Age-dependent differences in microglial responses to systemic inflammation are evident as early as middle age

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RUNNING TITLE: Microglial responses in middle-age

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ABSTRACT

Whereas age increases microglial inflammatory activities and impairs their ability to effectively regulate their immune response, it is unclear at what age these exaggerated responses begin. We tested the hypotheses that augmented microglial responses to inflammatory challenge are present as early as middle-age, and that repeated stimulation of primed microglia in vivo would reveal microglial senescence. Microglial gene expression was investigated in a mouse model of repeated systemic inflammation induced by intraperitoneal injection of bacterial lipopolysaccharide (LPS). Following LPS, microglia from middle-aged mice (9-10 months) displayed larger increases in Tnfα, Il-6 and Il-1β gene expression compared to young adults (2 months). Similar results were observed in the spleens of middle-aged mice indicating that exaggeration of both central and peripheral immune responses are already evident at early middle-age. Interestingly, despite greater pro-inflammatory responses to the first LPS challenge in the aged mice, there were no age-dependent differences in either microglia or spleen following a subsequent LPS dose, suggesting that animals at this age retain the ability to effectively control their immune response following repeated challenge. The exacerbated microglial immune response to systemic inflammation at early middle-age suggests that the CNS may be vulnerable to age-dependent alterations earlier than previously appreciated.
INTRODUCTION

Neuroinflammation, contributed to by microglia, is a hallmark of many neurodegenerative disorders, stroke and traumatic injuries (2, 5, 50, 55). Chronic or poorly controlled neuroinflammation is detrimental and accelerates neurodegeneration (43). Although microglia, CNS resident innate immune cells, have many beneficial activities, mounting evidence suggests that with aging, microglia undergo morphological and physiological changes that can lead to “para-inflammation” (reviewed in (22, 38, 54, 56)). This pro-inflammatory environment may increase susceptibility to neurodegenerative disorders and/or worsen ongoing pathology in the aging CNS. Indeed, age is the most significant risk factor for many neurodegenerative disorders, including Alzheimer’s disease and Parkinson’s disease.

Age-related morphological changes in microglia, typically in 18-20 month-old mice, are characterized by decreased ramification and perinuclear cytoplasm hypertrophy that are suggestive of increased pro-inflammatory activation. Elevated expression of several pro-inflammatory genes also support notion that microglia may be transitioning to an activated state with aging (14, 20, 30). A recent study revealed that microglia in the aged CNS have significantly reduced IL-4/IL-13 signaling that hinders their polarization towards the anti-inflammatory M2 phenotype (27). In addition to age-induced alterations in microglial pro-inflammatory characteristics, their ability to provide support and protection to neurons also deteriorates with age (48). Similar microglial transformations and para-inflammation found in animal models have also been observed in the aged human hippocampus, superior frontal gyrus and post-central gyrus (15). This post-mortem evidence of para-inflammation is strengthened by in vivo observations of increased microglial activation in healthy older human subjects (mean age 53 years) as assessed by positron emission tomography (PET) using R-[^11]C]PK11195, a ligand that preferentially binds to activated microglia in the CNS (45).
Interestingly, the biological onset of neurodegenerative disorders usually precedes clinical symptoms by several years to several decades (24), indicating that middle-age may be an especially sensitive or critical period for the development of these diseases. Thus, changes in microglia during the middle-age period, and its associated para-inflammation, may be important factors in the etiology of neurodegenerative disorders. Whereas age-related alterations in microglia are well-documented in animal models of aging (>18-20 month-old mice and rats) it is not clear at what age these changes begin to occur. Thus, in this study, we tested the hypotheses that: 1) microglial responses to an initial inflammatory challenge will be elevated in middle-aged mice (9-10 months) compared to young adult mice (2 months), and 2) microglia from middle-aged mice will display properties of senescence involving stronger pro-inflammatory responses to a subsequent systemic inflammatory challenge. While microglial responses to repeated inflammatory challenge have been previously tested *in vitro*, they have not to our knowledge been tested *in vivo*, or in aged animals. To test these hypotheses, we used a model of repeated systemic inflammation induced by intraperitoneal (i.p.) injection of bacterial lipopolysaccharide (LPS).

