



---

MSU Graduate Theses

---

Summer 2019

## Elucidating Immune Signaling of Influenza A Virus and *Aspergillus Fumigatus* Co-Infections through Pioneered Model Development

Meagan Danyelle Rippee-Brooks

Missouri State University, Rippee417@live.missouristate.edu

As with any intellectual project, the content and views expressed in this thesis may be considered objectionable by some readers. However, this student-scholar's work has been judged to have academic value by the student's thesis committee members trained in the discipline. 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.

---

Follow this and additional works at: <https://bearworks.missouristate.edu/theses>

 Part of the [Bacterial Infections and Mycoses Commons](#), [Comparative and Laboratory Animal Medicine Commons](#), [Fungi Commons](#), [Immunology of Infectious Disease Commons](#), [Laboratory and Basic Science Research Commons](#), [Medical Immunology Commons](#), [Medical Microbiology Commons](#), [Molecular Genetics Commons](#), [Pathogenic Microbiology Commons](#), [Respiratory Tract Diseases Commons](#), [Virology Commons](#), and the [Virus Diseases Commons](#)

### Recommended Citation

Rippee-Brooks, Meagan Danyelle, "Elucidating Immune Signaling of Influenza A Virus and *Aspergillus Fumigatus* Co-Infections through Pioneered Model Development" (2019). *MSU Graduate Theses*. 3430. <https://bearworks.missouristate.edu/theses/3430>

This article or document was made available through BearWorks, the institutional repository of Missouri State University. The work contained in it may be protected by copyright and require permission of the copyright holder for reuse or redistribution.

For more information, please contact [bearworks@missouristate.edu](mailto:bearworks@missouristate.edu).

**ELUCIDATING IMMUNE SIGNALING OF INFLUENZA A VIRUS AND  
*ASPERGILLUS FUMIGATUS* CO-INFECTIONS THROUGH PIONEERED MODEL  
DEVELOPMENT**

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

Meagan Danyelle Rippee-Brooks

August 2019

Copyright 2019 by Meagan Danyelle Rippee-Brooks

**ELUCIDATING IMMUNE SIGNALING OF INFLUENZA A VIRUS AND  
*ASPERGILLUS FUMIGATUS* CO-INFECTIONS THROUGH PIONEERED MODEL  
DEVELOPMENT**

Biology

Missouri State University, August 2019

Master of Science

Meagan Danyelle Rippee-Brooks

**ABSTRACT**

Bacterial co-infections with influenza A virus (IAV) are extremely serious and life-threatening. However, there exists limited understanding about the importance of fungal infections with IAV. Clinical case reports indicate that fungal co-infections do occur and suggest the IAV pandemic of 2009 had a propensity to predispose patients to secondary fungal infections more than previous IAV strains. IAV-fungal co-infections are marked by high mortality rates of 47 to 61% in previously healthy individuals between the ages of 20 and 60. Yet, the variables involved in this co-infection remain undetermined. I achieved effective recapitulation of this co-infection using a C57Bl/6 murine (mouse) model which resulted in similar morbidity and mortality rates seen in humans. Here, I proposed that an exacerbated immune response during infection with IAV and the opportunistic saprophytic ubiquitous fungal pathogen, *Aspergillus fumigatus*, induces the development of more severe pneumonia. I explored the possible mechanisms regulating inflammation at the cellular level. To do this, a cellular model was designed using primary mouse bone marrow derived macrophages (BMDMs) infected first with IAV and later co-infected with *A. fumigatus*. Our *in vitro* data indicated that IAV and fungal co-infections synergistically enhanced immune cell signaling and pro-inflammatory cytokine production through the caspase-1 containing inflammasome. Through various immunological techniques, I established that, during co-infection, AIM2 mediated maturation of caspase-1 facilitates the observed increase in production of pro-inflammatory cytokines. Interestingly, enhanced caspase-1 maturation is not due to increased NLRP3 inflammasome priming. NLRP3 expression actually diminishes over the course of infection, which could be explained through increased proteasomal degradation of NLRP3 through dysregulated DAPK1 signaling.

**KEYWORDS:** influenza a virus, *Aspergillus fumigatus*, H1N1 pandemic, secondary pneumonia, acute respiratory distress syndrome, co-infection, inflammasome, cytokine, reactive oxygen species, autophagy

**ELUCIDATING IMMUNE SIGNALING OF INFLUENZA A VIRUS AND  
*ASPERGILLUS FUMIGATUS* CO-INFECTION THROUGH PIONEERED MODEL  
DEVELOPMENT**

By

Meagan Danyelle Rippee-Brooks

A Master's Thesis  
Submitted to the Graduate College  
Of Missouri State University  
In Partial Fulfillment of the Requirements  
For the Degree of Master of Science, Biology

August 2019

Approved:

Christopher Lupfer, Ph.D., Thesis Committee Chair

Laszlo Kovacs, Ph.D., Committee Member

Kyoungtae Kim, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

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

I would like to first express how thankful I am that God has given me the opportunity to pursue higher education. It has been my life for 9 years to this date, and every day I am still learning.

I would like to express my sincere gratitude to my research advisor, Dr. Christopher Lupfer, who has helped me to remain faithful and diligent in producing my best work, always. He believed in my abilities as a student and a scientist and told me to never quit. I will never forget his mentorship and the patience that he provided. In addition, I would like to thank my committee members Dr. Laszlo Kovacs and Dr. Kyoungtae Kim for supporting me through this project by providing insightful ideas, laughs and a safe place.

I want to thank Missouri State's Department of Biology, Vivarium and the Graduate College for funding and supplying endless resources that were vital to my portion of this project.

I also want to thank Hazar Abu Salamah. She became my very best friend during my first year of research. She taught me most techniques that I know in the laboratory today. But most importantly, the laughs she provided were what I will always cherish! I cannot forget to mention Keslie Naffa. I couldn't have survived my second year of graduate school without her. I would also like to thank Abbi Brown, Chris Bogart, Jessi Reel and Jordan Fleetwood for their love and support too during this stage of graduate school.

I dedicate this thesis to my husband, Scott Brooks and my mother, Jackie Lea Jones because of their unending love, encouragement, patience and support during my educational journey. I also dedicate this work to those who truly believed in me. I've learned that, through everything, having a dedicated heart, you can accomplish anything you set your mind to!

## TABLE OF CONTENTS

Introduction	Page 1
Introductory Statement	Page 1
Influenza A Virus	Page 2
<i>Aspergillus fumigatus</i>	Page 6
Relevant Immune System	Page 10
IAV and <i>A. fumigatus</i> co-infections	Page 22
Problem Statements and Hypotheses	Page 24
Materials and Methods	Page 27
Overall Experimental Designs	Page 27
Animal Welfare	Page 27
Preparation of viral and fungal stocks	Page 28
<i>In vivo</i> Infection Schemes	Page 33
Differentiating Bone Marrow Derived Macrophages	Page 33
<i>In vitro</i> Infection Schemes	Page 34
Statistical Analysis	Page 38
Results	
Increase in Mortality and Morbidity	Page 39
Surge in pro-inflammatory cytokine production and casapase-1 activation during co-infection	Page 40
Exploring viable agonists contributing to enhanced NLRP3 or AIM2 mediated caspase-1 activation	Page 41
Intracellular pathogen-mediated antagonistic signals contribute to the ‘perfect storm’	Page 42
Discussion	Page 45
References	Page 51
Appendices	Page 101
Appendix A: IUCAC Application/Approval Letter	Page 101
Appendix B: Application to Use Live Vertebrate Animals: Continuation	Page 132

## LIST OF TABLES

Table 1. MHN Buffer Recipe	Page 80
Table 2. 1X Plaque Assay Medium Recipe	Page 80
Table 3. 2X Plaque Assay Medium Recipe	Page 80
Table 4. Bone Marrow Differentiating Medium Recipe	Page 81
Table 5. 1X RIPA Lysis Buffer Recipe	Page 81
Table 6. 4X SDS Loading Dye Recipe	Page 82
Table 7. 10X Tris Buffered Saline (TBS)	Page 82
Table 8. 1X Tris Buffered Saline Tween 20 (TBST)	Page 82
Table 9. 1X Tris/Glycine/SDS (Running) Buffer Recipe	Page 83
Table 10. 1X Tris/Glycine (Transfer) Buffer Recipe	Page 83
Table 11. Western Blot Primary Antibodies	Page 83
Table 12. Western Blot Secondary Antibodies	Page 84
Table 13. cDNA PCR Master Mix Recipe	Page 84
Table 14. Quantitative Real Time-PCR Primer Sequences	Page 84

## LIST OF EQUATIONS

Equation 1. Multiplicity of Infection for Influenza A virus and <i>Aspergillus fumigatus</i>	Page 85
Equation 2. General solving for pathogen stock volume	Page 85
Equation 3. Normalizing RNA concentrations	Page 85

## LIST OF FIGURES

Figure 1. Proposed IAV and <i>A. fumigatus</i> immune signaling pathways	Page 86
Figure 2. <i>In vivo</i> infection scheme, morbidity and mortality of infected WT mice	Page 87-88
Figure 3. <i>In vitro</i> infection scheme	Page 89
Figure 4. Generating an ELISA Standard Curve	Page 90
Figure 5. Co-infection with IAV and <i>A. fumigatus</i> results in overproduction of pro-inflammatory cytokines through enhanced caspase-1 activation	Page 91
Figure 6. Overproduction of IL-1 $\beta$ is NF- $\kappa$ B and pro-IL-1 $\beta$ independent	Page 92
Figure 7. Negating ROS as a source of direct inflammasome activation through cytoplasmic sensing	Page 93
Figure 8. Negating viable agonists contributing to the culmination of caspase-1 activation through inflammasome complex assembly	Page 94
Figure 9. Expression of DAPK1 during IAV and <i>A. fumigatus</i> co-infections	Page 95
Figure 10. Co-infecting pathogenic agents synergistically induce AIM2 inflammasome activation	Page 96
Figure 11. Preliminary <i>in vitro</i> knockout experiments reiterate that NLRP3 and AIM2 inflammasomes independently reach similar objectives	Page 97-98
Figure 12. Possible molecular signaling mechanisms left to explore	Page 99
Figure 13. IAV and <i>A. fumigatus</i> co-infections and the pivotal role of activated caspase-1	Page 100

## INTRODUCTION

Over the last century, the field of immunology, through great strides, has made meaningful contributions to science through prolonging and improving the quality of life. As our repertoire of knowledge about the immune system continues to evolve, interestingly, so does the causative agents of infectious diseases we must combat daily. Immunologists, as detectives, have plenty of clues left to investigate. As new pathogens emerge, old pathogens mutate and/or evolve and continue to infect the human population. Host-pathogen interactions are a large portion of understanding how populations succumb to disease. Some interactions are multivariate. Some diseases occur with multiple pathogens infecting the same host, a phenomenon known as co-infections. A co-infection is simply two pathogens successfully infecting the host in sequential order, and the order of infection is often essential. These interactions are the premise of the research to be elaborated further in this thesis.

Much like a thrilling mystery novel, there exists a constant struggle between the protagonist (the host) and the antagonist (the pathogen). The pathogen is consistently developing mischievous methods to invade or evade the host. Our story begins with a recent example in human history of a host-pathogen battle, the influenza A pandemic of 2009. Medical professionals and immunologists were puzzled by this pandemic, in particular, by an explicit population of young, previously healthy individuals who became ill and died—a population not noticeably plagued by pandemic strains in recorded history [1]. The primary pathogen was influenza A virus (IAV). IAV has claimed many lives throughout the last century [2]. IAV, after infecting the host, initiates a robust immune response, which results in taxing of the body's resources. Interestingly, IAV usually does not possess the virulence to kill someone on its own,

but the depletion of the hosts ability to clear this primary pathogen allows a secondary or opportunistic pathogen, such as a fungus, *Aspergillus fumigatus* for example, to occupy the host [3]. Together, these two pathogens are the culprits of a devastating disease with mortality rates ranging from 47 to 61% in these co-infected (but initially immunocompetent) individuals [4].

While IAV-bacterial co-infections are well defined, our understanding of co-infections with fungus as the major co-infecting agent is severely lacking. Interestingly, this co-infection still leaves medical professionals baffled by the lack of treatments to successfully prevent such high mortality rates over the last decade. Therefore, growing awareness of these co-infections arises from their occurrence, especially in recent years [5].

For scientists, the goal is to make a contribution to ‘solve’ this mystery or understand how these two antagonists successfully destroy the protagonist together in this increasingly prevalent viral-fungal co-infection. The development of the disease is likely associated with the specific immune responses to IAV and *A. fumigatus*. It must be noted that the response of the immune system to each of these pathogens is quite different. To generally understand this co-infection, one must first obtain a foundational understanding of these causative agents involved and their unique immune responses separately. Improved understanding of the pathogens themselves, and the immune responses they elicit, will facilitate new research into improving the effectiveness of available treatments.

## **Influenza A virus**

Influenza A virus (IAV), a common, but significant human respiratory pathogen, has affected us all in one way or another. Ranging from mild illness to life threatening infections, most of us have suffered from the seasonal flu.

**History.** IAV remains responsible for the killing of millions of people. The most notorious IAV pandemic was the devastating 1918-1919 H1N1 ‘Spanish Flu’ pandemic, and IAV *still* remains a global threat to human health *yearly* via seasonal pandemics [6]. Despite development of an ever-changing vaccine in response to mutating IAV existing for the last 70 years, seasonal and pandemic influenza infections remain prevalent. Other influenza pandemics have occurred but were less severe. Specifically, the most recent flu pandemic, the 2009 H1N1 ‘Swine Flu’, surfaced in the United States (USA) and Mexico [7]. The Swine Flu emerged in April and by June, the World Health Organization (WHO) classified this specific strain as a pandemic virus [8].

**Characteristics.** Belonging to the *Orthomyxoviridae* family, IAV is one of three genera of influenza viruses infectious to humans, influenza A, B and C [9]. IAV is an enveloped virus. The envelope is a lipid membrane surrounding the virion derived from the host cell plasma membrane [10]. The IAV genome consists of eight separate segments of negative-sense single-stranded RNA (entire genetic material). Two important glycoprotein antigens project from the virion’s surface: haemagglutinin, HA (of which there are 18 versions, H1 – H18) and neuraminidase, NA (with versions N1 –N11) [11]. The 18 variants of HA and 11 variants of NA glycoproteins contribute to further subdividing IAV [12]. IAV strains are referred to by the associated nomenclature through the HA and NA specific to the virus – H1N1 [13-15]. IAV has additional proteins including the nucleoprotein (NP), responsible for aiding in viral replication, and an internal coat of matrix proteins (M1 and M2), which function as mediators for exporting of viral ribonucleoproteins from the host cell nucleus and virus assembly and exit [16-18]. The remaining segments of the RNA genome of IAV encode for molecular machinery proteins known generally as polymerases. These polymerase complex proteins, polymerase basic proteins

(PB1/2) and polymerase acidic protein (PA) together are the subunits that form a trimeric viral RNA-dependent RNA polymerase [19-22]. Genome replication and mRNA transcription from the RNA viral genome by these RNA polymerases occurs within the nucleus of infected host cells [23]. Lastly, NS1 (nonstructural protein) and PB1-F2 (part of the viral RNA polymerase) are important in viral virulence through blocking antiviral immune responses (interferons) within the host cell [24-27].

**Gene Re-assortment.** The 2009 strain of IAV was unique. The genes involved were not previously identified together in any of the common reservoirs of influenza before –human, avian and other mammals, like swine. Therefore, this pandemic (H1N1) 2009 virus consisted of multiple re-assorted genes from diverse organism origins [28]. This pandemic (H1N1) 2009 virus is currently the only IAV strain with an animal reservoir that can sufficiently transmit infection to humans. The ability to transmit from an animal reservoir to humans has been associated with pandemic viruses reported throughout history and is one major reason why it is studied [29]. Recall the segmented genome of IAV mentioned previously. This segmented genome structure facilitates the exchange of genes between different strains of influenza that have infected the same host cell (antigenic shift). Such genetic re-assortment poses a significant risk of zoonotic infection through host switching [30]. Importantly, this pandemic (H1N1) 2009 virus underwent genetic translocation from three different IAV strains and expedited the generation of a *novel* pandemic strain of IAV in 2009 [31]. The genome of IAV H1N1 2009 was composed of one genetic segment from human IAV H3N2, two segments of avian IAV H1N1 and the remaining five segments originating from swine IAV H1N1 [32]. Thus, the addition of swine as a confirmed mixing vessel for influenza viruses contributes to increased host range and virulence of IAV in mammals [33-35].

**Transmission.** The 2009 H1N1 strain of IAV was also different from most seasonal IAVs, because it affected mainly teens, young adults and adults instead of the common propensity of most IAVs to affect the very young or elderly [36]. This pattern of infectivity was likely due to previous IAV (H1N1) strains circulating amongst the human population for decades prior to this specific exposure with the 2009 pandemic strain [37]. Transmission of IAV occurs normally through inhalation of virus-laden air-droplets, contact with contaminated objects, also called fomites, and by direct contact with infected individuals [38]. Post inhalation of aerosols, IAV, an obligate intracellular parasite, targets the pseudostratified columnar epithelial cells of the trachea and bronchial tree [39].

**Viral Factors Implicating Infection Severity.** Previous exhaustive research of IAV co-infections with bacterial pathogens, especially with *Streptococcus pneumoniae*, have highlighted the severity of a primary viral infection to be associated with the specifics of HA and NA surface antigen types randomly resulting from viral re-assortment [40-42]. Due to the specificity of HA binding to  $\alpha$ 2,6-sialylated glycans (cellular glycoprotein receptors) present on the cellular membrane of unique respiratory epithelia previously mentioned and the host protease activity does impact the site and development of the strain of IAV infection depending on the strain [43-44]. HA experiences a conformational change once the low pH within an endosome permits the successful penetration of the virion into the host cell. Highly pathogenic strains of IAV are cleaved inside host cells. NA must be complementary to and share the same receptor affinity of HA [45-46]. This NA affinity promotes ease of release of newly formed virion progeny through hydrolyzing the sialic acid to facilitate virion detachment from the host cell [47-48]. IAV can also produce a viral cytotoxin, PB1-F2. This cytotoxin actually has been linked to increasing inflammation and therefore host cell damage and actually the adherence of co/secondary

bacterial infecting agents, such as the mentioned *S. pneumoniae* [49-52]. Alarming, the predicted superinfections reported were of different types during the 2009-2010 flu season in comparison to the 1918 H1N1 pandemic due to the use of antibiotics, supportive measures (ventilators) and vaccinations for *S. pneumoniae*. Therefore, the predicted reduction in *S. pneumoniae* co-infection were correct, a decrease to 30% [53], but there was a shift to co-infection with *S. aureus* and *A. fumigatus*. The direct link of HA, NA and PB1-F2 and their involvement of co/secondary fungal infections with IAV have yet to be studied in detail.

**Pathogenesis.** An infection with IAV causes a broad range of signs and symptoms in infected individuals. Commonly, the signs or symptoms of a positive IAV infection includes runny nose, congestion, muscle and headaches, dizziness, fatigue or malaise and fever [54]. The pathogenesis of IAV can be quite mild to severe. Interestingly, 2009 H1N1 is reported to be characterized also by the observed increased replication and pathological changes in the lungs of nonhuman primates and *ex vivo* human lung tissues [55]. Such observations could contribute to explaining the ability of this strain of IAV to cause severe viral pneumonitis in humans [56]. 2009 H1N1 was predicted as a high-virulence virus like 1918 H1N1 and H5N1. High-virulence viruses such as these tend to infect pneumocytes and intra-alveolar macrophages [57-59]. Among the extensive list of co-infecting bacterial and other viral pathogens after IAV infection occurs, the 2009 pandemic strain of IAV is believed to have the propensity to predispose patients to opportunistic pathogens such as fungi like *Aspergillus fumigatus* [60].

### ***Aspergillus fumigatus* (*A. fumigatus*)**

*A. fumigatus* is a ubiquitous, airborne saprophytic fungus. Its airborne spores are highly prevalent, and it is essential in the recycling of environmental carbon and nitrogen within the

earth's soil by the decomposition organic debris [61]. Due to the ability of the innate immune systems of healthy individuals to eliminate the inhaled conidia spores and prevent infection with *A. fumigatus*, this fungus was considered an opportunistic weak pathogen *until* recently [62]. *A. fumigatus* is now considered to be there causal agent of the most common mold infection worldwide [63-64]!

**General Biology.** *A. fumigatus* is ubiquitous, not only because of its prevalent and easily dispersed spores, but also due to several factors such a cell wall structure and the physiology of the vegetative biofilm generated through metabolism and responses to stress. The fungus can grow and survive at a wide range of temperatures (~12°C to 37°C+) and pH (3.7 to 6.7) [65]. The conidia spores are easily airborne upon starvation of the fungus. These asexual spores detach from specialized hyphal head structures, or conidiophores, to eventually become airborne and spread through wind currents after disturbance [66]. Conidiophores produce the conidia and aid in the spore's transmission asexually. It was believed, until 2009, that *A. fumigatus* was reproducing exclusively asexually. But, conditions for sexual replication are explicit and non-physiological due to the ascospores (sexual cycle structure) remaining dormant until they successfully germinate above 65°C [67]. The hyphae of *A. fumigatus* are responsible for the formation of fungal colony growth, or mycelium formation, after germination begins under optimal temperature, pH and humidity [68]. The colony eventually forms a biofilm, the vegetative form of this filamentous fungi that is made of an extracellular matrix of hyphae composed of embedded septated multinucleated cells [69]. It must be noted here that forms of the cell wall of the fungus differ between the multiple stages (conidial, mycelial/hyphal, biofilm) of *A. fumigatus* [70-71].

The cell wall composition of *A. fumigatus* continuously adapts over the life cycle of the fungus as the cell cycles progress and the environment of the fungus is altered [72]. Understanding why and how these fungal cell wall configurations exist remains a major challenge. However, the organizational structure of the inner cell wall is thoroughly characterized. Generally, the inner portion of the cell wall is composed of cross-linked branches of  $\beta$ -1,3-glucan/ $\beta$ -1,4-glucan, galactomannan, galactosaminogalactan (GAG) and chitin – constituting the insoluble, alkali skeleton [73-74]. Beginning with the conidial stage, the dense layer around the conidia contains  $\alpha$ -1,3-glucan, melanin and RodA hydrophobin [75]. The hyphal cell wall, succeeding germination, changes and the melanin and rodlet layer are shed to allow hyphal growth to proceed [76]. It must be noted that depending upon the environmental conditions *A. fumigatus* is experiencing, the hyphae may continue to contain melanin despite the shedding of that specific layer [77]. Previous research has highlighted that the gene required for the biosynthesis of melanin, PKSP, was upregulated within the lung environment of immunocompromised mice [78-79]. Therefore, melanin still remains a significant area of study for understanding the virulence of *A. fumigatus* and the resulting fungal diseases which lead to aspergillosis. It is known that the extracellular matrix composed of GAG, galactomannan,  $\alpha$ -1,3-glucans and melanin facilitate the promotion of *A. fumigatus* infections [80].

For the metabolism of *A. fumigatus* to be effective, this fungus has adapted to a variety of nutritional requirements found in biological materials, also contributing to its ubiquity. The ability of the fungus to adapt to the vast variety of nutritional sources is associated with its wide range of protease and enzyme production. These proteases and enzymes allow not easily accessible nutrients to become obtainable. These proteases and enzymes facilitate the acquisition of essential cations from the host tissue such as iron and zinc [81]. Previous research highlighted

that the acquisition of nitrogen and carbon is crucial for initiating the early stages of infection [82]. Mutating the transcription factors that regulate these protease and enzyme genes (cross-pathway control protein A (CpcA), AreA and CreA) resulted in reduced virulence observed in murine *in vivo* experiments [83]. *A. fumigatus* is actually an obligate aerobe, meaning that the fungus must have oxygen to survive [84-85]. However, *A. fumigatus* has developed a mechanism of strain/stress fitness under low oxygen conditions – contributing to virulence of the fungus. It was reported that *A. fumigatus* actually induces a gene that correlates to the production of sterol, SRBA (sterol regulatory element-binding protein gene). This gene initiates the biosynthesis of ergosterol. Low sterol levels are confirmed in fungi like *A. fumigatus* when in a hypoxic environment. Hypoxia actually facilitates proteolytic cleavage through Sre1 due to a loss of acyl-CoA. The regulatory links between hypoxia adaptation, iron homeostasis and ergosterol biosynthesis remains with the fungal electron transport chain within the mitochondria and alcohol dehydrogenase [86-87, 289-290]. This, therefore, allows *A. fumigatus* to thrive in many, including the lungs of humans.

**Transmission.** *A. fumigatus* fungus profusely conidiates because of the thousands of conidia produced by the conidial head. Dissemination of the conidia is not complicated; it is simply done through disturbances usually caused by strong air currents in the environment. Due to their small size and buoyancy, the conidia, once in the air, remain there. Therefore, it would make sense that *at least* hundreds to thousands of asexual spores are inhaled per person per day [88]. Airborne conidia, with their microscopic sizes of 2 –3 microns, can be inhaled deep into the lungs where they reach and settle into the alveoli [89]. The lung tissues, although not a normal niche for *A. fumigatus*, provides an environment rich in organic compounds and essential metals for the fungus to feed upon. *A. fumigatus* is normally no danger to healthy ‘immunocompetent’

individuals due to the armory of defenses the innate immune system provides us. In immunocompetent individuals, resident alveolar macrophages, muco-ciliary action of the lung epithelial cells and nutritional immunity are just a few key mechanisms in host defense [90-92]. However, proteases and destructive enzymes permit the broad biological specificity of this fungus to achieve nourishment from within the human host. The explicit known proteases that *A. fumigatus* uses to enable growth include serine metalloproteinases, and aspartic proteases [93-95]. Not surprisingly, much more research is needed to understand which pivotal proteases and enzymes destroy the human host for *A. fumigatus*' survival.

*Aspergillus fumigatus* remains one of the most challenging and poorly understood pathogens that can infect humans. The complexity of this nomad pathogen accelerates the urgent need to grasp its interaction with the host immune response.

## **Relevant Immune System**

Maintaining homeostasis of the host is *the* vital role of the immune system, through the orchestration of cells, tissues and organs working together to protect and defend against the invasion of foreign agents. Intriguingly, all living things have an immune system of some kind. Humans, in particular, have complex immune systems, configured normally with sections of the innate and adaptive immune systems.

**Overview of Innate Immunity.** During the first critical hours and days after exposure to an invader, the innate immune system acting as the 'first line of defense', is responsible for initiating a biological response to harmful stimuli –known as inflammation.

Inflammation. The primary purpose of inflammation is the communication and migration of immune cells to the exact site where the trauma occurred. Without this response, the

infections, wounds and damage to tissue that the body experiences would not be able to heal efficiently. There exist five symptoms that are commonly known for acute inflammation, these include: redness, heat, swelling, pain and loss of function. So, what causes these five symptoms? The immune cells responding to a specific trauma release various chemical messages, known generally as inflammatory mediators [96-98]. These mediators include hormones: bradykinin and histamine. These two hormones cause a physiological reaction around the site of trauma known as vasodilation, therefore increasing blood flow to reach the injured tissue. Two of the five symptoms, redness and heat, are explained through this increase in blood flow due to the release of these mediators. The increased blood flow and vascular permeability in the injured area facilitates immune cell entry into the affected tissue. This process, scientifically known as diapedesis, is the movement of immune cells into tissue by passing through leaky blood vessel walls. Blood vessel leakiness and diapedesis are responsible for the swelling and pain experienced. Due to the pain, loss of function of the injured area results, causing the host to protect the affected portion of the body [99-106].

Innate Immune Cells. Commonly known as white blood cells or leukocytes, immune cells come in all shapes, sizes and functions. These differentiated leukocytes originate from hematopoietic stem cells [107-108]. There are subsections of leukocytes known as granulocytes, antigen presenting cells and lymphocytes. Here, I will mainly focus on cells involved with the innate immune system –specifically antigen presenting cells (APCs) and granulocytes. APCs include macrophages and dendritic cells, which coordinate their arrival at specific sites of trauma [109-110]. These phagocytes have the general role of the detection of foreign invaders or their particles –including carbohydrates, proteins and lipids –and literally presenting or ‘showing’ chopped up pieces of pathogens (antigens) to activate a lymphocyte (adaptive immune cells such

as T and B cells) [111-112]. How can these innate immune cells, specifically phagocytes, know that what they are presenting is a cause of danger to the host? The answer to this question lies in a host of proteins capable of detecting pathogen components not found in the human body.

Pathogen Associated Molecules. Foreign molecular components of a microorganism can come in all shapes and sizes and are usually referred to as pathogen associated molecular patterns, or PAMPs. PAMPs are molecules found in the pathogen, but not in the host, like cell wall components or specific pathogen proteins [113-116].

*PAMPs of IAV.* The pathogenic particles that trigger innate immunity in response to IAV include the following: host DNA released in response to death of neighboring cell by IAV, ssRNA and dsRNA from the virus, the virus M2 protein, viral PB1-F2 protein and the associated damage to the cell in response to viral replication all serve to activate the immune response [117-119].

*PAMPs of Aspergillus fumigatus.* Research in *A. fumigatus* virulence is currently a topic of interest due to its complexity described above. Several well-defined PAMPs of *A. fumigatus* include the carbohydrates present within the cell wall, specifically  $\beta$ -1,3-glucan, mannose-containing structures such as galactomannan, and chitins [120-122].

Pathogen Detectors. Interestingly, there exist specific immune receptors called pattern recognition receptors (PRRs) present in or on the host epithelial and immune cells that can distinguish foreign molecular markers or PAMPs and host cell damage associated molecular patterns (DAMPs), like extracellular ATP release. The specificity of PAMPs and DAMPs and their activation of exclusive PRRs remains dependent upon the pathogen causing the infection. In other words, IAV will activate a specific subset of PRRs that are different than the PRRs activated by *A. fumigatus*. There are a variety of PRRs such as Toll-like receptors (TLRs), retinoic

acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and absent-in-melanoma 2 (AIM2)-like receptors. Once these PRRs are activated by PAMPs and/or DAMPs, cellular signaling cascades within these cells result in phagocytosis, transcription of immune genes, activation of multiprotein complexes known as inflammasomes and the production of immune signaling molecules or cytokines [123-127].

*PRRs and IAV.* During IAV infection and genome replication, the 5' triphosphate (PPP) of uncapped-RNA is bound by a specific cytoplasmic sensor, RIG-I of the RLR family. 5' triphosphate containing RNA is not usually present in the cytoplasm of human cells. Human mRNA possesses a 5-methylguanine cap added during maturation, while tRNA and rRNA only have 5' monophosphates. Thus, viral RNA with a 5' triphosphate indicates to the cell that something is amiss. Deficiency in RIG-I is linked to an impaired antiviral response. Activated RIG-I interacts with a downstream adaptor protein, the mitochondrial antiviral-signaling protein (MAVS) [128-131]. Prior research has linked diminished levels of interferon production to a deficiency in MAVS causing increased difficulty fighting viral infections within *Mavs* knockdown mice [132]. TLRs are also utilized to recognize IAV, specifically TLR3, TLR7 and TLR8. These receptors of viral RNA reside in the endosome where they can sense any RNA that does not escape the endosome during viral infection. Activation of TLR3, 7, and 8 subsequently signals the adaptor proteins TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) and myeloid differentiation primary response gene 88 (MYD88) [133-136]. Whether it is the RIG-I – MAVS or the TLR – TRIF/MYD88 pathway, detection of viral RNA induces the production of pro-inflammatory cytokines and type I interferons [137].

*PRRs and Aspergillus fumigatus.* Of the PRR families, previous research has proved that *A. fumigatus* can activate CLRs, TLRs and various NLRs [138]. The best-characterized PRR activated via *A. fumigatus* is the CLR dectin-1 through the recognition of  $\beta$ -1,3-glucan moieties present on germinating or swollen conidia. Another CLR, dectin-2, can recognize the  $\alpha$ -mannans such as galactomannan. The recently discovered  $\alpha$ -mannans, constituents of the outer layer of the fungal cell wall, are responsible for masking  $\beta$ -glucans. There remains the possibility that once the conidia are inhaled by the host, the detection of conidia by dectin-2 precedes dectin-1 [139]. TLRs 2 and 4, located on the extracellular surface of the host cell, were shown in several studies to be key recognition PRRs of the host against *A. fumigatus*. However, their functions currently have not been fully elucidated due to conflicting reports. Presumably, TLR 2 detects chitin and TLR 4 detects  $\alpha$ -glucans. After Dectin-1 binds to fungal  $\beta$ -1,3-glucans, adaptor proteins, like caspase recruitment domain protein 9 (CARD9), and kinases, like spleen tyrosine kinase (Syk), ultimately result in the activation of similar signaling pathways that were discussed for TLRs, namely MYD88. For sake of simplicity, the cascade of activation and signaling initiates the transcription of pro-inflammatory genes, like cytokines, and genes required for cytokine maturation. [140-141].

Transcriptional Activation. The discussed adaptor proteins for PRR signaling involved in both IAV and *A. fumigatus* infections – MYD88, TRIF, MAVS and CARD9 - all mediate the downstream activation of one common family of transcription factors known as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B). PRRs and adaptor proteins regulate this family of five transcription factors. However, antigen receptors like PRRs are not the only means by which the NF- $\kappa$ B pathway is activated. Oxidative stress and the production and

sensing of cytokines by neighboring cells facilitate its activation too. NF- $\kappa$ B proteins have the ability to form distinct transcriptionally active homo- and heterodimeric complexes [142-143].

*NF- $\kappa$ B signaling.* The most recognized dimer of the NF- $\kappa$ B family is RelA (p65)/p50. The p65/p50 dimer in most unstimulated cells remains within the cytosol inactivated by a family of inhibitors known as I $\kappa$ B proteins ( $\alpha/\beta/\gamma$ ). When PRRs and adaptor proteins interact, like RIG-I – MAVS, this triggers a kinase cascade leading to activation of the p65/p50 dimer via phosphorylation of the serine residues of I $\kappa$ B by the I $\kappa$ B kinase (IKK) complex. Phosphorylation of I $\kappa$ B results in polyubiquitination at two lysine residues, 21 and 22 (Lys<sup>21</sup>/Lys<sup>22</sup>) [144-148]. Ubiquitination is a type of post-translational modification where ubiquitin proteins are used as a ‘tag’ to mark the specific protein for degradation via the proteasome complex [149-152]. Polyubiquitination of I $\kappa$ Bs are recognized by the multi-subunit 26S proteasome, where they are unfolded and degraded into small peptides to be recycled. This 26S proteasome degradation process releases NF- $\kappa$ B dimers, which translocate into the nucleus. These NF- $\kappa$ B dimers bind to promoters of inflammatory or immune-related genes through  $\kappa$ B motif interactions. NF- $\kappa$ B is a crucial mediator of pro-inflammatory gene induction. If the activation of downstream adaptor proteins and PRRs ceases, so will the transcription of pro-inflammatory associated gene expression [153-157]. Negative feed-back loops operate as check-points to excessive inflammation and the diseased states that could result from excessive inflammatory conditions [158-160].

*Interferon Production.* Interferons are important antiviral cytokines produced by innate immune cells to communicate the presence of a viral infection and initiate cellular responses to block virus replication [161]. To accomplish this, MAVS activates downstream antiviral signaling pathways through interferon regulator factors (IRFs), which are crucial for the

expression of type I interferons (specifically IFN- $\alpha$  and IFN- $\beta$ ) [162-164]. Endosomal TLR also induce the transcription of type I interferons through this method. Specifically, TLR receptor signaling and their activated adaptors results in IRF3 and IRF7 activation. IRF3 and 7 are transcription factors that induce expression of type I IFNs [165-166]. Once the interferons are made, they are released from the cell and bind in both paracrine and autocrine fashion to the IFN- $\alpha$  receptor (IFNAR) [167]. This canonical signaling pathway of type I IFNs activates the receptor-associated protein tyrosine kinases Janus kinase I (JAK1) and tyrosine kinase 2 (Tyk2). These two receptor-associated proteins facilitate the phosphorylation mediated activation of STAT1 and STAT2 (signal transducer and activator of transcription 1 and 2). STAT1 and STAT2 dimerize together and rapidly translocate to the nucleus. Once inside the nucleus, they bind with IFN-regulatory factor 9 (IRF9) to form a complex known as IFN-stimulated gene factor 3 (ISGF3) [168-170]. ISGF3 is a transcription factor complex that induces specific antiviral gene expression, like Mx1, that inhibits IAV RNA replication [171].

Cytokines. Immune signaling molecules called cytokines are cellular biochemicals released and sensed by cells to permit cells to communicate with each other [172]. Because of the significance of NF- $\kappa$ B within this investigation, I will use it as the primary example of a family of transcription factors that regulates the production of cytokines. NF- $\kappa$ B induces transcription of proinflammatory pyrogenic cytokines namely interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1-beta (IL-1 $\beta$ ) [173-174]. IL-1 $\beta$  is also a mediator in the regulation and production of TNF- $\alpha$  and IL-6. IL-1 $\beta$  has attracted considerable scientific attention in many diseased states due to its manifestation of innate immunity through inflammation. Usually, this cytokine functions in host defense, but when it is uncontrolled, its

continued expression is detrimental to survival. Activation of specific inflammasomes aids in the processing and secretion of IL-1 $\beta$  [175-176].

Inflammasome Activation. Of the variety of inflammasomes, here only the two closely related to our study will be discussed, the NLR Family Pyrin Domain Containing 3 (NLRP3) and Absent in melanoma 2 (AIM2) inflammasomes. Simply put, inflammasomes are caspase-activating molecular machines.

