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Protracted downregulation of CX₃CR1 on microglia of aged mice after lipopolysaccharide challenge

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Abstract

Fractalkine (CX₃CL1) to fractalkine receptor (CX₃CR1) interactions in the brain are involved in the modulation of microglial activation. Our recent findings indicate that there is microglial hyperactivity in the aged brain during an inflammatory challenge. The underlying cause of this amplified microglial response in the aged brain is unknown. Therefore, the purpose of this study was to determine the degree to which age-associated impairments of CX₃CL1 and CX₃CR1 in the brain contribute to exaggerated microglial activation after intraperitoneal (i.p.) injection of lipopolysaccharide (LPS). Here we show that CX₃CL1 protein was reduced in the brain of aged (18–22 mo) BALB/c mice compared to adult (3–6 mo) controls. CX₃CL1 protein, however, was unaltered by LPS injection. Next, CX₃CR1 levels were determined in microglia (CD11b⁺/CD45^{low}) isolated by Percoll-density gradient separation at 4 and 24 h after LPS injection. Flow cytometric and mRNA analyses of these microglia showed that LPS-injection caused a marked decrease of CX₃CR1 and a simultaneous increase of IL-1 β at 4 h after LPS injection. While surface expression of CX₃CR1 was enhanced on microglia of adult mice by 24 h, it was still significantly downregulated on a subset of microglia from aged mice. This protracted reduction of CX₃CR1 corresponded with a delayed recovery from sickness behavior, prolonged IL-1 β induction, and decreased TGF β expression in the aged brain. In the last set of studies BV2 microglia were used to determine effect of TGF β on CX₃CR1. These results showed that TGF β enhanced CX₃CR1 expression and attenuated the LPS-induced increase in IL-1 β expression.

Keywords

Microglia; Aging; Brain; Cytokines; Fractalkine; Inflammation

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1. Introduction

Microglia are part of the innate immune system in the CNS and play an important role in interpreting and propagating inflammatory signals in response to activation of the peripheral immune system (Davalos et al., 2005; Nguyen et al., 2002; Nimmerjahn et al., 2005). Several studies using older rodents, non-human primates, and humans indicate that the profiles of microglia and astrocytes become more inflammatory with age. For example, markers of glial reactivity or priming, such as major histocompatibility complex (MHC) class II, are increased in the brain during normal aging (Godbout and Johnson, 2006; Perry et al., 2003; Wynne et al., 2009). Recent studies in older rodents indicate that a potential consequence of a reactive glial profile is an exaggerated neuroinflammatory cytokine response to either central (Abraham et al., 2008; Huang et al., 2008) or peripheral (Chen et al., 2008; Godbout et al., 2005b; Henry et al., 2008; Henry et al., 2009) innate immune challenge. An exaggerated microglial response with age (Henry et al., 2009) is relevant because it is coupled with a myriad of complications including cognitive impairment (Barrientos et al., 2009; Barrientos et al., 2006; Chen et al., 2008), altered febrile response (Barrientos et al., 2009), exaggerated sickness behavior (Abraham et al., 2008; Godbout et al., 2005a; Huang et al., 2008), and protracted depressive-like behavior (Godbout et al., 2008). Collectively, these data point to an impaired ability to regulate microglia in the aged brain.

Recent data has begun to establish fractalkine (CX₃CL1) to fractalkine receptor (CX₃CR1) interactions as an important mechanism of microglial regulation. CX₃CL1 is a member of the CX₃C family of chemokines (Harrison et al., 1998; Nishiyori et al., 1998; Pan et al., 1997) and is one of the few chemokines that is highly expressed in the brain (Pan et al., 1997; Sunnemark et al., 2005; Tarozzo et al., 2003). CX₃CL1 exists both as a ~95 kDa membrane-bound ligand and a ~65 kDa soluble glycoprotein (Harrison et al., 1998). Complementary expression of CX₃CL1 on neurons and CX₃CR1 on microglia (Hughes et al., 2002; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998; Pan et al., 1997) has led to the hypothesis of a unique communication system where neurons constitutively express and release CX₃CL1 to modulate activation of microglia (Cardona et al., 2006).

In acute inflammatory conditions, CX₃CL1 and CX₃CR1 interact to attenuate microglial activation. For example, pretreatment with neutralizing anti-CX₃CL1 antibody exaggerates neuroinflammation after i.c.v. injection of LPS (Zujovic et al., 2001). In cultured microglia or mixed glia cultures, soluble CX₃CL1 attenuates LPS-induced production of TNF α , IL-6, and IL-1 β (Mizuno et al., 2003; Zujovic et al., 2000). Moreover, CX₃CR1-deficiency amplifies microglial IL-1 β expression, neurotoxicity, and mortality in CX₃CR1^{-/-} mice compared to CX₃CR1^{+/-} mice after repeated i.p. injections of lipopolysaccharide (LPS) (Cardona et al., 2006). Furthermore, in *ex vivo* microglial cultures, CX₃CR1 expression is up-regulated by the anti-inflammatory cytokine, transforming-growth-factor β (TGF β) (Chen et al., 2002), and is downregulated by LPS (Boddeke et al., 1999). The reduction in CX₃CR1 expression after exposure to LPS is associated with decreased sensitivity to CX₃CL1 (Boddeke et al., 1999). Collectively, these data indicate that CX₃CL1-CX₃CR1 interactions in the brain function to regulate the activation of microglia following an acute immune challenge.

Our recent findings indicate that there is amplified microglial activation in the aged brain after i.p. challenge with LPS (Henry et al., 2009). We hypothesize that age-associated changes in neural expression of CX₃CL1, microglial expression of CX₃CR1, or both, contribute to impaired regulation of microglia in the aged brain after LPS challenge. Therefore, the purpose of this study was to determine the degree to which expression of CX₃CL1 or CX₃CR1 was affected in the brain of adult and aged mice by peripheral LPS challenge. Here we show that CX₃CL1 was decreased in the brain of aged BALB/c mice, but was not altered by LPS injection. Moreover, we show that injection of LPS reduced surface expression of CX₃CR1 on microglia

of aged and adult mice and caused protracted downregulation of CX₃CR1 on microglia of aged mice. This LPS-induced reduction of CX₃CR1 on aged microglia corresponded with a delayed recovery from sickness behavior, prolonged microglial activation, and decreased TGF β expression in the brain of aged mice compared to adults. Furthermore, data from cultured BV2 microglia showed that TGF β enhanced CX₃CR1 expression and attenuated the LPS-induced increase in IL-1 β expression.