**METHODS**

**Animals**

Male ICR/CD1 mice were purchased from Charles River (Wilmington, MA, USA) and housed in AAALAC-accredited facilities under standard 12 hour light/dark cycles with water and food available *ad libitum*. To study the effect of age, 2 month-old (young) and 9-10 month old (middle-aged) adult mice were used (19). All experiments were conducted under protocols
approved by the University of Wisconsin Institutional Animal Care and Use Committee. All
efforts were taken to minimize the number of animals used for experimentation while allowing
the formation of statistically relevant observations.

LPS treatment: Lipopolysaccharide (LPS; E.Coli 011:B4, Sigma Chemical Co, MO, USA) was administered by i.p. injection at a dose of 5 mg/kg body weight. Control animals received i.p. injection of 100 µl PBS. Mice were sacrificed 3 or 24 hours after LPS or PBS administration, transcardially perfused with ice-cold PBS followed by spleen and brain dissection. Another group of mice received second dose of LPS (5 mg/kg) 24 hours after the first injection, followed by spleen and brain harvesting 3 hours later.

Microglial isolation

Microglia were isolated from brains as we have described in detail previously (36). Briefly: after perfusion with PBS, brains (including brain stem and cerebellum) were dissected, weighed and enzymatically digested using Trypsin supplemented with DNase I for 20 min. Myelin was removed by centrifugation in 30% Percoll in HBSS. Cell pellet was resuspended in IMAG buffer (PBS supplemented with 0.5% BSA and 2mM EDTA) with PE-anti-CD11b antibodies (Miltenyi Biotech, Germany) and incubated for 10 min at 4ºC. After washing, cells were incubated with anti-PE magnetic beads for 15 min. CD11b+ cells were separated in the magnetic field using MS columns (Miltenyi Biotech, Germany). Columns in the magnetic field were washed 5 times with IMAG buffer. After removing columns from the magnetic field, microglia were eluted by 1 ml of IMAG buffer. Cells were resuspended in TriReagent and frozen at -80º until further use.
**RNA extraction and qRT-PCR**

Total RNA was extracted from isolated microglial cells or spleen tissue using Tri-Reagent (Sigma, MO, USA) according to the manufacturer’s protocol. RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Invitrogen, Grand Island, NY). This was followed by quantitative PCR using SYBR Green solution (Applied Biosystems, CA, USA). Primer sequences are provided in Table 1. Gene expression was normalized to 18S levels and relative gene expression was determined by the ∆∆Ct method (28). Gene expression was considered undetectable if the C\textsubscript{T} values > 35 cycles. Data are expressed as fold change relative to the treatment indicated in each figure.

**Flow cytometry analysis**

After transcardial perfusion with ice-cold PBS, brains were dissected and homogenized using a glass-teflon homogenizer and filtered through 70 μm cell strainer. Cells were resuspended in IMAG buffer and stained with PE-conjugated CD11b (Miltenyi, Germany) and FITC-conjugated CD45 antibodies (BD Pharmingen) for 10 min at 4ºC. This CD45 antibody (30-F11 clone) reacts with all CD45 isoforms arising due to alternative splicing of exons 4, 5 and 6 (designated A, B, and C) and both alloantigens of CD45. After washing, cells were fixed with 1.6% PFA and analyzed by flow cytometry using a BD FACSCalibur. Cells were first gated based on FSC and SSC parameters to exclude doublets and cell debris. Cells were then gated on CD11b\textsuperscript{+} immunoreactivity. CD45 expression was examined on CD11b\textsuperscript{+} gated cells. Data (n=6-7) were analyzed using FlowJo software v.10. (TreeStar Inc.).

