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TITLE: Targeting Regulatory T Cells to Treat Chronic Migraine and Post-Traumatic Headache

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14. ABSTRACT

In this study we propose to test the hypothesis that that reduced regulation of immune homeostasis by regulatory T (Treg) cells at peripheral tissues contributes to the chronification of headache and cognitive impairment in chronic migraine (CM) and post-traumatic headache (PTH). The research objective is to validate Treg as a cellular target for novel, peripherally active therapy for CM and PTH, with mechanisms distinct from the existing treatment options.

We made significant progress during the last funding period. **First,** we have found that mild traumatic brain injury (mTBI) resulted in astroglial and microglial activation in hippocampus and cortex (Aim 1A), both were inhibited by the low-dose interleukin-2 (LD-IL-2) treatment, suggesting that LD-IL-2 mitigates mTBI-induced deficit through inhibition of inflammatory responses in the brain. **Secondly,** results from Aim 1B and 2B suggest that endogenous Treg cells differentially regulate CM- and PTH-related behaviors, suggesting that CM and PTH may have unique underlying mechanisms. **Thirdly**, we have tested the efficiency of Cre-mediated recombination in TG neurons from AvCreERT2-TβRIIf/f and AvCreERT2-IL10Rαf/f mice (Aim 2A) and planned for more pilot studies to improve the knockout efficiency. **Lastly**, we have found that the FDA-approved drugs simvastatin and vitamin D3 differentially modulate the number of Treg cells in peripheral blood and in tissues. This provided a foundation to test their effects, alone or in combination, on CM- and PTH-related behavioral and neuronal sensitization.

15. SUBJECT TERMS
Chronic migraine, post-traumatic headache, mild-traumatic brain injury, cognitive defect, regulatory T cell, trigeminal ganglion

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1. Introduction

In this study we propose test the hypothesis that that reduced regulation of immune homeostasis by regulatory T (Treg) cells at peripheral tissues contributes to the chronification of headache and cognitive impairment in chronic migraine (CM) and post-traumatic headache (PTH). The research objective is to validate Treg as a cellular target for novel, peripherally active therapy for CM and PTH, with mechanisms distinct from the existing treatment options. The scope of the research contains 3 Specific Aims: **1)** To investigate whether endogenous Treg cells regulate the development and maintenance of recurring headache and cognitive impairment in CM and PTH; **2)** To elucidate the mechanisms through which low-dose interleukin-2 (ld-IL2) treatment and Treg cell transfer reverse CM and PTH; and **3)** To determine whether FDA-approved drugs simvastatin and vitamin D3 are effective for CM, PTH and mild traumatic brain injury (mTBI)-induced cognitive impairment through targeting endogenous Treg cells.

2. Keywords

Chronic migraine (CM), post-traumatic headache (PTH), mild-traumatic brain injury mTBI), cognitive impairment, regulatory T (Treg) cell, low-dose interleukin-2 (ld-IL2), interleukin-10 (IL-10), transforming growth factor beta 1 (TGFβ1), trigeminal ganglion (TG) neurons, peripheral sensitization, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP)

3. Accomplishments

What were the major goals of the project?

Obtain IACUC and ACURO approvals on animal study **completed**

Specific Aim 1) To investigate whether endogenous Treg cells regulate the development and maintenance of recurring headache and cognitive impairment in CM and PTH.

Purchase C57BL/6J and DEREG mouse breeders from The Jackson Laboratory and establish the colonies to breed mice for experiments. **completed**

Aim 1-A Experiment 1. Using flow cytometry to characterize mTBI-induced changes of circulating Tregs and other immune cells. **70% completed**

Aim 1-A Experiments 2-3. Using immunohistochemistry to examine mTBI-induced changes of Tregs and other immune cells in dura, TG and hippocampus. **completed**

Aim 1-B To investigate whether depletion of endogenous Treg cells affects CM-related behaviors. **completed**

Specific Aim 2) To elucidate the mechanisms through which ld-IL2/Treg cells reverse CM and PTH.

Purchase TβRIIf/f, IL10Rαf/f and AvCreERT2 mouse breeders from The Jackson Laboratory, establish breeding colonies and generate AvCreERT2-TβRIIf/f and AvCreERT2-IL10Rαf/f mice for experiments. **80% completed**

Aim 2-A Experiments 1 and 5. To test the efficiency of Cre-mediated recombination in TG neurons. **50% completed**

Aim 2-B) Experiments 1-4. To test if neutralizing antibodies against TGFβ or IL10 abolishes the effects of ld-IL2 on mTBI-induced behavioral and neuronal changes. **40% completed**

Specific Aim 3) To determine whether FDA-approved drugs simvastatin and vitamin D3 are effective for CM, PTH and mTBI-induced cognitive impairment through targeting endogenous Treg cells.