2. Materials and 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 (3–6 and 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 mice is approximately 26 months (Morley and Trainor, 2001). To investigate changes that occur from adulthood to what is considered aged, 3–6-month-old (young adult) and 18–22-month-old (aged) male mice were used. Upon arrival, mice were individually housed as described above. 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.

2.2 Experimental Protocols

In the first study, adult (3–6 mo) BALB/c mice received an intraperitoneal (i.p.) injection of saline or *Escherichia coli* LPS (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO) and were sacrificed by CO₂ asphyxiation 4, 8, or 24 h later (n=6). This LPS dosage was selected because it elicits a proinflammatory cytokine response in the brain resulting in a transient sickness response in adult mice (Berg et al., 2004; Godbout et al., 2005b; Henry et al., 2008). The entire cortex and hippocampus were dissected out of the brain and homogenized separately for total RNA isolation/quantitative PCR (n=7–8) or CX₃CL1 protein analysis (n=4). In a related experiment, adult (3–6 mo) and aged (18–22 mo) male BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and a 1 mm coronal brain section (+0.38 mm from bregma) (Paxinos and Franklin, 2004) was used for CX₃CL1 protein analyses (n=6). The brain section was collected by cutting the intact brain using a rodent brain matrix (ASI instruments, Warren, MI) as a guide.

In the second study, adult (3–6 mo) BALB/c mice received an intraperitoneal (i.p.) injection of saline or LPS (0.33 mg/kg or 3.3 mg/kg) and were sacrificed by CO₂ asphyxiation 4 h later (n=6). Brains were homogenized and microglia were isolated by discontinuous Percoll density gradient. Microglia were used for total RNA isolation/quantitative PCR (n= 8) or for flow cytometric analyses of CD11b, IL-1 β , and CX₃CR1 expression (n=6–8).

In the third study, adult (3–6 mo) and aged (18–22 mo) male BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and social exploratory behavior was determined 0, 4, 8, 12, and 24 hours after i.p. injections (n=4). At 4 or 24 h after injections, mice were sacrificed and microglia were isolated by discontinuous Percoll density gradient. Microglia were used for total RNA isolation/quantitative PCR (n=6–8) or for flow cytometric analyses for CD11b and CX₃CR1 (n=7–8). In a subset of mice, a 1 mm coronal brain section (+0.38 mm from bregma) was collected and was used for CX₃CL1 protein analysis or mRNA analysis (IL-1 β , TGF β , IL-10, and IL-4) (n=8–10).

In the final study, BV2 microglia were pre-treated with vehicle or recombinant human TGF β (0.1 ng/ml; R&D Systems, MN) for 30 minutes and then treated with saline or LPS (100 ng/ml). BV2 microglia were collected 4 h later and used for total RNA isolation/quantitative PCR (IL-1 β and CX $_3$ CR1) or immunocytochemistry for CX $_3$ CR1 surface expression (n=12, three independent experiments).

2.3 BV2 Cell culture and treatment

BV2 microglia cell lines were cultured in growth medium (DMEM supplemented with 10% FBS, sodium bicarbonate 3.7 g/L, 200 mM glutamine, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 0.25 μ g/ml fungizone) as previously described (Abraham et al., 2008). Cultures were maintained at 37°C with 95% humidity and 5% CO $_2$ and growth medium was replenished every third day until confluence. For RNA isolation/qPCR experiments, cells were seeded at 5x10 5 cells per well in 12-well plates and allowed to adhere for 20 h. For immunocytochemistry experiments, cells were seeded at 2x10 5 cells per well in 24-well plates on poly-L-lysine coated glass coverslips and allowed to adhere for 20 h. Immediately before treatment, cultures were washed twice and supplemented with warm growth medium containing experimental treatments. Cell viability was measured by the MTS cell proliferation assay according to the manufacturer's instructions (Promega, Madison, WI).

2.4 Isolation of Microglia

Microglia were isolated from whole brain homogenates as described previously (Frank et al., 2006b; Henry et al., 2009). In brief, brains were homogenized in Hank's Balanced Salt Solution (HBSS, pH 7.4) by passing through a 70 μ m 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 (GE-healthcare, Uppsala, Sweden) 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 (Frank et al., 2006b). Cells were washed and then re-suspended in sterile HBSS. The number of viable cells was determined using a hemocytometer and 0.1% Trypan blue staining. Each brain extraction yielded approximately 3 x 10 5 viable cells. We have previously characterized these cells as approximately 85% CD11b $^{+}$ /CD45 low microglia with less than 1% CD11b $^{+}$ /CD45 high macrophages (Henry et al., 2009). These relative percentages of cells do not change with age or LPS (Henry et al., 2009). Based on this previous characterization, cells isolated by Percoll density separation are referred to as "enriched microglia".

2.4 RNA isolation and qPCR

RNA was isolated from discrete brain regions (e.g., cortex or hippocampus), 1mm coronal brain section (+0.38 mm from bregma), BV2 microglia or enriched microglia extracted by Percoll density gradient. For brain samples and BV2 microglia, total RNA was isolated using the Tri-Reagent protocol (Sigma, MO) and subjected to the DNA-free TM RNA clean up procedure (Ambion, TX). For Percoll isolated cells, RNA was isolated using the RNeasy plus mini kit (Qiagen, CA). In all RNA isolation procedures, RNA concentration was determined by spectrophotometry (Eppendorf, NY) and RNA was reverse transcribed to cDNA using an RT RETROscript kit (Ambion, TX).

Quantitative PCR was performed using the Applied Biosystems (Foster, CA) Taqman $^{\circledR}$ Gene Expression assay as previously described (Godbout et al., 2005b). In brief, cDNA was amplified by real time PCR where a target cDNA (e.g., CD11b, IL-1 β , CX $_3$ CL1, CX $_3$ CR1, TGF β 1, IL-4, and IL-10) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase) 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.

