TGFβ Increases CX3CR1 Expression and Attenuates Lipopolysaccharide (LPS) Induced Microglia Activation

A Senior Honors Thesis

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Abstract

Interactions between Fractalkine (CX_3CL1) and Fractalkine receptor (CX_3CR1) in the CNS function to modulate the activation of microglia. The Godbout Laboratory has recently reported that CX_3CR1 is down regulated on microglia after lipopolysaccharide (LPS) injection and that this down-regulation was protracted (24 h)on microglia of aged mice (20 mo) compared to adult mice (3- 6 mo). This extended reduction in receptor expression in aged microglia corresponded with exaggerated Interleukin 1-b expression and an impaired recovery from the behavioral symptoms of sickness**.** The underlying cause of the protracted down regulation of the Fractalkine receptor on microglia of aged mice is unknown but may be related to an age-related decrease in levels of the thanti-inflammatory cytokine, Transforming growth factor beta (TGFβ). Therefore, the purpose of this study was to determine the extent to which TGFβ is involved in the regulation of CX_3CR1 expression on microglia. In this study cultured BV2 microglia were used to determine effects of TGFβ and LPS on CX_3CR1 *in vitro*. Here it is shown that LPS treatment of BV2 cells are caused a reduction in CX_3CR1 and increase in Il;-6 in a dose dependent manner.. Moreover, culturing the BV-2 cells with LPS conditioned media was sufficient to cause a reduction in CX_3CR but did increase IL-6 secretion. These data indicate that CX_3CR1 goes down prior to the microglia being activated. In the next set of studies, TGFβ pretreatments enhanced CX_3CR1 expression and attenuated the LPS-induced increase in IL-1β expression. Last, ICV injection of TGFB enhanced expression of CX_3CR1. Taken together these data support the hypothesis that TGFB is important in up regulating CX_3CR1 and attenuating microglia activation.

1. Introduction

1.1 Aging and Neuroinflammation

According to the National Institute of Health, the number of people in the United States 65 years and older will increase by 20 million people by the year of 2024. (Jackson, 2009; Penninx 2003). Moreover, as the segment of US population 65 and older grows, the occurrence of neurobehavioral complications in the elderly is expected to increase dramatically. It is predicted that increased life expectancy will result in only a modest increase in health span, with a marked increase in years of poor or compromised physical and mental health.

A potential explanation for the higher incidence of mental health complications concomitant with peripheral infection is an age-related change in microglia function that impairs the normal bidirectional communication between the immune system and the brain. Bi-directional communication is necessary for mounting the appropriate immunological, physiological, and behavioral responses to immune stimulation. Understanding changes in microglia during normal aging is paramount because microglia play an integral role in receiving and propagating inflammatory signals generated in the periphery. For example, pathogens in the periphery activate the innate immune system by stimulating macrophages or monocytes causing production of inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-alpha). These cytokines use both neural (via the afferent vagus nerve) (Goehler, 1998; Konsman, 2000) and humoral pathways (via circumventricular organs (CVO) (Laflamme,1999; Lacroix, 1998) and direct transport (Banks, 1999) to communicate this innate immune activation to the brain. In turn, this peripheral signal induces microglia (and perivascular macrophages) to produce the same cytokines (Rivest, 2003), which target neuronal substrates

and elicit a sickness behavior syndrome that is normally adaptive and beneficial to the host (Kelley, 2003). A dysregulated inflammatory response in the aged brain, however, may cause neurobehavioral complications (Blasko, 2004) including cognitive dysfunction (Heyser, 1997), anorexia (Finck, 1997), and mood and depressive disorders (Musselman, 2001; Pollmacher, 2002; Capuron, 2003).

1.2 The role of microglia

Microglia are part of the innate immune system in the CNS and play an important role in receiving and propagating inflammatory signals in response to activation of the peripheral immune system (Nguyen, 2002). Microglia are bone marrow derived myeloid lineage cells (Hess, 2004) that are interspersed throughout the CNS, and represent approximately 10-15% of the total CNS cell population. In early development, myeloid cells migrate from the bone marrow to the brain parenchyma and terminally differentiate into microglia. During development of the CNS, microglia are responsible for the removal of apoptotic cells and in same cases may be responsible for inducing apoptosis (Besis, 2007). In the absence of inflammatory stimuli, microglia are in a resting state "surveying" their microenvironment (Davallos, 2005; Nimmerjahn, 2005). Once activated, microglia have macrophage-like capabilities including phagocytosis, inflammatory cytokine production, and antigen presentation (Hanisch, 2007). Because of the inflammatory potential of microglia, it is necessary that activation be tightly regulated. Neuroinflammatory processes are normally transient with microglia returning to a resting state as the immune stimulus is resolved (Biber, 2007).

1.3 Microglia and Fractalkine

One mechanism involved in microglia regulation in the CNS is the Fractalkine (CX_3CR1 or CX_3CL1) regulatory system. Fractalkine is one of the few chemokines that is highly expressed in the brain (Pan et al., 1997). Recently, interaction between CX_3CR1 and CX_3CL1 has been shown to be important in regulation of microglia. A complimentary relationship between CX_3CR1 on microglia and CX_3CL1 from neurons has been described (Cardona et al., 2006). This relationship involves the constant release of CX_3CL1 by neurons in order to modulate the activation of microglia. During acute infection, the interaction between CX_3CL1 and CX_3CR1acts to attenuate microglia activation. If mice are pretreated with an intracebroventricular injection of an anti-CX_3CL1 antibody prior to injection of LPS then the inflammatory response by microglia is exaggerated (Zujovic et al., 2001). Furthermore, primary microglia cell cultures treated with CX_3CL1 prior to LPS show an attenuated increase in pro-inflammatory cytokines (Zujovic et al., 2000). In addition, CX_3CR1 deficiency in mice causes an exaggeration in pro-inflammatory cytokines as well as mortality in CX_3CR1 mutant mice after being injected with LPS (Cardona et al., 2006).

Recent studies indicate that the primary function of the Fractalkine system in the CNS is to regulate microglia activation in an anti-inflammatory manner. Neurons constitutively express and release Fractalkine to provide specific restraint on microglia activation. In fact, the CNS concentration of Fractalkine is approximately 300 pg/mg (Cardona, 2006). Fractalkine modulates microglia activation both *in vitro* and i*n vivo*. For example, in microglia, mixed glia, and neuron cell cultures, soluble fractalkine attenuates LPS-induced production of TNF-alpha, IL-6, and IL-1 (Mizuno, 2003; Zujovic, 2000). Fractalkine is also neuro-protective against excitotoxic levels of glutamate (Limatola,2005), GP120 (Meucci, 1998) and Fas Ligand (Boehme, 2000).

Moreover, neuroinflammation caused by central (i.c.v.) LPS injection is exaggerated by pretreatment with a neutralizing anti-fractalkine antibody (Zujovic, 2001). Furthermore, repeated i.p. injections of LPS or MPTP, a model of Parkinson's disease, amplify neuroinflammation, neurotoxicity, and mortality in CX_3CR1 deficient mice ($CX_3CR1^{-/-}$) compared to heterozygote ($CX_3CR1^{+/-}$) controls (61).

The fractalkine system is influenced by neuroinflammatory events. For example, CX_3CR1 mRNA is up-regulated by anti-inflammatory cytokines including TGF-alpha (Chen, 2002), but is down-regulated by neuroinflammatory mediators such as LPS. LPS treatment causes a marked reduction of CX_3CR1 mRNA expression and decreased sensitivity to fractalkine in *ex vivo* rat microglia cultures. It has been proposed that in order to circumvent the neuroinflammation-induced reduction in CX_3CR1 expression on microglia, neurons increase the release of soluble fractalkine. In support of this notion, inflammatory stimuli cause the release of soluble fractalkine from neurons in vitro, but have no affect on fractalkine mRNA or total protein levels (Chapman, 2000). Moreover, increased levels of soluble fractalkine are detected in the CSF of patients with HIV-associated dementia (Erichson, 2003), which may be a sign of impaired microglia regulation. Taken together, these findings indicate that fractalkine modulates microglia activation and that impaired fractalkine/CX_3CR1 interactions may contribute to microglial dysregulation and corresponding neurobehavioral complications.

