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TITLE: MULTI-OMICS AND MITOCHONDRIAL DYSFUNCTION IN ACUTE LUNG I

PRINCIPAL INVESTIGATOR: Madesh Muniswamy, Ph.D.

CONTRACTING ORGANIZATION: The Univ. of TX Hlth Sci Ctr at San Antonio

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MULTI-OMICS AND MITOCHONDRIAL DYSFUNCTION IN ACUTE LUNG INJURY

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6. AUTHOR(S)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal addresses the FY18 PRMRP Topic Area of "Acute Lung Injury", utilizing novel approaches (metabolomics, lipidomics, and mass spec imaging) to identify the causes of sepsis-induced acute lung injury (ALI) and biomarkers which differentiate patients at risk of developing ALI from those not at risk. This project also proposes the development and testing of a potential new drug, BTP2, to reduce the incidence and/or severity of ALI.

15. SUBJECT TERMS

ATP – adenosine triphospate; mCa2+ – mitochondrial Ca2+; MCU – mitochondrial Ca2+ uniporter; ROS – reactive oxygen species

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REPORT OUTLINE

1. INTRODUCTION:

Multiple organ failure (MOF) is a major cause of death and reduced quality of life in the military theater and in civilian life. Causes include blunt trauma and shock-like states leading to ischemia and inflammation and impaired cellular function in multiple tissues. Limited mechanisms and tools are currently available to understand the progression of sepsis and trauma to ALI due to heterogeneity in patient populations. Our recent findings revealed that LPS-induced vascular permeability is initiated by LPS-induced overproduction of reactive oxygen species (ROS), which in turn leads to Ca^{2+} transients via modulation of both InsP3-dependent Ca^{2+} release and extracellular Ca^{2+} influx through STIM/Orai1. The resultant elevation in cytoplasmic Ca^{2+} leads to further mitochondrial Ca^{2+} overload via the mitochondrial Ca^{2+} uniporter (MCU), creating a positive feedback loop leading to the expression of multiple inflammatory molecules (lipids, metabolites, proteins) that contribute to vascular leakage and ultimately, to MOF. One of the major changes that occur during Multi-organ-failure (MOF) in severe sepsis is mitochondrial dysfunction. Under aerobic conditions, energy conserving molecules produced in the Tri-Carboxylic Acid cycle are utilized by the Electron Transport Chain to produce ATP in a process known as oxidative phosphorylation (OXPHOS). During sepsis, OXPHOS can become impaired, and organs rich in mitochondria become highly susceptible to injury. While oxygen deprivation is known to compromise mitochondrial function, it remains poorly understood how ion channels like MCU may act as a regulator of organ function during sepsis. To identify and establish the components that participate in LPS-mediated mitochondrial reprogramming and MOF, we will utilize multiple innovative approaches including, the newly designed small molecule MCU inhibitor, MCU knockout (***Cell Reports 2016***), CRISPR/Cas9-mediated MCU knock-in (***Cell Reports 2019***) mouse models, and multiomic tools coupled with systems biology.

2. KEYWORDS:

ALI – acute lung injury
ATP – adenosine triphospate
[Ca2+]m – mitochondrial calcium concentration
[Ca2+]c – cytosolic calcium concentration
CFU – colony forming units
EC – endothelial cell
ICAM 1 – intracellular adhesion molecule 1
KI – knock in
KO – knockout
LPS – lipopolysaccharide
mCa2+ – mitochondrial Ca2+
MCU – mitochondrial Ca2+ uniporter
MPMVECs – mouse primary microvascular endothelial cells
mROS – mitochondrial reactive oxygen species
OCR – oxygen consumption rate
Orai1 – Calcium Release-Activated Calcium Modulator 1
OxPhos – oxidative phosphorylation
ROS – reactive oxygen species
SOCE – store-operated calcium entry

VE-cre – transgenic mouse strain expression cre-recombinase under the vascular endothelial-cadherin promoter

3. ACCOMPLISHMENTS:

a. What were the major goals of the project?

Major Task 1 Delineate whether Orai1-mediated $[Ca^{2+}]_c$ elevation is associated with MCU-mediated $[Ca^{2+}]_m$ uptake and mROS generation during endotoxin stimulation.

Major Task 2 Delineate whether Orai1-mediated $[Ca^{2+}]_c$ elevation is associated with MCU-mediated $[Ca^{2+}]_m$ uptake and mROS generation in Orai1 and MCU KO cells.

Major Task 3 Determine whether MCU C96A mutation operates as a MCU-SSG mimetic, persistently activates the channel, and promotes $[Ca^{2+}]_m$ overload and EC death.

Major Task 4 Assessment of intracellular and extracellular metabolite and lipid profiles in ECs following LPS challenge.

