Central IL-4 promotes a neuroprotective and anti-inflammatory brain environment following peripheral LPS injection

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Abstract

Aging is associated with the loss of appropriate immune regulation resulting in increased inflammatory status within the central nervous system (CNS). Microglia, the innate immune cell of the CNS, are particularly sensitive to age-associated dysregulation and promote an exaggerated inflammatory response following a peripheral or central immune challenge in aged animals. Recently our lab found that after a peripheral immune challenge, microglia from adult, but not aged, mice upregulated interleukin (IL)-4 receptor-alpha (IL-4Rα), the receptor for the anti-inflammatory cytokine IL-4. The functional consequence of impaired IL-4Rα upregulation was insensitivity to the anti-inflammatory promoting effects of IL-4. Thus, to further characterize

potential consequences of impaired IL-4Rα upregulation on aged microglia, we conducted a series of studies investigating the extent to which a peripheral immune challenge with lipopolysaccharide (LPS) and central injection of IL-4 promoted IL-4Rα upregulation and the induction of anti-inflammatory and neuroprotective markers *in vivo*. We provide evidence that upregulation of IL-4Rα is necessary to promote a strong neurotrophin and arginase response following exposure to IL-4. Furthermore, we show that a functional consequence of exaggerated arginase expression in microglia is increased polyamine production resulting in high levels of CCL2 and increased recruitment of macrophages to the brain. This is important because IL-4 levels are typically elevated in the CNS following tissue damage. Thus, impaired IL-4Rα upregulation in aged mice may prevent an appropriate arginase and polyamine response necessary for tissue repair and growth following CNS injury.

Introduction

Aging is a normal physiological process associated with a gradual decline in immune regulation. Impaired immune regulation results in reduced immune function in the periphery and exaggerated inflammation in the central nervous system (CNS) (Dantzer et al., 2008; Corona et al., 2012). Increased inflammation in the CNS is associated with multiple neurological complications including delirium, prolonged sickness, and induction of depression. Impaired regulation of microglia, the innate immune cell of the CNS, is likely the cause of exaggerated neuroinflammation in the aged brain. Microglia are long-lived cells with little to no turnover for the life of the animal (Ajami et al., 2007; Ginhoux et al., 2010). Thus, these cells are particularly susceptible to age-related increases in oxidative stress and become more inflammatory with age (Godbout et al., 2005; Sierra et al., 2007). Our lab and others have shown that microglia from aged

mice, rats, hamsters, and humans have increased markers of activation and priming (Perry et al., 1993; Streit et al., 2004; Frank et al., 2006; Henry et al., 2009; Corona et al., 2012) associated with exaggerated inflammatory production after an immune challenge (Henry et al., 2009). Exaggerated immune activation results in increased inflammatory cytokine production and behavioral complications including cognitive impairment and depression-like behavior (Barrientos et al., 2006; Chen et al., 2008; Godbout et al., 2008).

Microglia may become dysregulated in the aged brain, in part, from a lack of appropriate regulation from anti-inflammatory mediators. Fractalkine (CX_3CL1), an abundant chemokine within the CNS, is critical in regulating a surveying and quiescent microglial state through the fractalkine receptor (CX_3CR1) (Corona et al., 2010; Jurgens and Johnson, 2010; Bachstetter et al., 2011). Following an immune challenge, CX_3CR1 is shunted away from the membrane of microglia allowing for inflammatory activation. During the resolution of the inflammatory response CX_3CR1 is upregulated on the microglial cell surface even beyond baseline levels (Wynne et al., 2010). Microglia from aged mice, however, show extended downregulation of CX_3CR1 resulting in prolonged inflammatory activation (Wynne et al., 2010). Confirmation that impaired CX_3CR1 upregulation results in prolonged microglial activation and induction of depression-like behavior was confirmed in CX_3CR1 knockout ($CX_3CR1^{-/-}$) mice in which a peripheral injection of lipopolysaccharide (LPS) promoted exaggerated and extended microglial activation compared to their heterozygous littermates (Corona et al., 2010).

Recently our lab demonstrated that the receptor for interleukin (IL)-4, IL-4 receptor-alpha (IL-4Rα), was also impaired on microglia from aged mice. Following a peripheral injection with LPS, microglia from adult, but not aged, mice significantly upregulated surface expression of IL-4Rα. The functional consequence of impaired IL-4Rα upregulation was reduced sensitivity to the

anti-inflammatory promoting effects of IL-4. Thus, LPS-activated microglia from aged mice had extended inflammatory gene expression (i.e., inducible nitric oxide synthase (iNOS)) and impaired upregulation of an IL-4 promoted gene (i.e., arginase) in the presence of IL-4 (Fenn et al., 2011). IL-4 is a classic anti-inflammatory cytokine that is primarily released by Th2-helper cells and best known for its promotion of antibody production in B-cells (Paul, 1991). Thus, the role of IL-4 within the CNS is still debated. Confusion surrounding IL-4 remains, in part, because there are normally very low to undetectable levels of IL-4 within the CNS (Lovett-Racke et al., 2000). A lack of IL-4 in the CNS makes it difficult to assess the functional consequence of increased IL-4Rα expression on microglia.

We conducted a series of studies investigating the role of IL-4 administration *in vivo* on microglial activation following inflammatory activation by LPS. Here we show that a central injection of IL-4 one hour following a peripheral injection with LPS did not ameliorate the sickness response, but did reduce circulating levels of IL-6 and decrease depression-like behavior. Within the brain, IL-4 tended to promote a more anti-inflammatory and reparative brain environment with a trend towards increased M2 microglial phenotype expression. LPS, as expected, promoted increased inflammation and more of an M1 microglial response. IL-4 following LPS promoted an exaggerated reparative microglial phenotype concomitant with exaggerated inflammatory cytokine production. Moreover, IL-4 and LPS co-treatment was required to induce microglial-specific arginase expression, which coincided with a significant shift in the brain chemokine profile resulting in increased recruitment of macrophages to the brain.

Methods

Animals

Adult (3-4 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 housed in groups of 3-5 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. At 3 months of age mice were individually housed under the same standard conditions until use. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Experimental Protocols

In the fist study, an indwelling cannlua was surgically implanted into the lateral ventricle of adult mice under ketamine anesthesia. Mice were allowed one week to recover. After one week mice received an intraperitoneal (ip) injection of saline or *Escherichia coli* lipopolysaccharide (LPS) (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO). One hour later mice received an intracerebroventricular (icv) injection of vehicle or recombinant mouse IL-4 (IL-4) (50 ng/ 2 µl; R&D, Minneapolis, MN). A subset of mice was euthanized 4 hours after LPS injection to evaluate plasma IL-6 levels (n=8). Another subset was assessed for sickness behavior using the social exploratory test 4, 8, 12, and 24 h after the LPS injection. Body mass and food intake were also assessed at these time-points (n=8). The final subset of mice was assessed for depressive-like behavior using the tail suspension test (TST) 24 h after LPS injection (n=14). Following the completion of the social exploratory and tail suspension tests mice were euthanized, blood was collected to evaluate plasma IL-6 levels, and the brain was collected. A 1 mm coronal section from the brain was collected through the icv injection site and the remaining tissue was used for microglial isolation. mRNA analysis of neuroinflammatory and microglial

regulation genes were determined in both the brain section and microglia by real time (RT)-PCR.

