Microglia depletion by administration of a colony-stimulating factor 1 receptor antagonist exacerbates inflammation-induced fatigue in mice

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Abstract
Background: Production of inflammatory mediators by activated microglial cells in the central nervous system is commonly considered to be responsible for the development of symptoms of depression including fatigue and lack of energy (anergia) in response to systemic inflammation.
Methods: In order to directly test the validity of this hypothesis, male C57BL/6 mice were treated orally with PLX5622, a specific antagonist of colony stimulating factor receptor to eliminate microglia before administration of a fatigue-inducing dose of lipopolysaccharide (LPS) according to a 2 (PLX5622 versus control diet) x 2 (LPS x control) factorial design. Fatigue was measured by decreased wheel running activity.
Results: Chronic administration of PLX5622 eliminated microglia and peripheral tissue macrophages. However, it did not abrogate the inducible expression of proinflammatory cytokines in the brain in response to LPS. Instead PLX5622 increased IL-6 and abrogated IL-10 response to LPS. PLX5622 had only moderate effects on the liver inflammatory response to LPS. In accordance with these neuroimmune effects, PLX5622-treated mice responded to LPS with the same level of decreased locomotor activity in a new environment as mice given the control diet. Further, PLX5622-treated mice displayed a prolonged decrease in running wheel activity. In addition, PLX5622-treated mice were less active in running wheels than mice given the control diet even in baseline conditions.
Conclusions: These findings reveal a role for microglia in the regulation of immune-to-brain signaling that is different from the one usually ascribed to it and call for a re-evaluation of the importance of microglia in the brain response to physical exercise.
Introduction
Highly convergent clinical and preclinical findings point to an involvement of the innate immune system in major depressive disorder(1). In the general population, depression is associated with elevated levels of biomarkers of inflammation(2–5). Longitudinal studies of patients treated with repeated doses of interferon-alpha for chemotherapy-resistant cancer or hepatitis virus C infection reveal that the affective-cognitive symptoms of depression emerge gradually from inflammation-induced symptoms of sickness characterized by fatigue, reduced appetite and sleep disorders(6, 7).
This has been confirmed in murine models of acute and chronic inflammation-induced depression-like behavior (8, 9) as well as in human studies of endotoxin- or typhoid vaccine-induced changes in mood (10–12). In addition, brain imaging studies show that the cortical and subcortical brain structures that are sensitive to inflammation are strikingly similar to those brain areas that are implicated in the mood, motivation, and cognitive deficits of patients with major depressive disorder (13, 14). Taken all together, these findings point to the possibility that inflammation plays a role in at least some subtypes of depression (15).

The exact subtypes of depression in which inflammation could play a role are not yet fully elucidated. Epidemiological studies carried out in the general population reveal that inflammation is more likely to be associated with somatic/neurovegetative symptoms of depression than with cognitive and affective symptoms (16–20). The most prominent symptoms associated with inflammation are represented by sleep disturbances, fatigue and loss of energy (anergia), and reduced motivation. The observation that the relationship between inflammation and depression is mainly driven by somatic symptoms has been confirmed in psychiatric populations (20) as well as in individuals at risk for depression because of bereavement (21) or HIV infection (22).

Experimental studies in animal models of inflammation confirm that activation of the innate immune system reduces activity, propensity to exercise and motivation in effort tasks (23). The mechanisms for these effects involve propagation of inflammation from the periphery to the brain via multiple pathways including afferent nerves, circulating immune mediators interacting with endothelial cells and macrophages in parts of the brain devoid of a fully functional blood-brain barrier, active transport of immune-derived molecules via the blood-brain barrier and, in some cases, trafficking of peripheral immune cells into the brain (9, 24–26). This results in the activation of brain microglia and the local production of inflammatory cytokines which, by acting directly or indirectly on neurons, modify brain functions.