During the Year 1, we have accomplished specific Aim 1A-1D. We have also partly completed Aim 1E.

b. What was accomplished under these goals?

During the Year 1, we have accomplished specific Aim 1A-1D. We have also partly completed Aim 1E.

Accomplishments:

The role of SOCE-mediated $[Ca^{2+}]_c$ elevation in MCU-mediated $[Ca^{2+}]_m$ uptake and mROS generation: As LPS-mediated TLR-signaling induces TNFα, we utilized TNFα (20 ng/ml) to identify the specific role of SOCE in ECs. Mouse pulmonary microvascular endothelial cells (MPMVECs) from VE-Cre, Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$, and MCU$^{\Delta EC}$ were challenged with TNFα (16 hrs). Although TNFα challenge altered $[Ca^{2+}]_c$ transients (**Fig. 1A & B**) and

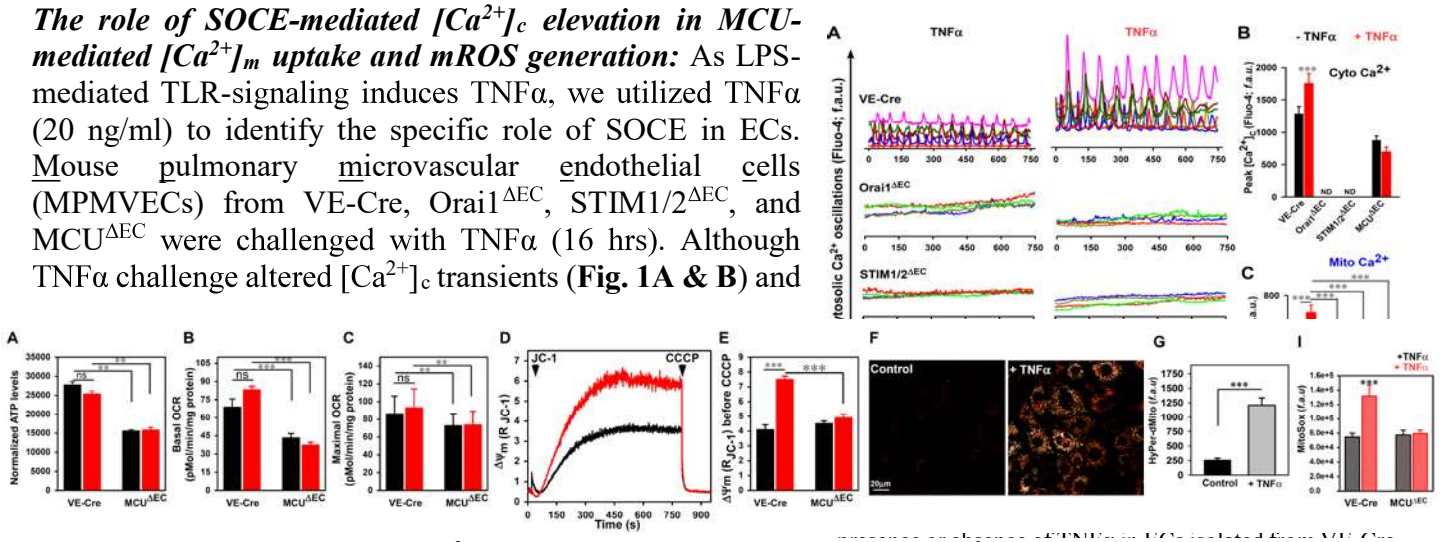

Fig. 2: Contribution of MCU-mediated Ca^{2+} in mROS generation. (A-C) *TNFα induced $[Ca^{2+}]_m$ uptake is uncoupled from OCR and ATP generation.* Quantification of cellular ATP (A), basal OCR (B), and maximal OCR (C) in VE-Cre, and MCU$^{\Delta EC}$ (n = 8) with and without TNFα stimulation. **(D and E)** *TNFα hyperpolarizes the mitochondrial membrane.* Representative traces of $\Delta\Psi_m$ in VE-Cre cells in the presence or absence of TNFα (D). Quantification of $\Delta\Psi_m$ in VE-Cre, and MCU$^{\Delta EC}$ (E). **(F-I)** TNFα induced mitochondrial hyperpolarization leads to mROS elevation. Confocal images of VE-Cre MPMVECs expressing Hyper-dMito, activated with or without TNFα (F). Quantification of Hyper-dMito fluorescence in MPMVECs with or without TNFα (G). Quantification of MitoSox fluorescence in VE-Cre, and MCU$^{\Delta EC}$ with or without TNFα challenge (I) (n = 8). **P<0.01; ***P<0.001