1.4 Microglial reactivity in aging.

Microglia have a very low turnover rate in the CNS, making them particularly sensitive to the effects of aging. Thus, aging may provide a brain environment where microglia become "primed" or "reactive" to peripheral immune challenge (Perry, 2003; Godbout, 2006). In support

of this notion, microglial expression of major histocompatibility complex (MHC) class II, a marker of microglia priming, is increased in brains of healthy aged humans, primates, and rodents. Moreover, increased expression of scavenger receptors (Wong, 2005), integrins (Stichel, 2007), Toll-like-Receptors (Letiembre, 2007), and astrocytic markers (Lee, 2000; Morgan 1999) are also detected in the brain of older rodents. It is noteworthy that the number of resident astrocytes and microglia is not increased in the brain with age (Lee, 2000; Long, 1998). Therefore, glial reactivity appears to increase in existing astrocyte and microglia populations. It is important to note that primed microglia are morphologically similar to activated microglia, but do not produce appreciable levels of inflammatory cytokines in this state (Perry, 2002).

In a BALB/c murine model of aging, the Godbout Lab has reported that a consequence of a reactive glial profile is an exaggerated neuroinflammatory cytokine response to either central (Abraham, 2008) or peripheral (Chen, 2008) innate immune challenges. For instance, primary mixed glia cultures and coronal brain sections established from the brain of aged rodents are hyper-responsive to LPS stimulation and produce more inflammatory cytokines (IL-1β & IL-6) compared to cultures established from adult brains (Xie, 2003; Yi, 2001). The peripheral stimulation of the innate immune system with LPS causes an exaggerated neuroinflammatory response and prolongs sickness behavior in aged (22-24 m) BALB/c mice. In this model, older mice show amplified and protracted IL-1β and IL-6 in the brain following LPS injection, which is paralleled by a protracted sickness response. The direct stimulation of microglia by intracerebroventricular (i.c.v.) administration of LPS causes amplified cytokine expression (IL-1β, IL-6, & TNF-alpha) and prolonged sickness behavior in aged (20-22 m) mice. Furthermore, our recent findings indicate that microglia of aged mice are primed (MHC II^{+}) and become hyperactive with excessive cytokine and IDO induction following peripheral LPS challenge. In

several rodent models of aging, the excessive neuroinflammatory cytokine response is coupled with a myriad of complications including cognitive impairment (Chen, 2008), exaggerated sickness behavior (Abraham, 2008), and protracted depressive-like behavior (Godbout, 2008). For example, the exaggerated inflammatory cytokine response in the brain of aged mice given LPS promotes depressive-like behavior that is exhibited even after the acute effects of LPS are resolved (Godbout, 2008). Collectively these findings indicate that exaggerated microglia responses in the aged brain lead to long-lasting neurobehavioral deficits.

1.4 The role of TGFβ in regulating CX_3CR1

Transforming growth factor beta (TGFβ) is a highly conserved protein which has a myriad of functions including the control cell growth and cell differentiation. It is a member of the TGFβ signaling family which includes activins, bone morphogenic protein, and nodal. It is known to be expressed in the midbrain (Falk, 2008). TGFβ is an anti-inflammatory cytokines that reduces activation of several inflammatory cells including macrophages and microglia**.** In the brain, TGFB is primarily produced by astrocytes. Recent findings have shown that CX_3CR1 expression is upregulated by (TGFβ) (Chen et al., 2002).

One of the primary mechanisms involved in intracellular TGFβ signaling involves the phosphorylation of the SMA and MAD related proteins (SMADs). This phosphorylation has been shown to be inhibited in epithelial cells by the novel ALK-5 inhibitor A-83-01 (Tojo et al., 2005). Another part of the TGFβ pathway utilizes the intracellular signaling of Phosphoinositide 3-kinase (PI3K). PI3K activity has been shown to be inhibited with the treatment of LY-294002 in rat hepatocytes.

2. Materials and Methods

2.1 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 μg/ml streptomycin, 0.25 μg/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 conditioned media experiments, cells were plated in 10 cm wells at a concentration of $5x10^6$ cells per well and allowed to adhere 20 hours. 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).

2.2 Experimental Protocols

In the first study, BV2 cells were treated with either saline or various levels of *Escherichia coli* LPS (serotype 0127:B8, Sigma, St. Louis, MO). Cellular supernatant was then extracted and centrifuged for 5 min at 3000g. Supernatant IL-6 and IL-1b protein levels were then determined by ELISA (R&D Systems, MN) (n=3). Absorbance (450 nm) was determined using a Bio-Tek synergy HT microplate reader (Bio-Tek Instruments, VT).

In the second study, BV2 cells plated in 10 cm wells were treated with 100 ng/mL of LPS. Select wells were replaced with fresh media 1 h after treatment with LPS and along with the rest of the cells were cultured for the rest of the 20 h at 37°C with 95% humidity and 5%

CO_2. The supernatant was then transferred to a new tube in order to ensure that there were no floating cells present. This supernatant was stored at -80°C until needed. Recipient cells plated in 1 cm wells were then treated with various dilutions of the warmed conditioned media in fresh media. The BV2 cells were then collected 4h later for RNA isolation/quantitative PCR (CX3CR1 and IL-1b) (n=3).

In the third study, BV2 microglia were pre-treated with vehicle or recombinant human TGFβ (0.1 ng/ml; R&D Systems, MN) for 30 minutes and then treated with saline or LPS (100 ng/ml). BV2 microglia were collect 4 h later and used for total RNA isolation/quantitative PCR (IL-1β and CX3CR1) or immunocytochemistry for CX3CR1 surface expression (n=12, three independent experiments).

In the fourth study, select wells were treated with either A-83-01 (Sigma, MO) (0.1-10 mM) or LY-294002 (Tocris, MO) (.1 or 25 uM) , or DMSO for 30 minutes. The cells were then treated with TGFβ (1 ng/mL) or saline and cultured for 4 h. The BV2 cells were then collected and used for total RNA isolation/quantitative PCR (IL-6 and CX3CR1).

In the final study, adult Balb/c mice (3 mo) were injected intracerebroventricularly (i.c.v.) with recombinant TGFβ. Mice were then injected intraperitoneally (i.p.) with 0.33 mg/kg LPS one hour after TGFβ injection. Mice were sacrificed at 4 or 24 h after LPS injection and microglia were isolated from the whole brain using a percoll density gradient. Total RNA was extracted from isolated microglia and qPCR was performed to determine expression of CX3CR1.

2.3 RNA isolation and qPCR

Total RNA was isolated from primary isolated microglia or BV2 microglia using the Tri-Reagent protocol (Sigma, MO) and subjected to the DNA-free™ RNA clean up procedure (Ambion, TX). 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® 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-1β, CX_3CL1, CX3CR1, TGFβ1, 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.

2.4 Immunocytochemistry on cultured BV2 microglia

BV2 microglia were plated in 24-well plates on poly-L-lysine coated glass cover slips 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 cover slips 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).

