The Role of the *Alternaria* Secondary Metabolite Alternariol in Inflammation

Shivani Grover

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science in Biological Sciences

Christopher B. Lawrence, Chair

Liwu Li

Stefan Hoops

December 2015

Blacksburg, VA

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ABSTRACT

Allergic inflammatory disorders of the airway like asthma and atopic asthma are complex, often long-term diseases that generate large public health and socioeconomic footprints especially in developed countries like US, UK and Australia. In 2009, approximately 8.2%, 24.6 million people in United States were affected by asthma. Currently 235 million people are affected by asthma worldwide and about 90% of those have allergic (atopic) asthma. An important factor in patients with allergic respiratory tract diseases is sensitization to fungi. Other risk factors for asthma include inhaled allergens that irritate the airways. Up to 70% of mold allergic patients have skin test reactivity to *Alternaria.* Alta1, an allergen produced by *A. alternata also* produces a prolonged and intense IgE mediated reaction in sensitized patients. Therefore *A. alternata* is not only a risk factor in development of asthma but also can lead to exacerbation of severe and potentially lethal asthma than any other fungus.

Despite the well-documented clinical importance of *Alternaria* in allergic airway diseases, little knowledge exists about the role of individual fungal genes and gene products in theses pathological states besides a small repertoire of allergens and proteolytic enzymes. Moreover, the importance of small, secreted molecules of fungal origin has not been explored whatsoever in regards to immune responses triggered by *Alternaria*. This study addresses the hypothesis that *Alternaria* derived small molecule's have immune modulatory properties. A major thrust of this project was to assess the role of *Alternaria* secondary metabolites that are synthesized by genes called polyketide synthases (PKS) in immune responses of lung epithelial cells.

ACKNOWLEDGEMENTS

First, I would like to thank my committee chair and advisor Dr. Christopher Lawrence for his guidance throughout my graduate education. I am grateful for all the opportunities he gave me, that have helped me grow as a scientist and also as a person. I am thankful for all his support and encouragement throughout this process. I will always carry the skills and values that I obtained from our work together.

Next, I would like to thank my fellow lab members. I would like to thank Brad Howard for always being there to teach me new techniques and discuss scientific ideas. I would also like to thank him for his guidance in navigating the steps of the graduate school. I would like to thank Tristan Hayes for encouraging me throughout the process of obtaining this degree. Finally, I thank both of them for making research fun during our time working together.

I would also like to thank my committee members Dr. Liwu Li and Dr. Stefan Hoops for their continued guidance, assistance and feedback on my research. I truly appreciate the time and effort they spent on helping me perform better.

Finally, I would like to thank my parents and my brother, for their eternal encouragement and support in my life. I would also like to thank my cousin, with whom I share a name, for all her support and encouragement through this process and always inspiring me to do better in life.

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LIST OF ABBREVIATIONS

- AhR: Aryl hydrocarbon receptor
- ARNT: Aryl hydrocarbon receptor nuclear translocator
- AZT: Azidothymidine
- AOH: Alternariol
- AME: Alternariol monomethyl ether
- ATS: Altenusin
- ATX: Altertoxins
- BEAS-2B: Bronchial lung epithelial cells
- CCL2: The chemokine (C-C motif) ligand 2
- cDNA: Complementary DNA
- CDK1: Cyclin Dependent Kinase 1
- CLL: Chronic lymphocytic leukemia
- DMSO: Dimethyl sulfoxide
- DPBS: Dulbecco's Phosphate-Buffered Saline
- DNA: Deoxyribonucleic acid
- ELISA: Enzyme linked immunosorbent assay
- FBS: Fetal bovine serum
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- GYEB: Glucose-yeast extract broth
- HPH: Hygromycin B phosphotransferase
- HYG: Hygromycin
- HT29: Human adenocarcinoma cells
- IL-1β: Interleukin-1 beta
- IL6: Interleukin 6

- IL8: Interleukin 8
- IgE: Immunoglobulin E
- LDH: Lactate dehydrogenase assay
- LPS: Lipopolysaccharide
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- MN: Micronucleus assay
- MLC: Mouse lymphoma cell line
- NAT: Nourseothricin
- NHBE: Human Bronchial Epithelial Cells from Normal and Diseased Donors
- PBS: Phosphate buffer saline
- PDA: Potato dextrose agar
- PKS: Polyketide synthase
- RNA: Ribonucleic acid
- ROS: Reactive Oxygen Species
- siRNA: RNA silencing
- SOT: Solid organ transplant
- TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin
- TEA: Tenuazonic acid
- TGF-β: Transforming growth factor beta
- Th2: T helper 2
- Th17: T helper 17
- TNF-α: Tumor necrosis factor alpha
- qRT-PCR: Quantitative Real Time Polymerase Chain Reaction
- XRE: Xenobiotic response element

CHAPTER I

Introduction to *Alternaria alternata*

Alternaria species are fungi widely distributed in nature. They can cause many plant diseases and are also weak parasites, saprophytes and endophytes. They are the principle contaminating fungi in wheat, barley and sorghum. They also occur in oilseeds such as sunflower, tomato, apples, olives and several other fruits and vegetables. *Alternaria* can grow at low temperatures and are a major contaminant and spoiler of food products. They produce many secondary metabolites that are toxic to plants and animals. *Alternaria* metabolites exhibit a variety of biological properties such as phytotoxicity, cytotoxicity and anti-microbial properties, which have generated considerable research interest worldwide. [1]

Alternaria metabolites, which are toxic to plants, are referred as phytotoxins and those toxic to animals are referred as mycotoxins. These secondary metabolites belong to different chemical classes like nitrogen containing compounds, steroids, quinones, pyrones, peptides, phenolics, and the fumonisin-like toxins. Some toxins also have disease prevention or treatment properties. For example, porritoxin from endophytic *Alternaria* species is a likely cancer chemo-preventive agent; depudecin from *Alternaria brassicicola* is an inhibitor of histone deacetylase. [2][1][3]

However, of all the mycotoxins known, only a few are subject to regular monitoring of contamination and level intake like alfatoxins, fumonisins, deoxyivalenol, zearlenone and ochratoxin A. Legal authorities from both food and feed industry acknowledge the importance of detecting the mycotoxin levels and identifying the effects of their contamination. [4]

The fungus *Alternaria alternata* is of the *Alternaria* genus and poses a major risk of causing diseases in humans and animals. It is also one of the most common airborne fungi. Up to 70% of mold allergic patients have skin test reactivity to *Alternaria*. There is a direct association between this fungus and asthma including increasing IgE levels. [5]

Allergic inflammatory disorders of the airway like asthma are complex, often long-term diseases that generate large public health and socioeconomic footprints. In 2009, approximately 8.2%, 24.6 million people in United States were affected by asthma. An important factor in patients with allergic respiratory tract diseases is sensitization to fungi. Alta1, an allergen produced by *A. alternata* produces a prolonged and intense IgE mediated reaction in sensitized patients. Therefore *A. alternata* is not only a risk factor in development of asthma but also can lead to exacerbation of severe and potentially lethal asthma than any other fungus.[6][7]

Thus, the prevalence of fungal allergies is much greater than expected and understanding of pathologies of such diseases has been slow.[5] Elucidating the effects of such fungi and their secondary metabolites on humans and animals will help shed light on the disease mechanism and underlying processes that drive them.

Alternaria alternata Mycotoxins

Alternaria species produces more than 70 phytotoxins but a small proportion of them are categorized as mycotoxins and act on human and animals. A few examples of these toxins from *A. alternata* include alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TEA) and altertoxins (ATX). Of these, AOH, AME and TEA are of particular interest as they are the major contaminants of most food and feed products and are known to have severe genotoxic and cytotoxic properties.

Currently, there are no regulations on *A. alternata* toxins in food and feed in the world. AOH and AME are generally found in grains like wheat and barley and grain based products, legumes, tomato and tomato products, sunflower seeds and sunflower oil, fruits and fruit products and in beer and wine. [1] Furthermore, mycotoxicosis is an important health problem in mild, humid and temperate climates and tropical countries as these places favor the growth of the mold *A. alternata* on food products, which are then consumed by humans and animals. In apple juice and other fruit beverages, AOH is found at concentrations ranging from less than 1 ηg/ml up to 6 ηg/ml, which corresponds to a concentration of 0.03μM. However, as of yet, no data concerning tissue levels of AOH exists in animals and human. [8]

Biosynthesis of Alternariol

In 2012, Fischer et al identified polyketide synthase J (PksJ) of the polyketide cluster as the gene responsible for the formation of AOH in fungi. PKSJ is a 2225 amino acid long, multi-domain and multi-functional protein. [9] The chemical structure of AOH is given in Figure 1. The compound is a dibenzopyrone derivative. The biosynthetic pathway for AOH synthesis is described in Figure 2. The pathway consists of malonate as building blocks with claisen-type condensations. [9] A polyketide biosynthesis pathway is a common route for the synthesis of many fungal secondary metabolites like AOH. Polyketides synthesized by *A. alternata* display a variety of distinguished structural features and biological activity like the benzopyrone ring. Not much progress has been made on gene level characterization of these mycotoxins. Fischer et al (2012) provided one of the first reports on genes responsible for biosynthesis of *Alternata* toxins especially AOH.

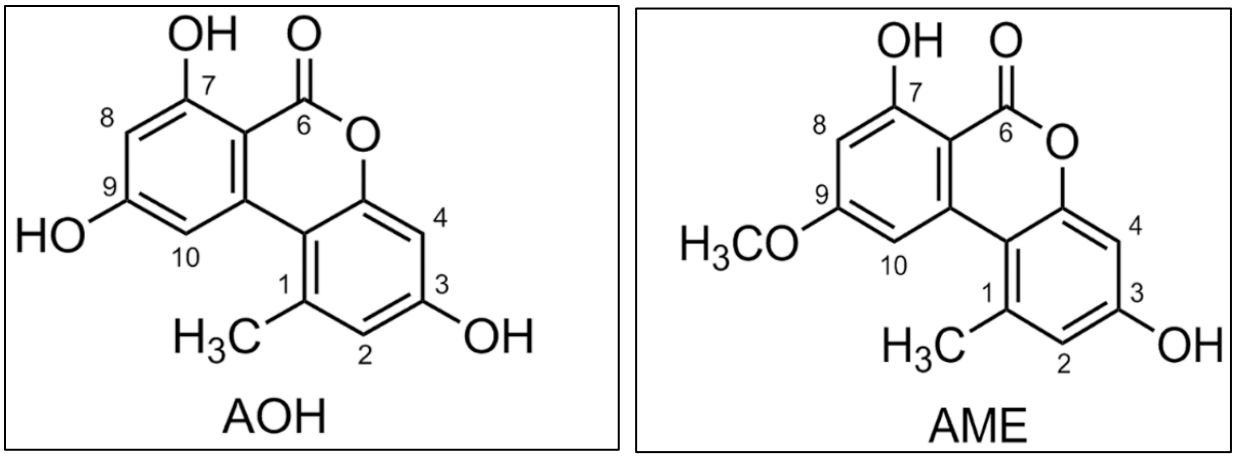


Figure 1. Chemical structure of alternariol (AOH) and alternariol monomethyl ether (AME). The compounds are dibenzopyrone derivatives. The one difference between the two mycotoxins is the methyl group at the 9th carbon atom. [67]

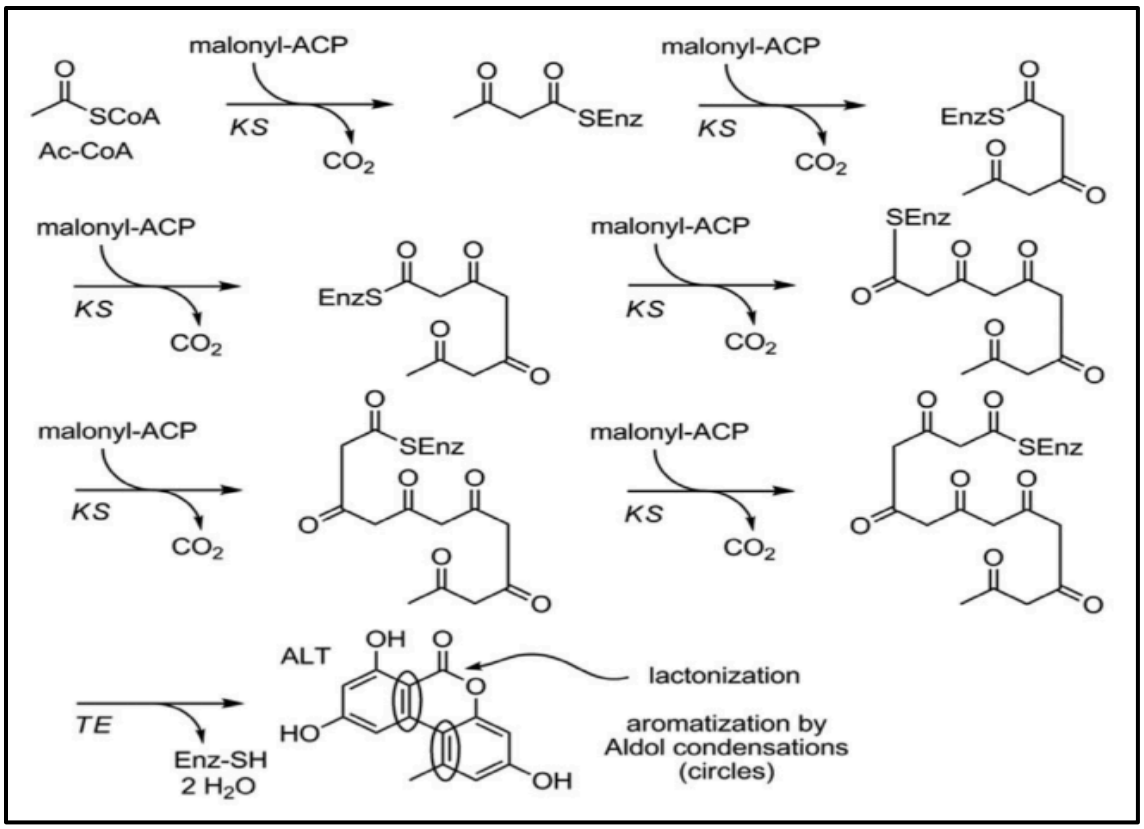


Figure 2. The biosynthetic pathway of alternariol and alternaria monomethyl ether production. [9]

Polyketide synthase J (PksJ) was identified to be the gene responsible for formation of AOH in fungus based on gene deletion and RNA silencing strategies. PKSJ is a 2225 amino acid long multifunctional protein. Gene expression levels of PksJ were highest at the seventh day, which fitted onto the growth pattern of AOH when compared to other Pks genes.

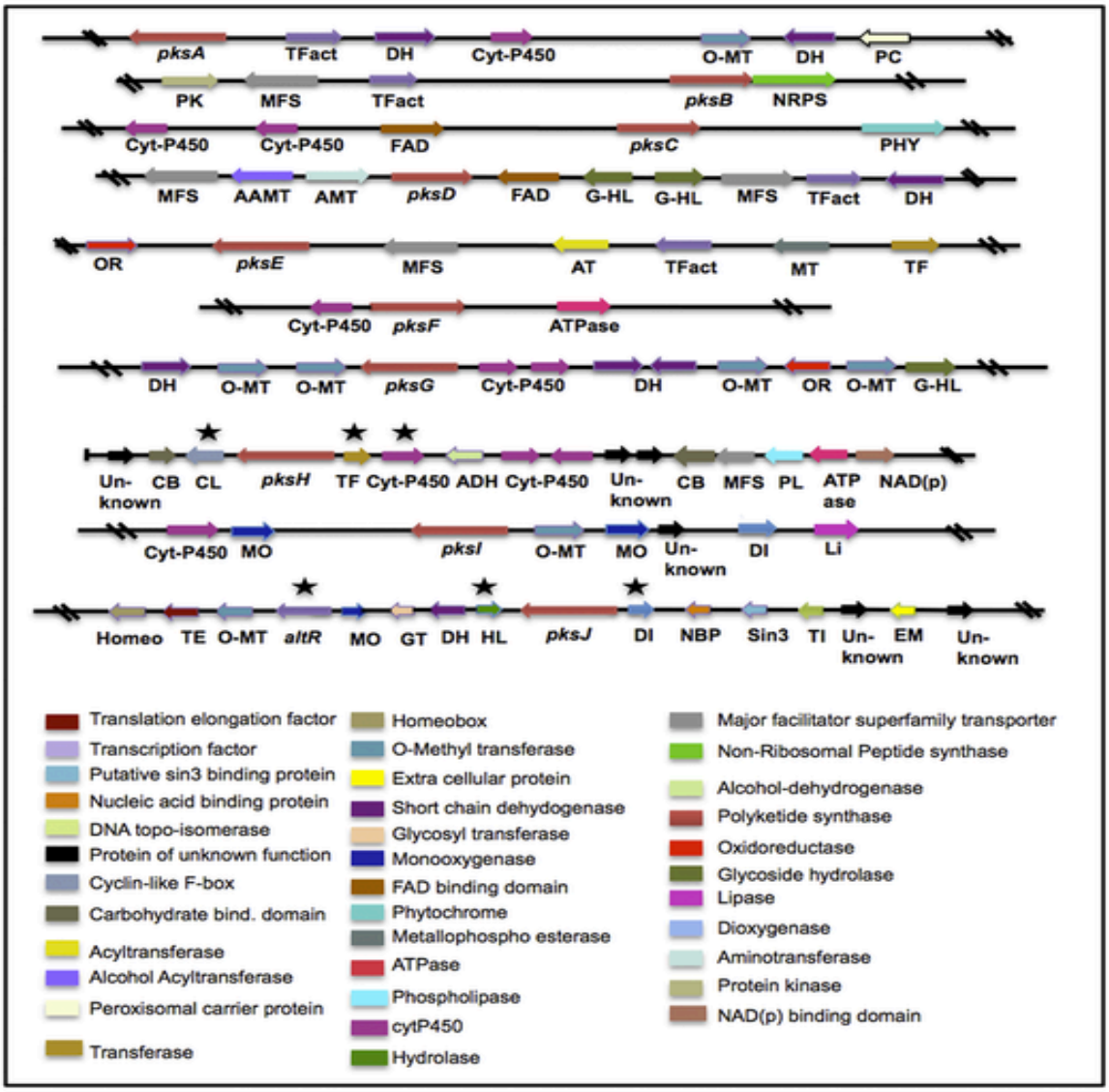


Figure 3. Predicted organization of polyketide biosynthesis gene clusters in *Alternaria alternata*. Each arrow indicates the direction of transcription deduced from the analysis of the nucleotide sequences. The genes are color coded according to domain patterns.[9]

Several genes are involved in the biosynthesis of a particular mycotoxin. More commonly, these genes are clustered together in the genome. A predicted architectural map of several transcription factors in the PKS cluster in *A. alternata* genome is given in Figure 3. [9]

Alternariol in Mammalian Cell Culture

The research on AOH advanced further in the 21st century. The very first experiments with pure AOH were carried out on chinese hamster V79 cell lines and human endometrial adenocarcinoma cells (Ishikawa cells). These were the first reports on the estrogenic and genotoxic potential of AOH. The genotoxic potential was further assessed by a micronucleus (MN) assay. Pronounced indication of MN in V79 cell line and slight induction in Ishikawa cells was demonstrated. Decrease in cell proliferation was also observed.[10]

A chicken embryo assay was conducted to measure the toxicity of *Alternaria* toxins including AOH. It was concluded that at maximal doses of 1000µg of AOH per egg, there was mortality or teratogenicity in the embryo.[11] Continued toxin studies were carried out on a test system on the mutagenic effects of AOH on chinese hamster V79 cells line as well as mouse lymphoma cell line (MLC). It was observed that viable cells depended on the concentration and plating efficiency, after treatment of both cell lines with up to 30µM of AOH for V79 cells and up to 20µM for MLC cells for 24 hours. This treatment reduced the number of viable cells to 35% in V79 cells and to 69% in MLC cells. There was also an increase in number of cells arrested in the G2/M phase (proliferation decrease) of cell cycle from 15% to approximately 62%, in AOH treated cells. Likewise in MLC, the rate increased from 21% to 37% indicating a cellular stress response. The findings suggested that there is not a complete block of cell cycle by AOH but a short reversible arrest in S phase with a delay in G2/M phase of the cell cycle. Extensive mutation frequency has also been observed, even at very low AOH concentrations (10µM) at HPRT gene locus (via Hypoxanthine-guanine phosphoribosyltransferase-measurement of

cytotoxicity assay) in V79 cells and in TK (via thymidine kinase assay) and locus in MLC cell lines supporting the hypothesis that it is a mutagen. [8]

