1 **Activated microglia from aged mice are less sensitive to anti-inflammatory feedback from**
2 **IL-4**
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14

15 **Abstract**

16 Aging is associated with increased inflammatory conditions both peripherally and
17 centrally. Our lab has previously shown that microglia, innate immune cells of the central
18 nervous system, become primed and reactive as a function of age. Following an inflammatory
19 stimulus these primed microglia show exaggerated and prolonged activation associated with an
20 extended sickness response and induction of depressive-like behavior. The purpose of this study
21 was to identify a mechanistic cause for prolonged microglial activation following immune
22 challenge. We report that following a peripheral injection of LPS, microglia from adult mice
23 upregulate both inflammatory (M1) and anti-inflammatory (M2c) gene expression, extending our

knowledge of the M2b monocyte phenotype. Furthermore, we demonstrate that activated microglia from aged mice show exaggerated expression of M1, M2a, and M2c related genes, rather than a definitive shift towards M1. Protein expression of the receptors for IL-10 (IL-10R1) and IL-4 (IL-4Rα) were assessed on microglia of adult mice following treatment with LPS. Microglia showed marked upregulation of IL-4Rα after LPS treatment, whereas expression of IL-10R1 did not change. Furthermore, activated microglia from aged mice failed to upregulate IL-4Rα. Treatment of activated microglia ex vivo with IL-4 promoted a down-regulation of M1 related gene, inducible nitric oxide synthase (iNOS) and an upregulation of M2 related gene, Arginase I (Arg) following LPS treatment in adult mice. Activated microglia from aged mice, however, maintained high levels of iNOS gene expression, though they still promoted Arg expression. Taken together, these results indicate that activated microglia from aged mice show impairments in the receptor and cell signaling pathways necessary for anti-inflammatory feedback from IL-4.

1. Introduction

Within the central nervous system (CNS), microglia are responsible for the induction of an innate immune response by receiving and propagating inflammatory signals (Nguyen et al., 2002). In the absence of inflammatory stimuli, microglia are in a resting state and involved in immune surveillance (Nimmerjahn et al., 2005). Once activated, microglia perform several macrophage-like functions including phagocytosis, inflammatory and anti-inflammatory cytokine production, and antigen presentation (Garden and Moller, 2006). Normally these neuroinflammatory changes are transient with microglia returning to a resting state as the immune stimulus is resolved.

1 Aging or neurological disease produces a brain environment where microglia become "primed or reactive" and show an exaggerated inflammatory response following peripheral immune activation (Perry et al., 2003; Godbout and Johnson, 2006). In support of this notion, we have reported that either central (Abraham and Johnson, 2008; Huang et al., 2008) or peripheral innate immune challenges (Godbout et al., 2005; Chen et al., 2008; Henry et al., 2009) lead to amplified and prolonged brain cytokine expression in aged BALB/c mice compared to adult controls. This exaggerated microglial activation is accompanied by amplified mRNA and intracellular protein expression of IL-1β (Henry et al., 2009) after treatment with lipopolysaccharide (LPS) (an activator of the innate immune response). An exaggerated microglial response with age is relevant because it is coupled with a myriad of complications including cognitive impairment (Barrientos et al., 2006; Chen et al., 2008; Barrientos et al., 2009), altered febrile response (Barrientos et al., 2009), exaggerated sickness behavior (Godbout et al., 2005; Abraham et al., 2008; Huang et al., 2008), and protracted depressive-like behavior (Godbout et al., 2008).

15 In an attempt to classify and study subsets of functionally diverse activation states in macrophage/microglia (monocytes) , they are divided broadly into M1 (classically activated) and M2 (alternatively activated) categories corresponding to T_H1 and T_H2 immune responses (Mantovani et al., 2004). M1 monocytes are elicited by interferon gamma (IFN-γ) + LPS and are generally pro-inflammatory producing interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), IL-6, IL-12, IL-23, and reactive oxygen- and nitrogen species (Colton, 2009). M2 monocytes are more diverse in their functional polarization and have been subdivided into M2a (alternative activation, or wound healing), M2b (regulate inflammation, pro- and anti-inflammatory), and M2c (classical deactivation, anti-inflammatory) activation states (Mantovani et al., 2004). M2a

1 monocytes are generally anti-inflammatory, elicited by IL-4 or IL-13 and identified by increased
2 expression of Arginase1 (Arg), chitinase3-like-3 (Ym-1), and mannose receptor (Mrc1) (Colton,
3 2009). M2b monocytes are both pro- and anti-inflammatory and elicited by immune complexes
4 and LPS. These monocytes are identified by a high production of inflammatory cytokines,
5 including IL-1β, but also produce high levels of IL-10 and protect the cell against LPS toxicity
6 (Mosser and Karp, 1999; Mosser and Edwards, 2008). M2c monocytes express IL-10, IL-4Rα,
7 and SOCS3, and are generally elicited by TGF-β or IL-10 (Colton, 2009), though certain
8 components can also be elicited by IL-4. Because of the potential for IL-4 to influence the anti-
9 inflammatory profile of microglia by promoting an M2a or M2c activation state, we decided to
10 investigate IL-4 signaling in the CNS as a function of age.

11 Detectible levels of IL-4 mRNA and protein have been found within the rodent CNS
12 (Szczepanik et al., 2001; Nolan et al., 2005). Moreover, reduced concentrations of IL-4 in the
13 aged brain have been shown to reduce long term potentiation (LTP), increase inflammation and
14 reduce neurogenesis in the hippocampus, and a reduced ability to perform in learning and
15 memory tasks (Maher et al., 2005; Nolan et al., 2005; Ziv et al., 2006; Derecki et al., 2010).
16 Addition of IL-4 to cultured neurons increases their expression of CD200, an important regulator
17 of microglia activation (Lyons et al., 2007). IL-4 has also been shown to suppress production of
18 monocyte-derived IL-1β (Hart et al 1989) and prevent LPS-induced neuronal cell injury by
19 decreasing microglia-derived production of TNF-α and nitric oxide (NO) (Chao et al 1993).
20 Further evidence of the anti-inflammatory properties of IL-4 are shown through the ability of IL-
21 4 treatments to alleviate animal models of inflammatory diseases such as arthritis (Miossec et al.,
22 1994; Joosten et al., 1996)

The reason for prolonged microglial activation in the aged brain after LPS is unknown, but may be related to a reduced sensitivity to anti-inflammatory feedback, including IL-4. Therefore, the purpose of this study was to determine the balance of M1 and M2 microglia in adult and aged mice and assess whether activated microglia from aged mice can respond to IL-4 and reduce inflammation following a peripheral LPS challenge. Here we show that peripheral LPS injection promoted an M1 and M2c microglial phenotype in adult mice and that this profile remained consistent in aged mice, though both M1 and M2c markers were elevated compared to adult controls. Furthermore, we show that following LPS adult mice enhance microglial surface expression of the receptor for IL-4 (IL-4Rα), and that this enhancement was not present in microglia from aged mice. A potential consequence of reduced IL-4Rα expression in activated microglia from aged mice was an inability to reduce LPS-associated iNOS gene expression in aged mice. Arginase was still promoted in activated microglia from aged mice indicating that a dysregulated IRS-2/PI3K/Akt pathway may be responsible for select insensitivity to IL-4 treatment.

2. Methods

2.1. Animals

Adult (3-6 months-old) BALB/c mice were obtained from a breeding colony kept in barrier-reared conditions in a specific-pathogen-free facility at the Ohio State University. Mice were individually housed in polypropylene cages and maintained at 25° C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. For age comparisons, male BALB/c mice (18-22 mo) were purchased from the National Institute on Aging specific-pathogen-free colony (maintained at Charles River Laboratories, Inc., MA). The median lifespan for BALB/c

1 mice is approximately 26 months (Morley and Trainor, 2001). To investigate changes that occur
2 from adulthood to what is considered aged, 3-6-month-old (young adult) and 18-22-month-old
3 (aged) male mice were used. Upon arrival, mice were individually housed as described above.
4 All procedures were in accordance with the National Institute of Health Guidelines for the Care
5 and Use of Laboratory Animals and were approved by The Ohio State University Institutional
6 Laboratory Animal Care and Use Committee.

7 **2.2 Experimental Protocols**

8 In the first study, adult male (3-4 mo) BALB/c mice received an intraperitoneal (i.p.)
9 injection of saline or *Escherichia coli* LPS (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis,
10 MO) and were euthanized 4 h or 24 h later (n=5-10). This LPS dosage was selected because it
11 elicits a proinflammatory cytokine response in the brain resulting in a transient sickness response
12 in adult mice (Berg et al., 2004; Godbout et al., 2005; Henry et al., 2008). In a related study,
13 adult male BALB/c mice were injected i.p. with vehicle or minocycline (50 mg/kg, Sigma, St.
14 Louis, MO) for three consecutive days (Henry et al., 2008) Twelve hours following the last
15 injection mice were injected i.p. with LPS or vehicle and were euthanized 4 h later (n=9). In both
16 sets of experiments, brains were homogenized and microglia were isolated by discontinuous
17 Percoll density gradient. Microglia were used for RNA isolation and analysis of M1, M2a and
18 M2c related genes by quantitative PCR.

19 In the next study adult (3 mo) male BALB/c mice received an i.p. injection of saline or
20 LPS (0.33 mg/kg) and microglia were isolated from whole brain homogenate 4 h or 24 h later.
21 Cells were stained with antibodies against CD11b and CD45 to gate specifically on microglia
22 ($CD11b^{+}/CD45^{low}$) and were also stained against IL-10R1 (n=4) or IL-4Rα (n=4-7).

