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14. ABSTRACT

The first goal was to determine the effects of TP-10 treatment in nerve and muscle recovery after sciatic nerve injury. Using sciatic nerve crush injury, we obtained reproducible damage as measured by muscle force and CatWalk gait analyses. To find the optimal dose of PDE10A inhibitor to improve these parameters, we used two different PDE10A specific inhibitors, MP-10 and TP-10. We optimized the dose using an in vitro macrophage model. Specifically, we stimulated inflammasome activation and pyroptosis to show the importance of PDE10A in macrophage mediated inflammation, inflammasome activation and pyroptosis (a specific type of programmed cell death that releases high amounts of inflammatory cytokines such as IL-b and IL-18. To stimulate pyroptosis, we incubated macrophages with lipopolysaccharide (LPS) and nigericin. Optimal protection was a 50% reduction in cell death with 3 μM MP-10 which correlated with a 60% reduction in IL-Ib release. We studied the process of pyroptosis that requires the clustering an adaptor protein called ASC in freshly isolated peritoneal macrophages. TP-10 at 3 μM inhibited ASC clustering and pyroptosis by 66%. The second goal was to define the effect of PDE10A inhibition on cytokine expression and after nerve injury. We performed immunohistochemistry on sham and crush injury sciatic nerve sections. There was a 5-fold increase in PDE10A after crush injury. Next, we performed a cytokine array on injured nerve and found significantly increased IL-1b, IL-18, and myeloperoxidase. We confirmed increases in these cytokines by performing an immunoblot. In summary, we showed that pyroptosis and “cytokine storm” were present after sciatic crush injury. TP-10 or MP-10 reduced inflammasome formation and activation.

15. SUBJECT TERMS

PDE10A and its inhibitors, inflammation, Macrophage, Sciatic Nerve Crush Injury, Gait Analysis, Pyroptosis

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1. INTRODUCTION:

The first goal was to determine the effects of TP-10 treatment in nerve and muscle recovery after sciatic nerve injury. Using sciatic nerve crush injury, we obtained reproducible damage as measured by muscle force and CatWalk gait analyses. To find the optimal dose of PDE10A inhibitor to improve these parameters, we used two different PDE10A specific inhibitors, MP-10 and TP-10. We optimized the dose using an in vitro macrophage model. Specifically, we stimulated inflammasome activation and pyroptosis to show the importance of PDE10A in macrophage mediated inflammation, inflammasome activation and pyroptosis (a specific type of programmed cell death that releases high amounts of inflammatory cytokines such as IL-b and IL-18. To stimulate pyroptosis, we incubated macrophages with lipopolysaccharide (LPS) and nigericin. Optimal protection was a 50% reduction in cell death with 3 μM MP-10 which correlated with a 60% reduction in IL-Ib release. We studied the process of pyroptosis that requires the clustering an adaptor protein called ASC in freshly isolated peritoneal macrophages. TP-10 at 3 μM inhibited ASC clustering and pyroptosis by 66%. The second goal was to define the effect of PDE10A inhibition on cytokine expression and after nerve injury. We performed immunohistochemistry on sham and crush injury sciatic nerve sections. There was a 5-fold increase in PDE10A after crush injury. Next, we performed a cytokine array on injured nerve and found significantly increased IL-1b, IL-18, and myeloperoxidase. We confirmed increases in these cytokines by performing an immunoblot. In summary, we showed that pyroptosis and "cytokine storm" were present after sciatic crush injury. TP-10 or MP-10 reduced inflammasome formation and activation.

2. KEYWORDS:

PDE10A, Inflammation, macrophage, sciatic nerve crush injury, gait analysis, pyrotosis, TP-10, MP-10

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: To determine the effects of TP-10 treatment in nerve and muscle functional recovery after sciatic nerve injury.

Aim 2: To determine the combined effects of TP-10 treatment and exercise training in neuropathic pain after nerve injury.

