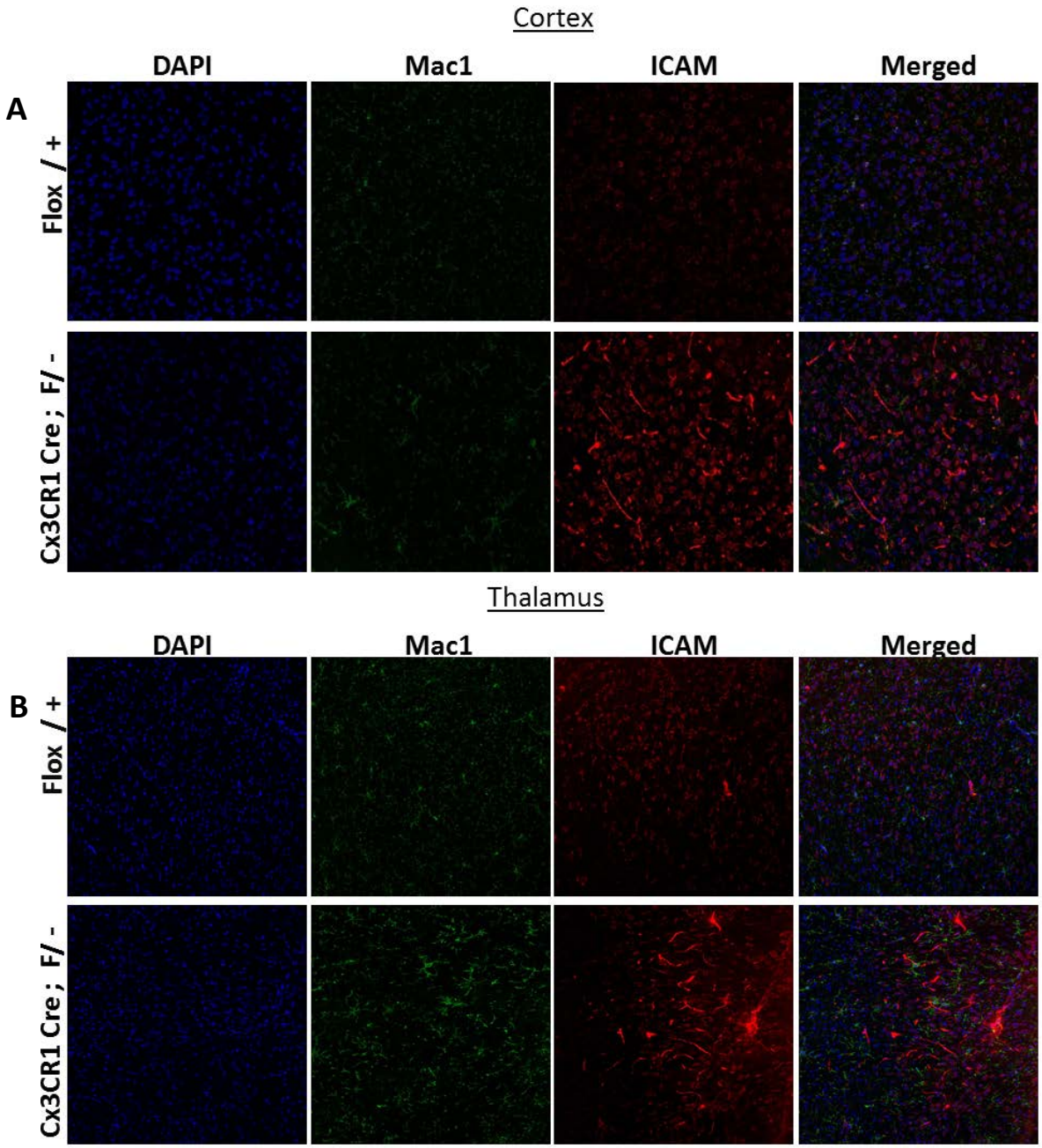


Fig.18: (A,B) Increase of ICAM-1 inflammatory marker in brain sections of Cx3CR1 KO mice. Representative superimposed confocal images (1 µm z-stacks of 8 images) of ICAM expression in Cortex of Cx3CR1 and control mouse model, in different brain areas. Images are zoomed out in incremental steps. (blue: DAPI; green:Mac1; red: ICAM). (A) Magnification x40, Zoom:0.75 (B) Magnification:x20, Zoom: 1.