resulted in sustained $[Ca^{2+}]_m$ uptake (**Fig. 1C**) in VE-Cre MPMVECs, this phenotype was not observed in Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$ or MCU$^{\Delta EC}$ ECs, providing further evidence of the link between SOCE-mediated $[Ca^{2+}]_c$ elevation and MCU-mediated $[Ca^{2+}]_m$ uptake. Because $[Ca^{2+}]_m$ stimulates the TCA cycle,[74] we asked whether elevated $[Ca^{2+}]_m$ during TNFα stimulation increases the oxygen consumption rate (OCR) and cellular ATP levels. Surprisingly, analysis of intracellular ATP levels showed no increase in ATP production (**Fig. 2A**). Further, OCR measurements using the Seahorse XF96 analyzer revealed no significant difference in basal or maximal OCR (**Fig. 2B &C**). Enigmatically, we observed TNFα stimulation hyperpolarized the mitochondria, as measured in freshly isolated MPMVECs permeabilized and loaded with a selective and sensitive indicator for $\Delta\Psi_m$ (JC-1) (**Fig. 2D&E**). Uncoupling of Ψ_m from ATP generation is expected to increase mitochondrial ROS (mROS) overproduction. Therefore, ECs were activated with or without TNFα and examined by confocal microscopy using mitochondria-targeted H_2O_2-sensitive, HyPer-Mito (genetically-encoded fluorescent sensor) (**Fig. 2F**). Consistent with its effect on Ψ_m, TNFα treatment led to mROS elevation in VE-Cre (**Fig. 2G & I**) but not MCU$^{\Delta EC}$ ECs (**Fig. 2I**). ***Collectively, our results indicate that during LPS stimulation, SOCE-mediated $[Ca^{2+}]_c$ elevation enables $[Ca^{2+}]_m$ uptake, activating the TCA cycle, uncoupled Ψ_m hyperpolarization, and mROS elevation, potentially revealing $[Ca^{2+}]$ elevation and consequently mROS generation as key factors for EC dysfunction.***