Specific Aim 1 **To determine the effects of TP-10 treatment on nerve and muscle functional recovery after sciatic nerve injury**	**Timeline** (Months)	*percentage of completion*	*Actual/ expected completion dates*
Major Task 1			

Optimize the dose of TP-10 on nerve and muscle functional recovery after nerve injury.			
Subtask 1 Perform sciatic nerve crush injury	1-2	100%	July 1, 2021
Subtask 2 Perform CatWalk gait analysis	3-4	100%	Aug 1, 2021
Subtask 3 Perform muscle force analysis	5-6	30%	Dec 1, 2021
Milestone Achieved: Identify TP-10 dose that maximally improves functional recovery	5-6	90%	Dec 1, 2021
Major Task 2 **Define the effect of PDE10A inhibition on cytokine expression and NLRP3 activation after nerve injury**			
Subtask 1 Perform immunohistochemistry for PDE10A on nerve	7-8	100%	Jan 31, 2022
Subtask 2 Perform cytokine array on nerve	9-10	80%	
Subtask 3 Perform protein assay for inflammasome components on nerve	11-12	100%	
Milestone(s) Achieved: TP-10 treatment reduces NLRP3 formation and activation	10-12	100%	

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Individuals funded by DOD are being trained to perform sciatic nerve crush injury and to measure motor function by CatWalk gait analysis. We performed sciatic nerve crush injury in 38 mice, and assessed motor recovery using CatWalk gait analysis to measure sciatic functional index (SFI) on days 7, 9, and 14 after injury with or without MP-10 (10 mg/kg injection, 3 hr after injury, once daily) Fig. 1.

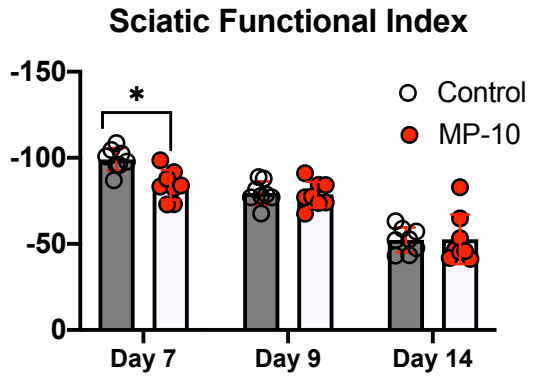

Fig 1. MP-10 treatment improved motor function after sciatic nerve injury.

To examine the effects of PDE10A inhibition on NLRP3 inflammasome activation, we measured cleaved IL-1β in the sciatic nerve by immunofluorescence 3 days after crush injury. We found that nerve injury dramatically increased IL-1β cleavage in sciatic nerve. MP-10 treatment significantly reduced this effect compared to vehicle group suggesting that PDE10A inhibition reduced inflammasome activation (Fig. 2A). (Green: Cleaved IL-1β, Blue: DAPI), representative image from 4 mice for each group). To examine muscle atrophy, we measured atrophy gene expression from gastrocnemius muscles on day 3 post-injury. Muscle atrophy is associated with increased expression of atrophy genes, such as myogenin and Gadd45a. Daily MP-10 treatment significantly decreased myogenin and Gadd45a mRNA expression in gastrocnemius muscle on day 3 after crush injury compared to vehicle (N=4 mice for each group). Fig. 2B-C.