In the next study, mice underwent the same experimental procedures as above. Following the icv injection with IL-4, mice were left undisturbed for 23 h (24 h after LPS injection). Mice were then perfused with ice cold saline followed by 4 % formaldehyde. Brains were collected, sliced, and stained by immunohistochemistry for Iba-1, arginase, and IL-4Rα (n=4).

In the final study, mice underwent the same experimental procedures as above. Following the icv injection with IL-4, mice were left undisturbed for 23 h (24 h after LPS injection). Mice were then euthanized and the brain was collected for microglial/macrophage isolation. Microglia/macrophages were stained with antibodies against CX_3CR1, CCR2, CD45, and CD11b and analyzed by flow cytometry. Microglia were differentiated from macrophages by CD45 expression (microglia: $CD11b^{+}/CD45^{low-}$; macrophages: $CD11b^{+}/CD45^{high}$) (n=6).

Intracerebroventricular cannulation surgery

The icv cannulation surgery was performed as previously described with a few modifications (Huang et al., 2008). In brief, mice were anesthetized using a mix of ketamine (100 mg/kg) and xylazine (10 mg/kg). The top of the head was shaved and sterilized with betadine and mice were positioned in a stereotaxic frame. A small incision was made on the cranium to expose the skull. A 26-guage stainless-steel guide cannula (Plastics One, Roanoke, VA) was placed in the lateral ventricle (stereotaxic coordinates from Bregma: Lat 1.2 mm; AP 0.5 mm; Hor 2.0 mm from the dura matter). Two anchoring cranial screws were inserted adjacent to the cannula and the cannula was secured with cranioplastic cement (Permanent Repair Acrylic Powder and Liquid, Henry Schein, Inc). A dummy cannula (Plastics one, Roanoke, VA) was inserted in the guide cannula to prevent occlusion and infection. Mice were injected

subcutaneously (sc) with Buprinex (111 g/kg) following surgery and again 24 h later. Mice were provided a minimum of seven days to recover prior to experimental treatments.

For icv injections, the indwelling cannlua was connected with sterile tubing to a Hamilton syringe. Injections of vehicle (0.1% bovine serum albumin in saline) or IL-4 (25 ng/µl) were administered in a 2 µl volume (total of 50 µl IL-4) using a KS Scientific precision syringe pump.

Behavioral Analyses

Social exploratory test: Mice were tested for sickness behavior using the social exploratory test 4, 8, 12, and 24 h after an ip injection with LPS as previously described (Huang et al., 2008). Briefly, a novel juvenile conspecific was introduced into the home cage of the test subject for 10 min. Behavior was video-taped (EthoVision XT 7.0, Noldus Information Technology) and the cumulative amount of time the subject mouse engaged in social investigation (sniffing, chasing, licking) was determined by a trained observer blind to experimental treatments (Bluthé et al., 1999). Baseline social behavior was determined at each time-point as the difference from *Saline-Vehicle* treated mice. Results are expressed as a percent decrease in time engaged in social behavior compared to the baseline measurement.

Tail suspension test*:* Mice were tested for depression-like behavior using the TST 24 h after LPS as previously described (Corona et al., 2010). In brief, mice were suspended by their tails by a piece of tape and hung in a 32 x 33 x 33 cm box for 10 min. Behavior was video taped (EthoVision XT 7.0, Noldus Information Technology) and the cumulative amount of time spent immobile was determined by a trained observer blind to experimental treatments. Results are expressed as total time immobile.

Microglial/Macrophage isolation

Microglia and macrophages were isolated from whole brain homogenates as previously described (Wynne et al., 2010; Fenn et al., 2011). In brief, brains were homogenized in phosphate buffered saline (PBS, pH 7.4) by passing through a 70 μm nylon cell strainer. Resulting homogenates were centrifuged at 600 g for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll at room temperature. A discontinuous Percoll density gradient was layered as follows: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 minutes at 2000 g and microglia were collected from the interphase between the 70% and 50% Percoll layers. Microglia were washed and re-suspended in PBS. Each brain extraction yielded approximately 3 x 10^5 viable cells. We have previously characterized these cells as roughly 85% $CD11b^+/CD45^{low}$ microglia (Henry et al., 2009), and 5-10% macrophages (Wohleb et al., 2011).

RNA isolation and qPCR

RNA was isolated from the 1 mm brain section of the injection site or from isolated microglia. For the brain section, total RNA was isolated using the Tri-Reagent protocol (Sigma, MO) and subjected to the DNA-free™ RNA clean up procedure (Ambion, TX). For isolated microglia, RNA was isolated using the PrepEase kit (USB, CA). In all RNA isolation procedures, RNA concentration was determined by spectrophotometry (Eppendorf, NY) and RNA was reverse transcribed to cDNA.

Quantitative PCR was performed using the Applied Biosystems (Foster, CA) Taqman® Gene Expression assay as previously described (Godbout et al., 2005). In brief, cDNA was amplified by RT-PCR where a target cDNA (e.g., IL-1β, BDNF, arginase) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ).

Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference from control. In the case of indoleamine 2,3-dioxygenase (IDO), results are expressed as relative units.

Flow cytometry

Cells were assayed for microglial cell surface antigens by flow cytometry as previously described (Henry et al., 2009; Fenn et al., 2011). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody. Next, enriched microglia were incubated with rat anti-mouse CX_3CR1-FITC, CD11b-PE, CD45-PerCP-Cy5.5, and CCR2-APC antibodies (eBioscience, CA). Expression of these surface receptors was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Ten thousand events characterized as microglia/macrophages were recorded. Microglia and macrophages were identified by $CD11b^{+}/CD45^{low}$ and $CD11b^{+}/CD45^{high}$ expression, respectively (Wohleb et al., 2011). For each antibody, gating was determined based on appropriate negative isotype stained controls. Flow data were analyzed using FlowJo software (Tree Star, CA).