**Statistical analysis**
Experiments were performed independently at least 2 times with 3-4 animals/group in each experiment. Results are expressed as mean ± SE. Since there was no difference in basal gene expression detected at 3 and 24hrs after PBS injection, both groups are combined in the “baseline” data shown on the graphs. Data were analyzed by one-way ANOVA followed by the Holm-Sidak or Fisher LSD post-hoc test using SigmaStat software (Systat, San Jose, CA, USA).

RESULTS

**Microglial cell number and basal gene expression does not differ between young and middle-aged naïve mice**

The yield of isolated microglia from the healthy brain was similar in young (1589 ± 363 cells/mg tissue) and middle-aged (1475 ± 420 cells/mg tissue) mice (Fig 1A). Microglia expressed low levels of CD45 as determined by flow cytometry, and there were no age-related differences (Fig 1B; left panels 0hr). We also evaluated basal gene expression of typical M1 and M2 markers. There were no age-dependent differences noted in basal gene expression (0 hr, first arrow) of the M1 markers inducible nitric oxide synthase (iNos; Fig 2A), tumor necrosis factor alpha (Tnfα; Fig 2B), interleukin (Il)-6 (Fig 2C) or Il-1β(Fig 2D). Also there were no differences in basal microglial gene expression (0 hr, first arrow) of the M2 markers Il-10 (Fig 3A), arginase-1 (Fig 3B) or Ym1 (Fig 3C) between young and middle-aged animals. Baseline gene expression of Toll-like receptor (Tlr) 2 (Fig 4A) and Tlr4 (Fig 4B) were also similar between both ages.

**Microglia from middle-aged mice exhibit stronger inflammatory responses to systemic administration of LPS**
Systemic inflammation was induced by intraperitoneal injection of LPS (5 mg/kg body weight). Microglia were examined at 3 and 24 hours after injection. In young mice, the total number of microglia in the brain increased by 27% (p<0.01) following 3 hours after LPS injection, and was further increased to 36% by 24 hours after LPS (Fig. 1A, solid line). In middle-aged mice, microglial cell number was not statistically increased at either 3 or 24 hours following LPS exposure (Fig 1A, dotted line), although there was a slight trend 17% and 9% towards an increase respectively, at each time point. There were no differences in CD45 protein levels analyzed by flow cytometry in microglia from young and middle-aged animals, and their response to LPS was also similar (Fig 1B-D). The lack of significant changes in young adult microglial CD45 levels at 3 or 24 hours after LPS injection suggests that macrophage influx is unlikely to be responsible for the increase in CD11b+ cell number observed in the brain at these time points.

In both young and aging mice, LPS-induced systemic inflammation resulted in significant upregulation of all M1 pro-inflammatory genes tested in microglia 3 hours after LPS administration (Fig 2). While there were no age-dependent differences in the magnitude of iNos up-regulation (Fig 2A), there were differences in the mRNA levels of the cytokines Tnfα (Fig 2B), Il-6 (Fig 2C) and Il-1β (Fig 2D) which were significantly higher in microglia from the older animals. By 24 hours post-LPS injection, the expression of all pro-inflammatory genes had returned close to baseline levels, although Tnfα and Il-1β still remained significantly elevated. The expression of the anti-inflammatory cytokine Il-10 followed a similar expression pattern to the pro-inflammatory cytokines with highest levels being observed 3 hours after LPS injection; however, there was no significant difference in Il-10 mRNA levels between young and middle-aged mice (p=0.073, n=6-7) (Fig 3A). By 24 hours after LPS injection, Il-10 mRNA levels had
returned to baseline levels in microglia from both ages. The expression of other markers of the
M2 phenotype arginase-1 (Fig 3B) and Ym1 (Fig 3C) was not significantly changed at 3 hours
after LPS in microglia from mice of either age. Interestingly, however, 24h after LPS injection
when Il-10 mRNA levels were significantly down-regulated from their peak at 3 hours post-
injection, the expression of arginase-1 was significantly upregulated by 14 and 22 times in
young and aging mice, respectively, whereas Ym1 expression was not affected.