The key role of brain microglia in the development of inflammation-induced behavioral alterations has been demonstrated by various approaches mainly aiming at counteracting the production and action of inflammatory cytokines (27) or at down-regulating microglia activation using minocycline (28, 29).
More recently, more targeted approaches have been proposed to eliminate microglia using genetic or pharmacological tools (30). Based on the observation that the development and survival of microglia critically depends on colony stimulating factor-1 receptor (CSF-1R) signaling (31), CSF-1R antagonists have been successfully developed and are now commonly used to eliminate microglia. Continuous administration of these molecules to mice via their food results in a gradual depletion of Iba-1 and CD68 positive microglia in the brain within a few days of treatment, which persists until cessation of treatment and is then followed by repopulation (30). The objective of the present study was to determine whether microglia activation plays a critical role in the fatigue and lack of energy induced by systemic administration of lipopolysaccharide (LPS) to mice. For this purpose, we used the brain penetrant CSF-1R antagonist PLX-5622 (32, 33). Despite the fact that chronic administration of PLX5622 eliminated tissue macrophages and microglia, mice treated with this compound still displayed depressed locomotor activity in response to LPS but engaged in less wheel running activity and took longer to recover from LPS-induced decrease in wheel running activity.

Animals And Methods

Mice

Male C57BL/6J mice (Jackson Labs) started being fed the control or PLX5622 diet at 10 weeks of age. They were maintained in the animal facility at 24°C and 50% humidity with a 12-h light:dark cycle and food and water ad libitum.

Treatments

PLX5622 was provided by Plexxikon Inc. (Berkeley, CA). It was formulated in standard AIN-76A rodent chow at a concentration of 1,200 mg/kg (Research Diets, New Brunswick, NJ) and provided ad libitum. Control mice were given standard AIN-76A rodent chow. LPS (serotype O127:B8; Sigma-Aldrich, St-Louis, MO) was prepared in a solution of phosphate-buffered saline (PBS) at a concentration of 50 mg/ml and injected intraperitoneally at the dose of 0.5 mg/kg. Control mice received an equivalent volume of PBS.

Behavioral testing

Mice were single housed with wireless low-profile running wheels (Med Associates, Fairfax, VT) to
measure voluntary wheel running, which was quantified as total number of rotations during the night. Running wheels were provided to mice for 10-12 days prior to the initial LPS or PBS treatment. Locomotor activity in a new environment was measured for 5 min after mice were individually placed in an empty rectangular arena (18.4 x 29.2 cm). Activity was recorded by video camera and distance traveled was quantified using Noldus Ethovision XT Software (Noldus Information Technology, Leesberg, VA).

**Experimental design**

The experiment was organized according to a 2 (PLX5622 diet vs. control diet) x 2 (LPS vs. PBS) factorial design with 6 mice per group. The PLX5622 diet or the control diet was administered during the entire duration of the experiment. Mice were group housed with their assigned experimental diet for 12 days before they were single housed and provided with running wheels for the rest of the experiment. LPS or PBS was administered 1 month after the start of experimental diets. Locomotor activity in a new cage was measured 3 h after LPS or PBS treatment and voluntary wheel running was assessed for 5 days after.

Just over a week later, mice were submitted to a cross-over treatment so that mice that had received PBS were given LPS and vice versa. They were euthanized for tissue collection 6 h later to assess the effects of PLX5622 on the inflammatory response to LPS.

**Tissue processing**

Mice were euthanized by exposure to CO\textsubscript{2}. Livers and brains were collected after intracardiac perfusion of PBS, snap frozen in liquid nitrogen, and stored at –80°C until analyzed. RNA was extracted using E.Z.N.A. Total RNA Isolation kit (Omega Bio-Tek, Norcross, GA). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) and analyzed by real-time PCR using TaqMan Gene expression assays. GADPH was used as a house keeping gene. Primers are listed in Table 1.

**Results**

**PLX5622 eliminates microglia in the brain but does not attenuate the brain inflammatory response to LPS**
The extent of microglia depletion was quantified by the expression of CX3CR1 and CD11b mRNA. In accordance with previous reports, PLX5622 abrogated the expression of these microglial markers in the brain (Fig. 1A, Table 2). Peripheral macrophages were also depleted by PLX5622 in the liver, as measured by the gene expression of CSF-1R and Cd11b (Fig. 1B, Table 2).

As expected, LPS increased the gene expression of IL-1b, TNF, IL-6 and the type I interferon responsive gene Oas1a in the brain and liver (Fig. 1C & D). LPS also increased the gene expression of IL-10 in the brain and liver. PLX5622 did not alter the brain inflammatory response to LPS with the exception of IL-6 mRNA which was more highly expressed in the brains of PLX5622-treated mice compared to the brains of control mice in response to LPS, and IL-10 mRNA which was no longer increased by LPS in the brains of PLX5622 mice (Fig. 1C). In the liver, PLX5622 attenuated the TNF, IL-10 and Oas1a response to LPS but had no significant effect on the response of other cytokines to LPS (Fig. 1D).

