Published in final edited form as:

J Neuroimmunol. 2018 November 15; 324: 90–99. doi:10.1016/j.jneuroim.2018.09.002.

Microglia priming by interleukin-6 signaling is enhanced in aged mice

Katherine M. Garner¹, Ravi Amin³, Rodney W. Johnson³, Emily J. Scarlett¹, Michael D. Burton^{1,2,*}

¹Laboratory of Neuroimmunology and Behavior, School of Behavioral and Brain Sciences, University of Texas at Dallas 800 W. Campbell Road, Richardson, TX 75080, United States

²Center for Advanced Pain Studies, University of Texas at Dallas, Richardson, TX 75080

³Laboratory of Integrative Immunology and Behavior, Animal Science Department, University of Illinois at Urbana-Champaign, Urbana, 7 Animal Sciences Lab 1207 W. Gregory Dr., Urbana, IL 61801, USA

Abstract

During peripheral infection, excessive production of pro-inflammatory cytokines in the aged brain from primed microglia induces exaggerated behavioral pathologies. While the pro-inflammatory cytokine IL-6 increases in the brain with age, its role in microglia priming is not known. This study examined the functional role of IL-6 signaling on microglia priming. Our hypothesis is that IL-6 signaling mediates primed states of microglia in the aged. An initial study assessed age-related alteration in IL-6 signaling molecules; sIL-6R and sgp130 were measured in cerebrospinal fluid of young and aged wild-type animals. Subsequent studies of isolated microglia from C57BL/6J (IL-6^{+/+}) and IL-6 knock-out (IL-6^{-/-}) mice showed significantly less MHC-II expression in aged IL-6^{-/-} compared to IL-6^{+/+} counterparts. Additionally, adult and aged IL-6^{+/+} and IL-6^{-/-} animals were administered lipopolysaccharide (LPS) to simulate a peripheral infection; sickness behaviors and hippocampal cytokine gene expression were measured over a 24 h period. Aged IL-6^{-/-} animals were resilient to LPS-induced sickness behaviors and recovered more quickly than IL-6^{+/+} animals. The age-associated baseline increase of IL-1 β gene expression was ablated in aged IL-6^{-/-} mice, suggesting IL-6 is a key driver of cytokine activity from primed microglia in the aged brain. We employed *in vitro* studies to understand molecular mechanisms in priming factors. MHC-II and pro-inflammatory gene expression (IL-1 β , IL-10, IL-6) were measured after treating BV.2 microglia with sIL-6R and IL-6 or IL-6 alone. sIL-6R enhanced expression of both pro-inflammatory genes and MHC-II. Taken together, these data suggest IL-6 expression throughout life is involved in microglia priming and increased amounts of IL-6 following peripheral LPS challenge are involved in exaggerated sickness behaviors in the aged.

*Corresponding author. Address: University of Texas at Dallas, BSB 10.546, 800 W. Campbell Rd. AD-14, Richardson, Texas 75080, USA, michael.burton@utdallas.edu (M.D. Burton).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical Abstract

Keywords

MHC-II; inflammation; IL-6 trans signaling; neuroimmunology

Introduction

Peripheral immune stimulation causes the production of pro-inflammatory cytokines, such as IL-6. The signal for these cytokines is transported to the brain via both neural and humoral pathways, including vagal afferents and direct crossing of the blood-brain barrier (Banks et al., 1994; Maier Steven F. et al., 2006; Poon et al., 2013; Quan, 2008). In the brain, microglial cells respond to signals from the periphery by producing pro-inflammatory cytokines, which then target neurons to elicit a sickness behavior response that is adaptive in nature (Dantzer Robert et al., 2006). Microglia appear to be the main cell in the brain that express the IL-6 receptor and potentially secrete IL-6 during peripheral immune stimulation (Burton et al., 2013). IL-6 knockout (IL-6^{-/-}) mice have shown an overall decrease in the number of activated brain macrophages associated with cortical lesions, suggesting a role for IL-6 in the orchestration of central nervous system inflammation (Penkowa et al., 1999). IL-6 in the central nervous system is also implicated in a myriad of behavioral pathways, including but not limited to neurodegeneration, astrogliosis, and changes in *c-fos* expression (Banks et al., 1994; Campbell et al., 1993; Vallières et al., 1997). In the brain of adult mice, IL-6 plays a pivotal role in mediating lipopolysaccharide (LPS)-induced sickness behaviors (Burton et al., 2011) as well as cognitive deficits (Sparkman et al., 2006; Wei et al., 2015). Although it is known that aged mice experience exaggerated sickness behaviors (Godbout et al., 2005; Kelley et al., 2013), it is yet unclear if IL-6 contributes to these behaviors.

In addition to the classical IL-6 pathway, where IL-6 binds its receptor on the cell membrane, there is an IL-6 trans-signaling pathway. In this pathway, IL-6 binds the soluble IL-6 receptor (sIL-6R) in extra cellular fluid; this complex then binds gp130 present on the cell membrane of any cell (Rose-John and Neurath, 2004). Therefore, an IL6R-/gp130+ cell is able to utilize IL-6 signaling (Rose-John and Neurath, 2004). Our previous work has identified this mechanism in sickness and cognitive behaviors in adult mice (Burton et al.,

2011; Burton and Johnson, 2012, p. 20). However, little has been done to investigate the effects of this mechanism on sickness and other behaviors in aged mice.

Microglia are derived from early myeloid lineage cells and represent approximately 10–12% of the total central nervous system cell population (Ginhoux et al., 2010). Normally, in the absence of any stimuli, microglia are quiescent and in an immune surveillance state (Nimmerjahn et al., 2005). Once activated, microglia possess a macrophage-like phenotype, including inflammatory cytokine production, phagocytosis, and antigen presentation (Ransohoff and Perry, 2009). This neuroinflammatory process is normally transient, with microglia returning to a resting state as the immune stimulus is resolved. However, in various brain environments, from neurodegenerative disease to aging, it is proposed that elements render microglia in a “primed” or “reactive” state, wherein a subsequent local or peripheral immune challenge causes an exaggerated and protracted cytokine production (d’Avila et al., 2018; Dilger Ryan N. and Johnson Rodney W., 2008; Godbout and Johnson, 2009; Norden and Godbout, 2013). Furthermore, markers of primed microglia, such as major histocompatibility complex class II (MHC-II) and CD68, are increased in the brain during pathology and normal aging (Burton et al., 2016; Frank et al., 2006a; Ogura et al., 1994; Safaiyan et al., 2016; Wong et al., 2005). Although typically confined to professional antigen presenting cells, MHC-II can be induced in other cells types if exaggerated amounts of pro-inflammatory substances, such as IL-6, are present in the extracellular environment (Holling et al., 2004). *Ex-vivo* or peripheral immune stimulation results in an exaggerated cytokine response in microglia that express higher levels of MHC-II in aged mice (Henry et al., 2008; Njie et al., 2012). Furthermore, studies have also shown IL-6 induced MHC-II expression in peripheral monocyte-derived cells (Shafer et al., 2002; Vassiliadis and Papadopoulos, 1995). Taken together, these findings suggest that enhanced microglial priming in aged populations may be due to the influence of IL-6.