23 The third study consisted of a series of experiments using cultures of microglia-like BV-2

1 cells. BV-2 cells were treated with saline or LPS (10 ng/ml) for 1 h followed by treatment with
2 either vehicle or recombinant IL-4 (R&D Systems; Minneapolis, MN) (20 ng/ml), or vehicle or
3 recombinant IL-10 (R&D Systems; Minneapolis, MN) (10 ng/ml), for an additional 3 h. RNA
4 was isolated and qPCR on M1 and M2 related genes was done. Next, BV-2 cells were treated
5 with saline or LPS (10 ng/ml) for 1 h followed by treatment with vehicle or IL-4 (20 ng/ml) for
6 45 min. Whole cell lysates were collected for western blot analysis of phospho-STAT6 and
7 STAT6.

8 The next study utilized a set of ex vivo microglial cell cultures. Adult (3-4 mo) male
9 BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg). After 4 h microglia were
10 isolated and plated on poly-L-lysine for 1 h. After 1 h cells were treated with vehicle or IL-4 (20
11 ng/ml) for 3 h and RNA was isolated and analyzed for iNOS, IL-1β, and Arg (n=9-10).

12 In the fifth study, adult (3-4 mo) or aged (18-22 mo) male BALB/c mice received an i.p.
13 injection of saline or LPS (0.33 mg/kg) and were euthanized 4 h or 24 h later. The brain was
14 collected and a 1 mm coronal brain section (+0.38 mm from bregma) (Paxinos and Franklin,
15 2004) was taken using a rodent brain matrix (ASI instruments, Warren, MI). Brain sections were
16 used for analysis of mRNA levels of IL-4 (n=5-14). The remainder of the brain was used for
17 microglia isolation. Microglia were then used for RNA isolation and qPCR analysis of M1, M2a
18 and M2c related genes (n=6-9). Using this same model microglia were isolated from adult (3-4
19 mo) and aged (20 mo) male BALB/c mice and stained with antibodies against CD11b and CD45
20 to identify microglia ($CD11b^{+}/CD45^{low}$), as well as an antibody against IL-4Rα (n=4-8) by flow
21 cytometry.

22 In the next study aged (20 mo) male BALB/c mice were given an i.p. injection of saline
23 or LPS (0.33 mg/kg). After 24 h microglia were isolated and plated on poly-L-lysine coated

1 plates for 1 h. Cells were then treated with vehicle or IL-4 (20 ng/ml) for 3 h and RNA was
2 isolated and iNOS, IL-1β, and Arg gene expression was determined using qPCR.
3 In the seventh study, BV-2 cells were treated simultaneously with vehicle or IL-4 (20
4 ng/ml) and DMSO or Ly-294-002 (20 nM) for 45 min. Whole cell lysates were then collected for
5 western blot analysis of phospho-STAT6, phospho-Akt, and STAT6.
6 **2.3. BV-2 cell culture**
7 BV-2 microglia cell lines were cultured in growth medium (DMEM (Bio-Whittaker,
8 Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT) sodium bicarbonate 3.7
9 g/L, 200 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml fungizone)
10 as previously described (Wynne et al., 2010) Cultures were maintained and incubated at 37°C
11 with 95% humidity and 5% CO_2 and growth medium was replenished every third day until
12 confluence. For RNA isolation/qPCR experiments, cells were seeded at $1x10^5$ cells per well in
13 24-well plates and allowed to adhere for 20 h. Immediately before treatment, cultures were
14 washed twice with serum free DMEM medium and supplemented with warm serum free DMEM
15 medium containing experimental treatments.
16 **2.4. Microglial isolation**
17 Microglia were isolated from whole brain homogenates as described previously (Henry et
18 al., 2009; Wynne et al., 2010). In brief, brains were homogenized in Hank's Balanced Salt
19 Solution (HBSS, pH 7.4) by passing through a 70 μm nylon cell strainer. Resulting homogenates
20 were centrifuged at 600 g for 6 min. Supernatants were removed and cell pellets were re-
21 suspended in 70% isotonic Percoll at room temperature. A discontinuous Percoll density gradient
22 was layered as follows: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged
23 for 20 minutes at 2000 g and microglia were collected from the interphase between the 70% and

1 50% Percoll layers. Microglia were washed and re-suspended in sterile HBSS. Each brain
2 extraction yielded approximately 3 x 10^5 viable cells. We have previously characterized these
3 cells as approximately 85% $CD11b^+/CD45^{low}$ microglia (Henry et al., 2009). Based on this
4 previous characterization, cells isolated by Percoll density separation are referred to as "enriched
5 microglia".

6 **2.5. RNA isolation and qPCR**

7 RNA was isolated from brain slice, BV-2 cell lines, or enriched microglia. For brain slice
8 and BV-2 cells, total RNA was isolated using the Tri-Reagent protocol (Sigma, MO) and
9 subjected to the DNA-free™ RNA clean up procedure (Ambion, TX). For enriched microglia,
10 RNA was isolated using the RNeasy plus mini kit (Qiagen, CA) or the PrepEase kit (USB, CA).
11 In all RNA isolation procedures, RNA concentration was determined by spectrophotometry
12 (Eppendorf, NY) and RNA was reverse transcribed to cDNA.

13 Quantitative PCR was performed using the Applied Biosystems (Foster, CA) Taqman®
14 Gene Expression assay as previously described (Godbout et al., 2005). In brief, cDNA was
15 amplified by RT-PCR where a target cDNA (e.g., IL-1β, iNOS, Arginase) and a reference
16 cDNA (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were amplified simultaneously
17 using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye
18 (NFQ). Fluorescence was determined on an ABI PRISM 7300-sequence detection system
19 (Applied Biosystems, CA). Data were analyzed using the comparative threshold cycle (Ct)
20 method and results are expressed as fold difference from control.

21 **2.6. Flow cytometry**

22 Cells were assayed for microglial cell surface antigens by flow cytometry as previously
23 described (Henry et al., 2008; Henry et al., 2009). In brief, Fc receptors were blocked with anti-

CD16/CD32 antibody. Next, enriched microglia were incubated with rat anti-mouse CD14-FITC, IL-4Rα-PE, IL-10R1-PE, CD45-PerCP-Cy5.5, and CD11b-APC antibodies (eBioscience, CA). Expression of these surface receptors was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Twenty thousand events were recorded and microglia were identified by $CD11b^{+}/CD45^{low}$ expression (Ford et al., 1995; Nair and Bonneau, 2006). For each antibody, gating was determined based on appropriate negative isotype stained controls. Microglia from aged mice were found to have extensive autofluorescence compared to microglia from adult mice, and therefore separate isotypes for adult and aged mice were used. Microglia from aged mice were confirmed to have elevated MHCII expression using this method validating the use of separate isotypes (data not shown) (Henry et al., 2009). Flow data were analyzed using FlowJo software (Tree Star, CA).

2.7. Western blot

Western blot analysis was performed as described previously (Nguyen and Benveniste, 2000). In brief, $1x10^6$ BV-2 cells were lysed in 500 µl of ice-cold 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 1 mM phenylmethylsufonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT) and 50 mM Tris-base, pH 7.4. Lysates were clarified and normalized using Biorad protein reagent. Proteins were resolved by SDS-PAGE (60 µg/lane) under reducing conditions in 8% gels and then electrotransferred to nitrocellulose membranes. Immunoreactive proteins were visualized using the indicated primary antibodies (anti-phospho-STAT6 (Cell Signaling, Beverly, MA) (1:1000) or anti-phospho-Akt (Cell Signaling, Beverly, MA) (1:1000)) and enhanced ECL reagents followed by autoradiography and densitometry. *Membrane Stripping and re-probing-* Nitrocellulose membranes were incubated for 60 minutes at room temperature with Thermo Scientific stripping

buffer (#21059, Waltham, MA). Stripped membranes were extensively washed with Tris buffered Saline (TBS)-0.01% Tween 20 and immunoreactive proteins were visualized using anti-STAT6 (R&D Systems, Minneapolis, MN) (1:2000) and enhanced ECL reagents followed by autoradiography and densitometry.

2.8. Ex-vivo microglia cultures

Adult and aged mice were given an i.p. injection of saline or LPS (0.33 mg/kg) and enriched microglia were collected using the Percoll gradient separation protocol outlined above 4 h or 24 h later. Cells were counted and plated ($1x10^5$ cells/well) on poly-L-lysine coated 24-well plates in serum free DMEM medium for 1 h. Microglia were then stimulated with vehicle or IL-4 (20 ng/ml) for an additional 3 h.

2.9. Statistical Analysis

To ensure a normal distribution, data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) statistical software (Cary, NC). To determine significant main effects and interactions between main factors, data were analyzed using one- (i.e. Age, Pretreatment, Treatment), two- (i.e. Age × Pretreatment, Age x Treatment, Pretreatment x Treatment) or three- (i.e. Age × Pretreatment x Treatment) way ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an *F*-protected *t*-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± standard error of the mean (SEM). Values were considered significant at P-values < 0.05 and a tendency at P-values ≤ 0.15.