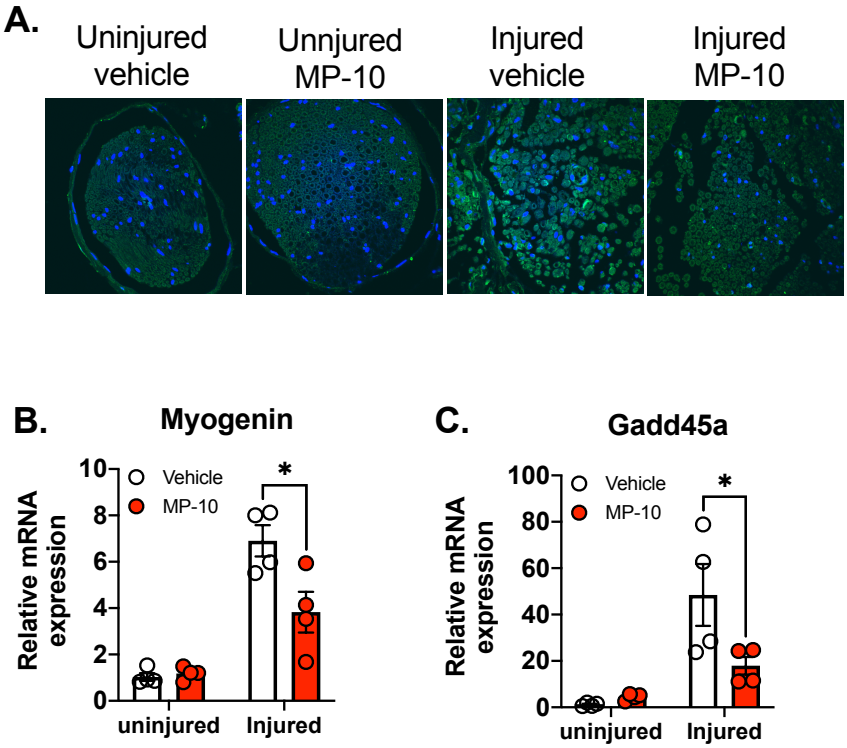

Fig 2. MP-10 treatment reduced nerve inflammation and prevented muscle atrophy

Inflammatory monocytes and resident tissue macrophages are key mediators of tissue repair, regeneration, and fibrosis during infections and sterile tissue injury. To understand the regulation and function of PDEs in macrophages, we screened for all PDE genes in both peritoneal and alveolar macrophages, and found that PDE4B and PDE10A were the two major PDEs highly induced by lipopolysaccharide (LPS) (Fig. 3A). To study the role of PDE10A in inflammasome activation and pyroptosis, we used two different PDE10A specific inhibitors, MP-10 and TP-10. TP-10, has been administered systemically in people with schizophrenia, as well as in mouse models of heart failure. MP-10 has been used in several human clinical trials to treat Huntington disease. Recently, we showed MP-10 treatment decreased wire injury-induced intima formation in femoral arteries. To show the importance of PDE10A in macrophage mediated inflammation, we co-incubated LPS with MP-10, followed by nigericin, in differentiated human THP-1 macrophages. Cell death was measured by real-time nucleic acid staining (SYTOXTM), and IL-1β release from the

medium was measured by ELISA. We found MP-10 treatment significantly decreased the magnitude of LPS-nigericin stimulated cell death and IL-1β release in a dose dependent manner (Fig. 3B-C) suggesting that PDE10A inhibition protects macrophages from LPS/nigericin-mediated pyroptosis. Upon NLRP3 inflammasome activation, the adaptor protein ASC is recruited by NLRP3 and forms large multimeric complexes, termed ASC specks. To characterize further the role of PDE10A in inflammasome activation, we studied its effect on formation of ASC specks. We overexpressed an ASC-GFP fusion protein in THP-1-differentiated macrophages and stimulated them with LPS followed by nigericin. PDE10A inhibition by TP-10 blocked ASC speck formation, as indicated by reduced ASC speck immunofluorescence (Fig. 3D). Therefore, we measured the effect of TP-10 on these parameters of inflammasome activation in peritoneal macrophages. TP-10 treatment dramatically decreased cleaved caspase-1, GSDMD, and IL-1β (Fig. 3E). To analyze the physiological effect of TP-10, we used ATP instead of nigericin to induce inflammasome activation. Again, PDE10A inhibition by TP-10 decreased IL-1β secretion (Fig. 3F), supporting the role of PDE10A in regulating inflammasome activation.

