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8. **ABSTRACT**
   - Epilepsy is a common, disabling problem in patients with tuberous sclerosis complex (TSC) and is usually intractable to available treatments. This Exploration-Hypothesis Development Award investigates the hypothesis that inflammation contributes to epileptogenesis in an animal model of TSC and that anti-inflammatory drugs may represent rational, effective therapies for epilepsy in TSC. In the first grant year, we have identified a series of inflammatory cytokines and chemokines, such as interferon IL-1beta and CXCL10, based on polymerase chain reaction, western blotting, and immunohistochemistry, which are elevated in a mouse model of TSC. Furthermore, these inflammatory markers could be reversed by the mammalian target of rapamycin (mTOR) inhibitor, rapamycin. These findings suggest that these inflammatory mediators could be involved in epileptogenesis in the mouse model and support the testing of anti-inflammatory agents that inhibit these mediators as potential treatments for epilepsy in the mice.

9. **SUBJECT TERMS**
   - Epilepsy, inflammation, tuberous sclerosis

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Introduction

Epilepsy is a very common, disabling neurological manifestation of Tuberous Sclerosis Complex (TSC), affecting up to 90% of individuals with TSC and causing significant morbidity and mortality [1]. Seizures in TSC patients are often refractory to the available medical and surgical therapies. While much progress has been made in understanding epilepsy in TSC, the specific pathophysiological mechanisms causing epileptogenesis in this disease are still largely unknown. Obtaining a better understanding of these mechanisms of epileptogenesis should lead to more effective therapies for seizures in TSC, including “antiepileptogenic” treatments to prevent epilepsy. Recently, the role of brain inflammation in the pathophysiology of various types of epilepsy has received increasing attention, especially in response to epileptogenic brain injuries [2]. Proinflammatory markers have been found to be activated in a variety of animal models of epilepsy, including chemoconvulsant and electrical kindling models of epilepsy, as well as in human tissue obtained from epilepsy patients, such as with mesial temporal sclerosis. Different types of inflammatory mediators and pathways have been implicated, including cytokines, the complement system, and cyclooxygenase/prostaglandins [2-4]. Furthermore, anti-inflammatory treatments targeting these pathways have begun to be explored. For example, selective pharmacological inhibition of the cytokine IL-1β production in astrocytes blocks kindled seizures in rats [5]. While activation of inflammatory pathways during epileptogenesis in response to acquired brain injury is perhaps not surprising, a relatively novel idea is that brain inflammation could also be important in the pathophysiology of developmental or genetic epilepsies. In fact, many of the inflammatory markers implicated in models of acquired epilepsy due to brain injury have also been found in brain specimens from patients with malformations of cortical development [6,7]. Furthermore, evidence for activation of proinflammatory pathways, such as IL-1β and components of the complement cascade, have been demonstrated in cortical tubers from TSC patients [7-9]. Many of the inflammatory reactions, such as in the cytokine system, appear to be most closely associated with glial cells, including reactive astrocytes and activated microglia.

While these pathological studies from human TSC brain specimens suggest a possible role of inflammatory mechanisms in epileptogenesis, whether inflammation is pathogenic, compensatory, or an epiphenomenon relative to the neurological manifestation of TSC is not known. In this Exploration-Hypothesis Development Award, we will test the novel hypothesis that inflammation promotes epileptogenesis in TSC. As glial cells are important mediators of inflammatory reactions, this will be achieved first by examining the expression of different components of inflammatory pathways, including cytokines and prostaglandin systems, in a knock-out mouse model of TSC (Tsc1GFAP CKO mice) [10], involving conditional inactivation of the Tsc1 gene primarily in glia (Task/Aim 1). Then, the effect of anti-inflammatory drugs, inhibiting these specific pathways, will be tested on the development of epilepsy in this mouse model (Task/Aim 2). This project has the potential to reveal novel information about the role of inflammation in epileptogenesis in TSC and to support novel therapeutic approaches involving anti-inflammatory agents for the neurological manifestations of TSC.