3. Results

3.1. LPS injection promotes M1 and M2c gene expression in enriched microglia

1 We have previously shown that microglia are more reactive in the brain of aged BALB/c
2 mice compared with adults (Henry et al., 2009; Wynne et al., 2010). To begin to determine M1
3 and M2 activation states of microglia after a peripheral injection of LPS, genes associated with
4 classical activation (M1), alternative activation (M2a), and classic deactivation (M2c)
5 (Mantovani et al., 2004) were determined. Adult (3 mo) male BALB/c mice were injected with
6 LPS and enriched microglia were collected 4 h or 24 h later by Percoll density gradient
7 separation (Henry et al., 2009). Fig.1A shows the relative mRNA expression of M1 (CD86,
8 (inducible nitric oxide synthase (iNOS) and IL-1β), M2a (mannose receptor (Mrc1), arginase I
9 (Arg), and chitinase3-like-3 (Ym-1)) and M2c (IL-10, IL-4Rα, suppressor of cytokine signaling
10 (SOCS)3) related genes 4 h after i.p. injection of LPS. As expected, LPS increased the levels of
11 M1-related gene expression in microglia (CD86 $P<0.05$, iNOS $P<0.0005$, IL-1β $P<0.0001$). LPS
12 injection, however, did not induce an increase in M2a related gene expression with the exception
13 of Ym-1 ($P<0.005$). Moreover, M2c related gene expression was also increased 4 h after LPS
14 injection (IL-10 $P<0.0001$, IL-4Rα $P<0.001$, SOCS3 $P<0.0001$). These results indicate that
15 inflammatory M1 and anti-inflammatory M2c related genes are both increased in microglia
16 within 4 h after peripheral injection of LPS.
17 Next, M1, M2a, and M2c related genes were determined 24 h after LPS corresponding to
18 the resolution of the sickness response in adult mice following acute LPS injection (Berg et al.,
19 2005). The overall LPS induced inflammatory (M1) gene induction was still increased in
20 microglia of adult mice 24 h after LPS compared to saline controls (IL-1β $P<0.0001$, iNOS
21 $P<0.01$), but was lower than the 4 h time-point ($P<0.05$) (Fig.1B). Similar to the results at 4 h,
22 Ym-1 was the only M2a-realted gene that was elevated 24 h following LPS treatment ($P<0.001$).
23 All M2c-related genes tested remained elevated at 24 h after LPS compared to saline controls

1 (IL-10 $P<0.05$, IL-4Rα $P<0.005$, SOCS3 $P<0.05$) (Fig. 1B) and did not significantly differ from
2 levels at 4 h ($P>0.05$). These data indicate that M1 related genes are reduced by 24 h after LPS
3 and that M2c gene expression was maintained.

4 Because minocycline pretreatment attenuates microglia activation and sickness behavior
5 associated with LPS injection (Henry et al., 2008), the degree to which minocycline attenuates
6 M1-related gene expression and enhances M2-related gene expression in microglia after LPS
7 challenge was determined. In this experiment, adult male BALB/c mice were injected with
8 minocycline for 3 consecutive days. Twelve hours following the last minocycline injection, mice
9 received an i.p. injection of saline or LPS and microglia were isolated 4 h later. Similar to the
10 mRNA results obtained in Fig.1A, LPS injection promoted an increase in microglial M1 related
11 genes (IL-1β $P<0.0001$) 4 h after LPS (Fig.1C). As expected, minocycline attenuated induction
12 of IL-1β mRNA 4 h after LPS (Minocycline x LPS: $F(1,33)=4.24$, $P<0.05$). In addition
13 minocycline significantly increased the M2a gene, Ym-1, when given prior to LPS treatment
14 (Minocycline x LPS: $F(1,30)=8.15$, $P<0.009$) and augmented for maintained the induction of the
15 M2c related genes (IL-10: Minocycline LPS, $P<0.05$ compared to Vehicle LPS and Minocycline
16 Saline; IL-4Rα: Minocycline LPS, $P>0.05$ compared to Vehicle LPS, $P<0.002$ compared to
17 Vehicle Saline; SOCS3: Minocycline LPS, $P>0.05$ compared to Vehicle LPS, $P<0.003$ compared
18 to Vehicle Saline) (Fig. 1C). These mRNA data indicate that minocycline attenuates M1 related
19 gene (IL-1β) mRNA expression and increases expression of M2 related genes (Ym-1 and IL-10)
20 in enriched microglia.

21 **3.2. IL-4Rα surface expression increased on microglia following LPS injection.**

22 Because LPS induced an increase in M2 gene expression in enriched microglia, we
23 investigated surface expression of two M2 promoting cytokine receptors, IL-4Rα and IL-10R1.

1 In these experiments adult (3 mo) mice were injected with saline or LPS and enriched microglia
2 were collected 4 h or 24 h later. Fig.2A shows representative bivariate dot plots of CD11b and
3 CD45 staining. Microglia were gated based on the $CD11b^{+}/CD45^{low}$ staining and IL-4Rα and IL-
4 10R1 surface expression was determined on microglia specifically. Representative histograms of
5 IL-4Rα -PE mean fluorescence intensity (MFI) (Fig.2B) and IL-10R1-PE MFI (Fig.2C) on
6 microglia 4 h and 24 h after LPS are shown. Overall LPS increased the MFI of IL-4Rα on
7 microglia, but did not significantly increase the MFI of IL-10R1. Fig.2D shows representative
8 bivariate dot plots of IL-4Rα staining on microglia ($CD11b^{+}/CD45^{low}$). Data from Fig.4D
9 confirm that IL-4Rα expression on microglia was markedly increased 4 h after LPS injection
10 (8.9% ± 2.7% to 45.4% ± 3.3% , main effect of LPS, $F(1,11)=65.45$, $P<0.0001$) and that this
11 increase was still maintained 24 h after LPS (39.3% ± 5.3%, main effect of LPS $F(1,6)=33.29$,
12 $P<0.003$). Fig.4E shows representative bivariate dot plots of IL-10R1 staining on microglia
13 ($CD11b^{+}/CD45^{low}$). These data show that IL-10R1 protein expression is similar to IL-4Rα basal
14 expression on microglia (13.1%), but was not increased on the surface after peripheral injection
15 of LPS (12.1% and 11.4% at 4 h and 24 h, respectively).

16 **3.3 LPS activated BV-2 cells are responsive to the anti-inflammatory effects of IL-4**

17 To better understand the differences of IL-4 and IL-10 on reversing microglia activation,
18 a series of BV-2 experiments were completed. In the first experiment, BV-2 cells were activated
19 with LPS and then treated with either recombinant IL-4 or IL-10. Table 1 shows that IL-4
20 treatment alone decreased mRNA levels of iNOS ($P<0.0001$) and increased mRNA levels of Arg
21 ($P<0.0001$), IL-10 ($P<0.0001$) and SOCS1 ($P<0.0001$). As expected, LPS treatment increased
22 mRNA levels of iNOS ($P<0.0001$) and IL-1β ($P<0.0001$). LPS also increased IL-10 ($P<0.0001$)
23 and SOCS1 ($P<0.0003$) and decreased Arg ($P<0.009$). These data are consistent with microglia

expression of M1 and M2 related genes after i.p. LPS injection as shown in Fig.1A. When LPS treated BV-2 were incubated with IL-4, IL-4 reduced the LPS dependent increase in mRNA levels of iNOS ($F(1,23)=338.91$, $P<0.0001$) and IL-1β ($F(3,23)=28.91$, $P<0.0001$), whereas it enhanced the LPS induced increase in SOCS1 mRNA expression ($F(1,11)=10.06$, $P<0.02$). The IL-4 dependent induction of mRNA levels of Arg and IL-10 were unaffected by LPS.

Whereas IL-4 was capable of reversing microglia M1 activation after LPS and promoted an M2 genotype, IL-10 had little effect in inhibiting an M1 phenotype following pre-treatment with LPS. For example, IL-10 enhanced the LPS induce expression of iNOS ($F(1,9)=23.76$, $P<0.002$) and IL-1β ($F(1,21)=2.57$, $P=0.13$). IL-10 treatment did not affect Arg mRNA expression and IL-10. IL-10 was able to enhance LPS induced SOCS3 expression ($F(1,22)=22.70$, $P<0.0001$). Taken together IL-10 did not seem to promote an anti-inflammatory microglial genotype with pre-treatment of LPS and exacerbated inflammation in this context.

To confirm that IL-4 was inducing the correct cellular signaling pathways within microglia Western blot analysis of STAT-6 phosphorylation was conducted. BV-2 cell cultures were treated with LPS for 1 h followed by IL-4 treatment for 45 min, and the phosphorylation state of STAT-6 was determined. The 45 min time point was based on STAT-6 phosphorylation time course after IL-4 stimulation (data not shown). Fig.3 shows a representative western blot from LPS and IL-4 treated BV-2 cells. These data demonstrate that IL-4 increases STAT-6 phosphorylation and this occurred independent of LPS treatment (main effect of IL-4 $F(1,11)=74.14$, $P<0.0001$).

3.4 IL-4 treated ex-vivo microglial cultures reduce iNOS and promote Arginase (Arg) expression

1 To determine the relative sensitivity of active microglia to direct IL-4 stimulation, adult
2 mice were injected with LPS and enriched microglia were collected 4 h later. These microglia
3 were cultured ex-vivo in the presence of vehicle or recombinant IL-4. Fig.4A shows that
4 enriched microglia collected from LPS treated mice had increased levels of iNOS ($P<0.0001$)
5 and IL-1β ($P<0.0001$) mRNA expression compared to saline controls. Following treatment with
6 IL-4, ex-vivo microglia showed a significant reduction in LPS-induced iNOS expression
7 ($F(1,30)=18.6$, $P<0.0002$). LPS induction of IL-1β mRNA levels, however, were unaffected by
8 IL-4 treatment. Fig.4B shows that Arg expression was markedly induced by the ex-vivo
9 stimulation of IL-4 ($P<0.0001$). Moreover, when microglia were activated in-vivo with LPS,
10 treatment ex-vivo with IL-4 significantly increased Arg gene expression compared to the Saline
11 IL-4 treated group ($F(1,36)=6.74$, $P<0.014$). Taken together, these data indicate that ex-vivo
12 stimulation of active microglia with IL-4 does not affect IL-1β transcripts, but does promote a
13 shift away from an M1, iNOS genotype towards an M2, Arg genotype.

