# *Impact of aging and senescence on phenotype and function of murine brain microglia in vitro and in vivo*

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## *List of abbreviations:*

| | |
|---|---|
| *DNA* | *Deoxyribonucleic acid* |
| *SIPS* | *Stress induced premature senescence* |
| *OIS* | *Oncogene-induced senescence* |
| *PAMPs* | *Pathogen-associated molecular pattern* |
| *DAMPs* | *Danger-associated molecular pattern* |
| *PRRs* | *pattern recognition receptors* |
| *SA-β-gal* | *Senescence-associated beta-galactosidase* |
| *SAHF* | *Senescence-associated heterochromatin foci* |
| *DNA-SCARS* | DNA segments with chromatin alterations reinforcing senescence |
| *SASP* | *Senescence-Associated Secretory Phenotype* |
| *IL* | *Interleukin* |
| *MCP-2* | *Monocyte Chemoattractant Protein 2* |
| *MIP1* | *Macrophage inflammatory protein* |
| *FGF* | *Fibroblast growth factor* |
| *EGF* | *Epidermal growth factor* |
| *VEGF* | *Vascular endothelial growth factor* |
| *LPS* | *Lipopolysaccharide* |
| *CNS* | *Central nervous system* |
| *TRAP* | *Telomerase Repeated Amplification Protocol* |
| *ATP* | *Adenosine triphosphate* |
| *BDNF* | *Brain-derived neurotrophic factor* |
| *ROS* | *Reactive oxygen species* |
| *TNF-α* | *Tumor necrosis factor alpha* |
| *iNOS* | *Nitric oxide synthase* |
| *PFKFB3* | *6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatases* |
| *TGF-β* | *Transforming growth factor beta* |
| *Arg1* | *Arginase 1* |
| *AD* | *Alzheimer disease* |
| *mTOR* | *mammalian target of rapamycin* |
| *AMPK* | *AMP-activated protein kinase* |
| *GMCSF* | *Granulocyte-macrophage colony-stimulating factor* |
| $H_2O_2$ | *Hydrogen peroxide* |
| *CCP* | *cytoplasmic chromatin fragments* |
| *HIV* | *Human immunodeficiency virus* |
| *CSFR1* | *Colony stimulating factor 1 receptor* |
| *PLX3397, PLX5622* | *CSF1 receptor inhibitors* |
| $CX_3CR_1$ | *chemokine receptor 1* |
| *β-amyloid* | *Amyloid beta* |

**Summary**

Aging is characterized by a progressive loss of cell, tissue and organ functions and is a main risk factor for many diseases. Recently it has been shown that aging is accompanied and possibly induced by accumulation of senescent cells. Senescent cells are cells which lost their proliferation potential but are hyperfunctional and secrete many potentially detrimental cytokines and growth factors. Senescence of macrophages, so called macroph-aging, seems to be especially involved in several age-related diseases like Alzheimer or atherosclerosis. Microglia cells as main immune cells and brain macrophages play an important role in brain development, homeostasis and defense against pathogens. The role of microglia in brain aging as well as the factors regulating their response to pathogens are not well understood. Whether microglial cells develop a senescent phenotype has also not been fully addressed.
The aims of this study were: 1) to develop an *in vitro* model to better characterize the senescent phenotype of murine brain microglia; 2) to determine the impact of aging and senescence on phenotype and function of microglia; 3) to evaluate the impact of pathogen-dose, maturation state, aging and senescence on microglial response *in vitro*.

We found that microglia develop a senescent phenotype after long-term culture characterized by telomere shortening, increased expression of $p16^{Ink4a}$, p21/p53, and altered response to the prototypical pathogen-associated molecular pattern (PAMP) LPS. Aged microglia *in vivo* showed increased p16 expression, enhanced activation and dysfunctional profiles, however did not show typical senescence-associated changes like DNA damage or p21/p53 pathway activation.

In order to further characterize the impact of aging on the immune response from murine microglia, we tested the effect of increasing doses of typical PAMPs (1fg-100 ng of LPS and 1fg-1µg for β-glucan) on naive, adult and aged microglia. Naive microglia showed a dose-dependent stimulation with adaptive responses, while adult or aged microglia did not exhibit a similar pattern. A stronger response after the repeated stimulation, so called sensitization, was only found in neonatal or naive microglia cells. Reduced responses after a second stimulation, so called tolerance occurred in all groups.

Our study shows that murine microglia respond to pathogen stimulation in a dose-dependent and unspecific manner, possibly involving a hormetic mechanism. These findings are highly relevant to the medical community since provoking trained responses might promote resistance against pathogens, which may be useful in cancer therapy or vaccine development. On the other hand,

tolerance induction might induce an anti-inflammatory response, and might influence the progression of autoimmune or hyperinflammatory diseases.

In conclusion, we found that aging of microglia was followed by cellular dysfunction but not by typical senescent changes as shown in other studies. Response of microglia to pathogen molecules was found to be dose-dependent, maturation and age-dependent but not pathogen specific.


***Zusammenfassung***

Das Altern geht mit einem fortschreitenden Funktionsverlust von Zellen, Gewebe und Organen einher und ist ein Hauptrisikofaktor für verschiedenste Krankheiten. Kürzlich konnte gezeigt werden, dass das Altern von der Akkumulation seneszierender Zellen begleitet und möglicherweise sogar induziert wird. Seneszierende Zellen definieren sich über den Verlust ihres Proliferationspotentials, sind allerdings überfunktionell und schütten vermehrt potenziell schädliche Zytokine und Wachstumsfaktoren aus. Die Seneszenz von Makrophagen, die als 'macrophaging' bezeichnet wird, scheint mit mehreren altersbedingten Erkrankungen wie Alzheimer oder Arteriosklerose assoziiert zu sein. Mikrogliazellen als wichtige Immunzellen und Hirnmakrophagen spielen eine wichtige Rolle bei der Entwicklung und Alterung des Gehirns sowie der Abwehr von Krankheitserregern. Die genaue Rolle von Mikrogliazellen im gealterten Gehirn ist allerdings aktuell noch immer unzureichend verstanden. Ebenfalls existieren bisher keine Untersuchungen darüber, ob Mikrogliazellen einen seneszenten Phänotyp entwickeln.

Ziel der Studie war die Entwicklung eines *in-vitro*-Modells zur Untersuchung des Seneszenzphänotyps muriner Mikroglia. Darüber hinaus sollte der Einfluss des Alterns auf phänotypische und funktionelle Veränderungen der Mikroglia von Mäusen *in vivo* bestimmt werden. Zuletzt wurden die Auswirkungen von Erregerdosis, Reifung, Alterung und Seneszenz auf die Reaktion der Mikrogliazellen untersucht.

Es stellte sich heraus, dass die Zellen der Mikroglia nach einer Langzeit-Kultivierung einen Seneszenzphänotyp entwickelten. Dieser war charakterisiert durch eine Verkürzung der Telomere, eine gesteigerte Expression von $p16^{Ink4a}$ und p21/p53 sowie einer veränderten Antwort auf Lipopolysaccharide (LPS), einem Pathogen-asoziierten-molekularen-Muster (PAMP). Gealterte Mikroglia zeigte *in vivo* erhöhte p16, Aktivierungs- und Dysfunktionsprofile, jedoch keine typischen seneszenten Veränderungen wie DNA-Schäden oder eine Aktivierung des p21/p53 Signalweges.

Um den Einfluss des Alterns auf die Immunantwort muriner Mikroglia zu charakterisieren, testeten wir Effekt ansteigender Dosen typischer PAMPs (1fg-100ng LPS und 1fg-1µg β-Glucan), auf naive, adulte und gealterte Mikroglia. Die naive Mikroglia zeigte eine dosisabhängige Stimulation mit adaptiven Reaktionen. Ähnliche Auswirkungen konnten in adulten und gealterten Zellen nicht nachgewiesen werden. Eine stärkere Reaktion nach wiederholter Stimulation, Sensibilisierung gennant, zeigte sich nur bei naiven neonatalen Mikrogliazellen. Reduzierte Reaktionen nach der zweiten Stimulation, auch als Toleranz bezeichnet, traten jedoch in allen Gruppen auf.

Unsere Studie zeigt, dass murine Mikroglia in Abhängigkeit der Dosis unspezifisch auf die Erregerstimulation reagiert und möglicherweise einen hormetischen Mechanismus beinhaltet. Diese Erkenntnisse können für die medizinische Fachwelt von hoher Relevanz sein, da das Auslösen trainierter Reaktionen die Resistenz gegen Krankheitserreger fördern kann. Dies könnte bei der Entwicklung von Impfstoffen oder Krebstherapien hilfreich sein. Auf der anderen Seite könnte eine Toleranzinduktion eine antientzündliche Reaktion auslösen und auf diese Weise das Fortschreiten von autoimmunen oder hyperinflammatorischen Erkrankungen beeinflussen.

Zusammenfassend konnten wir feststellen, dass die Alterung der Mikroglia mit einer zellulären Dysfunktion einhergeht, jedoch typische senenescente Veränderungen nicht auftraten. Die Reaktion von Mikroglia zeigte sich sowohl dosis- als auch reifungs- und altersabhängig, war jedoch nicht erregerspezifisch.

## *Introduction*

### *Aging and senescence*

Aging is currently defined as a multifaceted process with progressive loss of physiological functions over time, culminating in death of the individual (Tosato, Zamboni et al. 2007). Aging is the main risk factor for most of the chronic diseases accounting for the increasing morbidity, mortality, and health in both developed and developing countries (Kirkland and Tchkonia 2017). Increasing aging population represents an economical challenge for health care systems all over the world. Aging research investigates mechanisms of aging in order to develop strategies aimed to prevent age-related disorders and eventually death. There are currently several theories on the causes and mechanisms of aging discussed in the literature: 1) the oxidative theory of aging postulates that accumulation of oxidative radicals leads to macromolecular (DNA, proteins) damage in the cells and to cellular dysfunction (Harman 1956); 2) the Immune theory of aging focuses on decline in immune system function leading to increased vulnerability to infectious diseases with age (Walford 1964); 3) inflammaging and oxi-inflammaging theories postulate that aging is caused by increased accumulation of low level inflammation due to oxidative damage to the cells, eventually leading to tissue aging and disease (De la Fuente and Miquel 2009, Franceschi and Campisi 2014); 4) the programmed theory of aging indicates that aging results as a consequence of programmed gene on/off switching with age or due to an existing biological clock that controls the pace of aging through hormones (Longo, Mitteldorf et al. 2005); 5) the senescent theory of aging, one of the most recent aging theories, tries to unify all of them. According to this theory, there is a significant accumulation of senescent cells with age that induces loss of regeneration potential, inflammation and organ dysfunction (López-Otín, 2013, Bhatia-Dey, 2016).

Cellular senescence is defined as a stress response whereby cells lose their proliferation capacity in an irreversible manner (Campisi 2013). The concept was introduced more than 50 years ago for human diploid cells grown *in vitro* (Hayflick 1961, Hayflick and Moorhead 1961) and it has been mainly thought to represent an evolutionary mechanism of tumor suppression. Senescent cells are normally removed by immune cells in a process called immunosurveillance. It is thought that with age it comes to reduced removal of senescent cells, and accumulation of these cells may induce cellular dysfunction in neighboring cells and eventually whole organ or system dysfunction (Ovadya, Landsberger et al. 2018). Although senescence represents a crucial mechanism to prevent malignant transformation of damaged cells, accumulated senescent cells are able to support tumor cell growth and metastasis (Schosserer, Grillari et al. 2017). This fits

well to the antagonistic pleiotropy theory of aging, postulating that one mechanism that is beneficial in young individuals becomes detrimental in aged ones (Giaimo and d'Adda di Fagagna 2012). Cellular senescence is currently considered a hallmark of aging, and has been proposed as a major factor responsible for previously discussed aging-associated chronic and systemic low-level inflammation ("inflammaging") (Franceschi and Campisi 2014). Tissue macrophages seem to be the major players in this constant low level inflammation during aging, a process termed macroph-aging (Prattichizzo, Bonafè et al. 2016).

The current concept of cellular senescence has been extended to include four major types of senescence: replicative senescence characterized by telomere shortening, oncogene-induced senescence, mitochondrial-damage induced senescence, and DNA-damage induced senescence (Lowe, Horvath et al. 2016). More recently, the term premature cellular senescence (Stress-induced premature senescence or SIPS) has also been included (Sapieha and Mallette 2018, Schmeer et al. 2019).

Cell culture stress is a strong inductor of premature senescence without relevant telomere shortening, caused by contact with plastic and culture serum, and hyperphysiological oxygen levels (Campisi 2013). According to the inducing stressor, senescence can be divided into seven types: 1) DNA damage-induced senescence, caused by irreparable DNA damage often on the telomeres; 2) Oncogene-induced senescence (OIS), caused by oncogene activation; 3) Oxidative stress-induced senescence, caused by macromolecule damage as a result of excessive free radicals; 4) Chemotherapy-induced senescence, caused by inhibition of cyclin-dependent kinases; 5) Mitochondrial dysfunction-associated senescence, 6) Epigenetically induced senescence, caused by inhibitors of DNA methylases or histone deacetylases and 7) Paracrine senescence, induced by the senescence associated phenotype (SASP) produced by primary senescent cells (Hernandez-Segura, Nehme et al. 2018). Recently, clinically more relevant inducers of senescence including hyperglycemia, hypertension and high fat diet were discussed (van Deursen 2014). Interestingly senescent cells are characterized by a gain of function - senescent cells are hyperfunctional and characterized by hyperproduction of cytokines and other molecules. Aging, on the other hand, is characterized by loss of function. Hyperactive senescent cells may induce dysfunction or aging of healthy neighboring cells by promoting an inflammatory environment, Therefore, aging tissues may contain both senescent and aged cells (Schmeer et al., 2019).

Senescent cells share several common characteristics in addition to irreversible cell cycle arrest, e.g. (1) a flat, enlarged, often multinucleated morphology, (2) induction of senescence-associated β-galactosidase (SA-β-gal) activity, (3) appearance of senescence-associated heterochromatic foci (SAHF) and DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), (4) activation of tumor suppressor network, such as p16INK4a and p19ARF, and (5) secretion of pro-inflammatory mediators so called senescence associated secretory phenotype (SASP). Secreted factors included interleukins and chemokines, e.g. IL-1α/β, IL-6, growth factors, such as EGF and VEGF, and several matrix metalloproteinases (Rodier and Campisi 2011, Salminen, Kauppinen et al. 2012). Several of these markers have to be used in combination in order to investigate cellular senescence since none of them are fully specific.

*Microglia cells in the aging brain*

Microglia cells, the main brain macrophages, are involved in brain protection against infection but also in complex-processes like brain plasticity (Casano and Peri 2015). Microglia are long lived cells, originating from yolk sack primitive macrophages. They have a very low turnover and therefore are exposed for an extended time to the damaging agents like oxidative stress, cytokines but also antigen stimulation with pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Ginhoux and Prinz 2015). Furthermore, microglia prune synapses, a physiological process of removing unnecessary synapses/dendrites in development, however, possibly detrimental in aging (Paolicelli, Bolasco et al. 2011, Rajendran and Paolicelli 2018). Two recent studies reported a very long microglia lifespan between 15 and 41 months in the mouse brain, which seem to be brain region specific (Füger, Hefendehl et al. 2017, Tay, Mai et al. 2017). In the aging brain there is an excessive synapse loss and reduced brain plasticity which has been associated with well-known neurodegenerative disorders (von Bernhardi, Eugenín-von Bernhardi et al. 2015). Following activation, microglia exhibit changes characterized by alterations in their morphology, decreased branching, synthesis of certain cell surface and intracellular molecules, and increased proliferation (Perry and Teeling 2013, Morrison, Young et al. 2017). Simplistically, there are currently two states of microglia described: M1, characterized by production of pro-inflammatory factors and M2 associated with anti-inflammatory cytokine release. While data are still limited, some reports indicate that aging shifts the sensitivity of activated microglia towards a diminished anti-inflammatory and M2-promoting phenotype (Norden and Godbout 2013). Activated microglia cells in the aged brain produce cytokines which, in excessive amounts, may damage the sensitive brain tissue. Changes in long-lived microglia cells with brain aging could be therefore involved in decreased brain plasticity and

cognitive decline. The mechanisms by which microglia aging leads to decreased brain plasticity are not completely understood. Recently, it was found that microglia phagocytize synapses by a complement-based mechanism. Complement factors seem to accumulate at the synapses with age, leading to an increased microglia-based synapse removal (Stevens, Allen et al. 2007, Shi, Chowdhury et al. 2017).

Previous research has described microglia in aged brain as dystrophic or dysfunctional, with increased basal levels of activation. Dystrophic microglia are characterized by abnormalities in their cytoplasmic structure, such as deramified, atrophic, fragmented or unusually tortuous processes, frequently bearing spheroidal or bulbous swellings and membrane blebbing (Streit 2006).

Dystrophic or senescent microglia exhibiting reduced phagocytosis could be responsible for diminished removal of cell debris. On the other hand, the SASP could induce the dysfunction of neighboring neuronal cells. Neurons in the mouse brain are known to show certain senescence typical changes with age with increased DNA damage and p21 expression, possibly due to dysfunctional autophagy (Jurk et al. 2012, Moreno-Blas et al. 2019). It is feasible that neuronal senescence partially occurs as a bystander effect due to microglial senescence. Appearance of dystrophic microglia have been shown to precede neuron loss, synapse loss and tau pathology in Alzheimer disease as well as in Down`s Syndrome dementia (Streit et al., 2009, Xue and Streit 2011).

Microglia may thus be the primary drivers of impaired brain plasticity in the aged brain. Interestingly, there is very little known about changes in microglia with age. It is unclear whether microglia show signs of senescence under pathological conditions and due to aging, and one reason for this is the lack of a proper model to investigate aging of these cells. Furthermore, the role of microglia in the aging brain is still not well understood.

*Innate immune memory of microglia. Impact of pathogen dose*

As the resident macrophages of the CNS, microglia are the main cell type responsible for the innate immunity in the brain (Hanisch and Kettenmann 2007, Ransohoff and El Khoury 2015). Microglia are highly plastic and responsive after lesion or disease (Kettenmann, Kirchhoff et al. 2013). As mentioned before, activated microglia display characteristic morphological and functional changes. Similarly to other, innate immune cells such as dendritic cells or macrophages, microglia also express pattern recognition receptors (PRRs) which recognize

conserved molecular structures known as PAMPs (Janeway and Medzhitov 2002, Esen and Kielian, 2006; J. Immunol).

Until recently it was assumed that only the adaptive immune system possesses the ability to mount a memory response and, therefore improve the immunological reaction to a second infection. However, increasing evidence suggests that also the innate immune system shows adaptive memory like characteristics (Netea, Quintin et al. 2011). Several studies demonstrate that the innate immune system can also adapt after a previous challenge through metabolic changes and epigenetic reprogramming, a process that has been termed trained immunity or innate immune memory (Netea, Quintin et al. 2011, Netea, Joosten et al. 2016). This rather unspecific innate immune memory, characterized by increased or decreased production of proinflammatory mediators, was shown in mice strains with known deficiencies in adaptive immune response (Quintin, Cheng et al. 2014).

Classically, macrophages and macrophage-like cell lines have been used as a model for the assessment of innate immune reactions. Two archetypal PAMPs commonly used as stimulating agents are lipopolysaccharides (LPS), endotoxins found on the cell membranes of gram-negative bacteria and β-glucans, polysaccharides occurring in the cell walls of bacteria and fungi. Nonspecific enhanced (training) or diminished (tolerance) cytokine production in monocytes and macrophages upon secondary stimulation with different PAMPs, including LPS, was thought to be ligand specific (Ifrim, Quintin et al. 2014). Recently, short exposure to LPS was shown to induce tolerant macrophages while priming with β-glucans from the fungus *Candida albicans* induced a trained state (Cheng, Quintin et al. 2014, Saeed, Quintin et al. 2014). One study has shown that microglia stimulation *in vitro* only induces a tolerant state in these cells (Schaafsma et al. 2015). More recently it was shown that LPS stimulation *in vivo* may achieve both immune training and tolerance in microglia cells. The study also showed that peripheral LPS stimulation and microglia innate memory may influence brain pathology in diseases like stroke or Alzheimer's (Wendeln et al. 2018).

Moreover, the concept of hormesis has been recently revived due to fundamental discoveries in pharmacology and toxicology after applying very low doses of several substances (Calabrese and Mattson 2011, Schmidt, Schneble et al. 2014, Weis, Rubio et al. 2017). Whether hormetic mechanisms are involved in the adaptive responses to pathogens observed in cells from the innate immune system has not been fully addressed. A new study suggests that induction of adaptive responses in microglia might involve a hormetic mechanism (Lajqi et al. 2019). However,

the factors involved in the induction of innate immune responses in microglia are not yet fully investigated. In particular, the impact of maturation and/or age-related differences in dose-dependent responses of microglia still has to be elucidated.

Here, we hypothesized that microglia are able to respond in a ligand-specific and also dose-dependent as well as maturation dependent manner. Furthermore, we propose the existence of a hormetic mechanisms underlying the immune response to pathogens and stressors.

There is increasing evidence for an age-associated immunosenescence of the innate immune system (Gomez, Nomellini et al. 2008). Cells of the aging innate immune system are characterized by altered expression and/or function of innate immunity receptors and signal transduction, leading to defective activation and decreased chemotaxis, phagocytosis and intracellular removal of pathogens. In particular, *in vivo* studies show that the ability of aging macrophages and microglia to acquire a regulatory phenotype may be compromised (Rawji, Mishra et al. 2016). In particular, macrophages are less able to produce a functional pro-inflammatory response (Shaw, Goldstein et al. 2013). On the other hand, microglia exhibit an inflated pro-inflammatory response, a phenomenon referred to as microglia priming, which renders them more susceptible to a secondary stimulation (Perry and Teeling 2013, Rawji, Mishra et al. 2016).

In aged individuals, the secondary stimulus mostly arises from a systemic disease with an inflammatory component and might contribute to the progression of chronic neurodegenerative diseases (Perry and Holmes 2014). In addition, both senescent microglia and microglia from the aged mouse brain show an altered immune response after stimulation with LPS (Stojiljkovic, Ain et al. 2019).

A better characterization of the impact of trained immunity on the persistence of inflammation induced by PAMPs may help to explain why microglia can be either neuroprotective or neurotoxic, resulting in containment or disease progression (Hanisch and Kettenmann 2007) and would also provide new possibilities for intervention in aging and autoinflammatory disorders.

***Aims of the study***

The present study aimed to:

1) Determine the impact of aging and senescence on phenotypic and functional properties of brain microglia both *in vitro* and *in vivo*. For our study we developed an *in vitro* system to characterize microglia from newborn, adult, aged and senescent microglia by means of well recognized senescence markers including p16 $^{Ink4a}$, p21/p53, telomere length and telomerase activity, and inflammation as well as functional markers.

2) To evaluate the impact of maturation, aging, senescence and pathogen-dose on the innate immune response of microglia. Using our cell culture system, we analyzed the dose-dependent response of newborn, adult, aged and senescent microglia to known pathogen-associated molecular patterns (PAMPs) including LPS and β-glucan, and their capacity to develop an adaptive immune response in form of tolerance or sensitization.

The following hypotheses were tested:

1. Microglia become senescent *in vitro* after long-term culture due to telomere shortening and cell culture-associated stress
2. Microglia in the aging brain *in vivo* show a dysfunctional phenotype that differs from the typical senescence pattern *in vitro*
3. Microglia show a pathogen dose-dependent innate immune memory response after stimulation with different stressors
4. Maturation, aging and senescence differentially alter the innate immune response profile of microglia after repeated pathogen-induced stimulation

## Summary of the manuscripts

### Manuscript 1: Phenotypic and functional differences between senescent and aged murine microglia

Microglia cells have been reported to be involved in brain aging and neurodegenerative disorders; however, few studies have analyzed microglia senescence and the impact of aging on the properties of these cells. Here, we developed an *in vitro* senescence model and characterized the senescence phenotype of microglia by means of well-accepted markers, including telomere length, telomerase activity, expression of $p16^{INK4a}$, p21, p53, senescence-associated β-galactosidase and senescence associated secretory phenotype. Quantitative real-time polymerase chain reaction analysis and a Telomeric Repeat Amplification Protocol (TRAP) assay were used to measure telomere length and telomerase activity in senescent microglia. We found that microglia cells decrease their rate of proliferation, show telomere shortening and typical induction of the SASP after approximately 8-10 weeks *in vitro*. Interestingly, these senescent cells also increase migration rates without changes in the phagocytosis function. Senescence associated markers $p16^{Ink4a}$ as well as p21/p53 pathway were strongly activated in these cells. Additionally, we established methods in our lab to isolate microglia from adult and aged mice and to culture these cells for functional analyses. Microglia from aged mice showed $p16^{Ink4a}$ pathway activation without p21/p53. They also showed reduced phagocytosis and activation of SASP, however, at a much lower level. Furthermore, telomeres remained unaltered in aged microglia and there was no relevant sign of DNA damage. In contrast to senescent microglia, microglia from the aged brain did not show a decreased proliferation rate *in vivo* or *in vitro*.

