Ph.D.

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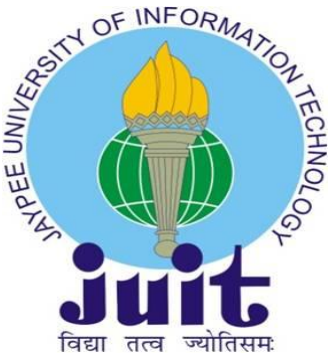
PROTEOME ANALYSIS OF HUMAN LUNG EPITHELIAL CELLS INTERACTING WITH *ASPERGILLUS TERREUS* CONIDIA AND STRUCTURE-FUNCTION RELATION OF nsSNPs IN INNATE IMMUNE RECEPTORS

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

RAMAN



Department of Biotechnology & Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

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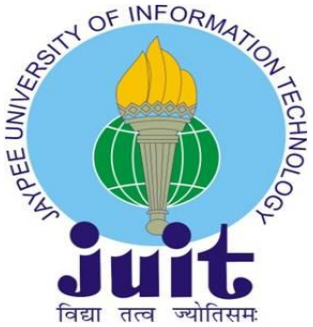

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DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of nsSNPs in innate immune receptors"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Jata Shankar**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.

Raman **Date:**

Enrollment No. 136558

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, India - 173234

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of nsSNPs in innate immune receptors"** submitted by **Mr. Raman** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor:

Dr. Jata Shankar **Date:**

Associate Professor

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, India - 173234

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Raman Thakur

ABSTRACT

The life-cycle of filamentous fungi consists of conidia, germinating conidia, hyphae and mycelium. Recently, filamentous fungus has gained importance among the opportunistic fungal pathogens. Germination is the key event in life-cycle of the filamentous fungus including *Aspergillus* species. The germination of the conidia in *Aspergillus* species is critical to establish invasive infections in immunocompromised individuals. Inhaled conidia often cause hypersensitive complications in immunocompetent host. Various studies using transcriptomic and proteomic approaches have been carried out to elucidate the biochemical pathways associated with germination. Such studies revealed that multiple genes and their products are active during germination of conidia in fungal system. However, *Aspergillus* species lack such studies at different conditions, morphological forms etc. Thus, objective (first) of current study was to establish a global proteome profile of germinating conidia of *Aspergillus terreus*. Our study reports proteins involved in translation, carbohydrate metabolism and transport that suggest their association during germination. Also, high expression of terrelysin in germination conidia may help to propose *A. terreus* specific diagnosis. Further, identification of proteins that are associated with mycotoxins production suggests their role in invasion of host tissues. The second objective of study was to explore the global proteome analysis of human lung epithelial cells (A549) during its interaction with conidia, as epithelial cells are the primary defense cells encountered by inhaled conidia. Interaction studies showed expressed proteins were involved in internalization of conidia or in immune response through Nuclear Factor-kB pathway. This pathway stimulates the production of IL-8 and, thus, could be evaluated further for biomarker target to control inflammation during lung infections. The third objective of current study was to predict the deleterious effect of non-synonymous SNPs on innate immune receptors such as dectin-1 and pentraxin-3 that recognize inhaled or germinating conidia. Dectin-1 is present on epithelial cells or on immune cells, whereas pentraxin-3 does surveillance in lung compartments. Seven high-risk non-synonymous-SNPs were present in CTLD-domain of dectin-1 and 4 non-synonymous SNPs were present in pentraxin domain of PTX-3 protein. These non-synonymous SNPs may provide a marker to screen susceptibility to *Aspergillus* associated infections. Thus, our study paved way to compare the proteins from germinating conidia of *Aspergilli* that may lead to development of better diagnosis or therapeutic targets. Along with this, NF-kB pathway could be a target to control inflammation during *A. terreus* or in other *Aspergilli* infections.

LIST OF ABBREVIATIONS

ABPA	Acute Bronchopulmonary Aspergillosis
AmB	Amphotericin B
BAL	Bronchial Lavage
BLAST	Basic Local Alignment Search Tool
CTLD	C-Type Lectin Domain
CD	Cluster of Differentiation
CCL	Chemokine Ligand
CXCL	Chemokine (C-X-C-Motif) Ligand
CBP	Clinical Break Points
CSF	Colony Stimulated Factor
cDNA	Complementary DNA
Ct	Threshold Cycle
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DDG	Gibbs free energy
ELISA	Enzyme Linked Immunosorbent Assay
ETBR	Ethidium Bromide
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
GO	Gene Ontology
HIV	Human Immunodeficiency Virus
HSP	Heat Shock Protein
IA	Invasive Aspergillosis
Ig	Immunoglobulin
IU	International Units
INF	Interferon

IL	Interleukin
ITRAQ	Isobaric Tag For Relative and Absolute Quantification
JAK	Janus kinase
KEGG	Kyoto Encyclopedia of Genes and Genomics
L	Liter
LC	Liquid Chromatography
MAPK	Mitogen Activated Protein Kinase
MIC	Minimum Inhibitory Concentration
MALDI	Matrix Assisted Laser Desorption/ Ionization
MS	Mass Spectrometry
Mg	Milligram
mM	Millimolar
MPKC	Mitogen Activated Protein Kinase C
MW	Molecular Weight
MHC	Major Histocompatibility Complex
nsSNP	Non-synonymous Single Nucleotide Polymorphism
NaCl	Sodium Chloride
NET	Neutrophils Extracellular Traps
NCCPF	National Collection Centre of Pathogenic Fungi
NCCS	National Centre for Cell Science
NCBI	National Centre for Biotechnology Information
PRR	Pattern Recognition Receptors
PAMPs	Pathogen Associated Molecular Patterns
PTX-3	Pentraxin-3
PCR	Polymerase Chain Reaction
PAGE	Polyacryilamide Gel Electrophoresis
PBS	Phosphate Buffer Saline

PPM	Part Per Million
PLGS	Protein Linux Global Server
PP	PolyPhen
PHD-SNP	Prediction of Human Diseased SNP
PDB	Protein Data Bank
qRT-PCR	Quantitative Real Time- Polymerase Chain Reaction
QTOF	Quadrupole Time of Flight
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
RMSD	Root Mean Square Deviation
SNP	Single Nucleotide Polymorphisms
SP	Surfactant Protein
SDS	Sodium Dodecyl Sulphate
SDA	Sabouraud Dextrose Agar
STAT	Signal Transducer and Activator of Transcription
SIFT	Sorting Intolerant from Tolerant
SNAP	Screening Non-Acceptable Polymorphisms
TLRs	Toll Like Receptors
TNF	Tumor Necrosis Factor
Th	T-Helper
TM	Template Modeling
UV	Ultra Violet
UniProt	Universal Protein Resource
UPLC	Ultra Performance Liquid Chromatography
µ	Micro
γ	Gamma
β	Beta
Δ	Delta
%	Percent

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INTRODUCTION

Over the last few decades, opportunistic fungal infections have increased worldwide. Among all fungal pathogens, *Aspergillus* species associated infections gained importance in humans and almost in all domestic animals to birds. They cause infections ranging from acute to systemic in humans as well as various allergic reactions such as ABPA, *Aspergillus* sinusitis, hypersensitivity pneumonitis and IgE-associated asthma. They are one of the most ubiquitous spore-bearing fungi that present in environment. Among all *Aspergilli*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus* cause severe fetal infections in humans having compromised immune system as well as in some immunocompetent individuals. In last decade, the number of immunocompromised patients increased due to transplantation, cancer, serious immunodeficiency diseases and other diseases such as HIV that made human prone to opportunistic fungal pathogens. Along with this situation, the emergence of drug resistance *Aspergilli,* further, complicated the *Aspergillus* associated infection scenario worldwide. Furthermore, the lacks of early diagnostic markers for *Aspergilli* pose a high risk to vulnerable human population. In last few years, *A. terreus* emerged as a leading causative agent of invasive aspergillosis in severely immunocompromised patients, especially in cancer patients. Further, the intrinsic resistance of *A. terreus* to Amphotericin B (AmB) complicates the treatment regime in infected patients that lead to worrisome in medical community. Germination is an imperative step for fungal pathogens to invade host tissues successfully and thus initiate an invasive infection in the host. Remolding of cell wall in fungi is important for conidial germination. As conidia start to germinate, the rodlet and melanin layer of conidia disappeared, and exposes proteins and polysaccharide that help in the establishment of successful fungal infections. So the proteins expressed during germination are important not only to decipher the mechanism of invasion into host tissues, but also can act as valuable early biomarkers for the diagnosis of *Aspergillus* species infections. Further, expressed proteins during conidial germination can be utilized as lead molecules for drug target and vaccine or allergen shot candidates for *Aspergillus* associated infections or allergies.

In humans, lungs are the primary organ site where inhaled conidia from *Aspergillus species* reside, and if it is successful in germination, establish invasive infection in the host. The bronchial and alveolar epithelial cells are the primary cells that

encounter these inhaled conidia and hinder the establishment of invasive infection. In immunocompromised persons, these cells play an important role in the blockade of infection due to the presence of fungal recognition receptors such as dectin-1, TLRs, etc. Pathogen Recognition Receptors (PRRs) recognize these fungal pathogens by their pathogen associated molecular patterns (PAMPs) and help to clear them by activation of effector molecules such as cytokines and chemokines or antimicrobial peptides. In immunocompromised patients, the phagocytic functions of immune cells are impaired and thus conidia stars to germinate. In such a condition, lung epithelial cells play an important defensive role against fungal growth or inhaled conidia of fungi. Further, people having respiratory disorders such as asthma, allergy and chronic obstructive respiratory diseases makes these individuals vulnerable to fungal manifestations. In these peoples, respiratory or lung epithelium cells are in direct contact with *Aspergilli*. Interaction of *A. fumigatus* with lung epithelial cells showed the production of various immune-regulatory molecules and activation of signaling pathways. Despite importance of lung epithelial cells, there is limited information available to understand the global proteome profile of lung epithelial cells interacting with *A. terreus*. Thus, it is important to decipher the proteins that are expressed by human lung epithelial cells during interaction with *A. terreus* that will shed light on the biochemical pathways/molecular events in these cells.

The genome sequencing of *Aspergillus* species have made possible to understand the biology of these fungal pathogens. Further, the high-throughput technologies such as microarray, RNA-seq helped to decipher critical factors or molecular pathways of these pathogens that are involved in virulence and invasion of the host tissues. The interaction studies *in-vitro* using various immune cells and *in-vivo* studies in mice models helped to understand the mechanism of pathogenesis of *Aspergillus species* and host immune response to their infections. However, the limited studies were carried out at the protein level on emerging *Aspergillus species* such as *A.terreus* during their morphological transition from conidia to germinating conidia. The proteins expressed during germination may shed light on virulence and invasive factors of this emerging fungal pathogen. Further, proteome studies will help to establish new early diagnostic markers as well as drug and vaccine candidates. On the other side, human immune system has different strategies to encounter these fungal pathogens. Immune systems of humans recognize these pathogens with the help of PRRs. The recognition of pathogens leads to

activate immune response to clear them from the host. In last decade, the single nucleotide polymorphisms (SNPs) gain importance due to their effect on the structure function of gene's products. Thus, the SNPs in pathogen recognition receptors may have deleterious effects on their structure that make individuals susceptible to pathogens.

In the personalized medicine era and emergence of next generation sequencing technologies not only help to sequence entire human individual's genome but also provide insight into human genomes such as Single nucleotide polymorphism. The central objective of the personalized medicine, population genetics and molecular biology is to characterize non-synonymous SNPs in genes associated with various biological functions. Thus, it seems to be imperative to analyze the SNPs in human population to understand the effect of these variations for individual's susceptibility to various infectious diseases. Further, the understanding of nsSNPs will not only provide the insight into disease susceptibility but also possible effects of the drugs or vaccines. Furthermore, the allergic disorders caused by *Aspergillus species* have gained importance due to their increased prevalence. Among allergic disorders, *Aspergillus species* cause ABPA, allergic Aspergillus induced sinusitis, hypersensitive pneumonitis and asthma induced by elevated level of IgE. Thus, it is a need of time to develop or come across with a promising therapy against *Aspergillus* induced health disorders.

REVIEW OF LITERATURE

2.1 *Aspergilli*

Fungi are eukaryotic organisms present in soil and environment. They are the most ubiquitous microorganisms with clinical importance and industrial applications [1]. There is a rise in infections caused by different fungal pathogens [2]. *Candida albicans* became the leading source of various infections [3, 4]. However, in last few years, *Aspergillus* species have emerged as a prominent opportunistic fungal pathogen due to rise in the immunocompromised host [2]. *Aspergillus* species are a ubiquitous fungi and gained medical importance. They are the leading opportunistic fungal pathogens. The genus *Aspergillus* contains more than 350 species, and the number is increasing every year with the identification of new species. Among all species, there are around 40 species of *Aspergilli* that can cause infections and allergic disorders in humans [5-7]. Among all *Aspergilli*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus* cause serious fetal infections in humans having compromised immune system as well as in some immunocompetent individuals [8]. In last decade, the number of immunocompromised patients increased due to organ transplantation, cancer, severe immunodeficiency diseases and other diseases such as HIV that made human prone to opportunistic fungal pathogens [9,10]. Along with this situation, the emergence of drug resistance *Aspergilli* further complicated the *Aspergillus* associated infections scenario worldwide [11,12]. Furthermore, the lack of early diagnosis of *Aspergillus* associated infections posses risk to vulnerable human population. In last few years, *A. terreus* emerged as a leading cause of invasive aspergillosis in severely immunosuppressive persons, especially in cancer patients [13,14]. Further, the intrinsic resistance of *A. terreus* to Amphotericin B complicates the treatment regime in infected patients who lead the worrisome in medical community [15]. The genome sequencing of different *Aspergillus* species have made advancement in understanding the biology of these fungal pathogens. Further, the high-throughput technologies such as microarray, RNA-seq helped to decipher critical factors or molecular pathways of these pathogens that help in virulence and invasion of the host tissues [16]. The interaction studies *in-vitro* using various phagocytic cells and *in-vivo* studies helped to understand the mechanism of pathogenesis of *Aspergillus* species and host immune response to their infections [17-21]. However, the limited studies were carried out at the protein level on emerging *Aspergillus* species such as *Aspergillus*

terreus during their morphological transition from conidia to germinating conidia. The proteins expressed during germination may shed light on virulence and invasive factors of this emerging fungal pathogen. Further, proteome studies will help to establish new early diagnostic markers as well as drug and vaccine candidates. On the other side, human immune system has different strategies to encounter these fungal pathogens. Immune systems of humans recognize these pathogens with the help of PRRs [7]. The recognition of pathogens leads to activate immune response to clear them from the host. In the last decade, the SNPs gained importance due to their effect on the structure function of genes [22]. Thus, the SNPs in pathogen recognition receptors have deleterious effects on their structure that make individuals susceptible to pathogens.

2.2 Taxonomy of *Aspergillus terreus*

Kingdom : Fungi
Phylum : Ascomycota
Order : Eurotiales
Family : Trichocomaceae
Genus : *Aspergillus*
Species : *terreus*

2.3 Distribution and morphology of *A. terreus*

Aspergillus terreus is a ubiquitous fungi having worldwide distribution. Its distribution in soil ranges from tropical to subtropical regions throughout the world. *A. terreus* distributes in environment through asexual spores called conidia that can grow at wide range of temperature (11 to 48°C). Further, the conidia of this species can tolerate high concentration of sodium chloride (NaCl), thus *A. terreus* also present in costal sand and salt marshes. *A. terreus* is a filamentous fungus with septate hyphae and produces two type asexual spores first are produced at the tip of vesicle present on conidiophores called Phialidic conidia or spores. Conidiophores begin from foot cells of supporting hyphae. Phialidic conidia are ultra small in size around 2µm. The second asexual spores are the globose-hyalinated conidia emerged from hyphae. Due to their tiny size, they remain suspended in environment and continuously inhaled by humans [23-25].