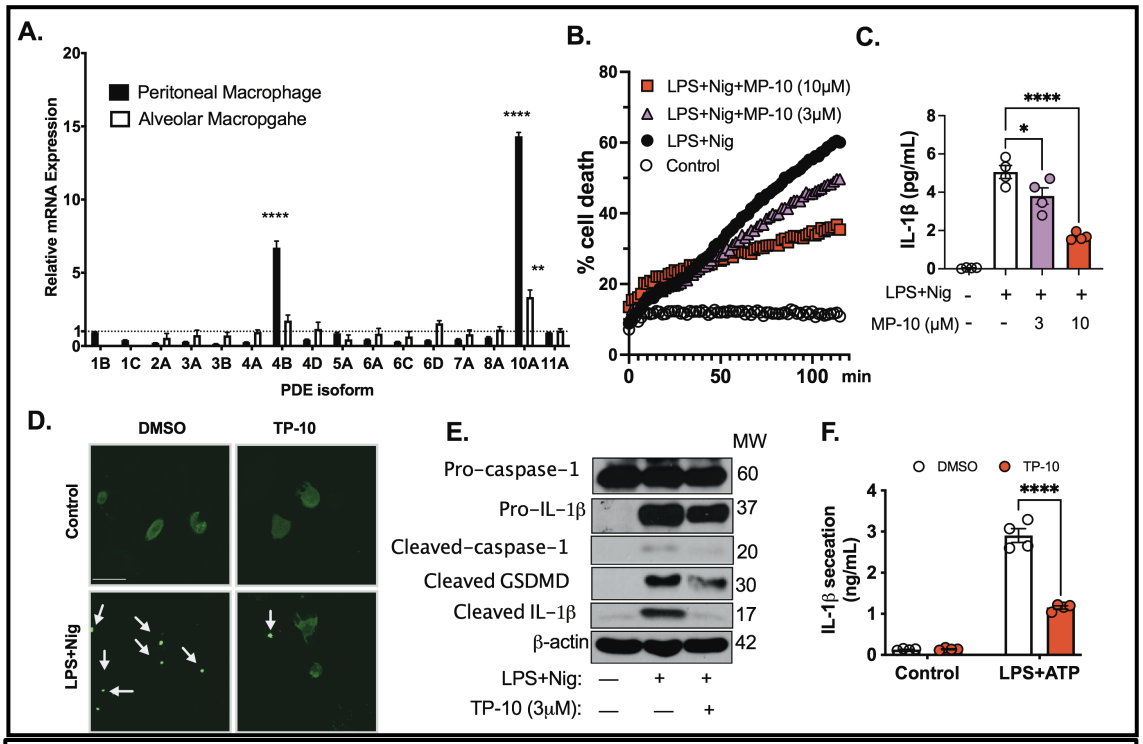

Fig. 3. PDE10A inhibition or knockout reduces inflammasome activation in LPS-stimulated macrophages. (A) Different PDE expressions after LPS (100ng/mL) stimulation for 6 hours in peritoneal macrophages (Black bar) and alveolar macrophages (White Bar), dashed line: no LPS.(B) THP-1 differentiated macrophages were stimulated with LPS (100 ng/mL) and co-incubated with MP-10 (3 or 10 μM) or vehicle (ethanol) for 3 hr followed by 2 hr nigericin (Nig, 6 μM) treatment. Real-time percentage cell death was determined by normalizing to maximal nuclear acid fluorescent intensity (0.1% Triton-X100 treated cells). (C) Peritoneal macrophages were stimulated with LPS (100 ng/mL) for 3hr followed by nigericin (Nig, 2 μM) stimulation for 1hr. IL-1β in the medium was measured by ELISA. (D) THP-1 ASC-GFP overexpressed macrophages were stimulated by LPS+Nig (nigericin) with or without TP-10. ASC speck formation was measured by confocal microscope. (Arrowheads indicate ASC specks (green)). Scale bar = 50 μm) (E) Peritoneal macrophages were stimulated with LPS for 3 hours followed by nigericin. Pro- and cleaved caspase-1, IL-1β and GSDMD were measured by western blots. (F) Peritoneal macrophages were stimulated with LPS for 3 hours followed by ATP (2mM). IL-1β in the medium was measured by ELISA. Data were expressed as mean ± SEM.

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

We hired a technician, Camila Lage Chavez, to assist with the injury and analysis experiments. She will attend medical school in the late summer; therefore she will not complete the project. However she was mentored by the postdoc working full time on this project, Chia Hsu, who taught her numerous techniques. Furthermore she states that the training of the sterile technique and surgery will benefit her as she goes through medical school. Also, her time in the lab made her interest in biomedical research increase significantly. Furthermore, she was able to assist in other projects analyzing and interpreting the data, and then participating in writing the paper. She already has one published paper and another one has been reviewed favorably. We hope that during future summer breaks she will be able to return to the lab to help complete the project.

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?