2.5 Protein Analysis of CX₃CL1

Protein levels of CX₃CL1 in a 1 mm coronal brain section (+0.38 mm from Bregma) or the whole hippocampus were determined by ELISA (R&D Systems, MN). Brain tissue was homogenized in ice-cold lysis buffer containing 1% Triton-X-100, 100 mM NaCl, 50 mM NaF, 1mM EGTA, 25 mM benzamidine, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM PMSF, and 50 mM Tris, pH 7.4. Lysates were clarified by centrifugation and total protein concentration was determined by Bio-Rad protein assay. Clarified lysates were assayed for CX₃CL1 according to the manufacturer's instructions. Absorbance (450 nm) was determined using a Bio-Tek synergy HT microplate reader (Bio-Tek Instruments, VT). Assays were sensitive to ~0.20 ng/ml of CX₃CL1 and inter- and intra-assay coefficients of variation were less than 0.1.

2.6 Microglial Staining and Flow Cytometry

Microglial surface antigens were stained as previously described (Henry et al., 2008; Henry et al., 2009). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, CA). Next, enriched microglia were incubated with rabbit anti-mouse CX₃CR1 and rat anti-mouse CD11b-APC antibodies (eBioscience, CA). Microglia were washed and then incubated with a FITC-conjugated goat anti-rabbit IgG secondary antibody (eBioscience, CA). For isotype controls, cells from each treatment group were combined and stained with non-specific, isotype-matched antibody (APC-conjugated anti-rat IgG or FITC-conjugated goat anti-rabbit IgG). Cells were washed and then resuspended in FACS buffer for analysis.

Staining for intracellular IL-1β was performed using the BD Cytfix/Cytoperm Plus fixation/permeabilization protocol (BD Biosciences, CA). To block secretion of cytokines, microglia were incubated in 10% FBS DMEM media containing brefeldin-A (BD Biosciences, CA) for 4 h at 37 °C. Next, enriched microglia were washed in FACS buffer (2% FBS in HBSS with 1mg/ml sodium azide) and Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, CA). After blocking, microglia were stained with a rat anti-mouse CD11b-APC antibody. Microglia were then fixed and permeabilized with BD Fixation/Permeabilization buffer for 20 min. Microglia were washed with BD Perm/Wash™ buffer, re-suspended in BD Perm/Wash™ buffer, and incubated with rabbit anti-mouse IL-1β-FITC (eBioscience, CA) for 30 min. Non-specific binding was assessed by using non-specific, isotype matched-conjugated antibodies. Microglia were washed twice in BD Perm/Wash buffer and resuspended in FACS buffer for analysis.

Antigen expression was determined using a Becton-Dickinson FACSCaliber four color flow cytometer. Ten thousand events were recorded. For each antibody, gating was determined based on appropriate negative isotype stained controls. Flow data were analyzed using FlowJo software (Tree Star, CA). For analysis of CX₃CR1 expression, cells were gated for positive CD11b expression and expression levels of CX₃CR1 were determined on this population. CD11b⁺ cells that exhibited FITC-fluorescence below the levels of the isotype control were considered CX₃CR1^{low} microglia. In some analyses, an additional gate was placed to divide the remaining cells into medium and high-expressing cells (CX₃CR1^{med} and CX₃CR1^{high}). All samples were analyzed with identical gates.

2.7 Immunocytochemistry on cultured BV2 microglia

BV2 microglia were plated in 24-well plates on poly-L-lysine coated glass coverslips and treated as described above. After treatment, media was aspirated and cells were washed 2 times.

Cells were blocked for 10 minutes with anti-CD16/CD32 antibody in PBS supplemented with 1% BSA and 5% normal goat serum. Cells were then incubated with rabbit anti-mouse CX₃CR1 (eBioscience, CA) diluted 1:250 in PBS for 20 minutes. Cells were then fixed in 4% paraformaldehyde for 10 minutes at room temperature before staining with alexafluor 594-conjugated secondary antibody (1:500) for 20 minutes. After staining, glass coverslips were mounted on superfrost plus glass slides (Fisher Scientific, PA) with fluoromount G (EMS, PA).

Images were captured at 20x using an epifluorescent Leica DM5000B microscope equipped with a Leica DFC300 FX camera and Leica imaging software. Fluorescent images were captured with identical exposure, gamma, and gain settings. Fluorescence intensity was measured using Image-Pro software (Media Cybernetics, Bethesda, MD).

2.8 Social Exploratory Behavior

To assess the motivation to engage in social exploratory behavior, a novel juvenile conspecific was introduced into the test subject's home cage for a 10-min period. Behavior was videotaped and the cumulative amount of time the subject engaged in social investigation was determined from the video records by a trained observer who was blind to the experimental treatments. Baseline social behavior was measured immediately before experimental treatment (time 0). Social behavior was determined as the amount of time that the experimental subject spent investigating (e.g., anogenital sniffing, trailing) the juvenile. Results are expressed as percent decrease in time engaged in social behavior compared to respective baseline measures.

2.9 Statistical Analysis

To ensure a normal distribution, data were subjected to Shapiro-Wilk test using Statistical Analysis Systems (SAS) statistical software (Cary, NC). Observations greater than 3 interquartile ranges from the first and third quartile were excluded in the subsequent analysis. To determine significant main effects and interactions between main factors, data were analyzed using one- (Age, Treatment, Time), two- (Age $\times$ Treatment, Treatment $\times$ Time) or three- (Age $\times$ Treatment $\times$ Time) 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 $\pm$ standard error of the mean (SEM).

3. Results

3.1 Age associated reduction of CX₃CL1 protein in the brain of aged mice

To determine the degree to which CX₃CL1 levels were altered in the brain following LPS injection, adult mice were injected i.p. with saline or LPS, and mRNA expression was determined in the hippocampus or cortex collected 4, 8, or 24 h later. The hippocampus and cortex were selected because the neurons of these regions express the highest levels of CX₃CL1 (Sunnemark et al., 2005; Taroazzo et al., 2003). CX₃CL1 mRNA and total protein levels were unchanged by LPS injection at the times tested in the hippocampus (Fig. 1A&B) and cortex (data not shown). Previous findings indicate that membrane-bound CX₃CL1 (~95kd) can be released from glutamate-overstimulated neurons as a soluble glycoprotein (~65 kDa) independent of mRNA transcription or total protein increase (Limatola et al., 2005). To exclude this possibility in our model, western blot analysis for CX₃CL1 was also performed. This assay did not reveal any significant change in membrane-bound or soluble CX₃CL1 protein (data not shown).