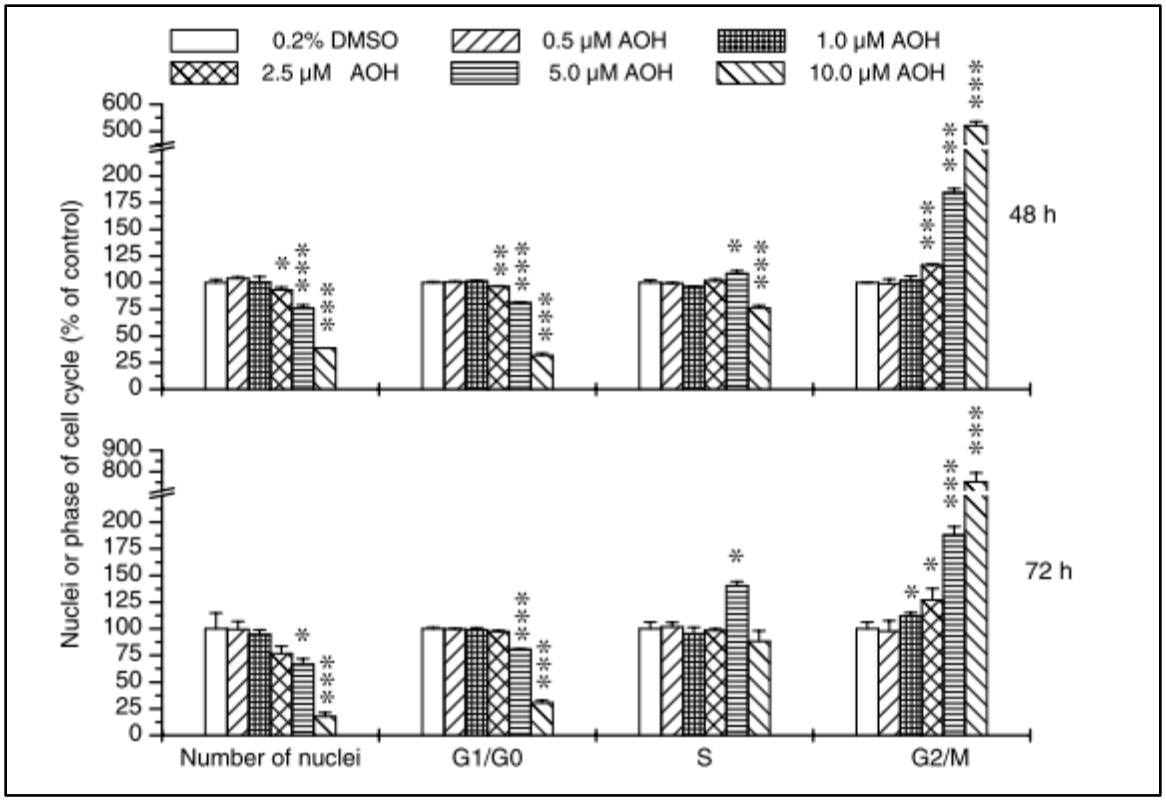


Figure 4. Cell cycle distribution of Ishikawa cells after treatment with various doses of AOH for 48-hours. [10]

Nitrosylation reactions are common in gut in also in food preserved with nitrite. The examination of the effect of nitrosylation on mutagenicity of *Alternaria* toxins showed some specificity to base pair mutagens at AT sites and not GC. Nitrosylated AOH showed increased direct acting mutagenicity at AT sites along with less toxicity in the examined cell lines. The treatment was conducted on *Ames Salmonella* mutation responsive strains and suggested production of reactive oxygen species and consequently oxygen damage in cellular systems and an opportunity to explore further. [12]

To study the effects of AOH on the reproductive performance in pigs, porcine granulosa cells were treated with AOH. It was documented that AOH decreased progesterone synthesis along with the viability and number of cultured porcine granulosa cells. Cell viability was more affected than cell number resulting in the conclusion that the toxin inhibited metabolic activity and proliferation rather than caused cell death. The concentration of toxin used was 12.5μM for a 24-hour treatment. The AOH toxin also had an inhibitory effect on the steroid progesterone. This conclusion was not a result of cell death because, after the removal of the toxin, the progesterone production reached levels that were equivalent to the untreated control. This suggests that AOH has inhibitory effects on follicular development and interferes with reproductive performance in swine and possibly other mammals. [13]

The studies conducted on *Alternaria* toxins continued in 2012. Hepa-1 cells were treated with pure AOH. It was documented that the metabolism of the toxin is affected by glucuronidation. More cell cycle arrest was observed in cells with beta-glucuronidase, which hydrolyzed the glucuronides generated, by the cell. This provides an excellent example that the metabolic fate of a toxin is an important determinant of the effects observed in vitro. Addition of beta-glucuronidase provides an excellent method for treatment with cell line with high activity of glucuronide formation. [14]

Both AOH and TEA caused significant damage to human adenocarcinoma cells (HT29). Increased oxidative stress signal in the HT29 cells analogous to the concentration of toxin in the culture was found. This represents the genotoxic potential of *Alternaria* toxins. [15] Further investigation on the genotoxic potential of AOH and whether oxidative stress contributes to it or not, revealed that while the toxin modulates ROS levels, it is completely unrelated to the DNA

damage levels in human adenocarcinoma cell line (HT29). It was further demonstrated that after treatment with AOH, cell cycle arrest takes place in the G2/M phase in HT29 cells. [16][17]

Intestinal systems are one of the primary targets of the *Alternaria* toxins including AOH and TEA. Human colon carcinoma cells were used to elucidate the mode of cell death mode utilized by AOH. A decrease in cell viability was observed in a dose dependent manner with doses ranging from 0μM to 200μM. Apoptotic cell death was also observed through p53 and caspase dependent pathways. Furthermore, apoptosis is triggered by mitochondrial intrinsic pathway resulting in loss of ionic homeostasis, matrix swelling and outer membrane rupture. Production of reactive oxygen species (ROS) following treatment of cells with AOH was not due to an early step in apoptosis but rather a late step, and due to mitochondrial alterations. These alterations may amplify the apoptotic process.[18]

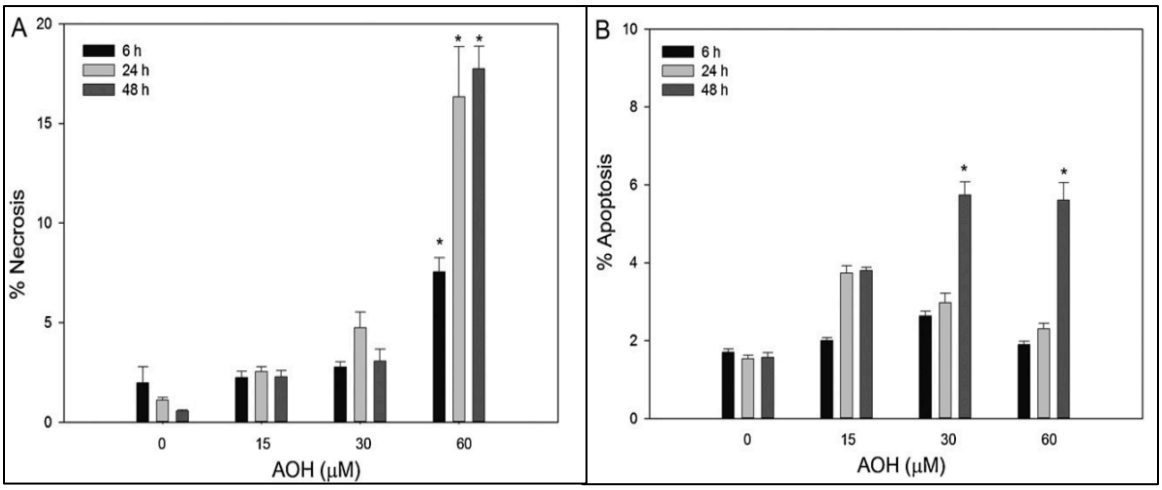


Figure 5. AOH induces cell death by necrosis. Murine cell lines (Raw 264.7) were treated with AOH doses from 0-60μM for 6hr, 24hr and 48hr. Increasing rate of cell death was observed.[19]

To understand the mechanism of action further, murine macrophage RAW 264.7 cells were treated with AOH. It was observed that AOH causes cytotoxicity. DNA strand breaks were found

as well as oxidative damage and cell cycle arrest, even though the oxidative damage was not directly linked to cell cycle arrest. [19] These findings support the previous study conducted by Lemaire et al (2012). [18] In this study, the investigators suggested ROS as a secondary product and not a primary response to inflammation. AOH also was found to have a cytotoxic effect on cultured *Glycine max* (soybean) cells and not just mammalian cells. [20]

All the studies discussed here suggest that AOH is a cytotoxic and genotoxic mycotoxin. Furthermore, among all the other toxins of *A. alternata*, AOH is a major food and food product contaminant whose mechanism of action needs to be elucidated. Hence, it can be hypothesized that AOH may play a role in inducing inflammation and further accentuate the IgE mediated effects of the major *Alternaria* Alt a 1 allergen in allergic airway disorders such as asthma. [21][22] Further investigations need to be carried out to fully characterize its effects and understand its mechanism of action.

Alternaria alternata and Tenuazonic Acid

In 1983, Griffin et al conducted a study of effects of *Alternaria* toxins including AOH on chicken embryo.[11] The chicken embryo assay was used as a measure of toxicity of selected mycotoxins. They concluded that secondary metabolite TEA induced embryonal death but no teratogenic affect over a dose range of 150 to 1500µg per egg.[11]

The toxic effects of TEA were studied on esophagus of mice. Forty 6-week old Swiss albino mice were given a dose of 25mg/kg/day of TEA and continued for 10 months. The results showed weight loss in mice after treatment with TEA. Electron microscopic examination of mice esophageal epithelia showed moderate to severe dysplasia, loss of nuclear polarity and pleomorphism in all the cells. A significant number of lesions were also noted on the esophageal mucosa of TEA treated mice compared to the control group. Furthermore, continuous exposure

of animals to TEA for 10 months resulted in precancerous changes in esophageal mucosa. Therefore, progression to esophageal cancer may occur with long-term exposure of mycotoxin TEA.[23]

The anti-carcinogenic potential of TEA was investigated on female Swiss albino mice. The mice had induced skin carcinogenesis. The animals treated with TEA had a longer period before development of tumor compared to the control. This may be due to TEA's ability to inhibit ornithine decarboxylase, which has an important part in tumor promotion. This indicates TEA's anti-carcinogenic potential though the complete mechanism has yet to be elucidated.[24]

Treatment of porcine granulosa cells with TEA resulted in the conclusion that TEA is not as active as AOH in reducing progesterone synthesis in the cells. This could be due to the difference in their chemical structures as AOH is a dibenzopyrone and TEA is a tetramic acid derivative. Furthermore, higher concentrations of AOH along with high AME and lower concentration of TEA resulted in a much stronger reduction in progesterone synthesis. This suggests that AOH is much stronger than TEA in influencing metabolic growth and in follicular development in swine even though other studies have suggested that TEA is more cytotoxic.[13]

Hence, TEA has higher toxicity levels than AOH. A single study suggested a link to the development of esophageal cancer, however TEA also has cytotoxic properties towards certain types of cancer cells. Further elucidation of its mechanism of action would shed more light on this phenomenon.

Alternaria alternata and Altenusin

Little is known about altenusin (ATS) except that it is a very unstable compound.[25] ATS showed marked DPPH radical scavenging activity at the IC50 value of 17.6 ± 0.23. It has moderate cytotoxic activity (IC50 25-35μM) when treated on HCT116 cancer cell line. Cytotoxic activity was measured by a sulforhodamine B (SRB) colorimetric assay.[26] ATS also has strong antimicrobial activity shown in a dilution assay against several drug-resistant pathogens (*E. coli, Enterococcus faecium, Enterococcus cloacae, Staphylococcus aureus, Streptococcus pneumonia, Pseudomonas aeruginosa, Klebsiella pneumonia, Candida albicans, Candida krusei, Aspergillus faecalis, and Aspergillus fumigatus*) at the minimal inhibitory concentration (MIC) of 31.25, 31.25, 62.5, 125, 62.5 and 125μg/ml respectively.[27] It is also a weak inhibitor of myosin light chain kinase (IC50=340μM), sphingomyelinase (IC50=28μM) and has moderate HIV-1- integrase inhibitory activity.[28] It also inhibits cell wall synthesis in *S. pombe and* has potent synergistic activity against *C. albicans* and thus, can be a potential anti-fungal lead compound.[29][30]

Alternaria alternata and Altertoxins

Altertoxins are some of the most abundant toxins produced by *Alternaria*. Presently there are 5 analogs (I- V) whose structures have been elucidated. They have acute toxicity and chronic effects that have not been elucidated yet. They are known inhibitors of HIV-1 virus with an activity more potent than even AZT.[31] They are mutagenic (Ames test) and are genotoxic and apoptotic. HT29 cells showed substantial DNA damage with the induction of formamidopyrimidine DNA glycosylase (FPG)-sensitive sites. A significant increase of cell cycle arrest at G0/G1 phase and inhibition of cell proliferation at 24 hours by a sulforhodamine B assay were also observed. Altertoxins are 50-times more potent cytotoxic and DNA damaging molecules in chinese hamster V79 cell lines than AOH.[32][33][3]

Aryl Hydrocarbon Receptor (AhR)

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that controls the expression of various environmental toxins most of which are man made contaminants. It has been studied in relation with various environmental contaminants like the xenobiotic TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Binding of the AhR to the ligand causes the translocation of the complex to the nucleus to bind with AhR nucleus translocator (ARNT). The AhR-ARNT complex then binds to various xenobiotic response elements (XRE's) and causes induction of various genes like the cytochrome P450 family. AhR is also involved in cell proliferation, differentiation and cytokine secretion. Several inflammatory response-related genes contain potential XRE boxes in their 5' flanking region. [34]

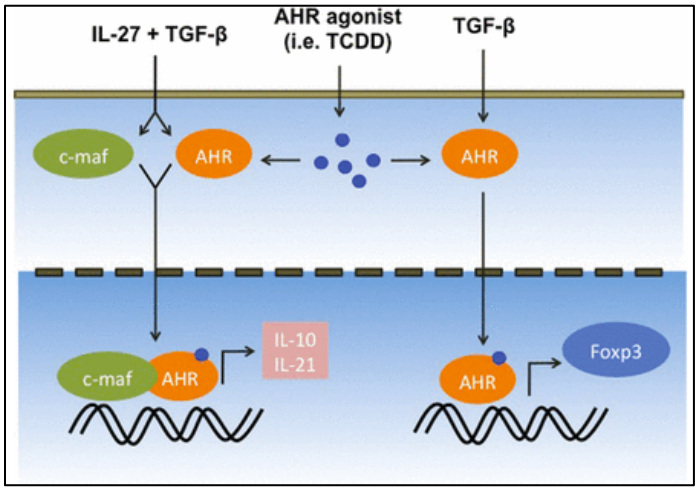


Figure 6. T cells polarizing conditions trigger the expression of transcription factors like AhR. It also interacts with Foxp3 promoter under Treg cell polarizing condition. [26]

AhR is usually considered an orphan receptor given that no endogenous ligand for it has been identified till now. The only endogenous role that has been identified for it is activation of drug metabolizing enzymes. Dietary substances can also readily activate AhR. Their ligands can act as both agonist and antagonist such as reseveratrol and galangin. As inflammation leads to suppression of drug metabolizing enzymes like cytochrome P450 family, the cytokines such as IL6, IL-1β, TNF-α and endotoxin LPS reduce AhR-ligand induced CYP1A1 activity in Hepa-1 cells yet don't alter the amount of AhR-ARNT bound to the promoter. This has been demonstrated with the

herbicide TCDD. AhR is also expressed under Th17 cell-polarizing conditions in which IL6 and TGF-β are markers but not in response to either cytokine alone.[34][35]

AhR also binds to environmental pollutants like automobile exhaust, tobacco smoke and industrial pollutants and causes their detoxification through UDP-glucuronosyl transferases and several CYPs. AhR and its deregulation of the main target for ligand-gene induction, CYP1A1, mediate the toxicity of these ligands. CYP1A1's xenobiotic metabolism results in the conversion of pro-carcinogen to carcinogens but can also protect many organs from carcinogens.[36]

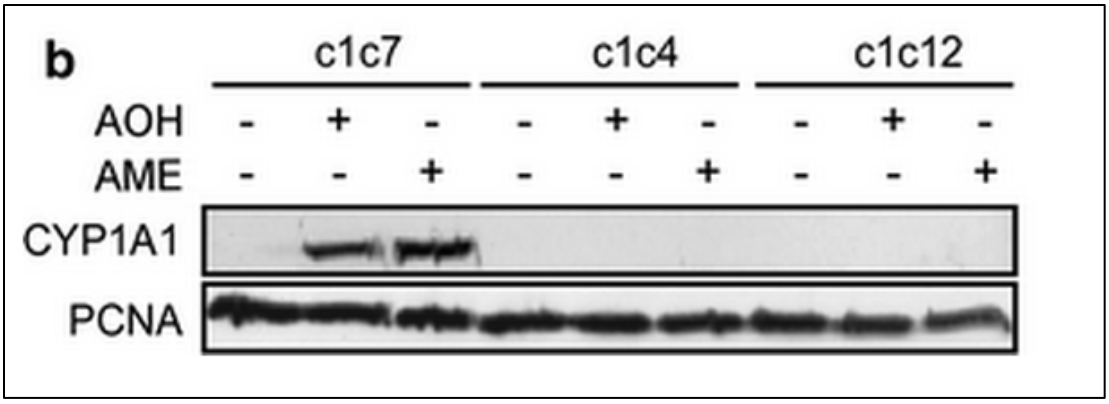


Figure 7. Effect of treatment of AOH on CYP1A1 induction in murine hepatoma cells with activated and inactivated AhR. The mouse hepatoma cells Hepa-1c1c7 have the functional AhR and the other two cell lines Hepa-1c1c4 and Hepa-1c1c12 are deficient in ARNT and AhR, respectively. In the presence of 40μM AOH and its derivative, AME for 24 hours, CYP1A1 was induced, while no expression was observed in AhR and ARNT deficient cell lines. [37]

Aryl Hydrocarbon Receptor (AhR) and Alternariol

The *Alternaria alternata* secondary metabolite AOH is a potential carcinogen. CYP450 family of genes is a major target of AhR-ARNT complex and mediates their hydroxylation and further metabolism. The highest expressed gene of the CYP450 family is CYP1A1. It has a highly continuous expression in lung and esophagus. AOH is a substrate of CYP1A1 and has a planar

structure that is similar to other AhR ligands. Since AOH is of major interest in inflammatory responses in lung cells, it can be hypothesized that it is xenobiotically metabolized by AhR. Further evidence of this was provided by treatment of AOH on murine hepatoma cells and measuring the expression of CYP1A1 in presence of activated and inactivated AhR. It was noted that the AhR induction of CYP1A1 did not mediate the main cytotoxic effect of AOH, but decrease in cell number and apoptosis in the presence of AOH is regulated by this receptor. [37]

Alternaria Infections in Immune-compromised and Transplant Patients: A Review of Case Studies and Treatment Methods

Alternaria species are fungi widely distributed in nature. As opportunistic pathogens, they can cause many plant diseases. They are also weak parasites, saprophytes and endophytes. The species is the principle contaminating fungi in several food and food products. *Alternaria* spores are the predominant spores in the atmosphere and act after inhalation. Fungal spores concentration in the atmosphere is 1000-fold more than pollen and can cause prolonged exposure overtime [6,38]. Its spores, while preferring warm and humid climate, can also grow at low temperatures and thus pose a major risk to humans and animals [1,2,39,40].

Alternaria alternata is a saprophyte and is also known to cause many opportunistic infections in humans. *Alternaria* infections are also important factors of morbidity and morality in immune-compromised and solid organ transplant (SOT). *Alternaria* is a common genus for invasive infection in transplants patients. About 33.3% of transplant patient die due to the fungal infection [41]. In this group, lung graft patients have the highest incidence of fungal infections. A table of incidence of fungal infections in organ transplants is given in Table 1.

