Pharmacological Depletion of Microglia Leads to a Dose-Dependent Reduction in Inflammation and Senescence in the Aged Murine Brain

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Abstract—Chronic macrophage activation was implicated as one of the main culprits for chronic, low-grade inflammation which significantly contributes to development of age-related diseases. Microglia as the brain macrophages have been recently implicated as key players in neuroinflammation and neurodegeneration in the aged brain. Microglial cell functions are indispensable in early development, however, activation or senescence of microglia in aging cells may be detrimental. Depletion of microglia using genetical or pharmacological approaches leads to opposite results regarding effects on brain cognition. In this study we pharmacologically depleted microglia using orally delivered low and high doses of the CSF1R inhibitor PLX5622 and assessed the expression levels of known inflammation markers (TNF-α, IL1-β, IL-6, IL-10), glia markers (Iba-1 and Gfap) and specific senescence marker p16 Ink4a in the aged murine brain. Our results indicate that treatment with low and high doses of PLX5622 leads to a dose-dependent depletion of microglial cells with similar levels in young and aged mice. We also show that treatment with low and high PLX5622 differentially affected cytokine levels in young and old brains. By using low doses we could achieve reduction in inflammation circumventing the astrocyte activation. Removal of microglia cells led to decreased expression of the senescence marker p16 Ink4a in the aged brain, indicating a relevant contribution of these cells to the expression of this marker and their senescent status in the healthy aging brain. Our results indicate that increased and detrimental brain inflammation in aged murine brain can be impaired by selectively reducing the microglial cell population. © 2022 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: microglia, senescence, depletion, PLX5622, aging, inflamming.

INTRODUCTION

Aging is a physiological process defined as progressive changes in an organism that lead to a decline of biological functions, and can develop without appearance of concurrent diseases (Mora, 2013). Currently, two of the most widely accepted theories of aging are the ‘inflammaging theory’ as described by Franceschi et al. (2000) as well as the extension of this theory adding oxidative damage of biomolecules as a cause of inflammaging called oxi-inflammaging theory of aging (De la Fuente and Miquel, 2009). These theories presume that constant stimulation of our innate immune cells with pathogens or endogenous cell debris leads to chronic, sterile low-grade inflammation which contributes to development of age-related diseases. More recently, a novel theory termed ‘senescent theory of aging’ was postulated, which attempts to unify all of the previous aging theories. According to this theory, there is a significant accumulation of senescent cells with age that induces loss of regeneration potential, inflammation and organ dysfunction (Lopez-Otin et al., 2013; Bhatia-Dey et al., 2016). Cellular senescence is defined as a stress response whereby cells lose their proliferation capacity in an irreversible manner (Campisi, 2013). Senescent cells are normally removed by immune cells in a process called immunosurveillance. However, with age immune cells become dysfunctional or senescent as well, leading to reduced removal of other senescent cells, spreading the senescence to other neighbouring cells and eventually leading to whole organ or system dysfunction (Ovadya et al., 2018). Tissue macrophages, as long lived innate immune cell population, seem to be the major players in this constant low level inflammation during aging, a process termed macroph-aging (Prattichizzo et al., 2016). Microglia, the resident macrophages of the CNS, have been implicated as key players in neuroinflammation in the aged brain (Norden and Godbout, 2013) and in neurodegeneration (for review see (Angelova and Brown, 2019)). Moreover, microglial senescence seems to switch these cells from neuropro-
tective in the young brain to neurotoxic in the aged brain (Luo et al., 2010).