We also analyzed the expression of Tlr2 and Tlr4 in microglia from young and middle-
age mice following systemic LPS administration. Tlr2 expression did not differ by age at
baseline (p=0.438); it was upregulated baseline 15 and 5 times after 3 and 24 hours of LPS,
respectively, in young mice (Fig 4A, gray bars), and 25 and 1.7 times in middle-aged animals
(black bars). On the contrary, LPS down-regulated microglial Tlr4 mRNA levels in mice of both
ages relative to expression at baseline (Fig 4B); basal Tlr4 levels did not differ by age (p=0.787).
We found that the expression of Tlr4 was decreased by 40% after 3 hours of LPS injection in
mice of both ages, an effect that was maintained for at least for 24 hours after LPS
administration.

Microglial responses to repeated systemic LPS administration are similar in young and
older mice

We hypothesized that since the cytokine response to the first dose of LPS was greater in
middle-aged microglia, that repeated stimulation of these primed microglia may reveal further
differences in responsiveness between young and middle-aged mice. Thus, a second dose of LPS
was delivered intraperitoneally 24 hours after the first injection when the expression of the pro-
inflammatory genes measured here had almost returned to baseline levels. Microglial responses
to this second LPS challenge were evaluated 3 hours later. Contrary to the responses to the first LPS dose, we did not observe an increase in microglial number following the second injection of LPS in mice of either age (Fig 1A). CD45 levels on CD11b+ cells as detected by flow cytometry were increased (Fig 1B-D), although there was no observable population of distinctly CD45^{high} cells that would suggest macrophage infiltration. Rather, our data suggest an upregulation of cell surface CD45 levels by resident microglia. The M1 inflammatory gene response of microglia to the second LPS challenge was at least as strong as it was to the first LPS injection (Fig 2A-D), but this time we did not observe statistically significant differences with age, with the exception of *Tnfa* gene expression (Fig. 2B) which was higher in middle-aged microglia. *Arginase-1* mRNA levels were high in both ages following the first LPS injection, and they remained upregulated after the second stimulation (Fig 3B). Although the expression of *Ym1* was unaffected by the first LPS injection, its mRNA levels were significantly increased after the second dose of LPS (Fig 3C), and no age-dependent differences were observed.

*Tlr2* (Fig 4A) and *Tlr4* (Fig 4B) expression was not different following the second LPS challenge (LPS 24+3h), and there was no difference observed with age. While *Tlr2* mRNA levels at 24 hrs were either returning to or were already at baseline both ages, the second LPS challenge induced an increase in *Tlr2* expression in microglia from both ages that was not different from the first LPS dose. Likewise, *Tlr4* expression remained below baseline at 24 hrs, and the subsequent challenge with LPS did not reduce *Tlr4* levels further; responses were similar in both ages.