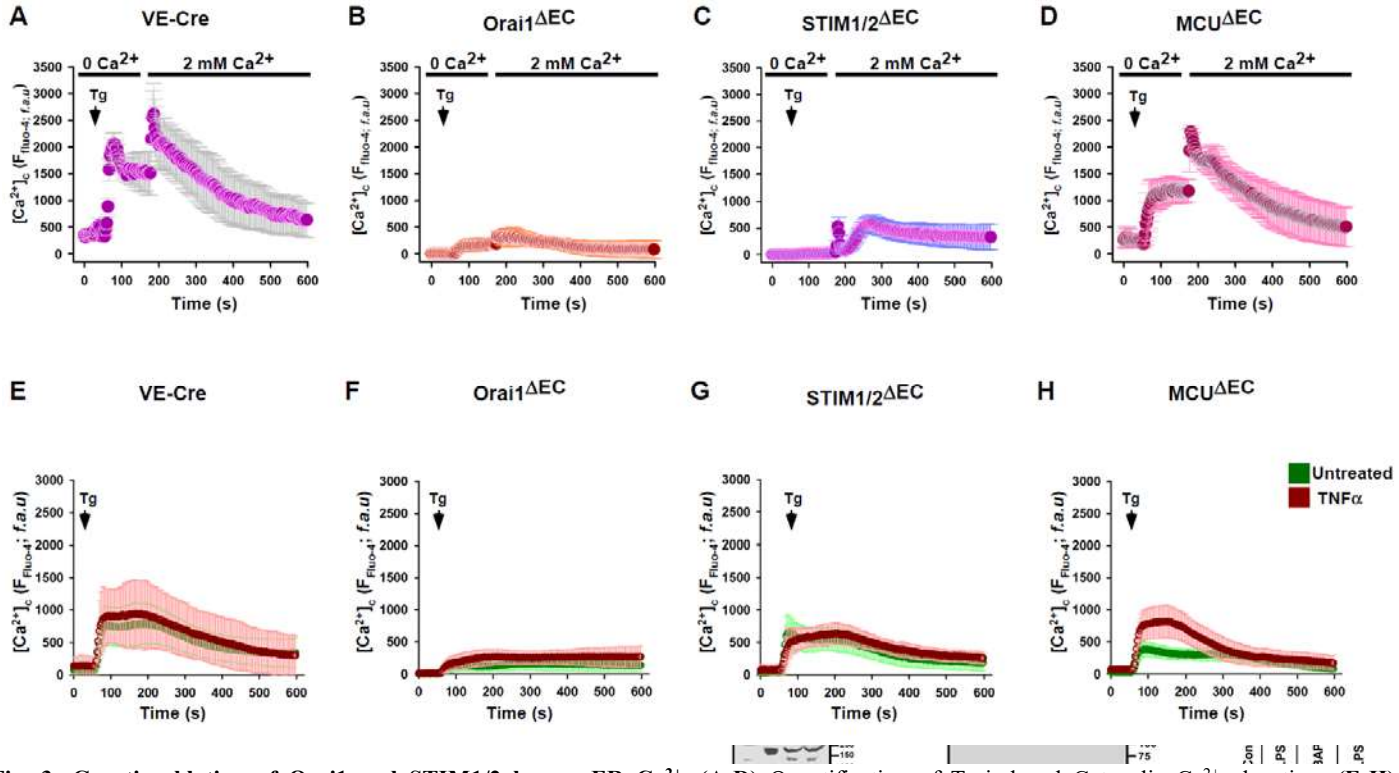

Fig. 3: Genetic ablation of Orai1 and STIM1/2 lowers ER Ca^{2+}. (A-D) Quantification of Tg-induced Cytosolic Ca^{2+} elevation. **(E-H)**. Quantification of Tg-induced Cytosolic Ca^{2+} elevation in ECs with or without TNFα pretreatment. (n = 3). Mean ± SEM.

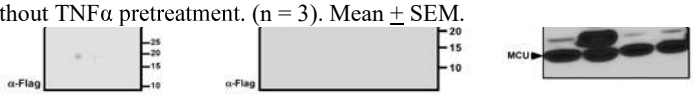

Fig. 4: Contribution of SOCE-mediated $[Ca^{2+}]_i$ transients in oxidizing MCU. LPS stimulation oxidizes MCU. (**A**) HPMVECs expressing MCU-FLAG (Ad-MCU) were exposed to menadione (10 μM), LPS (10 μg/ml) or GSH-depleting agent, BSO (200 μM) (n = 3). (**B**) Representative Western blots for the identification of reactive cysteines in MCU^{WT} and MCU^{C97A}. (**C**) HPMVECs expressing MCU-FLAG (Ad MCU) were exposed to LPS (10 μg/ml) with or without BAPTA (n = 3).

Assessment of SOCE in Orai1, STIM1/2, or MCU KO Endothelial Cells: To assess the SOCE, ECs isolaed isolated from VE-Cre, Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$, and MCU$^{\Delta EC}$ mice were cultured for 48 hrs in glass-bottom petri-dishes. ECs were loaded with cytosolic Ca^{2+} indicator fluo-4 to determine the SOCE-mediated $[Ca^{2+}]_c$ elevation. As expected, Tg-induced ER store depletion elicited a marked elevation of cytosolic Ca^{2+} upon 2 mM Ca^{2+} stimulation (**Fig. 3A**). This phenomenon was significantly suppressed in both Orai1 and STIM1/2

KO ECs (**Fig. 3B and 3C**). Interestingly, the SOCE activity was not significantly affected in MCU KO ECs (**Fig. 3D**). Since Orai1 and STIM1/2 KO ECs exhibited smaller Tg response, we next investigated whether TNF-□ □pretreatment alters ER Ca^{2+} level. As expected, Orai1 KO and STIM1/2 KO ECs showed less cytosolic Ca^{2+} accumulation upon Tg stimulation (**Figure 3E-EH**). TNF-□ pretreatment did not alter ER Ca^{2+} content indicating that loss of Orai1 or STIM1/2 lower ER Ca^{2+} level (**Figure 3E-EH**).

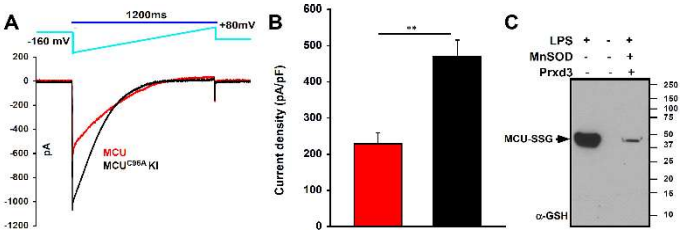

Fig. 5: MCU^{C96A} KI results in increased I_{MCU} (A and B) Mitoplasts prepared from WT and MCU^{C96A} ECs were subjected to electrophysiological recording of MCU current (n=6). (C) ECs treated with LPS exhibited MCU oxidation. Pretreatment with mitochondria antioxidant enzymes significantly reduced MCU oxidation.

***Blockade of extracellular Ca^{2+} entry prevents MCU oxidation*:** Recently we have shown that mROS generation driven by the $[Ca^{2+}]_m$ and $\Delta\Psi_m$ hyperpolarization explicitly oxidizes human MCU at Cys-97 (**Fig. 4A&B**). We hypothesized that the blockade of anterograde extracellular Ca^{2+} entry can reduce MCU oxidation, protecting EC dysfunction. In pilot experiments, we found that ECs pretreated with LPS (1 μg/ml, 4 hr) in an extracellular Ca^{2+}-free condition (BAPTA), markedly reduced LPS-induced MCU oxidation (**Fig. 4C**). We therefore predict that MCU oxidation is predominantly Ca^{2+} dependent, with SOCE required for MCU oxidation.