14 **3.5 Microglia from aged mice show a shifted M1 and M2c microglial profile**

15 We next sought to determine differences in overall M1 and M2 gene expression in
16 microglia from aged mice compared to adult controls. In these experiments, adult (3 mo) and
17 aged (20-22 mo) male BALB/c mice were i.p. injected with LPS and enriched microglia were
18 collected 4 h and 24 h later. Fig.5 shows the relative expression of M1 (iNOS and IL-1β), M2a-
19 (Arg, and Ym-1) and M2c- (IL-4Rα, SOCS3) related gene levels 4 h (Fig.5A) (n=6-8) and 24 h
20 (Fig.5B) (n=8-9) after LPS. Corresponding to previous work in our lab there was a significant
21 increase in M1-related iNOS ($P<0.05$) and IL-1β ($P<0.01$) gene expression following LPS. There
22 was also a significant Age x LPS interaction as microglia from aged mice show increased
23 induction of both M1 related genes (iNOS ($F(1,26)=4.58$, $P<0.05$) and IL-1β ($F(1,26)=4.13$,

P<0.05)). There was no significant difference in LPS induced M2a induction between age groups 4 h after LPS. LPS also induced significant upregulation of M2c related genes (IL-4Rα P<0.01, SOCS3 P<0.01). Aged mice treated with LPS also had a significant increase in IL-4Rα upregulation compared to adult mice treated with LPS (P<0.05) (Fig.2A). These data indicate that aged mice show an increase in both M1 and M2c gene expression following LPS treatment compared to adult controls.

Twenty-four h following LPS injections, microglia from aged mice maintained elevated expression of iNOS compared to adult controls (F(1,31)=8.37, P<0.01). IL-1β was also increased in 24 h following LPS compared to saline controls (P<0.0001), but this difference was independent of age. At the 24 h time-point, aged mice treated with LPS showed an upregulation in M2a gene expression compared to all other groups (Arg F(1,31)=6.40, P<0.05; Ym-1 F(1,31)=2.32, P=0.14), and maintained elevated M2c gene expression (IL-4Rα P<0.01, SOCS3 P<0.001) compared to saline controls, though these values were not different from adult mice treated with LPS. These results indicate that microglia from aged mice have exaggerated and prolonged expression of M1 related genes compared to microglia from adult mice, but show no deficits in the upregulation of anti-inflammatory, M2c related gene expression.

3.6 IL-4 mRNA expression changes following LPS

Adult (3 mo) and aged (18-22 mo) mice were treated with saline or LPS. After 4 h or 24 h a coronal brain slice through the prefrontal cortex was taken, total mRNA was isolated and evaluated for IL-4 gene-expression by RT-qPCR. Fig.6 shows that IL-4 mRNA levels were decreased in both adult and aged mice 4 h after LPS injection (P<0.001). IL-4 mRNA expression returned to baseline levels within 24 h after an injection of LPS (n=5-14).

3.7 Upregulation of IL-4Rα on the surface of microglia is impaired in aged mice

Because IL-4 RNA expression was independent of age, we turned to protein expression of IL-4Rα to determine whether an increase or maintenance in IL-4Rα gene expression following LPS in aged mice (Fig.5A,B) corresponded to an increase in protein expression on the surface of microglia. Microglia were stained with fluorochrome-conjugated antibodies to CD11b, CD45, and IL-4Rα. Microglia can be distinguished from other CNS cells by their $CD11b^{+}/CD45^{low}$ staining. For analysis of IL-4Rα on microglia, $CD11b^{+}/CD45^{low}$ stained cells were gated (Fig.2A), and the percentage of cells positive for IL-4Rα was determined for this population. Representative dot plots for IL-4Rα expression on microglia of adult and aged mice are shown in Fig.7A and Fig.7B, respectfully. IL-4Rα protein was elevated on the surface of microglia of adult mice 4 h and 24 h following LPS treatment compared to saline controls ($P<0.0001$), consistent with Fig.2D. Aged mice treated with saline had the same percentage of IL-4Rα positive microglial cells (10.0% ± 1.0%) as adult mice (10.2% ± 0.6%). Following activation with LPS, however, aged mice failed to upregulate the IL-4Rα at either the 4 h or 24 h time-point (4h $F(1,21)=47.34$, $P<0.0001$, 24h $F(1,24)=42.39$, $P<0.0001$) (Fig.7B). These results show that aged mice maintain the same expression of IL-4Rα on the surface of their microglia as adult mice under basal conditions, but show impaired upregulation of the receptor following activation with LPS.

3.8 Activated microglia from aged mice treated ex-vivo with IL-4 do not suppress iNOS expression

To better understand the functional consequence of reduced IL-4Rα on the surface of microglia from aged mice, a series of ex-vivo experiments were conducted to evaluate the role of IL-4 in mediating microglial profile changes independently and in the presence of inflammation. Aged (20 mo) male BALB/c mice were injected i.p. with LPS. After 24 h, corresponding to a

time-point when adult mice have recovered from neuronal inflammation but microglia from aged mice are still active (Henry et al., 2009), microglia were removed and plated on poly-L-lysine coated 24-well plates. Cells were treated with IL-4 for 3 h and RNA was collected for qPCR analysis. Adult mice treated with saline were used as statistical controls. Aging alone increased iNOS gene expression compared to adult controls ($P<0.05$) and this increase was maintained with LPS treatment ($P=0.07$). Aged mice treated with saline responded to IL-4 by reducing iNOS gene expression back to levels of the adult control mice. Aged mice treated with LPS, however, showed no reduction of iNOS following treatment with IL-4 (Age x LPS x IL-4 interaction: $F(2,57)=3.63$, $P=0.06$). Much like the adult mice, aged mice showed an increase in IL-1β gene expression with LPS and treatment with IL-4 ex-vivo did not change IL-1β levels for either saline treated or LPS treated mice (Fig.8A). Aged mice treated with saline also responded to IL-4 showing a significant increase in Arg expression compared to adult controls. Furthermore, activated microglia from aged mice also showed a marked induction of Arg gene expression following IL-4 treatment (Fig.8B). Together these data indicate that microglia from aged mice are able to respond to IL-4 and promote a shift away from iNOS expression towards Arg expression in a basal condition. Following activation of microglia from aged mice with LPS, however, gene expression of Arg is still induced by IL-4 but these microglia are unable to reduce LPS-induced iNOS gene expression following IL-4 treatment ($P>0.05$).

3.9 Activated microglia from aged mice may have impaired PI3K activation following treatment with IL-4

To address why activated microglia from aged mice show induction of Arg but no inhibition of iNOS following treatment with IL-4, cell signaling pathways used by IL-4 were examined in BV-2 cell cultures. BV-2 cells were treated simultaneously with IL-4 and Ly-294-

002, an inhibitor of the PI3K/Akt pathway, and analyzed by western blot for phospho-STAT-6 and phospho-Akt (Fig.9A). When IL-4 binds to IL-4Rα, both the JAK-1/STAT-6 as well as the IRS-2/PI3K/Akt pathways are activated. Aged mice may show capable induction of Arg and impaired down-regulation of iNOS because of an in-tact JAK-1/STAT-6 pathway and an impaired IRS-2/PI3K/Akt pathway. Western blot analysis revealed that treatment with IL-4 and Ly-294-002 was able to promote STAT-6 phosphorylation while inhibiting Akt phosphorylation (Fig.9A,B).

4. Discussion

Previous studies from our lab and others have reported that aged mice show prolonged sickness behavior, (Godbout et al., 2005; Huang et al., 2008), cognitive impairment (Chen et al., 2008), and depressive-like behavior (Godbout et al., 2008), following peripheral or central immune challenge. We have also shown that these behavioral deficits correspond to exaggerated and protracted microglial activation in aged mice (Henry et al., 2009; Wynne et al., 2010). Here we extend our previous studies and support our hypothesis that age-associated neurobehavioral deficits following immune challenge are a result of dysregulated microglial activation. First, we show that shortly following LPS treatment microglia upregulate both M1 and M2c related genes and that after 24 h, the M1 genotype is diminished whereas the M2c genotype is maintained. Administration of minocycline accelerates this shift towards an M2c genotype. Next, we show that in response to LPS, IL-4Rα is markedly upregulated on the surface of microglia in adult mice both 4 h and 24 h after treatment. IL-10R1 expression was not changed in response to LPS. Furthermore, IL-4, but not IL-10, treatment following LPS promoted a decrease in M1 and an increase in M2 related genes in BV-2 cell cultures and this was recapitulated in ex vivo studies

1 from adult microglia. In aged animals both M1 and M2 related genes were significantly
2 upregulated in response to LPS compared to adult controls. However, microglia from aged mice
3 did not upregulate IL-4Rα surface expression. This was significant because following treatment
4 with IL-4, activated microglia from aged mice showed induction of M2 related genes, but did not
5 show a decrease in M1 related genes. BV-2 cell cultures studies showed that a possible
6 explanation for this is the inhibition of the IL-4 initiated IRS-2/PI3K/Akt pathway in activated
7 microglia of aged mice.

8 First, our studies show that microglia from adult mice simultaneously increase M1 and
9 M2c related gene expression 4 h following an injection of a low dose of LPS (Fig.1A). These
10 results correspond with an induction of inflammation and M1 related genes following a low dose
11 of LPS (Godbout et al., 2005). It is also known that monocytes can be driven to an M2b
12 phenotype by LPS, characterized by an increase in IL-1β and a concomitant increase in IL-10
13 production (Mosser and Edwards, 2008). Our studies show a clear increase in the M2c genotype
14 at a time when inflammation is also present suggesting the induction of an M2b rather than an
15 M1 response after LPS (Ledeboer et al., 2002). From our method of collecting microglia from
16 whole brain homogenate, however, it is unclear whether the same microglia are upregulating M1
17 and M2 markers (M2b), or whether there are separate and distinct populations of M1 and M2c
18 microglia distributed throughout the brain (Kigerl et al., 2009). In addition to the 4 h data, our
19 results show that 24 hours after LPS injection, expression of M1 related genes is reduced
20 compared to 4 h whereas M2c related gene induction is maintained or increased (Fig.1B). This
21 corresponds with a resolution of sickness behavior in adult mice following a low dose of LPS
22 (Godbout et al., 2005; Huang et al., 2008). Furthermore, injections of minocycline for 3 days
23 prior to the injection of LPS reduced M1 related gene expression and promoted or maintained

M2c gene induction. We have reported that treatment with minocycline accelerates recovery from LPS by ameliorating the sickness response and reducing microglial IL-1β production (Henry et al., 2008). Here we suggest that amelioration of the sickness response occurs because minocycline promotes a microglial shift from an M1 to an M2c phenotype.

