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Sodium Pyruvate Ameliorates Influenza A Virus Infection In Vitro and In Vivo

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SODIUM PYRUVATE AMELIORATES INFLUENZA A VIRUS INFECTION

IN VITRO* AND *IN VIVO

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Jessica Morgan Reel

May 2021

SODIUM PYRUVATE AMELIORATES INFLUENZA A VIRUS INFECTION *IN VITRO* AND *IN VIVO*

Biology

Missouri State University, May 2021

Master of Science, Biology

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ABSTRACT

Pyruvate is produced in duplicate at the end of glycolysis in addition to ATP and NADH. Pyruvate is the metabolite of choice in most cells, whether obtained exogenously or endogenously. Recently we found that the addition of pyruvate's conjugate base, sodium pyruvate, to cell culture media dampened the immune response to influenza A virus (IAV) infection in cultured innate immune cells. Thus, I decided to investigate the mechanism and potential for treatment of IAV. *In vitro* using bone marrow derived macrophages that were infected with IAV we found that adding sodium pyruvate to the media decreased immune signaling pathways through a decrease in pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α). Additionally, exogenous sodium pyruvate added to the infection media of macrophages, diminished the mitochondrial reactive oxygen species production, without inhibiting virus replication *in vitro*. To investigate the metabolite's effects *in vivo,* we used C57Bl/6J mice to establish a model for sodium pyruvate treatment during IAV infection. We used a moderate infectious dose of IAV at 250PFU. We began by injecting the mice twice daily with diluted sodium pyruvate. While overall animal activity increased, no differences in proportional weight loss between saline controls and sodium pyruvate treated groups were observed. IAV is a respiratory virus and sodium pyruvate a metabolite of choice for cells; therefore, targeting the treatment to the respiratory tract with nebulizer treatments three times a day showed a significant difference (Days 7-14 post infection) in proportional weight loss. Sodium pyruvate treated mice were found to lose less mass, consume more chow, and feel better overall. Sodium pyruvate nebulized mice had decreased viral titer and decreased pro-inflammatory cytokines 7 days post-infection as well. Conclusively, sodium pyruvate ameliorates IAV infection *in vitro* and *in vivo*.

KEYWORDS: influenza A virus, inflammation, inflammasome, pyruvate, NOD-like receptor (NLR), metabolite, immunometabolism

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

ACKNOWLEDGEMENTS

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Disclaimer

This work is the result of a collaborative effort with Hazar Abusamalah (spelled Hazzar Abysalamah in BearWorks Institutional Repository, MSc graduate thesis, May 2018). In my second chapter, Hazar and I worked collaboratively to publish this manuscript (now in press). We both used C57Bl/6J mice to obtain bone marrow for BMDM cell culture, influenza A/PR/8/34 H1N1, sodium pyruvate (NaPyr), and media/additives for all experiments. Additionally, Hazar's thesis includes methods used throughout the project by both her and I. Hazar's data for cell death, mitochondrial damage (reactive oxygen species), and viral replication were used to publish this manuscript, and are included here (page 29, Figure 1 D-G). Hazar tested the effects of different glycolysis inhibitors, 2 deoxy-glucose or hemagglutinin. She then tested *E. coli* and *Aspergillus fumigatus*, where I tested for Poly I:C, and both looked at LPS+ATP as alternative infections. Additionally, I tested for intracellular ATP and lactate production and interferon-β production in samples. I replicated ELISA and western blot samples to increase the appropriate sample size for statistical analysis. Our theses differ in the methods by Hazar's IAV cultivation, her testing the difference in glycolysis inhibitors and infection of BMDM with *Aspergillus fumigatus*. This is replaced in my thesis by intracellular ATP, lactate production, and treatment/infection with Poly I:C. Consequently, the results vary the same as the methods. In summation, I continued the *in vitro* work in BMDMs. In addition, I introduced the work in mice, *in vivo*, which can be found in my third chapter (second manuscript, now in press). Chapter 3 is solely my work, built on top of Hazar and my collaboration of *in vitro* work. Both studies suggest that NaPyr may be an alternative therapy to IAV infection.

Background

Influenza A Virus (IAV), commonly known as the flu virus, has an impact on human life annually. IAV continues to plague the population and while anti-viral treatments are available, they are prone to viral resistance by mutations in the viral RNA. The influenza vaccine is available annually, but the most recent vaccine covers only four variants of the thousands of influenza variants in the world [1] and only 48.4% of the United States population 18 years or older received the vaccine in the 2019-2020 flu season [2]. Years where the vaccination rates are low puts a heavy burden on anti-viral therapies to help with the IAV infections. However, those therapies target specific components of the virus and are prone to viral resistance.

From our study, one potential alternative to these anti-viral treatments would be the metabolite sodium pyruvate (NaPyr). Pyruvate (Pyr) is the end product of glycolysis. Pyruvate has two fates for energy production. When oxygen is present aerobic glycolysis occurs, which then can be used for energy production via a series of pathways, the citric acid cycle, electron transport chain, and oxidative phosphorylation, which ends in the most efficient energy production [3]. Alternatively, glycolysis can occur anaerobically where lactate dehydrogenase converts pyruvate to lactate. Lactate can then be exported out of the cell. However, anaerobic glycolysis leads to decreased ATP efficiency through lactic acid fermentation [4]. Therefore, using aerobic glycolysis and oxidative pathways is more efficient leaving Pyr as an essential component of cellular metabolism (Figure 1). Using a universal metabolite, such as Pyr, to enhance the innate immune response would decrease the concerns that are observed from using other anti-viral treatments that target specific components or intricacies of the virus. Targeting specific pieces of the virus is likely to lead to virus mutation, which ultimately renders the anti-viral treatment useless against the resistant virus.

This project hails from a moment of serendipity, in which two different bottles of media were used to culture innate immune cells. These two experiments yielded differing results between the same treatment groups based on the media used. The only difference was that one bottle of media contained NaPyr, and the other did not. From this preliminary observation, it was found that the addition of NaPyr to the media seemed to dampen the immune response to influenza A virus (IAV) in macrophages. From there, we continued to investigate the possible mechanisms in which NaPyr might be immunomodulating the disease associated with IAV infection. Hazar Abusalamah's groundwork was the foundation of the *in vitro* work and demonstrated that NaPyr does have an effect on inflammasome activation and subsequent immunomodulatory responses in murine bone marrow derived macrophages (BMDM) [5]. Recognizing some of the mechanism *in vitro*, we continued to develop a mouse model for treatment with NaPyr over the course of IAV infection.

Influenza Virus

 How influenza invades. There are four types of influenza virus; three of which are currently infectious to humans: A, B, and C [6]. Influenza C causes only mild illness, but influenza A and B are the perpetrators for our annual flu season. IAV is well known to cause deadly pandemics, because of its zoonotic capabilities, such as the 1918 Spanish flu, or the 2009 H1N1 pandemic [7]. Part of the *Orthomyxoviridae* family, IAV has a segmented genome with eight segments of negative sense viral RNA that codes its 10 structural proteins and some non-structural proteins [8, 9]. The virus is encapsulated in a lipid viral envelope, which is derived from the host cell's phospholipid bilayer, which it acquires upon exit and serves to protect all the viral components from the external environment while the virus moves to its next

destination [10]. Protruding from the lipid viral envelope are two primary surface glycoproteins. When IAV encounters a cell, hemagglutinin (HA), one of two primary surface glycoproteins, must bind with sialic acid, IAV's receptor molecule on the target cell [11]. Once HA has fused with sialic acid the virus becomes endocytosed and is now trapped within the endosome inside of the cell, where the endosome is directed to travel close to the nucleus [12, 13]. The endosome becomes acidic which activates the M2 proton channel protein [14]. The lipid viral envelope and endosome then fuse, and a pore is formed for the viral ribonucleoprotein particles (RNPs) to be released into the cytoplasm. The RNA is then translocated to the nucleus [12, 15]. While in the nucleus, transcription and replication of the viral genome begins. Once the genomic replication has been completed, the virus needs to make it to the cell's membrane to become enveloped. Neuraminidase (NA), the other primary surface glycoprotein, is an enzyme that helps the virus escape the infected host cell. NA cleaves the terminal α-sialic acid residues that remain on the cell's surface to prevent subsequent infection by daughter virions, as well as helping the newly formed virions move on to infect new cells or even new hosts [16]. Another suggested potential use of NA is that it may aid in HA fusion in human airway epithelium [17].

Naming and genomic changes. The two primary surface glycoproteins, HA and NA, are the method in which we name IAVs, for example H1N1 or H7N15. This is important, because IAVs are prone to antigenic drift and antigenic shift. Antigenic drift is recognized as random minor changes within the HA and NA protein structures [16]. These changes can be caused by subtle mistakes made during viral replication. In turn, antigenic shift is known as major changes in the in the protein structures. These changes are large reassortments of different IAV subtypes that happen when two different IAVs infect the same host cell at the same time. For instance, if the H1N1 and H7N15 viruses just mentioned infected the same cell, they could reassort creating

H1N15 or H7N1 viruses. Both drift and shift can result in disease outbreaks; however, shift is more likely to create combinations that the naïve human immune system has never seen. Therefore, antigenic shift is more likely to evade the immune response and result in pandemics, such as the 2009 H1N1 pandemic [16].

The Immune System and How it Recognizes IAV

    Innate Immune Cells. The immune system is comprised of two parts: the innate and the adaptive immune system. The innate immune system is the first line of defense of the two-phase immune system. Many cells are considered part of the innate immune system and are able to detect foreign particles, such as dust, viruses, bacteria, etc. These cells belong to a larger class of cells known as leukocytes, or white blood cells, and are derived from hematopoietic stem cells. Leukocytes include antigen presenting cells, such as dendritic cells and macrophages, as well as lymphocytes [18]. The response from the cells of the innate immune system is typically robust and indirect. For example, macrophages can be embryonic yolk-sac or embryonic hematopoietic stem cell (HSC) derived and persist as resident macrophages in the tissue of choice or can be recruited from HSC found in the bone marrow [19, 20]. Macrophages, also contain the inflammasome, which can promote the secretion of many pro-inflammatory signaling molecules, known as cytokines, which ultimately induce inflammation, circulate through the host, and potentially cause tissue damage. Macrophages are also phagocytes, known as "the big eater", which engulf foreign particles and digest them [21, 22]. Through Major Histocompatibility Complex (MHC) class I, which is present on all nucleated cells, and MHC class II, which is present on "professional" antigen presenting cells, the macrophage activates the adaptive immune system's lymphocytes [23]. Lymphocytes are a subset of leukocytes that

includes B and T cells. The T cell is activated through the junction of the T cell receptor and the MHC class I and II on the antigen presenting cell (APC) and further stimulated by cytokines (Figure 2) [24].

Pathogen Recognition and Inflammasome Activation. Pattern Recognition Receptors (PRRs) are present on the surface of epithelial and immune cells that discern the difference between self and non-self-materials. PRRs are poised to detect Pathogen Associated Molecular Patterns (PAMPs), such as viral RNA from IAV. Toll like receptors (TLR) are subsets of PRRs which contain leucine-rich repeat domains (LRRs) and upon stimulation send signals inside of the cell to stimulate an immune response [25]. TLR7, an endosomal receptor, is essential in identifying single stranded RNA (ssRNA). TLR3, another intracellular receptor, is a potent identifier of double stranded RNA (dsRNA) within the endosome and is valuable in mounting an immune response from respiratory tract cells [26]. Additionally, the activation of TLR3 and TLR7 can lead to the activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) [27]. Activation of either TLR3 or 7 triggers the signaling cascade with Myeloid differentiation primary response gene 88 (MyD88) with Interleukin-1 receptor associated kinase 1 (IRAK4) and TNF receptor-associated factor 6 (TRAF6), which ultimately activates NF-κB, which leads to the production of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α [28, 29]. Another NF-κB activator is the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). Unlike TLR3 and 7 in the endosome, RIG-I is important for sensing negative sense ssRNA of IAV in the cytoplasm. Once RIG-I senses the IAV's ssRNA, it interacts with the mitochondrial adaptor signaling protein, MAVS, which can then induce the production of type I interferons and other antiviral genes [30]. NF-κB and many diverse stimuli, including mitochondrial reactive oxygen species (ROS), are potent activators of the NOD-like receptor

containing pyrin 3 (NLRP3) inflammasome. The NLRP3-inflammasome (Figure 3) is initiated

by binding with the adaptor protein apoptosis-associated speck-like protein containing caspase

activation and recruitment domain (ASC) [31], which recruits pro-Caspase-1 and pro-IL-1β for

processing to their active forms [32, 33]. Active Caspase-1 induces pyroptosis, a form of

inflammatory programmed cell death, as well as causing the enzymatic cleavage of pro-IL-1β

and pro-IL-18 to their active forms of IL-1β and IL-18 [33, 34]. Both cytokines go on to create

an inflammatory response to infection.

The immune system and metabolism interface. Glycolysis is a well-regulated pathway

in which glucose-6-phosphate is broken down to produce ATP, NADH, and pyruvate. Pyruvate

then has two primary fates, oxidative phosphorylation or lactic acid production. When oxygen is

present, pyruvate is shuttled to the mitochondria where it enters the tricarboxylic acid cycle

(TCA) as Acetyl-CoA and can be used to produce more ATP (Figure 1). IAV increases glucose

uptake and aerobic glycolysis in infected cells to enhance glucose metabolism, which

demonstrates IAV's dependency on the host cell's machinery [35, 36]. Pyruvate can be

fermented to lactic acid, which has been shown to have antagonistic effects on RIG-I's anti-viral

functions by binding to MAVS [4]. An alternative branch of glycolysis is the pentose phosphate

pathway (PPP); the PPP is known to aid with the production of sugars that make up nucleic

acids, but not ATP. During IAV infection, we observe an enhancement in the PPP, as it helps the

virus replicate its own nucleotides, which results in a decrease of cellular protein production. In

addition to the decreased cellular proteins, a decrease in lipids is observed as well. Driven by the

virus's replication, cells increase the biosynthesis of lipids, as the virus takes a piece of the

phospholipid bilayer with it as it exits, and pro-inflammatory lipids increase in response to IAV

infection [35-37]. Overall, IAV infection diminishes a cell's resources to replicate itself and to

mount an immune response to the infection. Those resources are necessary for the cell to maintain homeostasis before, during, and after the cell is infected.

Questions and Hypotheses

To combat issues such as viral resistance, mutation, and antigenic drift/shift caused by the use of anti-viral treatments, other treatments need to be explored. As viral resistance gains a foothold and the virus variant spreads, it renders the old anti-viral treatment useless. Hence there is a need for more extensive research on universal metabolites, such as Pyr, and their ability to combat viral infections, such as IAV. Universal metabolites with antioxidant capacity could, potentially, be used prophylactically without the fear of anti-viral resistance. More extensive studies were, and are, necessary to determine the ability and efficacy of NaPyr to act as an immunomodulator and ameliorator of IAV symptoms, both *in vitro* and *in vivo*.

I proposed the two following hypotheses regarding NaPyr's ability to dampen the immune response to IAV infection:

Hypothesis one: Infecting murine bone marrow derived macrophages (BMDMs) with IAV and treating them with varying concentrations of NaPyr (2µL, 5µL, and 10µL) would yield differing immunomodulatory effects, because the additional NaPyr will fill the metabolic deficiency caused by the virus's replication preventing cellular damage and reducing immune signaling. Alternatively, treating cells with NaPyr may increase lactic acid, which subsequently inhibits RIG-I activation, as noted above, resulting in decreased immune signaling.