Systemic inflammation induced by i.p. LPS is more severe in older mice
Many peripheral immune cell types can make cytokine(s) that promote neuroinflammation following peripheral administration of an inflammatory stimulus such as LPS. Because many of these cell types are found in the spleen, we analyzed the expression of inflammatory cytokines in splenocytes as an indicator of the general peripheral immune response to reflect the contributions of as many individual peripheral immune cell populations as possible. Whereas basal pro-inflammatory gene expression in the spleen was similar in mice of both ages, with regard to \textit{Ifn$\gamma$}(Fig 5A), \textit{Il-1$\beta$} (Fig 5B) and \textit{Tnf$\alpha$} (Fig 5C) mRNA levels, there were differences in the magnitude of the responses between young and aging mice. The first dose of LPS induced a strong up-regulation of \textit{Ifn$\gamma$}(Fig 5A) in spleens of mice from both ages, but the response in the older mice was significantly higher than in the younger mice (70 vs 25 times, respectively) 3 hours after LPS injection. The aging animals also had significantly higher mRNA levels of \textit{Il-1$\beta$} compared to the young adults (Fig 5B), but there were no age-dependent differences in the expression of \textit{Tnf$\alpha$} (Fig 5C). By 24 hours post-LPS injection, all pro-inflammatory genes had significantly returned back to baseline levels in both ages. Interestingly, the second LPS injection did not induce expression of \textit{Ifn$\gamma$} or \textit{Tnf$\alpha$} (Fig 5A, C) in the spleen like the first dose, but \textit{Il-1$\beta$} mRNA was significantly upregulated in both young and middle-aged mice by ~2-fold (Fig 5B).

**DISCUSSION**

In the present study we investigated how microglial responses to acute and repeated systemic inflammation are affected by aging. Our data suggest that microglial responses to systemic inflammation experienced at critical periods beginning in middle-age may have profound consequences on CNS vulnerability to neurodegenerative and other neural disorders.
associated with neuroinflammation and microglial activation. Microglial senescence, dystrophy, increased expression of pro-inflammatory cytokines and dysregulated immune responses are evident in very old (>20 month) mice and rats, but some transitioning to the inflammatory phenotype is already present in 12 month-old animals (6, 14, 27, 30, 38, 48, 54, 59). Here we show that some exacerbated pro-inflammatory responses occur as early as 9-10 months of age, and that these exaggerated responses are evident in both splenocytes and microglia, suggesting that age-dependent central and peripheral immune responses are affected earlier than previously thought.

Although there were no significant age-related differences detected in basal gene expression, or in the number of microglia in the young and middle-aged adult brain, we found two major differences in microglial responses to acute systemic inflammation induced by LPS between young and middle-aged animals. First, after LPS injection, there was about a 30% increase in the number of CD11b+ cells in the CNS in young but not older mice. That CD45 levels after LPS treatment were unchanged in CD11b+ cells suggests that proliferation of resident microglia, rather than infiltration of peripheral macrophages, are likely responsible for this increase in microglial cell number. Since an increase in microglial number was not observed to the same degree in middle-aged mice following LPS treatment, microglia from the older mice may have a decreased proliferative capacity, but additional studies are needed to further test this idea. Second, microglia from the older mice mounted a significantly stronger inflammatory response to the first peripheral LPS challenge than microglia from the younger mice. But by 24 hours after a single LPS injection, the expression of all pro-inflammatory genes examined was significantly down-regulated in both young and middle-aged mice, suggesting that the inflammatory response was transient and well-regulated in animals of both ages. Tnfα and Il-1β
mRNA levels appeared to remain higher than baseline in the middle-aged mice, perhaps suggesting that the age related changes in the regulation of neuroinflammation are already beginning.

Because microglial inflammatory responses in the aging brain are prolonged and often dysregulated (27, 38), we were interested in whether priming microglia with acute systemic inflammation might reveal possible age-dependent differences in their responses to subsequent activation with a second dose of LPS. Repeated stimulation of microglia with LPS has been done in immortalized BV2 cells, and a senescent phenotype involving heterochromatic foci and replication arrest was observed (58); pro-inflammatory genes were not assessed. However, in primary microglia (neonatal-derived), LPS induction of TNF-α and iNOS was successively decreased after each LPS challenge (1). Our observations here in adult microglia in vivo indicate that microglial pro-inflammatory responses to the second LPS challenge are just as strong as the first. Moreover, we found that microglia from both young and middle-aged mice mounted equally strong pro-inflammatory responses to a second LPS challenge. Some differences were noted in the expression of the M2a markers arginase-1 and Ym1 in response to the first and the second LPS injections in mice of both ages; they were higher after the second dose than the first. In macrophages, this phenotype is associated with tissue repair and growth stimulation (18). The upregulation of these M2a molecules in microglia after the second LPS dose suggests that microglia may be polarizing towards the anti-inflammatory phenotype, perhaps to promote tissue healing after repeated inflammatory episodes. Interestingly, the Il-10 response in young and middle-aged adult microglia were not different after either LPS dose, suggesting that the exacerbated IL-10 signaling that has been reported in geriatric mice (18-20 months) (23) does not yet occur in the middle-aged (9-10 month-old) animals used here despite the disparities in
some pro-inflammatory cytokines that are already apparent at this age. These data suggest
dichotomous regulation of pro- and anti-inflammatory cytokines during aging.