The ICAM1 expressioninthe Cx3CR1 KO was highly elevated in extended parts of the brain. The images taken for cell-type specific KO, compared to the control mice, depicted an inflammatory situation that derives from cellautonomousresponse of microglia. Subsequently, this response seems to cause monocyte recruitment from the bloodstream leading to a more systemic inflammatory reaction (Fig.18). However, for antibody quanitification, some more animals need to be included.

4.6. Circulating macrophages specific ERCC1 disruption as a control

Our lab, besides Cx3CR1 KO, investigates the role of conditional Ercc1 knock out in bone marrow-derived macrophages (BMDMs). Deletion of Ercc1, in this model, is under the control of lysozymeM (LyzM or Lyz2) promoter, known to be mainly expressed in circulating macrophages.

A set of confirming experiments follows, in order to show that:

A) Bone Marrow derived macrophages do not affect out microglia culture.

B) LysozymeKO, does not affect tissueresidentmacrophagepopulationsandinthisparticularcase, themicrogliapopulation.

To begin with, we examined the percentage of Mac1 positiveErcc1 negative cells, isolated from non-perfusedadultbrains (Fig.19A).

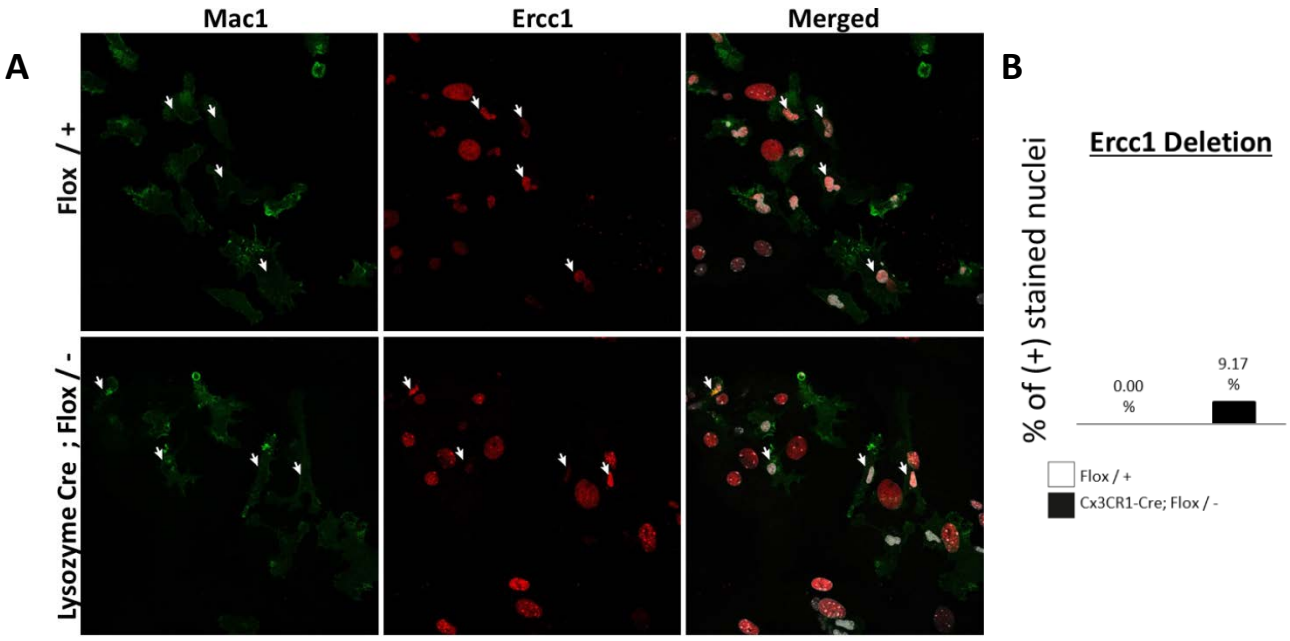


Fig.19: Macrophage-specific deletion of Ercc1. (A) Representative confocal images depict Ercc1 deletion in mixed population for Lyz2 KO and Control, at DIV 10. (white: DAPI; green: Mac1; red: Ercc1). (B) Quantitative analysis for Ercc1 Deletionof Lyz2-Cre;Flox/- and Flox/+ microglia (Lyz2 KO: 218 cells/ 2 coverslips, CONTROL: 30 cells/ 2 coverslips).Magnification x40, Zoom : 0.75.