Hypothesis two: Since the treatment of IAV-infected BMDMs with NaPyr dampens the immune response, then I hypothesized that administering NaPyr to C57Bl/6J mice infected with IAV would lead to an improvement in health of infected animals and improved survival.
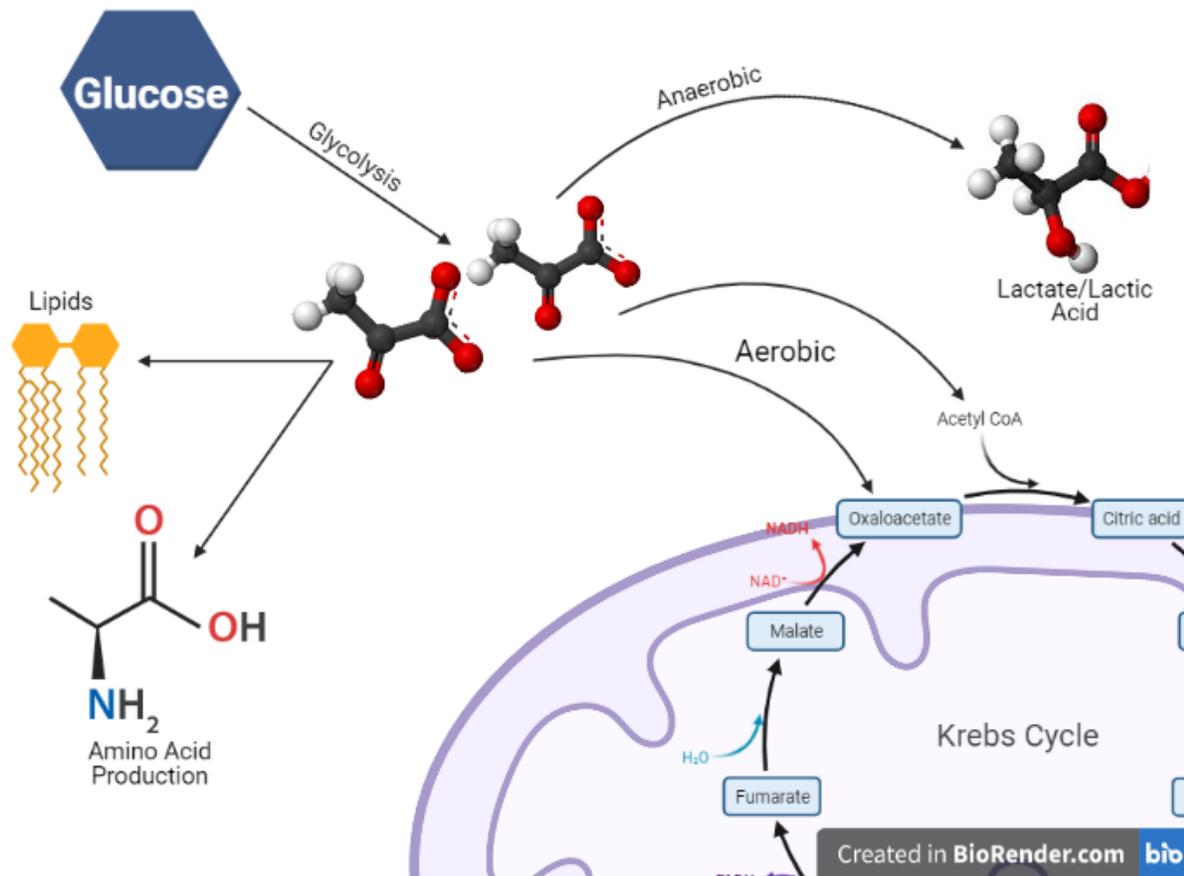
Figure 1: Pyruvate is produced by glycolysis and can be shuttled to produce many other things.
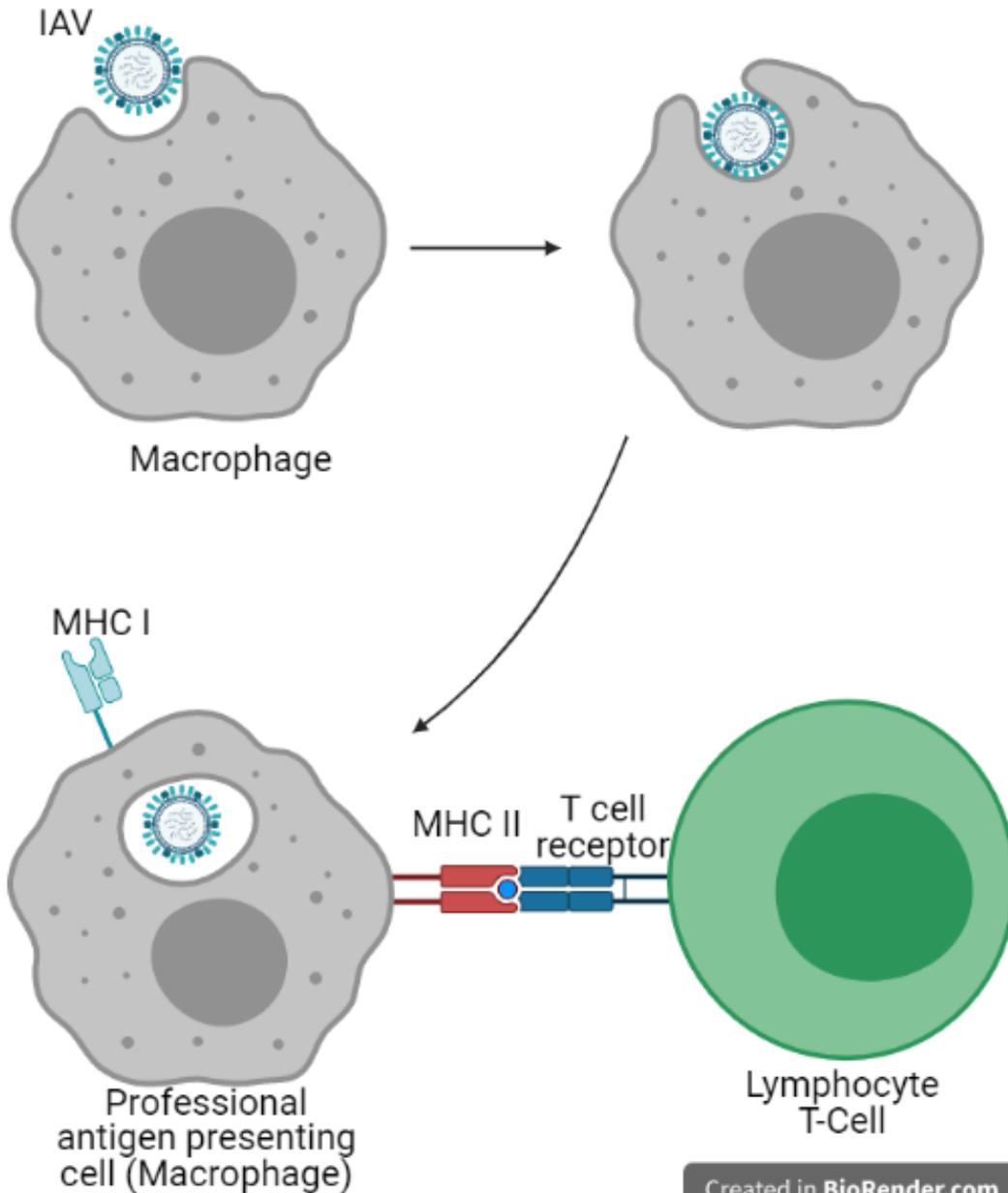
Figure 2: Macrophage (APC) phagocytosing and displaying IAV antigen to a T cell via the MHC II/T cell receptor complex.
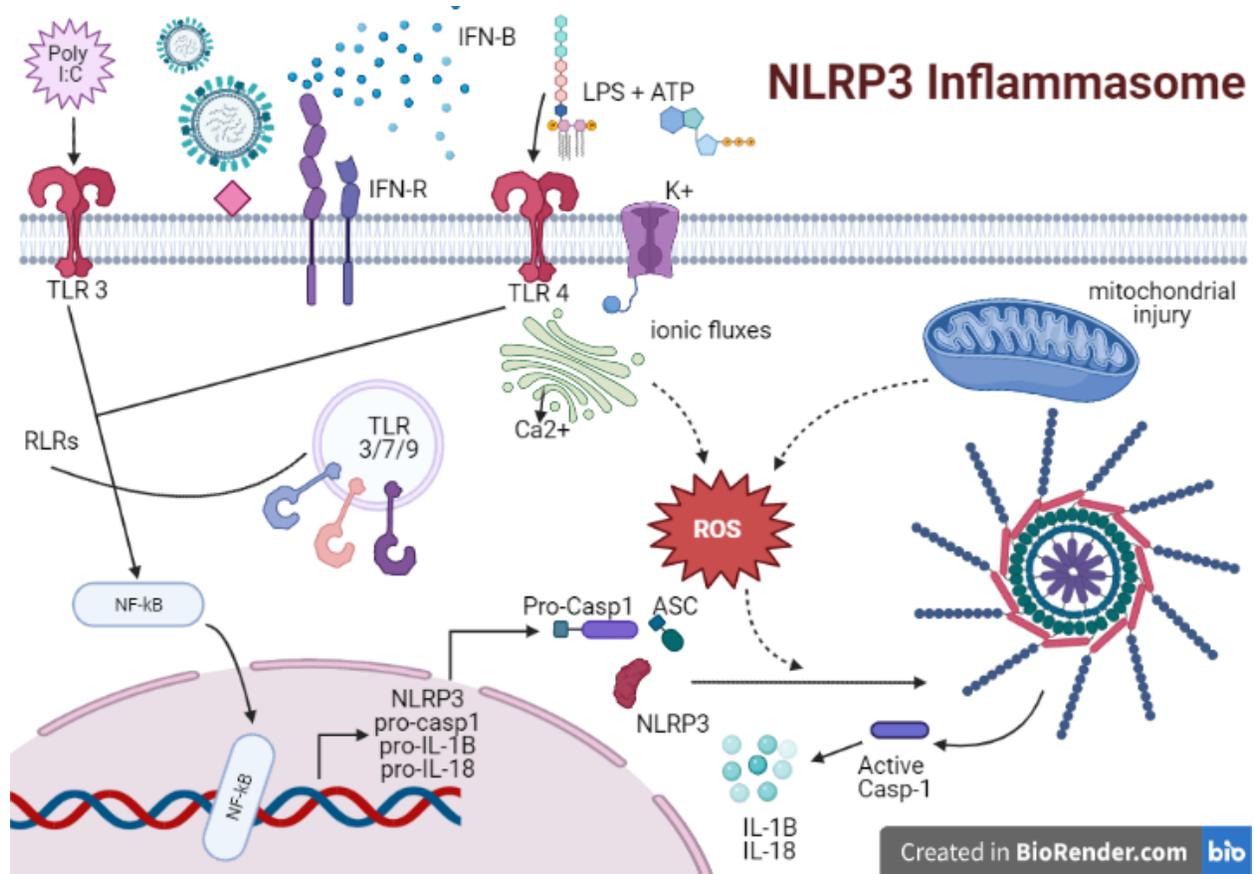
Figure 3: The NLRP3 inflammasome and how the 3 *in vitro* activators/infections initiate inflammasome induced inflammation.
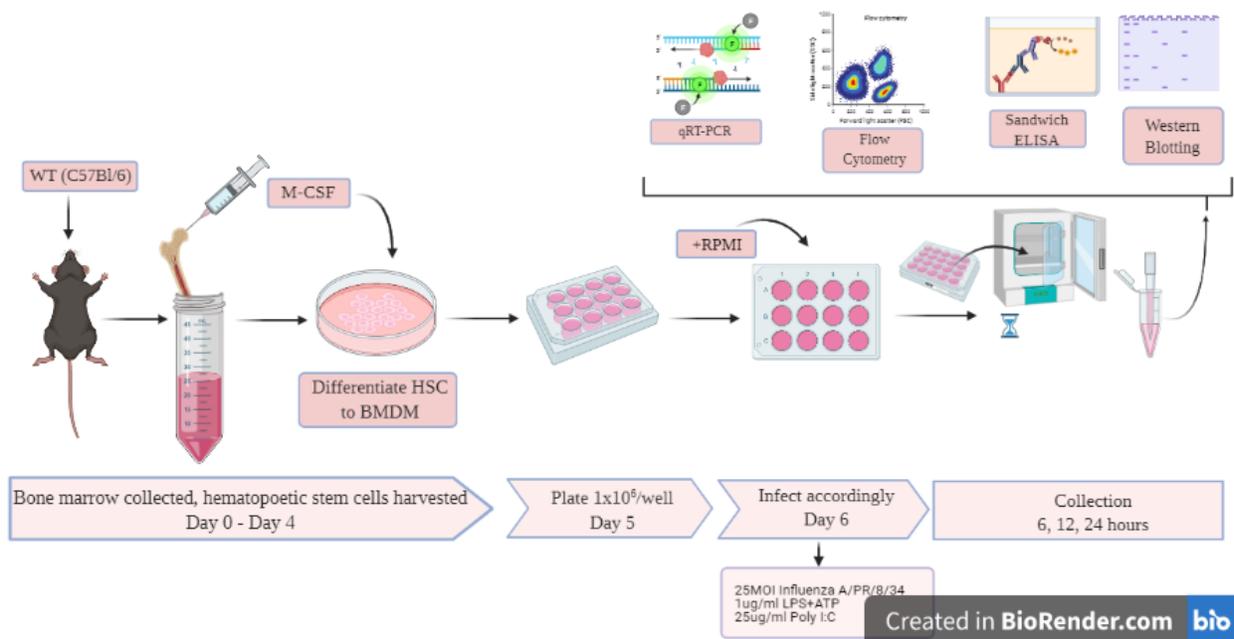
Figure 4: *In vitro* infection schemes to produce bone marrow derived macrophages, infect, and analyze samples.
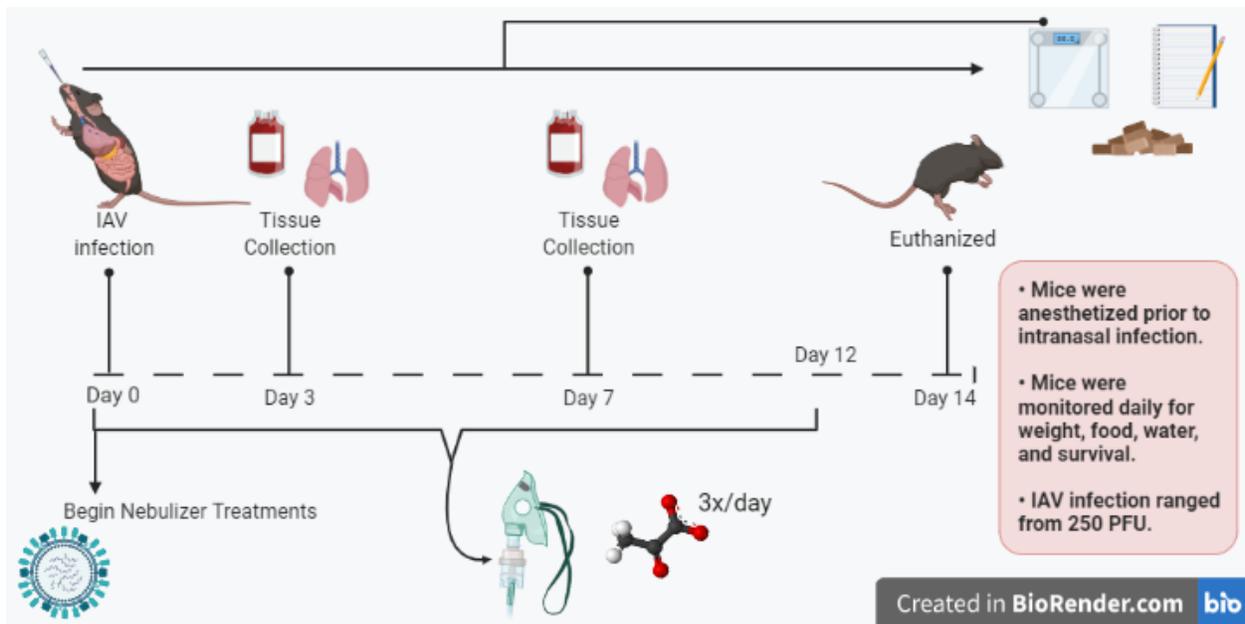
Figure 5: *In Vivo* schemes for nebulizer treatments and tissue collections.

PYRUVATE AFFECTS INFLAMMATORY RESPONSES OF MACROPHAGES DURING INFLUENZA A VIRUS INFECTION

Abstract

Pyruvate is the end product of glycolysis and transported into the mitochondria for use in the tricarboxylic acid (TCA) cycle. It is also a common additive in cell culture media. We discovered that inclusion of sodium pyruvate in culture media during infection of mouse bone marrow derived macrophages with influenza A virus impaired cytokine production (IL-6, IL-1β, and TNF-α). Sodium pyruvate did not inhibit viral RNA replication. Instead, the addition of sodium pyruvate alters cellular metabolism and diminished mitochondrial reactive oxygen species (ROS) production and lowered immune signaling. Overall, sodium pyruvate affects the immune response produced by macrophages but does not inhibit virus replication.

Introduction

Pyruvate (Pyr) ($C_3H_4O_3$) is a central molecule in cellular metabolism. In addition to the typical glycolysis-to-TCA pathway [1], Pyr can be derived from lactate taken up from outside the cells or synthesized intracellularly from amino acids [2, 3]. Instead of entering the TCA cycle, anaerobic glycolysis can occur (fermentation) where Pyr is reduced into lactate in order to regenerate NAD^+. In rapidly dividing cells, like some immune cells or cancer cells, this also occurs even when oxygen is present (aerobic glycolysis/Warburg effect) [4]. Although energetically less favorable, aerobic glycolysis facilitates metabolite production necessary for rapid cell division, such as amino acid and nucleic acid synthesis [5]. Reports have shown that IAV infection severely alters metabolism including amino acid and lipid metabolism [6].

The innate immune system has germline-encoded pattern-recognition receptors (PRRs). These sensors are capable of recognizing microorganisms that invade the host [7]. PRRs can bind to pathogen-associated molecular patterns (PAMPs) such as RNA from viral genomes [8]. Detection of PAMPs by PRRs activates a variety of immune signaling pathways resulting in cytokines production, increased phagocytosis and cell death. However, these responses can be modulated by metabolic processes. When retinoic acid inducible gene-I (RIG-I) is activated by cytoplasmic viral RNA, it moves to the mitochondria, where it interacts with mitochondrial antiviral signaling protein (MAVS) [9]. MAVS then recruits adaptors proteins at the mitochondria forming the MAVS signalosome, which activates the transcription factors IRF3/7 and NF-κB [10]. However, lactate can inhibit this pathway, thus dampening inflammation during viral infection [11].

The inflammasome is another immune signaling pathway that forms a multiprotein complex, which activates the cysteine protease caspase-1 [12]. Active caspase-1 then activates the inflammatory cytokines interleukin (IL)-1β and IL-18 [13]. Inflammasome activation by NOD-like receptor containing a pyrin 3 (NLRP3) is somewhat unique, as its main activation signals are cellular damage including oxidative stress and potassium efflux [14, 15]. Intriguingly, NLRP3 appears to be tuned-in to the metabolic state of cells through glycolysis [16, 17].

Pyr is well studied in metabolism, but its role in the immune response is not. During the course of infecting macrophages with IAV, we noted that different brands of cell culture media with different nutrient compositions affected the magnitude of the immune response. In particular, the inclusion of sodium pyruvate (NaPyr) in culture media inhibited immune signaling during IAV infection. Here we show that NaPyr added to BMDM cell culture media inhibits the release of important pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. In addition to these

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findings, we observed that addition of NaPyr does not inhibit viral replication, rather it suppresses the immune response to IAV through altering metabolism and ROS production.

Materials and Methods

Animal Welfare. WT C57BL/6J mice were bred and raised in the Temple Hall Vivarium at Missouri State University. Mice were euthanized via CO_2 asphyxiation and cervical dislocation, and bone marrow collected for differentiation into macrophages. All breeding and experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines (protocols 16.009, Appendix C and 19.019, Appendix A), the AVMA Guidelines on Euthanasia, NIH regulations (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal Welfare Act of 1966.

Generation of Bone Marrow Macrophages. Bone Marrow Derived Macrophages (BMDM) were produced by harvesting bone marrow from the femur and tibia of 7-14-week-old C57BL/6J mice. Bone marrow cells were then grown for 5 days in bone marrow differentiation media (BMDM media), which consisted of Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS + 1% Pen/Strep + 1% Non-essential amino acids (NEAA) and supplemented with L929 cell conditioned media. L929 cell conditioned medium contains Macrophage colony-stimulating factor (M-CSF) and was produced by growing L929 cells in DMEM+ 10% FBS+ 1% Pen/Strep for 10 days and then filtering the media via a 0.2μm filter.

On day 5 of BMDM growth, cells were scraped and re-plated into 12-well plates at $1x10^6$ cells/well in 1ml BMDM media and incubated overnight to allow cells time to adhere to the plates. Macrophages were used the following day for experiments as described below.

Virus Production. The strain of IAV used in all experiments is influenza A/PR/8/34 H1N1 (Appendix B). In order to generate virus, we inoculated pathogen-free hen's eggs with 1000 PFU of IAV. Three days post inoculation, the allantoic fluid was harvested, centrifuged to remove debris, and frozen at -80°C for later use.

Viral Plaque Assay. To determine the viral titer of NaPyr treated or non-treated IAV infected BMDM, IAV plaque assays were performed using Madin-Darby Canine Kidney (MDCK) cells seeded at $3x10^5$ cells/well in 12-well plates in DMEM+ 5% FBS+ 1%Pen/Strep. 10-fold dilutions of the virus were prepared in MEM without FBS. MDCK cells were washed with PBS twice and 100µl of each virus dilution was added to duplicate wells in 12-well plates and incubated at 37°C and 5% CO_2 for one hour. Semisolid overlay was prepared as previously described [18]. To allow for virus replication, TPCK-trypsin was added to a final concentration of 1.0 µg/ml. After the full hour of incubation, infection medium was removed from the 12-well plates, and 2ml of the warm overlay with TPCK trypsin was added to each well and allowed to solidify. Plates were turned upside down and incubated for 3 days. After 3 days, the overlaid agar was removed, and plaques counted after staining with 1% crystal violet in methanol.

Enzyme-Linked Immunosorbent Assay (ELISA). Cell culture supernatants collected from infected and control BMDM were analyzed for IL-1β, TNF- α, and IL-6. ELISA kits were purchased from Ebioscience (88-7013-88, 88-7324-88, 88-7064-88) and assays performed according to the manufacturer's recommendations. Plates were read at 450nm on a microplate reader (BioTek ELx808).

Western Blotting. Cell lysates were collected by adding RIPA buffer with protease and phosphatase inhibitors (Thermo Scientific, PIA32959, PIA32957) to BMDM treated and/or infected as indicated. 4x SDS loading dye was then added to samples, which were boiled for 20

minutes and resolved by SDS-PAGE and then transferred to PVDF membranes. Western blotting

for caspase-1 (caspase-1 p45 and p20), phosphorylated IκBα and total IκBα were then performed

by incubating membranes in primary antibody diluted in 5% milk in TBST overnight at 4 ^{0}C

(See Table 1 for a list of antibodies). The next day, membranes were washed 3x in TBST buffer

and incubated for 45 minutes in secondary antibody diluted in 5% milk in TBST (Table 1).

Membranes were washed again, and images obtained using Super Signal West Fempto substrate

(ThermoFisher, A53225) and an Azure C300 digital imaging system.

Cell Death and ROS. Macrophages were plated in 12-well plates and infected and/or

treated as indicated. After 24 hours, and 30 minutes before collecting samples, cells were stained

with a mitochondrial specific ROS sensitive dye (2.5nM Mitosox; ThermoFisher, M36008) or a

cell death stain (5 mM SYTOX-red; ThermoFisher, S34859). After 30 minutes, the media was

removed and 1ml of PBS was added to each well and the macrophages were scraped off the

wells. Cells were analyzed on an ACURI C6 or Attune NxT flow cytometer. Cells (10,000 per

sample) were analyzed for fluorescence intensity and percentage of cells positive for each dye.

Analysis of Gene Expression. BMDM were infected and treated as indicated and

samples were collected at 6, 12, and 24 hours after infection. Media was removed and 500µl

Trizol (Invitrogen, AM9738) was added to samples and incubated for 5 minutes at room

temperature. RNA was then isolated according to the manufactures protocol. All samples were

normalized to 200ng/µl RNA in nuclease-free water. The High Capacity cDNA Reverse

Transcriptase kit (Thermo Fisher Scientific 436881) was then used to convert 1ug RNA into

cDNA. Then, cDNA was diluted 1:5 in nuclease-free water, and 5 µl cDNA was used per

reaction to perform qRT-PCR with the DyNAmo HS SYBR Green qPCR master mix (Thermo

Scientific 00596849) according to the manufacturer's instructions using a STRATAGENE-Mx3005P PCR machine. (See Table 2 for Primer Sequences).