The LPS model of systemic inflammation used here is a common inflammatory stimulus
used in rodent neuroimmune aging research and our results are readily comparable with existing
literature from older aged animals. Also important, LPS treatment induces a similar profile of
inflammatory markers, and mirrors the exaggerated microglial responses observed during aging
in other models of neuroinflammation that are induced for example, by beta amyloid
overexpression (25, 29, 32) or ischemia (26, 31, 40). Lastly, LPS is clinically relevant in its own
right since gram negative bacterial infections are the most common in geriatric populations (16,
34, 35). The present results using this model of inflammation provide important information for
future mechanistic studies aimed at elucidating the relationship between chronic inflammation
and altered neural immune system function with age.

Sex is a risk factor for many neurodegenerative disorders (41, 42, 51). We have
previously shown that basal microglial expression of the cytokines evaluated here did not differ
between males and females at similar ages (4 and 12 months) (14), and our unpublished
observations in independent male and female primary microglial cultures indicate that LPS
responsiveness of the genes assessed here does not differ by sex. However, in light of the present
results demonstrating that exaggerated microglial responses to inflammation are already evident
at middle-age, it will be necessary to assess females in future studies, to determine if sexually
dimorphic interactions are revealed when age and systemic inflammation are combined in vivo.

Another interesting observation in our study is the downregulation of microglial
expression of Tlr4 and the up-regulation of Tlr2 after systemic administration of LPS. We found
that Tlr2 mRNA was upregulated in microglia from both young and middle-aged mice. This is
consistent with previous observations in microglia from geriatric (20-24 months) BALB/c mice after systemic LPS administration (23); effects on Tlr4 were not presented. Exposure of macrophage or microglial cultures to LPS (which strongly binds TLR4 and weakly binds TLR2) downregulates Tlr4 and upregulates Tlr2 expression (12, 57). However, the changes we observed in microglial Tlr expression due to systemic LPS delivery in vivo are likely mediated by mechanisms independent of TLR4-LPS binding because peripherally administered LPS does not cross the blood brain barrier (BBB) (4, 47).

Whereas LPS does not directly cross the BBB, it can increase BBB permeability (4, 47). In addition, BBB epithelial cells can directly respond to LPS by producing large amounts of cytokines such as IL-1β that can be released into the CNS to propagate neuroinflammation (47, 52). We found that LPS injection resulted in a transient up-regulation of the pro-inflammatory genes Ifnγ, Tnfα and Il-1β in the spleen, surrogate measures of peripheral immune responses, which were higher in older animals, suggesting that LPS may also cause greater systemic inflammation in middle-aged animals like it does in the CNS. IFNγ is produced primarily by NK cells, and Th1-polarizing CD4 and CD8 T cells that are important cellular activators of macrophages (44). IL-1β and TNFα can be produced by many innate immune cell types, but macrophages are a major source of these inflammatory cytokines (49). Interestingly, the expression levels of Ifnγ and Tnfα were not upregulated in the spleen following the second LPS challenge like they were after the first; Il-1β was increased equally in mice of both ages after the second LPS dose.