Next, we sought to determine CX₃CL1 protein levels in the brain of aged mice after LPS injection. In this related but separate experiment, adult and aged mice were injected with saline or LPS i.p. and total CX₃CL1 protein levels were determined in brain homogenate from a 1

mm coronal brain section through the prefrontal cortex (+0.38 mm from bregma) collected either 4 or 24 h later. The 24 h time point corresponds with the time when older mice show extended behavioral symptoms of sickness after LPS injection compared to adults (Godbout et al., 2005a; Godbout et al., 2008). The coronal brain section samples were collected prior to microglial extraction and corresponding analyses (shown in the subsequent figures). This protocol allowed for comparison of CX₃CL1 protein levels in the same mice that social exploratory behavior and microglial mRNA and protein analyses were determined.

Fig. 1C shows that there was a significant age-dependent decrease in CX₃CL1 protein in the brain of aged mice collected 24 h later (Age, $F(1,23) = 32.7$, $p < 0.0001$). Similar results were also obtained at 4 h after LPS (data not shown). Although there was a main effect of age on CX₃CL1 levels at the 4 and 24 h time points, there was not a significant main effect of LPS treatment. Collectively, these data indicate that CX₃CL1 protein is decreased in the brain of aged mice and that i.p. injection of 0.33 mg/kg LPS does not affect mRNA or total protein levels of CX₃CL1.

3.2 Peripheral injection of LPS decreased CX₃CR1 and increased IL-1 β expression in microglia

CX₃CR1 is highly expressed on microglia and plays a role in modulating their responses (Boddeke et al., 1999; Boehme et al., 2000; Hughes et al., 2002; Mizuno et al., 2003), the effect of peripheral injection of LPS on microglial CX₃CR1 expression, however, has not been determined *in vivo*. Thus, we investigated the extent to which CX₃CR1 was altered on microglia following peripheral challenge with LPS. In this experiment, adult mice were injected with LPS and mRNA levels of several microglia-related genes (CD11b, IL-1 β , and CX₃CR1) were determined in enriched microglia collected 4 h later. This 4 h time point after LPS injection was selected because it is a time when microglia actively produce cytokines (Henry et al., 2009) and when mice display the behavioral symptoms of sickness (Godbout et al., 2005b). Fig. 2A shows that CX₃CR1 mRNA expression in enriched microglia was markedly reduced after peripheral LPS injection (LPS, $F(1,30) = 26.82$, $p < 0.001$). Moreover, peripheral injection of LPS increased IL-1 β mRNA expression in enriched microglia at 4 h after injection (Fig. 2B; LPS, $F(1,30) = 33.11$, $p < 0.0001$). Fig. 2C shows that CD11b mRNA expression was unaffected by LPS injection.

Because peripheral LPS injection caused a marked reduction of CX₃CR1 mRNA in enriched microglia at 4 h (Fig. 2A), we next determined CX₃CR1 surface expression on microglia after LPS injection. In this experiment adult mice were injected i.p. with saline or LPS (0.33 or 3.3 mg/kg) and CX₃CR1 surface expression was determined by flow cytometry 4 h later. Fig. 2D shows representative bivariate dot plots of CD11b and CX₃CR1 staining. LPS injection caused a dose-dependent decrease in CX₃CR1 surface protein on microglia (Fig. 2F; LPS, $F(2,23) = 23.9$, $p < 0.001$). The representative histogram of CX₃CR1-FITC mean fluorescence intensity (MFI) confirmed that CX₃CR1 expression was reduced on a subset of microglia from that received LPS (3.3mg/kg) (Fig. 2G). Intracellular IL-1 β staining was also determined in CD11b⁺ microglia (Fig. 2E). Fig. 2E&H show that LPS injection caused a dose-dependent increase in IL-1 β protein levels in microglia (LPS, $F(2,11) = 16.6$, $p < 0.001$). Taken together, these findings indicate that LPS injection led to increased intracellular IL-1 β protein and decreased CX₃CR1 surface expression on microglia in the adult brain.

3.3 Peripheral LPS injection caused protracted sickness behavior and prolonged downregulation of CX₃CR1 mRNA on microglia of aged mice

We have previously reported that LPS injection caused exaggerated IL-1 β production by microglia of aged mice (Henry et al., 2009) and that the behavioral symptoms of sickness (i.e., anorexia, lethargy, and social withdrawal) persist longer in aged mice compared to adults

(Godbout et al., 2005b). Because CX₃CL1 modulates the activation of microglia, impairment in the regulation of CX₃CR1 may contribute to exaggerated and prolonged microglial activation in response to inflammatory stimuli. To confirm the age-associated differences in the recovery from sickness behavior, a subset of adult and aged mice (n = 4) were injected i.p. with LPS and social exploratory behavior was determined 0, 4, 8, 12, and 24 h after injection. As expected, aged mice showed an impaired recovery from the LPS-induced sickness behavior (Fig.3A; Age x LPS x Time, $F(4,15)=6.29$, $p < 0.001$). Fig.3B shows CD11b mRNA levels in enriched microglia were unaffected by either LPS or age. Fig.3C shows that enriched microglia from the brain of aged mice 24 h after LPS had the highest levels of IL-1 β mRNA compared to all other treatments (Age x LPS interaction, $F(1,31)=4.1$, $p < 0.05$).

Next, mRNA expression of CX₃CR1 in microglia of adult and aged mice 4 and 24 h after LPS injection was determined. Fig.3D shows that LPS injection decreased CX₃CR1 mRNA expression in microglia independent of age (LPS, $F(1,30)=17.15$, $p < 0.005$) at 4 h after injection. At 24 h after LPS injection, CX₃CR1 mRNA expression returned to baseline in microglia of adult mice, but it was still reduced in microglia of aged mice (Fig.3E; Tendency for Age x LPS interaction, $F(1,27)=2.4$, $p = 0.1$).