Endotoxin-derived oxidants promote S-glutathionylation of MCU at Cysteine 97: Our data indicate that oxidation of human MCU at C97 or mutation of its mouse homologue at position C96 results in higher MCU activity (**Fig. 5A&B**). Finally, we used a mitochondrial antioxidant (AdMnSOD+Prdx3) to demonstrate that mROS, specifically, is required for LPS-induced formation of MCU-SSG (**Fig. 5C**).

Genetic ablation of Orai1, STIM1/2, and MCU alters mitochondrial bioenergetics: We next examined whether deletion of SOCE machineries (Orai1 or STIM1/STIM2) or MCU alters mitochondrial energetic status during

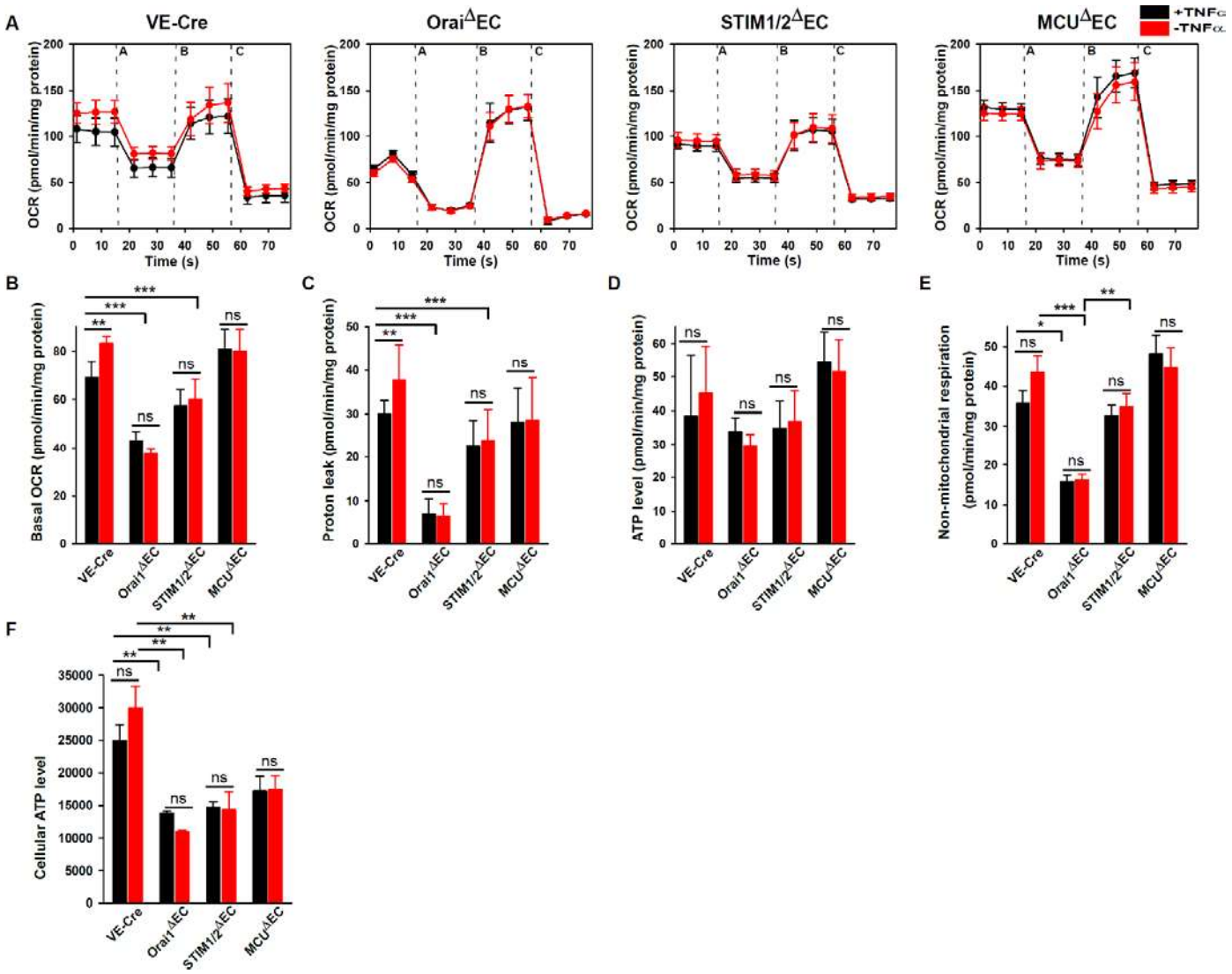

Fig. 6: Endothelial deletion of Orai1, STIM1/2, and MCU alters mitochondrial bioenergetics. (A) Mitochondrial oxygen consumption rate (OCR) cures of VE-Cre, Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$, and MCU$^{\Delta EC}$ endothelial cells with or without pretreatment with TNF-α. (**B-E**) Bar graphs show basal, proton leak, ATP, and non-mitochondrial respiration rates. (**F**) Quantification of cellular ATP levels. (n = 3). Mean ± SEM. *P<0.05, **P<0.01; ***P<0.001, ns; not significant.

