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PRINCIPAL INVESTIGATOR: Keisa Mathis

CONTRACTING ORGANIZATION: University of North Texas Health Science Center

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13. SUPPLEMENTARY NOTES

This technical report is a part of a request to transfer this grant to another institution, namely the University of Texas Southwestern Medical Center.

14. ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease with chronic inflammation as a life-threatening feature. While currently available immunosuppressive therapy for SLE patients result in globally adverse side effects, other therapies involving biologics that target specific immune mediators/pathways are still being tested. A novel, safe therapeutic option to combat uncontrolled inflammation in patients with SLE would provide relief and may lead to better outcomes. The cholinergic anti-inflammatory pathway is a neuro-immune mechanism that regulates inflammation. In this pathway, activation of the efferent vagus nerve increases splenic nerve activity and ultimately inhibits the release of inflammatory mediators from splenic immune cells; therefore, the cholinergic anti-inflammatory pathway may be an important therapeutic target against systemic inflammation. There are current clinical trials testing the efficacy of implantable vagal nerve stimulators in reducing pain in SLE, and pharmacologic stimulation of the cholinergic anti-inflammatory pathway may also be a viable option. However, because the safety of these therapies is unknown, alternative methods to stimulate the cholinergic anti-inflammatory pathway at different levels (e.g., the splenic nerve) could provide novel therapeutic alternatives. Interestingly, a recent study demonstrated that exposure to left kidney ultrasound increases splenic nerve activity due to the close proximity of the kidney, spleen and splenic nerve, and results in a reduction of immune cell infiltration in the kidneys of mice subjected to renal ischemia-reperfusion injury. Based on this provocative ultrasound study and my exciting preliminary data, we have generated an overall hypothesis that splenic nerve stimulation reduces the production and release of pro-inflammatory cytokines from splenic immune cells via the cholinergic anti-inflammatory pathway. This would therefore attenuate systemic inflammation and protect from renal inflammation, renal oxidative stress and the development of renal injury in SLE. We planned to test this hypothesis with the following aims: 1) To test the hypothesis that increased splenic nerve activity is anti-inflammatory and prevents renal injury in SLE mice; and 2) To test the hypothesis that exposure to renal ultrasound increases splenic nerve activity and therefore reduces chronic inflammation and subsequently renal injury in SLE mice.

We were able to initiate studies using a more recently described mode of ultrasound delivery, LIPUS or low-intensity pulsed ultasound, that has been shown to be effective in other disease models. We planned to substitute LIPUS for the proposed rental and shipment of the Philips IU22 Ultrasound Unit from United Medical Instruments. Our plan was to first test whether this ultrasound works in our hands. Female C57BL/6J mice (Jackson Labs) were randomized into Control, LPS, Pre-treatment, and Post-treatment groups. Pre-treatment mice were given LIPUS (using Mettler Electronics portable ultrasound machine Sonicator740X) under anesthesia for 20 min for five consecutive days. All mice were then intraperitoneally injected with LPS (1 mg/kg dissolved in saline), apart from Control mice which received 0.1 mL saline. One hour after LPS injection, Post-treatment mice were given LIPUS for 20 min. Three hours after LPS injection, all mice were humanely euthanized, and tissues were harvested for analysis. LPS mice had significantly increased plasma TNF-α (280 ± 37 pg/mL vs. 0.07 ± 0.00 pg/mL; P < .0001) and IL-6 in plasma (96124 ± 6688 pg/mL vs. 425 ± 181 pg/mL; P < .0001), spleen (0.77 ± 0.16 pg/μg vs. 0.17 ± 0.04 pg/μg; P = .0303), and cortex (0.50 ± 0.07 pg/μg vs. 0.01 ± 0.00 pg/μg; P = .0009) compared to Control mice. Compared to LPS mice, Pre-treatment mice had reduced plasma TNF-α (183 ± 15 pg/mL vs. 280 ± 37 pg/mL; P = .0370) and plasma IL-6 (63656 ± 8441 pg/mL vs. 96124 ± 6688 pg/mL; P = .0134). Post-treatment mice also had reduced plasma IL-6 (47432 ± 1182 pg/mL vs. 96124 ± 6688 pg/mL; P = .0008) compared to LPS mice.In conclusion, five days of LIPUS treatment before an LPS challenge successfully reduced systemic TNF-α and IL-6, while a single LIPUS treatment after an LPS challenge also successfully reversed systemic IL-6. These findings demonstrate the potential for LIPUS as a therapeutic for reducing and reversing systemic inflammation in vivo.