In a previous study, we found a conserved aging-related signature of senescence and inflammation in different tissues and species, characterized by an increase in typical inflamming markers, pro- and anti-inflammatory cytokines and growth factors, as exemplified in aged mice brains (Barth et al., 2019). More recently, we found that microglia cells become dysfunctional, increase secretion of factors belonging to so called senescence associated secretory phenotype (SASP) and express the senescence marker p16Ink4a both in vitro and in vivo in the aging brain (Stojilkovic et al., 2019). p16Ink4a is an established senescence marker and has already been used to target and remove senescent cells from different tissues (Baker et al., 2016). Several groups found profound inflammatory changes in the brain of a tau AD mouse model (Bennett et al., 2018). Interestingly, partial depletion of microglia cells led to improved cognition also in an AD mouse model (Spangenberg et al., 2016). On the other hand, complete genetic depletion of the microglial cell population was found to cause a cytokine storm and aggravation of cognitive dysfunction (Parkhurst et al., 2013; Bruttger et al., 2015) Keeping the levels of age associated inflammation low seems to be crucial for healthy aging and longevity, as shown in studies on centenarians and in long lived mice (Brooks-Wilson, 2013; Barth et al., 2019). Whether microglial depletion in aged mice brain leads to a decrease in inflammation levels remains an open question. Moreover, whether removal of microglia affects the expression of senescence markers in the brain is also not known.

Here we pharmacologically depleted microglia using different doses of the CSF1R inhibitor PLX5622 and assessed the expression levels of known inflammation markers and of the specific senescence marker p16Ink4a in the aged murine brain. Our results indicate that treatment with low and high doses of PLX5622 leads to a dose-dependent depletion of microglial cells with similar levels in young and aged mice. We also show that treatment with low and high PLX5622 differentially affected cytokine levels in young and old brains. Interestingly, by using low doses we could achieve reduction in inflammation circumventing the astrocyte activation. Removal of microglia cells led to decreased expression of the senescence marker p16Ink4a in the aged brain, indicating a relevant contribution of these cells to the expression of this marker and their senescent status in the healthy aging brain.

**EXPERIMENTAL PROCEDURES**

**Animals**

All animal experiments were conducted in accordance with the German legislation on the protection of animals and were approved by the local animal welfare committee (02-006/16). The CSF1R inhibitor PLX5622 was provided by Plexxikon and formulated in standard chow by Research Diets at 300 mg/kg or 1200 mg/kg. Young adult (3-month-old) and aged (22–24-month-old) males from a C57BL/6J locally inbred mouse strain were used. Mice were maintained on standard chow containing different doses of PLX5622 for 7 days. Animals were sacrificed by an overdose of isoflurane anesthesia. Brains were carefully extracted after transcardial perfusion with ice cold PBS for 5 min. Left brain hemisphere was snap frozen in dry ice and used for RNA isolation, and the right brain hemisphere was used for brain sectioning and immunohistochemistry. Number of used animals per experiment is reported in the supplementary Table S1.

**Immunostaining procedure**

The right brain hemispheres were cut into 18 µm slices. Slices were fixed for 20 min with 4% PFA. To block non-specific staining, slices were incubated with 10% donkey serum (NDS) in 0.3% Triton-X in PBS for 2 h at room temperature. Unconjugated primary antibody was diluted in dilution buffer (2% NDS, 1% BSA, and 0.3% Triton-X in PBS) and incubated overnight at 4 °C. We used antibodies raised against the microglial marker Iba1 (ionized calcium-binding adapter molecule 1; dilution 1:500; WAKO; Neuss, Germany AB_2314667). Alexa 488 conjugated secondary (Jackson Immunoresearch, West Grove, PA, USA) antibody was diluted in dilution buffer (1:500). DAPI solution was added to each well and incubated for 5 minutes at room temperature.

The images were taken with an Axioplan2 Imaging microscope (40x air objective; Zeiss, Oberkochen, BW, Germany) coupled to an AxioCam HRc camera (Zeiss). At least 10 random images per brain region (motor cortex, hippocampus or white matter) were taken, cells counted and analyzed.