2.5 Cannulation surgeries and intracerebroventricular injections of TGFβ

The i.c.v. cannulation was performed as previously described with a few modifications (Godbout et al., 2005a). In brief, mice were deeply anesthetized using ketamine and xylazine (100 mg and 10 mg/kg BW i.p., respectively) and the surgical site was shaved and sterilized. Mice were positioned in a stereotaxic instrument so that the plane formed by the frontal and parietal bones was parallel to the table top. An incision, 1.5 cm in length, was made on the cranium to reveal the bregma and a 26-guage stainless-steel guide cannula was placed in the lateral cerebral ventricle using the following stereotaxic coordinates: Lat 1.6 mm; and A-P 1mm to the bregma; and Hor −2mm from the dura mater. Two anchoring cranial screws were inserted adjacent to the cannula and the cannula was secured with cranioplastic cement. A dummy cannula was inserted in the guide cannula to prevent occlusion and infection. Mice were injected subcutaneously with Buprinex (111 g/kg BW) following surgery and then again 12 h later. Mice were provided a minimum of 7 d to recover before any treatment was administered. Accurate cannula placement was confirmed by injecting trypan blue dye and examining the dye diffusion throughout the ventricles.

For i.c.v. injection into the lateral ventricle, the indwelling cannula was connected with sterile tubing to a Hamilton syringe and injections were administered using a KS Scientific

precision syringe pump. TGFβ was injected i.c.v. into the lateral ventricle at a dose of 100ng in 2 μl at a rate of 1 μl/minute.

2.6 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 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 (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. The Godbout Lab has 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".

3. Results

3.1 Treatment of BV2 cells with LPS causes an increase in pro-inflammatory signaling and a subsequent decrease in CX_3CR1 levels.

The treatment of primary microglia cells results in a decrease in CX_3CR1. Therefore, an aim was to determine the effect that LPS had on BV2 microglia cell lines. Since LPS injection is known to cause a pro-inflammatory response in mice microglia, IL-6 and IL-1b protein levels present in the cell supernatant were analyzed 4 h and 24 h after treatment with LPS (Figure 1). The 4 h time point was chosen because this is the time point when microglia actively produce cytokines (Henry et al., 2009) and the 24 h time point was chosen as it is the time point in which aged mice show extended behavior symptoms of sickness (Godbout et al., 2005b). Both IL-1b and IL-6 protein production was increased after 4 h and 24 h in an LPS dose dependant manner. Thus, the BV2 cell line used in the present study has a similar inflammatory profile to microglia cells en vivo.

The next aim was to determine the effect that LPS had on the expression of CX_3CR1 in BV2 cells (Figure 2C). After treatment with LPS for 4 h, there was a significant decrease in CX_3CR1 mRNA levels. Treatment with LPS caused a significant increase in IL-1b mRNA levels after 4 h (Figure 2D). This increase in IL-1b mRNA levels correlates well with the amount of IL-1b protein found in the supernatant 4 h post LPS treatment. In order to determine whether or not the decrease in CX_3CR1 levels is caused by prolonged exposure to LPS, a set of experiments were designed which were aimed at limiting the amount of LPS exposure to BV2 cells while not limiting their exposure to any cellular products potentially produced as a response to LPS exposure (Figure 3). In this experiment, the wash out (W/O) conditioned media (CM) is used as a model for limited LPS exposure.

In figure 3A, there is an exaggerated increase in CX_3CR1 at low concentrations of wash out conditioned media compared to the normal conditioned media. This suggests that there may be a factor which increases CX_3CR1 levels in BV2 cells with limited LPS exposure and that this factor is present in a higher concentration within the first hour after LPS exposure. Figure 3B shows us that the increase in IL-1b mRNA levels can be attenuated by limited LPS exposure, as can be expected.

3.2 TGFβ can attenuate the pro-inflammatory response in LPS treated BV2 cells as well as exaggerate the expression of CX_3CR1.

As previously found, the treatment of BV2 cells with LPS caused a decrease in CX_3CR1 mRNA levels. However, this decrease in CX_3CR1 mRNA levels has been shown to be attenuated in primary microglia pre-treated with TGFβ. Therefore, this study aimed to determine the effect that TGFβ had on both pro-inflammatory cytokine mRNA levels as well as CX_3CR1 levels. Figures 2A and 2B shows that BV2 cells treated with TGFβ exhibit a simultaneous increase in CX_3CR1 and decrease in IL-1b mRNA levels. The next aim was to determine the effect that TGFβ had on the cellular response to LPS. Figures 2C and 2D show that the decrease in CX_3CR1 mRNA levels caused by LPS was attenuated by TGFβ treatment. In fact, CX_3CR1 mRNA levels increased in cells treated with both TGFβ and LPS. Furthermore, the increase in IL-1b mRNA levels caused by LPS treatment was attenuated by prior treatment to TGFβ.

TGFβ is able to attenuate the pro-inflammatory response to LPS so the next goal was to evaluate the amount of CX_3CR1 surface expression after treatment with LPS and TGFβ. Fig.4E shows representative staining for CX_3CR1. In saline-treated BV2 cells, CX_3CR1 was detected as a distinct ring around the membrane of the cell (Fig.4A.i). In LPS-treated BV2 cells, there was

less CX_3CR1 on the membrane of the cells compared to saline controls (Fig.4A.iii&B; LPS). Moreover, TGFβ enhanced CX_3CR1 surface expression (Fig.4A.v&B; TGFβ) and post hoc analysis revealed that TGFβ attenuated the LPS-induced reduction of CX_3CR1 surface expression.

3.3 Treatment of BV2 cells with the novel inhibitors A-83-01 and LY 294002 does not attenuate the cellular response to TGFβ.

As exposure to TGFβ alone is sufficient to increase CX_3CR1 levels and to attenuate the pro-inflammatory response caused by LPS, this study sought to determine if inhibiting certain pathways involved in TGFβ signaling was capable of limiting the potency of TGFβ treatment. A-83-01 effectively decreases the amount of SMAD phosphorylation involved in TGFβ signaling. Therefore, BV2 cells were treated with either A-83-01 or DMSO prior to treatment with TGFβ or saline (Figures 5A and 5B). Unexpectedly, pre-treatment with A-83-01 caused an increase in both CX_3CR1 and IL-6 mRNA expression. The next aim was to inhibit PI3K activity in BV2 cells using LY 294002, a novel inhibitor shown to inhibit PI3K activity in hepatocytes (Figures 5C and 5D). However, pre-treatment with LY 294002 also seemed to cause an increase in CX_3CR1 mRNA expression (Figure 5C). Unlike the increase in IL-6 mRNA levels experienced with the A-83-01 pre-treatment of BV2 cells, LY 294002 seemed to cause a dose dependant decrease in IL-6 levels in absence of TGFβ

3.4 Intracerebroventricular injection of TGFβ causes an increase in CX_3CR1 mRNA levels.

Injection of TGFβ into the lateral ventricle resulted in an increase in CX_3CR1 mRNA

levels after both 4 h and 24 h. This corresponds to the increase of CX_3CR1 in BV2 microglia cells. Unfortunately, this study was not effective in determining the pro-inflammatory cytokine production as the vehicle contains hydrochloric acid which causes an increase in pro-inflammatory cytokine levels alone.

4. Discussion

In aged mice, there is prolonged microglial activation following acute immune challenge with LPS. As the main interpreter and propagator of cellular signaling between the CNS and the periphery, microglia play an important role in regulating the response to infection and other challenges to the immune system. Microglia in the aged brain exhibit a pro-inflammatory profile that may be responsible for the susceptibility to long term dysfunction and neuronal deficits in the elderly population. The pro-inflammatory characteristics of the aged brain are best portrayed by the increase in inflammatory cytokine production, most notably IL-1b and IL-6. The propagation of IL-1b and IL-6 signaling by activated microglia is known to stimulate behavioral changes, increase in body temperature, and produce changes in homeostasis. The regulation of microglia activation is controlled, in part, by the interaction between CX_3CR1 and CX_3CL1. This interaction inhibits the migration of proinflammatory cells (Pachot, 2008). TGFβ signaling is important in several areas of growth and development. TGFβ increases the amount of CX_3CR1 expression. Concurrently, TGFβ levels are lower in the aged brain than in the adult brain. Therefore, one can suspect that the deficiency of TGFβ in the aged brain may relate to lower CX_3CR1 levels and an overall decrease in the ability to regulate pro-inflammatory signaling.