We wanted to further investigate the role of the M2 phenotype in the resolution of neuroinflammation. Therefore, we looked at the expression of receptors for two M2 promoting cytokines, IL-4 and IL-10, and the affect of these cytokines on dampening microglial activation. We report that microglia showed low levels of IL-10R1 expression and that these levels did not change with a peripheral injection of LPS (Fig.2B). This was consistent with previous studies (Ledeboer et al., 2002). In contrast, we report that following an injection of LPS microglia from adult mice markedly increase expression of IL-4Rα from 8.9% to 45.4% (Fig.2A). To our knowledge an increase in the expression of IL-4Rα on microglia specifically after LPS has not been shown. This is consistent, however, with studies showing global increases in IL-4Rα expression in the spinal cord 24 h after spinal cord injury (Lee et al., 2010). To see if the response to IL-10 and IL-4 mirrored the changes in receptor expression, we conducted a series of BV-2 cell culture studies. Cells were treated with LPS for 1 h followed by IL-10 or IL-4 for 3 h. IL-10 was unable to affect the level of inflammatory gene induction and, other than SOCS3, did not promote an increase in M2c related genes. IL-4 treatment, however, reduced IL-1β and iNOS gene expression and strongly promoted both M2a and M2c related genes (Table 1). Western analysis of phosphorylation of STAT-6 confirmed that IL-4 strongly induces STAT-6 phosphorylation in microglia and that cell signaling was not impaired with LPS pre-treatment (Fig.3). Collectively, these date show that microglia from adult mice are more responsive to the anti-inflammatory effects of IL-4, potentially because of an increase in the IL-4Rα surface

1 expression following LPS. IL-10 has been shown to be a potent effecter of anti-inflammation in
2 the brain (Spera et al., 1998), but may promote these effects mainly through astrocytic regulation
3 of microglia. In fact, astrocytes maintain significantly higher levels of IL-10R1 expression
4 compared to microglia (Ledeboer et al., 2002).

5 In contrast to the BV-2 cell cultures, our ex vivo studies showed that IL-4 had no effect
6 on microglial gene expression of IL-1β. IL-4 was, however, able to reduce iNOS gene expression
7 and promote a significant induction in the M2a related gene, Arg (Fig.4). In this model microglia
8 were activated in vivo by a peripheral injection of LPS and isolated 4 h later during the height of
9 inflammation (Godbout et al., 2005; Huang et al., 2008). Microglia were then treated ex vivo
10 with IL-4 for an additional 3 h. The lack of effect on IL-1β expression ex vivo was surprising,
11 but remains consistent with studies showing that inhibition of IL-4 in a model of spinal cord
12 injury had no effect on IL-1β levels but still promoted exacerbation of microglial activation (Lee
13 et al., 2010). Others have shown that pre-treatment with IL-4 strongly reduces some M1 markers
14 (IL-6), while having no or marginally effects on others (IL-1β, TNF-α) (Szczepanik et al., 2001).

15 Following our findings that IL-4 treatment following the induction of inflammation can
16 reduce select M1 genes and strongly promote an M2a phenotype, we wanted to investigate how
17 M1 and M2 gene expression as well as surface expression of IL-4Rα in microglia was affected
18 by aging. It has been suggested that prolonged microglial activation in aged animals may result
19 from a transcriptional imbalance in M1 and M2 gene induction, with aged mice showing reduced
20 M2 gene and protein induction (Ye and Johnson, 2001). In the current study we show that
21 prolonged microglial activation in aged mice may not result from an imbalance in the
22 transcription of M1 and M2 related genes, but rather from a reduced sensitivity to IL-4 anti-
23 inflammatory feedback. Similar to previous findings, we show that IL-1β induction is

1 significantly increased 4 h after LPS compared to adult controls (Henry et al., 2009; Wynne et
2 al., 2010). Expression of iNOS was also increased in microglia from LPS treated aged mice at
3 this time point. Interestingly, aged mice also showed a significant increase in M2c related gene
4 expression 4 h after LPS. These levels were significantly higher than M2c gene induction in
5 adult controls (Fig.5A). Twenty-four h after LPS aged mice maintained a significant increase in
6 iNOS gene expression indicating a prolonged inflammatory phenotype. Aged mice also
7 maintained high levels of M2c gene expression and showed significant induction of M2a gene
8 expression at this time point (Fig.5B). An increase in both M1 and M2 gene expression in aged
9 mice is consistent with previous studies in our lab demonstrating that an age-associated increase
10 in IL-1β protein secretion by microglia was accompanied by an increase in IL-10 protein
11 secretion (Henry et al., 2009). An increase in the M2a phenotype suggests that IL-4 may be
12 acting on microglia from aged mice at this time point to cause induction of these genes. It may
13 also be indicative of cellular injury cause by the significant exacerbation of IL-1β induction
14 following LPS. Arg is strongly upregulated in mononuclear cells following cellular injury
15 (Ochoa et al., 2001; Kigerl et al., 2009) and has also been found to be increased in the
16 hippocampus with aging alone (Liu et al., 2003), and may indicate an active repair process
17 occurring in the brains of aged mice.

18 A key finding in this study was that even though levels of IL-4Rα gene expression were
19 significantly increased in aged mice following LPS compared to adult controls, aged mice
20 showed impaired upregulation of IL-4Rα protein expression on the surface of microglia
21 following LPS treatment (Fig.7). Previous studies in our lab have shown that protein expression
22 of another important receptor for microglial regulation, CX3CR1, is impaired on microglia of
23 aged mice (Wynne et al., 2010). CX3CR1 protein surface expression on microglia is

1 downregulated in response to LPS in both adult and aged mice. Twenty-four h after LPS,
2 however, surface expression of CX3CR1 has returned to baseline levels in adult mice but
3 remains downregulated in aged mice. When cells were permeabilized and stained for CX3CR1
4 no differences were observed between aged groups. This suggests that rather than a
5 transcriptional problem, microglia from aged mice have impairment in translocation of receptors
6 to the membrane surface. Whether microglia from aged mice have impairment in the
7 translocation of IL-4Rα to the membrane surface was not investigated in the present study.

8 Aged mice were then given a peripheral injection of LPS and microglia were extracted 24
9 h later to evaluate responsiveness to IL-4 t treatment. This time point is associated with
10 prolonged activation in microglia from aged mice (Henry et al., 2009; Wynne et al., 2010). Aged
11 mice injected with saline showed a appropriate response to IL-4 because iNOS gene expression
12 was reduced and Arg gene expression was increased (Fig. 8). Interestingly, following activation
13 with LPS, microglia from aged mice no longer showed IL-4 mediated reduction in iNOS
14 expression indicative of reduced IL-4 anti-inflammatory responsiveness. It is important to note,
15 however, that activated microglia from aged mice still showed a large induction of Arg gene
16 expression following IL-4 treatment (Fig.8). This suggests an impairment in the IL-4 cell
17 signaling cascade in activated microglia from aged mice. The binding of IL-4 to IL-4Rα and
18 either the common γc chain or IL-13Rα induces two main signaling cascades: the JAK-1/STAT-
19 6 pathway and the IRS-2/PI3K/Akt pathway (Nelms et al., 1999). Phosphorylation of STAT-6
20 causes the transcription factor to dimerize and translocate to the nucleus to induce expression of
21 IL-4 mediated genes, including Arg. Phosphorylation of PI3K by the docking protein, IRS-2, can
22 have several different. Using a BV-2 cell culture model we show that inhibition of PI3K through
23 Ly-294-002 allows for IL-4 induced phosphorylation of STAT-6, but inhibits the

1 phosphorylation of Akt (Fig.9). How activation of microglia from aged mice may prevent the
2 activation of PI3K in response to IL-4 is unknown.

3 The role of IL-4 signaling in the brain is still unclear. From the current studies we have
4 established that following pre-treatment with an inflammatory stimulus, microglia from adult
5 mice upregulate the receptor for IL-4. Furthermore, if IL-4 is present these microglia show a
6 response with a strong shift from an M1 to an M2a genotype. In accordance, IL-4 pre-treatment
7 has been shown to be anti-inflammatory pushing monocytes into an alternative activated
8 phenotype and reducing levels of nitric oxide, IFN-γ, and IL-6 (Paludan et al., 1999; Szczepanik
9 et al., 2001; Bluthé et al., 2002). Furthermore, IL-4 knockout mice show exacerbated sickness
10 behavior and an increase in IL-1β production by mixed glial cultures in response to LPS (Lyons
11 et al., 2009). IL-4 has also been shown to exacerbate inflammation indicating a immune-
12 enhancing component of IL-4 signaling. When added concurrently with an inflammatory
13 stimulus, IL-4 increases IL-1β driven IL-6 levels (Pousset et al., 1999), and can exacerbate LPS
14 induced sickness behavior (Bluthé et al., 2002). Moreover, IL-4 has been shown to induce the
15 upregulation of MCP-1 (CCL2) (Szczepanik et al., 2001), a key signal in the recruitment of
16 inflammatory peripheral monocytes into the brain (Boddeke et al., 1999). The role of in vivo IL-
17 4 signaling was not addressed in the current paper, but inferences based on ex vivo cultures can
18 be made. It is clear that following inflammation, IL-4 did not affect IL-1β gene expression by
19 microglia. We interpret these results to suggest that IL-4 does little to change the course of
20 sickness behavior following immune activation as IL-1β is the predominant cytokine responsible
21 for the sickness response (Konsman et al., 2002). However, based the decrease in iNOS gene
22 expression and concurrent induction of Arg, microglia may be taking on a role of promoting
23 neuroplasticity and neurogenesis (Chao et al., 1993; Cai et al., 2002). In support of this notion,