Immunohistochemical Staining

Mice were euthanized by CO_2 and immediately transcardially perfused with ice cold saline followed by 4% formaldehyde. Brains were collected and post-fixed in 4% formaldehyde for 24 h followed by 30% sucrose for another 24 h. Brains were frozen in dry-ice cooled isopentane and stored at -80° C until sectioned. Brains were sectioned (10 μm) using a Microm HM550 cryostat (Mikron Instruments) and slide-mounted. Sections of the injection site and those immediately adjacent were used for staining and analysis. Only animals with confirmed injection tracts into the lateral ventricle were used for analysis. Sections were heated for 30-45 min at 37°

C for antigen retrieval and blocked with 5DFTP^{+} (5% normal donkey serum (NDS), 0.1% fish gelatin, 1% bovine serum albumin (BSA), 0.1% Triton x-100). Sections were stained for goat anti-mouse arginase I (1:200; Santa Cruz Biotechnology) and incubated over night at 4° C. A flurochrome-conjugated secondary antibody (Alexa Fluor, anti-goat 546) was used for 1 h at room temperature. Sections were blocked for a second time with 5% NDS/1%BSA and incubated overnight in rabbit anti-mouse Iba-1 (1:1000; Wako Chemicals) and mouse anti-mouse IL-4Rα (1:100; Santa Cruz Biotechnology). Fluorochrome-conjugated secondary antibodies (Alexa Fluor, goat anti-rabbit 488 and goat anti-mouse 633) were then applied for 1 h at room temperature. Sections were cover-slipped with Fluoro-Gel (Electron-Microscopy Sciences, Hatfield, PA) and stored at -20° C. Images were taken with a Zeiss 510 Meta confocal microscope and analyzed using LSM Image Browser Zhen and Metamorph Analysis software.

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. Pretreatment and Treatment) and two- (i.e. Pretreatment x Treatment) way ANOVA using the General Linear Model procedures of SAS. For social exploration, data were analyzed by repeated measures ANOVA. 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 ≤ 0.1.

Results

Icv IL-4 did not ameliorate sickness behavior, but did reduce plasma IL-6 levels and depressive-like behavior.

The dose of LPS selected induces a transient sickness response with mice showing reduced social exploratory behavior and food intake for less than 24 h (Corona et al., 2010). Because increased anti-inflammatory signaling in the brain (i.e., IL-10) leads to an ameliorated sickness response (Bluthé et al., 1999), we examined whether central injection of IL-4 would also improve recovery following a peripheral injection of LPS. Adult mice were injected ip with saline or LPS and one hour later injected icv with vehicle or IL-4. Mice were tested for social exploratory behavior as a measure of sickness response 4, 8, 12, and 24 h after the LPS injection. Body mass and food intake measurements were also collected at these time-points. As expected, LPS caused a significant reduction in social exploration, independent of IL-4 treatment (Main effect of LPS: $F(1,32)=23.05$, $P<0.0001$) (Fig.1A). Furthermore, icv IL-4 treatment did not reduce body mass loss or increase food intake, but rather may have potentiated these factors of the sickness response (Main effect of LPS: $F(1,37)=137.76$, $P<0.0001$; Main effect of IL-4: $F(1,37)=6.76$, $P<0.02$) (Fig.1B).

Our previous studies demonstrate that plasma IL-6 levels are significantly elevated 4 h after a peripheral LPS injection, but return close to baseline within 24 hours (Godbout et al., 2005). We report that post-treatment with an icv injection of IL-4 prevented the LPS-induced increase in plasma IL-6 when assessed 4 h after the LPS injection (Fig.1C). Although there was not a reported interaction between LPS and IL-4 ($P=0.14$), Post-hoc analysis revealed that *LPS-Vehicle* was significantly different ($P<0.04$) from all other groups, and *LPS-IL-4* was not significantly different from *Saline-Vehicle* controls.

Because reduced IL-6 has been connected with reduced depression-like behavior

(Godbout et al., 2008; Howren et al., 2009), we investigated depression-like behavior 24 h after the LPS injection in mice treated ip with saline or LPS followed by icv vehicle or IL-4. This time-point was selected because social exploration behavior had returned to baseline in both LPS treatment groups (Fig.1A). Depression-like behavior was significantly reduced by IL-4, irrespective of LPS treatment (Main effect of IL-4: $F(1,53)=6.37$, $P<0.02$). These effects are similar to observations following treatments with anti-depressant medications (Steru et al., 1985).

Icv IL-4 following a peripheral injection with LPS exaggerated brain arginase and CX_3CR1 mRNA expression, and reduced IDO expression 24 h after LPS.

Following the findings that a central injection of IL-4 prevented the LPS-associated increase in plasma IL-6 with a concomitant reduction in depression-like behavior, we investigated the profile of mRNA expression at the IL-4 injection site in the brain 24 h after the LPS injection. We hypothesized that IL-4 would promote an anti-inflammatory, M2 microglial phenotype with the promotion of neuroprotective markers, whereas LPS would promote an inflammatory, M1 microglial phenotype with the reduction of neuroprotective markers. Furthermore, because our previous studies showed that LPS promotes IL-4Rα upregulation on microglia (Fenn et al., 2011), we hypothesized that many factors induced by IL-4 would be exaggerated by pre-treatment with LPS. To assess IL-4 responsiveness we determined the mRNA expression of brain derived neurotrophic factor (BDNF) (an important neurotrophin for neuronal health and protection), arginase (an anti-inflammatory marker associated with IL-4 signaling), CX_3CR1 (a receptor located on brain microglia important for reduced inflammatory activity), and indoleamine 2,3-dioxygenase (IDO) (an enzyme implicated in promoting depression-like behavior).

Neither LPS nor IL-4 had any effect on BDNF levels 24 h after LPS injection. Arginase, however, was strongly induced in the *Saline-IL-4* group ($P<0.0003$) and reduced in the *LPS-Vehicle* group ($P=0.06$). Importantly, co-treatment with LPS and IL-4 resulted in exaggerated arginase expression well above levels promoted by IL-4 (LPS x IL-4 interaction: $F(1,47)=4.38$, $P<0.05$). From our previous studies we know that LPS treatment initially reduces CX_3CR1 expression on microglia and that by 24 h after LPS, levels return to baseline or increase slightly above baseline. Twenty-four hours after LPS, CX_3CR1 expression in the *LPS-Vehicle* group was indeed elevated over *Saline-Vehicle* controls ($P<0.0001$) and this was further promoted in the *LPS-IL-4* group (Tendency for LPS x IL-4 interaction: $F(1,47)=2.84$, $P=0.1$). IDO expression is strongly correlated with increased depression-like behavior. Nonetheless, increased depression-like behavior in mice treated with LPS was not observed (Fig.1D). Reduced depression-like behavior compared to baseline, however, was observed following treatment with IL-4 independent of pretreatment and this corresponded with reduced IDO expression in *LPS-IL-4* co-treated mice (Fig.2D).

Icv IL-4 promoted microglial BDNF, arginase, IL-1β and IL-4Rα mRNA expression 24 h after a peripheral injection of LPS.

Previous studies by our lab and others have demonstrated that increased neuroinflammation and associated behavioral complications stem from activation of the innate immune cell of the brain, microglia (Henry et al., 2009; Corona et al., 2010; Williamson et al., 2011; Wohleb et al., 2011). Because microglia are also important producers of BDNF and potentially the M2-related factor arginase, microglia from the whole brain were assessed for BDNF, arginase, IL-1β, and IL-4Rα mRNA expression 24 h after LPS. It is critical to point out, however, that a coronal brain slice of the injection site was removed prior to microglial isolation.

Whereas whole brain BDNF mRNA expression was not affected by LPS or IL-4 treatment, in microglia, BDNF levels were increased after IL-4 (Main effect of IL-4: $F(1,35)=6.59$, $P<0.02$). Moreover, compared to IL-4 alone ($P=0.054$ compared to *Saline Vehicle*), LPS and IL-4 co-treatment significantly upregulated BDNF expression ($P<0.05$) (Fig.3A).