Examination of Cellular ATP. BMDM were infected and treated as indicated, with IAV infected samples collected at 12 and 24 hours post infection. LPS + ATP samples were collected at 4 hours post LPS treatment with ATP added for the final 30 minutes. Samples were analyzed using a StayBrite Highly Stable ATP Bioluminescence Assay kit (BioVision, K791-100) according to the kit instructions. To collect cell samples, the culture supernatant was removed, cells were washed 3x with 5ml DPBS, and then, 100µl of 1X RIPA buffer was added to each well. Cells lysate was collected and centrifuged at 12,000xg for 30 seconds. 10 µl of each cell lysate was pipetted into enzyme/buffer mix, and then analyzed quickly using the GloMax Jr. by ProMega on the GloBrite module, in RLUs.

Lactate Production Assay. BMDM were infected and treated as indicated and the supernatant collected at 24 hours post infection. The supernatant samples were then centrifuged at 14,000xg for 1 minute to remove cell debris. Samples were analyzed using the Eton Bioscience L-Lactate Assay kit I (SKU# 1200011002) according to kit directions. Plates were read at 490nm on a microplate reader (BioTek ELx808).

In Vitro Pyruvate Treatment. $1x10^6$ macrophages were plated in 12-well plates overnight. The next day, cells were washed 2x with PBS. Then, 200µl of RPMI 1640 without serum or NaPyr and with L-glutamine was added to BMDM. Then 2.5×10^7 PFU of IAV (25MOI) was added to some wells and other wells were left uninfected as controls. Uninfected controls and IAV infected BMDM were also either untreated or treated with 1mM NaPyr (HyClone, SH30239.01). Plates were incubated at 37 ^{0}C and 5% CO_2 for two hours with shaking. Then, 200µl RPMI +20% FBS was added to each well. The delayed addition of FBS is required

for IAV infection of BMDM. Additional NaPyr was added to appropriate wells to maintain the 1mM concentration. Samples were collected at 6, 12, and 24 hours after IAV infection.

To test the effects of NaPyr on the immune response of BMDM to other stimuli, $1x10^6$ BMDM were plated per well in 12-well plates. The next day, BMDM were washed 2x with PBS and 400µl of RPMI 1640 with L-glutamine + 10% FBS, but without NaPyr was added to each well. Some wells were treated with 1µg/ml LPS for 4 hours with inclusion of 5mM ATP (Sigma, L3129 and Acros, 102800100) for the last 30 minutes. Some wells were also treated with 1, 2 or 5mM NaPyr. Samples were collected at the end of 4 hours of treatment. Poly I:C samples were treated with 25µg/ml as indicated above and supernatants collected 24 hours post treatment.

Statistical Analysis. Statistical analysis was performed using GraphPad PRISM6. Comparison of 2 conditions was performed using the 2-sided student's t-test. Comparison of multiple conditions was performed using the One-Way ANOVA with Tukey's post-hoc test. A p-value <0.05 was considered statistically significant.

Results

NaPyr Affects the Immune Response, Not virus Replication. In discussion with other researchers (personal communication, Teneema Kurikose, St Jude Children's Research Hospital), we discovered the use of media from different suppliers impacted the magnitude of the immune response by bone marrow derived macrophages (BMDM) during IAV infection. Specifically, BMDM infected with IAV in Dubelco's Modified Eagle's Medium (DMEM) purchased from Sigma Aldrich (Catalogue # D5671) produced elevated cytokine responses compared to IAV infected BMDM cultured in DMEM purchased from ThermoFisher Scientific (Corning MT10013CV or Gibco11995040). We also noted that bone marrow derived dendritic

cells (BMDC) generally produce higher cytokine levels than BMDM in response to IAV infection, but BMDC are typically cultured in RPMI 1640. We examined the composition of these media and determined that NaPyr was associated with lower immune responses. Therefore, we infected BMDM with IAV in RPMI1640 medium with and without NaPyr. Our data demonstrate that addition of NaPyr significantly impaired cytokine production by BMDM infected with IAV (Figure 1A-C).

We next examined virus replication by collecting cell culture media from infected BMDMs 24h after infection and performing viral plaque assays. BMDM are refractory to infection with some strains of IAV [19], but similar levels of functional virions were recovered from BMDMs in our model with or without NaPyr treatment (Figure 1D). As virion production was low overall, via qRT-PCR we further confirmed that NaPyr did not affect virus growth or its ability to infect macrophages by examining viral RNA levels (IAV M1 and NP genes). NaPyr did not inhibit the replication of virus RNA, demonstrating that NaPyr does not affect cytokine responses by inhibiting IAV replication (Figure 1E-F). In addition, NaPyr treatment of IAV infected BMDM had no effect on cell death (Figure 1G).

NaPyr Inhibits immune signaling pathways. We examined cytokine gene expression by qRT-PCR at 6, 12, and 24h after IAV infection. NaPyr did have an inhibitory effect on gene expression in IAV infected BMDM compared to virus infected BMDM cultured in the absence of NaPyr (Figure 2A-D). We performed western blotting on cell lysates from BMDM infected with IAV and treated with NaPyr but did not observe any significant differences in the activation of NF-κB (phospho-IκBα) (Figure 2E-F). However, NaPyr inhibited caspase-1 activation in BMDM infected with IAV (Figure 2G-H).

To determine if NaPyr treatment broadly inhibited immune signaling, we treated BMDM with lipopolysaccharide (LPS) and adenosine triphosphate (ATP), which is a potent activator of the NLRP3 inflammasome [20]. Intriguingly, LPS+ATP treated BMDM cultured with NaPyr produced similar amounts of IL-1β, IL-6, and TNF compared to control infected cells (Figure 3A-C). Furthermore, caspase-1 activation was not inhibited, even at higher doses of NaPyr than used with IAV (Figure 3D-E).

To determine if NaPyr's anti-inflammatory properties were linked to viral ligands as opposed to bacterial ligands, we stimulated BMDM with the TLR3 ligand poly I:C (PIC). Interestingly, BMDM treated with PIC and cultured with NaPyr produced similar amounts of IL-6 compared to the untreated controls (Figure 3F).

Anti-inflammatory effects of NaPyr are associated with altered metabolism in BMDM. Previously, NaPyr was reported to be an antioxidant with potential therapeutic uses in a variety of inflammatory diseases [21, 22]. NLRP3 activation in many instances is dependent on reactive oxygen species (ROS) and mitochondrial damage [23-25]. NF-κB signaling can also be modulated by ROS [26]. Thus, we examined the antioxidant capacity of NaPyr in BMDM infected with IAV or LPS+ATP treated BMDM by staining with the mitochondrial ROS sensitive dye MitoSOX. IAV infection increased mitochondrial ROS generation, and addition of NaPyr lowered mitochondrial ROS during IAV infection (Figure 4A-B). During LPS+ATP treatment, mitochondrial ROS was elevated, but NaPyr had no effect on ROS in this setting (Figure 4A, C). These results indicate that NaPyr inhibits ROS in a context specific manner.

IAV replication requires a massive metabolic burst to produce not only the viral nucleotides and proteins for virus replication, but also the antiviral immune responses of the cell. Previous research has shown that IAV induces a unique and elevated catabolic profile including

increased lactate production [27]. Elevated lactate levels have been reported to inhibit RIG-I

signaling [11], which could explain our observations. We examined lactate production from

BMDM and observed a significant increase in lactate, a byproduct of elevated metabolism,

caused by IAV infection alone, but this was not enhanced by the addition of NaPyr (Figure 4D).

We thus hypothesized that NaPyr may fulfil a metabolic need during IAV infection, as opposed

to the formation of a byproduct. We examined intracellular ATP production by BMDMs infected

with IAV and found that IAV infection results in increased ATP levels over uninfected BMDM

or LPS+ATP treated BMDM (Figure 4E). Importantly, NaPyr treatment was able to transiently

boost ATP output from BMDM to match the need seen in IAV infected cells (Figure 4E). As the

ATP needs of IAV infected cells were not copied by LPS+ATP, NaPyr may specifically decrease

mitochondrial ROS during IAV infection by balancing metabolic stress.

Discussion

The ability of metabolites to affect the immune response to infection is an important area

of research with implications for preventing and treating disease. Recent research shows that

changes in metabolism in cells of the immune system can affect diseases such as influenza,

cancer, diabetes and more [28-30]. Our data clearly indicate that treatment of IAV infected

macrophages with NaPyr can reduce cytokines production (IL-1β, TNF-α, and IL-6). However,

NaPyr does not affect virus titer or RNA replication in macrophages. Instead, NaPyr alters the

immune function of the macrophages.

Antioxidants that can prevent mitochondrial damage also prevent NLRP3 inflammasome

activation and release of IL-1β from infected cells [23, 24]. Previous reports indicate that NaPyr

can decrease inflammation by its antioxidant potential [21, 31, 32]. Although NaPyr may

function as a ROS scavenger, we further propose that NaPyr reduces metabolic stress and ROS generation in an infection or disease specific manner. Specifically, pyruvate is taken into cells and bypasses many of the regulatory checkpoints for energy metabolism such as glucose transporters and phosphofructokinase [33, 34]. It can be directly transported into the mitochondria for use in the TCA cycle and ATP production or used in anabolic pathways [35]. Thus, addition of NaPyr to cells increases ATP production, as we observed, and likely affects additional metabolic pathways. In our model, we propose that decreased mitochondrial ROS is thus a secondary, but important, anti-inflammatory effect of NaPyr treatment. There are also additional factors that may affect the ability of NaPyr to inhibit other stimuli. In the case of LPS+ATP treatment, the treatment duration is much shorter than IAV (only 4 hours for LPS+ATP instead of 24 hours for IAV). Thus, intrinsic differences in the timing and pathways of the different stimuli may further impact the effects of NaPyr and should be examined further.

In conclusion, NaPyr affects cytokine production by inhibiting inflammatory signaling pathways and not by affecting virus growth or cell death in macrophages. Metabolic pathways are important for cellular activation and have documented roles in immune signaling and immune cell function [4, 6, 16, 17, 27]. Understanding the effects NaPyr has on the immune response to IAV and other infections will help elucidate the immune response in general and determine if certain nutrients can improve the immune response. Furthermore, severe IAV infection in human patients is associated with a metabolic crisis (especially depleted ATP) resulting in multi organ failure [36]. As pyruvate clearly increases ATP production, decreases ROS and limits inflammation during IAV infection, it is worth examining as a potential therapeutic option. Finally, severe infections with IAV and the current COVID19 pandemic are both associated with a "cytokine storm" that results in severe immunopathology [37, 38].

Corticosteroids are used in severe cases to suppress this overt inflammation. Importantly, the

only drug to date that has demonstrated clinical benefit for COVID19 is dexamethasone [39, 40].

Unfortunately, corticosteroids may leave the host susceptible to outgrowth of the initial pathogen

or to secondary infection [41]. As we observed no significant change in virus replication in this

model, NaPyr may have therapeutic benefit for severe IAV and other infections where excessive

inflammation is a key factor and this research warrants further investigation.

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Table 1. Antibodies.

Antibodies	Catalog Number
Rabbit anti-mouse β-Actin	Cell Signaling Technology, 8457S
Rabbit anti-mouse IκB-α	Cell Signaling Technology, 9242S
Rabbit anti-mouse Phospho-IκB-α	Cell Signaling Technology, 2859S
Mouse anti-mouse Caspase-1	Adipogen, 661228
Anti-Rabbit-HRP secondary	Jackson Immuno Res.111-035-144
Anti-mouse-HRP secondary	BioRad, HAF007

Table 2. Primer Sequences.