A. alternata can cause invasive infections such as keratomycosis, cutaneous alternariosis, paranasal sinusitis, granulomatous pulmonary nodule, peritonitis and phaeohyphomycosis. *A. alternata* can also affect patients that are immune-compromised by HIV. A 31-year-old man with AIDS developed necrotic lesions in nasal septum due to the fungus *A. alternata*. The patient was effectively treated with surgical excision and amphotericin B. This suggests the importance of innate cell-mediated immunity in host defense against this organism [42].

Solid Organ Transplant	Rate of Incidence (%)
Lung	7.9
Heart	3.4
Liver	3.1
Kidney	1.1
Pancreas	0.7

Table 1. Rate of Incidence of fungal infections in solid organ transplant. [43]

Alternaria alternata and Cutaneous Infections

Nearly 4.5-6% of organ transplant patients are prescribed with tacrolimus. However, this application is not without risks as about 66-67% of patients developed fungal infections due to its usage. The incidence of *Alternaria* fungal infections has increased the mortality rate of the patients. Cutaneous alternariosis is an opportunistic infection that occurs in patients being treated with systemic corticosteroids and in a few rare cases in patients with HIV. High cortisol levels induce fragility in cutaneous lesions that permit direct infections from fungi like *A. alternata* and *A. infectoria* [44]. The treatment methods are also not standardized and can be

difficult. *A. alternata* is also reported to be partially unresponsive to amphotericin B, miconazole, itraconazole, ketoconazole and imazalil [45].

Patients with cutaneous *A. alternata* infections (Alternariosis) and on tacrolimus monotherapy show poor response to surgical excision and itraconazole alone. Reduction of immunosuppressive drug dosage provides better results. In *Alternaria* infections surgical excision followed by treatment with amphotericin B provides a more effective therapy. Voriconazole provided an effective treatment response to *A. alternata* skin lesions in liver transplant patients as seen in a 62 year old patient with hepatic cirrhosis with a history of hepatocarcinoma [46]. *Alternaria* infections are also harder to diagnose based on histopathology or morphology alone. DNA testing provides a more effective diagnosis [47].

Another 60-year old male patient reported skin lesions nine months after a heart transplant due to dilated cardiac myopathy with an underlying squamous cell carcinoma. The lesions were later identified to be *A. alternata* hyphae. *Alternaria* was also cultured from the broncho-alveolar lavage in the left lung with computed tomography angiography after a progressive dyspnea was reported. The patient was first treated with reduced tacrolimus, an immunosuppressant, levels and daily dose of 400mg voriconazole and then changed to 800mg posaconazole upon persistent infection in the lung. The treatment was effective and no relapse was seen after 2 months [48].

A 56-year-old cardiac transplant patient developed an *Alternaria* skin infection 9 months after surgery. This case illustrates the difficulties in treating invasive *Alternaria* infections and a unique case of treatment of fungal infections with curettage and cautery in absence of anti-fungal therapy. Initial treatment of oral fluconazole 200mg for 5 weeks was unsuccessful. One year after onset of skin infection, skin biopsy showed progression with hyperkeratosis and pseudo-epitheliomatous hyperplasia with a dermal granulomatous infiltrate. After unsuccessful

treatment with itraconazole, intravenous methylprednisolone and an increased dose of tacrolimus and mycophenolate mofetil, the infection was treated with curettage and cautery and double freeze-thaw cryotherapy. [49]

Alternaria infections are also common in children when on an immunosuppressive regimen as in the case of a 12-year-old male patient with Fanconi's anemia was reported to have an *Alternaria* infection 33 days after allogeneic hematopoietic stem cell transplantation. An anti-fungal prophylaxis treatment was performed with 600mg posaconazole orally and caspofungin for 4 days before the transplant. Skin biopsy of the nodules seen in the lower limb identified them as invasive *A. alternata* hyphae infection as the culprit. A treatment combination of posaconazole and liposomal amphotericin B provided complete resolution of skin lesions. These results raise the question of most appropriate drug for prophylaxis treatment as well as the importance of the synergy of several drugs for treatment of *A. alternata* infections. [50]

Cutaneous infections with *Alternaria* usually occur on the extremities. Invasive fungal infections by *A. alternata and A. infectoria* are becoming more common as the rate of organ transplants grow along with increased use of immune suppressive regimens. In chronic lymphocytic leukemia (CLL), the patient is heavily immune compromised. CLL itself is associated with immune deficiency due to loss of both cell mediated and humoral immunity. A 58-year old male farmer was admitted complaining of fever, rigors and night sweats with a greenish blue nodule on the right hand. With prior history of chemotherapy and immunotherapy due to CLL, the patient was at considerable risk of death by an opportunistic infection. The fungal elements on the nodule were identified to be *A. alternata*. The nodule invaded the subcutaneous tissue and had to be surgically removed. The surgical bed was then irrigated with amphotericin B. Oral anti-fungal's like voriconazole and posaconazole failed to have any effect prior to surgery. In soft tissue infections like this, medical therapy seems to be failing in treating an aggressive fungal infection. This case suggests that a combination of surgical and anti-fungal therapy is

recommended for immune compromised patients for successful outcomes. Identifying the fungal species is also very important for optimal treatment of systemic infections [51].

Another 65-year old male liver transplant patient developed an invasive *A. infectoria* infection. The patient was successfully treated with fluconazole. A combinatorial therapy comprising of anti-fungal azole based drugs and a reduction of immune suppressive drugs seems to be the corner stone for invasive fungal infections in solid organ transplant patients [52].

Persistent thermotherapy was applied in the rare case of a patient with a subcutaneous infection with an underlying history of renal transplant. Amphotericin B could not be used because of the potential renal toxic effects. Warmth therapy proved to be more effective in this case and the fungal colonies were reduced after six months of therapy [53].

Alternaria alternata and Phaeohyphomycosis

A 65-year-old male Caucasian patient with a history of a liver transplant within 4 months and under immunosuppressive therapy reported nodules on the right leg and dorsal of the left hand. Microscopic analysis identified the biopsy isolates as *Alternaria* spp. even though there was slight difference in the biopsy material from the hand and the leg. Molecular sequencing and corresponding analysis identified *A. alternata* as the species in the leg and *A. infectoria* as the species in the hand. The infection was defined as Phaeohyphomycosis and is one of the first cases of cutaneous co-infection with two different species of *Alternaria* in the world. The patient treatment consisted of surgical excision and oral itraconazole. No relapse was reported. [43]

Phaeohyphomycotic infections are also increasing prevalent in immune compromised patients. It manifests clinically as lesions or ranges up to disseminated infections. Treatment options

involve Itraconazole for subcutaneous infections but if the infection is systemic, amphotericin B is required [54].

Alternaria alternata in Corneal Transplants

Keratomycosis was detected in 21 cases of infection of the eye. All of the cases were limited to cornea. After a corneal transplant, a 53-year-old Japanese woman was reported to have contracted an ulcer in the right eye. *A. alternata* was detected in the culture of the ulcerated tissue. Five drugs were used for treatment: Thimerosal, Pimaricin, Amphotericin B and Nystatin. Out of these, Thimerosal was most effective. [55]

Another case of *Alternaria* associated keratomycosis was reported in a 66-year old female patient with the corneal transplant of the right eye. A second keratoplasty was performed as the consequence of corneal melting by the fungal infection. A local and systemic anti-fungal treatment resulted in complete resolution of the fungus and minimized the risk of permanent eye loss. [56] A record of opportunistic infections caused by *Alternaria* species is given in table 2.

Patient Details	*Alternaria* infection	Immune Defect	Treatment	Outcome	Ref.	Organism
65/M	Phaeohyphomycosis	Liver transplant, Tacrolimus immune suppressive therapy and diabetes	Itraconazole	No Relapse	[43]	*A. alternata and A. infectoria*
31/M	Visceral and mucosal infections	AIDS	Amphotericin B	No Relapse	[42]	*A. alternata*
62/M	Cutaneous	Liver Transplant	Voriconazole	No relapse	[46]	*A. alternata*
66/M	Cutaneous	Liver Transplant due to hepatic carcinoma	Surgical excision, Tacrolimus	No relapse	[47]	*A. alternata*
60/M	Cutaneous and Pulmonary infection	Heart transplant due to dilated cardiac myopathy	Posaconazole	No Relapse	[48]	*A. alternata*
55/M	Cutaneous alternariosis	Cardiac Transplant	Intravenous methylprednisolone, Tacrolimus, Cryotehrapy,Curettage and cautery	Recurrent	[49]	*A. alternata*
12/M	Invasive Alternariosis	Allogeneic hematopoietic stem cell transplantation for Fanconi anaemia	Posaconazole and amphotericin B	Recurrent	[50]	*A. alternata*
53/F	Keratomycosis	Corneal Transplant	Thimerosal, Pimaricin, Amphotericin B and Nystatin	No Relapse	[55]	*A. alternata*
66/F	Keratitis	Corneal Transplant	Keratoplasty, cefazolin	No Relapse	[56]	*A. alternata*
70/M	Cutaneous	Cadaveric renal transplantation , ulceration and vascular graft rejection	Itraconazole	cerebrovascular accident	[45]	*A. alternata*
58/M	Progressive subcutaneous infection	Chronic Lymphocytic Leukemia	Surgical Excision and Posaconazole	Recurrent	[51]	*A. alternata*
65/M	Multiple crusty ulcerative skin	Liver Transplant	Fluconazole	No Relapse	[52]	*A. Infectoria*

	lesions					
55/M	Cutaneous	Renal Transplant	Thermotherapy	No relapse	[53]	*A. alternata*
61/M	Cutaneous alternariosis	Renal Transplant	Amphotericin B wet-packing and systemic anti-fungal therapy with oral voriconazole	Recurrent	[57]	*A. alternata*
10/F	Rhinocerebral zygomycosis	Allogeneic stem cell transplantation for severe aplastic anaemia	Surgical Excision, liposomal amphotericin B and posaconazole	No Relapse	[58]	*A. alternata*
47/M	Cutaneous Alternariosis	CREST (calcinosis, Raynaud's phenomenon, oesophageal dysfunction, sclerodactyly and telangiectasia) syndrome with pulmonary hypertension	itraconazole	No Relapse	[59]	*A. alternata*
6/M	Granulomas with fungal elements	Aplastic anemia presented with generalized erythematous papules	Anti-fungals	No Relapse	[60]	*A. alternata*

Table 2. A summary of case studies involving invasive infections caused by *Alternaria* species and their underlying defect.

Statement of Objectives

Hypothesis: *Alternaria alternata* is involved in development and exacerbation of allergic airway disorders including asthma, chronic rhinosinusitis and rhinitis. Several *Alternaria* secondary metabolites have been shown to have cytotoxic and genotoxic properties. We hypothesize that *A. alternata* toxins alternariol (AOH) and alternariol monomethyl ether (AME) induce potent inflammatory responses in mammalian bronchoalveolar epithelial cells and mouse macrophages.

Specific Aim 1: **Investigate the dynamic interplay between the inflammatory/anti-inflammatory properties of *Alternaria* secondary metabolites and immune barriers in mammalian respiratory mucosa in vitro.** Here, we aim to explore and further advance the knowledge about the cellular and innate immune responses to AOH and AME. Human lung epithelial and mouse macrophage cells will be utilized to characterize the response. Our approach will involve mRNA and protein level quantification and analysis.

Specific Aim 2: **Elucidate the cellular mechanism of action of *Alternaria alternata* secondary metabolites in mammalian lung epithelium in vitro.** In order to initially investigate the receptor mechanism of AOH mycotoxin metabolism, we will investigate the role of the aryl hydrocarbon receptor (AhR). AhR is the primary target for xenobiotic metabolism in vertebrates. We will utilize an RNA silencing approach in human lung epithelial cells and mouse hepatoma cell lines with knockout strains of AhR and related protein ARNT to elucidate the mechanism of action of the AOH immune-modulatory response. Additionally, Pks-encoding genes identified in the draft genome of *A. alternata* are postulated to be the primary candidates of mycotoxin production in this fungus. We will knock out the PksJ gene shown previously to be responsible

for AOH and AME production. We will investigate the innate immune responses in lung cells generated by wildtype and pksJ gene disruption mutants.

CHAPTER II

The Effects of *Alternaria alternata* Metabolite Alternariol Exposure on LPS Induced Inflammation in Human Lung Epithelial Cells

Abstract

Sensitivity to the airborne fungus *Alternaria alternata* (common mold) is believed to be a common cause of allergic asthma. Epidemiological studies worldwide indicate that *Alternaria* sensitivity is closely linked with the development and exacerbation of allergic airway disorders such as asthma, allergic rhinitis, and chronic rhinosinusitis. Therapies are limited by the lack of knowledge about the role of individual fungal gene products in airway responses. The *Alternaria* mycotoxin alternariol is a genotoxic and cytotoxic molecule and a major contaminant of most food, grains and feed products. We hereby present a study where we explored the hypothesis of alternariol having immune-modulatory properties. It led us to the discovery that alternariol is strongly anti-inflammatory. We have investigated alternariol's dynamic interplay between innate immunity and human bronchoalveolar epithelial cells. Dose dependent immune assays have provided the key doses needed to suppress LPS-induced inflammation. We have also profiled the response of putative mutant fungal spores that no longer produce alternariol in comparison to the wild type spores on lung epithelium. This model has also been used to investigate alternariol's mechanism of action in relation to aryl hydrocarbon receptor. Greater understanding of the role of alternariol in allergic asthma may lead to improved treatment strategies as well as a potential drug target for inflammatory syndromes.

An Introduction to *Alternaria alternata* and Asthma

Alternaria species are fungi widely distributed in nature. They can cause many plant diseases as pathogens and are also weak parasites, saprophytes and endophytes. They are the principle

contaminating fungi in several food and food products. *Alternaria* spores are the predominant spores in the atmosphere and act after inhalation. Fungal spores concentration in the atmosphere is 1000-fold more than pollen and can cause prolonged exposure overtime. [38][6] Spores while preferring warm and humid climate, can grow at low temperatures and thus pose a major risk to humans and animals. [1,3,31,61]

Allergic inflammatory disorders of the airway like asthma and atopic asthma are complex, often long-term diseases that generate large public health and socioeconomic footprints especially in developed countries like US, UK and Australia. In 2009, approximately 8.2%, 24.6 million people in United States were affected by asthma. Chronic asthma, being under diagnosed and under treated severely limits an individual activities for the lifetime. Risk factors for asthma include inhaled allergens that irritate the airways. Currently 235 million people are affected by asthma worldwide and about 90% of those have allergic (atopic) asthma. [54] Atopic asthma is a hallmark of a potent Th2 adjuvant activity.

Another important factor in patients with allergic respiratory tract diseases is sensitization to fungi. Up to 70% of mold allergic patients have skin test reactivity to *Alternaria.* Alta1, an allergen produced by *A. alternata also* produces a prolonged and intense IgE mediated reaction in sensitized patients. Therefore *A. alternata* is not only a risk factor in development of asthma but also can lead to exacerbation of severe and potentially lethal asthma than any other fungus. [6,7,62,63]

Alternaria alternata Secondary Metabolites

Fungal mycotoxins are products of their secondary metabolism that can evoke a toxic response in vertebrates. These secondary metabolites belong to different chemical classes like nitrogen containing compounds, steroids quinones, pyrones, peptides, phenolics, and the fumonisin-like

toxins. Why fungi produce such substances is not entirely clear as they are not relevant for cell function but they may be in part used for survival in a highly competitive environment and might be form of chemical warfare during natural selection. These toxins can enter the body through skin, mucous, airways and ingestion. Constant exposure can lead to hypersensitivity and mycotoxicosis leading to a potentially compromised immune system and onset of other illness and infection (HIV, kidney and liver damage).[64,65] However, of all the mycotoxins known, only a few are subject to regular monitoring of contamination and level intake like alfatoxins, fumonisins from *fusarium*, deoxyivalenol, zearlenone and ochratoxin-A. Legal authorities from both food and feed industry acknowledge the importance of detecting the mycotoxin levels and identifying the effects of their contamination.[4]

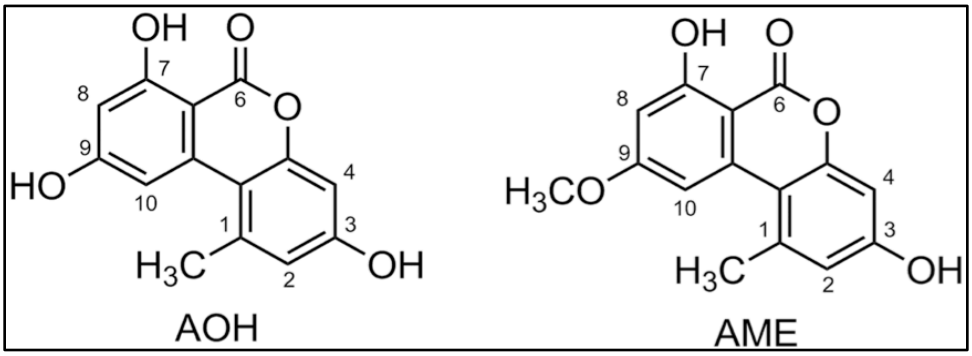


Figure 8. **Chemical structure of alternariol (AOH) and alternariol monomethyl ether (AME).**

Endophytic fungi are also an important resource of potential therapeutic lead compounds. Ever since the discovery of penicillin in 1929, the importance of elucidating the potential of fungal secondary metabolites in host-fungal pathosystems is beyond question.[66] *Alternaria* metabolites exhibit a variety of therapeutic and biological properties such as phytotoxicity, cytotoxicity, anti-HIV and anti-microbial properties, all of which have generated considerable research interest worldwide. Porritoxin from *Alternaria* species is a likely cancer chemo-preventive agent and depudecin from *A. brassicicola* is an inhibitor of histone deacetylase. [1,3,31,61]

The most well studied *Alternaria* toxin AOH has been detected in most foods and grains at high concentrations. Foods such as apples, apple products, mandarins, olives, pepper, tomatoes, oilseed, sunflower seeds, sorghum, wheat, edible oils, citrus fruits, melons, pears, prune nectar, raspberries, red currant, carrots, barley, oats, red wine and lentils are known to be frequently contaminated with AOH. The maximum levels reported are in the range of 1-103 µg/kg with higher levels in food products visibly rotted with *A. alternata*.[67] However, as of yet, no data concerning tissue levels of AOH exists in animals and human.[3] Previous studies have shown that AOH and related molecule AME are genotoxic, estrogenic, clastogenic and mutagenic in vitro, but the cytotoxic, inflammatory and genetic effects of these small molecules on lung cells is poorly understood.[17]

AOH causes mutagenicity and cytotoxicity in Chinese hamster V79 cells. An increase in cell cycle arrest in G2/M phase (proliferation decrease) of AOH treated cells has also been observed. This is an indicative of cellular stress response. Extensive mutation frequency was also observed at very low AOH concentrations (10uM) at HPRT gene locus (via Hypoxanthine-guanine phosphoribosyl transferase measurement of cytotoxicity) in V79 cells and in TK (via Thymidine kinase assay) in MLC cell lines.[3] AOH is also known to cause formation of micronucleus (MN) in V79 and human endometrial adenocarcinoma cell line (Ishikawa cells).[10,19] Treatment of AOH on murine macrophage cell line RAW 264.7 showed cytotoxicity, DNA strand breakage as well as oxidative damage and cell cycle arrest in G2/M phase.[19] Human adenocarcinoma cells (HT29) treated with AOH indicated that while the toxin modulates ROS levels, it is completely unrelated to the DNA damage levels.[16]

Alternariol and Aryl Hydrocarbon Receptor (AhR)