Evidence from previous studies also shows that microglia from aged mice are potent producers of IL-6 (Godbout and Johnson, 2004; Ye and Johnson, 1999). Decreasing the amount of IL-6 after or during peripheral stimulation also decreases the amounts of other pro-inflammatory cytokines (Godbout et al., 2004). Further findings show that exaggerated pro-inflammatory cytokines in aged mice interact with the brain microenvironment, leading to more severe sickness behaviors (Godbout et al., 2005), depressive-like behaviors (Godbout et al., 2008), and deficits in hippocampal-dependent learning and memory when compared with younger animals (Barrientos et al., 2006; Chen et al., 2008). Interestingly, when the pro-inflammatory arm of IL-6 is inhibited during peripheral immune stimulation, aged mice are refractory to cognitive deficits (Burton et al., 2011). Although these data lay the groundwork to support the notion that microglial priming via MHC-II plays a central role in exaggerated neuroinflammation and behavioral deficits, IL-6-specific involvement during aging has yet to be determined. This study therefore seeks to bridge multiple gaps, including establishing a causative role of IL-6 signaling in the protracted sickness phenotype associated with aging.

Experimental Procedures

BV.2 microglial cell culture

The murine microglia cell line, BV.2 (a gift from Linda Van Eldik, Northwestern University, Evanston, IL; used at UIUC) has been used as a model to investigate the neuroimmune system (Jang et al., 2008; Zorina et al., 2010). Cells were maintained in 150-cm² tissue culture flasks (BD Falcon, Franklin Lakes, NJ) in Dulbecco's Modified Eagle's Media (DMEM) (Bio-Whittaker, Cambrex, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 200 mM glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator under 5% CO₂. Confluent cultures were passed by trypsinization. Cells were centrifuged (5 minutes (min) at 27°C, 200 × *g*) and culture medium was removed. In all experiments, cells were re-suspended in DMEM supplemented with 10% FBS and seeded in six-well plates (BD Falcon, Franklin Lakes, NJ) at a population of 5 × 10⁵ cells per well overnight at 37°C in a humidified incubator under 5% CO₂ before treatments. Cells were treated with sterile saline containing 0.1% bovine serum albumin (BSA) (vehicle) or 25ng/mL sIL-6R (R&D systems, Minneapolis, MN) for 1 hour (h) followed by treatment with recombinant 100–1000pg/mL IL-6 (R&D systems, Minneapolis, MN) or 100ng/mL LPS (serotype 0127:B8, obtained from Sigma, St. Louis, MO) for 12–24hr. Cells were then washed with ice cold phosphate buffered saline (PBS) and prepared for flow cytometric analysis or gene expression. BV.2 microglial cells were assayed for the surface markers Cd11b and MHC-II as described previously, with a few modifications (Burton et al., 2011). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA) in a PBS/1% BSA/ sodium azide solution and incubated with anti-CD11b APC and anti- MHC-II PE (eBioscience, San Diego, CA), fluorescently labeled isotype antibodies for APC and PE (eBioscience, San Diego, CA), and unstained samples were used for controls. Expression of surface receptors was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Thirty thousand events were collected; flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Animal Studies

Adult (3–5 months) and aged (22–24 months) male C57BL/6 (IL-6^{+/+}) and IL-6 knockout B6.129S2-Il6tm1 Kopf/J (IL-6^{-/-}) (Kopf et al., 1994) mice were used. All mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were 2-months old upon receipt. Mice were housed in polypropylene cages and maintained at 21°C under a reverse-phase 12-h light-dark cycle with *ad libitum* access to water and rodent chow. At the end of each study, mice were examined post mortem for gross signs of disease (e.g., tumors or splenomegaly). Data from mice determined to be unhealthy were excluded from the analysis (<5%).

All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee.

Experimental protocols

Mice were handled 1–2 minutes each day for 7 days before experimentation to acclimate them to handling. To assess the effects of systemic LPS on sickness behavior and pro-inflammatory gene expression in the hippocampus, mice were injected intraperitoneally (IP) with sterile saline or 3.30 mg/kg body weight (100 µg) LPS (serotype 0127:B8, obtained from Sigma, St. Louis, MO). An array of sickness behaviors that assess motivation and survivability measures (locomotor activity, food intake, and weight loss) (Kent et al., 1992) were recorded starting 4 hr after LPS administration and continued until 24 hr. Mice were killed by CO₂ asphyxiation 24 hr later. The brain was rapidly removed and dissected to obtain hippocampal tissue. Hippocampal tissue was snap frozen in liquid nitrogen and stored at –80o C for later analysis. In some cases, microglia from whole brain, to be used in flow cytometry experiments, were also isolated from these animals.

Microglia isolation

In experiments for flow cytometry, microglia from whole brain were isolated as described previously, with few modifications (Cardona et al., 2006; Henry et al., 2009). Mice were euthanized by CO₂ asphyxiation, and whole brains were collected and stored in sterile PBS. Brains were homogenized by passage through a 70 µm cell strainer in Dulbecco's Phosphate Buffered Salt Solution (DPBS) supplemented with 0.2% glucose. Resulting homogenates were centrifuged at 600 × g for 6 min at 10°C. Supernatants were removed and cell pellets were re-suspended in a 70% isotonic Percoll (GE-healthcare, Uppsala, Sweden) supplemented with phenol red (0.01%) at room temperature. A discontinuous Percoll density gradient of 70%, 50%, 35%, and 0% isotonic Percoll was set up. The gradient was centrifuged for 20 min at 2000 × g and microglia were collected from the interphase between the 70% and 50% Percoll layers (Frank et al., 2006b). Cells were washed with DPBS and then re-suspended in a flow buffer consisting of PBS/0.5% BSA/0.01% sodium azide solution. The number of viable cells was determined using a hemacytometer and 0.1% trypan blue staining; each isolation yielded approximately 3 ×10⁵ viable cells.

Flow Cytometry

Flow cytometric analysis of microglial surface markers was performed as described previously, with a few modifications (Henry et al., 2008). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA) in flow buffer. The cells were then incubated with anti-CD11b-Allophycocyanin (APC), anti-CD45-Fluorescein isothiocyanate (FITC), and anti-MHC-II-Phycoerythrin (PE) antibodies (eBioscience, CA); fluorescently labeled isotype antibodies for APC, FITC, and PE (eBioscience, San Diego, CA), and unstained samples were used as controls. Expression of surface receptors was determined using a Becton-Dickinson LSR II Flow Cytometer (Red Oaks, CA). Thirty thousand events were collected and microglia were identified by CD11b⁺ and CD45^{low} expression (Ford et al., 1995). Flow data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Behavioral tests

Locomotor activity: Mice were maintained in their home cage. Locomotor activity was video recorded during 5 min intervals using a camera mounted approximately 91.0 cm directly above the center of the cage floor. Tests were conducted during the dark phase (between 07:00 and 19:00) of the light/dark cycle under infrared lighting to aid video recording. Baseline behavior was taken just before LPS treatment (0 µg or 100 µg IP); measurements taken 4, 6, 8, and 24 hr afterwards were considered to be sickness behaviors. Videos were tracked to by Ethovision (Noldus, Leesburg, VA) software to record total distance moved. Additionally, body weight and food intake were measured at each time point over the 24 hr period.