Senescent and aged microglia also exhibited differential activation profiles and altered responses to LPS or ATP stimulation.

In this first part of the study, we have shown that microglial senescence *in vitro* and aging *in vivo* are not identical processes, since aged microglia do not show typical senescent-associated changes.

**Manuscript 2**: Impact of maturation and pathogen-dose on immune adaptive responses in murine brain microglia

The concept of immune innate memory in macrophages and monocytes has recently been established, also in the mature brain (Netea et al. 2016). However, innate immune memory in microglia cells and the putative factors determining its emergence have not been fully investigated yet. In particular, the role of maturation and aging for emergence of immunological memory within microglial cells, which constitute the first line of innate host defense response inside the CNS, is still unknown.

One reason for this is the lack of a suitable model to investigate microglial immune memory *in vitro*. Here, we implemented the so called 'two hit' *in vitro* model already developed for macrophages (Saeed, Quintin et al. 2014). We evaluated the effect of repeated challenge with varying doses of typical pathogen-associated molecular patterns (PAMPs) including LPS (1fg-100ng) and β-glucan (1fg-1µg) on the emergence of innate adaptive memory like responses in microglia *in vitro*. Additionally, we evaluated the impact of the maturation state of microglia on induction of innate immune memory in these cells.

We found that repeated administration of LPS to cultivated primary microglia induced pathogen-dose- and developmental-dependent changes in gene expression and protein levels of pro- and anti-inflammatory mediators, BDNF, ROS, and led to a metabolic rewiring in both young neonatal as well as in adult microglia cells.

A stronger response after the second stimulation (second hit), so called sensitization, was only observed in neonatal or naive microglia cells. Priming with ultra-low (1fg/ml) LPS significantly increased levels of pro-inflammatory mediators TNF$\alpha$, IL-6 and iNOS, as well as BDNF and PFKFB3, whereas ROS levels were decreased in naive microglia derived from newborn mice, indicating induction of trained immunity and metabolic rewiring. In contrast, mature microglia derived from adult mice failed to show such a sensitization response.

Reduced responses after second stimulation, so called tolerance developed in both groups. Stimulation with high doses of LPS led to a several fold down-regulation of pro-inflammatory cytokines and iNOS in both newborn and mature microglia, indicating induced immune tolerance. Furthermore, there were specific differences in the induction of anti-inflammatory mediators and M2 microglia state including IL-10, IL-4, TGF$\beta$ and Arg-1 in naive and adult microglia after a high

dose LPS. This indicated induction of tolerance associated with a M2-anti-inflammatory phenotype only in naive microglia.

In summary, naive microglia showed a fine-tuned immune memory as compared to mature microglia, as indicated by LPS and β-glucan-dose-dependent changes in pro-and anti-inflammatory response, resulting in trained immunity after priming with ultra-low LPS doses but tolerance after priming with high doses. Naive microglia cells are apparently prone to activate their proinflammatory response even after mild systemic inflammatory challenge, albeit exhibit robust anti-inflammatory capability in case of reinforced systemic inflammation. Maturation of microglia led to a lack of response to ultra-low LPS stimulation but to development of immune tolerance at high doses. We show here that naive microglia primed with ultra-low LPS doses acquire a trained character and immune memory possibly associated with a long-term dysfunctional phenotype in certain neurodevelopmental diseases, and a strong stimulus leads to an immunosuppressed phenotype, possibly in order to prevent excessive damage to the sensitive brain tissue, in the case of recurrent local inflammation.

**Manuscript 3 (Review): Pathogen-Induced Hormetic Responses**

Infectious diseases due to microorganisms are still one of the leading causes of death all over the world (Dye 2014). The constant contact with pathogenic microorganisms or saprophytes shapes the immune system and enables a broad range of different responses. The microbiome, consisting of trillions of bacteria, and mycobiome have been recently found to be involved in maturation not only of peripheral gut immune cells but also microglia (Erny, Hrabě de Angelis et al. 2015). Changes in the microbiome have been connected to several age-related diseases including neurodegeneration (Vogt, Kerby et al. 2017). On the other hand, pathogenic bacteria induce inflammatory responses by stimulation of innate immune cells. They are recognized by so called PAMPs using PRRs and induce a cascade of signaling pathways leading to appropriate responses and microorganism removal (Janeway and Medzhitov 2002). Innate immune cells are the first line of defense against invading pathogens and they support adaptive immune cells, which later mediate specific responses to pathogens and develop an immune memory. This interplay between our immunity and microorganisms attracts scientific attention due to the prime importance of infectious diseases in human medicine.

In this review, we discuss four major aspects embedding pathogen attacks to men into the ecological reaction pattern of higher organisms:

(1) Pathogenic microorganisms typically provoke hormetic responses of the affected organism, i.e., stimulatory effects at 'low doses' and tolerance or cell damage at high pathogen load.

(2) The stimulatory effects of pathogens comprise increased resistance and tolerance responses of the innate immune system.

(3) Analysis of signaling processes induced by pathogens in the affected host tissue discloses novel options for the treatment of infectious diseases.

(4) Responses are dose-dependent and not pathogen-specific.

In this review we also discussed some ideas about their medical relevance, and main recent discoveries regarding the innate immune memory.

One study has shown that stimulation with *Candida albicans* led to increased survival in mice after a subsequent lethal infection with this pathogen (Quintin, Saeed et al. 2012). β-glucan alone could induce a sensitization effect and this has been thought to be responsible for the so called 'trained immunity' (Di Luzio and Williams 1978, Quintin, Saeed et al. 2012). On the other hand, stimulation with LPS induces so called tolerance or paralysis of the immune response, a process well known for long time (Ziegler-Heitbrock 1995). It was thought that β-glucan induces training while LPS can induce only tolerance, which we have shown not to be accurate (Lajqi et al., 2019;

Lajqi, Stojiljkovic et al. in preparation). Whether pathogens will induce training or tolerance depends on the dose, time of exposure, and nature of the stimulus. Findings about the hormetic character of responses to microorganisms are highly relevant for medicine. In particular, inducing trained responses might promote resistance against pathogens, which may be useful in cancer therapy, vaccine development or infection treatment, especially in elderly and frail population. Tolerance induction might provoke an anti-inflammatory response, possibly useful in autoimmune diseases or in hyperinflammatory phase in sepsis.

Neurodegenerative diseases, like Alzheimer's disease (AD) have been also connected to hyperactive innate immune system (Sarlus and Heneka 2017, Wendeln, Degenhardt et al. 2018). We have shown that both aged and senescent microglia are hyperactive (Stojiljkovic, Ain et al. 2019). Also here, induction of tolerance may be useful in preventing excessive response of these cells after repeated insult.

Investigating the role of metabolic changes and reprogramming of our innate immune system is highly relevant, and a clear understanding of mechanisms and key players in the signaling cascade could enable specific targeting not only with microorganisms but also with drugs that mimic their effect by stimulating the same pathways. Further investigations of key pathways like autophagy, mTOR, AMPK, DNA damage-induced stress responses, and their interplay are needed as a reasonable strategy to design targeted therapies selectively inducing trained immunity or tolerance responses.

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# Phenotypic and functional differences between senescent and aged murine microglia

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ABSTRACT

Microglia, the key innate immune cells in the brain, have been reported to drive brain aging and neurodegenerative disorders; however, few studies have analyzed microglial senescence and the impact of aging on the properties of microglia. In the present study, we characterized senescence- and aging-associated phenotypes of murine brain microglia using well-accepted markers, including telomere length, telomerase activity, expression of $p16^{INK4a}$, p21, p53, senescence-associated β-galactosidase, and a senescence-associated secretory phenotype. Quantitative real-time polymerase chain reaction analysis and a Telomeric Repeat Amplification Protocol assay indicated shortened telomeres and increased telomerase activity in senescent microglia, whereas telomeres remained unaltered and telomerase activity was reduced in aged microglia. Senescent microglia upregulated $p16^{INK4a}$, p21, and p53, whereas acutely isolated microglia from the aged brain only exhibited a modest upregulation of $p16^{INK4a}$. Senescent microglia showed decreased proliferation, while it was unchanged in aged microglia. Furthermore, microglia at late passages strongly upregulated expression of the senescent marker senescence-associated β-galactosidase. Senescent and aged microglia exhibited differential activation profiles and altered responses to stimulation. We conclude that microglia from the aged mouse brain do not show typical senescent changes because their phenotype and functional response strongly differ from those of senescent microglia in vitro.


## 1. Introduction

Microglia are the main innate immune cells of the central nervous system. In the healthy brain, they are responsible for continuous and active surveillance of brain parenchyma (Davalos et al., 2005). In addition, microglia are involved in cognitive processes, including learning and memory, via the promotion of learning-dependent synapse formation and resolution (Parkhurst et al., 2013). Microglia live long and exhibit little turnover during their lifetime, which exposes them to potentially damaging mediators, such as cytokines, oxidative species, and eicosanoids, produced in response to environmental stressors (von Bernhardi et al., 2015). Furthermore, microglia may contribute to brain aging and destructive neurodegenerative processes (Chinta et al., 2015; Luo et al., 2010). In the aging brain, microglia become less functional, with an impairment of the ability to support neuronal functions (Streit et al., 2004, 2014). Aged microglia have an altered phenotype characterized by deramification, a bulbous shape and a swelling of processes, particularly in patients with Alzheimer's disease (Streit et al., 2009).

Replicative stress associated with telomere shortening or stress induced by radiation may cause irreversible proliferation arrest referred to as cellular senescence (Hayflick, 1965; Rodier and Campisi, 2011). Senescent cells, although not dividing, are metabolically active; they are characterized by changes in morphology and a "senescence-associated secretory phenotype" (SASP), with an altered pattern of release of growth factors and cytokines (Coppé et al., 2010; Salminen et al., 2012). Cellular senescence in vitro has been proposed to recapitulate the aging process or loss of regenerative capacity of cells in vivo (Campisi and d'Adda di Fagagna, 2007). When cultured alone, murine brain microglia exhibit an age-like phenotype after 2 weeks (Caldeira et al., 2014). Furthermore, the telomere length has been reported to be reduced in microglia after 4 weeks in vitro and in vivo in the aging rat brain (Flanary et al., 2007, Flanary and Streit, 2004).

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Data regarding age-associated changes in murine brain microglia are sparse. There is a lack of evidence for a direct link between the expression of well-known senescence markers, changes in telomere length, and functional alterations in aging and senescent microglia. A rigorous comparison of aging- and senescence-associated cellular changes in microglia in vitro and in vivo is missing. An understanding of microglial aging is important because it may enable the development of strategies aimed at enhancing microglia neuroprotective function or slowing down or even preventing microglial age-associated dysfunction.

This study aimed to characterize microglial aging and senescence based on the expression pattern of well-accepted senescence markers, including telomere length and $p16^{INK4a}$, p21, p53, senescence-associated β-galactosidase (SA-β-Gal) and telomerase activity. For this purpose, we investigated microglia both in vitro and after acute isolation from the aging mouse brain *ex vivo*. Our study indicates that phenotypically and functionally, microglial senescence in vitro differs from microglial aging in the murine brain in vivo.

## 2. Materials and methods

### 2.1. Animals

Newborn (P1-P3), young adult (3-month-old) and aged (24-month-old) males from a C57Bl/6J locally inbred mouse strain were used. In addition, we used aged-matched $Cx3Cr1^{gfp/+}$ mice kindly provided by Prof. Reinhard Wetzker at the Institute of Molecular Cell Biology in Jena. All experiments were conducted in accordance with the German legislation on the protection of animals. Animals were sacrificed by an overdose of isoflurane anesthesia; brains were carefully extracted after transcardial perfusion with ice cold phosphate-buffered saline for 5 minutes. Mixed cultures from neonatal brains (P1-P3) were prepared and microglia were enriched as previously described (Giulian and Baker, 1986; Saura et al., 2003). For *ex vivo* analyses, microglia from adult and aged mouse brains were acutely isolated and enriched with a 35/75% Percoll gradient according to Njie et al. (2012). Long-term cultures of adult microglia were established as previously described (Moussaud and Draheim, 2010). The purity of the microglia ranged from 94% to 98%, as confirmed by staining with the microglial marker ionized calcium-binding adapter molecule 1 (Iba1, 1:500, Waco Chemicals, Germany, 019-19,741, AB_2314667).

### 2.2. Development of an in vitro approach to evaluate microglial senescence

To induce microglial senescence in vitro, we performed serial passaging of mixed astrocyte-microglia cultures, without additional growth factors. After seeding, cells were allowed to grow until they reached approximately 80% confluence (typically 1–2 days before reaching full confluence). After 4-5 passages (1:2 ratio), mixed cultures failed to reach confluence even after 1 month of incubation at 37 °C and 5% $CO_2$ with ambient or 3% $O_2$ and regular media changes. Based on this finding, the following senescence protocol was established: microglia were enriched from 2-week-old primary cultures by gently shaking or mild trypsinization (early passage), and the remaining cells were split in a 1:2 ratio 4-5 times. Microglia were harvested again after 6–8 weeks (late passage) by mild trypsinization and compared with microglia that originated from the same primary cultures. For functional studies, enriched microglia cells were pelleted by centrifugation at 500 × g for 5 minutes and seeded in 24-well plates overnight before stimulation was performed. Purified microglia cells were used for all downstream analyses (RNA, DNA, protein isolation).

### 2.3. Telomere length measurement

The telomere length was determined using a real-time quantitative polymerase chain reaction–based method and primers as previously described (O'Callaghan and Fenech, 2011). Briefly, genomic DNA was extracted from isolated microglia using the NucleoSpin tissue kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The reaction was conducted in a 25 μL volume with 5 μL of template containing 20 ng of DNA, 12.5 μL of SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 0.5 μL of each primer (final 100 nM), and 6.5 μL of water for a final reaction volume of 25 μL. The acidic ribosomal phosphoprotein P0 (*36b4*) was used as the housekeeping gene. All samples were run in duplicate. In each run, a standard curve and a negative control were included. The thermal cycling profile for both amplicons began with a 95 °C incubation for 10 minutes, followed by 40 cycles of 90 °C for 15 seconds and 60 °C for 1 minute. Detection and quantification were conducted with a Rotor gene cycler and Rotor gene Q software (Qiagen, Hilden, Germany). The relative telomere length was calculated using the delta-delta Ct method (Livak and Schmittgen, 2001).

### 2.4. Analysis of average telomere length, autofluorescence, and cell cycle by flow cytometry and FISH

The telomere length was also determined using Flow cytometry–fluorescent in situ hybridization (Flow-FISH). Briefly, enriched microglia cell cultures at early and late passages were harvested and pelleted via centrifugation at 500 × g for 5 minutes. Average telomere length and cell cycle phase analyses were conducted using Flow-FISH with a PNA-FITC kit (DAKO, Hamburg, Germany) according to the manufacturer's instructions. For the analysis, after excluding the doublets, different cell cycle phases were determined based on the DNA content as measured by the intensity of propidium iodide staining. The telomere length was determined by calculating the fluorescence intensity with the following formula: median fluorescence intensity of cells with PNA-FITC probe − median fluorescence intensity of cells without probe. Analysis was performed using FCS Express software (De Novo Software, Glendale, CA, USA). In parallel, a cell cycle analysis via propidium iodide nuclear staining was performed using the Multicycle option of the software. In addition, we measured autofluorescence of unstained microglia cells from early and late passages in the PL-1 (FITC) channel. For the analysis of autofluorescence in microglia from brain slices, random images were taken with a confocal laser scanning microscope (LSM 710, Zeiss, Germany), and colocalization of the microglia marker Iba1 in the green (A488) and red (unstained) channels was evaluated.

### 2.5. Telomerase activity

The telomerase activity was measured using a quantitative polymerase chain reaction (qPCR)-based kit (TRAPeze-kit S7710, Millipore, Darmstadt, Germany) following the manufacturer's instructions. Briefly, cell lysates were prepared using CHAPS buffer. Protein determination was performed using the Bradford method (Bradford, 1976). The reaction was performed in a 96-well plate with a Thermocycler Q Tower 2.2 (Analytic, Jena, Germany) using 1 μg of protein of lysate/reaction.

### 2.6. Immunostaining procedure and quantification of p16 immunoreactivity

Microglia cells (~30.000) cultured on coverslips were fixed for 20 minutes with 4% paraformaldehyde. The nonspecific staining was blocked for 2 h using 10% donkey serum (NDS). Unconjugated primary antibody was diluted in the dilution buffer (2% NDS, 1%

BSA, and 0.3% Triton-X in phosphate-buffered saline) and incubated overnight at 4 °C. Rhodamine or Alexa 488–conjugated secondary (Jackson ImmunoResearch, West Grove, PA, USA) antibody was diluted in the dilution buffer 1:500. DAPI solution was added to each well and incubated for 5 minutes at room temperature. Coverslips were embedded with Fluoromount G (SouthernBiotech, Birmingham, Ala, USA). We used antibodies raised against the microglial marker Iba1 (dilution 1:500; WAKO; Neuss, Germany AB_2314667), p21 (cyclin-dependent kinase inhibitor 1A; dilution 1:100; F-5, Santa Cruz, Dallas, Texas, USA AB_628073), 53bp1 (1:3000, ab36823, Abcam, Cambridge, UK, AB_722497), and anti-p16 (1:50, M-156:sc1207, Santa Cruz Biotechnologies, AB_632106), Cd11b (1:100, 550282, BD Biosciences, San Jose, CA, USA, AB_393577), F4/80 (T-2006, BMA Biomedicals, Switzerland, AB_1227368), isolectin B4 (1:100, Vector Laboratories, Burlingame, CA, USA, AB_2336489). SA-β-Gal staining was performed as previously described (Debacq-Chainiaux et al., 2009).

p16 fluorescence intensity was analyzed in images taken with an Axioplan2 Imaging microscope (40× air objective; Zeiss, Oberkochen, BW, Germany) coupled to an AxioCam HRc camera (Zeiss). Microglia isolated from early and late passages or from 3-month-old and 24-month-old brains (n = 4–5) were allowed to attach for 30 minutes to poly-L-lysine-coated coverslips, fixed, and immunostained with the anti-p16 antibody. At least 10 random images per coverslip with an average cell number of 314 ± 24 were taken and analyzed. Cell nuclei were identified as the region of interest using the DAPI staining, and the intensity of the p16 signal within the region of interest was calculated as "integrated density per nucleus" using the ImageJ software. Fluorescence intensity values were normalized to the nuclei size, and data are presented as fold change to early passage or 3-month-old microglia. DAPI nuclei area was used to analyze the changes of the nuclei size due to senescence and age.

### 2.7. Proliferation analysis

Early passage and late passage cells were cultured in 24-well plates using high glucose Dulbecco's Modified Eagle's medium with 10% fetal bovine serum (FBS). For bromodeoxyuridine (BrdU) incorporation, cells were incubated with 10 μm of BrdU (Sigma, B9285, St. Louis, MO, USA) for 1 h or 24 h; the cells were fixed, and detection was performed using anti-BrdU antibody staining (Bio-Rad AbD Serotec, OBT0030, Hercules, California, USA, AB_609568). In addition, we used freshly isolated microglia cells and stained them with a BrdU pulse for 1 h and 24 h. To detect microglia in mixed cultures or in vivo, cells were costained with the anti-Iba1 antibody. Analysis was performed using fluorescence microscopy and calculating the percentage of Iba1-positive cells that were also BrdU positive. To determine the percentage of microglia cells proliferating under steady-state conditions in the mouse brain in vivo, we costained 40-μM thick free floating slices for the proliferation marker Ki-67 (1:250, NB110-89717, Novus Biologicals, Littleton, CO USA, AB_1217074) and the microglia marker Iba1 (1:250, ab5076, Abcam, Cambridge, UK, AB_2224402). The percentage of Ki-67–positive microglia cells was determined in at least 10 random images of the somatosensory cortex and hippocampus taken with an Axioplan 2 Imaging microscope (40x air objective; Zeiss, Oberkochen, BW, Germany) coupled to an AxioCam HRc camera (Zeiss). We used the same Ki67 antibody and protocol for determining Ki67+ microglia cells in early and late passages.

### 2.8. Analysis of gene expression with qPCR

To determine gene expression levels, RNA was extracted from isolated cells using QIAzol reagent (Qiagen). The RNA concentration, quality, and integrity were determined using a NanoDrop (Thermo scientific, Waltham, MA, USA) and QIAxcel Systems (Qiagen). cDNA was synthetized from 500 ng of RNA/reaction using a RevertAid First Strand cDNA Synthesis kit (Thermo scientific). 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, Hprt,* and *Hmbs* were used for normalization. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The *p16* PCR products were separated on a 2% agarose gel containing ethidium bromide; bands were visualized under UV light and photographed. The primers used are listed in Supplementary Table S1.

### 2.9. Cytokine determination using ELISA

To evaluate the cytokine levels in cultured microglia, 50,000 cells were seeded in a 24-well plate and stimulated with lipopolysaccharide (LPS) (1 μg/mL) for 12 and/or 24 hours. The protein levels of the cytokines tumor necrosis factor alpha (TNFα), interleukin-10 (IL-10), pro–interleukin 1 beta (IL-1β), and interleukin-6 were determined using commercially available ELISA kits from Affymetrix (eBioscience, San Diego, CA, USA) following the instructions supplied by the manufacturer. To determine the release of IL-1β, 100,000 cells/well in a 24-well plate were used and stimulated with LPS (1 μg/mL) for 12 hours before adenosine triphosphate (ATP) (5 mM) was added for an additional 30 minutes. The cytokine concentration was assessed colorimetrically using a Thermomax plate reader (Molecular devices, Sunnyvale, CA, USA).

### 2.10. Western blot analysis

Cells were lysed in an ice-cold Tris buffer pH 7.4 (50 mM Tris, 2 mM EDTA, 1 mM EGTA) containing 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $Na_3VO_4$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μL/mL protease inhibitor. Lysate proteins were solubilized in Laemmli buffer and separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (25 μg of lysate protein/lane). Membranes were blocked with 5% nonfat milk powder in TBS-Tween (0.1%). The blots were immunostained with primary antibodies overnight at 4 °C. The membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature, and proteins were visualized with enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK). Protein bands were evaluated via densitometry using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The primary antibodies included anti-p53 (1:1000, sc-81168, Santa Cruz Biotechnologies, Dallas, TX, USA, AB_1126972), anti-p16 (1:200, M-156:sc1207, Santa Cruz Biotechnologies, AB_632106), anti-p16 (1:500, 10883-1-AP, Proteintech, Rosemont, IL, USA, AB_2078303), anti-p16 (1:500, PA5-20379, Millipore, AB_11157205) anti-phospho p53-Ser15 (1:500, 9284s, Cell Signaling, Danvers, MA, USA AB_331464), and anti-β-actin (1:5000, 4970s, Cell Signaling, AB_2223172), anti-53BP1 (1:2500, ab36823, Abcam, Cambridge, UK, AB_722497), anti-phospho pRb-Ser780 (1:1000, 9307, Cell Signaling, Danvers, MA, USA AB_330015), anti-pRB (1:300, 554136, BD Biosciences, San Jose, CA, USA, AB_395259), anti-cyclin A (1:300, H-3: sc-271645, Santa Cruz Biotechnologies, Dallas, TX, USA, AB_10707658), anti-phospho Chk2-Thr68 (1:500, 2661, Cell Signaling, Danvers, MA, USA, AB_331479), anti-Chk2 (dilution 1:200; A-11, Santa Cruz, Dallas, Texas, USA, AB_2721962), and p21 (cyclin-dependent Kinase Inhibitor 1A; dilution 1:100; F-5, Santa Cruz, Dallas, Texas, USA AB_628073).

### 2.11. Migration/chemotaxis assay

Briefly, 20,000 cells were resuspended in a serum-free media and allowed to migrate for 24 hours in a 24-well plate containing inserts (8 μm Transwell, Millipore, Billerica, MA, United States). Cell migration was stimulated by the addition of 10 μM ATP to the lower chamber. Inserts were fixed in methanol, nonmigrated cells were removed using a cotton swab, and the remaining cells that migrated through were stained with DAPI. Photos from random fields were obtained and analyzed under 40× magnification with an Axiovert 40 CFL microscope from Zeiss (Jena, Germany). Results are expressed as the mean cell migration/cm$^2$.