It is interesting to consider that IL-1β may play an important role in microglial responsiveness to systemic inflammation since microglial responses in the CNS followed Il-1β expression in the spleen after both doses of LPS. IL-1β, associated with the pathology of many
neurodegenerative diseases, can traverse the BBB (3) and propagate inflammatory responses within the CNS (13, 46). Of note, it has been suggested that there is an amplification of the immune response within the aged CNS that is independent of the peripheral immune system (39). Young adult inbred BALB/c mice (2-6 months) had greater circulating levels of IL-1β than old mice (20-24 months) following stimulation with systemic LPS, whereas brain IL-1β levels were higher in the geriatric mice (21). While our data also suggest an amplification of microglial activities (including \( \text{IL-1}\beta \)) in middle-aged mice, \( \text{IL-1}\beta \) mRNA levels in the spleen and microglia were similarly elevated. Reasons for this difference may involve age, as the mice in our study are considerably younger (9-10 months old) than the geriatric mice used in the above study. Further, the mice in our study were an outbred strain (ICR/CD1), so there may be also be strain-dependent differences in the interactions between the peripheral and central immune systems; we have previously reported important differences in microglial responses between inbred and outbred mouse strains (37).

Systemic inflammation is associated with many conditions such as atherosclerosis, obesity, metabolic syndrome, type II diabetes and others. Several epidemiologic studies suggest that populations affected by these diseases have significantly higher risks for neurodegenerative disorders (8, 10, 33), implying an important link between systemic inflammation and neurodegeneration. Indeed systemic inflammation induces cognitive dysfunction, memory disruption, neurodegeneration, neuroinflammation and exacerbates neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases, multiple sclerosis and amyotrophic lateral sclerosis (7, 9, 11, 17, 53). Understanding how interactions between the peripheral immune system and microglia are influenced by age is critical if we are to begin to identify novel targets to manipulate microglial activities during neurodegenerative disease.
In conclusion, our results demonstrate that exaggerated microglial inflammatory responses to acute systemic inflammation are already evident at middle-age (9-10 months) in mice. These age-related microglial responses to an initial dose of LPS mirrored age-associated responses observed in the spleen, an important component of the peripheral immune system. We did not find augmented pro-inflammatory responses to a subsequent LPS challenge in microglia (or splenocytes) from either age group, suggesting that microglial senescence is not yet apparent at this age. We did note however, that while M1 pro-inflammatory genes displayed age-dependent differences to the first LPS challenge, anti-inflammatory M2 genes did not, suggesting a previously unreported disparity in the effects of aging on M1 and M2 microglial gene expression. More studies are needed to fully understand how acute and chronic systemic inflammation, conditions which are highly prevalent in middle-aged and older populations, affect microglia in the aging CNS. We suggest that the exposure of microglia to systemic inflammation at critical periods such as middle-age, may set the stage for CNS vulnerability to neurodegeneration, and other neural disorders associated with neuroinflammation.

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FIGURE LEGENDS

**Fig 1:** Microglial number, expression of CD11b, and CD45 after intraperitoneal LPS injection. Young adult and middle-aged mice were injected with PBS or LPS (5mg/kg, i.p.) at times 0 and 24 hours (arrows). Microglia were immunomagnetically isolated at 0, 3 and 24 after the first LPS injection, and 3 hours after the second PBS or LPS injection (24+3h). The yield of isolated microglia are shown in (A). Cells were also analyzed by flow cytometry for cell surface expression of CD11b and CD45 (B). Representative CD45 fluorescence intensity histograms (C) and CD45 mean fluorescent intensity was quantification (D) is shown. One symbol, p<0.05; two symbols, p<0.01; three symbols, p<0.001; * time point vs. baseline (0h) in young mice; † time point vs. baseline (0h) in middle-aged mice; # young adult vs. middle-aged mice at the indicated time point; ‡ time point vs. all other time points in young adults; †† time point vs. all other time points in middle-aged adults.