Experiment 1. Using flow cytometry to examine whether simvastatin and/or vitamin D3 increases circulating Treg cells and whether they alter the number of other immune cells. **70% completed**

Experiment 2. Using immunohistochemistry to examine simvastatin- and/or vitamin D3-induced changes of Tregs and other immune cells in dura, TG and hippocampus. **50% completed**

What was accomplished under these goals?

1) We have completed experiments 2-3 proposed in Aim 1A. Using immunohistochemistry, we quantified mTBI-induced changes of Tregs, $CD3^+CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, $Iba1^+$ macrophages/microglia as well as $GFAP^+$ astrocytes in dura, TG, cervical/medullary dorsal horn, hippocampus and cortex).

We found that, 7 days after mTBI, there was no significant changes in the number of Tregs, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, or $Iba1^+$ macrophages/microglia in mouse dura, TG or cervical/medullary dorsal horn.

However, 3 days after mTBI (Fig. 1A), the density of $GFAP^+$ astrocytes was significantly increased in both hippocampal CA1 and cortical regions (Fig. 1B, D-E, sham versus mTBI+saline), indicating astroglial activation. The density of Iba1+ microglia was increased in the cortex but not hippocampus (Fig. 1C, F-G, sham versus mTBI+saline). In sham mice, microglia displayed a resting morphology with small soma and long and thin processes (Fig. 1B, sham). After mTBI, cortical microglia showed an activated morphology, characterized by a larger cell body with shorter and thicker processes (Fig. 1C, mTBI+saline).

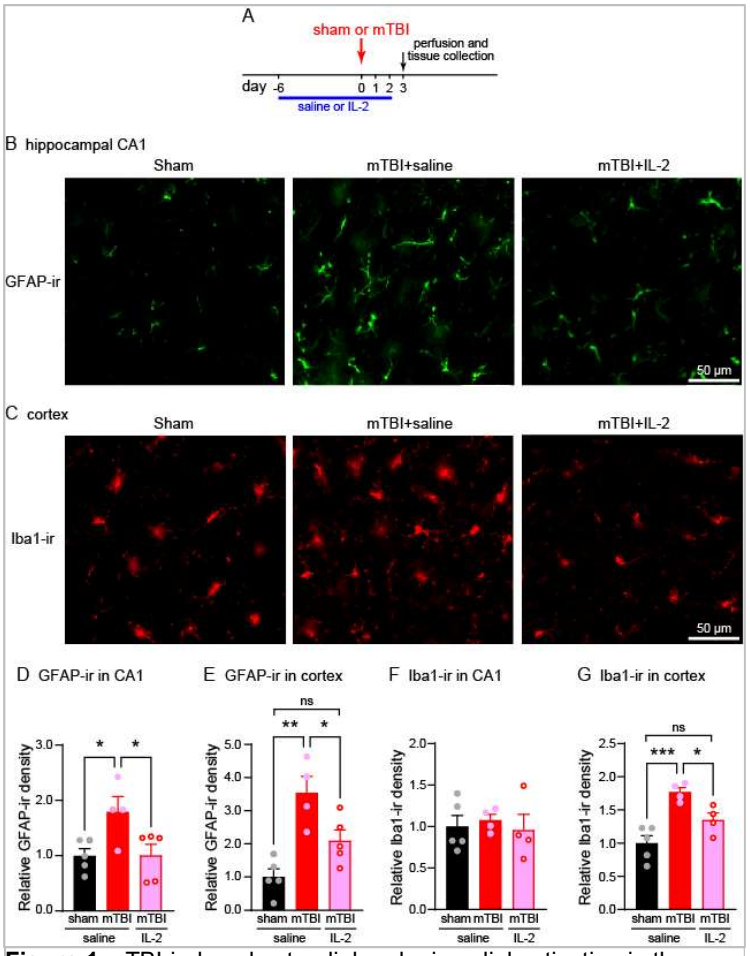

Figure 1 mTBI-induced astroglial and microglial activation in the hippocampus and cortex is inhibited by LD-IL-2 treatment.

Notably, in mice that received low-dose interleukin-2 (LD-IL-2) treatment (Fig. 1A), mTBI-induced increase in GFAP-ir and Iba1-ir densities were completely inhibited (Fig. 1D-G, mTBI+IL-2 versus sham and mTBI+saline). Cortical microglia maintained a resting morphology (Fig. 1C, mTBI+IL-2). These results support the model that LD-IL-2 mitigates mTBI-induced deficit through inhibition of inflammatory responses in the brain.