**Analysis of gene expression with qPCR**

To determine the gene expression levels, RNA was extracted from the brain using Qiazol reagent (Qiagen). The RNA concentration, quality and integrity were determined using a Nanodrop (Thermoscientific, Waltham, MA, USA) and a QIAxcell Systems (Qiagen). cDNA was synthetized from 500 ng of RNA/reaction using a RevertAid First Strand cDNA Synthesis kit (Thermoscientific). qPCR was performed using a LightCycler 480 SYBR Green kit (Roche, Germany). Detection and quantification were conducted with a Rotor gene cycler and Rotor gene Q software (Qiagen). The housekeeping genes Gapdh and Hmbs were used for normalization. The relative gene expression was calculated using the 2-DDCT method (Livak and Schmittgen, 2001). The primers used are listed in Supplementary Table S2.

**Statistical analysis**

Data are presented as box-and-whiskers plots, where the "box" depicts the 25th and 75th quartiles, the horizontal line is the median and the whiskers show the 5th and 95th percentile.

Comparison between experimental groups and conditions were analyzed with two-way analysis of
variance in all cases for the factors "Group" (two different age groups) and "Treatment" which consists of untreated, low-dose treated and high-dose treated mice. In all cases, interaction between both factors were analyzed. Post-hoc comparisons were made with the Holm–Sidak test. Statistical analysis was performed using Sigma plot version 12.5 (Systat, San Jose, CA, USA). P values < 0.05 were considered significant.

RESULTS

Treatment with PLX5622 leads to a dose-dependent reduction in microglia numbers in motor cortex, hippocampus and brain white matter from young and aged brains

As shown in Fig. 1 from mice control vs. PLX5622 treated animals, there was a significant dose-dependent reduction of microglia cells after 7 days of treatment with the CSF1R inhibitor PLX5622. Treatment with 300 mg/kg or 1200 mg/kg led to significant reduction of microglia cells numbers in the motor cortex of young mice by 44% and 84.4% and in aged mice by 32% and 80% respectively (Fig. 1). There were no significant differences in depletion efficiency in motor cortex between young and aged mice. The body weight of animals was not significantly affected by PLX5622 treatment and the weight was for young animals 27.592 ± 0.86 vs 28.8 ± 0.84 g, for aged animals 30 ± 1.84 g vs 32.26 ± 1.91 g, (n = 5–6). The chow intake in control chow treated animals vs. PLX5622 treated animals was 2.84 g vs. 2.45 g per animal per day.

In the hippocampus, treatment with 300 mg/kg or 1200 mg/kg led to significant reduction of microglia cell numbers of young mice by 35.7% and 77.3% and in the aged mice by 37.7% and 71% respectively (Fig. 2). There were also no significant differences in depletion efficiency in hippocampus between young and aged mice. The brain weight of animals was not significantly affected by PLX5622 treatment and the weight was for young animals 27.592 ± 0.86 vs 28.8 ± 0.84 g, for aged animals 30 ± 1.84 g vs 32.26 ± 1.91 g, (n = 5–6). The chow intake in control chow treated animals vs. PLX5622 treated animals was 2.84 g vs. 2.45 g per animal per day.

Depletion of microglia in aged mice brains led to a dose-dependent reduction in IL-6 and IL-10 expression to levels observed in young untreated mice, with no significant changes in expression of Tnf-α. Treatment with the high dose led to a significant decrease in Tgf-β in the aged murine brain (Fig. 3).

Treatment with low doses of PLX5622 leads to a reduced Iba1 expression avoiding astrocyte activation

Analysis of microglia and astrocyte activation markers indicated a significant increase in Gfap and Iba1 expression in untreated aged murine brains. Gfap has been described as the best marker of reactive astrogliosis (Pekny and Nilsson, 2005). Treatment of young or old mice with low and high dose led to a significant decrease in Iba1 expression compared to the untreated group, with no significant differences in efficiency between low and high dose groups (Fig. 4).

Neither treatment with low nor with high dose led to differences in Gfap expression in young mice. Treatment of aged mice with high but not with low doses led to a 4-fold increase in Gfap expression, indicating astrocyte activation (Fig. 4).

Depletion of microglia did not lead to significant changes in IL-1β expression in the young murine brain (Fig. 4). Interestingly, the low but not high PLX5622 dose led to a significant decrease in IL-1β expression in the aged murine brain (Fig. 4).