Cytokine mRNA measurement by quantitative real-time PCR

Total RNA from BV.2 cells or mouse hippocampi was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO.). A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real time PCR was performed using the Applied Biosystems (Foster, CA) Assay-on Demand Gene Expression protocol as previously described (Krzyszton et al., 2008). In brief, cDNA was amplified by PCR where a target cDNA (IL-6, Mm00446190_m1; IL-1β, Mm00434228_m1; TNF-α, Mm00443258_m1; and IL-10, Mm0089636_g1; MHC-II, Mm00439221_m1) and a reference cDNA (glucose-3 phosphate dehydrogenase, Mm99999915_g1) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed in triplicate under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold difference.

ELISA

Roughly 20ul of cerebrospinal fluid was collected from the cisterna magna of adult (3–5 months) and aged (22–24 months) mice. ELISAs for sIL-6R and sgp130 (R&D Systems) were conducted according to kit protocol.

Statistical analysis

All data were analyzed using the ANOVA routine in Statview and MIXED procedure of the Statistical Analysis System software (SAS Inst., Cary, NC). Data were subjected to a univariate analysis to ensure normality. BV.2 average data was subjected to a two-way ANOVA (pre-treatment × treatment). Locomotor data were subjected to an ANOVA (age × genotype × treatment × time) using repeated measures in which time (0, 4, 6, 8, and 24 h) was a within subjects measure, and age and treatment (LPS or sterile saline) and genotype were between subjects measures; for ease of reading, data have been split by genotype in graphs. Weight loss, food intake, microglia, and cytokine mRNA data were analyzed using a three-way ANOVA (age × genotype × treatment). Post hoc Bonferroni tests for across group

comparisons were used to determine if treatment means were significantly different from one another ($p < 0.05$). All data are presented as mean $\pm$ standard error of the mean (SEM).

Results

IL-6 signaling is enhanced in the brains of aged mice

IL-6 is a potent modulator of inflammation that is increased during normal aging (Wei et al., 1992; Young et al., 1999). However, it is unclear if this leads to functional differences. We therefore examined differences in signaling molecules between adult and aged mice in the absence of inflammatory stimulus. The cerebrospinal fluid of aged mice had increased amounts of sIL-6R compared with adult counterparts, although adult and aged mice did not produce significantly different amounts of its inhibitor, sgp130 (Figure 1). Furthermore, the brains of aged mice had elevated amounts of Cd11b⁺/MHC-II⁺ cells, indicative of primed microglia. However, aged IL-6 knockout (IL-6^{-/-}) mice exhibited lower amounts of Cd11b⁺/MHC-II⁺ cells than their wild type (IL-6^{+/+}) counterparts, although there was no significant genotype difference in adult mice (Figure 2C). Taken together, these findings suggest that aging enhances susceptibility to IL-6, specifically IL-6 trans-signaling. It also posits a role for aging alone being capable of priming microglia.

Microglia from aged IL-6^{-/-} mice are refractory to priming

In aging, there is a population of microglia that are in a primed state; studies have found that both mRNA and protein for MHC-II, the marker of this primed state, are basally upregulated during aging (Frank et al., 2006a; Henry et al., 2009) and may be a major pre-disposing factor for the exaggerated pro-inflammatory cytokine production seen in aging (Henry et al., 2009) (Figure 2). Because IL-6 has been shown to be capable of inducing MHC-II (Shafer et al., 2002; Vassiliadis and Papadopoulos, 1995), we investigated the effects of IL-6 throughout life by isolating microglia from adult and aged IL-6^{+/+} and IL-6^{-/-} mice, stained for CD11b, CD45, and MHC-II, and analyzed them by flow cytometry (Figure 2). To verify our microglia population, we profiled the isolated cells for a CD11b⁺/CD45^{low} cell population in representative two-color dot blots for adult (Figure 2A) and aged (Figure 2B) mice (Ford et al., 1995). Upon identifying microglia cells, we then gated our population for CD11b⁺/MHC-II⁺ for each age and genotype (Figure 2 A & B, bottom panels). Despite the strain difference from previous studies (BALB/c vs. C57 B/6) (Henry et al., 2009), the results are consistent in that the number of MHC-II expressing microglia is increased in IL-6^{+/+} aged mice; about 20% versus 4% in adult mice (Figure 2C). Interestingly, the microglia from aged IL-6^{-/-} mice displayed a significantly reduced number of MHC-II expressing microglia, down to roughly 11% ($p < 0.05$) (Figures 2 A, B, & C), while there was no difference in MHC-II expression in IL-6^{-/-} versus IL-6^{+/+} adult mice (Figures 2A and 2C).

IL-6- *trans*-signaling induced MHC-II and pro-inflammatory gene expression in BV.2 microglia

While previous studies have shown IL-6 alone has an effect on MHC-II expression (Shafer et al., 2002), recent studies implicate IL-6 trans-signaling in pro-inflammatory processes (Greenhill et al., 2011). We believe this to be important in microglia activation and MHC-II

upregulation. To assess if IL-6 trans-signaling is involved in MHC-II induction on microglial cells, MHC-II cell surface expression was examined after BV.2 microglia were pre-treated 1 hr with vehicle or sIL-6R then treated 12 hr with 0, 100, or 1000 pg/mL IL-6. Vehicle and sIL-6R treated microglial BV.2 cells express a similar amount of MHC-II, roughly 50% of the cells (Figures 3A and 3B). Pre-treatment with sIL-6R for 1 hr significantly increased the induction of MHC-II expressing cells to 100 pg/mL IL-6 ($p<0.01$) (Figures 3A and 3B); this dose of IL-6 alone did not significantly induce MHC-II expression. Figure 3B indicates a ceiling effect, where 1000 pg/mL of IL-6 maximally induces MHC-II expression on microglia cells at 80–90% of the cells, regardless of the presence of sIL-6R. However, in the absence of sIL-6R, this maximum dose of IL-6 was required to induce a change in the amount of MHC-II expressed on individual cells. We also observed significant differences in IL-6 evoked gene expression in the presence of sIL-6R (Figures 4A, B, & C). Microglia treated with LPS alone saw little or no changes in gene expression in the absence of sIL-6R; however, the same treatment in the presence of sIL-6R showed an increase in the expression of these pro-inflammatory genes (Figures 4 D, E, & F). these findings suggest that classic and *trans*-signaling on microglia readily regulate microglia activation or priming.

IL-6 is involved in recovery from LPS-induced sickness behavior in aged animals

Primed microglia produce exaggerated amounts of pro-inflammatory cytokines, including IL-6, that induce exacerbated and prolonged sickness behaviors. Given the results from our and others' studies (Burton et al., 2011; Nguyen et al., 2012), we investigated the effects of IL-6 and LPS-induced sickness behaviors in the aged. Adult and aged IL-6^{+/+} and IL-6^{-/-} mice were administered IP LPS; locomotor activity (Figures 5A and 5B), body weight, and food intake (Figures 5C–F) were used as measures of sickness. As expected, LPS-treated IL-6^{+/+} adult and aged mice showed a time-dependent decrease in locomotor activity ($p<0.001$). Adult and aged LPS-treated IL-6^{-/-} mice also displayed a decrease in locomotor activity for the 4 and 6 hr time points; although their vehicle-treated counterparts did have a decrease in locomotor activity, it was not statistically different from baseline (Figures 5B). In figure 5A, LPS induced modest sickness in adult IL-6^{+/+} mice where LPS-induced sickness behavior returned to baseline by 24 hr post-injection; however, aged IL-6^{+/+} mice were still impaired at 24 hr, consistent with prior studies (Godbout et al., 2005). The LPS-induced depression of locomotor activity in IL-6^{+/+} adult and aged mice was ameliorated in the IL-6^{-/-} animals at the 8 hr time point, a moderate difference in the adult, and a dramatic improvement in the aged (Figure 5B). Although the locomotor behaviors of LPS-treated IL-6^{-/-} aged mice were statistically lower from saline-treated animals at the 4 and 6 hr time point, the depression of locomotor activity was not as severe as IL-6^{+/+} counterparts. The initial depression of locomotor activity can be attributed to LPS-induced IL-1 β production, which is implicated in the onset of sickness (Abraham and Johnson, 2009). While the LPS-induced sickness response was not inhibited in adult IL-6^{-/-} mice, these animals exhibited a recovery to LPS treatment at 8 hr post LPS, similar to the aged mice. Additionally, IL-6^{-/-} mice had significant mitigating effects on the LPS-induced weight loss ($p<0.05$, Figures 5C and 5E) seen in the aged cohort, whereas the weight loss was not exaggerated as in the IL-6^{+/+} mice. However, the lack of IL-6 did not have similar effects in adult mice. Moreover, LPS-induced reductions in food intake for both IL-6^{+/+} and IL-6^{-/-} aged mice were significantly lower than LPS-treated adult counterparts (Figures 5D and 5F). The food intake

data showed no difference in adult IL-6^{-/-} mice, similar to the weight loss data. These data suggest that IL-6 is important for maintaining sickness behavior, and aged animals appear to be more sensitive to the effects of LPS-induced IL-6 production.

IL-6 is implicated in reduced levels of baseline IL-1 β and MHC-II gene expression in aged animals

Previous studies have shown that MHC-II activated microglia are mainly responsible for the exaggerated production of IL-1 β (Henry et al., 2009). Our earlier results suggest that a life devoid of IL-6 reduces the amount MHC-II expressed on microglia. We then proposed to assay for gene expression of markers of inflammation IL-1 β , TNF- α , MHC-II, and IL-6 in hippocampal tissue 24 hr after LPS treatment. We chose to use the hippocampus, because it is enriched in microglia and a region of the brain that is sensitive to LPS-induced pro-inflammatory cytokines. Our data are consistent with previous studies (Godbout et al., 2005) and show a basal age-related upregulation of IL-1 β , IL-6, and MHC-II expression in IL-6^{+/+} mice. However, in IL-6^{-/-} mice, this upregulation of IL-1 β (Figure 6A) and MHC-II (Figure 6C) is significantly decreased. Upon LPS treatment there was no significant overall age $\times$ genotype $\times$ LPS interaction in IL-1 β , TNF- α , or MHC-II gene expression. This signifies that while IL-6 is not the only cytokine important for microglia priming and exaggerated cytokine production, it is an integral component.

Discussion

Peripheral immune-to-brain communication causes the upregulation of various pro-inflammatory cytokines, including IL-6. During normal aging, primed microglia are responsible for the exaggerated and prolonged cytokine production and the ensuing behavioral response during a peripheral infection (Godbout et al., 2005; Godbout and Johnson, 2009); making it a necessity to understand the involvement of individual cytokines on microglial priming and behavioral output, during aging. These data extend previous works, with several novel findings. First, we show that IL-6 *trans*-signaling is an important pathway for MHC-II induction in microglia, as well as upregulation of IL-1 β and IL-10. Secondly, there is an age-related increase of molecules important to IL-6 signaling. An age-related increase of MHC-II expression was decreased in aged IL-6^{-/-} microglia. Next, we showed the exaggerated sickness behavior response seen by our lab and others (Burton et al, 2013; Godbout et al, 2005) in aged mice after peripheral LPS injection was mitigated in IL-6^{-/-} mice. Lastly, baseline age-related induction of IL-1 β and MHC-II was decreased in aged IL-6^{-/-} mice. These data further demonstrate that MHC-II plays a role in microglial hyperactivity in the aged brain and indicate that IL-6 is a significant contributor to the microglia-induced exaggeration in neuroinflammation in aged mice following peripheral immune stimulation.

An important finding of this study was that the age-related increase of MHC-II expression in microglia and hippocampal tissue was down-regulated in aged IL-6^{-/-} mice (Figures 2B and 6C). While an age-related increase in MHC-II gene expression in the brain of aged mice and rats has previously been reported (Frank et al., 2006a; Godbout et al., 2005), the current study provides novel evidence of an IL-6 dependent mechanism for increased MHC-II

mRNA in hippocampus and protein expression on microglia isolated from aged mice. The data show approximately 20% of microglia from aged IL-6^{+/+} mice were positive for MHC-II expression compared to 11% in the aged IL-6^{-/-} animals (Figures 2A and 2B). Furthermore, the hippocampal tissue of aged IL-6^{-/-} mice displayed half of the baseline gene expression seen in aging (Figure 6C). These data indicate that IL-6 is involved in the primed phenotype in microglia from aged mice.

Microglia priming is implicated in various other models of peripheral stimulation. For instance, in the ME7 murine model of prion disease, peripheral administration of LPS induces an upregulated pro-inflammatory cytokine response in microglia, which elicits exaggerated sickness behavior, and accelerates progression of the disease (Combrinck et al., 2002; Cunningham et al., 2009). Similar studies in other rodent models of chronic neurodegenerative disease, such as amyotrophic lateral sclerosis (Evans et al., 2013; Nguyen et al., 2004, 2002), show the disease is responsible for a priming effect and a subsequent signal from the periphery elicits a microglial response that is greater in magnitude than individual stimuli alone. This interaction lays the basis to our understanding on how infection serves as a risk factor for exaggerated behavioral manifestations in patients with chronic neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Dilger and Johnson, 2008; Doorn et al., 2012; Holmes et al., 2003). Utilizing BV.2 microglia, we were able to observe that sIL-6R plays a role in priming, or MHC-II upregulation. Using two stimuli, IL-6 and LPS, we were able to observe the biological range of gene expression from microglia when pre-treated with sIL-6R and a more specific stimulus (IL-6) or a broad-spectrum stimulus (LPS) (Figure 4). This is likely influenced by IL-6-*trans* signaling, as the presence of sIL-6R greatly enhanced changes in cytokine gene expression. These data are consistent with *in vivo* studies examining the effects of sIL-6R and LPS on gene expression (Burton et al., 2011), as well as studies examining overall changes of cortical cytokine levels following LPS insult in aged and adult mice (Henry et al., 2009).