**PLX5622 does not block the fatigue inducing effects of LPS**

Statistics on the effects of PLX5622 and LPS on body weight and behavior are summarized in Table 3. LPS administration induced body weight loss (24 h vs. baseline, p<0.01) and decreased locomotor activity in a new environment 3 h after treatment (Fig. 2A & B). These effects were not modified by PLX5622. During the week preceding LPS treatment, mice fed the diet supplemented with PLX5622 ran on average 20% less than mice fed the control diet (p=0.001) and responded to LPS with a prolonged suppression of voluntary wheel running that lasted 3 days (PLX5622 x LPS interaction: p<0.05 for day 2 & 3) instead of only 1 day for the mice receiving the control diet (LPS effect on day 1: p<0.001) (Fig. 2C).

**Discussion**

The present results show that despite its ability to deplete microglia and peripheral macrophages chronic oral administration of PLX5622 had very limited effects on the peripheral and central inflammatory response to LPS. Therefore, it is not surprising that this treatment was unable to abrogate the decrease in locomotor activity and voluntary wheel running that developed in response to LPS. These findings indicate that the fatigue inducing effects of systemic inflammation are not
primarily mediated by microglial activation in PLX5622-treated mice.

As already reported in previous studies on CSF-1 receptor antagonism(30, 31, 33), administration of the CSF-1 receptor antagonist PLX5622 for 4 weeks resulted in the near complete elimination of microglia in the central nervous system and a significant depletion of macrophages in the spleen and liver. At the periphery, PLX5622 had only moderate effects on the LPS-induced increase in gene expression of proinflammatory cytokines in the spleen and liver. This is probably due to the fact that CSF-1R antagonism specifically depletes tissue macrophages but does not affect pro-inflammatory monocytes recruited from the bone marrow, dendritic cells, or neutrophils which can all contribute to the peripheral inflammatory response(34). In the brain, elimination of microglia by PLX5622 should have attenuated the neuroinflammation induced by LPS and its behavioral consequences. In accordance with this prediction, there are already several publications showing that depletion of microglia by PLX5622 protects from neuroinflammation(35–38) and prevents behavioral alterations in response to cranial irradiation(38), repeated social defeat(39), partial sciatic nerve ligation(40) and experimental autoimmune encephalomyelitis(37). In addition, antibody-mediated neutralization of peripheral macrophage CSF-1R was reported to block the development of sickness behavior measured by reduced locomotor activity and body weight loss in response to CD40 activation, a model of autoimmune disease(41). In contrast with these positive findings, a number of studies reveal that microglial depletion is not always neuroprotective. In mice infected with prions, administration of PLX5622 accelerated disease progression(42). In the same manner, PLX5622 increased viral load and enhanced mortality in a number of murine models of viral infection(43–45). This protective role of microglia was also apparent in the progression of neurodegeneration in APP-PS1 transgenic mice(46), the extent of excitotoxic injury in a model of brain injury induced by cerebral ischaemia(47) and the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine (MPTP)(48).

The problem with most of the studies described above is that they are often descriptive and do not provide a clear explanation of what the elimination of microglia by CSF-1 receptor antagonists actually accomplishes. In the first study to show that CSF1 receptor antagonism eliminates microglia in a reversible way, mice were treated with a low dose of LPS (0.25 mg/kg) after only 7 days of the
CSF-1R antagonist PLX3397, and brains were collected 6 h after LPS but without any intracardiac perfusion of PBS or saline to eliminate residual blood(31). While this study showed that PLX3397 attenuated IL-1β and reversed TNF mRNA expression in response to LPS, it had only limited effects on other inflammatory markers, with no effect on IL-6 mRNA expression in response to LPS. In a model of Parkinson’s disease caused by administration of MPTP, a 21-day administration of PLX3397 increased the gene expression of proinflammatory cytokines in response to MPTP in the striatum of mice(48). Flow cytometry analysis of chemokines and proinflammatory cytokines in astrocytes from the substantia nigra and striatum revealed that PLX5622 significantly increased the IL-6 and TNF response to MPTP. These findings can be interpreted to suggest that microglia cells down regulate the astrocytic response to inflammatory insults. There is already evidence that astrocytes from mice treated chronically with the CSF1 receptor antagonist PLX3397 to deplete microglia still respond to LPS in vivo by developing a reactive A1 phenotype(49). This is probably facilitated by the lack of IL-10 from microglial origin as this anti-inflammatory cytokine normally lowers the proinflammatory profile of LPS-activated astrocytes(50).