Upon activation of primary microglia, there is a significant decrease in CX_3CR1. This reduction is also seen in BV2 cells (Supplemental Figure). Treatment with TGFβ alone was able to increase CX_3CR1 levels in both BV2 cells and microglia in vivo (Figures 2,4,6). Treatment with TGFβ was also able to attenuate the reduction of CX_3CR1 in BV2 cells after LPS exposure.

The proinflammatory response to LPS exposure in BV2 cells is relatable to that found in the brain. There is a clear increase in the protein levels of both IL-1b and IL-6 in cultures of BV2

cells treated with LPS. Furthermore, this increase is experienced in the absence of prolonged LPS exposure. This suggests that there is an LPS initiated factor which is responsible for the downstream regulation of inflammatory signaling. In a previous study, the levels of CX_3CR1 in BV2 cells treated with LPS increases at lower doses and then decrease in a dose dependant manor after the amount of LPS reaches a certain level. The decrease in CX_3CR1 mRNA expression corresponds to the resulting increase in IL-6 levels. This seems to suggest that it is the decrease in CX_3CR1 mRNA levels that is leading to the increase in pro-inflammatory cytokine production. However, the cause of the immediate increase in CX_3CR1 mRNA levels is not entirely known. It can be speculated that this initial increase in CX_3CR1 levels is similar to the response found in the normal brain after acute immune challenge. After severe infection or prolonged exposure to immune challenge, CX_3CR1 function may, as a result, degenerate. This functional loss of CX_3CR1 at high levels of inflammation could be one of the reasons for the susceptibility found in the elderly population.

While treatment with TGFβ is sufficient in increasing CX_3CR1, it can not yet be said if it is directly responsible for the increase in CX_3CR1 after acute immune challenge. The TGFβ inhibitor studies tried thus far have been unable to attenuate the effect of TGFβ on CX_3CR1. This may be because the inhibitors used in this study did not target the appropriate pathway of TGFβ signaling involved in CX_3CR1 transcription. Alternatively, there may be another parallel pathway initiated simultaneously with TGFβ that is responsible for the increase in CX_3CR1 mRNA. It is likely that both of these alternatives are possible. Little is known yet about the pathway involved in Fractalkine signaling. However, as seen in this study and in previous studies, TGFβ has a role in brain regulation. Meaning, TGFβ up-regulation in the brain may lead to a repressed response to inflammation in the aged brain which may also lead to a decrease in

the neurological deficits and disorder found in the elderly population.

Figures:

Figure 1.

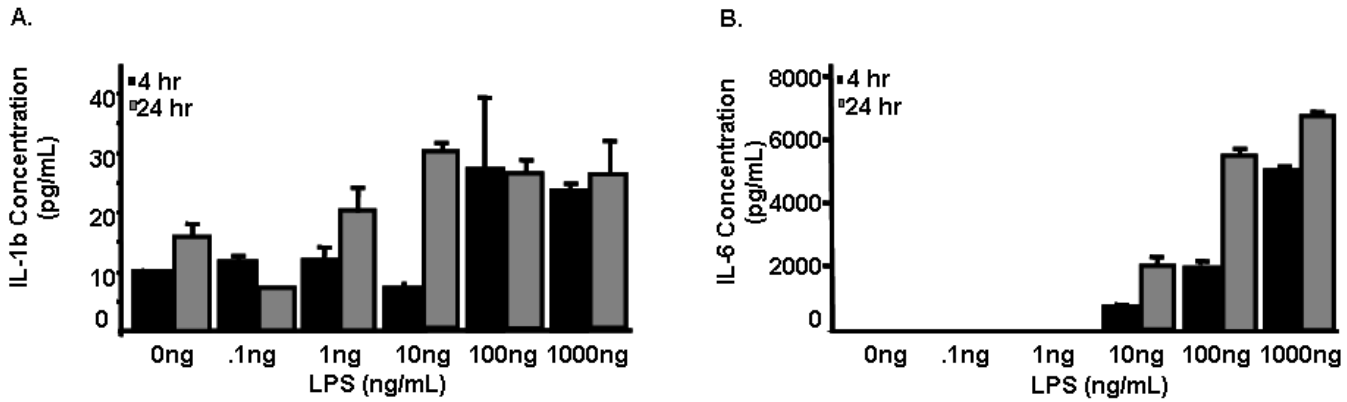


Figure 2.