A study using IL-6 knock-out mice showed that IL-6 is important for peripheral immune stimulated-sickness behavior in adult mice (Nguyen et al., 2012). Furthermore, studies that blocked the pro-inflammatory arm of IL-6 signaling facilitated recovery of LPS-induced sickness behavior in adult mice (Burton et al., 2011). However, these previous studies did not examine the effects of IL-6 on sickness behaviors in aged mice. The present study expands on the work done in adult mice, finding that aged sickness behavior in response to peripheral LPS injection in IL-6^{-/-} mice was blunted. Previous findings in aged wild type (i.e. IL-6^{+/+}) mice show delayed recovery from LPS-induced sickness behaviors compared to their adult counterparts (Godbout et al., 2005), we anticipated that peripheral LPS injection would be associated with a protracted decrease in locomotor activity and an exaggeration in weight loss and a decrease in food intake over a 24 hr period regardless of genotype, albeit reduced in IL-6^{-/-} mice. Interestingly, both adult and aged IL-6^{-/-} mice displayed a recovery from LPS-induced depression of locomotor activity at 8 hr post LPS (Figure 5B), whereas adult IL-6^{+/+} mice had recovered from LPS-induced sickness behaviors by 24 hr. However, aged IL-6^{+/+} mice continued to exhibit a significant decrease in locomotor activity, body weight, and food intake at the 24 hr timepoint, implicating a role for IL-6 signaling in exaggerated sickness behaviors. Body weight measurements from aged

IL-6^{-/-} mice taken 24 hr after LPS injection were not different from the adult LPS treated groups, further indicating that aged IL-6^{-/-} animals do not show the exaggerated behavioral phenotype (Figure 5E). Contrary to our expectations, food intake from aged LPS-injected IL-6^{-/-} mice significantly decreased compared to their adult counterparts (Figure 5F), presumably because IL-1 β compensates for IL-6 to act in the hypothalamus to control food intake (Harden et al., 2008).

A final original finding was that the baseline upregulation of gene expression of IL-1 β and MHC-II observed in aging was decreased in the hippocampus of aged IL-6^{-/-} mice (Figures 6A and 6C). Although treatment with LPS increased the amounts of IL- β mRNA to comparable levels in aged mice regardless of genotype, similar amounts of IL-1 β mRNA were observed in adult mice regardless of genotype as well. These findings suggest a lifelong expression of IL-6 is capable of priming microglia independent of IL-1 β . In aging, primed microglia exhibit constitutive expression of IL-1 β (Henry et al., 2009), which is associated with neurological disease (Griffin and Mrak, 2002) and behavioral deficits (Abraham and Johnson, 2009). Conversely, disruption of IL-1 β activity is linked to reduced lesion volume post-stroke (Schielke et al., 1998) as well as restoring LPS-induced behavioral deficits (Zhu et al., 2010); therefore, the basal downregulation we observed could be indicative of an overall improvement in the aged phenotype. In addition, this downregulation also displays a possible new mechanism of IL-6 - IL-1 β interaction. While other studies have shown that IL-1 β influences IL-6 (Cahill and Rogers, 2008), our data provide evidence of a bi-directional interaction. Furthermore, as no significant differences of IL-1 β were observed between adult IL-6^{+/+} and IL-6^{-/-} mice, it is unlikely that the genotypes experience different sensitivities to LPS. Taken together, our findings indicate that IL-6 signaling contributes to both microglial priming and the immunological and behavioral deficits seen after peripheral LPS challenge in the aged.

In conclusion, the present study demonstrates that IL-6 is involved in the pronounced age-related increase in MHC-II protein in microglia, likely via the trans-signaling pathway. Moreover, IL-6 augments the behavioral response to peripheral LPS injection associated with exaggerated microglial activation, and induction of pro-inflammatory IL-1 β in aging. These data are significant because they show the importance of IL-6 to microglia priming in the aged brain and provide further evidence that MHC-II activated microglia are significant contributors to the exaggerated neuroinflammation and behavior in aged mice following peripheral LPS challenge.

Acknowledgements

This work was supported by NIH grant: K22NS096030 (MDB) and The University of Texas STARS program research support grant (MDB).

Glossary

CD11b

integrin family member, involved in the complement pathway, leukocyte adhesion, and inflammatory response mediation

Cluster of differentiation 45 (CD45)

transmembrane protein found on hematopatic cells that assists in their activation

Cluster of differentiation 68 (CD68)

protein highly expressed in cells of monocyte lineage, including macrophages

Cytokine

substance secreted by certain cells of the immune system that affects other cells

Interleukin 1 β (IL-1 β)

cytokine implicated in fever production

Interleukin 6 (IL-6)

pro-inflammatory cytokine secreted by macrophages to induce immune response

Lipopolysaccharide (LPS)

major component of gram-negative bacteria membrane

Major histocompatibility complex class II (MHC-II)

set of proteins used to present antigen to T cells

Signal transducer and activator of transcription 3 (STAT3)

transcription factor that responds to cytokines, in particular IL-6

Tumor necrosis factor α (TNF- α)

cytokine implicated in the acute phase reaction and systemic inflammation

Abbreviations

IL-6	Interleukin-6
LPS	lipopolysaccharide
sIL-6R	soluble IL-6 receptor
gp130	glycoprotein 130
sgp130	soluble glycoprotein 130
MHC-II	major histocompatibility complex class II
IL-1β	interleukin-1 β
TNF-α	tumor necrosis factor alpha
CD45	cluster of differentiation 45
CD68	cluster of differentiation 68
STAT3	Signal transducer and activator of transcription 3