The possibility that reactive A1 astrocytes induced by LPS take over in the absence of microglia is consistent with the observation that in our study brain IL-6, a cytokine mainly produced by astrocytes during neuroinflammation(51), was the only cytokine of which the gene expression in response to LPS was enhanced by PLX5622. Of note, there is a consistent literature on the existence of a positive relationship between IL-6 and fatigue(52) with evidence of improvement in fatigue scores in response to anti-IL-6 agents(53). Another candidate cytokine for fatigue is type I interferon(7). As LPS induces type I interferons as well as classical proinflammatory cytokines, we assessed this possibility by measuring the expression of Oas1a, a type I interferon responsive gene. As expected, LPS increased the gene expression of Oas1a at the periphery and in the brain. However, PLX5622 attenuated this effect in the liver but did not modify it in the brain.

Another mechanism for the lack of attenuation of neuroinflammation by PLX5622 could be an enhanced trafficking of immune cells into the brain of microglia depleted mice. However, this is unlikely to account for the present results as PLX3397 treatment did not compromise the integrity of
the blood-brain barrier, based on blue Evans coloration exclusion(31). In addition, in situations in which there was evidence of increased infiltration of lymphocytes in the brain of microglia-depleted mice, genetic elimination of lymphocytes did not modify the increased sensitivity of microglia depleted mice to neurodegeneration(48).

No abnormalities at baseline in spontaneous behavior, motor coordination, locomotor activity or learning and memory have been described in mice treated with CSF-1R antagonists(30, 31). However, there has been no attempt to assess the effect of microglial elimination on the ability of mice to engage in strenuous exercise, as measured by running wheel activity or by treadmill running. Our results show that PLX5622 decreased the amount of voluntary wheel running at baseline by about 20%. It is possible to interpret this finding by what is already known concerning the involvement of microglia in the beneficial effects of physical exercise. In particular, microglial activation within the neurogenic niche has been shown to mediate the beneficial effects of running wheel activity on hippocampal neurogenesis in the adult or aged mouse brain(54, 55). In addition, wheel running has been reported to induce microglia proliferation in the adult murine cortex, which could play a role in the positive effects of physical exercise on neurological health(56, 57). Our observation of a significant decrease in voluntary wheel running activity in microglia depleted mice is consistent with this hypothesis.

Conclusion
In conclusion, the results of the present study cast doubt on an exclusive role of microglia activation in the fatigue inducing effects of systemic inflammation and call for a re-evaluation of the importance of microglia in the brain response to physical exercise.

Abbreviations
Cd11b
Cluster of differentiation 11b
CSF-1
Colony stimulating factor 1
CSF-1R
Colony stimulating factor 1 receptor
CX3CR1
CX3C chemokine receptor 1
E.Z.N.A.
Registered commercial name
IL-1β
Interleukin-1beta
IL-6
Interleukin-6
IL-10
Interleukin-10
LPS
Lipopolysaccharide
mRNA
Messenger ribonucleic acid
Oas1a
2′-5′-oligoadenylate synthase 1A
PBS
Phosphate-buffered saline
PCR
Polymerase chain reaction
TNF
Tumor necrosis factor-alpha

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Tables
Table 1 – List of primers
### Table 2 – Effects of PLX and LPS on gene expression of markers of microglia/macrophages and proinflammatory cytokines. F values (F(1,20)) from 2 (PLX diet vs. control diet) x 2 (LPS vs. control) ANOVA with 6 mice/group. NS = non-significant, * p<0.05, ** p<0.01, ***p<0.001