*The NLRP3 inflammasome.* Anatomically, the best characterized inflammasome, NLRP3, once activated, is constructed of the following protein: NLRP3 binds to an adaptor protein known as ASC (apoptosis-associated speck-like protein containing a CARD), which in turn binds pro-caspase-1 [177-179]. Caspase-1 is a protease. The integration of pro-caspase-1 allows the multiprotein complex to cleave itself, and cleaved caspase-1 is fully functional with the ability to cleave cytokines such as inactive pro-IL-1 $\beta$  into mature functional IL-1 $\beta$ , thereby inducing inflammation [180-181]. Active caspase-1 also cleaves and activates Gasdermin D, which is a membrane pore forming protein, causing a version of cell death known as pyroptosis. The NLRP3 inflammasome requires a two-step activation process. First, PAMPs activate TLRs resulting in increased expression of NLRP3 and pro-IL-1 $\beta$ . Second, DAMPs or PAMPs, such as potassium efflux, lysosomal-damage, uric acid, silica, aluminum, whole pathogens, mitochondrial damage, cholesterol crystals and amyloid- $\beta$  induce a conformational change and/or post-translational modifications of NLRP3, which results in its activation and binding to ASC [182-184]. A non-canonical method of activation is also perpetuated through the binding of LPS in the cytoplasm of cells to another caspase, caspase-11. Active caspase-11 then facilitates the activation of the NLRP3 inflammasome complex. Both the canonical and non-canonical

methods of activation lead to inflammasome complex assembly and activation of caspase-1 with cell death and inflammation as the final consequences [185-188].

*The AIM2 inflammasome.* The AIM2 inflammasome is a cytoplasmic sensor of double-stranded DNA molecules of self and foreign (pathogens) origin [189-191]. AIM2 has two domains that structurally define it: one HIN domain and one pyrin domain (PYD). The HIN domain of this specific ALR recognizes dsDNA. The HIN domain can have several distinct sub-classes: HIN-A, HIN-B and HIN-C [192-195]. Of these sub-classes, previous research has proposed that they may differ in function due to minor differences in structure, therefore they may bind DNA at different affinities. AIM2 also forms an inflammasome capable of activating IL-1 $\beta$  and inducing pyroptosis through the cleavage of caspase-1 [196-197].

*Caspase-1.* Caspase-1 is one of the many inflammatory cysteine-aspartic proteases characterized in mammals (specifically mice and humans). Caspases begin as inactive zymogens (pro-caspases) that can be activated through cleavage following activation by a suitable stimulus [198-199]. This activation is based not only on proximity to other caspase molecules, but also affected by post-translational modifications. The inflammatory caspases, like caspase-1, are termed 'inflammatory' because they activate substrates like pro-IL-1 $\beta$  and cause cell death in a manner that releases cytosolic contents that potentiate inflammation [200-202].

As already revealed, biologically active IL-1 $\beta$  is a pleiotropic cytokine that influences multiple cell types to enable inflammation. Without inflammasomes activating IL-1 $\beta$ , the intensity of inflammatory responses would diminish. Therefore, excessive or inappropriate inflammatory responses could be controlled or remediated by inhibiting the inflammasome [203].

**Pathogen Immune Evasion.** The evolutionary arms race between pathogen and host is an enduring battle. Due to the selective pressure imposed by the immune system, pathogens such as IAV and *A. fumigatus* constantly evolve and have established an assortment of techniques to evade the immune system.

**Influenza A virus.** IAV, although very small and simple, contains a collection of elegant mechanisms to evade the immune system; it is a master tactician. Beginning with NS1 protein expression, IAV can inhibit the cascade needed in the initial production of type I IFN production, thereby acting as an effective suppressor of host antiviral immunity [204]. By doing this, IAV sets itself up for mass replication events, therefore spreading the virus throughout the current host and impairing the quality of the upcoming adaptive immune response. NS1 inhibits RIG-I-mediated signaling through halting ubiquitination, therefore preventing the production of type I IFNs [205-206]. PB1-F2 has been shown to boost mitochondrial ROS and calcium efflux through the inhibition of MAVS [207-209]. PB2 and PA together cap-snatch. Cap-snatching is the process that IAV does to ‘steal’ the cap off host mRNA by cleavage of the 5’-methyl guanidine cap of host mRNA and transferring it to viral mRNA. This stolen host cap is then used as a primer to begin transcription of viral mRNAs and to make the viral mRNA undetectable by RIG-I [210].

*IAV and phagocytosis impairment.* IAV infection primes the hosts airways for co/secondary infections [211-213]. With understood bacterial co-infections, such as with *S. pneumoniae*, the respiratory impairments are well characterized [215-216]. These published data have shown that influenza infections result in increased and prolonged bacterial growth and a reduced ability of macrophages to effectively clear bacteria due to reduced phagocytic activity [217-219]. This reduction in the ability of macrophages to effectively phagocytize pathogens

during IAV infections is quite common –proven now even in fungal infections! Oliveira et al. determined that IAV at the peak of infection, around day 3 in their established murine model, causes an impairment of phagocytosis of yeast-like fungus, *Cryptococcus gattii*. This host-vulnerability factor is also demonstrated previously in cases of bacterial co-infections too [220-223]. Phagocytosis must occur rapidly in order to halt dying cells from exposing damage associated molecular patterns, or DAMPs and prevent excessive inflammation [224-225]. Macrophage activity during IAV infection is suppressed through inhibitory receptors like CD200, disruption of normal ciliary clearance in the lung and by decreasing concentrations of T cell-derived interferon (IFN- $\gamma$ ) through type 1 interferon production (IFN- $\alpha/\beta$ ) [226-227]. Perhaps not only bacterial clearance is affected by this. Perhaps fungal pathogens could take advantage of these diminished phagocytic responses as well? This hypothesis is supported by research into heritable genetic defects that demonstrate deadly fungal infections are mediated by macrophage activation defects, along with intact T-cell activation therefore leading to reduced macrophage fungicidal activity [228-230].

*Aspergillus fumigatus*. Usually, the innate immune system eliminates *A. fumigatus* through the coordination action of alveolar macrophages, neutrophils and NK cells. These cells help to kill conidia in the lungs. In addition, the pseudostratified columnar epithelium integrated with goblet (mucus producing) and ciliated cells creates a mucus barrier that is constantly being swept up and out of the airways to prevent colonization of the lungs [231-232]. However, if there is significant impairment of the immune system through corticosteroid use, AIDS, immunosuppressive medication, genetic abnormalities or, as I hypothesize, acute IAV infections, *A. fumigatus* can effectively infiltrate and infect the host. *A. fumigatus* can be shielded so that the fungus can evade the immune system. For example, the surface of *A. fumigatus* conidia consists

of coats of melanin and hydrophobin to literally act as a shield to protect PAMPs from recognition by PRRs. Melanin not only envelopes the conidial cell wall to protect the conidia from oxidative stress and desiccation, but it also aids in preventing phagocytes from engulfing and killing *A. fumigatus* conidia through inhibition of a specific form of phagocytosis termed LAP [239-242]. When conidia germinate, they shed the surface layers of melanin and hydrophobin and expose PAMPs such as  $\beta$ -1,3-glucans. Other fungal proteins, including GAG, gliotoxin and fumagillin (toxins), have evolved to poison host cells and prompt apoptosis. GAG also specifically inhibits a mechanism employed by neutrophils called neutrophil extracellular traps or NETs and it can inhibit IL-1 $\alpha/\beta$  signaling [233-238].

*A. fumigatus*, *Autophagy and DAPK1*. Autophagy is an elaborate cellular self-eating process essentially conserved to allow the degradation and recycling of really anything the cell wants to dispose of. Simply, both forms of autophagy –canonical and non-canonical- result in formation of a double-membrane structure containing the sequestered cytoplasmic material (also called the autophagosome) [243-245]. Ultimately, fusion of the autophagosome with a lysosome is permitted by Rab and SNARE proteins. Rab proteins, specifically Rab7, interact with tethering factors, and Q-SNARE STX17. Together, they allow the fusion events of the autophagosome with lysosomes, resulting in the autophagolysosome [246-250]. The canonical pathway of autophagy involves four steps: initiation (mediated by the ULK1 complexes), nucleation (BECLIN1-PtdIns3KC3-ATG14L complexes), elongation and closure (ATG12-ATG5 and LC3-PE conjugation systems) [251-252]. In regard to infections with *A. fumigatus*, I will focus on LC3-associated phagocytosis (lipidated microtubule-associated protein 1 light chain 3(LC3)-associated phagocytosis or LAP). This specialized form of autophagy links PRR signaling with phagosome development, inflammation and host defenses [253-254]. Observed as

mechanistically distinct in comparison to canonical autophagy, LAP is regulated by the protein Rubicon, which actually inhibits canonical autophagy. This inhibition of canonical autophagy is due to the formation of the Atg14L-containing Beclin-1/VPS34Class III PI3K complex. Rubicon activates LAP through association with a unique UVRAG-containing Beclin-1/VPS34Class III PI3K complex on the phagosome [255-256]. Fundamental requirements of LAPosome formation occur through PI3P (phosphoinositol-3-phosphate –a lipid) and reactive oxygen species (ROS) production. Rubicon facilitates the process of LAPosome formation by activation of class III PI3K and stabilization of NADPH-complex formation through the connection of the p22 $phox$  subunit, which is required for ROS production. LAP recently has surfaced as an integral anti-inflammatory pathway and contributes to host defense against extracellular pathogens, including *A. fumigatus* through recognition of  $\beta$ -glucans through Dectin-1 [256-259]. Therefore, experiencing a defect in LAP would cause phagocytes to experience impeded killing mechanisms and increased susceptibility for *A. fumigatus* infections. In an effort to determine the molecular mechanisms responsible for anti-inflammatory responses upon activation of LAP, researchers identified IFN- $\gamma$  mediated expression of death-associated protein kinase 1 (DAPK1) signaling as a promising role in the control of *A. fumigatus* infections [260-261]. DAPK1, a calmodulin-regulated serine-threonine kinase, is also a Janus molecule that is regulated by JAK/STAT-independent IFN- $\gamma$  signaling. It seems that DAPK1 associates with *A. fumigatus* LAPosomes and then induces proteasomal degradation of NLRP3 via ubiquitination of NLRP3 by FBXL2. When NLRP3 is degraded, the immune response is muted, and overt inflammation is avoided. This flourishing area of research holds much promise in the application of fungal immunology [262-263].

## **IAV and *A. fumigatus* Co-infections**

Co-infections, by common definition, occur when two or more pathogens simultaneously infect a host [264]. Co-infections begin with a primary pathogen, here it is IAV. IAV successfully infiltrates the host and distracts or depletes the host's array of immune defenses. This is optimal for an opportunistic pathogen, such as *A. fumigatus*, to infect the same host. Co-infections with IAV and *A. fumigatus* are becoming more prevalent, or at least more recognized. It is believed that, in general, an inappropriate immune response through excessive inflammation from a cytokine storm results in the rising rates of morbidity and mortality seen with IAV and *A. fumigatus* co-infections [265].

**Clinical case reports.** Since the 2009 H1N1 Pandemic, there has been an increasing awareness about influenza-associated-aspergillosis (IAA) and the propensity that IAV has to predispose patients to secondary fungal infections more than previous IAV strains. There exists *limited* understanding about the consequences of fungal infections with IAV. Patients who experience severe influenza A are increasingly susceptible to more severe pneumonia [266-272]. Dr. Mitsuru Toda and her team from the US Centers for Disease Control and Prevention (CDC) Mycotic Diseases Branch recently shared results of a study they presented at ID Week 2018 analyzing cases of invasive aspergillosis as a risk factor for patients with current influenza. What they found was *prominent* –greater than one-third of patients documented with IAV-*Aspergillus* co-infections lacked immunosuppressive conditions, like AIDS, that are typically associated with invasive aspergillosis [273]. This indicates that previously healthy people are developing invasive aspergillosis after even mild infections with influenza. Before Dr. Toda's findings that more than one-third of the 57 cases in the USA did not have a documented immunosuppressive condition, published clinical case reports were sporadic. The sporadic nature of individual case

reports made it difficult to determine the significance of IAA, especially among previously immunocompetent hosts. Of the documented 57 cases in the literature, 92.9% of the cases were associated with IAV. Of those, 82.33% were due to the H1N1 subtype. It must be noted here that since 2010, the majority of cases of IAA were due to H1N1 [273].

To further extend the awareness, van der Veerdonk and colleagues observed similar trends in the UK. An analysis of 68 IAA cases reported in the literature before 2005 demonstrated the mortality rate was 47%. IAA in critically ill patients with influenza during the flu season in the Netherlands from December 2015 to April 2016 reported IAA cases had a significantly higher rate of up to 61%, and this was despite antiviral and antifungal therapies. This mortality rate among affected patients without traditional risk factors of invasive aspergillosis was not lower compared to patients with known low, intermediate, or high risk for an invasive fungal disease state. It seems that the use of corticosteroids might not be as strong as a predisposing factor contributing to IAA illness as alleged previously [274-276]. Another extraordinary observation in these documented IAA infections in immunocompetent individuals is that their age ranged from roughly 20 to 80 years old. This also eliminates another preconceived predisposing factor –age—which is associated with lower immune function in the very young and very old [277].

The reasons for the intense rise in the number of recently published cases of IAA is currently unclear. Hypotheses from the scientific community on this topic range from the evolution of more virulent IAV strains (exemplified by 2009 H1N1 pandemic strain), the observed severity of damage to respiratory mucosa reported possibly allowing for increased fungal invasion, and generally greater reporting over time. Exposure to *A. fumigatus* at a critical

time of both reduced mucosal and systemic immune defenses could also lead to the observed diseased state [278], but it is not clear why exposure to *A. fumigatus* has increased.

### **Problem Statements and Hypotheses**

In order to understand IAA, the virus, fungus and host must be examined. Similarly, any model to study IAA must include all players. Previously, research has focused on each pathogen and the host response separately. Collaboration with medical mycologists, virologists and immunologists could aid in the exploration of crucial immune pathways utilized by viral and fungal pathogens to successfully invade tissue and evade the host's immune system. My scientific study here will bring increased awareness of IAA as an early complication of IAV infections through the knowledge provided through investigating a novel model of IAV-*A. fumigatus* co-infection. As a result, hopefully, more awareness, prompt diagnoses and initiation of suitable treatments can be implemented in future seasonal flu outbreaks.

I discovered through preliminary experiments that co-infections of macrophages with IAV and *A. fumigatus* results in an immense increase in the production of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6) and active caspase-1-p20 in comparison to IAV and *A. fumigatus* alone.

**Problem Statements.** There are two major roadblocks to furthering our understanding of IAA: a lack of an animal model for *in vivo* studies, and a lack of understanding of the immune response and how this and other factors may contribute to IAA. The objective of my research was to determine the cause of the increase in pro-inflammatory cytokine levels during an IAV and *A. fumigatus* co-infection and to effectively recapitulate this co-infection within a murine model. Previously, researchers were able to generate a different model IAV-fungal co-infection

with *Cryptococcus neoformans* and *C. gattii*. Their investigation showed that acute IAV infection predisposes mice to *C. neoformans* and *C. gattii* infections, with the highest morbidity and mortality rates occurring if mice were co-infected with the fungus on day 3 after IAV [220].

Based on our preliminary research and the IAV- *C. neoformans/gattii* mouse model, I narrowed down our research to two testable and answerable questions:

- *In vivo*
  - What variables are required for development of a co-infection model in mice? Specifically, what doses of IAV and *A. fumigatus* and how many days apart should the infections be to induce the highest morbidity and mortality of mice?
- *In vitro*
  - Why is there an enhanced activation of caspase-1-p20 and cytokines during IAV-*A. fumigatus* co-infections and does this cause the high morbidity and mortality in humans?

**Hypotheses.** Through the establishment of working *in vitro* and *in vivo* models, I propose the following two hypotheses to explain the preliminary observations of enhanced pro-inflammatory cytokine production, caspase-1 activation and high morbidity and mortality rates seen in humans with IAA:

- *In vivo*
  - If IAV predisposes humans or mice to *A. fumigatus* infection, then infecting mice with IAV should allow for infection with *A. fumigatus* (it does not normally infect mice even at very high doses), and IAV infection

coupled with *A. fumigatus* infection should result in more severe disease than either infection alone.

- *In vitro*
  - If caspase-1 activation is increased during co-infection compared to single infection with IAV or *A. fumigatus*, then factors regulating activation of AIM2 and NLRP3 must be enhanced, including AIM2 and NLRP3 gene expression and activation signals like ROS.

## MATERIALS AND METHODS

In order to carefully elucidate immune signaling as a whole during infections, the use of models to simulate infections in humans within a model organism or cell line is preferable. IAV and *A. fumigatus* co-infections were recapitulated in both *in vitro* and *in vivo* models in this research. Figure 1, a visual representation of the complexity of IAV and *A. fumigatus* co-infections and where the science led me during my thesis project.

### Overall Experimental Designs

The experiments in mice and cell culture were performed with a mouse adapted strain of IAV – influenza A/PR/8/34 H1N1 virus and Type 293 *A. fumigatus* (ATCC MYA-4609). Mice were infected with various concentrations of IAV and *A. fumigatus* alone or co-infected with both pathogens on various days and monitored for symptoms of morbidity (weight loss) and their mortality. Cell cultures of mouse-derived macrophages were also infected with IAV and *A. fumigatus* alone or co-infected with both pathogens. Cell culture samples were collected to check cytokine and associated protein and gene production through activation. Specifically, analyzation of the signaling proteins with the predicted pathogen recognition receptor pathways that *could* be involved. Lastly, data normalization and statistical analysis of these forms of data were completed using Microsoft Excel and GraphPad Prism Version 7.0.

### Animal Welfare

All mice used in these experiments were of the pathogen-free mice (*Mus musculus*) C57Bl/6 genetic background including knockouts of *Nlrp3*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> originated from The

Jackson Laboratory and bred in-house within Missouri State's vivarium. All breeding, experimentation, and data collection were performed in accordance to the Institutional Animal Care and Use Committee (IACUC)–specific accepted protocols utilized for living mouse and cell culture experiments were February 17, 2016; approval #16.015 with continuation on February 19, 2019 (Appendix A) and July 26, 2018; approval #2018-07 (Appendix B), the AVMA Guidelines on Euthanasia, NIH regulation (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal Welfare Act of 1966 (Appendix A).

### **Preparation of fungal and viral stocks**

Viral and fungal infectious agents utilized received prior approval for this product was obtained from the Institutional Biosafety Committee (IBC) on (October 23<sup>rd</sup>, 2015) (Appendix B). Both pathogens were grown and used within the laboratory.

**Preparing an *A. fumigatus* stock.** *A. fumigatus* used in this study was a laboratory pathogenic species, gifted from Dr. Thirumala-Devi Kannetganti, St. Jude Children's Research Hospital. Sabouraud Dextrose Agar (better known as 'SabDex' or SDA) was made per manufacture (ThermoFisher Scientific, catalog #CM0041R) recommendations. The agar was poured into T175 cm<sup>2</sup> tissue culture flasks (ThermoFisher Scientific) and cooled cap-off inside a sterilized certified biosafety cabinet. Five-hundred  $\mu$ L of *A. fumigatus* conidia in glycerol stock (Af293, ATCC MYA-4609) was pipetted into the flasks with cooled agar and incubated at 37°C/5% CO<sub>2</sub> for approximately 7 to 14 days or until the fungus was thick and black in color. The live asexual spores of *A. fumigatus* were harvested within a certified sterilized biosafety cabinet by pouring approximately 10 mL of sterile 1X Phosphate Buffered Saline (PBS) (prepared from 10X ThermoFisher Scientific PBS, pH 7.4, RNase-free, catalog#AM9625) to each

tissue culture flask filled with thick black fungus. The flasks were re-capped and shaken vigorously to suspend spores in the PBS. The PBS-spore mixtures from both flasks were pooled together into one 50 mL centrifuge tube. Tween 80 was supplemented to the falcon tube with the PBS and spores at a total concentration of 0.1% v/v of Tween 80 (Sigma-Aldrich, CAS#9005-65-6) The Tween 80 was used to reduce surface tension between spores and water to aid in the precipitation of spores out of the PBS-spore mixture. The spore mixture was centrifuged at 2000 rpm for 10 minutes. The supernatant (PBS) was decanted as bio-hazard waste and 5 mL of 50% glycerol (Sigma-Aldrich, CAS#56-81-5) in Brain Heart Infusion (BHI) broth (prepared according to the manufacture, Sigma-Aldrich, CAS#53286) was added to the concentrated *A. fumigatus* spores. Next, the fungal stock was aliquoted in low temperature cryovials at 500  $\mu$ L and frozen at -80°C.

Determining Fungal Stock Concentration via CFUs. Approximately 25 mL of SDA was poured per plate into 10 petri dishes to eventually determine colony forming units (CFUs) of the spore stock through a CFU assay. The agar was kept at room temperature until solidified into a sterilized biosafety cabinet. Ten-fold fungal serial dilutions of the stock were performed with 900  $\mu$ L of PBS and 100  $\mu$ L of fungal stock prepared previously. Then, 100  $\mu$ L of each dilution, from  $10^{-1}$  to  $10^{-7}$ , were dispensed onto each plate as one-drop on each SDA plate (1 plate per dilution), liquid was spread and plate incubated upside down at 37 °C/5% CO<sub>2</sub> for 24 to 48 hours or longer –until there were visible fungal colonies to count. Once colonies were determined ready by inspection, they were counted visually. The dilution with colonies within a range of 30-300 was selected to perform a back-dilution calculation to obtain the CFU/mL.

**Preparing viral stocks.** To prepare viral stocks, two methods were utilized to grow and purify the stock – these include growth of the IAV strain A/Puerto Rico/08/1934 (H1N1)

(hereafter referred to as 'PR8') via chicken eggs and MDCK cells. Following growth and aliquoting of the PR8, the concentration of the virus was determined through plaque forming units (PFUs) generated from a viral plaque assay technique, which will also be explained below.

Growing an IAV Stock in Chicken Eggs. Fertilized chicken eggs were provided by a local chicken breeder. The eggs were incubated in a humidified 37.5°C incubator and flipped twice a day for 10 to 12 days (or until the candled eggs showed a viable chick: dark shadow within the egg with blood vessels, movement, and an intact air sac). Viable eggs were infected inside a sterilized certified biosafety cabinet. Eggs were first decontaminated by spraying 70% ethanol on the exterior of the egg shell. Then, a hole was made about 1cm above the air sac line with a sterile 18-gauge needle. Next, using a 1 mL insulin syringe and a 1 inch 22-gauge needle, 100µL of virus solution diluted to  $10^4$  PFU, was injected into each egg at a 45-degree angle through the previously made hole. Super glue was used to patch the hole and the developing eggs were stored again in a humidified incubator at 37.5°C and flipped twice a day for 72 hours.

After 72 hours, the eggs were pulled from the incubator and visualized with a flashlight to check viability of the developing chick embryo. If the chick inside the egg was still alive, indicated by movement of the chick, the eggs were placed inside a 4°C refrigerator for at least 1 hour to stop the heart and blood flow of the developing chick. After 1 hour, the eggs were placed inside a sterilized certified biosafety cabinet and sanitized with 70% ethanol to prevent any contamination from the shell to the fluid within the egg containing the influenza A virus. Using sanitized forceps, the shell of the eggs was chipped away over the air sac. The allantoic membrane was peeled aside and the clear allantoic fluid was pipetted into sterile 50 mL centrifuge tubes. These tubes were kept on ice and spun down at 4,000 x g for 10 minutes at 4°C to pellet unwanted debris. The clear supernatant from the tubes was transferred into new 50 mL

tubes and kept on ice. The clarified allantoic fluid (supernatant) containing the influenza A virus was then aliquoted and stored at  $-80^{\circ}\text{C}$  for further use. The debris-pellet and eggs were disposed in a BSL-2 waste container.

#### Growing an IAV Stock in MDCK Cells and Purification via Ultracentrifugation.

Alternative methods of virus stock preparation were utilized in hopes of increasing stock concentrations and making a larger amount of a stock. Madin-Darby Canine Kidney (MDCK) cells (a gift from Dr. Paul Thomas, St. Jude Children's Research Hospital) were grown until confluent (about 3 days) within a T175 tissue culture flask. The MDCK cells were washed twice with PBS and infected with PR8 at a concentration of  $2.5 \times 10^6$  PFU diluted in 5 mL of 1X plaque assay media per T175 flask. These PR8 infected flasks were incubated at  $37^{\circ}\text{C}/5\% \text{CO}_2$  for one hour with intermittent shaking every 10 minutes. After the 1-hour incubation, the PR8-containing 1X plaque media was removed from the infected flasks and an additional 20 mL of 1X plaque media was pipetted into each flask. To allow the virions of PR8 to mature, 20  $\mu\text{L}$  of TPCK trypsin was aliquoted into each infected flask. The infected flasks were stored again at  $37^{\circ}\text{C}/5\% \text{CO}_2$  for approximately 72 hours or until 85% of the cells were dead. The virus-laden 1X plaque media was transferred from the infected flask to 50 mL centrifuge tubes and vortexed for 5 minutes to detach any virions from cells and put them into the media. These now vortexed tubes were centrifuged at  $2,000 \times g$  for 10 minutes to pellet out the MDCK cells. Ultra-centrifuge tubes were preloaded with 3 mL of 5% sucrose in MHN buffer (Table 1). The virus-laden media was gently overlaid atop the sucrose buffer. Tubes were ultra-centrifuged in a JS-24 rotor at  $4^{\circ}\text{C}$  at 23,000 rpm for 1 hour. Tubes were placed on ice in the biosafety cabinet and all but approximately 3 mL of supernatant was removed from each tube, leaving the virus pellet. The remaining 3 mL of media from each tube was pooled together into one ultra-centrifuge tube and

ultra-centrifuged again at the same conditions previously described. Once ultra-centrifugation was completed a second time, all but 3 mL of supernatant was again carefully removed. The remaining 3 mL of media and the virus pellet were transferred to and vortexed in a 15 mL centrifuge tube for 10 minutes to resuspend the virus pellet into solution. The virus stock was aliquoted at approximately 30  $\mu$ L for each 1.5 mL tube and stored at  $-80^{\circ}\text{C}$  until use.

Determining Viral Stock Concentration via Plaque Assay. In order to obtain the approximate number of infectious virions in a sample, the plaque assay technique was utilized. Approximately 48 hours before beginning the plaque assay for IAV, MDCK cells were seeded in 12-well culture plates at  $3 \times 10^5$  cells/well within 1 mL of 1X DMEM +10% FBS + 1% Pen/Strep. On the day of the plaque assay, ten-fold dilutions of the viral stocks (grown in eggs or concentrated by ultracentrifugation) were prepared in 1X plaque assay medium (See Table 2). Next, MDCK cells were washed twice with about 1 mL of 1X PBS per well each time. Then, 100  $\mu$ L/well of the virus dilutions were added to duplicate wells within the 12-well plates. These plates were incubated at  $37^{\circ}\text{C}/5\% \text{CO}_2$  for 1 hour with intermittent shaking every 10 minutes (to spread the media containing the diluted virions across the cells and to prevent the cells from drying). During the last 20 minutes of plate incubation (40 minutes into the hour incubation period), the agarose overlay was prepared: 2% SeaPlaque low melting point agarose (Bio Whittaker, catalog #50100) in  $\text{dH}_2\text{O}$  was microwaved, cooled to a temperature around 37 to  $42^{\circ}\text{C}$  and was mixed together with pre-warmed ( $37$  to  $42^{\circ}\text{C}$ ) 2X plaque assay medium (Table 3) at a 1:1 ratio. TPCK-trypsin was added to the overlay mixture at a final concentration of 1.0  $\mu\text{g}/\text{mL}$ . Following the 1-hour incubation, the infection medium was aspirated from each well and 2 mL of the warm agar/plaque medium overlay was added to each well. The agar was allowed to harden completely within the biosafety cabinet with lids ajar on the plates (to prevent unwanted

condensation) before flipping the infected plates over and incubating them at 37°C/5% CO<sub>2</sub> for 72 hours. After 3 days, the agar plugs were scooped out of the wells and plates stained with 1% crystal violet in methanol. The stain was incubated at room temperature within the biosafety cabinet for around 30 seconds to 1 minute and was removed. The wells were then rinsed twice with water. After drying upside on paper towels, the plaques were read by counting clearings visually observed in the wells.

### ***In vivo* Infection Schemes**

In order to understand the causes and contributing factors associated with human case-study-based morbidity and mortality, I sought to develop a mouse model using the C57Bl/6J mouse strain. To effectively do this, an *in vivo* method was created from previous work with another misunderstood viral-fungal co-infection, IAV and *C. neoformans* and *C. gatii* [220]. At least eight-week old mice were anesthetized on day 0 by intraperitoneal injection with 80 mg/kg Ketamine and 8 mg/kg Xylazine diluted in 1X PBS. Mice were infected with approximately 250 to 300 PFU of PR8 intranasally in a volume of 30 µL of 1X PBS. Mice were mock infected, single infected with PR8 or *A. fumigatus* or co-infected depending on their assigned groups on days 3, 5 and 7 with 10,000 to 10,000,000 CFU of *A. fumigatus* intranasally in a volume of 30 µL of 1X PBS (220). At all-time points, mice were monitored at least once daily for weight loss and food/water availability. Mice were euthanized when they achieved 30% or greater weight loss and/or became moribund (Fig 2A).

### **Differentiating Bone Marrow Derived Macrophages**

To effectively understand, characterize and simulate this viral-fungal co-infection in cell culture, bone marrow derived macrophages were utilized. L-929 cell conditioned media – which contains the growth factor M-CSF (macrophage-colony stimulating factor) – was made by incubating L929 cells in DMEM (Dulbecco’s Modified Eagle Medium), 10% FBS (Fetal Bovine Serum) and Penicillin/Streptomycin for 10 days, and then 0.45  $\mu\text{m}$  filtering the medium. Bone marrow was collected from the femur and tibia of WT, *Nlrp3*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> mice and differentiated for 5 days in tissue culture dishes (150mm x 25mm) in the presence of bone marrow differentiation media (BMDM –recipe in Table 4) media containing L-929 cell conditioned medium. On day 5, now differentiated macrophages from bone marrow hematopoietic stems cells were removed by scraping, counted using a hemocytometer, and seeded into 12-well plates at a concentration of  $1 \times 10^6$  cells/well within 1 mL of BMDM medium. These macrophages were incubated overnight to allow cells to adhere to the plastic wells. The next day, the macrophages were used for *in vitro* cell infection experiments (Fig 3A).

### ***In vitro* Infection Schemes**

Macrophages in 12-well plates were washed twice with 1 mL of 1X PBS per well and 200  $\mu\text{L}$  of Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Corning, catalog #10-040e) added to each well. To add the correct volume of pathogen stock to a designated number of cells (Equation 2), multiplicity of infection (MOI –Equation 1) was calculated. Macrophages were either mock infected, single infected with either pathogen, or co-infected with both pathogens 3 hours apart. Mock infected macrophages were treated the same as macrophages single or co-infected, but without a pathogen. Single infected macrophages were treated with 10 MOI of PR8 at 0 hour or 1 MOI of *A. fumigatus* at 3 hours into infection followed by incubation.

Co-infected macrophages were treated with 10 MOI of PR8 at 0 hour and 1 MOI of *A. fumigatus* at 3 hours into infection followed by an hour incubation. During periods of incubation, the plates were stored at 37°C/5% CO<sub>2</sub> and the plates were intermittently shaken every 15 to 30 minutes to disperse the infectious media over the cells and to prevent the cells from drying. At 4 hours into infection, 200 µL RPMI with 20% FBS was pipetted into all wells (Fig 3B) and plates returned to the incubator until their designated sample collection time-point. Samples were collected at 6-, 12-, 18- or 24-hour time points by removing the supernatant for ELISA assays and lysing the cells with 1X RIPA buffer and 4X SDS loading dye (recipes in Table 5 and Table 6, respectively). The supernatant samples were kept at -20°C and cell lysates were kept at -80°C until their analysis.

**Enzyme-linked Immunosorbent Assay (ELISA).** Cell culture supernatants for the cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-6 were examined via ELISA. Mouse Ready-SET-Go ELISA kits (eBioscience; catalog #s 88-7013, 88-7324, 88-7064, respectively) were used according to the manufacturer's instructions except that all antibodies and streptavidin-HRP were diluted to 1:500 instead of 1:250. The standard curve was prepared by two-fold dilutions ranging from 1000 pg/mL to 31.25 pg/mL (Fig 4). Upon completion of the assay, plates were immediately read at 450 nm using a BioTek ELx808 microplate reader.

**SDS-PAGE and Immunoblotting.** Intracellular protein expression and activation in cell lysate samples was determined via Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting. On day 1, the cell lysate samples with 4X SDS loading dye added were thawed and boiled at 95°C for approximately 20 minutes. Samples were loaded into 12% polyacrylamide gels and electrophoresed at 100V for two hours in 1X Tris/Glycine/SDS (running) buffer (recipes for buffers in Table 7, 8 and 9). After gel

electrophoresis, the gels were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (GE Health Life Sciences, catalog #10600023) at 40V for 50 minutes (Table 10). After the transfer run was complete, PVDF membranes were blocked in blocking buffer (5% milk in 1X TBST) on a shaker at room temperature for 1-hour. Blocking buffer was discarded and replaced with protein-specific primary antibodies diluted in 5% milk in 1X TBST (recipe from Table 8 and 11). Membranes in antibody solution were incubated on a shaker at 4°C overnight. Day 2: The diluted primary antibody was removed and saved, and the membranes were washed 3 times with approximately 10 mL of 1X TBST for 10 to 20 minutes shaking per wash at room temperature. After the last wash was discarded, the membranes were incubated in primary antibody-specific secondary HRP conjugated antibodies diluted in 5% milk + TBST (Table 12). Membranes were incubated at room temperature for around 45 minutes with gentle shaking. After the secondary antibody was removed and saved, the membranes were washed 4 times with 10 mL of 1X TBST at room temperature with vigorous shaking for approximately 10 to 20 minutes for each wash. After the last wash was discarded, fresh 1X TBST was added to each membrane and membranes were transferred to a clean container where they were treated for one minute with 1 mL of substrate per membrane (Super Signal West Femto Maximum Sensitivity Substrate; ThermoFisher Scientific, catalog #34096). Separated protein bands were visualized using the Azure Biosystems C300 digital imaging system.

**Mitochondrial Damage via Flow Cytometry.** To determine possible causation of the enhanced activation of caspase-1-p20, macrophages were stained with fluorescent dyes designed to detect mitochondrial damage through reactive oxygen species production originating from the mitochondria (MitoSOX; ThermoFisher, catalog #M36008). Macrophages were mock, single or co-infected as described above in the ‘In vitro Infection Schemes’ section. At 18- or 24-hours but

30 minutes before collecting the samples, 25  $\mu$ L of MitoSOX diluted in RPMI was pipetted into each well (at a final concentration of 2.5 nM MitoSOX). Each flow cytometry experiment had at least one control well left unstained. The samples in the plates were left in the biosafety cabinet to incubate in the dark at room temperature for approximately 30 minutes. After the 30-minute incubation, the media of each sample was replaced with 1 mL of warmed (37°C) PBS.

Macrophages were scraped off the plates with a 200  $\mu$ L pipette tip (turned upside-down). The macrophages, now lifted into the PBS, were transferred into a 1.5 mL centrifuge tube and analyzed on the Attune NxT Flow Cytometer using the BL3 channel for MitoSOX. Per sample, 10,000 events were analyzed for fluorescence intensity and percent positivity for the dye.

**RNA Isolation.** Gene expression of hypothesized genes that could play key roles in the production of inflammatory cytokines were examined against  $\beta$ -Actin as the control: *Aim2* and *Dapk1*. Macrophages were prepared and infected as previously described. At 6-, 12- and 24-hours after infection, the supernatant was removed (kept for ELISA) and 500  $\mu$ L of TRIZOL (Invitrogen, catalog #AM97381) was added to each well and incubated for 5 minutes at room temperature to extract the total mRNA from the samples. TRIZOL lysed cell samples (0.5 mL) were transferred to labeled 1.5 mL centrifuge tubes and 100  $\mu$ L of chloroform was added to each sample. Tubes were shaken vigorously by hand for about 15 seconds and incubated at room temperature for 3 minutes. Tubes were centrifuged at 12,000 x g for 15 minutes at 4°C. The clear aqueous phase (top layer) was carefully pipetted off and transferred to new labeled tubes. Next, the addition of isopropanol (250  $\mu$ L) was used to precipitate out the nucleic acids. The samples were shaken by hand and incubated at room temperature for 10 minutes before centrifugation at 12,000 x g for 10 minutes at 4°C. All of the supernatant was removed but approximately 5  $\mu$ L. This was done to avoid discarding the RNA pellet. The sample RNA pellets were washed with

75% ethanol (500  $\mu\text{L}$ ) to remove excess salt content from the pellet. Tubes were vortexed briefly for 5 seconds and centrifuged at  $7,500 \times g$  for 5 minutes at  $4^\circ\text{C}$ . The ethanol was removed and dried cap-open within a vacuum centrifuge. Once the pellet was almost completely dried, 20  $\mu\text{L}$  of nuclease-free water was pipetted into each tube to reconstitute the RNA pellet back into solution. The concentrations of the RNA samples were measured using an Implen Nanophotometer. All samples were normalized to 200  $\text{ng}/\mu\text{L}$  by adding additional nuclease-free water to each sample (Equation 3).

**cDNA Synthesis and qRT-PCR.** Due to the instability of RNA, this nucleic acid must be reverse transcribed into complementary DNA (cDNA) to be used. The High Capacity cDNA Reverse Transcriptase Kit (ThermoFisher Scientific, catalog #436881) was used to reverse-transcribe mRNA into cDNA according to the manufacturer's instructions using 1  $\mu\text{g}$  total RNA (Table 1). cDNA samples were diluted 1:5 (80  $\mu\text{L}$  of nuclease-free water to the 20  $\mu\text{L}$  of amplicon solution). 5  $\mu\text{L}$  of the diluted cDNA samples and 2-fold standard curve samples were analyzed via quantitative real-time PCR (qRT-PCR) utilizing the DyNAmo HS SYBR Green qPCR Kits (ThermoFisher Scientific, catalog #F.410L) according to the manufacturer's directions (see Table 13 for cDNA Master Mix recipe). Primers (forward and reverse) for  *$\beta$ -Actin*, *Aim2*, and *Dapk1* were used to test gene expression (A15612T) (see Table 14). The instrument used for data acquisition was a STRATAGENE-Mx3005P PCR machine.