Expression analysis of the growth factor BDNF indicates a significant decrease in the untreated aged murine brain as compared to the young cohort. Interestingly, only treatment with the high dose of PLX5622 led to an increase in Bdnf expression in the aged murine brain (Fig. 4).

Treatment of mice with the PLX5622 leads to a reduced p16<sup>ink4a</sup> expression in a dose-independent manner

Expression analysis of the senescence marker p16<sup>ink4a</sup> shows a 5-fold increase in the untreated aged murine brain as compared to the young group. Neither treatment with low nor with high dose led to differences in p16<sup>ink4a</sup> expression in young mice brains. Treatment of aged mice with low dose led to a significant decrease of p16<sup>ink4a</sup> expression compared to untreated group, with no significant differences between low or high dose treated groups (Fig. 5).

DISCUSSION

Here we found a dose-dependent microglial depletion after delivery of the CSFR1 PLX5622 to young and old
Fig. 1. Dose-dependent effect of PLX-5622 on microglia cell numbers in murine cortex. (A) Number of microglia per mm² in young and aged murine brain untreated, treated with low dose (300 mg/kg) or with high dose (1200 mg/kg) PLX-5622. (B) Representative images of cortex brain slices from young and aged mice untreated or treated with low (300 mg/kg) or high (1200 mg/kg) dose of PLX5622. ***p < 0.001, two way ANOVA. Boxes represent the median and the 25th and 75th quartiles and the whisker showing the 5th and 95th percentile, (n = 3–4).

Fig. 2. Dose-dependent effect of PLX-5622 on microglia cell numbers in murine hippocampus. (A) Number of microglia per mm² in young murine brain untreated, treated with low dose (300 mg/kg) or with high dose (1200 mg/kg) PLX5622. (B) Representative images of hippocampus brain slices from young and aged mice untreated or treated with low (300 mg/kg) or high (1200 mg/kg) dose of PLX5622. ***p < 0.001, two way ANOVA. Boxes represent the median and the 25th and 75th quartiles and the whisker showing the 5th and 95th percentile, (n = 3–4).
mice cohorts. Treatment with 300 mg/kg PLX5622 led to 32–44% removal of microglia in different brain regions while 1200 mg/kg reached 69–84% levels of depletion, in agreement with previous results (Dagher et al., 2015). Our data also indicate increased microglia numbers in the aged brain, possibly due to brain atrophy but also due to an increased inflammatory state which is in agreement with 2 recently published studies (Hefendehl et al., 2014, Elmore et al., 2018). In the first study, researchers found a significantly increased number of microglia cells in the murine cortex (approximately 14%) by using the nearest neighbour analysis (Bennett et al., 2018). In the later study there was an increase in the density (or cell number) of Iba1+ cells in the hippocampus by around 56% (Spangenberg et al., 2016). Our results show no difference in efficiency of depletion comparing young and aged mice treated with either low or high dose PLX5622, indicating similar sensitivity of aged microglia cells to CSF1R blockade. As shown in our study, treatment with the low dose of PLX5622 reduced the number of microglia in the aged brain to levels observed in the young untreated mice. A recent study showed that similar effects may be achieved, at least in the short term by complete removal and repopulation of microglia cells (Elmore et al., 2018). However, in contrast to our findings, microglia replacement in the aged brain did not affect expression of cytokines or chemokines belonging to SASP, indicating the presence of local extrinsic factors and cues which controls the levels of microglial activation (Elmore et al., 2018).