### 2.12. Phagocytosis assay

To evaluate the phagocytotic activity from microglia, 20,000 cells were incubated with 4 μL of fluorescent latex beads (L3030, Sigma) in 500 μL of the media for 30 minutes in the media containing 0.1% FBS (Pan Biotech, Aidenbach, Germany). Cells were fixed in 4% paraformaldehyde solution, stained for Iba1, and analyzed with a confocal LSM 710 microscope from Zeiss (Jena, Germany). The phagocytosis index was calculated as previously described (Krabbe et al., 2013).

### 2.13. Statistical analysis

Data are presented as the mean ± SEM, and n represents the number of independent experiments.

Data were initially analyzed using the Shapiro-Wilk normality test and Brown-Forsythe or Levene's test for equal variances. If the data were normally distributed, Student's t-test and one-way analysis of variance with Holm-Sidak correction were used for analysis. If the data were not normally distributed, the Mann-Whitney *U* test and Kruskal-Wallis one-way analysis of variance on ranks with Dunn's correction were used. Statistical analysis was performed using SPSS Statistics 22 software package (SPSS Inc, Chicago, IL, USA) and Sigma plot version 12.5 (Systat, San Jose, CA, USA). *p* values of 0.05 or less were considered significant. Detailed statistical analyses are presented in Supplementary File.

## 3. Results

### 3.1. Microglia enter replicative arrest after long-term culture

To investigate microglial senescence, we established a long-term protocol for mixed cultures prepared from neonatal mouse brains (P1-P3). Cultures were repeatedly split until proliferation ceased. After 5-6 passages, the cell number remained constant and cells did not reach confluence even after one month in culture, which indicated cell cycle arrest (Fig. 1A and B). To determine the contribution of environmental stress to microglial senescence in vitro, some microglia cultures were kept under low oxygen conditions (3%) (Fig. 1A). In late passages, microglia underwent morphological changes, which resembled an activated-like phenotype (Fig. 1C and Fig. S1a and b). The microglia cells showed a larger cell body, less branching, and a two-fold increase in nuclei size (Figs. S1 and S2). Microglia proliferation was analyzed after 2 weeks (early passage) and after 8–10 weeks (late passage). One hour after BrdU delivery, as many as 17.66 ± 0.73% of microglia cells entered the cell cycle at early passage, compared with only 2.19 ± 0.98% of cells at late passages (Fig. S3), and this ratio was similar after 24h BrdU incorporation (Fig. 1D). Cells with altered morphology colocalized with nonproliferating entities (Fig. 1C and Fig. S1). Decreased cell proliferation was further confirmed by the reduced number of Ki67$^+$ cells in late passages as compared with early passages (51.42 ± 0.65% vs. 13.16 ± 0.91%; Fig. 1F, Fig. S4). Concomitant with this proliferation decrease, we identified a significant increase in the number of cells in the $G_0/G_1$ cell cycle phase (54.0 ± 1.99 vs. 74.7 ± 1.91; Fig. S5a), which further indicates cell cycle arrest in most of the cell population. Furthermore, mRNA expression levels of the proliferation marker *Ki67*, present in all cell cycle phases with the exception of $G_0$, were also decreased at late passages (Fig. S5b). In addition, cyclin A expression and levels of phosphorylated pRb protein were higher in early passages, indicating active microglia proliferation only in young cells (Fig. 1E, Fig. S6a). Using an antibody which allows to discriminate the phosphorylated and nonphosphorylated pRb protein forms by the band size of the protein on the gel, we found the phosphorylated pRb to be the main form present in early passages, whereas the most abundant form in late passages was the nonphosphorylated one (Fig. 1E). On the contrary, expression of cyclin D1 was not altered at late passages (Fig. S6b).

### 3.2. Microglia show no change in proliferation rates with age in vivo

Microglia from the aged brain showed an altered phenotype characterized by increased size and reduced length of processes (Fig. S7a); however, we did not observe a difference in the size of the nuclei (Fig. S7a and b). To analyze the microglia cell proliferation, we evaluated the levels of cell division markers Ki-67 and BrdU. Surprisingly, in the cortex of aged mice brains, we found a higher number of microglia expressing Ki-67 as compared with 3-month-old brains (1.07 ± 0.3% vs. 0.14 ± 0.007%) suggesting slightly increased cell cycle activity in aged microglia. (Fig. 2A and B). However, the mRNA expression levels of *Ki-67* were the same in microglia isolated from 3-month-old and 24-month-old brains (Fig. S8). To further analyze the proliferative potential of microglia *ex vivo*, we stained freshly isolated microglia for 1 h and 24 h with a BrdU pulse and determined the number of BrdU-positive cells. After 1 h and 24 h, there were no significant differences in the number of proliferating microglia cells; however, there was again a clear trend toward a higher proliferation of microglia from 24-month-old brains (0.44 ± 0.1% vs. 0.62 ± 0.15% after 1 h pulse; 0.43 ± 0.06% vs. 1.39 ± 0.68% after 24 h pulse, Fig. 2C, Fig. S9a and b). Taken together, these results suggest that most of microglia from both 3 and 24 months old brains are quiescent. The long-term proliferative capacity of microglia extracted from 24-month-old brains was further evaluated using a mixed culture protocol (Moussaud and Draheim, 2010). The purity of the acutely isolated microglia obtained by shaking from mixed cultures was >98%, as analyzed with Iba1, CD11b, F4/80, and lectin staining. We found that microglia from aged mice can be cultured for extended periods of time and that the number of cells obtained from astrocyte monolayers during 2 months *ex vivo* was the same for cultures obtained from adult (3-month-old) and aged (24-month-old) brain tissues (Fig. S10). This indicates that microglia from aged brains still maintain a proliferative capacity. In support to this, levels of cyclin A were increased in microglia isolated from aged mice, but still much lower than cyclin expression in microglia in vitro (Fig. 2D, Fig. S6a). We could not detect expression of the phosphorylated form of the pRb protein in these cells, probably due to the very low number of microglia cells proliferating in the aged brain in vivo. The unphosphorylated form of the protein was expressed in 3-month-old and 24-month-old microglia with no significant differences due to age (Fig. 2D). Similar as for microglia from early and late passages, cyclin D1 expression was not altered (Fig. S6c).

### 3.3. Analysis of senescence markers in microglia after long-term culture and in the aged brain in vivo

Expression of SA-β-Gal, a marker of cell senescence, was increased in microglia after long-term culture compared with the early time points (Fig. 3A and B). Although we were not able to specifically detect SA-β-Gal in microglia from the aged brain, and a

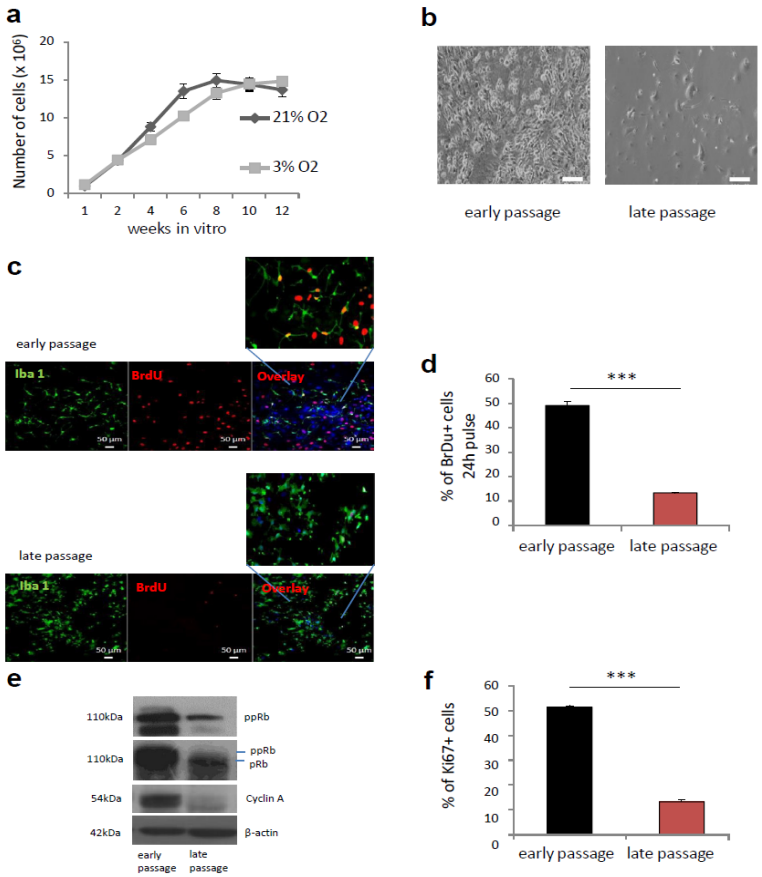


**Fig. 1.** Effect of long-term coculture on astrocyte-microglia replicative activity. (a) Growth curve of mixed astrocyte-microglia cocultures. Cells were cultured in a medium containing 10% FBS, under ambient (21%) or 3% $O_2$ atmosphere, and cell counting was performed before the cells were split. Microglia were separated from astrocytes after 2 weeks (early passage) and after 8–10 weeks (late passage) for further analysis. (b) Representative images of mixed cultures at early and late passages. (c) Representative image of BrdU incorporation assay on mixed cultures at early and late passages. Microglia cells were stained for Iba1 (green) and BrdU (red) at early and late passages. For nuclear staining, DAPI was used (blue). (d) Analysis of microglia cell proliferation in cocultures assessed by BrdU incorporation assay 24 hours after BrdU delivery. (e) Representative Western blots showing expression levels of proliferation markers using a phospho pRb-Ser780–specific antibody, both unphosphorylated pRb and phospho pRb antibody, cyclin A antibody in microglia at early and late passages with loading controls (β-actin). (f) Analysis of microglia in cocultures assessed by Ki67 labeling. ***$p < 0.001$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3–5), scale bars 100 µm. Abbreviations: FBS, fetal bovine serum; Iba1, ionized calcium-binding adapter molecule 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

quantification was not carried out, this senescence marker was seemingly increased in slices from aged brains (24 months) (Fig. 3C).

As we found increased SA-β-Gal levels in microglia from both late passages and in slices from 24-month-old mice, we further evaluated the putative senescent phenotype of these cells by measuring the expression of senescence markers p21, p16, and p53. To characterize microglia acutely isolated from aged brains, we used a protocol that yielded a >94% highly enriched microglia cell population (Njie et al., 2012). The number of p21-positive cells in late passages increased, whereas p21 was almost not detectable in early passages (44.4 ± 2.81% vs. 5.9 ± 0.92; $p < 0.001$; Fig. 4A, Fig. S11a). In contrast to the in vitro situation, we found no p21-positive microglia cells in the aged brain in vivo (Fig. 4B, Fig. S11b). In support of this finding, the mRNA and protein levels of p21 were significantly upregulated only in late passaged microglia in vitro with no changes observed under *ex vivo* conditions (Fig. 4C–F).

Next, we analyzed protein levels and relative mRNA expression of the cyclin-dependent kinase inhibitor 2A, also referred to as $p16^{ink4a}$, or generally as p16, a well-established senescence marker. Protein and relative mRNA levels were increased at late passages compared with early time points (Fig. 4E, G, I and Fig. S12a, b, c), and also in aged microglia (from 24-month-old brains) *ex vivo* (Fig. 4F, H, J and Fig. S13a, b, c) . Interestingly, low expression of p16 protein was also found in early passages in vitro and in 3-month-old microglia *ex vivo*. This was confirmed by immunocytochemistry, indicating that p16 is ubiquitously expressed in all microglia cells (Fig. 4G and H, Figs. S12a, b, S13a, b). The observed increase in nuclear p16 in late passage microglia in vitro and aged microglia cells *ex vivo* was quantified by fluorescence intensity measurements (Fig. S12c, S13c), which confirmed findings from quantitative PCR and Western blotting. When compared with microglia at late passages (used as a positive control), p16 expression levels in aged

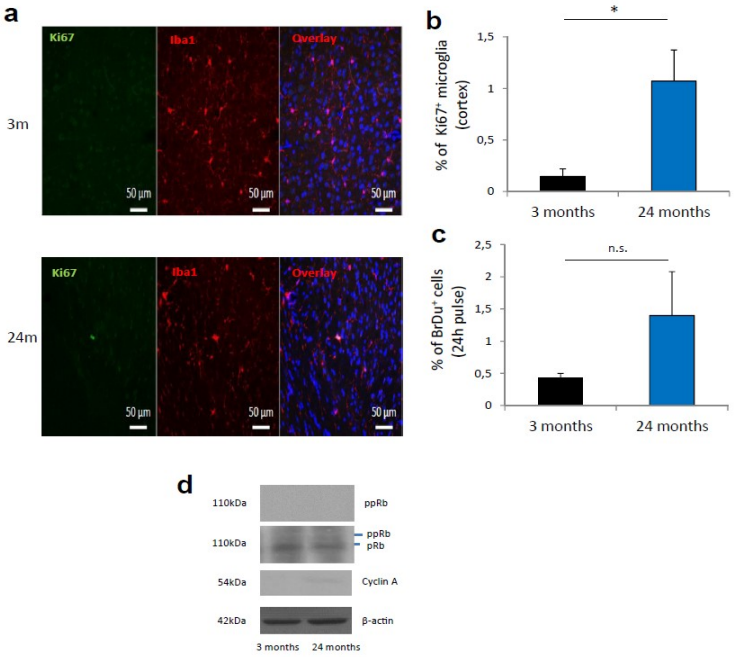


**Fig. 2.** Expression of proliferation markers in microglia in vivo. (a) Representative images showing colocalization of Ki-67 (green) and Iba1 (red) in microglia cells of brain slices (cortex) from 3-month-old and 24-month-old mice. (b) Analysis of Ki-67$^+$ microglia cells in the cortex of 3-month-old and 24-month-old brains. (c) Analysis of microglia cell proliferation *ex vivo* after acute isolation from 3-month-old and 24-month-old whole brains assessed by BrdU incorporation assay 24 hours after BrdU delivery. (d) Representative Western blots showing expression levels of proliferation markers using acutely isolated microglia from 3-month-old and 24-month-old mice brains (phospho pRb-Ser780–specific antibody, both unsphosphorylated pRb and phospho pRb antibody, cyclin A, and loading control β-actin antibody). $^*p < 0.05$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3). Abbreviation: Iba1, ionized calcium-binding adapter molecule 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

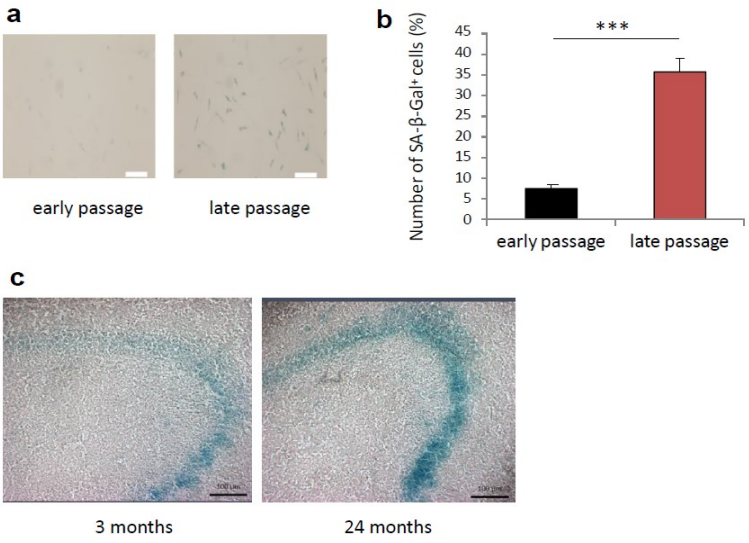


**Fig. 3.** Expression of SA-β-Gal in long-term cultures in vitro and in brain slices in vivo. (a) Representative images of microglia at early and late passages stained for SA-β-Gal. (b) Analysis of the number of SA-β-Gal$^+$ cells in early and late passages. (c) Representative image of slices from 3-month-old and 24-month-old brains stained for SA-β-Gal, scale bars 100 µm. $^{***}p < 0.001$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3). Abbreviation: SA-β-Gal, senescence-associated β-galactosidase.

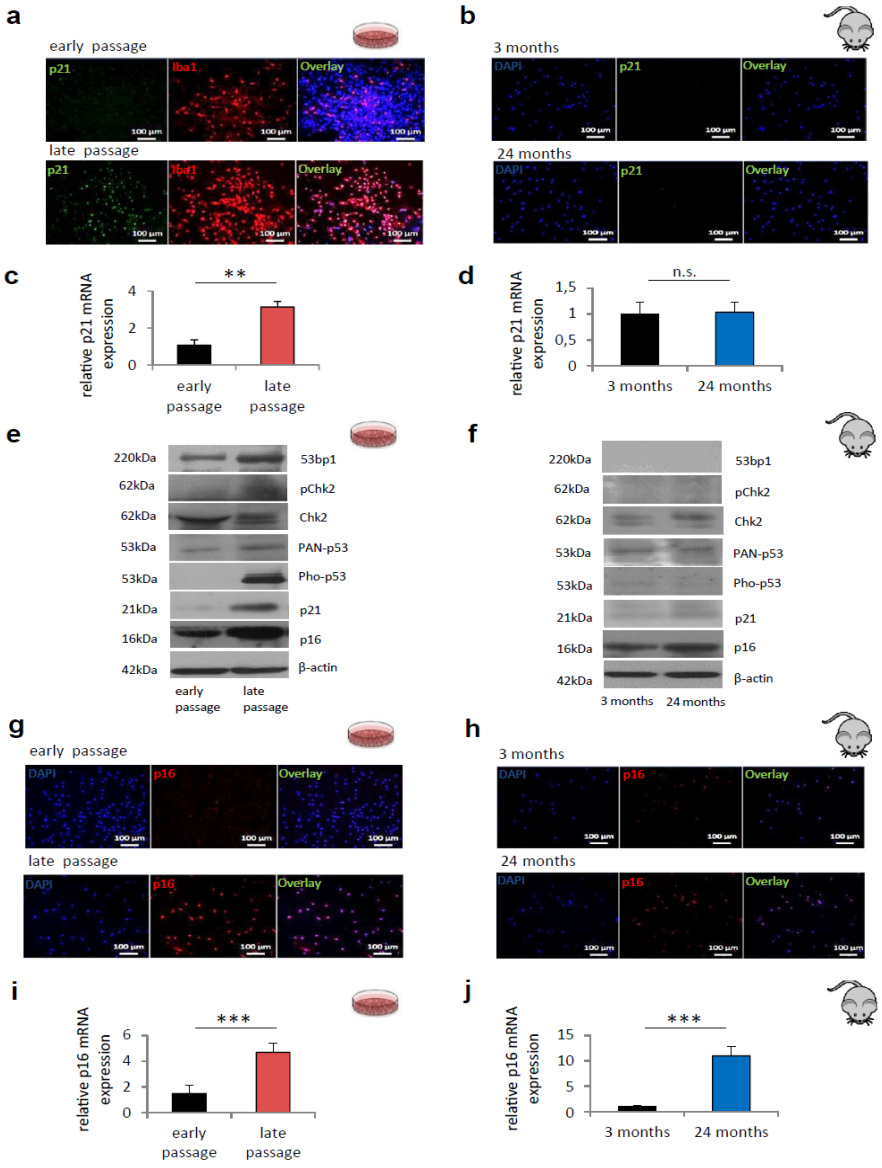


**Fig. 4.** Levels of senescence markers in microglia after serial passaging in vitro and after acute isolation *ex vivo*. (a) Representative images of microglia cells at early and late passages stained for Iba1 (red), p21 (green), and DAPI (blue). (b) Representative images of p21 immunofluorescence staining *ex vivo*. Microglia cells were isolated from 3-month-old and 24-month-old brains and stained for p21 (green) and DAPI (blue). (c) Expression levels of p21 in early and late passaged microglia. (d) Expression levels of p21 in acutely isolated microglia from 3-month-old and 24-month-old mice brains. (e) Representative Western blots of microglia at early and late passages showing expression levels of senescence and DNA-damage markers (53bp1, phospho Chk2-Thr68, Chk2, PAN-p53, phospho p53, p21, and p16). (f) Representative Western blots of acutely isolated microglia from 3-month-old and 24-month-old mice brains showing expression levels of senescence and DNA-damage markers (53bp1, phospho Chk2-Thr68, Chk2, PAN-p53, phospho p53, p21, and p16). (g) Representative images of p16 immunofluorescence staining in vitro. Microglia cells at early and late passages were stained for p16 (red) and DAPI (blue). (h) Representative images of p16 immunofluorescence in vivo. Microglia cells were isolated from 3-month-old and 24-month-old brains and stained for p16 (red) and DAPI (blue). (i) Expression levels of *p16* in early and late passaged microglia. (j) Expression levels of *p16* in freshly isolated microglia from 3-month-old and 24-month-old mice brains. $^{**}p < 0.01$, $^{***}p < 0.001$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3–7), scale bars 100 μm. Abbreviations: Iba1, ionized calcium-binding adapter molecule 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microglia was significantly lower (Fig. S14a), which corresponds to Western blot findings (Fig. 4E and F, Fig. S14b). The reason for differences in p16 expression levels may come from the sustained oxidative stress in vitro.

Because our finding regarding the age-independent p16 expression in microglia is in agreement with a recent report indicating that p16 is physiologically expressed in tissue macrophages and can be regulated by different M1 stimuli (Hall et al., 2017), we stimulated late passaged microglia with LPS and IFNγ for 72 h and measured p16 mRNA expression in these cells. Although we found no significant differences, there was a trend towards increased p16 expression in stimulated microglia (n = 3–4, Fig. S14c).

We subsequently evaluated the protein and mRNA levels of the tumor suppressor protein p53 in microglia both in vitro and in vivo. The levels of the phosphorylated active p53 protein were enhanced (~250 fold) in microglia at late passages with no differences *ex vivo* (Fig. 4E and F). Similarly, the mRNA levels of *p53* were higher than the control conditions in vitro, with no changes *ex vivo* (Fig. S15a and b).

In addition, we found signs of increased DNA damage in late passaged microglia as indicated by increased protein levels of 53bp1, Chk2, and pChk2 (Fig. 4E). However, we found no differences in levels of these proteins in microglia obtained from adult and aged brain tissues (Fig. 4F). Furthermore, we observed 53bp1 foci formation in long-term cultured microglia (Fig. S16a), but not in microglia isolated from aged mice, suggesting no significant DNA damage or double strand breaks occurring in microglia with age (Fig. S16b), as compared with late passage microglia. In addition, we also observed a dramatic increase in autofluorescence in microglia at late passages and aged microglia (24 months old) (Fig. S17a and b, Fig. S18a and b), possibly associated with accumulation of lipofuscin or other undegraded proteins known to accumulate in the aged central nervous system.

### 3.4. Telomere length and telomerase activity are differentially regulated in senescent and aged microglia

The telomere length in microglia was determined by a reproducible real-time quantitative PCR assay (Cawthon, 2002; O'Callaghan and Fenech, 2011). A list of the primers used for real-time PCR reactions is provided in Supplementary Table S1. In vitro, late passages were associated with a strong reduction of the telomere length (at 6–8 weeks and 8–10 weeks in culture) compared with early passages (Fig. 5A and Fig. S19a and b). There was no difference in the telomere length between the two late time points (Fig. 5A). The telomerase activity in vitro showed cyclical activity with an increase at 6–8 weeks and returning to basal levels at 8–10 weeks (Fig. 5B).

Aging of microglia did not significantly alter telomere length, as indicated in Fig. 5C. The telomerase activity was reduced in microglia acutely extracted from aged brains compared with adult brains. Notably, in contrast to young microglia, it did not increase when these cells were cultured for 6–8 weeks (Fig. 5D). Similar to microglia from young brains, telomeres shortened when microglia from adult and/or aged brains were cultured for 6–8 weeks (Fig. 5E). The dynamics of telomere shortening was similar for all groups with an approximately identical slope coefficient (-0.7313x + 2.49 for 3-month-old vs. -0.7317x + 2.56 for microglia cultured from 24-month-old brains).

### 3.5. Senescence and aging are associated with microglial activation

To further characterize senescent and aged microglial cells, we analyzed the functional characteristics by determining the mRNA expression of typical activation markers, growth factors, and cytokines. Senescent microglia at late passages were activated, as indicated by higher expression levels of *Cd68*, *Cd14*, *Tlr2*, *Tlr7*, and *Trem2* (Fig. 6A). Toll-like receptors 2 and 7 (Tlr2 and Tlr7) are involved in effective innate immune responses to pathogens and danger signals associated with inflamed or damaged tissues. Coreceptor CD14 interacts with TLR2 and TLR4 for signal transduction and is crucial for an effective microglia response (Janova et al., 2016). TREM2 is a receptor involved in microglia activation and phagocytosis. In addition, senescent cells also exhibited an inflammatory and secretory phenotype, as indicated by increased expression levels of *IL-1β* and anti-inflammatory cytokines and growth factors, such as *Il-10 and Tgf-β*. The expression of *Bdnf*, an important growth factor involved in neuronal plasticity, tended to decrease. We also found a significant increase in senescence and microglial activation markers in microglia cultured in 3% $O_2$ excluding the possibility that microglia senescence in vitro is only due to inappropriate culture conditions and increased environmental stress (Fig. S20).