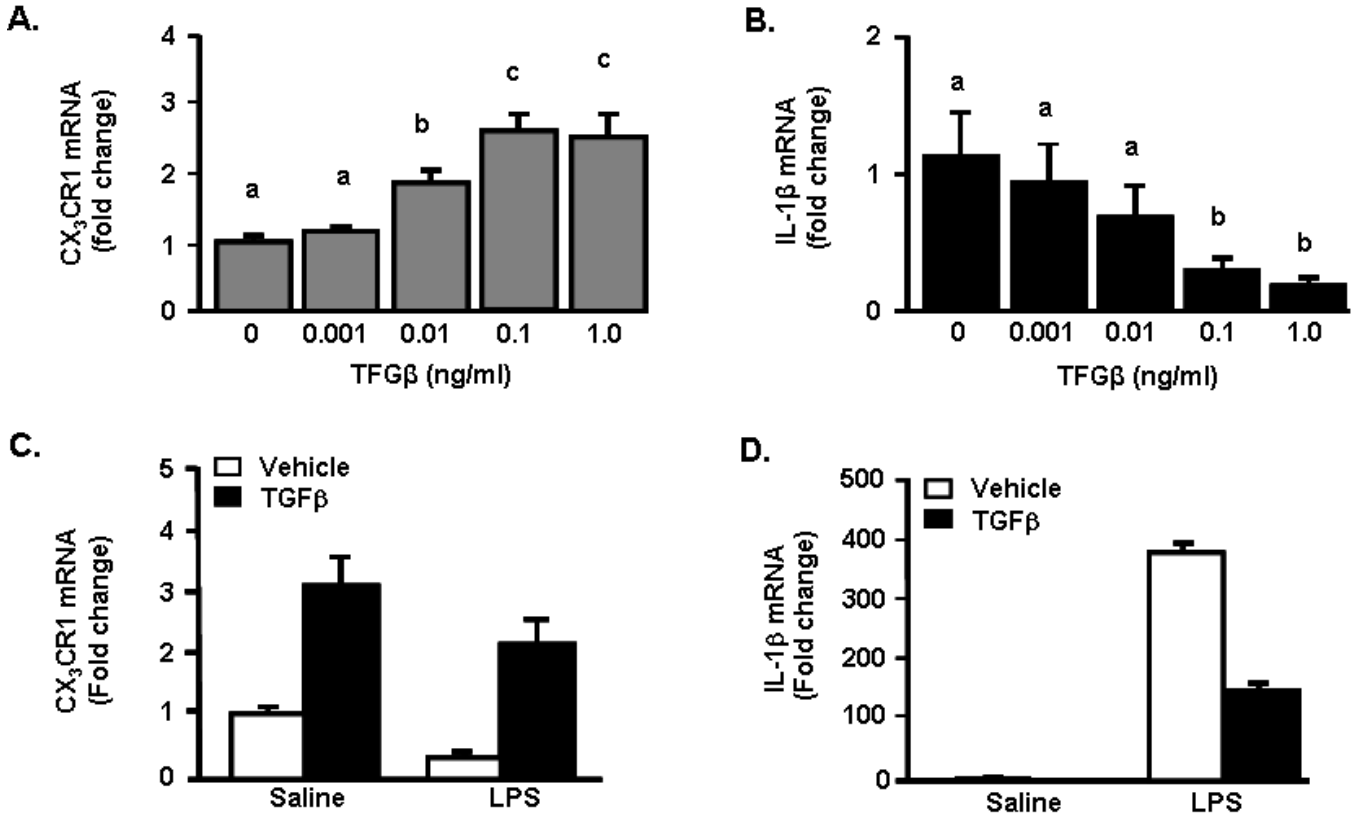

A.
CX3CR1 mRNA (fold change)
0
1
2
3
4
a
a
b
c
c
0
0.001
0.01
0.1
1.0
TFGβ (ng/ml)
B.
IL-1β mRNA (fold change)
0
1
2
a
a
a
b
b
0
0.001
0.01
0.1
1.0
TFGβ (ng/ml)
C.
CX3CR1 mRNA (Fold change)
0
1
2
3
4
5
Vehicle
TGFβ
Saline
LPS
D.
IL-1β mRNA (Fold change)
0
100
200
300
400
500
Vehicle
TGFβ
Saline
LPS

Figure 3.

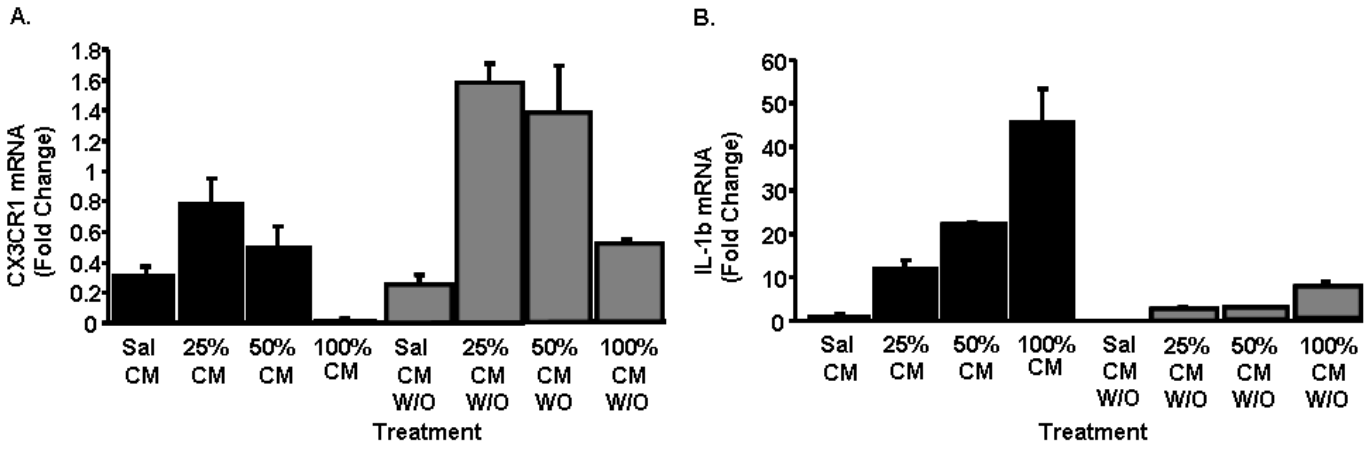
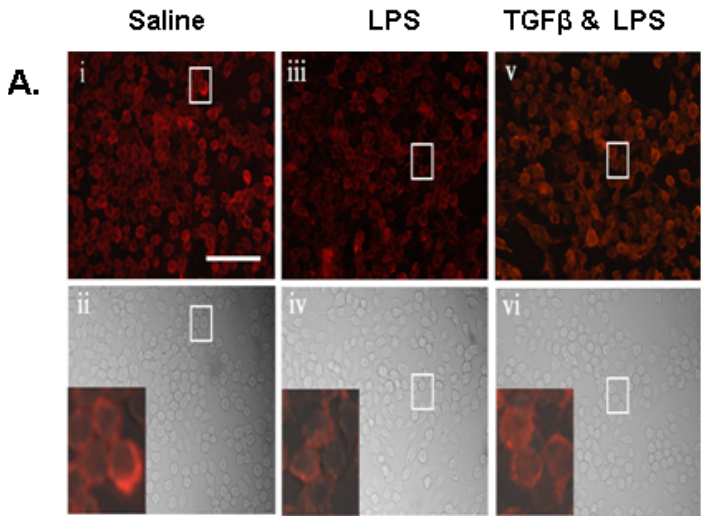

A.
CX3CR1 mRNA (Fold Change)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sal CM
25% CM
50% CM
100% CM
Sal CM W/O
25% CM W/O
50% CM WO
100% CM W/O
Treatment
B.
IL-1b mRNA (Fold Change)
60
50
40
30
20
10
0
Sal CM
25% CM
50% CM
100% CM
Sal CM W/O
25% CM W/O
50% CM W/O
100% CM W/O
Treatment

Figure 4.


A.
Saline
LPS
TGFβ & LPS
i
iii
v
ii
iv
vi

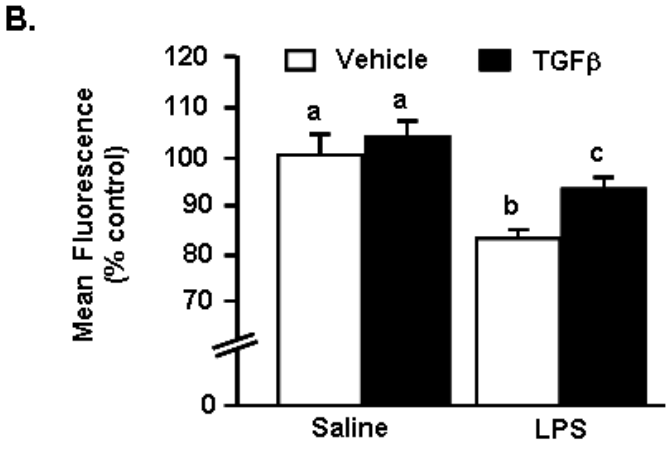

B.
Vehicle
TGFβ
Mean Fluorescence (% control)
120
110
100
90
80
70
0
a
a
b
c
Saline
LPS

Figure 5.