Body

Task 1/Specific Aim 1: To determine whether inflammatory pathways, including cytokines (IL-1β, IL-6 and TNF-α) and cyclooxygenase/prostaglandins, are abnormally activated in a mouse model of TSC (months 1-12).

Subtask 1a. Immunocytochemistry studies will assess the expression of IL-1β, IL1-R, IL-6, TNF-α, and Cox-2 in Tsc1GFAP CKO and control mice.

Subtask 1b. Western blot studies will assess the expression of IL-1β, TNF-α and Cox-2 in Tsc1GFAP CKO and control mice.

Subtask 1c. ELISA studies will assess the expression of IL-1β in Tsc1GFAP CKO and control mice.
We completed the experiments in Task 1. First, in preparation for this project, we had conducted pilot studies using quantitative polymerase chain reaction (q-pcr) to screen a large number of inflammatory markers, including the cytokines and prostaglandins listed above, as well as other related mediators of inflammation, such as chemokines. Four week old control and Tsc1\(^{GFAP}\) CKO mice were compared, correlating with the age that typically coincides with the onset of epilepsy in the KO mice. mRNA levels of the cytokine IL-1β and IL-6, as well as of the chemokine CXCL10, were upregulated in Tsc1\(^{GFAP}\) CKO mice, compared with controls. In contrast, IL1-R, TNF-α and Cox-2 mRNA were not significantly different between the KO mice and controls.

For subtask 1a, consistent with the pilot q-pcr studies, immunohistochemical staining showed dramatically increased expression of IL-1β in cortex and hippocampus of Tsc1\(^{GFAP}\) CKO mice (Fig. 1). Similarly, IL-6 and CXCL10 immunostaining was increased in Tsc1\(^{GFAP}\) CKO mice, but there was no difference in IL1-R, TNF-α and Cox-2 (data not shown). For subtask 1b, quantitative western blot analysis also confirmed that the protein levels of IL-1β and CXCL10 (Fig. 2), but not TNF-α and Cox-2, were significantly higher in Tsc1\(^{GFAP}\) CKO mice than that of control mice. In addition, the increased IL-1β and CXCL10 expression was reversed by rapamycin, indicating that the mammalian target of rapamycin (mTOR) signaling pathway is an upstream mediator of this inflammatory response (Fig. 2). Finally, for subtask 1c, ELISA studies also confirmed an increase in IL-1β in the Tsc1\(^{GFAP}\) CKO mice (5.9 ± 0.2 relative units versus 4.3 ± 0.4 in control mice; p < 0.05 by t-test).

Overall, our studies in Task 1 demonstrated that a subset of cytokines and chemokines, but not prostaglandins, is abnormally activated in Tsc1\(^{GFAP}\) CKO mice, which correlates with the onset of epilepsy in these mice. We hypothesize that these inflammatory mediators may contribute to epileptogenesis and neuropathological abnormalities in Tsc1\(^{GFAP}\) CKO mice. These results helped in the selection of anti-inflammatory agents targeting specific inflammatory mediators to test for efficacy against epilepsy in Task 2, as described below.

**Task 2/Specific Aim 2:** To determine whether anti-inflammatory agents targeting cytokine or prostaglandin production prevent epilepsy in a mouse model of TSC (months 13-24)

Subtask 2a. Immunocytochemistry and western blot studies will be performed to assess the effect of cytokine inhibitors and cyclooxygenase inhibitors on expression of cytokine or Cox-2 in Tsc1\(^{GFAP}\) CKO mice (months 13-15; ~30 mice total).

Subtask 2b. Histological studies will be performed to determine the effect of cytokine inhibitors and cyclooxygenase inhibitors on histological abnormalities of Tsc1\(^{GFAP}\) CKO and mice (months 16-18; ~30 mice total).