Main reasons for impaired cognition in the aged brain are increased levels of brain inflammation and dysfunctional anti-inflammatory response especially to Tgf-β (Von Bernhardi et al., 2015). Here, we confirmed increased levels of pro- and anti-inflammatory markers in the aged mouse brain. Interestingly, we observed a clear dose-dependent reduction of these cytokines, indicating a need for accurate titration studies in order to assess beneficial effects of microglia depletion, without the detrimental contribution of cytokine storm or astrocyte activation. A recent study showed that near-complete microglia depletion using a genetic model approach leads to cytokine storm accompanied by activation of astrocytes and the remaining microglia population (Bruttger et al., 2015). Using the pharmacological approach reduces the cytokine storm, probably due to co-deletion of peripheral

![Fig. 3. Dose-dependent effect of PLX-5622 on cytokine levels in young and aged murine brains. Depicted are expression levels of IL-6, TNF-α, IL-10 and Tgf-β in young murine brain untreated (blue box), treated with low dose (300 mg/kg, red box) or high dose (1200 mg/kg, green box) and in aged murine brain untreated (violet box), treated with low dose (300 mg/kg, orange box) or with high dose (1200 mg/kg, black box) PLX5622. *p < 0.05, **p < 0.01, ***p < 0.001, two way ANOVA. Boxes represent the median and the 25th and 75th quartiles and the whisker showing the 5th and 95th percentile, (n = 4–6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
immune system including macrophages, however, it still leads to significant astrocyte activation, possibly due to phagocytosis of apoptotic microglial cells (Elmore et al., 2014). Complete removal of microglia cells was found to be detrimental after traumatic spinal cord injury (Fu et al., 2020), coronavirus infection (Sariol et al., 2020), stroke (Szalay et al., 2016) and for synaptic plasticity in the adult mouse brain (Parkhurst et al., 2013). Beneficial effects following 90% microglia depletion were described after brain hemorrhage or after radiation brain injury, as reviewed elsewhere (Han et al., 2017). On the other hand, no detrimental effects were described after partial depletion using PLX5622 and even a decreased tau propagation and improvement in cognition in novel object recognition test in AD mice brain models (Parkhurst et al., 2013). 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Fig. 5. Effect of PLX-5622 treatment on senescence marker expression in young and aged murine brains. Expression levels of the senescence marker p16 in young murine brains untreated (blue box), treated with low dose (300 mg/kg, red box) or with high dose (1200 mg/kg, green box) PLX5622 and expression levels of the senescence marker p16 in aged murine brain untreated (violet box), treated with low dose (300 mg/kg, orange box) or with high dose (1200 mg/kg, black box) PLX5622. ***(p < 0.001, two way ANOVA. Boxes represent the median and the 25th and 75th quartiles and the whisker showing the 5th and 95th percentile, (n = 4–6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dysregulation in aged or AD model mice (Von Bernhardi et al., 2015). A further decrease of Tgf-β could only be detrimental as it is one of the regulatory mechanisms secondary to cell activation in response to inflammatory conditions (Herrera-Molina and von Bernhardi, 2005). The low dose treatment would probably lead to depletion of cells being highly dependent on CSF1 stimulation for their survival. Remaining microglia cells may have higher CSF1R expression or survive in a CSF1-independent manner (e.g. by IL-34).

Microglia are the most relevant immune cells in the brain, playing a crucial role in brain development, in the process of pruning and maturation of synapses (Elmore et al., 2014). Removal of microglia in the early critical period of development, even removal of single receptors important in signaling or phagocytosis like CX3CR1 or P2Y12, impairs brain plasticity months later (Rogers et al., 2011, Sipe et al., 2016). However, microglia may become detrimental with age due to increased levels of SASP or senescence marker expression. Recently we performed a comprehensive analysis of all major senescence markers in murine brain microglia in vivo (Stojilkovic et al., 2019). The SASP was significantly increased as well as the senescence marker p16Ink4a, without changes in the p21/p53 senescence pathway (Stojilkovic et al., 2019). Several findings indicate that tissue macrophages are the most relevant cell population involved in low level inflammation or para-inflammation, and show a cytokine profile which is very similar to the SASP (Childs et al., 2016). These low levels of inflammation are then probably involved in pathogenesis of most age-related diseases, like neurodegeneration and atherosclerosis. This process has been coined as macroph-aging (Prattichizzo et al., 2016). We recently evaluated the expression levels of senescence- and inflammation-related genes that consistently and similarly change during aging in a tissue-specific or unspecific manner in several species, using whole genome RNA sequencing (Barth et al., 2019). The data showed that most relevant common genes being consistently increased with age in several tissues of most of the species evaluated were macrophage-associated genes (e.g. Marco, cd40). In the brain we found cd68 and cybb as the most relevant and higher expressed genes, both of them being microglial specific. Indeed, removal and repopulation of microglia led to normalisation of cd68 expression. Again, a high dose did not lead to further decrease in cd68 levels in the aged brain with reduced lipofuscin levels in microglial cells (O’Neil et al., 2018). Here again, the response to LPS and so-called primed state of the ‘new’ microglia was unchanged, pointing out to the important role of the microenvironment and further indicating the need for a continuous depletion or inhibition of microglial cells for beneficial effects on the long-term (Elmore et al., 2018).