| Target molecule | PLX                  | LPS                  | PLX x L       |
|-----------------|----------------------|----------------------|---------------|
| Brain CX3CR1   | F(1,20)=684***       | F(1,20)=5.29*        | F(1,20)=6*    |
| Brain CD11b    | F(1,20)=333***       | F(1,20)=9.61**       | F(1,20)=1.4   |
| Brain IL-1b    | F(1,20)=1.43 NS      | F(1,20)=9.70**       | F(1,20)=1    |
| Brain TNF      | F(1,20)=0.75 NS      | F(1,20)=21.1***      | F(1,20)=0.1  |
| Brain IL-6     | F(1,20)=0.30 NS      | F(1,20)=7.56*        | F(1,20)=4    |
| Brain IL-10    | F(1,20)=1.10 NS      | F(1,20)=3.79         | F(1,20)=4    |
| Brain OAS1a    | F(1,20)=0.29 NS      | F(1,20)=14.7***      | F(1,20)=0.4  |
| Liver CSF-1R   | F(1,20)=23.9***      | F(1,20)=12.3**       | F(1,20)=4    |
| Liver CD11b    | F(1,20)=3.22 NS      | F(1,20)=17.3***      | F(1,20)=2.4  |
| Liver IL-1b    | F(1,20)=0.41         | F(1,20)=27.1***      | F(1,20)=4    |
| Liver TNF      | F(1,20)=7.96**       | F(1,20)=39.1***      | F(1,20)=7    |
| Liver IL-6     | F(1,20)=2.49 NS      | F(1,20)=23.6***      | F(1,20)=2    |
| Liver IL-10    | F(1,20)=9.62**       | F(1,20)=15.2***      | F(1,20)=9    |
| Liver OAS1a    | F(1,20)=4.15 NS      | F(1,20)=28.9***      | F(1,20)=4    |

### Table 3 – Effects of PLX on body weight, locomotor activity in a new cage and voluntary wheel running response to LPS. F values from 2 (PLX diet vs. control diet) x time ANOVA for body weight and voluntary wheel running before LPS treatment, from 2 (PLX diet vs. control diet) x 2 (LPS vs. control)
ANOVA for locomotor activity in a new cage, and from 2 (PLX diet vs. control diet) x 2 (LPS vs. control) ANOVA with 6 mice/group with time as a repeated factor for body weight loss, and voluntary wheel running. NS non-significant, * p<0.05, ** p<0.01, ***p<0.001

|                          | PLX | LPS | PLX x LPS | Time | PLX x time | LPS x time |
|--------------------------|-----|-----|-----------|------|------------|------------|
| Body weight              | F(1,22)=0.200 NS | | | F(6,132)=3.85** | F(6,132)=1.86 NS |
| LPS effect on body weight| F(1,20)=0.342 NS | F(1,20)=1.14 NS | | F(2,40)=65.2*** | F(2,40)=1.57 NS | F(2,40)=31.0*** |
| LPS effect on activity new cage | F(1,20)=0.186 NS | F(1,20)=1.36 NS | F(1,20)=16.8*** | | |
| Pre-LPS wheel running    | F(1,22)=18.4*** | | | F(6,132)=44.3*** | F(6,132)=0.803 NS |
| LPS effect on wheel running | F(1,20)=275*** | F(1,20)=7.45* | F(1,20)=1.67 NS | F(5,100)=4.24** | F(5,100)=25.8*** |

Declarations

**Availability of data and materials**

The datasets collected and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

RD has received honoraria from Pfizer USA and from Danone Nutricia Research France for work that is not related to the present study. All remaining authors declare no competing interests.

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**Compliance with Ethical Standards**

All protocols were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.
Authors’ Contributions

EGV: conception, design of the work; acquisition, analysis, and interpretation of data; drafting of the work and revised it.

FGB: acquisition and analysis of data

RD: conception, design of the work; drafting of the work and revised it

All authors have approved the submitted version and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Figures
Figure 1

Effects of PLX and LPS on relative mRNA expression of Cd11b and CX3CR1 in the brain (A), Cd11b and CSF1R in the liver (B), IL-1b, TNF, IL-6, IL-10 and Oas1a in the brain (C) and liver (D). Mean +/- standard deviation, n=6/group, * p<0.05, **p<0.01, ***p<0.001. CTL = control diet, PLX = diet supplemented with PLX5622.
Figure 2

Effects of PLX and LPS on body weight expressed as percent change from the baseline, locomotor activity in a new environment measured as distance traveled (cm) and wheel running activity measured by total number of rotations per night at baseline and during 5 days after LPS administration. Mean +/- standard deviation, n=6/group, *p<0.05, ** p<0.01, ***p<0.001. CTL = control diet, PLX = diet supplemented with PLX5622.