Subtask 2c. Video-EEG studies will be performed to determine the effect of cytokine inhibitors and cyclooxygenase inhibitors on the development of epilepsy and interictal EEG abnormalities in Tsc1\(^{GFAP}\) CKO mice (months 19-24; ~30 mice total).

Based on the results in Task 1, we selected anti-inflammatory agents targeting cytokine and chemokine production to determine whether such drugs could inhibit epilepsy and the associated inflammatory and pathological abnormalities in Tsc1\(^{GFAP}\) CKO mice. First, we attempted to inhibit IL-1β, using an IL-1β-specific antibody, canakinumab, obtained from Novartis. Unfortunately, pilot pharmacokinetic studies testing various doses of canakinumab failed to find an inhibitory effect on the increased IL-1β expression in the cortex of Tsc1\(^{GFAP}\) CKO mice. As canakinumab does not pass through the blood brain barrier, we performed ancillary studies to determine blood-brain barrier breakdown in Tsc1\(^{GFAP}\) CKO mice and found that there is limited breakdown of the blood-brain barrier at early ages when the drug needs to be administered to have antiepileptogenic effects. Thus, we concluded that canakinumab would not be an effective antiepileptogenic agent with systemic application in our mouse model. As an alternative approach, we have recently obtained a IL-1 receptor antagonist, kineret, which does
penetrate the blood-brain barrier, and initial experiments are in progress to test its antinflammatory and antiepileptogenic properties in Tsc1GFAPCKO mice.

As a broader approach, we have tested other compounds, particularly epicatechins, which inhibit both IL-1β and the chemokine CXCL10. First, for subtask 2a, treatment of Tsc1GFAPCKO mice with epicatechin-3-gallate (ECG) starting at 3 weeks of age, which is just prior to typical onset of epilepsy in these mice, significantly inhibited the subsequent increased expression of IL-1β and CXCL10 in Tsc1GFAPCKO mice (Fig. 3). For subtask 2b, ECG treatment at least partially reduced glial proliferation in cortex and hippocampus that occurs in Tsc1GFAPCKO mice (Fig. 4). Finally, for subtask 2c, ECG treatment caused a small but significant improvement in seizure frequency and survival in the Tsc1GFAPCKO mice (Fig. 5). Overall, the findings from Task 2 indicate that inflammatory mechanisms involving specific cytokines and chemokines may be involved in epileptogenesis and neuropathological abnormalities in Tsc1GFAPCKO mice and provide proof-of-principle that anti-inflammatory treatments targeting these mechanisms may be a rational therapeutic approach for epilepsy and other neurological manifestations of TSC.

Key Research Accomplishments
We have obtained the following experimental results in this research project:

- Quantitative qPCR demonstrates that mRNA expression of the cytokines IL-1β and IL-6 and the chemokine CXCL10 is elevated in Tsc1GFAPCKO mice, compared with controls. In contrast, IL1-R, TNF-α and Cox-2 mRNA expression is unchanged.
- Immunohistochemical studies show increased IL-1β, IL-6 and CXCL10 immunostaining in neocortex and hippocampus of Tsc1GFAPCKO mice, but there was no difference in IL1-R, TNF-α and Cox-2.
- Quantitative western blot analysis also confirmed that the protein levels of IL-1β and CXCL10, but not TNF-α and Cox-2, were significantly higher in Tsc1GFAPCKO mice than that of control mice.
- The increased IL-1β and CXCL10 expression was reversed by rapamycin, indicating that the mammalian target of rapamycin (mTOR) signaling pathway is an upstream mediator of this inflammatory response.
- ELISA studies also confirmed an increase in IL-1β in the Tsc1GFAPCKO mice.
- Treatment with the epicatechin, ECG, decreased the elevated IL-1β and CXCL10 expression and at least partially inhibited abnormal glial proliferation in Tsc1GFAPCKO mice.
- ECG caused a small, but significant, improvement in seizure frequency and survival of Tsc1GFAPCKO mice.