### Statistical Analysis

For *in vitro* experiments with cytokine production or ROS production a one-way ANOVA with Tukey's post hoc analysis was performed using GraphPad Prism 7 software (GraphPad Prism 7 Software, San Diego, CA, USA). For *in vitro* gene expression experiments

during certain collection time-points, a two-way ANOVA with Tukey's post hoc analysis was performed using GraphPad Prism 7 software. For weight loss during *in vivo* experiments, a two-way ANOVA with Tukey's post hoc analysis was performed using GraphPad Prism 7 software. For survival during *in vivo* experiments, survival analysis was performed using the Kaplan-Meier Survival Plot with LogRank Test using GraphPad Prism 7 software. Any visual representations within figures representing immune signaling or cellular mechanisms were completed with the latest version of the academic membership of BioRender.com.

## RESULTS

Although there are numerous case reports of humans contracting IAV-*A. fumigatus* co-infections, there is no animal model to study the causes and contributing factors involved. For an animal model to be relevant, it should demonstrate similar morbidity and mortality rates observed in humans. As with any disease, effectively providing an *in vivo* model holds promise for understanding the causes of the disease as well as developing future treatments or vaccines.

### Increased Morbidity and Mortality

I initially infected mice intranasally with a low dose of IAV (300 PFU), which causes no death and only minor weight loss, and then intranasally co-infected the same mice on different days after the IAV infection with  $10^7$  CFU of *A. fumigatus* (Fig 2A). Control groups present in this experiment that were singly infected with IAV on day 0 (but mock co-infected on day 3) and singly infected with *A. fumigatus* on day 3 (but mock infected on day 0), showed little or no weight loss and almost no mortality (Fig 2B-C). However, mice coinfecting on day 3 after IAV infection almost all died by day 10. Interestingly, mice coinfecting on day 5 or day 7 after IAV had much less mortality (Fig 2B-C). After solidifying the ideal co-infection day as day 3, a separate experiment was done to determine the lethal dose of *A. fumigatus* needed to really cause severe illness during co-infection. Mice were infected with 300 PFU of PR8 intranasally on day 0. Then, mice were infected or co-infected on day 3 with a fungal dose ranging from  $10^4$  to  $10^7$  of *A. fumigatus* CFUs. All mice were monitored for weight loss and survival until day 14 after the initial flu infection. I observed that  $10^4$  CFU of *A. fumigatus* was just as efficient as  $10^7$  CFU at causing similar mortality during co-infection (Fig 2D-E). It should be noted that mice infected

with *A. fumigatus* alone, without prior IAV infection, scarcely lost any weight even at an infectious dose of  $10^7$  CFU (Fig 2D-E). *A. fumigatus* alone actually had significant increases in weight loss on various days compared to IAV (Fig 2D). This confirms previous reports that even a high dose of  $10^7$  *A. fumigatus* spores alone does not pose a threat to immunocompetent mice and humans [285]. Instead, the initial infection of mice with IAV must affect the immune response to fungal pathogens and render the mice more susceptible to the co-infection.

### **Surge in pro-inflammatory cytokine production and caspase-1 activation during co-infection**

In addition to developing the mouse model for studying the factors involved in co-infection *in vivo*, I also examined the immune signaling pathways involved during co-infection in mouse bone marrow derived macrophages (Fig 3A-C). I examined the cytokines produced by macrophages either mock, single or co-infected for 24 hours and found a complex ‘cytokine storm’ exists. Specifically, there was a dramatic increase in the level of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  during co-infection of IAV and *A. fumigatus* compared to mock or single infected samples (Fig 5A-5C). In addition, IL-1 $\beta$  must be cleaved by caspase-1 to become active, I performed western blots of macrophage cell lysates either mock, single, or coinfecting and found that caspase-1 activation is dramatically elevated during co-infection. (Fig 5D).

**Inflammasome priming stages and the overproduction of IL-1 $\beta$ .** IL-1 $\beta$  is produced as an inactive precursor which must be cleaved to become fully active. The observed enhanced IL-1 $\beta$  production during co-infection could, therefore, result due to increased expression of pro-IL-1 $\beta$  or enhanced activation of IL-1 $\beta$  by caspase-1 within the inflammasome (Fig 6A). Previously, bacterial co-infections with IAV and *S. pneumoniae* showed similar heightened cytokine

production compared to preliminary observations of IAV and *A. fumigatus* co-infections [281-282]. During IAV-bacterial co-infection, increased IL-1 $\beta$  production was associated with enhanced production of pro-IL-1 $\beta$  (283-284). Interestingly, this is exactly the opposite seen with co-infections with IAV and *A. fumigatus*. Pro-IL-1 $\beta$  expression was not different between IAV infected and IAV-fungal coinfecting macrophages (Fig 6B).

Also, there was no increase in the activation of the transcription factor NF- $\kappa$ B that controls pro-IL-1 $\beta$  gene expression (Fig 6B), as was seen with IAV-bacterial co-infection [283-284]. NF- $\kappa$ B also controls the gene expression of the inflammasome activator NLRP3 [287]. In agreement, there was no difference in NLRP3 in IAV or coinfecting cells (Fig 7A). Thus, I concluded that increased expression of pro-IL-1 $\beta$  and NLRP3 is not responsible for enhanced caspase-1 activation and overproduction of IL-1 $\beta$ .

### **Exploring viable agonists contributing to enhanced NLRP3 or AIM2 mediated caspase-1 activation**

As the first, or priming step, of NLRP3 inflammasome activation was not affected by co-infection, I examined other possibilities. The second step in activation of the inflammasomes is usually provided by PRRs sensing a diverse group of agonists that can trigger the specific activation of the inflammasome in question. For example, NLRP3 can recognize ROS produced by cell damage from both IAV and *A. fumigatus*, and AIM2 detects DNA in the cytoplasm [278]. I hypothesized that if there were two pathogens, then there could be twice as much ROS or other cell damage. Alternatively, the co-infection could affect AIM2 sensing of fungal DNA. Either of these could result in increased inflammasome activation and more cytokine production.

**Negating ROS as a source of direct inflammasome activation through cytoplasmic sensing.** To determine if the production of ROS facilitates the activation of NLRP3, upstream of caspase-1 activation during co-infection, macrophages were infected as before, and mitochondrial ROS was examined 18 hours into infection via flow cytometry. Based on median fluorescence intensity (MFI), or the median level of fluorescence detected within macrophages that have experienced mitochondrial damage, co-infected samples do not provide evidence that ROS is a primary factor in the observed over activation of caspase-1. In comparison to untreated samples, IAV singly infected samples produced the highest amount of ROS along with the observed highest percentage of ROS afflicted macrophages (Fig 7B-G). The amount of ROS production and percentage of ROS afflicted macrophages for the *A. fumigatus* singly infected and co-infection samples were not significant different from mock infected controls (Fig 7B-G). Interestingly, there are many reports of *A. fumigatus* inducing ROS production in host cells [286-287]. Other reports show that melanin, within the *A. fumigatus* cell wall, can actually function as a physiological redox buffer through binding metal ions and free electrons. Either way, an overproduction of ROS does not occur in the co-infection samples in our experiments and suggests that other mechanisms for enhanced caspase-1 activation must be at play (Fig 8).

### **Intracellular pathogen-mediated antagonistic signals contribute to the ‘perfect storm’**

Upon further research of the possible mechanisms for NLRP3 mediated inflammasome activation, several reports of a specific kinase, DAPK1, surfaced [262, 279-280]. DAPK1 actually drives the FBXL-2-dependent proteasomal degradation of NLRP3 during *A. fumigatus* infections (see Fig 9C). This was a promising primary lead into understanding exactly how the two pathogens are interacting with each other and the macrophages.

**IAV-mediated suppression of DAPK1 gene expression promotes NLRP3 activation.**

To examine the role of DAPK1, qRT-PCR for gene expression of DAPK1 was performed on IAV, *A. fumigatus* and co-infected samples. In agreement with previous reports, *A. fumigatus* only infected macrophages showed an 8-fold increase in DAPK1 expression compared to the base-line in uninfected macrophages (Fig 9B). IAV, on the other hand, suppressed the expression of DAPK1 by four-fold compared to the uninfected macrophages (Fig 9B). Surprisingly, coinfecting macrophages at 6h after infection also has suppressed DAPK1 expression, but this did increase by 24h (Fig 9B). This suggests that IAV suppresses DAPK1 expression early during co-infection, which may prevent the proteasomal degradation of NLRP3 (Fig 9A) and allow for a more potent NLRP3 mediated inflammasome response during co-infection, even if NLRP3 is not more highly expressed by NF- $\kappa$ B (Fig 7A).

**Co-infecting pathogens synergistically induce AIM2 expression.** The infection of macrophages with *A. fumigatus* can also activate the AIM2 inflammasome [278]. I examined AIM2 gene expression using qRT-PCR and found that IAV infection alone results in highly induced AIM2 gene expression (Fig 10A). However, fungal infection did not greatly induce AIM2 expression (Fig 10A). Finally, coinfecting macrophages did show high AIM2 expression, especially at earlier time points (Fig 10A). Thus, infection with IAV appears to induce AIM2 gene expression, and, although AIM2 cannot detect IAV directly, because it is an RNA virus, the elevated AIM2 expression caused by IAV may then cause more inflammasome activation once the fungal co-infection occurs through the proposed pathway in Figure 10B.

**Preliminary *in vitro* knockout experiments reiterate that NLRP3 and AIM2 inflammasomes are both involved.** Finally, to verify the role of AIM2 and NLRP3 during co-infection, I infected macrophages from mice that are genetically deficient in *Aim2* and *Nlrp3*. I

infected these macrophages as previously and performed ELISAs. I found that the dramatic increase in IL-1 $\beta$  and other cytokines during co-infection was muted in both the *Nlrp3*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> macrophages (Fig 11A-F). I also observed less caspase-1 activation in *Nlrp3*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> macrophages (Fig 11G). Overall, these data provide preliminary but essential insight into the inner-workings of IAV and *A. fumigatus* co-infections at the animal and molecular level and will help pave the way for future research. As IAV and *A. fumigatus* co-infections continue to gain attention, these integral cell-signaling pathways (shown in Fig 12) will promote thinking and discourse into how these co-infections can be prevented, diagnosed and treated.

## DISCUSSION

In the last decade, the importance of IAV predisposing patients to opportunistic pathogens has become a critical area of research, mainly due to the implications for increasing prevention and improving the prognosis of these co-infections. Interestingly, recent research shows that people currently infected with IAV are confirmed to have susceptibility to opportunistic pathogens of fungal origin, in particular *C. neoformans* and *C. gattii* [220]. However, before beginning this experimental investigation of IAV and *A. fumigatus*, only clinical case reports provided information about these co-infections. Extensive research has led to advanced understanding regarding how to prevent and treat IAV-bacterial co-infections [284]. However, similar knowledge is severely lacking in IAV and *A. fumigatus* co-infections. Not only are immunocompromised individuals at risk to developing this devastating condition, but so are healthy people without previous serious illnesses prior to becoming infected with IAV (H1N1). Relevant research to IAV or *A. fumigatus* infections alone were used as preliminary clues to piece together the mystery of this co-infection [271-277]. However, it was discovered that these pathogens attack the host differently and there is plenty of work left to do.

My goal for this project, through developing an *in vivo* and *in vitro* model, was to interrogate *how* the pathogens attack the host, resulting in the observed robust immune response. My *in vivo* data clearly indicates that co-infecting with a low dose of IAV (250 to 300 PFU) on day 0 and  $10^4$  to  $10^7$  CFUs on day 3 results in high morbidity (% weight loss) and the highest observed mortality (% survivorship). Therefore, my *in vivo* hypothesis was supported. In addition, my *in vitro* data insightfully indicates that when co-infecting WT murine macrophages with IAV at hour 0 and *A. fumigatus* at hour 3, there is enhanced inflammation. However, this is

not from heightened activation of NF- $\kappa$ B and pro-IL-1 $\beta$  gene transcriptional priming nor was higher ROS as a result of mitochondrial damage directly activating the NLRP3 inflammasome. Instead, my *in vitro* WT murine macrophage data demonstrated a vigorous production of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and heightened caspase-1 activation through an upregulated transcriptional activation of AIM2 and down regulated DAPK1. It must be noted that DAPK1 expression facilitates the proteasomal degradation and/or recycling of NLRP3. Thus, less DAPK1 could mean there is no way to shut off or eliminate NLRP3 activation, resulting in more caspase-1 activation. In addition, the increased expression of AIM2 could result in increased detection of fungal DNA in coinfecting macrophages and enhanced caspase-1 activation. In all, my data indicate that PAMPs from the pathogens and the host signaling pathways are a balancing-act between the activation of DAPK1, NLRP3 and AIM2.

As previously mentioned, NLRP3 and AIM2 are inflammasomes that function as cytoplasmic sensors of damage or ‘stuff’ in the case of NLRP3 and dsDNA in the case of AIM2. I thought that through damage elicited by IAV attacking macrophages, ROS production would occur. There are other research and review reports mentioning that *A. fumigatus* also induces ROS production in immune cells too [279, 287-288]. These previous reports led me to predict that the heightened activation of caspase-1-p20 and downstream inflammatory cytokine production was due more mitochondrial damage and released ROS induced by both pathogens combined. This, in turn, led me to formally hypothesize that the damage and the recognition of the fungal DNA would activate the AIM2 inflammasome along with the NLRP3 inflammasome. Thus, both inflammasomes would be working ‘over-time’ to try to eliminate the pathogens. This excessive inflammasome activation would result in an over-production of downstream mediators of cytokine production and release. Although I did observe more AIM2 expression, I did not

observe more ROS. Instead, I now propose that DAPK1 may actually mediate NLRP3 function through the proteasomal degradation of NLRP3.

IFNs and damage to infected macrophages could contribute to the activation of the NLRP3 inflammasome over the course of infections. When IAV infects the macrophages, interferons can be produced (IFN- $\alpha/\beta$  and also some reports of IFN- $\gamma$ ) and signal in an autocrine manner through interferon receptors like IFNAR. Transcription factors STAT1/STAT2 lead to the downstream expression of DAPK1. DAPK1 can then inhibit the function of the NLRP3 inflammasome via E3 ligase mediated polyubiquitination and subsequently the protein-complex will be chopped up and recycled in the proteasome [262]. This would prevent excessive inflammation during infection [280]. However, IFN production by the host cells will block further virus replication. Thus, the NS1 protein is used by IAV to block further IFN production. Consequently, DAPK1 expression is reduced or even suppressed during IAV infection. The opposite is observed for *A. fumigatus*. In *A. fumigatus* singly infected samples, the production of IFNs induces the expression of DAPK1, as previously seen in related research [262, 280]. Intriguingly, during co-infection, IAV appears to override *A. fumigatus*, resulting in less DAPK1 during co-infection, likely due to IAV NS1 protein initially halting IFN production. Although DAPK1 suppression during co-infection could result in more NLRP3 activation, this avenue of research needs further exploration for complete validation.

AIM2 recognizes fungal DNA and activates the corresponding inflammasome. IAV also induces AIM2 expression but cannot directly activate AIM2. Instead, IAV infection can result in necrotic cell death and dsDNA release from host cells that then activates AIM2. During co-infection, the increased AIM2 expression could result in more inflammasome activation due to enhanced AIM2 recognition of fungal DNA and host cell DNA from dead cells. Overall, the

increased activation of NLRP3 and AIM2 simultaneously during co-infection could result in increased inflammasome activation which is facilitating caspase-1 activation and release of cleaved IL-1 $\beta$ . The use of genetic knockouts for *Aim2* and *Nlrp3* support that these inflammasomes together facilitate the production of mature IL-1 $\beta$ , because either knockout resulted in lower levels of pro-inflammatory cytokines during co-infection (Fig 11A – 11F). Therefore, during co-infection, the signals expressed between the pathogens and the host are not properly regulated, explaining the rapid and inappropriate immune response observed. These data caused me to accept part of my initial *in vitro* hypothesis that NLRP3 and AIM2 inflammasomes are activated together. However, these data caused me to reject the other part of this hypothesis due to the production of ROS or *how* the inflammasomes were becoming activated.

After developing the *in vivo* mouse model and studying the pathways for enhanced caspase-1 activation and release of pro-inflammatory cytokines, many questions still remain, and even more questions have surfaced. To begin with, what DAMPs are contributing to the activation stage of the NLRP3 and AIM2 inflammasomes. It does not appear to be mitochondrial ROS. My prediction is either a potassium efflux from within the cell's cytoplasm or ROS from ER damage is/are the DAMP(s) that is/are the direct stimuli leading to NLRP3 activation. Experiments are currently in the works for confirming this. Another current planned experiment is to determine which IFN, especially if IFN- $\gamma$ , actually is produced by macrophages through transcriptional gene regulation via qRT-PCR of singly infected and co-infected samples. This would bridge further understanding DAPK1's and IFN- $\gamma$ 's roles during this co-infection. Also, how does IAV inhibit DAPK1 and what would happen to immune signaling during co-infection in a *Dapk1* knockout mouse? In the *in vivo* model, I wanted to really understand why co-

infecting mice on day 3 with *A. fumigatus* produces the observed high morbidity and mortality rates. Previous research of other IAV co-infections highlight that the infective viral titers are highest during this time-point during IAV infection with H1N1 [220]. The ultimate unanswered question is why do mice and humans die from this co-infection? Is it due to increased inflammation, as seen in our macrophages, or are the pathogens growing out of control? I need to reexamine how low of a dose of spores can be given to elicit the same morbidity and mortality rates (can I go lower than  $10^4$  CFU?). Also, the dissemination of the pathogens in the body and organ pathologies should be considered. There are previous data within several clinical case reports that also identify that some patients who succumbed to this co-infection were previously on corticosteroids [266, 268]. What formula, strength and duration of corticosteroids and how does this effect the immune system during these co-infections? Could administering a common antiviral prevent or delay the co-infection if caught early enough? Could a possible explanation to decreased ROS production in co-infection samples in comparison to singly infected samples be due to reduced macrophage fungicidal activity through possible IFN- $\gamma$  exposure/production?

To sum up, a ‘perfect storm’ results from IAV and *A. fumigatus* co-infections, leading to the observed high morbidity and mortality seen in clinical case reports, and this was recapitulated in my *in vivo* mouse model (Fig 13). The ‘perfect storm’ may be caused by increased activation of inflammasomes, specifically AIM2 and NLRP3. This affects the downstream protease caspase-1 and cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  expression. DAPK1 would normally decrease NLRP3 inflammasome activation, but during co-infection, IAV inhibits DAPK1, which results in higher NLRP3 activation. IAV also induces AIM2, which further induces AIM2 inflammasome activation. Hopefully, in the case of these IAV and *A. fumigatus*

co-infections, our research will grow the awareness and need to help the people who experience these robust immune responses annually during seasonal IAV pandemics.

## REFERENCES

1. Lat A, Bhadelia N, Miko B, Yoko Furuya E, Thompson III GR. Invasive Aspergillosis after Pandemic (H1N1) 2009. *Emerg Infect Dis.* 2010; 16: 971-973. <https://doi.org/10.3201/eid1606.100165> PMID: [20507748](https://pubmed.ncbi.nlm.nih.gov/20507748/)
2. Doshi P. Trends in Recorded Influenza Mortality: United States, 1900-2004. *Am J Public Health.* 2008; 98(5):939-945. <https://doi.org/10.2105/AJPH.2007.119933> PMID: [18381993](https://pubmed.ncbi.nlm.nih.gov/18381993/)
3. Van de Veerdonk FL, Kolwijck E, Lestrade PPA, Hodiament CJ, Rijnders BJ, van Paassen J, et al. Influenza-associated Aspergillosis in Critically Ill Patients. *Am J Respir Crit Care Med.* 2017; 196(4):524-527. <https://doi.org/10.1164/rccm.201612-2540LE> PMID: [28387526](https://pubmed.ncbi.nlm.nih.gov/28387526/)
4. Van de Veerdonk FL, Kolwijck E, Lestrade PPA, Hodiament CJ, Rijnders BJ, van Paassen J, et al. Influenza-associated Aspergillosis in Critically Ill Patients. *Am J Respir Crit Care Med.* 2017; 196(4):524-527. <https://doi.org/10.1164/rccm.201612-2540LE> PMID: [28387526](https://pubmed.ncbi.nlm.nih.gov/28387526/)
5. Rosa K. Severe Influenza May Be a Risk Factor for Aspergillosis. 2018. Available from: <https://www.contagionlive.com/news/severe-influenza-may-be-a-risk-factor-for-aspergillosis/>
6. Institute of Medicine (US) Forum on Microbial Threats. The Story of Influenza. In: Knobler SL, Mack A, Mahmoud A, Lemon SM: editors. *The Threat of Pandemic Influenza: Are We Ready? Workshop Summary.* Washington (DC): National Academies Press (US); 2005:7-57. <https://doi.org/10.17226/11150> PMID: [20669448](https://pubmed.ncbi.nlm.nih.gov/20669448/)
7. Centers for Disease Control and Prevention. Influenza (Flu): 2009 H1N1 Pandemic 2017. Available from: <https://www.cdc.gov/flu/pandemic-resouces/2009-h1n1-pandemic.html>
8. Al-Muharrmi Z. Understanding the Influenza A H1N1 2009 Pandemic. *Sultan Qaboos Univ Med J.* 2010; 10(2):187-195. PMID: [21509228](https://pubmed.ncbi.nlm.nih.gov/21509228/)
9. Centers for Disease Control and Prevention. Influenza (Flu): Types of Influenza Viruses. 2017. Available from: <https://www.cdc.gov/flu/about/viruses/types.htm>
10. Taubenberger JK, Morens DM. The Pathology of Influenza Virus Infections. *Annu Rev Pathol.* 2008; 3:499-522. <https://doi.org/10.1146/annurev.pathmechdis.3.121806.154316> PMID: [18039138](https://pubmed.ncbi.nlm.nih.gov/18039138/)

11. Kosik I, Yewdell JW. Influenza Hemagglutinin and Neuraminidase: Yin-Yang Proteins Coevolving to Thwart Immunity. *Viruses*. 2019; 11(4):346. <https://doi.org/10.3390/v11040346> PMID: [3101429](https://pubmed.ncbi.nlm.nih.gov/3101429/)
12. Kosik I, Yewdell JW. Influenza Hemagglutinin and Neuraminidase: Yin-Yang Proteins Coevolving to Thwart Immunity. *Viruses*. 2019; 11(4):346. <https://doi.org/10.3390/v11040346> PMID: [3101429](https://pubmed.ncbi.nlm.nih.gov/3101429/)
13. Al-Muharrmi Z. Understanding the Influenza A H1N1 2009 Pandemic. *Sultan Qaboos Univ Med J*. 2010; 10(2):187-195. PMID: [21509228](https://pubmed.ncbi.nlm.nih.gov/21509228/)
14. Jester BJ, Uyeki TM, Patel A, Koonin L, Jerigan DB. 100 Years of Medical Countermeasures and Pandemic Influenza Preparedness. *Am J Public Health*. 2018; 108(11):1469-1472. <https://doi.org/10.2105/AJPH.2018.304586> PMID: [30252525](https://pubmed.ncbi.nlm.nih.gov/30252525/)
15. World Health Organization. What is the Pandemic (H1N1) 2009 Virus? 2010. Available from: <https://www.who.int/csr/disease/swineflu/>
16. Bouvier NM, Palese P. The Biology of Influenza Viruses. *Vaccine*. 2008; 26(Suppl 4):D49-D53. <https://doi.org/10.1016/j.vaccine.2008.07.039> PMID: [1923160](https://pubmed.ncbi.nlm.nih.gov/1923160/)
17. Zhirnov OP, Manykin AA, Rossman JS, Klenk HD. Intravirion cohesion of matrix protein M1 with ribonucleocapsid is a prerequisite of influenza virus infectivity. *Virology*. 2016; 492:187-196. <https://doi.org/10.1016/j.virol.2016.02.021> PMID: [17855515](https://pubmed.ncbi.nlm.nih.gov/17855515/)
18. Rossman JS, Lamb RA. Influenza virus assembly and budding. *Virology*. 2011; 411(2):229-236. <https://doi.org/10.1016/j.virol.2010.12.003> PMID: [21237476](https://pubmed.ncbi.nlm.nih.gov/21237476/)
19. Perez DR, Donis RO. Functional analysis of PA binding by influenza a virus PB1: Effects on polymerase activity and Viral Infectivity. *J Virol*. 2001; 75(17):8127-8136. <https://doi.org/10.1128/JVI.75.17.8127-8136.2001> PMID: [11483758](https://pubmed.ncbi.nlm.nih.gov/11483758/)
20. Gao Q, Chou YY, Doganay S, Vafabakhsh R, Ha T, Palese P. The Influenza a virus PB2, PA, NP, and M segments play a pivotal role during genome packaging. *J Virol*. 2012; 86(13):7043-7051. <https://doi.org/10.1128/JVI.00662-12> PMID: [22532680](https://pubmed.ncbi.nlm.nih.gov/22532680/)
21. Gonzalez S, Ortin J. Characterization of Influenza Virus PB1 Protein Binding to Viral RNA: Two Separate Regions of the Protein Contribute to the Interaction Domain. *J Virol*. 1999; 73(1):631-637. PMID: [9847368](https://pubmed.ncbi.nlm.nih.gov/9847368/)
22. Boivin S, Cusack S, Ruigrok RWH, Hart DJ. Influenza A Virus Polymerase: Structural Insights into Replication and Host Adaptation Mechanisms. *J Bio Chem*. 2010; 285(37):28411-28417. <https://doi.org/10.1074/jbc.R117531> PMID: [20538599](https://pubmed.ncbi.nlm.nih.gov/20538599/)

23. Te Velthuis AJ, Fodor E. Influenza virus RNA Polymerase: insights into the mechanisms of viral RNA synthesis. *Nat Rev Microbiol*. 2016; 14(8):479-93.  
<https://doi.org/10.1038/nrmicro.2016.87> PMID: [27396566](https://pubmed.ncbi.nlm.nih.gov/27396566/)
24. Marc D. Influenza virus non-structural protein NS1: interferon antagonism and beyond. *J Gen Virol*. 2014; 95(Pt 12):2594-2611. <https://doi.org/10.1099/vir.0.069542-0> PMID: [25182164](https://pubmed.ncbi.nlm.nih.gov/25182164/)
25. Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, et al. The Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon at the Level of the MAVS Adaptor Protein. *PLoS Pathog*. 2011; 7(6):1-16.  
<https://doi.org/10.1371/journal.ppat.1002067> PMID: [21695240](https://pubmed.ncbi.nlm.nih.gov/21695240/)
26. Hale BG, Albrecht RA, Garcia-Sastre A. Innate immune evasion strategies of influenza viruses. *Future Microbiol*. 2010; 5(1):1-29. <https://doi.org/10.2217/fmb.09.108> PMID: [20020828](https://pubmed.ncbi.nlm.nih.gov/20020828/)
27. Le Goddic R, Bouguyon E, Chevalier C, Vidic J, Da Costa B, Leymarie O, et al. Influenza A Virus Protein PB1-F2 Exacerbates INF- $\beta$  Expression of Human Respiratory Epithelial Cells. *J Immunol*. 2010; 185(8):4812-4823.  
<https://doi.org/10.4049/jimmunol.0903952> PMID: [28613140](https://pubmed.ncbi.nlm.nih.gov/28613140/)
28. Shao W, Li X, Goraya MU, Wang S, Chen JL. Evolution of Influenza A Virus by Mutation and Re-Assortment. *Int J Mol Sci*. 2017; 18(8):1650.  
<https://doi.org/10.3390/ijms18081650> PMID: [28783091](https://pubmed.ncbi.nlm.nih.gov/28783091/)
29. Jilani TN, Jamil RT, Siddiqui AH. H1N1 Influenza (Swine Flu). StatPearls Publishing. 2019. Accessed from: <https://www.ncbi.nlm.nih.gov/books/NBK513241/>
30. Parrish CR, Holmes EC, Morens DM, Park EC, Burke DS, Calisher CH, et al. Cross-Species Virus Transmission and the Emergence of New Epidemic Diseases. *Microbiol Mol Biol Rev*. 2008; 72(3):457-470. <https://doi.org/10.1128/MMBR.00004-08> PMID: [18772285](https://pubmed.ncbi.nlm.nih.gov/18772285/)
31. Wiikramaratna PS, Sandeman M, Recker M, Gupta S. The Antigenic Evolution of Influenza: Drift or Thrift? *Philos Trans R Soc Lond B Bio Sci*. 2013; 368(1614):1-9.  
<https://doi.org/10.1098/rstb.2012.0200> PMID: [2332423](https://pubmed.ncbi.nlm.nih.gov/2332423/)
32. Shao W, Li X, Goraya MU, Wang S, Chen JL. Evolution of Influenza A Virus by Mutation and Re-Assortment. *Int J Mol Sci*. 2017; 18(8):1-13.  
<https://doi.org/10.3390/ijms18081650> PMID: [28783091](https://pubmed.ncbi.nlm.nih.gov/28783091/)
33. Biggerstaff M, Cauchemez S, Reed C, Gambhir M, Finelli L. Estimates of the Reproduction Number for Seasonal, Pandemic, and Zoonotic Influenza: A Systematic Review of the Literature. *BMC Infect Dis*. 2014; 14(480):1-20.  
<https://doi.org/10.1186/1471-2334-14-480> PMID: [25186370](https://pubmed.ncbi.nlm.nih.gov/25186370/)

34. Vijaykrishna D, Poon LLM, Ma SK, Li OTW, Cheung CL, Smith GJD, et al. Re-assortment of Pandemic H1N1/2009 Influenza A Virus in Swine. *Science*. 2010; 328(5985):1529. <https://doi.org/10.1126/science.1189132> PMID: [20558710](https://pubmed.ncbi.nlm.nih.gov/20558710/)
35. Urbaniak K, Markowska-Daniel I, Kowalczyk A, Kwit K, Pomorska-Mol M, Fracek B, et al. Re-assortment Process After Co-infection of Pigs with Avian H1N1 and Swine H3N2 Influenza Viruses. *BMC Veterinary Research* 2017; 13(1):215. <https://doi.org/10.1186/s12917-017-1137-x> PMID: [28688454](https://pubmed.ncbi.nlm.nih.gov/28688454/)
36. Smith W, Andrewes CH, Laidlaw PP. A Virus Obtained from Influenza Patients. *The Lancet*. 1933; 222:66-68. Available from: [https://www.thelancet.com/journals/lancet/article/PIIS0140-6736\(00\)78541-2/fulltext](https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(00)78541-2/fulltext)
37. Neumann G, Kawaoka Y. Transmission of Influenza A Viruses. *Virology*. 2015; 234-246:234-246. <https://doi.org/10.1016/j.virol.2015.03.009> PMID: [25812763](https://pubmed.ncbi.nlm.nih.gov/25812763/)
38. Salomon R, Webster RG. The Influenza Virus Enigma. *Cell*. 2009; 236(3):402-410. <https://doi.org/10.1016/j.cell.2009.01.029> PMID: [19203576](https://pubmed.ncbi.nlm.nih.gov/19203576/)
39. Van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RAM, Osterhaus ADME, et al. Human and Avian Influenza Viruses Target Different Cells in the Lower Respiratory Tract of Humans and Other Mammals. *Am J Pathol*. 2007; 171(4):1215-1223. <https://doi.org/10.2353/ajpath.2007.070248> PMID: [17717141](https://pubmed.ncbi.nlm.nih.gov/17717141/)
40. McCullers JA. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat Rev Microbio*. 2014; 12(4):252-62. <https://doi.org/10.1038/nrmicro3231> PMID: [24590244](https://pubmed.ncbi.nlm.nih.gov/24590244/)
41. Smith AM, McCullers JA. Secondary bacterial infections in influenza virus infection pathogenesis. *Curr Top Microbiol Immunol*. 2014; 385:327-56. [https://doi.org/10.1007/82\\_2014\\_394](https://doi.org/10.1007/82_2014_394) PMID: [25027822](https://pubmed.ncbi.nlm.nih.gov/25027822/)
42. Robinson KM, Kolls JK, Alcorn JF. The immunology of influenza virus-associated bacterial pneumonia. *Curr Opin Immunol*. 2015; 34:59-67. <https://doi.org/10.1016/j.coi.2015.02.002> PMID: [25723597](https://pubmed.ncbi.nlm.nih.gov/25723597/)
43. Steinhauer DA. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology*. 1999; 258(1):1-20. <https://doi.org/10.1006/viro.1999.9716> PMID: [10329563](https://pubmed.ncbi.nlm.nih.gov/10329563/)
44. Samji T. Influenza A: understanding the viral life cycle. *Yale J Biol Med*. 2009; 82(4):153-159. PMID: [20027280](https://pubmed.ncbi.nlm.nih.gov/20027280/)
45. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: influenza virus receptors in the human airway. *Nature*. 2006; 440(7038):435-436. <https://doi.org/10.1038/440435a> PMID: [16554799](https://pubmed.ncbi.nlm.nih.gov/16554799/)

46. Kash JC, Taubenberger. The role of viral, host, and secondary bacterial factors in influenza pathogenesis. *Am J Path.* 2015; 185(6):1528-1536. <https://doi.org/10.1016/j.ajpath.2014.08.030> PMID: [25747532](https://pubmed.ncbi.nlm.nih.gov/25747532/)
47. Zambon MC. The pathogenesis of influenza in humans. *Rev Med Virol.* 2001; 11(4):227-241. PMID: [11479929](https://pubmed.ncbi.nlm.nih.gov/11479929/)
48. Baum LG, Paulson JC. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. *Virology.* 1991; 180(1):10-15. [https://doi.org/10.1016/0042-6822\(91\)90003-t](https://doi.org/10.1016/0042-6822(91)90003-t) PMID: [19846642](https://pubmed.ncbi.nlm.nih.gov/19846642/)
49. Iverson AR, Boyd KL, McAuley JL, Plano LR, Hart ME, McCullers JA. Influenza virus primes mice for pneumonia from *Staphylococcus aureus*. *J Infect Dis.* 2011; 203(6):880-888. <https://doi.org/10.1093/infdis/jiq113> PMID: [21278211](https://pubmed.ncbi.nlm.nih.gov/21278211/)
50. Lee PH, Bird N, MacKenzie-Kludas C, Mansell A, Kedzierska K, Brown L, et al. Induction of memory cytotoxic T cell to influenza A virus and subsequent viral clearance is not modulated by PB1-F2-dependent inflammasome activation. *Immunol Cell Biol.* 2016; 94(5):439-446. <https://doi.org/10.1038/icb.2015.115> PMID: [26667784](https://pubmed.ncbi.nlm.nih.gov/26667784/)
51. Conenello GM, Palese P. Influenza A virus PB1-F2: a small protein with a big punch. *Cell Host Microbe.* 2007; 2(4):207-209. <https://doi.org/10.1016/j.chom.2007.09.010> PMID: [18005736](https://pubmed.ncbi.nlm.nih.gov/18005736/)
52. Morris DE, Clearly DW, Clarke SC. Secondary Bacterial Infections Associated with Influenza Pandemics. *Front Microbiol.* 2017; 8:1041. <https://doi.org/10.3389/fmicb.2017.01041> PMID: [28690590](https://pubmed.ncbi.nlm.nih.gov/28690590/)
53. Lee EH, Wu C, Lee EU, Stoute A, Hanson H, Cook HA, et al. Fatalities associated with the 2009 H1N1 influenza A virus in New York City. *Clin Infect Dis.* 2010; 50(11):1498-1504. <https://doi.org/10.1086/652446> PMID: [20420514](https://pubmed.ncbi.nlm.nih.gov/20420514/)
54. Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. *Curr Opin Immunol.* 2012; 23(4):481-486. <https://doi.org/10.1016/j.coi.2011.07.016> PMID: [2184185](https://pubmed.ncbi.nlm.nih.gov/2184185/)
55. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature.* 2009; 460(7258):1021-1025. <https://doi.org/10.1038/nature08260> PMID: [19672242](https://pubmed.ncbi.nlm.nih.gov/19672242/)
56. Bautista E, Chotpitayasunondh T, Gao Z, Harper SA, Shaw M, Tyeki TM, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *N Engl J Med.* 2010; 362(18):1708-1719. <https://doi.org/10.1056/NEJMra1000449> PMID: [20445182](https://pubmed.ncbi.nlm.nih.gov/20445182/)