Because arginase is strongly promoted by IL-4, its expression may rely heavily on the expression of IL-4Rα. Thus, we investigated arginase mRNA expression in microglia following LPS and IL-4 treatment. Indeed, *Saline-IL-4* groups tended to have an increase in arginase expression ($P=0.056$). *LPS-Vehicle* treatment, however, significantly reduced arginase expression ($P<0.004$) (Fig.3B). These findings are consistent with the promotion of an M2 microglial phenotype by IL-4 and an M1 phenotype by LPS (Mantovani et al., 2004; Mosser and Edwards, 2008). Furthermore, microglia from mice co-treated with LPS and IL-4 maintained high arginase expression that was significantly upregulated from *Saline-Vehicle* controls ($P<0.03$). Together, these data indicate a strong arginase response with IL-4 (Main effect of IL-4: $F(1,42)=3.82$, $P<0.02$) that may have been promoted by pretreatment with LPS.

Our previous study found that whereas IL-4 reduced IL-1β levels in a microglial cell line (BV2 cells), it had no effect on IL-1β mRNA expression in primary microglia from adult mice, and may have potentiated IL-1β in aged microglia (Fenn et al., 2011). Similar to these findings IL-4 did not reduce LPS-induced IL-1β production (Main effect of LPS: $F(1,44)=14.05$, $P<0.0005$), and rather significantly increased IL-1β production (LPS x IL-4 interaction: $F(1,44)=5.40$, $P<0.03$) (Fig.3C). Although, increased expression of IL-1β in the *LPS-IL-4* group did not coincide with exaggerated or prolonged sickness behavior in these mice (Fig.1A).

IL-4Rα is a factor that was previously characterized as an M2c, or strictly anti-

inflammatory, factor upregulated on classically deactivated microglia. Our lab and others have published that immune challenges including LPS (Fenn et al., 2011) and traumatic CNS injury (Kigerl et al., 2009) strongly promote microglial IL-4Rα surface expression. Whether this is to induce a negative feed-back loop to promote the return of microglia to a surveying and quiescent state, or whether this is a marker of increased microglial activation, are unknown. Four h after LPS, both LPS treated groups had increased IL-4Rα mRNA expression (Main effect of LPS: $F(1,32)=10.14$, $P<0.004$; data not shown). Nonetheless, 24 h after an LPS injection the only group that maintained elevated levels of IL-4Rα expression was the *LPS-IL-4* group (LPS x IL-4 interaction: $F(1,44)=4.49$, $P<0.05$) (Fig.3D). Elevated IL-4Rα expression on *LPS-IL-4* treated microglia corresponded to maintained IL-1β upregulation in addition to increased markers of tissue repair (arginase) and neuroprotection (BDNF) in this group.

Icv IL-4 following a peripheral injection of LPS strongly promotes arginase expression exclusively in microglia.

Although microglial expression of arginase tended to increase following co-treatment with LPS and IL-4, it was not to the levels found in the brain section. Thus, to determine the cell-type responsible for the exaggerated arginase expression, mice were perfused 24 h after LPS and the brain was sectioned and stained for Iba-1 (microglia), arginase, and IL-4Rα. Sections at the injection site and immediately adjacent to the injection site were used. Arginase positive cells were almost exclusively Iba-1 positive indicating that arginase expression was restricted to microglia (Fig.4A). Furthermore, whereas arginase was slightly promoted by icv injection of IL-4 alone ($P=0.12$), it was markedly induced following co-treatment with both LPS and IL-4 (LPS x IL-4 interaction: $F(1,14)=23.72$, $P<0.0005$) (Fig.4B). Exaggerated arginase expression was likely a result of elevated IL-4Rα expression following LPS treatment (Fig.4B). Although we

could not confirm exaggerated IL-4Rα expression by IHC, this was likely because of the high levels of background staining produced by the antibody. When sections adjacent to the injection site were evaluated, microglia positive for arginase were only noted around the ventricles and only in *LPS-IL-4* treated mice (Fig. 4C).

Icv IL-4 following a peripheral injection of LPS promotes the polyamine pathway important for chemokine production and macrophage recruitment to the CNS.

Despite the well characterized roles of arginase in the periphery associated with the urea cycle and tissue repair, the role of arginase in the CNS is still unclear (Lange et al., 2004). One potential implication of exaggerated arginase expression in microglia is the potentiation of the polyamine pathway. While the role of polyamines in the CNS is also poorly understood, a recent paper proposed that induction of microglial CCL2 is polyamine-dependent (Puntambeckar et al., 2010). CCL2 is an important chemokine that strongly attracts peripheral monocytes, specifically differentiated $CCR2^{+}$ macrophages, to sites of inflammation in the CNS (Babcock et al., 2003). To identify whether the functional consequence of an icv IL-4 injection was to promote polyamine production and CCL2 upregulation, we investigated mRNA expression of another enzyme important in the arginase-polyamine pathway, ornithine decarboxylase (ODC). ODC converts the metabolic substrate of arginase, ornithine, into the first of the three primary polyamines, putrescine. Indeed, LPS alone induced an increase in ODC mRNA expression (Main effect of LPS: $F(1,38)=2.06$, $P<0.05$) (Fig.5A). Thus, a combined effect of exaggerated arginase production by *LPS-IL-4* co-treatment and upregulated ODC following LPS treatment could markedly promote polyamine production.

To assess the effects of increased polyamine production we evaluated mRNA expression of CCL2 and another chemokine important for macrophage extravasation into the CNS,

CX_3CL1. *Saline-IL-4* treatment promoted a decrease in CCL2 production compared to *Saline-Vehicle* controls 24 h after saline/LPS injection (P=0.10). Furthermore, while *LPS-Vehicle* did not significantly increase CCL2 24 h after injection, *LPS-IL-4* treatment significantly elevated CCL2 expression above all other groups (LPS x IL-4 interaction: $F(1,44)=10.10$, $P<0.003$) (Fig.5B). Increased CCL2 production supports an arginase-dependent exaggeration in polyamine production in the CNS following LPS and IL-4 co-treatment. Induction of polyamines may also promote a chemokine profile shift in the CNS. In concert with increased CCL2 production, *LPS-IL-4* treated mice had a significant reduction in CX_3CL1 expression compared to *Saline-Vehicle* mice ($P<0.005$) (Fig.5C).

To evaluate how these shifted chemokine profiles influenced macrophage association with the brain, microglia/macrophages were isolated from whole brain tissue of mice injected ip with saline or LPS and icv with vehicle or IL-4, 24 h after the LPS injection. Corresponding with an increase in CCL2 expression in the brain of *LPS-IL-4* mice, co-treatment with LPS and IL-4 promoted increased accumulation of macrophages with the brain (LPS x IL-4 interaction: $F(1,30)=3.67$, $P=0.067$) (Fig.5D,E).