Reportable Outcomes
- A poster abstract of the data from this project has been submitted to the American Epilepsy Society. If accepted, the poster will be presented at the annual meeting of the American Epilepsy Society in December 2014 in Seattle, WA.
- A full manuscript of the data from this project is currently in preparation, with anticipated submission to a neuroscience journal within 1-2 months.

Conclusion
We have demonstrated that specific inflammatory cytokines and chemokines are abnormally activated during epileptogenesis in a mouse model of TSC. In contrast, prostaglandins, at least as assayed by Cox-2 expression, did not appear to be similarly involved. Treatment with an anti-inflammatory drug that inhibited specific cytokine and chemokine expression inhibited pathological abnormalities, decreased seizures, and improved survival in this mouse model. Overall, these findings implicate inflammatory mechanisms in epileptogenesis and neuropathological abnormalities in Tsc1GFAPCKO mice and provide proof-
of-principle that anti-inflammatory treatments targeting these mechanisms may be a rational therapeutic approach for epilepsy and other neurological manifestations of TSC.

References

Appendices
None

Supporting Data/Figures

Figure 1

![Figure 1](image)

Fig. 1 Increased IL-1β expression in Tsc1GFAPCKO mice assayed by immunohistochemistry. Representative images of immunohistochemical staining for IL-1β expression is dramatically increased in neocortex of Tsc1GFAPCKO mice (right) compared with control mice (left). **Green:** IL-1β; **Blue:** TO-PRO-3 nuclear stain.
**Figure 2**

A

![Image](image1.png)

B

![Image](image2.png)

Fig. 2 Increased IL-1β and CXCL10 expression in Tsc1GFAPCKO mice assayed by western blotting. Tsc1GFAPCKO mice had increased IL-1β (A) and CXCL10 (B) expression in cortical homogenates compared with control mice. Treatment with rapamycin inhibited these increases in IL-1β and CXCL10 in Tsc1GFAPCKO mice. *p<0.05, versus Cont + Veh by one-way ANOVA; # p<0.05, ### p<0.001 versus KO + Veh by one-way ANOVA (n = 7-11 mice/group). The ratio of CXCL10 or IL-1beta/beta-actin was normalized to the vehicle-treated control group. Cont = control, KO = Tsc1GFAPCKO, Veh = vehicle, Rap = Rapamycin.

**Figure 3**

A

![Image](image3.png)

B

![Image](image4.png)

Fig. 3 Increased IL-1β and CXCL10 expression in Tsc1GFAPCKO mice is inhibited by ECG. Tsc1GFAPCKO mice had increased IL-1β (A) and CXCL10 (B) expression in cortical homogenates compared with control mice. Treatment with ECG inhibited these increases in IL-1β and CXCL10. ***p<0.001 versus Cont + Veh; ### p<0.001 versus KO + Veh.

**Figure 4**

A

![Image](image5.png)

B

![Image](image6.png)

Fig. 4 Glia proliferation in Tsc1GFAPCKO mice is inhibited by ECG. Representative images (A) and quantitative analysis demonstrate that Tsc1GFAPCKO mice (KO + Veh) have increased GFAP-positive (red) astrocytes in neocortex and hippocampus compared with control mice (Cont + Veh). ECG at least partially reverses the increased glial proliferation in KO mice (KO + ECG). ECG treatment was started at 3 weeks of age and histological analysis performed at 7 weeks of age. Red: GFAP; Blue: TO-PRO-3 nuclear stain. *p<0.05, ** p<0.001 versus KO + Veh by two-way ANOVA (n = 8 mice/group). #p<0.05, ## p<0.01 versus KO + Veh by two-way ANOVA (n = 8 mice/group).
Fig. 5  ECG treatment causes a small, but significant decrease in seizure frequency (A) and survival (B) of Tsc1<sup>GFP</sup>CKO mice. ECG treatment was started at 3 weeks of age and maintained for the lifetime of the mice. *p<0.05 by one-way ANOVA in A and by Chi-Square test in B.