Primers	Sequence 5′--3′
B-Actin forward	GGCTGTATTCCCCTCCATCG
B-Actin reverse	CCAGTTGGTAACAATGCCATGT
IL-1β forward	GACCTTCCAGGATGAGGACA
IL-1β reverse	AGCTCATATGGGTCCGACAG
TNF-α forward	CATCTTCTCAAAATTCGAGTGACAA
TNF-α reverse	TGGGAGTAGACAAGGTACAACCC
IL-6 forward	TCCAGTTGCCTTCTTGGGAC
IL-6 reverse	GTACTCCAGAAGACCAGAGG
IFN-β forward	GCCTTTGCCATCCAAGAGATGC
IFN-β reverse	ACACTGTCTGCTGGTGGAGTTC
IAV M1 forward	TGAGTCTTCTAACCGAGGTC
IAV M1 reverse	GGTCTTGTCTTTAGCCATTCC
IAV NP forward	CTCGTCGCTTATGACAAAGAAG
IAV NP reverse	AGATCATCATGTGAGTCAGAC
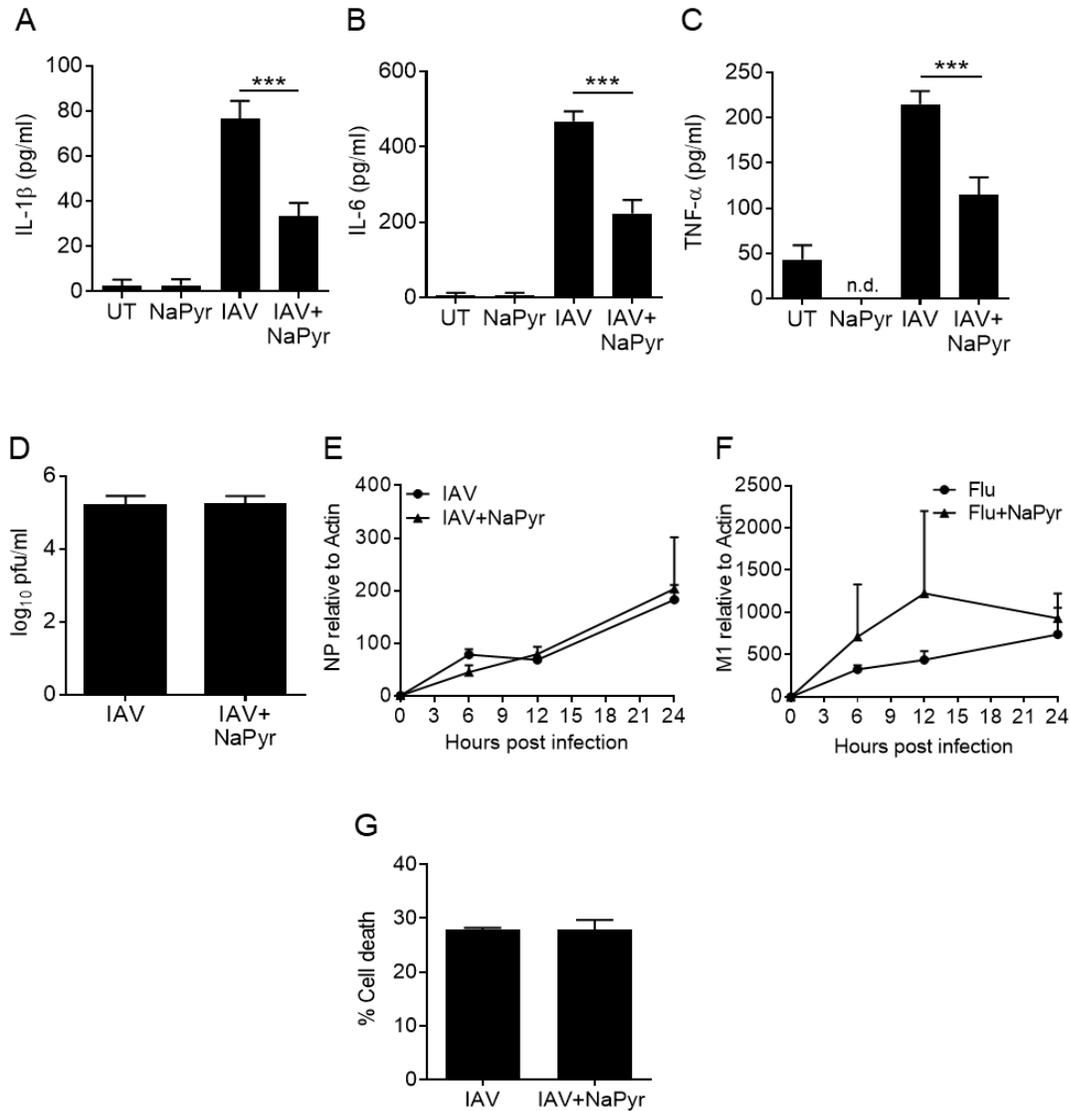
Figure 1: Pyruvate inhibits cytokine responses but not virus replication. BMDM were mock infected (untreated = UT) or infected with 10MOI influenza A/PR8/34/H1N1 virus (IAV) in the presence or absence of sodium pyruvate (NaPyr). After 24 hours, cell culture supernatants were collected and examined for cytokine expression by ELISA (A-C) or examined for virus titer by plaque assay (D). Total RNA was isolated from BMDM at the indicted time points after IAV infection in the presence or absence of NaPyr. RNA was transcribed into cDNA and qRT-PCR performed for the indicated viral genes (E-F). BMDM were infected for 24h in the presence or absence of NaPyr and stained with Sytox-Red then examined by flow cytometry for percentage of cell death (G). Data are representative of 2-4 independent experiments with n=2-3 wells per experiment. Statistical significance was determined using the students T-test for single comparisons, one-way ANOVA with Tukey post-hoc for multiple comparisons. *** $p < 0.001$
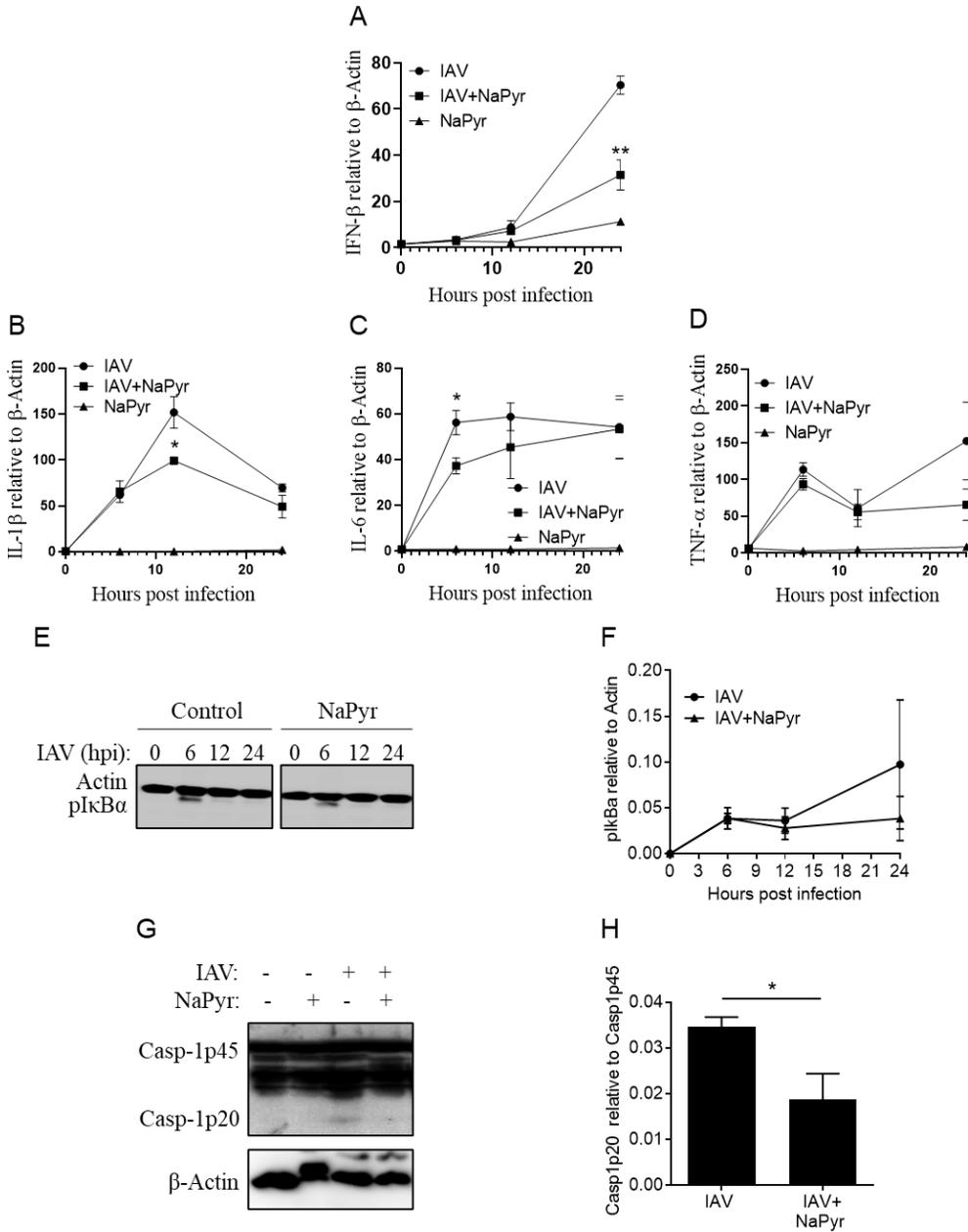
Figure 2: Pyruvate inhibits immune signaling during IAV infection.
BMDM were mock infected (untreated = UT) or infected with 10MOI IAV with or without NaPyr. Total RNA was isolated from BMDM at the indicted time points after IAV infection in the presence or absence of NaPyr. RNA was transcribed into cDNA and qRT-PCR performed for the indicated cytokine genes (A-D). Cell lysates were collected at the indicated time points after IAV infection of BMDM in the presence or absence of NaPyr and examined by western blotting for NF-κB activation (phosphorylated-IκBα) (E-F) or caspase-1 activation (Casp-1p20) after 24h (G-H). Data are representative of 2-4 independent experiments with n=2-3 wells per experiment. Statistical significance was determined using the students T-test for single comparisons, one-way ANOVA with Tukey post-hoc for multiple comparisons. * p<0.05, ** p<0.01
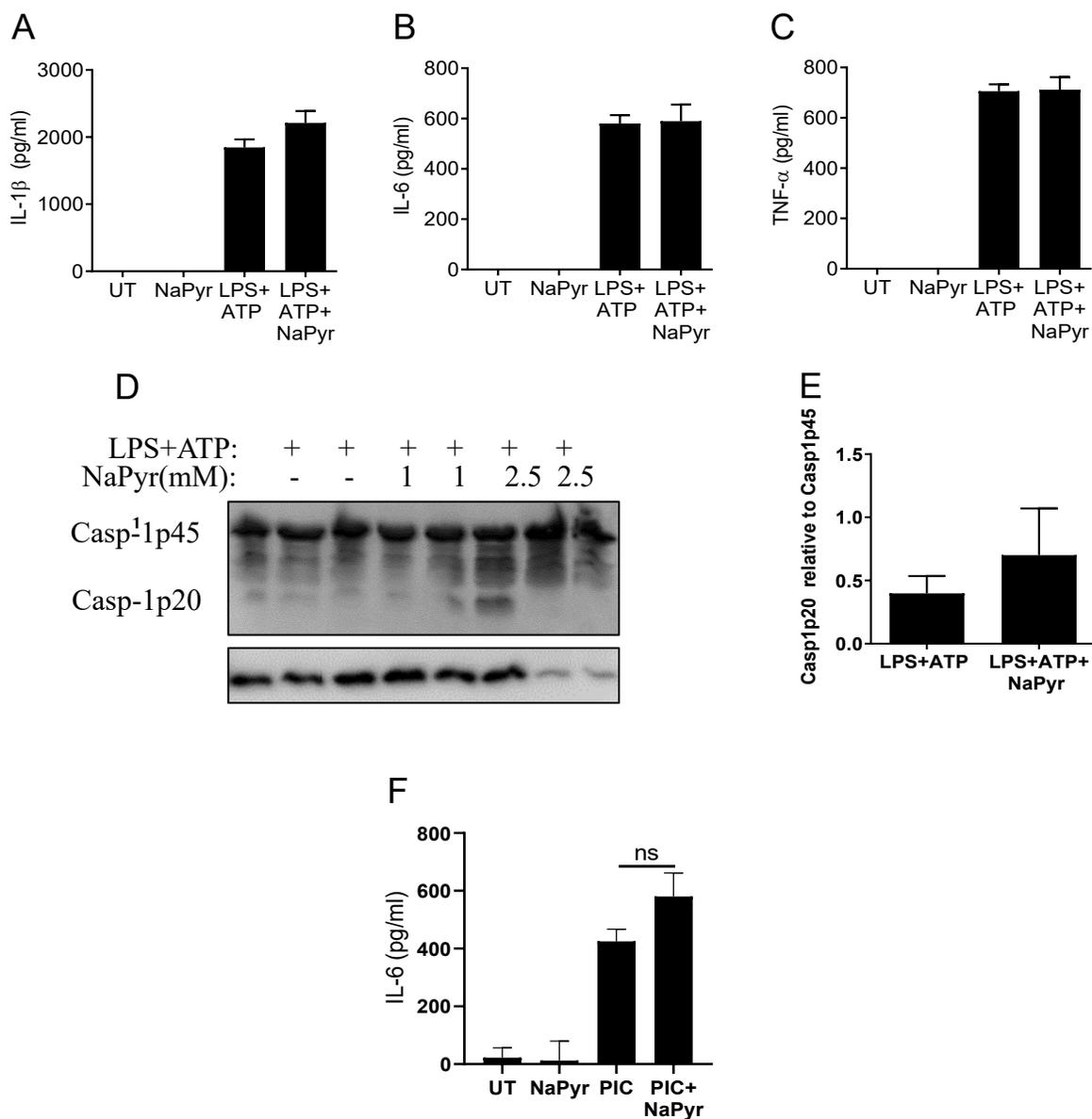
Figure 3: Immune responses to LPS+ATP are not affected by NaPyr.
BMDM were mock treated (untreated = UT) or treated with 1μM LPS for 3.5h and then treated with 5mM ATP for 0.5h in the presence or absence of NaPyr. Culture supernatants and cell lysates were collected after the total 4h treatment and examined by ELISA for cytokine production (A-C) or by western blot for caspase-1 activation (Casp-1p20) (D-E). BMDM were mock treated (untreated = UT) or treated with 25μM poly I:C (PIC) in the presence or absence of NaPyr (F). Data are representative of 2-4 independent experiments with n=2-3 wells per experiment. Statistical significance was determined using the students T-test test for single comparisons, one-way ANOVA with Tukey post-hoc for multiple comparisons. No results were significantly different.
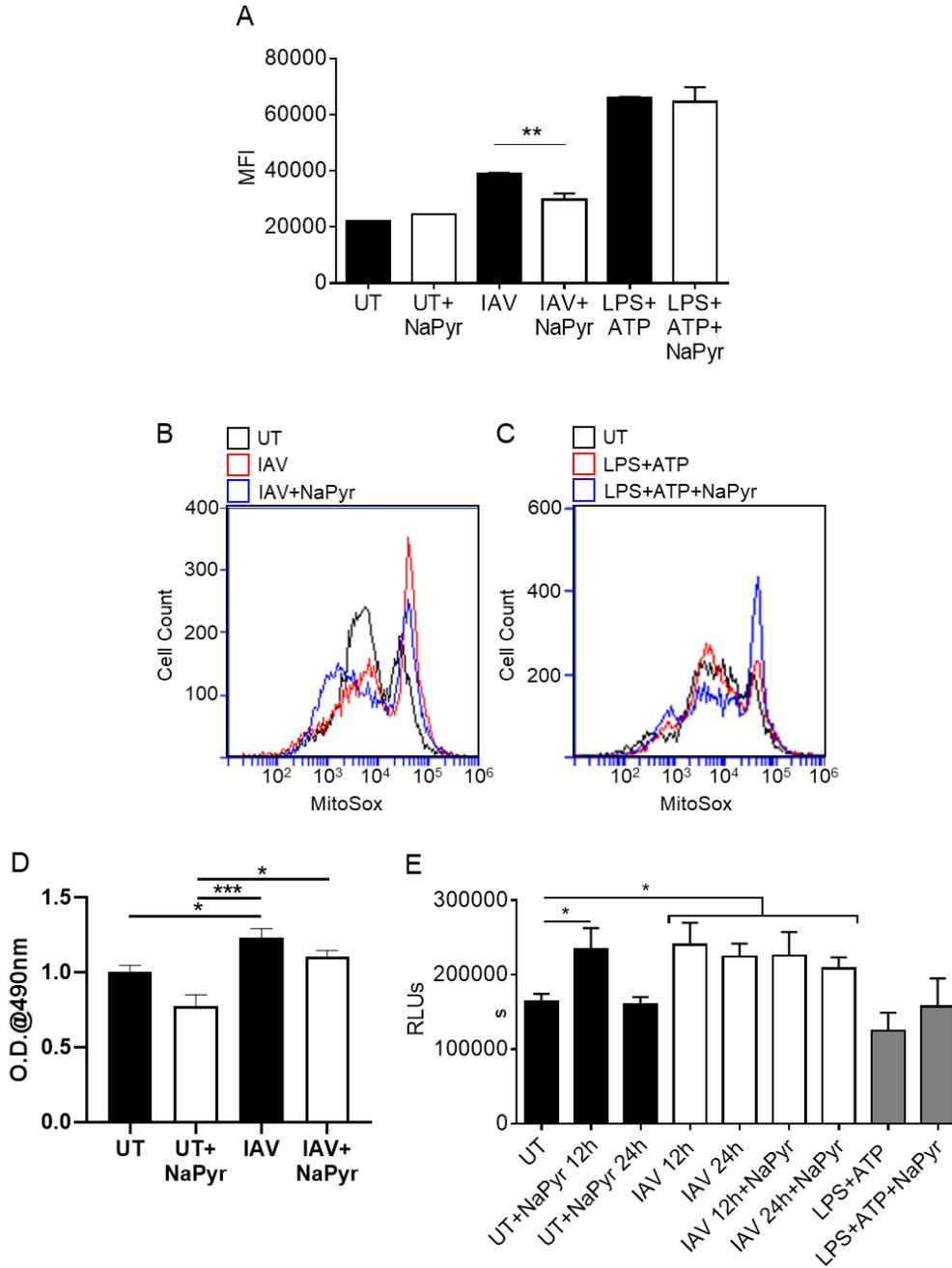
Figure 4: Altered immune signaling is associated with ROS and ATP levels.
BMDM were mock infected (untreated = UT) or infected with 10MOI IAV for 24h or treated with 1μM LPS for 3.5h and then treated with 5mM ATP for 0.5h, in the presence or absence of NaPyr. Cells were then stained with MitoSox and mitochondrial ROS levels determined by flow cytometry (MFI=Median Fluorescence Intensity) (A-C). Cell supernatants were examined for lactate (D) and lysates were also examined for ATP levels (E). Data are representative of 3-4 independent experiments with n=2-3 wells per experiment. Statistical significance was determined using the students T-test for single comparisons, one-way ANOVA with Tukey post-hoc for multiple comparisons. * p<0.05, ** p<0.01, *** p<0.001

SODIUM PYRUVATE AMELIORATES INFLUENZA A INFECTION IN VIVO

Abstract

Influenza A virus (IAV) causes seasonal epidemics annually and pandemics every few decades. Most antiviral treatments used for IAV are only effective if administered during the first 48 h of infection and antiviral resistance is possible. Therapies that can be initiated later during IAV infection and that are less likely to elicit resistance will significantly improve treatment options. Pyruvate, a key metabolite, and an end product of glycolysis, has been studied for many uses, including its anti-inflammatory capabilities. Sodium pyruvate was recently shown by us to decrease inflammasome activation during IAV infection of Bone-Marrow Derived Macrophages. Here, we investigated sodium pyruvate's effects on IAV *in vivo*. We found that nebulizing mice with sodium pyruvate decreased morbidity and weight loss during infection. Additionally, treated mice consumed more chow during infection, indicating improved symptoms. There were notable improvements in pro-inflammatory cytokine production (IL-1β) and lower virus titers on day 7 post-infection in mice treated with sodium pyruvate compared to control animals. We conclude that pyruvate acts on the host immune response and metabolic pathways, and not directly on the virus. Our data demonstrate that sodium pyruvate is a promising treatment option that is safe, effective, and unlikely to elicit antiviral resistance.

Keywords: pyruvate; inflammation; influenza A virus; antiviral

Introduction

Influenza A virus (IAV) causes seasonal epidemics and periodic pandemics with significant morbidity and mortality. In the 2019-2020 flu season, the United States Center for

Disease Control and Prevention (CDC) estimated 38 million IAV infections and 22,000 deaths. The most prevalent virus of the 2019-2020 season was the 2009 pandemic IAV (H1N1). Notably, during this season, was a higher rate of infections among children aged 0-4 and adults aged 18-49 years than in other recent seasons [1]. During pandemics, the emergence of novel viruses can cause severe complications with increased morbidity and mortality [2]. Due to the novelty of pandemic viruses, vaccines must be redesigned. Anti-viral therapies exist to treat IAV [3]. However, viral resistance to these therapies is always possible. Therefore, treatments that alter the host response to IAV infection and are less likely to result in evolution of resistance are desirable.

Studies have shown that IAV hijacks cellular metabolisms to increase viral replication [4, 5]. Pyruvate (Pyr) ($C_3H_4O_3$) is a central metabolite and key component in energy metabolism and cellular respiration. Pyr can enter directly into the mitochondria to produce ATP via the tricarboxylic acid cycle (TCA), which bypasses many of the metabolic regulatory pathways that control glycolysis [6, 7]. Mitochondrial oxidative phosphorylation is the most efficient way to produce ATP for cells. However, Pyr can also be used to make amino acids or be reduced to lactate via fermentation or the Warburg Effect [8, 9]. Reduction of Pyr is used to replenish NAD^+ and increase uptake of necessary nutrients for rapidly dividing cells, such as immune and cancer cells [8-10]. The end goal is rapid proliferation, not energy efficiency, in most of these cases [10].

Pyr in its many forms (ethyl Pyr, pyruvic acid, pyruvate anion, sodium pyruvate, etc.) has been found to have many antioxidant-like benefits in several body's systems. The molecule seems to be well tolerated in the body with little to no toxicity [11]. Ringer's ethyl pyruvate has been used primarily for its increased stability in solution, however hypertonic sodium pyruvate

has been found to more effectively protect against inflammation and stress during injury events [12]. Additionally, Pyr has been found to have a plethora of beneficial effects on the cardiac system [13-15]. Moreover, increasing extracellular Pyr in the brain has been found to decrease neuronal death during traumatic brain injury events [16, 17] and be protective against neurotoxic compounds [18]. When administered to mice of various ages, Pyr increased glycogen stores and brain energy metabolites, which could help with diseases such as Alzheimer's [19]. Also, Pyr decreases epithelial permeability, inflammation, and bacterial translocation during intestinal ischemic reperfusion (I/R) events [20, 21] Decreased damage during I/R events has also been shown to be beneficial during organ transplantation because of decreased organ damage [22] and damage caused by rejection following transplantation [23]. In addition to having benefits in body systems, NaPyr has been shown to help with organ storage for transplant surgeries by decreasing cell death, overall improved metabolism during cold storage [24, 25], and increasing graft metabolism [26]. Red blood cell (RBC) storage has been known to generate reactive oxygen species (ROS), but the addition of NaPyr to the storage media decreases ROS and increase antioxidant enzymatic, SOD, activity which leads to an overall higher RBC recovery rate [27]. RBCs stored with varying levels of Pyr maintains 2,3-DPG production for longer periods of storage [28]. Bone and tissue inflammation models have shown that Pyr treatment leads to less destructive disease via anti-inflammatory properties [29, 30]. In relation to infectious disease, sodium pyruvate (NaPyr) ($C_3H_4NaO_3$) can improve herpes simplex 2 virus infection *in vivo*, and our lab recently reported that NaPyr can regulate inflammation during IAV infection *in vitro* [31, 32].

In our previous study, we observed in mouse bone marrow derived macrophages (BMDM) that NaPyr has anti-inflammatory capabilities through altered metabolism [31]. The

addition of NaPyr to BMDM decreased mitochondrial damage in response to IAV infection. These findings led us to further investigate NaPyr's potential anti-viral and anti-inflammatory capabilities in a mouse model of IAV infection. Here we show that nebulizing NaPyr *in vivo* in WT C57BL/6J mice leads to decreased weight loss and increased chow intake over the course of IAV infection. Seven days p.i., animals treated with NaPyr displayed decreased pro-inflammatory cytokines (IL-1β) in the lungs and decreased virus replication.

Materials and Methods

Animal Welfare. WT C57BL/6J mice were bred and raised in the Temple Hall Vivarium at Missouri State University (Appendix C). Mice were euthanized via CO_2 asphyxiation and cervical dislocation or cardiac puncture at humane end points for tissue collection. All breeding and experiment protocols were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines (protocols 19.005, Appendix C and 19.019, Appendix A), the AVMA Guidelines on Euthanasia, NIH regulations (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal Welfare Act of 1966.

Virus Production. The strain of IAV used in all experiments was influenza A/PR/8/34 H1N1 (PR8) (Appendix B). PR8 stocks were generated by infecting pathogen-free hen's eggs with 1000 PFU of PR8. Following a 3-day incubation, the allantoic fluid was harvested, centrifuged to remove debris, and stored at -80°C for later use.

In Vivo Infection and NaPyr Treatments. Mice were anesthetized on Day 0 via intraperitoneal injection of 80 mg/kg of Ketamine and 8 mg/kg of Xylazine. Mice were then infected intranasally with approximately 250 PFU of influenza A/PR/8/34 H1N1, diluted in 30 mL of phosphate buffered saline (PBS). Mice used for subcutaneous (Sub-Q) injections of NaPyr

were injected with 110mg/kg of body weight daily, divided into two doses, morning and evening. Mice that were treated with nebulized NaPyr were treated with Emphycorp's clinical grade N115 (20mM NaPyr), or with 10 mM NaPyr (Fisher Bioreagents, BP356-100) diluted in PBS, or treated with PBS alone as control. Mice were treated three times a day for 20-minute per treatment. All mice were monitored for food/water availability and weighed daily for weight loss and/or becoming moribund. Mice were euthanized on day 14, or day of sacrifice for tissue samples. Chow intake was also monitored by weighing the food daily and averaging the change in food mass by the number of animals per cage.

Tissue Collection and Processing. Mice sacrificed for tissue samples on day 3 and day 7 p.i. were euthanized via CO_2 asphyxiation and cardiac puncture as an adjunct. Lungs were taken from sacrificed mice for processing. Lungs were weighed and homogenized through a 70 mm cell strainer (Fisherbrand, 22363548) with a final volume of 4 mL of RPMI 1640 without serum and without NaPyr (Hyclone, SH30027.01) per tissue sample. Samples were then centrifuged and aliquoted for future use.

Flow Cytometry for Innate and Adaptive Immune Cells. Lung homogenates were centrifuged at 400xg for 7 minutes to achieve cell pellet. After removal of the supernatant for other assays, red blood cells were lysed with ACK lysis buffer. Dead cells and debris were then removed by centrifugation in 37.5% Percoll (GE Healthcare, 17-0891-02) at 2000g for 20 minutes. Cells were then stained with fluorescent antibodies (Table 1). Samples were run on the Accuri C6 Flow Cytometer.

Viral Plaque Assay. The IAV plaque assay was performed using MDCK cells seeded at $2x10^5$ cells/well in 12-well plates in DMEM+ 5% FBS+ 1%Pen/Strep. Ten-fold dilutions of the virus were prepared in RPMI 1640. MDCK cells were washed with PBS twice and 100µl of each

virus dilution added to wells in 12-well plates and incubated at 37°C and 5% CO_2 for one hour. Semisolid overlay was prepared as previously described [33]. TPCK-trypsin was added to a final concentration of 1.0 µg/ml. After a full hour of incubation, infection medium was removed from 12-well plates, 2ml of the warm overlay with TPCK trypsin was added to each well and allowed to solidify. Plates were turned upside down and incubated for 3 days at 37°C and 5% CO_2. After incubation, the overlay was removed, and plaques counted after staining with 1% crystal violet in formalin.