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that controls the expression of various environmental toxins most of which are man made contaminants. It has been studied in relation with various environmental contaminants like the xenobiotic TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Binding of the AhR to the ligand causes the translocation of the complex to the nucleus to bind with AhR nucleus translocator (ARNT). The AhR-ARNT complex then binds to various xenobiotic response elements (XRE's) and causes induction of genes like the cytochrome P450 family.[34] AhR is a potential receptor for AOH and AME. CYP450 family of genes that are a major target of AhR-ARNT complex might mediate their hydroxylation and further metabolism. The most expressed gene of the CYP450 family is CYP1A1 that has a highly continuous expression in lung and esophagus. AOH is a substrate of CYP1A1 and has a planar structure that is similar to other AhR ligands. Since AOH is of major interest in inflammatory response in lung cells, it can be hypothesized that AhR xenobiotically metabolizes AOH. Further evidence of this was substantiated by the treatment of AOH on murine hepatoma cells and the observed increase in expression of CYP1A1 in presence of activated and inactivated AhR.[35,37]

Alternaria alternata and Polyketide Synthase (PKS) Cluster of Genes

In a recently published study, it was established that the gene PksJ, in the polyketide synthase cluster of genes in *Alternaria* is responsible for biosynthetic pathway for AOH and AME formation. Although the cytotoxic and genotoxic effects of *Alternaria* mycotoxins are somewhat known, virtually nothing is known about their interaction with the mammalian immune system. In the draft genome of *Alternaria alternata,* 10 PKS-encoding genes were identified (Lawrence et al, unpublished). PksJ is the AOH and AME encoding genes, while PksA is the melanin biosynthesis (albino) gene. All PKS genes share similar architecture.[9]

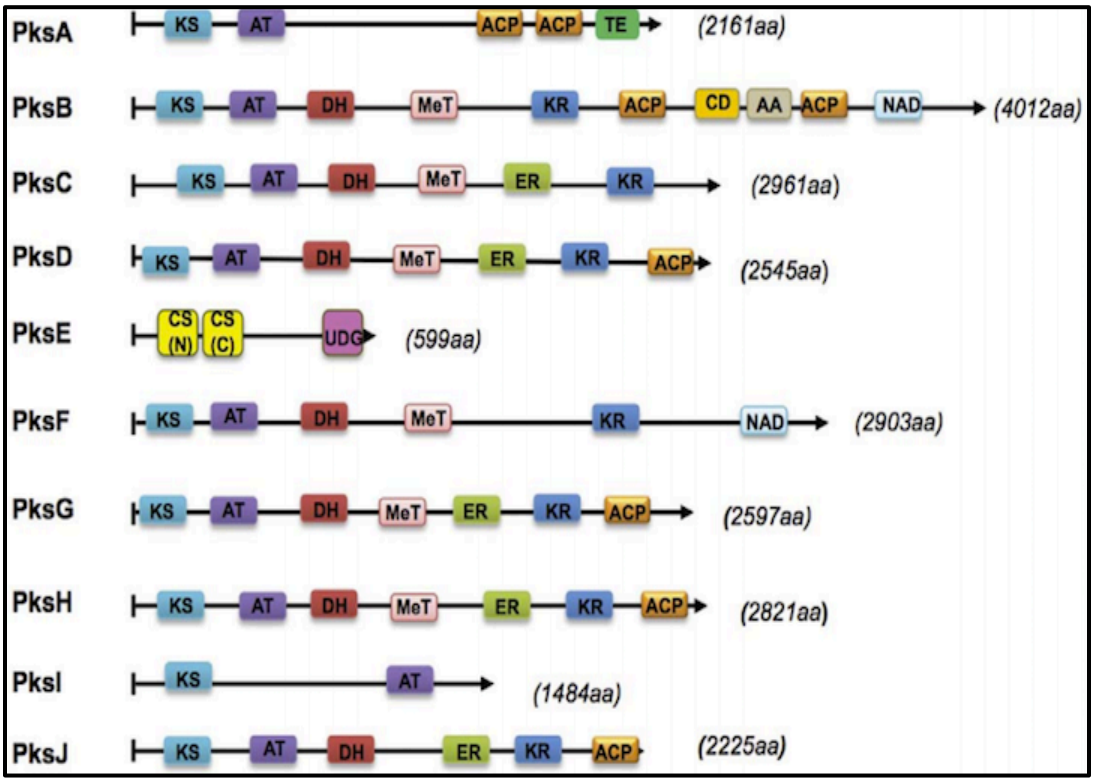


Figure 9. Architecture of PKS cluster of genes in *A. alternata*. KS, b-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; MT, methyltransferase; ER, enoyl reductase; KR, ketreductase; ACP, acyl carrier protein, CD, condensation domain; AA, Amino acid adenylation domain; CS:Chalon- and Stilben- Synthase (N)/(C); UDG: Uracil DNA Glycolase Superfamily; NAD, NAD binding domain.[9]

Conclusion

This study presents the first attempt at providing an experimental framework to decipher and define the immune-modulatory and clinical importance of the fungal secondary metabolite AOH/AME. We have studied the dynamic interplay between AOH and innate immunity in an immune suppressive manner by using the bacterial endotoxin lipopolysaccharide (LPS) to induce inflammation.

Since LPS is a potent trigger in the pathogenesis of sepsis and septic shock, this provides another avenue for therapeutic LPS suppression.[68] Additionally, despite the well-documented

clinical relevance of *Alternaria* airway allergens, none of its small molecules have been studied in regards to lung epithelium. The genetics behind the mycotoxins and the response generated by pathogenic spores in fungal-host interactions have also been largely ignored until now. Last, we will explore the immunogenicity on the entire organism (spores) in airway mucosa, making it highly pathologically relevant in regards to innate immunity and patient therapies for clinical outcomes based on inflammatory issues.

Materials and Methods

Secondary Metabolites: Alternariol, Alternariol Monomethyl Ether, Lipopolysaccharide and RO-3306

Alternariol (AOH) (Cayman Chemical) was diluted in 1mg/ml DMSO. Alternariol monomethyl ether (AME) (Sigma-Aldrich) was diluted in 1mg/ml methanol. Ultrapure bacterial endotoxin Lipopolysaccharide (Sigma-Aldrich), cell culture grade, was diluted in 1mg/ml phosphate buffered saline (PBS). RO-3306 (Sigma-Aldrich) was dissolved in 1mg/ml DMSO. The stock solutions were stored in sterile eppendorf tubes at -20°C until needed. The stock solutions were diluted to their final concentrations in the culture medium.

Cell Culture and Cell Lines

BEAS-2B's, a secondary bronchial lung epithelial cell line and mouse macrophage raw 264.7 cell lines were maintained in RPMI-1640 culture medium (Fisher Scientific) with 10% heat inactivated fetal bovine serum (FBS) (Fisher Scientific) and 1% penicillin-streptomycin (Thermo Scientific) in round bottom tissue culture treated plates (Fisher Scientific). The cells were incubated in 5% CO_2 at 37°C. Cells were starved for 2 hours to 16 hours before treatment with secondary metabolites in RPMI-1640 media with 1% penicillin-streptomycin. Cells were seeded at a density of 500,000 cells/well in 6-well tissue culture plates for treatment.

Cytokine and Chemokine Profiling and Quantification using ELISA

The cells in 6-well plates were seeded in 1.5ml RPMI-1640 media and cells in 12-well plates were seeded in 1ml RPMI-1640 media. BEAS-2B's were seeded on the plates in triplicates and after an overnight incubation at 37°C and 5% CO_2, washed with DPBS (Fisher Scientific). The cells were then placed in the starve media for 2 hours and after that, washed again with DPBS before being placed in fresh RPMI-1640 media. The secondary metabolites and LPS were then

added to the media. Cells were then incubated for 24 hours. The resulting supernatant and cells were collected and stored at -80°C. The protein levels in the cells were analyzed with enzyme linked immunosorbent assay (ELISA) kits (Biolegend and eBiosciences) following the instructions of the manufacturer. The absorbance was recorded with a microplate reader at 450nm. The concentration of cells was determined using a hematocytometer.

Alternaria alternata Growth Conditions and DNA and RNA Isolation

The fungus *Alternaria alternata* were first grown on potato dextrose agar media plates (PDA) for 7-10 days until fully conidiated. The spores were then collected and added to flasks containing glucose-yeast extract broth (GYEB). The subsequent hyphae were harvested after 2 days and freeze dried in a Labconco FreeZone 4.5 L Console Freeze-Dry System for 48 hours. DNA isolation for the fungus was then performed using the CTAB method. Total RNA isolation from cell culture was performed using the RNeasy mini kit (Qiagen) with an on-column DNase digestion step (Qiagen) following the instructions of the manufacturer. A NANODROP 1000 Spectrophotometer was used to determine the concentration of each RNA sample. The concentration of spores for treatment was determined using a hematocytometer. A concentration of 100,000 spores/well was used for our study.

Quantitative Real-Time PCR and cDNA Synthesis

The RNA samples isolated from the cell culture treatment were used as background material for the RT assays. The samples were processed into cDNA following manufactures instructions (Bioline Tetro cDNA synthesis kit) and stored at -20°C. All the qRT-PCR reactions for the biological triplicates were performed as technical duplicates using the cDNA as template. GAPDH was used as a control housekeeping gene for all experiments as it has a continuous expression in mammalian cell lines. A BIO-RAD iQ ^{TM}5 Multicolor Real-Time PCR Detection

System machine was used to conduct the qRT-PCR reaction. All reactions were carried out at 20µl volume with SYBR Green (Bioline) as the fluorescent reporter molecule. Relative fold change in gene expression was calculated using the 2(-Delta Delta C(T)) method and Pfaffl equation by normalization to GAPDH.

Generation of pksJ and pksA Mutant Constructs and Fungal Transformation

Using *Alternaria alternata* genomic DNA as a template, a 1000bp fragment from both PksA and PksJ gene was amplified using Accuzyme DNA polymerase (Bioline) in a thermocycler. The fragment was then transformed into pCB1636 vector into *E.coli* using the heat shock method. The pCB1636 contains a ~1.7kb hygromycin B phosphotransferase (*hph*) gene cassette. We then used the M13 primer pair to amplify the 2.7kb construct containing the Pks gene and hph gene from the bacterial genome. The PCR products were purified with the QIAquick PCR purification kit (Qiagen). The fungal transformation was then carried out as previously described by Lawrence et al.[69] The primer pair PksJ-Fwd and PksJ-Rev was used to amplify a 1kb fragment from the PksJ gene. The primer pair PksA-fwd and PksA-rev was used to amplify a 1kb fragment for the PksA gene. The primer sequences are given in supplementary table 1.

Cell Based Assays

The 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) solution was added to 50µl of RPMI-1640 starve media harvested from cells treated with AOH at the 24 hour time point. The plates were incubated at 37°C for 4 hours for the reduction of MTT formazon. 100µl of DMSO was then used to stop the reaction. Absorbance was measured at a wavelength of 570nm using a micro plate reader. The Lactate Dehydrogenase (LDH) assay was performed using the Pierce™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific). Cells were seeded at a density of 10,000 cells/well in 100µl RPMI-1640 media, in 96 well flat bottom plates and

incubated overnight at 37°C and 5% CO_2. After a 24-hour treatment, 50µl of media from each well was transferred to a new plate and 50µl's of LDH reaction mixture was added. After a 30-minute incubation, the reaction was stopped and absorbance was measured at 490ηm and 680ηm. To measure LDH activity, absorbance at 490ηm was subtracted from absorbance at 680ηm. All treatments were performed in biological replicates and technical triplicates. Percent cytotoxicity was calculated using the formula:

$$\%\ Cytotoxicity\ = \frac{Compound - treated\ LDH\ activity - Spontaneous\ LDH\ activity\ DH\ activity}{Maximum\ LDH\ activity - Spontaneous\ L} \times 100$$

Confocal Microscopy

The surface morphology of bronchial lung epithelial cells was imaged by a Nikon Eclipse TE2000-U Inverted microscope provided by Virginia Bioinformatics Institute. Cells were seeded at a density of 500,000 cells/wells and treated with 10µM AOH for 24 hours before imaging.

RNA Silencing

The cells were seeded at a density of 125,000 cells/well. Silencing of AhR receptor was performed using a target specific 19-25 nucleotide siRNA designed to knockdown its gene expression (Santa Cruz Biotechnology). The siRNA reagent was mixed with HiPerFect Transfection Reagent (Qiagen) for transfection. Two consecutive doses of 1nm of siRNA for 24 hours were performed to achieve a knockdown efficiency of 70%. The cells were then treated with the desired doses of secondary metabolites and endotoxin LPS. All experiments were performed in biological triplicates and with a scrambled control. Primer pair human AHR_F and human AHR_R used for checking knockdown efficiency is given in supplementary table 1 and as previously described. [70]

Statistical Analysis

All tests were performed as biological triplicates and technical triplicates. Standard deviation was calculated from among biological replicates. The difference between individual treatment groups was validated, by using an unpaired Student's *t* test for independent samples, including, LPS alone and LPS stimulation in presence of AOH. P value < 0.05 was regarded as statistically significant.

Results and Discussion

We aimed to observe the response of the mammalian innate immune system by quantifying cytokine and chemokine inflammatory markers upon AOH treatment on airway epithelial cells. Cytokine IL6 and chemokines IL8 and MCP-1/CCL2 are highly induced in many inflammatory diseases, including chronic obstructive pulmonary disease, rheumatoid arthritis and inflammatory bowel disease and are specific to Th2 allergic inflammation.[71]

We first evaluated Interleukin-6 (IL6) and Interleukin-8 (IL8) protein production as well as gene expression levels after treatment of bronchial lung epithelial cells (BEAS-2B's) with AOH. The cells were incubated with 25μM, 50μM and 100μM AOH for 6 hours, 12 hours and 24 hours. The subsequent protein production was quantified and analyzed with Enzyme Linked Immunosorbent Assay (ELISA) (Data not shown).

The gene expression levels were evaluated with quantitative Real-time PCR (Data not shown). At 6 hours, no IL6 and IL8 protein induction was observed. IL6 gene expression was then, observed to be down regulated. Similar observations were then detected at 12 hours and 24 hours after introduction of AOH to cells. This data is summarized in Table 3. We also considered the possibility that AOH may not be able to stimulate the primary inflammation markers (IL6 and IL8) and hence, searched for other cytokine and chemokine markers that might be stimulated by AOH. We subsequently conducted ELISA's for TSLP, TNF-α, IL-1β, IL-10 and TGF-β, but no induction was observed (Data not shown).

Fungi are complex organisms. One part of them might be hyper-inflammatory while the other might be completely anti-inflammatory. Upon discovering the down regulating mechanism of action of AOH, we delved further into elucidating this mechanistic phenomenon by using a highly pro-inflammatory bacterial cell wall endotoxin Lipopolysaccharide (LPS) as a control.

AOH although cytotoxic, showed strong immunosuppressive properties by suppressing the hyper-inflammatory response of LPS.

AOH Dose	Time	IL6 (Protein)	IL6 (Gene)	IL8 (Protein)	IL8 (Gene)
25μM, 50μM, 100μM	6 hrs	No Induction	Down-Regulation	No Induction	Down-Regulation
25μM, 50μM, 100μM	12 hrs	No Induction	Down-Regulation	No Induction	Down-Regulation
25μM, 50μM, 100μM	24 hrs	No Induction	Down-Regulation	No Induction	Down-Regulation

Table 3. Summary of alternariol (AOH) dosage and treatment conditions for BEAS-2B cells. No IL6 and IL8 protein level production was observed. IL6 and IL8 were then, found to be down regulated upon stimulation with alternariol in BEAS-2B cells.

Analysis of Alternariol and Alternariol Monomethyl Ether's Immune Response on Lung Epithelium and Mouse Macrophages

In the presence of 10μM AOH and (+/-) 10μg/ml of LPS at 24 hours, the levels of IL6, IL8 and MCP-1/CCL2 went down several folds (Figure 10). While at a similar dose of AME, 10μM, cytokine and chemokine protein levels were reduced only half as much as AOH leading to the conclusion that both AOH and AME have immunosuppressive properties, but AOH is the more potent molecule of the two. This experimental design provided further evidence of AOH's immune suppressive properties. We repeated this experimental design with mouse macrophage Raw 264.7 cell line and observed similar results. LPS induced IL6 was completely suppressed at a dose of 10μM AOH. LPS induces a much higher amount of IL6 in mouse macrophages

than human lung epithelial cells and hence, a much more stronger response was suppressed with this experimental design (Figure 10).

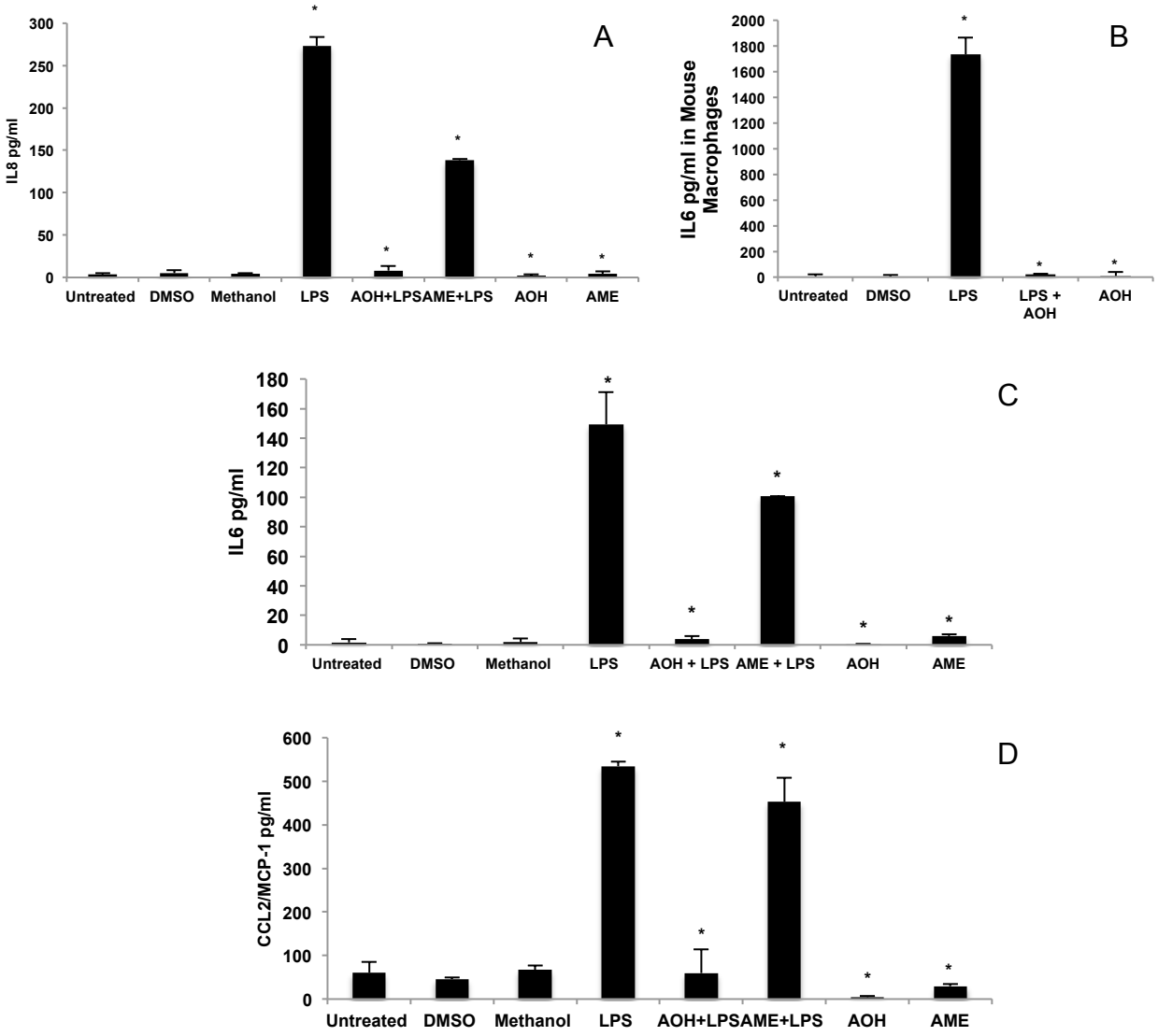


Figure 10. Treatment of airway epithelium cells by alternariol (AOH), alternariol monomethyl ether (AME) and LPS. BEAS- 2B airway epithelium cells and RAW 264.7 mouse macrophages at a density of 5 x 10^5 cells/well were treated with 10μM of AOH and 10μM of AME in presence and absence of 10μg of LPS and incubated for 24hrs. Under normal conditions at 37°C, 5% CO^2 cells treated with AOH showed a marked suppression of cytokine levels both in presence and absence of LPS. (A) IL8 BEAS-2B (B) IL6 Mouse Macrophage (C) IL6 BEAS-2B (D) CCL2 BEAS-2B released. An * indicates $p < 0.05$ according to Student's *t*-test.