inflammation. To test this, we isolated ECs from VE-Cre, Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$, and MCU$^{\Delta EC}$ mice and plated for oxygen consumption rate (OCR) studies (**Fig. 6A**). As expected, ECs from VE-Cre showed normal OCR, however Orai1 or STIM1/STIM2 deletion exhibited lower basal OCR as well as spare capacity (**Fig. 6B-E**). Although OCR levels were altered in Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$ ECs under basal condition, TNF-a pretreatment did not significantly alter the basal OCR (**Fig. 6A-E**). Having observed the OCR changes in Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$ ECs, we next assessed the cellular ATP levels with or without TNF- pretreatment. Remarkably, basal cellular ATP levels were reduced in three KO ECs (Orai1$^{\Delta EC}$, STIM1/2$^{\Delta EC}$, and MCU$^{\Delta EC}$) (**Fig. 6F**). Interestingly, TNF- did not alter the cellular ATP levels (**Fig. 6F**) indicating that SOCE mediated Ca^{2+} entry is essential for mitochondrial bioenergetics.

Assessment of intracellular and extracellular metabolite and lipid profiles in ECs following LPS challenge: To assess the dynamism of intracellular metabolites and lipid species, VE-Cre, Orai1$^{\Delta EC}$, and MCU$^{\Delta EC}$ mice were challenged with LPS (5mg/Kg) for 24 hours. Serum was collected before and after LPS challenge from

the above described animal models. Additionally, multiple organs were collected following LPS challenge for multi-omics studies.

Methodology:

Measurement of cytosolic and mitochondrial Ca^{2+} dynamics

ECs were plated on 25-mm glass coverslips and loaded with 5 μM Fluo-4/AM in extracellular medium (ECM). For simultaneous measurement of $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} uptake, cells were loaded with 2 μM rhod-2/AM (Invitrogen) and 5 μM Fluo-4/AM in ECM followed by an additional 10-min incubation in a dye-free medium. Coverslips were placed in a chamber and mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) and maintained at 37°C on an inverted microscope (Axio Observer [Carl Zeiss, Inc.]). Spontaneous cytosolic Ca^{2+} oscillations were recorded every 3 s using a laser-scanning confocal system (510 Meta; Carl Zeiss, Inc.) equipped with an Argon ion laser source at 488- and 568-nm excitation using a 63× oil objective. Images were acquired using either Lasersharp or ZEN 2008 software (Carl Zeiss, Inc). Images were analyzed and quantitated using ImageJ (National Institutes of Health) and a custom-made software (Spectralyzer). To assess Ca^{2+} entry, Ca^{2+}-free ECM was used in conjunction with 0.5 mM EGTA. 2 mM Ca^{2+} was added as indicated.

Mitochondrial reactive oxygen species measurement

Primary ECs isolated from control and KO mice were grown on 25-mm fibronectin-coated glass coverslips overnight and ECs were transiently transfected with mitochondria-targeted H_2O_2-sensitive, HyPer-Mito plasmid construct. 48 hours post-transfection, fluorescence changes were monitored (ex/em 488/515 nm) using a laser-scanning confocal system (510 Meta; Carl Zeiss, Inc.) equipped with an Argon ion laser source at 488- and 568-nm excitation using a 63× oil objective. Images were acquired using either Lasersharp or ZEN 2008 software (Carl Zeiss, Inc). Images were analyzed and quantitated using ImageJ (National Institutes of Health).

MCU-mPEG Gel-Shift Assay

ECs overexpressing MCU-FLAG were exposed to LPS and cell proteins were precipitated with 10% w/v TCA in acetone. TCA precipitated samples were collected by centrifugation at 12,000 rpm for 10 mins. The protein precipitate was dissolved in strong denaturing buffer (DB) (200mM Tris-HCl (pH 8.5), 10mM EDTA, 0.5% SDS and 6M Urea) containing iodoacetamide to block free thiols followed by TCA precipitation. The resultant pellet was resuspended in DTT to reduce any oxidized cysteines and TCA precipitation was again performed for DTT removal. The free cysteines liberated by this treatment were subjected to 0.4 mM methoxypolyethylene glycol (MW 5 kDa; mPEG5)-maleimide incubation for 30 min. mPEG5-conjugated MCU was resolved on SDS-PAGE and probed with FLAG antibody to visualize the molecular weight shift.