Discussion

Microglia from adult, but not aged, mice markedly upregulate surface expression of IL-4Rα following a peripheral immune challenge (Fenn et al., 2011). To establish the functional consequence of impaired IL-4Rα upregulation, we injected mice ip with saline or LPS and, one hour later, injected mice icv with vehicle or IL-4. We demonstrate that upregulation of IL-4Rα is necessary to induce an appropriate response to elevated levels of IL-4 in the brain, but does not impact the progression of microglial activation relating to the sickness response. For example,

central IL-4 did not ameliorate sickness behavior corresponding to maintained and elevated IL-1β expression, but did reduce levels of circulating IL-6 and depression-like behavior. In the brain, arginase expression was markedly induced only in mice that received both LPS and IL-4 injections and this was restricted to microglia. Increased arginase corresponded to increased markers of microglial regulation and neuroprotection, and reduced expression of the depression-associated enzyme, IDO. Finally, increased arginase corresponded with polyamine-related gene induction (i.e., CCL2) and the recruitment of peripheral macrophages to the brain.

IL-4 is normally very lowly expressed or not-detectible in the adult brain (Lovett-Racke et al., 2000). Some studies have reported identifiable levels of IL-4 mRNA and protein within the hippocampus related to improved LTP, memory and learning, and neurotrophin production (Maher et al., 2005; Nolan et al., 2005; Lyons et al., 2007; Derecki et al., 2010). The cells producing IL-4 in the homeostatic brain, however, have not been identified and whether these results can be attributed to IL-4 remain controversial. Elevated CNS IL-4 is noted, however, after traumatic injury (Kim et al., 2000; Lee et al., 2010). Traumatic injury also induces high levels of IL-4Rα expression (Kigerl et al., 2009). The functional consequence of elevated IL-4Rα following an inflammatory challenge and exposure to high levels of IL-4 (mimicking a state of CNS trauma) is unknown. This is important because aged individuals consistently suffer increased morbidity and mortality following CNS trauma (Fassett et al., 2007; Anderson et al., 2009) and this may be related to impaired IL-4Rα upregulation.

Icv administration of IL-4 did not improve the sickness response in mice pre-injected with LPS. This was not altogether surprising considering that IL-4 promoted IL-1β induction in LPS-treated mice. IL-1β is the primary inflammatory cytokine thought to regulate sickness behavior (Dantzer et al., 2008). This finding also coincided with previous work showing that a

simultaneous administration of central IL-4 and peripheral LPS resulted in an exaggerated sickness response (Bluthé et al., 2002). Thus, administration of IL-4 in the presence of inflammation may not promote a strict anti-inflammatory microglial phenotype. This is important when discussing the use of IL-4 in moderating inflammatory activation. While IL-4 *in vitro* may promote an anti-inflammatory and reparative M2a monocyte phenotype (Mantovani et al., 2004), the alternative activation profile promoted by IL-4 in the presence of ongoing inflammation may exaggerate some inflammatory cytokines leading to exacerbated tissue damage.

Icv injection of IL-4, however, did lead to reduced levels of IL-6 in the plasma corresponding to reduced depression-like behavior. This may have resulted from reduced IDO expression from microglia. IDO is an important enzyme responsible for restricting extracellular tryptophan levels and diverting them to sources unavailable for infiltrating pathogens. In the brain the role of IDO is less clearly defined and may actually divert tryptophan away from important neurotransmitters, including serotonin, and promote the creation of neuroactive metabolites responsible for increased oxidative stress and depression-like behavior (Dantzer et al., 2008; O'Connor et al., 2008; Haroon et al., 2012). Restricting activation of IDO promotes increased microglial regulation, reduced neuroinflammation, and ameliorates depression-like behavior (unpublished findings; (O'Connor et al., 2008)). IL-4 has been shown to reduce IDO in the peripheral monocytes (Musso et al., 1994), but a novel role for IL-4 in the CNS may be reducing damaging levels of IDO and restricting the development of depression-like behavior.

Other microglial regulatory factors that were upregulated by co-treatment with LPS and IL-4 were CX_3CR1 and IL-4Rα. As expected, CX_3CR1 was increased over baseline in *LPS-Vehicle* mice 24 h following the LPS injection, but was further increased in *LPS-IL-4* mice.

Exaggerated CX_3CR1 in the brains of *LPS-IL-4* mice appeared a sign of increased microglial regulation rather than merely a compensatory mechanism, as CX_3CR1 levels 4 h after LPS were not different between *LPS-Vehicle* and *LPS-IL-4* groups (data not shown).

Although BDNF levels were not changed in a gross brain section at the site of IL-4 injection, IL-4 did promote increased BDNF expression in microglia. Microglial-specific BDNF expression is particularly important in the context of synaptic plasticity. Microglia are intimately associated with neuronal synapses and involved with synaptic pruning (Tremblay et al., 2011). Increased BDNF specifically in microglia could promote improved synaptic function which coincides with a role for IL-4 in LTP and memory and learning (Maher et al., 2005; Derecki et al., 2010).

The most significant change noted in microglia and the brain following co-treatment with LPS and IL-4 was a marked upregulation of arginase mRNA and protein expression. Because significant increases in arginase expression were only noted in microglia from *LPS-IL-4* mice, LPS-associated IL-4Rα upregulation is likely necessary to initiate an IL-4 dependent arginase response in the CNS. The role of arginase in the CNS is still not well defined. While some have used arginase to identify "alternative activated" microglia and macrophages implicated in tissue repair (Gordon, 2003; Schwartz, 2003), others have focused on the ability of arginase to inhibit iNOS activity (Lange et al., 2004), while others have focused on the role of arginase in overcoming axonal growth inhibition (Cai et al., 2002). In particular, the groups focusing on axonal growth inhibition have identified the functional role of arginase is to produce polyamines. Arginase is the rate-limiting enzyme for the conversion of L-arginine to the polyamines, putrescine, spermidine, and spermine. The pathway includes the conversion of L-arginine to ornithine and urea by arginase, and the subsequent metabolism of ornithine to putrescine by

ornithine decarboxylase (ODC) (Soulet and Rivest, 2003). In addition to their potential roles in regulating axonal growth and neuronal health, polyamines may also be important for the regulation of chemokine profiles in the CNS (Puntambekar et al., 2011). In particular, CCL2 was found to be polyamine-dependent. This is important because CCL2 is the primary chemokine for recruiting peripheral macrophages to the CNS (Babcock et al., 2003). To confirm an increase in the polyamine pathway we investigated ODC mRNA expression following LPS and IL-4 treatments. ODC was increased in both LPS-treated groups, irrespective of IL-4 treatment. This corresponds with previous research demonstrating increased ODC in the brain following inflammatory stimuli (Soulet and Rivest, 2003; Puntambekar et al., 2011). Thus, following LPS and IL-4 co-treatment, both arginase and ODC are upregulated and likely promote a strong polyamine response. Indeed when we investigated mRNA levels of CCL2 in the brain, only *LPS-IL-4* mice had significant increases in CCL2 levels. In fact, IL-4 alone decreased CCL2 levels demonstrating that IL-4 exposure in the presence of inflammation promotes a separate microglial phenotype than when exposed to IL-4 alone.