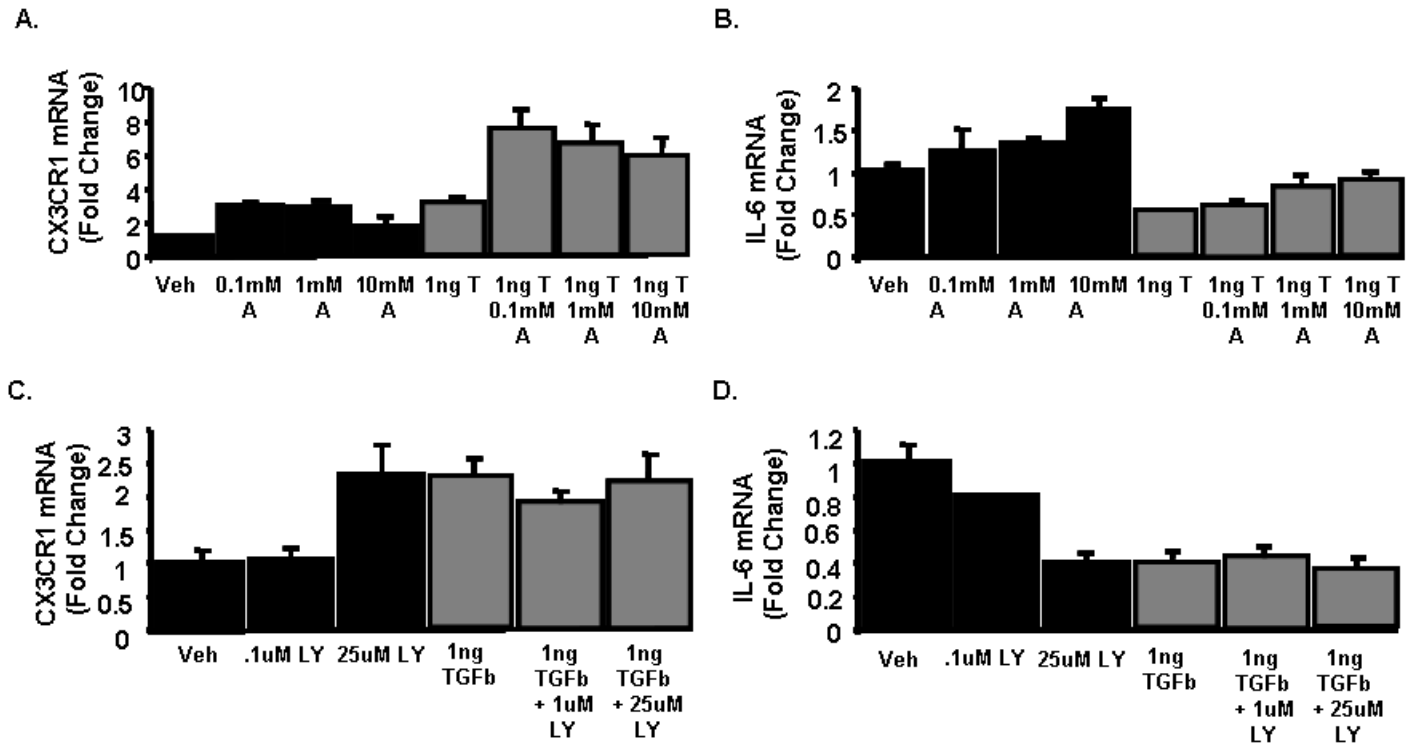
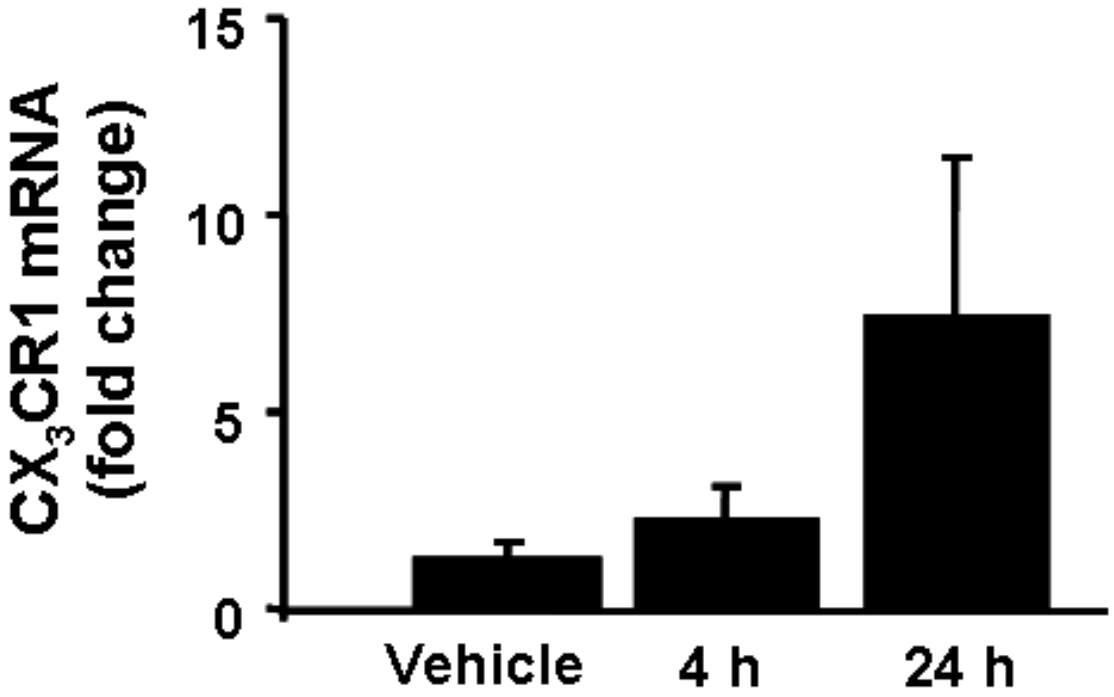
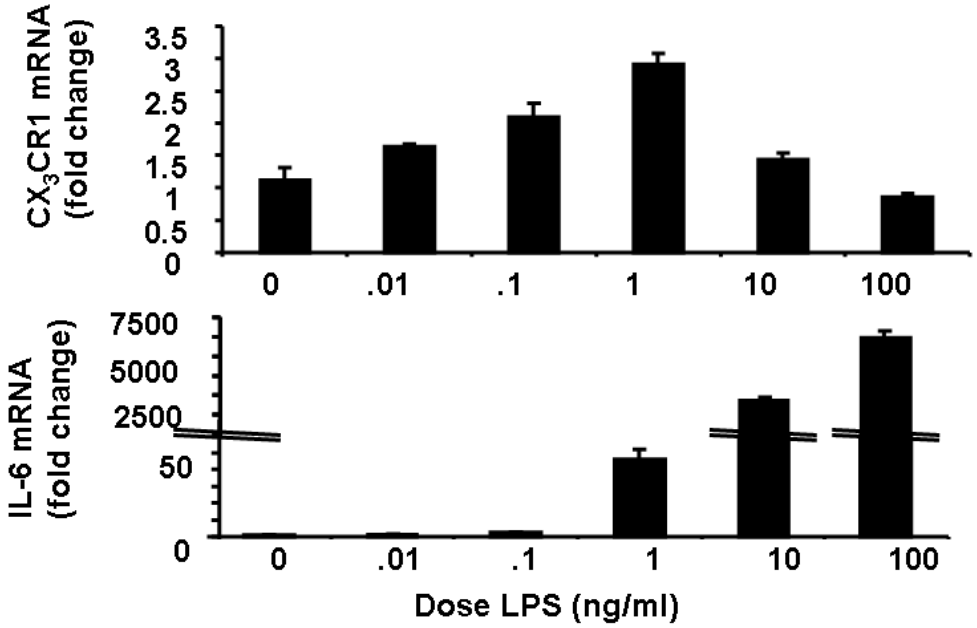

A.
CX3CR1 mRNA (Fold Change)
10
8
6
4
2
0
Veh
0.1mM A
1mM A
10mM A
1ng T
1ng T 0.1mM A
1ng T 1mM A
1ng T 10mM A
B.
IL-6 mRNA (Fold Change)
2
1.5
1
0.5
0
Veh
0.1mM A
1mM A
10mM A
1ng T
1ng T 0.1mM A
1ng T 1mM A
1ng T 10mM A
C.
CX3CR1 mRNA (Fold Change)
3
2.5
2
1.5
1
0.5
0
Veh
.1uM LY
25uM LY
1ng TGFb
1ng TGFb + 1uM LY
1ng TGFb + 25uM LY
D.
IL-6 mRNA (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
Veh
.1uM LY
25uM LY
1ng TGFb
1ng TGFb + 1uM LY
1ng TGFb + 25uM LY

Figure 6.



Supplemental Figure



BV2 cells were treated with indicated doses of LPS. Cells were extracted at 4hr after LPS treatment and total RNA was isolated. mRNA expression of A) CX3CR1 and B) IL-6 were determined. Bars represent the mean ±SEM.