2.4 Diseases or manifestations cause by *A. terreus*

Due to rise in immunocompromised conditions in individuals, *A. terreus* has gained importance as a rising opportunistic fungal pathogen. It causes life threatening IA in severely immune-compromised individuals. It became the most important cause of invasive aspergillosis in cancer patients as reported by various hospitals worldwide [13, 14]. The high mortality rate due to *A. terreus* infections appears because of severe immune suppression, persistent in immune cells, virulent factors and resistance to AmB, a standard antimycotic drug [13, 21, 26]. Further, inadequate diagnostic marker against *A. terreus* may also be a reason to the current scenario. The immuno-competent individuals easily eliminated the inhaled conidia of *Aspergilli*, but the conidia of *A. terreus* reside inside immune cells for long time due to this macrophages transported inhaled conidia to other secondary organs [27]. When the immune system of any individual compromised, these persisted conidia start to germinate and cause infection in such persons. *A. terreus* causes superficial to systemic infection in humans. Among systemic infections, it causes invasive aspergillosis in immunocompromised patients [28]. Whereas among the superficial infections, it infects skin and toenail onychomycosis is the most frequently reported infections caused by *A. terreus*, which infects toe in humans [29]. Apart from infections in immunocompromised individuals, *A. terreus* causes hypersensitive complications in immunocompetant host such as ABPA, rhinitis, allergic sinusitis and pneumonitis [30]. Delay in diagnosis of *A. terreus* associated invasive infection leads to tissue destruction and, often a fatal outcome. In addition to invasive aspergillosis, it causes aspergilloma ("colonization" of existing pulmonary cavities in tuberculosis treated patients [31]. Further, lack of diagnostic markers and limited knowledge of proteins expressed during germination, which can be targeted as diagnostic markers, complicates the infection caused by *A. terreus*. Therefore, studies on the *A. terreus* germinating conidia and proteins expressed by them are essential to understand the biology, biochemical mechanism and the virulent factors of this fungus.

2.4.1 Invasive aspergillosis

Invasive aspergillosis (IA) is a systemic infection caused by *Aspergilli* in immunocompromised patients. Among *Aspergillus* species, *A. fumigatus, A. flavus* and *A. terreus* are the main cause of IA [1]. The frequency rate of IA associated with *A. terreus*

has increased in last few years [32]. Further, *A. terreus* resistance to AmB complicated the IA that leads to the high mortality in infected persons. IA mainly occurs in those individuals who undergo organ transplants or having acute leukemia and other forms of cancer or on chemotherapy [15,28]. Today, invasive aspergillosis is the leading infection in cancer patients caused by *Aspergilli*, and its actual incidence in patients is underestimated due to poor or low sensitive diagnostic methods. Other major risk factors of invasive aspergillosis are the neutropenia and dysfunctioning of receptors, which recognize *Aspergilli* such as lectins, TLRs and pentraxin-3 (soluble receptors circulate inside lung compartments) present on various cells [8]. Thus, the clear understating of receptor functions and SNPs present in genes, which encode these proteins are influential and can be utilized as biomarker for invasive aspergillosis in patients those undergo organ transplants. Further, it is also significant to understand the interaction of conidia and germinating conidia of *Aspergilli* with lung epithelial cells and other immune cell which surveillance the lungs for better understanding of immune response and pathophysiology of invasive aspergillosis in immunocompromised hosts [33]. There are different forms of invasive aspergillosis classified as (1) chronic acute invasive aspergillosis (2) invasive rhino sinusitis aspergillosis (3) obstructive bronchial aspergillosis and disseminated form of invasive aspergillosis of kidney, heart, brain and skin. The symptoms of invasive aspergillosis can vary with the infected organs at the primary site, i.e. lung. Patients develop cough, production of sputum, dyspnoea and fever that is unresponsive to antibiotics. Patients can also develop chest pain and further hemoptysis [34]. Invasive aspergillosis develops after germination of inhaled conidia of *Aspergilli*. So, it is important to study the proteins and metabolites expressed during germination of *Aspergillus* conidia that may help in the invasion of host tissue, and some of them could be further utilized for the development of early diagnostic marker for *Aspergillus species* specific invasive aspergillosis [35,36]. Thus, the improved diagnosis of invasive aspergillosis may help in better antifungal therapy in case of IA implicated by *A. terreus.*

2.4.2 Acute Bronchopulmonary Aspergillosis

Acute bronchopulmonary aspergillosis (ABPA) is a type of hypersensitive reaction caused by *Aspergillus species*. ABPA is one of the most severe complications of respiratory tract and frequently takes place in individuals having atopic asthma and affected with cystic fibrosis [37]. *A. fumigatus* is the leading *Aspergilli* which cause

ABPA but now *A. terreus* has also gained importance as a causative agent of ABPA [1, 30, 38]. There are various reports that suggested *A. terreus* as an upcoming cause of ABPA. The ABPA in asthmatic and cystic fibrosis patients is characterized as an eosinophilia, pulmonary opacities and bronchial asthma. The courses of ABPA complications are the same as the classical asthma having unique pathophysiological conditions caused by T-cell immune response [39]. If the ABPA is remaining untreated, it leads to respiratory failure in infected patients [37]. Thus, diagnosis of ABPA is essential, and also it is imperative to understand the role of lung epithelial cells during their interaction with *Aspergilli* for better treatment.

2.4.3 Aspergilloma

Aspergilloma is basically a ball of developing mycelia of fungi that grow in preexisting cavities of lung [40]. These cavities are occurring after tuberculosis or its treatment, lung disorders and obstructive paranasal sinuses. Aspergilloma is a non-invasive form of aspergillosis may also develop inside immunocompetant hosts. It consists of fungal hyphae that are embedded in protein matrix having sporulating fungal structure at the periphery in the cavity inside lungs. Aspergilloma reported after hemoptysis, which is caused by rupturing of blood vessel, and sometimes it may be fatal [34].

2.5 Diagnosis

Invasive aspergillosis (IA) diagnosis remains a challenging work, and it is difficult to early diagnose IA in immunocompromised patients [41]. The histological examinations of the lung tissues and biopsy samples from lungs are the standard methods for diagnosis of IA. But observations of histological examination can vary with patient type such as those having transplant or cancers [42]. The histopathological examination of lung tissues allows the detection of septate hyphae along with positive culture of biopsy samples for *Aspergillus species*. The positive sputum samples from immunocompetent hosts are insignificant because *Aspergillus* species always found with no-clinical symptom in immunocompetent hosts [43]. Further, the immunocompromised individuals having positive sputum samples considered for invasive aspergillosis, especially those having leukemia or undergone stem cell transplantation [44]. Blood cultures from patients having invasive aspergillosis are rarely positive and having no significant use [45]. Also chest

radiographs are insignificant for early conformation of IA because other filamentous fungal infections of lungs have the same radiographs of chest along with tuberculosis [46]. The recent advancements have allowed the detection of *Aspergilli* antigens in body fluids. Recently sandwich ELISA is available for the detection of cell wall components from *Aspergilli* such as β-glucan and glactomannan. FDA has approved the glactomannan detection ELISA assay for the identification of IA with threshold rate of 0.5 ng/ml concentration in serum of patients [47]. The major limitations of this assay are less sensitivity (71%) false-positive results due to the glactomannan presence in food products and indistinguishable with other fungi such as *Fusarium* and *Zygomycetes* [48,49]. PCR is another method used to diagnose invasive aspergillosis, which detected the *Aspergillus* DNA in serum and BAL fluid in infected patients. The specificity and sensitivity of this assay can vary and ranged from 55-95% and 67-100% respectively [50-51]. The major drawback of this method is that it cannot discriminate in colonization and infection of *Aspergilli*. Further, this method is limited to limited laboratories. FDA also approved the detection of β-glucan in serum of infected patients, and this test is highly sensitive and precise to detect deep invasive fungal infections. However, still it is difficult to diagnose the *Aspergilli* at the species level, which will further help for better therapeutic treatments. So there is a need to develop species-specific diagnostic methods. The diagnosis of aspergilloma is based on the chest radiographs along with serological methods described above. The chest radiograph clearly indicates the occurrence of fungal balls in pre-existing cavities [52]. Nevertheless, it is always advisable to have serological diagnosis along with the chest radiographs because some conditions mimic the aspergilloma conditions such as neoplasm, granulomatosis with polyangiitis and hydatid cyst [53-54]. For the diagnosis of ABPA, serological tests and radiography is used. In clinical outcomes, the ABPA patients always have asthma, chest pain, constant fever, expectorating sputum and wheezing [55]. The patients having ABPA diagnose with elevated serum IgE level, i.e., IgE>1000 IU/mL and peripheral eosinophils more than 1000/μL. Thus the minimum criteria used to diagnose ABPA is person having asthma, positive skin test for *Aspergillus* antigens and showing a high IgE level in serum [34].

2.6 Immune response to *Aspergilli*

Host response against *Aspergillus* associated infection is of great significance for effective treatment and control of the disease condition [56]. Understandings of host's

interactions with pathogens are essential to determine the extent of damage and disease depending upon the immunological conditions of any individual [57]. Studies in mice have shown that both innate and adaptive immune system determine the protection of mice against *Aspergillosis* [58]. The inhalation of *Aspergillus* conidia by humans initiates their entrapment by alveoli and if conidia are not cleared from lungs, they start to germinate and establish infection in lungs and also disseminated to other organs like kidney and brain [59]. Recognition of *Aspergilli* conidia or germinating conidia is accomplished by a diverse group of PRRs that recognize various conserved fungal cell wall moieties called PAMPs including proteins, carbohydrates, and nucleic acids. Immune response against *Aspergilli* depends critically on PRRs [60]. After recognition of *Aspergillus* PAMPs, the PRRs induce intracellular signaling that activates immune cells and secretion of immune-regulatory molecules instructs the evolving immune response [61]. PRRs are vital components to recognize fungi include TLRs, C-type lectins and pentraxin-3 receptors. They are expressed on the cell membranes of distinct immune cells where they sense specific moieties present on *Aspergilli* germinating conidia surface and developing hyphae [62]. TLRs activate signaling pathways through interaction with different adaptor proteins, including My88. This adaptor protein activates the mitogen-associated protein kinase (MAPKs), interferon regulatory factor (IRF) and nuclear factor-kβ (NF-kβ) and induces the production of cytokines and reactive oxygen species [8,62]. Whereas the C-type lectins (dectin-1) signal through immunoreceptor tyrosine-based activation motif (ITAM), leading to the activation of NF-kβ signaling pathway [63]. Thus, induces the production of cytokines. The first line of defense against respiratory tract pathogens is the innate immune cells (alveolar macrophages, neutrophils and antigen presenting cells such as dendritic cells or monocytes) which recognize respiratory pathogens, including conidia of fungi using these receptors that present on their surface [64]. In human lungs, alveolar macrophages are the important phagocytic cells which provide the defense against conidia of *Aspergillus species* that reach to lung alveoli [27]. After the recognition of *Aspergillus* conidia through PRRs, they initiate the phagocytosis of inhaled conidia and start their killing through acidification of phagolysosome and antimicrobial enzymes. However, sometimes conidia escape the phagocytosis due to heavy melanin content present in conidia by inhibiting acidification of phagolysosome [65]. Further, macrophages produce various cytokines (INF, TNF) that attract other immune cells at the site of infection [66]. *A. terreus* conidia have unique ability to persist

for long time in alveolar macrophages, thus they transported to secondary organs through macrophages and execute wait and watch function [21, 27]. When individual having *A. terreus* conidia at secondary sites become immunocompromised, they start to germinate and imitate infections. If fungal conidia and hyphae escape the macrophage, there is another first-line phagocytic cell, which clears fungal spores and hyphae that are neutrophils. Neutrophils are the immune cells, which also help in eliminating fungal spores and hyphae from infected area. In lungs neutrophils recruited to the site of infection by cytokines, mostly by IL-8 and trigger the inflammatory response which clears the fungal hyphae [67]. So, the people suffering from neutropenia are on high risk of invasive aspergillosis. *In-vitro*, studies and animal model showed that neutrophils are important for clearance of fungal infection. Neutrophils attach to the fungal hyphae and their degranulation leading to inflammation, which further clear the fungus from infected area [68, 69]. Neutrophils cells recognize fungi through pathogen recognition receptor, including toll-like receptor and dectin-1. These cells are also able to clearing fungal hyphae through neutrophil's extracellular traps (NET) which are formed after autolysis of neutrophils, cause release of their DNA into surrounding environment to hinder the progress of infection [70]. In addition to these defense strategies, neutrophil's granules contain the different type of antimicrobial compound such as defensins, lysozyme, lactoferrin and proteases [71]. Mostly, conidia are resistant to neutrophils attack but recent studies show that these immune cells encountered the conidia through lactoferrin mediated iron sequestration. Iron is essential for immune cell's functions, so if iron is sequestered by immune cells, they clear the fungal conidia because there is no free iron for conidia germination and if iron is sequestered by fungi, then they escape the first line of defense [68]. Transcriptomic studies on *A. fumigatus* conidia interacting with neutrophils revealed the complex response of gene associated with oxidative stress. Using these studies, the genes which encode catalases and superoxide dismutase were found up-regulated [68]. *A. terreus* conidia interaction with neutrophils is not completely understood and needs further investigations. The interaction of *Aspergilli* conidia with macrophages and neutrophils starts inflammatory immune response thus attracts antigen presenting cells at the site of infections from surrounding tissues or blood by secreting cytokines and chemokines [8]. The primary antigen presenting cells are the monocytes and dendritic cells. Monocytes are separated into a subpopulation on the basis of their markers such as $CD14^{+}$ $CD16^{+}$ and $CD14^{+}CD16^{-}$. The 90% of these cells are

$CD14^{+}CD16^{-}$ and phagocytose the *Aspergilli* conidia and secrete a cytokine TNF-α [72]. Further, it has been suggested that through PRRs (dectin-1, pentraxin3 and TLRs) they can trigger T-cell immune response (adaptive immunity) [64]. Transcriptomic studies of monocytes interacting with *A. fumigatus* reported the up-regulation of the genes that encode pentraxin-3 and chemokine receptors (CCL3, CCL20, CXCL2 and CCL4) [73]. Further, other antigen presenting cells, i.e. dendritic cells surveillance the human system in immature form, when they interact with pathogens and become mature. These cells present the microbial antigens on their surface and activate T-cell adaptive immune response [64]. The interaction study on mature dendritic cells and conidia of *A. fumigatus* using transcriptomic approach reported the up-regulation of genes, which encode dectin-1, pentraxin-3, CCL20, IL-1B and IL-8 [73]. The direct interaction of dendritic cells with *A. fumigatus* conidia suggested the 48% phagocytosis of conidia after two hours of interaction [74]. Dendritic cells are the most important cells which bridge the innate and adaptive immunity. By presenting pathogen's antigens on their surface, they activate the adaptive branch of immune response. They modulate the T-cell immune response to either protective (Th1 or Th17) or distractive (Th2 or Th9) during *Aspergillus species* infections [75]. Adaptive immune response through Th1 helper cells is associated with an increase in IL-12 and INF-ϒ cytokines [76]. If, the cytokine INF-ϒ level increases initially during invasive aspergillosis, it suppresses the activity of other cytokines IL-4 and IL-17, thus inhibit the activation of Th2 and Th17 T-cells [77]. Whereas, if IL-4 dominates during the early infection state of *Aspergilli*, it starts the activation of Th2 cells by inhibiting the IL-12 and thus activates the inflammatory response through eosinophil and basophiles activation [78, 79]. Role of several chemokines, chemokine receptors and other immune cells has also been reported in response to invasive pulmonary aspergillosis [1]. Further, there is diverse T-helper immune response during invasive aspergillosis and allergic boncho-pulmonary aspergillosis [19, 20, 80]. However, there is limited of knowledge about cell mediate immune response, especially T-helper cell immune response to *A. terreus*.