Ercc1 was present in 90.8% of microglia cells, 68.35% of which demonstrated weaksignal (Fig.19B). To make sure that nuclear sign was not noise, imageswerecaptured under the same conditions for Cx3CR1 KO, butnosignalwasreceived.

The measurement result showed that a part of BM-derived macrophages (meninges, choroidplexusandperivascularspaces macrophages), were not effectively removed from the brain. The ten percent of circulatedmacrophageswillbereducedifperfusedmicebrain are used for isolation procedure, according to DeHaas, A.H.*etal*., 2007.

As there are references (Prinz, M., and Priller, J., 2014) forlowtomediumLyz2 expression in microglia, Lyz2KO isolated cell cultures were stained with pATM and γH2Axantibodies. The quantification for LysozymeKO showed almost the same cell percentage for (a) γH2Ax expression (27%) and (b) γH2Ax cytoplasmic detection (5.8%) as Control(Fig.20C).

Moreover pATM, showednocytoplasmicandnuclear differences between Lyz2 KO and control. FigureD depicts Fold-Change for intensity fluorescence of cytosolic and nuclear microglia (Fig.20D).

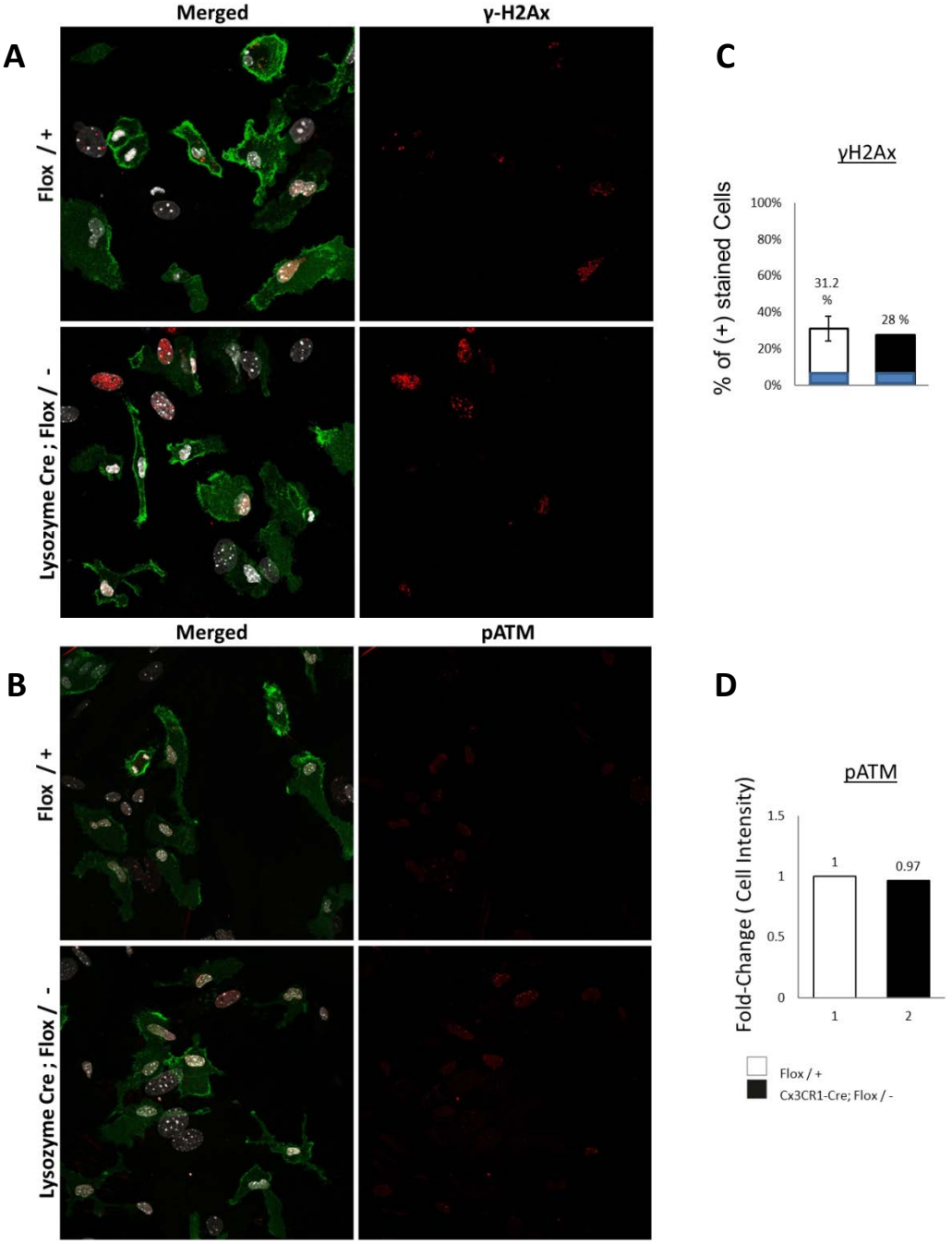


Fig20.: Ercc1 deletion in Circulating Macrophages does not alternate expression ofγH2Ax andpATM for microglia, at 10th DIV. (A,B) Confocal projections for Mac1 positive cells of control and Lyz2 KO mice stained for (A) γ-H2Ax and (B) pATM. (white :DAPI; green: Mac1; red: γH2Ax/ pATM). (C,D) Quantitative analysis for (C) γH2Ax microglia (Lyz2 KO: 137 cells/ 1 coverslips, control: 305 cells/ 4 coverslips) and (D) pATM (Lyz2 KO: 137 cells/ 1 coverslips, control:

150 cells/ 3 coverslips) Data are representative of one experimental repeat.(A) Magnification: x63, Zoom: 1 (B) Magnification x63, Zoom: 0.75.

To characterize microglia morphology and disclose possible differences between Lysozyme-Cre; Flox/- and Flox/+ genotypes, adult mouse brains (3½ to 5 month-old) were immunostained with Mac1 (Fig.21A).

Brain cryosections were further immunostained with ICAM1, so a potent upregulated expression was to be depicted (Fig.21B).