57. Rynda-Apple A, Robinson KM, Alcorn JF. Influenza and Bacterial Superinfection: Illuminating the Immunologic Mechanisms of Disease. *Infect Immun*. 2015; 83(10):3764-3770. <https://doi.org/10.1128/IAI.002980-15> PMID: [2621621](https://pubmed.ncbi.nlm.nih.gov/2621621/)
58. Ghebrehewet S, MacPherson P, Ho A. Influenza. *BMJ* .2016; 355:i6258. <https://doi.org/10.1136/bmj.i6258> PMID: [27927627](https://pubmed.ncbi.nlm.nih.gov/27927627/)
59. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical Signs and Symptoms Predicting Influenza Infection. *Arch Intern Med*. 2000; 160(21):3243-3247. PMID: [11088084](https://pubmed.ncbi.nlm.nih.gov/11088084/)
60. Kolwijck E, Scheper H, Beubing J, Kuijper EJ, van der Hoeven JG, Verweij PE. Invasive Pulmonary Aspergillosis in Influenza. *Ned Tijdschr Geneeskd*. 2015; 159:A7431. PMID: [25714763](https://pubmed.ncbi.nlm.nih.gov/25714763/)
61. Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin Microbiol Rev*. 2009; 22(3): 447-465. <https://doi.org/10.1128/CMR.00055-08> PMID: [19597008](https://pubmed.ncbi.nlm.nih.gov/19597008/)
62. Bennett JW, Klich MA, editors. *Aspergillus*, biology and industrial application. Butterworth-Heinemann. 1992; 249-267. Available from: <https://pubag.nal.usda.gov/download/24476/PDF>
63. Chazalet V, Debeaupuis JP, Sarfati J, Lortholary J, Ribaud P, Shah P, et al. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. *J Clin Microbiol*. 1998; 36(6):1494-1500. PMID: [9620367](https://pubmed.ncbi.nlm.nih.gov/9620367/)
64. Debeaupuis JP, Sarfati J, Chazalet V, Latge JP. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. *Infect Immun*. 1997; 65(8):3080-3085. PMID: [9234757](https://pubmed.ncbi.nlm.nih.gov/9234757/)
65. Kwon-Chung KJ, Sugui JA. *Aspergillus fumigatus* –What Makes the Species a Ubiquitous Human Fungal Pathogen? *PLoS Pathog*. 2013; 9(12):e1003743. <https://doi.org/10.1371/journal.ppat.1003743> PMID: [24348239](https://pubmed.ncbi.nlm.nih.gov/24348239/)
66. Latge JP. *Aspergillus fumigatus*, a saprotrophic pathogenic fungus. *Mycologist*. 2003; 17(2):56-61. [https://doi.org/10.1017/S0269-915X\(03\)00209-X](https://doi.org/10.1017/S0269-915X(03)00209-X)
67. O’Gorman C, Fuller H, Dyer P. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature*. 2009; 457(7228):471-474. <https://doi.org/10.1038/nature07528> PMID: [19043401](https://pubmed.ncbi.nlm.nih.gov/19043401/)
68. Krijghsheld P, Bleichrodt R, van Veluw GJ, Wang F, Muller WH, Dijksterhuis, et al. Development of *Aspergillus*. *Stud Mycol*. 2013; 74(1):1-29. <https://doi.org/10.3114/sim0006> PMID: [23450714](https://pubmed.ncbi.nlm.nih.gov/23450714/)

69. Twumasi-Boateng K, Yu Y, Chen D, Gravelat FN, Nierman WC, Sheppard DC. Transcriptional profiling identifies a role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite cluster in *Aspergillus fumigatus*. Eukaryotic Cell. 2009; 8(1):104-115. <https://doi.org/10.1128/EC.00265-08> PMID: [19028996](https://pubmed.ncbi.nlm.nih.gov/19028996/)
70. Reichhardt C, Ferreira JA, Joubert ML, Clemons KV, Stevens DA, Cegelski L. Analysis of the *Aspergillus fumigatus* biofilm extracellular matrix by solid-state nuclear magnetic resonance spectroscopy. Eukaryot Cell. 2015; 14(11):1064-1072. <https://doi.org/10.1128/EC.00050-15> PMID: [26163318](https://pubmed.ncbi.nlm.nih.gov/26163318/)
71. Zhao Y, Su H, Zhou J, Feng H, Zhang KQ, Yang J. The APSES family proteins in fungi; characterizations, evolution and functions. Fungal Genet Biol. 2015; 81:271-280. <https://doi.org/10.1016/j.fgb.2014.12.003> PMID: [25534868](https://pubmed.ncbi.nlm.nih.gov/25534868/)
72. Yoshimi A, Miyazawa K, Abe K. Cell wall structure and biogenesis in *Aspergillus* species. Biosci Biotechnol Biochem. 2016; 80(9):1700-1711. <https://doi.org/10.1080/09168451.2016.1177446> PMID: [27140698](https://pubmed.ncbi.nlm.nih.gov/27140698/)
73. Yoshimi A, Miyazawa K, Abe K. Function and Biosynthesis of Cell Wall  $\alpha$ -1,3-Glucan in Fungi. J Fungi (Basel). 2017; 3(4):63. <https://doi.org/10.3390/jof3040063> PMID: [29371579](https://pubmed.ncbi.nlm.nih.gov/29371579/)
74. Arana DM, Prieto D, Roman E, Nombela C, Alonso-Monge R, Pla J. The role of the cell wall in fungal pathogenesis. Microb Biotechnol. 2009; 2(3):308-320. <https://doi.org/10.1111/j.1751-7915.2008.00070.x>. PMID: [21261926](https://pubmed.ncbi.nlm.nih.gov/21261926/)
75. Valsecchi I, Dupres V, Stephen-Victor E, Guijarro JI, Gibbons J, Beau R, et al. Role of Hydrophobins in *Aspergillus fumigatus*. J Fungi (Basel). 2018; 4(1):2. <https://doi.org/10.3390/jof4010002> PMID: [29371496](https://pubmed.ncbi.nlm.nih.gov/29371496/)
76. Brand A. Hyphal Growth in Human Fungal Pathogens and Its Role in Virulence. Int J Microbiol. 2012; 2012:517529. <https://doi.org/10.1155/2012/517529> PMID: [22121367](https://pubmed.ncbi.nlm.nih.gov/22121367/)
77. Mousavi B, Hedayati MT, Hedayati N, Ilkit M, Syedmousavi S. *Aspergillus* species in indoor environments and their possible occupational and public health hazards. Curr Med Mycol. 2016; 2(1):36-42. <https://doi.org/10.18869/acadpub.cmm.2.1.36> PMID: [28681011](https://pubmed.ncbi.nlm.nih.gov/28681011/)
78. Heinekamp T, ThywiBen A, Macheleidt J, Keller S, Valiante V, Brakhage AA. *Aspergillus fumigatus* melanins: interference with the host endocytosis pathway and impact on virulence. Front Microbiol. 2012; 3:440. <https://doi.org/10.3389/fmicb.2012.00440> PMID: [2334679](https://pubmed.ncbi.nlm.nih.gov/2334679/)
79. Brakhage AA. Systemic fungal infections caused by *Aspergillus* species: epidemiology, infection process and virulence determinants. Curr Drug Targets. 2005; 6:875-886. <https://doi.org/10.2174/138945005774912717> PMID: [16375671](https://pubmed.ncbi.nlm.nih.gov/16375671/)

80. Gravelat FN, Beauvais A, Liu H, Liu H, Lee MJ, Snarr BD, et al. *Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal  $\beta$ -glucan from the immune system.* PLoS Pathog 2013; 9(8):e1003575.  
<https://doi.org/10.1371/journal.ppat.1003575> PMID: [23990787](https://pubmed.ncbi.nlm.nih.gov/23990787/)
81. Ghazaei C. Molecular Insights into Pathogenesis and Infection with *Aspergillus fumigatus*. Malays J Med Sci. 2017; 24(1):10-20.  
<https://doi.org/10.21315/mjms2017.24.1.2> PMID: [28381925](https://pubmed.ncbi.nlm.nih.gov/28381925/)
82. Ries LNA, Steenwyk JL, Alves de Castro P, de Lima PBA, Almeida F, de Assis LJ, et al. Nutritional Heterogeneity Among *Aspergillus fumigatus* Strains Has Consequences for Virulence in a Strain- and Host- Dependent Manner. Front Microbiol. 2019; 10: 854.  
<https://doi.org/10.3389/fmicb.2019.00854> PMID: [31105662](https://pubmed.ncbi.nlm.nih.gov/31105662/)
83. Krappmann S, Braus GH. Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. Medical Mycology. 2005; 43 Suppl 1:S31-40.  
<https://doi.org/10.1080/13693780400024271> PMID: [16110790](https://pubmed.ncbi.nlm.nih.gov/16110790/)
84. Grahl N, Shepardson KM, Chung D, Cramer Jr RA. Hypoxia and fungal pathogenesis: to air or not to air? Eukaryot Cell. 2012; 11(5): 560-570. <https://doi.org/10.1128/EC.00031-12> PMID: [22447924](https://pubmed.ncbi.nlm.nih.gov/22447924/)
85. Willger SD, Grahl N, Cramer Jr RA. *Aspergillus fumigatus* metabolism: Clues to mechanisms of in vivo fungal growth and virulence. Med Mycol. 2009; 47(Suppl 1):S72-S79. <https://doi.org/10.1080/13693780802455313> PMID: [19253141](https://pubmed.ncbi.nlm.nih.gov/19253141/)
86. Willger SD, Puttikamonkul S, Kim KH, Burritt JB, Grahl N, Metzler LJ, et al. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. PLoS Pathog. 2008; 4(11):e1000200. <https://doi.org/10.1371/journal.ppat.1000200> PMID: [18989462](https://pubmed.ncbi.nlm.nih.gov/18989462/)
87. Field FJ, Born E, Murthy S, Mathur SN. Gene expression of sterol regulatory element-binding proteins in hamster small intestine. J Lipid Res. 2001; 42(1):1-8. PMID: [11160359](https://pubmed.ncbi.nlm.nih.gov/11160359/)
88. Hospenthal DR, Kwon-Chung KJ, Bennett JE. Concentrations of airborne *Aspergillus* compared to the incidence of invasive aspergillosis: lack of correlation. Med Mycol. 1998; 36(3):165-168. PMID: [9776829](https://pubmed.ncbi.nlm.nih.gov/9776829/)
89. Hoselton SA, Samarasinghe AE, Seydel JM, et al. An inhalation model of airway allergic response to inhalation of environmental *Aspergillus fumigatus* conidia sensitized BALB/c mice. Med Mycol. 2010; 48(8):1056-1065.  
<https://doi.org/10.3109/13693786.2010.485582> PMID: [20482452](https://pubmed.ncbi.nlm.nih.gov/20482452/)

90. Nicod LP. Lung defences: an overview. *Eur Respir Rev.* 2005; 14(95):45-50. <https://doi.org/10.1183/09059180.05.00009501>
91. Janssen WJ, Stefanskii AL, Bochner BS, Evans CM. Control of Lung Defense by Mucins and Macrophages: Ancient defense mechanisms with modern functions. *Eur Respir J.* 2016; 48(4):1201-1214. <https://doi.org/10.1183/13993003.00120-2015> PMID: [27587549](https://pubmed.ncbi.nlm.nih.gov/27587549/)
92. Whitesett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol.* 2015; 16(1):27-35. <https://doi.org/10.1038/ni.3045> PMID: [25521682](https://pubmed.ncbi.nlm.nih.gov/25521682/)
93. Robinson BW, Venaille TJ, Mendis AH, McAleer R. Allergens as proteases: An *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment. *J Allergy Clin Immunol.* 1990; 86(5):726-731. [https://doi.org/10.1016/s0091-6749\(05\)80176-9](https://doi.org/10.1016/s0091-6749(05)80176-9) PMID: [2229838](https://pubmed.ncbi.nlm.nih.gov/2229838/)
94. Watson DS, Feng X, Askew DS, Jambunathan K, Kodukula K, Galande AK. Substrate Specificity Profiling of the *Aspergillus fumigatus* Proteolytic Secretome Reveals Consensus Motifs with Predominance of Ile/Leu and Phe/Tyr. *PLoS One.* 2011; 6(6):e21001. <https://doi.org/10.1371/journal.pone.0021001> PMID: [21695046](https://pubmed.ncbi.nlm.nih.gov/21695046/)
95. Hogan LH, Klein BS, Levitz SM. Virulence Factors of medically important fungi. *Clin Microbiol Rev.* 1996; 9(4):469-488. <https://doi.org/10.1128/CMR.9.4.469> PMID: [8894347](https://pubmed.ncbi.nlm.nih.gov/8894347/)
96. Koh TJ, DiPietro LA. Inflammation and wound healing: The role of the macrophage. *Expert Rev Mol Med.* 2011; 13:e23. <https://doi.org/10.1017/S1462399411001943> PMID: [21740602](https://pubmed.ncbi.nlm.nih.gov/21740602/)
97. Gonzalez AC, Costa TF, Andrade ZA, Medrado ARAP. Wound healing – A literature review. *An Bras Dermatol.* 2016; 91(5):614-620. <https://doi.org/10.1590/abd1806-4841.20164741> PMID: [27828635](https://pubmed.ncbi.nlm.nih.gov/27828635/)
98. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of Inflammation: What controls Its Onset? *Front Immunol.* 2016; 7(160):1-18. <https://doi.org/10.3389/fimmu.2016.00160> PMID: [27199985](https://pubmed.ncbi.nlm.nih.gov/27199985/)
99. Adulkhaleq LA, Assi MA, Abdullah R, Zamri-Saad M, Taufiq-Yap YH, Hezme MNM. The crucial roles of inflammatory mediators in inflammation: A review. *Vet World.* 2018; 11(5):627-635. <https://doi.org/10.14202/vetworld.2018.627-635> PMID: [29915501](https://pubmed.ncbi.nlm.nih.gov/29915501/)
100. Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. *Nat Rev Mol Cell Biol.* 2010; 11(5):366-378. <https://doi.org/10.1038/nrm2889> PMID: [20414258](https://pubmed.ncbi.nlm.nih.gov/20414258/)

101. Muller WA. Getting Leukocytes to the Site of Inflammation. *Vet Pathol.* 2013; 50(1):7-22. <https://doi.org/10.1177/0300985812469883> PMID: [23345459](https://pubmed.ncbi.nlm.nih.gov/23345459/)
102. Carmen CV, Sage PT, Sciuto TE, de la Fuente MA, Geha RS, Ochs HD, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity.* 2007; 26(6):784-797. <https://doi.org/10.1016/j.immuni.2007.04.015> PMID: [17570692](https://pubmed.ncbi.nlm.nih.gov/17570692/)
103. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol.* 2007; 7(9):678-689. <https://doi.org/10.1038/nri2156> PMID: [17717539](https://pubmed.ncbi.nlm.nih.gov/17717539/)
104. Mullet WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol.* 2003; 24(6):327-334. [https://doi.org/10.1016/S1471-4906\(03\)00117-0](https://doi.org/10.1016/S1471-4906(03)00117-0) PMID: [12810109](https://pubmed.ncbi.nlm.nih.gov/12810109/)
105. Petri B, Bixel GM. Molecular events during leukocyte diapedesis. *FEBS J.* 2006; 273(19):4399-4407. <https://doi.org/10.1111/j.1742-4658.2006.05439.x> PMID: [16965541](https://pubmed.ncbi.nlm.nih.gov/16965541/)
106. Sage PT, Carmen CV. Settings and mechanisms for transcellular diapedesis. *Front Biosci.* 2009; 14:5066-5083. PMID: [19482605](https://pubmed.ncbi.nlm.nih.gov/19482605/)
107. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. *Immunobiology: The Immune System in Health and Disease.* 5th edition. New York: Garland Science; 2001. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK10757/>
108. Meinderts SM, Baker G, van Wijk S, Beuger BM, Geissler J, Jansen MH, et al. Neutrophils acquire antigen-presenting cell features after phagocytosis of IgG-opsonized erythrocytes. *Blood Adv.* 2019; 3(11):1761-1773. <https://doi.org/10.1182/bloodadvances.2018028753> PMID: [31182561](https://pubmed.ncbi.nlm.nih.gov/31182561/)
109. Samokhvalov IM. Deconvoluting the ontogeny of hematopoietic stem cells. *Cell Mol Life.* 2014; 71(6):957-978. <https://doi.org/10.1007/s00018-013-1364-7> PMID: [23708646](https://pubmed.ncbi.nlm.nih.gov/23708646/)
110. NIH: NIAID; 2014. Immune Cells. Available from: <https://www.niaid.nih.gov/research/immune-cells>
111. Tagliani E, Shi C, Nancy P, Tay CS, Pamer EG, Erlebacher A. Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. *J Exp Med.* 2011; 208(9):1901-1916. <https://doi.org/10.1084/jem.20110866> PMID: [21825019](https://pubmed.ncbi.nlm.nih.gov/21825019/)
112. Weiss U. Inflammation. *Nature.* 2008; 454(7203):427. <https://doi.org/10.1038/454427a> PMID: [18650912](https://pubmed.ncbi.nlm.nih.gov/18650912/)
113. Parkn J, Cohen B. An overview of the immune system. *Lancet.* 2001; 357(9270):1777-1789. [https://doi.org/10.1016/S0140-6736\(00\)04904-7](https://doi.org/10.1016/S0140-6736(00)04904-7) PMID: [11403834](https://pubmed.ncbi.nlm.nih.gov/11403834/)

114. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev.* 2009; 22(2):240-273. <https://doi.org/10.1128/CMR.00046-08> PMID: [19366914](https://pubmed.ncbi.nlm.nih.gov/19366914/)
115. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol.* 2004; 5(10):987-995. <https://doi.org/10.1038/ni1112> PMID: [15454922](https://pubmed.ncbi.nlm.nih.gov/15454922/)
116. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002; 20:197-216. <https://doi.org/10.1146/annurev.immunol.20.083001.084359> PMID: [11861602](https://pubmed.ncbi.nlm.nih.gov/11861602/)
117. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell.* 2006; 124(4):783-801. <https://10.1016/j.cell.2006.02.015> PMID: [16497588](https://pubmed.ncbi.nlm.nih.gov/16497588/)
118. Schmolke M, Garcia-Sastre A. Evasion of Innate and Adaptive Immune Responses by Influenza A Virus. *Cell Microbiol.* 2011; 12(7):873-880. <https://doi.org/10.1111/j.1462-5822.2010.01475.x>. PMID: [20482552](https://pubmed.ncbi.nlm.nih.gov/20482552/)
119. Chen X, Liu S, Goraya MU, Maarouf M, Huang S, Chen JL. Host Immune Response to Influenza A Virus Infection. *Front Immunol.* 2018; 9:320. <https://doi.org/10.3389/fimmu.2018.00320> PMID: [29556226](https://pubmed.ncbi.nlm.nih.gov/29556226/)
120. Jensen S, Thomsen AR. Sensing of RNA Viruses: a Review of Innate Immune Receptors Involved in Recognizing RNA Virus Invasion. *J Virol.* 2012; 86(6):2900-2910. <https://doi.org/10.1128/JVI.05738-11> PMID: [22258243](https://pubmed.ncbi.nlm.nih.gov/22258243/)
121. Sales-Campos H, Tonani L, Cardoso CR, Kress MR. The Immune Interplay between the Host and the Pathogen in *Aspergillus fumigatus* Lung Infection. *BioMed Res Inter.* 2013; 2013:693023. <https://doi.org/10.1155/2013/693023> PMID: [23984400](https://pubmed.ncbi.nlm.nih.gov/23984400/)
122. Gresnigt MS, Bozza S, Becker KL, Joosten LA, Abdollahi-Roodsaz S, van der Berg WB, et al. A Polysaccharide Virulence Factor from *Aspergillus fumigatus* Elicits Anti-inflammatory Effects through Induction of Interleukin-1 Receptor Antagonist. *PLoS Pathog.* 2014; 10(3):e1003936. <https://doi.org/10.1371/journal.ppat.1003936> PMID: [24603878](https://pubmed.ncbi.nlm.nih.gov/24603878/)
123. Amarsaikhan N, Templeton SP. Co-recognition of  $\beta$ -glucan and chitin and programming of adaptive immunity to *Aspergillus fumigatus*. *Front Microbiol.* 2015; 6:344. <https://doi.org/10.3389/fmicb.2015.00344> PMID: [25954267](https://pubmed.ncbi.nlm.nih.gov/25954267/)
124. Newton K, Dixit VM. Signaling in Innate Immunity and Inflammation. *Cold Spring Harb Perspect Biol.* 2012; 4(3):a006049. <https://doi.org/10.1101/cshperspect.a006049> PMID: [22296764](https://pubmed.ncbi.nlm.nih.gov/22296764/)

125. Carrillo JLM, Garcia FPC, Coronado OG, Garcia MAM, Cordero JFC. Physiology and Pathology of Innate Immune Response Against Pathogens. *Phys and Path of Immunol*. 2017; 99-134. <https://doi.org/10.5772/iintechopen.70556>
126. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int J Mol Sci*. 2018; 19(1):E92. <https://doi.org/10.3390/ijms19010092>. PMID: [29286292](https://pubmed.ncbi.nlm.nih.gov/29286292/)
127. Schaefer L. Complexity of Danger: The Diverse Nature of Damage-associated Molecular Patterns. *J Biol Chem*. 2014; 289(51):35237-35245. <https://doi.org/10.1074/jbc.R114.619304> PMID: [25391648](https://pubmed.ncbi.nlm.nih.gov/25391648/)
128. Goraya MU, Wang S, Munir M, Chen JL. Induction of innate immunity and its perturbation by influenza viruses. *Protein Cell*. 2015; 6(10):712-721. <https://doi.org/10.1007/s13238-015-0191-z> PMID: [26206138](https://pubmed.ncbi.nlm.nih.gov/26206138/)
129. Kell AM, Gale Jr M. RIG-I in RNA virus recognition. *Virology*. 2015; 479-480:110-121. <https://doi.org/10.1016/j.virol.2015.02.017> PMID: [25749629](https://pubmed.ncbi.nlm.nih.gov/25749629/)
130. Loo YM, Gale Jr M. Immune signaling by RIG-I-like receptors. *Immunity*. 2011; 34(5):680-692. <https://doi.org/10.1016/j.immuni.2011.05.003> PMID: [21616437](https://pubmed.ncbi.nlm.nih.gov/21616437/)
131. Wang R, Zhu Y, Lin X, Ren C, Zhao J, Wang F, et al. Influenza M2 protein regulates MAVS-mediated signaling pathway through interacting with MAVS and increasing ROS production. *Autophagy*. 2019; 15(7):1163-1181. <https://doi.org/10.1080/15548627.2019.1580089> PMID: [30741586](https://pubmed.ncbi.nlm.nih.gov/30741586/)
132. Holm CK, Rahbek SH, Gad HH, Bak RO, Jakobsen MR, Jiang Z, et al. Influenza A virus targets a cGAS-independent STING pathway that controls enveloped RNA viruses. *Nat Comm*. 2016; 7:10680. <https://doi.org/10.1038/ncomms10680> PMID: [26893169](https://pubmed.ncbi.nlm.nih.gov/26893169/)
133. Varga ZT, Grant A, Manicassamy B, Palese P. Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon by Binding to MAVS and Decreasing Mitochondrial Membrane Potential. *Virology*. 2012; 86(16):8359-8366. <https://doi.org/10.1128/JVI.01122-12> PMID: [22674996](https://pubmed.ncbi.nlm.nih.gov/22674996/)
134. Dalpke AH, Helm M. RNA mediated toll-like receptor stimulation in health and disease. *RNA Biol*. 2012; 9(6):828-842. <https://doi.org/10.4161/ma.20206> PMID: [22617878](https://pubmed.ncbi.nlm.nih.gov/22617878/)
135. Xagorari A, Chlichlia K. Toll-Like Receptors and Viruses: Induction of Innate Antiviral Immune Responses. *Open Microbiol J*. 2008; 2:49-59. <https://doi.org/10.2174/1874285800802010049> PMID: [19088911](https://pubmed.ncbi.nlm.nih.gov/19088911/)
136. Biondo C, Lentini G, Beninati C, Teti G. The dual role of innate immunity during influenza. *Biomedical Journal*. 2019; 42(1):8-18. <https://doi.org/10.1016/j.bj.2018.12.009> PMID: [30987709](https://pubmed.ncbi.nlm.nih.gov/30987709/)

137. Pizzolla A, Smith JM, Brooks AG, Reading PC. Pattern recognition receptor immunomodulation of innate immunity as a strategy to limit the impact of influenza virus. *J Leukoc Biol.* 2017; 101(4):851-861. <https://doi.org/10.1189/jiib.4MR0716-290R> PMID: [27810944](https://pubmed.ncbi.nlm.nih.gov/27810944/)
138. Hotz C, Roetzer LC, Huber T, Saiiler A, Oberson A, Treinies M, et al. TLR and RLR Signaling Are Reprogrammed in Opposite Directions after Detection of Viral Infection. *J Immunol.* 2015; 195(9):4387-4395. <https://doi.org/10.4049/jiimmunol.1500079> PMID: [26392465](https://pubmed.ncbi.nlm.nih.gov/26392465/)
139. Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabanes FJ, et al. Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. *Stud Mycol.* 2007; 59:31-37. <https://doi.org/10.3114/sim.2007.59.04> PMID: [18490955](https://pubmed.ncbi.nlm.nih.gov/18490955/)
140. Gresnigt MS, Netea MG, van de Veerdonk FL. Pattern recognition receptors and their role in invasive aspergillosis. *Ann N Y Acad Sci.* 2012; 1273:60-7. <https://doi.org/10.1111/j.1749-6632.2012.06759.x>. PMID: [23230838](https://pubmed.ncbi.nlm.nih.gov/23230838/)
141. Bidula S, Schelenz S. A Sweet Response to a Sour Situation: The Role of Soluble Pattern Recognition Receptors in the Innate Immune Response to Invasive *Aspergillus fumigatus* Infections. *PLoS Pathog.* 2016; 12(7):e1005637. <https://doi.org/10.1371/journal.ppat.1005637> PMID: [27415780](https://pubmed.ncbi.nlm.nih.gov/27415780/)
142. Patin EC, Thompson A, Orr SJ. Pattern recognition receptors in fungal immunity. *Semin Cell Dev Bio.* 2019; 89:24-33. <https://doi.org/10.1016/j.semcdb.2018.03.003> PMID: [29522806](https://pubmed.ncbi.nlm.nih.gov/29522806/)
143. Oeckinghaus A, Ghosh S. The NF- $\kappa$ B family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol.* 2009; 1(4):a000034. <https://doi.org/10.1101/cshperspect.a000034> PMID: [20066092](https://pubmed.ncbi.nlm.nih.gov/20066092/)
144. Baeuerle PA, Henkel T. Function and activation of NF- $\kappa$ B in the immune system. *Annu Rev Immunol.* 1994; 12:141-179. <https://doi.org/10.1146/annurev.iy.12.040194.001041> PMID: [8011280](https://pubmed.ncbi.nlm.nih.gov/8011280/)
145. Yu Y, Wan Y, Huang C. The Biological Function of NF- $\kappa$ B (p50) and its Potential as an Anti-Cancer Target. *Curr Cancer Drug Targets.* 2013; 9(4):566-571. PMID: [19519322](https://pubmed.ncbi.nlm.nih.gov/19519322/)
146. Sen R, Smale ST. Selectivity of the NF- $\kappa$ B Response. *Cold Spring Harb Perspect Biol.* 2010; 2(4):a000257. <https://doi.org/10.1101/cshperspect.a000257> PMID: [20452937](https://pubmed.ncbi.nlm.nih.gov/20452937/)
147. Dev A, Iyer S, Razani B, Cheng G. NF- $\kappa$ B and Innate Immunity. *Curr Top in Microb and Immunol.* 2011; 349:115-43. [https://doi.org/10.1007/82\\_2010\\_102](https://doi.org/10.1007/82_2010_102) PMID: [20848362](https://pubmed.ncbi.nlm.nih.gov/20848362/)

148. Saccani S, Pantano S, Natoli G. Modulation of NF- $\kappa$ B activity by exchange of dimers. *Mol Cell*. 2003; 11(6):1563-74. PMID: [12820969](https://pubmed.ncbi.nlm.nih.gov/12820969/)
149. Hayden MS, Ghosh S. Shared Principles in NF- $\kappa$ B Signaling. *Cell*. 2008; 132(3):344-362. <https://doi.org/10.1016/j.cell.2008.01.020> PMID: [18267068](https://pubmed.ncbi.nlm.nih.gov/18267068/)
150. Lecker SH, Goldberg AL, Mitch WE. Protein Degradation by the Ubiquitin-Proteasome Pathway in Normal and Disease States. *J Am Soc Nephrol*. 2006; 17(7):1807-1819. <https://doi.org/10.1681/ASN.2006010083> PMID: [16738015](https://pubmed.ncbi.nlm.nih.gov/16738015/)
151. Corn JE, Vucic D. Ubiquitin in inflammation: the right linkage makes all the difference. *Nat Struct Mol Biol*. 2014; 21(4):297-300. <https://doi.org/10.1038/nsmb.2808> PMID: [24699077](https://pubmed.ncbi.nlm.nih.gov/24699077/)
152. Tokunaga F. Linear ubiquitination-mediated NF- $\kappa$ B regulation and its related disorders. *J Biochem*. 2013; 154(4):313-323. <https://doi.org/10.1093/jb/mvt079> PMID: [23969028](https://pubmed.ncbi.nlm.nih.gov/23969028/)
153. Weissman AM, Shabek N, Ciechanover A. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat Rev Mol Cell Biol*. 2011; 12(9):605-20. <https://doi.org/10.1038/nrm3173> PMID: [21860393](https://pubmed.ncbi.nlm.nih.gov/21860393/)
154. Collins PE, Mitxitorena I, Carmody RJ. The Ubiquitination of NF- $\kappa$ B Subunits in the Control of Transcription. *Cells*. 2016; 5(23):1-27. <https://doi.org/10.3390/cells5020023> PMID: [27187478](https://pubmed.ncbi.nlm.nih.gov/27187478/)
155. Liu S, Chen ZJ. Expanding role of ubiquitination in NF- $\kappa$ B signaling. *Cell Res*. 2011; 21(1):6-21. <https://doi.org/10.1038/cr.2010.170> PMID: [21135871](https://pubmed.ncbi.nlm.nih.gov/21135871/)
156. Wu L, Pu Z, Feng J, Li G, Zheng Z, Shen W. The ubiquitin-proteasome pathway and enhanced activity of NF- $\kappa$ B in gastric carcinoma. *J Surg Oncol*. 2007; 97(5):439-444. <https://doi.org/10.1002/jso.20952> PMID: [18163448](https://pubmed.ncbi.nlm.nih.gov/18163448/)
157. Kempe S, Kestler H, Lasar A, Wirth T. NF- $\kappa$ B controls the global pro-inflammatory in endothelial cells: evidence for the regulation of a pro-atherogenic program. *Nucleic Acids Res*. 2005; 33(16):5308-19. <https://doi.org/10.1093/nar/gki836> PMID: [16177180](https://pubmed.ncbi.nlm.nih.gov/16177180/)
158. van Essen D, Engist B, Natoli G, Saccani S. Two modes of transcriptional activation at native promoters by NF- $\kappa$ B p65. *PLoS Biol*. 2009; 7(3):e73. <https://doi.org/10.1371/journal.pbio.1000073> PMID: [19338389](https://pubmed.ncbi.nlm.nih.gov/19338389/)
159. Liu YZ, Wang YX, Jiang CL. Inflammation: The Common Pathway of Stress-Related Diseases. *Front Hum Neurosci*. 2017; 11(316):1-11. <https://doi.org/10.3389/fnhum.2017.00316> PMID: [28676747](https://pubmed.ncbi.nlm.nih.gov/28676747/)

160. Kotas ME, Medzhitov R. Homeostasis, Inflammation, and Disease Susceptibility. *Cell*. 2015; 160(5):816-827. <https://doi.org/10.1016/j.cell.2015.02.010> PMID: [25723161](https://pubmed.ncbi.nlm.nih.gov/25723161/)
161. Ricklin D, Lambris JD. Complement in Immune and Inflammatory Disorders: Pathophysiological Mechanisms. *J Immunol*. 2013; 190(8):3831-3838. <https://doi.org/10.4049/jimmunol.1203487> PMID: [23564577](https://pubmed.ncbi.nlm.nih.gov/23564577/)
162. Samuel CE. Antiviral Actions of Interferons. *Clin Microbol Rev*. 2001; 14(4):778-809. <https://doi.org/10.1128/CMR.14.4.778-809.2001> PMID: [11585785](https://pubmed.ncbi.nlm.nih.gov/11585785/)
163. Pichlmair A, Reis e Sousa C. Innate Recognition of Viruses. *Immunity*. 2007; 27(3):370-383. <https://doi.org/10.1016/j.immuni.2007.08.012> PMID: [17892846](https://pubmed.ncbi.nlm.nih.gov/17892846/)
164. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I and Mda5-mediated type I interferon induction. *Nat Immunol*. 2005; 6:981-988. <https://doi.org/10.1038/ni1243> PMID: [16127453](https://pubmed.ncbi.nlm.nih.gov/16127453/)
165. Seth R, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*. 2005; 122:669-682. <https://doi.org/10.1016/j.cell.2005.08.012> PMID: [16125763](https://pubmed.ncbi.nlm.nih.gov/16125763/)
166. Reis e Sousa C. Toll-like receptors and dendritic cells: For whom the bug tolls. *Semin Immunol*. 2004; 16:27-34. PMID: [14751761](https://pubmed.ncbi.nlm.nih.gov/14751761/)
167. Uematsu S, Akira S. Toll-like receptors and Type I interferons. *J Bio Chem*. 2007; 282(21):15319-15323. <https://doi.org/10.1074/jbc.R700009200> PMID: [17395581](https://pubmed.ncbi.nlm.nih.gov/17395581/)
168. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem*. 1998; 67:227-264. <https://doi.org/10.1146/annurev.biochem.67.1.227> PMID: [9759489](https://pubmed.ncbi.nlm.nih.gov/9759489/)
169. Seif F, Khoshmirsafa M, Aazami H, Mohsenzadegan M, Sedighi G, Bahar M. The role of JAK-STAT signaling pathway and its regulators and the fate of T helper cells. *Cell Commun Signal*. 2017; 15(23):1-13. <https://doi.org/10.1186/s12964-017-0177-y> PMID: [28637459](https://pubmed.ncbi.nlm.nih.gov/28637459/)
170. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol*. 2015; 14(1):36-49. <https://doi.org/10.1038/nri3581> PMID: [24362405](https://pubmed.ncbi.nlm.nih.gov/24362405/)
171. Majoros A, Platanitis E, Kernbauer-Holz E, Rosebrock F, Muller M, Decker T. Canonical and Non-canonical Aspects of JAK-STAT Signaling: Lessons from Interferons for Cytokine Responses. *Front Immunol*. 2017; 8(29):1-13. <https://doi.org/10.3389/fimmu.2017.00029> PMID: [28184222](https://pubmed.ncbi.nlm.nih.gov/28184222/)

- 172.Hsu SF, Su WC, Jeng KS, Lai MM. A Host Susceptibility Gene, DR1, facilitates influenza A Virus Replication by Suppressing Host Innate Immunity and Enhancing Viral RNA Replication. *J Virol.* 2015; 89(7):3671-3682.  
<https://doi.org/10.1128/JVI.03610-14> PMID: [25589657](https://pubmed.ncbi.nlm.nih.gov/25589657/)
- 173.Heldin CH, Lu B, Evans R, Gutkind JS. Signals and Receptors. *Cold Spring Harb Perspect Biol.* 2016; 8(4):a005900. <https://doi.org/10.1101/cshperspect.a005900> PMID: [27037414](https://pubmed.ncbi.nlm.nih.gov/27037414/)
- 174.Oster W, Cicco NA, Klein H, Hirano T, Kishimoto T, Lindemann A, et al. Participation of the cytokine interleukin 6, tumor necrosis factor-alpha, and interleukin 1-beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *J Clin Invest.* 1989; 84(2):451-7. <https://doi.org/10.1172/JCI114186> PMID: [2788173](https://pubmed.ncbi.nlm.nih.gov/2788173/)
- 175.Dinarello CA. Overview of the IL-1 family in inflammation and acquired immunity. *Immunol. Rev* 2018; 281(1):8-27. <https://doi.org/10.1111/imr.12621> PMID: [29247995](https://pubmed.ncbi.nlm.nih.gov/29247995/)
- 176.Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget.* 2018; 9(6):7204-7218.  
<https://doi.org/10.18632/oncotarget.23208> PMID: [29467962](https://pubmed.ncbi.nlm.nih.gov/29467962/)
- 177.Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the Eye of the Cytokine Storm. *Microbiol Mol Biol Rev.* 2012; 76(1):16-32.  
<https://doi.org/10.1128/MMBR.05015-11> PMID: [22390970](https://pubmed.ncbi.nlm.nih.gov/22390970/)
- 178.Stehlik C, Lee SH, Dorfleutner A, Stassinopoulos A, Sagara J, Reed JC. Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. *J Immunol.* 2003; 171(11):6154-63.  
<https://doi.org/10.4049/jimmunol.171.11.6154> PMID: [14634131](https://pubmed.ncbi.nlm.nih.gov/14634131/)
- 179.Compan V, Martin-Sanchez F, Baroja-Mazo A, Lopez-Castejon G, Gomex AI, Verkhratsky A, et al. Apoptosis-associated speck-like protein containing a CARD forms specks but does not activate caspase-1 in the absence of NLRP3 during macrophage swelling. *J Immunol.* 2015; 194(3):1261-73. <https://doi.org/10.4049/jimmunol.1301676> PMID: [25552542](https://pubmed.ncbi.nlm.nih.gov/25552542/)
- 180.Vanaja SK, Rathinam VAK, Fitzgerald KA. Mechanisms of inflammasome activation: recent advances and novel insights. *Trends in Cell Biol.* 2015; 25(5):308-315.  
<https://doi.org/10.1016/j.tcb.2014.12.009> PMID: [25639489](https://pubmed.ncbi.nlm.nih.gov/25639489/)
- 181.Franchi L, Eigenbrod T, Munoz-Planillo R. The Inflammasome: A Caspase-1 Activation Platform Regulating Immune Responses and Disease Pathogenesis. *Nat Immunol.* 2009; 10(3):241-247. <https://doi.org/10.1038/ni.1703> PMID: [19221555](https://pubmed.ncbi.nlm.nih.gov/19221555/)

182. Creagh EM. Caspase crosstalk: integration of apoptotic and innate immune signaling pathways. *Trends Immunol.* 2014; 35(12):631-640.  
<https://doi.org/10.1016/j.it.2014.10.004> PMID: [25457353](https://pubmed.ncbi.nlm.nih.gov/25457353/)
183. Tsuchiya K, Nakajima S, Hosojima S, Thi Nguyen D, Hattori T, Manh Le T, et al. Caspase-1 initiates apoptosis in the absence of gasdermin D. *Nat Comm.* 2019; 10(1):2091. <https://doi.org/10.1038/s41467-019-09753-2> PMID: [31064994](https://pubmed.ncbi.nlm.nih.gov/31064994/)
184. Rathkey JK, Benson BL, Chirieleison SM, Yang J, Xiao TS, Dubyak GR, et al. Live-cell visualization of gasdermin D-driven pyroptotic cell death. *J Biol Chem.* 2017; 292(35):14649-14658. <https://doi.org/10.1074/jbc.M117.797217> PMID: [28726636](https://pubmed.ncbi.nlm.nih.gov/28726636/)
185. Malik A, Kanneganti TD. Inflammasome activation and assembly at a glance. *J Cell Sci* 2017; 130(23):3955-3963. <https://doi.org/10.1242/jcs.207365> PMID: [29196474](https://pubmed.ncbi.nlm.nih.gov/29196474/)
186. Yi YS. Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology.* 2017; 152(2):207-217. <https://doi.org/10.1111/imm.12787> PMID: [28695629](https://pubmed.ncbi.nlm.nih.gov/28695629/)
187. Cheng KT, Xiong S, Ye Z, Hong Z, Di A, Tsang KM, et al. Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. *J Clin Invest.* 2017; 127(11): 4124-4135. <https://doi.org/10.1172/JCI94495> PMID: [28990935](https://pubmed.ncbi.nlm.nih.gov/28990935/)
188. Tang D, Kang R, Berghe TV, Vandenabeele P, Froemer G. The molecular machinery of regulated cell death. *Cell Res.* 2019; 29(5):347-364. <https://doi.org/10.1038/s41422-019-0164-5> PMID: [30948788](https://pubmed.ncbi.nlm.nih.gov/30948788/)
189. He Y, Hara H, Nunex G. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in Biochem Sci* 2016; 41(12):1012-1021.  
<https://doi.org/10.1016/j.tibs.2016.09.002> PMID: [27669650](https://pubmed.ncbi.nlm.nih.gov/27669650/)
190. Lugrin J, Martinon F. The AIM2 inflammasome: Sensor of pathogens and cellular perturbations. *Immunol Rev.* 2018; 281(1):99-114. <https://doi.org/10.1111/imr.12618> PMID: [29247998](https://pubmed.ncbi.nlm.nih.gov/29247998/)
191. Kesavardhana S, Kanneganti TD. Mechanisms governing inflammasome activation, assembly and pyroptosis induction. *Int Immunol.* 2017; 29(5):201-210.  
<https://doi.org/10.1093/intimm/dxx018> PMID: [28531279](https://pubmed.ncbi.nlm.nih.gov/28531279/)
192. Sharma D, Kanneganti TD. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *JCB* 2016; 213(6):617-629.  
<https://doi.org/10.1083/jbc.201602089> PMID: [27325789](https://pubmed.ncbi.nlm.nih.gov/27325789/)
193. Jin T, Perry A, Jiang J, Smith P, Curry JA, Unterholzner L, et al. Structures of The HIN Domain: DNA Complexes Reveal Ligand Binding and Activation Mechanisms of The

- AIM2 Inflammasome and IFI16 Receptor. *Immunity*. 2013; 36(4):561-571.  
<https://doi.org/10.1016/j.immuni.2012.02.014> PMID: [22483801](https://pubmed.ncbi.nlm.nih.gov/22483801/)
194. Shaw N, Liu ZJ. Role of the HIN Domain in Regulation of Innate Immune Responses. *Mol Cell Biol*. 2014; 34(1):2-15. <https://doi.org/10.1128/MCB.00857-13> PMID: [24164899](https://pubmed.ncbi.nlm.nih.gov/24164899/)
195. Lu A, Li Y, Yin Q, Ruan J, Yu X, Egelman E, et al. Plasticity in PYD assembly revealed by cryo-EM structure of the PYD filament of AIM2. *Cell Discov*. 2015; 1:15013.  
<https://doi.org/10.1038/celldisc.2015.13> PMID: [26583071](https://pubmed.ncbi.nlm.nih.gov/26583071/)
196. Lu A, Magupalli VG, Ruan J, Yin Q, Atianand MK, Vos MR, et al. Unified Polymerization Mechanism for the Assembly of ASC-Dependent Inflammasomes. *Cell*. 2014; 156:1193-1206. <https://doi.org/10.1016/j.cell.2014.02.008> PMID: [24630722](https://pubmed.ncbi.nlm.nih.gov/24630722/)
197. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. 2013; 13(6):397-411. <https://doi.org/10.1038/nri3452> PMID: [23702978](https://pubmed.ncbi.nlm.nih.gov/23702978/)
198. Matyszewski M, Morrone SR, Sohn J. Digital signaling drives the assembly of the AIM2-ASC inflammasome. *PNAS*. 2018; 115(9):E1963-E1972.  
<https://doi.org/10.1073/pnas.1712860115> PMID: [29440442](https://pubmed.ncbi.nlm.nih.gov/29440442/)
199. Shi Y. Caspase activation, inhibition, and reactivation: A mechanistic view. *Protein Sci* 2004; 13(8):1979-1987. <https://doi.org/10.1110/ps.04789804> PMID: [15273300](https://pubmed.ncbi.nlm.nih.gov/15273300/)
200. Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J*. 2004; 384(Pt 2):201-232.  
<https://doi.org/10.1042/BJ20041142> PMID: [15450003](https://pubmed.ncbi.nlm.nih.gov/15450003/)
201. Brough D, Rothwell NJ. Caspase-1-dependent processing of pro-interleukin-1 $\beta$  is cytosolic and precedes cell death. *J Cell Sci*. 2007; 120(Pt 5):772-781.  
<https://doi.org/10.1242/jcs.03377> PMID: [17284521](https://pubmed.ncbi.nlm.nih.gov/17284521/)
202. Chang HY, Yang X. Proteases for Cell Suicide: Functions and Regulation of Caspases. *Microbiol Mol Biol Rev*. 2000; 64(4):821-846. <https://doi.org/10.1128/mmbr.64.4.821-846.2000> PMID: [11104820](https://pubmed.ncbi.nlm.nih.gov/11104820/)
203. Kelly P, Meade KG, O'Farrelly C. Non-canonical Inflammasome-Mediated IL-1 $\beta$  Production by Primary Endometrial Epithelial and Stromal Fibroblast Cells Is NLRP3 and Caspase-4 Dependent. *Front Immunol*. 2019; 10(102):1-17.  
<https://doi.org/10.3389/fimmu.2019.00102> PMID: [30804935](https://pubmed.ncbi.nlm.nih.gov/30804935/)
204. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol*. 2010; 7(2):99-109. <https://doi.org/10.1038/nrmicro2070> PMID: [19148178](https://pubmed.ncbi.nlm.nih.gov/19148178/)

205. Guo Z, Chen LM, Zeng H, Gomez JA, Plowden J, Fujita T, et al. NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. *Am J Respir Cell Mol Biol*. 2007; 36(3):263-9. <https://doi.org/10.1165/rcmb.2006-0283RC> PMID: [17053203](https://pubmed.ncbi.nlm.nih.gov/17053203/)
206. Hsu ACY. Influenza Virus: A Master Tactician in Innate Immune Evasion and Novel Therapeutic Interventions. *Front Immunol*. 2018; 9:743. <https://doi.org/10.3389/fimmu.2018.00743> PMID: [29755452](https://pubmed.ncbi.nlm.nih.gov/29755452/)
207. Liu Y, Olganier D, Lin R. Host and Viral Modulation of RIG-I-Mediated Antiviral Immunity. *Front Immunol*. 2017; 7:662. <https://doi.org/10.3389/fimmu.2016.00662> PMID: [28096803](https://pubmed.ncbi.nlm.nih.gov/28096803/)
208. Anand SK, Tikoo SK. Viruses as Modulators of Mitochondrial Functions. *Adv Virol*. 2013; 2013:738794. <https://doi.org/10.1155/2013/738794> PMID: [24260034](https://pubmed.ncbi.nlm.nih.gov/24260034/)
209. Moreno-Altamirano MMB, Kolstoe SE, Sanchez-Garcia FJ. Virus Control of Cell Metabolism for Replication and Evasion of Host Immune Responses. *Front Cell Infect Microbiol*. 2019; 9:95. <https://doi.org/10.3389/fcimb.2019.00095> PMID: [31058096](https://pubmed.ncbi.nlm.nih.gov/31058096/)
210. Wang R, Zhu Y, Lin X, Ren C, Zhao J, Wang F, et al. Influenza M2 protein regulates MAVS-mediated signaling pathway through interacting with MAVS and increasing ROS production. *Autophagy* 2019; 15(12):1163-1181. <https://doi.org/10.1080/15548627.2019.1580089> PMID: [30741586](https://pubmed.ncbi.nlm.nih.gov/30741586/)
211. Lin W, Qiu P, Jin J, Liu S, Ul Islam S, Yang J, et al. The Cap Snatching Segmented Negative Sense RNA Viruses as a Tool to Map the Transcription Start Sites of Heterologous Co-infecting Viruses. *Front Microbiol*. 2017; 8:2519. <https://doi.org/10.3389/fmicb.2017.02519> PMID: [29312219](https://pubmed.ncbi.nlm.nih.gov/29312219/)
212. Li N, Ren A, Wang X, Fan X, Zhao Y, Gao GF, et al. Influenza viral neuraminidase primes bacterial co-infection through TGF- $\beta$  –mediated expression of host cell receptors. *PNAS*. 2015; 112(1):238-243. <https://doi.org/10.1073/pnas.1414422112> PMID: [2553543](https://pubmed.ncbi.nlm.nih.gov/2553543/)
213. Stegemann-Koniszewski S, Behrens S, Boehme JD, Hochnadel I, Riese P, Guzman CA, et al. Respiratory Influenza A Virus Infection Triggers Local and Systemic Natural Killer Cell Activation via Toll-Like Receptor 7. *Front Immunol*. 2018; 9:245. <https://doi.org/10.3389/fimmu.2018.00245> PMID: [29497422](https://pubmed.ncbi.nlm.nih.gov/29497422/)
214. Le Goffic R, Leymarie O, Chevalier C, Rebours E, Da Costa B, Vidic J, et al. Transcriptomic Analysis of Host Immune and Cell Death Responses Associated with the Influenza A Virus PB1-F2 Protein. *PLoS Pathog*. 2011; 7(8):e1002202. <https://doi.org/10.1371/journal.ppat.1002202> PMID: [21901097](https://pubmed.ncbi.nlm.nih.gov/21901097/)

215. Metzger DW, Sun K. Immune Dysfunction and Bacterial Co-Infections following Influenza. *J Immunol.* 2013; 191(5):2027-2052.  
<https://doi.org/10.4049/jimmunol.1301152> PMID: [23964104](https://pubmed.ncbi.nlm.nih.gov/23964104/)
216. Sharma-Chawla N, Sender V, Kershaw O, Gruber AD, Volckmar J, Henriques-Normark B, et al. Influenza A Virus Infection Predisposes Hosts to Secondary Infection with Different *Streptococcus pneumoniae* Serotypes with Similar Outcome but Serotype-Specific Manifestation. *Infect Immun.* 2016; 84(12):3445-3457.  
<https://doi.org/10.1128/IAI.00422-16> PMID: [27647871](https://pubmed.ncbi.nlm.nih.gov/27647871/)
217. Bellingshausen C, Rohde GGU, Savelkoul PHM, Wouters EFM, Stassen FRM. Viral-bacterial interactions in the respiratory tract. *J Gen Virol.* 2016; 97(12):3089-3102.  
<https://doi.org/10.1099/jgv.0.000627> PMID: [27902340](https://pubmed.ncbi.nlm.nih.gov/27902340/)
218. Cooper GC, Pounce ZC, Wallington JC, Bastidas-Legarda LY, Nicholas B, Chidomere C, et al. Viral Inhibition of Bacterial Phagocytosis by Human Macrophages: Redundant Role of CD36. *PLoS One.* 2016; 11(10):e0163889.  
<https://doi.org/10.1371/journal.pone.0163889> PMID: [27701435](https://pubmed.ncbi.nlm.nih.gov/27701435/)
219. Uribe-Querol E, Rosales C. Control of Phagocytosis by Microbial Pathogens. *Front Immunol.* 2017; 8:1368. <https://doi.org/10.3389/fimmu.2017.01368> PMID: [29114249](https://pubmed.ncbi.nlm.nih.gov/29114249/)
220. Atri C, Guerfali FZ, Laouini D. Role of Human Macrophage Polarization in Inflammation during Infectious Diseases. *Int J Mol Sci.* 2018; 19(6):1801.  
<https://doi.org/10.3390/ijms19061801> PMID: [29921749](https://pubmed.ncbi.nlm.nih.gov/29921749/)
221. Oliveira LVN, Costa MC, Magalhaes TFF, Bastos RW, Santos PC, Carneiro HCS, et al. Influenza A Virus as a Predisposing Factor for Cryptococcosis. *Front Cell Infect Microbiol.* 2017; 7:419. <https://doi.org/10.3389/fcimb/2017.00419> PMID: [29018774](https://pubmed.ncbi.nlm.nih.gov/29018774/)
222. Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *Lancet Infect Dis.* 2006; 6(5):303-12.  
[https://doi.org/10.1016/S1473-3099\(06\)70466-2](https://doi.org/10.1016/S1473-3099(06)70466-2) PMID: [16631551](https://pubmed.ncbi.nlm.nih.gov/16631551/)
223. Wu T, Mao H, Ling MT, Chow KH, Ho PL, Tu W, et al. Successive influenza virus infection and *Streptococcus pneumoniae* stimulation alter human dendritic cell function. *BMC Infect Dis.* 2011; 11:201. <https://doi.org/10.1186/1471-2334-11-201> PMID: [21771345](https://pubmed.ncbi.nlm.nih.gov/21771345/)
224. Cauley LS, Vella AT. Why is co-infection with influenza virus and bacteria so difficult to control? *Discov Med.* 2015; 19(102):33-40. PMID: [25636959](https://pubmed.ncbi.nlm.nih.gov/25636959/)
225. Venereau E, Ceriotti C, Bianchi E. DAMPs from Cell Death to New Life. *Front Immunol.* 2015; 6:422. <https://doi.org/10.3389/fimmu.2015.00422> PMID: [26347745](https://pubmed.ncbi.nlm.nih.gov/26347745/)

226. Yoon KW. Dead cell phagocytosis and innate immune checkpoint. *BMB Rep.* 2017; 50(10):496-503. <https://doi.org/10.5483/bmbrep.2017.50.10.147> PMID: [28768566](https://pubmed.ncbi.nlm.nih.gov/28768566/)
227. Moreira-Teixeira L, Sousa J, McNab FW, Torrado E, Cardoso F, Machado H, et al. Type I IFN Inhibits Alternative Macrophage Activation during Mycobacterium tuberculosis Infection and Leads to Enhanced Protection in the Absence of IFN- $\gamma$  Signaling. *J Immunol.* 2016; 197(12):4714-4726. <https://doi.org/10.4049/jimmunol.1600584> PMID: [27849167](https://pubmed.ncbi.nlm.nih.gov/27849167/)
228. Denney L, Ho LP. The role of respiratory epithelium in host defense against influenza virus infection. *Biomed J.* 2018; 41(4):218-233. <https://doi.org/10.1016/j.bj.2018.08.004> PMID: [30348265](https://pubmed.ncbi.nlm.nih.gov/30348265/)
229. Lionakis MS, Iliev I, Hohl TM. Immunity against fungi. *JCI Insight* 2017; 2(11):e93156. <https://doi.org/10.1172/jci.insight.93156> PMID: [28570272](https://pubmed.ncbi.nlm.nih.gov/28570272/)
230. Gow NAR, Netea MG. Medical mycology and fungal immunology: new research perspectives addressing major world health challenge. *PTRSLBBS.* 2016; 371:1709. <https://doi.org/10.1098/rstb.2015.0462> PMID: [28080988](https://pubmed.ncbi.nlm.nih.gov/28080988/)
231. Andrews T, Sullivan KE. Infections in Patients with Inherited Defects in Phagocytic Function. *Clin Microbiol Rev.* 2003; 16(4):597-621. <https://doi.org/10.1128/CMR.16.4.597-621.2003> PMID: [14557288](https://pubmed.ncbi.nlm.nih.gov/14557288/)
232. Svirshchevskaya E, Zubkov D, Mouyna I, Berkova N. Innate Immunity and the Role of Epithelial Barrier During *Aspergillus fumigatus* Infection. *Curr Immunol Rev.* 2012; 8(3):254-261. <https://doi.org/10.2174/157339512800671985> PMID: [23255875](https://pubmed.ncbi.nlm.nih.gov/23255875/)
233. Park SJ, Mehrad B. Innate Immunity to *Aspergillus* Species. *Clin Microbiol Rev.* 2009; 22(4):535-551. <https://doi.org/10.1128/CMR.00014-09> PMID: [19822887](https://pubmed.ncbi.nlm.nih.gov/19822887/)
234. Hohl TM, Feldmesser M. *Aspergillus fumigatus*: Principles of Pathogenesis and Host Defense. *Eukaryot Cell.* 2007; 6(11):1953-1963. <https://doi.org/10.1128/EC.00274-07> PMID: [17890370](https://pubmed.ncbi.nlm.nih.gov/17890370/)
235. Rambach G, Blum G, Latge JP, Fontaine T, Heinekamp T, Hagleitner M, et al. Identification of *Aspergillus fumigatus* Surface Components That Mediate Interaction of Conidia and Hyphae with Human Platelets. *J Infect Dis.* 2015; 212(7):1140-9. <https://doi.org/10.1093/infdis/jiv191> PMID: [25810442](https://pubmed.ncbi.nlm.nih.gov/25810442/)
236. Hernandez-Chavez MJ, Perez-Garcia LA, Nino-Vega GA, Mora-Montes HM. Fungal Strategies to Evade the Host Immune Recognition. *J Fungi (Basel).* 2017; 3(4):51. <https://doi.org/10.3390/jof3040051> PMID: [29371567](https://pubmed.ncbi.nlm.nih.gov/29371567/)
237. Behnsen J, Hartmann A, Schmalzer J, Gehrke A, Brakhage AA, Zipfel PF. The Opportunistic Human Pathogenic Fungus *Aspergillus fumigatus* Evades the Host

- Complement System. *Infect Immun.* 2008; 76(2):820-827.  
<https://doi.org/10.1128/IAI.01037-07> PMID: [18039838](https://pubmed.ncbi.nlm.nih.gov/18039838/)
238. Dasari P, Shopova IA, Stroe M, Wartenberg D, Martin-Dahse H, Beyersdorf N, et al. Aspf2 From *Aspergillus fumigatus* Recruits Human Immune Regulators for Immune Evasion and Cell Damage. *Front Immunol.* 2018; 9:1635.  
<https://doi.org/10.3389/fimmu.2018.01635> PMID: [30166981](https://pubmed.ncbi.nlm.nih.gov/30166981/)
239. Heinekamp T, Schmidt H, Lapp K, Pahtz V, Shopova I, Koster-Eiserfunke N, Kruger T, et al. Interference of *Aspergillus fumigatus* with the immune response. *Semin Immunopathol.* 2015; 37(2):141-152. <https://doi.org/10.1007/s00281-014-0465-1> PMID: [25404120](https://pubmed.ncbi.nlm.nih.gov/25404120/)
240. ThywiBen A, Heinekamp T, Dahse HM, Schmalder-Ripcke J, Nietzsche S, Zipfel PF, et al. Conidial Dihydroxynaphthalene Melanin of the Human Pathogen Fungus *Aspergillus fumigatus* Interferes with the Host Endocytosis Pathway. *Front Microbiol.* 2011; 2:96.  
<https://doi.org/10.3389/fmicb.2011.00096> PMID: [21747802](https://pubmed.ncbi.nlm.nih.gov/21747802/)
241. Wasylnka JA, Moore MM. Uptake of *Aspergillus fumigatus* Conidia by Phagocytic and Nonphagocytic Cells In Vitro: Quantitation Using Strains Expressing Green Fluorescent Protein. *Infect Immun.* 2002; 70(6):3156-3163. <https://doi.org/10.1128/iai.70.6.3156-3163.2002> PMID: [12011010](https://pubmed.ncbi.nlm.nih.gov/12011010/)
242. Schmidt H, Vlaic S, Kruger T, Schmidt F, Balkenhol J, Dandekar T, et al. Proteomics of *Aspergillus fumigatus* Conidia-containing Phagolysosomes Identifies Processes Governing Immune Evasion. *Mol Cell Proteomics.* 2018; 17(6):1084-1096.  
<https://doi.org/10.1074/mcp.RA117.000069> PMID: [29507050](https://pubmed.ncbi.nlm.nih.gov/29507050/)
243. Wong SSW, Rani M, Dodagatta-Marri E, Ibrahim-Granet O, Kishore U, Bayry J, et al. Fungal melanin stimulates surfactant protein D-mediated opsonization of and host immune response to *Aspergillus fumigatus* spores. *J Biol Chem.* 2018; 293(13):4901-4912. <https://doi.org/10.1074/jbc.M117.815852> PMID: [29414772](https://pubmed.ncbi.nlm.nih.gov/29414772/)
244. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. *Autophagy.* 2018; 14(2):207-215. <https://doi.org/10.1080/15548627.2017.1378838> PMID: [28933638](https://pubmed.ncbi.nlm.nih.gov/28933638/)
245. Ryter SW, Cloonan SM, Choi AM. Autophagy: A Critical Regulator of Cellular Metabolism and Homeostasis. *Mol Cells.* 2013; 36(1):7-16.  
<https://doi.org/10.1007/s10059-013-0140-8> PMID: [23708729](https://pubmed.ncbi.nlm.nih.gov/23708729/)
246. Codogno P, Mehrpour M, Proikas-Cezanne T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nat Rev.* 2012; 13(1):7-12.  
<https://doi.org/10.1038/nrm3249> PMID: [22166994](https://pubmed.ncbi.nlm.nih.gov/22166994/)

247. Nakamura S, Yoshimori T. New insights into autophagosome–lysosome fusion. *J Cell Sci.* 2017; 130(7):1209-1216. <https://doi.org/10.1242/jcs.196352> PMID: [28302910](https://pubmed.ncbi.nlm.nih.gov/28302910/)
248. Diao J, Liu R, Rong Y, Zhao M, Zhang J, Lai Y, et al. ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature.* 2015; 520(7548):563-6. <https://doi.org/10.1038/nature14147> PMID: [25686604](https://pubmed.ncbi.nlm.nih.gov/25686604/)
249. Kumar S, Jain A, Farzam F, Jia J, Gu Y, Choi SW, et al. Mechanism of Stx17 recruitment to autophagosomes via IRGM and mammalian Atg8 proteins. *J Cell Bio.* 2018; 217(3):997-1013. <https://doi.org/10.1083/jcb.201708039> PMID: [29420192](https://pubmed.ncbi.nlm.nih.gov/29420192/)
250. Nair U, Jotwani A, Geng J, Gammoh N, Richerson D, Yen WL, et al. SNARE Proteins Are Required for Macroautophagy. *Cell.* 2011; 146(2):290-302. <https://doi.org/10.1016/j.cell.2011.06.022> PMID: [21784249](https://pubmed.ncbi.nlm.nih.gov/21784249/)
251. Ganley IG. Autophagosome maturation and lysosomal fusion. *Essays Biochem.* 2013; 55(1):65-78. <https://doi.org/10.1042/bse0550065> PMID: [24070472](https://pubmed.ncbi.nlm.nih.gov/24070472/)
252. Nakatogawa H. Two ubiquitin-like conjugation systems that mediate membrane formation during autophagy. *Essays Biochem.* 2013; 55:39-50. <https://doi.org/10.1042/bse0550039> PMID: [24070470](https://pubmed.ncbi.nlm.nih.gov/24070470/)
253. Hurley JH, Schulman BA. Atomistic Autophagy: The Structures of Cellular Self-Digestion. *Cell.* 2014; 157(2):300-311. <https://doi.org/10.1016/j.cell.2014.01.070> PMID: [24725401](https://pubmed.ncbi.nlm.nih.gov/24725401/)
254. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-Associated Phagocytosis and Inflammation. *J Mol Biol.* 2017; 429(23):3561-3576. <https://doi.org/10.1016/j.jmb.2017.08.012> PMID: [28847720](https://pubmed.ncbi.nlm.nih.gov/28847720/)
255. Herb M, Gluschko A, Schramm M. LC3-associated phagocytosis – The highway to hell for phagocytosed microbes. *Semin Cell Dev Biol.* 2019; S1084-9521(19)30014-X. <https://doi.org/10.1016/j.semcdb.2019.04.016> PMID: [31029766](https://pubmed.ncbi.nlm.nih.gov/31029766/)
256. Oikonomou V, Renga G, De Luca A, Borghi M, Pariano M, Puccetti M, et al. Autophagy and LAP in the Fight against Fungal Infections: Regulation and Therapeutics. *Mediators Inflamm.* 2018; 2018:6195958. <https://doi.org/10.1155/2018/6195958> PMID: [29692681](https://pubmed.ncbi.nlm.nih.gov/29692681/)
257. Cadwell K. Crosstalk between autophagy and inflammatory signaling pathways: balancing host defense and homeostasis. *Nat Rev Immunol.* 2017; 16(11):661-675. <https://doi.org/10.1038/nri.2016.100> PMID: [27694913](https://pubmed.ncbi.nlm.nih.gov/27694913/)
258. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis (LAP) reveals distinct roles for Rubicon,

- NOX2, and autophagy proteins. *Nat Cell Bio* 2015; 17(7):893-906.  
<https://doi.org/10.1038/ncb3192> PMID: [26098576](https://pubmed.ncbi.nlm.nih.gov/26098576/)
- 259.Schille S, Crauwels P, Bohn R, Bagola K, Walther P, van Zandbergen G. LC3-associated phagocytosis in microbial pathogenesis. *Int J Med Microbiol*. 2018; 308(1):228-236. <https://doi.org/10.1016/j.ijmm.2017.10.014> PMID: [29169848](https://pubmed.ncbi.nlm.nih.gov/29169848/)
- 260.Yang CS, Lee JS, Rodgers M, Min CK, Lee JY, Kim HJ, et al. Autophagy Protein Rubicon Mediates Phagocytic NADPH Oxidase Activation in Response to Microbial Infection or TLR Stimulation. *Cell Host Microbe*. 2012; 11(3):264-76.  
<https://doi.org/10.1016/j.chom.2012.01.018> PMID: [22423966](https://pubmed.ncbi.nlm.nih.gov/22423966/)
- 261.Elbadawy M, Usui T, Yamawaki H, Sasaki K. Novel Functions of Death-Associated Protein Kinases through Mitogen-Activated Protein Kinase-Related Signals. *Int J Mol Sci*. 2018; 19(10):3031. <https://doi.org/10.3390/ijms19103031> PMID: [30287790](https://pubmed.ncbi.nlm.nih.gov/30287790/)
- 262.Salazar F, Brown GD. Antifungal Innate Immunity: A Perspective from the Last 10 Years. *J Innate Immun*. 2018; 10(5-6):373-397. <https://doi.org/10.1159/000488539> PMID: [29768268](https://pubmed.ncbi.nlm.nih.gov/29768268/)
- 263.Oikonomou V, Moretti S, Renga G, Galosi C, Borghi M, Pariano M, et al. Noncanonical Fungal Autophagy Inhibits Inflammation in Response to IFN- $\gamma$  via DAPK1. *Cell Host Microbe*. 2016; 20(6):744-757. <https://doi.org/10.1016/j.chom.2016.10.012> PMID: [27889463](https://pubmed.ncbi.nlm.nih.gov/27889463/)
- 264.Ali MF, Dasari H, Van Keulen VP, Carmona EM. Canonical Stimulation of the NLRP3 Inflammasome by Fungal Antigens Links Innate and Adaptive B-Lymphocyte Responses by Modulating IL-1 $\beta$  and IgM Production. *Front Immunol*. 2017; 8:1504.  
<https://doi.org/10.3389/fimmu.2017.01504> PMID: [29170665](https://pubmed.ncbi.nlm.nih.gov/29170665/)
- 265.Singer M. Pathogen-pathogen interaction. *Virulence*. 2010; 1(1):10-18.  
<https://doi.org/10.4161/viru.1.1.9933> PMID: [21178409](https://pubmed.ncbi.nlm.nih.gov/21178409/)
- 266.Liu Q, Zhou YH, Yang ZQ. The cytokine storm of severe influenza and development of immunomodulatory therapy. *Cell Mol Immunol*. 2016; 13(1):3-10.  
<https://doi.org/10.1038/cmi.2015.74> PMID: [26189369](https://pubmed.ncbi.nlm.nih.gov/26189369/)
- 267.Arias M, Santiago L, Vidal-Garcia M, Redrado S, Lanuza P, Comas L, et al. Preparations for Invasion: Modulation of Host Lung Immunity During Pulmonary Aspergillosis by Gliotoxin and Other Fungal Secondary Metabolites. *Front Immunol*. 2018; 9:2549. <https://doi.org/10.3389/fimmu.2018.02549> PMID: [30459771](https://pubmed.ncbi.nlm.nih.gov/30459771/)
- 268.van de Veerdonk FL, Kolwick E, Lestrade PP, Hodiamont CJ, Rijnders BJ, van Paassen J, et al. Influenza-associated Aspergillosis in Critically Ill Patients. *Am J Respir Crit Care Med*. 2017; 196(4):524-527. <https://doi.org/10.1164/rccm.201612-2540LE> PMID: [28387526](https://pubmed.ncbi.nlm.nih.gov/28387526/)

269. Ku YH, Chan KS, Yang CC, Tan CK, Chuang YC, Yu WL. Higher mortality of severe influenza patients with probable aspergillosis than those with and without other co-infections. *JFMA* 2017; 116(9):660-670. <https://doi.org/10.1016/j.jfma.2017.06.002> PMID: [28647219](https://pubmed.ncbi.nlm.nih.gov/28647219/)
270. Crum-Cianflone NF. Invasive Aspergillosis Associated with Severe Influenza Infections. *OFID* 2016; 3(3):ofw171. <https://doi.org/10.1093/ofid/ofw171> PMID: [27704024](https://pubmed.ncbi.nlm.nih.gov/27704024/)
271. Latge JP. *Aspergillus fumigatus* and Aspergillosis. *Clin Microbiol Rev.* 1999; 12(2):310-350. PMID: [10194462](https://pubmed.ncbi.nlm.nih.gov/10194462/)
272. Agarwal R, Chakrabarti A, Shah A, Gupta D, Meis JF, Guleria R, et al. Allergic bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic and classification criteria. *Clin Exp Allergy.* 2013; 43(8):850-873. <https://doi.org/10.1111/cea.12141> PMID: [23889240](https://pubmed.ncbi.nlm.nih.gov/23889240/)
273. Alshabani K. Invasive pulmonary aspergillosis in patients with influenza infection: Report of two cases and systematic review of the literature. *Expert Rev Respir Med* 2015; 9(1):89-96. <https://doi.org/10.1586/17476348.2015.996132> PMID: [25547335](https://pubmed.ncbi.nlm.nih.gov/25547335/)
274. Rosa K. Is Severe Influenza a Risk Factor for Aspergillosis? *Contagion Live: Infection Diseases Today* 2018. Available from: <https://www.contagionlive.com/news/severe-influenza-may-be-a-risk-factor-for-aspergillosis>
275. Beumer MC, Koch RM, van Beuningen D, OudeLashof AM, van de Veerdonk FL, Kolwijck E, et al. Influenza virus and factors that are associated with ICU admission, pulmonary co-infections and ICU mortality. *J Crit Care.* 2019; 50:59-65. <https://doi.org/10.1016/j.jcrc.2018.11.013> PMID: [30481669](https://pubmed.ncbi.nlm.nih.gov/30481669/)
276. Schauwvlieghe A, Rijnders B, Philips N, et al. Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study. *Lancet Resp Med.* 2018; 6(10):782-792. [https://doi.org/10.1016/S2213-2600\(18\)30274-1](https://doi.org/10.1016/S2213-2600(18)30274-1) PMID: [30076119](https://pubmed.ncbi.nlm.nih.gov/30076119/)
277. Center for Disease Control and Prevention (CDC). Influenza-Associated Intensive-Care Unit Admissions and Deaths –California, September 29, 2013-January 18, 2014. *Morbidity and Mortality Weekly Report (MMWR)* 2014; 63(07):143-147. Available from: <https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6307a2.htm>
278. Alshabani K, Haq A, Miyakawa R, Palla M, Soubani AO. Invasive pulmonary aspergillosis in patients with influenza infection: report of two cases and systematic review of the literature. *Expert Rev Respir Med.* 2015; 9(1):89-96. <https://doi.org/10.1586/17476348.2015.996132> PMID: [25547335](https://pubmed.ncbi.nlm.nih.gov/25547335/)

279. Karki R, Man SM, Malireddi RKS, Gurung P, Vogel P, Lamkanfi M, et al. Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against *Aspergillus* infection. *Cell Host Microbe*. 2016; 17(3):357-368. <https://doi.org/10.1016/j.chom.2015.01.006> PMID: [25704009](https://pubmed.ncbi.nlm.nih.gov/25704009/)
280. Zhang J, Hu MM, Shu HB, Li S. Death-associated protein kinase 1 is an IRF3/7-interacting protein that is involved in the cellular antiviral immune response. *Cell Mol Immunol*. 2014; 11(3):245-252. <https://doi.org/10.1038/cmi.2013.65> PMID: [24531619](https://pubmed.ncbi.nlm.nih.gov/24531619/)
281. Akoumianaki T, Chamilos G. DAPK1 Keeps the Peace in Antifungal Inflammation. *Cell Host Microbe*. 2016; 20(6):695-697. <https://doi.org/10.1016/j.chom.2016.11.008> PMID: [27978429](https://pubmed.ncbi.nlm.nih.gov/27978429/)
282. Rudd JM, Ashar HK, Chow VT, Teluguakula N. Lethal Synergism between Influenza and *Streptococcus pneumoniae*. *J Infect Pulm Dis*. 2016; 2(2). <https://doi.org/10.16966/2470-3176.114> PMID: [27981251](https://pubmed.ncbi.nlm.nih.gov/27981251/)
283. Wolf AI, Strauman MC, Mozdzanowska K, Whittle JR, Williams KL, Sharpe AH, et al. Co-infection with *Streptococcus pneumoniae* Modulates the B Cell Response to Influenza Virus. *J Virol*. 2014; 88(20):11995-12005. <https://doi.org/10.1128/JVII.01833-14> PMID: [25100838](https://pubmed.ncbi.nlm.nih.gov/25100838/)
284. Rodriguez AE. Enhanced Production of Pro-IL-1 $\beta$  Contributes to Immunopathology during the Co-infection of Influenza a Virus and *Streptococcus pneumoniae*. MSU Graduate Theses. 2018; 3242:1-96. Available from: <https://bearworks.missouristate.edu/cgi/viewcontent.cgi?article=4259&context=theses>
285. Rodriguez AE, Bogart C, Gilbert CM, et al. Enhanced IL-1 $\beta$  production is mediated by TLR2-MYD88-NLRP3 signaling axis during co-infection with influenza A virus and *Streptococcus pneumoniae*. *PLoS One*. 2019; 14(2):e0212236. <https://doi.org/10.1371/journal.pone.0212236> PMID: [30794604](https://pubmed.ncbi.nlm.nih.gov/30794604/)
286. Paulussen C, Hallsworth JE, Alvarez-Perez S, Nierman WC, Hamill PG, Blain D, et al. Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. *Microb Biotechnol*. 2017; 10(2):296-322. <https://doi.org/10.1111/1751-7915.12367> PMID: [27273822](https://pubmed.ncbi.nlm.nih.gov/27273822/)
287. Lupfer CR, Anand PK, Liu Z, Stokes KL, Vogel P, Lamkanfi M, et al. Reactive Oxygen Species Regulate Caspase-11 Expression and Activation of the Non-canonical NLRP3 Inflammasome during Enteric Pathogen Infection. *PLoS Pathog*. 2014; 10(9):e1004410. <https://doi.org/10.1371/journal.ppat.1004410> PMID: [25254654](https://pubmed.ncbi.nlm.nih.gov/25254654/)
288. Lupfer CR, Kanneganti TD. The role of inflammasome modulation in virulence. *Virulence*. 2012; 3(3):262-270. <https://doi.org/10.4161/viru.20266> PMID: [22546900](https://pubmed.ncbi.nlm.nih.gov/22546900/)

289. Chung D, Haas H, Cramer RA. Coordination of hypoxia adaptation and iron homeostasis in human pathogenic fungi. *Front Microbiol.* 2012; 3:381.  
<https://doi.org/10.3389/fmicb.2012.00381> PMID: [23133438](https://pubmed.ncbi.nlm.nih.gov/23133438/)
290. Grahl N, Cramer Jr RA. Regulation of Hypoxia Adaptation: An Overlooked Virulence Attribute of Pathogenic Fungi? *Med Mycol.* 2010; 48(1):1-15.  
<https://doi.org/10.3109/13693780902947342> PMID: [19462332](https://pubmed.ncbi.nlm.nih.gov/19462332/)

Table 1. MHN Buffer Recipe.

Ingredients	Amount
1 M MgSO <sub>4</sub>	24.65 g
50 mM HEPES	11.915 g
150 mM NaCl	0.876 g
Double distilled H <sub>2</sub> O	Fill to 100 mL

Table 2. 1X Plaque Assay Medium Recipe.

Ingredients	Amount
2X MEM medium	10.00 mL
Molecular grade H <sub>2</sub> O	10.00 mL

Table 3. 2X Plaque Assay Medium.

Ingredients	Amount
Molecular grade H <sub>2</sub> O	31.00 mL
10X MEM	10.00 mL
100X Glutamine	1.00 mL
7.5% Bovine Serum Albumin (BSA)	4.00 mL
Penicillin/Streptomycin (10,000 U/mL)	1.00 mL
Sodium Bicarbonate	3.00 mL

Table 4. Bone Marrow Differentiating Medium Recipe.

Ingredients	Amount
DMEM	300.00 mL
L-929 Medium	150.00 mL
Heat Inactivated FBS	50.00 mL
Penicillin/Streptomycin (10,000 U/mL)	5.00 mL
Non-Essential Amino Acids (NEAA)	5.00 mL

Table 5. 1X RIPA Lysis Buffer Recipe.

Ingredients	Final Concentration
NaCl	150 mM
EDTA, pH 8.0	5 mM
Tris, pH 8.0	50 mM
NP-40 (IGEPAL CA-630)	1.00 %
Sodium Deoxycholate	0.50 %
SDS	0.10%
Distilled Water	N/A
100X Halt Protease Phosphatase Inhibitor Cocktail (Thermo Scientific #78442)	1X

Table 6. 4X SDS Loading Dye Recipe.

Ingredients	Final Concentration
Tris-Cl, pH 6.8	200 mM
SDS	8.00% (w/v)
Bromophenol Blue	0.40 % (w/v)
Glycerol	40.00 %
$\beta$ -Mercaptoethanol	400 mM

Table 7. 10X Tris Buffered Saline (TBS).

Ingredients	Amount
Tris Base	24.50 g
Sodium Chloride	87.65 g
diH <sub>2</sub> O	Fill to 1,000 mL

Table 8. 1X Tris Buffered Saline Tween 20 (TBST).

Ingredients	Amount
10X TBS (made previously)	100.00 mL
diH <sub>2</sub> O	Fill to 1,000 mL
Tween 20	0.50 mL

Table 9. 1X Tris/Glycine/SDS (Running) Buffer.

Ingredients	Amount
10X Tris/Glycine/SDS (made previously)	100.00 mL
diH <sub>2</sub> O	Fill to 1,000 mL

Table 10. 1X Tris/Glycine (Transfer) Buffer.

Ingredients	Amount
diH <sub>2</sub> O	900.00 mL
10X Tris/Glycine (made previously)	100.00 mL
Methanol	200.00 mL

Table 11. Western Blot Primary Antibodies.

1° Ab	Catalog #	Manufacturer
Anti-caspase-1 (p20) (mouse)	AG-20B-0042-C100	Adipogene
Anti-β-Actin (rabbit)	D6A8	Cell Signaling Technologies
α-Tubulin (rabbit)	TU-02	Santa Cruz Biotechnology
Pro-IL-1β (rabbit)	D3H1Z	Cell Signaling Technologies
(Total) IκB-α (rabbit)	9242	Cell Signaling Technologies
(Phosphorylated) IκB-α, Ser32 (rabbit)	14D4	Cell Signaling Technologies
NLRP3 (rabbit)	D4D8T	Cell Signaling Technologies
Caspase-8 (mouse)	1C12	Cell Signaling Technologies

Table 12. Western Blot Secondary Antibodies.

2° Ab	Catalog #	Manufacturer
Anti-rabbit HRP	111-035-144	Jackson Immuno Research Laboratories
Anti-mouse HRP	HAF007	R&D Systems

Table 13. cDNA PCR Master Mix Recipe.

Ingredients	Amount (Kit w/o Inhibitor)
10X RT Buffer	2.00 µL
25X dNTP Mix (100mM)	0.8 µL
10X RT Random Primers	2.00 µL
Multiscribe™ Reverse Transcriptase	1.00 µL
Nuclease-free H <sub>2</sub> O	9.2 µL

Table 14. Quantitative Real Time-PCR Primer Sequences.

Specific Gene	Forward Primer	Reverse Primer
<i>β-Actin</i>	5' GGC TGT ATT CCC CTC CAT CG 3'	5' CCA GTT GTT AAC AAT CGG ATG A 3'
<i>Aim2</i>	5' GAT TCA AAG TGC AGG TGC GG 3'	5' TCT GAG GCT TAG CTT GAG GAC 3'
<i>Dapk1</i>	5' CCT GGG TCT TGA GGC AGA TA 3'	5' TCG CTA ATG TTT CTT GCT TGG 3'

Equation 1. Multiplicity of Infection for Influenza A virus (IAV) and *Aspergillus fumigatus*. The desired MOI of IAV to infect macrophages was 10 per macrophage. The desired MOI of *A. fumigatus* to infect macrophages was 1 per macrophage.

$$1 \text{ or } 10 \text{ MOI} = \frac{\# \text{ of pathogens}}{\# \text{ of cells}}$$

Equation 2. General solving for pathogens stock volume to add. The volume of pathogen stock to add depends upon the number of cells present per well multiplied by the desired (previously calculated) MOI and divided by the known concentration of the pathogen stock solution.

$$\text{Volume of pathogen stock to add} = \frac{\# \text{ of cells in well} \times \text{MOI}}{[\ ] \text{ of pathogen stock}}$$

Equation 3. Normalizing RNA concentrations. RNA concentrations were measured using a nanodrop and normalized using the following general calculation.

$$(C1)(V1) = (C2)(V2 - 19\mu\text{L})$$

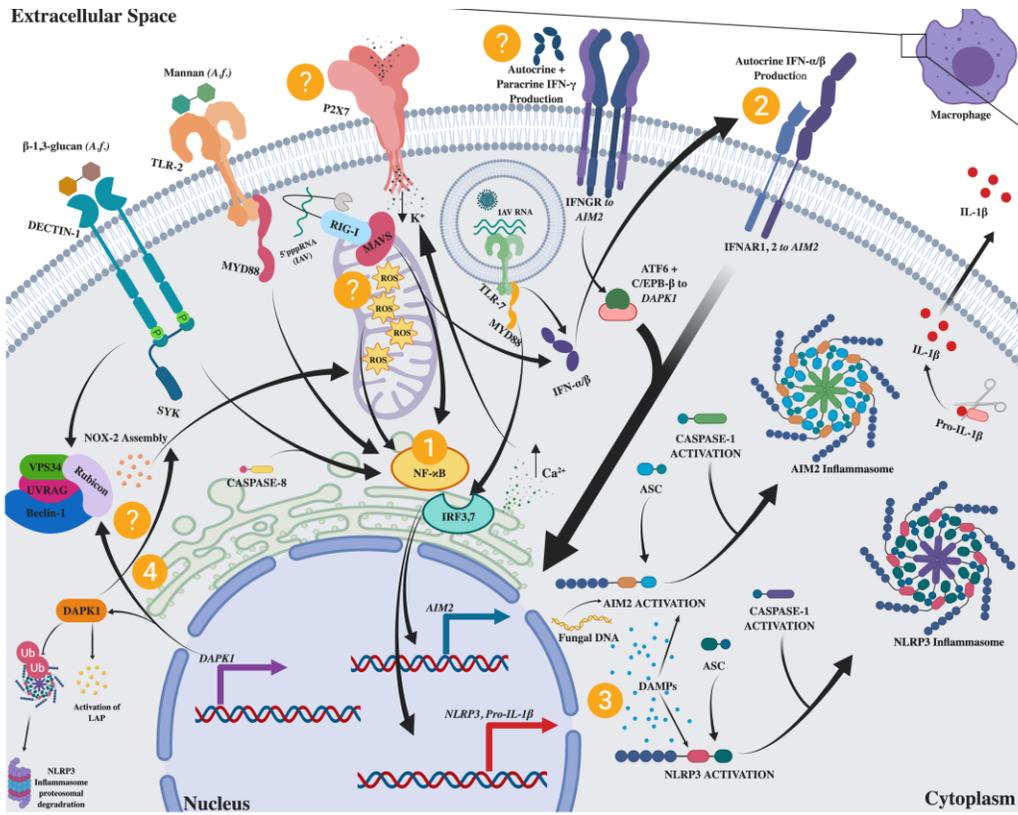


Figure 1. Proposed IAV and *A. fumigatus* immune signaling pathways. 1) Priming stage via NF- $\kappa$ B activation. 2) Autocrine IFN production. 3) Damaged associated molecular patterns (DAMPs) inflammasome sensing and activation. 4) LC3-associated phagocytosis (LAP) activation. ? = undetermined involvement. Visual created in BioRender.com.

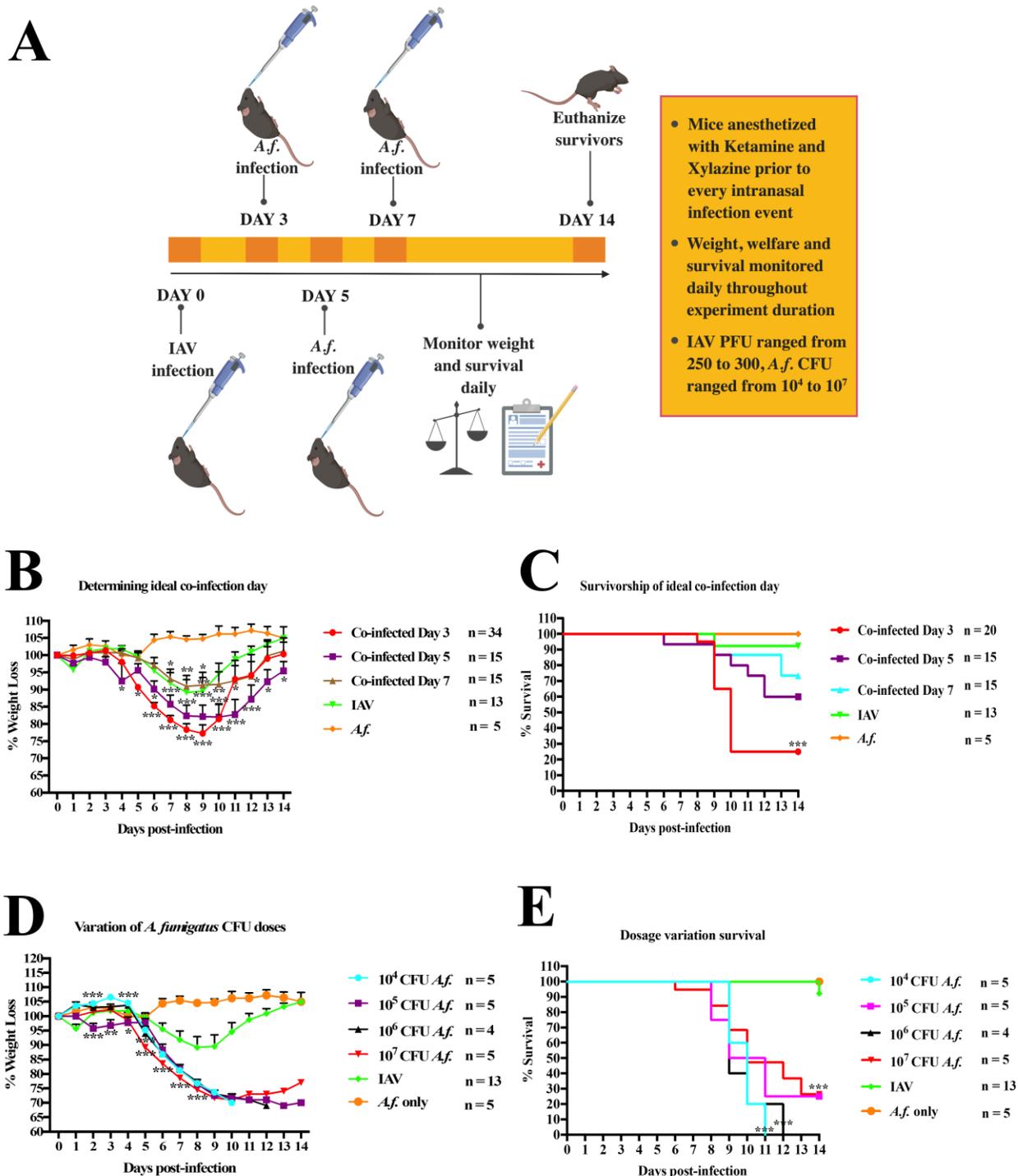


Figure 2. *In vivo* infection scheme, morbidity and mortality of infected WT mice. A) Outline of *in vivo* infection scheme created via BioRender.com. B) Weight loss in WT mice that were singly or co-infected on various days. C) Mortality in mice that were singly or co-infected on various days. D) Weight loss in WT mice infected with IAV alone, *A. fumigatus* at  $10^7$  CFU alone, or IAV co-infected with various CFU dosages of *A. fumigatus* on day 3. E) Mortality in mice infected with IAV alone, *A. fumigatus* at  $10^7$  alone, or IAV co-infected with various CFU

doses of *A. fumigatus* on day 3. B-E) Data were combined from 2 to 3 experiments, total n is indicated. Two-way ANOVA using Tukey's post hoc analysis was utilized for statistical comparison for weight loss and Kaplan-Meier Survival Plot with LogRank Test was utilized for survival data. p values: <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*)).

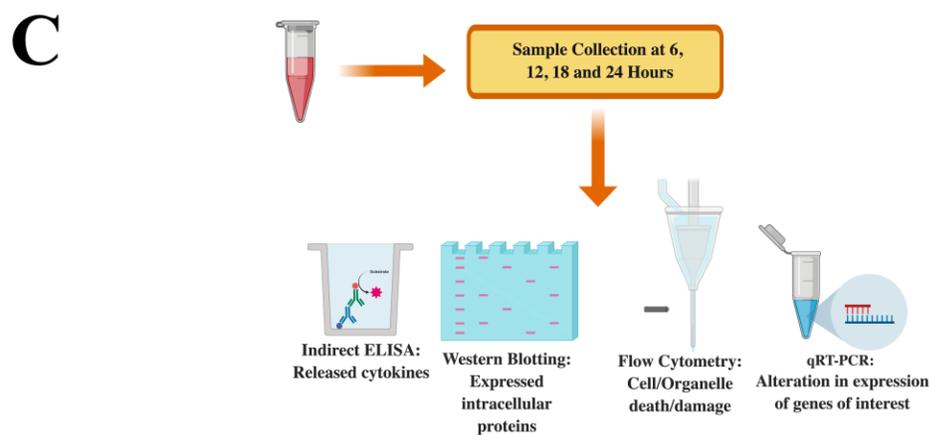
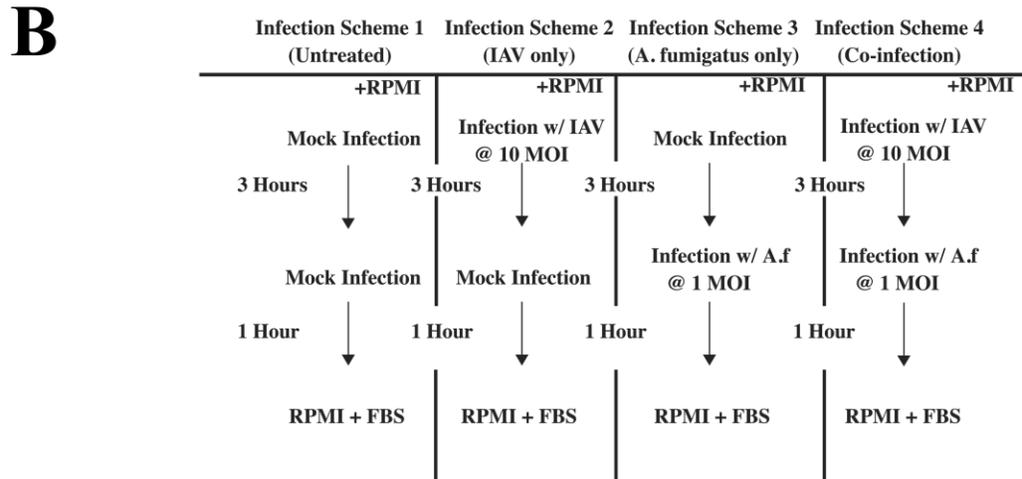
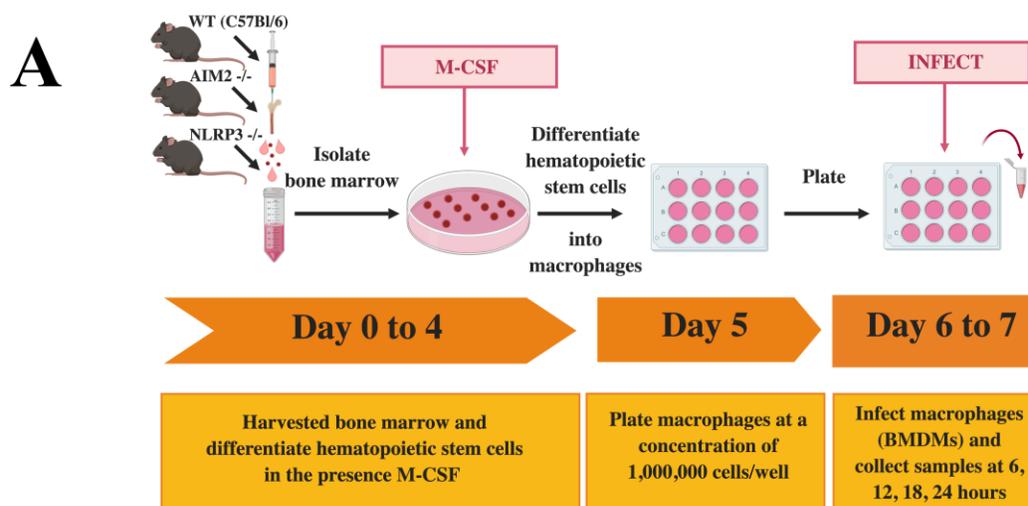


Figure 3. *In vitro* infection scheme. A) Process for generating macrophages from murine bone marrow for *in vitro* infections. B) *In vitro* infection schemes. C) Sample analysis workflow. All visuals created in BioRender.com.

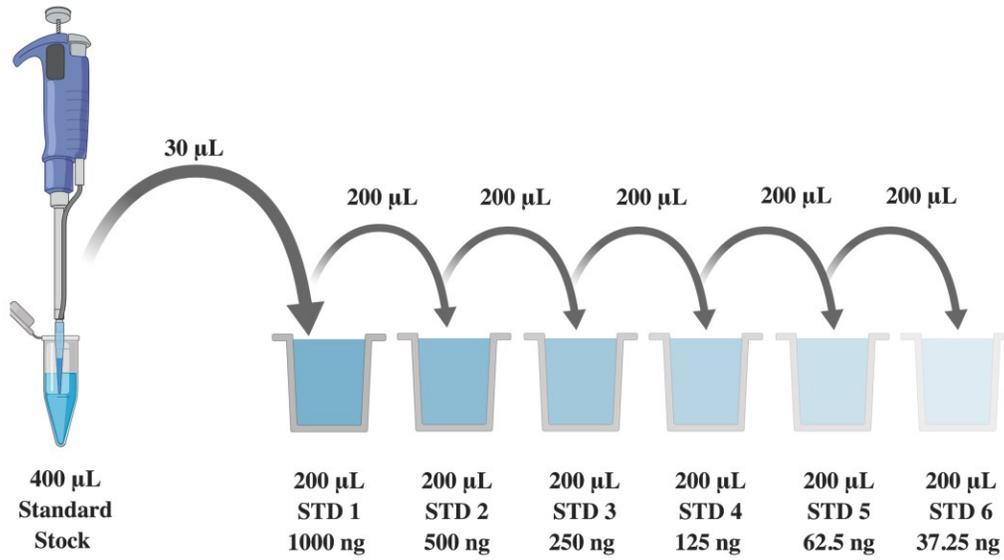


Figure 4. Generating an ELISA standard curve. Graphical representation via BioRender.com of the process of generating an ELISA standard curve via serial dilution.

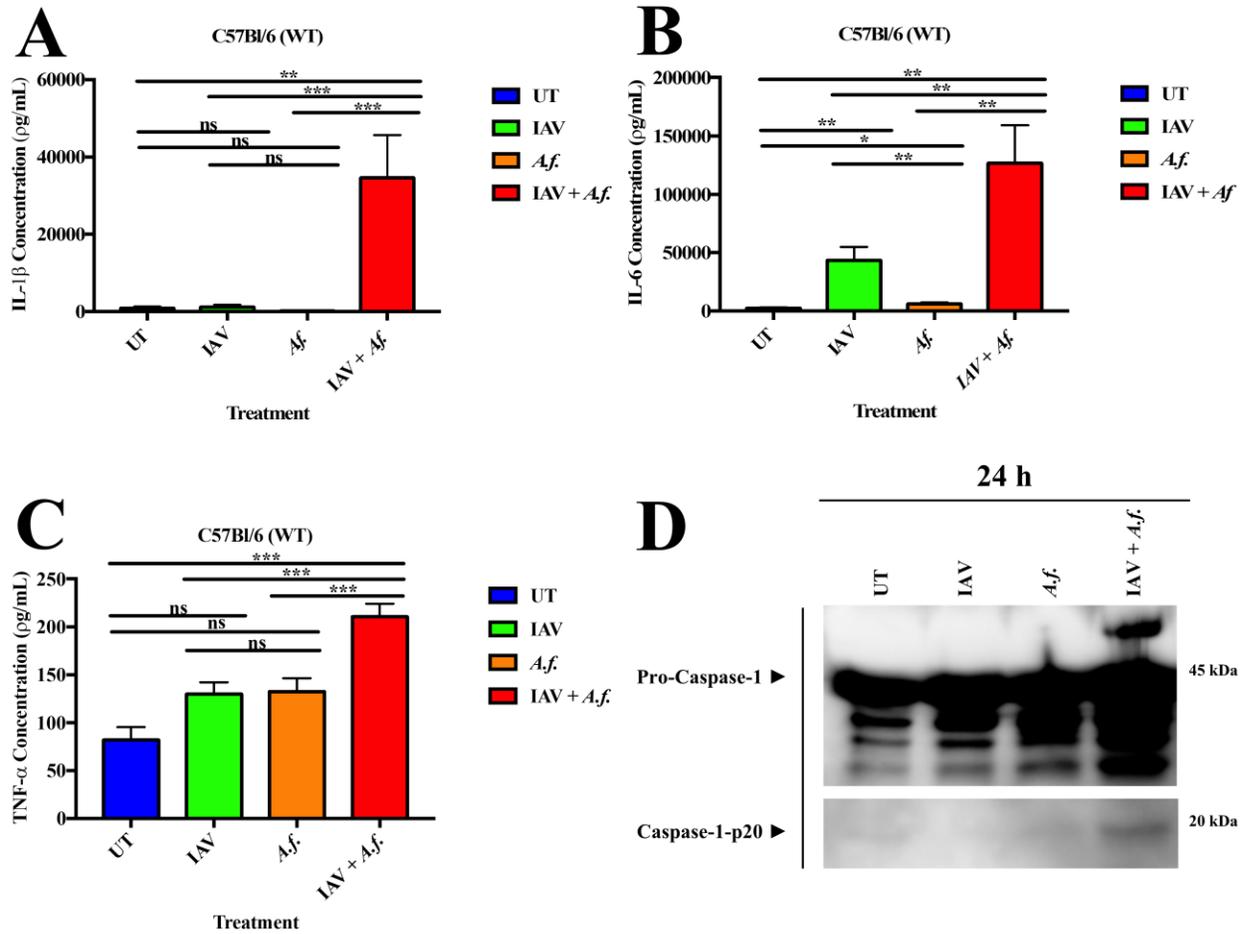


Figure 2. Co-infection with IAV and *A. fumigatus* results in overproduction of pro-inflammatory cytokines through enhanced caspase-1 activation. A-C) ELISAs were completed on supernatant from *in vitro* samples collected with one of the previously stated infection schemes. Data present 2-3 independent experiments using n=2 per experiment. One-way ANOVA using Tukey's post hoc analysis was utilized for statistical comparison. ns: not significant, p-values: <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*). D) Protein levels of pro-caspase-1 and active caspase-1-p20 were measured using western blot analysis from differentiated macrophages infected as indicated for 24-hours.

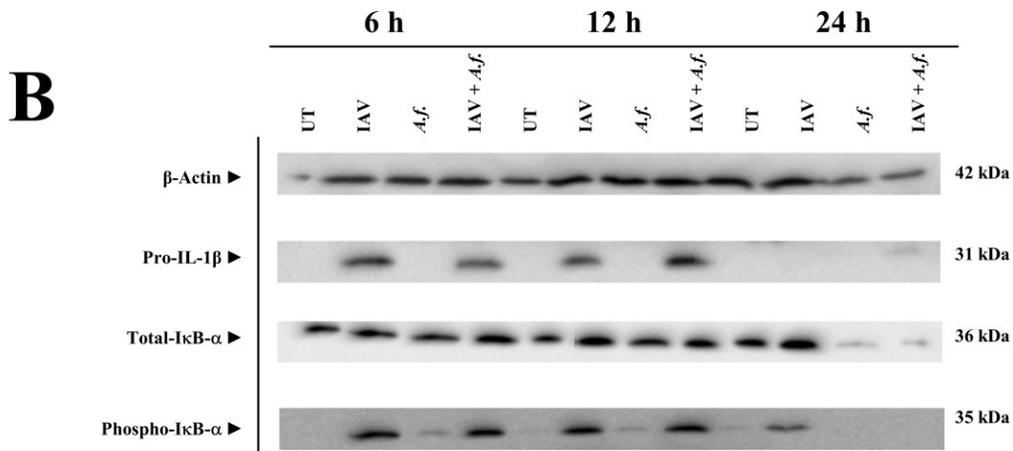
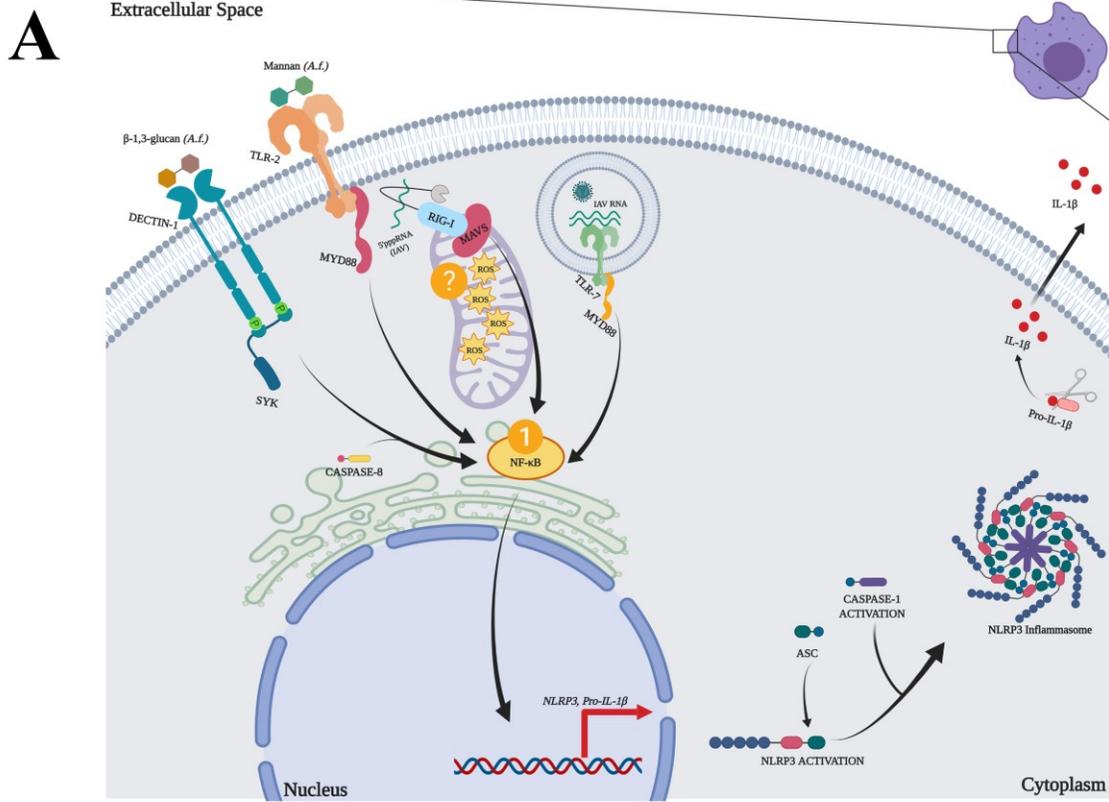


Figure 3. Overproduction of IL-1 $\beta$  is NF- $\kappa$ B and pro-IL-1 $\beta$  expression independent. A) Focused NF- $\kappa$ B activation pathways that could prime inflammasome activation created in BioRender.com. B) Protein levels of pro-IL-1 $\beta$ , total I $\kappa$ B- $\alpha$  and phosphorylated I $\kappa$ B- $\alpha$  were measured using western blot analysis from samples collected at 6-, 12-, or 24-hours after the indicated infections.  $\beta$ -Actin was used as a control.

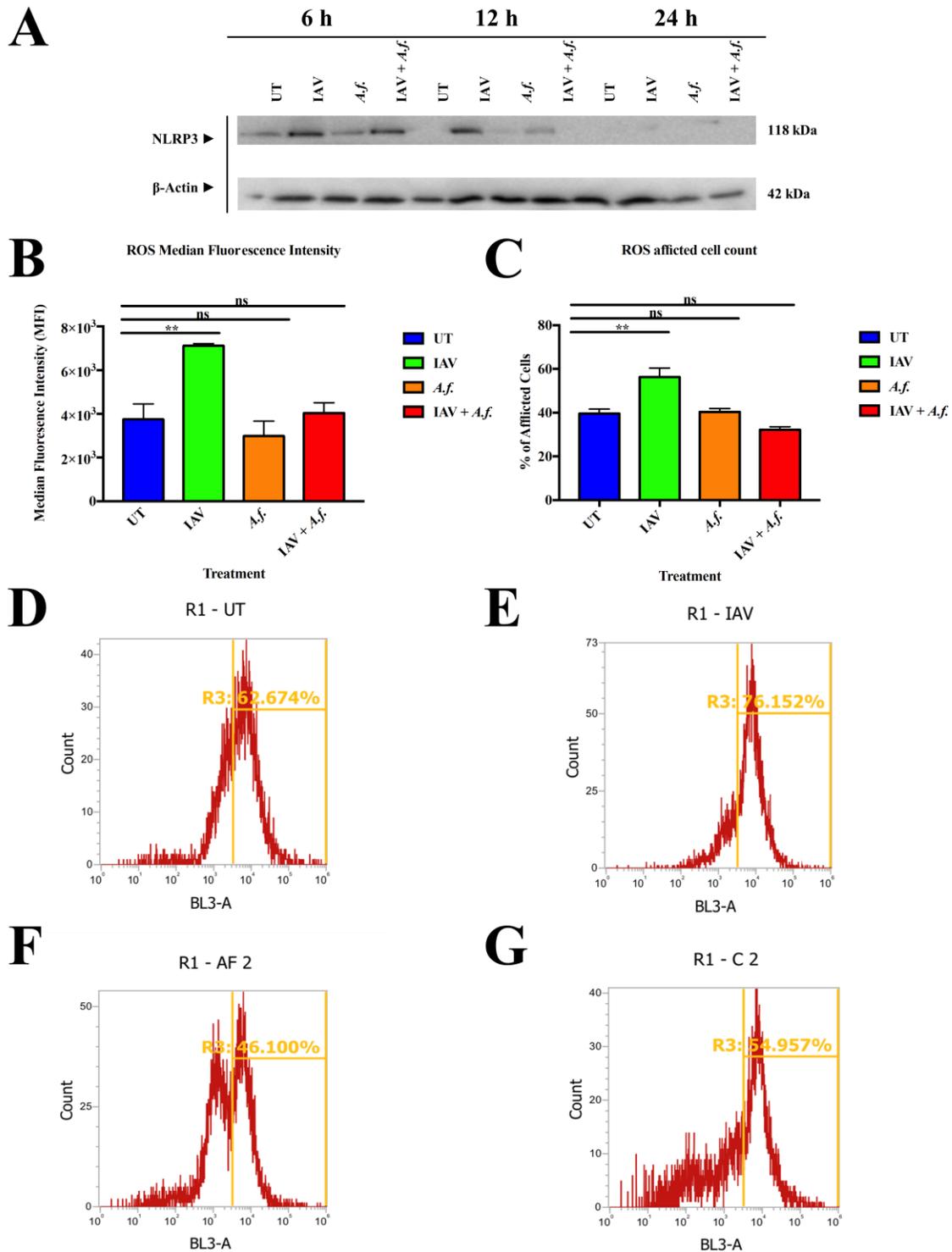


Figure 4. Negating ROS as a source of direct inflammasome activation. A) NLRP3 and  $\beta$ -Actin protein expression using western blot analysis. B-G) ROS median fluorescence intensity (MFI) and percentage of ROS afflicted cells were analyzed by flow cytometry. One-way ANOVA using Dunnett's multiple comparisons test. ns: not significant, p values:  $<0.05$  (\*),  $<0.01$  (\*\*),  $<0.001$  (\*\*\*)

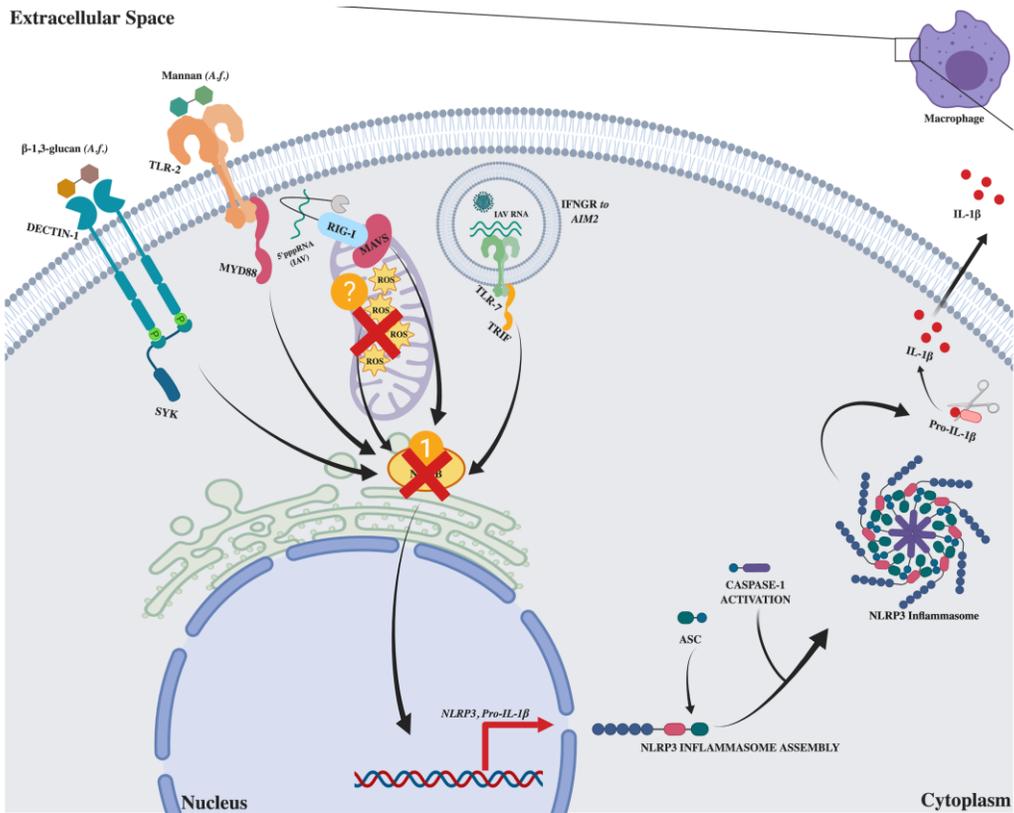


Figure 5. Negating viable agonists contributing to the culmination of caspase-1 activation through inflammasome complex assembly. Recall the complete proposed signaling pathways that could be involved in the enhanced expression of activated caspase-1 and overproduction of pro-inflammatory cytokines (Figure 1). Here, updated pathways via BioRender.com show that NF-κB dependent immune signaling does not contribute to the inflammasome activation seen at 24-hours in co-infection samples. The production of DAMPs through mtROS as a NLRP3 inflammasome activator has also been negated here.

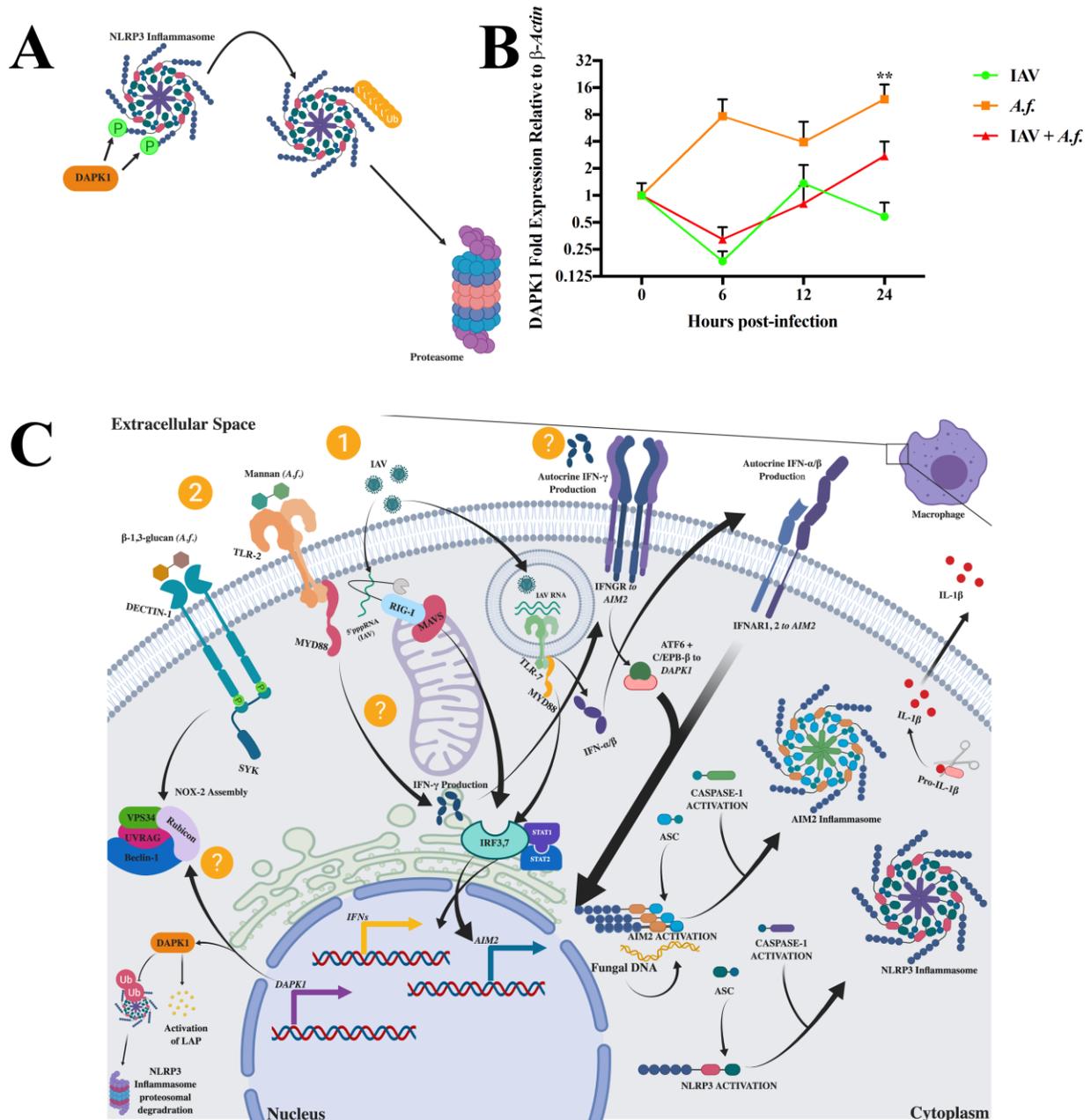


Figure 9. Expression of DAPK1 during IAV + *A. fumigatus* co-infections. A) Visual explaining DAPK1 and NLRP3 interaction. B) mRNA from macrophage samples at 6-, 12-, or 24-h.p.i. examined for DAPK1 gene expression by qRT-PCR. DAPK1 mRNA was normalized relative to  $\beta$ -Actin. One-way ANOVA using Tukey's post hoc analysis was used for statistical comparison. p values: <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*). C) Visual proposed involvement of DAPK1's role during this co-infection created via BioRender.com.

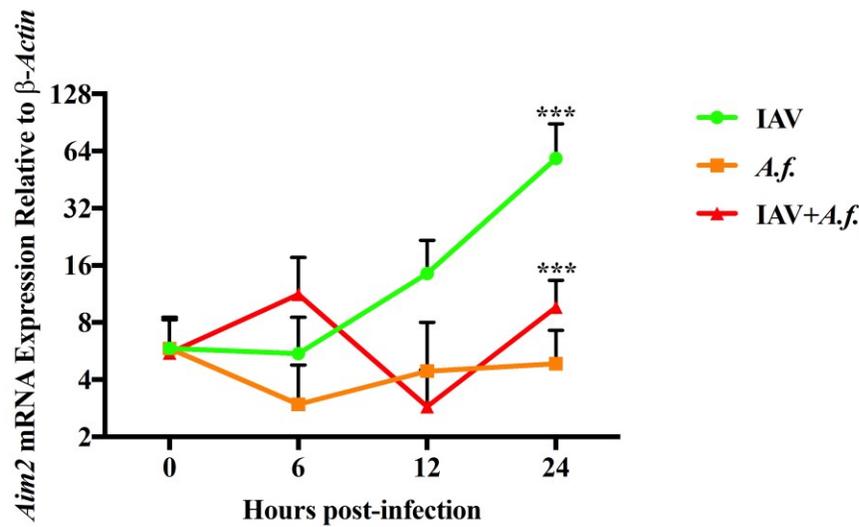
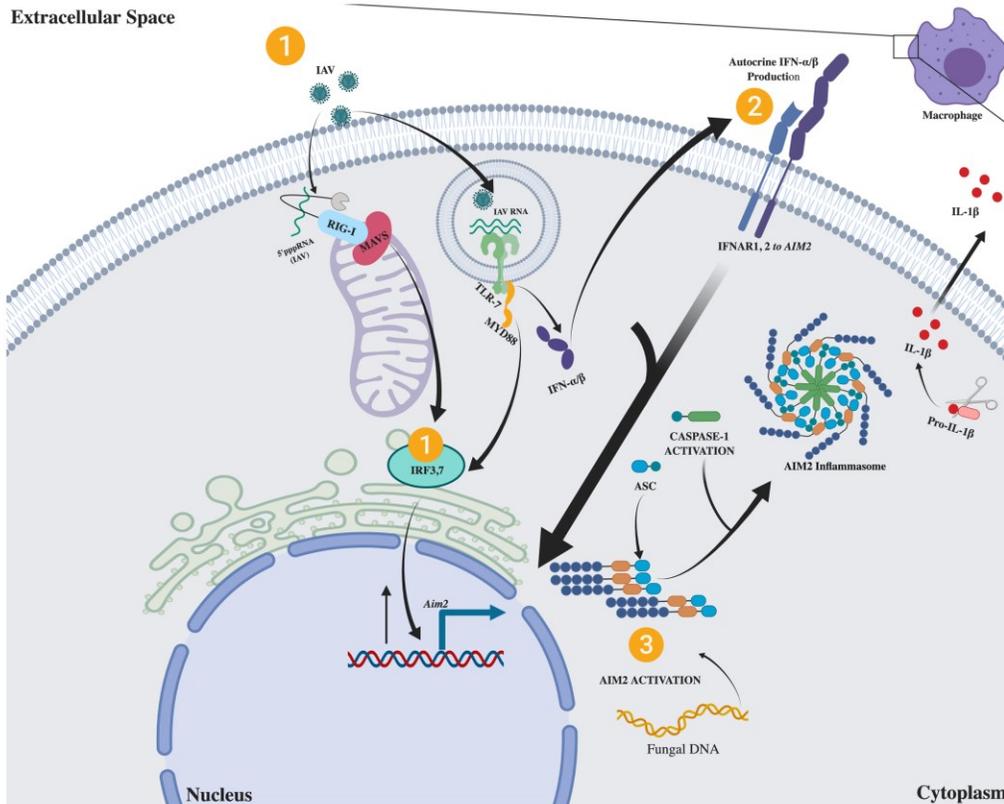
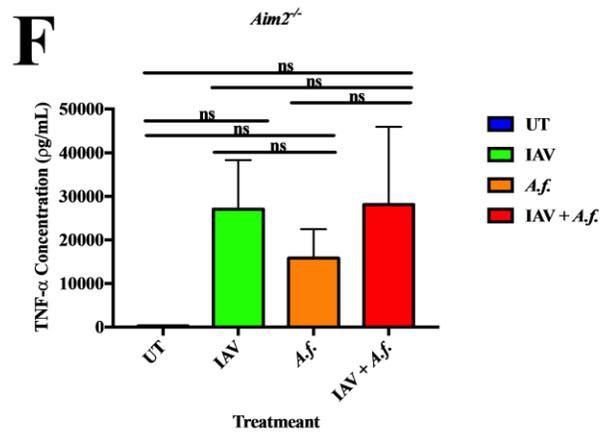
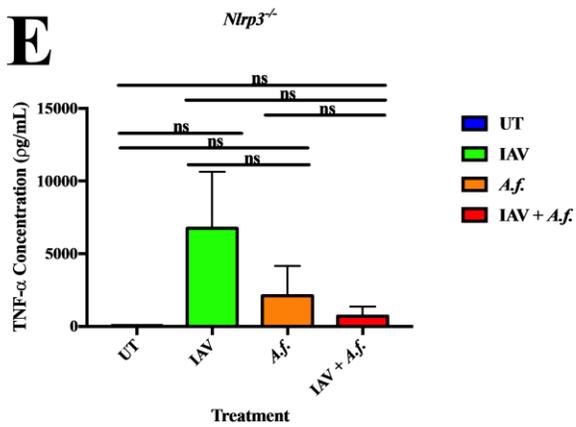
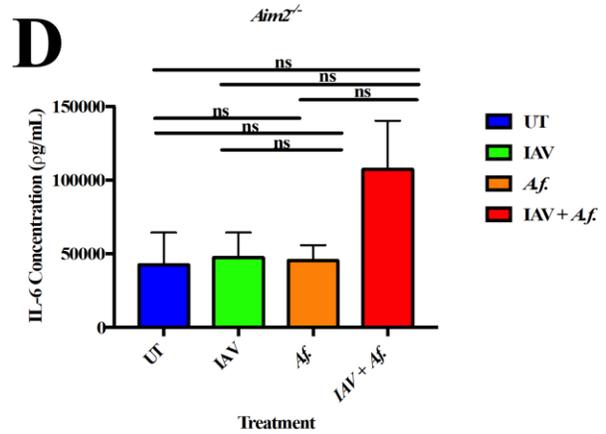
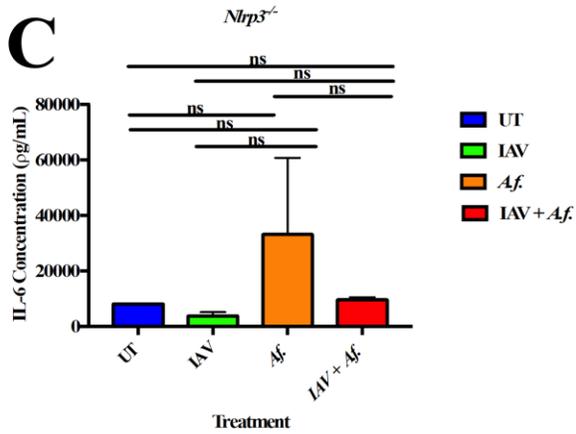
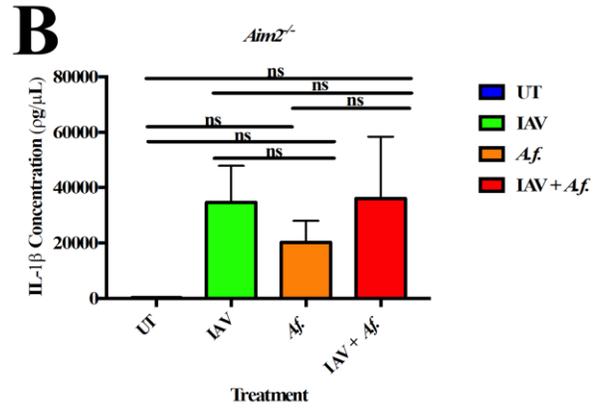
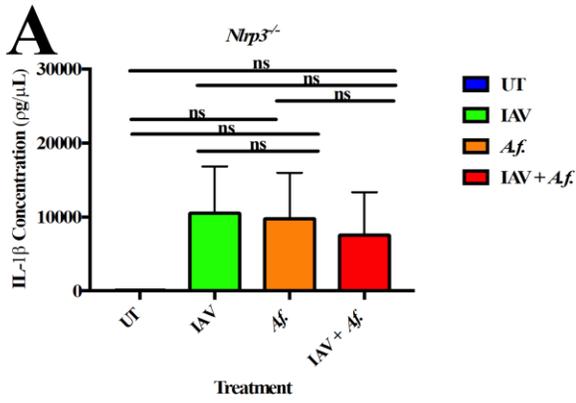
**A****B**

Figure 10. Co-infecting pathogenic agents synergistically induce AIM2 inflammasome activation. mRNA from differentiated macrophage samples collected at 6-, 12-, or 24-h.p.i. with the indicated pathogens examined for AIM2 gene expression by qRT-PCR. AIM2 mRNA was normalized relative to  $\beta$ -Actin. One-way ANOVA using Tukey's post hoc analysis used for statistical comparison. p values: <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*)). B) Visual of proposed involvement of AIM2 during this co-infection created via BioRender.com.



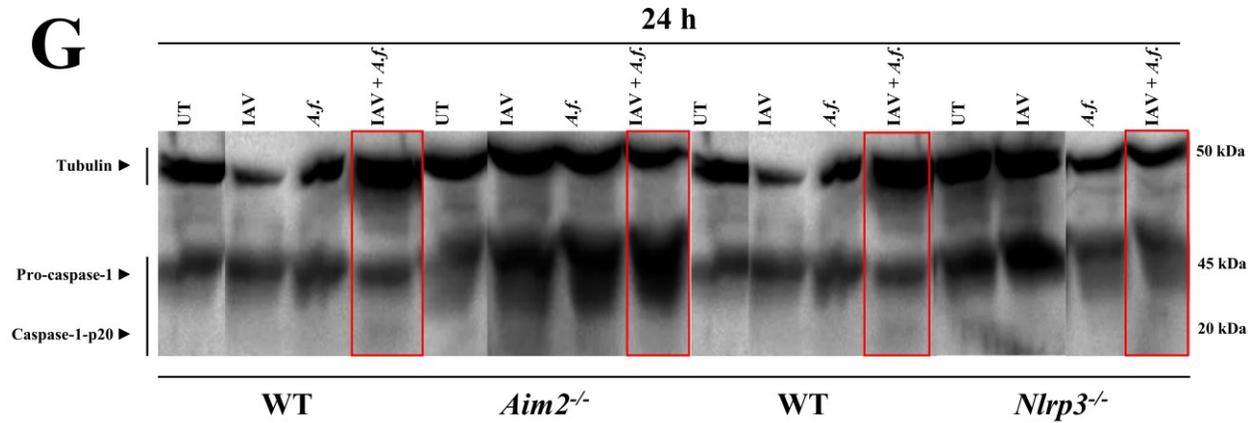


Figure 11. Preliminary *in vitro* experiments reiterate that NLRP3 and AIM2 inflammasomes independently reach similar objectives. A-F) Differentiated *Nlrp3*<sup>-/-</sup> and *Aim2*<sup>-/-</sup> knockout macrophage supernatants were collected from one of the previously stated infection schemes and analyzed via ELISA for IL-1 $\beta$ , IL-6 and TNF- $\alpha$  cytokines. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  cytokine concentrations were not significantly increased during co-infection compared to uninfected or singly infected samples. Data presented 2-3 independent experiments using n=2 per experiment. One-way ANOVA using Tukey's post hoc analysis was utilized for statistical comparison. ns: not significant, p-values: <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*). G) Protein levels of pro-caspase-1 and active caspase-1-p20 were measured using western blot analysis from differentiated macrophages of the indicated genotype infected for 24-hours. Tubulin was used as a control.

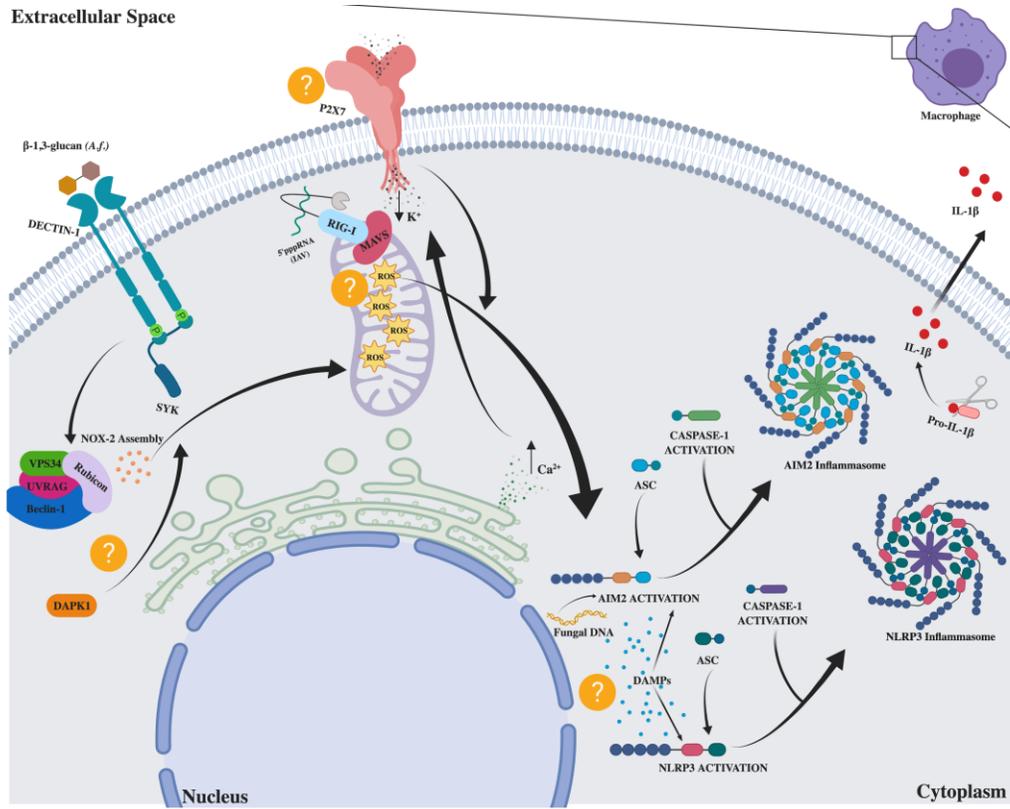


Figure 12. Possible molecular signaling mechanisms left to explore. Visual representation via BioRender.com of the possible molecular mechanisms to examine in the future based on previous research.

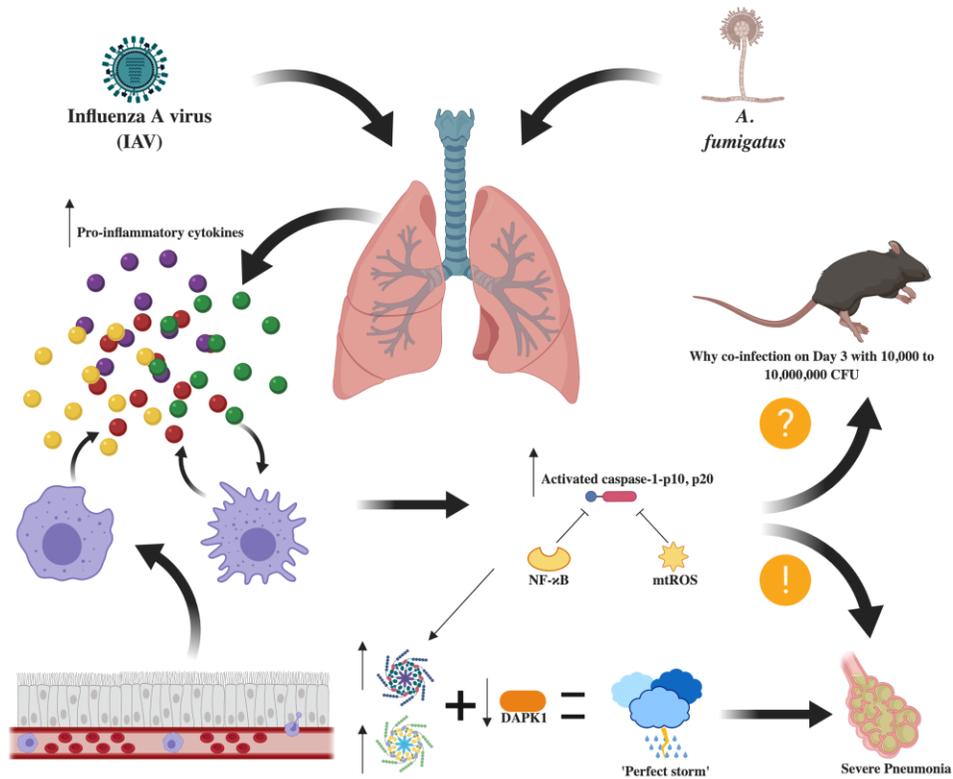


Figure 13. IAV and *A. fumigatus* co-infection and the pivotal role of activated caspase-1. Understanding of the molecular mechanisms of IAV and *A. fumigatus* co-infections were severely lacking prior to this thesis research. Through development of both *in vivo* and *in vitro* models, a foundational understanding of the inappropriate immune signaling involved. This inappropriate immune signaling can begin to effectively explain the resulting high percentages of morbidity and mortality reported with these co-infections. Hopefully this research will begin to increase the attention and lead to innovative treatments soon. Visual created in BioRender.com.

## APPENDICES

### APPENDIX A

**IACUC -2018-7**

Generated On July 26, 2019

#### Study Details

<b>IACUC #</b>	IACUC -2018-7
<b>Principal Investigator</b>	Christopher Lupfer
<b>Official Title</b>	Determining Factors Involved in Viral-Fungal Coinfections
<b>Short Title</b>	Viral-Fungal Coinfection
<b>Approval Date</b>	07/05/2018
<b>Expiration Date</b>	07/05/2021
<b>Status</b>	Approved
<b>Sponsors</b>	--
<b>Units</b>	--

#### Study History

Type	Review Type	Decision
Amendment	Administrative Review	Approved
Initial Application	Full Committee Review	Approved

#### Key Study Contacts

Name	Email	Role	Date Assigned
Christopher Bogart	cab5@live.missouristate.edu	Investigator	04/16/2019 8:23 PM
Christopher Lupfer	christopherlupfer@missouristate.edu	Principal Investigator	06/18/2018 3:20 PM
Christopher Lupfer	christopherlupfer@missouristate.edu	Primary Contact	06/18/2018 3:21 PM
Christopher Lupfer	christopherlupfer@missouristate.edu	Study Creator	06/18/2018 3:17 PM
Jessica Reel	jessica19@live.missouristate.edu	Investigator	04/16/2019 8:23 PM
Jordan Fleetwood	jordan987@live.missouristate.edu	Investigator	04/16/2019 8:23 PM
Meagan Rippee-Brooks	rippee417@live.missouristate.edu	Investigator	06/28/2018 2:17 PM

## Amendment

### 1. Amendment Title

This title should be a title that reflects the nature of changes to the submission (i.e., Personnel Addition Fall 2018)

Addition of student workers

### 2. Check applicable sections that have been modified

- Investigator Information
- Investigator Training
- Funding
- Submission Overview
- Submission Details
- Animal Use Justification
- Animal Acquisition and Care
- Procedural Overview
- Breeding Details
- Surgical Details
- Wildlife Details

### 3. Provide a brief overview of changes to the submission

*I.e., Investigator Information: I am adding personnel for the fall semester.*

*Procedural Overview: Animals were going to be purchased from Charles River Laboratories, but animals are not available so I am now asking to purchase animals from Jackson Laboratory.*

I need to add two students to the protocol.

## Vertebrate Animal Certification

### # Does the submission you are proposing involve the manipulation of live, vertebrate animals?

- Yes
- No

## Investigator Assurances

### # Statement of Investigator Assurances

1. I agree to provide information that is accurate and, to the best of my knowledge, conforms to all applicable Public Health Service (PHS), United States Department of Agriculture (USDA), and Missouri State University (MSU) policies on the care use of animals in research, testing, and teaching.
2. I agree not to proceed with any portion of this submission until I receive written approval from the MSU Institutional Animal Care and Use

Committee (IACUC).

3. I affirm that all procedures will be performed only by personnel trained in the humane care, handling, and use of animals and that all personnel will abide by the recommendations of MSU's Occupational Health and Safety Program (OHSP).
  4. I agree, following submission approval, to submit desired changes to the IACUC for review and approval prior to implementation of changes.
  5. I agree to provide required documentation (i.e., licenses, permits, annual reviews, etc.) to the Office of Research Administration (ORA) throughout the submission approval period.
  6. I agree to allow inspection of animal activities (including facilities and/or laboratories) by the Attending Veterinarian, ORA staff, and IACUC.
  7. I agree to comply promptly if informed of policy violations.
- By checking this box, all personnel associated with this submission understand and agree to the Statement of Investigator Assurances.

**Investigator Information**

- # If you can not locate a person using the "Find" button, please request access at [Cayuse Logon Request](#).  
For additional help, email [IACUC@missourisate.edu](mailto:IACUC@missourisate.edu).

# **Principal Investigator**

Name	Email
Christopher Lupfer	christopherlupfer@missouristate.edu

- # **Co-Principal Investigator(s)**  
*Co-PI(s) will be required to certify the submission (in addition to the PI). This person will also be included on all correspondence related to this project.*

Name	Email
--	

- # **Primary study contact(s)**  
*This person(s), in addition to the PI and Co-PI, will be included on all correspondence related to this project.*

Name	Email
Christopher Lupfer	christopherlupfer@missouristate.edu

# **Other Investigator(s)**

Name	Email
Jordan Fleetwood	jordan987@live.missouristate.edu
Jessica Reel	jessica19@live.missouristate.edu
Christopher Bogart	cab5@live.missouristate.edu
Meagan Rippee-Brooks	riptide417@live.missouristate.edu

**Investigator Training**

# To access online training documents, please go to [CITI Training](#).

# **General Training**

*Attach the general training (i.e., Researcher - Agricultural, Researcher - Biomedical, Researcher - Wildlife) certificate for all investigators listed.*

Filename	Uploaded By	Date Uploaded
Reel - Researcher Biomedical.pdf	Christopher Lupfer	04/09/2019 7:22 PM
Fleetwood - Researcher Biomedical.pdf	Christopher Lupfer	04/09/2019 7:22 PM

# **Species-Specific Training**

*Attach species-specific training (i.e., Working with Mice) certificate(s) for all investigators listed.*

Filename	Uploaded By	Date Uploaded
Reel- Working with Mice.pdf	Christopher Lupfer	04/09/2019 7:23 PM
Fleetwood- Working with Mice.pdf	Christopher Lupfer	04/09/2019 7:23 PM

# **Survival Surgery Training**

*Attach the survival surgery training (i.e., Reducing Pain and Distress in Laboratory Mice and Rats) certificate for all investigators listed.*

**NOTE:** At this time, only personnel involved with rodents that will undergo surgical procedures or a procedure that will cause more than momentary/slight pain and distress are required to take an online training for survival surgery.

Filename	Uploaded By	Date Uploaded
Reel - Reducing Pain and Distress in Laboratory Mice and Rats.pdf	Christopher Lupfer	04/09/2019 7:23 PM
Fleetwood - Reducing Pain and Distress in Laboratory Mice and Rats.pdf	Christopher Lupfer	04/09/2019 7:23 PM

# **If applicable, supporting training documentation may be attached.**  
*I.e., Hands-on training (document or video), another training system certificate, etc.*

Filename	Uploaded By	Date Uploaded
--		

### Funding

**1. Are submission activities funded?**

- No  
 Yes  
 Anticipated (this project is being submitted for funding, but has not yet been awarded)

**1A. Funding source(s).**

NIH

**1B. Funding source(s) deadline.**

02/25/2019 6:00 AM

### Submission Overview

**1. Choose the class of submission.**

- Agricultural  
 Biomedical  
 Wildlife

**2. Choose the type of submission.**

- Research  
 Teaching

**2A. Choose the type of research submission activity.**

*Note: You may choose both (i.e., you are conducting an activity where you manipulate a pregnant animal and/or study the progeny).*

- Breeding  
 Experimental

**3. Is this submission a continuation?**

*Note: A previously approved submission has expired and/or has been terminated and you would like to continue similar work.*

- Yes  
 No

### Submission Details

**1. What potential benefits (i.e., to society, education, or animals) may result from this research submission?**

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]. 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].

Despite the complexity of coinfections, there are key players which, if better understood, would provide scientists and medical practitioners the knowledge needed to design and implement the most effective treatment options. Growth and dissemination of the pathogens to the lower airways plays a clear role in disease progression and treatment with antiviral and antibacterial drugs provides some improvement in disease outcomes. However, an inappropriate immune response contributes significantly to the pathogenesis of these coinfections too. Inhibition of Interferon- $\gamma$  actually results in reduced bacterial numbers in the lungs and less weight loss in conjunction with antibiotic treatment [10]. Our own research has elucidated the regulation and function of the inflammasome and the cytokine Interleukin-1 $\beta$  (IL-1 $\beta$ ) during coinfection of mice with IAV and *S. pneumoniae*. We discovered that IL-1 $\beta$  contributes to lung pathology and that lowering IL-1 $\beta$  levels results in improved recovery time in mice. These results point to alterations in the immune system as major players in the pathogenesis of coinfections and that absolute pathogen titers are not the only cause of lung damage in this disease.

**2. Research submission goals and objectives.**

*Explanation should be in terms comprehensible to a non-scientist and, ideally, understandable to a high school senior.*

Our continuing research will examine additional immune signaling pathways involved in lung inflammation during coinfections as well as examine the importance of viral-fungal coinfections in mice. Specific Aims:

1: Examine the immune signaling pathways active in non-bacterial coinfections.

Coinfections in general are highly understudied, but very little is known concerning viral-fungal coinfection. Therefore, in this aim, we will examine the immune signaling pathways involved during the

coinfection of influenza A virus and *Aspergillus fumigatus*.

2: Develop an *in vivo* model for viral fungal coinfection in mice.

No animal model exists for studying the coinfection of IAV and *Aspergillus fumigatus*, although many case reports indicate such coinfections do occur in clinical patients [19]. These clinical reports may also help us develop an animal model, as they suggest that certain strains of IAV have a greater propensity to facilitate fungal coinfection (REF). Also, the use of corticosteroids may predispose patients to

secondary fungal infection (REF). Thus, we will examine all such factors as we seek to develop a mouse model of IAV-*Aspergillus fumigatus* coinfection.

**3. Provide a concise overview of the experimental design (including manipulations and treatments).**

*Explain in terms comprehensible to a non-scientist and, ideally, understandable to a high school senior.*

Bone marrow isolation:

For the isolation of bone marrow, 6-12 week old mice will be euthanized by CO2 asphyxiation and cervical dislocation. Subsequently, tibia and femurs will be removed and bone marrow flushed from bones.

Infection:

There is currently no animal model for the coinfection of IAV and *A. fumigatus*. Therefore, we will initially test several viruses and time points for coinfection to determine the one that is most effective. In brief, 5 WT C57BL/6j mice per group will be anesthetized using 80mg/kg ketamine and 8mg/kg xylazine administered via intraperitoneal injection. Groups of mice will be infected in one of 11 ways:

- (1) 125 PFU of influenza A/PR/8/34 H1N1 alone
- (2) 107 CFU of *A. fumigatus* alone
- (3-6) 125 PFU of influenza A/PR/8/34 H1N1 and then coinfecting on either day 3, 5, 7 or 9 with 107 CFU of *A. fumigatus*
- (7) 125 PFU of influenza A/California/04/09 H1N1 alone
- (8-11) 125 PFU of influenza A/California/04/09 H1N1 and then coinfecting on either day 3, 5, 7 or 9 with 107 CFU of *A. fumigatus*

Based on our previous experience with IAV infections and coinfections, severe disease should only be observed in coinfecting mice. During viral-bacterial coinfection, this typically occurs 24-48 hours after coinfection. However, a recent viral-fungal coinfection model using *Cryptococcus neoformans* found that mice began to succumb to infection about 4 days after coinfection [1]. Thus, all mice will be weighed and monitored daily for disease severity and animals will be euthanized when they reach >30% weight loss or become moribund to determine differences in survival for up to 21 days after the initial influenza A virus infection. We selected the influenza A/PR/8/34 H1N1 virus because it is known to predispose mice to coinfection with other pathogens (mainly bacterial) and is well mouse adapted. On the other hand, we selected the influenza A/California/04/09 H1N1 virus because it is a human clinical isolate that still replicates well in mice and several clinical case reports suggest this virus has a propensity to predispose human patients to fungal coinfection [2].

One potential difficulty we foresee is that mice are highly resistant to pulmonary infection with *A. fumigatus* alone. If our initial infection schemes are not successful, then we will repeat these experiments but administer corticosteroids beginning on day 3 after influenza A virus infection (when mice first display clinical signs of illness). We have selected corticosteroids as their use is a proposed risk factor for IAV-fungal coinfection [2].

Once we determine the virus, timing, and conditions of the coinfection that produces severe disease, we will then determine the overall weight loss and mortality (30% weight loss, or moribund) by infecting 10 mice per group with a single pathogen or coinfecting them and monitoring them for 21 days. We will also examine the immune response in 6 mice per group infected with a single pathogen versus coinfection (3 groups of mice) on day 1 and day 3 after coinfection. This will include euthanizing mice and collecting blood, lung, spleen, liver and brain to determine pathogen burden and cytokine/chemokine levels. These tissues will also be examined for cellular infiltration and lung pathology. Finally, RNAseq will be performed to examine the overall immune signature of gene expression.

- 1. van de Veerdonk F.L., et al., Influenza-associated Aspergillosis in Critically Ill Patients." *American Journal of Respiratory and Critical Care Medicine*, 196(4), pp. 524-527
- 2. Lat A, et al., Invasive aspergillosis after pandemic (H1N1) 2009. *Emerg Infect Dis*. 16(6):971-3.

# **If applicable, supporting materials to clarify methods and/or techniques described in the submission design may be attached.**

*I.e., Graphs, images, videos, etc.*

Filename	Uploaded By	Date Uploaded
--		

# **If applicable, supporting documentation such as consent forms, surveys, etc may be attached.**

Filename	Uploaded By	Date Uploaded
--		

**Animal Use Justification**

- 1. For each animal requested, assign an approximate number and pain category. Please also consider the definition of distress when assigning animals to pain categories.**

*Pain categories are based on the USDA Criteria*

**Category B** - Animals are those that are being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but have not yet been used for such purposes, however minor.

**Category C** - Animals are those that are subjected to procedures that involve no pain or distress, or procedures that would not require the use of pain-relieving drugs. (i.e. animal behavior or routine injections and blood samples).

**Category D** - Animals are those subjected to potentially painful procedures for which anesthetics, analgesics, or tranquilizers will be used. (i.e. surgery with appropriate anesthesia and postoperative analgesia).

**Category E** - Animals are those subjected to painful or stressful procedures without the use of anesthetics, analgesics, or tranquilizers. (i.e. Lethal dose studies or pain studies that do not allow pain-relieving agents).

Distress

An aversive state in which an animal fails to cope or adjust to various stressors with which it is presented. Distress does not require that the stressor induce an immediate and observable pathologic or behavioral alteration; both the duration and intensity of the state are important considerations when trying to prioritize attention to and treatment of animal distress.

Animal	Approximate Number	Pain Category				
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129S6-Nlrp3tm1Bhk/j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129S6-Nlrp3tm1Bhk/j	Mouse	100	<input type="radio"/> B <input type="radio"/> C <input type="radio"/> D <input checked="" type="radio"/> E
Name	Type					
B6.129S6-Nlrp3tm1Bhk/j	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.12P2-Aim2Gt(CSG445)Byg/j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.12P2-Aim2Gt(CSG445)Byg/j	Mouse	100	<input type="radio"/> B <input type="radio"/> C <input type="radio"/> D <input checked="" type="radio"/> E
Name	Type					
B6.12P2-Aim2Gt(CSG445)Byg/j	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>C57BL/6j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	C57BL/6j	Mouse	100	<input type="radio"/> B <input type="radio"/> C <input type="radio"/> D <input checked="" type="radio"/> E
Name	Type					
C57BL/6j	Mouse					

- 1A. Provide scientific justification for Pain Category E.**

The proposed research will examine the morbidity, mortality, and immune response to viral and fungal coinfections. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response. Therefore, no such drugs can be employed in this protocol. We have incorporated the following humane end points. Any animal that loses greater than 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 in those animals. Finally, corticosteroid drug doses, if needed for predisposing the mouse to secondary infection, will be based upon previously published research to avoid toxicity.

**2. For each animal requested, explain features (i.e., anatomic, physiologic, etc.) that make it a desirable model for this submission.**

The purpose of this study is to examine the immune response to infectious pathogens. In immunology, the mouse is the preferred species as there is a wealth of 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. First, mice are easy to handle, house and 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 such as 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*<sup>-/-</sup>, *Nlrp3*<sup>-/-</sup> and more. Finally, the mouse has already been established as a model for the study of multiple infectious diseases of human importance.

**3. Explain why the number of animals requested is warranted.**

*If possible, provide a statistical power justification of sample size. For complex designs, including a flow chart or table of group size, time frame, and other relevant information may be very useful. Consider the 3 R's:*

- **Replacement** - replacing animals with non-animals models (i.e., computer models, in vitro assays, or cell culture, etc.).
- **Reduction** - reducing animal numbers (i.e., pilot study to estimate variability, proper statistical analysis, performing experiments simultaneously, etc.).
- **Refinement** - refining procedures to reduce pain and distress in animals (i.e., new anesthetics/analgesics, pain-relieving drugs, etc.).

All animal models using intranasal infection such as influenza A virus or *A. fumigatus* 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. For this amendment, we are only requesting additional mice in 3 groups WT, Nlrp3-/- and Aim2-/- mice for category E. From the initiation of this protocol, we have discovered that 300pfu of IAV followed 3 days later by coinfection with  $10^7$  CFU of *A. fumigatus* results in the most consistent and severe disease. Thus, all experiments from now on will be conducted with this timing and dose. From our in vitro experiments with bone marrow macrophages, we have also found that AIM2 and NLRP3 appear to be important for immune signaling and cytokine production. Thus, we are requesting an additional 276 WT mice, for category E. We are also requesting 276 Nlrp3-/- and 276 Aim2-/- mice for category E. We still plan to conduct the experiments in our initial proposal, but now propose to examine survival, morbidity, and immune responses in WT mice compared to Nlrp3-/- and Aim2-/- knockout mice to determine if these gene deletions contribute to the immune response of this coinfection. We will perform weight loss and survival experiments with 10 mice per group in 2 independent experiments (2 experiments x 3 groups (IAV, fungus, coinf.) x 10 mice = 60 mice per genotype). We will examine the immune response in all 3 genotypes of mice infected with a single pathogen versus coinfection (IAV, fungus, coinf.) on day 1, 2 and 4 after coinfection. We anticipate needing 6 mice per group at each collection point and the experiments will be repeated 2 independent times (2 experiments x 3 collection points x 3 infection groups x 6 mice = 108 mice per genotype). In order to obtain lungs for histopathological examination and RNAseq, we will also need to repeat this experiment three additional times requiring 108 more mice per genotype. In all, 276 mice for each genotype will be needed for this amendment. Mice used in this portion of the proposed research will be bred in house.

**4. Indicate the resources and information used to verify the submission does not unnecessarily duplicate previous animal activities and/or that less painful or stressful procedures are unavailable.**

- Literature database
- Expert consultation and/or personal experience
- Taxon-specific and/or research specific guidelines

**4A. Literature database information.**

Date of Search	Database Searched	Years Covered
05/24/2018 5:00 AM	Pubmed	1900-2018

**4B. Provide keywords used and a summary of search results.**

Keywords:

Influenza, Coinfection, Animals, inflammasome (Pubmed) 2 publications found  
Influenza, Coinfection, Animals, IL-1 (Pubmed) 4 publications found  
Influenza, Coinfection, Animals (Pubmed) 252 publications found  
Influenza, Aspergillus, Animals (Pubmed) 38 publications found

Summary of search results:

Many deaths attributed to influenza A virus (IAV) infection are the result of secondary infection with bacteria, but recently, physicians have reported an increase in the number of coinfections with fungal pathogens, especially *Aspergillus Fumigatus*. In fact, recent estimates suggest that more than 40-60% of all patients that acquire a coinfection with influenza A virus and *A. fumigatus* die from the infection (ref. 1). I propose to examine the immune response to this coinfection using both in vitro and in vivo models. We have already developed an in vitro model that has provided us with important preliminary data that warrants further examination in vivo. However, there is no in vivo model for this viral-fungal coinfection. Of the 296 publications found in my Pubmed search 0 publications were found where there was an in vivo model. Thus this model is greatly needed to understand how this coinfection between influenza A virus and *A. fumigatus* can happen and how the immune system responds so that we can begin to understand how to prevent it and treat it. Thus, the research proposed here 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 (ref. 2). 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 and *S. pneumoniae* coinfection and 1 publication has also used mice for developing a model of IAV and *Cryptococcus* fungal coinfection suggesting that mice can be used for viral-fungal coinfection model development (ref. 3).

References:

1. van de Veerdonk F.L., et al., Influenza-associated Aspergillosis in Critically Ill Patients." American Journal of Respiratory and Critical Care Medicine, 196(4), pp. 524-527
2. F. Menga, N. H. Wua, A. Nerlichb, G. Herrlera, P. Valentin-Weigandb and M. Seitzb. Dynamic Virus-Bacterium Interactions in a Porcine Precision-Cut Lung Slice Coinfection Model: Swine Influenza Virus Paves the Way for *Streptococcus suis* Infection in a Two-Step Process. Infect Immun, 2015. 83(7): p. 2806-15
3. Oliveira L.V.N., et al., Influenza A Virus as a Predisposing Factor for Cryptococcosis. Front Cell Infect Microbiol. 2017; 7: 419.

**4A. Provide a summary of any resources (i.e., other researchers, conferences attended, etc.) and/or personal experiences that contributed to the development of this submission.**

*Information should include: name of consultant(s) and background, specific date(s) of consultation, type of meetings, experiences of activities similar to the proposed submission, etc..*

There is no expert in this field as no mouse model has been developed. There are some clinical experts such as Dr. Frank van de Veerdonk, whom I have collaborated with in the past. Dr. Jon McCullers, Chair of the Department of Pediatrics, University of Tennessee Health Sciences Center is an expert in the field of influenza A virus and bacterial coinfections and has agreed to collaborate with me on this research.

**# If applicable, supporting documentation such as articles, conference abstracts, meeting minutes, etc. may be attached.**

Filename	Uploaded By	Date Uploaded
--		

**Animal Acquisition and Care**

**1. Animal Source(s).**

- Acquired from an organization/institution
- Departmental transfer
- Purchased from vendor
- Wild-caught

**1A. Specify organization/institution and provide a point of contact.**

Organization/Institution	Point of contact
Missouri State University	Vivarium

**1A. Specify vendor(s).**

Jackson Labs

**2. Animal Holding Location.**

**Dedicated Housing Area** - Facilities designed to provide housing specifically for animals and managed by dedicated staff (i.e., University Vivarium).

**Satellite Housing Area** - A location outside of a dedicated housing area that houses USDA covered species for greater than 12 hours or any other vertebrate species greater than 24 hours.

Animal		Holding Area			
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.12P2-Aim2Gt(CSG445)Byg/j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.12P2-Aim2Gt(CSG445)Byg/j	Mouse	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Name	Type				
B6.12P2-Aim2Gt(CSG445)Byg/j	Mouse				
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129S6-NlRp3tm1Bhk/j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129S6-NlRp3tm1Bhk/j	Mouse	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Name	Type				
B6.129S6-NlRp3tm1Bhk/j	Mouse				
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>C57BL/6j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	C57BL/6j	Mouse	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Name	Type				
C57BL/6j	Mouse				

**2A. Specify the dedicated holding area.**

*I.e., JVIC Vivarium, Temple Vivarium - Mammal Holding Room 2, Temple Vivarium - Aquatic Holding Room 2 & Aquatic Procedure Room, Darr - Horse Barn.*

Temple-mammal holding rooms 3 and 4.

**2B. Describe how animals will be monitored and maintained.**

*I.e., Animal staff will provide animal husbandry and care per approved standard operating procedures (SOP's), Animal staff will check animals, but researcher will perform husbandry, Researchers will provide animal husbandry and care per approved SOP's.*

Animal staff will provide animal husbandry and care per approved standard operating procedures (SOP's). However, when animals are infected, researchers will provide animal husbandry and care per approved SOP's.

**3. Animal ID method.**

*I.e., Tattooing, ear punch, pit tag.*

Tattooing

**4. Will animals have special housing requirements?**

*Housing that deviates from approved regulatory requirements and/or standard operating procedures (I.e., macroenvironment space is smaller than standard).*

- Yes  
 No

**5. Will animals have special care and maintenance requirements?**

*Care and maintenance that deviates from approved regulatory requirements and/or standard operating procedures (I.e., Macroenvironment not changed regularly, animals not checked daily, etc.).*

- Yes  
 No

**6. Will animals have special dietary requirements?**

*Dietary restrictions and/or dietary provisions that deviate from approved regulatory requirements and/or standard operating procedures (I.e., food an fluid regulation).*

- Yes  
 No

**6A. Please explain.**

Autoclaved food

**7. Explain provisions for handling sick animals.**

If animals are found moribund, Vivarium staff should contact the PI.

Note: When animals are infected per study design, they are expected to become sick and will be monitored at least daily for single infections and twice daily for coinfections. No additional contact is necessary for sick animals. However, if infected animals are moribund, please contact PI immediately and such animals will be euthanized.

**8. Explain provisions for handling deceased animals.**

If animals are found dead, Vivarium staff should contact the PI. Dead animals can be placed in the freezer if not infected. Dead animals that are infected should be left in the cage/room until the PI and/or submission personnel can retrieve them.

Note: infected carcasses will be taken to the PI's BSL-2 laboratory for storage.

## Procedural Overview

**1. Will animals undergo prolonged restraint?**

*Physical restraint is the use of manual or mechanical means to limit some or all of an animal's normal movement for the purpose of examination, collection of samples, drug administration, therapy, or experimental manipulation. Restraint devices should be suitable in size, design, and operation to minimize discomfort, pain, distress, and the potential for injury to the animal and personnel. Typically, animals may be restrained for brief periods, usually minutes, in many procedural applications.*

*Prolonged restraint would be anything that doesn't aim to achieve the above definition.*

- Yes  
 No

**2. Will animals undergo surgical procedures?**

*A surgical procedure is a procedure that penetrates a body cavity or that produces a permanent handicap.*

- Yes  
 No

**3. Describe criteria for removal of animals prior to the experimental endpoint.**

Animals that are intended for use for the collection of bone marrow that show any signs of sickness or developmental abnormalities will be euthanized immediately. 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.

**4. Will animals be euthanized?**

- Yes  
 No

**4A. Detail method(s) of euthanasia.**

CO2 followed by cervical dislocation/thoracotomy.

## Initial Application

### Vertebrate Animal Certification

- # **Does the submission you are proposing involve the manipulation of live, vertebrate animals?**
- Yes
- No

### Investigator Assurances

- # **Statement of Investigator Assurances**
1. I agree to provide information that is accurate and, to the best of my knowledge, conforms to all applicable Public Health Service (PHS), United States Department of Agriculture (USDA), and Missouri State University (MSU) policies on the care use of animals in research, testing, and teaching.
  2. I agree not to proceed with any portion of this submission until I receive written approval from the MSU Institutional Animal Care and Use Committee (IACUC).
  3. I affirm that all procedures will be performed only by personnel trained in the humane care, handling, and use of animals and that all personnel will abide by the recommendations of MSU's Occupational Health and Safety Program (OHSP).
  4. I agree, following submission approval, to submit desired changes to the IACUC for review and approval prior to implementation of changes.
  5. I agree to provide required documentation (i.e., licenses, permits, annual reviews, etc.) to the Office of Research Administration (ORA) throughout the submission approval period.
  6. I agree to allow inspection of animal activities (including facilities and/or laboratories) by the Attending Veterinarian, ORA staff, and IACUC.
  7. I agree to comply promptly if informed of policy violations.
- By checking this box, all personnel associated with this submission understand and agree to the Statement of Investigator Assurances.

### Investigator Information

- # **If you can not locate a person using the "Find" button, please request access at [Cayuse Logon Request](#).**
- For additional help, email [IACUC@missourisate.edu](mailto:IACUC@missourisate.edu).
- # **Principal Investigator**

Name	Email
Christopher Lupfer	christopherlupfer@missouristate.edu

# **Co-Principal Investigator(s)**

*Co-PI(s) will be required to certify the submission (in addition to the PI). This person will also be included on all correspondence related to this project.*

Name	Email
--	

# **Primary study contact(s)**

*This person(s), in addition to the PI and Co-PI, will be included on all correspondence related to this project.*

Name	Email
Christopher Lupfer	christopherlupfer@missouristate.edu

# **Other Investigator(s)**

Name	Email
Meagan Rippee-Brooks	ripee417@live.missouristate.edu

<b>Investigator Training</b>
------------------------------

# To access online training documents, please go to [CITI Training](#).

# **General Training**

*Attach the general training (i.e., Researcher - Agricultural, Researcher - Biomedical, Researcher - Wildlife) certificate for all investigators listed.*

Filename	Uploaded By	Date Uploaded
Lupfer - Working with IACUC.pdf	Allison Overmyer	06/22/2018 5:18 PM
Rippee-Brooks - Researcher Biomedical.pdf	Allison Overmyer	06/28/2018 5:54 PM

# **Species-Specific Training**

*Attach species-specific training (i.e., Working with Mice) certificate(s) for all investigators listed.*

Filename	Uploaded By	Date Uploaded
Rippee-Brooks - Working with Mice.pdf	Allison Overmyer	06/28/2018 5:53 PM

# **Survival Surgery Training**

*Attach the survival surgery training (i.e., Reducing Pain and Distress in Laboratory Mice and*

Rats) certificate for all investigators listed.

**NOTE:** At this time, only personnel involved with rodents that will undergo surgical procedures or a procedure that will cause more than momentary/slight pain and distress are required to take an online training for survival surgery.

Filename	Uploaded By	Date Uploaded
Lupfer - Reducing Pain and Distress in Laboratory Mice and Rats.pdf	Allison Overmyer	06/22/2018 5:19 PM
Rippee-Brooks - Reducing Pain and Distress in Laboratory Mice and Rats.pdf	Allison Overmyer	06/28/2018 5:53 PM

# **If applicable, supporting training documentation may be attached.**

*I.e., Hands-on training (document or video), another training system certificate, etc.*

Filename	Uploaded By	Date Uploaded
--		

**Funding**

**1. Are submission activities funded?**

- No
- Yes
- Anticipated (this project is being submitted for funding, but has not yet been awarded)

**1A. Funding source(s).**

- American Lung Association
- NIH

**1B. Funding source(s) deadline.**

12/21/2017 6:00 AM

**Submission Overview**

**1. Choose the class of submission.**

- Agricultural
- Biomedical
- Wildlife

**2. Choose the type of submission.**

- Research
- Teaching

**2A. Choose the type of research submission activity.**

*Note: You may choose both (i.e., you are conducting an activity where you manipulate a pregnant animal and/or study the progeny).*

- Breeding  
 Experimental

**3. Is this submission a continuation?**

*Note: A previously approved submission has expired and/or has been terminated and you would like to continue similar work.*

- Yes  
 No

**Submission Details**

**1. What potential benefits (i.e., to society, education, or animals) may result from this research submission?**

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]. 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].

Despite the complexity of coinfections, there are key players which, if better understood, would provide scientists and medical practitioners the knowledge needed to design and implement the most effective treatment options. Growth and dissemination of the pathogens to the lower airways plays a clear role in disease progression and treatment with antiviral and antibacterial drugs provides some improvement in disease outcomes. However, an inappropriate immune response contributes significantly to the pathogenesis of these coinfections too. Inhibition of Interferon-g actually results in reduced bacterial numbers in the lungs and less weight loss in conjunction with antibiotic treatment [10]. Our own research has elucidated the regulation and function of the inflammasome and the cytokine Interlukine-1 $\beta$  (IL-1 $\beta$ ) during coinfection of mice with IAV and *S. pneumoniae*. We discovered that IL-1 $\beta$  contributes to lung pathology and that lowering IL-1 $\beta$  levels results in improved recovery time in mice. **These results point to alterations in the immune system as major players in the pathogenesis of coinfections and that absolute pathogen titers are not the only cause of lung damage in this disease.**

**2. Research submission goals and objectives.**

*Explanation should be in terms comprehensible to a non-scientist and, ideally, understandable to a high school senior.*

Our continuing research will examine additional immune signaling pathways involved in lung inflammation during coinfections as well as examine the importance of viral-fungal coinfections in mice.

**Specific Aims:**

**1: Examine the immune signaling pathways active in non-bacterial coinfections.**

Coinfections in general are highly understudied, but very little is known concerning viral-fungal coinfection. Therefore, in this aim, we will examine the immune signaling pathways involved during the

coinfection of influenza A virus and *Aspergillus fumigatus*.

**2: Develop an *in vivo* model for viral fungal coinfection in mice.**

No animal model exists for studying the coinfection of IAV and *Aspergillus fumigatus*, although many case reports indicate such coinfections do occur in clinical patients [19]. These clinical reports may also help us develop an animal model, as they suggest that certain strains of IAV have a greater propensity to facilitate fungal coinfection (REF). Also, the use of corticosteroids may predispose patients to

secondary fungal infection (REF). Thus, we will examine all such factors as we seek to develop a mouse model of IAV-*Aspergillus fumigatus* coinfection.

**3. Provide a concise overview of the experimental design (including manipulations and treatments).**

*Explain in terms comprehensible to a non-scientist and, ideally, understandable to a high school senior.*

**Bone marrow isolation:**

For the isolation of bone marrow, 6-12 week old mice will be euthanized by CO<sub>2</sub> asphyxiation and cervical dislocation. Subsequently, tibia and femurs will be removed and bone marrow flushed from bones.

**Infection:**

There is currently no animal model for the coinfection of IAV and *A. fumigatus*. Therefore, we will initially test several viruses and time points for coinfection to determine the one that is most effective. In brief, 5 WT C57BL/6j mice per group will be anesthetized using 80mg/kg ketamine and 8mg/kg xylazine administered via intraperitoneal injection. Groups of mice will be infected in one of 11 ways:

- (1) 125 PFU of influenza A/PR/8/34 H1N1 alone
- (2) 10<sup>7</sup> CFU of *A. fumigatus* alone
- (3-6) 125 PFU of influenza A/PR/8/34 H1N1 and then coinfecting on either day 3, 5, 7 or 9 with 10<sup>7</sup> CFU of *A. fumigatus*
- (7) 125 PFU of influenza A/California/04/09 H1N1 alone
- (8-11) 125 PFU of influenza A/California/04/09 H1N1 and then coinfecting on either day 3, 5, 7 or 9 with 10<sup>7</sup> CFU of *A. fumigatus*

Based on our previous experience with IAV infections and coinfections, severe disease should only be observed in coinfecting mice. During viral-bacterial coinfection, this typically occurs 24-48 hours after coinfection. However, a recent viral-fungal coinfection model using *Cryptococcus neoformans* found that mice began to succumb to infection about 4 days after coinfection [1]. Thus, all mice will be weighed and monitored daily for disease severity and animals will be euthanized when they reach >30% weight loss or become moribund to determine differences in survival for up to 21 days after the initial influenza A virus infection. We selected the influenza A/PR/8/34 H1N1 virus because it is known to predispose mice to coinfection with other pathogens (mainly bacterial) and is well mouse adapted. On the other hand, we selected the influenza A/California/04/09 H1N1 virus because it is a human clinical isolate that still replicates well in mice and several clinical case reports suggest this virus has a propensity to predispose human patients to fungal coinfection [2].

One potential difficulty we foresee is that mice are highly resistant to pulmonary infection with *A. fumigatus* alone. If our initial infection schemes are not successful, then we will repeat these experiments but administer corticosteroids beginning on day 3 after influenza A virus infection (when mice first display clinical signs of illness). We have selected corticosteroids as their use is a proposed risk factor for IAV-fungal coinfection [2].

Once we determine the virus, timing, and conditions of the coinfection that produces severe disease, we will then determine the overall weight loss and mortality (30% weight loss, or moribund) by infecting 10 mice per group with a single pathogen or coinfecting them and monitoring them for 21 days. We will also examine the immune response in 6 mice per group infected with a single pathogen versus coinfection (3 groups of mice) on day 1 and day 3 after coinfection. This will include euthanizing mice and collecting blood, lung, spleen, liver and brain to determine pathogen burden and cytokine/chemokine levels. These tissues will also be examined for cellular infiltration and lung pathology. Finally, RNAseq will be performed to examine the overall immune signature of gene expression.

1. van de Veerdonk F.L., et al., "Influenza-associated Aspergillosis in Critically Ill Patients." *American Journal of Respiratory and Critical Care Medicine*, 196(4), pp. 524-527
2. Lat A, et al., "Invasive aspergillosis after pandemic (H1N1) 2009." *Emerg Infect Dis*. 16(6):971-3.

# **If applicable, supporting materials to clarify methods and/or techniques described in the submission design may be attached.**  
*I.e., Graphs, images, videos, etc.*

Filename	Uploaded By	Date Uploaded
--		

# If applicable, supporting documentation such as consent forms, surveys, etc may be attached.

Filename	Uploaded By	Date Uploaded
--		

**Animal Use Justification**

1. For each animal requested, assign an approximate number and pain category. Please also consider the definition of distress when assigning animals to pain categories.

*Pain categories are based on the USDA Criteria*

**Category B** - Animals are those that are being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but have not yet been used for such purposes, however minor.

**Category C** - Animals are those that are subjected to procedures that involve no pain or distress, or procedures that would not require the use of pain-relieving drugs. (i.e. animal behavior or routine injections and blood samples).

**Category D** - Animals are those subjected to potentially painful procedures for which anesthetics, analgesics, or tranquilizers will be used. (i.e. surgery with appropriate anesthesia and postoperative analgesia).

**Category E** - Animals are those subjected to painful or stressful procedures without the use of anesthetics, analgesics, or tranquilizers. (i.e. Lethal dose studies or pain studies that do not allow pain-relieving agents).

Distress

An aversive state in which an animal fails to cope or adjust to various stressors with which it is presented. Distress does not require that the stressor induce an immediate and observable pathologic or behavioral alteration; both the duration and intensity of the state are important considerations when trying to prioritize attention to and treatment of animal distress.

Animal	Approximate Number	Pain Category				
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>C57BL/6j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	C57BL/6j	Mouse	40	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
C57BL/6j	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>C57BL/6j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	C57BL/6j	Mouse	496	<input type="radio"/> B <input type="radio"/> C <input type="radio"/> D <input checked="" type="radio"/> E
Name	Type					
C57BL/6j	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129-Card9tm1xlin/j</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129-Card9tm1xlin/j	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129-Card9tm1xlin/j	Mouse					

<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129-Mavstm1Zjc/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129-Mavstm1Zjc/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129-Mavstm1Zjc/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129P2-IL18tm1Aki/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129P2-IL18tm1Aki/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129P2-IL18tm1Aki/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129S1-Tlr7tm1Flv/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129S1-Tlr7tm1Flv/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129S1-Tlr7tm1Flv/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129s6-Clec7atm1Gdb/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129s6-Clec7atm1Gdb/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129s6-Clec7atm1Gdb/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129S6-Nlrp3tm1Bhk/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129S6-Nlrp3tm1Bhk/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129S6-Nlrp3tm1Bhk/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129-Tlr2tm1Kir/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129-Tlr2tm1Kir/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129-Tlr2tm1Kir/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.12P2(SJL)-Myd88tm1.1Defr/</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.12P2(SJL)-Myd88tm1.1Defr/	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.12P2(SJL)-Myd88tm1.1Defr/	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>Trif</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	Trif	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
Trif	Mouse					

<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.12P2-Aim2Gt(CSG445)Byg/J</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.12P2-Aim2Gt(CSG445)Byg/J	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.12P2-Aim2Gt(CSG445)Byg/J	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>Tlr3</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	Tlr3	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
Tlr3	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>Rigl</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	Rigl	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
Rigl	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>B6.129S7-IL1r1tm1lmx/J</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	B6.129S7-IL1r1tm1lmx/J	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
B6.129S7-IL1r1tm1lmx/J	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>NOD.129S2(B6)-Casp1tm1SeshCasp4del/LtJ</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	NOD.129S2(B6)-Casp1tm1SeshCasp4del/LtJ	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
NOD.129S2(B6)-Casp1tm1SeshCasp4del/LtJ	Mouse					
<table border="1"> <thead> <tr> <th>Name</th> <th>Type</th> </tr> </thead> <tbody> <tr> <td>lfnar</td> <td>Mouse</td> </tr> </tbody> </table>	Name	Type	lfnar	Mouse	10	<input type="radio"/> B <input checked="" type="radio"/> C <input type="radio"/> D <input type="radio"/> E
Name	Type					
lfnar	Mouse					

**1A. Provide scientific justification for Pain Category E.**

The proposed research will examine the morbidity, mortality, and immune response to viral and fungal coinfections. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response. Therefore, no such drugs can be employed in this protocol. We have incorporated the following humane end points. Any animal that loses greater than 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 in those animals. Finally, corticosteroid drug doses, if needed for predisposing the mouse to secondary infection, will be based upon previously published research to avoid toxicity.

**2. For each animal requested, explain features (i.e., anatomic, physiologic, etc.) that make it a desirable model for this submission.**

The purpose of this study is to examine the immune response to infectious pathogens. In immunology, the mouse is the preferred species as there is a wealth of 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.

First, mice are easy to handle, house and 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 such as 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<sup>-/-</sup>, Nlrp3<sup>-/-</sup>, Myd88<sup>-/-</sup>, caspase1<sup>-/-</sup>, Trif<sup>-/-</sup>, Card9<sup>-/-</sup>, Ifnar1<sup>-/-</sup>, Clec7a<sup>-/-</sup> and more. Finally, the mouse has already been established as a model for the study of multiple infectious diseases of human importance.

**3. Explain why the number of animals requested is warranted.**

*If possible, provide a statistical power justification of sample size. For complex designs, including a flow chart or table of group size, time frame, and other relevant information may be very useful. Consider the 3 R's:*

- **Replacement** - replacing animals with non-animals models (i.e., computer models, in vitro assays, or cell culture, etc.).
- **Reduction** - reducing animal numbers (i.e., pilot study to estimate variability, proper statistical analysis, performing experiments simultaneously, etc.).
- **Refinement** - refining procedures to reduce pain and distress in animals (i.e., new anesthetics/analgesics, pain-relieving drugs, etc.).

All animal models using intranasal infection such as influenza A virus or *A. fumigatus* 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 C:**

Specific Aim 1 of my research is to investigate the molecular mechanisms involved in the synergistic immune response to coinfection with influenza A virus and *A. fumigatus* fungus. To accomplish this, we will need 40 WT mice and 10 mice from each KO mouse strain for the generation of bone marrow derived macrophages and for harvesting of alveolar macrophages. The cells will then be infected *in vitro*. We propose to test primary macrophages derived from mice in two phases. (1) WT, Myd88<sup>-/-</sup>, Trif<sup>-/-</sup>, Mavs<sup>-/-</sup>, Casp1<sup>-/-</sup>, Card9<sup>-/-</sup>, Ifnar<sup>-/-</sup> mice; (2) WT, Nlrp3<sup>-/-</sup>, Aim2<sup>-/-</sup>, Tlr2<sup>-/-</sup>, Tlr3<sup>-/-</sup>, Tlr7<sup>-/-</sup>, Rlg1<sup>-/-</sup>, Clec7a<sup>-/-</sup>, IL1r1<sup>-/-</sup> and IL18<sup>-/-</sup> mice. Therefore, **190** animals will be required to address the objectives of this aim over the three years of the protocol. Animals used in this category will be bred in house, purchased from Jackson Labs, or obtained from collaborators at other institutions.

**Category E:** Over the three years of the protocol, it is estimated that we will require **496** mice for category E. As there is no animal model for IAV-A. fumigatus coinfection, we will conduct some preliminary experiments to determine the most effective doses of pathogen and timing of infection. WT C57BL/6J mice will be infected with either Influenza A/PR/8/34 H1N1 virus or the Influenza A/California/04/2009 virus at 125 PFU per mouse to cause mild disease. Then, mice will be divided into four different groups and coinfecting with 10<sup>7</sup> CFU of *A. fumigatus* on either day 3, 5, 7 or 9 after the initial influenza A virus infection (2 viruses x 4 time points = 8 groups). Alternatively, three more groups of mice will be infected with only influenza A virus (either califomia/09 or PR8) or only *A. fumigatus* to compare their weight loss and survival. To accomplish the objectives of this research, we will need approximately **220** mice. We will perform 2 independent survival experiments with 5 mice in each of the 11 groups (2 independent experiments x 11 different infection groups x 5 mice = **110** mice). Alternatively, mice may not show overt clinical signs during this coinfection, as mice are highly resistant to *A. fumigatus* infection alone. We will thus repeat this experiment but administer corticosteroids beginning on day 1 after influenza A virus infection, as this is a proposed risk factor for IAV-fungal coinfection (van de Veerdonk F.L., et al., Influenza-associated Aspergillosis in Critically Ill Patients." American Journal of Respiratory and Critical Care Medicine, 196(4), pp. 524-527). This would then require an additional **110** mice.

Once we determine the virus strain, timing of coinfection and necessity for corticosteroids for coinfection that produces severe disease, will repeat the weight loss and survival experiments with 10 mice per group 2-more times (2 experiments x 3 groups x 10 mice = **60** mice we will examine the immune response in mice infected with a single pathogen verses coinfection (3 groups of mice) on day 1 and day 3 after coinfection. We anticipate needing 6 mice per group at each collection point and the experiments will be repeated 3 independent times (3 experiments x 2 collection points x 3 groups x 6 mice = **108** mice). In order to obtain lungs for histopathological examination and RNAseq, we will also need to repeat this experiment three additional times requiring **108** more mice. In all, **276** mice will be needed for this section. WT mice used in this portion of the proposed research will be purchased from Jackson Laboratories or bred in house.

**4. Indicate the resources and information used to verify the submission does not unnecessarily duplicate previous animal activities and/or that less painful or stressful procedures are unavailable.**

- Literature database
- Expert consultation and/or personal experience
- Taxon-specific and/or research specific guidelines

#### 4A. Literature database information.

Date of Search	Database Searched	Years Covered
05/24/2018 5:00 AM	Pub Med	1900 - 2018

#### 4B. Provide keywords used and a summary of search results.

##### Keywords

- Influenza, Coinfection, Animals, inflammasome (Pubmed) 2 publications found
- Influenza, Coinfection, Animals, IL-1 (Pubmed) 4 publications found
- Influenza, Coinfection, Animals (Pubmed) 252 publications found
- Influenza, Aspergillus, Animals (Pubmed) 38 publications found

##### Summary of search results

Many deaths attributed to influenza A virus (IAV) infection are the result of secondary infection with bacteria, but recently, physicians have reported an increase in the number of coinfections with fungal pathogens, especially *Aspergillus Fumigatus*. In fact, recent estimates suggest that more than 40-60% of all patients that acquire a coinfection with influenza A virus and *A. fumigatus* die from the infection (**ref. 1**). I propose to examine the immune response to this coinfection using both *in vitro* and *in vivo* models. We have already developed an *in vitro* model that has provided us with important preliminary data that warrants further examination *in vivo*. However, there is no *in vivo* model for this viral-fungal coinfection. Of the 296 publications found in my Pubmed search 0 publications were found where there was an *in vivo* model. Thus this model is greatly needed to understand how this coinfection between influenza A virus and *A. fumigatus* can happen and how the immune system responds so that we can begin to understand how to prevent it and treat it. Thus, the research proposed here 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 (**ref. 2**). 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 and *S. pneumoniae* coinfection and 1 publication has also used mice for developing a model of IAV and Cryptococcus fungal coinfection suggesting that mice can be used for viral-fungal coinfection model development (**ref. 3**).

##### References

1. van de Veerdonk F.L., et al., "Influenza-associated Aspergillosis in Critically Ill Patients." American Journal of Respiratory and Critical Care Medicine, 196(4), pp. 524-527
2. F. Menga, N. H. Wua, A. Nerlichb, G. Herrlerc, P. Valentin-Weigandb and M. Seitzb. Dynamic Virus-Bacterium Interactions in a Porcine Precision-Cut Lung Slice Coinfection Model: Swine Influenza Virus Paves the Way for Streptococcus suis Infection in a Two-Step Process. Infect Immun, 2015. 83(7): p. 2806-15
3. Oliveira L.V.N., et al., Influenza A Virus as a Predisposing Factor for Cryptococcosis. Front Cell Infect Microbiol. 2017; 7: 419.

**4A. Provide a summary of any resources (i.e., other researchers, conferences attended, etc.) and/or personal experiences that contributed to the development of this submission.**

*Information should include: name of consultant(s) and background, specific date(s) of consultation, type of meetings, experiences of activities similar to the proposed submission, etc..*

There is no expert in this field as no mouse model has been developed. There are some clinical experts such as Dr. Frank van de Veerdonk, whom I have collaborated with in the past. Dr. Jon McCullers, Chair of the Department of Pediatrics, University of Tennessee Health Sciences Center is an expert in the field of influenza A virus and bacterial coinfections and has agreed to collaborate with me on this research.

**# If applicable, supporting documentation such as articles, conference abstracts, meeting minutes, etc. may be attached.**

Filename	Uploaded By	Date Uploaded
--		

**Animal Acquisition and Care**

**1. Animal Source(s).**

- Acquired from an organization/institution
- Departmental transfer
- Purchased from vendor
- Wild-caught

**1A. Specify organization/institution and provide a point of contact.**

Organization/Institution	Point of contact
TBD	TBD

**1A. Specify what department, a point of contact, and the approved IACUC submission from which a transfer will occur.**

Department	Point of Contact	Approved IACUC Submission
BIO	Lupfer	16-015.0

**1A. Specify vendor(s).**

Jackson Laboratory

**2. Animal Holding Location.**

**Dedicated Housing Area** - Facilities designed to provide housing specifically for animals and managed by dedicated staff (i.e., University Vivarium).

**Satellite Housing Area** - A location outside of a dedicated housing area that houses USDA covered species for greater than 12 hours or any other vertebrate species greater than 24 hours.

Animal	Holding Area
--------	--------------

<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
C57BL/6j	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129S6-Nlrp3tm1Bhkj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
NOD.129S2(B6)-Casp1tm1SeshCasp4del/Ltj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129-Tlr2tm1Kirj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129S1-Tlr7tm1Flvj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.12P2(SJL)-Myd88tm1.1Defrj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.12P2-Aim2Gt(CSG445)Bygj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6;129-Mavstm1Zjcj	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Tlr3	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Rigl	Mouse	
		<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area

<b>Name</b>	<b>Type</b>	
B6.129S7-IL1r1tm1lmx/j	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
Trif	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129s6-Clec7atm1Gdb/j	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129P2-IL18tm1Aki/j	Mouse	
<b>Name</b>	<b>Type</b>	<input checked="" type="checkbox"/> Dedicated housing area <input type="checkbox"/> Satellite housing area
B6.129-Card9tm1xlin/j	Mouse	

**2A. Specify the dedicated holding area.**

*I.e., JVIC Vivarium, Temple Vivarium - Mammal Holding Room 2, Temple Vivarium - Aquatic Holding Room 2 & Aquatic Procedure Room, Darr - Horse Barn.*

Temple Vivarium - mammal holding rooms

**2B. Describe how animals will be monitored and maintained.**

*I.e., Animal staff will provide animal husbandry and care per approved standard operating procedures (SOP's), Animal staff will check animals, but researcher will perform husbandry, Researchers will provide animal husbandry and care per approved SOP's.*

Animal staff will provide animal husbandry and care per approved standard operating procedures (SOP's). However, when animals are infected, researchers will provide animal husbandry and care per approved SOP's.

**3. Animal ID method.**

*I.e., Tattooing, ear punch, pit tag.*

Tattooing

**4. Will animals have special housing requirements?**

*Housing that deviates from approved regulatory requirements and/or standard operating procedures (I.e., macroenvironment space is smaller than standard).*

Yes

No

**5. Will animals have special care and maintenance requirements?**

*Care and maintenance that deviates from approved regulatory requirements and/or standard operating procedures (I.e., Macroenvironment not changed regularly, animals not checked daily,*

etc.).

- Yes  
 No

**6. Will animals have special dietary requirements?**

*Dietary restrictions and/or dietary provisions that deviate from approved regulatory requirements and/or standard operating procedures (i.e., food and fluid regulation).*

- Yes  
 No

**6A. Please explain.**

Myd88-deficient proposed in this study must be maintained on oral antibiotics (Sulfatrim/Sulfamethoxazole) in the drinking water due to increased susceptibility to bacterial infections

Dosage: 10m/500ml water

**7. Explain provisions for handling sick animals.**

If animals are found moribund, Vivarium staff should contact the PI.

*Note:* When animals are infected per study design, they are expected to become sick and will be monitored at least daily for single infections and twice daily for coinfections. No additional contact is necessary for sick animals. However, if infected animals are moribund, please contact PI immediately and such animals will be euthanized.

**8. Explain provisions for handling deceased animals.**

If animals are found dead, Vivarium staff should contact the PI. Dead animals can be placed in the freezer if not infected. Dead animals that are infected should be left in the cage/room until the PI and/or submission personnel can retrieve them.

*Note:* infected carcasses will be taken to the PI's BSL-2 laboratory for storage.

**Procedural Overview**

**1. Will animals undergo prolonged restraint?**

*Physical restraint is the use of manual or mechanical means to limit some or all of an animal's normal movement for the purpose of examination, collection of samples, drug administration, therapy, or experimental manipulation. Restraint devices should be suitable in size, design, and operation to minimize discomfort, pain, distress, and the potential for injury to the animal and personnel. Typically, animals may be restrained for brief periods, usually minutes, in many procedural applications.*

*Prolonged restraint would be anything that doesn't aim to achieve the above definition.*

- Yes  
 No

**2. Will animals undergo surgical procedures?**

*A surgical procedure is a procedure that penetrates a body cavity or that produces a permanent handicap.*

- Yes
- No

**3. Describe criteria for removal of animals prior to the experimental endpoint.**

Animals that are intended for use for the collection of bone marrow that show any signs of sickness or developmental abnormalities will be euthanized immediately.

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.

**4. Will animals be euthanized?**

- Yes
- No

**4A. Detail method(s) of euthanasia.**

CO2 followed by cervical dislocation/thoracotomy

## APPENDIX B

<b>Application to Use Live Vertebrate Animals</b>	PI: Christopher L Lupfer	Page: 1 of 9
	Dept: Biology	
	IACUC ID: 19-005.0	Web ID: 462

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

<b>Yes</b> 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.
---

<b>Submission History for Continuation:</b>
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**

PI: Christopher L Lupfer Page: 2 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

**1. Personnel Information**

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

**2. Funding**

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

**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:

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 wealth of 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 and 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 such as 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<sup>-/-</sup>, Nlrp3<sup>-/-</sup>, Myd88<sup>-/-</sup>, caspase1<sup>-/-</sup> 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:

Approval Date: 2/19/2019

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

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

**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<sup>-/-</sup>, IL18<sup>-/-</sup>, IFNg<sup>-/-</sup>, Nlrp3<sup>-/-</sup> and Aim2<sup>-/-</sup> 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 <sup>-/-</sup>	54	100
Nlrp3 <sup>-/-</sup>	54	100
Aim2 <sup>-/-</sup>	54	100
IL18 <sup>-/-</sup>	54	100
IFNg <sup>-/-</sup>	54	100

**Application to Use Live Vertebrate Animals**

PI: Christopher L Lupfer Page: 4 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

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

Pain Category Animals	
B	1478
C	0
D	0
E	0
Total = 1478	

Justification for Category E:

4. Transfer of Existing Animals: Yes

If Yes, Indicate the IACUC ID: 16-015.0

**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 Medline:
- No 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

Approval Date: 2/19/2019

## Application to Use Live Vertebrate Animals

PI: Christopher L Lupfer Page: 5 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

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 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

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.

### 2. If this application is a continuation of an ongoing project, state concisely how these goals differ from those in the original 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.

Approval Date: 2/19/2019

## Application to Use Live Vertebrate Animals

PI: Christopher L Lupfer Page: 6 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

**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 housed in wire bar lid rat cages to allow cohousing of more mice per cage.

Approval Date: 2/19/2019

**Application to Use Live Vertebrate Animals**

PI: Christopher L Lupfer Page: 7 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

**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:

Approval Date: 2/19/2019

**Application to Use Live Vertebrate Animals**

PI: Christopher L Lupfer Page: 8 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

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:

2. Animals experiencing unrelieved pain or distress prior to the endpoint, as defined by institutional policy, must be humanely 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<sub>2</sub>

Approval Date: 2/19/2019

**Application to Use Live Vertebrate Animals**

PI: Christopher L Lupfer Page: 9 of 9  
Dept: Biology  
IACUC ID: 19-005.0 Web ID: 462

**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 my 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 discuss 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: 2/19/2019