Mitoplast Patch-Clamp Recording

Mitoplast patch-clamp recordings were performed at 30°C as detailed previously with the following modifications. Freshly prepared mitoplasts from MCUWT or MCUC97A ECs were plated on the Cell-Tak–coated coverslips and mounted on the microscope. The isolated mitoplasts were bathed in a solution containing sodium gluconate (150 mM), KCl (5.4 mM), CaCl2 (5 mM), and HEPES (10 mM) (pH 7.2). The pipette solution contained sodium gluconate (150 mM), NaCl (5 mM), sucrose (135 mM), HEPES (10 mM), and EGTA (1.5 mM) (pH 7.2). After formation of GΩ seals (20 to 35 MΩ), the mitoplasts were ruptured with a 200- to 400-mV pulse for 2 to 6 ms. Mitoplast capacitance was measured (2.5 to 3.0 pF). After capacitance compensation, mitoplasts were held at 0 mV and I_{MCU} was elicited with a voltage ramp (from −160 to 80 mV, 120 mV/s). Samples were discarded if the break-in took longer than 5 s after addition of 5 mM Ca2+. Currents were recorded using an Axon200B patchclamp amplifier with a Digidata 1320A acquisition board (pCLAMP 10.0 software; Axon Instruments). The bath solution (5 mM Ca^{2+}) was chosen on the basis of previous measurements.

Mitochondrial oxygen consumption rate

Primary ECs were plated on 96 well Agilent Seahorse XF Cell Culture Microplates. For all primary EC experiments, the microplates were coated with fibronectin prior to cell addition. Primary ECs were plated at a density of 5×10^5 cells per well. Cells were plated in their normal growth media overnight. Media was changed to Seahorse XF Cell Mito Stress Test Kit (Agilent) assay media supplemented with glucose, glutamine, pyruvate concentrations equivalent to that of the growth media 1 hour before the experiment start time. After media change, per manufacturer instructions, cells were placed in a CO2-free incubator for 1 hour. Oxygen consumption rate (OCR) was measured at 37°C in an XF96 extracellular flux analyzer (Seahorse Bioscience, Agilent), which had been previously calibrated using Seahorse XF Calibrant solution (Seahorse Bioscience, Agilent) in a CO_2-free incubator overnight. Respiratory chain inhibitors were then loaded into the XF96 flux analyzer, and during the run, added sequentially to cells at indicated time points. Primary cells received oligomycin, FCCP, and a mixture of antimycin A and rotenone at concentrations of 2 □M, 5 □M, and 1 □M, respectively. For treatment conditions, LPS was purchased from Sigma-Aldrich (Cat #: E. coli O111:B4). Data was collected using Agilent Seahorse Wave 2.6.1 Desktop software and exported to GraphPad Prism version 7 for analysis.

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

Nothing to Report

d. **How were the results disseminated to communities of interest?**

Nothing to Report

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

We will comprehensively analyze changes in metabolites and lipids from cell extracts and conditioned media from endothelial cells of Control (VE-Cre) and KO (Orai1ΔEC and MCUΔEC) mice before and after LPS treatment. Upon completion of **Aim 1**, we will test and define whether MCU as a critical regulator of EC survival and vascular inflammation during sepsis (**Aim 2**). Here, our goal is to establish the in vivo relevance of this relationship towards control of pathogen-induced pulmonary vascular dysfunction. K. pneumoniae is a gram-negative bacterium that causes respiratory infections in human and animal hosts and produces significant quantities of LPS, which is thought to be a major factor in ALI. To assess the role of SOCE and MCU-dependent Ca^{2+} signaling in triggering vascular inflammation and lung injury experiments in this sub-aim will focus on studying the response of mice exhibiting EC-specific loss of Orai1 and MCU to K. pneumoniae. To test our hypothesis, we will use Orai1 and MCU EC KO mice which will demonstrate whether these mice are susceptible or resistant to bacterial infection.

4. IMPACT:

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

Nothing to Report

b. **What was the impact on other disciplines?**

Nothing to Report

c. **What was the impact on technology transfer?**

Nothing to Report

d. **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS:

a. **Changes in approach and reasons for change**

Nothing to report. .

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report

c. **Changes that had a significant impact on expenditures**

Co-Investigator, Dr. Subramanya Srikantan left the institution at the end July 2020. We're actively recruiting to fill his role on the project.