Dysfunctional tissue macrophages like microglia cannot be easily removed. Indeed, they are known as Trojan horses in many infectious disease since infected macrophages, the largest phagocytes, are not removed and spread the infection to other cells and tissues (e.g. HIV, tuberculosis) (Herbein et al., 2002; Guirado et al., 2013). Chronically activated or senescent microglia, which reinforce inflammation in the brain, are very little or not replaced by cells from the periphery (Füger et al., 2017). As a long-lived cell type, they are exposed to several well-known stressors for a prolonged period of time. We could only speculate that microglia, the brain macrophages, may spread low level inflammation and senescence to neighboring cells. Here we showed that microglia depletion either with low or high doses of PLX5622 led to a significant decrease in p16^INK4A expression. Again, a high dose did not lead to further decrease in p16^INK4A mRNA expression, suggesting that astrocyte or microglia activation due to massive apoptotic cell death induced by high doses of PLX5622 blocked further beneficial effects.

Our study indicates that treatment with low and high doses of PLX5622 leads to a dose-dependent depletion of microglial cells with similar levels in young and aged mice. Furthermore, treatment with low and high PLX5622 differentially affected cytokine levels in young and old brains. A low dose of PLX5622 achieved a reduction in inflammation avoiding the astrocyte activation. Removal of microglia cells led to decreased...
expression of the senescence marker p16\textsuperscript{ink4a} in the aged brain, indicating a relevant contribution of these cells to the expression of this marker and their senescent status in the healthy aging brain. Limitations of the present study are the lack of protein data as well as the lack of behavioral data and neuronal markers.

Drugs used in the study are already available in several clinical trials investigating novel therapies for cancers or autoimmune diseases, and are found to be relatively safe (Cannanile et al., 2017). Other drugs like minocycline, which inhibit microglial function, are currently in clinical trials to treat diseases like depression, where microglia activation is presumed (Rosenblat and McIntyre, 2018). Therefore, microglial removal or inhibition might soon become clinically relevant as a side effect, or a therapeutic target of several drugs. Further research on the role of microglial cells in human diseases and age-related pathologies will allow the development of novel therapeutic strategies. Several of the above-mentioned studies used low doses of PLX5622 to deplete microglia for several weeks without serious side effects to the mice health. We may speculate that indispensable microglia functions in early development become dispensable in adulthood and even detrimental in the aged brain.

DISCLOSURE
The authors report no conflicts of interest.

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CRediT authorship contribution statement
Milan R. Stojiljkovic: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Visualization.
Christian Schmeer: Conceptualization, Methodology, Validation, Resources, Visualization, Supervision, Project administration. Otto W. Witte: Conceptualization, Methodology, Validation, Resources, Project administration.