1 IL-4 knockout mice have been shown to have reduced learning and memory and this is restored
2 by adoptive transfer of $CD4^+$, IL-4 producing T-cells, in addition to transfer of macrophages that
3 have been co-cultured with $CD4^+$, IL-4 producing T-cells (Derecki et al., 2010; Kiyota et al.,
4 2010; Derecki et al., 2011). Furthermore, IL-4 has been shown to promote increases in BDNF
5 production by glia indicating a role for neuroplasticity (Derecki et al., 2010). Therefore,
6 microglial cells may be upregulating surface expression of IL-4Rα following an inflammatory
7 stimulus in preparation for seeing a $CD4^+$, IL-4 producing T-cell. In this respect, a failure of
8 microglia from aged mice to upregulate IL-4Rα could explain the increase in cognitive deficits
9 observed following immune activation in these mice. Alternatively, an increase in IL-4Rα
10 surface expression could be a compensatory mechanism to recover from a decrease in IL-4
11 expression following inflammation. We show that IL-4 gene expression is reduced in the brain of
12 both adult and aged mice 4 h after an injection of LPS (Fig.6). This is consistent with a reduction
13 in IL-4 protein 4 h after LPS in rats (Nolan et al., 2005). However, we also show that IL-4 levels
14 return to baseline by within 24 h of LPS treatment, a time point when IL-4Rα remains elevated
15 on microglia (Fig.2A). Similarly, following spinal cord injury IL-4 was found in high
16 concentrations in the spinal cord at a time when IL-4Rα expression was also elevated (Lee et al.,
17 2010). Therefore, it appears that the induction of IL-4Rα is an active process potentially to affect
18 neurogenesis or neuroplasticity following inflammation.

19 Aged mice may also show prolonged microglial activation because of a decrease in the
20 level of IL-4 protein in the brain of aged mice (Nolan et al., 2005). This is likely a component of
21 microglial dysregulation with age, but our results also demonstrate that even if IL-4 is present,
22 aged mice would show impairments in their response to IL-4 and remain in an M1, activated
23 state (Fig.8A).

1 In conclusion, the present study demonstrates that adult mice show a clear microglia
2 specific induction of IL-4Rα following an injection of LPS that is absent in aged mice.
3 Furthermore, whereas activated microglia from adult mice show a shift from an M1 to an M2a
4 phenotype in response to IL-4, activated microglia from aged mice are unable to reduce LPS-
5 induced M1 gene expression following IL-4. We interpret these data to suggest that activated
6 microglia from aged mice show prolonged activation because of a reduced sensitivity to anti-
7 inflammatory feedback from IL-4.
8
9 **5. Figure Legends**

10 **Figure 1: Peripheral LPS injection increased M1 and M2 mRNA expression in microglia.**
11 Adult mice were injected with saline or LPS i.p. and mRNA levels of M1, M2a and M2c related
12 genes were determined from enriched microglia isolated A) 4 h or B) 24 h later (n=6-8). Bars
13 represent the mean ± SEM. Means with * are significantly different ($P<0.05$) from Saline
14 controls. C) Adult mice were injected with vehicle or minocycline for three consecutive days. On
15 the third day mice were injected with saline or LPS i.p and mRNA levels of M1, M2a, and M2c
16 related genes were determined from enriched microglia isolated 4 h later (n=9). Bars represent
17 the mean ± SEM. For each mRNA transcript, means with different letters are significantly
18 different ($P<0.05$) form each other.

19 **Figure 2: Peripheral LPS injection increased microglial surface expression of IL-4Rα, but**
20 **not IL-10R1.** Adult mice were injected with saline or LPS i.p. and enriched microglia were
21 collected by Percoll isolation. A) Representative bivariate dot plot of Percoll isolated cells gated
22 on CD11b^{+}/CD45^{low} (86%) and CD11b^{+}/CD45^{high} (6%) expression for microglia and
23 macrophages, respectively. All analyses were done on cells gated as microglia. B,C)

1 Representative MFI histograms of IL-4Rα and IL-10R1 staining, respectively. D-E)
2 Representative bivariate dot plots of microglia stained for IL-4Rα (n=4-7) and IL-10R1 (n=4),
3 respectively, following treatment and LPS for 4 or 24 h. Bars represent the mean ± SEM. Means
4 with * are significantly different (P<0.001) from saline controls.
5 **Figure 3: IL-4 promotes phosphorylation of STAT-6 independent of LPS.** BV-2 cells were
6 plated and pre-treated with LPS or saline for 1 h followed by treatment with vehicle or IL-4 for
7 45 min. Cell lysates were collected and analyzed for phospho-STAT-6 and total STAT6 levels
8 by western blot (n=3). Bars represent the mean ± SEM.
9 **Figure 4: Ex vivo microglia from adult mice treated with IL-4 show a reduction in LPS-**
10 **induced iNOS gene expression and promote Arginase (Arg) expression.** Adult (3 mo) mice
11 were given an i.p. injection of saline or LPS. After 4 h microglia were isolated and treated with
12 vehicle or IL-4 for 3 h. mRNA transcripts of A) M1 related genes iNOS and IL1β, and B) M2
13 related gene Arg were evaluated (n=9-10). Bars represent the mean ± SEM. Means with different
14 letters are significantly different (P<0.05) from each other.
15 **Figure 5: Aged mice show an increase in M1, M2a and M2c gene induction following LPS**
16 **compared to adult controls.** Adult (3-4 mo) and aged (18-22 mo) mice were given an i.p.
17 injection of LPS or saline and mRNA levels of M1, M2a, and M2c related genes were
18 determined by qPCR from enriched microglia isolated A) 4 h or B) 24 h after LPS injection
19 (n=6-9). Bars represent the mean ± SEM. Means with * are significantly different (P<0.05) from
20 saline controls. Means with # are significantly different (P<0.05) from saline controls and means
21 with an *.
22 **Figure 6: mRNA levels of IL-4 decrease 4 h after LPS and return to baseline 24 h after**
23 **LPS, independent of age.** Adult and aged mice were given an i.p. injection of LPS or saline and

1 mRNA levels of IL-4 from a coronal brain slice through the pre-frontal cortex were determined
2 by qPCR 4 h and 24 h after LPS injection (n=5-14). Means with * are significantly different
3 ($P<0.05$) from saline controls.

4 **Figure 7: Microglia from aged mice show impaired upregulation of IL-4Rα following LPS.**
5 A) Adult (3-4 mo) and B) aged (18-22 mo) mice were given an i.p. injection of saline or LPS and
6 enriched microglia were collected by Percoll gradient isolation 4 h and 24 h later (n=4-8).
7 Microglial surface expression of IL-4Rα is shown in representative bivariate dot plots. Bars
8 represent the mean ± SEM. Means with * are significantly different ($P<0.0001$) from saline
9 controls.

10 **Figure 8: Activated microglia from aged mice fail to down regulate LPS-induced iNOS**
11 **expression but show strong Arginase (Arg) expression following IL-4 treatment.** Aged (20
12 mo) mice were given an i.p. injection of saline or LPS. Adult (3 mo) mice treated with saline
13 were used as a reference control. After 24 h, an a population of enriched microglia were isolated
14 and treated with vehicle or IL-4 for 3 h. mRNA levels of A) M1 related genes iNOS and IL-1β
15 and B) M2 related gene Arge are shown. Bars represent the mean ± SEM.

16 **Figure 9: BV2 cells treated with Ly-294-002 show maintained levels of phospho-STAT-6,**
17 **but reduced levels of phospho-Akt following IL-4 treatment**. BV2 cells were treated
18 simultaneously with vehicle or IL-4 and DMSO or Ly-294-002 for 45 min (n=4). Representative
19 western blots for levels of phospho-STAT-6, phospho-Akt, and STAT-6. B) Bars represent mean
20 ± SEM. All phosphorylated blots were compared to STAT-6 as a reference.

21

22 **Table 1: IL-4, but not IL-10, promotes an M2, anti-inflammatory genotype in BV2 cells**
23 **following LPS stimulation.** BV-2 cells were plated and pre-treated with saline or LPS for 1 h

followed by treatment with vehicle or IL-4 for 3 h. After 3 h mRNA was isolated and analyzed for M1, M2a, and M2c gene expression using qPCR (n=6). Numbers represent the mean value and means with different letters are significantly different (P<0.05) from each other.

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1 FIGURE 1
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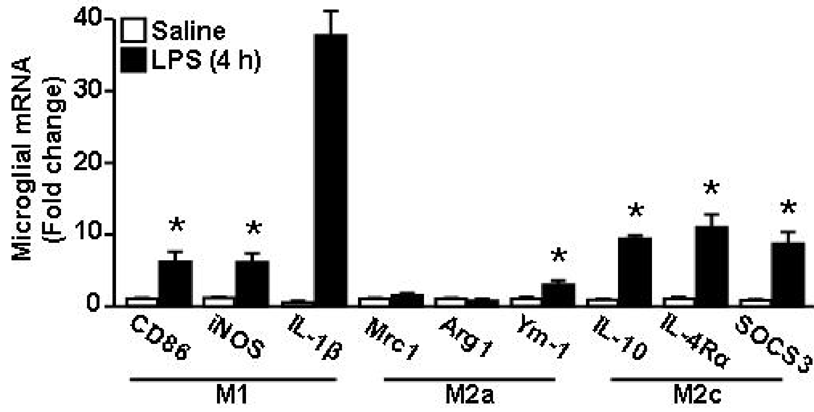
3 A.