15. SUBJECT TERMS

lupus, SLE, ultrasound, inflammation, cholinergic anti-inflammatory pathway

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INTRODUCTION

Lupus is an autoimmune disease that causes long-term inflammation in organs like the kidney and the skin. The long-term inflammation also contributes to the chronic pain and fatigue that most lupus patients experience. The symptoms of lupus are currently treated with medication that reduce inflammation, like steroids, but long-term steroid therapy also causes other unwanted harm to the body. The goal of our proposal is to develop a new therapeutic option, ultrasound, to decrease inflammation with no side effects. The rational basis for this proposal is that the proposed animal studies will aid in advancing the understanding of aberrant inflammatory mechanisms in SLE. We anticipate that our results will confirm the therapeutic potential of splenic nerve stimulation via ultrasound in SLE. These studies are beyond an incremental advancement since they could potential lead to a novel therapy for chronic inflammation in SLE. The proposed studies are immediately translational as they use a commonly accepted mouse model of human SLE and pulse ultrasound would be a safe unconventional therapy for SLE.

KEYWORDS

Lupus, systemic lupus erythematosus, SLE, ultrasound, inflammation, cholinergic anti-inflammatory pathway,

ACCOMPLISHMENTS

What were the major goals of the project? The major goals of this project as stated in the approved SOW are the following (dates are adjusted as actual start of the project was delayed 2-3 months):

Specific Aim	Timeline (Month #)	Personnel	Estimated Completion Date	Percentage Completed and/or Other Notes
Experiment preparation				
Obtain any additional UNTHSC Institutional Animal Care and Use Committee (IACUC) approval	Month 1	Dr. Mathis	September 2022	100%;
Order mice and allow to age for the study. *NZBWF1* (systemic lupus erythematosus; SLE) and *NZW* (control) strains are ordered from Jackson Laboratories at up to 6 weeks of age and not used until 20 or 30	N/A	Dr. Mathis	October 2023	Variable; Mice were ordered to start the aging process; however, the PI planned

weeks, therefore, there will be a delay (from award start date) before initiating first set of animal studies. Mice orders 7 orders; n=60/orders #1-6 (30 SLE and 30 controls) will occur every two months after the 1st order (i.e., April, June, August, October, December 2022; February, April 2023 (n=48 on April 2023 order #7). Female and male mice required for acute and chronic electrical and ultrasound induced splenic stimulation based on power analysis as described is 408.				ahead for the upcoming lab move, estimating lab would have to be packed up by May 2023. To move things along C57 mice were ordered in January 2023 to collect data on the LIPUS method as discussed in next section
Acquire ultrasound rental for Specific Aim 2	Month 9-17	Dr. Mathis	May 2024	100%; We acquired the Mettler Electronics portable ultrasound machine Sonicator740X with funds other than this grant in order to conduct feasibility studies discussed below
Milestone(s) Achieved: Animals scheduled for order/arrival and aging at the institution until ready to use for experiments	N/A	Mathis		
Specific Aim 1: To test the hypothesis that increased splenic nerve activity is anti-inflammatory and prevents renal injury in SLE mice.				
Initiate studies investigating the impact of acute electrical splenic nerve stimulation in female control and SLE mice only at two time points (n=96) (Question 1). Complete sample preparation, data analysis and interpretation.	Up month 9 (including mice aging)	Dr. Mathis Dr. Ma Dr. Lima Stubbs	September 2023	0%; Dr. Lima left the university due to family duties. Did not complete studies due to move.
Initiate studies investigating the impact of chronic splenic nerve stimulation via designer receptors exclusively activated by designer drugs (DREADDs) in female and male mice (n=96) (Question 2). Complete sample preparation, data analysis and interpretation.	Up to month 15 (including mice aging)	Dr. Mathis Dr. Ma Dr. Lima Stubbs	October 2023	10%; Preliminary studies testing efficacy of injecting DREADDs into superior cervical ganglion. These studies were not completed by the deadline due to the move to the new institution
Milestone(s) Achieved: 1) Complete animal studies; 2) Complete data analysis; 3) Present work at national conference; 4) Prepare manuscript	N/A	Dr. Mathis Dr. Ma Dr. Lima Stubbs		
Specific Aim 2: To test the hypothesis that exposure to renal ultrasound increases splenic nerve activity and therefore reduces chronic inflammation and subsequently renal injury in SLE mice.				
Initiate studies investigating the impact of acute ultrasound in female control and SLE mice only at one time point (n=72) (Question 1). Complete sample preparation, data analysis and interpretation.	Month 15-17 (including mice aging)	Dr. Mathis Dr. Ma Dr. Lima Stubbs	May 2024	33% Completed studies described below in control mice injected

				with LPS as described below.
Initiate studies investigating the impact of chronic pulsed ultrasound in female and male mice (n=144) (Question 2). Complete sample preparation, data analysis and interpretation.	Month 17-21 (including mice aging)	Dr. Mathis Dr. Ma Dr. Lima Stubbs	September 2024	20%; Initiated studies in control mice injected with LPS as described below.
Milestone(s) Achieved: 1) Complete animal studies; 2) Complete data analysis; 3) Present work at national conference; 4) Prepare manuscript	N/A	Dr. Mathis Dr. Ma Dr. Lima Stubbs		