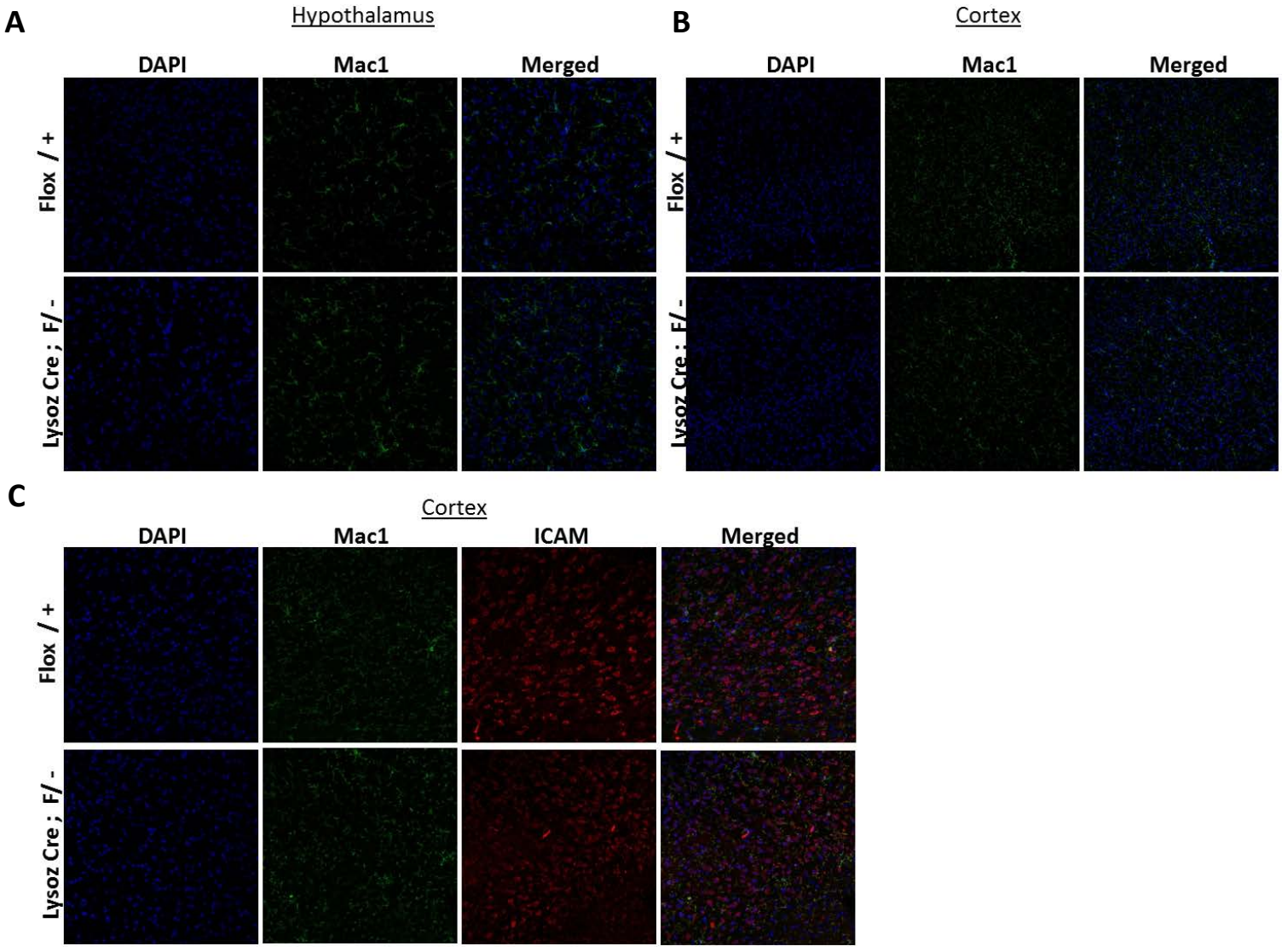


Fig.21: Lyz2 KO mice exhibit ramified microglial morphology, while ICAM1 expression remains unaffected. (A-C) Superimposed confocal images (1 μm z-stacks of 8 images) of (A,B) quiescent microglia (C) ICAM expression, for Lysozyme KO and control cryo-sections in different brain areas. (A) Magnification x40, Zoom: 0.75. (B) Magnification: x20, Zoom: 0.75 (C) ICAM1 Magnification: x40, Zoom: 0.75

Visual observation of Lysozyme KO and wt images from different brain regions, in both cases, showed characteristics indicative of quiescent microglia. Respectively, LysozymeKOstaining for ICAM1, had a similar pattern to control staining.

CHAPTER V:

DISCUSSION

On one hand, defectiveTC-NER patients demonstrate premature ageing syndromes that are characterized by neurological abnormalities. One the other hand, microglia is crucial in the progression of multiple neurodegenerative diseases, including Alzheimer, Parkinson and Huntigton. Until today, there have been studies, supporting that defective DNA Damage repair mechanisms in the neurons can cause phenotypic and functional alterations in microglia, which in turn associate with neurodegeneration. However, intrinsic microglia responses for repair defective pathways remain unclear. In this work, we attempt to trigger the onset of CNS-specific pathology by deleting the *Ercc1* gene in innate immune cells of the CNS. The experiments were performed on adult animals (from 3 ½ to 8 month-old) in order to observe the time-dependent cellular responses of Cx3CR1 KO mice.

GammaH2Ax histone is a sensitive marker for DSBs, mainly induced by ionizing radiation (IR), but also caused by ATR (ATM and Rad3-related) in response to UV radiation. This modified histone serves as a binding site for repair enzymes and checkpoint proteins (Strozyk, E. and Kulms, D., 2013). Likewise, FANCI protein is recruited to ICLs and has a critical role in orchestrating their detection and repair. FANCI belong to the FA pathway that displays extensive crosstalk with other repair mechanisms, including homologous recombination (HR) and NER (Lopez-Martinez, D. et al., 2016). Therefore, the enhanced nuclear presence of FANCI and γH2Ax staining in Cx3CR1 KO, suggests multiple DNA Damage and decreased efficiency of repair mechanisms in the absence of ERCC1-XPF incision. Furthermore, γH2Ax and to a lesser degree FANCI protein presence were detected in the cytoplasm.
This possibly facilitates a time-dependent effect, as when we treated control microglia with MMC, inordertoobserve acute DNA Damage-induced responses, we identified increased proteins amounts in the nucleus, but failed to detect any cytoplasmic γH2Ax and FANCI presence.

ATM is a DNA damage sensor, which becomes activated through phosphorylation in response to genotoxic stress. Its role is involved in both nucleus response initiation and cytoplasm DDR meditation. In the case of Cx3CR1 KO microglia, we once more detected elevated pATM in the nucleus and either diffuse or in conjugates protein presence in the cytoplasm. Respectively with results of factors that mark DNA damages, exposure of MMC on cells, raised the nucleus level of protein but was not able to replicate the increased cytoplasmic levels of Cx3CR1 KO.
Furthermore, pATM is guarding the integrity of DNA, by participating in interplays with innate immunity and autophagy pathways. Nonetheless, in our attempt to identify induction of autophagy, we found no alteration in the basal levels of LC3β marker between Cx3CR1 KO and control mice.

To build a hypothetical crosstalk with immune responses throughout our work, we will regress to a previous paper from our lab, where adipocyte-specific Ercc1 knock-out mice, relative toourresults, displayed increased levels of cytosolic pATM. The study had revealed an essential role for pATM in linking the DDR signaling to the transcriptional activation of pro-inflammatory cytokines, as it was found that indirect involvement of the protein causes disengagement of co-repressor complexes from promoters of *Il6, Tnf*α and *Kc* genes and induction of their expression(Karakasilioti, I. et al., 2013).

In an attempt to further justify the cytosolic response of Cx3CR1 KO, we take into account that DNA factors were shown to form granules inside the cells (apart from diffuse pATM response). Like in similar cases of microglia cultures,we should consider that the effect may depict increase in phagocytosisMoussaud, S., and Draheim, H. J. in 2010 published figures of adult microglia that had re-gain amoeboid properties and phagocytosed latex beads in cell culture, (Fig. 4e,f). Intracellular beads distribution pattern is similar to our cytoplasmic protein aggregates pattern. Accordingly, we have to confirm the identity of granules through double-label staining for DDRFactors and Lysosomal-associated proteins, to exclude the possibility that cytosolic protein detection indicates increased phagocytosis of dying cells.
Related to aforementioned literature, it is possible that CX3CR1 KO microglial cells secrete higher pro-inflammatory cytokine levels, gradually intoxicating the cultural environment. To conclude, it is noteworthy that different morphology and cytoplasmic response of microglia is observed depending on whether they lie in cell aggregates or are sparsely distributed.

The microglial morphology was examined in vivo, in a tissue-context level. Results showed vulnerability to age-dependent accumulation of DNA damages, since cells displayed a transition to activated form (enlarged cell soma, thickened processes). This distinct phenotype is referred in previous studies as “hypertrophic” “reactive” or “intermediate” **(**Ayoub AE and Salm AK, 2003; Gomide, G. et al., 2005; Karperien, A. et al., 2013). The questions, then, arise as to in what extent interactions between microglia and neurons are disrupted and what are the defects in synaptic plasticity. In Raj, A.D.D. et al., 2014 study, microglia from the hypomorphic mouse, Ercc1$^{-/\Delta}$, were examined. The observation of adult (4-month-old) brain sections showed a primed microglial phenotype - similar to our case - and enhanced phagocytosis, but non-significant mortality (verification by Caspase3 staining). Moreover,treatment with LPS, elevated the number of activated microglia cells and pro-inflammatory TNFα, IL1β and IL6 responses, while Ercc1$^{-/\Delta}$microglia transcriptome, pointed to upregulated functions of: cell migration, inflammatory response, activation of microglia, phagocytosis (in support to previous paragraph) and adhesion of immune cells. The last process once more is in accordance with our data, as ICAM 1 increased expression, supports the recruitment of circulating immune cells, and attributes the effects solely to microglia autonomous responses.

Microglia cultivate and monitor neuronal circuits connectivity, as well as they are responsible to fight pathogens, cancer cells and enhance memory. In the last few years, more and more research teams bring their attention to microglia due to its vital role in neuro-inflammation and neurodegenerative diseases. The age-related effects of those cells are based on the non-tightly regulated phagocytosis or their reduced function and efficiency to respond to stimuli.

Oxidative stress greatly affects the brain due to high-grade oxygen metabolism and lipid content as compared to other organs. Evidence acquired from recent reports (Raj D.D.A. et al., 2014; Jaarsma, D. et al., 2011; BorgesiusN.Z. et al., 2011) convey the tissue degeneration with DNA Damage repair defects in neuronal environment, that resulted in microglia activation, progressive phagocytosis and an impact in neural activity. In our work, there are still players missing to solve the puzzle of Ercc1 defective intrinsic DDR, however, this Master thesis gives first indications that microglia autonomous reactions may leadtochronicinflammationandunderlyingaging.

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CHAPTER VI:

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