To identify whether increased CCL2 levels promoted increased macrophage association with the brain we isolated microglia/macrophages from the brain and evaluated these cells using flow cytometry. Microglia and macrophages were differentiated based on CD45 expression with microglia being $CD11b^{+}/CD45^{low}$ and macrophages being $CD11b^{+}/CD45^{high}$. Corresponding with significantly elevated CCL2, *LPS-IL-4* mice had significantly increases in the number of macrophages associated with the brain. To evaluate whether other major brain chemokines were altered by IL-4 and LPS treatment, we examined mRNA levels of CX_3CL1. CX_3CL1 is not only an important chemokine for microglial regulation, but is also thought to be important for perivascular macrophage extravasation into the CNS (Geissmann et al., 2003; Prinz and Priller,

2010). Interestingly, CX_3CL1 levels were significantly reduced in the brains of *LPS-IL-4* treated mice. First, this finding may be responsible for the exaggerated induction of CX_3CR1 in this group observed earlier, and second, this may be important for the phenotype of the recruited macrophages. A recent study identified that $CX_3CR1^{-/-}$ mice had improved tissue sparing and increased functional recovery following a spinal cord injury compared to heterozygous litter mates (Donnelly et al., 2011). Furthermore, improved recovery was associated with increased recruitment of $CCR2^+$ macrophages to the site of injury. This study identified that CX_3CR1^+ macrophages were $iNOS^+$ and increased tissue damage whereas $CCR2^+$ macrophages were $iNOS^-$ associated with increased tissues repair. Thus, an increase in CCL2 and decrease in CX_3CL1 may promote the recruitment of a specific subset of macrophages to the CNS important for tissue repair.

Together these results have implications for CNS trauma in which both IL-4 and IL-4Rα expression is increased in the presence of marked neuroinflammation. Because *LPS-IL-4* co-treatment strongly promoted BDNF, arginase, and CCL2 expression, inflammatory-induced upregulation of IL-4Rα may be necessary to mount appropriate tissue repair processes in response to IL-4 in the CNS. Thus, a failure to upregulate IL-4Rα on microglia of aged mice may result in reduced sensitivity to IL-4 following CNS trauma. Reduced sensitivity to IL-4 could result in impaired upregulation of neurotrophins, reduced arginase expression, and increased recruitment of more inflammatory, CX_3CR1^+ macrophages to the CNS. Moreover, because polyamines are also implicated in bypassing axonal growth inhibition, impaired IL-4Rα on aged microglia may promote increased axonal dieback and worsened functional outcome after CNS trauma.

Figure Legends

Figure 1. Icv IL-4 reduced plasma IL-6 levels and depressive-like behavior independent of sickness behavior. Mice received an ip injection of saline or LPS followed by an icv injection of vehicle or IL-4 one hour later. A) Social exploratory behavior was evaluated prior to the peripheral injection and 4, 8, 12, and 24 hours after LPS and B) total body mass loss and food intake over the 24-hour period is reported (n=8). C) A subset of mice was euthanized at 4 and 24 h after LPS to collect blood and evaluate plasma levels of IL-6 by ELISA (n=8-12). D) A final subset of mice was tested for depression-like behavior 24 h after LPS using the tail suspension test (TST) (n=14). Bars represent the mean ± SEM. Asterisks indicate significant difference ($P<0.05$) compared to *Saline-Vehicle* controls. Asterisks with a line under them (1A) denote significance ($P<0.05$) of both LPS-treated groups compared to *Saline-Vehicle* controls. Means with different letters (a,b,c) are significantly different ($P<0.05$) from each other.

Figure 2. Co-treatment with LPS and IL-4 promoted exaggerated arginase and CX_3CR1 expression and reduced IDO expression. Mice received an ip injection of saline or LPS followed by an icv injection of vehicle or IL-4 one hour later. Following the behavioral tests shown in Figure 1, mice were euthanized and a brain slice at the site of IL-4 injection was collected for mRNA analysis (n=8). mRNA expression levels of A) BDNF, B) arginase, C) CX_3CR1, and D) IDO are reported. Bars represent the mean ± SEM. Means with different letters (a,b,c) are significantly different ($P<0.05$) from each other. N.d. values are not-determined.

Figure 3. Co-treatment with LPS and IL-4 promoted increased BDNF, arginase, IL-1β, and IL-4Rα expression. Mice received an ip injection of saline or LPS followed by an icv injection of vehicle or IL-4 one hour later. Following the behavioral tests shown in Figure 1, mice were

euthanized and microglia were isolated for mRNA analysis (n=9-11). mRNA expression levels of A) BDNF, B) arginase, C) IL-1β, and D) IL-4Rα are reported. Bars represent the mean ± SEM. Means with different letters (a,b,c) are significantly different ($P<0.05$) from each other.

Figure 4. Co-treatment with LPS and IL-4 promoted arginase protein expression exclusively in microglia. Mice received an ip injection of saline or LPS followed by an icv injection of vehicle or IL-4 one hour later. After 24 h mice were trancardially perfused and brains were fixed, sectioned, and stained for Iba-1 to denote microglia, arginase, and IL-4Rα (n=4). A) Representative images from the site of icv injection for Iba-1, arginase, IL-4Rα and co-labeled staining (20x). Single cells are enlarged images of microglia indicated by arrow. B) Quantification of the percent of microglia that were arginase positive and triple positive for Iba-1, arginase, and IL-4Rα evaluated by DIA using Metamorph software. C) Representative images from the lateral ventricle in sections adjacent to the injection site for Iba-1, arginase, and co-labeled staining. Single cells are enlarged images of microglia indicated by arrow. Bars represent the mean ± SEM. Means with different letters (a,b) are significantly different ($P<0.05$) from each other.

Figure 5. LPS increased brain ODC expression, and co-treatment with LPS and IL-4 increased CCL2 mRNA and lowered CX_3CL1 mRNA expression with increased association of macrophages with the brain. Mice received an ip injection of saline or LPS followed by an icv injection of vehicle or IL-4 one hour later. After 24 h mice were euthanized and microglia/macrophages were isolated from whole brain tissue and used for flow cytometry analysis of macrophage number. A) ODC, B) CCL2, and C) CX_3CR1 mRNA expression in the brain were evaluated from mRNA collected from previous studies (Figure. 2) (n=8). D) Representative dot plot showing the characterization of macrophages ($CD11b^{+}/CD45^{high}$). E)

Quantification of the percent of macrophages associated with the brain (n=6). Bars represent the mean ± SEM. Means with different letters (a,b,c) are significantly different ($P<0.05$) from each other.