Enzyme-Linked Immunosorbent Assay (ELISA). Supernatant from homogenized lung tissue samples were analyzed for IL-1β and IL-6. ELISA kits were purchased from Ebioscience (88-7013-88, 88-7064-88) and assays performed according to the manufacturer's recommendations. Plates were read at 450nm on a microplate reader (BioTek ELx808). Cytokine levels were normalized to lung mass/ml.

Statistical Analysis. Statistical analysis was performed using GraphPad PRISM9. For *In Vivo* weight loss and chow consumption during experiments, a two-way ANOVA was performed (time and treatment effects). For viral titer, cytokine and cell populations analysis, a Student's t-test was performed. A p-value <0.05 was considered statistically significant.

Results

NaPyr is not toxic In Vivo. N115 is a clinical grade nasal spray containing 20mM NaPyr that has undergone safety and phase I, phase II and phase III clinical trials. The FDA is currently reviewing the administration of N115 for use in COPD patients with Idiopathic Pulmonary Fibrosis, or Idiopathic Pulmonary Fibrosis Patients alone (EmphyCorp, Cellular Sciences Inc FDA submissions). Patient surveys indicated that use of N115 may decrease the incidence,

symptoms, and duration of respiratory infections too. Millions of patients have been treated with N115 nasal spray in over 200 hospitals globally, which includes pregnant women, patients with allergic rhinitis, COPD patients, sinusitis, and patients with pulmonary fibrosis, with no adverse events reported. The use of the nasal spray in these patients demonstrates its safety and efficacy and the ability of NaPyr to reduce nasal congestion and inflammation. In a Phase III Placebo Controlled Clinical Trial with Idiopathic Pulmonary Fibrosis Patients, the N115 nasal spray demonstrated a statistically and clinically significant increase in nasal nitric oxide, the volume of air exhaled at the end of the first second of forced expiration (FEV-1), SaO2, the volume of air exhaled during forced expiration (FVC), FEV-1/FVC ratios (52% to 86%). N115 reduced hypoxemia, and it also reduced lung inflammation, inflammatory cytokines, and coughing. Other studies confirm the safety of supplementation with NaPyr [11, 17, 34].

Based on these promising data, we sought to examine the effectiveness of NaPyr for treatment of IAV infection. We conducted preliminary toxicity experiments using nebulized NaPyr at 10mM and 1M concentrations made in-house using Fisher Bioreagents NaPyr (BP356-100) diluted in PBS, or just PBS for a control. We found no noticeable weight loss in mice treated with 10mM NaPyr. However, 1M NaPyr treatment did show some slight decline in weight of mice, but this was likely due to the cloud produced by nebulizing 1M NaPyr, which was thick like chalk dust and difficult to breathe (Figure 1A). Overall, NaPyr was not found to be toxic at the concentrations used for the treatment of IAV infected mice.

Nebulized NaPyr improves weight loss in IAV infected mice. Our previous research with NaPyr *in vitro* established its immunomodulatory properties [31]. We, therefore, examined its effects in mice infected with IAV. WT C57BL/6J mice (n = ???) were infected with 250PFU of influenza A/PR/8/34 H1N1 virus and injected sub-cutaneously (Sub-Q) with 55mg/kg of

NaPyr twice a day for 14 days (n = ??) and compared to PBS injection controls (n = ??). Although injection of NaPyr resulted in increased food intake early and late during infection, it did not significantly improve weight loss in IAV infected mice (Figure 2A-B). Thus, we began looking for an alternative, more direct, administration method during infection. Aerosols are used frequently for treatment of lower respiratory infections, more specifically, viral pneumonia [35]. Hence, we hypothesized that a nebulization model would be a more direct route to the site of infection. treating mice three times a day with nebulized 10mM NaPyr for approximately 15-minute intervals, resulted in less weight loss and increase in food intake (Figure 2C-D:these data are combined from 2 independent experiments with n=4-5 mice per treatment group per experiment). Two-way ANOVA $p=0.0399$, 0.0043, 0.0116, and 0.0363 for days 5-8 in Figure 2C, Two-way ANOVA $p=0.0199$ and 0.0093 for days 2-3 in Figure 2D in relation to indicated treatment).

N115 decreases weight loss and increases chow intake during IAV infection. As stated above, EmphyCorp manufactures a stable 20 mM NaPyr nasal spray (N115). In Phase I/II/III clinical trials, N115 has demonstrated promising results in decreasing lung inflammation in COPD and Idiopathic Pulmonary Fibrosis patients. Using N115, we examined potential toxicity, but observed no difference in weight loss between uninfected WT C57BL/6J mice treated with N115 compared to PBS controls (Figure 3A). Next, we examined weight loss in mice infected with 250PFU of influenza A/PR/8/34 H1N1 virus and treated three times a day for 20-minute intervals. Our results indicate that nebulizing mice with N115 over the course of 12 days of IAV infection decreased weight loss (days 7-14) and increased chow intake (days 9-10), compared to the PBS controls (Figure 3B-3C). (Weight loss and chow intake data are combined from 3 independent experiments with n=4-6 mice per treatment group per independent

experiment. Two-way ANOVA p=0.0127, 0.0012, 0.0002, <0.0001, 0.0005, 0.0046, 0.0233, 0.0311 for days 7-14 respectively in Figure 3B. Two-way ANOVA p=0.0492 and 0.0335 for days 9-10 in Figure 3C when looking at treatments day by day).

As N115 improved weight loss, we next examined the cause for improved weight loss. We investigated viral titers by plaque assay on Day 3, before weight loss, and Day 7 p.i., just as the N115 treated mice started to show improvement in weight loss. We found that there was significantly less virus in the lungs of N115 treated mice compared to PBS treated controls on Day 7 (Figure 3D-E). (Viral titer data are combined from 2 individual experiments with 3-5 mice per treatment group per experiment. Statistical significance was determined using a Student's T-test, p=0.0172 for figure 3E.)

As previously reported *in vitro* [31], we also observed significantly less IL-1β levels in the lungs of N115 treated mice (Figure 4A-B). Despite lower IL-1β levels, most leukocyte numbers were similar in the lungs of N115 and PBS treated mice, except for inflammatory monocyte numbers, which were elevated in the N115 mice (Figure 4C-D). Overall, N115 appears to decrease disease during IAV infection by decreasing virus titers and lowering inflammatory cytokine levels. (Data in Figures 4A-D are combined from 2 independent experiments with n=3-5 mice per experiment. Statistical significance was determined using a Student's T-test. Figure 4A p=0.00672 for IL-1β; Figure 4B p=0.0351 for IL-1β; Figure 4C p=0.0442 for inflammatory monocytes).

Discussion

Due to the evolution of antiviral or antibiotic resistance, the development of therapies that target host pathways to disrupt pathogen replication or disease is an avenue worthy of exploration. Some cellular metabolites can alter inflammation or pathogen replication, but our data suggest that the route of administration is important. Our data indicates that sub-Q injection of NaPyr does not influence IAV induced weight loss. However, nebulizing NaPyr does have a significant impact on weight loss, virus titer, and cytokine production during IAV infection *in vivo* in mice. Since Pyr can be rapidly absorbed by virtually any cell, injected NaPyr is likely taken up by other cells before reaching the target cells in the IAV infected lung [36, 37]. Most IAV antiviral treatments target specific proteins within the virus. These proteins are prone to mutations and resistance to such drugs. Certain strains of IAV are known to be resistant to the M2 and neuraminidase inhibitors [38]. Since NaPyr affects cellular metabolism and inflammation instead of directly targeting virus replication, there is a much lower chance that the virus will develop resistance to NaPyr treatment. Influenza and COVID-19 are known to cause mortality and morbidity in the elderly and immunocompromised. However, it is often forgotten that both diseases afflict children, usually with mild symptoms. In rare cases, there is mortality caused by complications during IAV infection. Seasonally, influenza causes 7,000-26,000 hospitalizations in children under five years old [39]. COVID-19 this year has resulted in 3,240 hospitalizations in school-aged children [40]. As of 23-Feb-2021, 51 children, aged less than 18, have died in the United States from complications with COVID-19 [40]. Comparatively, the CDC has reported a range of 37-188 deaths annually in children under five years old from complications caused by influenza infections [39]. Our data in this manuscript clearly demonstrate that N115 improves influenza disease. Furthermore, we have preliminary data that

suggest it may work similarly during other respiratory virus infections including COVID19/SARS-CoV-2. Proactive treatment with NaPyr is not toxic and could be of benefit to children that are afflicted by many respiratory viruses.

As previously noted *in vitro*, the addition of NaPyr reduces mitochondrial ROS, in turn reducing mitochondrial damage during the course of IAV infection [31]. This reduction could indicate decreased NLRP3 inflammasome activation, which would further explain why IL-1β is lower in the mice treated with N115. Further studies would be necessary to verify that NaPyr is modulating the NLRP3 inflammasome *in vivo*. However, *in vitro* we demonstrated that addition of NaPyr to the infection media of BMDMs led to lowered caspase-1 activation and decreased pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) [31]. Presumably, this would be a similar mechanism *in vivo* to be explored with mice treated with N115. Future directions would include investigating whether nebulized NaPyr:

    a) influences lasting immunity following IAV infection,

    b) alters or diminishes lung damage caused by IAV infection,

    c) protects against severe disease cases,

    d) is acting as an immunomodulator as we demonstrated *in vitro*.

It is also not clear how NaPyr affects virus replication. We reported that NaPyr does not affect virus replication in macrophages *in vitro* [31], and we have examined IAV replication in MDCK cells *in vitro* and found no effect of NaPyr either (data not shown). One possible explanation is that NaPyr alters immune cell function, such as enhanced T_h1 responses. This would agree with the timing observed in our experiments where virus titers were only lower on Day 7 and weight loss was improved after day 7 too. Alternatively, NaPyr may elicit a response from respiratory epithelial cells that is antiviral such as NO production or increased interferon

responses. Duplicating the viral replication in both respiratory epithelial cell lines and other innate immune cells is needed to further address these questions. Another potential avenue would be to explore carbon tracing to determine where NaPyr is being shuttled during the IAV infection. More studies would have to be done to confirm NaPyr, or N115's ability to ameliorate IAV infection in other model organisms such as ferrets. Notably, N115 is currently in human clinical trials for a multitude of inflammatory lung diseases including chronic obstructive pulmonary disorder and COVID-19. These clinical trials may also shed light on the mechanisms involved during IAV infection.

In conclusion, we show that nebulizing mice with sodium pyruvate decreased morbidity and weight loss during infection. Additionally, treated mice consumed more chow during infection indicating improved disease symptoms. There were notable improvements in pro-inflammatory cytokine production (IL-1β) and lower virus titers on days 7 post infection (p.i.) in mice treated with NaPyr compared to control animals. As NaPyr appears to act on the host immune response and metabolic pathways and not directly on the virus, sodium pyruvate is a promising treatment option that is safe, effective, and unlikely to elicit antiviral resistance.

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Table 1. Fluorescent antibodies used for FACS staining in preparation for flow cytometry.

Fluorophore	Monocyte Stain	Cat#	Lymphocyte Stain	Cat#
FITC	CD11c	35-0114-U100	CD4	35-0042-U100
PE	Gr1	50-5931-U100	CD8	100707
PerCP 5.5	CD3ε	65-0031-U100	CD3ε	65-0031-U100
APC	CD11b	20-0112-U100	CD19	115511

Antibodies were purchased from Tonbo (San Diego, CA, USA) or Biolegend (San Diego, CA, USA).
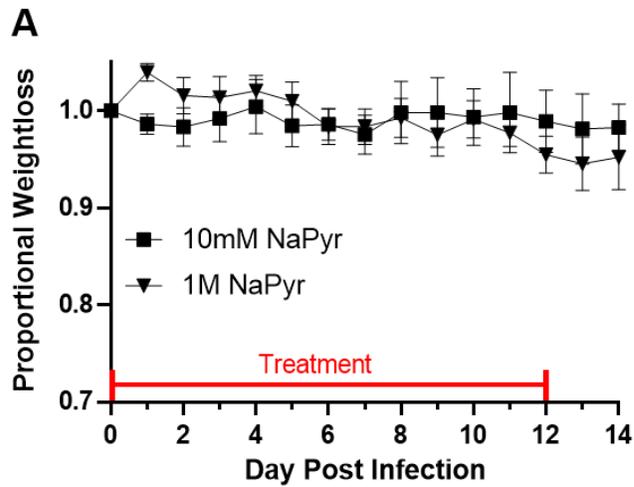
Figure 1. Sodium pyruvate (NaPyr) shows no toxicity in mice. WT C57BL/6J mice were nebulized 3 times daily for 15 min per treatment with 10 mM and 1 M concentrations of NaPyr diluted in phosphate buffered saline (PBS) for 14 days to determine toxicity and weight differences between treatment groups. Data are representative of one experiment with $n = 5$ mice per treatment group.
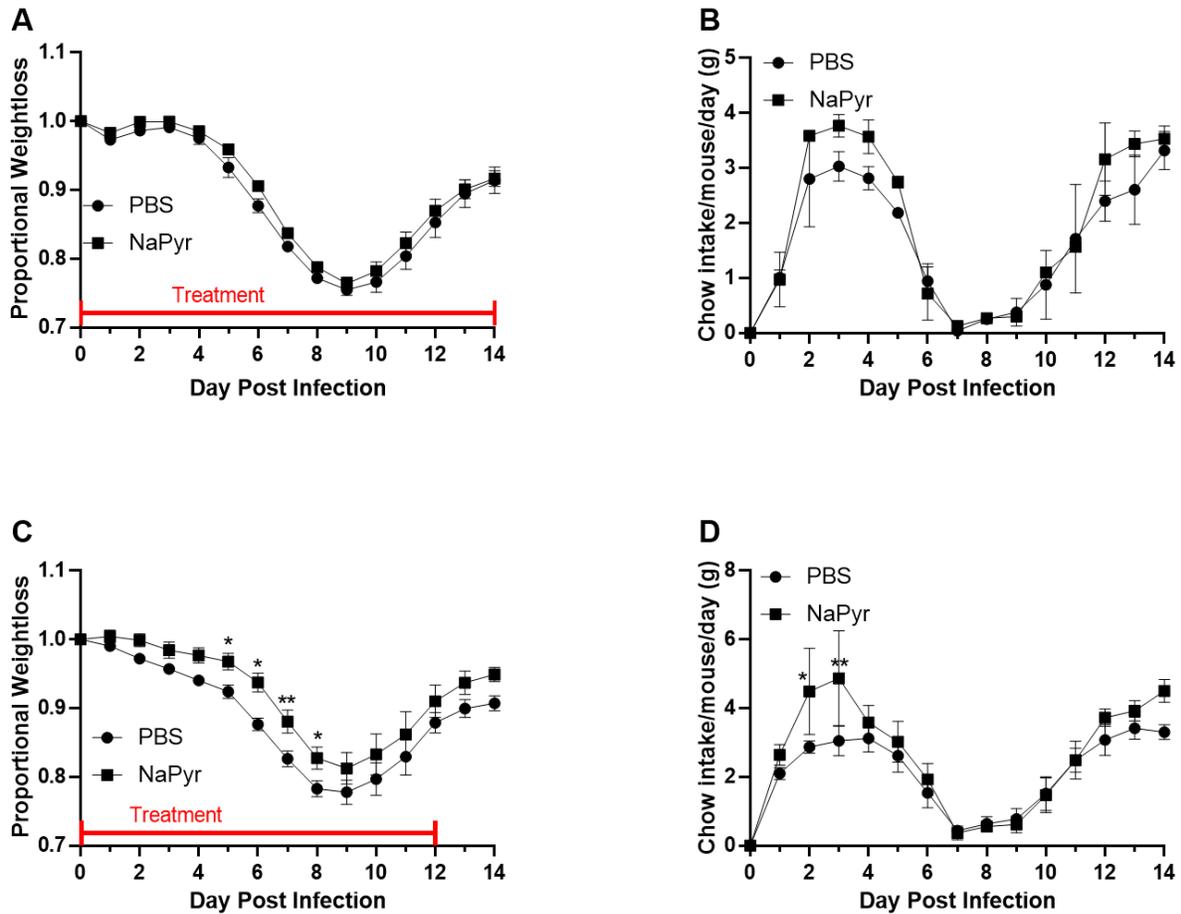
Figure 2. Effects of injection or nebulization of NaPyr on influenza A virus (IAV) infection. WT C57BL/6J mice were infected intranasally with 250 PFU of influenza A/PR/8/34 H1N1. Mice were treated as indicated and monitored daily for 14 days to determine survival and weight differences between treatment groups. (A) Weight loss was examined in mice injected Sub-Q with 55 mg/kg NaPyr twice a day for 14-days compared to PBS injected mice. (B) Average chow intake over the 14-day IAV infection of both Sub-Q NaPyr treated and PBS treated mice. (C) Mice were treated 3 times a day with either nebulized 10 mM NaPyr or nebulized PBS as control. Weight loss differences viewed over the 14-day IAV infection of both NaPyr treated and PBS treated mice. (D) Average chow intake over the 14-day IAV infection of both nebulized 10 mM NaPyr and PBS treated mice. Data are representative of 2–3 individual experiments with $n = 4$–5 mice per treatment group per independent experiment. Statistical significance was determined using a Two-way ANOVA with Fisher LSD post-hoc for multiple comparisons. * $p < 0.05$, ** $p < 0.01$.
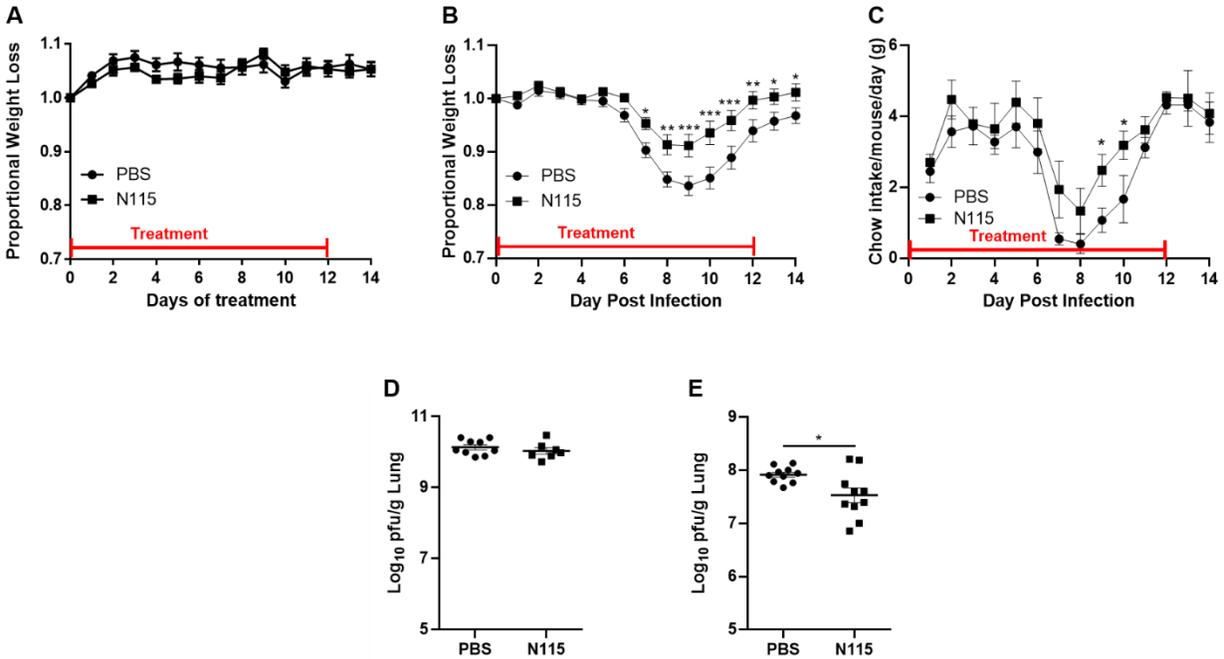
Figure 3. Nebulized N115 improves weight loss and virus titer in mice infected with IAV. WT C57BL/6J mice were treated with either nebulized 20 mM NaPyr (N115) or nebulized PBS as control 3 times a day for 20 min/treatment to test for toxicity (A). WT C57BL/6J mice were infected intranasally with 250 PFU of influenza A/PR/8/34 H1N1. Mice were treated with either nebulized 20 mM NaPyr (N115) or nebulized PBS as control 3 times a day for 20 min/treatment. (B) Mice were monitored daily for 14 days to determine weight differences between treatment groups. (C) Average chow intake over the 14-day IAV infection of both N115 treated and PBS treated mice. (D–E) Viral titer was assessed by plaque assay on day 3 (D) and day 7 (E) p.i. from lung homogenates. Weight loss and chow intake data are representative of 3 independent experiments with n = 4–6 mice per treatment group per independent experiment. Viral titer data are representative of 2 individual experiments with 3–5 mice per treatment group per individual experiment. Statistical significance was determined using a Two-way ANOVA with Fisher LSD post-hoc for multiple comparisons, and Student's t-test for single comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
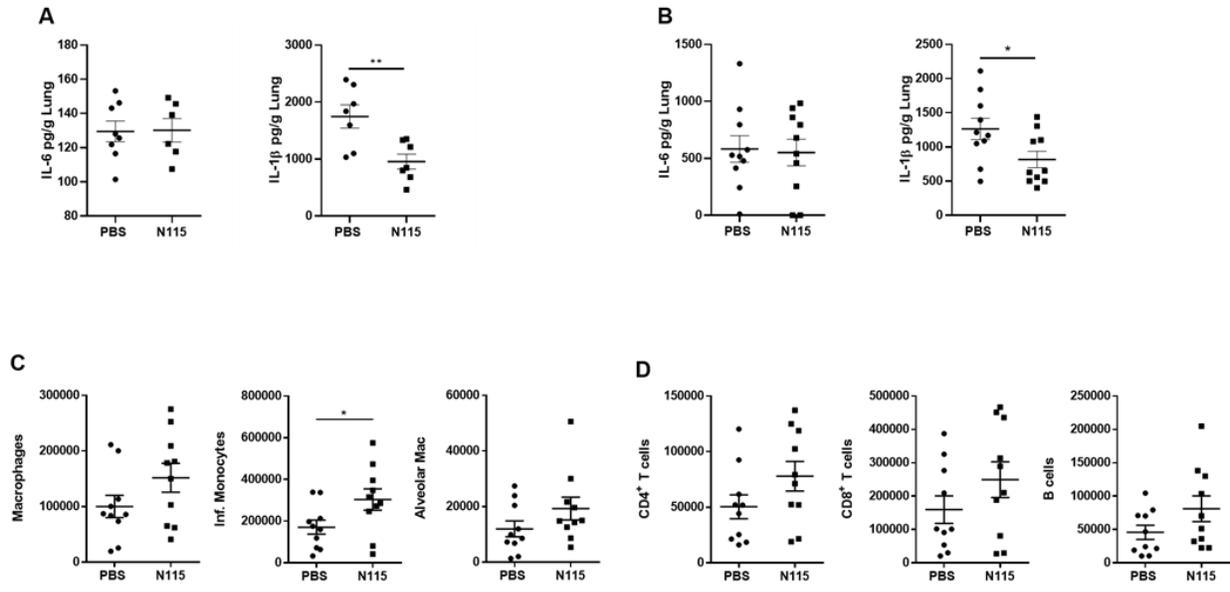
Figure 4. N115 treatment modulates inflammatory responses during IAV infection. WT C57BL/6J mice were anesthetized and infected with 250 PFU of influenza A/PR/8/34 H1N1. Mice were treated 3 times a daily for 20 min/treatment with either nebulized 20 mM NaPyr (N115) or nebulized PBS as control. Mice were euthanized on day 3 (A) or day 7 p.i. (B–D) for tissue collection. Lung samples were then homogenized and examined via ELISA for cytokine production (A–B) or cellular infiltration into the lungs by flow cytometry (C–D). Data are combined from 2 independent experiments with n = 3–5 mice per experiment. Statistical significance was determined using a Student's *t*-test for single comparisons. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