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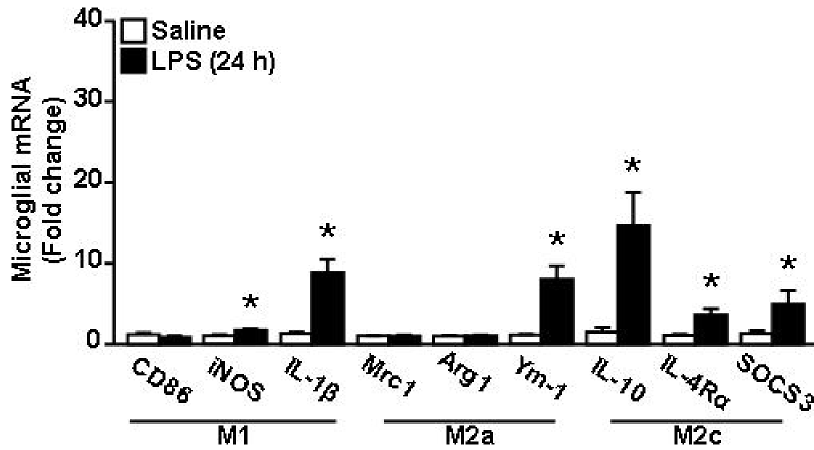

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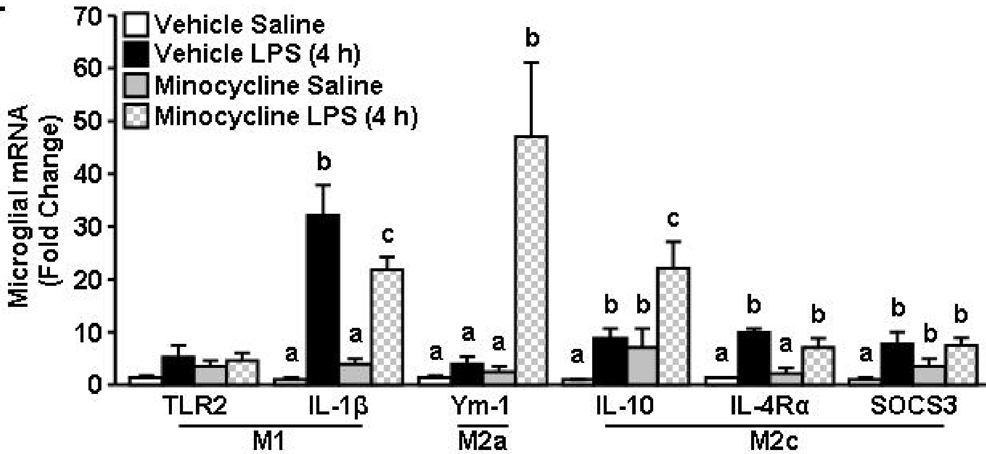

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FIGURE 2

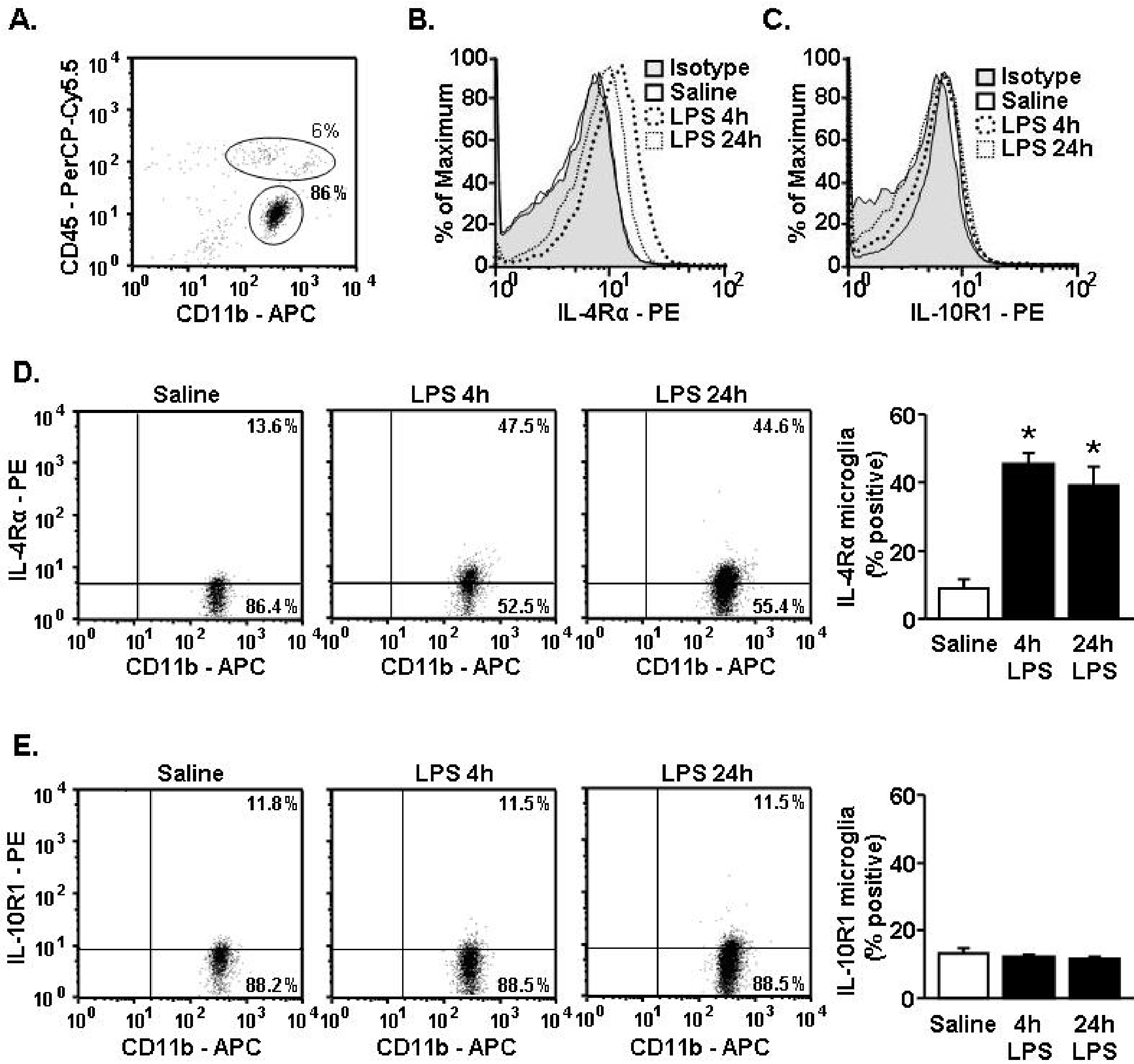

TABLE 1

	mRNA	Saline Vehicle	Saline IL-4	LPS Vehicle	LPS IL-4
		IL-4 Treatment			
M1	iNOS	1.00 ± 0.4 (a)	0.28 ± 0.02 (b)	9.37 ± 0.22 (c)	3.88 ± 0.14 (d)
	IL-1b	1.00 ± 0.02 (a)	0.80 ± 0.04 (a)	21.76 ± 0.64 (b)	16.54 ± 0.68 (c)
M2	Arg	1.02 ± 0.09 (a)	29.38 ± 2.37 (b)	0.68 ± 0.05 (c)	28.80 ± 1.92 (b)
	IL-10	1.01 ± 0.07 (a)	3.22 ± 0.22 (b)	1.84 ± 0.07 (c)	3.11 ± 0.22 (b)
	Socs1	1.00 ± 0.03 (a)	58.56 ± 6.42 (c)	3.06 ± 0.17 (b)	85.63 ± 4.57 (d)

TABLE 2

	mRNA	Saline Vehicle	Saline IL-10	LPS Vehicle	LPS IL-10
		IL-10 Treatment			
M1	iNOS	1.11 ± 0.23 (a)	1.78 ± 0.63 (a)	5.48 ± 0.28 (b)	11.55 ± 1.21 (c)
	IL-1b	1.01 ± 0.07 (a)	2.50 ± 0.86 (a)	13.97 ± 2.22 (b)	20.67 ± 2.10 (c)
M2	Arg	1.34 ± 0.60	3.40 ± 1.63	0.86 ± 0.30	2.59 ± 1.64
	IL-10	1.18 ± 0.32 (a)	1.87 ± 0.43 (a,b)	1.45 ± 0.24 (a,b)	2.60 ± 0.55 (b)
	Socs3	1.03 ± 0.12 (a)	3.44 ± 0.98 (a,c)	3.16 ± 0.39 (b)	11.16 ± 1.43 (c)

FIGURE 3

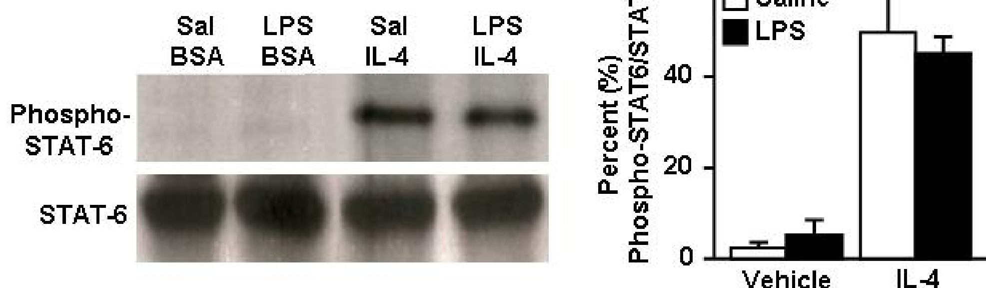

FIGURE 4

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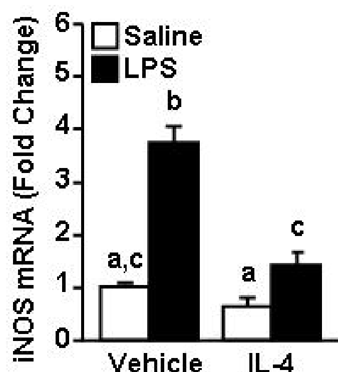