Gene Expression Analysis of Alternariol's Effect on Mammalian Lung Epithelium

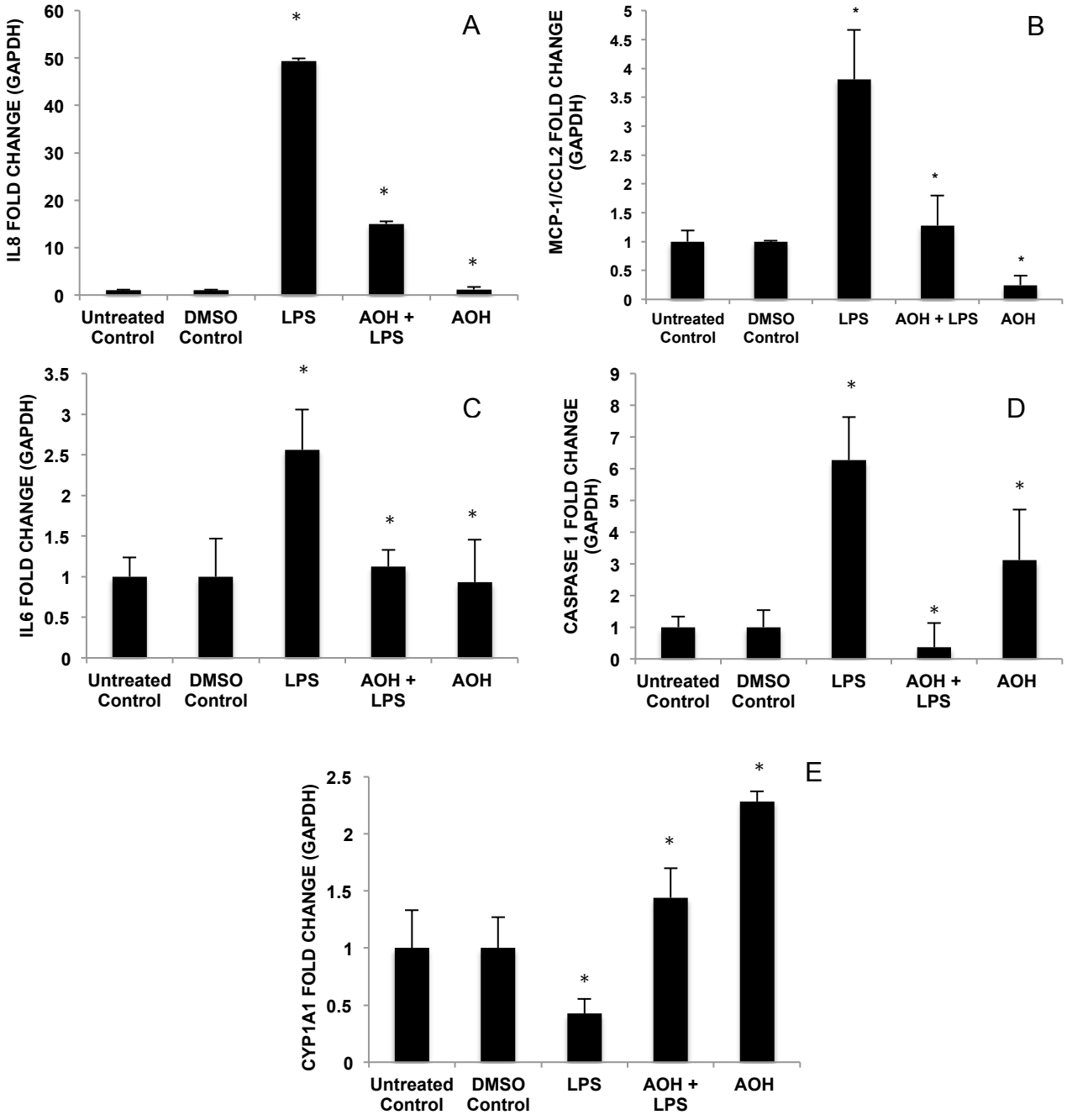


Figure 11. Quantitative Real-Time PCR analysis of airway epithelium. BEAS-2B cells seeded at a density of 500,000 cells/well were treated with 10μM AOH and 10μg LPS for 24 hours. The resulting RNA was harvested and quantified with qRT-PCR. Each graph here demonstrates the up regulation and down regulation (fold change) of gene expression by normalization with the control GAPDH. (A) IL8 (B) CCL2 (C) IL6 (D) Caspase 1 (E) CYP1A1 fold change. An * indicates $p < 0.05$ according to Student's *t*-test.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to detect gene expression changes induced by AOH, in the presence and absence of LPS. Chemokine and cytokine gene expression profiles by normalization to the control housekeeping gene GAPDH were generated in this study for a deeper look at AOH (10μM dose) phenotype response after a 24-hour treatment in the presence and absence of 10μg LPS. LPS induced IL6 levels were reduced 2-fold in presence of AOH. IL6 levels detected in the presence of AOH alone were equivalent to the control. No down regulation was detected in this treatment group. Chemokines IL8 and CCL2 followed a different pattern. IL8 level showed a 4-fold decrease in LPS induced inflammation in the presence of AOH. While CCL2 qRT-PCR showed a similar decrease of LPS induced inflammation, it showed additional down regulation of the gene in the presence of AOH alone. Furthermore, we analyzed caspase 1. Caspase 1 aids in the formation of mature peptides for inflammatory cytokines interleukin-1β and interleukin-18 and is also involved in cell death and inflammasome (NLRP1 multi-molecular complex) formation.[72,73] An AOH dose of 10μM down regulated caspase 1 by almost 5-fold in our experimental design. This provides further credence to AOH ability to suppress the innate immune response without cell death (Figure 11).

Dose Dependent Analysis of Alternariol and Bacterial Lipopolysaccharide

We evaluated greater variations of phenotype changes by testing varying doses of AOH and LPS. AOH is highly immune suppressive in a dose-dependent manner. IL8 protein levels were observed for AOH activity in BEAS-2B's at 24-hour treatments. AOH doses of 10ηM, 100ηM, 1μM, 5μM and 10μM were analyzed for protein level quantification. We observed a dose-dependent decrease in LPS (10μg) induced inflammation in lung epithelial cells. Although in all the above-mentioned doses, IL8 was not detected with AOH alone, significant LPS induced IL8 suppression was observed starting at 5μM dosage. A dose of 10μM showed the highest amount

of IL8 suppression. IL8 levels at 10μM were equivalent or less than the levels in untreated cells (Figure 12).

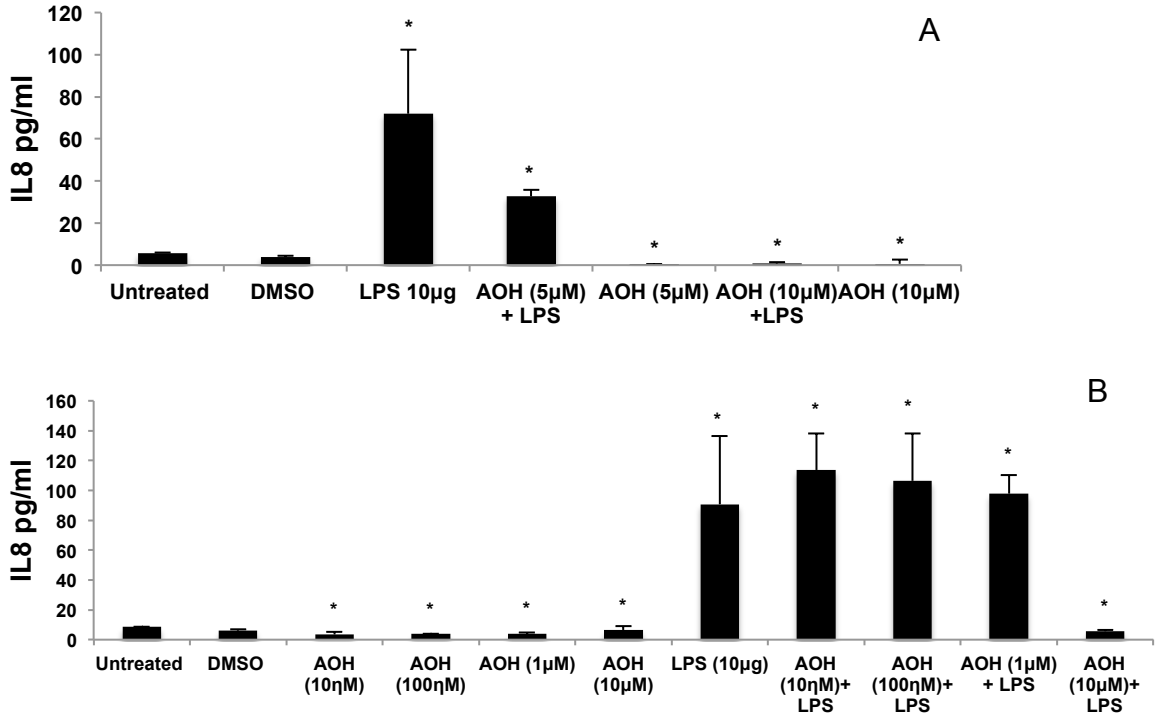


Figure 12. Dose dependent response of airway epithelium cells after treatment with AOH and LPS. (A) BEAS-2B cells were treated with (10ηM-10μM) of AOH in presence and absence of 10μg of LPS to measure IL8 levels released. Cell densities were 5 x 10^5 cells/well and were incubated for 24 hours under normal conditions at 37°C, 5% CO_2 after treatment. (B) BEAS-2B cells were treated with (5μM-10μM) of AOH in presence and absence of 10μg of LPS to measure IL8 levels released. Cell densities were 5 x 10^5 cells/well and were incubated for 24 hours under normal conditions at 37°C, 5% CO_2 after treatment. An * indicates $p < 0.05$ according to Student's *t*-test.

To further investigate the dose dependent response of AOH, we conducted an experiment to quantify bacterial endotoxin LPS doses on bronchial lung epithelial cells (BEAS-2B). Our previous experimental design of a 24-hour cell treatment was applied here to evaluate protein levels of IL6 and IL8. We tested doses including 10ηg, 100ηg, 500ηg, 1μg, 5μg and 10μg. A dose of 10μg of LPS produced 132pg/ml of IL6 and 221pg/ml of IL8. With these results, we

validated the doses of 10µg of LPS and 10µM of AOH as sufficiently substantiated for further mechanistic elucidation of secondary metabolite response in lung epithelium (Figure 13).

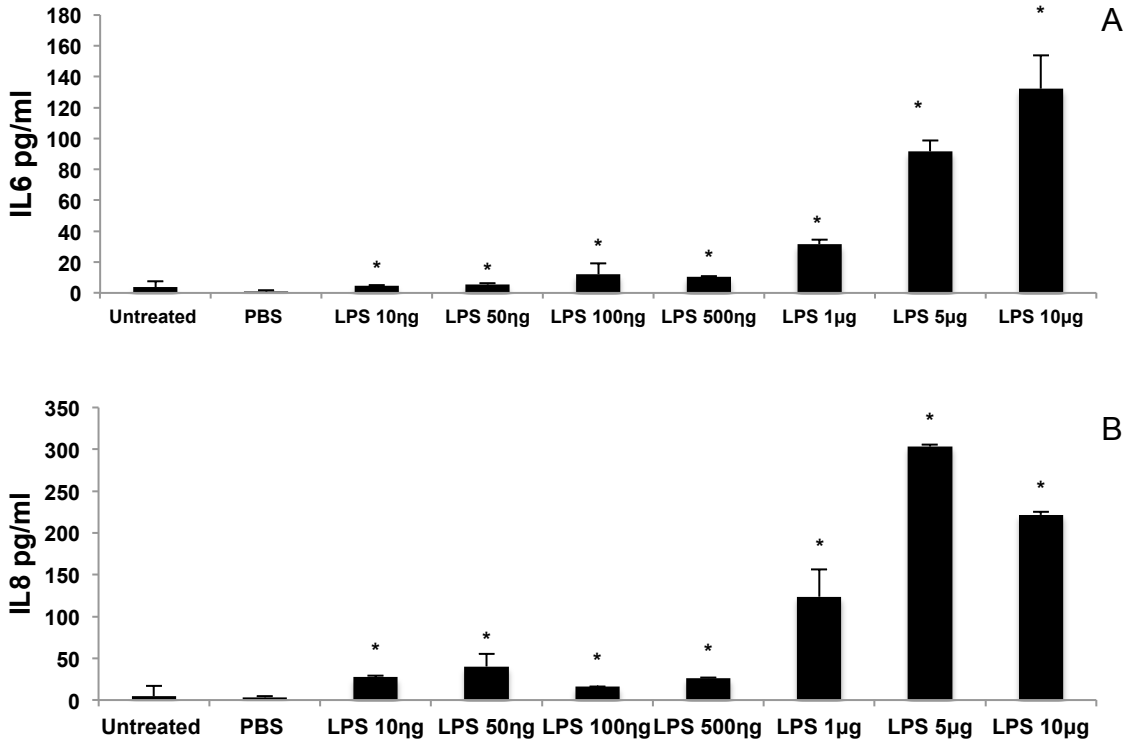


Figure 13. Dose dependent response of airway epithelium to LPS. LPS was added to BEAS-2B cells at a density of 500,000 cells/well for 24 hours. (A) IL6 measured by ELISA. (B) IL8 measured by ELISA. An * indicates $p < 0.05$ according to Student's *t*-test.

To test whether AOH induced immune suppression is dependent on the time of LPS addition; we treated BEAS-2B cells with AOH and added LPS 2 hours later. No change was observed. A range of doses of AOH (10ηM to 100µM) and AME (1-30µM) were tested on BEAS-2B cells. All showed a marked decrease in IL6, IL8 and MCP-1/CCL2 in the presence and absence of LPS (Supplementary Figure 1).

Cell Surface Morphology in Response to Alternariol

Multiple morphological changes were also observed in the cells after treatment with AOH. BEAS-2B cells treated with AOH showed a marked change after 24hrs (Figure 13). The cells show clear morphological changes in response to stress. The cells were observed, to be more spread out and had elongated arms. We conclude that the cell cycle arrest at the G2/M phase causes cells to be stressed and therefore, results in this morphology change.

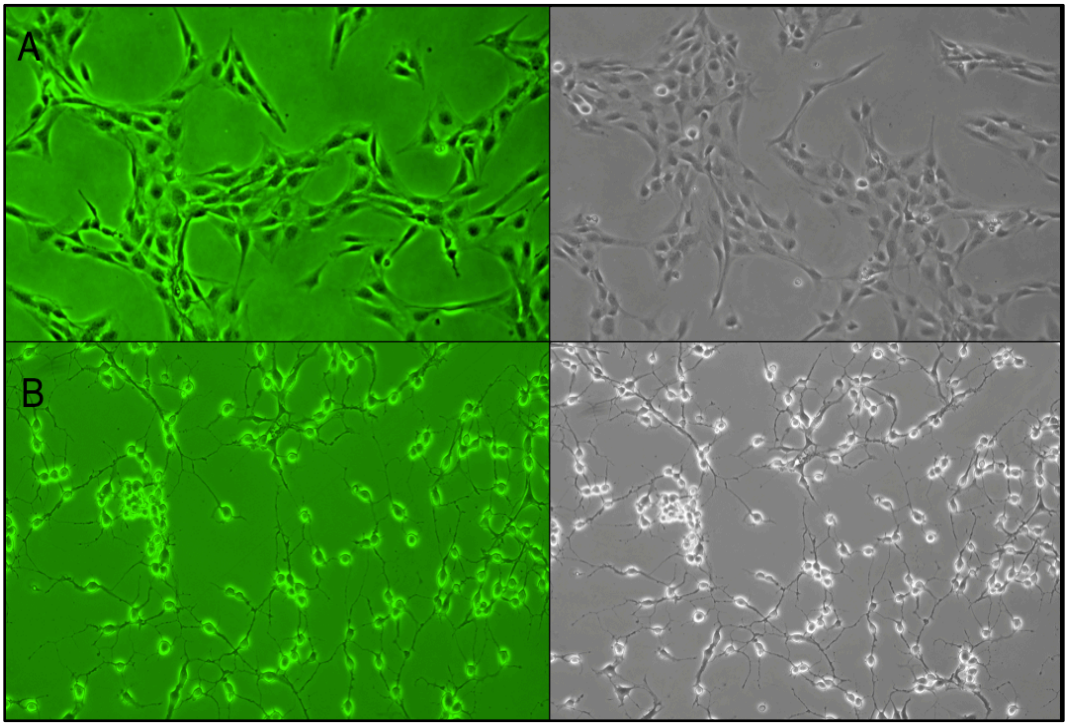


Figure 14. Human airway epithelial cells in presence of *A. alternata* toxins. BEAS-2B cells were incubated with 10μM of mycotoxin alternariol (AOH) for 24 hours under normal conditions at 37°C, 5% CO^2. The images were taken with confocal microscopy with a cell density of 5 x 10^5 cells/well. (A) Untreated BEAS-2B cells at 24-hours. (B) BEAS-2B with 10μM AOH at 24 hours

Alternariol Response Analysis Based on Cell Based Assays

Previous studies have emphasized on AOH's ability to cause cell death and cell cycle arrest. Hence, the colorimetric assay MTT was performed at doses ranging from 1μM to 100μM of AOH. In the MTT assay, the yellow MTT is reduced to purple formazon in the mitochondria of living cells. This happens when mitochondrial reductase enzymes are active as in a living and viable cell. Cell proliferation was 56% at 10μM of AOH. It reduced to 23% at 20μM. At 100μM, only 12% of the cells were proliferating.

A lactate dehydrogenase (LDH) assay measures LDH released into the media by dead cells. Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different types of cells and is released when the plasma membrane is damaged. An LDH assay was performed for apoptosis and necrosis quantification. Less than 10% cell death was detected at 10μM. Collectively, the MTT and LDH assays suggest that cell cycle arrest suppresses LPS induced inflammation by causing the cessation of cell proliferation, when they are exposed to AOH in vitro.[74]

Alternariol Response Analysis Based on Cell Cycle Arrest in Lung Epithelium

The previous experiments have reinforced the hypothesis that AOH possesses immune suppressive properties that are separate from its cell death and cell cytotoxicity. The 10μM dose, we have identified causes less than 10% cell death and 50% reduction in proliferation. The reduction in cell proliferation is an intrinsic property of AOH, caused by the short and yet reversible arrest in the G2/M phase of the cell cycle.

To differentiate whether the cell cycle arrest is the cause of immune suppression observed in our experimental design, we used the compound RO-3306, a selective ATP-competitive

inhibitor of CDK1. Cyclin Dependent Kinase 1 (CDK1) is a typical serine/threonine kinase that controls the progression of cell cycle through each checkpoint (courtesy of the Cimini Lab, Virginia Tech). The compound RO-3306 has been identified to cause cell cycle arrest at the G2/M phase, similar to AOH at a dose of 10μM.[75]

Hence, we treated BEAS-2B cells with 10μM AOH and 10μM RO-3306 in the presence and absence of inflammation induced by 10μg LPS. We profiled IL6 and IL8 protein levels for our analysis. The data showed that RO-3306 followed a similar pattern as AOH and caused the suppression of LPS induced IL8 but is only half as potent as AOH. No IL8 induction was seen in cell treated with RO-3306 alone. No IL6 was detected in this experimental design but no suppression was observed either (Figure 16).