The easiest way to prevent influenza infection is to get vaccinated. The influenza vaccine was 45% effective during the 2019-2020 influenza season [38]. If we recall, only 48.4% of the United States population, 18 years or older, received the vaccine in the 2019-2020 influenza season [2]; therefore, there is an inherent need for other options to ameliorate the disease associated with IAV infection. These include but are not limited to anti-viral therapies, home remedies, and corticosteroid treatments.

As of 17-Mar-2021, there are four pharmaceutical anti-viral treatments that are FDA approved for treatment of IAV on the market: oseltamivir, zanamivir, peramivir, and baloxavir marboxil [39]. All of these treatments must be administered early in the infection and can be prescribed prophylactically in unvaccinated individuals [39, 40]. Oseltamivir, zanamivir, and peramivir are effective on influenza A and B and work by inhibiting the neuraminidase (NA) on the virus's surface, thus preventing, or dysregulating, the release of the viral particles and reinfection inhibition caused by the NA during functional viral replication [41-44]. Viral resistance has been observed in each of these three therapies [41, 45-48]. Targeting a different part of the influenza virus, baloxavir marboxil, prevents transcription of the viral RNA [49]. Interestingly, a combination therapy of an NA inhibitor and baloxavir marboxil leads to decreased viral titer in mice at multiple time points post infection. This could lead to a potent combination therapy option up to 96 hours post infection [50]. Previously, M2 inhibitors, rimantadine and amantadine were used to treat IAV infection and worked by preventing viral replication because they altered the M2 ion channels. The caveat to both of these treatments is they only functioned on influenza A virus, not influenza B virus. Many strains of IAV are now

resistant to both rimantadine and amantadine. The United States no longer recommends either M2 inhibitor for treatment of IAV [43].

Home remedies would include quarantining during infection, staying hydrated, rest, and over the counter pain relievers [51]. Each of these treatments have their own set of downfalls. Additionally, corticosteroid treatment decreases inflammation and is a potent immunomodulator, but has been shown to have adverse effects in severe cases of IAV infection [44]. In addition, using corticosteroid treatment compromises the immune response and leaves the body prey to other opportunistic pathogens, creating coinfections [52-55].

The current pharmaceutical antiviral remedies are limited by their capacity to become like the M2 inhibitors insofar that they could be phased out due to antigenic drift or shift. This sets the stage, and the need, for alternative antiviral therapies that are not as susceptible to antiviral resistance. As noted, the anti-viral treatments above are for the use of influenza viruses, and in some instances only specific strains of influenza virus. Antioxidants have long been studied for their ROS scavenging abilities and their influence on influenza infection in cases of antiviral resistance. Antioxidants scavenge the superoxide produced by innate immune cells, namely macrophages, thus reducing ROS and oxidative stress observed during infection [56-58]. Treating with antioxidants may also treat other viruses, such as SARS-CoV-2, that induce cytokine storms or hyper ROS production. NaPyr has been shown to have antioxidant properties [59-61], but we did not observe any benefit in adding NaPyr to *in vitro* studies with other pathogens, such as E. coli. Sodium pyruvate has been shown to have anti-inflammatory activity within moderate and chronic models of inflammation [62-65]. Additionally, Pyr is secreted into serum of cultured cells exposed to H2O2, suggesting Pyr is naturally exported to act as an antioxidant [59, 66].

However, many questions still surround our findings. We observed *in vitro* that overall ROS and mitochondrial ROS are decreased; more tests are necessary to determine if this is the case *in vivo*. *In vitro* we experimented on terminally differentiated macrophages, which are innate immune cells, but what cells are being affected *in vivo* is still a mystery. Potentially, this could be answered by testing cytokine levels, viral replication, and ROS production in other epithelial cell lines, such as lung epithelial cells. Nitric oxide, which is produced by neutrophils at the onset of influenza infection, could also be targeted by sodium pyruvate's antioxidant properties. *In vivo,* there are many things that could still be done, testing for interferons and what exactly NaPyr is immunomodulating in the whole system. Another interesting feature of our *in vitro* work is that in the BMDMs, we observed a significant decrease in interferon (IFN)-β production, which reduces antigen presentation and allows for immune suppression [67]. This decrease in BMDMs would explain the decreased viral titer seen on day 7 *in vivo* due to increased T cell responses, but what does lasting immunity to IAV look like with NaPyr treatment? Presumably the treatment would provide the same immunity as an untreated infection or any other treated infection, but with a decrease in IFN- β and decreased viral titer, is the adaptive immune system being primed appropriately for subsequent infections? Noting that according to our data, adaptive immune cells are slightly elevated in mice treated with NaPyr during IAV infection, which would potentially mean a more robust adaptive immune response. Thus, leaving a more potent antibody response to subsequent IAV infections. Overall, our findings indicate that NaPyr is a potential therapeutic treatment for IAV with little chance of developing antiviral resistance and no negative side effects for the host.

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APPENDICES

Appendix A

Application to Use Live Vertebrate Animals Dept: Biology

PI: Christopher L Lupfer   Page: 1 of 15

IACUC ID: 19-019.0   Web ID: 500

Title: Anti-inflammatory function of sodium pyruvate during influenza A virus infection

Species: Mouse (Other)
Application Type: New Application
Multiple Species: No
Total Animal Number: 264 (Non-ORC - Bred)

Office Use Only

IACUC ID: 19-019.0-A

Renewal Date:

Yes 4.1 REQUIRED - Check this box in order to access Section 4.1, Alternatives to Proposed Procedures. Failure to check this box may result in protocol review delays.

Submission History for New Application:

06/13/2019 - Submitted
06/26/2019 - Under Review
07/03/2019 - Approved
07/03/2019 - Complete

Approval Date: 6/28/2019

Application to Use Live Vertebrate Animals Dept: Biology

1. Personnel Information

Personnel		Roles	Techniques
Name:	**Christopher L Lupfer**	Email Contact	Anesthesia - Administering
Dept:	152024 - Biology	Laboratory Coordinator	Anesthesia - Monitoring
Campus Box:	901 S National Ave Temple 254 Springfield MO 65897-0027	Official Contact Principal Investigator	CO_2 with Physical Euthanasia
			Handling and Restraint
Phone:	417-836-6887		Injectable Anesthesia
Email:	christopherlupfer@missouristate.edu		Intraperitoneal Injection
			Weighing and Measuring
			Intranasal infection with influenza A virus.
			administration of treatment in drinking water and food.
Name:	**Jessica Reel**	Student Investigator	Anesthesia - Administering
Dept:	152024 - Biology		Anesthesia - Monitoring
Campus Box:	901 S National Avenue Springfield MO 65897-0027		CO_2 with Physical Euthanasia
			Handling and Restraint
Phone:			Injectable Anesthesia
Email:	jessica19@live.missouristate.edu		Intraperitoneal Injection
			Weighing and Measuring
			Intranasal infection with influenza A virus. administration of treatment in drinking water and food.
Name:	**Jordan Fleetwood**	Student Investigator	Anesthesia - Administering
Dept:	152024 - Biology		Anesthesia - Monitoring
Campus Box:	901 S National Ave Springfield MO 65897-0027		CO_2 with Physical Euthanasia
			Handling and Restraint
Phone:			Injectable Anesthesia
Email:	jordan987@live.missouristate.edu		Intraperitoneal Injection
			Weighing and Measuring
			Intranasal infection with influenza A virus. administration of treatment in drinking water and food.

2. Funding

Funding Source	Agency Deadline	Funding Period	Grant Number
National Institutes of Health	10/25/2019	3 years	

3. Scientific Justification for Animal Species

1. Justify the species to be used by indicating:

   This is a new model. (Veterinarians available for consultation on new model development.) **No**

   The results will be directly applicable to the health, care or welfare of this species. **No**

   Other Justification? **Yes**

If Yes, Explain:

>   The purpose of this study is to examine the effects of the natural metobolite sodium pyruvate on the immune response to influenza A virus infection. In immunology, the mouse is the prefered

species as there is a wealth of knowledge regarding the mouse immune system and there are established molecular and diagnostic tools, such as antibodies, for detecting mouse cytokines.

2. **Features of the species (e.g. anatomic, physiologic, genetic, etc.) that make it desirable for this model.**

>   The mouse is prefered for the study of infectious disease and immunology for several reasons. First, mice are

easy to handle, house and physicaly manipulate. Second, mice are the prefered species as there is a wealth

ofknowledge regarding the mouse immune system and there are established molecular and diagnostic tools such as antibodies for detecting mouse cytokines. Finally, the mouse has already been established as a model for the study of multiple infectious diseases of human importance, including influenza A virus.

3. **Will the PI conduct the same experiment in multiple species? No**

>   If Yes, Explain:

4. Reduction, Refinement, Replacement, and Animal Numbers

1. Reduction, Refinement, and Replacement

a. Replacing vertebrate animals

>   **No** Are there computer simulation, non-living, or in vitro alternatives to the proposed use of animals described in your application?

>   If Yes, Explain:

>>   Although we have performed initial examination of the immune signaling pathways in vitro in cell lines, cells in isolation cannot recapitulate the complexity of the immune system of an entire living organism. Therefore, the conclusions of any in vitro immunology experiment must be validated in vivo. The determination of therapeutic efficacy requires the examination of these drugs in an animal model.

b. Refining experimental procedures to minimize pain or distress

>   **Yes** endpoints in the design of the experiment? Did you consider the use of pain-relieving drugs, or procedures that avoid or minimize discomfort, distress and pain, and humane

>>   If No, Explain:

>>   The proposed research will examine the anti-inflammatory effects of sodium pyruvate on the immune response to influenza A virus and determine if there is any therapeutic benifit. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response or add a confounding variable to the examination of therapeutic efficacy. Therefore, no such drugs can be employed in this protocol while still acheiving the research objectives. However, we have incorporated the following humane end points. Any animal that loses greater that 30% starting body weight or is moribund will be immediately euthanized. In addition, we have conducted preliminary experiments in cell culture in vitro to determine the potential signaling pathways involved and will only perform in vivo experiments to examine those

pathways. Finally, drug doses will be based upon previously published research to avoid toxicity.

c. Reduction in the number of animals Specify the methods used for reducing the number of animals that were considered in the design of the proposed experiments.

Yes Rational selection of group size (e.g., pilot studies to estimate variability, power analysis) **Yes**

Careful experimental design (e.g., appropriate choice of control groups)

Yessharing tissues with other investigators) Maximize use of animals (e.g., selecting the minimal number of animals per group required for statistical verification,

Yes Minimize the loss of animals (e.g., good post-operative care, avoidance of unintended breeding)

For any of the above items not checked, please provide a brief comment about why the option is not appropriate.

2. Using the specifics of your experimental plan, justify the number of animals requested for each pain category (B, C, D, E).

All animal models using intranasal infection such as influenza A virus or S. pneumoniae are inherently variable with standard deviations consistently around 30% of the mean. To arrive at the numbers of mice proposed here, we have taken into account past experience with intranasal infection models over the last 10 years as well as the number of animals typically used in the published literature. The effect size for the type of research designs employed in this protocol averages a Cohen's f = .40; therefore, using G*Power for the various designs proposed, with a Power = .80; Alpha = .05, a minimum of 6 animals is required per experiment. In the case of survival challenge experiments or experiments with multiple groups, more animals may be required for confidence in the interpretation of the results (e.g. n=10 per group for survival experiments). The justification for mice is as follows. Category D:

All mice in category D will be used to investigate the in vivo effecacy of treating mice with sodium pyruvate as an anti-inflammatory drug. Two groups will be examined during infection with influenza A virus: (1): a placebo group (daily subQ saline injection), (2): sodium pyruvate treatment group (daily subQ injection of 2mg/kg). This dose of sodium pyruvate has already been shown to be safe in mice in other disease models. We will need 66 WT mice per treatment or control group. However, the placebo group (66 mice) will be included in category E, the remaining
66 mice are included here in category D. The 66 mice in each treatment group will be used for examination of survival (3 independent replicates x 10
mice per replicate = 30 mice) and examination of immune responses on day 3 and day 7 after
infection (3 independent replicates x 6 mice per time point x 2 non-repeat measure time points = 36). It is possible that the subQ route of sodium pyruvate administration is not ideal for influenza A virus infection. Thus, we request an additional 66 mice to examine the treatment of mice with nebulized sodium pyruvate. Therefore, a total of 132 mice are classified as category D for the examination of the anti-inflammatory effects of sodium pyruvate. WT mice used in this portion of the proposed research will be bred in house.

Category E:

All mice in category E will be used to investigate the in vivo effecacy of treating mice with sodium pyruvate as an anti-inflammatory drug. As mentioned for category D, Two groups of mice will be examined during infection with influenza A virus: (1): a placebo group (daily subQ saline injection), (2): sodium pyruvate treatment group (daily subQ injection of 2mg/kg), The placebo control mice are included in category E, as they will recieve no interventional treatment. We will need 66 WT mice for the control group. The 66 mice in will be used for examination of survival (3 independent replicates x 10 mice per replicate = 30 mice) and examination of immune responses on day 3 and day 7 after infection (3 independent
replicates x 6 mice per time point x 2 non-repeat measure time points = 36). It is possible that the subQ route of sodium pyruvate administration is not ideal for influenza A virus infection. Thus, we request an additional 66 mice to examine the treatment of mice with nebulized sodium pyruvate. Therefore, a total of 132 mice are classified as category E for the examination of the anti-inflammatory effects of sodium pyruvate. WT mice used in this portion of the proposed research will be bred in house.

3. Estimate the following animal number totals required for this study during the three-year approval period.

Pain Category	Animals
B	0
C	0
D	132
E	132
	Total = **264**

Justification for Category E:

    The proposed research will examine the effects of sodium pyruvate on the immune response to influenza A virus infections. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response or add a confounding variable to the examination of therapeutic efficacy. Therefore, no such drugs   can be employed in this protocol. We have incorporated the following humane end points. Any animal that

    loses greater that 30% starting body weight or is moribund will be immediately euthanized. In addition, we have conducted preliminary experiments in cell culture in vitro to determine the potential signaling pathways involved and will only perform in vivo experiments relative to those pathways. Finally, drug doses will be based upon previously published research to avoid toxicity.

4. **Transfer of Existing Animals: Yes**   If Yes, Indicate the IACUC ID: **19.005**

4.1 Alternatives to Proposed Procedures

1. **Details about the search for alternatives**

    a. Names of searched databases and the date when the search was conducted:

        No   Agricola:
        No   AWIC:
        No   Biosis:
        No   Cab Abstracts:
        No   Crisis:
        No   Embase:
        Yes Pub Med: **06/11/19 No** Medline: **No**
        NTIS:
        No   Psychlit:
        No   Scisearch:
        No   Toxline:
        No   Pascal: **No**
        Other:

        If Yes, Explain:

    b. Keywords used in the database searches:

    Influenza and pyruvate: This search returned 28 results, and none have examined the affect of treating influenza A virus infection with pyruvate. one article (Respir Investig. 2016 Sep;54(5):312-9.) discusses that treatment of patients with drugs that enhance pyruvate metabolism (inhibitors of pyruvate dehydrogenase kinase 4) may have therapeutic benifit, but no one has examined the dirrect treatment with sodium pyruvate. c. Years Searched: 1900-2019

    d. Resources used in addition to the computer database search:

    Information Services and other Literature Sources:

No Animal Welfare Information Center
No Lab Animal Welfare Bibliography (NLM)
No Laboratory Animal Science Journal
No Alternatives to Laboratory Animals Journal (FRAME, U.K.)
No Quick Bibliography Series (AGRICOLA)
No Peer Review

   If Yes, Explain:

No Other

   If Yes, Explain:

Other Methods or Sources Used:
   No Direct contact with investigators in field

   If Yes, Explain:
   No Consultation with expert in the area of investigation

   If Yes, Explain:
   No Other methods or sources

   If Yes, Explain:

5. Details of Animal Use:

1. **Goals and objectives of your research**

Influenza A virus infection results in hundreds of thousands of deaths world wide every year. Although a vaccine exists, it is often poorly effective, and is too expensive for use in the developing world. We accidentally discovered that the addition of sodium pyruvate to the cell culture medium of cells infected with influenza A virus results in supression of the immune response, with lower levels of immune signaling molecules called cytokines. Subsequent research is needed to determine if injection or inhalation of sodium pyruvate may help to diminish the inflammation and symptoms associated with influenza A virus infection. We propose to examine the effects of sodium pyruvate treatment during influenza A virus infection in mice. If sodium pyruvate proves benificial, then it is a natural and inexpensive treatment for a disease that is a global problem.