2.7 Airway epithelial cells

Due to the tiny size of *Aspergilli* conidia, they easily reach to the lung alveoli. It has been reported that every individual inhale several conidia daily, and they can be isolated from sputum of immunocompetent hosts [1]. In lungs, conidia of *Aspergilli* come in the contact

with epithelial cells either bronchial or alveolar lung epithelial cells. Airway cells are not only considered as physical barrier against respiratory tract pathogens, but now they are the important member of cells, which defend against fungal pathogens [81]. Lung epithelial cells are categorized into two types; type-I cells that constitute about 95% of surface of lung's alveoli, whereas type-II cells only represent 5%. Type-II cells are the important cell type of alveolar surface because they secret surfactant proteins or also they are progenitor of type-I cells type [82]. Further, now type -II lung epithelial cells consider to be the first cells which interact with fungal pathogens and initiate immune response. Various studies have been carried out on alveolar type-II cells (A549-carcinoma cell type) interacting with *A. fumigatus* conidia, and these studies demonstrated that A549 cell internalized conidia and activates downstream immune response against inhaled conidia [83]. Furthermore, these cells provide the model system to study the host pathogen interaction, especially for respiratory tract pathogens. Previously, it has been reported that the internalization of conidia initiated by rearranging cytoskeleton of these cells by activating phospholipase D and cadherins proteins [83,84]. After internalization of conidia, they activate innate immune response. The interaction of inhaled conidia of *Aspergilli* with lung epithelial cells is multifaceted and highly complex and also serious implication at later stages of *Aspergillus* related infections. Lung epithelial cells are the early player of immune response to fungal pathogens [85]. These cells also express chemokines, and cytokines thus modulate the innate and adaptive immunity. Previous, interaction studies on epithelial cells (A549) with *A. fumigatus* conidia using RNA-seq technique revealed that they expressed the genes which involve in hindrance of oxidative damage to cells and repair system (microsomal glutathione S-transferase 1) [86]. In addition, these cells also express the PPR dectin-1 and also secret pentraxin-3 protein that function as an opsonin and activates classical complement pathway by binding with C1q component. The recognition of conidia through dectin-1 receptor initiates synthesis of pro-inflammatory cytokine IL-8 and attracts immune cells at the location of pathogen recognition [85]. Moreover, it has been suggested that the loss of dectin-1 from lung epithelial cells induce the lung damage [87]. Many studies have shown the key role of these cell types at early stages of infection caused by respiratory pathogens. Thus, it is necessary to study the early interaction of *Aspergillus* conidia or germinating conidia with epithelial cells to understand the establishment of *Aspergillus* infection or *A. terreus* associated infections in high-risk patients.

2.8 Therapies for infections caused by *A. terreus*

2.8.1 Treatment of invasive aspergillosis

The invasive aspergillosis caused by different *Aspergilli* is difficult to treat because *Aspergillus* species differ in susceptibility to antifungal drugs [88]. In last decade, voriconazole is the recommended drug used to treat the invasive aspergillosis along with standard drug Amphotericin B (AmB) [89,90]. The other alternative therapy is lipid formulation of AmB and micafungin [91]. In spite of antifungal treatment of invasive aspergillosis, the results are disappointing, with high mortality rate ranging from 27% to 80% [92]. Different efforts have been made for establishing the clinical breakpoints (CBPs) for antifungal drugs like wise azoles, echinocandins and for AmB [93]. In last few years, European Committee on Antimicrobial Susceptibility Testing has set up the clinical break point for antifungal drugs for their resistance and susceptibility to *Aspergillus* species [94]. But in the absence of CBPs these drugs are used in combinations, and their significances are still not fully understood. AmB is the choice of antifungal drug that is used for treatment of IA from many years [95]. However, *A. terreus* has inherent resistance to this drug and mechanism behind resistance is not fully understood [15]. Recently, it has been reported that the heat-shock proteins and oxidative stress may involve in *A. terreus* resistance against AmB [96]. The minimum inhibitory concentration of AmB for *A. terreus* is >2 mg/L. Thus, AmB has poor efficacy against invasive aspergillosis (IA) caused by *A. terreus* [26]. Further, AmB has serious side effects such as decrease in glomerular filtration, hypokalemia, hepatotoxicity and cell toxicity [97]. The clinical studies have suggested the high rate of failure of AmB therapy (80-90%) against IA caused by *A. terreus* [98]. Further, the lipid formulation of AmB significantly not increases the success of this drug therapy [99]. Thus, the AmB is not a choice of therapy for *A. terreus* infections. Voriconazole is the first line of therapy against IA caused by *A. terreus* [100]. The voriconazole treatment failure is 52.9% as compared to other drugs having a failure rate as 65.3%. Further, the *A. terreus* acquired the resistance against Voriconazole. *A. terreus* gain a mutation M217I in Cyp51 which leads to high MIC of this drug against *A. terreus* [101]. The other choices of drugs used for the treatment of IA caused by *A. terreus* are posaconazole and echinocandins and these drugs are reported as more promising against IA caused by *A. terreus* [102, 103]. The failure rate of antifungal therapies for the treatment of IA caused by *A. terreus* is multi-factorial. The patients who

suffer from IA caused by *A. terreus* are immunocompromised and on co-mediation (chemotherapy in case of cancer patients). Thus co-meditation causes drug-drug interactions that come up with failure of antifungal treatment.

2.8.2 Treatment of acute bronchopulmonary aspergillosis

Acute bronchopulmonary aspergillosis (ABPA) or other hypersensitive aspergillosis disorders such as allergic rhinitis and allergic sinusitis affects those persons having asthma or cystic fibrosis [37]. They can affect immunocompetent host. These hypersensitive disorders mainly occur due to hyper-immune response or inflammatory immune response [38]. So to control or treat allergic aspergillosis oral steroidal drugs are used to suppress hyper or inflammatory immune response [37]. The treatment using corticosteroids helps in reduction of IgE and eosinophils. Along with corticosteroids, itraconazole has been also given to patients affected with ABPA to reduce the burden of *Aspergilli*. But, major risk of corticosteroids use for immune-suppression, it makes individuals susceptible to fungal infections [104]. Now a day, voriconazole tried with steroids and its use significantly improves the ABPA condition in cystic fibrosis and asthmatic patients [34, 105].

2.8.3 New antifungal drugs or compounds and targets for *Aspergillus species* infections

The high mortality and morbidity of infections caused by fungal pathogens, especially *Aspergillus* species are associated with limited antifungal drugs and their toxicity to patients [106]. Further, to identify new drug targets for antifungal drugs against fungal pathogens are challenging due to fungal cell similarities with human cells [107, 108]. The most targets for antifungal drugs are cell wall or membrane components along with transcription inhibition. Thus, it is the need of time to investigate novel drug targets against fungal pathogens [106]. Further, most of the pathogenic fungi acquired the resistance mechanisms against existing antifungal drugs by development of fungal bio-films and over-expression of efflux pump proteins [109,110]. Thus, there is a need of new strategies to enhance antifungal treatment such as by developing new formulation of existing drugs, discovery of new drugs and better carrier molecules like nanoparticles can be utilized to deliver an antifungal drug at the sites of infection. Calcineurin signaling pathway emerged as a new anti-aspergillus target. Calcineurin protein is related to

activation of Calcineurin signaling pathway that associated with various biological processes in *Aspergilli* or other fungal pathogens such as *Candida* [111]. It regulates the fungal cell morphogenesis, stress response and antifungal resistance. It also regulates the biosynthesis of ergosterol, β-glucan and chitin. Recently, triphenylethylene suggested a new compound that inhibits the calcineurin pathway by acting on calmodulin, which activates calcineurin [112,113]. Further, heat shock proteins such as Hsp-90 and inhibition of microtubule synthesis emerged as new antifungal targets [114]. Furthermore, in last decade new antifungal formulations and structure modifications have been used to develop less toxic antifungal drugs. A derivative of AmB, *N*-methyl-*N*-D-fructosyl AmB methylester (MFAME) has been developed with less toxicity to humans [115]. Echinocandins are the newest antifungal drugs that have been approved for antifungal treatments. This class of drugs inhibits the biosynthesis of cell wall glucan of fungal pathogens [90]. Further new azole compounds have been synthesized to treat aspergillosis and to combat resistance *A. fumigatus* strains such as PC945 and PC1244 [116]. These compounds are designed to take through inhalation to combat invasive aspergillosis. In addition, a novel echinocandin, CD101 has been developed to fight against serious fungal infections such as invasive aspergillosis. However, this drug is under clinical development and showed promising results against candidemia [117]. Furthermore, another novel antifungal compound F901318 is under clinical development for the cure of fatal fungal infections [118]. Despite the enormous efforts to control antifungal resistance or develop new antifungal compounds and modification in existing drugs are insufficient to solve toxicity of drugs and resistance acquired by fungal pathogens. Thus, there is a need of alternative therapies in combination with antifungal drugs such as immunotherapy [119, 120].

2.8.4 Immunotherapy against *Aspergillus* associated disease.

Aspergillus associated infections mainly occur in immunocompromised individuals or persons having genetic deficiency related to immune response [121]. Thus, the immunotherapy is an alternative strategy to improve the host immune response that leads to the better outcome of infections associated with *Aspergillus species* [122]. In the last decade, progress has been made to understand the antifungal immune response against major fungal pathogens that help to develop immune molecules or cell based immunotherapy to *Aspergillus* associated infections or for candidiasis [106]. Surfactant

proteins present in lungs have gained attention as a primary immune modulator in aspergillosis [123-124]. Previously, Maden *et al* demonstrated that surfactant proteins-A and D involves in clearance of *A. fumigatus* conidia with the help of alveolar macrophage and neutrophils [125]. Further, they reported that therapeutic use of surfactant proteins and mannan bonding lectin in the murine model of aspergillosis reduces that *A. fumigatus* burden and suppresses the production of IgE and eosinophil recruitment, thus help in controlling ABPA [125]. Pentraxin-3 protein furthermore gains importance as a potential immunotherapeutic agent against aspergillosis [126]. It recognizes *Aspergillus* conidia and amplifies the immune response. It was previously recognized that its deficiency in humans makes them susceptible to invasive and allergic aspergillosis. Further, in mice studies, it has been elucidated that the early exogenous administration of pentraxin-3 protein enhances clearance of *A. fumigatus* conidia and increase survival rate of infected mice [127]. Thus, pentraxin protein can be utilized as an immunomodulatory molecule in patients having invasive or allergic aspergillosis. Further, different immunomodulatory molecules, mainly cytokines have been used as immunotherapeutic agents to treat fungal infections. Colony Stimulating Factors (CSF) and interferon-γ have been utilized to control invasive aspergillosis [128-129]. Cytokine interferon-γ modulates the T-helper cell immune response to protective Th1 cells [128]. Thus, they help to clear fungal pathogens from infected hosts. In last few years, IL-37 cytokine discovered to control the inflammasome in murine aspergillosis [130]. Thus, the immunotherapeutic properties of this cytokine can be explored in aspergillosis patients. T-cells also gain attention as immunotherapeutic agents for fungal infections. *Aspergillus specific* T-helper cells (Th1 cells) can be utilized to control *Aspergillus* associated infection, especially invasive aspergillosis. The immunogenic epitopes such as *A. fumigatus* cell wall component glucanase Crf1 can be utilized to induce Th1 immune response [131]. Further, bioengineering of T-cell specific receptors that recognize *Aspergillus species* cell wall components will open a new way to treat aspergillosis. Thus, in the future, the manipulation of immune response using cytokines in combination with antifungal drugs may help in development of better therapeutic strategies for *Aspergillus* associated infections.

2.9 Single nucleotide polymorphisms and pathogen recognition receptors

With upcoming individual based therapy, affordable and accessible next generation sequencing technology not only helps to sequence entire genome but also may provide insight into single nucleotide polymorphisms (SNPs). The central objective of the personalized medicine, population genetics and molecular biology is to characterize SNPs in gene associated with various irregular biological functions [132]. SNPs are the single base change in the coding or non-coding parts of genes, and their presence has been located in the human genome after every 200-300 bp [133]. Annotation of sequenced human genome, so, for identified at ~500000 SNPs in coding part of human genome [134]. SNPs are categorized into synonymous-SNPs and non-synonymous SNPs (nsSNPs) depending upon amino acid changes. The changes in the amino acids due to nsSNPs affect coded proteins by altering their stability or functions. The effect on protein's functions due to nsSNPs was reported in various human diseases [135,136]. Further, U.S. has initiated the "precision medicine initiative" to sequence 1 million individuals' genome, and U.K also proposed to sequence 100,000 human genomes [137]. Thus, the data available from these projects would reveal individual person's variability in terms of single nucleotide polymorphism that has to be correlated to disease susceptibility and drug or vaccine responses. Therefore, the understanding of nsSNPs not only provides the insight into disease susceptibility but also highlights the drug targets and bio markers. In last few years, many studies have been carried out on deleterious nsSNPs in relation to genes, and their product associated with immune response. Owing to various deleterious nsSNPs effects, individuals are on risk to diverse infections, inflammatory and immune system-related disorder [135,138,139]. The early recognition of the fungal pathogens is crucial for activation of immune response and elimination of inhaled fungal conidia [8]. The immune cells activate by PRRs which recognize PAMPs, mainly β-glucan and galactomannan. The innate immunity receptors which are present on neutrophils, alveolar macrophages, dendritic cells and epithelial cells are important along with soluble receptors [7]. Among cell bound receptors, C-type lectin family member, dectin-1 and DC-SIGN are influential for effective immune response against *Aspergilli* [140]. Further, soluble receptors such as surfactant proteins-A or D, MBL, and pentraxin-3 proteins also consider as important for clearance of *Aspergillus* conidia from lungs [141-143]. Recent

studies have been demonstrated that dectin-1 protein receptors selectively recognize *A. fumigatus* by binding to fungal cell wall carbohydrate and mediate their internalization by immune and lung epithelial cells [144]. Further, these receptor proteins also activate adaptive immune response (Th1 and Th17 antifungal immune response) [145].

Human dectin-1 protein is a transmembrane receptor protein (Type-II) present on innate immune cells or lung epithelial cells. It contains an extracellular C-type lectin domain (CTLD) or a carboxyl-terminal domain, stalk, transmembrane domain and an intracellular immunoreceptor tyrosine-based activated motif (ITAM) [146]. Primary sequence analysis of dectin-1 receptor protein suggested that it is a 28-kDa membrane protein [63]. C-type lectin domain functions as a PRR that involves in the recognition of conserved carbohydrate moieties present on the microbial cell wall called PAMPs such as β-glucan, a major constituent of the cell wall of *Aspergillus* and *Candida* species [146, 147]. β-glucan is a carbohydrate moiety of the filamentous fungal cell wall that exposed to extracellular environment during cell wall remolding in germinating conidia of fungi and at mycelia stage of fungi. In dimorphic fungi such as Paracoccidioides, β-glucan is predominately present on the cell wall of mycelia whereas yeast stage contains more of α-glucan [148]. Currently, β-glucan is an important test for the diagnoses of invasive fungal infections [149,150]. Other then fungal pathogens, it has been suggested that dectin-1 receptor also recognizes *Mycobacterium tuberculosis* by unknown ligand molecule [151]. Further, Heyl *et al* showed that nontypeable *Haemophilus influenza* was also recognized by dectin-1 receptor [152]. Interaction of dectin-1 receptor with β-glucan leads to the phosphorylation of ITAM motif dectin-1 receptor [146]. The dectin-1 receptor interacts with other proteins such as TLR-2 and SyK. SyK kinase initiates the downstream signaling and activates NF-kβ, which further regulates effector function of innate immunity by activating various cytokines and chemokines [152]. Dectin-1 receptor activates the NF-kβ pathways that lead to the production of immuno-regulator molecules such as pro-inflammatory cytokines, IL1a, IL12, and IL-18 and chemokines (CXCL10, CCL3, and CCL2) [152]. These immune-regulatory molecules mediated the recruitment of neutrophils and macrophages at the site of infection and thus clear the fungal pathogens. Further, dectin-1 receptor works in collaboration with TLRs likewise, TLR-2, 4 or TLR-6 and mediates the production of IL-6 and TNF [140]. Therefore, this finding suggests that dectin-1 is an important receptor which helps to clear *Aspergilli* at the site of infection and modulation of proper immune response. The availability of human genome

and better sequencing facilities help to decipher the association of several polymorphisms in human immune receptors associated with pathogen recognition. If, these single nucleotide polymorphisms are present in coding regions and promoter region of genes, which encodes PRRs, there may be defective receptor production or less expression of these receptors on immune cell. Thus, these conditions can make individual those having SNPs in PRRs susceptible to *Aspergillus* associated infections. Among soluble receptors of innate immune response pentraxin-3 protein, surfactant proteins and MBL are important for fungal pathogen clearance [153-154]. Pentraxin-3 protein gains importance because its deficiency makes individuals susceptible to fungal infection; especially those undergo stem cell transplants or having cancer. The deficiency of this protein in these patients occurs due to presence of SNPs in the promoter region of gene, which encode this protein [155]. Pentraxin proteins are the evolutionary conserved proteins that play an important role in innate immunity by acting as PRRs against microbial pathogens [156]. The pentraxin proteins are categorized on the basis of their sizes into long and short pentraxins. The long pentraxins represent the pentraxin-3, pentraxin-4 and neuronal pentraix-1 or 2 proteins, whereas short pentraxins are serum amyloid proteins and C-reactive proteins [126]. These proteins represent by a pentraxin-domain at carboxyl terminus, and they also contain multi-domain sites at N-terminus. Short pentraxin proteins are well studied, and they have the abilities to recognize bacterial (*Salmonella* species and *Pseudomonas aeruginosa*) and fungal pathogens (*Aspergillus* species) [157]. In last decade, pentraxin-3 is recognized as an important player of innate immune response against respiratory pathogens. Human pentraxin-3 protein is encoded by a gene, which is present on the chromosome number-3 (q22-28). Human pentraxin-3 gene consists of three exons, and third exon encodes pentraxin domain. Pentraxin-3 protein acts as soluble PRR and it surveillance the lung compartments. If it encounters any pathogen during surveillance, it acts as an opsonin [158]. Human pentraxin-3 protein is produced by various cells likely alveolar macrophages, neutrophils and antigen presenting cells (DCs) after interaction with inflammatory mediators of immune response, likewise, lipopolysaccharide of bacterial cell wall, *A. fumigatus* conidial glactomannan, interlukin-1 and tumor-necrosis factor-α respectively [145]. Pentraxin-3 inhibits the colonization of respiratory tract and lung by various microorganisms included fungi such as *C. albicans* and *A. fumigatus* and virus like *influenza virus* [159]. The various studies based on human population helped to identify potential SNPs that may be considered as early

biomarkers for *Aspergillus* associated infections in immunocompromised patients and such SNPs presence can also increase the risk of invasive aspergillosis. Previously, Cunha *et al* reported the occurrence of SNP rs-16910526 (Y238X) in the c-type lectin domain of dectin-1 protein associated with *Aspergillus* and *Candida* infections [155]. Further, other SNPs in dectin-1 (rs3901533 and rs7309123) were found to be involved in invasive pulmonary aspergillosis [160]. Thus the patients carrying these SNPs in dectin-1 gene have increased risk of *Aspergillus* associated infections. Different efforts have been made to reveal the effect of these SNPs on protein structure and functions [137,161,162]. The presence of SNPs in genes which encode surfactant proteins showed significant associations with ABPA patients in an Indian population [154]. However, still, there is a large number of SNPs data available to discover effects of SNPs/ on protein's function and structure is the huge task. An effective alternative to these hurdles is various *in-silico* or molecular dynamic approaches to elucidate biochemical severity of the amino acid substitution, as well as the protein sequences and/or structural information, thus provide a more feasible approach for phenotype prediction.