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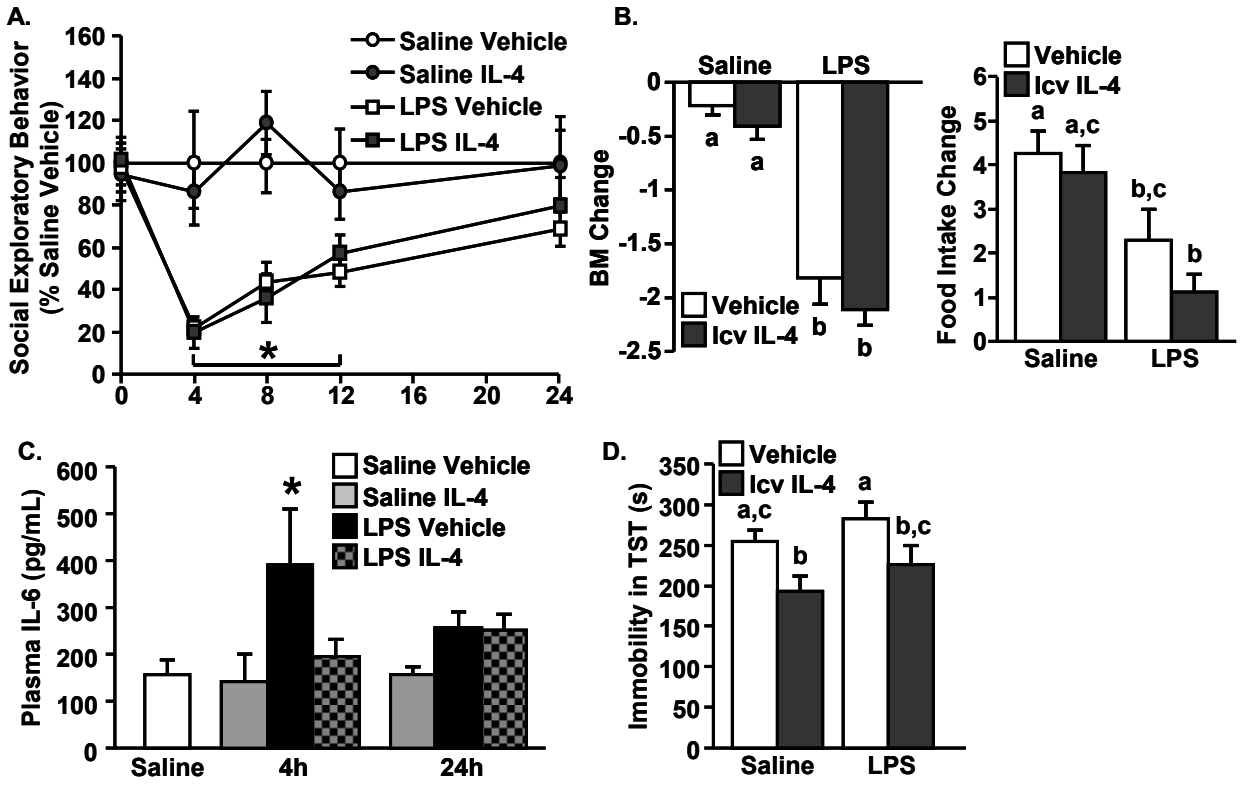
Figure 1



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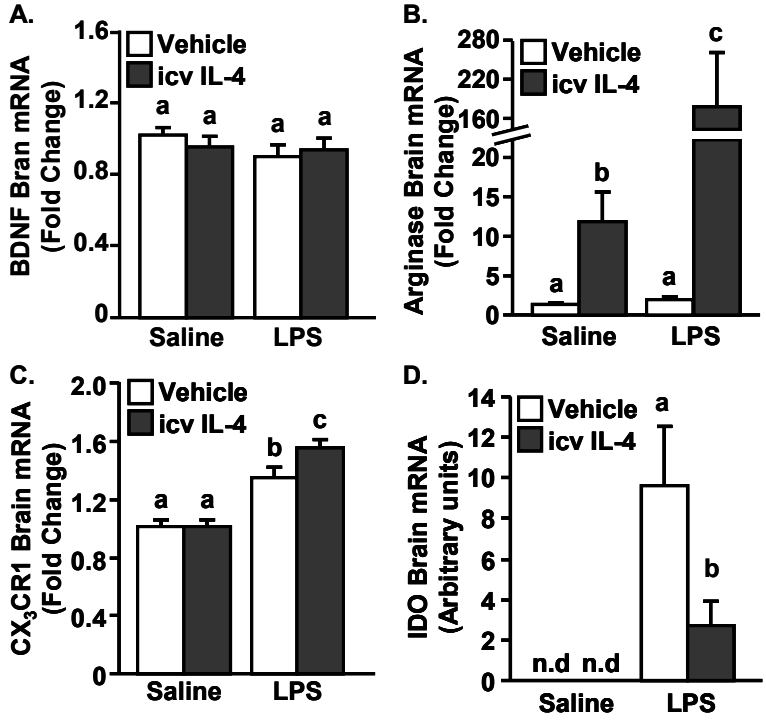
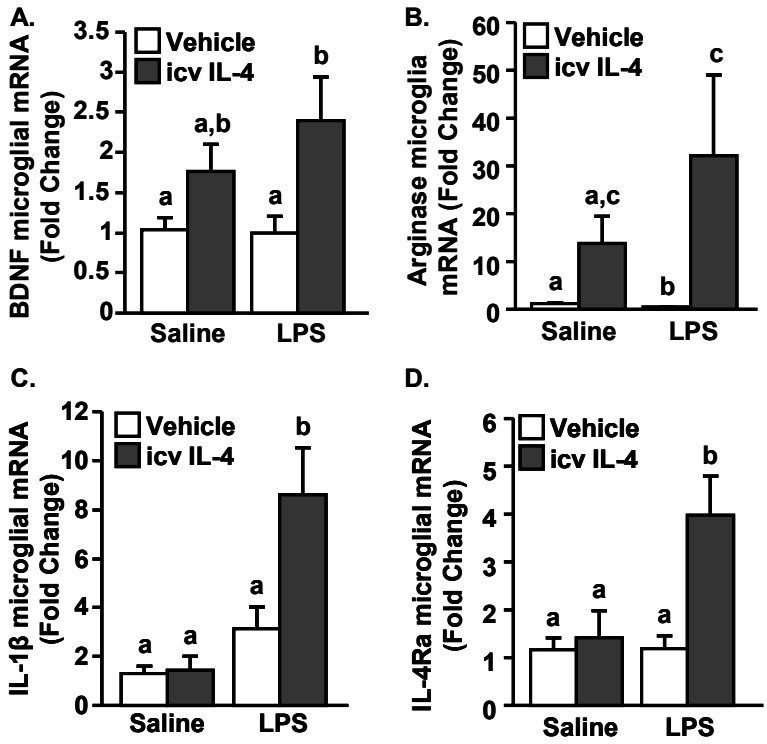


Figure 3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Saline
LPS
B.
Arginase microglia mRNA (Fold Change)
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a,c
b
c
C.
IL-1β microglial mRNA (Fold Change)
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a
a
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D.
IL-4Ra microglial mRNA (Fold Change)
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a
a
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Figure 4