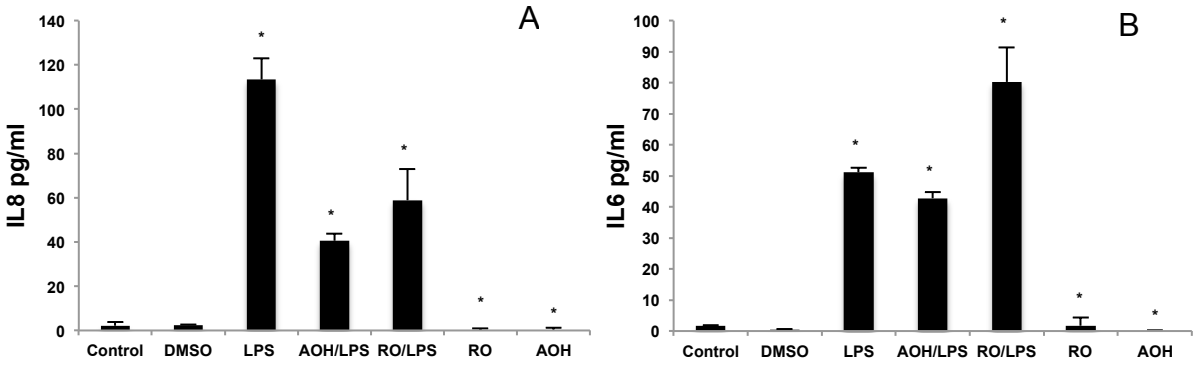


Figure 15. Treatment of airway epithelium cells by AOH, RO-3306 and LPS. BEAS-2B cells were seeded at a density of 5 x 10^5 cells/well were treated with 10μM of AOH and 10μM of RO-3306 in presence and absence of 10μg of LPS and incubated for 24hrs. Under normal conditions at 37°C, 5% CO^2 cells treated with AOH and RO-3306 showed a marked suppression of cytokine levels both in presence and absence of LPS. (A) IL8 (B) IL6 released. An * indicates $p < 0.05$ according to Student's *t*-test

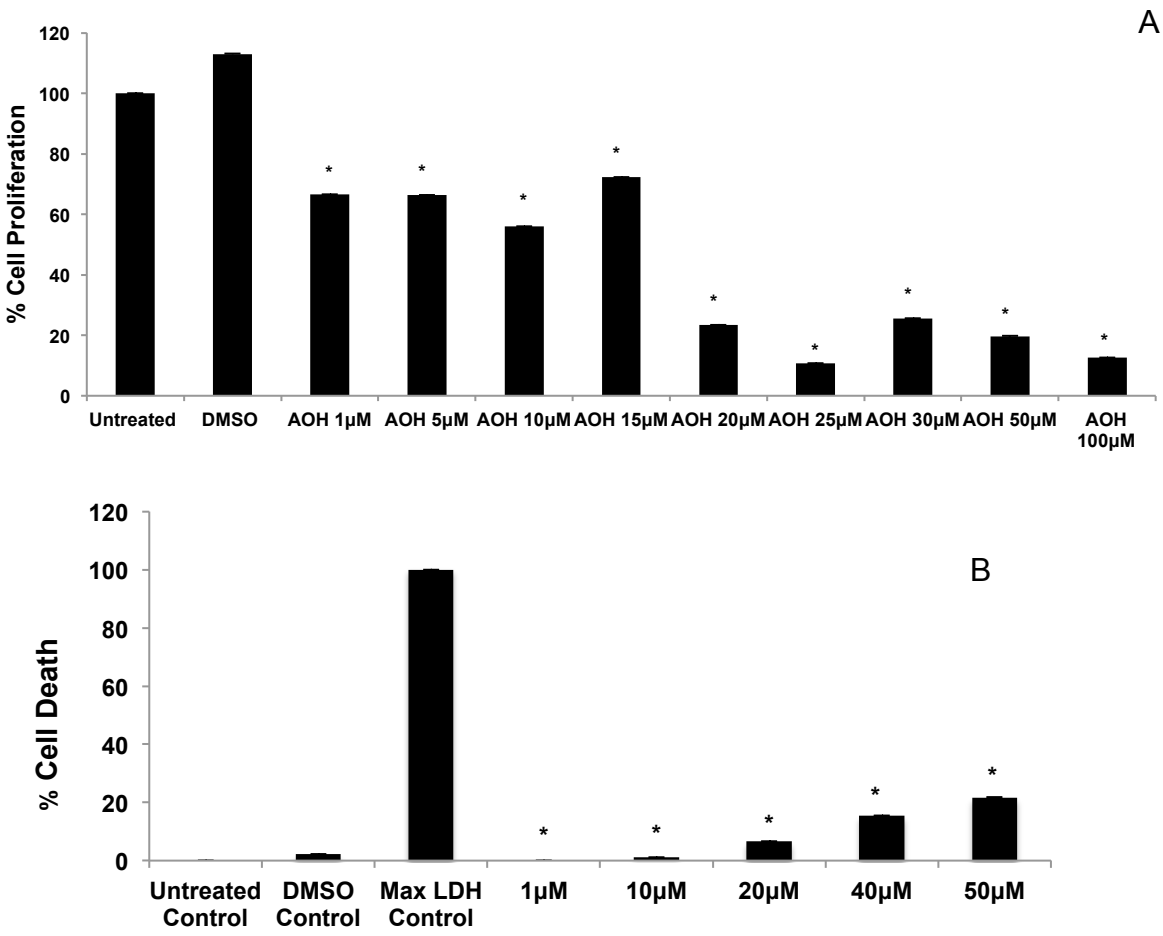


Figure 16. Cell proliferation and cell death analysis of airway epitheliums response to AOH. (A) An exhaustive dose dependent analysis of cell proliferation of BEAS-2B cells after treatment with alternariol was performed by MTT assay. Cells were seeded at a density of 500,000 cells/well for 24 hours (B) A dose curve of Lactate dehydrogenase (LDH) assay to measure the amount of LDH released by a dead cells upon treatment with alternariol for 24 hours at a cell density of 20,000 cells/well. An * indicates $p < 0.05$ according to Student's *t*-test.

Alternariol PKS Gene Disruption Analysis

In the putative architecture of the polyketide synthase cluster of genes identified in the *Alternaria alternata* draft genome, PksJ was identified as the gene responsible for AOH and its related compound AME biosynthesis. The genes pksA *(*melanin biosynthesis) and pksJ (AOH and AME) were knocked out of the *A. alternata* genome by gene disruption followed by protoplast

transformation (Figure 17). The gene-disrupted mutants have the added capability of growing on hygromycin rich media. The pksA mutant is utilized in this study as a positive control for successful mutation.

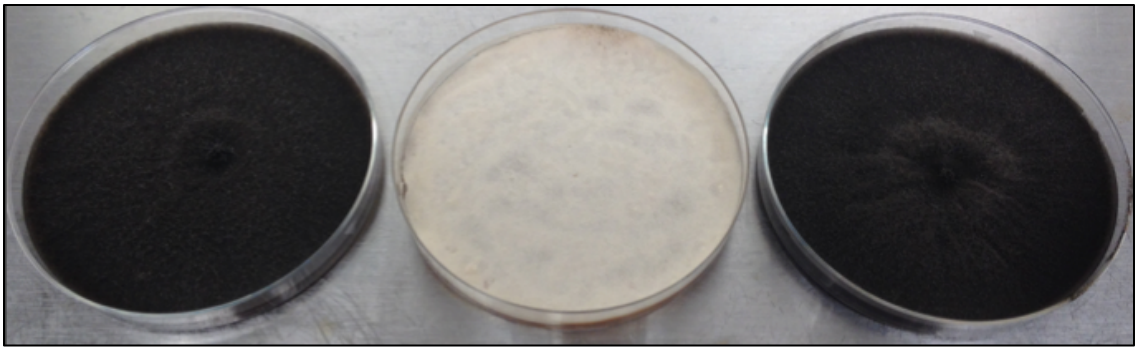


Figure 17. In vitro growth of *A. alternata* wild type, *pksA* and *pksJ* mutant spores on potato dextrose agar. The wild type is on the left. The Δ*pksA* strain is in the middle and white due to loss of melanin in cell wall. The strain on the right is the alternariol and alternariol monomethyl ether deficient mutant (Δ*pksJ*).

Cytokine (IL6) and chemokine (IL8) production significantly increased when lung epithelial cells were treated with *Alternaria* spores (100,000 spores/well) from the putative pksJ knockout (AOH and AME) strain compared to wild type (Figure 18). The lung epithelial cells are 200% more responsive to the putative mutant spores than the wild-type spores. This reinforces the hypothesis that AOH and AME are immunosuppressive molecules. The putative pksA mutant caused a lesser induction of IL6 and IL8 in comparison to wild-type spores. Hence, the putative AOH production knockouts exhibit higher pro-inflammatory activity than the wild type.

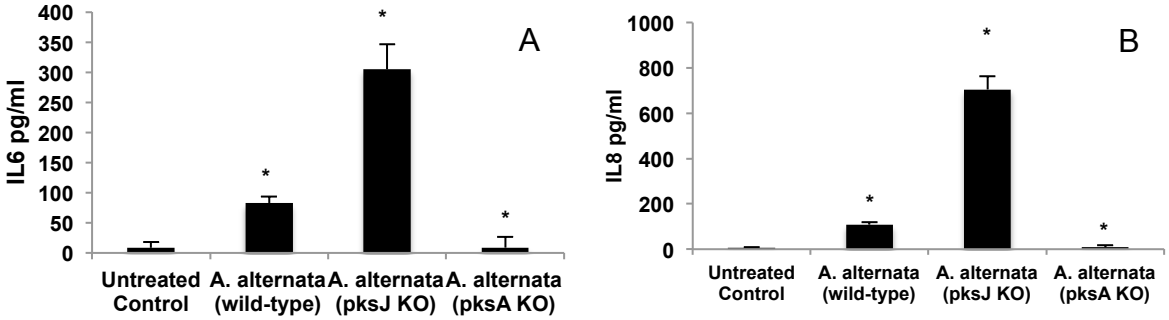


Figure 18. Cytokine induction following treatment of airway epithelium with fungal spores. BEAS-2B cells were treated with 1x10 spores of wild type and mutant *A. alternata* for 24 hours. Cell density was 500,000 cells/well. Cells were starved for 24 hours prior to treatment. (A) IL6 and IL8 (B) levels measured by enzyme-linked immunosorbent assay (ELISA). An * indicates $p < 0.05$ according to Student's *t*-test.

Aryl Hydrocarbon Receptor Analysis and Alternariol's (AOH) Mechanism of Action

To profile the mechanism of AOH action inside the cells and to gain an understanding of AOH's curious immune suppressive response in lung epithelial cells, we hypothesized that Aryl Hydrocarbon Receptor (AhR) is the target receptor for AOH inside the cell that triggers downstream signaling. RNA silencing was used to knockdown AhR in BEAS-2B's. After a transitory knock down induced with a 1ηM dose, cells were treated with pure AOH in the presence and absence of LPS for 24-hours to study, if the immune suppression was affected. It was concluded that AOH naturally down regulates AhR rather than being its receptor as no change in response was observed that correlated with the silenced gene (Figure 19).

We next quantified gene expression levels of CYP1A1. CYP1A1 induction is closely related to AhR production. We detected a 2-fold increase in CYP1A1 levels in cell treated with AOH

(Figure 11).

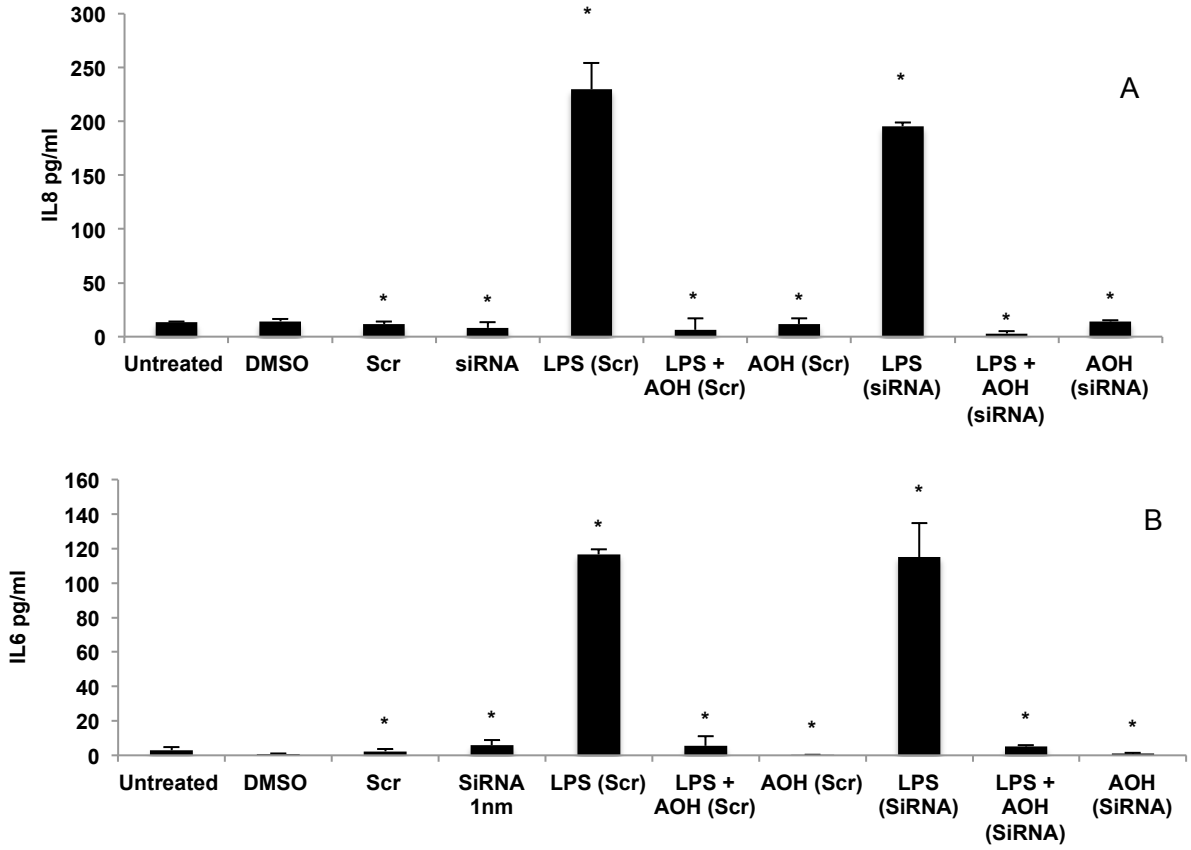


Figure 19. RNA silencing of AhR followed by treatment with AOH in BEAS-2B. Cells were seeded with a density of 150,000 cells/well. Cells were treated with AhR siRNA for 24 hours twice to successfully knockout AhR. (A) IL8 (B) IL6 released upon treatment with 10μM AOH and 10μg LPS for 24 hours. An * indicates $p < 0.05$ according to Student's *t*-test.

Hence, we attempted to isolate whether the cell death inducing property of AOH is conducted through AhR and not immune suppression. No significant difference was detected to conclude any substantial impact from AhR (Figure 20). Furthermore, we used mouse hepatoma cell lines with knocked out AhR and ARNT receptor to shed further light onto the mechanism of action. The data analyzed was inconclusive (Supplementary Figure 3). Hence, no direct correlation was observed between alternariol-associated innate immunological activity and Aryl Hydrocarbon Receptor.

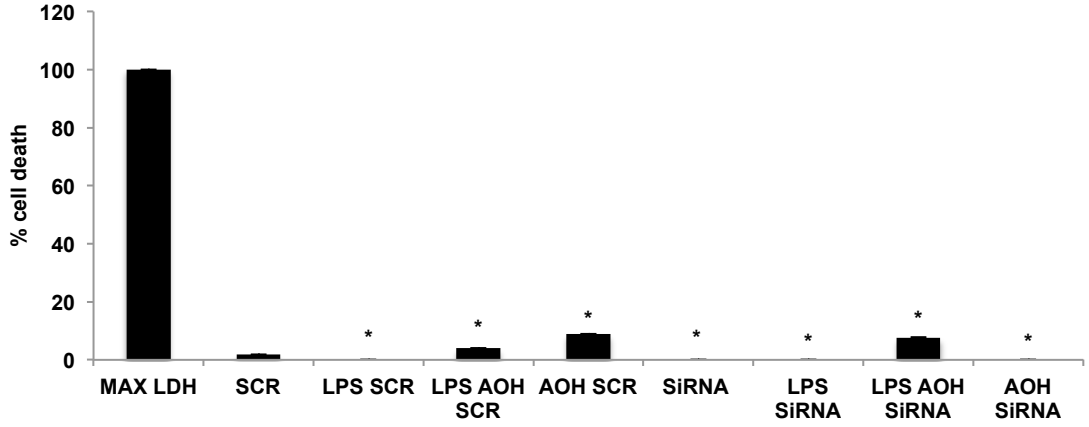


Figure 20. An LDH assay performed on AhR silenced airway epithelium. An LDH assay was performed to demonstrate changes in cell death dependent upon AhR knockdown in cells on treatment with 10μM AOH and 10μg LPS. No change was observed relative to AhR knockdown. An * indicates $p < 0.05$ according to Student's *t*-test.

CHAPTER III

Conclusion and Future Directions

Conclusion

Alternariol is present in contaminated food and feed products at very high concentration when compared to other mycotoxins. *Alternaria alternata* is also a major allergen producing fungus and is a health risk for exacerbation of asthma and allergy in humans. Fungal secondary metabolites are present in contaminated food and feed products at very high concentrations. Hence, it is important to characterize the activity of alternariol in the lungs. Higher levels of decrease in inflammatory cytokine's observed in preliminary studies indicate that alternariol has strong anti-inflammatory properties.

Collectively, our data suggest that high levels of decrease in inflammatory cytokines observed, is associated with cell cycle arrest at G2, resulting in the suppression of LPS induced Inflammation. The cell proliferation and cell death assays conducted in this study raise our understanding of the cytotoxic effects of this compound at various doses. The cell proliferation assay suggests that alternariol decreases cell viability by almost 50% in 10uM dose. This implies further association with cell cycle arrest. The cell death assay advocates that alternariol is highly cytotoxic to lung epithelial cells at a dose of 20uM or higher. But the 10μM dose utilized in this study provides evidence that the cell death at the concerned dose is minimal and hence, has no effect on alternariol's immune suppressive properties.

This was further investigated by the experimental design involving the CDK1 inhibitor RO-3306. Therefore, we conclude that cell cycle arrest caused by alternariol in turn causes the suppression of LPS induced inflammation in mammalian respiratory epithelium and mouse

macrophage models. The dose dependent analysis provides dose standards for treatment and experimental validation of alternariol induced anti-inflammatory activity. The increase in pro-inflammatory cytokines released by cells upon treatment with putative mutants of alternariol and alternariol monomethyl ether reinforces the conclusion that these secondary metabolites suppress LPS induced inflammation. To summarize, we have characterized several alternariol properties at the innate immunology level. Further work will provide more insight on its mechanism of action.

Future Directions

The 10 putative PKS-encoding genes identified in the draft genome of *A. alternata* are postulated to be the primary candidates of mycotoxin production in fungi. In the future, we aim to explore the expression levels of these genes in fungal spore DNA upon treatment with lung epithelium and mouse macrophages in more detail. We aim to predict the genes for specific mycotoxins as well as profile the innate immune response generated. This will give us a very accurate view of the gene cluster while it is actively involved in the infection process. A metabolite profiling of *Alternaria* fungal extracts in the presence of human cells will help identify which toxins are the most abundant agents of immune modulation in vitro as well as link them to possible PKS genes responsible for their production. A gas chromatography–mass spectrometer (GC-MS) can be used for metabolite profiling of fungal extracts. We also aim to further characterize the pksJ KO strain by creating a complement mutant that can proliferate in hygromycin (HYG) and nouroseothricin (NAT) rich medium. A gene deletion based approach can be utilized to make the construct that will be inserted through protoplast. The resulting KO strains and complement strains can be analyzed for KO efficiency by southern blots (Roche). The fungal strain extracts can also be quantified for toxin production to further substantiate our fungal molecular biology data.

Characterizing the immune response and other biological activity in primary lung epithelial cells (NHBE) may provide further insights into the immune modulatory property of alternariol because primary cells have a very distinct physiology from secondary cells. Furthermore, utilizing computational approaches to model mechanism of action of *A. alternata* mycotoxins in humans at the cellular and molecular level might prove to be very insightful. Immune modeling is an excellent tool for revealing critical targets in an immune response. COPASI and Cell Designer can be used to create a multi-cell level model to decipher mycotoxin action on lung epithelium. Parameter estimation can be carried out by laboratory-generated data. Simulations of the model will allow us to predict which signaling pathways specific triggers affect. This will lead to a deeper understanding of the inflammatory response leading to better experimental results.

REFERENCES

1. Panel E, Chain F. Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. *EFSA J 2011*. 2011;9(10). doi:10.2903/j.efsa.2011.2407.