Source: Angela Wynne

Figure Legends:

Figure 1. Presence of LPS in growth Media of BV2 cells causes an increase in the amount of pro-inflammatory cytokine production. BV2 cells were treated with LPS (0-1000ng) for 4 h and 24 h. A) IL-1b or B)IL-6 protein levels were determined from an ELISA performed on the cell supernatant. Bars represent SEM (n=3).

Figure 2. TGFβ enhanced CX3CR1 expression and attenuated IL-1b 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). In a related study, BV2 cells were incubated with vehicle or TGFβ (0.1 ng/ml) for 30 min before LPS (100ng/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).

Figure 3. BV2 cells treated with Conditioned Media from BV2 cells that had a limited exposure to LPS causes an increase in CX3CR1 mRNA levels and an attenuated increase in IL-1b mRNA levels compared to BV2 cells treated with conditioned media from BV2 cells with prolonged LPS exposure. Media was conditioned in 10 cm well plates and treated with either saline or LPS (100ng). Select conditioning wells (W/O) were washed out with PBS and replaced with fresh media 1 h after LPS treatment. Conditioned media was then diluted in fresh media (0-100%) and placed on BV2 cells. The cells were collected 4 h later in order to measure A) CX3CR1 mRNA levels or B) IL-1b mRNA levels. Bars represent SEM (n=3).

Figure 4. The effects of LPS on surface expression of BV2 microglia cells can be attenuated by pre-treatment with TGFβ. BV2 cells were treated for 30 minutes with TGFβ (0 or 1 ng) followed by treatment with LPS (100 ng) or saline. 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).

Figure 5. Treatment of BV2 microglia cells with the SMAD inhibitor A-83-01 and the PI3K inhibitor LY 294002 prior to TGFβ exposure does little to attenuate the effects of TGFβ. Cells were treated with A&B) A-83-01 (0.1-10 mM; "A"), C&D) LY 294002 (0.1-25 uM; "LY") , or DMSO (10 uL; "Veh") for 30 minutes. Cells were then treated with TGFβ (1 ng) or saline. CX3CR1 mRNA expression (Figures A&C) and IL-6 mRNA expression (Figures B&D) were determined 4 h later. Bars represent SEM (n=3).

Figure 6. I.C.V injection of TGFβ in Balb/C mice causes an increase in CX3CR1 expression. Balb/c mice were surgically implanted with an indwelling cannula in the left lateral ventricle. 100ng/2ul of TGFB was injected into the brain and mice were sacrificed 4 or 24 h after injection. Microglia were isolated and mRNA expression of CX3CR1 was determined. Bars represent the mean ±SEM.

References

Abraham, J., Jang, S., Godbout, J. P., Chen, J., Kelley, K. W., Dantzer, R., and Johnson, R. W. (2008) Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. *Neurobiol Aging* 29, 614-621

Banks, W. A. (1999) Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. *J Neurovirol* 5, 538-555

Barrientos RM, Frank MG, Hein AM, Higgins EA, Watkins LR, Rudy JW, Maier SF. 2009. Time course of hippocampal IL-1 beta and memory consolidation impairments in aging rats following peripheral infection. *Brain, behavior, and immunity* 23(1):46-54.

Barrientos RM, Higgins EA, Biedenkapp JC, Sprunger DB, Wright-Hardesty KJ, Watkins LR, Rudy JW, Maier SF. 2006. Peripheral infection and aging interact to impair hippocampal memory consolidation. *Neurobiol Aging* 27(5):723-732.

Bessis A, Bechade C, Bernard D, Roumier A. 2007. Microglial control of neuronal death and synaptic properties. *Glia* 55:233–238.

Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007) Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci* 30, 596-602

Blasko, I., Stampfer-Kountchev, M., Robatscher, P., Veerhuis, R., Eikelenboom, P., and Grubeck-Loebenstein, B. (2004) How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. *Aging Cell* 3, 169-176

Boehme, S. A., Lio, F. M., Maciejewski-Lenoir, D., Bacon, K. B., and Conlon, P. J. (2000) The chemokine fractalkine inhibits Fas-mediated cell death of brain microglia. *J Immunol* 165, 397-403

Capuron, L., Neurauter, G., Musselman, D. L., Lawson, D. H., Nemeroff, C. B., Fuchs, D., and Miller, A. H. (2003) Interferon-alpha-induced changes in tryptophan metabolism. relationship to depression and paroxetine treatment. *Biol Psychiatry* 54, 906-914

Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R, Lee JC, Cook DN, Jung S, Lira SA, Littman DR, Ransohoff RM. 2006. Control of microglial neurotoxicity by the Fractalkine receptor. *Nat Neurosci* 9(7):917-924.

Chapman, G. A., Moores, K., Harrison, D., Campbell, C. A., Stewart, B. R., and Strijbos, P. J. (2000) Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J Neurosci* 20, RC87

Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. 2008. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain, behavior, and immunity* 22(3):301-311.

Chen, S., Luo, D., Streit, W. J., and Harrison, J. K. (2002) TGF-beta1 upregulates CX3CR1 expression and inhibits fractalkine-stimulated signaling in rat microglia. *J Neuroimmunol* 133, 46-55

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 9(1):46–56.

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L., and Gan, W. B. (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* 8, 752-758

Erichsen, D., Lopez, A. L., Peng, H., Niemann, D., Williams, C., Bauer, M., Morgello, S., Cotter, R. L., Ryan, L. A., Ghorpade, A., Gendelman, H. E., and Zheng, J. (2003) Neuronal injury regulates fractalkine: relevance for HIV-1 associated dementia. *J Neuroimmunol* 138, 144-155

Falk, Sven. 2008. Brain Area-Specific Effect of TGF-β Signaling on Wnt-Dependent Neural Stem Cell Expansion Cell Stem Cell 8;2(5):472-83

Finck, B. N., and Johnson, R. W. (1997) Anorexia, weight loss and increased plasma interleukin-6 caused by chronic intracerebroventricular infusion of interleukin-1beta in the rat. *Brain Res* 761, 333-337

Frank, M. G., Barrientos, R. M., Biedenkapp, J. C., Rudy, J. W., Watkins, L. R., and Maier, S. F. (2006) mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiol Aging* 27, 717-722

Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW. 2005b. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. Faseb J 19(10):1329-1331.

Godbout, J. P., and Johnson, R. W. (2006) Age and neuroinflammation: a lifetime of psychoneuroimmune consequences. *Neurol Clin* 24, 521-538

Godbout J.P., Johnson R.W.: Interleukin-6 in the aging brain. *J Neuroimmunol* 147. (1 2): 141-144.2004

Godbout JP, Moreau M, Lestage J, Chen J, Sparkman NL, J OC, Castanon N, Kelley KW, Dantzer R, Johnson RW. 2008. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system.

Goehler, L. E., Gaykema, R. P., Hammack, S. E., Maier, S. F., and Watkins, L. R. (1998) Interleukin-1 induces c-Fos immunoreactivity in primary afferent neurons of the vagus nerve. *Brain Res* 804, 306-310

Hanisch, U. K., and Kettenmann, H. (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10, 1387-1394

Henry CJ, Huang Y, Wynne AM, Godbout JP. 2009. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. *Brain, behavior, and immunity* 23(3):309-317.

Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT, Sheridan JF, Godbout JP. 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. *Journal of neuroinflammation* 5:15.

Heyser, C. J., Masliah, E., Samimi, A., Campbell, I. L., and Gold, L. H. (1997) Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *Proc Natl Acad Sci U S A* 94, 1500-1505

Huang Y, Henry CJ, Dantzer R, Johnson RW, Godbout JP. 2008. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. *Neurobiol Aging* 29(11):1744-1753.

Kelley, K. W., Bluthe, R. M., Dantzer, R., Zhou, J. H., Shen, W. H., Johnson, R. W., and Broussard, S. R. (2003) Cytokine-induced sickness behavior. *Brain Behav Immun* 17, 112-118.