References

- Abraham J, Johnson RW, 2009 Central inhibition of interleukin-1 β ameliorates sickness behavior in aged mice. *Brain. Behav. Immun* 23, 396–401. 10.1016/j.bbi.2008.12.008 [PubMed: 19152833]
- Banks WA, Kastin AJ, Gutierrez EG, 1994 Penetration of interleukin-6 across the murine blood-brain barrier. *Neurosci. Lett* 179, 53–56. [PubMed: 7845624]
- Barrientos RM, Higgins EA, Biedenkapp JC, Sprunger DB, Wright-Hardesty KJ, Watkins LR, Rudy JW, Maier SF, 2006 Peripheral infection and aging interact to impair hippocampal memory consolidation. *Neurobiol. Aging* 27, 723–732. 10.1016/j.neurobiolaging.2005.03.010 [PubMed: 15893410]
- Burton MD, Johnson RW, 2012 Interleukin-6 trans-signaling in the senescent mouse brain is involved in infection-related deficits in contextual fear conditioning. *Brain. Behav. Immun., Aging, Brain, Behavior, and Immunity* 26, 732–738. 10.1016/j.bbi.2011.10.008
- Burton MD, Rytch JL, Amin R, Johnson RW, 2016 Dietary Luteolin Reduces Proinflammatory Microglia in the Brain of Senescent Mice. *Rejuvenation Res.* 19, 286–292. 10.1089/rej.2015.1708 [PubMed: 26918466]
- Burton MD, Rytch JL, Freund GG, Johnson RW, 2013 Central inhibition of interleukin-6 trans-signaling during peripheral infection reduced neuroinflammation and sickness in aged mice. *Brain. Behav. Immun* 30, 66–72. 10.1016/j.bbi.2013.01.002 [PubMed: 23354002]
- Burton MD, Sparkman NL, Johnson RW, 2011 Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. *J. Neuroinflammation* 8.
- Cahill CM, Rogers JT, 2008 Interleukin (IL) 1 β Induction of IL-6 Is Mediated by a Novel Phosphatidylinositol 3-Kinase-dependent AKT/I κ B Kinase α Pathway Targeting Activator Protein-1. *J. Biol. Chem* 283, 25900–25912. [PubMed: 18515365]
- Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L, 1993 Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc. Natl. Acad. Sci* 90, 10061–10065. 10.1073/pnas.90.21.10061 [PubMed: 7694279]
- Cardona AE, Huang D, Sasse ME, Ransohoff RM, 2006 Isolation of murine microglial cells for RNA analysis or flow cytometry. *Nat. Protoc* 1, 1947–1951. 10.1038/nprot.2006.327 [PubMed: 17487181]
- Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW, 2008 Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain. Behav. Immun* 22, 301–311. 10.1016/j.bbi.2007.08.014 [PubMed: 17951027]
- Combrinck MI, Perry VH, Cunningham C, 2002 Peripheral infection evokes exaggerated sickness behavior in pre-clinical murine prion disease. *Neuroscience* 112, 7–11. 10.1016/S0306-4522(02)00030-1 [PubMed: 12044467]
- Cunningham C, Campion S, Lunnon K, Murray CL, Woods JFC, Deacon RMJ, Rawlins JNP, Perry VH, 2009 Systemic Inflammation Induces Acute Behavioral and Cognitive Changes and Accelerates Neurodegenerative Disease. *Biol. Psychiatry* 65, 304–312. [PubMed: 18801476]
- d'Avila JC, Siqueira LD, Mazeraud A, Azevedo EP, Foguel D, Castro-Faria-Neto HC, Sharshar T, Chrétien F, Bozza FA, 2018 Age-related cognitive impairment is associated with long-term neuroinflammation and oxidative stress in a mouse model of episodic systemic inflammation. *J. Neuroinflammation* 15, 28 10.1186/s12974-018-1059-y [PubMed: 29382344]
- Robert Dantzer, Bluthé Rose-Marie, Gheusi Gilles, Sandrine Cremona, Sophie Layé, Patricia Parnet, Kelley Keith W., 2006 Molecular Basis of Sickness Behavior. *Ann. N. Y. Acad. Sci* 856, 132–138. 10.1111/j.1749-6632.1998.tb08321.x
- Dilger Ryan N, Johnson Rodney W, 2008 Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. *J. Leukoc. Biol* 84, 932–939. 10.1189/jlb.0208108 [PubMed: 18495785]
- Doorn KJ, Lucassen PJ, Boddeke HW, Prins M, Berendse HW, Drukarch B, van Dam A-M, 2012 Emerging roles of microglial activation and non-motor symptoms in Parkinson's disease. *Prog. Neurobiol* 98, 222–238. 10.1016/j.pneurobio.2012.06.005 [PubMed: 22732265]

- Evans MC, Couch Y, Sibson N, Turner MR, 2013 Inflammation and neurovascular changes in amyotrophic lateral sclerosis. *Mol. Cell. Neurosci.*, Neuroinflammation in neurodegeneration and neurodysfunction 53, 34–41. 10.1016/j.mcn.2012.10.008
- Ford AL, Goodsall AL, Hickey WF, Sedgwick JD, 1995 Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4⁺ T cells compared. *J. Immunol* 154, 4309–4321. [PubMed: 7722289]
- Frank MG, Barrientos RM, Biedenkapp JC, Rudy JW, Watkins LR, Maier SF, 2006a mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiol. Aging* 27, 717–722. 10.1016/j.neurobiolaging.2005.03.013 [PubMed: 15890435]
- Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF, 2006b Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: Immunophenotypic and functional characteristics. *J. Neurosci. Methods* 151, 121–130. 10.1016/j.jneumeth.2005.06.026 [PubMed: 16125247]
- Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M, 2010 Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science* 330, 841–845. 10.1126/science.1194637 [PubMed: 20966214]
- Godbout JP, Berg BM, Kelley KW, Johnson RW, 2004 α -Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. *J. Neuroimmunol.* 149, 101–109. 10.1016/j.jneuroim.2003.12.017 [PubMed: 15020070]
- Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW, 2005 Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system | The FASEB Journal. *FASEB J.* 19, 1329–1331. [PubMed: 15919760]
- Godbout JP, Johnson RW, 2009 Age and Neuroinflammation: A Lifetime of Psychoneuroimmune Consequences. *Immunol. Allergy Clin. North Am.*, Psychoneuroimmunology 29, 321–337. 10.1016/j.iac.2009.02.007
- Godbout JP, Johnson RW, 2004 Interleukin-6 in the aging brain. *J. Neuroimmunol, Neuroimmune Circuits and Infectious Disease: Proceedings of the 9th Conference of the Society for NeuroImmune Pharmacology* 147, 141–144. 10.1016/j.jneuroim.2003.10.031
- Godbout JP, Moreau M, Lestage J, Chen J, Sparkman NL, Connor JO, Castanon N, Kelley KW, Dantzer R, Johnson RW, 2008 Aging Exacerbates Depressive-like Behavior in Mice in Response to Activation of the Peripheral Innate Immune System. *Neuropsychopharmacology* 33, 2341–2351. 10.1038/sj.npp.1301649 [PubMed: 18075491]
- Greenhill CJ, Rose-John S, Lissilaa R, Ferlin W, Ernst M, Hertzog PJ, Mansell A, Jenkins BJ, 2011 IL-6 Trans-Signaling Modulates TLR4-Dependent Inflammatory Responses via STAT3. *J. Immunol* 186, 1199–1208. 10.4049/jimmunol.1002971 [PubMed: 21148800]
- Griffin W Sue T, Mrak Robert E., 2002 Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. *J. Leukoc. Biol* 72, 233–238. 10.1189/jlb.72.2.233 [PubMed: 12149413]
- Harden LM, Plessis I. du, Poole S, Laburn HP, 2008 Interleukin (IL)-6 and IL-1 β act synergistically within the brain to induce sickness behavior and fever in rats. *Brain. Behav. Immun* 22, 838–849. 10.1016/j.bbi.2007.12.006 [PubMed: 18255258]
- Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT, Sheridan JF, Godbout JP, 2008 Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. *J. Neuroinflammation* 5, 15 10.1186/1742-2094-5-15 [PubMed: 18477398]
- Henry CJ, Huang Y, Wynne AM, Godbout JP, 2009 Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1 β and anti-inflammatory IL-10 cytokines. *Brain. Behav. Immun* 23, 309–317. 10.1016/j.bbi.2008.09.002 [PubMed: 18814846]
- Holling TM, Schooten E, van Den Elsen PJ, 2004 Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. *Hum. Immunol* 65, 282–290. 10.1016/j.humimm.2004.01.005 [PubMed: 15120183]