2. Lou J, Fu L, Peng Y, Zhou L. Metabolites from alternaria fungi and their bioactivities. *Molecules*. 2013;18(5):5891-5935. doi:10.3390/molecules18055891.

3. Fleck SC, Burkhardt B, Pfeiffer E, Metzler M. Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol Lett*. 2012;214(1):27-32. doi:10.1016/j.toxlet.2012.08.003.

4. Streit E, Schwab C, Sulyok M, Naehrer K, Krska R, Schatzmayr G. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins (Basel)*. 2013;5(3):504-523. doi:10.3390/toxins5030504.

5. Knutsen AP, Bush RK, Demain JG, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol*. 2012;129(2):280-291; quiz 292-293. doi:10.1016/j.jaci.2011.12.970.

6. Sanchez H, Bush RK. A review of Alternaria alternata sensitivity. *Rev Iberoam Micol*. 2001;18(2):56-59.

7. Hedayati MT, Arabzadehmoghadam A, Hajheydari Z. Specific IgE against Alternaria alternata in atopic dermatitis and asthma patients. *Eur Rev Med Pharmacol Sci*. 2009;13(3):187-191.

8. Brugger E-M, Wagner J, Schumacher DM, et al. Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. *Toxicol Lett*. 2006;164(3):221-230. doi:10.1016/j.toxlet.2006.01.001.

9. Saha D, Fetzner R, Burkhardt B, et al. Identification of a polyketide synthase required for alternariol (AOH) and alternariol-9-methyl ether (AME) formation in Alternaria alternata. *PLoS One*. 2012;7(7):e40564. doi:10.1371/journal.pone.0040564.

10. Lehmann L, Wagner J, Metzler M. Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food Chem Toxicol*. 2005;44(3):398-408. doi:10.1016/j.fct.2005.08.013.

11. Griffin GF, Chu FS. Toxicity of the Alternaria metabolites alternariol, alternariol methyl ether, altenuene, and tenuazonic acid in the chicken embryo assay. *Appl Environ Microbiol*. 1983;46(6):1420-1422.

12. Schrader TJ, Cherry W, Soper K, Langlois I. Further examination of the effects of nitrosylation on Alternaria alternata mycotoxin mutagenicity in vitro. *Mutat Res*. 2006;606(1-2):61-71. doi:10.1016/j.mrgentox.2006.02.008.

13. Tiemann U, Tomek W, Schneider F, Müller M, Pöhland R, Vanselow J. The mycotoxins alternariol and alternariol methyl ether negatively affect progesterone synthesis in porcine granulosa cells in vitro. *Toxicol Lett*. 2009;186(2):139-145. doi:10.1016/j.toxlet.2009.01.014.

14. Burkhardt B, Jung S a, Pfeiffer E, Weiss C, Metzler M. Mouse hepatoma cell lines differing in aryl hydrocarbon receptor-mediated signaling have different activities for glucuronidation. *Arch Toxicol*. 2012;86(4):643-649. doi:10.1007/s00204-011-0789-8.

15. Schwarz C, Kreutzer M, Marko D. Minor contribution of alternariol, alternariol monomethyl ether and tenuazonic acid to the genotoxic properties of extracts from Alternaria alternata infested rice. *Toxicol Lett*. 2012;214(1):46-52. doi:10.1016/j.toxlet.2012.08.002.

16. Tiessen C, Fehr M, Schwarz C, et al. Modulation of the cellular redox status by the Alternaria toxins alternariol and alternariol monomethyl ether. *Toxicol Lett*. 2013;216(1):23-30. doi:10.1016/j.toxlet.2012.11.005.

17. Solhaug A, Holme J a, Haglund K, et al. Alternariol induces abnormal nuclear morphology and cell cycle arrest in murine RAW 264.7 macrophages. *Toxicol Lett*. 2013;219(1):8-17. doi:10.1016/j.toxlet.2013.02.012.

18. Bensassi F, Gallerne C, Sharaf El Dein O, Hajlaoui MR, Bacha H, Lemaire C. Cell death induced by the Alternaria mycotoxin Alternariol. *Toxicol In Vitro*. 2012;26(6):915-923. doi:10.1016/j.tiv.2012.04.014.

19. Solhaug a, Vines LL, Ivanova L, et al. Mechanisms involved in alternariol-induced cell cycle arrest. *Mutat Res*. 2012;738-739:1-11. doi:10.1016/j.mrfmmm.2012.09.001.

20. de Souza GD, Mithöfer A, Daolio C, Schneider B, Rodrigues-Filho E. Identification of Alternaria alternata Mycotoxins by LC-SPE-NMR and Their Cytotoxic Effects to Soybean (Glycine max) Cell Suspension Culture. *Molecules*. 2013;18(3):2528-2538. doi:10.3390/molecules18032528.

21. Asturias J a., Ibarrola I, Ferrer A, et al. Diagnosis of Alternaria alternata sensitization with natural and recombinant Alt a 1 allergens. *J Allergy Clin Immunol*. 2005;115(6):1210-1217. doi:10.1016/j.jaci.2005.02.012.

22. Aden E, Weber B, Bossert J, et al. Standardization of Alternaria alternata: extraction and quantification of alt a 1 by using an mAb-based 2-site binding assay. *J Allergy Clin Immunol*. 1999;104(1):128-135.

23. Yekeler Ha of TE of AT on E of M by L and EM, Bitmiş K, Özçelik N, Doymaz MZ, Çalta M. Analysis of Toxic Effects of Alternaria Toxins on Esophagus of Mice by Light and Electron Microscopy. *Toxicol Pathol*. 2001;29(4):492-497. doi:10.1080/01926230152499980.

24. Antony M, Shukla Y, Janardhanan KK. Protective effect of tenuazonic acid against dimethyl benz(a)antracene-induced skin carcinogenesis in mice. *Teratog Carcinog Mutagen*. 2002;22(4):309-314. doi:10.1002/tcm.10032.

25. Noser J, Schneider P, Rother M, Schmutz H. Determination of six Alternaria toxins with UPLC-MS/MS and their occurrence in tomatoes and tomato products from the Swiss market. *Mycotoxin Res*. 2011;27(4):265-271. doi:10.1007/s12550-011-0103-x.

26. Xiao J, Zhang Q, Gao Y, Tang J, Zhang A, Gao J. Secondary Metabolites from the Endophytic Botryosphaeria dothidea, and Their Antifungal, Antibacterial, Antioxidant, and Cytotoxic Activities. *J Agric Food Chem*. 2014.

27. Kjer J, Wray V, Edrada-Ebel R, et al. Xanalteric acids I and II and related phenolic compounds from an endophytic Alternaria sp. isolated from the Mangrove plant Sonneratia alba. *J Nat Prod*. 2009;72(11):2053-2057. doi:10.1021/np900417g.

28. Singh SB, Jayasuriya H, Dewey R, et al. Isolation, structure, and HIV-1-integrase inhibitory activity of structurally diverse fungal metabolites. *J Ind Microbiol Biotechnol*. 2003;30(12):721-731. doi:10.1007/s10295-003-0101-x.

29. Johann S, Rosa LH, Rosa C a., et al. Antifungal activity of altenusin isolated from the endophytic fungus Alternaria sp. against the pathogenic fungus Paracoccidioides brasiliensis. *Rev Iberoam Micol*. 2012;29(4):205-209. doi:10.1016/j.riam.2012.02.002.

30. Phaopongthai J, Wiyakrutta S, Meksuriyen D, Sriubolmas N, Suwanborirux K. Azole-synergistic anti-candidal activity of altenusin, a biphenyl metabolite of the endophytic fungus Alternaria alternata isolated from Terminalia chebula Retz. *J Microbiol*. 2013;51(6):821-828. doi:10.1007/s12275-013-3189-3.

31. Bashyal BP, Wellensiek BP, Ramakrishnan R, Faeth SH, Ahmad N, Gunatilaka a. a. L. Altertoxins with potent anti-HIV activity from Alternaria tenuissima QUE1Se, a fungal endophyte of Quercus emoryi. *Bioorg Med Chem*. 2014;22(21):6112-6116. doi:10.1016/j.bmc.2014.08.039.

32. Stack ME, Prival MJ. Mutagenicity of the Alternaria metabolites altertoxins I, II, and III. *Appl Environ Microbiol*. 1986;52(4):718-722.

33. Schwarz C, Tiessen C, Kreutzer M, Stark T, Hofmann T, Marko D. Characterization of a genotoxic impact compound in Alternaria alternata infested rice as Altertoxin II. *Arch*

Toxicol. 2012;86(12):1911-1925. doi:10.1007/s00204-012-0958-4.

34. Nguyen NT, Hanieh H, Nakahama T, Kishimoto T. The roles of aryl hydrocarbon receptor in immune responses. *Int Immunol*. 2013;25(6):335-343. doi:10.1093/intimm/dxt011.

35. Beischlag T V, Luis Morales J, Hollingshead BD, Perdew GH. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit Rev Eukaryot Gene Expr*. 2008;18(3):207-250.

36. Solaimani P, Damoiseaux R, Hankinson O. Genome-wide RNAi high-throughput screen identifies proteins necessary for the AHR-dependent induction of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci*. 2013;136(1):107-119. doi:10.1093/toxsci/kft191.

37. Schreck I, Deigendesch U, Burkhardt B, Marko D, Weiss C. The Alternaria mycotoxins alternariol and alternariol methyl ether induce cytochrome P450 1A1 and apoptosis in murine hepatoma cells dependent on the aryl hydrocarbon receptor. *Arch Toxicol*. 2012;86(4):625-632. doi:10.1007/s00204-011-0781-3.

38. van Leeuwen WS. Bronchial Asthma in Relation to Climate. *Proc R Soc Med*. 1924;17(Ther Pharmacol Sect):19-26.

39. Fleck SC, Burkhardt B, Pfeiffer E, Metzler M. Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol Lett*. 2012;214(1):27-32. doi:10.1016/j.toxlet.2012.08.003.

40. Knutsen AP, Bush RK, Demain JG, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol*. 2012;129(2):280-291; quiz 292-293. doi:10.1016/j.jaci.2011.12.970.

41. McCarty TP, Baddley JW, Walsh TJ, et al. Phaeohyphomycosis in transplant recipients: Results from the Transplant Associated Infection Surveillance Network (TRANSNET). *Med Mycol*. 2015;53(5):440-446. doi:10.1093/mmy/myv018.

42. Wiest PM, Wiese K, Jacobs MR, et al. Alternaria infection in a patient with acquired immunodeficiency syndrome: case report and review of invasive alternaria infections. *Rev Infect Dis*. 9(4):799-803.

43. Brás S, Sabino R, Laureano A, et al. Cutaneous infection by different Alternaria species in a liver transplant recipient. *Med Mycol Case Rep*. 2015;8:1-4. doi:10.1016/j.mmcr.2015.01.004.

44. Machet L, Jan V, Machet MC, Vaillant L, Lorette G. Cutaneous alternariosis: role of corticosteroid-induced cutaneous fragility. *Dermatology*. 1996;193(4):342-344.

45. Cutaneous infection with Alternaria alternata complicating immunosuppression: successful treatment with itraconazole. *Br J Dermatol*. 1998;138(2):354-356. doi:10.1046/j.1365-2133.1998.02091.x.

46. Luque P, García-Gil FA, Larraga J, et al. Treatment of cutaneous infection by Alternaria alternata with voriconazole in a liver transplant patient. *Transplant Proc*. 2006;38(8):2514-2515. doi:10.1016/j.transproceed.2006.08.031.

47. Pereiro M, Pereiro Ferreirós MM, De Hoog GS, Toribio J. Cutaneous infection caused by Alternaria in patients receiving tacrolimus. *Med Mycol*. 2004;42(3):277-282.

48. Sečníková Z, Jůzlová K, Vojáčková N, et al. The rare case of Alternaria alternata cutaneous and pulmonary infection in a heart transplant recipient treated by azole antifungals. *Dermatol Ther*. 27(3):140-143. doi:10.1111/dth.12096.

49. Gilmour TK, Rytina E, O'Connell PB, Sterling JC. Cutaneous alternariosis in a cardiac transplant recipient. *Australas J Dermatol*. 2001;42(1):46-49. doi:10.1046/j.1440-0960.2001.00473.x.

50. Ferreira I de S, Teixeira G, Abecasis M. Alternaria alternata invasive fungal infection in a patient with Fanconi's anemia after an unrelated bone marrow transplant. *Clin Drug Investig*. 2013;33 Suppl 1:S33-S36. doi:10.1007/s40261-012-0018-0.

51. Kpodzo DS, Calderwood MS, Ruchelsman DE, et al. Primary subcutaneous Alternaria alternata infection of the hand in an immunocompromised host. *Med Mycol*. 2011;49(5):543-547. doi:10.3109/13693786.2011.555848.

52. Coussens E, Rogge S, Haspeslagh M, et al. Cutaneous infection by Alternaria infectoria in a liver transplant recipient: a case report. *Acta Gastroenterol Belg*. 2014;77(2):256-258.

53. Torres-Rodríguez JM, González MP, Corominas JM, Pujol RM. Successful thermotherapy for a subcutaneous infection due to Alternaria alternata in a renal transplant recipient. *Arch Dermatol*. 2005;141(9):1171-1173. doi:10.1001/archderm.141.9.1171-b.

54. Garcia-Diaz JB, Baumgarten K. Phaeohyphomycotic infections in solid organ transplant patients. *Semin Respir Infect*. 2002;17(4):303-309. doi:10.1053/srin.2002.36448.

55. Ando N, Takatori K. Keratomycosis due to Alternaria alternata corneal transplant infection. *Mycopathologia*. 1987;100(1):17-22.

56. Konidaris V, Mersinoglou A, Vyzantiadis T-A, Papadopoulou D, Boboridis KG, Ekonomidis P. Corneal Transplant Infection due to Alternaria alternata: A Case Report. *Case Rep Ophthalmol Med*. 2013;2013:589620. doi:10.1155/2013/589620.

57. Hsu C-C, Chang S-S, Lee P-C, Chao S-C. Cutaneous alternariosis in a renal transplant recipient: a case report and literature review. *Asian J Surg*. 2015;38(1):47-57. doi:10.1016/j.asjsur.2012.08.010.

58. Sörensen J, Becker M, Porto L, et al. Rhinocerebral zygomycosis in a young girl undergoing allogeneic stem cell transplantation for severe aplastic anaemia. *Mycoses*. 2006;49 Suppl 1:31-36. doi:10.1111/j.1439-0507.2006.01300.x.

59. Del Palacio A, Gómez-Hernando C, Revenga F, et al. Cutaneous Alternaria alternata infection successfully treated with itraconazole. *Clin Exp Dermatol*. 1996;21(3):241-243.

60. Mirkin LD. Alternaria alternata infection of skin in a 6-year-old boy with aplastic anemia. *Pediatr Pathol*. 14(5):757-761.

61. Lou J, Fu L, Peng Y, Zhou L. Metabolites from Alternaria fungi and their bioactivities. *Molecules*. 2013;18(5):5891-5935. doi:10.3390/molecules18055891.

62. Hong SG, Cramer RA, Lawrence CB, Pryor BM. Alt a 1 allergen homologs from Alternaria and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genet Biol*. 2005;42(2):119-129. doi:10.1016/j.fgb.2004.10.009.

63. Kobayashi T, Iijima K, Radhakrishnan S, et al. Asthma-related environmental fungus, Alternaria, activates dendritic cells and produces potent Th2 adjuvant activity. *J Immunol*. 2009;182(4):2502-2510. doi:10.4049/jimmunol.0802773.

64. Auger PL, Gourdeau P, Miller JD. Clinical experience with patients suffering from a chronic fatigue–like syndrome and repeated upper respiratory infections in relation to airborne molds. *Am J Ind Med*. 1994;25(1):41-42. doi:10.1002/ajim.4700250110.

65. Corrier DE. Mycotoxicosis: Mechanisms of immunosuppression. *Vet Immunol Immunopathol*. 1991;30(1):73-87. doi:10.1016/0165-2427(91)90010-A.

66. Deshmukh SK, Verekar SA, Bhave S V. Endophytic fungi: a reservoir of antibacterials. *Front Microbiol*. 2014;5:715. doi:10.3389/fmicb.2014.00715.

67. Ostry V. Alternaria mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J*. 2008;1(2):175-188. doi:10.3920/WMJ2008.x013.

68. Opal SM. Endotoxins and other sepsis triggers. *Contrib Nephrol*. 2010;167:14-24. doi:10.1159/000315915.

69. Kim K-H, Willger SD, Park S-W, et al. TmpL, a transmembrane protein required for intracellular redox homeostasis and virulence in a plant and an animal fungal pathogen.

PLoS Pathog. 2009;5(11):e1000653. doi:10.1371/journal.ppat.1000653.

70. Portal-Nuñez S, Shankavaram UT, Rao M, et al. Aryl hydrocarbon receptor-induced adrenomedullin mediates cigarette smoke carcinogenicity in humans and mice. *Cancer Res*. 2012;72(22):5790-5800. doi:10.1158/0008-5472.CAN-12-0818.

71. Andersson M, Downs S, Mitakakis T, Leuppi J, Marks G. Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr Allergy Immunol*. 2003;14(2):100-105. doi:10.1034/j.1399-3038.2003.00031.x.

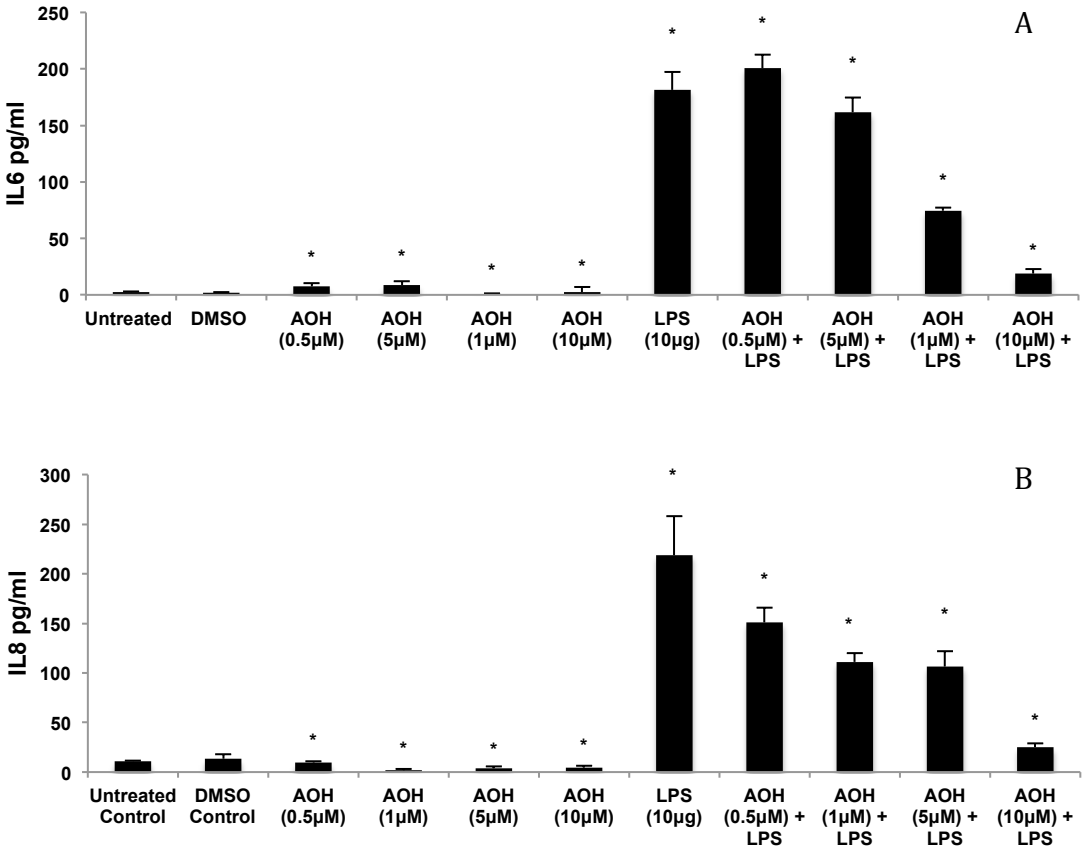
72. Martinon F, Burns K, Tschopp J. A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-β. *Mol Cell*. 2002;10(2):417-426. doi:10.1016/S1097-2765(02)00599-3.