**Fig 2:** Microglial M1 pro-inflammatory gene mRNA levels after intraperitoneal LPS injection. Young adult and middle-aged mice were injected with PBS or LPS (5mg/kg, i.p.) at times 0 and 24 hours (arrows). Microglia were immunomagnetically isolated at 0, 3 and 24 after the first LPS injection, and 3 hours after the second PBS or LPS injection (24+3 hours). qRT-PCR was used to evaluate the expression of iNos (A), Tnfα (B), Il-6 (C) and Il-1β (D). One symbol, p<0.05; two symbols, p<0.01; three symbols, p<0.001; * time point vs. baseline (0h) in young adult mice; † time point vs. baseline (0h) in middle-aged mice; # young adult vs. middle-aged mice at the indicated time point.
**Fig 3:** Microglial M2 anti-inflammatory gene mRNA levels after intraperitoneal LPS injection. Young adult and middle-aged mice were injected with PBS or LPS (5mg/kg, i.p.) at times 0 and 24 hours (arrows). Microglia were immunomagnetically isolated at 0, 3 and 24 after the first LPS injection, and 3 hours after the second PBS or LPS injection (24+3 hours). qRT-PCR was used to evaluate the expression of *Il-10* (A), *Arginase-1* (B) and *Ym1* (C). One symbol, p<0.05; two symbols, p<0.01; three symbols, p<0.001; * time point vs. baseline (0h) in young adult mice; + time point vs. baseline (0h) in middle-aged mice.

**Fig 4:** Microglial *Tlr2* and *Tlr4* mRNA levels after intraperitoneal LPS injection. Young adult and middle-aged mice were injected with either PBS (baseline) or LPS (5mg/kg, i.p.) at times 0 and 24 hours. Microglia were immunomagnetically isolated at 3 and 24 after the first LPS injection, and 3 hours after the second LPS injection (24+3h). qRT-PCR was used to evaluate the expression of *Tlr2* (A) and *Tlr4* (B). One symbol, p<0.05; two symbols, p<0.01; * time point vs. baseline (0h) in young mice; + time point vs. baseline (0h) in middle-aged mice.

**Fig 5:** Inflammatory gene mRNA levels in the spleen after intraperitoneal LPS injection. Young adult and middle-aged mice were injected with either PBS or LPS (5mg/kg, i.p.) at times 0 and 24 hours (arrows). Spleens were harvested at 0, 3 and 24 after the first LPS injection and 3 hours after a second PBS or LPS injection (24+3 hours). qRT-PCR was used to evaluated the expression of *Ifnγ*(A), *Il-1β* (B) and *Tnfα* (C). One symbol, p<0.05; two symbols, p<0.01; three symbols, p<0.001; * time point vs. baseline (0h) in young adult mice; + time point vs. baseline
(0h) in middle-aged mice; # p=0.059, young adult vs. middle-aged mice at the indicated time point.


Table 1. Primer sequences.

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<th>Gene</th>
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<th>Reverse primer (5'→3')</th>
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Fig 1

A

**Figure 1.** Graph showing the number of CD11b+ cells per mg tissue over time for young and middle-aged groups. The solid line represents the young group, and the dashed line represents the middle-aged group. Arrows indicate the time points for LPS treatment. Statistical significance is indicated by asterisks: **p < 0.01, #p < 0.05.**
Young

Middle-aged

Fig 1
Fig 1
Fig 2
Fig 2
Fig 3
Fig 4

A

TLR2 mRNA (fold change)

Young
Middle-aged

Baseline LPS 3h LPS 24h LPS 24+3h

B

TLR4 mRNA (fold change)

Young
Middle-age

Baseline LPS 3h LPS 24h LPS 24+3h
Fig 5
Fig 5

C

\[ \text{TNF}\alpha \text{ mRNA [fold change]} \]

-2
0
2
4
6
8
10

time [h]

Young

Middle-aged

**

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Fig 5