- Holmes C, El-Okli M, Williams AL, Cunningham C, Wilcockson D, Perry VH, 2003 Systemic infection, interleukin 1 β , and cognitive decline in Alzheimer's disease. *J. Neurol. Neurosurg. Amp Psychiatry* 74, 788 10.1136/jnnp.74.6.788
- Jang S, Kelley KW, Johnson RW, 2008 Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. *Proc. Natl. Acad. Sci* 105, 7534 10.1073/pnas.0802865105 [PubMed: 18490655]
- Kelley KW, O'Connor JC, Lawson MA, Dantzer R, Rodriguez-Zas SL, McCusker RH, 2013 Aging leads to prolonged duration of inflammation-induced depression-like behavior caused by *Bacillus Calmette-Guérin*. *Brain. Behav. Immun* 32, 63–69. 10.1016/j.bbi.2013.02.003 [PubMed: 23454036]
- Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Köhler G, 1994 Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368, 339–342. 10.1038/368339a0 [PubMed: 8127368]
- Krzyszton CP, Sparkman NL, Grant RW, Buchanan JB, Broussard SR, Woods J, Johnson RW, 2008 Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. *Am. J. Physiol.-Regul. Integr. Comp. Physiol* 295, R1109–R1114. 10.1152/ajpregu.90302.2008 [PubMed: 18650318]
- Maier Steven F, Goehler Lisa E, Fleshner Monika, Watkins Linda R., 2006 The Role of the Vagus Nerve in Cytokine-to-Brain Communication. *Ann. N. Y. Acad. Sci* 840, 289–300. 10.1111/j.1749-6632.1998.tb09569.x
- Nguyen K, D'Mello C, Le T, Urbanski S, Swain MG, 2012 Regulatory T cells suppress sickness behavior development without altering liver injury in cholestatic mice. *J. Hepatol.* 56, 626–631. 10.1016/j.jhep.2011.09.014 [PubMed: 22027577]
- Nguyen MD, D'Aigle T, Gowing G, Julien J-P, Rivest S, 2004 Exacerbation of Motor Neuron Disease by Chronic Stimulation of Innate Immunity in a Mouse Model of Amyotrophic Lateral Sclerosis. *J. Neurosci.* 24, 1340 10.1523/JNEUROSCI.4786-03.2004 [PubMed: 14960605]
- Nguyen MD, Julien J-P, Rivest S, 2002 Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat. Rev. Neurosci* 3, 216–227. 10.1038/nrn752 [PubMed: 11994753]
- Nimmerjahn A, Kirchhoff F, Helmchen F, 2005 Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. *Science* 308, 1314–1318. 10.1126/science.1110647 [PubMed: 15831717]
- Njie eMalick G., Boelen E, Stassen FR, Steinbusch HWM, Borchelt DR, Streit WJ, 2012 Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiol. Aging* 33, 195.e1–195.e12. 10.1016/j.neurobiolaging.2010.05.008
- Norden DM, Godbout JP, 2013 Review: Microglia of the aged brain: primed to be activated and resistant to regulation. *Neuropathol. Appl. Neurobiol* 39, 19–34. 10.1111/j.1365-2990.2012.01306.x [PubMed: 23039106]
- Ogura K, Ogawa M, Yoshida M, 1994 Effects of ageing on microglia in the normal rat brain: immunohistochemical observations. *Neuroreport* 5, 1224–1226. [PubMed: 7919169]
- Penkowa M, Moos T, Carrasco J, Hadberg H, Molinero A, Bluethmann H, Hidalgo J, 1999 Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice. *Glia* 25, 343–357. 10.1002/(SICI)1098-1136(19990215)25:4<343::AID-GLIA4>3.0.CO;2-V [PubMed: 10028917]
- Poon DC-H, Ho Y-S, Chiu K, Chang RC-C, 2013 Cytokines: How important are they in mediating sickness? *Neurosci. Biobehav. Rev* 37, 1–10. 10.1016/j.neubiorev.2012.11.001 [PubMed: 23153795]
- Quan N, 2008 Immune-To-Brain Signaling: How Important are the Blood–Brain Barrier-independent Pathways? *Mol. Neurobiol.* 37, 142–152. 10.1007/s12035-008-8026-z [PubMed: 18563639]
- Ransohoff RM, Perry VH, 2009 Microglial Physiology: Unique Stimuli, Specialized Responses. *Annu. Rev. Immunol.* 27, 119–145. 10.1146/annurev.immunol.021908.132528 [PubMed: 19302036]
- Rose-John S, Neurath MF, 2004 IL-6 trans-Signaling: The Heat Is On. *Immunity* 20, 2–4. 10.1016/S1074-7613(04)00003-2 [PubMed: 14738759]