To compare the properties of senescent microglia in vitro with microglia from the aging brain, we analyzed the same markers in acutely isolated cells from aged brains ex vivo. These microglia also exhibited an activated phenotype, as confirmed by higher expression levels of *Cd68* and *Tlr2* (Fig. 6B). In addition, the expression of *Il-1β*, *Tnf-α*, and *Tgf-β* was increased, which indicates an activation of both proinflammatory and anti-inflammatory pathways. *Tlr7*, one of the pattern recognition receptors for RNA involved in antiviral responses, was decreased. *Bdnf* was increased, which may reflect a compensatory mechanism to support neurons in an inflammatory environment (Fig. 6B). The expression of *Cx3cr1*, an important receptor for microglia activation and microglia-neuron crosstalk, was decreased in aged microglia (Fig. 6B). In summary, activation markers and cytokines in aged microglia were less increased compared with senescent cells and exhibited differences in the pattern of activation (see proposed model, Fig. 9).

### 3.6. Senescence and aging induce a dysfunctional phenotype in microglia

We subsequently analyzed the functional activity of senescent microglia and microglia from aged brains (24 months). We determined the migration and phagocytosis function and the responses to the stimulators LPS and ATP. There were several notable differences between the activation of senescent and aged microglia by LPS. This was true for TNF-α, interleukin-6, IL-1β, and IL-10 (Fig. 7A–F). Interestingly, release of IL-1β in response to ATP exhibited a completely opposite behavior: in vitro, there was an increased release of IL-1β from senescent microglia, whereas the same protein was decreased in supernatants from aged microglia *ex vivo* (Fig. 7E).

In senescent microglia, the migration rate increased (3-fold) after ATP stimulation (Fig. 8A). In accordance with this finding, the mRNA expression of the ATP receptors *P2x4* and *P2x7*, which are involved in cell migration and inflammasome activation (Horvath and DeLeo, 2009), was enhanced at late passages compared with early time points (3-fold and 4-fold, respectively; Fig. S21a and b). In contrast to senescent cells, the migration of microglia from aged brains was not increased but tended to decrease (Fig. 8B). The decreased response to ATP by aged microglia may be a result of a decrease in the purinergic receptor expression *P2x4* (Fig. S21c and d). The phagocytic capacity of aged cells was significantly reduced (Fig. 8D). Similarly, senescent microglia tended to exhibit an impaired phagocytosis of fluorescent beads (Fig. 8C).

The main findings of this study and the proposed model explaining differences between the process of microglial senescence in vitro and microglial aging in vivo are summarized in Fig. 9 and Fig. S22.

## 4. Discussion

Knowledge regarding microglial aging and senescence remains limited. Several recent publications use the term "microglia

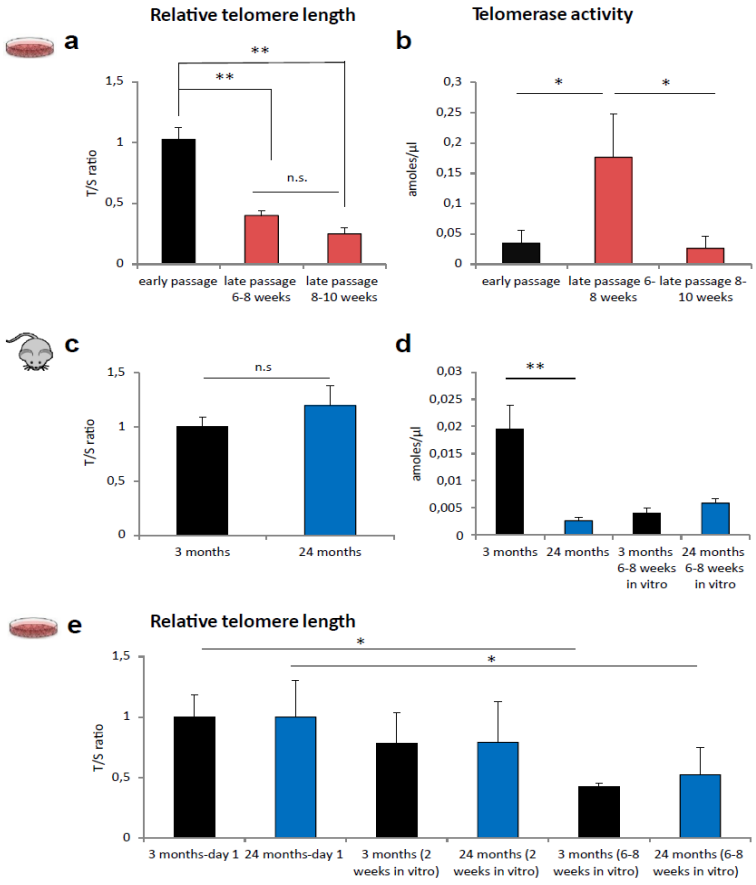


**Fig. 5.** Analysis of telomere length and telomerase activity in microglia both in vitro and ex vivo. (a) Telomere length measurement in microglia at early and late passages (after 6–8 weeks and after 8–10 weeks). (b) Telomerase activity in microglia in vitro at early and late passages (after 6–8 weeks and after 8–10 weeks). (c) Telomere length measurement in acutely isolated microglia from 3-month-old and 24-month-old mice brains. (n = 6). (d) Telomerase activity of microglia isolated from 3-month-old and 24-month-old brains after 6–8 weeks in culture. (e) Telomere length of microglia isolated from 3-month-old and 24-months-old brains after 2 and 6–8 weeks in culture. $^{*}p < 0.05$, $^{**}p < 0.01$, two-sided t-test and one-way ANOVA with Holm-Sidak post hoc test. Bars represent the mean ± SEM (n = 3–6).

senescence" (Flanary et al., 2007; Streit et al., 2009); however, a systematic analysis of typical senescent markers in microglia has not previously been undertaken. One reason for this may be the lack of adequate in vitro models to investigate microglia senescence. In addition, microglial properties in the aged brain are difficult to analyze because of complex isolation and culturing protocols. Here, we developed an in vitro approach to investigate the typical "Hayflick" replicative senescence of brain microglia (Hayflick, 1965). The current findings indicate that the pattern of microglial senescence in vitro is different from the pattern these cells exhibit in the aging brain in vivo.

Microglia in vitro cannot survive longer than 2–3 weeks without support from astrocytes. For this reason, we developed a reproducible coculture protocol and obtained highly enriched and viable Iba1-positive microglia populations. As expected for non-proliferative cells, the G0/G1 phase arrest was increased in senescent microglia, whereas the proliferation markers were decreased, which indicates a proliferative arrest. In accordance with these findings, the proportion of SA-β-gal–positive cells, a well-established marker for senescence, was increased 9-fold in microglia at late passages. We therefore termed these cells "senescent microglia." Our in vitro approach has important advantages compared with previous studies: Flanary et al., 2007 induced telomere shortening in cultured microglia by stimulating them with growth factors for 32 days, whereas Caldeira et al. (2014) left microglia alone in culture for 2 weeks. These interventions decrease microglia viability (Flanary and Streit, 2004; Saura, 2007). In addition, this approach reduces microglia purity as a result of the faster astrocyte proliferation, which is always present to some degree at the onset of cultures (Saura, 2007; Saura et al., 2003; Tomozawa et al., 1996). Our protocol benefits from a constant interaction and support from astrocytes, without additional granulocyte macrophage colony-stimulating factor stimulation, which has been shown to change the cell phenotype (Re et al., 2002).

To analyze whether microglia in the aging brain in vivo also exhibit similar signs of senescence as in vitro, we used freshly extracted microglia from 24-month-old mouse brains, referred to here as "aged microglia." These microglia exhibited similar rates of proliferation as microglia from adult brains when cultured for 2 months, thus indicating a preserved proliferating capacity.

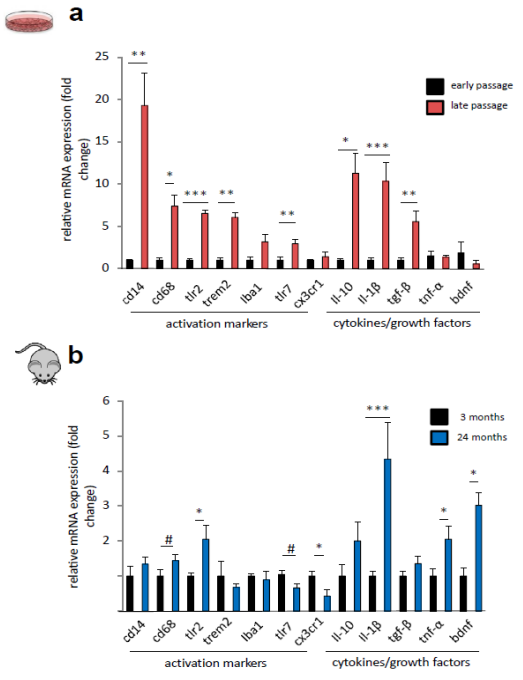


**Fig. 6.** Expression levels of microglial activation and SASP-associated markers in senescent and aged microglia analyzed by qPCR. (a) Activation markers and cytokines/growth factors forming SASP, in early versus late passaged microglia. (b) Activation markers and cytokines/growth factors forming SASP in microglia from 3-month-old and 24-month-old brains. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-sided *t*-test. # $p < 0.05$, one-sided *t*-test. Bars represent the mean ± SEM (n = 3–7). Abbreviations: SASP, senescence-associated secretory phenotype; qPCR, quantitative polymerase chain reaction.

Furthermore, there was an increase in the number of Ki-67–positive microglia. Although we cannot exclude the possibility of a partial in vitro selection process that allows only nonsenescent cells to proliferate, our data indicate that most microglia in the old murine brain are not senescent. We found cyclin A slightly increased in acutely isolated microglia from the aged brain. This represents a novel finding and might be associated with a cell cycle–independent function of this protein during development and aging (Gygli et al., 2016); however, it deserves further research to evaluate for a possible mechanism.

Cellular senescence classically involves two main cell cycle regulatory pathways: p16 and p21/p53. Interestingly, we identified striking differences between senescent microglia in vitro and "aged microglia" from old brains. The p21/p53 pathway was highly upregulated during the senescence process in culture; however, this upregulation was not identified in the healthy aging brain.

This pathway is mainly activated by DNA damage, which could be telomere associated; thus, we assessed DNA damage and telomere length in mice and in cultures under low oxidative stress conditions.

In our cell culture model, murine microglia contained shortened telomeres after several passages, as determined with both qPCR and Flow-FISH, and this was accompanied by increased DNA damage. This finding is in agreement with previous findings for rat microglia reported by Flanary et al. (2007). Interestingly, we did not find telomere shortening in the aged murine microglia, which is in contrast to the results from Flanary et al. (2007) for microglia from the aging rat brain. One potential explanation for this finding may be the interspecies differences in the telomere length. It has been reported that inbred mouse strains, such as the strain used in this study, have 5 times longer telomeres than rats (Bedoyan et al., 1996). Accordingly, telomere shortening should not have an influence on aging or the lifespan in mice.

With regard to the discrepancy between the telomere length in senescent and aged microglia, we assume that this is a result of the strong difference in cell proliferation. In vitro, approximately 40% of early passage microglia cells were cycling at the time of our analysis. In contrast, only 0.1%-1% of microglia proliferate at a given time point in the normal adult murine brain in vivo, which is in accordance with a previous study (Lawson et al., 1992). Recent data indicate that the median lifespan of microglia cells in vivo is at least 15 months, limiting microglial proliferative capacity to only 1-2 cell divisions/cell for a lifetime in the healthy mouse brain (Füger et al., 2017). To test our hypothesis, that telomere shortening in vitro results from increased cell proliferation, we cultured adult and aged microglia for 2 months using the same mixed culture protocol as for neonatal microglia. As expected, long-term cultured microglia showed significant telomere attrition. Telomere shortening observed in vitro may be due to replicative stress induced by increased proliferation because microglia normally show a low proliferation rate and low turnover under physiological conditions. Long telomeres, as present in mice, are particularly prone to replicative stress. In particular, a form of telomere rapid deletion termed telomere trimming has been associated with length dynamics of long telomeres (Pickett and Reddel, 2012). Telomere rapid deletion is compatible with continued cell proliferation. On the contrary, there was no significant telomere shortening or DNA damage in vivo, which would explain the lack of p21/p53 pathway upregulation. In support of our hypothesis, this pathway, together with telomere shortening, was also upregulated in microglia from telomerase-deficient mice (Raj et al., 2015), which links telomere shortening and DNA damage to the p21/p53 pathway.

As the telomere length is critically maintained by telomerase, we determined whether activity of this enzyme is altered in cultured senescent microglia and in aged microglia in vivo. After 6 weeks in vitro, the telomerase activity was increased in senescent microglia; thus, it was negatively associated with the telomere length dynamics shown here. The activity returned to the baseline levels after 10 weeks. Whether telomerase increase is caused by telomere shortening observed in vitro remains to be investigated. These cyclical changes in the telomerase activity have also been described in rats, which suggests a similar mechanism of telomerase regulation in both species (Flanary and Streit, 2004). In aged microglia, the telomerase activity was significantly decreased compared with adult microglia. However, there were no differences after culturing these cells for 6–8 weeks (Fig. 5D). Because telomerase is known to have many noncanonical extratelomeric protective functions, this finding should be further investigated (Martínez and Blasco, 2011).

As indicated, in addition to the p21/p53 pathway, the p16 pathway is associated with aging of different tissues, including the brain cortex; however, the cell types involved remain unknown (Krishnamurthy et al., 2004). Here, we identified a substantial increase in the p16 expression in microglia cells extracted from aged mouse brains and senescent cultures. Importantly, *p16* expression and function during aging has been shown to be independent of the telomere status (Rayess et al., 2012; Rheinwald et al., 2002); thus, we propose p16 as a candidate marker associated with both in vitro microglial senescence and in vivo microglial aging. Interestingly, some findings indicate that p16 may have CDK4/6-independent roles, like in macrophage M1/M2 polarization or anti-

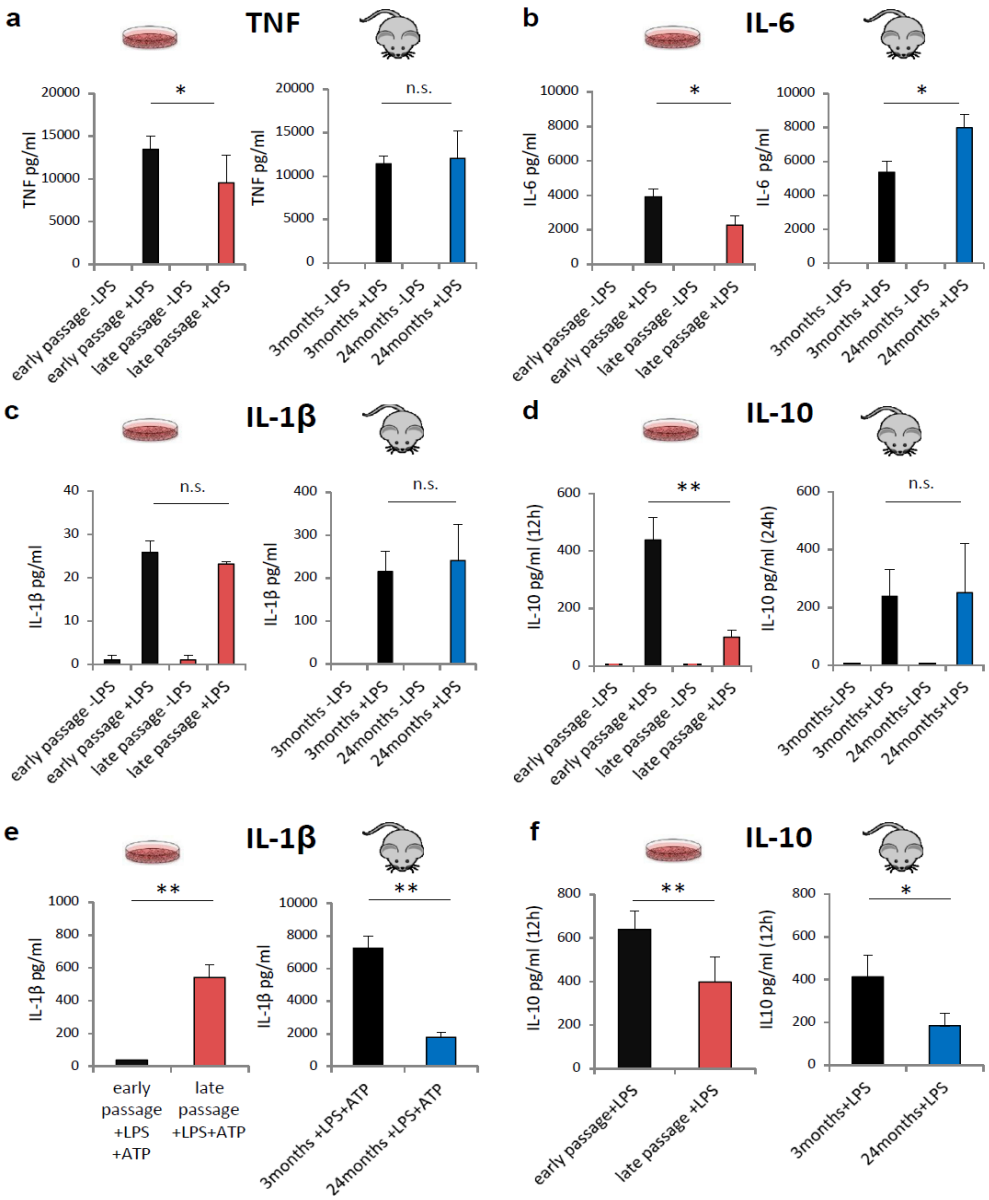


**Fig. 7.** Effect of LPS and ATP stimulation on senescent and aged microglia. (a) TNF-α release 24 hours after LPS stimulation. (b) IL-6 release 24 hours after LPS stimulation. (c) IL-1β release measured 24 hours after LPS stimulation. (d) IL-10 release measured 24 hours after LPS stimulation. (e) IL-1β release measured 24 hours after LPS and ATP stimulation (5 mM) for 30 additional minutes. (f) IL-10 release 12 hours after LPS stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3–7). Abbreviations: ATP, adenosine triphosphate; LPS, lipopolysaccharide.

inflammatory roles through accelerated IRAK1 degradation in these cells (Cudejko et al., 2011; Murakami et al., 2012). More recently, it was found that macrophages express p16 and SA-β-gal under physiological conditions (Hall et al., 2017), and this can be changed by polarization stimuli like LPS, IFN-γ, or IL-4. We found that microglia, the brain macrophages, express p16 in young and in aged cells, thus indicating that it may also play different roles in microglia apart from senescence, like in cell polarization. However, we could not observe significant difference of p16 expression after LPS or IFN-γ stimulation, which could be due to the low number of repetitions or because of using already senescent cells in this experiment. As inflammatory stimuli are known to induce p16 expression (Campisi and d'Adda di Fagagna, 2007; Coppé et al., 2011; Cudejko et al., 2011), we speculate that increased p16

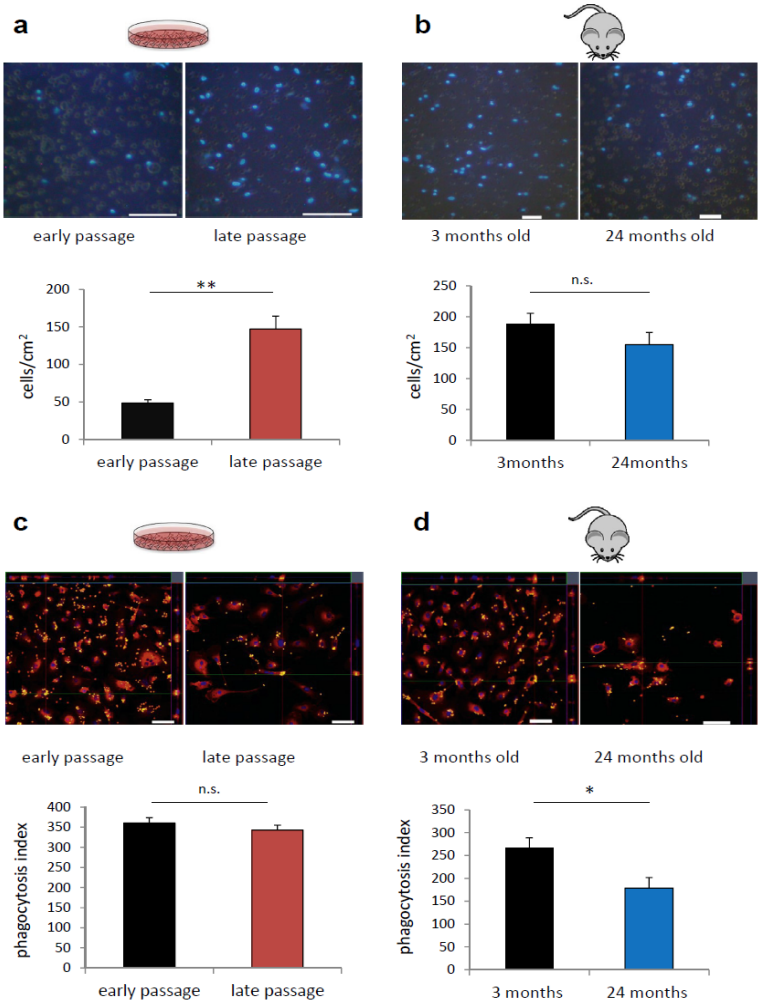


**Fig. 8.** Migratory and phagocytotic activity of senescent microglia and microglia from the aged mouse brain. (a) Migratory activity/chemotaxis of microglia in early and late passages after stimulation with ATP 10 μM. To detect migrated cells, microglia were stained with DAPI (blue). (b) Migratory activity/chemotaxis of microglia from 3-month-old and 24-month-old brains after stimulation with ATP 10 μM. To detect migrated cells, microglia were stained with DAPI (blue). (c) Representative images confirming the presence of beads inside microglia. Phagocytosis index of early and late passaged microglia. Cells were stained for Iba1 (red). (d) Representative images and analysis of phagocytotic activity from young (3 months) and aged (24 months) microglia. Cells were stained for Iba1 (red). $^{*}p < 0.05$, $^{**}p < 0.01$, two-sided *t*-test. Bars represent the mean ± SEM (n = 3–4), scale bars 100 μm, for Figure 8b 50 μm. Abbreviation: ATP, adenosine triphosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expression is due to increased SASP in the aged brain, possibly playing a role in SASP suppression, as shown previously (Coppé et al., 2011). Data from p16 levels by Western blot and immunofluorescence correlated well. The mRNA and protein expression, however, were much higher in senescent cells in vitro, possibly due to replicative stress as previously mentioned. The exact role of p16 in aged microglia requires further evaluation.

A recent study by Baker et al. (2016) indicated that the removal of p16-positive cells is beneficial for the surrounding tissue, delays aging, and increases the lifespan of mice (Baker et al., 2016). Our finding regarding the expression of p16 in microglia from the aged brain suggests that this approach probably also targeted p16-positive tissue macrophages and microglia. Indeed, treatment of aged mice with clodronate, a known macrophage and microglia removing agent, leads to decreased p16 expression (Hall et al., 2016). Furthermore, removal of microglia from the Alzheimer's brain was found to improve cognitive performance (Dagher et al., 2015). In particular, amyloid-β is known to induce increased expression of senescence markers in glial cells (Bhat et al., 2012), and patients with Alzheimer's disease exhibit profound dystrophic changes in microglia (Streit et al., 2009). Thus, removal of dystrophic/or p16-positive microglia may contribute to an improved cognitive performance and retard disease progression.