2.10 Proteomics of *Aspergilli* and the significance

The releases of *Aspergillus* species genomes have opened the way for post-genomic studies and increase the knowledge against this important fungal genus [16]. After the availability of genomes of *Aspergilli* or other fungi lead to the study of proteome of filamentous fungi. In last few years, the proteome studies on fungi have got attention due to their emergence as opportunistic pathogens of humans, plant pathogens and their increased use in industrial biotechnology applications [163,164]. The analysis of proteomic is the global assessment of proteins expressed by any organism. The evaluation of these expressed proteins provides the systematic understating of events or functions or organisms at the cellular and molecular level [163]. The release of *Aspergilli* genomes led to the various transcriptomic studies on *Aspergilli*, especially on *A. fumigatus* and *A. terreus* that provide the comprehensive knowledge about these opportunistic pathogens [165,166]. In comparison to transcriptomic studies, the global proteomic studies on *Aspergillus species* are relatively less on emerging fungal pathogens such as *A. terreus*. The majority of proteomic studies on *A. fumigatus* were done using 2-dimension electrphoresis and MALDI and thus not provided the complete set of proteins expressed by these fungi [167-169]. However, the proteomic studies of *Aspergillus* catching up in

recent years and with the availability of modern proteomic approaches, there is a great promise of increasing knowledge on proteomics of *Aspergilli* [170]. With the introduction of multidimensional chromatographic techniques with mass-spectrometry such as LC-MS/MS helped to detect the proteins that are underrepresented on 2D-gels such as proteins having extreme pI, hydrophobic proteins and some membrane proteins [170,171]. Proteomic studies on *A. fumigatus* helped to elucidate the pathogenic mechanism. Further, the proteomic studies have been used to discover new antigens and response of antifungal drugs to *Aspergilli*, especially to *A. fumigatus* [168,172,173]. In recent years, the proteomic studies on morphological forms of *Aspergilli* or other fungal pathogens have gained importance because at different stages of growth, they express unique proteins that may involve in pathogenesis [174-176]. The identification and characterization of antigenic proteins of pathogens are the primary requirement of vaccine and diagnostic method development. The development of vaccines for emerging fungal pathogens is the alternative treatment strategies to control fungal infections in high-risk population (immunocompromised individuals) [177-178]. For the identification of immune-reactive antigenic proteins of fungal pathogens with human and murine antibodies in serum, the SDS-PAGE or 2D gel electrophoresis and immunobloting techniques remain the gold standards [170,179]. Previously, various studies were performed to screen the human or murine serum for anti-Aspergillus antibodies, mainly IgE and IgG for identification of allergic aspergillosis as well for development of passive immunotherapy for invasive aspergillosis. Gautam *et al* blotted the patient's sera (*A. fumigatus* sensitized asthmatic patients) with the culture filtrate for *A. fumigatus*. They have identified 11 novel antigens of *A. fumigatus,* and major antigens were chitosanase and an extracellular arabinase [180]. Further, Glaser *et al* reported PhiA protein as major antigen that recognized by immunoglobin E from the serum of ABPA patients [181]. Singh *et al* also identified 3 IgG and 63 IgE reactive antigens from *A. fumigatus* blot with pooled sera of ABPA patients. They have also identified 35 more antigenic proteins and most of them were belonged to carbohydrate metabolism, stress proteins, transport and energy conservation [182]. Furthermore, various murine and rabbit models of invasive aspergillosis were used to identify antigenic proteins from *A. fumigatus* [183-184]. In last decade, the antifungal resistance mechanisms have been developed by *Aspergilli* and there is a need of new antifungal drugs [90]. Therefore, for the identification of new drug targets, proteomic approaches may be helpful and can be utilized to study the molecular

interaction of antifungal drugs with *Aspergilli* (Resistance vs. susceptible) [185-186]. Previously, some proteomics studies were performed on *A. fumigatus* to see the interaction with antifungal drugs. Gautam *et al* used the proteomic approach (2D-gel electrophoresis) to study the molecular interaction of AmB with *A. fumigatus*. They identified various proteins of *A. fumigatus* affected by exposure to AmB. Most of these proteins were involved in protein transport, primary metabolism, biosynthesis of ergosterol and heme pathway. They also observed the downregulation of RodB protein, a hydrophobin involved in cell wall integrity and upregulation of Mn-superoxide dismutase [168]. Whereas, treatment of *A. fumigatus* with caspofugin drug has led to a different protein profile as compared to AmB treatment. Various ribosomal proteins were found to be increased in response to caspofugin and mitochondrial hypoxic-responsive protein observed significantly downregulated. Thus, this protein suggested as a potential biomarker against caspofugin resistance strains of *A. fumigatus* [187]. Further, Blatzer *et al* reported that the exposure of AmB to *A. terreus* (resistance and sensitive strains) increases the Ssb a member of Hsp-70 subfamily in AmB resistance strain of *A. terreus* demonstrate by using 2D electrophoresis followed by mass spectroscopy for identification of protein. Therefore, they suggested the role of Hsp-70 in *A. terreus* resistance against AmB [96]. Conidia of *Aspergilli* are the main reproductive bodies that give rise to new individual in response to favorable environmental conditions [188]. After inhalation, if conidia are not cleared from the respiratory tract, they usually find suitable environment conditions in respiratory tract or lungs. They start the biochemical and genetic machinery to become germinating conidia or finally form masses or hyphae called the mycelium [189]. Thus, germination of conidia of *Aspergilli* is a crucial step to establish infection in immunocompromised individuals. The proteins expressed during their morphological transition from conidia to germinating conidia are important to elucidate their mechanism of pathogenesis or colonization of host organs [174]. Further, the proteins expressed during germination can provide early diagnostic markers for *Aspergillus species*, and some of them can be utilized as a vaccine candidate [172]. So, it is important to decipherer the global proteome profile of *Aspergillus species* conidia or germinating conidia. Previously, few studies have been carried out for the identification of proteins represented by germinating conidia of *A. fumigatus* using 2D-gel electrophoresis. Previous studies using 2D-PAGE identified various proteins of *A. fumigatus* that were involved in early development of conidia such as those functions in translation

machinery, transport, ATP synthesis and respiratory metabolism [168,190]. Cagas *et al* previously profiled the *A. fumigatus* proteome over different time points using iTRAQ and simultaneously comparing proteome profile with transcriptomic analysis. A total 231 proteins were identified. The most abundant protein was hydrophobin RodA. Additionally, they have identified 23 proteins that were specific to dormant conidia [172]. Further, Barreira *et al* used the difference gel electrophoresis approach to study the surface proteins of germinating conidia or hyphae of *A. fumigatus*. Using this technique, they have identified 63 differentially expressed proteins between germinating conidia or hyphae. In germinating conidia most of the expressed proteins were from biosynthetic pathways and in case of hyphae, they were from metabolic pathways or most of them have unknown functions. Barreira *et al,* further, identified two putative drug target proteins; translation factor (eEF3) and CipC-like protein with no similarity with human proteins [185]. Furthermore, Suh *et al* studied the developmental stage specific proteome of *A. fumigatus*. They have carried out the proteome analysis of conidia, isotropic conidia, germ tube and pre-septation hyphae using a shotgun proteomic approach. Suh *et al* have identified 375 unique proteins and also identified the developmental stages specific markers. Among specific biomarkers of different stages, likewise, Grg1 protein (0 hr), Hsp-90 binding co-chaperone and CipC protein (4 hr), 40s ribosomal protein s19 (8 hr germinating conidia) and ribosomal associated protein Stm1 [174]. Furthermore, Tiwari *et al* studied the proteins expressed by *A. flavus* germinating conidia using shotgun approach, i.e. LC-MS/MS. A total 416 proteins were identified using qToF-LC-MS/MS analysis. Most of the characterized proteins were involved in cell wall biosynthesis, protein synthesis, and carbohydrate metabolism. Tiwari *et al* also suggested that the MAPK signaling pathway play a critical role during germination of *A. flavus* conidia [176]. Currently, there is no proteomic study available on conidia or germinating conidia of *A. terreus* an emerging fungal pathogen. Thus, the studies on *A. terreus* protein profiling at different morphotypes will help to compare proteomic data across *Aspergillus species* that will lead to identification of species-specific diagnostic markers as well as pan fungal vaccine candidates. With the advancement of proteomic study tools such as a shotgun, proteomic approach based on LC-MS/MS holds the promise to study the quantitative or global proteome profile of *Aspergilli* at specific developmental stages to identify unique proteins that can be utilized as diagnostic antigens, drug targets or vaccine candidates [191-192]. Further, it is important to study the proteins expressed by host lung

epithelial cells during interaction with *Aspergillus* conidia to understand the pathophysiology of *Aspergillus* related infections. But there are few studies available and most of them were carried using transcriptomic analysis. Previously, some interaction studies using RNA-seq established that the human lung epithelial cells contact with *A. fumigatus* conidia lead to internalization of conidia by lung epithelial cells [86,193]. Further, it is unclear how lung epithelial cells respond to *A. terreus* conidia or germinating conidia. So it is important to understand the mechanism or pathways/ proteins expressed by lung epithelial cells during interaction that may involve in the clearance of *A. terreus* conidia from lungs after inhalation.

Keeping in view the gap existing in *A. terreus* morphogenesis, response of lung epithelial cells to *A. terreus* conidia and deleterious effect of nsSNPs in innate immune receptors, the current work on "**Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of nsSNPs in innate immune receptors**" was undertaken with the following objectives.

Objectives:

1. To elucidate the proteins/enzymes or biochemical pathway of *Aspergillus terreus* conidia during the germination.
2. Global proteome analysis of human lung epithelial cells (A549) in response to *A. terreus* conidia
3. Structural-functional relation of deleterious nsSNPs in pentraxin-3 (a soluble receptor) and in dectin-1 (a cell bound receptor) that recognize *Aspergilli* conidia during their phenotypic transition to germinating conidia

To elucidate the proteins/enzymes or biochemical pathway of *Aspergillus terreus* conidia during the germination

3.1 Introduction

Aspergillus species are the emerging threat to immunocompromised patients as well as for immunocompetent individuals [194-195]. Among all recognized *species* of *Aspergillus, A. fumigatus* is one of the most isolated *Aspergilli* from clinical samples but in recent year's *A. terreus* has gained importance as emerging fungal pathogen worldwide particularly in cancer hospitals [27,196]. The frequency of invasive aspergillosis caused by *A. terreus* differs from 1% to 30% [21]. Even in some medical centers such as Houston, Texas, and Innsbruck, Austria, *A.terreus* interestingly emerges as a leading cause of invasive aspergillosis among all opportunistic fungal pathogens [27]. With the increase of incidence of infection caused by *A. terreus* and having high *in-vitro* resistance to AmB (Minimum Inhibitory Concentration > 2mg/l) and high mortality (51% versus 30%) of invasive aspergillosis in comparison to non-terreus *Aspergillus* species lead to worrisome in the medical community [197] .

The conidia of *A. terreus* are ubiquitously present in the air and every day hundreds of these conidia inhaled by each individual. Due to their tiny size they successful reach to alveoli of lungs through respiratory tract [21]. In immunocompetent persons, these inhaled conidia are eliminated by circulating immune cells but in immunosuppresed persons, those having neutropenia, leukemia and bone marrow transplantation, they are not efficiently eliminated by innate immune cells and remain there, followed by germination under suitable conditions [7,27,197]. The transitions of conidia to the germinating conidia lead to formation of hyphae and network of hyphae, called mycelia. They are the long finger like projection emerged from germinating conidia and directly interact with lung epithelial cells as well start invasion into the host tissues and blood vessels within hours of colonization or days [85]. The proteins/enzymes/metabolites expressed in germinating conidia are considered crucial for the successful invasion of infected human host tissues or for pathogenesis of *Aspergilli* [33,86,198]. Thus, the study on proteins expressed by germinating conidia is important step to understand the biology of *A. terreus* morphogenesis and for the development of better drug, vaccine or diagnostic markers.

With availability of limited transcriptomic and proteomic data, accessibility to sequenced genomes of *Aspergilli,* there may be help to expedition in discovery of new drug, vaccine and diagnostic marker for *Aspergilli*-associated infections [170,199]. Looking at the importance to decipher the proteins expressed during transition from conidia to germinating conidia, various proteomics studies have been carried out on *A. fumigatus* and *A. flavus* [168,174,200,201]. Most of these studies were carried out using 2D gel electrophoresis followed by MALDI-TOF for identification of separated protein spots on gels. These studies helped to identify various protein expressed during morphogenesis of *Aspergillus* species but lack the sensitivity for protein quantification. Thus, little is known about dynamic range of proteins expressed during germination of conidia. The negligible studies have been carried out to interpret the proteins/enzymes or various molecular mechanisms engage during morphological transition of *A. terreus*. Being an emerging fungal pathogen and having intrinsic resistance to AmB, it is imperative to study the proteins expressed by *A. terreus* during morphogenesis from dormant conidia to germinating conidia. Thus, the objective was taken to uncover the proteins that are expressed during the dormant conidia and in germination stage of *A. terreus*.

3.2 Material and Methods

3.2.1 *Aspergillus terreus* strain and culture conditions

Aspergillus terreus strain (NCCPF 860035) used in the current study was procured from NCCPF, Chandigarh, India. This strain was isolated from induced sputum of a patient. The growth of *A. terreus* (NCCPF 860035) was maintained on SDA media (HiMedia Pvt. Ltd, Mumbai, India) over the period of 1 week at a temperature of 37°C. After that the conidia were collected by gentle rubbing the surface of media plates having fungal growth using PBS (pH 7.4) with 0.05 % Tween-20. The suspension of conidia was collected and filtered to remove hyphae and mycelia. Further, the conidial sample after filtration was centrifuged at 1800 x *g* for 5 min at 4°C and collected conidial pellet was washed to remove medium component using ice cold PBS (pH 7.4) Further, light microscopy was carried out to check the purity of obtained conidial suspension. They were further stored in refrigerator (4°C) and some of the aliquots of conidial pellets were frozen using liquid nitrogen and stored at -80°C freezer for protein isolation.

3.2.2 *Aspergillus terreus* morphotypes identification

Dulbecco's Modified Eagle Medium (DMEM), pH 7.4 (HiMedia, India) was used to inoculate *A. terreus* conidia and fed with 10% FBS (HiMedia, India). The viability of *A. terreus* conidial was assessed on SDA medium (HiMedia, India) plates prior to inoculation. To determine the germination time point of *A. terreus* conidia, the inoculated DMEM medium flasks were incubated at 37°C with 120 rpm. At each hour of incubation, the conidial transition to germinating conidia was monitored using light microscope (Olympus, India). The different developmental changes or stages of *A. terreus* conidia were noted as dormant conidia, expanding or swollen conidia, germ tube conidia and hyphae. More than 90% germination of *A. terreus* conidia was calculated by counting 200 conidia in triplicate under light microscope.