Konsman, J. P., Luheshi, G. N., Bluthe, R. M., and Dantzer, R. (2000) The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals; a functional anatomical analysis. *Eur J Neurosci* 12, 4434-4446

Laflamme, N., Lacroix, S., and Rivest, S. (1999) An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood-brain barrier in response to a systemic and localized inflammation but not during endotoxemia. *J Neurosci* 19, 10923-10930

Lacroix, S., Feinstein, D., and Rivest, S. (1998) The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. *Brain Pathol* 8, 625-640

Lee, C. K., Weindruch, R., and Prolla, T. A. (2000) Gene-expression profile of the ageing brain in mice. *Nat Genet* 25, 294-297

Letiembre, M., Hao, W., Liu, Y., Walter, S., Mihaljevic, I., Rivest, S., Hartmann, T., and Fassbender, K. (2007) Innate immune receptor expression in normal brain aging. *Neuroscience* 146, 248-254

Limatola, C., Lauro, C., Catalano, M., Ciotti, M. T., Bertollini, C., Di Angelantonio, S., Ragozzino,D., and Eusebi, F. (2005) Chemokine CX3CL1 protects rat hippocampal neurons against glutamate-mediated excitotoxicity. *J Neuroimmunol* 166, 19-28

Long, J. M., Kalehua, A. N., Muth, N. J., Calhoun, M. E., Jucker, M., Hengemihle, J. M., Ingram, D. K., and Mouton, P. R. (1998) Stereological analysis of astrocyte and microglia in aging mouse hippocampus. *Neurobiol Aging* 19, 497-503

Meucci, O., Fatatis, A., Simen, A. A., Bushell, T. J., Gray, P. W., and Miller, R. J. (1998) Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Natl Acad Sci U S A* 95, 14500-14505

Mizuno, T., Kawanokuchi, J., Numata, K., and Suzumura, A. (2003) Production and neuroprotective functions of fractalkine in the central nervous system. *Brain Res* 979, 65-70

Morgan, T. E., Xie, Z., Goldsmith, S., Yoshida, T., Lanzrein, A. S., Stone, D., Rozovsky, I., Perry, G., Smith, M. A., and Finch, C. E. (1999) The mosaic of brain glial hyperactivity during normal ageing and its attenuation by food restriction. *Neuroscience* 89, 687-699.

Mouton, P. R., Long, J. M., Lei, D. L., Howard, V., Jucker, M., Calhoun, M. E., and Ingram, D. K. (2002) Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Res* 956, 30-35

Musselman, D. L., Lawson, D. H., Gumnick, J. F., Manatunga, A. K., Penna, S., Goodkin, R. S., Greiner, K., Nemeroff, C. B., and Miller, A. H. (2001) Paroxetine for the prevention of depression induced by high-dose interferon alfa. *N Engl J Med* 344, 961-966

Nicolle, M. M., Gonzalez, J., Sugaya, K., Baskerville, K. A., Bryan, D., Lund, K., Gallagher, M., and McKinney, M. (2001) Signatures of hippocampal oxidative stress in aged spatial learning- impaired rodents. *Neuroscience* 107, 415-431

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314-1318

Nguyen MD, Julien JP, Rivest S. 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat Rev Neurosci 3(3):216-227.

Ogura, K., Ogawa, M., and Yoshida, M. (1994) Effects of ageing on microglia in the normal rat brain: immunohistochemical observations. *Neuroreport* 5, 1224-1226

Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC, Gearing D. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* 387(6633):611-617.

Pachot et al. 2008. Decreased Expression of the Fractalkine Receptor CX3CR1 on Circulating Monocytes as New Feature of Sepsis-Induced Immunosuppression. *The Journal of Immunology* 180 :6421 -6429.

Perry, V. H., Cunningham, C., and Boche, D. (2002) Atypical inflammation in the central nervous system in prion disease. *Curr Opin Neurol* 15, 349-354

Perry, V. H., Matyszak, M. K., and Fearn, S. (1993) Altered antigen expression of microglia in the aged rodent CNS. *Glia* 7, 60-67

Perry, V. H., Newman, T. A., and Cunningham, C. (2003) The impact of systemic infection on the progression of neurodegenerative disease. *Nat Rev Neurosci* 4, 103-112.

Pollmacher, T., Haack, M., Schuld, A., Reichenberg, A., and Yirmiya, R. (2002) Low levels of circulating inflammatory cytokines--do they affect human brain functions? *Brain Behav Immun* 16, 525-532

Ragozzino, D., Di Angelantonio, S., Trettel, F., Bertollini, C., Maggi, L., Gross, C., Charo, I. F., Limatola, C., and Eusebi, F. (2006) Chemokine fractalkine/CX3CL1 negatively modulates active glutamatergic synapses in rat hippocampal neurons. *J Neurosci* 26, 10488-10498

Rivest S.: Molecular insights on the cerebral innate immune system. *Brain Behav Immun* 17. (1): 13-19.2003; Abstract

Sheffield, L. G., and Berman, N. E. (1998) Microglial expression of MHC class II increases in normal aging of nonhuman primates. *Neurobiol Aging* 19, 47-55

Shoba LN, Newman M, Liu W, Lowe WL Jr. 2001. LY 294002, an inhibitor of phosphatidylinositol 3-kinase, inhibits GH-mediated expression of the IGF-I gene in rat hepatocytes. *Endocrinology* 142(9):3980-6.

Sloane, J. A., Hollander, W., Moss, M. B., Rosene, D. L., and Abraham, C. R. (1999) Increased microglial activation and protein nitration in white matter of the aging monkey. *Neurobiol Aging* 20, 395-405

Stichel, C. C., and Luebbert, H. (2007) Inflammatory processes in the aging mouse brain: participation of dendritic cells and T-cells. *Neurobiol Aging* 28, 1507-1521

Streit, W. J., and Sparks, D. L. (1997) Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. *J Mol Med* 75, 130-138

Streit W, Xue QS. 2009. Life and Death of Microglia. J Neuroimmune Pharmacol 4:371–379.

Tansey M, Goldberg M. 2010. Neuroinflammation in Parkinson's disease: Its role in neuronal death and implications for therapeutic intervention. Neurobiology of disease 37(10)510-518.

Tojo M, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, Node M, Imamura T. 2005. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Science* 96(11): 791-800.

Wilson C.J., Finch C.E., Cohen H.J.: Cytokines and cognition—the case for a head-to toe inflammatory paradigm. *J Am Geriatr Soc* 50(12): 2041-2056.2002;

Abstract.
Wong, A. M., Patel, N. V., Patel, N. K., Wei, M., Morgan, T. E., de Beer, M. C., de Villiers, W. J., and Finch, C. E. (2005) Macrosialin increases during normal brain aging are attenuated by caloric restriction. *Neurosci Lett* 390, 76-80
Wynne AM, Henry CJ, Godbout JP. 2009. Immune and behavioral consequences of microglial reactivity in the aged brain. *Integrative and Comparative Biology* 49(3):254-266.
Xie, Z., Morgan, T. E., Rozovsky, I., and Finch, C. E. (2003) Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. *Exp Neurol* 182, 135-141
Ye, S. M., and Johnson, R. W. (2001) An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. *Neuroimmunomodulation* 9, 183-192
Zujovic, V., Benavides, J., Vige, X., Carter, C., and Taupin, V. (2000) Fractalkine modulates TNF-alpha secretion and neurotoxicity induced by microglial activation. *Glia* 29, 305-315
Zujovic, V., Schussler, N., Jourdain, D., Duverger, D., and Taupin, V. (2001) In vivo neutralization of endogenous brain fractalkine increases hippocampal TNFalpha and 8-isoprostane production induced by intracerebroventricular injection of LPS. *J Neuroimmunol* 115, 135-143