3.4 Prolonged downregulation of CX₃CR1 surface expression on aged microglia at 24 h after peripheral injection of LPS

Based on the mRNA results we next determined surface expression of CX₃CR1 in adult and aged microglia after LPS challenge. In this experiment adult and aged mice were injected with saline or LPS, CD11b and CX₃CR1 levels were determined on microglia collected by Percoll separation 4 or 24 h after injection. Fig.4A shows that the same percentage of CD11b⁺ cells were used in the analysis for each treatment and age group. Representative bivariate dot plots of CD11b and CX₃CR1 staining at 4 and 24 h after LPS injection are shown in Fig.4B&C, respectively. At 4 h after LPS injection, both adult and aged mice showed a significant increase of CX₃CR1^{low} microglia (Fig.4B&D); main effect of LPS, $F(1,29)=257.85$, $p < 0.0001$). At 24 h after LPS injection, CX₃CR1 expression was recovered to baseline levels in adult mice, but was still markedly reduced on microglia of aged mice (Fig.4C; Age x LPS, $F(1,29)=14.66$, $p < 0.001$). Furthermore, this significant age x LPS interaction was dependent on time after LPS injection (Age x LPS x time interaction, $F(1,51)=10.49$, $p < 0.01$). Taken together, these data indicate that the reduction of CX₃CR1 on the surface of microglia was protracted in the aged brain.

3.5 Enhanced expression of CX₃CR1 on microglia of adult mice at 24 h after LPS injection

In addition to a recovery to baseline levels, the bivariate dot plots in Fig.4C indicated that total expression of CX₃CR1 on microglia of adult mice was enhanced at 24 h after LPS injection. To confirm this observation, CX₃CR1-FITC fluorescence was determined and gated based on low, medium, and high CX₃CR1-FITC staining. Representative histograms of adult and aged microglia at 24 h after LPS injection with the low, medium, and high designations are shown in Fig.5A&B. Fig.5C illustrates the relative percentages of CX₃CR1-expressing microglia for each treatment group. The average MFI of CX₃CR1-FITC fluorescence and the average relative percentages of CX₃CR1-expressing microglia for each treatment group are provided in Fig.5D. There was a significant increase of CX₃CR1 MFI on microglia of adult mice at 24 h after LPS compared to all other groups (Age x LPS interaction, $F(1,37)=4.3$, $p < 0.05$). Consistent with Fig.4C, a significant population of CX₃CR1^{low} microglia was still present from aged mice treated with LPS (Fig.5B&D). It is important to mention that there was also a modest increase in the CX₃CR1^{high} population in aged mice 24 h after LPS ($9.6 \pm 1.3\%$) compared to the adult ($1.3 \pm 0.2\%$) and aged saline controls ($4.7 \pm 0.6\%$). The percentage of CX₃CR1^{high} microglia, however, was highest in the microglia of adult mice ($15.6 \pm 2.4\%$) and this Adult LPS group was the only group that exhibited an increase in overall MFI (Fig.5D,

Age x LPS interaction, $F(1,37)=9.15, p < 0.05$). Taken together, these data indicate that while CX₃CR1 surface expression was globally enhanced on microglia of adult mice 24 h after LPS injection, it was still significantly reduced on a subset of microglia of aged mice.

3.6 TGF β mRNA levels were augmented in the adult brain 24 h after LPS

Surface expression of CX₃CR1 was still significantly lower in a subset of microglia from aged mice 24 h after LPS challenge, while it was enhanced on microglia of adult mice at this time. Because CX₃CR1 is modulated by cytokines, mRNA levels of several cytokines (IL-1 β , IL-4, IL-10, and TGF β) were determined in brain homogenate derived from a coronal brain section (+0.38 mm from bregma) collected prior to the extraction of brain microglia used in the above experiments (Fig.4). Coronal brain sections were collected from the same mice used in the experiments shown in Figs.3&4. As expected, IL-1 β mRNA was the highest in the brain of aged mice 24 h after LPS (Fig.6A; Tendency for Age x LPS interaction, $F(1,22)=3.7, p = 0.07$). Fig.6B shows that LPS decreased IL-4 mRNA levels independent of age (LPS, $F(1,28)=6.48, p < 0.05$). IL-10 mRNA levels were increased by LPS, independent of age (Fig.6C). It is important to note that IL-10 mRNA was undetected in the brain of saline-treated mice. Fig. 6D shows that TGF β was increased in the brain of adult mice 24 h after LPS (LPS, $F(1,27)=19.27, P < 0.001$), but TGF β mRNA expression was not increased in the brain of aged mice 24 h after LPS (age x LPS interaction, $F(1,27)=6.28, p < 0.02$). These data indicate that 24 h after LPS there were higher mRNA levels of IL-1 β , but lower mRNA levels of TGF β in the brain of aged mice compared to adults.

3.7 TGF β enhanced CX₃CR1 expression and attenuated the LPS-induced increase in IL-1 β in BV2 microglia

To begin to understand the potential effect of less TGF β in the brain of aged mice after LPS injection, a series of cell culture experiments using BV2 microglia were completed. In the first experiment, BV2 cells were treated with recombinant human TGF β and mRNA levels of CX₃CR1 and IL-1 β were determined. Fig.7A&B shows that TGF β treatment simultaneously increased CX₃CR1 mRNA expression (LPS, $F(5,23)=9.9, p < 0.0001$) and decreased IL-1 β mRNA expression in a dose-dependent manner (LPS, $F(5,23)=3.85, p < 0.05$). In the second experiment, BV2 cells were incubated with TGF β and then were treated with LPS (Fig.7C&D). As anticipated, mRNA of levels CX₃CR1 were enhanced by TGF β (TGF β , $F(1,36)=56.4, p < 0.0001$) and reduced by LPS (LPS, $F(1,36)=10.3, p < 0.01$). Moreover, TGF β pretreatment reversed the LPS-induced reduction in CX₃CR1. In fact, the enhancement of CX₃CR1 mRNA by TGF β was still maintained in the LPS-treated BV2 cells. Fig.7D shows that TGF β pretreatment attenuated the LPS-induced increase in IL-1 β (TGF β x LPS, $F(1,31)=163.65, p < 0.0001$).

Next, surface expression of CX₃CR1 was determined on BV2 cells after TGF β and LPS treatments. Fig.7E shows representative staining for CX₃CR1. In saline-treated BV2 cells, CX₃CR1 was detected as a distinct ring around the membrane of the cell (Fig.7E.i). In LPS-treated BV2 cells, there was less CX₃CR1 on the membrane of the cells compared to saline controls (Fig.7E.iii&F; LPS, $F(1,48)=6.79, p < 0.05$). Moreover, TGF β enhanced CX₃CR1 surface expression (Fig.7E.v&F; TGF β , $F(1,48)=26.46, p < 0.0001$) and post hoc analysis revealed that TGF β attenuated the LPS-induced reduction of CX₃CR1 surface expression ($p < 0.05$).

4. Discussion

We have previously reported that stimulation of the peripheral innate immune system in aged BALB/c mice caused exaggerated microglial activation with amplified mRNA and intracellular protein expression of IL-1 β (Henry et al., 2009). The exaggerated

neuroinflammation and microglial activation was paralleled by prolonged sickness behavior (Godbout et al., 2005b), impaired working memory (Chen et al., 2008), and protracted depressive-like behavior (Godbout et al., 2008). The results of the current study support our hypothesis that impaired CX₃CL1-CX₃CR1 mediated regulation of microglia contributes to the exaggerated microglial activation in the brain of aged mice after peripheral immune challenge. First, we show there was significantly less CX₃CL1 protein in the brain of aged mice compared to adults (Fig.1). Second, peripheral challenge with LPS caused a simultaneous reduction of CX₃CR1 (mRNA and surface expression) and an induction of IL-1 β (mRNA and intracellular protein expression) in microglia (Figs.2–4). Third, our data indicate that i.p. injection of LPS caused an extended sickness response, prolonged induction of IL-1 β , and a protracted reduction of CX₃CR1 on microglia of aged mice compared to adults (Figs.3–5). Fourth, TGF β mRNA was increased in the brain of adult mice 24 h after LPS, but was not up-regulated in the brain of aged mice (Fig.6). Last, studies with BV2 microglia show that TGF β enhanced surface expression of CX₃CR1 and attenuated IL-1 β expression following an LPS challenge (Fig.7).

Several reports using rodent models of aging indicate that the brain environment becomes more proinflammatory with age (Frank et al., 2006a; Godbout et al., 2005a; Henry et al., 2009; Lyons et al., 2009; Sierra et al., 2007). Consistent with this hypothesis, we show that there was an age-dependent reduction in CX₃CL1 protein (Fig.1C). A potential explanation for less CX₃CL1 in the brain of aged mice is a reduction in total fractalkine produced by neurons. This may be caused by increased neuronal loss with aging (Woodruff-Pak et al., 2010) or functional changes in aged neurons associated with impaired oxidative or calcium homeostasis (Toescu, 2005). Whatever the cause, reduced levels of CX₃CL1 in the brain of aged mice may contribute to a reduced ability to regulate microglial activation after LPS challenge. In support of this notion, other studies show that soluble CX₃CL1 attenuated LPS-induced production of TNF α , IL-6, and IL-1 β in microglia, mixed glia, and neuron/glia co-cultures (Mizuno et al., 2003; Zujovic et al., 2000). Moreover, *in vivo* studies show that pretreatment with a neutralizing anti-CX₃CL1 antibody led to exaggerated neuroinflammation (i.e., TNF α and 8-isoprostane production) after i.c.v. injection of LPS (Zujovic et al., 2001). Our results are also consistent with recent studies showing that CX₃CL1 was decreased in the brain of 22 mo Wistar rats (Lyons et al., 2009) and 22 mo Fisher 344 rats (Bachstetter et al., 2009). In these studies, the age-associated increase in MHC II⁺ microglia was attenuated following either a single i.c.v. injection of CX₃CL1 (Lyons et al., 2009) or a 7-day infusion of CX₃CL1 (Bachstetter et al., 2009). We have reported an age-associated increase of MHC II expression on microglia of BALB/c mice (CD11b⁺/CD45^{low}) and that LPS challenge elicited a robust induction of IL-1 β in these MHC II⁺ microglia (Henry et al., 2009). Thus, a reduction in CX₃CL1 protein in older BALB/c mice may contribute to the increased number of MHC II⁺ microglia detected in our model (Henry et al., 2009). In addition, we interpret these current findings to suggest that a reduction in CX₃CL1 in the aged brain (Fig.1C) contributes to the amplified microglial activation following peripheral LPS injection in a BALB/c model of aging (Figs.3&4).

It is important to note that the function of CX₃CL1 is dependent on the nature of the inflammatory stimulus. For instance, expression of CX₃CL1 is markedly enhanced in several rodent models of neurodegenerative diseases (Huang et al., 2006; Sunnemark et al., 2005), ischemia (Denes et al., 2008) and traumatic CNS injury (Harrison et al., 1998; Rancan et al., 2004). In these examples, increased CX₃CL1 is associated with recruitment of peripheral immune cells into the CNS. Moreover, intra-parenchymal injection of high levels of CX₃CL1 is associated with increased microglial activation (Hughes et al., 2002; Shan et al., 2009). Therefore, neuronal or glial release of significant levels of CX₃CL1 may be a mechanism by which microglia and other CX₃CR1⁺ immune cells are recruited to damaged areas within the CNS. In the present study, however, LPS injection did not increase total mRNA or protein levels of CX₃CL1 in the hippocampus (or cortex) at 4, 8, or 24 h after LPS injection (Fig.1).

Furthermore, we did not detect a significant influx in either macrophages (CD45^{high}, CD11b⁺) or T-cells (CD3⁺, CD45⁺) in Percoll isolated cells from adult mice at 24 or 72 h after LPS injection (data not shown). Thus, in the context of an acute immune activation, our data support the idea that CX₃CL1-CX₃CR1 interactions function to regulate the activation of microglia (Cardona et al. 2006; Lyons et al. 2009a).

Microglia are modulated by CX₃CL1 and therefore a change in the ligand/receptor interaction must occur to circumvent this regulation by neurons. A relevant finding of this study was that activation of microglia in response to a peripheral injection of LPS was associated with decreased surface expression of CX₃CR1 (Fig.2). Independent of the age comparisons, this microglial specific reduction in surface expression of CX₃CR1 after i.p. injection of LPS is a novel finding. Our data indicate that there is less CX₃CR1 on the surface of microglia after LPS injection (Figs.2, 4&5) to respond to the constitutively produced CX₃CL1 (Fig.1). These findings are consistent with a previous study showing that LPS caused a marked reduction of CX₃CR1 mRNA expression and decreased sensitivity to CX₃CL1 in *ex vivo* microglial cultures (Boddeke et al., 1999). Moreover, in our studies, peripheral injection of LPS caused a dose-dependent reduction in CX₃CR1 that corresponded with an induction of IL-1 β in microglia of adult mice (Fig.2). This inverse relationship between IL-1 β and CX₃CR1 was also apparent in the studies with BV2 microglia (Fig.7). Taken together, we interpret these data to suggest that the CX₃CR1^{low} microglia represent a more active subset of microglia.

A key finding of this study was that downregulation of CX₃CR1 surface expression on microglia of aged mice was protracted compared to adults 24 h after LPS injection (Fig.4C and Fig.5). The protracted down-regulation of CX₃CR1 on microglia of aged mice was associated with extended IL-1 β expression (Fig.3C) and prolonged sickness behavior (Fig.3A). We hypothesize that protracted reduction of CX₃CR1 on microglia plays a direct role in the prolonged production of IL-1 β and subsequent neurobehavioral deficits in aged mice. In support of this notion, microglia from LPS-treated CX₃CR1-deficient (CX₃CR1^{-/-}) mice express higher levels of IL-1 β mRNA compared to CX₃CR1^{+/-} controls. Injection of microglia isolated from LPS-treated CX₃CR1^{-/-} mice into the brain of a wild-type mouse caused neuronal cell death, which was blocked by co-injection of IL-1 receptor antagonist (Cardona et al., 2006). Another study showed that blocking CX₃CR1 in the brain of adult rats with an anti-CX₃CR1 antibody caused increased IL-1 β production that was paralleled by deficits in neurogenesis (Bachstetter et al., 2009). These collective findings indicate that either loss or significant downregulation of CX₃CR1 on microglia leads to extended expression of IL-1 β after the microglia have become activated.

It is important to mention that along with an increased CX₃CR1^{low} population there was also an increased CX₃CR1^{high} population in aged mice 24 h after LPS compared to saline treated mice (Fig.5D). This is not unexpected considering the 24 h time-point after LPS in the aged brain represents a transitional time when inflammation is still present, but is moving towards resolution (Godbout et al, 2005). The CX₃CR1 staining in microglia supports this conclusion. For example, in the adult brain at 24 h after LPS, the inflammation has dissipated and microglial expression of CX₃CR1 is uniformly higher than in any other group (Fig.5D). In the aged mice, brain inflammation and sickness behavior are lower than they were at 4 h but both are still present 24 h after LPS (Fig.3A&C, Fig.6A; (Godbout et al., 2005b) and this is paralleled by the presence of a significant CX₃CR1^{low} population of microglia (Figs.4C & 5D). This is relevant because a persistent population of CX₃CR1^{low} microglia in the brain of aged mice after LPS is consistent with an active phenotype. Collectively these data support the hypothesis that impaired fractalkine-mediated regulation of microglia in the brain of aged mice contributes to exaggerated neuroinflammation and the corresponding extension of sickness and depressive-like behaviors (Godbout et al., 2005; Godbout et al., 2008).

It is important to highlight that along with transcriptional regulation, translation and translocation into the membrane are also important levels of regulation for chemokine receptors (Kershaw et al., 2009). This may explain the apparent inconsistency between CX₃CR1 mRNA and protein data. For example, while there was a marked increase of CX₃CR1 surface expression on adult microglia 24 h after LPS (Fig.5), this protein increase was detected in the absence of a corresponding increase in mRNA expression (Fig.3E). Other chemokine receptors, including CXCR4, are sequestered in intracellular vesicles and can be translocated to the membrane independent of transcription (Forster et al., 1998). It is plausible that a similar mechanism can increase CX₃CR1 surface expression on microglia.

The cause of the protracted reduction of CX₃CR1 expression on microglia of aged mice 24 h after LPS is unknown, but it may be related to an imbalance of pro- and anti-inflammatory cytokines. For example, higher IL-1 β mRNA was detected in microglia (Fig.3C) as well as in homogenate derived from a coronal brain section (Fig.6A). Because several anti-inflammatory cytokines, including IL-10, IL-4, and TGF β , attenuate microglial activation (Wynne et al., 2009) mRNA levels of these genes were determined in homogenate derived from a coronal brain section at 24 h after LPS (Fig.6). Of the three, only TGF β mRNA levels were significantly affected by both LPS and age. For example, TGF β mRNA levels were increased in brain homogenate from adult mice 24 h after LPS, but were not increased by LPS in aged mice (Fig. 6D). Moreover, the enhancement of TGF β mRNA levels in the adult brain at 24 h after LPS corresponded with an overall increase in MFI of CX₃CR1 surface expression that was not present in the microglia of aged mice (Figs.5D). In a previous study using microglia cultures, TGF β enhanced mRNA levels of CX₃CR1 (Chen et al., 2002). Consistent with this previous finding, pretreatment with TGF β directly increased CX₃CR1 mRNA and protein expression on BV2 microglia (Fig.7A&E). Furthermore, these data show that pretreatment with TGF β enhanced CX₃CR1 expression and attenuated the LPS-induced increase in IL-1 β (Fig.7C&D). Last, a recent study showed that lower TGF β expression in the brain of aged mice was associated with exaggerated IL-1 β and prostaglandin E₂ levels in a model of chronic inflammatory arthritis (Wu et al., 2008). Taken together, restoration or enhancement of CX₃CR1 on the surface of microglia after activation is compromised in the brain of aged mice and it is plausible that this is dependent on the levels of TGF β .

In conclusion, the present study demonstrates that there was a reduction of CX₃CL1 protein in the aged brain and that LPS induced a protracted reduction of CX₃CR1 surface expression on microglia in the aged brain. We interpret these data to indicate that there are two potential age-associated problems in CX₃CL1-CX₃CR1 mediated regulation of microglia. First, decreased levels of CX₃CL1 are likely associated with the exaggerated pro-inflammatory response of microglia after LPS injection. Second, a failure to restore CX₃CR1 receptor expression after it has been downregulated leads to an incomplete resolution of the microglia-driven inflammatory signal.

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Fig. 1. Total CX₃CL1 was reduced in the aged brain

Adult mice (3–6 mo) were injected with saline or LPS i.p. and A) CX₃CL1 mRNA or B) CX₃CL1 protein levels were determined in the hippocampus collected 4, 8, or 24 h later. Bars represent the mean $\pm$ SEM (n=6). C) Adult (3–6 mo) and aged (18–22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and protein levels of CX₃CL1 were determined from a 1 mm coronal brain section collected 24 h later. Bars represent the mean $\pm$ SEM (n=6). Means with different letters (a or b) are significantly different ($p < 0.05$) from each other.

Fig. 2. Peripheral LPS injection increased IL-1 β mRNA expression and decreased CX₃CR1 expression in microglia

Adult mice (3–6 mo) were injected with saline or LPS i.p. and A) CX₃CR1, B) IL-1 β , and C) CD11b mRNA levels were determined from enriched microglia isolated 4 h later (n=8). Means with different letters (a or b) are significantly different ($P < 0.05$) from each other.

Representative bivariate dot plots of Percoll isolated cells stained with antibodies for D) CD11b and CX₃CR1 or E) CD11b and intracellular IL-1 β . Cells were gated for CD11b expression. F) Average percentage of cells that were CD11b⁺/CX₃CR1^{Low}. Bars represent the mean $\pm$ SEM (n=8). Means with different letters (a, b, or c) are significantly different ($P < 0.05$) from each other. G) Representative histogram of CX₃CR1-FITC fluorescence in microglia from a saline and LPS (3.3mg/kg) treated mouse. H) Average percentage of cells that were CD11b⁺/IL-1 β ⁺. Bars represent the mean $\pm$ SEM (n=4). Means with different letters (a, b, or c) are significantly different ($P < 0.05$) from each other.

Fig. 3. LPS injection caused a reduction of CX₃CR1 mRNA in microglia of adult and aged mice Adult (3–6 mo) and aged (18–22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and A) social exploratory behavior was determined 0, 4, 8, 12, and 24 h later (n=4). Means marked with * are significantly different from baseline controls and means marked with ‡ are significantly different from *Adult LPS* ($p < 0.05$). Adult and aged mice were injected i.p. with saline or LPS and B) CD11b and C) IL-1 β mRNA levels were determined from microglia isolated 24 h later. Mice were treated as above and CX₃CR1 mRNA expression was determined from microglia isolated D) 4 h or E) 24 h after LPS injection. Bars represent the mean $\pm$ SEM (n=7–8). Means with different letters (a, b, or c) are significantly different ($p < 0.05$) from each other.

Fig. 4. Protracted reduction of CX₃CR1 surface expression on microglia of aged mice after LPS injection

Adult (3–6 mo) and aged (18–22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and CX₃CR1 surface expression was determined on microglia. A) Percentage of CD11b positive microglia from each treatment group that were used in the analysis. Bars represent the mean $\pm$ SEM (n=7). Representative bivariate dot plots of Percoll isolated cells stained with antibodies for CD11b and CX₃CR1 and gated for positive CD11b expression at B) 4 and C) 24 h after LPS injection. D) Average percentage of cells that were CD11b⁺/CX₃CR1^{Low}. Bars represent the mean $\pm$ SEM (n=7). Means with different letters (a or b) are significantly different ($p < 0.05$) from each other

Fig. 5. Enhanced CX₃CR1 surface expression on microglia of adult mice after LPS injection
 Adult (3–6 mo) and aged (18–22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and CX₃CR1 surface expression was determined on microglia. Representative histograms of CX₃CR1-FITC fluorescence on CD11b⁺ cells from A) adult and B) aged mice 24 h after injection. C) Relative percentages of CX₃CR1-low, medium, or high-expressing microglia. D) Mean fluorescence intensity (MFI) and percentage of CX₃CR1-low, medium, or high-expressing microglia. Values represent the mean ± SEM (n=6–8). Means with different letters (a, b, or c) are significantly different ($p < 0.05$) from each other.

Fig. 6. Attenuated TGF β mRNA levels in the aged brain 24 h after LPS injection

Adult (3–6 mo) and aged (18–22 mo) BALB/c mice were injected i.p. with saline or LPS (0.33 mg/kg) and A) IL-1 β , B) IL-4, C) IL-10, and D) TGF β mRNA levels were determined from a 1 mm coronal brain section collected 24 h later. Bars represent the mean $\pm$ SEM (n=6–8).

Means with different letters (a, b, or c) are significantly different ($p < 0.05$) from each other.

Fig. 7. TGFβ enhanced CX₃CR1 expression and attenuated IL-1β expression in BV2 microglia after LPS stimulation

BV2 microglia were incubated with TGFβ (0–1.0 ng/ml) and A) CX₃CR1 and B) IL-1β mRNA expression was determined 4 h later. Bars represent the mean ± SEM (n=6). Means with different letters (a, b, or c) are significantly different ($p < 0.05$) from each other. In a related study, BV2 cells were incubated with vehicle or TGFβ (0.1 ng/ml) for 30 min before LPS (100 ng/ml) treatment and C) CX₃CR1 and D) IL-1β mRNA levels were determined in cells collected 4 h later. Bars represent the mean ± SEM (n=8, 2 independent experiments). Means with different letters (a, b, or c) are significantly different from each other ($p < 0.05$). BV2 cells were treated as above and CX₃CR1 surface expression was determined 4 h later. E) Representative CX₃CR1 staining in saline-treated (panels i & ii), LPS-treated (panels iii & iv), and TGFβ/LPS-treated BV2 microglia (panels v & vi). Top panels show fluorescent images of CX₃CR1⁺ staining and the bottom panels show corresponding bright field images. The inset images in the bottom panels represent merged fluorescent and brightfield images of the white outlined boxes. Scale bar = 100 μm. F) Average fluorescence intensity of CX₃CR1 staining. Bars represent the mean ± SEM (n=12, 3 independent experiments). Means with different letters (a, b, or c) are significantly different from each other ($p < 0.05$).