- Safaiyan S, Kannaiyan N, Snaidero N, Brioschi S, Biber K, Yona S, Edinger AL, Jung S, Rossner MJ, Simons M, 2016 Age-related myelin degradation burdens the clearance function of microglia during aging. *Nat. Neurosci* 19, 995–998. 10.1038/nn.4325 [PubMed: 27294511]
- Schielke GP, Yang G-Y, Shivers BD, Betz AL, 1998 Reduced Ischemic Brain Injury in Interleukin-1 β Converting Enzyme—Deficient Mice. *J. Cereb. Blood Flow Metab* 18, 180–185. 10.1097/00004647-199802000-00009 [PubMed: 9469161]
- Shafer LL, McNulty JA, Young MRI, 2002 Brain Activation of Monocyte-Lineage Cells: Involvement of Interleukin-6. *Neuroimmunomodulation* 10, 295–304. 10.1159/000069973 [PubMed: 12759566]
- Sparkman NL, Buchanan JB, Heyen JRR, Chen J, Beverly JL, Johnson RW, 2006 Interleukin-6 Facilitates Lipopolysaccharide-Induced Disruption in Working Memory and Expression of Other Proinflammatory Cytokines in Hippocampal Neuronal Cell Layers. *J. Neurosci* 26, 10709–10716. 10.1523/JNEUROSCI.3376-06.2006 [PubMed: 17050710]
- Vallières L, Lacroix S, Rivest S, 1997 Influence of Interleukin-6 on Neural Activity and Transcription of the Gene Encoding Corticotrophin-releasing Factor in the Rat Brain: An Effect Depending Upon the Route of Administration. *Eur. J. Neurosci* 9, 1461–1472. 10.1111/j.1460-9568.1997.tb01500.x [PubMed: 9240403]
- Vassiliadis S, Papadopoulos GK, 1995 IL-6-mediated MHC class II induction on RIN-5AH insulinoma cells by IFN- γ occurs via the G-protein pathway. *Mediators Inflamm.* 4, 374–379. 10.1155/S0962935195000615 [PubMed: 18475668]
- Wei J, Xu H, Davies JL, Hemmings GP, 1992 Increase of plasma IL-6 concentration with age in healthy subjects. *Life Sci.* 51, 1953–1956. 10.1016/0024-3205(92)90112-3 [PubMed: 1453878]
- Wei P, Liu Q, Li D, Zheng Q, Zhou J, Li J, 2015 Acute nicotine treatment attenuates lipopolysaccharide-induced cognitive dysfunction by increasing BDNF expression and inhibiting neuroinflammation in the rat hippocampus. *Neurosci. Lett* 604, 161–166. 10.1016/j.neulet.2015.08.008 [PubMed: 26259694]
- Wong AM, Patel NV, Patel NK, Wei M, Morgan TE, de Beer MC, de Villiers WJS, Finch CE, 2005 Macrosialin increases during normal brain aging are attenuated by caloric restriction. *Neurosci. Lett* 390, 76–80. 10.1016/j.neulet.2005.07.058 [PubMed: 16157452]
- Ye S-M, Johnson RW, 1999 Increased interleukin-6 expression by microglia from brain of aged mice. *J. Neuroimmunol.* 93, 139–148. 10.1016/S0165-5728(98)00217-3 [PubMed: 10378877]
- Young DG, Skibinski G, Mason JI, James K, 1999 The influence of age and gender on serum dehydroepiandrosterone sulphate (DHEA-S), IL-6, IL-6 soluble receptor(IL-6 sR) and transforming growth factor beta 1 (TGF- β 1) levels in normal healthy blood donors. *Clin. Exp. Immunol* 117, 476–481. 10.1046/j.1365-2249.1999.01003.x [PubMed: 10469050]
- Zhu C-B, Lindler KM, Owens AW, Daws LC, Blakely RD, Hewlett WA, 2010 Interleukin-1 Receptor Activation by Systemic Lipopolysaccharide Induces Behavioral Despair Linked to MAPK Regulation of CNS Serotonin Transporters. *Neuropsychopharmacology* 35, 2510–2520. 10.1038/npp.2010.116 [PubMed: 20827273]
- Zorina Y, Iyengar R, Bromberg KD, 2010 Cannabinoid 1 Receptor and Interleukin-6 Receptor Together Induce Integration of Protein Kinase and Transcription Factor Signaling to Trigger Neurite Outgrowth. *J. Biol. Chem* 285, 1358–1370. 10.1074/jbc.M109.049841 [PubMed: 19861414]

Highlights

- IL-6 trans-signaling is involved in microglia priming.
- Microglia in aged mice are more susceptible to IL-6-induced priming.
- Ablation of IL-6 signaling significantly reduces exaggerated LPS-induced sickness behaviors in aged animals.

Figure 1. Pro-inflammatory soluble IL-6 receptor, but not soluble gp130, is increased in aged animals.

ELISA was performed on Cerebral Spinal Fluid (CSF) collected from the cisterna magna of adult and aged animals (n=6–7; samples plated in duplicate). The CSF of naïve aged animals contained increased amounts of sIL6-R compared to their adult counterparts. Amounts of sgp130 were not different between groups. Means with different letters are significantly different from each other ($p < 0.05$).

Figure 2. IL-6 signaling exaggerates markers of microglial priming in aged mice. Microglia were collected from adult and aged IL-6^{+/+} or IL-6^{-/-} animals (n=6–8) and underwent flow cytometry. Representative dot blots from (A) naïve adult (B) and aged IL-6^{+/+} and IL-6^{-/-} animals show how microglia were identified by Cd11b⁺/Cd45^{low} staining (top panels). Aged IL-6^{-/-} animals had significantly less Cd11b⁺/MHC-II⁺ animals than their IL-6^{+/+} counterparts (bottom panel). (C) Percentages of Cd11b⁺/MHC-II⁺ cells were not significantly different between adult IL-6^{+/+} and IL-6^{-/-} animals (*= p < 0.05).

Figure 3. sIL-6R enhances IL-6 induced MHC-II expression in BV.2 microglia.

(A) Representative dot blot of MHC-II expression from BV.2 microglial cells (n=3–5 replicates) treated with IL-6 (12hr) in the presence or absence of sIL-6R (25ng/mL; 1 hr pre-) (bottom panel). (B) MHC-II expression is enhanced when cells are treated with 100pg/mL of IL-6 in the presence of sIL-6R, but not 100pg/mL of IL-6 alone. A ceiling effect is reached at 1000 pg/mL of IL-6, regardless of the presence of sIL-6R. (C) This ceiling effect is confirmed by mean fluorescence intensity (MFI) data. Means with different letters are significantly different from each other ($p < 0.05$).

Figure 4. Effects of sIL-6R on cytokine gene expression in BV.2 microglia.

BV.2 cells (n=3–5 replicates) were treated with (A–C) IL-6 (100pg/mL) or (D–F) LPS

(100ng/mL) for 8hr in the presence or absence of sIL-6R (25ng/mL, 1hr pre-treated).

Cytokine gene expression was significantly increased in the presence of sIL-6R after treatment with both IL-6 and LPS in all genes. sIL-6R was required for changes in gene expression of (A) IL-1 β and (B) IL-10 after treatment with IL-6. Although LPS was able to induce changes in gene expression in the absence of sIL-6R (D–F), the presence of sIL-6R greatly enhanced these changes. Means with different letters are statistically different from each other ($p < 0.05$).

Figure 5. Ablation of IL-6 signaling reduces differences in age-related sickness behaviors.

Adult and aged (A) IL-6^{+/+} and (B) IL-6^{-/-} animals were given intraperitoneal Lipopolysaccharide (LPS) as a peripheral immune challenge. Locomotor activity, food intake, and body weight were measured as indicators of the sickness response. (A) Aged IL-6^{+/+} animals (n=8–10) exhibited prolonged depression of locomotor behavior at 24hr. (B) This response was eliminated in IL-6^{-/-} animals (n=8–9), (*= p < 0.05). (F) Although LPS-treated aged IL-6^{-/-} animals consumed less food than their adult counterparts, overall body weight was not different (E). LPS-treated aged IL-6^{+/+} animals consumed less food (D) and weighed less (C) than their adult counterparts. Means with different letters are statistically different from each other, (p < 0.05).

Figure 6. The presence of IL-6 affects basal mRNA levels of inflammatory markers in aged animals.

mRNA from the hippocampal tissue of adult and aged animals (n=6-7) 24hr after LPS treatment was measured. (A) WT Aged animals had basally increased IL-1 β mRNA levels that was ameliorated in the aged IL-6^{-/-} animals. However, this difference did not persist after LPS treatment; aged animals of both genotypes showed similar increases in IL-1 β mRNA (A). Furthermore, LPS-induced changes in TNF- α mRNA in aged animals regardless of genotype (B). Lastly, Aged IL-6^{+/+} animals had a basal increase in MHC-II mRNA and aged IL-6^{-/-} animals had lower levels of MHC-II mRNA similar to adult IL-6^{+/+} animals. LPS treatment did not affect levels of MHC-II mRNA in aged animals of either genotype (C). Adult animals had no differences in inflammatory marker mRNA, regardless of treatment or genotype. Means with different letters are statistically different from each other, (p < 0.05).