What was accomplished under these goals? The major activity included testing the efficacy of the portable ultrasound. This was not a major difference to the proposed studies, but an important one as technology has changed and advances in the field showed efficacy of this method in reducing inflammation in other disease models [Gouda SAA et al. Life Sci. 2023;1;314:121338 and Lin CY et al. Int J Mol Sci 2022; 23(21), 13387]. We ordered mice to conduct a study using an alternative ultrasound protocol to the one described called low-intensity pulsed ultrasound (LIPUS) using the Mettler Electronics portable ultrasound machine Sonicator740X . It has been shown that LIPUS exposure preceding renal ischemia/reperfusion injury activates the cholinergic anti-inflammatory pathway (CAP) via the splenic nerve, leading to reduction of inflammatory responses in kidneys and spleens, so we tested this anti-inflammatory potential in our hands using mice. This was an alternative to the ultrasound machine rental proposed and we needed to test whether this mode of ultrasound was effective in reducing inflammation in mice. Additionally, we aimed to determine if LIPUS treatment after an acute inflammatory challenge can be reversed.

Female *C57BL/6J* mice (Jackson Labs) were randomized into Control, LPS, Pre-treatment, and Post-treatment groups. Pre-treatment mice were given LIPUS (using Mettler Electronics portable ultrasound machine Sonicator740X) under anesthesia for 20 min for five consecutive days. All mice were then intraperitoneally injected with LPS (1 mg/kg dissolved in saline), apart from Control mice which received 0.1 mL saline. One hour after LPS injection, Post-treatment mice were given LIPUS for 20 min. Three hours after LPS injection, all mice were humanely euthanized, and tissues were harvested for analysis.

LPS mice had significantly increased plasma TNF-α (280 ± 37 pg/mL vs. 0.07 ± 0.00 pg/mL; $P < .0001$) and IL-6 in plasma (96124 ± 6688 pg/mL vs. 425 ± 181 pg/mL; $P < .0001$), spleen (0.77 ± 0.16 pg/µg vs. 0.17 ± 0.04 pg/µg; $P = .0303$), and cortex (0.50 ± 0.07 pg/µg vs. 0.01 ± 0.00 pg/µg; $P = .0009$) compared to Control mice. Compared to LPS mice, Pre-treatment mice had reduced plasma TNF-α (183 ± 15 pg/mL vs. 280 ± 37 pg/mL; $P = .0370$) and plasma IL-6 (63656 ± 8441 pg/mL vs. 96124 ± 6688 pg/mL; $P = .0134$). Post-treatment mice also had reduced plasma IL-6 (47432 ± 1182 pg/mL vs. 96124 ± 6688 pg/mL; $P = .0008$) compared to LPS mice.*

In summary, five days of LIPUS treatment before an LPS challenge successfully reduced systemic TNF-α and IL-6, while a single LIPUS treatment after an LPS challenge also successfully reversed systemic IL-6. These findings demonstrate the potential for LIPUS as a therapeutic for reducing and reversing systemic inflammation *in vivo*. We concluded that our future studies should indeed use the LIPUS method.

These studies took place February-June 2023, with planning, execution, and finally data collection and analysis. The data collected will be presented in an upcoming meeting. The information attained in this preliminary study was crucial in understanding if LIPUS could be a viable route of ultrasound therapy in lupus mice, which is the

next phase of our proposed studies. Since the lab was planning to move in July 2023, we did not order the lupus mice before our move. If granted the transfer, we will immediately gain IACUC approval (application already in process) and then order mice so that we can continue proposed studies.

The unmet goals were due to the lab shutting down in May of 2023 to start the process of moving to UT Southwestern. My start date at UTSW was 7/17/2023. We currently in the process of submitting the animal protocol.

What opportunities for training and professional development has the project provided? Nothing to report

How were the results disseminated to communities of interest? The abstract for the study has been written and finalized and will be submitted to the American Physiological Summit in November. This is an important audience as there are many American Physiological Society investigators interested in the control of inflammation.

What do you plan to do during the next reporting period to accomplish the goals? We will continue the studies proposed if the grant is transferred. We are still excited about these studies and especially since we saw an effect in our hands in control mice treated with LPS.

IMPACT

Nothing to report

CHANGES/PROBLEMS

There were no significant changes to the proposed aims. The major change is the move to the new institution and the request for transfer, which has delayed progress.

PRODUCTS

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Viet Dinh
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Mr. Dinh conducted the ultrasound project described
Funding Support:	UNTHSC Graduate School

Name:	Dr. Rong Ma

Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Dr. Ma provided guidance on renal function measurements
Funding Support:	DOD Idea Award

Name:	Keisa Mathis
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Mr. Dinh conducted the ultrasound project described
Funding Support:	UNTHSC Graduate School

SPECIAL REPORTING REQUIREMENTS

Nothing to report

APPENDICES

Nothing to report