2) We have made good progress on experiment 1 proposed in Aim 1A. Dr. Hotchkiss and Dr. Unsinger used flow cytometry to profile time-dependent changes of immune cells by mTBI in the peripheral blood. They found that mTBI did not consistently alter the number/frequency of $CD115^+$ and $CD11b^+$ monocytes in the peripheral blood of either male or female C57BL/6J mice.

We have established breeding colonies for C57BL/6J and DEREG mice (Aim 1). However, due to the shortage of DEREG mice, the experiment of comparing total $CD3^+$ T cells as well as T cell subpopulations ($CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and $Foxp3^+CD25^+CD4^+$ Treg cells) in the peripheral blood will be postponed (see section 5 for details).

3) We have completed Aim 1B. We treated DEREG mice with 0.5 μg diphtheria toxin (DT) for two

consecutive days every 6 days to achieve selective and persistent depletion of endogenous Treg cells in dura and TG. We found that DT-mediated Treg depletion did not alter the magnitude or the duration of behavioral sensitization resulting from repeated administration of nitroglycerin (NTG, a reliable trigger of migraine in patients). This, along with our previous observation about the lack of effects of CD25 antibody-mediated Treg depletion on NTG-induced sensitization, indicate that the endogenous Treg cells do not contribute to the development and resolution of CM.

4) We have generated AvCreERT2-TβRIIf/f and AvCreERT2-IL10Rαf/f mice but the colonies were rather small. We are expanding the colony to meet the needs for behavioral experiments proposed in Aim 2A.

We have initiated experiments 1 and 5 in Aim 2A. We found that tamoxifen treatment resulted in about 50% reduction of the number of TG neurons expressing TβRII orIL10Rα, respectively. We are trying to alter the tamoxifen regimen (higher dose or longer duration) to see if this improves the efficiency of Cre-mediated recombination. This will delay the experiments proposed in Aim 2A (see section 5 for details).

5) In a control study for Aim 2B, we found that a neutralizing antibody against TGFβ prolonged mTBI-induced behavioral sensitization. We are testing whether neutralizing antibody against IL10 had similar effect on mTBI-induced behaviors. If yes, this will suggest that endogenous Treg cells are functionally important for the resolution of mTBI-induced sensitization.

6) In Experiment 1 of Aim 3A, Dr. Hotchkiss and Dr. Unsinger showed by flow cytometry that DEREG mice that received simvastatin or vitamin D3 alone had a small increase in Treg cells in the peripheral blood. However, **in Experiment 2 of Aim 3A**, simvastatin or vitamin D3 treatment alone did not significantly increase the number of Treg cells in dura or TG. These results suggest that simvastatin and vitamin D3 differentially regulate the abundance of circulating and resident Treg cells. This may explain the effects (or the lack) of simvastatin and/or vitamin D3 on CM- and PTH-related sensitization.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We have updated the SOW to reflect the change of experimental schedule based on the results obtained so far and the availability of transgenic mice.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Actual or anticipated problems or delays and actions or plans to resolve them:

Due to the limited numbers of DEREG, AvCreERT2-TβRIIf/f and AvCreERT2-IL10Rαf/f mice produced in our colony, we are experiencing delay in many of the proposed experiments. We plan to significantly expand the colony to address this issue. We have attached a revised SOW to reflect the change of plan.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Publications, conference papers, and presentations

Journal publications

Nothing to report

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers and presentations

Nothing to report

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Yu-Qing Cao, Ph.D, Principal Investigator (3 months): Dr. Cao is responsible/for the overall administration and direction of the project.

Leandro Flores do Nascimento, Ph.D, Postdoc (12 months): Dr. Nascimento has established mouse breeding colonies for Specific Aim 1 (C57BL/6J and DEREG mice) and Specific Aim 2(TβRIIf/f, IL10Rαf/f and AvCreERT2 mice). He has also made good progress on Aims 1A and 1B.

Lily Feng, B.S., Research Technician II (4 months): Ms. Feng has assisted Dr. Nascimento to establish mouse breeding colonies for Specific Aims 1 and 2. She also assisted with the immunohistochemistry experiments in Aim 1A, 2A and 3A.

Roli Simoes, Ph.D., Postdoctoral researcher (12 months): Dr. Simoes has made good progress on immunohistochemistry experiments in Aim 2A and 3A.

Sun Ryu 6.0 CM

Richard S Hotchkiss, M.D., Collaborator (.6 month): Dr. Hotchkiss has provided guidance and supervision on using flow cytometry and ELISA to profile immune cells in Specific Aims 1A, 2B and 3A.

Jacqueline Unsinger, Ph.D., Senior Scientist (5 months): Dr. Unsinger has made good progress on flow cytometry experiments in Aims 1A and 3A.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

Nothing to report