3.2.3 Large scale culture for germinating conidia

Three flasks of DMEM medium supplemented with 10% FBS were used to inoculate viable conidia of *A. terreus* and incubated in a shaker at 37°C at 120 rpm. The conidia transition was monitored until more than 90% germinating conidia were achieved. After achieving more than 90% germination, the medium was separated by centrifuging samples at 15000 x *g* for 15 minutes at 4°C. Further, traces of medium components in collected pellets of germinating conidia were removed by repetitive washing of pellets at least three times using chilled PBS (pH 7.4). The washed samples of germinating conidia were frozen with liquid nitrogen and stored in ultra freezer (-80°C) for further use.

3.2.4 Total protein extraction

Germinating conidia from two independent biological replicates were snap chilled using liquid nitrogen. Then freezed samples were grinded to fine powder using mortar pestle [174]. Total proteins from samples were extracted at 4°C in sodium phosphate buffer pH 7.4 (50 mM) having 2 mM EDTA, 0.2 mM DTT, and 1mM PMSF with continuous stirring for 3 hours. Then samples were subjected to refrigerated centrifugation 15000 x *g* for 20 minutes. After centrifugation supernatants from both samples were transferred to new tubes and further subjected for overnight precipitation at -20°C using 5% tricholoracetic acid. Next day, overnight precipitated proteins were washed 4 times using chilled acetone for to remove any residues of tricholoracetic acid. Then pellets of proteins were dried and dissolved in required amount of protein rehydration buffer (2% CHAPS, 8 M urea, 25 mM DTT) [168]. Thereafter concentration of proteins was calculated with the

help of Bradford method followed by storage at -80°C until further use [202]. Simultaneously, the proteins from conidia were also extracted as discussed above.

3.2.5 SDS -Polyacrylamide Gel Electrophoresis of extracted proteins

Extracted proteins from conidia and germinating conidia (used from two biological replicates) were analyzed using SDS-PAGE gel (12%). Methanol and chloroform precipitation method was used to precipitate the equal amount of proteins from each sample. The obtained pellet after precipitation was dissolved in Laemmli sample buffer having fresh added β-mercaptoethanol. Further, each sample along with protein marker was completely dissolved by heating at 95°C for 5 minutes in water bath. A 12% SDS-PAGE gel was prepared using Mini-PROTEAN Electrophoresis System-Bio-Rad and samples were electrophoresised at 70V in staking gel and at 100V in separating gel. After protein separation, gel was stained with the help of Coomassie blue stain and then destains to remove any background dye and thereafter scanned using gel scanner software from Bio-Rad (G800 gel scanner software) [201].

3.2.6 Protein digestion

The samples of proteins from germinating conidia were prepared in 50 mM ammonium bicarbonate to final concentration of 1mg/ml. Thereafter, the proteins dissolved in ammonium bicarbonate were identified using quadrapole Time of Flight-Liquid Chromatographic-MS/MS analysis. The protein samples (100µl) were precipitated using acetone and then proteins reduction was done at 56°C using 10 mM dithiothreitol for one hour. After that reduced protein samples were alkylated using 55 mM IDA (iodoacetate) for 45 minutes in dark at room temperature followed by trypsin digestion at 37°C overnight. Next day digested proteins or peptides were eluted with formic acid (0.1%). To check the complete digestion of proteins into peptides the digested protein samples were analyzed by SDS-PAGE [201].

3.2.7 Quadrupole Time of Flight-LC-MS/MS

The digested protein samples were separated using Acquity Waters UPLC system coupled with qTOF-LC-MS/MS (Waters, Corporation). The peptides of proteins were separated for 60 minutes via BEHC18 column (Size of column, 2.1mm x 150 mm x 1.7um) using buffer A and B that constitutes 0.1% formic acid and acetonitrile or 0.1% formic acid respectively at a flow rate of 200 µL per minute. Subsequent to peptide separation, they were ionized using Electrospray Ionization (ESI) at 275°C with a spray

volume of 2 kV. Generated mass spectra of peptides were obtained using automated MS/MS mode [201]. Then obtained mass spectra of peptides were analyzed using PLGS from Waters Corporation. Data analysis was done by Mass Lynx 4.1 WATERS on the basis of SEQUEST search algorithm with more than 95% identity. Following parameters were used to obtain data; one missed cleavage of enzyme trypsin, modification of cysteine residue with carbamidomethyl and methionine oxidation having peptide and fragment tolerance of 100 & 200 ppm respectively. The obtained data was searched using UniProt database against *Aspergillus terreus*.

3.2.8 Gene ontology

The gene ontology annotations of predicted proteins were done using BLAST2go algorithm. To get the broader GO terms such as cellular components, molecular function and biological function, the GO slimmer tool was used. Further, some of the proteins involved in secondary metabolite pathways were annotated with the help of UniProt database [203].

3.2.9 Protein interaction network for expressed proteins from major biosynthetic pathways

After the Gene Ontology, the protein interaction network was mapped with the help of STRING database (version 10). The interactome study was done to predict the interaction among different proteins that may involve in various cellular or biochemical pathways. The proteins involved in carbohydrate metabolism, signal transduction, amino acid metabolism and other pathways were subjected to STRING databases to predict their cellular interaction. The protein interaction was done in various modes (action view mode, interactive mode, confirmation or evidence view mode and confidence mode) to get the appropriate interactions among different proteins that were involved in various cellular or biochemical pathways [204].

3.2.10 Total RNA extraction from *Aspergillus terreus* conidia and germinating conidia

The extraction of total RNA was carried out using TRIzol reagent (Invitrogen, USA) from two independent biological replicates from each morphotypes of *A. terreus*. The contaminated gDNA was eliminated by treating the isolated RNA using DNase (Qiagen GmbH, Germany). Then extracted RNA was subjected to quality and quantity check at A260nm/A280nm ratio using spectrophotometer (Thermo Scientific, NanoDrop 1000

spectrophotometer). Further, total extracted RNA was electrophoresed through 1.5% agarose and gel was stained with EtBr to check the intact bands of 18S and 28S ribosomal RNA, by visualizing gel at 302 nm using UV transilluminator [148].

3.2.11 Synthesis of cDNA from total RNA

Verso-cDNA synthesis kit (Thermo Scientific, USA) was used to synthesize the complementary DNA strand (cDNA) as per given instructions. A total 1µg RNA from both conidia and germinating was used for the synthesis of cDNA. The cDNA was synthesized by performing initial reverse transcription step at 42°C for 30 min. After that the inactivation of reagents were done at 95°C for 2 min. The synthesis of cDNA template was checked through PCR of housekeeping gene (40S ribosomal protein gene).

3.2.12 Comparative expression analysis of selected genes using qRT-PCR

The qRT-PCR reactions were performed on BIO-RAD CFX96 machine (BIORAD, USA). To check the expression of some of the selected genes, the primers were designed using NCBI based tool Primer-Blast [205]. To perform quantitative expression study, a total 1.5 µl of cDNA was used in 20 µl volume reaction and primers final concentration were 20 picomole. Master-Mix contain dye sybr green from Thermo Scientific, USA was used to perform all reactions in triplicates using cDNA from two independent replicates. The reaction conditions were setup as: 95°C as initial denaturation for 3 minutes followed by 39 cycles of 95°C for 10 sec, 49°C for 45 sec, 72°C for 30 second. Simultaneously, a multi-curve was also performed on same reactions to verify the primers specificity. 40S ribosomal protein gene was used as reference gene because no significant difference was obtained in its expression in conidia and germinating conidia [206]. The expressions of genes were quantified using "comparative ΔΔCt" method [168].

3.3 Results

3.3.1 Germ tube formation time point identification for *Aspergillus terreus* conidia

For the identification of germination time for *A. terreus* conidia, they were inoculated in DMEM medium and evaluated after each hour of incubation under microscope. During first 9 hours of incubation there was no significant development observed in conidia. Then, they isotropically expanded and became swollen at 11 hours of incubation (Figure-3.1). The polarized growth of conidia was observed after 13 hours of incubation in

DMEM medium. At 16 hours of incubation more than 90% *A. terreus* conidia were in germinating phase as determined by counting them under microscope. After germination they continued to grow and developed into hyphae followed by network of hyphae called mycelium (Figure-3.1).

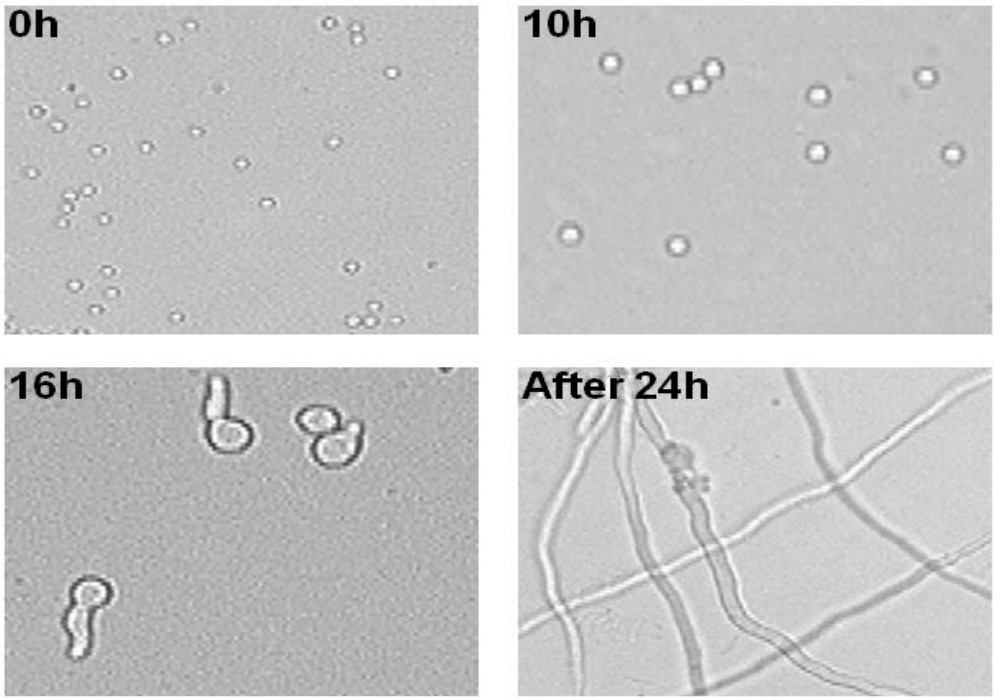


Figure-3.1: Morphological transition of *A. terreus* from conidia to mycelium

3.3.2 Identification of total proteins expressed by *Aspergillus terreus* germinating conidia

For the identification of expressed proteins, the extracted proteins from germinating conidia were subjected to quadrupole Time of Flight-Liquid Chromatography-MS/MS analysis. The proteins were identified from obtained spectra of peptides using Protein Linux Global Server software from WATERS Corporation. The obtained data was searched against *A. terreus* proteins dataset in UniProt protein database. After analyzing data, 374 proteins were identified and these proteins were specific to *A. terreus.* Further, among 374 proteins, 74 were identified without any functions or uncharacterized. The amino acid coverage of identified proteins was ranging from 4.2% to 80.8% with a Protein Linux Global Server score of 8.2 to 2993.3. Simultaneously, the isoelectric points of recognized proteins were ranging from 4 to 11 and molecular weights were 12949 to 228116 Da. Further, identified proteins were subjected to BLAST2go software to give

them gene ontology (GO) functions. Then each protein was assigned to a single gene ontology (GO) term. The gene ontology terms are divided into cellular components, biological processes and molecular functions. The cellular components of gene ontology (GO) revealed that most of the identified proteins were from cytosolic region (19.62%) followed by nuclear region (16%), mitochondrial (5.90%) and ER regions (7.50%). The other proteins from cellular components were given in figure-3.2.

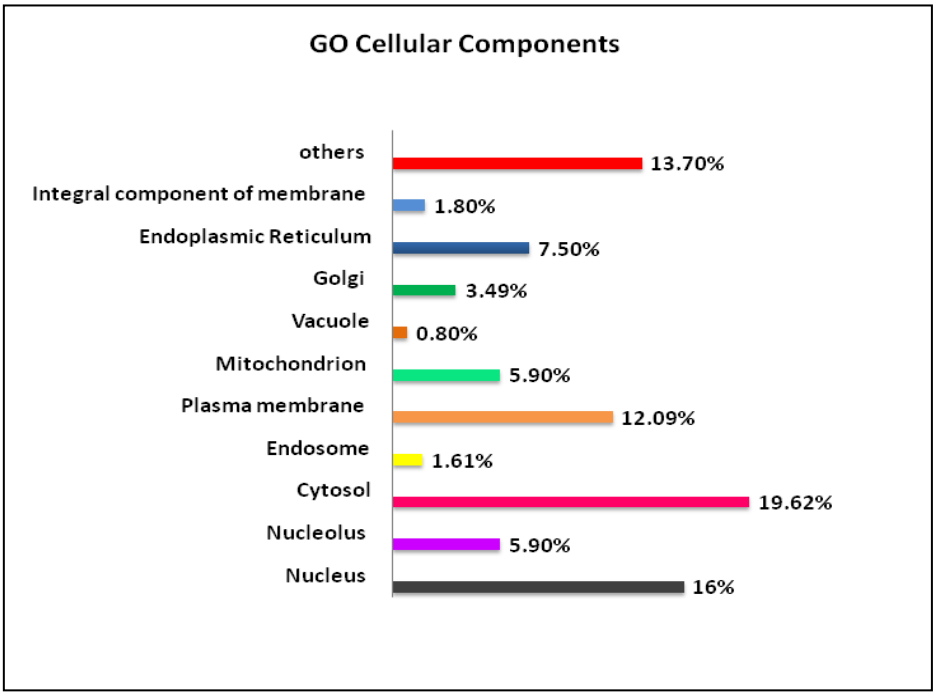


Figure-3.2: Cellular functions of expressed proteins from germinating conidia using GO enrichment

Further, these identified proteins were belonged to following biological processes likewise cell wall organization (3.01%), protein biosynthesis (4.01%) and transport (6.6%), RNA processing (10.70%), carbohydrate metabolism (6.02%) and rest of the biological processes were shown in figure-3.3. Along with cellular components and biological function, the identified proteins from germinating conidia were belonged to different molecular functions such as transferase activity (7.02%), ATP binding (10.36%), hydrolase activity (13.04%), DNA/ RNA binding capacity (14.7%), and other proteins were revealed in figure-3.4.