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

Nothing to report.

e. **Significant changes in use or care of human subjects**

Nothing to Report

f. **Significant changes in use or care of vertebrate animals.**

Nothing to Report

g. **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS:

Publications, conference papers, and presentations

- **Journal publications.**

1. Neeharika Nemani, Zhiwei Dong, Cassidy C Daw, Travis R Madaris, Karthik Ramachandran, Benjamin T Enslow, Cherubina S Rubannelsonkumar, Santhanam Shanmughapriya, Varshini Mallireddigari, Soumya Maity, Pragya SinghMalla, Kalimuthusamy Natarajanseenivasan, Robert Hooper, Christopher E Shannon, Warren G Tourtellotte, Brij B Singh, W Brian Reeves, Kumar Sharma, Luke Norton, Subramanya

Srikantan, Jonathan Soboloff, **Muniswamy Madesh**. Mitochondrial pyruvate and fatty acid flux modulate MICU1-dependent control of MCU activity. ***Science Signaling*** 2020 Apr 21;13(628):eaaz6206. (doi: 10.1126/scisignal.aaz6206).

2. Edmund J Carvalho, Peter B Stathopulos, **Muniswamy Madesh**. Regulation of Ca^{2+} exchanges and signaling in mitochondria. ***Current Opinion in Physiology*** 2020 (In Press). Available online 23 August 2020

3. Cassidy C. Daw, Karthik Ramachandran, Benjamin T. Enslow, Soumya Maity, Brian Bursic, Matthew J. Novello, Cherubina S. Rubannelsonkumar, Ayah H. Mashal, Joel Ravichandran, Terry M. Bakewell, Weiwei Wang, Kang Li, Travis R. Madaris, Christopher E. Shannon, Luke Norton, Soundarya Kandala, Jeffrey Caplan, Subramanya Srikantan, Peter B. Stathopulos, W. Brian Reeves, **Muniswamy Madesh**. Lactate elicits ER-mitochondrial Mg^{2+} dynamics to integrate cellular metabolism. ***Cell*** 2020 (Accepted).

- **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

a. **What individuals have worked on the project?**

Name:	Madesh Muniswamy
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-6745-9092
Nearest person month worked:	2
Contribution to Project:	Dr. Muniswamy organizes and coordinate weekly meetings with the lab members to ensure the incorporation of all data into a cohesive conceptual model. Dr. Muniswamy conceived and designed the study. He analyzed and interpreted experimental data and wrote the manuscript.
Funding Support:	R01 GM109882; R01 HL086699; R01HL142673

Name:	Ramana Subramanya Srikantan
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-1810-6519
Nearest person month worked:	5
Contribution to Project:	Dr. Srikantan propagated and purified MCU Adenovirus, maintain EC-specific MCU KO and Orai1 KO mice, and a recently generated constitutively active MCU CRISPR KI mouse. He worked closely with pre- and post-doctoral researchers in the Muniswamy lab, on the animal studies.
Funding Support:	R01 GM109882; R01 HL086699

Name:	*Cassidy Daw*
Project Role:	*Graduate Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-3441-0425*

Nearest person month worked:	*4*
Contribution to Project:	Miss Daw was responsible for molecular characterization of the proposed mouse models and their breeding. She performed experiments to understand molecular mechanisms of mitochondrial Ca2+ uptake with emphasis on the effects of Ca2+ on transcription factor activation during vascular inflammation and on protein-protein interactions.
Funding Support:	T32 AI 138944

Name:	Venkata Soundarya Kandala
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	0000-0002-3776-7737
Nearest person month worked:	6
Contribution to Project:	Miss Kandala was responsible for molecular characterization of the proposed mouse models, maintenance of animal colony and their breeding, surgical procedures and vascular inflammation assessment.
Funding Support:	N/A

Name:	Karthik Ramachandran
Project Role:	Postdoctoral Research Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0003-3673-3559
Nearest person month worked:	4
Contribution to Project:	Dr. Ramachandran primarily performed confocal imaging experiments linking store-operated Ca2+ entry, mitochondrial Ca2+ uptake, mROS generation, and mitochondrial functional studies.
Funding Support:	R01 GM109882; R01 HL086699

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

New Funding:

R01 GM135760 (PI: Muniswamy) **9/01/2020 - 8/31/2022** **2.04 Cal-Mos**
NIH/NIGMS **$200,000 (direct/year)**
Essential Role for SPG7 in Mitochondrial Permeability Transition Pore Assembly and Function

The major goals are:

- Define the molecular mechanism of SPG7-CypD interaction and PTP assembly.
- Investigate the mechanism of redox-mediated activation of SPG7 and CypD-SPG7 interaction in PTP formation.
- Define the contribution of SPG7 to PTP activation and cell death.

b. **What other organizations were involved as partners?**

N/A

8. SPECIAL REPORTING REQUIREMENTS

a. COLLABORATIVE AWARDS: This is a unique progress report submitted by Partnering PI, Madesh Muniswamy.

b. QUAD CHARTS: N/A

9. APPENDICES: N/A