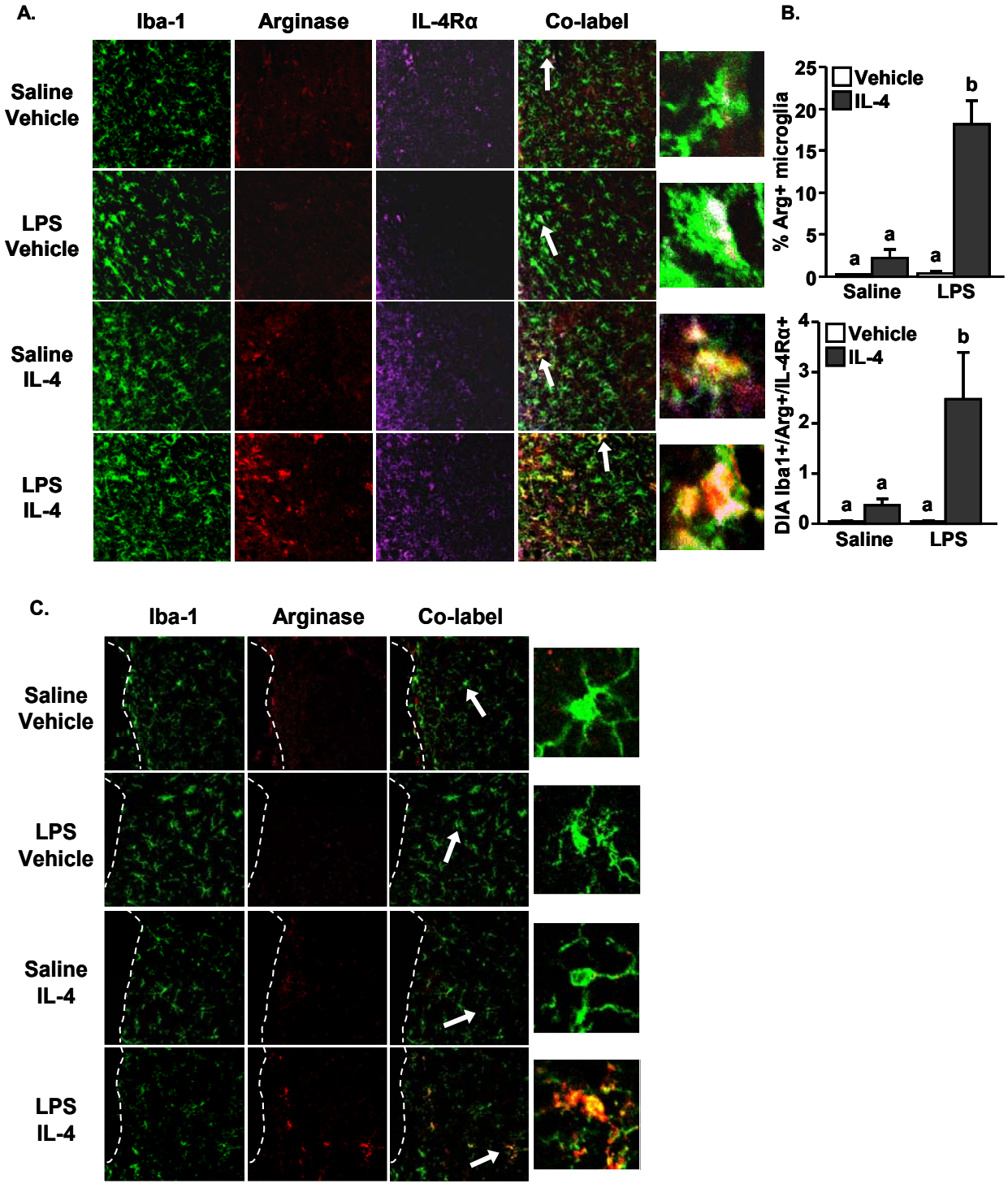
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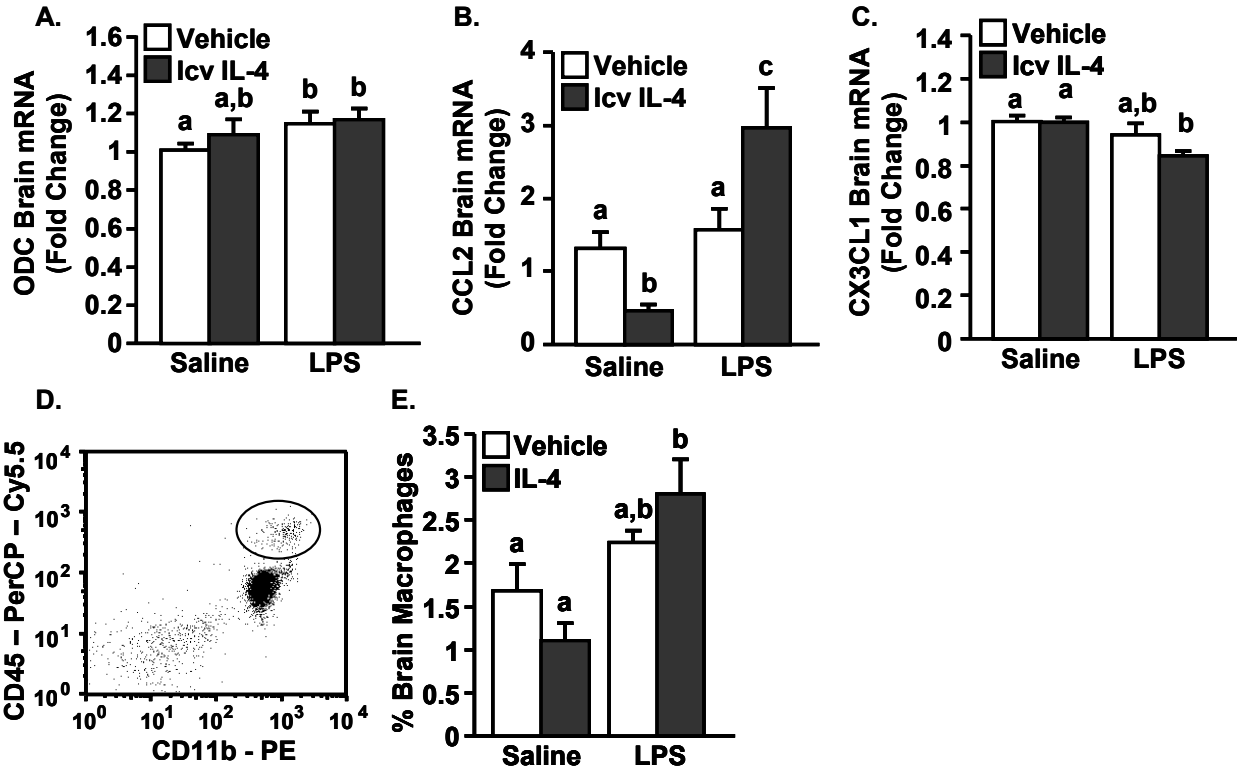
Figure 5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Vehicle
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Saline
LPS
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CD11b - PE
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