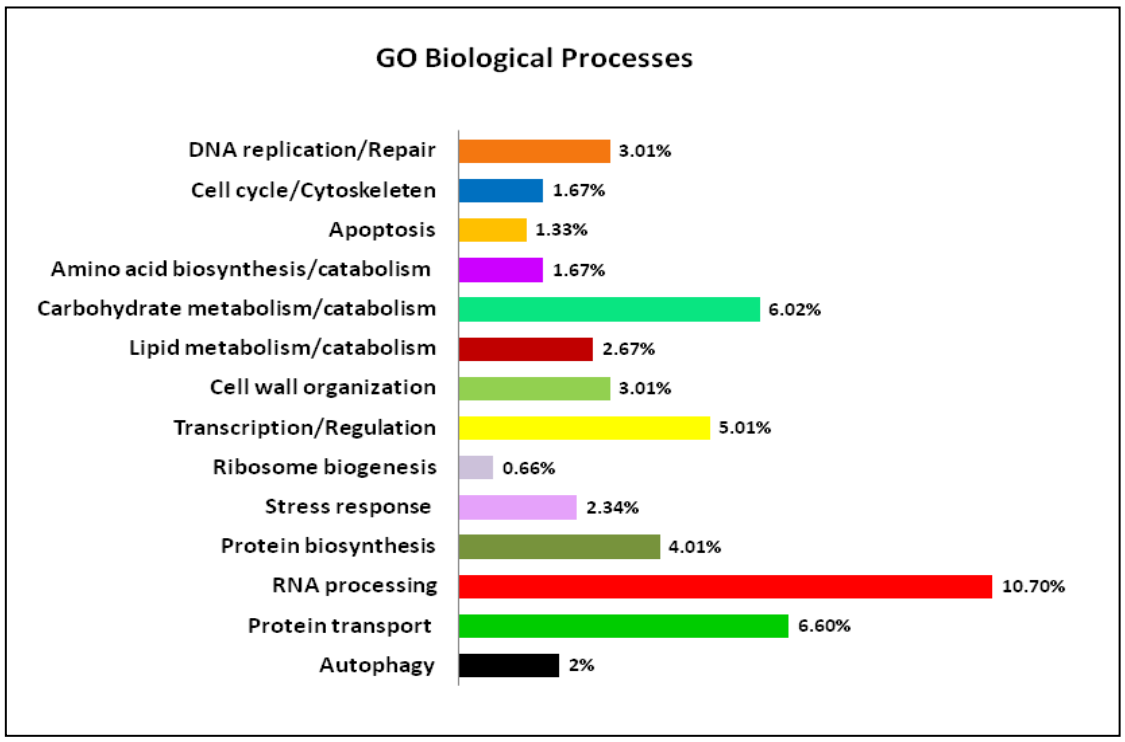


Figure-3.3: Biological functions of expressed proteins from germinating conidia using GO enrichment

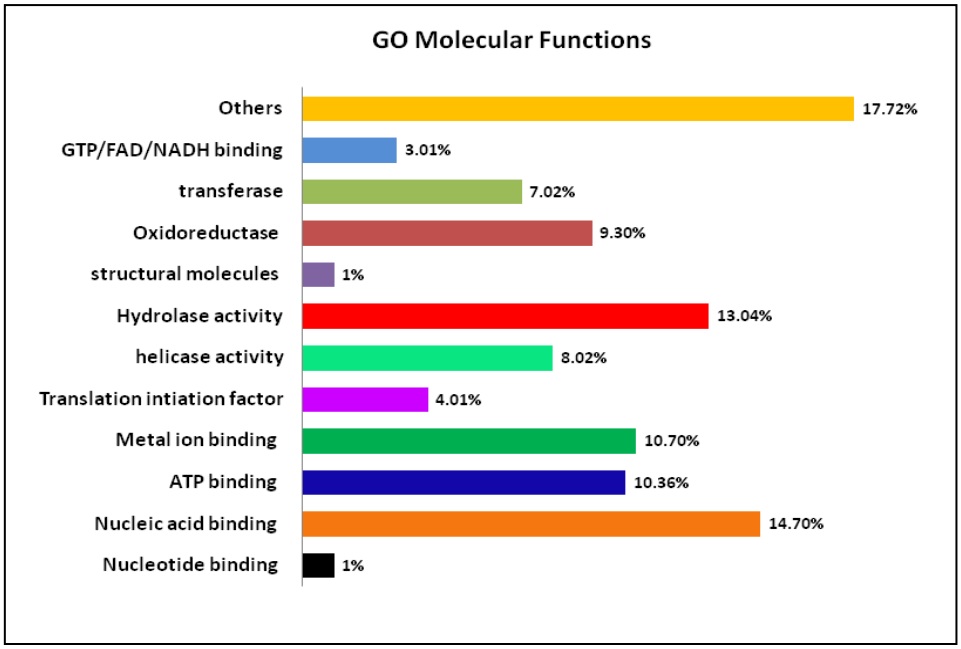


Figure 3.4: Molecular functions of expressed proteins from germinating conidia using GO enrichment

Other general functions of identified protein were revealed by UniProt data base and these proteins were belonged to secondary metabolite production, lipid and carbohydrate metabolism, energy production and transport of molecules (Appendix-II-Table-3.1).

3.3.3 Conidial proteins identification

A total 42 conidia protein of *A. terreus* were identified, out of which 17 were uncharacterized. Most of these proteins were belonged to lipid and carbohydrate metabolism, protein biosynthesis and secondary metabolite production (Appendix-II-Table-3.2).

3.3.4 Categories of proteins having functional associations with germination of conidia

The expressed proteins data analysis helped in identification of various proteins that were involved in wide range of metabolic pathways and biochemical event that occurs during germination of conidia.

3.3.5 Biosynthesis of proteins and metabolism of amino acids

After data analysis, we have identified various biochemical events or pathways that involve during germination of conidia such as DNA replication, transcription, protein synthesis, post translational modification, transport. The biosynthesis of proteins during germination of conidia needs amino acids that can be incorporate into proteins. The proteins such as pentafunctional AROM polypeptide, ornithine carbamoyltransferase, methylthioribulose-phosphate dehydratase and acetyltransferase were identified that help in synthesis of poly-aromatic amino acids, methionine and arginine. Along with these proteins various translation factors that initiate biosynthesis of proteins were also identified (Appendix-II-Table-3.3). Data from germinating conidia suggest that amino acid synthesis specifically arginine required during germination.

3.3.6 Proteins associated with cell wall reorganization/remolding and modification

The proteins/enzymes such as glucan β-glucosidase, α/β-glucosidases and endopolygalacturonase have been observed during germination that involve in degradation of cell wall components. Thus, they help in reorganization or remolding of cell wall during germination. Apart from these enzymes, those involved in carbohydrate catabolism were also observed. *Aspergilli* species cell wall mainly composed of α, β-glucan and chitin. Thesefore, the orthologs of enzymes that function in synthesis of cell wall components such as chitin synthase, β-glucan synthase and endopolygalacturonase B were also identified (Table -3.1). Thus, these observations suggest that there is modification of cell wall during germination.

Table-3.1: **Proteins that function in cell wall re-organization**

UniProt ID	Description	Function	PLGS score
Q0CMU3	Probable endopolygalacturonase I	Cell wall organization and modification	459.94
Q0CI96	Probable glucan endo-1,3-beta-glucosidase btgC	Cell wall organization and modification	271.67
Q0CFF7	Probable pectin lyase F-1	Cell wall organization and modification	241.71
Q0CW83	MYND-type zinc finger protein samB	Cell wall organization and modification	208.56
Q0C8Z0	Probable glucan endo-1,6-beta-glucosidase B	Cell wall organization and modification	178.19
Q0C7K7	Probable rhamnogalacturonate lyase B	Cell wall organization and modification	168.68
Q0C8Z0	Probable glucan endo-1,6-beta-glucosidase B	Cell wall organization and modification	178.19
Q0D0P5	Mitogen-activated protein kinase hog1	Cell wall organization and modification	156.79
Q0CR35	Probable glucan 1,3-beta-glucosidase A	Cell wall organization and modification	86.64
Q0C9B4	Putative metallocarboxypeptidase ecm14	Cell wall organization and modification	31.25

3.3.7 Expression of Virulent proteins of *A. terreus*

In this study the different proteins that act as virulent factors were identified in germinating conidia such as mpkC, cgrA, myoA an essential protein, creB and pepP (Table-3.2).

Table-3.2: List of proteins that act as virulent factors

S.No	UniProt ID	Gene Name	Description	PLGS Score	Virulence factor reported in *A. fumigatus*
1	Q0C7E6	***cgrA***	rRNA-processing protein	514.84	[Bhabhra A *et al*, 2005]
2	Q0CEX5	***myoA***	Myosin-1	208.58	[Renshaw H *et al*, 2016]
3	Q0CFJ0	***mep***	Extracellular metalloproteinase	191.10	[Hohl TM *et al*, 2007]
4	Q0D0P5	***hog1***	Mitogen-activated protein kinase hog1	156.79	[Bruder NAC *et al*, 2016]
5	Q0CIC7	***mpkC***	Mitogen-activated protein kinase mpkC	143.47	[Bruder NAC *et al*, 2016]

The homology of these proteins was also matched with *A. fumigatus* virulent proteins using BLASTp. In total 5 key virulent proteins were identified. Further, the knockout studies in *A. fumigatus* confirmed their virulent properties and they also help *A. fumigatus* in invasion of host tissues [207, 211].

3.3.8 Identification of enzymes that involve in biosynthesis pathways of secondary metabolite

Various secondary metabolites such as mycotoxins (geodin and terretonin) and industrial important components lovastatin or terrein are synthesis by *A. terreus.* We have observed various enzymes such as Trt1 (terpene cyclase), Trt3 (FAD-binding monooxygenase), Trt5 (methyltransferase), Trt6 (cytochrome P450 monooxygenase), Trt7 (dioxygenase), Trt9 (dehydrogenase) and Trt14 (isomerase) that involve in production of terretonin mycotoxin (Table-3.3).

Table-3.3: List of identified proteins that involve in terretonin biosynthesis

UniProt ID	Gene name	Protein name	PLGS score	Coverage
Q0C8A3	***trt5***	Methyltransferase trt5	249.04	51.61
Q0C8A5	***trt3***	FAD-binding monooxygenase trt3	143.18	22.06
Q0C8A7	***trt1***	Terpene cyclase trt1	141.98	17.64
Q0C8A2	***trt14***	Isomerase trt14	122.00	51.40
Q0C8A1	***trt6***	Cytochrome P450 monooxygenase trt6	121.76	34.1
Q0C898	***trt9***	dehydrogenase trt9	102.16	38.62
Q0C8A0	***trt7***	Dioxygenase trt7	71.96	38.11

Along with these enzymes we have also observed enzymes that function in biosynthetic pathway of geodin production such as dihydrogeodin oxidase, glutathione S-transferase, ACP thioesterase, glutathione S-transferase and rest of the enzymes were given in table-3.4. Also other proteins/enzymes that involve in biosynthesis of cholesterol lowering drug lovastatin and an anti-cancerous biomolecule terrein were expressed during germinating reported in Appendix-II-Table-3.4

Table-3.4: List of identified proteins that involve in geodin biosynthesis

UniProt ID	Gene name	Description	PLGS score
Q0CCY4	***gedB***	Atrochrysone carboxyl ACP thioesterase	371.26
P0DOB2	***gedH***	Anthrone oxygenase	102.60
Q0CCY5	***gedA***	O-methyltransferase	255.05
P0DOB3	***gedI***	decarboxylase gedI	148.02
Q0CCY3	***gedC***	Atrochrysone carboxylic acid synthase	133.79
Q0CCY0	***gedE***	Glutathione S-transferase-like protein	74.94
Q0CCX9	***gedF***	Monoogygenase	69.60
Q0CCX5	***gedK***	Questin oxidase	36.54
Q0CCX4	***gedL***	Sulochrin halogenase	291.27
Q0CCX6	***gedJ***	Dihydrogeodin oxidase	217.07

3.3.9 Interaction network of proteins identified from germinating conidia of *Aspergillus terreus*

The interactome network of expressed proteins was generated by using STRING database. The proteins network was generated to observe the interaction among expressed proteins to deduce their biological functions. The categories of proteins from biological process were used to produce interaction network. The proteins that didn't show any interaction with other proteins were removed from interaction network (Appendix-Figure-3.1). Most of these interacting proteins were associated with cell wall organization/ remolding, protein biosynthesis and transport, cell cycle, RNA processing and carbohydrate metabolism. The catalogue of interacting proteins is given in Appendix-II-Table-3.5.

3.3.10 Selective genes expression analysis between conidia and germinating conidia

For the quantification of expression of selected genes (Table-3.5) which encode the proteins that function in carbohydrate metabolism, initiation of protein biosynthesis, geodin biosynthesis pathway and a virulence factor terrelysin, quantitative RT-PCR was done. Using quantitative RT-PCR, the expression of genes likewise *pcy, terrelysin, tif35*

and *gedJ* was observed. A significant higher expression of these genes were observed which encode terrelysin (885.28 folds), pyruvate carboxylase (*pcy*-181.01 folds), translation initiation factor (*tif35*-148 folds) and dihydrogeodin oxidase (*gedJ* -26.53 folds) in germinating conidia as compare to conidia of *A. terreus* (Table-3.5). The list of primers used for quantification of expression of selected genes are given in table-3.6

Table-3.5: Comparative gene expression analysis between conidia and germinating conidia using quantitative Real-Time-PCR

S.No	Gene Name	Protein description	Fold difference Germinating conidia vs. conidia
1	*ATEG_03556*	Terrelysin	885.28
2	*tif35*	Eukaryotic translation initiation factor 3 G	148
3	*pcy*	Pyruvate carboxylase	181.01
4	*gedJ*	Dihydrogeodin oxidase	26.53

Table-3.6: List of primers

Accession No	Name	Primer
XM_001212734	Terrelysin	Forward 5'-ATCCATATCCGCGACCGT-3' Reverse 5'-GTCAGGGCTTTCTTCTCATCC-3'
XM_001213603	Eukaryotic translation initiation factor 3 G	Forward 5'-GTCGTGTCACCAGAGTATTCC-3' Reverse 5'-GGATAAGGTGGCGATAACCG-3'
AF097728	Pyruvate carboxylase	Forward 5'-GACGACAAGAAGGCCTCGAT-3' Reverse 5'-GAGGATCTCCCTTCTTCACCT-3'
XM_001218105	40S ribosomal protein S1	Forward 5'-CATTGGCCGTGAGATCGAG-3' Reverse 5'-CCCTTGTCATCGGTGGTAGA-3'
XM_001217596	Dihydrogeodin oxidase	Forward 5'-GTACTTACGACTGTGACCCG-3' Reverse 5'-GAAGTTATGCCCGTCGATCC-3'

3.4 Discussion

A. terreus is frequently appears as a clinical isolates in hospitals around the world. The epidemiological studies were carried out in different hospitals such as at Anderson Cancer hospital, Texas, USA and Innsbruck University Hospital, Austria [15]. In India, 6.6% prevalence rate of *A. terreus* isolates was observed in a 4 year study at a hospital in New Delhi [197]. *A. terreus* often causes fatal infections in patients those having severe compromised immune response. The inhalations of *A. terreus* conidia cause persistence in immune cells such as macrophages that leads to dissemination of this fungal pathogen to other secondary organs [212, 213]. The diagnosis of *Aspergilli* infections during early stages are difficult due to insufficient or lack of diagnostic markers or methods. For the diagnosis of *Aspergillus species* infections various antigens associated with cell wall of *Aspergilli* are used as compared to antibody detection based methods. The antibody based detection methods are not designated as better choice for the diagnosis of *Aspergillus* associated infections because in immunocompromised host there is poor antibody response to *Aspergilli* [214]. Further, *A. terreus* has intrinsic resistance to AmB that makes it important to diagnose at early stage of infection [215, 216]. So it can be treated with standard antimycotic drugs. Thus, to get better diagnosis regime, new drug targets, diagnostic markers, and vaccine candidates, the identification of proteins expressed during formation of germ tube from conidia is essential. The proteins involved in germination or expressed at the time of germination may help to deduce new diagnostic markers and further help to highlight the common invasive factors in *Aspergillus* species associated to invasion of host tissues. However, there is limited information regarding the proteins expressed during *A. terreus* conidia germination. Thus, in this objective, a total 374 proteins were identified in germinating conidia of *A. terreus*, out of these, only 299 had biological functions and rest of them were uncharacterized. Whereas in conidia, only 53 proteins were identified and out of these 17 were uncharacterized. The reason for less expressed proteins in conidia may be dormancy. The dormant conidia are metabolic less active and likely to have less number of proteins to carry out biological function as comparison to germinating conidia, which are metabolically more active due to the onset of germination and morphogenesis [189].