Here, we identified similarities, and important differences, between senescent microglia in vitro and microglia from the aged

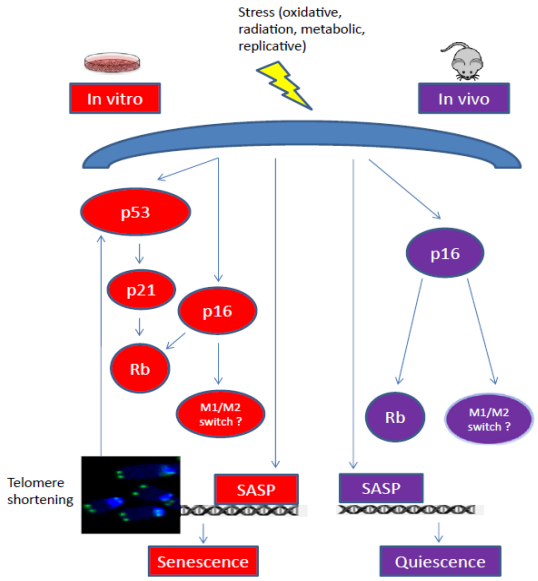


**Fig. 9.** Aging versus senescence of microglia. Proposed model for senescence- and aging-associated changes in microglia in vitro and in vivo. Serial passaging in vitro induced telomere shortening, DNA damage, and upregulation of p53/p21 and p16, whereas only p16 expression and SASP were observed in vivo/*ex vivo*. Abbreviation: SASP, senescence-associated secretory phenotype.

brain. The cytokine *IL-1β* and the activation markers *Cd68* and *Tlr2* were increased in both the senescence and aging process, with a substantially stronger increase in vitro. We presume that this may be a result of the extreme conditions in vitro, including higher oxidative stress or contact with serum, which are not typically present in the aging brain. Caldeira et al. (2014) reported that "old" microglia populations in vitro exhibit a decrease in the number of NF-kB–positive cells and a decrease in the *Tlr2* expression. Therefore, they concluded that microglia exhibit a deactivated "age-" like phenotype. In contrast, our findings and the findings from another study by Norden and Godbout (2013) suggest increased toll-like receptor expression and cell activation. We determined that only senescent microglia overexpress *Cd14* and *Trem2*, which are involved in both the innate immune response and phagocytosis (Henjum et al., 2016; Janova et al., 2016). Unexpectedly, genes like *Bdnf*, *Tlr7*, *P2x4*, and *P2x7* exhibited a completely opposite pattern of regulation in senescent and aged microglia. This finding further confirms our hypothesis of different processes occurring in our in vitro senescence model compared with healthy brain aging.

We identified a downregulation of the *Cx3cr1* receptor in aged microglia. The interaction between CX3CL1 and its receptor CX3CR1 is one of the most important neuronal "off" signals to regulate microglial activity (Kettenmann et al., 2011). This pathway was not regulated in senescent microglia likely because of the absence of neurons in the cultures. Complementary to this finding, we identified an increase of *Bdnf* only in aged microglia, which may be a compensatory neuroprotective mechanism in a state of increased inflammation.

Microglia are the main immune cells in the brain; thus, we also evaluated their responses to LPS and ATP. The microglial response to LPS was decreased in late passages, which confirms the previous observation that repeated microglia extraction by shaking impairs the response to LPS (Floden and Combs, 2007). In contrast, aged microglia exhibited an increased response, which confirmed the previous finding of so called "primed" microglia with age, as reported by others (Norden and Godbout, 2013). An opposite response was also identified when the cells were challenged with ATP, one of the most important damage-associated molecular pattern stimuli in the brain. We assume that with age, microglia become tolerant to ATP by downregulating the expression of the ATP receptors, and the analysis of *P2x4* and *P2x7* receptor expression confirmed this hypothesis.

In addition to the activation profile, we analyzed two other functions associated with microglial activity, that is, migration and phagocytosis. Phagocytosis was impaired in aged microglia, whereas this function was preserved in senescent microglia. Migration was increased in senescent microglia, which correlates well with purinergic receptor expression. In aged microglia, a tendency toward a decreased migration was identified, which is in agreement with previous observations (Hefendehl et al., 2014).

In summary, we identified striking differences between microglial changes in vitro, leading to a senescent phenotype, and alterations that occur with aging in the murine brain (Fig. S22).

Unlike senescence in vitro, aged microglia do not show telomere shortening and activation of the p21/p53 pathway. We conclude that microglia cells from the aged brain in vivo are dysfunctional but not senescent because their phenotype and functional responses strongly differ from that of senescent microglia in vitro.

Harsh conditions, including high oxygen levels, "cell culture shock," excess nutrients and metabolites, and nonphysiological contact with blood serum, may lead to DNA damage and accelerated senescence, telomere shortening and augmented SASP, which, in turn, reinforces senescence in vitro. The in vitro approach developed in this study is a valuable senescence model for evaluating potential mechanisms and developing strategies to prevent/postpone microglia dysfunction. However, a thorough systematic analysis indicates that different mechanisms are involved in replicative senescence in vitro and aging in vivo, which in mice is predominately telomere independent. A better understanding of microglia aging is of great interest because it may lead to novel approaches to the prevention and treatment of major age-related neurodegenerative diseases.

## Disclosure

The authors report no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2018.10.007.

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**Manuscript II**

# Innate immune memory-like responses in brain microglia are regulated by developmental state and pathogen dose

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**Running title:** Maturation-dependent microglial adaptive responses

**Keywords:** Microglia, LPS, β-glucan, trained immunity, tolerance, maturation

## Abstract

Brain microglia, the innate immune cells of the central nervous system, were found to develop features of adaptive immune memory, with implications for brain pathologies. However, factors involved in emergence and regulation of these opposing responses in microglia have not been fully addressed. Recently, we showed that microglia from the newborn brain display trained immunity and immune tolerance after repeated contact with pathogens in a dose-dependent manner. Here, we evaluated the impact of developmental stage on adaptive immune responses of brain microglia after repeated challenge with ultra-low (1 fg/ml) and high (100 ng/ml) doses of the endotoxin LPS *in vitro*. We found that priming of naive microglia derived from newborn but not mature murine brain with ultra-low LPS significantly increased levels of pro-inflammatory mediators TNFα, IL-6 and iNOS, as well as BDNF and PFKFB3, whereas ROS levels were decreased, indicating induction of trained immunity and associated metabolic reprogramming. In contrast, stimulation with high doses of LPS led to a robust down-regulation of pro-inflammatory cytokines and iNOS in both newborn and mature microglia, indicating induced immune tolerance. In addition, high LPS doses upregulated anti-inflammatory mediators including IL-10, IL-4, TGF-β and Arg-1 in newborn microglia. Our study shows that microglia from the newborn brain exhibit a remarkable fine-tuned innate immune memory compared with mature microglia. The trained character after priming with ultra-low LPS doses might be associated with dysfunctional phenotypes observed in certain neurodevelopmental diseases, whereas the immunosuppressed phenotype following stimulation with a high LPS dose might develop in order to prevent excessive damage after recurrent inflammation.

## 1. Introduction

Microglia are the resident macrophage population and the principal cell type responsible for innate immunity in the central nervous system (CNS) and constitute the first line of defense against invading pathogens (Streit and Kincaid-Colton, 1995;Davalos et al., 2005;Nimmerjahn et al., 2005;Hanisch and Kettenmann, 2007;Puntambekar et al., 2008;Ransohoff and Cardona, 2010;Kettenmann et al., 2011;Salter and Stevens, 2017). In addition, microglia play an important role in the development of the neuronal network by eliminating surplus brain cells or subcellular structures, especially synapses (Salter and Stevens, 2017). Microglia contribute considerably to maintenance of tissue homeostasis, neuroplasticity and neuroprotection on the basis of their considerable adaptive capacity and ability to detect alterations in their surveilled microenvironment (Wolf et al., 2017). Accordingly, microglia are activated by most pathologic events and changes in brain homeostasis. Their phenotype varies from pro-inflammatory to anti-inflammatory and tissue-supportive, thereby displaying specific adaptive functions, including migration towards injury, phagocytosis, antigen presentation and synapse remodelling (Eggen et al., 2013;Gertig and Hanisch, 2014;Ransohoff, 2016). Importantly, there is growing evidence indicating that microglial cells may not retransform to a completely naive status after activation, and may remain as 'post-activated' or 'primed' microglia, which could have a neuropathological relevance (Hanisch and Kettenmann, 2007). In the same context, it was recently demonstrated that peripheral inflammatory insults in adult mice induce long-term alterations in microglial response, with two possible outcomes: either enhanced activation or suppressed activation, thereby exacerbating or alleviating brain pathology in mouse models (Schaafsma et al., 2015;Wendeln et al., 2018;Neher and Cunningham, 2019). These two phenotypes seem to conform to the concept of innate immune memory, as demonstrated for the peripheral immune system (Ifrim et al., 2014;Netea et al., 2016).It is known that innate immune memory in microglia is mediated by epigenetic and transcriptional changes (Wendeln et al., 2018), however, how these opposed phenotypes are regulated is yet not well understood. Microglia display characteristic gene expression profiles during different phases of development, in order to regulate brain homeostasis, and that prenatal and early postnatal brain microglia have a different morphology than microglia in the adult brain (Bennett et al., 2016;Matcovitch-Natan et al., 2016). Furthermore, no substantial gene expression overlap was found between lipopolysaccharide (LPS)-stimulated microglia from the adult and from the control neonatal brain (Bennett et al., 2016). Therefore, not only (micro)environmental conditions but also the inherent genetics may influence microglial fate throughout the organismic development. These findings raise the question whether the developmental or maturation state of microglia affects their cellular responses also in the context of innate immune memory.

Not only the developmental state but also the pathogen dose seems to influence the type of cellular response after repeated challenge. Nonspecific enhanced (training) or diminished (tolerance) cytokine production in monocytes and macrophages upon secondary stimulation with different PAMPs (prototypical pathogen-associated molecular pattern), including LPS, was often found to be dependent on the ligand concentration (Baker et al., 2014;Ifrim et al., 2014;Morris et al., 2014). A previous study in our lab demonstrated a pathogen dose-dependency in phagocytotic and proliferative response of brain microglia after repeated LPS challenge *in vitro* (Schmidt et al., 2014). Moreover, we recently provided evidence for a pathogen-dose-dependent activation of adaptive immune responses in newborn microglia

*in vitro* (Lajqi et al., 2019). These findings strongly suggest the existence of hormetic mechanisms underlying the immune response to pathogens and stressors.

Here, we investigated the effect of the developmental state on adaptive responses of newborn and adult murine brain microglia after repeated challenge with LPS.

Our results indicate that newborn but not mature microglia primed with LPS at ultra-low doses develop a trained immune response characterized by a pro-inflammatory phenotype after renewed challenge with a "standard" high LPS dose. In contrast, repeated challenge of newborn or mature microglia with a high-dose of LPS results in a tolerant (anti-inflammatory) state. Furthermore, we found that induction of innate immunity in newborn microglia was associated with a metabolic reprogramming, as already shown for other cells from the innate immune system (Bekkering et al., 2018). These findings clearly indicate that in brain microglia trained immunity but not immune tolerance is developmentally regulated.

## 2. Materials and Methods

*Animals*

Neonatal (P0-P3) and young adult (3 months old) C57Bl/6 male mice (from a locally inbred mouse strain) were used. All experiments were carried out in accordance with the German legislation on protection of animals and with permission of the local animal welfare committee. Adult animals were sacrificed by an overdose of isoflurane anesthesia and brains were carefully removed after transcardial perfusion with ice cold PBS for 5 min. Neonatal primary microglial cells were isolated from cerebral cortex of newborn mice as described previously (Schmidt et al., 2013;Lajqi et al., 2019).

Microglial cultures were maintained in Dulbecco's Modified Eagle's Medium (SIGMA #06429, endotoxin tested) and 10% heat-inactivated fetal bovine serum (FBS, SIGMA-Aldrich #F7524, endotoxin tested and sterile-filtered). After 14 days, adherent microglial cells were separated from astrocytes by adding PBS-EDTA solution and carefully shaking. After harvesting, microglial cells were seeded in adherent well plates. Long-term cultures of adult microglia were performed as previously described (Moussaud and Draheim, 2010;Stojiljkovic et al., 2019). Purity of microglia was always in the range of 95-98%, as confirmed by specific Iba1 staining.

*Microglia stimulation*

In order to induce adaptive responses, microglial cells were seeded in 12-well plates (75 000 cells/well). Microglia were stimulated twice following a two-step ("two-hit") protocol as described elsewhere (Ifrim et al., 2014;Schaafsma et al., 2015). Microglia were first stimulated ("primed") with different doses of LPS ("first hit"; 1 fg/mL – 100 ng/mL; *E. coli* serotype 055:B5 obtained from Sigma-Aldrich, St. Louis, USA) for 24h. Cells were re-stimulated 6 days after the first challenge by a fixed dose of LPS ("second hit"; 100 ng/mL).

We divided our naive and mature microglia cells into 4 groups: The first group was represented by unstimulated microglia (US Group). The second group included unprimed microglia (UP Group, without the "first hit" on day 1 but stimulated on day 6 with a fixed dose of LPS, 100ng/ml). The third group (ULP

group) was represented by microglia stimulated with an ultra-low dose of LPS on day 1 (1 fg/mL – 100 ng/mL) and re-stimulated at day 6 with a fixed dose of LPS, (100ng/ml). The fourth group was the high-dose-primed group (HP group), stimulated with a high dose of LPS (100ng/ml) at day 1 and re-stimulated at day 6 with fixed dose of LPS 100ng/ml). To evaluate the effects of repeated stimulation with LPS, we normalized the data to the UP Group (in the case of mRNA expression). The US Group served as a negative control. To evaluate the developmental differences in microglial responses, we additionally compared naive microglia to adult microglia within the four mentioned groups.

*Measurement of the protein concentration*

Total protein concentration was determined using the Pierce™ 660 nm Protein Assay Kit (#22662) from Thermo Fisher Scientific (Massachusetts, USA). Ionic detergent compatibility reagent (IDCR) (#22663, Thermo Fischer Scientific) was used in order to reduce interference. Absorbance was measured at 660 nm using a TECAN Infinite 200 Plate reader (Tecan, Switzerland). Protein concentration was then calculated based on the values of the standard curve.

*Cytokine determination*

Cytokine levels in supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits for TNF-α (#430902), IL-6 (#431302) and IL-10 (#431412) obtained from BioLegend (San Diego, CA). The absorbance was determined with a VersaMax Microplate Reader (Molecular Devices, USA) at 450 nm and a second reference wavelength at 570 nm. Cytokine levels of TNF-α, IL-6 and IL-10 were normalized against the protein concentrations of each sample, and depicted as pg/µg of total protein

*Real-time qPCR*

To determine gene expression levels, total RNA was extracted using QIAzol Lysis Reagent (#79306) purchased from Qiagen (Hilden, Germany). RNA concentration and quality were checked by using a Nanodrop ND-1000 machine (Peqlab, Erlangen, Germany). cDNA was synthetized using RevertAid First Strand cDNA Synthesis kit (#K1612) from Thermo Fisher Scientific (Waltham, MA, USA). qPCR reaction was performed by using LightCycler 480 SYBR Green. Primers used in the study are depicted in **Table 1**. Housekeeping genes GAPDH and HMBS were used for normalization. Relative gene expression was calculated by the comparative CT method (Livak and Schmittgen, 2001).

**Table 1.** RT-PCR primers used in the study

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| ***Tnf-α*** | CTGTAGCCCACGTCGTAGC | TTGAGATCCATGCCGTTG |
| ***Gapdh*** | CATGGCCTTCCGTGTTTCCTA | CCTGCTTCACCACCTTCTTGAT |
| ***IL-10*** | ACCAGCTGGACAACATACTGC | TCACTCTTCACCTGCTCCACT |

| | | |
|---|---|---|
| ***IL-1β*** | GGCAGGCAGTATCACTCATT | AAGGTGCTCATGTCCTCAT |
| ***IL-4*** | TGGGTCTCAACCCCCAGCTAGT | TGCATGGCGTCCCTTCTCCTGT |
| ***Arg1*** | TCACCTGAGCTTTGATGTCG | CTGAAAGGAGCCCTGTCTTG |
| ***Tgf-b*** | TGCTTCAGCTCCACAGAGAA | TACTGTGTGTCCAGGCTCCA |
| ***BDNF*** | GACAGTATTAGCGAGTGGGTCA | CCTTTGGATACCGGGACTTT |
| ***Pfkfb3*** | GGAGAGGTCAGAGGATGCAAA | GCTGTTGATGCGAGGCTTTT |
| ***IL-6*** | CCTCTCTGCAAGAGACTTCCATCCA | GGCCGTGGTTGTCACCAGCA |
| ***Hmbs*** | GTTGGAATCACTGCCCGTAA | GGATGTTCTTGGCTCCTTTG |

*Measurement of reactive oxygen species (ROS)*

ROS were measured using the H2DCFDA-assay. The assay is based on the use of 2',7'-Dichlordihydrofluorescein-diacetat (H2DCFDA; #D399, Thermo Fisher Scientific, Waltham, MA, USA), a membrane-permeable reduced form of fluorescein which reacts with reactive oxygen species thereby emitting fluorescence light. For this, microglial cells were seeded into white clear bottom 96-well plates (30,000 cells/well). After becoming adherent, cells were stimulated, as described above. For measurement, the medium was aspirated and 200 µl of H2DCFDA-solution (stock 50 mM 1:1000 in 10 mM HEPES/$CaCl_2$) was added and cells were incubated for 20 min at 37°C. Thereafter, cells were carefully washed twice with an HEPES/$CaCl_2$ solution. Measurement of intracellular ROS levels was performed at 485 nm excitation and 535 nm emission using a TECAN Infinite 200 Plate reader (Tecan, Switzerland).

*Lactate production measurement*

Supernatants from microglial culture were used to measure lactate production by sequential enzymatic reactions (according to (Lin et al., 1999)). Briefly, lactate is converted by lactate oxidase (LO; #L0638, Sigma-Aldrich) to pyruvate and $H_2O_2$. In a second reaction, the chromogenic substrate ABTS (#A1888, Sigma-Aldrich) is converted to a colored dye, catalyzed by horseradish peroxidase (HRP; #77332, Sigma-Aldrich) in the presence of $H_2O_2$ and measured at 405 nm. Lactate levels were normalized for protein concentrations of each sample.

*Statistical analysis*

Statistical analysis was carried out using SigmaPlot Software (SigmaPlot Software, San Jose, USA). Data are presented as scatter plots showing means ± SEM. Experimental groups (adult vs. naive) were compared using one-way ANOVA, followed by Holm-Sidak post-hoc test. Comparisons between groups (treatment vs. unprimed state) were performed with two-way analysis of variance (Two-way ANOVA), followed by Holm-Sidak post-hoc test. Differences were considered significant when $P<0.05$

## 3. **Results**

*Effect of repeated stimulation with LPS on cytokine and iNOS levels in newborn and mature microglia*

Expression and protein levels of pro-inflammatory cytokines TNF-α and IL-6 were evaluated after repeated LPS stimulation in newborn and mature microglia. As expected, unstimulated microglia (US), expressed low cytokine levels (Fig. 1A-D). Unstimulated mature microglia showed higher levels of TNF-α and IL-6 as compared with mature microglia (Fig. 1B and D). Single challenge with 100 ng/mL LPS (UP) led to a marked increase in levels of TNF-α and IL-6 in microglia in both groups. Microglial priming with the ultra-low LPS dose (ULP) followed by a standard fixed LPS dose 6 days later further increased levels of both TNF-α (Fig. 1A,B) and IL-6 (Fig. 1C,D) in newborn microglia, indicating trained immunity. No such effect was observed in mature microglia after stimulation (Fig. 1B and D), which is in accordance with findings from other tissue macrophages (Linehan and Fitzgerald, 2015). These results may suggest that induction of trained immunity leads to a M1-pro-inflammatory like phenotype in newborn microglia. In contrast, priming with LPS at high doses (HP group) led to a robust downregulation of pro-inflammatory cytokines in both groups 6 days after renewed challenge with LPS 100 ng/mL, indicating induced immune tolerance. A similar effect was observed for expression of iNOS mRNA, with newborn microglia displaying both trained immunity after priming with ultra-low LPS doses and tolerance after priming with high LPS concentrations (Fig. 1E). Mature microglia did not show a significant response neither at ultra-low nor at high LPS doses as compared with the unprimed group.

In order to determine whether induction of trained immunity or tolerance shifts the microglial phenotype to a M2-anti-inflammatory state, we also assessed levels of anti-inflammatory cytokines IL-10, IL-4, Arg-1 and TGF-β in newborn and mature microglia. Single challenge with LPS 100 ng/mL markedly increased levels of IL-10 (Fig. 2A,B) but not IL-4, Arg-1 or TGF-β (Fig. 2C,E) in both microglial populations. Interestingly, priming with ultra-low LPS doses had no effect on newborn microglia, but significantly increased IL-4 gene expression in mature microglia (Fig. 2C). Priming with high LPS doses increased levels of IL-10, IL-4, TGF-β and Arg-1 in newborn, but not in mature microglia (Fig. 2A-E). This indicates that induction of tolerance in newborn microglia is associated with a M2-anti-inflammatory phenotype.

*Effect of repeated stimulation with LPS on ROS and BDNF levels in newborn and mature microglia*

Trained immunity has been associated with increased levels of reactive oxygen species (ROS) and therefore we evaluated the impact of maturation and LPS-dose on ROS production in microglia from newborn and mature mice brains. As shown in Fig. 3A, unstimulated mature microglia showed 82% higher ROS levels as compared with newborn naive microglia. Single stimulation with LPS (priming) significantly increased ROS levels in both microglia populations (Fig. 3A). Priming with ultra-low LPS doses followed by a second challenge 6 days later further increased ROS levels in newborn but not in mature microglia. This response further supports induction of trained innate immunity only in newborn microglia. In contrast, priming with high LPS doses reduced ROS levels in both cell populations, supporting induction of a tolerance state. Given the widespread functional role of brain-derived neurotrophic factor (BNDF)

released by microglia in physiological processes involved in learning and memory (Parkhurst et al., 2013) and also in pathological events like neuronal disinhibition of the intrinsic inhibitory system after peripheral nerve injury, causing neuropathic pain (Beggs et al., 2012;Ferrini and De Koninck, 2013), we evaluated the impact of the developmental state on gene regulation of microglial BNDF after repeated challenge with LPS. Priming with ultra-low LPS dose increased BNDF expression in newborn microglia but strongly reduced it in adult microglia. Priming newborn microglia with LPS at a high dose led to a diminished response after the second LPS stimulus compared with the unprimed state. In mature microglia, high-dose priming with LPS further decreased BDNF expression. Interestingly BDNF levels remained significantly higher in naive microglia when compared to mature microglia after stimulation with LPS at both ultra-low and high doses, possibly indicating a higher plasticity and stronger neuroprotective response of these cells in an inflammatory environment.

*Effect of repeated challenge with LPS on levels of metabolic enzymes in newborn and mature microglia*

Since metabolic reprogramming has been found to be a crucial step for the induction of trained immunity in peripheral innate immune cells (Dominguez-Andres et al., 2019), we assessed the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB)3, the rate limiting enzyme of glycolysis and lactate production, in both newborn and mature microglia. (PFKFB)3-driven macrophage glycolytic metabolism was found to be a crucial component of their innate immune response (Jiang et al., 2016). As shown in Fig. 3C, there was no significant difference in expression of the glycolytic enzyme PFKFB3 between unstimulated microglia obtained from newborn and mature mice. Priming with the ultra-low LPS dose induced a marked increase in PFKFB3 gene expression in newborn as well as adult microglia (Fig. 3C). In contrast, priming with LPS at a high-dose reduced PFKFB3 gene expression in newborn but not in adult microglia (Fig. 3C).

*Effect of repeated challenge with βglucan on cytokine levels in newborn and mature microglia*

In order to further characterize the impact of maturation on microglial response after repeated challenge with PAMPs, cells were primed with different doses of β-glucan, followed by a stimulation with LPS (100 ng/mL) at day 6. As shown in supplementary figure1A, priming with β-glucan induced a similar pro-inflammatory response as LPS in newborn but not mature microglia. Similar as for LPS, priming with ultra-low *βglucan* doses further increased levels of TNF-α and IL-6 and high concentrations reduced release of both cytokines in newborn microglia (Suppl. Fig. 1A,B). In contrast, mature microglia did not show a significant response to *βglucan*. These findings suggest a pathogen-mediated induction of trained immunity and tolerance only in newborn microglia in a dose-dependent manner, as previously shown for LPS.

## 4. **Discussion**

Our study supports recent evidences indicating that brain microglia are able to develop an innate immune memory after repeated immune challenge, probably involving an hormetic mechanism (Wendeln et al., 2018;Lajqi et al., 2019). Furthermore, we show here for the first time that the pattern of adaptive responses in microglia is also regulated by the developmental state of these cells. In particular, we found that ultra-low PAMP levels prime microglia derived from newborn mice towards an enhanced pro-

inflammatory state as evidenced by an increase in levels of TNF- α and IL-6, ROS production and iNOS expression, which reflects induction of trained innate immunity, as already described for other immune cells from the periphery (Netea et al., 2016;Bauer et al., 2018). In contrast, microglia derived from mature mice brains did not exhibit a similar reinforced response after priming with ultra-low doses of prototypical PAMPs This findings are in line with previous studies on primary microglia isolated from adult mice indicating a dose-dependent immune tolerance but not priming, after stimulation with low doses of LPS (5–50 pg/ml) (Schaafsma et al., 2015). Intriguingly, a higher LPS dose found to activate a robust pro-inflammatory response after a single administration (Kannan et al., 2013;Frister et al., 2014;Schaafsma et al., 2015;Matt et al., 2016), induced immune tolerance independent of the developmental state of microglia after repeated challenge.

Current work identifies metabolic reprogramming as a key hallmark of innate immunity along with immune cell activation. It is characterized by a fine-tuned pattern of metabolic regulations that become rewired after repeated immunological challenges, probably via epigenetic modifications (Arts et al., 2016;Van den Bossche et al., 2017). Although the proposed causal relations between immunometabolic rearrangements and sustained adaptive responses are still not proven for microglial cells, we recently showed that systematic application of LPS dose-dependently induces innate immune memory-like responses in newborn microglia (Lajqi et al., 2019). Long-term immune memory in microglia was also clearly demonstrated *in vivo* after LPS administration in a mouse model of Alzheimer's pathology (Wendeln et al., 2018). The study provided evidence for both trained immunity (in response to singular LPS challenge) and immune tolerance (after repeated consecutive challenge with LPS), as indicated by altered microglial cytokine release within several days after treatment. This altered response had a marked impact on neuropathology several months later. In line with such evidences, we found here that trained immunity driven by repeated LPS challenges in newborn microglia was associated with a shift towards aerobic glycolysis as the dominating immunometabolic process herein. Both, induction of the glycolytic activator PFKFB3 and increased lactate production suggest a possible TCA-cycle remodelling, probably by itaconate, as previously described elsewhere (Lampropoulou et al., 2016). Furthermore, itaconate pathway was found to play a central role linking innate immune tolerance and trained immunity in human monocytes after stimulation withβ-glucan (Dominguez-Andres et al., 2019).This metabolic switch feeds glycolysis-dependent pathways, especially the pentose phosphate pathway which supports inflammatory responses by generating amino acids for protein synthesis, ribose for nucleotides and NADPH for the production of reactive oxygen species by NADPH oxidase (Nagy and Haschemi, 2015;Lampropoulou et al., 2016;Borst et al., 2018). A comparable immunometabolic switch towards aerobic glycolysis occurred in mature microglia after priming with the ultra-low LPS dose and subsequent LPS stimulation as well, however, this did further increase pro-inflammatory cytokine levels.

These findings highlight the particular importance of trained immunity in microglia because of their longevity and it may be responsible for permanent modification of their molecular profile (Prinz and Priller, 2014;Tay et al., 2017).

Markers of the so-called alternative or neuroprotective microglial phenotype (also known as M2), characterized by the release of anti-inflammatory molecules including IL-4, IL-10 and TGF- α as well as neurotrophic factors (e.g. BDNF), showed opposing responses owing to differential priming and

maturation. Gene expression of the anti-inflammatory cytokines was not modulated by ultra-low LPS priming. However, pre-stimulation of microglial cells derived from newborn mice with the standard LPS dose exhibited an enhanced gene expression of the anti-inflammatory cytokines, whereas mature microglia remained unchanged. Mature microglia did not show increased levels of BDNF in an inflammatory environment, which might explain reduced neuronal and synapse recovery after inflammatory injury in the adult brain.

Interestingly, these data underscore the inherent neuroprotective capacity of immature microglia, presumably relevant in order to compensate for the vulnerability of the immature brain to the effects of inflammation (Hagberg et al., 2015). In line with these findings, inflammatory challenge by ultra-low LPS doses sensitized microglia which responded with upregulation of BNDF expression. On the contrary, priming with high-doses induced a downregulation. Studies on the effect of development and dose of PAMPs on microglia priming and BDNF expression or release were till now missing.

Release of reactive oxygen species was significantly altered after a challenge with LPS in microglia obtained from newborn mice compared with mature microglia, and showed a dose-dependent response after priming (e.g. trained immunity and immune tolerance). These results are in line with the regulation in gene expression of iNOS also found in newborn microglia (Fig. 1E) whereby newborn microglia showed enhanced gene expression in response to ultra-low LPS priming but immune tolerance when cells were primed with the standard LPS dose. Mature microglia did not show trained immunity but tolerance, however, levels of ROS and iNOS were already higher in unstimulated and unprimed microglia as compared with newborn microglia. Even if the biological relevance of these findings cannot be drawn from our data, it has to be considered that the immature brain appears to be especially vulnerable against oxidative stress. There is compelling evidence that high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentrations of antioxidants, and increased availability of “free” redox-active iron are responsible this effect (Siddappa et al., 2002;McQuillen and Ferriero, 2004;Saugstad, 2005).

Our finding that even ultra-low LPS doses (e.g., in the femtomolar range) are able to prime microglia raises the question whether this PAMP challenge is of physiological relevance, or it might result from particular experimental conditions. Previous studies revealed that as few as 100 invading Gram-negative bacteria, corresponding to femtomoles of endotoxins, are already able to stimulate host responses (Beutler and Rietschel, 2003;Weiss, 2003;Freudenberg et al., 2008). LPS is a well-characterized PAMP found in the outer leaflet of the outer membrane of most Gram-negative bacteria. The structurally unique lipid A region of LPS is the principal determinant of this pro-inflammatory activity. LPS-immune cell activation is mediated by activation of TLR4 (Poltorak et al., 1998;Raetz and Whitfield, 2002) and by caspases of the non-canonical inflammasome, as a cytosolic LPS-recognition system (Shi et al., 2014). While the cytosolic LPS sensing mechanism has still to be identified, extracellular or intra-vacuolar LPS effect via TLR4 have been largely elucidated (Rosadini and Kagan, 2017;Weiss and Barker, 2018). Potent activation of TLR4 by LPS requires initial interactions of LPS-binding protein (LBP) and CD14 with LPS-rich interfaces followed by extraction and transfer of individual LPS monomers first to CD14 (Gioannini et al., 2004;Prohinar et al., 2007;Ryu et al., 2017). Interestingly, enhanced TLR4 reactivity has been linked to a “two hit” response in a mouse model of thermal injury whereby burn mice showed an increased response when challenged

with LPS 7 days after injury (Murphy et al., 2005). Whether increased reactivity of TLR4 might underlie the potentiating effects found in newborn mice after priming with ultra-low LPS doses diserves further investigation.

Activation of MD-2/TLR4 by LPS requires binding of an individual LPS molecule (LPS monomer) to MD2/TLR4 (Prohinar et al., 2007) and dimerization of the LPS.MD-2.TLR4 ternary complex (Park et al., 2009). Recent studies revealed that as few as 25 LPS-MD-2.TLR4 complexes per cell can trigger measurable pro-inflammatory responses, implying very efficient dimerization of these ternary complexes (Teghanemt et al., 2013). Therefore, priming effects with ultra-low PAMPs doses might elicit intracellular regulatory mechanisms important for innate immune memory.

Divergent immune responses observed between naïve and mature microglia might be explained on the basis of significant differences found in the gene signature of neonatal (so called pre-microglia) and mature microglia (Butovsky et al., 2014;Matcovitch-Natan et al., 2016). Neonatal mouse microglia are still immature and lack the typical microglial gene signature and are more similar to primitive macrophage populations (Butovsky et al., 2014). Recently, microbiome-microglia interactions were shown to be an important mechanism involved in the process of microglia maturation (Erny et al., 2015). While adult microglia are continuously exposed to microbiome products like short-chain fatty acids, naive microglia isolated from newborn mice lack such a priming effect. Therefore, mature microglia might be already "pre-primed", which would partially explain increased increased levels of ROS and TNF-α/IL-6 observed in unstimulated microglia, and the lack of response after priming with LPS or β-glucan at ultra-low doses. This results support previous findings in our lab showing that *in vitro* and *in vivo* maturation and aging processes lead to priming of microglial cells with increased basal expression of inflammatory cytokines (Stojiljkovic et al., 2019). Such observations implicate that stimulation or infection during the critical period of microglia development might have a crucial impact on the vulnerability to later brain pathologies after a second hit event.

Findings from the present study are particularly relevant in the context of neuropathological conditions, as accumulating evidences point to a role of innate immune memory (trained immunity vs. tolerance) in neuroinflammation in association with neuropsychiatric issues including autism spectrum disorders (ASD) [for review see (Jyonouchi, 2019)].

In summary, we found a differential response to repeated PAMP challenges between naive microglia obtained from newborn murine brain and adult microglia, leading to induced innate immune memory responses. Whereas naive microglia appear to be prone for an orchestrated pro-inflammatory response in a dose-dependent manner, resulting in trained immunity after ultra-low dose priming by PAMPs, mature microglia did not show trained immunity. In contrast, priming with high-LPS doses induced immune tolerance in both naive and mature microglia. Induction of trained immunity was accompanied by a metabolic reprogramming, in agreement with previous studies.

## 5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 6. Author Contributions

TL, MS, RB, RW and CS contributed to the conception and design of the study; DLW provided the C. albicans derived β-glucan; TL and MS performed the experiments; TL, RB and MS performed the statistical analyses; CS and RB wrote the manuscript; OWW and RW critically discussed the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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**Figure legends:**

Figure 1.

Effect of maturation and repeated LPS stimulation on pro-inflammatory responses of murine brain microglia. Microglia isolated from newborn (orange) and adult (green) mice were primed initially by ultra-low (ULP, 1 fg/mL) or high (HP, 100 ng/mL) doses of LPS, followed by a second stimulation (day 6) with 100 ng/mL LPS. The data were normalized and compared to unprimed microglia (UP Group-without any stimulation at day 1 with stimulation at day 6 with fixed dose of LPS 100ng/ml). As negative control unstimulated microglia was used (US Group-without LPS or β-glucan stimulation neither at day 1 or at day 6). RNA samples (6h) and supernatants (24h) were collected after the 2nd stimulation and analyzed for gene expression of TNFα (A, n=5-6), IL-6 (C, n=4-5) and iNOS (E, n=4-5) and cytokine production of TNFα (B, n=7-8) and IL-6 (D, n=6-8) by ELISA (normalized to total protein concentration). Data are shown as scatter dot plots as means + SEM, # $p < 0.05$ vs. unprimed conditions within each age group (naive or mature), § $p < 0.05$ vs. adult microglia within each stimulation condition (US, UP, ULP, HP).

Figure 2.

Effect of maturation and repeated LPS stimulation on anti-inflammatory responses of murine brain microglia. Microglia isolated from newborn (orange) and adult (green) mice were primed initially by ultra-low (ULP, 1 fg/mL) or high (HP, 100 ng/mL) doses of LPS, followed by a second stimulation (day 6) with 100 ng/mL LPS. The data were normalized and compared to unprimed microglia (UP Group-without any stimulation at day 1 with stimulation at day 6 with fixed dose of LPS 100ng/ml). As negative control unstimulated microglia was used (US Group-without LPS or β-glucan stimulation neither at day 1 or at day 6). RNA samples (6h) and supernatants (24h) were collected after the 2nd stimulation and analyzed for gene expression of IL-10 (A, n=4-5), IL-4 (C, n=3-4), Arg-1 (D, n=3-4) and TGF-β (E, n=3-5) and cytokine production of IL-10 (B, n=5-6) by ELISA (normalized to total protein concentration). Data are shown as

scatter dot plots as means + SEM, # p <0.05 vs. unprimed conditions within each age group (naive or mature), § p <0.05 vs. adult microglia within each stimulation condition (US, UP, ULP, HP).

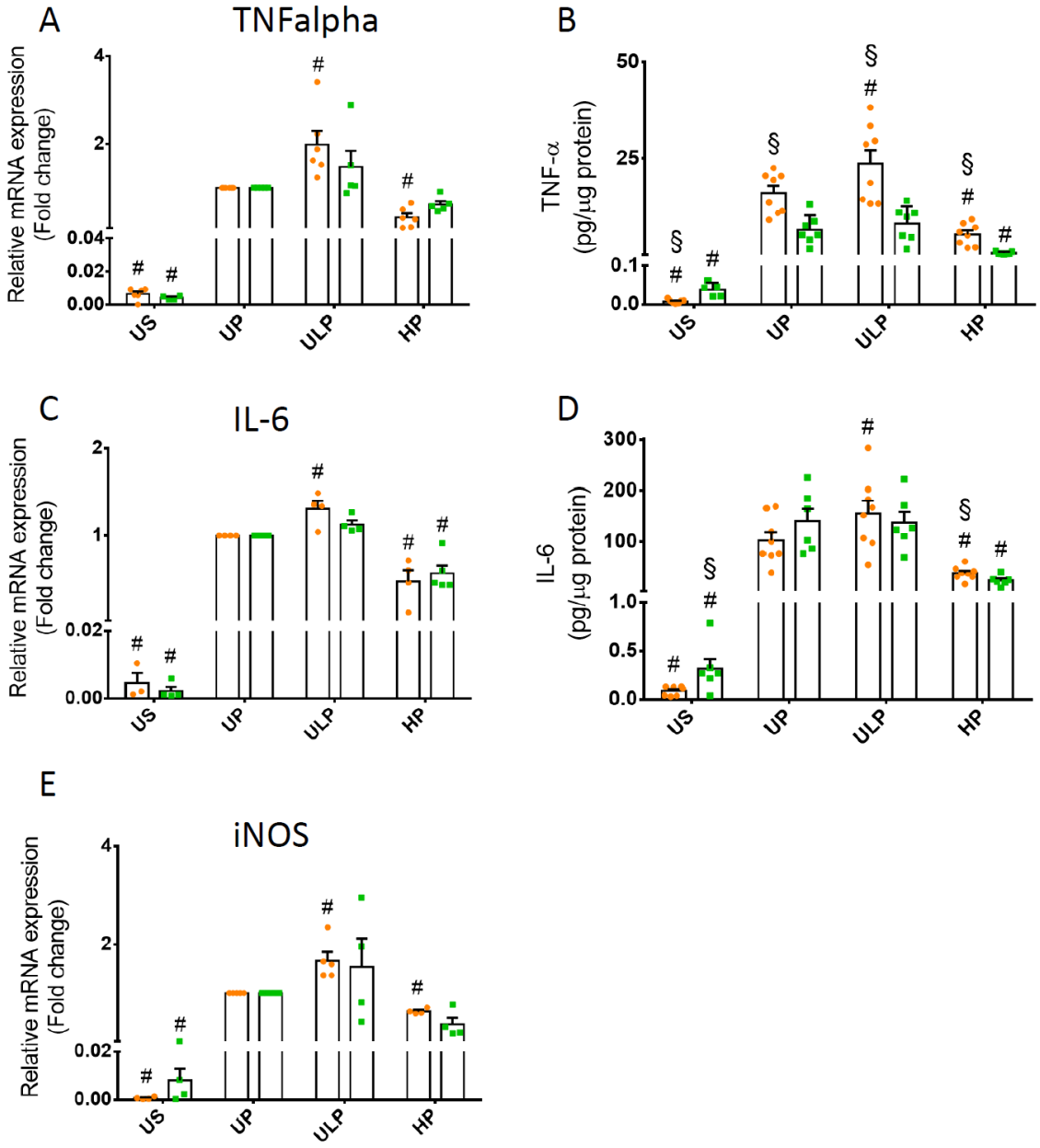
Figure 3.

Effect of maturation and repeated LPS stimulation on ROS production, BDNF and metabolic rewiring of murine brain microglia. Microglia isolated from newborn (orange) and adult (green) mice were primed initially by ultra-low (ULP, 1 fg/mL) or high (HP, 100 ng/mL) doses of LPS, followed by a second stimulation (day 6) with 100 ng/mL LPS. The data were normalized and compared to unprimed microglia (UP Group-without any stimulation at day 1 with stimulation at day 6 with fixed dose of LPS 100ng/ml). As negative control unstimulated microglia was used (US Group-without LPS or β-glucan stimulation neither at day 1 or at day 6). RNA samples (6h) and supernatant (24h) were collected after the 2nd stimulation and analyzed for ROS production (A, n=3-4) and gene expression of BDNF (B, n=4-5) and PFKFB3 (C, n=4) as well as lactate concentration in supernatant (D, n=5-6). Data are shown as scatter dot plots as means + SEM, # p <0.05 vs. unprimed conditions within each age group (naive or mature), § p <0.05 vs. adult microglia within each stimulation condition (US, UP, ULP, HP).

Supplementary Figure S1.

Effect of maturation and repeated .-glucan stimulation on pro-inflammatory responses of murine brain microglia. Microglia isolated from newborn (orange) and adult (green) mice were primed initially by ultra-low (ULP, 100 fg/mL) or high (HP, 1 µg/mL) doses of β-glucan, followed by a second stimulation (day 6) with 100 ng/mL LPS. The data were normalized and compared to unprimed microglia (UP Group-without any stimulation at day 1 with stimulation at day 6 with fixed dose of LPS 100ng/ml). As negative control unstimulated microglia was used (US Group-without LPS or β-glucan stimulation neither at day 1 or at day 6). Supernatants were collected 24h after the 2nd stimulation and analyzed for cytokine production of TNF. (A, n=4-8) and IL-6 (B, n=5-8) by ELISA (normalized to total protein concentration). Data are shown as scatter dot plots as means + SEM, # p <0.05 vs. unprimed conditions within each age group (naive or mature), § p <0.05 vs. adult microglia within each stimulation condition (US, UP, ULP, HP)

Fig. 1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TNFalpha
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2
0.04
0.02
0.00
US
UP
ULP
HP
B
TNF-α (pg/μg protein)
50
25
0.1
0.0
C
IL-6
Relative mRNA expression (Fold change)
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1
0.02
0.00
D
IL-6 (pg/μg protein)
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200
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E
iNOS
Relative mRNA expression (Fold change)
4
2
0.02
0.00

Fig. 2

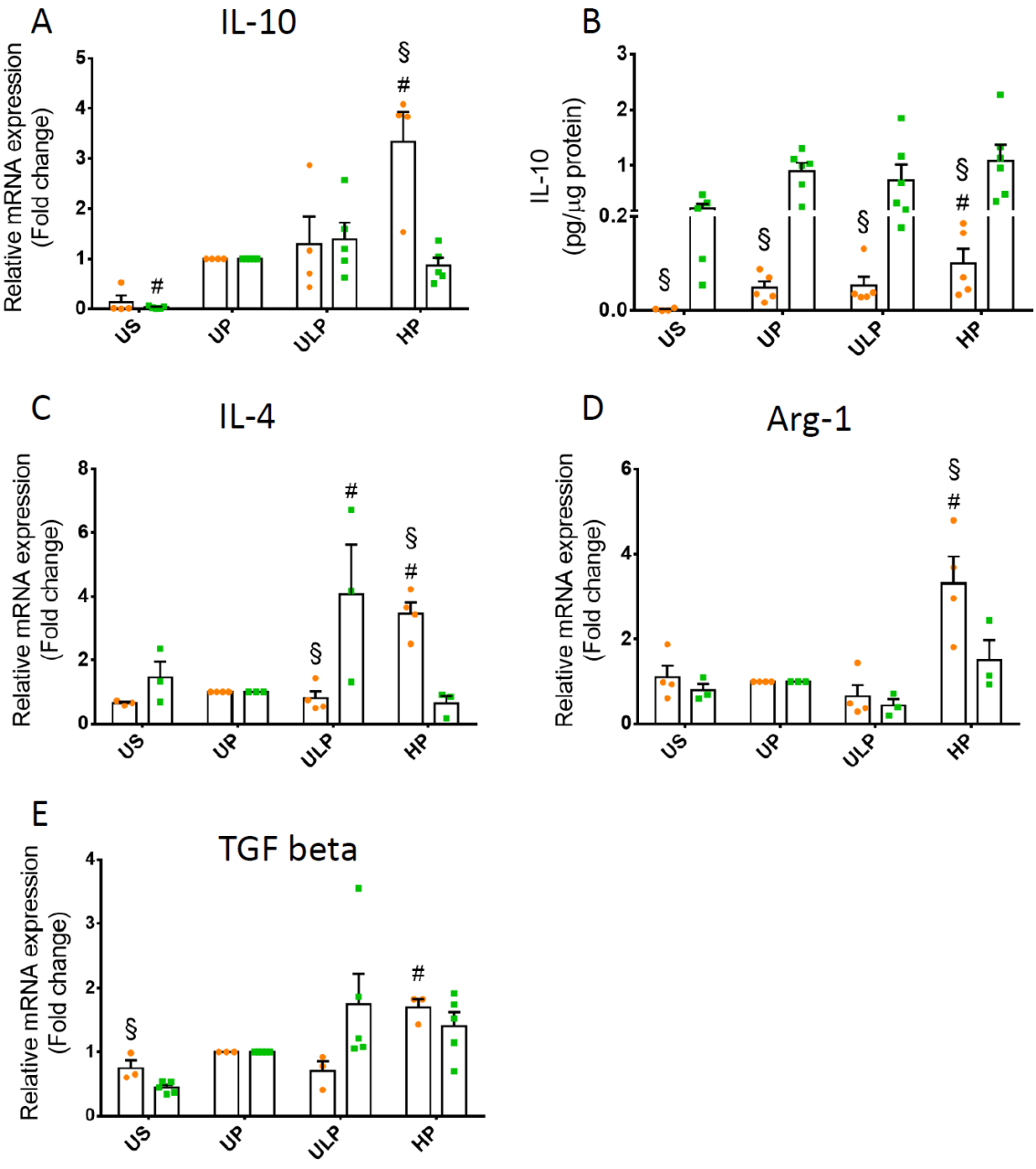
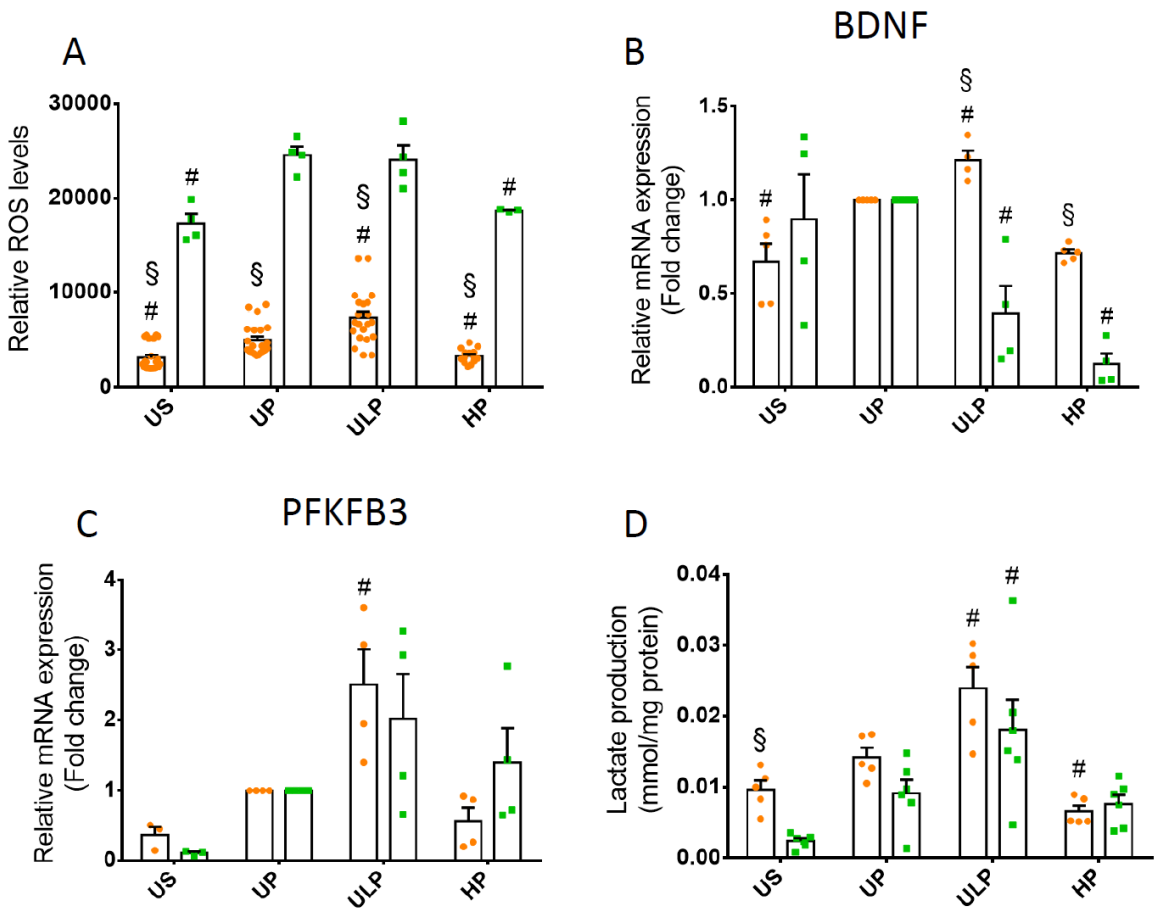

A
IL-10
Relative mRNA expression (Fold change)
B
IL-10 (pg/μg protein)
C
IL-4
Relative mRNA expression (Fold change)
D
Arg-1
Relative mRNA expression (Fold change)
E
TGF beta
Relative mRNA expression (Fold change)
US
UP
ULP
HP

Fig. 3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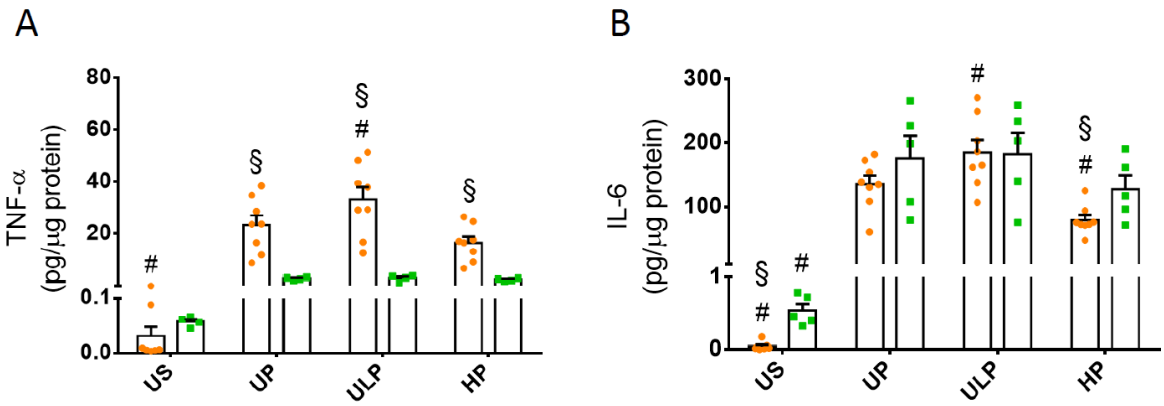
Relative ROS levels
30000
20000
10000
0
US
UP
ULP
HP
B
BDNF
Relative mRNA expression
(Fold change)
1.5
1.0
0.5
0.0
C
PFKFB3
Relative mRNA expression
(Fold change)
4
3
2
1
0
D
Lactate production
(mmol/mg protein)
0.04
0.03
0.02
0.01
0.00

Suppl. Fig. 1


A
TNF-α (pg/µg protein)
80
60
40
20
0.1
0.0
US
UP
ULP
HP
B
IL-6 (pg/µg protein)
300
200
100
1
0

**Manuscript III**

Chapter 14

# Pathogen-Induced Hormetic Responses*

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## 14.1 INNATE IMMUNE MEMORY AS A NEW PARADIGM

The mammalian immune system comprises complex interactions of innate immune cells, which build the first line defense against invading pathogens and adaptive immune cells, which mediate specific responses to pathogens and develop an immune memory. Together these reactions are facilitating an adapted immune reaction to repeated infection [1]. Invading pathogens are recognized by their pathogen-associated molecular patterns (PAMPs) binding to pattern recognition receptors (PRR) on innate immune cells [2]. This interaction triggers conserved signal cascades, induces the production of cytokines and chemokines, and provokes an appropriate adaptive immune response leading to the development of an immune memory [3]. Interestingly, organisms, e.g., plants, which lack an adaptive immune system display adaptive responses to reinfection. Consequently, they must possess a kind of innate immune memory [4].

Recently, a long-term memory of the mammalian innate immune system was considered by Quintin et al. The authors showed that mice pretreated with a nonlethal dose of *Candida albicans* were protected from a subsequent infection with lethal doses of this fungal pathogen 7 days after the first infection [5]. The survival of these mice was independent of T- and B-cell action since the effect disappeared in monocyte-deficient mice [5]. This phenomenon is referred as trained immunity opposing the well-known endotoxin-induced immune tolerance. Innate immune tolerance is characterized by reduced production of pro inflammatory cytokines in response to a secondary infectious stimulus. In contrast, trained immunity shows a strong proinflammatory response to a secondary stimulus, which persists for more than 1 week after the first pathogen challenge [6].

The new dogma of the trained immunity or innate immune memory was further analyzed in vitro by pretreating human peripheral blood mononuclear cells with the fungal cell wall compartment β-glucan. This molecule binds to the Dectin-1 receptor inducing an antifungal defense response in macrophages, potentially involving the tyrosine kinase Syk as an essential signaling mediator [7,8]. β-Glucan binding to Dectin-1 was shown to provoke Syk-independent or -dependent signaling pathways. Stimulation of the Syk-dependent pathway leads to IL-6 and IL-10 release, whereas the independent pathway induces TNFα most probably via crosstalk with TLR2 and TLR6 signaling [7,8]. Furthermore, the Syk-dependent pathway provokes the production of reactive oxygen species (ROS) in order to activate antimicrobial defense [7].

In contrast to innate immune training, the phenomenon of endotoxin tolerance has been known for a long time and has been well characterized (reviewed in [9]). It plays an important role during sepsis, a syndrome that occurs due to a maladaptive response to infection accompanied by organ failure [10]. The tolerance state is usually correlated to increased mortality and a high risk of secondary infections [10]. In vitro innate immune tolerance is mostly induced by

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lipopolysaccharides (LPS), which are usually exposed on the cell wall of gram negative bacteria such as *Escherichia coli*. LPS is recognized by a PRR called Toll-like receptor 4 (TLR4). Upon TLR4 binding several signaling cascades are induced leading to transcriptional changes via activation of NFκB or MAPK pathways.

Taken together training and tolerance emerge as alternative responses of the innate immune system to PAMPs and pathogenic bacteria. Evidently, specific PAMPs can induce tolerance, whereas others induce training. Until now the mechanistic background of these opposing effects of PAMPs on innate immune cells is unknown. However in the next section, we will introduce some ideas about the important role of pathogen dose on training and tolerance development.

## 14.2 TRAINED RESISTANCE AND TOLERANCE RESPONSES ARE SPECIFIED BY PATHOGEN DOSE

The current literature describes the occurrence of trained resistance and tolerance responses of the innate immune system as opposite effects induced by specific PAMPs. Hence, β-glucan has been identified as the prototypic inducer of trained immunity, whereas LPS appears as the typical mediator of tolerance responses of innate immune cells.

However, a recent study might lead to a novel understanding of the contrasting effects of PAMPs on innate immune memory. Ifrim et al. used a two-hit approach to analyze the dose-dependent effects of PAMPs on monocyte immune response [11]. Initial treatment with low doses of LPS followed 1 week later with a medium-dose LPS provoked increased production of the proinflammatory cytokine TNFα as compared to the immediate effect of a single dose of LPS. In evident agreement with the training paradigm, the initial LPS challenge at the given dose sensitized macrophages. In contrast to the trained immunity effects of low doses of LPS, initial treatment of the cells with higher doses induced tolerance development, i.e., significantly decreased production of TNFα in comparison to the "one-hit" control. In our laboratory, similar dose-dependent development of training and tolerance responses have been observed after treatment of monocytes with β-glucan (Jentho et al., unpublished data). Together, these data suggest a general long-term reaction pattern of innate immune cells, which predominantly depends on the PAMP dose sensed by the cell.

Pathogen dose-dependent development of two differential hormetic responses of the innate immune system may add to the current understanding of hormesis. Both trained immunity as well as tolerance represents vital functional patterns of the organism stressed by pathogens. Whereas trained immunity induces resistance reactions that reduce the number of pathogens, tolerance to infection indicates maintenance and repair responses that act without directly targeting pathogens [12–14].

Understanding the molecular mechanism of these differential adaptive responses of the innate immune system seems of major interest and represents a promising area for the development of novel approaches for the prevention and treatment of infectious diseases.

## 14.3 MOLECULAR SIGNATURES OF TRAINED IMMUNITY AND TOLERANCE RESPONSES

Molecular signaling responses to pathogen attacks have been mostly separately investigated, either for training or for tolerance development. In line with the dynamics of adaptive responses of the innate immune system, immediate signaling reactions caused by pathogens and enduring processes have been analyzed. Accordingly, signaling responses to pathogen attacks involve initial fast track signaling processes, alterations in the expression pattern and epigenetically fixed responses.

As mentioned above *immediate signaling processes* initiating pathogen-induced training responses have been elaborated by recent work on β-glucan effects on the innate immune system. Both in vitro and in vivo data indicate involvement of the mechanistic target of rapamycin (mTOR) signaling pathway in the induction of trained immunity and the corresponding epigenetic changes [15]. mTOR acts as the central mediator of anabolic processes [16]. Current understanding of signaling reactions involved in tolerance development is less developed. AMP-activated protein kinase (AMPK), a key molecule in cellular energy expenditure that is also involved in the induction and control of tolerance responses, might play a prominent role. In contrast to mTOR, AMPK activity is induced at low cellular energy level mediating maintenance and repair reactions in the affected cells [17]. Stimulation of AMPK activity by the antidiabetic drug metformin has been shown to suppress LPS-induced TNFα production by macrophages [18]. This supports the idea that AMPK exerts crucial functions in the control of tolerance responses. In addition, strong metabolic stimulation in the presence of large LPS concentration induces accumulation of $NAD^+$, which in turn activates sirtuin deacetylases, leading to inhibition of gene transcription [19]. The proposed central function of the evolutionary conserved mTOR signaling in trained resistance and the relevance of AMPK in tolerance development conjoin pathogen-induced reactions with a variety of different cellular responses. Both signaling proteins control a multitude of physiological responses to

diverse environmental stressors [16]. Environmental challenges such as heat, cold, toxins, or radiation possess the ability to induce anabolic reactions via mTOR. Alternatively, the same stimuli can provoke catabolic and maintenance responses controlled by AMPK. Hence, the identification of mTOR and AMPK as central mediators of innate immune responses integrates training and tolerance responses of innate immune cells to a general response pattern of cells and organs toward environmental stressors. The signaling reactions induced by pathogens and other environmental stressors merge inside the affected cells and provoke similar responses. Thus, resistance and tolerance appear as consecutive steps of hormetic responses of cells and organs to increasing doses of PAMPs and other stressors. mTOR and AMPK fulfill antagonistic functions in the control of these reactions. The mutual ability of mTOR and AMPK to inhibit each other [16] complies with a central function of these mediators in the adjustment of opposing trained immunity or tolerance responses of the affected cells.

*Expression control* represents an inherent element of pathogen-induced hormetic responses of innate immune cells. Saeed et al. recently showed distinct long-term effects of LPS on cytokine production [20]. In contrast to β-glucan pretreated macrophages, these cells revealed more transcriptional and epigenetic similarities to undifferentiated monocytes than to macrophages [20]. Epigenetic and transcriptomic analysis revealed LPS-dependent induction of genes involved in the immune responses and repression of genes associated with cellular differentiation. In comparison to this effect, the β-glucan trained cells showed an increase in epigenetic regulations of genes involved in lipid biosynthesis, metabolism, and lysosome pathway. Interestingly, β-glucan is able to reverse the LPS-induced epigenetic silencing of specific promoters and restores the secretion of cytokines as well as the transcription of genes involved in lipid biosynthesis [21].

*Epigenetically fixed long-term alterations* seem to be distinctive for trained macrophages. Accordingly, β-glucan-trained cells exhibit a specific histone acetylation and methylation pattern including H3K27 acetylation and H3K4 trimethylation. The changes in the H3K27 acetylation and H3K4me3 correlated to transcriptional changes of several gene clusters including the promoters of central metabolism mediators like mTOR [15]. Likewise, promoters of enzymes involved in the glycolysis were also epigenetically modified 1 week after the β-glucan treatment. These activated innate immune cells exhibit a typical change of oxidative phosphorylation to aerobic glycolysis characterized by increased glucose consumption as well as lactate production [15,22]. These recent data exemplify the extraordinary importance of epigenetic processes in the enduring responses of (innate immune) cells.

The ability of immune and parenchyma cells to react to pathogenic stimuli is evolutionary conserved from flies to humans [23]. In particular, myeloid cells like macrophages express a huge panel of receptors (PRRs) to sense threats from fungal, viral, or bacterial origin, e.g., Toll-like receptors TLR2, TLR3, and TLR4, respectively [24]. All stimuli-induced PRR-signaling cascades converge in the nucleus where transcription factors (TFs) act on chromatin and modulate transcriptional output [25]. However, TF activity depends highly on chromatin accessibility which is modulated by epigenetic marks, and thus, chromatin-modifying enzymes [26]. Interestingly, recent studies showed that encounter of monocytes with PRR ligands shapes their epigenetic profile creating a memory-like structure that persisted in differentiated macrophages (preconditioning) [11,20]. Importantly, sepsis as a chronic inflammatory disease is characterized at first by hyperinflammation, but nonreactive immune cells are causing mortality in late stages of disease (immunoparalysis) [27]. Thus, studying pathogen-induced hormetic responses and related epigenetic reprogramming might be the key to understand adverse outcomes of patients suffering from inflammatory diseases.

Changes in epigenetic marks are observable during monocyte to macrophage transition [20,28]. Differentiation is fulfilled by switching of around 3000 H3K27 acetylation marks in promoter and enhancer regions, leading to increased transcription [20]. Additionally, monomethylated histones H3K4 at promoter sites become trimethylated (H3K4me3) [20]. In contrast, demethylation of DNA occurs in distant enhancer regions but not at transcriptional start sites via DNA demethylase TET2 leading to cytoskeletal remodeling and increased phagocytosis in macrophages [28]. These processes are concomitant with recruitment of TFs to promoters and enhancers such as Krueppel-like factors, NFκB family members, and AP-1 generating a different TF repertoire during monocyte to macrophage transition [20,28]. As demonstrated in LPS preconditioning experiments, cytokine production of monocytes and microglia that experienced subsequent TLR2 or TLR3 stimulation was reduced [11,29]. On a molecular basis, LPS preconditioning was paralleled with less H3ac and H3K4me3 marks at proinflammatory promoters, while no altered expression or phosphorylation of TLR4 signal transducers was detectable [29]. These data raised the hypothesis that long-term epigenetic alterations are crucial for long-living innate immune cells like microglia that are characterized by low turnover numbers in contrast to monocytes which are permanently replenished from bone-marrow progenitor cells [29].

Ongoing investigations of the dynamics of pathogen-induced hormetic responses of innate immune cells increasingly disclose the complex signaling processes involved. Valuable targets for therapeutic treatment of infectious diseases become increasingly evident.

## 14.4 MEDICAL RELEVANCE

Innate immune cells are the first line of defense against pathogens. Whether pathogens will induce training or tolerance depends on the dose, time of exposure, and nature of exposure. These effects fit to the hypothesis of hormesis, which is of significant importance in medicine. As reviewed in Calabrese et al. , there are a number of examples relevant for medical practice where low doses of antibiotics actually promoted bacteria growth, low doses of cytostatics promoted cancer growth, and low doses of toxin arsenite increased proliferation of lymphocytes [30]. Low to moderate physical activity is known to be beneficial in preventing several diseases [31]. Low doses of alcohol might be beneficial in decreasing overall mortality from cardiovascular diseases [32]. These hormesis like effects may motivate to further elucidate and understand the mechanisms behind training and tolerance in order to use them for medical treatments. It seems reasonable that low or high doses of pathogen or pathogen products could be used therapeutically. Provoking training responses might promote resistance reactions against pathogens, while tolerance induction might induce antiinflammatory cytokines. The following paragraphs are aimed to review the relevance of trained resistance and tolerance responses in human medicine.

The extraordinary importance of the mechanistic understanding of training and tolerance responses becomes apparent in tumor treatment. Sensitization and strengthening the response of the innate immune system is of high relevance in oncology. In cancer patients repeated LPS injections were shown to induce antitumor immunity [33]. However, repeated application of LPS induces tolerance with decreased responses and corresponding loss of therapeutic efficacy. Interestingly, adding IFN-$\gamma$ was shown to restore the antitumor responses of monocytes [34,35]. Another example of medical application of trained immunity is usage of BCG vaccine as a first line of therapy for the treatment of superficial forms of bladder cancer approved by the FDA (U.S. Food and Drug Administration) [36].

One of the main targets of vaccination is the frail population like elderly, newborns, patients with chronic diseases, cancer patients, or immunosuppressed patients. The role of innate immune memory in vaccine development has been reviewed by Toepfer et al. [37]. First, unspecific training with pathogen products was shown to induce broad defense against several unrelated pathogens. Long-term epigenetic changes in innate immune cells might provoke long-term protection against reinfection with the pathogen. Substances inducing training responses could be used as adjuvants to enhance the response to vaccine especially in vulnerable populations. Finally, trained immunity could increase the efficacy of some low-efficiency vaccines and solve the problem of highly antigen diverse infections like HIV, Hepatitis C virus (HCV), and influenza. Vaccination would be also important in newborns where specific immunity is still not developed, and could be supportive to prevent deaths from respiratory syncytial virus (RSV) infection, an important cause of death in newborns [38]. On the other hand, vaccine from pathogen products that induce tolerance could be beneficial in autoimmune diseases to halt hyperinflammation.

Recent data reveal that pathogens and pathogen products like live culture preparation of the Bacillus of Calmette and Guerin (BCG) strain of *Mycobacterium bovis* vaccine is known to induce nonspecific protection against unrelated infections and pathogens [39]. Additionally, it was found that BCG vaccine has been associated with an overall reduction in mortality, which could not be assigned only to the defense against mycobacterium infections [40,41]. Other vaccines like measles and smallpox have been reported to induce similar beneficial effects [42]. It has been proven experimentally that the BCG-induced protective effects are realized by the innate immune system since T- and B-cell-deficient mice were still protected to reinfection [43].

Several infectious pathogen products have been found to sensitize or desensitize the immune system. Interaction between immune system and normal commensal bacteria and fungi is necessary for maturation of our immune system [42]. A recent publication elucidated the role of skin microbiome and mycobiome in activating and educating host immunity thus adding to the knowledge about the interplay between immune systems and microbiota [44]. Bacteria, fungi, and their products seem to be able to modulate our innate immune system and to decrease mortality in some diseases. Quintin et al. showed that nonlethal doses of *C. albicans* pretreatment prevent death of mice after lethal dose restimulation of *C. albicans* and this effect was monocyte dependent [5]. Additionally, other cells of the innate immunity like $\gamma\delta$ T cells of innate immune system showed memory like responses after infection with *Staphylococcus aureus* and were protective after reinfection [45]. Moreover, recent investigations suggest that innate immune memory, mediated by specific cellular and molecular programs, contributes to the localized host defense in recurrent skin and skin structure infection caused by methicillin-resistant *S. aureus* (MRSA) [46]. These insights support the development of targeted immunotherapeutic strategies to address the challenge of MRSA infection [46]. Besides, parasite *Nippostrongylus brasiliensis* primes the macrophages and induces a faster clearance of reinfection, independent from specific immunity but dependent on macrophages and neutrophils [47]. Consequently, long-lived macrophages have been suggested as a possible target for developing vaccines against helmints.

Enduring hormetic responses to pathogen and pathogen products could also be observed in sepsis. It is characterized by burst of pro- and antiinflammatory cytokine release to infecting pathogen which could lead to tissue injury, organ dysfunction, septic shock with a mortality rate of 15−50% [48]. The septic syndrome is affected not only by many endogenous factors like age and medications but also by the status of the innate immune system [49]. Hyperinflammation is induced by pathogen or pathogen products from gram positive (lipoprotein), gram negative bacteria (LPS), or fungi (zymozan) [49]. Thus, an acute problem in septic syndrome is hyperinflammation, which could probably be targeted by pathogen products that induce tolerance responses. This idea has been practiced in mice where pretreatment of mice with 50 μg/kg of LPS induced survival benefit after a sepsis induction using CLP [50]. Although tolerance may be protective mechanism in some situations, it may be detrimental by induction of immunosuppression. Main cause of death in sepsis survivors is long-term immune paralysis, which is characterized by apoptosis of immune cells, impaired response to antigen stimulation, and increased antiinflammatory response, all of these making patients susceptible to opportunistic infections [51]. Pretreatment of mice with ultra-low doses of LPS (5 ng/kg) induced increased immune response after sepsis induction and mortality of mice [50]. Training effect of ultra-low doses of LPS was tested in the tolerance phase to decrease immunoparalysis. Additionally, training human or mouse monocytes with β-glucan leads to stronger response after second stimulation by LPS [5]. Furthermore, pretreatment of mice with low doses of β-glucan decreased mortality after fungal sepsis induction in mice [5]. Also, β-glucan sensitized innate immunity has been found to be protective after sepsis induction with *S. aureus* [52]. It is not clear whether β-glucan pretreatment would increase or decrease the immune response in vivo because another study reported that β-glucan pretreatment reduces LPS-induced TNF production in mice proposing this agent as a treatment option with negligible side effects [53]. Additionally, as mentioned before, breaking of tolerance with IFN-γ in sepsis survivors appears a logic option where paralyzed immune system is the main cause of death [35]. Furthermore, not only pathogens but also cytokines can induce trained immunity [54] and the cytokine IL-1β induced protection against mortality from *Pseudomonas aeruginosa* infection [55]. Recently, one study suggested usage of gold nanoparticles for induction of trained immunity [56]. Inducing trained immunity with cytokines or nanoparticles could be of high medical importance, because they are already tested and used in some diseases.

In conclusion, enduring sensitization and tolerance of innate immune cells might have important effects on the susceptibility of a host to infections and should be further studied and exploited for the medical benefit of the patients.

Allergies and autoimmune diseases are inadequate responses to external and body's own antigens. In both the cases restoring tolerance is one of the main aims in the therapy. Antigen-specific tolerance is the basis for immunotherapy with allergen extracts to treat allergic disease. Pollinex Quattro (Allergy Therapeutics, West Sussex, UK) is a vaccine containing MPLA, combined with ragweed pollen extract and is used for the treatment of seasonal allergic rhinitis in Europe, where main mechanism is inducing tolerance and a shift from IL-5/Th2 to IFN-y/Th1 response [57]. This approach has also been suggested as an option for treating autoimmune diseases. An antigen-specific immunotherapeutic approach would allow preservation of protective immune cells and maintain their normal immune surveillance functions, while specifically targeting the T/B cells thought to be a possible therapeutic strategy [58]. However, while successful in mice, clinical trials for inducing antigen-specific tolerance in humans until now failed [59]. One of the possible reasons for treatment failure was disregarding the importance of innate arm of the immune system in pathogenesis of autoimmune diseases. Indeed cells of monocyte/macrophage system are involved in producing chemokines, cytokines, T/B cell activation, infiltration, and stronger response [3]. Additionally, γδ T cells have been found to play crucial role in autoimmunity. This cell type provides an early source of innate IL-17, which promotes antimicrobial peptide production but is also involved in many autoimmune diseases because of amplification of Th17 cells loop [60]. Moreover, autoimmune syndromes appeared in animals lacking immature dendritic cells, normally responsible for tolerance induction [61]. Disarming innate immunity could be suggested as one possible strategy. Support for this comes from the findings that long-term treatment of mice with helminths-induced immunosuppressive macrophages which later prevented development or at least suppressed experimental autoimmune encephalitis (EAE) [62,63]. This approach has been shown to be efficient in treatment of allergies in mice and undergoing clinical trials [38]. It was known that infection with *Bordetella pertussis* induces decreased cytokine release after second infection with influenza viruses and prevents mortality [64]. Recently, same pathogen has been found to induce IL-10-mediated antiinflammatory responses and attenuate EAE in mice [65,66]. It is possible that some of these pathogens induce long-term epigenetic changes in monocytes of trained or tolerant state. This has been shown to be true for infection with *Listeria monocytogenes* [67].

Neurodegenerative diseases, like Alzheimer's disease (AD), or atherosclerosis have been described as a consequence of hyperactive innate immune system [68]. Tissue macrophages like microglia or Kupffer cells in the liver are long-lived cell populations [69] where one stimulation could lead to lifelong memory. Recently, it was shown that one stimulation with LPS leads to tolerance responses in microglia [29]. Our own unpublished data show that this effect is dose

dependent and that microglia cells, but probably also other tissue macrophages can become primed with lower doses of PAMPs and respond stronger to restimulation (Lajqi and Stojiljkovic, 2017 unpublished data). This could be clinically relevant as it is known that in the state of disease or in aging microglia cells are hyperactive [70,71]. Innate immune memory in the brain has been reported as nongenetic modifier of the disease [42]. It was shown in myeloid progenitor cells that epigenetic memory induced by training could be transferred to the progeny [72] and long-lived cells with low turnover like microglia could be especially vulnerable to priming events. Inflammasomes were found to be active in the atherosclerotic plaques and induced by hypercholesterolemia [73]. Not only pathogens but also metabolic changes like high cholesterol, LDL, or hyperglycemia could induce long-term reprogramming of monocytes/macrophages [74,75]. Lifestyle habit like western type diet is also known to induce activation of microglia cells [76] and could be involved in pathogenesis of neurodegenerative diseases via microglia priming.

Main mechanisms by which tolerance and sensitization/training are mediated are activation of two key metabolic sensors and regulators AMPK and mTOR pathway [15]. Possibly not only pathogens but also other substances which could activate or block these pathways in immune cells could induce similar responses. For example, blocking mTOR with rapamycin decreases release of cytokines after LPS stimulation in vitro and in vivo, rescues mice from staphylococcal infections-induced shock and improves cognition in mice sepsis survivors [77–79]. In the same direction, fasting prior to initiation of sepsis, which is known to activate AMPK, significantly decreased mortality in mice [80]. The more specific AMPK activator AICAR showed similar beneficial effects in mice after sepsis induction [81]. On the other hand, metformin, a pleiotropic drug which also activates AMPK, however induced detrimental effects on mice survival [15]. These effects could depend on the decreased immune response after AMPK activation to LPS stimulation [82]. Different mechanisms of AMPK activation, off-target effects, dose, and application time might have provoked these opposite results.

A recent study suggests that moderate genotoxic stress would induce survival genes which could be beneficial for survival after second stimulation [83]. Indeed, septic mice treated with anthracyclines have lower mortality after sepsis induction because of decreased inflammatory response [84]. As a plausible explanation induction of repair and maintenance responses by the genotoxic stressor and competitor suppression of energy consuming inflammatory responses has been proposed [83]. This concept is confirmed by suppressed inflammation after DNA topoisomerase 1 inhibition [85]. In vivo, topoisomerase 1 inhibition therapy decreased mortality rate by 70%–90% caused by exacerbated inflammation in three mouse models: acute bacterial infection, liver failure, and virus-bacteria coinfection [85]. Moreover, whole body irradiation-induced DDR response increases survival of mice with polymicrobial sepsis [86]. It is also known that most of the hormesis effects of microbial pathogens and other stressors are strongly connected to autophagy induction, which seems an important mediator of tolerance responses. Several studies have shown that removal of crucial autophagy genes leads to absence of hormesis effects [84,87]. Further investigation of autophagy, mTOR, AMPK, DNA-damage-induced stress responses, and their interplay seems as a reasonable strategy in designing targeted therapies inducing trained immunity or tolerance responses.

To sum up, current insights in pathogen-induced hormetic responses pave the way for novel treatment options of diseases caused by either hyper- or hypoactive innate immune system. In a state of immunosuppression, drugs that induce robust immune responses are needed. On the other hand, in a state of autoimmune diseases, drugs that induce tolerance are of special relevance. It is important however to identify molecular and cellular mechanisms of trained immunity to different pathogens and to test these substances in clinical settings. Moreover, it seems important to elucidate the duration of induced innate immune memory and enduring protection in patients. In conclusion, there is enormous potential to exploit novel findings in the field of innate immune memory to investigate and produce safer vaccines and better drugs against infectious diseases or cancer. Moreover, there is a demand in studies on autoinflammatory diseases in which immunity is modulated. Induction of tolerance in sensitized innate immune cells during chronic inflammation in the brain and blood vessels could be a way to treat neurodegenerative diseases (i.e., Alzheimer's, Parkinson's) and atherosclerosis. Not only the pathogens but also their products, structural analogs or substances, which are able to mimic training or tolerance responses could be used to develop eligible targeted therapies.

## 14.5 CONCLUSIONS

The examples outlined in this section reveal the significant translational potential of research activities directed to a better understanding of resistance and tolerance reactions of the innate immune system and their embedding in the hormesis concept. Definition of the pathogen doses and of the mechanisms shaping innate immune responses in specific stress response patterns will likely be instrumental for these emerging investigations.

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### *Closing Discussion*

Most if not all of age-related brain pathologies, including Alzheimer's and Parkinson disease, are accompanied by activation of microglia cells, the main immune cell type in the brain (Rogers, Mastroeni et al. 2007). When activated, microglia can reinforce neuroinflammation, ultimately leading to synapse and neuronal loss, and well-known age-associated cognitive decline. The disappointing results of several phase III clinical trials for therapies directed to the above-mentioned neurodegenerative disorders, clearly indicate the need for the development of new approaches to identify candidate mechanisms involved in age-related degenerative diseases. Since one hallmark of the aging process is the aberrant accumulation of senescent cells, targeting these cells has an enormous biomedical interest. In this context, targeting senescent cells in mice was shown to be beneficial in several age-related diseases followed by senescent cell accumulation like atherosclerosis, diabetes, osteoarthritis, neurodegenerative and idiopathic lung fibrosis (Childs, Durik et al. 2015).

Taking into account that the prevalence of neurodegenerative disorders is over 60% in the population older than 80 years, and that aging is the main risk factor for these diseases, understanding the mechanisms involved in brain aging and the contribution of individual cell populations including microglia, is of great importance. Previous studies reported priming, activation and increased ROS production in aged mice microglia (Njie, Boelen et al. 2012). Furthermore, microglia in the aging brain have been described as dystrophic or possibly senescent (Streit 2006). However, it is still unclear whether these cells really become senescent, express accepted senescent markers, and whether senescence-associated changes in this cell population are then relevant in neurodegeneration. A study fully addressing the phenotype and expression of senescent markers in the aged microglia was missing. It is still unclear whether microglia are involved in the pathogenesis of major neurodegenerative disorders or are only activated as a consequence of the pathological microenvironment. Two studies have shown that signs of microglial activation and dysfunction appear well before development of amyloid plaques, a typical marker of Alzheimer's disease (Streit, Braak et al. 2009, Boza-Serrano, Yang et al. 2018). These studies unravel a relevant role of microglia cells in neurodegeneration, however, the role of microglia cells in the healthy aging brain is still not completely understood.

Senescent cells can be identified on the basis of a specific set of hallmarks including telomere shortening, expression of p16 $^{Ink4a}$, p21, p53, SA-β-galactosidase as well as proliferation arrest and DNA damage. Importantly, available evidence indicates that expression of only one senescent marker is not enough to identify a cell as senescent and rather a combination of

markers, including irreversible blockade of cell proliferation and functional changes, are needed. One possible reason for the scarcity of studies addressing microglial senescence is the lack of suitable *in vitro* and *in vivo* models. Therefore, here we developed a senescent model *in vitro* and compared changes in senescent microglia *in vitro* with changes occurring in microglia in the healthy aged murine brain. In addition, we evaluated the impact of aging and senescence on the innate immune memory in microglia. In this study, we describe for the first time how the maturation state of microglia affects the pathogen-dose-dependent activation of adaptive tolerance and sensitization responses in these cells.
Our study significantly contributes to the understanding of phenotypical and functional changes that take place in senescent and aged murine microglia.

*Development of a senescent model in vitro*

There are several different models described in the literature to induce cell senescence. One of these models, the replicative senescence model, is based on the Hayflick limit of cell proliferation (Hayflick and Moorhead 1961). Other models require application of different stressors to the cells (oxidative with $H_2O_2$ or genotoxic by using cytotoxic drugs) and therefore are not physiologically relevant (Hernandez-Segura, Nehme et al. 2018). In order to induce replicative senescence, cells have to replicate several times till reaching their replication limit, due to critical telomere shortening. A first problem in developing a microglia replicative senescent model is the fact that when pure isolated microglia cells *in vitro* do not proliferate, and after 7-14 days start losing their viability and become dysfunctional (Flanary and Streit 2004, Caldeira, Oliveira et al. 2014). Another important aspect is that after only 4-12h *in vitro,* when cultured alone, microglia cells lose their typical signature, acquiring a completely different expression profile and making it difficult to compare data from *in vitro* and *in vivo* analyses (Bohlen, Bennett et al. 2017). In order to continuously proliferate as well as to keep their phenotype, microglia cells require growth factors produced by astrocytes (Bohlen, Bennett et al. 2017). However, delivery of growth factors like GM-CSF to keep microglia viable or to stimulate proliferation may change their phenotype (Esen and Kielian 2007). Therefore, we first cultured microglia isolated from neonatal mice brains in a more ‘physiological’ mixed cell culture, which has been previously described to contain astrocytes, olygodendrocytes and olygodendrocyte precursor cells (Saura 2007, de Vellis and Cole 2012). In this environment microglia cells showed initially a high proliferation rate of about 40%. We then separated a part these young microglia and used them to perform phenotypical and functional analyses. The remaining mixed cultures were splitted several times until we observed a significant

reduction in the proliferation rates. In order to determine whether this proliferation arrest was associated with a senescent or a quiescent state, we evaluated the expression of several well-established senescence markers and showed that approximately 50% of the cells entered the senescent state. Non-proliferating senescent microglia were SA-β-gal and p21 positive, and showed telomere shortening. Interestingly, migration rates of senescent microglia were increased, correlating with increased purinergic receptor expression. Basal expression levels of cytokines were increased, however, response to LPS was significantly reduced. This pattern of increased basal activation and reduced LPS response is also found in aging macrophages (Albright, Dunn et al. 2016). This obvious similarity may be due to the fact that young neonatal microglia are more similar to the primitive macrophages than to the microglia in the adult mice brain (Matcovitch-Natan, Winter et al. 2016).

Interestingly, although expression of $p16^{Ink4a}$ was significantly increased in aged microglia, young microglia also showed a relevant basal expression of this marker. In this context, a recent study by Hall et al. (2017) showed that expression of $p16^{INK4a}$ and SA-β-Gal in macrophages was acquired as part of a reversible response to physiological immune stimuli. Importantly, macrophages were highly proliferative but lacked other properties of cellular senescence. In line with these findings, we found significant expression of $p16^{INK4a}$, both in newborn brain microglia and in microglia isolated from young adult brains. We therefore suggest that $p16^{INK4a}$ as well as SA-β-Gal cannot be used as only and reliable markers to define microglial senescence. For the purpose of our study, we defined senescent microglia as cells showing replicative arrest, telomere shortening and high expression levels of p21 and p53 in addition to $p16^{INK4a}$.

In conclusion, we developed a reproducible senescent model which may be used to further study microglial senescence and putative mechanisms involved. This is highly relevant for the establishment of therapeutic strategies aimed to prevent or postpone senescence development in this cell type.

*Aging microglia are not senescent*

It is becoming clear that senescent cells accumulate with aging. However, senescent cells appear also during development and are not specifically age-related. This so called ‘acute senescence’ is beneficial in wound healing or kidney development (van Deursen 2014). Acute senescence is a well-defined program where senescent cells appear, provide the necessary function and are removed within days. Recently a so called chronic or ‘deep senescence’ has been described, characterized by activation of p53 and transposable elements, opening of heterochromatic

regions and extrusion of cytoplasmic chromatin fragments (CCP) leading to chromatin loss (Sturmlechner, Durik et al. 2016). These deep senescent cells seem to be more relevant in age related pathologies and therefore constitute a real therapeutic target. One important difference between senescence cells and aged cells is that aged cells generally show reduced or loss of all functions whereas senescent cells show a hyperfunctional phenotype. Indeed, in our study, senescent microglial cells showed increased migration rates, while aged cells showed a tendency towards decrease.

One challenging possibility is that acute senescent cells just age as every other cell type, acquiring a dysfunctional phenotype which becomes detrimental. Another possibility is that they accumulate with age due to reduced removal, are exposed to different stressor for longer periods of time, consequently enter deep senescence and then become detrimental and disease relevant. This possibility deserves further investigation and could lead to new therapeutic avenues to reduce the negative impact of cellular senescence in aging tissues.

It is unclear which cell types are responsible for age-related diseases and which ones may become senescent with age. It is possible that senescent cells are involved in many age-related diseases. Recent studies show that several cell types including non-proliferative neurons or adipocytes may acquire senescent phenotypes *in vitro* and *in vivo* (Jurk, Wang et al. 2012, Ghosh, O'Brien et al. 2018). Some findings indicate that tissue macrophages are the most relevant cell population for induction of low level inflammation or parainflammation, and show a cytokine profile which is very similar to the SASP (Childs, Baker et al. 2016). These low levels of inflammation are then probably involved in pathogenesis of most of the age-related diseases, like neurodegeneration and atherosclerosis. This process has been coined as macroph-aging (Prattichizzo, Bonafè et al. 2016). Interestingly, it is currently unknown whether macrophages or brain microglia become senescent or just dysfunctional with age. Therefore, we determined expression levels of senescence markers in brain microglia at different ages. We established an acute isolation protocol to extract microglia from the adult brain tissue within 1h, thus reducing the possibility of phenotypical changes. Similarly as for microglia from the young brain, we established a mixed culture protocol in order to maintain these cells for a longer time and obtain comparable results in functional tests like migration and phagocytosis. Aged microglia showed increased expression of cytokines with exacerbated response to LPS. Aged cells also displayed increased expression of $p16^{INK4a}$, however, they did not show expression of other typical markers of senescence. As already mentioned, the problem is that microglia probably change the $p16^{INK4a}$ expression as a response to physiological stimulation due to an inflammatory environment and it

is not specifically senescence related. Furthermore, aged microglia showed significant lipofuscin accumulation. Indeed, lipofuscin accumulation was recently found to be a relevant factor of microglia dysfunction in the case of brain disease (Safaiyan, Kannaiyan et al. 2016).

Proliferation of microglia isolated from aged mice brains was not decreased *in vivo* or *ex vivo*, and speaks against development of a senescence phenotype. Here, we have shown that microglia from aged brain have a dysfunctional phenotype, however, do not express a typical senescence signature.

One interesting and important question remains of how aged or dysfunctional microglia cells are physiologically removed. 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 (ex. HIV, tuberculosis) (Herbein, Coaquette et al. 2002, Guirado, Schlesinger et al. 2013). Chronically activated microglia, which reinforce inflammation in the brain, are very little or not replaced by cells from the periphery (Füger, Hefendehl 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 or dysfunction to neighboring cells.

Therefore, removing and replacing dysfunctional microglia in the aged brain is currently a major focus of interest. Removal of dysfunctional microglia would require directed strategies to specifically eliminate these cells. A recent study indicates that removal of microglia using the $CX_3CR_1^{CreER/+}$:$R26^{iDTR/+}$mouse model leads to repopulation by the remaining pool of microglia, or by cells from the periphery, only in case of blood brain barrier damage (Bruttger, Karram et al. 2015). Microglial removal and repopulation has also been achieved using the CSFR1 inhibitors PLX3397 or PLX5622 (Elmore, Najafi et al. 2014, Spangenberg, Lee et al. 2016). In the aged mouse brain, newly repopulated cells immediately re-acquire a similar dysfunctional phenotype with a pro-inflammatory signature probably due to the pro-inflammatory microenvironment (Elmore, Hohsfield et al. 2018). The same study also showed that a single removal and repopulation of the aged microglia led to a certain improvement in cognitive flexibility. Another study showed that microglia depletion and repopulation reversed lysosome enlargement and lipofuscin accumulation in the aged microglia (O'Neil, Witcher et al. 2018). Here again, the response to LPS and so-called primed state of the 'new' microglia was unchanged, pointing out the important role of the microenvironment.

Further studies showed that continuous removal of microglia without repopulation was beneficial in a neurodegenerative AD mouse model, as indicated by reduced amyloidal plaque formation and cognitive improvement (Spangenberg, Lee et al. 2016, Spangenberg, Severson et al. 2019). Furthermore, neurogenesis, dendritic spine formation as well as long-term potentiation were improved in aged microglia depleted mice (Elmore, Hohsfield et al. 2018). 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 (Cannarile, Weisser 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, microglia 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 performed microglia depletion for several weeks without serious side effects to the mice health. We may speculate that indispensable microglia role in early development becomes dispensable in adulthood and possibly even detrimental in the aged brain.

It would be also important to consider therapies to target tissue microenvironment and not only senescent cells, in order to prevent reappearance of these cells after depletion. As aging is the leading risk factor for most relevant chronic diseases and disabilities, including stroke, heart diseases, cancer, dementia, osteoporosis, arthritis, diabetes, metabolic syndrome, kidney failure, blindness, and frailty, these therapies could then be utilized to prevent or postpone the occurrence of these inflammation-mediated and aged-related diseases (Galatro, Holtman et al. 2017).
We found no relevant expression of p21 and DNA damage in the normal brain aging tissue, however, other studies have shown that microglia express this senescence marker in a mouse model of neurodegeneration (Raj, Jaarsma et al. 2014, Ritzel, Doran et al. 2019). A recent study showed accumulation of senescent microglia after traumatic brain injury, possibly in order to support the tissue repair (Ritzel, Doran et al. 2019). It is known that senescence cells may support wound healing (Demaria, Ohtani et al. 2014). Repeated small spontaneous injuries of the blood vessels may therefore be responsible for microglia activation and deregulation in diseases like vascular dementia. Senescence of microglia seems not to occur in the healthy murine brain aging (Stojiljkovic, Ain et al. 2019), however, may still be relevant in human brain aging, after repeated traumatic brain injuries and in neurodegenerative diseases. Indeed, a recent study has shown

that microglia aging was significantly different in humans and mice, probably due to longer lifetime of these cells (Galatro et al. 2017).

Unraveling the mechanisms involved in age-related degenerative diseases and development of therapies aimed to delay disease-associated pathophysiological alterations has become an important focus of interest for the biomedical community. Since cellular senescence or dysfunction seems to play a major role in age-associated changes, both under physiological and pathophysiological conditions, defining cellular mechanisms leading to microglia dysfunction is critical for the treatment of age-related brain pathologies.

*Pathogen-dose dependent response of microglia*

Accumulating evidence indicates that the innate immune system may develop long-term memory in the form of trained innate immunity or tolerance (Netea, Quintin et al. 2011). Innate immune memory is increasingly becoming the focus of research not only in the immunology field. Elucidating mechanisms involved in training and/or tolerance of innate immune system could have a significant impact on our understanding of age-associated disorders where the hyperactive innate immune system, including macrophages, is implicated. Microglia priming early in lifetime followed by a second challenge is thought to be involved in neurodegenerative as well as neuropsychiatric disorders later in life (Perry and Holmes 2014).

The response of innate immune cells to repeated stimulation may be increased, so called trained immunity or ‘sensitization’, or decreased termed as ‘tolerance’, and these innate immune memory involves stable epigenetic changes and a metabolic switch (Guirado, Schlesinger et al. 2013, Ifrim, Quintin et al. 2014, Netea, Joosten et al. 2016). It was thought that development of tolerance or sensitization was substance-specific; for example, β-glucan induces sensitization while LPS induce only tolerance (Schaafsma, Zhang et al. 2015, Netea, Joosten et al. 2016). Our hypothesis was that microglia sensitization/tolerance is dose-dependent and not substance-dependent. It is also unclear whether cells show a similar response at different maturation stages of development. In order to evaluate these open questions, we evaluated dose- and time-dependent effects of LPS and β-glucan on naive and adult microglia, and assessed immune, metabolic and functional responses of microglia after repeated challenge with variable doses of these activators (1fg/ml to 1µg/ml). We found that microglia adaptive responses to repeated challenge are both dose- and maturation-dependent. Interestingly, mature microglia were unable to develop features of sensitization. On the contrary, challenge with higher doses led to tolerance state independent of age. A major finding here is that the maturation process affects microglia ability to become

sensitized, but not tolerant. Our results show for the first time that trained immunity and tolerance can be induced by the same activator (LPS and β-glucan) in a dose-dependent manner, following a classical hormetical pattern. Our study showed that young microglia are highly plastic to different PAMPs stimulation while mature microglia is not.

In our study, the dose-dependent responses of young microglia followed a hormetic where very low doses increase and high doses reduce cellular responses (Calabrese and Mattson 2011). It is currently unclear whether these femtomolar ultra-low doses of LPS are clinically relevant. However, previous studies show that even super low doses of LPS in the circulation may have effects on the health in connection with chronic infections, smoking or drinking (Goto, Edén et al. 1994, Lira, Rosa et al. 2010). Another study confirmed that even as few as 100 bacteria or 25 LPS monomers may induce a relevant response (Teghanemt, Weiss et al. 2013). Our study as well as the recent study by Lajqi et al (2019) show that even femtomolar concentrations may produce relevant physiological response (Lajqi, Stojiljkovic et al. in preparation).

Low dose stimulation with 1fg LPS did not have an effect on so called alternative activation. An anti-inflammatory response, as shown by increased levels of IL-10, was only present in naive microglia after stimulation with high doses of LPS, probably as a mechanism to prevent further damage to a repeated challenge in an inflamed tissue. Our preliminary data show that aged and senescent microglia do not show adaptive responses, which may be due to the fact that aged microglia are already primed and resistant to regulation (Norden and Godbout 2013, Stojiljkovic et al. in preparation). Whether modulation of training or tolerance in neonatal, adult or aged microglia may have an effect on age related neuroinflammation and dysfunctional microglial phenotype remains to be investigated.

One of the beneficial effects of microglia on brain plasticity is mediated by BDNF released by these cells (Parkhurst, Yang et al. 2013). Single stimulation with a low dose of LPS induced increased BDNF levels in naive microglia but decreased it in the adult counterparts. This fact may be relevant in different responses to inflammatory stimuli in the young and adult brain. While the young brain is able to promote BDNF-dependent plasticity and repair in the case of increased inflammation, the mature brain seems to be unable to do so. The role of BDNF in the adult and aged brain in the case of neuroinflammation has to be further evaluated. Microglia-centered therapies should therefore focus on supporting microglial beneficial roles like BDNF production, and inhibition of detrimental roles in hyperinflammation.

A recent study has shown that inducing microglia tolerance may be beneficial, while inducing training may be detrimental in the case of β amyloid pathology (Wendeln, Degenhardt et al. 2018).

The possible explanation for the different responses of naive and adult microglia may be a different gene signature of the two cell populations. Up to postnatal day 14, naive or immature microglia are more similar to primitive yolk sac macrophages and acquire a typical microglial signature later during development (Matcovitch-Natan, Winter et al. 2016). The study speculates that naive microglia have a more macrophage-like than microglia-like phenotype. Additionally, microbiome-microglia interactions seem to be important for the maturation process (Erny, Hrabě de Angelis et al. 2015). We postulate that mature or aged microglia as compared to immature naive microglia (P0-P3) may be already pre-primed by products from the gut microbiome. Any stimulation of immature microglia during this critical maturation period in development may have long-term effects on microglia phenotypes (Matcovitch-Natan, Winter et al. 2016).
Expanding the knowledge on pathogen-induced hormetic responses (like here to LPS, β-glucan) may open the way to novel treatment options for hyperactive or hypoactive immune responses.
In immunosuppressed patients, treatment with substances which induce trained immunity is needed, in autoimmune diseases inducing long-term tolerance to the specific antigen would constitute a desirable therapeutic target. The role of the innate immune system and its cellular components in these diseases has to be further investigated, as well as mechanisms involved, in order to test the putative therapeutic compounds in pre- and clinical settings.

Innate immune memory duration in microglia is still unclear. Can one single challenge during the early embryonic period be enough to induce a lifelong memory in microglia and lead to neurodevelopmental and neurodegenerative diseases? A recent study confirmed that tolerance or sensitization of microglia had an effect on neuropathology months later (Wendeln, Degenhardt et al. 2018). These results highlight the relevance of microglial training due to their longevity and thus, the long-term effects of one single challenge.
Knowledge acquired from studies on innate immune memory should be used for developing better drugs and vaccines. Inducing tolerance in hyperactive immune cells in normal aged brain or during chronic inflammation in the brain and blood vessels could be a way to treat neurodegenerative diseases (i.e., Alzheimer's, Parkinson's) and atherosclerosis. A better understanding of tolerance and sensitization may lead to development of new drugs in pathologies like sepsis, where both processes occur simultaneously.

There is still a long way before translation to the clinical settings.

In conclusion, our study revealed changes in microglia occurring over time *in vitro* and *in vivo.* We describe a novel senescence *in vitro* model which may be used for further studies aimed to develop new senolytic and microglia centered therapies. We found that aged microglia *in vivo* showed a dysfunctional activated phenotype and increased p16 $^{Ink4a}$ expression, whose function in microglia cells needs to be further evaluated. Additionally, we found that responses to different stressors like LPS and β-glucan were not only dose-dependent but also maturation- and age-dependent. Understanding of microglia senescence, age related changes, as well as inflammatory responses and memory of these cells is relevant for the elucidation of factors and mechanisms involved in the physiology and pathophysiology of brain aging and neurodegenerative diseases.

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Ort, Datum Unterschrift des Verfassers

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