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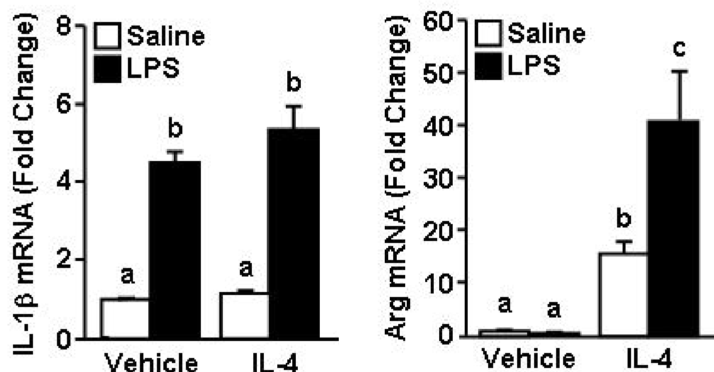

1 FIGURE 5
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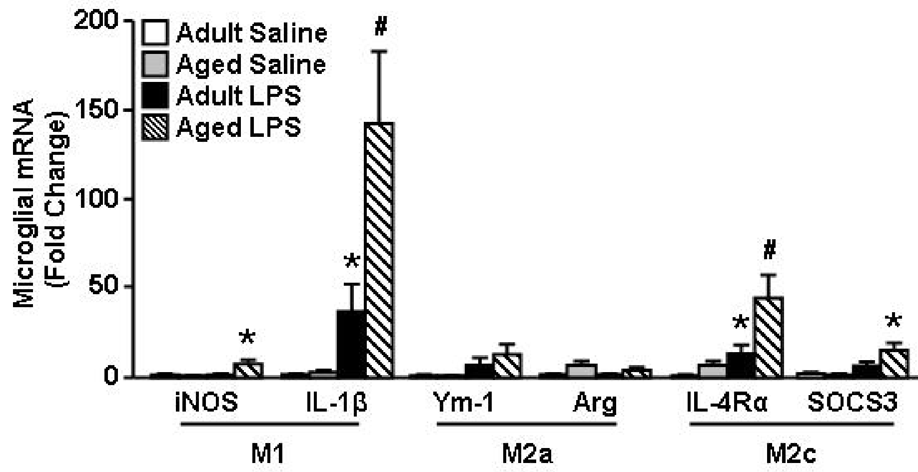

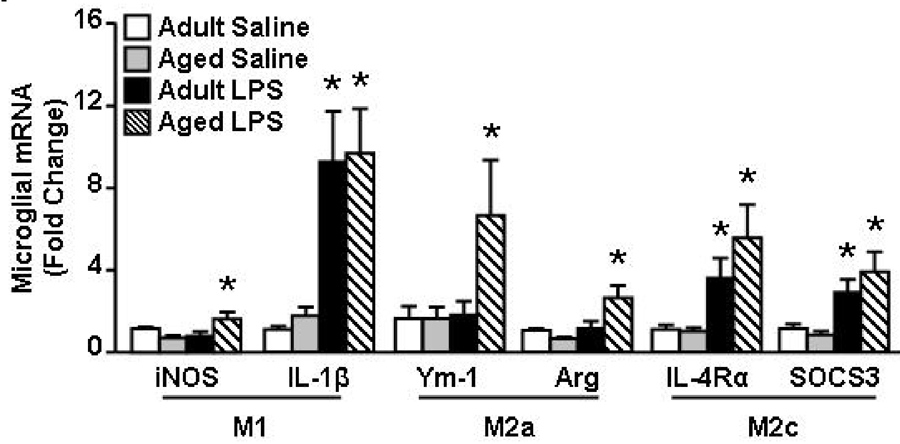

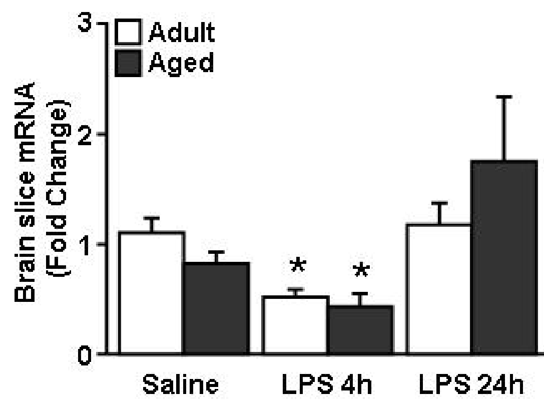

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FIGURE 7

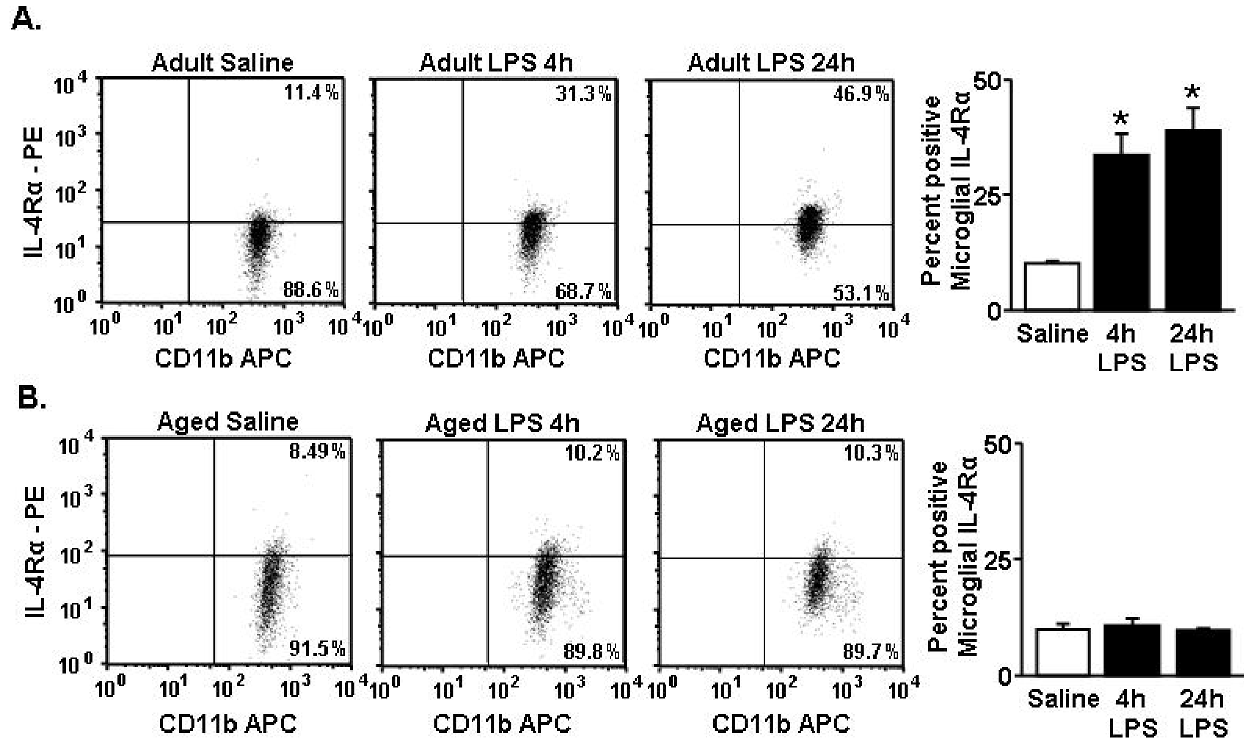
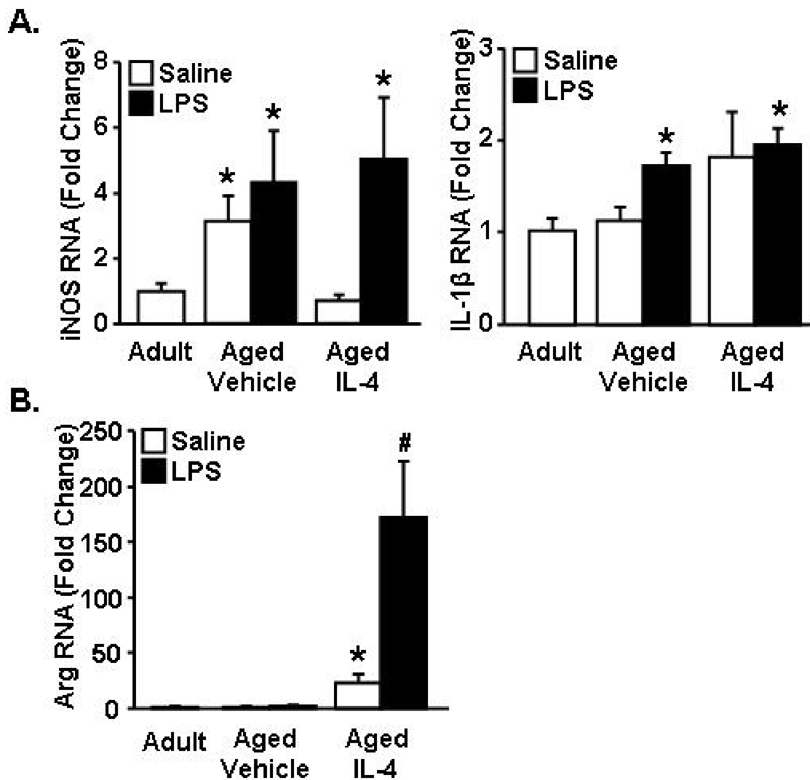


FIGURE 8



1 **FIGURE 9**