If this application is a continuation of an ongoing project, state concisely how these goals differ from those in the original
2. **application and what was accomplished during the prior approval period. If this is a new project, please indicate so.**

This is a new project.

Provide a concise overview of the experimental manipulations and treatments conducted on animals.
This description should allow the IACUC reviewer to understand exactly what will be done to all animals from entry into the
3. **experiment to the endpoint of the study.**

For the infection of mice with influenza A virus, 8-12 week old mice will be anesthetized by intraperitoneal injection of 80 mg/kg ketamine and 8 mg/kg xylazine. Following injection of mice with ketamine/xylazine, mice are allowed to become completely sedated for 5 minutes and sedation verified by pinching the paws. If no response is elicited, then mice are

inoculated intranasally with influenza A virus in 30µl phosphate buffered saline. Mice will be monitored daily. In allexperiments mice will recieve daily subQ injections of saline or 2mg/kg sodium pyruvate in saline. For survival experiments,

mice will be monitored for 14 days. Mice that lose >30% of their starting body weight or that are moribund will be euthanized immediately. Additional, mice will also be euthanized by CO_2 asphyxiation on day 3 or day 7 after infection and blood collected by cardiac puncture. Lung, spleen and lymph nodes will also be collected for examination of pathology, cellular infiltration and immune cytokine responses and viral titers.

6. Animal Care

1. **Animal ID Method:**
   No   Ear Tag
   No   Ear Punch
   No   Microchip

No Not Applicable

NoNo TattooToe Clip

Yes Other

If Yes, Explain:

Marking the tail with a sharpie.

2. **How will animals be monitored and maintained?**

All animals will be housed in the Missouri State Iniversity Managed Vivarium and maintained under the standard operating proceedures establishedd for that facility and species. Facility conditions and monitoring typically includes:

Temperature ~72-75F

12h/12h light/dark cycle

Humidity between 30-70%
Air changes from 10-15 per hour

Food and water provided ad libitum

Cage changes once per week

Animals that are infected with influenza A virus will be monitored daily by the PI or student on the IACUC protocol. The PI or emergency contact will perform after hours, weekend and holiday monitoring as needed during infection studies.

If special monitoring has been arranged with DLAM facility supervisor, provide DLAM contact name:

3. **Should ORC contact the PI or the emergency contact if animals are found dead?** Yes

4. **Indicate requests for special handling of sick and dead animals.** As mice used in these experiment will be infected with

influenza A virus, they are expected

to become sick and will be monitored at least daily for. No additional contact is necessary for infected sick animals. However, if infected animals are moribund, please contact me immediately and such animals will be euthanized.

5. **Special Housing**

Will any special housing or care be necessary? Yes

If Yes, describe and list any deviations from standard ORC husbandry procedures, Guide recommendations or special animal care needs.

All animals need to be housed in microisolator cages and all infected animals need to be housed under ABSL2 conditions.

6. **Special Diets**

Are special diets, additives to food and/or water, or antibiotics needed? No

If Yes, Describe and List Agents:

7. **Describe endpoints (time points, tumor sizes etc.) and/or the maximum time length of study.**

Mice used for infections will be infected at 8-12 weeks of age and the maximum duration of infection will be 14 days.
Some mice will be euthanized on day 3 or day 7 after infection with Influenza A virus to collect blood,

lung and lymphoid organs for examination of the immune system response and pathology. Mice to be used for survival experiments will be kept for a maximum of 14 days. All surviving mice will then be euthanized. During infections, mice that are moribund or that lose >30% of their starting body weight will be euthanized immediately.

8. Describe the criteria used to determine when an animal should be removed from the study prior its endpoint. During infections, I

have occasionally (1/100 infected mice) observed ataxia, presumably due to encephalitis. Such

mice will be immediately euthanized. Mice with severe infection will also present with hypothermia just prior to death. These mice will also be euthanized immediately.

9. Will animals be euthanized as part of the study? **Yes** If No, Describe the final disposition:

If Yes, Answer all of the following questions:

Euthanasia Methods

Yes CO2-compressed carbon dioxide gas in cylinders and a physical method
No Barbiturate overdose

If Yes, Specify Dosage and Route:

No Overdose of Gas Anesthetic

If Yes, Specify Agent:

No Anesthesia - followed by physical euthanasia

If Yes, Specify Agent, Dosage, and Route:

If any of the above have been checked, indicate the physical methods that are used to ensure animals are dead:
Cervical Dislocation,Thoracotomy (Open the chest cavity using sharp scissors or scalpel),Collection of vital organs performed if tissues are needed for experimental purposes

No Cervical Dislocation performed with no anesthesia

If Yes, Justify:

No Decapitation performed with no anesthesia

If Yes, Justify:

No Other Methods

If Yes, Describe:

10. Would the PI be willing to make available extra animal tissues or organs to other PI's?
Yes

7. Anticipated Animal Pain & Distress

1. Are there any clinical, behavioral, or physiological manifestations expected to result from experimental manipulation?
Yes

If Yes, Answer all questions in this section.

a. Expected clinical and/or behavioral signs of pain and distress in animals:

Yes Decreased weight
Yes Changes in food/water consumption
Yes Decreased ambulation
Yes Ruffled fur
No Skin abnormality
No Urinary problems
Yes Hunched posture
No Paw guarding
No Porphyrin Staining
Yes Lethargy
Yes Diarrhea
No Other

If Yes, Explain:

b. Methods of dealing with the above complications:

No Analgesics
No Anesthetics

No Sedation or tranquillization
Yes No Increased beddingOther

If Yes, Explain:

Agents used in dealing with complications:

Animals experiencing unrelieved pain or distress prior to the endpoint, as defined by institutional policy, must be humanely
2. euthanized, unless an exception to policy is requested and approved. Is exception required?

Yes

If Yes, Answer all questions in this section.

a. Criteria for euthanasia that will be used in this exception:

Mice will be euthanized if they are moribund or lose >30% of their starting body weight. Additionally, just
prior to mortality, mice become hypothermic. Any mice that do not meet the qualifications of being moribund
or losing 30% of their weight will be euthanized if they are hypothermic. Hypothermic mice will be examined
by handling (they feel cold). Finally, some mice (about 1/100) will develop ataxia as a result of the infection
and ensuing encephalitis. These mice will also be euthanized immediately.

b. Scientific justification for not using an earlier endpoint:In our studies, we are examining the anti-inflammatory
affects of sodium pyruvate. The use of
pain relieving or anti-inflammatory drugs, in addition to sodium pyruvate, would confound the interpretation
of our results in these studies.

A weight loss of 30% or moribund behavior are commonly accepted endpoints in the field of influenza Avirus

infection, as mice that lose less than 30% of their starting weight routinely recover and

the assessment of mortality cannot be correlated. Although death as an endpoint is not used here, we do
need a reliable correlate, for which the 30% weight loss cutoff has been shown to correlate well. In addition,
mice may become moribund prior to death but before the 30% weight loss cutoff and will thus be
euthanized.

8. Request for Exception to Regulation or Policy

8.1 Exception Information

1. Description of exception:
Due to the examination of the immune response and the testing of novel therapeutics during influenza A virus
infection, it is not possible to treat animals with any additional anti-inflammatory or analgesic drugs other

thansodium pyruvate, to alleviate the pain or discomfort of the infections.

2. Rationale (provide scientific justification and/or justification based on animal welfare):
In the human population, influenza A virus infection results in increased morbidity and mortality
[1]. Although vaccines and antiviral drugs exist to treat or prevent influenza A virus
infections, these are still of limited efficacy due to the fact that the virus constantly mutates, and there is a
disregulated immune response leading to immune cell and edema infiltration into the lung exacerbates
pneumonia [2]. Multiple lines of evidence point to exacerbated inflammation as a key factor. Additionally,
influenza A virus infection results in altered metabolism [3]. This requires more energy/ATP production. Based on
its known functions, and our preliminary experiments in cell culture in vitro, sodium pyruvate may be able to
increase ATP production from cells and decrease inflammatory cytokine production.

Because of the nature of these experiments, the study of the immune response and examination of therapeutic benefit of sodium pyruvate, we cannot administer any treatment of drugs that would alter or inhibit the immune response or
inflammation. All analgesic and anti-inflammatory drugs affect the immune response and cannot be used. As mortality is a potential outcome from influenza A virus infection in the human clinical setting, it is necessary to determine if the immune signaling pathways or treatments proposed in this study affect mortality and morbidity. We will use 30% weight loss or moribund responses as surrogates of mortality. Again, the inclusion of drugs to alleviate pain or discomfort would impair the interpretation of the proposed experiments.

References

1. Matias, G., R. Taylor, F. Haguinet, C. Schuck-Paim, R. Lustig, and V. Shinde. 2014. Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status. *Influenza and Other Respiratory Viruses* 8: 507–515

2. Zheng, J., and S. Perlman. 2018. Immune responses in influenza A virusand human coronavirus infections: an ongoing battle between the virus and host. *Current Opinion in Virology* 28: 43–52.

3. Kido H, Indalao IL, Kim H, Kimoto T, Sakai S, Takahashi E. (2016) Energy metabolic disorder is a major risk factor in severe influenza virus infection: Proposals for new therapeutic options based on animal model experiments. Respir Investig. 2016 Sep;54(5):312-9.

3. Potential adverse effects/clinical signs resulting from exception:
   Animals that are infected with influenza A virus will experience flu-like-symptoms, including

impaired breathing, weight loss, ruffled fur, decreased movement, malaise, hunched posture and in

some instances diarrhea and ataxia.

4. Specify which animals in the approved protocol will be affected:

All animals infected with influenza virus will be included in this exception.

Exception Approval Status: Approved   6/29/2019

12. Items not covered in other parts of the application

	PI:	Christopher L Lupfer	Page: 11 of 11
Application to Use Live Vertebrate Animals	Dept:	Biology	
	IACUC ID: 19-019.0		Web ID: 500

Approval Date: 6/28/2019

Application Certification

I agree to the following statements. Signify your agreement by signing at the bottom.

- I certify that I am familiar with and assure compliance in this Project with the legal standards of animal care and use established under the Federal and State laws and the policies on animal welfare of the National Institutes of Health and the University of Cayuse.
- I assume responsibility for ensuring that all persons working with animals on this project are familiar with and are trained in relevant animal procedures and that they will comply with established laws and policies regarding animal care and use. Applications will not be approved for investigators that have taken the IACUC orientation but have not completed required Laboratory Animal Coordinator certification. Contact the IACUC office to arrange training.
- I will appoint a Laboratory Coordinator to manage all animal use in the lab. I will ensure that the Coordinator receives required training and certification. I will ensure that after being certified, the Coordinator or IACUC representative will train and certify all individuals working with animals in the lab.
- I certify that all individuals working with animals on this project will register with the University Employee Occupational Health Clinic (UEOHC) by completing and submitting the "Research Animal Handlers & Animal Caretakers" medical history questionnaire (each individual who works with animals must complete the questionnaire during online orientation - UEOHC will assess the PI a processing fee).
- I certify the following: the research proposed herein is not unnecessarily duplicative of previously reported research; appropriate non-animal alternatives for this research do not exist; no alternatives to the potentially painful and/or distressful procedures conducted in this project exist. I have indicated methods used to make these determinations in the appropriate section of this animal use application. I will secure IACUC approval before changing procedures or personnel associated with this study (including adding personnel) I assure that I and personnel under by direct supervision will use the animals acquired for the activity described herein solely for said purpose. I also certify that if live animals are shared with other PIs or are used in any procedure other than those described in this application, I will provide the details in the form of a written amendment to the original application prior to their use.
- I acknowledge that veterinary care will be administered to moribund animals or animals experiencing more than momentary or slight pain or distress. Division of Laboratory Animal Medicine (DLAM) veterinary staff will attempt to contact me regarding the care of treatment of a moribund animal, but will institute treatment or euthanasia, as needed, if PI cannot be reached.
- I assure the IACUC and the Missouri State University that the general procedures involving animals described in my grant application have been described in the animal use application and submitted to the IACUC for review.
- I assure that I have read "Notes on Euthanasia" of animals used in research and understand how it applies to animals in this animal use application.

NOTE: Consultation of a DLAM veterinarian regarding space allocation is recommended prior to submission of application. IACUC approval of application does not assure DLAM space availability. Please contact DLAM for pre-study strategy meeting prior to ordering animals to dicuss availability of housing.

NOTE: Cell lines that have been passaged in animals or maintained using animal serum may contain murine viruses that can alter the outcome of the study and may cause an outbreak of disease among other mice. ATTCC does not screen cell lines for murine pathogens. Cell lines that have been passaged in animals or grown in media containing rodent serum should be tested for murine pathogens prior to use in animals. Please contact DLAM for more information on testing of your cell lines.

PI Signature	Date
Co-PI Signature	Date

Approval Date: 6/28/2019

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Appendix B

Missouri State UNIVERSITY

MEMORANDUM OF UNDERSTANDING & AGREEMENT (MUA) FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENTS

All MUA'S can be submitted electronically to researchadministration@missouristate.edu or submitted as a hard copy to the ORA in Carrington 405. A signed copy must be provided. *Biosafety in Microbiological & Biomedical Laboratories (BMBL)* should be used as a reference when completing this MUA (see http://www.cdc.gov/biosafety/publications/bmbl5/).

A. General Information

Date: June 12th, 2018

Researcher Name: Christopher Lupfer

Researcher Title: Assistant Professor

Phone: 6-6887

Department: Biology

Office Bldg & Room #: Temple Hall, room 254

Laboratory Bldg & Room #: Temple Hall, room 232

Granting Agency:

Grant Number (if applicable):

Title of Grant or Project: Examining different viral pathogens during respiratory viral infection

B. Project Information

1. **Describe the experiments involving biohazard(s). Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it.**

Coinfections that occur during an influenza A virus (IAV) infection are often severe and life threatening. As early as the 1918 influenza pandemic, researchers and clinicians have recognized that infection with IAV predisposes individuals to a more severe form of pneumonia resulting from secondary bacterial infections [1]. Overall, the likelihood of death from IAV and bacterial coinfection is at least twice that found in severe cases of IAV alone [1-4]. S. aureus and S. pneumoniae bacteria are among the most common coinfecting agents [5]. However, coinfection following IAV infection can occur with other pathogens including other viruses and fungal pathogens. In fact, over the last decade, numerous case reports demonstrate that viral-fungal coinfections deserve more scrutiny as a potential source of severe respiratory disease [19].

Secondary infections after an influenza A virus infection can lead to severe pneumonia. Although many discoveries have been made regarding the immune response during coinfection, these experiments have mainly focused on viral-bacterial coinfection. To date, there is no mouse model of IAV-*Aspergillus fumigatus* fungal coinfection. Therefore, in order to begin to examine the genetic, environmental and pathogen associated virulence factors relevant to this disease, we need to develop an animal model. The availability of an animal model will also allow us to target specific pathways of inflammation, or to test the benefit of antiviral or antifungal drugs and begin to move these toward clinical development.

As no animal model exists for studying the coinfection of IAV and *Aspergillus fumigatus*, we intend to examine the ability of different subtypes and strains of IAV to cause fungal coinfection. Some human clinical case reports indicate specific strains of IAV are associated with fungal coinfection more than other strains [19]. In particular, the influenza A/California/04/2009 H1N1 strain that caused the human influenza pandemic in 2009 appears to result in more fungal coinfections. Therefore, we plan to use this strain of influenza, or other strains from the 2009 pandemic to develop a new mouse model of IAV-fungal coinfection. We will infect mice or cells from mice with this virus or the PR8 strain of IAV and determine if one strain predisposes the mice to a secondary infection with the fungus *Aspergillus fumigatus*. Furthermore, we will examine the ability of other viruses to cause coinfection including human Respiratory Syncytial Virus (hRSV). All pathogens will be provided by Drs. Paul Thomas, Thirumala-Devi Kanneganti, and Jon McCullers at St. Jude Children's Research Hospital or attained from ATCC/BEI resource program.

2. **Provide an assessment of the physical containment required for the experiments.**

The influenza A/California/04/2009 H1N1 virus, human Respiratory Syncytial Virus (hRSV) and *Aspergillus fumigatus* fungus are all biosafety level 2 pathogens (BSL2). As such, the likelihood of transmission to humans is low to moderate. *Aspergillus fumigatus* is a common environmental mold and poses little health risk except for patients with allergies. hRSV and the California 09 influenza A virus are human pathogens and can cause infection in humans. However, both of these viruses are not serious in health humans. hRSV in adults causes cold-like symptoms. Although contracting the flu is a severe infection, the flu vaccine protects against the influenza A/California/04/2009 H1N1 strain and all students and laboratory personnel will be vaccinated or not allowed to conduct this research. In keeping with BSL2 guidelines, all experimental procedures will be conducted in a Class II biosafety cabinet and all personnel working with these pathogens must be wearing appropriate personal protective equipment. Proper handling of infectious cultures or samples must be observed. Proper decontamination of research equipment, and personal protective equipment must be followed. Also, proper personal hygiene in the laboratory environment must be maintained to prevent accidental contamination or infection.

3. **Describe the facilities and specific procedures which will be used to provide the required levels of containment.**

Temple 232 contains the space and designated equipment necessary for working with RSV, influenza A virus, bacteria and *Aspergillus fumigatus* fungus. This includes a tissue incubator with sealed air-tight door, designated centrifuge, and a class II biosafety cabinet (BSC). In addition, for work involving animals, the vivarium in Temple Hall has a procedure room with a BSC and is available for work with BSL2 level pathogens.

All procedures will be performed in the BSC present in the lab in Temple 232 or in the procedure room of the vivarium. All samples collected will only be opened and handled in a BSC. The BSC will be decontaminated prior to and following all procedures using either 70% ethanol or 10% bleach, which must remain on the surface for 5 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags placed in secondary containers and autoclaved prior to being discarded. Any liquid cultures will be collected in sealed containers containing bleach at a final concentration of at least 10% to inactivate pathogens. Samples collected during experiments may be handled outside of the safety cabinet if contaminating pathogens have been killed by either incubation for a minimum of 5 minutes with formaldehyde > 1% or methanol > 50%, incubation in solutions containing at least 10% bleach >5 minutes can also be used to decontaminate samples. Finally, heating at >95 degrees Celsius for at least 15 minutes (for example, boiling samples in Laemmli buffer prior to SDS-PAGE analysis) may also be used to ensure pathogens are destroyed. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

All personnel working with pathogens will wear disposable latex or nitrile gloves, a laboratory coat and eye protection. These must be worn at all times while working with infectious agents or potentially contaminated samples or cultures. Long pants and close toed shoes are also required. Prior to exiting the BSC, gloves must be removed and placed in the biohazard bag or decontaminated with 70% ethanol or 10% bleach. Samples to be removed from the BSC for transport or storage must likewise be decontaminated. No eating (including chewing gum), drinking, applying cosmetics or contact lenses is allowed in the laboratory even when work with infectious cultures is not taking place.

In addition to these procedures and precautions, all individuals working directly with these pathogens will be required to receive and be current on their seasonal influenza vaccine, as this does protect against the influenza A/California/04/2009 H1N1 virus.

4. **Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.**

When samples need to be transported, they should first be inactivated by chemical (RIPA buffer) or heat inactivation as described above. A secondary container should also be used if the sample contains any liquid (small paint can filled with paper towels etc.). If live cultures or samples containing potentially live organisms must be transported, then samples must be sealed in a shatter resistant container (such as a threaded-cap polypropylene plastic test tube) and the outside of the container decontaminated with 70% ethanol or 10% bleach. PPE including lab coat, eye protection and nitrile gloves will be worn when transporting live cultures. After experiments are complete or before leaving the lab, PPE will be removed and hands washed for 30 seconds with soap and warm water.

5. Describe the waste disposal procedures expected to be used during this experiment.

For BSL2 conditions, the BSC will be decontaminated prior to and following all procedures using either 70% ethanol or 10% bleach, which must remain on the surface for 5 minutes. All contaminated materials (pipet tips, gloves, vials, old samples, etc.) will be disposed of in biohazard bags in secondary containers in the biosafety cabinet, sealed and autoclaved prior to being discarded. Any liquid cultures, stocks or samples will be collected in sealed containers containing bleach at a final concentration of at least 10% to inactivate pathogens and then autoclaved. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution. PPE including lab coat, eye protection and nitrile gloves will be worn during any procedure using infectious agents or when decontaminating a surface or handling any waste. After experiments or decontamination procedures are complete or before leaving the lab, PPE will be removed and hands washed for 30 seconds with soap and warm water.