REFERENCES
Angelova DM, Brown DR (2019) Microglia and the aging brain: are senescent microglia the key to neurodegeneration? J Neurochem 151:676–688.
Asai H, Ikezu S, Tsuonoda S, Medalla M, Luebke J, Haydar T, Wolozin B, Butovsky O, Kügel S, Ikezu T (2015) Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nat Neurosci 18:1584–1593.
Baker DJ, Childs BG, Dink M, Wijers ME, Sieben CJ, Zhong J, A. Saltness R, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD, van Deursen JM (2016) Naturally occurring p16\textsuperscript{ink4a}-positive cells shorten healthy lifespan. Nature 530:184–189.

Barth E, Srivastava A, Stojiljkovic M, Framh C, Aker H, Witte OW, Marz M (2019) Conserved aging-related signatures of senescence and inflammation in different tissues and species. Aging (Albany NY) 11:8556–8572.
Bennett RE, Bryant A, Hu M, Robbins AB, Hopp SC, Hyman BT (2018) Partial reduction of microglia does not affect tau pathology in aged mice. J Neuroinflammation 15:311–311.
Bhatia-Dey N, Kanherkar RR, Stair SE, Makarev E, Csoka AB (2016) Cellular Senescence as the Causal Nexus of Aging. Frontiers in Genetics 7.
Brooks-Wilson AR (2013) Genetics of healthy aging and longevity. Hum Genet 132:1233–1338.
Bruttiger J, Karram K, Wörtge S, Regen T, Marini F, Hoppmann N, Klein M, Blank T, Yona S, Wolf Y, Mack M, Finteaux E, Müller W, Zipp F, Binder H, Bopp T, Prinz M, Jung S, Waisman A (2015) Genetic cell ablation reveals clusters of local self-renewing microglia in the mammalian central nervous system. Immunity 43:92–106.
Campisi J (2013) Aging, cellular senescence, and cancer. Annu Rev Physiol 75:685–705.
Cannanile MA, Weisser M, Jacob W, Jegg A-M, Ries CH, Rüttinger D (2017) Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. J Immunother Cancer 5:53.
Childs BG, Baker DJ, Wijshake T, Conover CA, Campisi J, van Deursen JM (2016) Senescent fetal neural cells are deleterious at all stages of atherosclerosis. Science 354:472–477.
Dagher NN, Najafi A, Kayala KMN, Elmore MRP, White TE, Medeiros R, West BL, Green KN (2015) Colony-stimulating factor 1 receptor inhibition prevents microglial plaque association and improves cognition in 3xTg-AD mice. J Neuroinflammation 12:139.
De la Fuente M, Miquel J (2009). An update of the oxidation-inflammation theory of aging: the involvement of the immune system in oxi-inflamm-aging. Curr Pharmaceut Des 15:3003-3026.
Elmore MRP, Hoshfield LA, Kramár EA, Soreq L, Lee RJ, Pham ST, Najafi AR, Spangenberg EE, Wood MA, West BL, Green KN (2018) Replacement of microglia in the aged brain reverses cognitive, synaptic, and neuronal deficits in mice. Aging Cell 17: e12832.
Elmore MP, Najafi A, Koike M, Dagher N, Spangenberg E, Rice R, Kitazawa M, Matsuzawa B, Nguyen H, West B, Green K (2014) Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron 82:380–397.
Franceschi C, Bonafé M, Valensin S, Olivier F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann New York Acad Sci 908:244–254.
Fu H, Zhao Y, Hu D, Wang S, Yu T, Zhang L (2020) Depletion of microglia exacerbates injury and impairs function recovery after spinal cord injury in mice. Cell Death Dis 11:528.
Füger P, Hefendehl JK, Veeraraghavalu K, Wendeln AC, Schlosser C, Obermüller U, Wegenast-Braun BM, Neher JJ (2017) Microglia turnover with aging and in an Alzheimer’s model via long-term in vivo single-cell imaging. 20:1371-1376.
Guirado E, Schlesinger LS, Kaplan G (2013) Macrophages in tuberculosis: friend or foe. Semin Immunopathol 35:563–583.
Han J, Harris RA, Zhang X-M (2017) An updated assessment of microglia depletion: current concepts and future directions. Mol Brain 10:25–25.
Hefendehl JK, Neher JJ, Sühs RB, Kohsaka S, Skodras A, Jucker M (2014) Homeostatic and injury-induced microglia behavior in the aging brain. Aging Cell 13:60–69.
Herbein G, Coaquette A, Perez-Bercoff D, Pancino G (2002) Colony-stimulating factor 1 receptor inhibition prevents microglial plaque association and improves cognition in 3xTg-AD mice. J Neuroinflammation 12:139.
De la Fuente M, Miquel J (2009). An update of the oxidation-inflammation theory of aging: the involvement of the immune system in oxi-inflamm-aging. Curr Pharmaceut Des 15:3003-3026.

DISCLOSURE
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Milan R. Stojiljkovic: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Visualization.
Christian Schmeer: Conceptualization, Methodology, Validation, Resources, Visualization, Supervision, Project administration. Otto W. Witte: Conceptualization, Methodology, Validation, Resources, Project administration.

REFERENCES
Angelova DM, Brown DR (2019) Microglia and the aging brain: are senescent microglia the key to neurodegeneration? J Neurochem 151:676–688.
Asai H, Ikezu S, Tsuonoda S, Medalla M, Luebke J, Haydar T, Wolozin B, Butovsky O, Kügel S, Ikezu T (2015) Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nat Neurosci 18:1584–1593.
Baker DJ, Childs BG, Dink M, Wijers ME, Sieben CJ, Zhong J, A. Saltness R, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD, van Deursen JM (2016) Naturally occurring p16\textsuperscript{ink4a}-positive cells shorten healthy lifespan. Nature 530:184–189.
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. Cell 153:1194–1217.

Luo XG, Ding JQ, Chen SD (2010) Microglia in the aging brain: relevance to neurodegeneration. Mol Neurodegener 5:12.

Mora F (2013) Successful brain aging: plasticity, environmental enrichment, and lifestyle. Dial Clin Neurosci 15:45–52.

Norden DM, Godbout JP (2013) Review: microglia of the aged brain: primed to be activated and resistant to regulation. Neuropathol Appl Neurobiol 39:19–34.

O’Neil SM, Witcher KG, McKim DB, Godbout JP (2018) Forced turnover of aged microglia induces an intermediate phenotype but does not rebalance CNS environmental cues driving priming to immune challenge. Acta Neuropathol Commun 6:129–129.

Prattichizzo F, Bonafé M, Olivier F, Franceschi C (2016) Senescence associated macrophages and “macroph-aging”: are they pieces of the same puzzle? Aging (Albany NY) 8:3159–3160.

Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, Grimmig BA, Weeber EJ, Bickford PC, Gemma C (2011) CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. J Neurosci 31:16241–16250.

Rosenblat JD, McIntyre RS (2018) Efficacy and tolerability of minocycline for depression: A systematic review and meta-analysis of clinical trials. J Affect Disord 227:219–225.

Sariol A, Mackin S, Allred M-G, Ma C, Zhou Y, Zhang Q, Zou X, Abrahante JE, Meyerholz DK, Perlman S (2020) Microglia depletion exacerbates demyelination and impairs remyelination in a neurotropic coronavirus infection. Proc Natl Acad Sci 117:24464–24474.

Spangenberg EE, Lee RJ, Najafi AR, Rice RA, Elmore MRP, Burton-Jones M, West BL, Green KN (2016) Eliminating microglia in Alzheimer’s mice prevents neuronal loss without modulating amyloid-β pathology. Brain 139:1265–1281.

Stojilkovic MR, An Q, Bondeva T, Heller R, Schmeer C, Witte OW (2019) Phenotypic and functional differences between senescent and aged murine microglia. Neurobiol Aging 74:56–69.

Szalay G, Martinecz B, Lénárt N, Kőrnyei Z, Orsolits B, Judák L, Császári E, Fekete R, West BL, Katona G, Rózsa B, Dénes A (2016) Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. Nat Commun 7:11499.

APPENDIX A. SUPPLEMENTARY DATA
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