73. Mariathasan S, Newton K, Monack DM, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*. 2004;430(6996):213-218. doi:10.1038/nature02664.

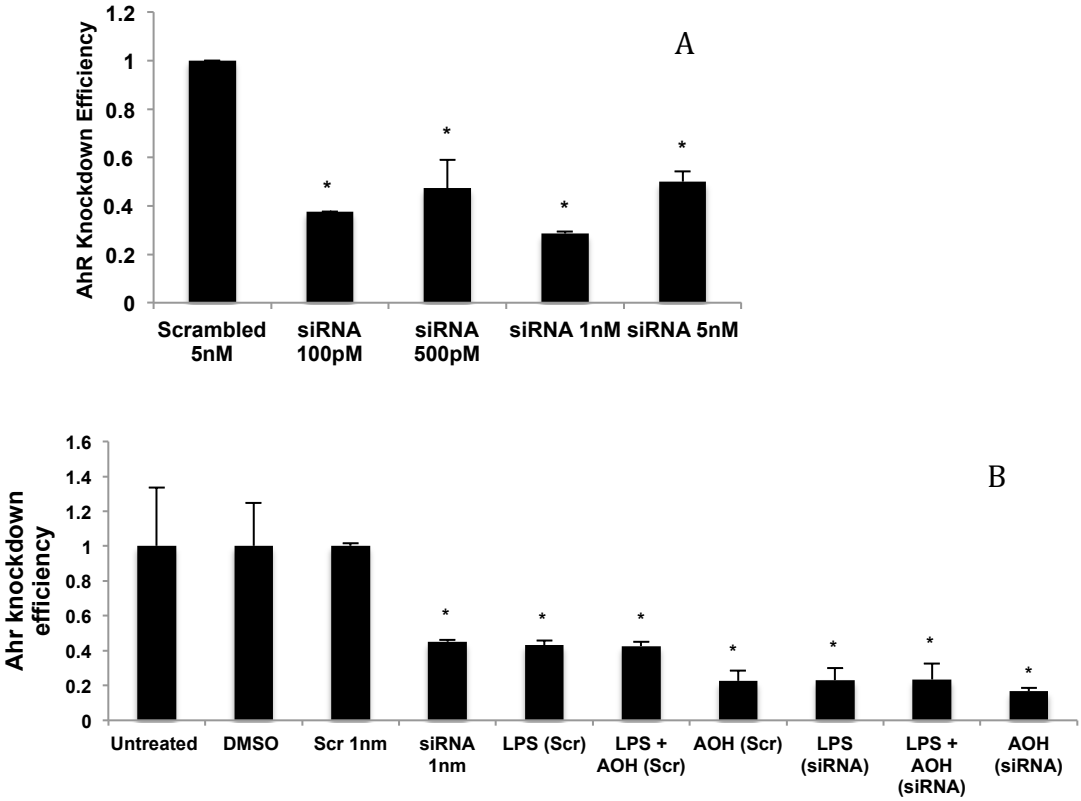
74. Berridge M V, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev*. 2005;11:127-152. doi:10.1016/S1387-2656(05)11004-7.

75. Vassilev LT, Tovar C, Chen S, et al. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc Natl Acad Sci U S A*. 2006;103(28):10660-10665. doi:10.1073/pnas.0600447103.

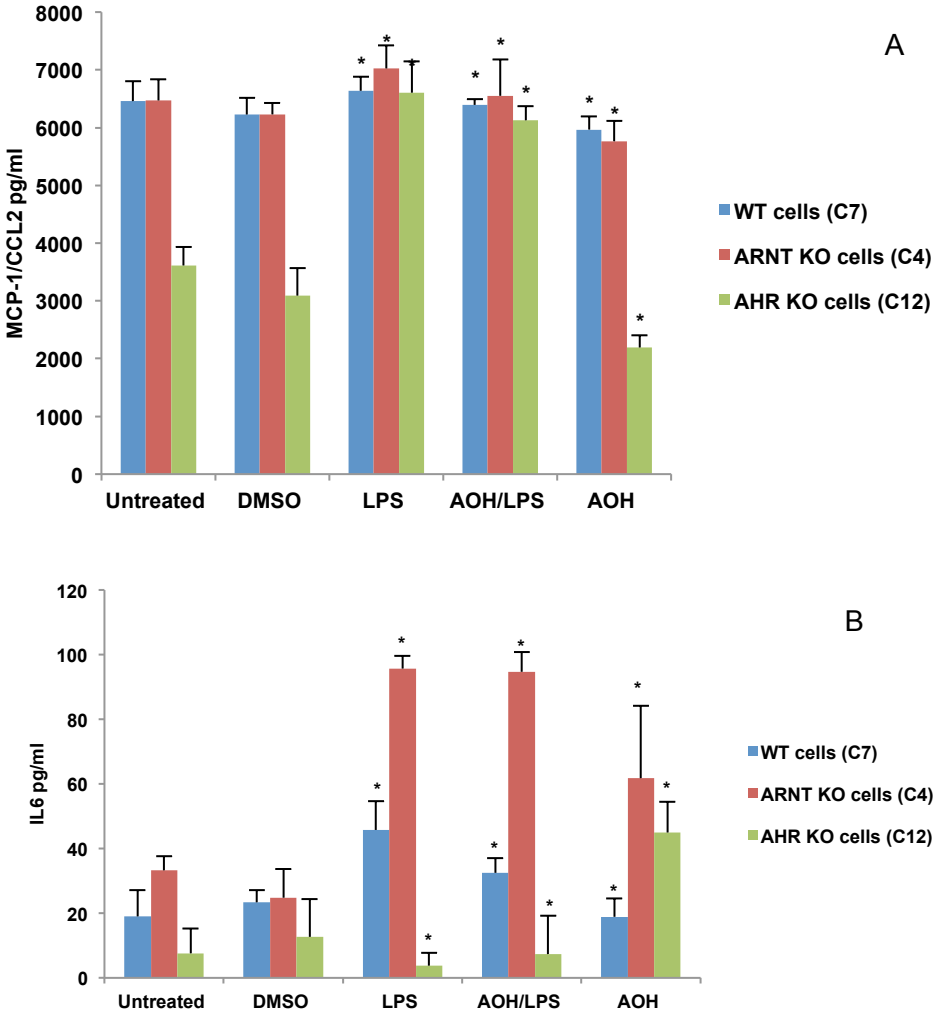
APPENDIX



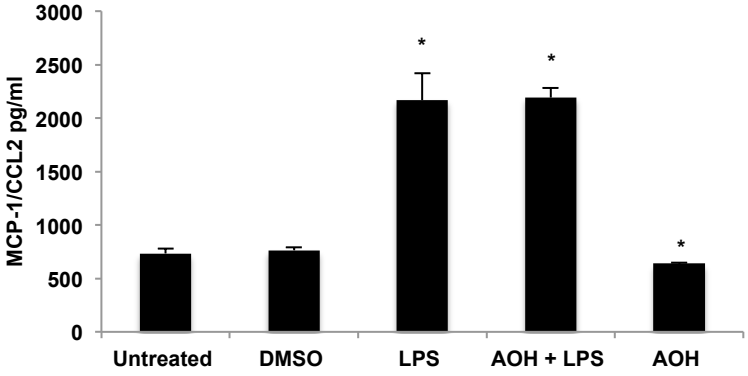
Supplementary Figure 1. Dose Dependent analysis of AOH response with LPS added 2 hours after AOH. BEAS-2B's were seeded at a density of 500,000 cells/well. Cells were treated with 0.5-10μM of AOH in presence and absence of 10μg of LPS to measure the cytokines levels released. Cell densities were 5 x 10^5 cells/well and were incubated for 24 hours. Under normal conditions at 37°C, 5% CO_2 cells treated with AOH showed a marked suppression of cytokines level both in the presence and absence of LPS (A) IL6 measured by ELISA. (B) IL8 measured by ELISA. An * indicates $p < 0.05$ according to Student's *t*-test.



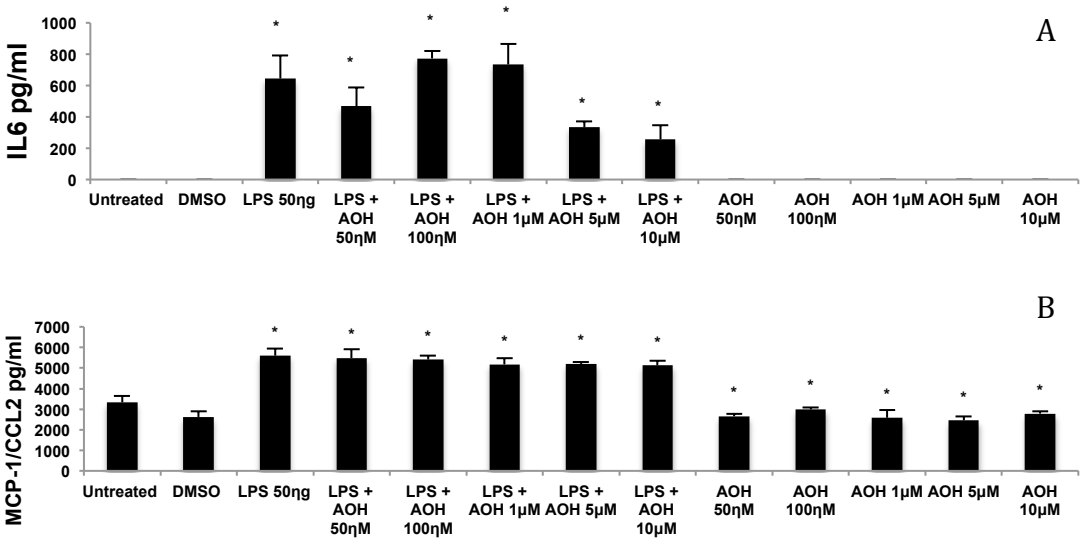
Supplementary Figure 2. qRT-PCR analysis of doses for AhR Silencing. Cells were seeded with a density of 150,000 cells/well. Cells were treated with AhR siRNA for a 24 hours twice to successfully knockout AhR. (A) Preliminary dose curve performed to elucidate the dose needed for AhR silencing in lung epithelium (B) AhR knockdown verified with qRT-PCR upon treatment with 10μM AOH and 10μg LPS for 24 hours. An * indicates $p < 0.05$ according to Student's *t*-test.



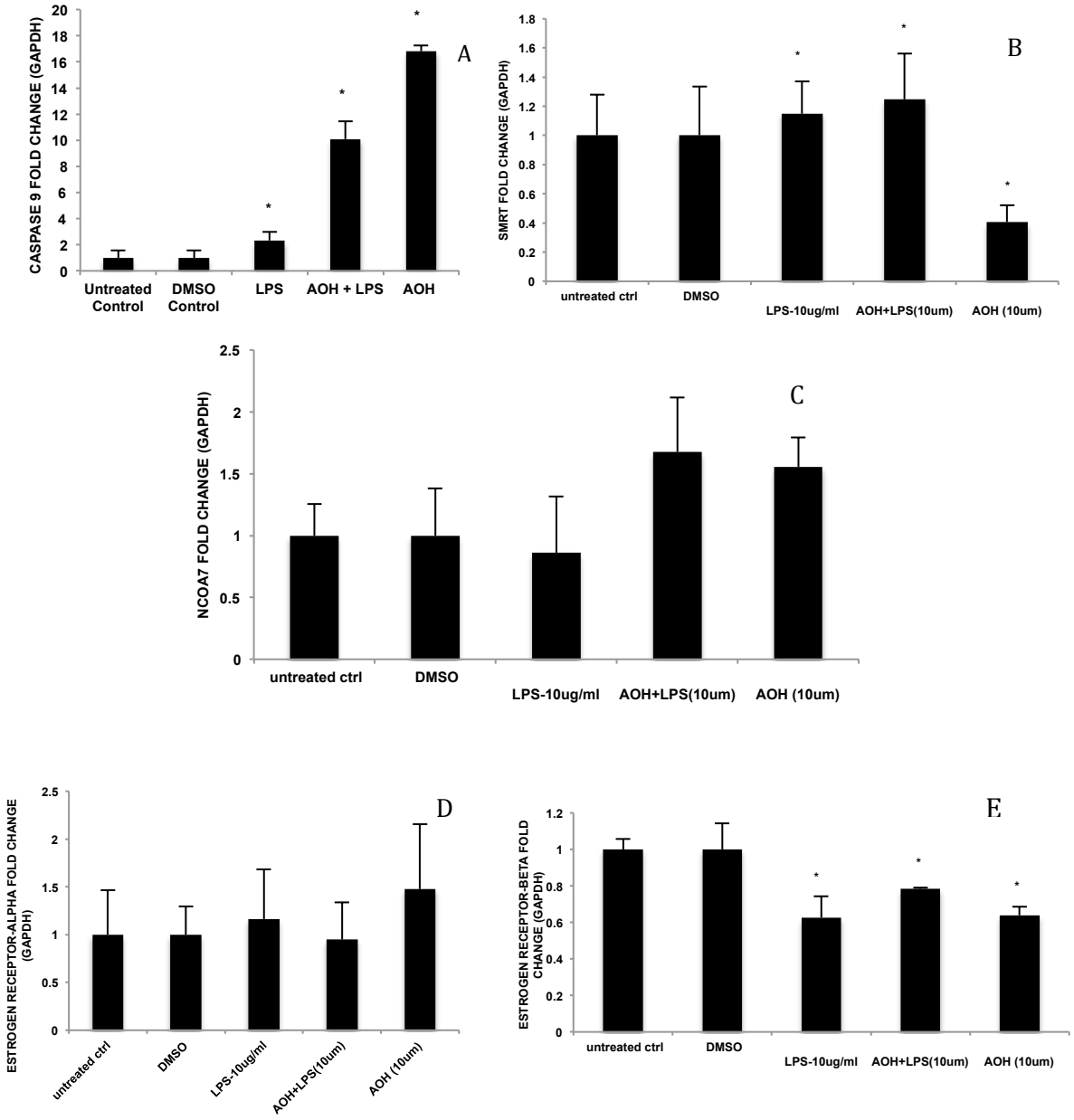
Supplementary Figure 3. Alternariol immune modulatory effects on mouse hepatoma wild type cells (Hepa-1c1c7), cells with silenced AhR receptor (Hepa-1c1c12), silenced ARNT receptor (Hepa-1c1c4).[37] Cell were seeded at a density of 5 x 10^5 cells/well and were incubated for 24 hours under normal conditions at 37°C, 5% CO_2. Cells were treated with10μM of AOH in presence and absence of 10μg of LPS to measure the cytokines levels released with ELISA. No marked change was observed relative to receptor silencing. The data was inconclusive. (A) CCL2 (B) IL6 released. An * indicates $p < 0.05$ according to Student's *t*-test.



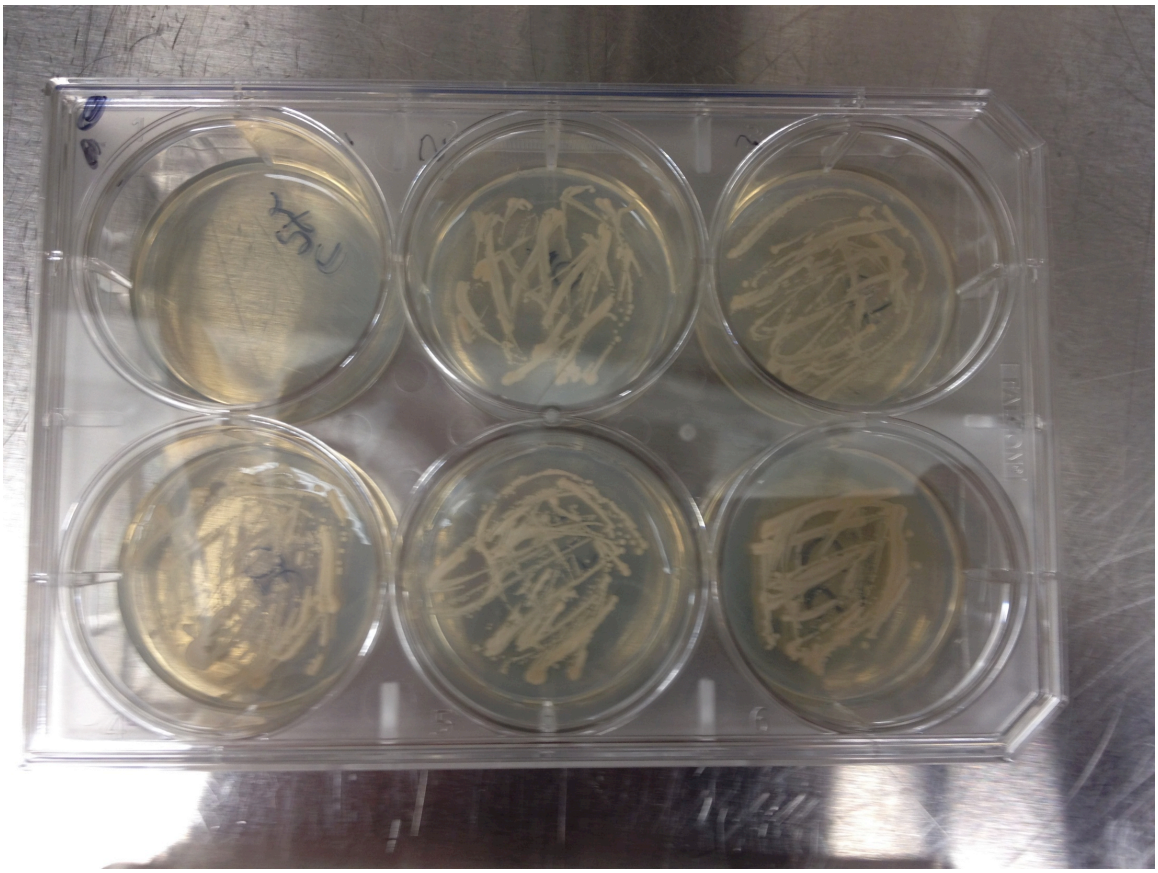
Supplementary Figure 4. Mouse Hepatoma cells with knocked out AhR receptor showed no change in CCL2 levels relative to silenced receptor. Cell were seeded at a density of 5 x 10^5 cells/well and were incubated for 24 hours under normal conditions at 37°C, 5% CO_2. Cells were treated with 10μM of AOH in presence and absence of 10μg of LPS to measure CCL2 levels released with ELISA. No marked change was observed relative to receptor silencing. The data was inconclusive. An * indicates $p < 0.05$ according to Student's *t*-test.



Supplementary Figure 5. Dose dependent response of mouse macrophages after treatment with AOH and LPS. Cells were treated with (50ηM-10μM) of AOH in presence and absence of 50ηg of LPS to measure the cytokine and chemokine levels released. Cell densities were 5 x 10^5 cells/well and were incubated for 24 hours under normal conditions at 37°C, 5% CO_2 after treatment. (A) IL6 (B) CCL2 released. An * indicates $p < 0.05$ according to Student's *t*-test.



Supplementary Figure 6. Quantitative Real-Time PCR analysis of airway epithelium. BEAS-2B's were seeded at a density of 500,000 cells/well and were treated with 10μM AOH and 10μg LPS for 24 hours. The resulting RNA was harvested and quantified with qRT-PCR. Each graph here demonstrates the up regulation and down regulation (fold change) of gene expression in comparison with the control GAPDH. (A) Caspase 9 (B) SMRT (C) NCOA7 (D) Estrogen Receptor-Alpha (E) Estrogen Receptor-Beta fold change. An * indicates $p < 0.05$ according to Student's *t*-test.



Supplementary Figure 7. Alternariol treatment with *Schizosaccharomyces pombe.* No changes were observed in growth between control and treatment groups. A dose of 10μM AOH was used here.

Primer	Sequence
PksJ-Fwd	5’ CTGCAGTATGCCCCTTACGAAGTTGG 3’
PksJ-Rev	5’ GAATTCGGCCGCTGAAGTCATAGAAC 3’
PksA-fwd	5’ GAATTCGGATCCACTCTCGCTCTCAC 3’
PksA-rev	5’ GGATCCGAGGACCACTGATGGTTAGG 3’
human AHR_F	5' TGGTTGTGATGCCAAAGGAAG 3'
human AHR_R	5' GACCCAAGTCCATCGGTTGTT 3'

Supplementary Table 1. Primers used during this study