6. Is this a select agent? If yes, contact the Office of Research Administration (ORA).
No

7. Please list all students, staff and faculty involved with this project. CITI Biosafety training is mandatory for all personnel working with biohazards prior to final IBC approval.

Christopher Lupfer
Meagan Rippee-Brooks

CITI training for biohazards and blood borne pathogens has and will be completed and passed prior to participation in any procedures using Influenza A virus.

8. The undersigned agree to certify the following conditions of the proposed research:
    a. The information above is accurate and complete. We agree to accept responsibility for training of all laboratory workers involved in the project. We agree to comply with the CDC requirements pertaining to shipment and of hazardous biological materials. We are familiar with and agree to abide the provisions of the Missouri State University policies and procedures applicable to experiments involving biohazards.
    b. We understand that only the organisms specified are covered by this MUA, and work with other organisms or types of biohazards may require other MUAs.
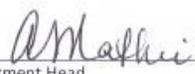
_____   8-20-18   _____   8-21-18
Principal Investigator   Date   Department Head   Date

9. The Institutional Biosafety Committee has determined, based on information provided the principal investigator, that:

   a. No special medical surveillance (other than usual University health programs) is required for the project described in this MUA

   b. The following specific medical surveillance procedures must be carried out, for individuals listed by name, before commencing the project described in this MUA:

10. We certify that the Missouri State University Institutional Biosafety Committee has reviewed the proposed project and has found in to be in compliance with Missouri State University's policies and procedures applicable to experiments involving biohazards.

_____   8/21/2018
MSU IBC Chair or Representative   Date

Appendix C

Application to Use Live Vertebrate Animals Dept: Biology

IACUC ID: 19-005.0   Web ID: 462

Title: Mouse Breeding 2019-2021	**Office Use Only**
Species: Mouse (Knockout and Other)	
Application Type: Continuation - 16-015.0	IACUC ID: 19-005.0-A
Multiple Species No	
Total Animal Number: 1478 (ORC,Non-ORC - Bred,Other Institutio Source:Approved Vendor)	Renewal Date: 02/2022
cSt. Jude Children's R,Other	

Yes 4.1 REQUIRED - Check this box in order to access Section 4.1, Alternatives to Proposed Procedures. Failure to check this box may result in protocol review delays.

Submission History for Continuation:

01/22/2019 - Submitted
01/25/2019 - Under Review
02/26/2019 - Approved
02/26/2019 - Complete
02/18/2022 - Renewal Date

Approval Date: 2/19/2019

Application to Use Live Vertebrate Animals Dept: Biology

1. Personnel Information

Personnel		Roles	Techniques
Name:	**Christopher L Lupfer**	Email Contact Laboratory Coordinator	CO2 with Physical Euthanasia
Dept:	152024 - Biology	Official Contact	Genotyping - Tail Clip
Campus Box:	901 S National Ave Temple 254 Springfield MO 65897 0027	Principal Investigator	Handling and Restraint
Phone:	417-836-6887		Sexing
Email:	ChristopherLupfer@missouristate.edu		Weighing and Measuring
Name:	**Meagan Rippee**	Student Investigator	CO2 with Physical Euthanasia
Dept:	152024 - Biology		Handling and Restraint
Campus Box:	Springfield MO 65897-0027		Weighing and Measuring
Phone:			
Email:	rippee417@live.missouristate.edu		

2. Funding

Funding Source	Agency Deadline	Funding Period	Grant Number
National Institutes of Health	**Feburary 25, 2019**	anticipated	

3. Scientific Justification for Animal Species

Application to Use Live Vertebrate Animals Dept: Biology

1. Justify the species to be used by indicating:

This is a new model. (Veterinarians available for consultation on new model development.) **No**

The results will be directly applicable to the health, care or welfare of this species. **No**

Other Justification? **Yes**

If Yes, Explain:

Most mice that will be bred on this protocol will be used on other IACUC approved protocols to examine the immune response to infectious pathogens.

In immunology, the mouse is the preferred species as there is a wealthof knowledge regarding the mouse immune system, there are established molecular and

diagnostic tools such as antibodies for detecting mouse cytokines, and genetic manipulation of mice for the generation of knockout mice is more established than in any other species.

In some instances, mice will be kept as replacement breeders or transferred to other PI's.

2. Features of the species (e.g. anatomic, physiologic, genetic, etc.) that make it desirable for this model.

The mouse is preferred for the study of infectious disease and immunology for several reasons. First, mice are easy to handle, house an physically manipulate. Second, mice are the preferred species as there is a wealth of

knowledge regarding the mouse immune system and there are established molecular and diagnostic tools suchas

antibodies for detecting mouse cytokines. Third, genetic manipulation of mice for the generation of knockout mice is more established than in any other species. Knockout mice for the proposed research already exist, including Aim2-/-, Nlrp3-/-, Myd88-/-, caspase1-/- and more. Finally, the mouse has already been established as a model for the study of multiple infectious diseases of human importance.

3. Will the PI conduct the same experiment in multiple species? **No**   If Yes, Explain:

4. Reduction, Refinement, Replacement, and Animal Numbers

1. Reduction, Refinement, and Replacement

a. Replacing vertebrate animals

No Are there computer simulation, non-living, or in vitro alternatives to the proposed use of animals described in your application?

If Yes, Explain:

b.   Refining experimental procedures to minimize pain or distress

Yes endpoints in the design of the experiment? Did you consider the use of pain-relieving drugs, or procedures that avoid or minimize discomfort, distress and pain, and humane

If No, Explain:

c.   Reduction in the number of animals Specify the methods used for reducing the number of animals that were considered

in the design of the proposed experiments.

Yes Rational selection of group size (e.g., pilot studies to estimate variability, power analysis)

Yes Careful experimental design (e.g., appropriate choice of control groups)

Yes sharing tissues with other investigators)   Maximize use of animals (e.g., selecting the minimal number of animals per group required for statistical verification,

Yes Minimize the loss of animals (e.g., good post-operative care, avoidance of unintended breeding)

For any of the above items not checked, please provide a brief comment about why the option is not appropriate.

2. Using the specifics of your experimental plan, justify the number of animals requested for each pain category (B, C, D, E).

Category B:

Mice for category B are for the use of breeding. Mice will be bred starting at 8 weeks of age. Females will be rebred 2-6 weeks after weaning of the previous litter. To provide sufficient mice for our studies, we will need 6 female breeders and 3 male breeders of each knockout genotype. 2 females will be caged together in a harem and one male will be used to impregnate a harem of females. The 3 males and 6 harem females will need to be replaced every 6 months during the 3 years and this will be done by using non-sibling weanlings from previous litters. Therefore, during the 3 years of this protocol, we will need 54 breeders of each of the following genotypes: IFNAR-/-, IL18-/-, IFNg-/-, Nlrp3-/- and Aim2-/- which are all mutants of the C57BL/6 mouse strain (referred to as WT). We will also need twice as many WT breeders (12 females and 6 males to be replaced every 6 months x 3 years = 108 mice) due to our study design where more WT mice will be used as controls and for development of flu-fungal coinfection models. In total, 378 mice will be used for breeding. The total number of mice to be weaned is 1100 mice. These numbers are summarized in the table below. 60 of the WT mice that are weaned will be available for other PIs at MSU to use. All other mice that are weaned will be used to support our other research protocols. Pups not transferred to other protocols or used as new breeders in this protocol will be euthanized prior to weaning at day 21.

Genotype	Number of breeders (3 years)	Number of mice to wean (3 years)
WT	108	600 (540 for PI & 60 for other PI's)
IFNAR-/-	54	100
Nlrp3-/-	54	100
Aim2-/-	54	100

IL18-/- IFNg-/-	54 54	100 100

Application to Use Live Vertebrate Animals Dept: Biology

4. **Transfer of Existing Animals: Yes**

If Yes, Indicate the IACUC ID: **16-015.0**   IACUC ID: 19-005.0   Web ID: 462

4.1 Alternatives to Proposed Procedures

1. Details about the search for alternatives

   a. Names of searched databases and the date when the search was conducted:

No	Agricola:	
No	AWIC:	
No	Biosis:	
No	Cab Abstracts:	
No	Crisis:	
No	Embase:	
Yes	Pub Med:	01/22/19

   No No Medline: NTIS:

No	Psychlit:
No	Scisearch:
No	Toxline:
No	Pascal: **No**
	Other:

   If Yes, Explain:

   b. Keywords used in the database searches:

   Keywords:

   - Influenza, Coinfection, Animals, inflammasome (Pubmed) 2 publication found
   - Influenza, Coinfection, Animals, IL-1 (Pubmed) 5 publications found
   - Influenza, Coinfection, Animals (Pubmed) 274 publications found
   - Influenza, Aspergillus, Animals (Pubmed) 39 publications found

   Summary of literature searched:

   The animals in this protocol are for breeding of more animals to be used in support of my research on viral and fungal coinfections. The breeding techniques are up-to-date. Although no experiments will be performed on the breeders, the following information supports the use of live animals and thus the need for breeding animals for my research.

   Many deaths attributed to influenza A virus (IAV) infection are the result of secondary bacterial or fungal infections termed coinfections. Although some research has been performed with viral-bacterial infections, there is currently only 1 publication on viral-fungal coinfection [1] and no publications specifically looking at influenza and Aspergillus fumigatus coinfection.

   Thus, the mice that will be breed on this protocol will support research that has no overlap with previous studies and the findings have direct clinical application as well as adding to our fundamental understanding of the pathogenesis of coinfections.

   In examining alternatives to animals, my database search provided 1 example of a lung tissue explant model for the study of coinfections. However, this model was limited to examination of the physiology of the coinfection, namely tissue damage, and pathogen replication. It was not able to recapitulate the immune response to coinfection as a live animal model would. In our preliminary research, we have also generated a novel cell culture model to study the immune signaling pathways that are involved during coinfection. However, our in vitro model uses a single immune cell (Macrophage) in isolation and once again, this does not recapitulate the complexity of the entire immune system or the physiology of pneumonia. Based on our database search, we conclude that there are no alternatives to the use of animals for studying the immune response to coinfection. Furthermore, we have chosen the mouse as a model because of the availability of reagents for studying the immune response in mice and the availability of genetic knockout mice. Mice are also the most established animal model used for IAV coinfection studies with 166 of the 274 publications using mice.

   References

   1. Oliveira LVN, et al. (2017) Influenza A Virus as a Predisposing Factor for Cryptococcosis. Front Cell Infect Microbiol. 2017 Sep 26;7:419. doi: 10.3389/fcimb.2017.00419. eCollection 2017. c. Years Searched:

1900-2019

d. Resources used in addition to the computer database search:

Information Services and other Literature Sources:

No Animal Welfare Information Center
No Lab Animal Welfare Bibliography (NLM)
No Laboratory Animal Science Journal
No Alternatives to Laboratory Animals Journal (FRAME, U.K.)
No Quick Bibliography Series (AGRICOLA) **No**
Peer Review

If Yes, Explain:

No OtherIf Yes, Explain:

Other Methods or Sources Used:

No Direct contact with investigators in field

        If Yes, Explain:
No Consultation with expert in the area of investigation

        If Yes, Explain:
No Other methods or sources

        If Yes, Explain:

5. Details of Animal Use:

1. Goals and objectives of your research

The primary goal is to produce mice to support other IACUC approved research protocols. Therefore, the mice on this protocol will be used strictly for breeding and no direct experimentation is planned. Mice to be used for experiments will be transferred accordingly.

If this application is a continuation of an ongoing project, state concisely how these goals differ from those in the original
2. application and what was accomplished during the prior approval period. If this is a new project, please indicate so.

This is a continuation of our previous breeding protocol. Our initial Breeding protocol supported research on viral-bacterial coinfections. This breeding protocol will support our viral-fungal research protocols.

Provide a concise overview of the experimental manipulations and treatments conducted on animals.
This description should allow the IACUC reviewer to understand exactly what will be done to all animals from entry into the
3. experiment to the endpoint of the study.

For breeding, homozygous male mice will be mated with homozygous female mice for a particular genetic background. Mice will be bred starting at 8 weeks of age. Females will be rebred 2-6 weeks after weaning of the previous litter. To provide sufficient mice for our studies, we will need 6 female breeders and 3 male breeders of each knockout genotype and 12 females and 6 males for WT (C57BL/6J) mice. 2 females will be caged together in a harem and one male will be used to impregnate a harem of females. The males and harem females will need to be replaced every 6 months during the 3 years and this will be done by using non-sibling weanlings from previous litters. Mice will be used for breeding and pups will be

used to support other projects.
For weaning, pups will be weaned at 21 days of age. To ensure that breeding is being maintained properly, we will periodically collect a 1mm tail snip from pups prior to weaning on day 21 of age. At weaning, pups will be sexed and will be transferred to other protocols. If mice will not be transferred to other protocols or used as future breeders, then they will be euthanized prior to 21 days of age.

6. Animal Care

1. Animal ID Method:
   No Ear Tag
   Yes Ear Punch
   No Microchip
   No Not Applicable

Yes Tattoo **No**
   Toe Clip
   No Other

   If Yes, Explain:

2. How will animals be monitored and maintained?

   All animals will be housed in the Missouri State University managed Vivarium and maintained under the standard operating procedures established for that facility and species. Facility conditions and monitoring typically includes:

   Temperature ~72-75F
   12h/12h light/dark cycle
   Humidity between 30-70%
   Air changes from 10-15 per hour
   Food and water provided ad libitum
   Cage changes at least once per week
   Daily monitoring by Vivarium staff and weekly by the Attending Veterinarian

   If special monitoring has been arranged with DLAM facility supervisor, provide DLAM contact name:

3. Should ORC contact the PI or the emergency contact if animals are found dead? **Yes**

4. Indicate requests for special handling of sick and dead animals. If animals become sick or are found dead, please contact the PI. The AV and/or Manager should also be contacted.

   Sick animals will be evaluated by the AV and recommendations for treatment or euthanasia will be given. Dead animals will be stored in the refrigerator in the event a necropsy needs to be preformed.

5. Special Housing

   Will any special housing or care be necessary? **Yes**

   If Yes, describe and list any deviations from standard ORC husbandry procedures, Guide recommendations or special animal care needs.

   All animals need to be housed in standard wire bar lid mouse cages. Mice may alternatively be hoursed in wire bar lid rat cages to allow cohousing of more mice per cage.

90

6. Special Diets

Are special diets, additives to food and/or water, or antibiotics needed? **Yes**

If Yes, Describe and List Agents:

Breeding mice will be fed on a breeding diet.

7. Describe endpoints (time points, tumor sizes etc.) and/or the maximum time length of study.

Mice will be used for breeding for on average for 6 months (not more than 1 year) and then euthanized. Pups will be weaned and transferred to other protocols at 21 days (and used between 7-12 weeks of age), given to other PI's, or euthanized if they cannot be used.

8. Describe the criteria used to determine when an animal should be removed from the study prior its endpoint.

If any breeders become sick or do not breed, they will need to be examined and euthanized if they can no longer serve as breeders.

9. Will animals be euthanized as part of the study? Yes

If No, Describe the final disposition:

If Yes, Answer all of the following questions:

Euthanasia Methods

Yes   **CO2-compressed carbon dioxide gas in cylinders and a physical method**

No   **Barbiturate overdose**

If Yes, Specify Dosage and Route:

No   **Overdose of Gas Anesthetic**

If Yes, Specify Agent:

No   **Anesthesia - followed by physical euthanasia**

If Yes, Specify Agent, Dosage, and Route:

If any of the above have been checked, indicate the physical methods that are used to ensure animals are dead:

Cervical Dislocation,Decapitation

No   **Cervical Dislocation performed with no anesthesia**

If Yes, Justify:

No   **Decapitation performed with no anesthesia**

If Yes, Justify:

No   **Other Methods**

If Yes, Describe:

10. Would the PI be willing to make available extra animal tissues or organs to other PI's?

Yes

7. Anticipated Animal Pain & Distress

1. Are there any clinical, behavioral, or physiological manifestations expected to result from experimental manipulation?

No

> If Yes, Answer all questions in this section.
>
> a. Expected clinical and/or behavioral signs of pain and distress in animals:
>
> No Decreased weight
> No Changes in food/water consumption
> No Decreased ambulation
> No Ruffled fur
> No Skin abnormality
> No Urinary problems
> No Hunched posture
> No Paw guarding
> No Porphyrin Staining
> No Lethargy
> No Diarrhea
> No Other
>
> If Yes, Explain:
>
> b. Methods of dealing with the above complications:
>
> No Analgesics
> No Anesthetics
> No Sedation or tranquilization
> No Increased bedding
> No Other
>
> If Yes, Explain:
>
> Agents used in dealing with complications:

Animals experiencing unrelieved pain or distress prior to the endpoint, as defined by institutional policy, must be humanely
2. euthanized, unless an exception to policy is requested and approved. Is exception required?

No

> If Yes, Answer all questions in this section.
>
> a. Criteria for euthanasia that will be used in this exception:
>
> b. Scientific justification for not using an earlier endpoint:

12. Items not covered in other parts of the application

Hazardous Agents

- CO_2

Application Certification

I agree to the following statements. Signify your agreement by signing at the bottom.

- I certify that I am familiar with and assure compliance in this Project with the legal standards of animal care and use established under the Federal and State laws and the policies on animal welfare of the National Institutes of Health and the University of Cayuse.
- I assume responsibility for ensuring that all persons working with animals on this project are familiar with and are trained in relevant animal procedures and that they will comply with established laws and policies regarding animal care and use. Applications will not be approved for investigators that have taken the IACUC orientation but have not completed required Laboratory Animal Coordinator certification. Contact the IACUC office to arrange training.
- I will appoint a Laboratory Coordinator to manage all animal use in the lab. I will ensure that the Coordinator receives required training and certification. I will ensure that after being certified, the Coordinator or IACUC representative will train and certify all individuals working with animals in the lab.
- I certify that all individuals working with animals on this project will register with the University Employee Occupational Health Clinic (UEOHC) by completing and submitting the "Research Animal Handlers & Animal Caretakers" medical history questionnaire (each individual who works with animals must complete the questionnaire during online orientation - UEOHC will assess the PI a processing fee).
- I certify the following: the research proposed herein is not unnecessarily duplicative of previously reported research; appropriate non-animal alternatives for this research do not exist; no alternatives to the potentially painful and/or distressful procedures conducted in this project exist. I have indicated methods used to make these determinations in the appropriate section of this animal use application. I will secure IACUC approval before changing procedures or personnel associated with this study (including adding
- personnel) I assure that I and personnel under by direct supervision will use the animals acquired for the activity described herein solely for said purpose. I also certify that if live animals are shared with other PIs or are used in any procedure other than those described in this application, I will provide the details in the form of a written amendment to the original application prior to their use.
- I acknowledge that veterinary care will be administered to moribund animals or animals experiencing more than momentary or slight pain or distress. Division of Laboratory Animal Medicine (DLAM) veterinary staff will attempt to contact me regarding the care of treatment of a moribund animal, but will institute treatment or euthanasia, as needed, if PI cannot be reached.
- I assure the IACUC and the Missouri State University that the general procedures involving animals described in my grant application have been described in the animal use application and submitted to the IACUC for review.
- I assure that I have read "Notes on Euthanasia" of animals used in research and understand how it applies to animals in this animal use application.

NOTE: Consultation of a DLAM veterinarian regarding space allocation is recommended prior to submission of application. IACUC approval of application does not assure DLAM space availability. Please contact DLAM for pre-study strategy meeting prior to ordering animals to dicuss availability of housing.

NOTE: Cell lines that have been passaged in animals or maintained using animal serum may contain murine viruses that can alter the outcome of the study and may cause an outbreak of disease among other mice. ATTCC does not screen cell lines for murine pathogens. Cell lines that have been passaged in animals or grown in media containing rodent serum should be tested for murine pathogens prior to use in animals. Please contact DLAM for more information on testing of your cell lines.

PI Signature	Date
Co-PI Signature	Date