To avoid human immune response *A. terreus* utilized different strategy. The inhaled conidia of *A. terreus* persist in immune cells thus escape from host immune surveillance. When the immune system of infected persons compromised or severely

suppressed, these inhaled conidia start to germinate and invade host lungs or other secondary organs. The proteins or factors expressed by *Aspergilli* are required for invasion of host organs or tissues. There are different virulent proteins identified in *A. fumigatus* that act as virulence factors. In this study, we have identified various proteins in *A. terreus* germinating conidia that help in invasion of host tissue and previously identified as virulent factors. In *A. terreus* germinating conidia, myosin-1, mpkC, and rRNA-processing protein and hog1 proteins were identified. These all proteins were previously reported as virulent factors in knock-out studies of *A. fumigatus* [207,210]. The proteins those having functions in carbohydrate metabolism likewise hog1, rhamno-galacturonate lyase B, β-glucan synthase component FKS1 and glucan endo-beta-glucosidase B were observed. Previously, it has been reported that the swollen and germ-tube containing conidia of *A. terreus* showed β-glucan on re-organized cell wall and help in recognition of these conidia by lung macrophages. Thus, the recognition of *A. terreus* swollen conidia initiates their phagocytosis that lead to their prolonged persistence in these immune cells [27]. Using quantitative Real–Time PCR, elevates expression of genes that encode pyruvate carboxylase (*pcy*) and eukaryotic translation initiation factor (*tif35*) in germ-tube containing conidia as compared to conidia of *A. terreus* was observed (Table-3.6). The proteins encoded by these genes involve in initiation of protein biosynthesis (*tif35*) and carbohydrate metabolism (*pcy*). Thus, this observation suggests germination of *A. terreus* conidia requires carbohydrate metabolism and protein biosynthesis. Previous studies suggested that the conidia require protein synthesis, carbohydrate and respiratory metabolism to exit dormancy. Further, the interaction network of proteins involves in protein biosynthesis (rps0, tif34, tif1) and their transport (sec13) as well as those involved in rRNA processing (rr3, dbp10 & dbp3) help conidia to exit dormancy and initiate there germination. Comparative analysis of *A. terreus* proteome data with *A. flavus* and *A. fumigatus* suggests that the germination of their conidia require the initiation of carbohydrate metabolism, protein biosynthesis and respiration [172,174,201]

Further, the proteins that function in biosynthetic pathways of secondary metabolite production such as mycotoxins (Geodin and Terretonin) and citreoviridin, terrein and lovastatin were identified in germinating conidia. The higher expression of gene *gedJ* was observed using quantitative Real-Time PCR in germinating conidia. This gene encodes an enzyme dihydrogeodin oxidase that involve in biosynthesis pathway of

geodin mycotoxin. Thus, the study concluded that geodin synthesis may be active in germinating conidia. Therefore, *A. terreus* mycotoxin roles during germination need evaluations. Studies suggested that mycotoxin can be used as diagnostic markers for fungal infections. Ozdemir *et al* reported the use of fungal toxin for the diagnosis of fungal infection but they did not test their results on clinical samples. Hence, other studies are required to screen mycotoxins and to study their kinetics in patient's sera samples. Also Koo *et al* [217] suggested the use of volatile secondary metabolites for diagnosis of fungal infections. Thus, various secondary metabolites of *A. terreus* may be explored for the development of non-invasive diagnostic markers for its associated infections.

Previously, it has been reported that various *Aspergilli* such as *A. fumigatus* and *A. flavus* synthesize enzymes or proteins that involve in production of mycotoxins (Gliotoxins and Aflatoxin) during germination of conidia [201,218,219]. These mycotoxins participate in invasion of infected individuals. The expression of proteins that involve in production of various mycotoxins of *A. terreus* were also identified in current study. Thus, these observations suggested that they may involve in invasion of host tissues. Slesiona *et al*, observed the degeneration of liver cells in leucopenic mice infected with *A. terreus*. They reported these observations after 48 hours of infection but the germinating conidia were obtained at 5 day of infection. So it indicates that *A. terreus* may produce secondary metabolites prior to germination of conidia that may involve in fatty liver degeneration disease [220-221].

We have also observed another virulent protein terrelysin that functions as a hemolysin.This protein may contribute in pathogenesis of *A. terreus* [222]. The gene encoding terrelysin showed high expression in germinating conidia (885.28 fold) as compared to conidia of *A. terreus* as quantified by Real-Time PCR. Studies on terrelysin showed that it produces in *A. terreus* at early stages of growth and its production decrease during formation of mycelium onward. It has been also suggested that this protein may diffuse to extracellular environment or in medium from germinating conidia or hyphae [222]. Being a hemolysin, it lysis the RBCs and that releases the iron into blood. This free iron is utilized by fungi for their development and survival in human host. So, it clearly suggested that terrelysin is a key virulence factor produced by *A. terreus* during early growth or invasion of host tissues. The high expression of terrelysin protein during early growth make it suitable diagnostic marker [223]. Thus, terrelysin can investigate as a diagnostic target or marker for *A. terreus* related infections. So in conclusion, in this

objective various proteins or enzymes that may involve in production of secondary metabolites were observed. Apart from this, various virulent proteins have been reported that may involve in invasion of host tissues.

Global proteome analysis of human lung epithelial cells (A549) in response to *A. terreus* conidia

4.1 Introduction

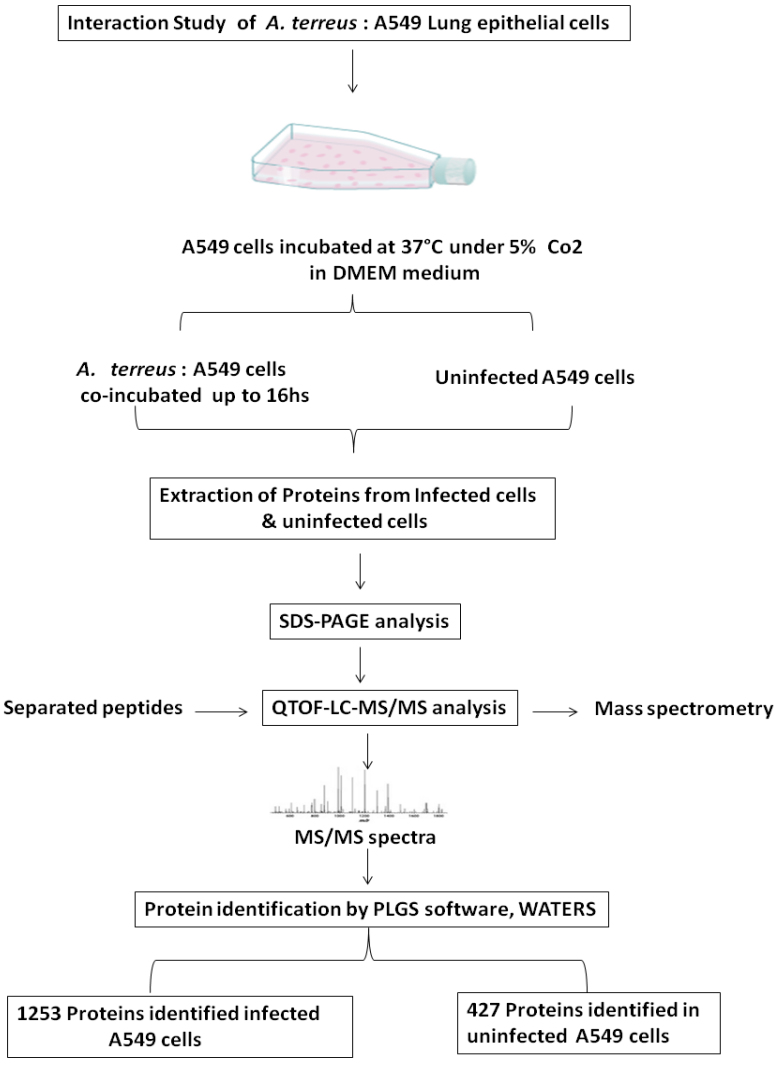
Being an emerging fungal pathogen, *A.terreus* causes superficial to invasive opportunistic infections in immunocompromised persons [195]. The infections associated with *A. terreus* ranges from IA to allergic manifestations such as ABPA in immunocompetant individuals [196]. *A. terreus* strains are frequently isolating from clinical settings, where individuals on chemotherapy (cases of cancer), severe immune suppression (in case of organ transplant individuals), or having neutropenia and HIV infection [27,224]. Denning *et al* reported the alarming situation of *Aspergillus* associated infections in pulmonary tuberculosis patients and individuals having asthma. After the treatment of chronic pulmonary tuberculosis, annually at least 372,385 treated individuals got infected with pulmonary aspergillosis worldwide [10]. Apart from this situation, approximately 4,837,000 individuals develop ABPA among 193 million active asthma patients [225]. However, in India, Agarwal *et al.* reported that out of 27.6 million asthmatic adults, 1.38 million individuals implicated with ABPA [226]. *A. terreus* is an emerging threat to immunocompromised patients. In some of the medical centers, especially those involving in cancer treatment, it emerged as a leading cause of IA among all causative agents. Further, *in-vitro* resistance to AmB and high mortality rate IA as compared to non-terreus species lead worrisome in medical community [15,197].

Every individual inhales hundreds of *A. terreus* conidia and these inhaled conidia easily cleared by healthy persons [7,27]. However, the persons having severed immunosupression due to chemotherapy or phagocytic cells with impaired functions are not able to eliminate inhaled conidia. In such persons or even in immunocompetant host the inhaled conidia encounter the lung epithelial cells [21]. Thus airway epithelial cells are the first which interact with conidia and help to clear them by activating immune-regulatory molecules [27]. Further, in last decade, there is steadily increase in immunosuppressive patients due to raise in cancer cases or transplantations cases [224, 227,228]. Thus, it is significant to study the role of epithelial cells interaction with *A. terreus* to predict the outcome of infection caused by it or to understand the patho-physiological conditions during initiation of infection [57,229]. Previously, RNA-seq or

DNA microarray techniques were used to carry out the interaction studies on *A. fumigatus* with airway epithelial cells, macrophages, dendritic cells and neutrophils [18,33,86,230,231]. Such studies help to gain the information about the role of various cells during their interaction with *Aspergilli* only at mRNA level. Therefore, there is need to carry out studies at functional level using proteomic approaches. Further, these studies will provide the catalogues of proteins that activate various cellular pathways during infection and may help to manipulate treatment regime against *Aspergilli* associated infections.

Thus, to get the global profile of proteins expressed by A549 cells infected with *A. terreus*, qTOF-LC-MS/MS was used [232]. The overview of methodology used to accomplish this objective is given in figure-4.1.

Figure-4.1: The overview of methodology used to accomplish objective 2



4.2 Material and Methods

4.2.1 *Aspergillus terreus* strain and culture conditions

Aspergillus terreus strain (NCCPF 860035) was used in the interaction study and procured from NCCPF, Chandigarh, India. This strain was isolated from induced sputum of a patient. The growth of *A. terreus* (NCCPF 860035) was maintained on SDA media (HiMedia Pvt. Ltd, Mumbai, India) over the period of 1 week at a temperature of 37°C. After that the conidia were collected by gentle rubbing the surface of media plates having fungal growth using PBS (pH 7.4) with 0.05 % Tween20. The suspension of conidia was collected and filtered to remove hyphae and mycelia. Further, the conidial sample after filtration was centrifuged at 1800 x *g* for 5 min and collected conidial pellet was washed to remove medium component using ice cold PBS (pH 7.4) Further, light microscopy was carried out to check the purity of obtained conidial suspension. They were further stored in refrigerator (4°C) and some of the aliquots of conidial pellets were frozen using liquid nitrogen and stored -80°C freezer for protein isolation.

4.2.2 Culture of A549 cells

A549 cells were procured from NCCS, Pune, India and maintained in DMEM medium fed with 10% FBS. They were incubated at 37°C under humidified conditions and 5% CO_2 until they reached to confluency. Thereafter, they were transferred to new culture flask (25 cm^2). The cells were separated from pervious flasks by treating them with culture grade trypsin contained in PBS (pH 7.4). Further, these flasks were maintained at pervious conditions for 7 days. These new cultured flasks were utilized for interaction studies with *A. terreus***.**

4.2.3 A549 cells interaction with *Aspergillus terreus* for proteome analysis

A549 cells in a cell density of 1x 10^6 cells were cultured in tissue culture flask and they were incubated for 18 hours in serum free medium prior to infection with *A. terreus*. Then lung epithelial cells were infected with *A. terreus* viable conidia in a ratio of 1:10 [86]. Then interaction assay flasks were maintained in an incubator at 37°C under humidified conditions with 5% CO_2. At each hours of interaction, the assay flasks were observed under inverted microscope for germ tube formation of conidia. At 16 hours of incubation, the germ tube formation was observed in all area of flasks. Therefore interaction assay were stopped and all cells were collected. Also control flasks of A549 cells without *A.*

terreus conidia were maintained at same conditions. All cells were collected by gentle scarping. The medium of collected cells from both control and interacting cells was removed by centrifugation. Further, the collected cells were washed with PBS (pH 7.4) to remove any medium residue. Then obtained pellets of cells were snapped chilled with liquid nitrogen and store in ultra-freezer for proteins extraction.

4.2.4 Total protein extraction

Total proteins from samples were extracted at 4°C in sodium phosphate buffer pH 7.4 (50 mM) having 2 mM EDTA, 0.2 mM DTT, and 1 mM PMSF with continuous stirring for 3 hours. Then samples were subjected to refrigerated centrifugation 15000 rpm for 20 minutes. After centrifugation supernatants from both samples were transferred to new tubes and further subjected for overnight precipitation at -20°C using 5% trichloroacteic acid. Next day, overnight precipitated proteins were washed using chilled acetone for 4 times to remove any residues of tricholoracetic acid. Then pellets of proteins were dried and dissolved in required amount of protein rehydration buffer (2% CHAPS, 8 M urea, 25 mM DTT) [168]. Thereafter, the concentration of proteins was calculated with the help of Bradford method followed by storage at -80°C until further use [202]. The quality of extracted proteins was checked with 12% SDS-PAGE and further quality protein samples were used for proteome analysis [201].

4.2.5 Protein digestion

The samples of proteins from interacting assay and control assay were prepared in 50mM ammonium bicarbonate to final concentration of 1mg/ml. Thereafter, the proteins dissolved in ammonium bicarbonate were identified using quadrapole Time of Flight-Liquid Chromatographic-MS/MS analysis. The protein samples (100µl) were precipitated using acetone and then proteins reduction was done at 56°C using 10 mM dithiothreitol for one hour. After that reduced protein samples were alkylated using 55 mM IDA (iodoacetate) for 45 minutes in dark at room temperature followed by trypsin digestion at 37°C overnight. Next day digest proteins or peptides of proteins were eluted to formic acid (0.1%). To check the complete digestion of proteins into peptides the digested protein samples were analyzed by SDS-PAGE [201].

4.2.6 Quadrapole Time of Flight-LC-MS/MS

The digested protein samples were separated using Acquity Waters UPLC system coupled with qTOF-LC-MS/MS (Waters, Corporation). The peptides of proteins were separated for 60 minutes via BEHC18 column (Size of column, 2.1mm x 150 mm x 1.7um) using buffer A and B that constitutes 0.1% formic acid and acetonitrile or 0.1% formic acids respectively at a flow rate of 200 µL per minute. Subsequent to peptide separation, they were ionized using Electrospray Ionization (ESI) at 275°C with a spray volume of 2 kV. Generated mass spectra of peptides were obtained using automated MS/MS mode [201]. Then obtained mass spectra of peptides were analyzed using PLGS from Waters Corporation. Data analysis was done by Mass Lynx 4.1 WATERS on the basis of SEQUEST search algorithm with more than 95% identity. Following parameters were used to obtain data; one missed cleavage of enzyme trypsin, modification of cysteine residue with carbamidomethyl and methionine oxidation having peptide and fragment tolerance of 100 & 200 ppm respectively. The obtained data was searched using UniProt database against *Homo sapiens*.

4.2.7 Gene ontology

The gene ontology annotations of predicted proteins were done using BLAST2go algorithms. To get the broader GO terms such as cellular components, molecular function and biological function, the GO slimmer tool was used [203].

4.2.8 Protein interaction network of proteins involved in various biological functions during interaction of A549 cells

After the Gene Ontology, the protein interaction network was developed with the help of STRING database (version 10). The interactome study was done to predict the interaction among different proteins that may involve in various cellular or biochemical pathways. The proteins that were involved in signaling transcription, transport, cytoskeleton, immune response and other functions were subjected to STRING databases to predict their cellular interaction. The protein interaction was done in various modes (action view mode, interactive mode, confirmation or evidence view mode and confidence mode) to get the appropriate interactions among proteins that were involve in various cellular functions.

4.2 Results

4.3.1 Identification of proteins from human lung epithelial cells (A549) interacting with *A. terreus* conidia

A total 1253 proteins were identified during the interactions that are expressed in human lung epithelial cells in comparison to 427 proteins in uninfected lung epithelial cells. The identified proteins sequence coverage ranged from 10% to 45% and Protein Linux Global server (PLGS) score were ranged from 50 to 1250. Further, the isoelectric point (pI) ranged from 4.09 to 12.25 and MW of proteins ranged from 2403 to 247926 Da. Furthermore, in control lung epithelial cells (A549), a total 427 proteins were identified that were expressed by these cells under same condition as adopted for interaction assay. The PLGS score was ranged from 10.00 to 892.06 and MW (Da) ranged from 9983 to 246194. Further, we observed 63 proteins that are common in control cells and interacting lung epithelial cells (Appendix-II Table-4.1). Thus, proteins that were expressed in A549 cells upon interaction with *A. terreus* conidia were selected for gene ontology analysis to reveal the response of these cells against *A. terreus*. Gene ontology functions were attributed to 1253 from human lung epithelial proteins using BLAST2go software. Expressed proteins were assigned single gene ontology terms as biological process, cellular components and molecular functions. Among the expressed proteins, biological processes include proteins from cytoskeleton rearrangement (7%), transcriptional process (25%), signaling (13%), transportation (10%), microtubule based movement (6%), immune response (3%) and other biological function proteins (21%), figure-4.2.