For specific aim 1, task 1 we need to perform additional catwalk gait analysis, muscle force analysis and treatment of more animals with MP-10. If we have time we will also compare a dose of 3 mg/kg vs 10 mg/kg. We also need to perform additional measurements of SFI, muscle protein atrophy and muscle force analysis.

4. IMPACT:

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

We have shown that crush injury of the peripheral sciatic nerve causes a rapid increase in inflammation mediated by specific cytokines such as IL-1b. We found a critical role for an enzyme called PDE10A that decreases intracellular cyclic AMP. Furthermore, we found that a drug inhibitor of PDE10A called MP-10 was able to inhibit the level of inflammation as measured by expression of IL-1b. We also found that MP-10 improved SFI, a measure of the sciatic nerve function. MP-10 decreased the muscle atrophy of the gastrocnemius showing that PDE10A mediated signal events were important in impaired muscle function.

What was the impact on other disciplines?

The data we present will extend to other disciplines in 2 ways. 1) The data show that nerve injury is associated with inflammation which is specifically regulated by the enzyme PDE10A. Because the inflammatory proteins such as IL-1b were inhibited by the specific PDE10A inhibitors, MP-10 and TP-10, they establish a new intracellular mediator for inflammation. 2) The demonstration that components of the NLRP3 inflammasome mediate the production of inflammatory cytokines extends the signals that induce inflammation in the nervous system. These results will influence neuroscience and immunology.

What was the impact on technology transfer?

Nothing to report.
The impact on commercial technology will be to develop additional PDE10A inhibitors that are more potent and tissue specific.

What was the impact on society beyond science and technology? ”

Nothing to report.

5. CHANGES/PROBLEMS:

We changed TP-10 to MP-10 for in vivo experiments because of the difference in cost, at the time of the grant submission, MP-10 was not readily available. We show that the dose response relationship for inhibiting PDE10A by MP-10 was similar to TP-10. Because they are related to each other we did not consider this a significant change.

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

1) Because we utilized the catwalk gait equipment of Dr. Chris Proschel we were affected by his technicians being infected with COVID-19 that limited our time to perform catwalk gait analysis.
2) We found more variability in the catwalk gait analysis than expected. We will have to increase the number of animals that we will study to achieve significant results.

Changes that had a significant impact on expenditures

The project has remained on budget.

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

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

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.

Hsu CG, Chavez CL, Zhang C, Sowden M, Yan C, Berk BC. The lipid peroxidation product 4-hydroxynonenal inhibits NLRP3 inflammasome activation and macrophage pyroptosis. Cell Death Differ. 2022. Online ahead of print.

Hsu CG, Li WJ, Chavez CL, Zhang C, Sowden M, Berk BC. Pnpt1 mediates NLRP3 inflammasome activation by MAVS and metabolic reprogramming in macrophages. Cell Mol Immunol. Under Revision.

- **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**

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Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Chia Hsu
Project Role: Postdoc Student
Research Identifier: 0000-0002-1133-4116
Nearest person month worked: 12
Contribution to Project: Dr. Hsu has designed the experiments, performed the surgeries, performed immunoblots, IHC, and catwalk gait analysis. He has trained the technician, Camila Lage Chavez, on the procedures above.
Funding Support:

Name: Camila Lage Chavez
Project Role: Lab Technician
Research Identifier: 0000-0002-5307-1908
Nearest person month worked: 6
Contribution to project: Camila has helped Dr. Hsu with performing the surgeries, as well as immunoblots, IHC and catwalk gait analysis.
Funding support:

Name: Mark Sowden
Project Role:
Research Identifier: 0000-0002-7824-0915
Nearest person month worked: 1
Contribution to project: Dr. Sowden has designed the experiments in collaboration with Dr. Hsu.
Funding support:

Bradford Berk
Project Role: PI
Research Identifier: 0000-0002-2767-4115
Nearest person month worked: 3
Contribution to project: Dr. Berk has designed the experiments, analyzed the data and troubleshooted with Dr. Hsu, Dr. Sowden and Camila.
Funding support: University endowment

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

Closed
NIH R01 HL 049192
Berk (PI) 08/01/2016 – 07/31/2020
Cyclophilin A: Novel Mediator of Pulmonary Arterial Hypertension.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: