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At the March 5, 2010 UW-Madison Chemistry Department Colloquium, Prof. Bassam Z. Shakhashiri, the director of the Wisconsin Initiative for Science Literacy (WISL), encouraged all UW-Madison chemistry Ph.D. candidates to include a chapter in their Ph.D. thesis communicating their research to non-specialists. The goal is to explain the candidate's scholarly research and its significance to a wider audience that includes family members, friends, civic groups, newspaper reporters, program officers at appropriate funding agencies, state legislators, and members of the U.S. Congress.

Over 50 Ph.D. degree recipients have successfully completed their theses and included such a chapter.

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Whisper with Lipids: Fungal Oxylipins in Development and Host Interactions in *Aspergillus fumigatus*

By

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CHAPTER 5: A Dance in the Fungal Woods:

Signaling Lipids in Fungal Disease and Development

I have written this chapter to tell the stories of my Ph.D. research and the personal experience behind it to a broader audience with minimal to no science background. I firmly believe that it is to our society's own interest to help its citizens better understand science and use scientific knowledge to guide our actions, live a heathier life, and create more sustainable solutions to problems like climate change, worldwide famine, and pandemics. Indeed, communicating cutting-edge science to the public is a fundamental component of our responsibilities as scientists. I am using this opportunity to tell my family and friends about my scientific journey and share a glimpse of my research discoveries to those who wonder about biology yet find it hard to digest. Lastly, I thank the Wisconsin Initiative for Science Literacy at UW-Madison for providing this platform to train future scientists to shoulder the vital responsibility, and for sponsoring and supporting the creation of this chapter.

If you have ever strolled in the woods after a good rain, had a tasty loaf of bread or a sip of beer, or thrown away spoiled food from the back of your fridge, you must have seen mushrooms, tasted yeast, and smelled mold. You are no stranger to fungi, nor are they to you. In 2015, when I started my graduate study in microbiology at the University of Wisconsin-Madison, I didn't know that fungi and I could be tied to each other, nor had I imagined that I would spend the next five years thinking about molds, caring for them day and night, and trying to understand how they grow and make us sick.

My journey started when Dr. Nancy Keller, now my Ph.D. advisor, brought me to her laboratory on the first day we met. While the three rooms were stuffed with cold glassware and busy-running instruments, a walk in this lab felt like a tour in the garden of fungi, where I saw them grow in a variety of forms, producing vibrant colors, distinct shapes, and at times, a mixture

of scents. Later as I journeyed through the fungal biology hidden in this garden, those shapes would tell me how fungi control their growth, reproduction and interactions with human-beings, and the colors and scents would indicate the natural chemicals they produce and release to adapt to their ever-changing environments. In nature, fungi live in our gut and on our skin, dwell in the soil and our trash bins, and tangle with plant roots. Even though laboratory conditions cannot accurately capture these natural niches that fungi occupy, they provide a variety of easily manipulatable yet highly controlled conditions that reveal the important biological principles of how fungi live, adapt to environments, interact with other lives on earth, and occupy almost every corner on earth.

Fungi belong to a group of micro-scale organisms, commonly known as microbes (Fig. 1A). Microbes are often too tiny to see and require magnification of over hundreds of times to be detected by our naked eyes. Microbes have different lifestyles: some of them can live freely, some occasionally infect and live in another organism such as a plant or animal (we call a “host”), and others strictly reside in a host to survive and grow. Viruses, such as coronavirus and influenza virus, heavily rely on infecting humans to survive. Thus, in communities with dense human populations and frequent human-human contact, they can hop between individuals and find new hosts to expand the viral population. In contrast to viruses, many bacteria and fungi can function freely: they absorb nutrients from the environment, generate their own energy, send and receive environmental signals, and produce the next generation of microbes.

Like a building contains a collection of rooms, organisms like plants, animals, and microbes (except for viruses) consist of one or more of its basic units called cells. Each cell is an independent, living entity: It is structurally separated from the environment and performs essential functions required for its survival, growth, adaptation, and continuity. While the original life formed over 4 billion years ago contained only one single cell, in evolutionary history, fungi arose

to become the first kingdom of lives that contained more than more cells. Only a small group of fungi, including baker's yeast, is composed of only one cell, the rest, such as mushrooms and molds, are made of many cells (Fig 1B). Despite that both human and fungi contain multiple cells, the many cells within a fungus don't form distinct tissues or organs, instead these cells form different developmental stages with distinct characteristics.

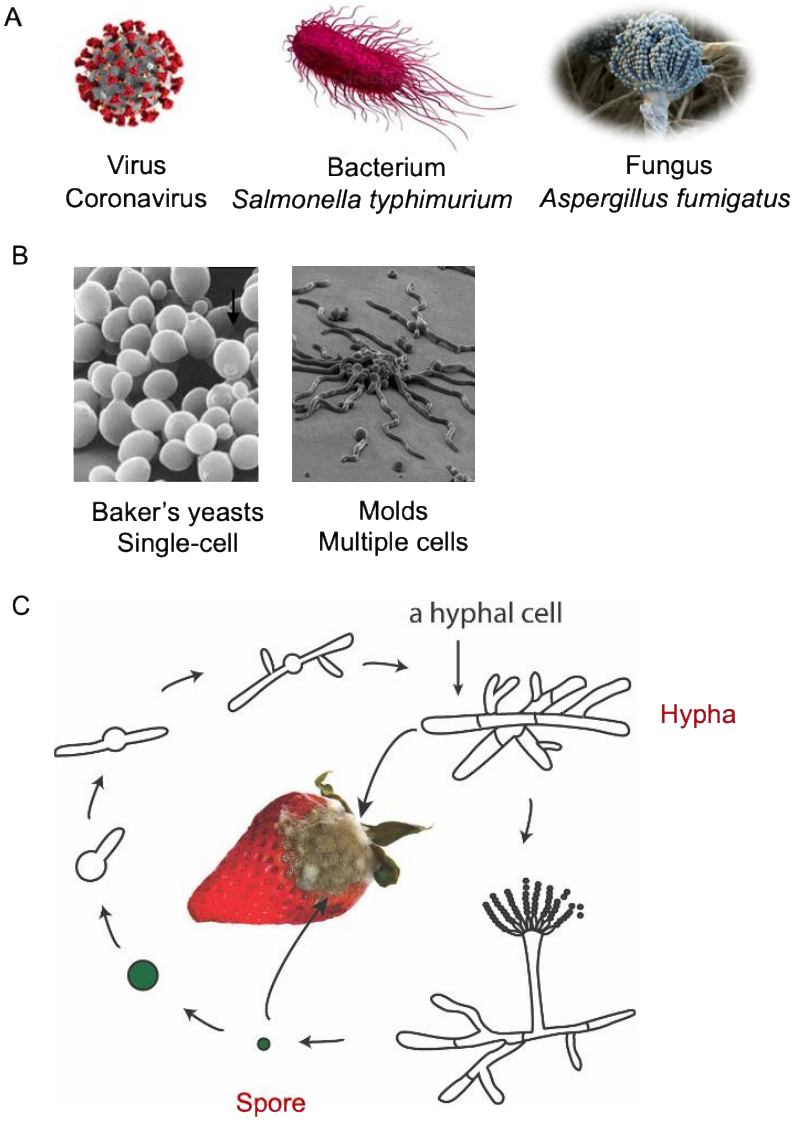

Fig 1. (A). Microbes that pose great health threats to human, including the virus coronavirus that causes mild to severe respiratory illnesses, the bacterium *Salmonella typhimurium* that causes diarrhea, and the fungus *Aspergillus fumigatus* that causes life-threatening lung infections. **(B).** Images of single-celled baker's yeast and multi-cell structures of mold. For the yeast, each round-shaped entity is a cell whereas for molds, each long filamentous structure is a fungus that consists of multiple cells. **(C).** The common lifecycle of filamentous fungi, such as the grey mold *Botrytis cinerea* found on strawberries. Spore germinates and grow to form hypha, which further develops to produce more spores. A hyphal cell is indicated on one end of the main growth axis of a hypha.

Two of these developmental stages are commonly present in fungi: spore and hypha. These structures can be well-observed on a spoiled fruit (Fig 1C). The white, fluffy structures growing

on rotten raspberries or strawberries are composed of the most characteristic structure of fungi, hypha (Fig. 1C). Hyphae are filamentous structures containing multiple longitudinally aligned cells, thus giving these fungi their collective name "filamentous fungi." *Aspergillus* is one genus of filamentous fungi that my research group studies in-depth. The blue-greenish spots on a soft, smelly apple or strawberry contain billions of spores (Fig. 1C). Spores are often circular, pigmented, single-celled, and commonly dispersed in the air. When filamentous fungi grow on decomposing organic matter, such as dead plants and animals, and food exposed to the open air, they undergo a cyclic lifecycle transitioning between spore and hypha (Fig. 1C). Analogous to plant seeds, fungal spores generally remain dormant when floating in the air or on object surfaces. While a seed planted on a well-nourished and watered soil can soon germinate and grow into a seedling, under sudden presence of favorable conditions such as on the skin of a fruit, spores can quickly sense the rich amount of sugars, switch to a fast-growing mode and germinate (Fig. 1C). Development of a spore into hypha is highly directional: a spore first germinates and extends rapidly along one major growth axis, followed by side branches stemming from the main filament (Fig. 1C). This directional, temporally and spatially regulated growth dynamic represents an early form of cell differentiation, of which we have limited understanding. And this is what I have been scrutinizing throughout my study.

My curiosity about fungal development drew my attention to one class of molecules. Fatty acids, also referred to as lipids or fats in more complex forms, should not sound unfamiliar to our society. Bad fats have claimed lives and disabled individuals especially in high- and middle-income countries by causing heart attacks, strokes, and diabetes. Newer studies have linked bad fats to a higher risk of developing asthma and cancer. Good fats, such as omega-3 and omega-6 fatty acids, commonly found in fish, nuts and dietary supplements, protect heart, brain and skin

health. The critical truth is, we can't live without fats. While we consume fats from daily diets, our body constantly generates a diverse group of modified fatty acids, called oxylipins, to support our immune, circulatory, and respiratory systems. Oxylipins are important for both maintaining the steady state of our cells and for responding to dangers surrounding us, such as when we catch a cold from bacterial or viral infection. Oxylipins are produced widely in animals and plants, as well as in microbes like fungi and bacteria. Some oxylipins can be produced by both animals and fungi, while others are produced only in animals or fungi. Intriguingly, a research group at the University of California-San Francisco has found that bacteria within our gut harbor genes to modify human oxylipins, which could promote early onset of allergies in newborns[1,2]. Oxylipins may be the strings that connect us with other organisms on earth, and central to my research, they may link our health to filamentous fungal pathogens universally present in nature.

How do scientists study cells and fats in cells?

To study the functions of a cell, we need to understand its composition. It might strike you that cells within you and me, a mushroom, a llama, and an oak tree are made out of the exact same classes of molecules that sustain similar functions in our cells. Through these molecules, cells perform their "living" functions – they breathe, they uptake nutrients and metabolize them, they shed wastes, and they reproduce. These processes are executed primarily through the concerted actions of four classes of large molecules: nucleic acids (DNA or RNA), proteins, sugars (carbohydrates), and fats (lipids). The same or similar molecules in different species can perform different cellular tasks depending on many factors, including when and where they are made in the cell and how they interact with other molecules to support cellular functions.

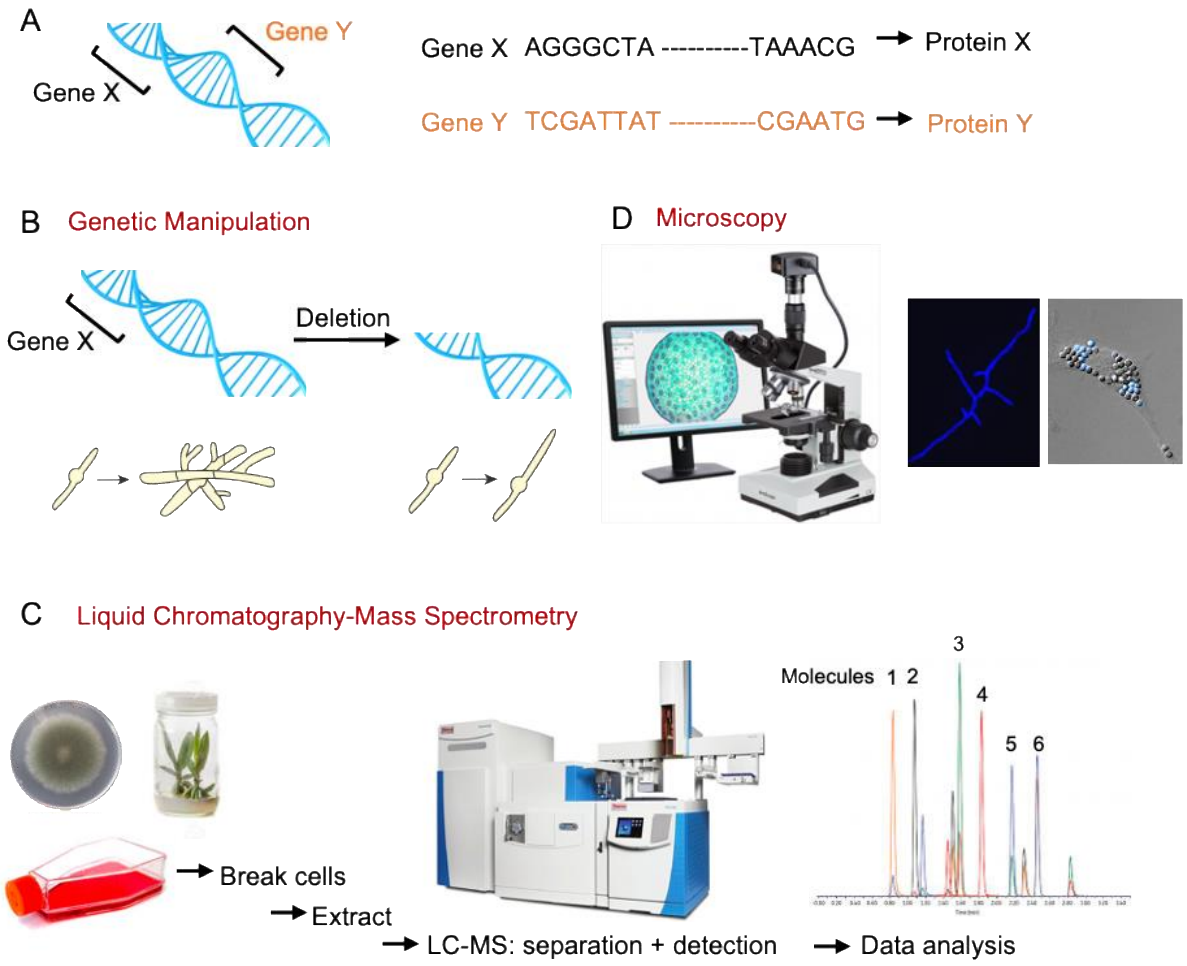

Fig 2. Biotechnologies used in my Ph.D. research. (A). Linear organization of genes on a part of a DNA molecule. A single DNA molecule includes thousands of such genes. Gene X and Gene Y, which contain different sequences of A, T, G, C, can produce different proteins X and Y. **(B).** A simplified illustration of genetic manipulation, such as deletion of a gene X in fungi. To investigate the biological relevance of any gene in hyphal growth, we can delete a gene and study whether cells without gene X shows altered branching as illustrated. **(C).** The workflow of liquid chromatography-mass spectrometry (LC-MS) to analyze molecules from various types of cells or tissues from fungi, plant, and animal, etc. Methods in extraction, separation and detection can be optimized to analyze molecules of interest. **(D).** Microscopy is frequently employed to observe microbes and animal cells. Two images shown are: hypha of *A. fumigatus* (left) and an animal immune cell with engulfed spores (right).

Technology propels scientific discoveries and vice versa. Since the structure of DNA was proposed in 1953 by Watson and Crick, rapid evolution of genetic and genomic technologies have brought a golden age of life sciences. Scientists have been able to understand that inheritable

information is carried on DNA molecules in the form of genes (Fig. 2A, left panel). If we consider a DNA molecule as a book, then each gene is one page of that book, and information stored in a gene is akin to stories within that page. Like a book page contains aggregates of words, formed into lines of texts to delineate the stories, a gene contains genetic codes, designated as A, T, G, C linked into sequences to confer information. The unique organization of A, T, G, C within each gene leads to production of a unique protein (Fig. 2A, right panel). Having found this relationship between gene and protein and the linear genetic code arrangements, biologists started to decipher the mystery embedded in genes in all kingdoms of life on earth, primarily through genetic modification (Fig. 2B). We can delete a specific gene X, change the A, T, G, C arrangement in gene X, or insert new sequences to gene X within cells. We then compare the modified cells to the un-modified cells to identify the process that gene X regulates. Though revolutionary in biological research, this approach is less powerful in studying carbohydrates and lipids, as the structures of these molecules are more versatile, less predictable, and harder to manipulate. Luckily, with recent advances in liquid chromatography (LC), a molecule-separating technology, coupled with mass spectrometry (MS), a molecule-detecting technology, we can separate, detect and identify tens of thousands of proteins, carbohydrates, lipids and other small molecules with distinct structures in a cell (Fig. 2C).

My research became a reality through genetic manipulation and LC-MS analysis, which allowed us to describe how fungal cells produce oxylipins, access pure oxylipins in small yet sufficient quantities to determine their cellular functions and discover genes that regulate development of filamentous fungi. In addition, microscopy, a modern technology for viewing, recording, and analyzing cells through a microscope (Fig. 2D), has tremendously expanded our

ability to characterize fungal growth before it is detectable by our naked eyes. Without these tools, we couldn't delve deep inside a cell to understand fungi and their relevance to us.

Serendipity, frustrated student, and one long road ahead

One of the most exciting aspects of science is probably that it's endless; in a laboratory, it means that graduate students and postdoctoral researchers seeking answers for one important question often end with more questions to be passed to the next researcher to solve. Studying fungal development was not my plan since day one in the lab, indeed, it was not until one year after I joined that I started chasing after an exciting finding made by a previous graduate student, Gregory Fischer. I only knew that it was a serendipitous observation from his email to me a few years later when I finally made a leap in explaining his initial finding.

One morning, Greg entered the dark lab to use a microscope to check the *Aspergillus fumigatus* culture he had grown the night before. He grew the fungus in two different liquid solutions, both containing the same essential nutrients that support fungal development. The two solutions only differed by one factor: one solution contained a small amount of oxylipin, the other didn't. Then he saw something astonishing - *A. fumigatus* hyphae that grew in the oxylipin-containing solution appeared vastly different from those that grew in the solution without oxylipin– they generated a crazy number of side branches. He followed with a second experiment growing the fungus in different solutions with increasing amounts of oxylipins. The more oxylipins that were present, the more branches he observed (Fig. 3A, 3B).

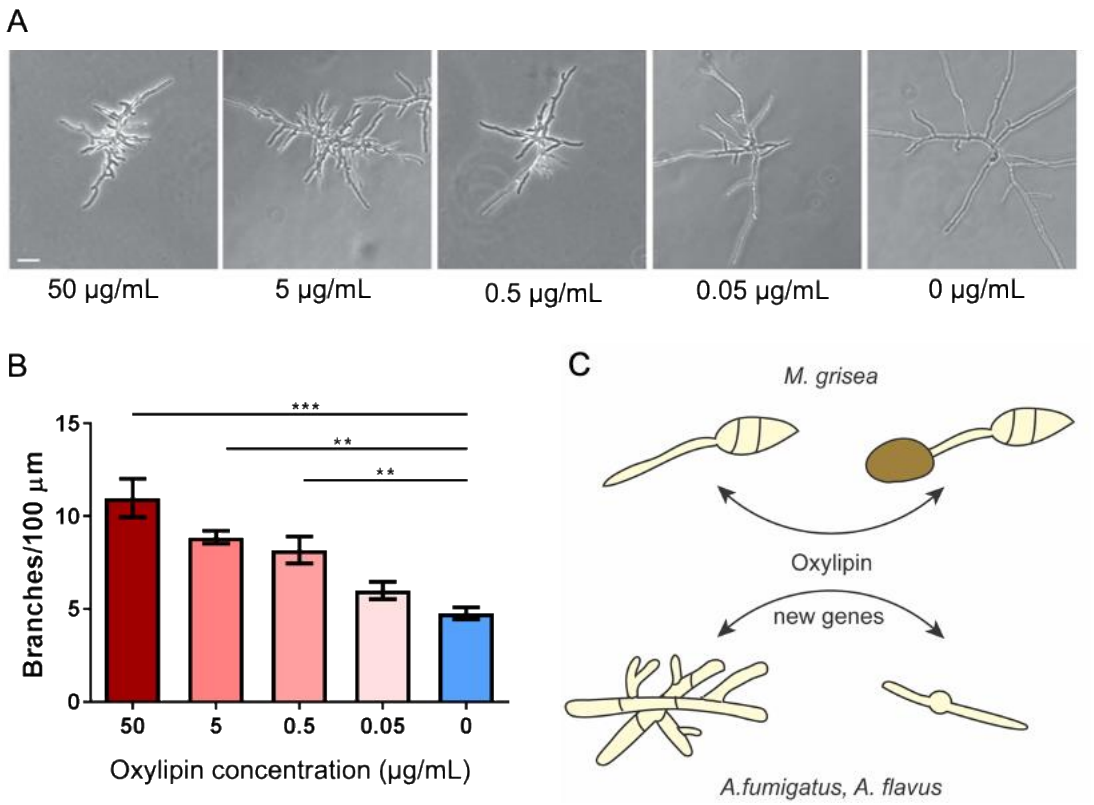

Fig 3. Oxylipin induces *A. fumigatus* branching. (A). Spores of *A. fumigatus* were co-incubated in standard fungal growth solution with increasing concentrations of oxylipins and microscopic images were acquired at 20 hours after fungal growth. **(B)**. Quantification of the degree of branching in the same experiment shown in **(A).** Branching was represented as the number of side branches per 100 µm of leading hypha (growing axis). 100 µm is one tenth of 1 mm. Values represent means ± standard error (a measurement of variation). Any two-group comparisons with asterisks indicates the two groups are statistically different. **(C).** An illustration of the major findings from studying the effect of oxylipin on hyphal growth in three species of filamentous fungi, *A. fumigatus*, *A. flavus*, and *M. grisea*.

So, why did this observation excite us?

Hyphal branching is a common occurrence in filamentous fungi, an evolutionarily conserved trait. Such orderly branched growth patterns are even commonly found in plant and animal tissues[3]. Branched growth in fungi supports nutrient absorption in soil and allows fungal pathogens to infect animals and plants. Using genetic manipulation and microscopy that I described earlier (Fig. 2B, 2D), scientists have found proteins and small molecules located at the

tips of the side branches as well as the main growth axis of a hypha to direct this type of growth[4]. Yet many questions remain unresolved, such as when and how fungal branching is triggered.

I designed the first set of experiments to start addressing these big questions. Knowing that calcium abundance and distribution in hyphae was linked with directional hyphal growth from other studies, my advisor and I decided to measure if intracellular calcium levels in fungal hyphae changed in the presence of oxylipin. Setting up the experiment turned out to be a non-trivial, even painstaking task: No lab nearby had done such an experiment – two research groups that had performed such an experiment in fungi were located in the United Kingdom and China and were using different fungi and protocols. We had to find advanced equipment that could measure the change in the calcium level in milliseconds. Finally, in the real experiment, I didn't get data consistent with the published findings. In other words, my first big attempt failed after months. We decided with heavy hearts that I was not going to pursue this hypothesis. To our further disappointment, none of the genes that were reported to influence branching showed any change as a result of the oxylipin, leaving us no apparent target to pursue further.

Failures are pervasive in science and are probably the most faithful friend to Ph.D. students trying to solve biological puzzles. The decision to forgo these hypotheses brought me frustration, self-doubt, and hesitation to continue this research. Fortunately, after more than a year, when another student started pursuing a similar research question, I decided to re-evaluate the biology of fungal branching in an alternative, holistic way. We collaborated, designed our experiment after rounds of debate, and modified our approach to chase down genes, including all unknown ones, that are important in responding to our branching-inducing oxylipin.

The new route urged me to hop on a rather steep learning curve to perform analysis of large datasets, create methods to find genes responsible for branching with improved efficiency, and

detect other effects of oxylipins beyond branching induction. By collaborating with Dr. Michael Bromley's lab in the University of Manchester and with help from many of my colleagues, we have identified new genes that regulate *A. fumigatus* hyphal branching. In addition, one of the genes was suggested to respond to calcium signals in other studies.

Through collective efforts, my research team has established that a specific oxylipin regulates hyphal growth in different ways (Fig. 3C): It induces hyphal branching in *Aspergillus* species, including the opportunistic fungal pathogen *A. fumigatus* and the carcinogenic plant pathogen *Aspergillus flavus*; it also induces formation of the infectious structure in the most devastating rice pathogen *Magnaporthe grisea*. We further identified new genes required for the oxylipin regulation of *A. fumigatus* branching (Fig. 3C). Most excitingly to me, many more questions can be asked and answered: Among many other genes that are influenced by the genes we identified, which of them have led to increased hyphal branching? As fungal pathogens show pronounced directional growth during infection and invasion of human and plant tissues, how does oxylipin affect disease progression in these hosts? How do these *A. fumigatus* genes affect progression of the devastating diseases caused by this fungus? How does oxylipin regulate growth in *M. grisea* and affect its infection of rice plants? These exciting questions will be pursued in the upcoming years by a collaborative group of researchers in our laboratory.

Shared language: oxylipins in clearing fungal infection

Research has provided insights into how microbes benefit and harm our health and helped us create solutions to alleviate human sufferings, including the many we are witnessing in the ongoing COVID-19 pandemic. I have witnessed sufferings and threats from malaria and tuberculosis in impoverished communities in sub-Saharan Africa, discussed antibiotic resistance

with clinicians and clinical laboratory microbiologists at UW Hospital & Clinics, and led teams in community-focused programs to prevent worm infection and promote awareness of microbial diseases. I believe that science is a bridge leading to human wellbeing.

Patients with HIV/AIDS, cancer, and asthma, and those seeking organ transplants or corticosteroid therapy, have to worry about *A. fumigatus*. Spores of *A. fumigatus* are well-equipped to survive harsh environments involving drought, heat, cold, UV radiation, etc., and are small enough to infect deeper regions of human lungs, establishing severe diseases in vulnerable individuals. *A. fumigatus* causes various forms of aspergillosis among individuals with either under- or over-reactive immune systems, including HIV/AIDS patients, patients who receive long-term corticosteroid therapy or recent solid organ transplants, asthmatic patients, and patients who have tuberculosis-related lung lesions. Germinated spores and hyphae further damage lung tissues by invading the intercellular space, producing toxins and triggering uncontrolled immune reactions. Existing aspergillosis treatments were either too toxic for these sick patients or not effective to drug-resistant *A. fumigatus*. In other words, we need new antifungals that work. Considering the natural history of aspergillosis, inhibiting spore germination and enhancing killing of inhaled spores could be the next solutions to treating aspergillosis.

My next set of discoveries may be a result of serendipity, wild guesses, and poorly managed curiosity. In working with oxylipins that control fungal branching, I noticed two structurally similar oxylipins, one unique to filamentous fungi, the other made in humans. More fascinating to me, both of these oxylipins influence fungal branching, yet in opposite ways. Why does *A. fumigatus* respond to an oxylipin signal made by humans? Can the reverse be true, that humans sense the presence of a fungal oxylipin? If a two-way communication through oxylipin is present,

what does that mean to a patient with an ongoing *A. fumigatus* infection, where the patient's immune cells meet the fungal cells and fungal oxylipins?

If I could only do one experiment to know if fungal oxylipin is important in infection, what would it be? At first, I was overwhelmed by my excitement to find the answer and my fear of missing the biology through a poorly designed or mal-performed test. After conducting many thought experiments, I created a method slightly modified from another experiment that I had established and repetitively done and thus was confident in performing it well. I obtained cells from mouse bone marrow and grew these cells for days in a special nutrient solution until they matured and became a type of immune cells called macrophages, giving them the ability to recognize, engulf and kill fungal spores. To determine whether fungal oxylipin has an effect on these macrophage cells, I grew the macrophages in two separate solutions, one that contained fungal oxylipin and another that didn't. After another 18 hours, I infected these two groups of macrophages with *A. fumigatus* spores and performed a series of procedures to assess the spore-killing ability of the two groups of macrophages (Fig. 4A).

After a few days, when I finally counted the number of live spores at the end of this procedure, my data showed me that macrophages exposed to the oxylipin were better killers of *A. fumigatus* spores. In other words, the fungal oxylipin helped animal immune cells fight *A. fumigatus*! I later repeated this experiment and confirmed the finding. So, how do these macrophages sense the fungal oxylipin, one they are incapable of making? Then next, how do they take action and enhance their spore killing efficiency after they detect the fungal oxylipin?

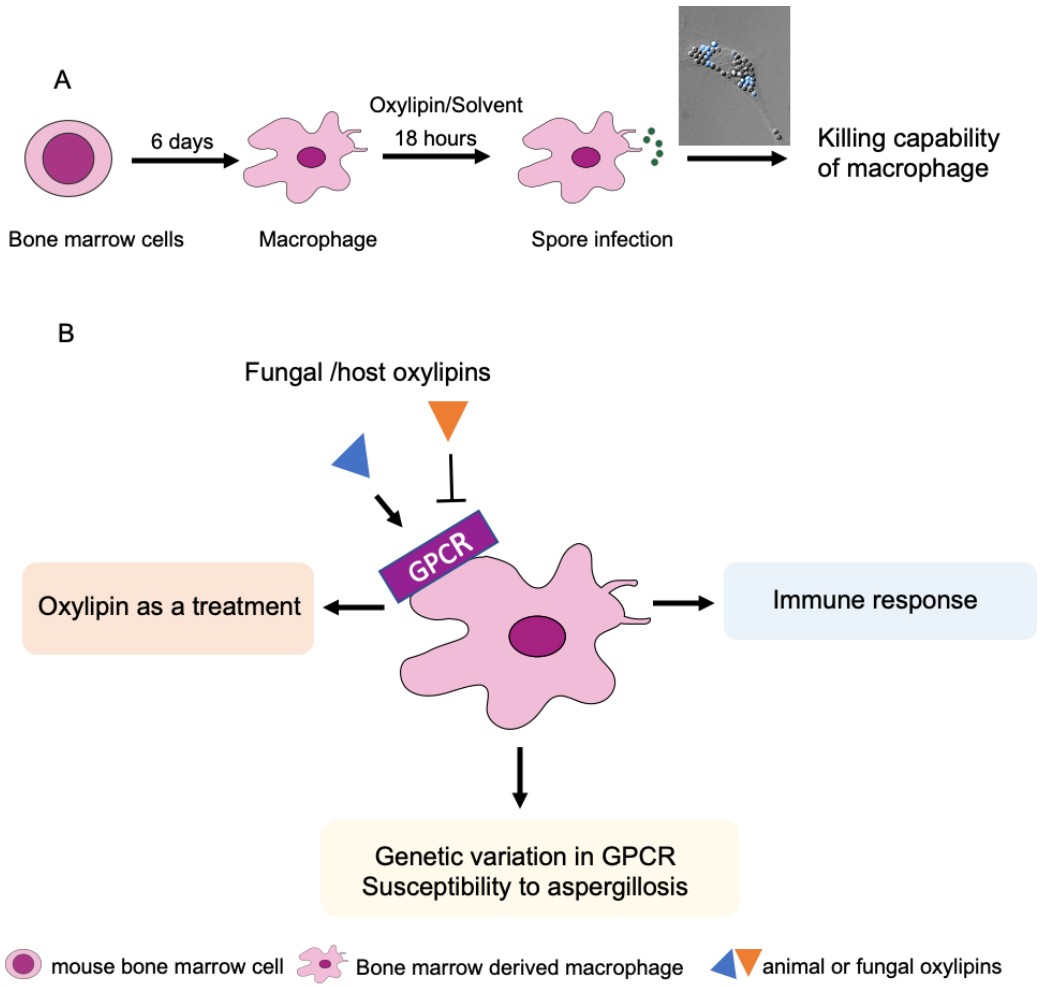

Fig 4. Assessment of fungal oxylipin for its effect on immune cells. (A). The experimental workflow of assessment of fungal oxylipin effect on macrophage killing of spores. Mouse bone marrow cells were extracted and cultured for 6 days into macrophages. Macrophages were treated with oxylipin or solvent (as a no-oxylipin comparison) for 18 hours and infected with spores. After 3 hours, spore-infected macrophages were processed to quantify the percentage of live spores, which was used to calculate the macrophage killing efficiency. An image illustrating macrophage engulfing spores is shown. **(B).** Identification of oxylipin binding to macrophage G-protein coupled receptor suggests new directions for future research on fungal disease biology and antifungal discovery.

All cells are enclosed by a membrane that separates the interior of the cell from the outside environment. Besides separating the relatively stable inner cell from the ever-changing outer environment, the cell membrane also serves as a hub for communication – this membrane is actively engaged in exchanging “messages” between the inside and the outside of the cell through

molecules on it. One class of these molecules is surface receptors. Acting as a cell's antenna, surface receptors constantly sense potential threats and trigger responses within the cell. For example, receptors on immune cells bind to molecules derived from tumor cells or invading pathogens to know that they should defend against these abnormal invaders. Macrophage receptors that recognize spore surface molecules send a message "Spores are here!" to direct actions by other molecules within the macrophage to engulf and kill invading spores.

One type of these surface receptors on human immune cells is called G-protein coupled receptors (GPCRs). Human GPCRs represent the largest and best studied family of surface receptors, with 20-40% of them being targeted by drugs to treat various human conditions. If GPCRs are locks on a door, molecules that can bind to GPCRs are keys that are shaped to fit in these locks. Intriguingly to us, many oxylipins produced by our body are the keys to our own GPCR locks, guiding us to a wild hypothesis: Fungal oxylipins, which are similar in shape to some human oxylipins, can also bind to human GPCR(s). Next, when fungal oxylipin binds to the GPCR(s) located on the surface of our macrophages, those macrophages become more effective at killing spores. But how can we find the GPCR(s) that does the job, out of more than 800 different GPCRs that can be made in human cells?

After comparing the structural similarity of human and fungal oxylipins, delving into published studies on human GPCRs, and sending emails and phone calls to seek technical consultations, we decided on a standard test to confirm oxylipin binding to our best guessed GPCR candidate. We made a risky decision and spent $1,300 on a commercial test consisting of 1ml of specially engineered cells and a few tubes of special solutions. To me, the stakes were especially high because I had to finish my experiments in one day and one trial, before the extremely delicate cells died. Finding no one nearby with such experience, I had to trust myself, gather all the

information required, and check every calculation and procedure time and again. Finally, at 10 pm after more than 12 hours of work, when I plotted my graphs and completed all my calculations, I found, to my knowledge, the first human GPCR that binds to fungal oxylipin! Everything that happened after that moment became blurry to me. Maybe I fell asleep in spite of the excitement, after exhausting all my nerves.

Together these results could suggest that *A. fumigatus* can produce oxylipins that steer disease outcome, or that humans sense *A. fumigatus* through its oxylipin. If this is the case, we may find why *A. fumigatus* is a more lethal pathogen in some people than others by analyzing human genetic variations in GPCRs. Maybe we can explain why certain variants of *A. fumigatus* are more devastating than others by examining their oxylipins. Maybe we can find such GPCR(s) and modify their actions to confer better resistance to *Aspergillus* infection. Maybe we can use oxylipins to treat aspergillosis and even other respiratory infections (Fig. 4B). What I know for sure is that the possibilities created by my scientific inquiries will become the next students' Ph.D. dissertation and reveal a new mechanism for fungal regulation of host immune response.

Fungal disease remains a globally neglected public health crisis while quietly posing a death toll to 1.5 million human lives annually[5]. 501 amphibian species have been endangered due to amphibian chytridiomycosis caused by a single fungal pathogen *Batrachochytrium dendrobatidis* in the past 50 years[6]. In the meantime, fungi bring wonders to human society. They have been turned into the most delicious cuisines, they are medicinal powerhouses, and they are the workhorses for the next generation of sustainable biofuels, biomaterials, and bioremediation agents to degrade human-made wastes[7]. The world of fungi has just started to be revealed and the

power of understanding the biology of fungi and the countless associations they make to the rest of the living world could be unfathomable.

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