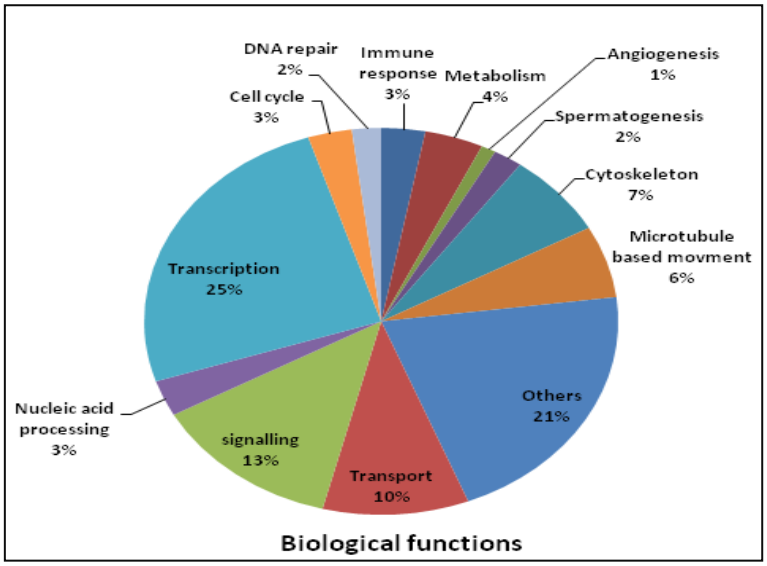


Figure: 4.2: Biological functions of expressed proteins from interaction assay using GO enrichment

Further the gene ontology classification of proteins during interaction having molecular functions showed expressed proteins from kinase activity (5.85%), transferases (3.12%), hydrolases (1.36%), nucleic acid binding activity (23.43%), ATP binding (13.28%), ion binding (28.71%), receptor activity (1.36%) and other proteins functions shown in figure-4.3.

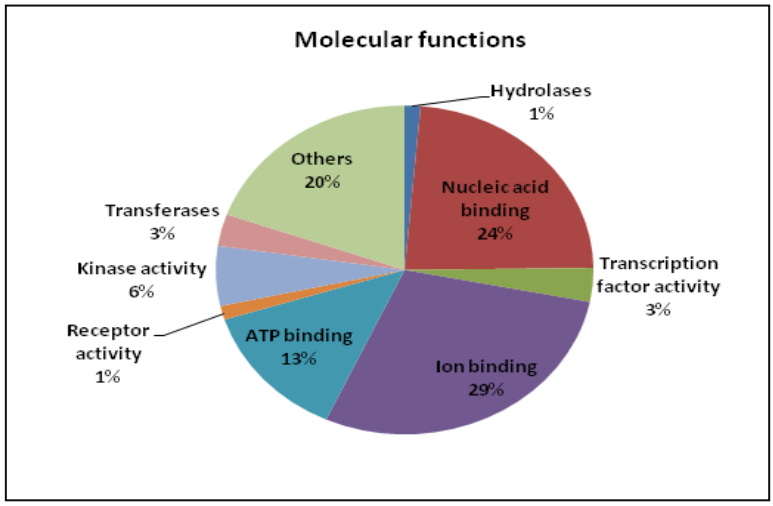


Figure-4.3: Molecular functions of expressed proteins from interaction assay using GO enrichment

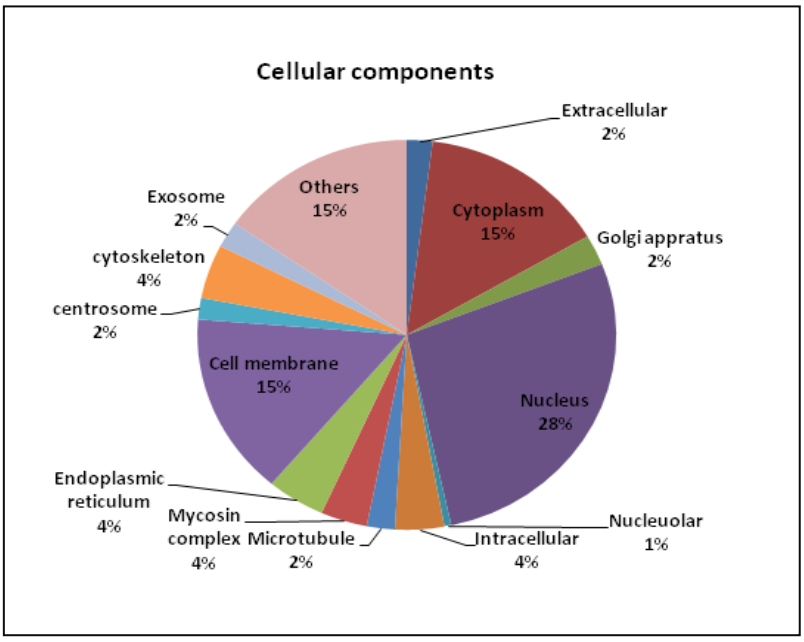


Figure-4.4: Cellular functions of expressed proteins from interaction assay using GO enrichment

Furthermore cellular component analysis showed the majority of expressed proteins from lung epithelial cells upon interaction with *A. terreus* belonged to nucleus (27.51%),

cytoplasm (14.56%), cell membrane (15.10%), golgi apparatus (2.5%), endoplasmic reticulum (4.49%), and others shown in figure-4.4.

4.3.2 Proteins associated with various functions of A549 cells upon interaction with *A. terreus*

Analysis of expressed proteins in A549 (lung epithelial cells) upon interaction with *A. terreus* revealed proteins involved in various functions and biological pathways are categorized as follow.

4.3.3 Lung epithelial cells proteins involved in internalization of *A. terreus* conidia

Proteins that may function in internalization of fungal conidia during interaction with human lung epithelial cells were observed. Among these proteins, we have identified phospholipase D, phospholipase B1, cadherin-24, protocadherin gamma-A8, protocadherins and clathrin proteins. The identification of these proteins suggested that internalization of *A. terreus* conidia by human lung epithelial cells. Further, we have also observed proteins from endosomal compartment, golgi apparatus and endoplasmic reticulum. Thus, the identification of these proteins suggested the trafficking of in internalized conidia to other compartments of lung epithelial cells. The list of proteins is given in table-4.1

Table-4.1: Proteins list involved in various biological functions during interaction with *A. terreus*

Accession number	Protein name	PLGS Score
Signaling		
H7BYL6	A-kinase anchor protein 9	225.64
A0A024R998	Protein phosphatase 1	215.22
F8W9P4	Phosphatidylinositol 4,5-bisphosphate 3-kinase	190.42
Q9UQC2	GRB2-associated-binding protein 2	183.25
Q04759	Protein kinase C theta type	162.62
A0A0A0N0M2	Tyrosine-protein kinase	156.49

Q6P4F7	Rho GTPase-activating protein 11A	145.60
B4DYG5	Phosphatidylinositol 4-kinase alpha	145.22
O75628	GTP-binding protein REM 1	140.32
A0A087WTD7	A-kinase anchor protein 13	136.17
P33402	Guanylate cyclase soluble subunit alpha-2	125.17
Q13972	Ras-specific guanine nucleotide-releasing factor 1	92.79
Q15283	Ras GTPase-activating protein 2	84.93
O00443	Phosphatidylinositol 4-phosphate 3-kinase C2 domain	63.14
A7KAX9	Rho GTPase-activating protein 32	52.28
B3KN77	Phosphodiesterase	52.38
B2RAH5	Protein phosphatase 1 regulatory subunit	53.23
P57078	Receptor-interacting serine/threonine-protein kinase 4	78.84
P42224	Signal transducer and activator of transcription 1-alpha/beta	70.26
P52333	Tyrosine-protein kinase JAK3	80.67
A0A0A0N0M2	Tyrosine-protein kinase OS=Homo sapiens GN=JAK1	156.49
Cytoskeleton		
H7C2C6	Girdin	293.03
B3KSQ7	Drebrin 1, isoform CRA_d	247.49
P15311	Ezrin	212.15
Q59GM4	PTK2B protein tyrosine kinase 2 beta	206.54
A0A024RBH2	Cytoskeleton-associated protein 4	199.54
Q59EF6	Calpain 2	112.74
A0A075B7B1	Desmuslin	111.34

Q702N8	Xin actin-binding repeat-containing protein 1	104.43
O60504	Vinexin	87.83
Q5JV20	Nesprin-1	84.12
Q0ZGT2	Nexilin	83.16
B7Z2M7	Kinesin-like protein	214.48
E7EVA0	Microtubule-associated protein	256.65
H9KV85	Ninein	204.11
E9PGW9	Pericentriolar material 1 protein	188.78
Q4KMP8	KIF6 protein	188.39
Transport		
Q149M6	Exophilin 5	405.26
A0A0A0MRV0	Ribosome-binding protein 1	359.44
H0Y6I0	Golgin subfamily A member 4	310.96
Q9NY06	Integral membrane transporter protein	233.40
V9HWE4	Epididymis luminal protein 161	215.74
A0A087WXL6	Vacuolar protein sorting 11	173.73
Q9UL25	Ras-related protein Rab-21	168.53
O95528	Solute carrier family 2	166.72
B2RDK3	Oxysterol-binding protein	163.11
Q99747	Gamma-soluble NSF attachment protein	153.96
Q9UPT6	C-Jun-amino-terminal kinase-interacting protein 3	150.34
P63010	AP-2 complex subunit beta	146.31
Q8WXP9	Oxysterol-binding protein	142.37
Q92738	USP6 N-terminal-like protein	139.85
A6NIM6	Solute carrier family 15 member 5	137.50

O75843	AP-1 complex subunit gamma-like 2	122.57
A6NNN8	sodium-coupled neutral amino acid transporter 8	88.66
Q0VD83	Apolipoprotein B receptor	82.03
Q14524	Sodium channel protein type 5 subunit alpha	70.56
Metabolism		
B2RAG5	Dihydroxyacetone phosphate acyltransferase	190.39
Q8IW92	Beta-galactosidase-1-like protein 2	172.61
Q0MQR4	Poly (ADP-ribose) glycohydrolase	144.74
E9PKF3	Acetyl-CoA acetyltransferase	144.41
P06737	Glycogen phosphorylase	131.30
A7LFL1	Phosphotransferase	88.6
O60502	Protein O-GlcNAcase	83.78
O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase	79.51
Q3SY69	Mitochondrial 10-formyltetrahydrofolate dehydrogenase	73.67
Q6ZQY3	Acidic amino acid decarboxylase GADL1	69.01
P49915	GMP synthase [glutamine-hydrolyzing	55.96
Motor proteins		
A0A0G2JMZ6	Kinesin-like protein	480.83
F5H6I8	Unconventional myosin-XVIIIb	253.27
A0A0U4BW16	Non-muscle myosin heavy chain 9	224.15
B4DEQ1	Kinesin-like protein	268.30
F8WAR6	Kinesin-like protein	261.01
Q5XUU0	Ninein isoform 6	256.75
E7EVA0	Microtubule-associated protein	256.65

E9PN67	Ninein	233.99
E9PGW9	Pericentriolar material 1 protein	188.78
Q4KMP8	KIF6 protein	188.39
A0A024R3Z1	Microtubule-associated protein	148.94
Q9Y623	Myosin-4	89.86
Cell cycle		
Q5T200	Zinc finger CCCH domain-containing protein 13	242.12
O75400	Pre-mRNA-processing factor 40 homolog A	146.55
P51957	Serine/threonine-protein kinase Nek4	144.89
Q9BX26	Synaptonemal complex protein 2	88.88
Q5TZA2	Rootletin	81.11
Q9C099	Leucine-rich repeat and coiled-coil domain-containing protein 1	73.11
Q96NB3	Zinc finger protein 830	69.71
Q8TCY9	Up-regulator of cell proliferation	59.28
Q8TDY2	RB1-inducible coiled-coil protein 1	54.48
Spermatogenesis		
Q96L12	Calreticulin-3	258.44
A0A060L980	Sperm associated antigen 9	216.75
A0A024R0I2	Synaptonemal complex protein 1	186.42
Q9NSV4	Protein diaphanous homolog 3	161.28
Q96JN2	Coiled-coil domain-containing protein 136	145.02
Q9UKQ2	Disintegrin and metalloproteinase domain-containing protein 28	125.29
Q8IWV8	E3 ubiquitin-protein ligase UBR2	69.04

Cell migration		
D6W5D1	KIAA1212, isoform CRA_a	339.39
H7C2C6	Girdin	293.03
O60271	C-Jun-amino-terminal kinase-interacting protein 4	137.64
Q0ZGT2	Nexilin	83.16
Q9H7D0	Dedicator of cytokinesis protein 5	61.07
Transcription		
Q9H609	Zinc finger protein 576	318.19
Q5TEC3	Zinc finger protein 697	284.36
P29084	Transcription initiation factor IIE subunit beta	232.47
Q96PQ6	Zinc finger protein 317	207.33
Q03938	Zinc finger protein 90	202.69
F2Z2R5	Chromodomain-helicase-DNA-binding protein 5	192.10
Immunity		
P01031	Complement C5	50.09
A0A0K0KRG9	MHC class II antigen	109.55
P48740	Mannan-binding lectin serine protease 1	69.57
A0A0A7E7T3	Endoplasmic reticulum aminopeptidase 1	69.56
Q13123	Protein Red	123.05
A0A087WT25	Tubby-like protein	307.45
Angiogenesis		
B4E173	Plexin domain-containing protein 1	165.4
Q59GM4	PTK2B protein tyrosine kinase 2 beta	206.54
P35916	Vascular endothelial growth factor receptor 3	226.29
A0A024R8E5	Collagen, type V, alpha 1, isoform CRA_a	254.73

Apoptosis		
Q9UL42	Paraneoplastic antigen Ma2	219.09
Q92851	Caspase-10	286.35
A0A0S2Z3Z5	Caspase 10 apoptosis-related cysteine peptidase	302.65
Others		
Q59EA4	Phospholipase D	10.04
B2RWP8	Phospholipase B1	61.62
Q86UP0	Cadherin-24	66.77
Q9Y5G5	Protocadherin gamma-A8	55.54
Q9HCL0	Protocadherin-18	78.45
O14917	Protocadherin-17	90.78
Q9BZA7	Protocadherin-11	113.56
Q9Y2D8	Afadin- and alpha-actinin-binding protein	132.67
Q00610	Clathrin heavy chain 1	78.45
A0A087WX41	Clathrin heavy chain 2	90.56

4.3.4 Proteins associated with cytoskeleton rearrangement during interaction with *A. terreus* conidia

The internalization initiation by lung epithelial cell is associated with rearrangement of cytoskeleton. Thus, we have observed various proteins that were involve in cytoskeleton rearrangement such kinesin-like protein; activity regulated cytoskeleton-associated protein 4, nexilin, pericentriolar material 1 protein, girdin, vinexin, KIF6 protein etc. We have also observed microtubule based movement and motor proteins involved in rearrangement of cytoskeleton of lung epithelial cells during interaction with *A. terreus.* The list of proteins is given in table-4.1.

4.3.5 Proteins associated with immune response

Upon A549 cells interaction with *A. terreus,* we have observed the expression of various protein related to immune functions or that are involve in activation of various pathways e.g, immune-regulatory molecules. We also observed complement component C5, MHC class II antigen, mannan-binding lectin serine protease, tubby-like protein (involve in phagocytosis) and other proteins such as tyrosine-protein kinase, STAT1-alpha/beta and RIPK4 that involve in the activation of signaling pathways associated with immune-regulatory molecules such as cytokines and chemokines. Thus, the identification of these proteins in interacting cells revealed the active of immune response to *A. terreus* by lung epithelial cells.

4.3.6 Protein-protein interactome of identified proteins of A549 cells upon interaction with *A. terreus*

The functions of proteins can be determined by its interaction with other proteins, thus protein- protein interaction has been performed. Based on our analysis, proteins from biological functional were identified during A549: AT interaction, these proteins were involved in signaling transcription, transport, cytoskeleton, immune response and others were subjected to interactome network evaluation. The interaction network analysis was performed with the help of STRING Database 10 [204]. In the interaction data, the non-interacting proteins were excluded and presented in appendix-I- figure-4.1 that showed the interaction of various proteins with each other and information for total interacting proteins is given in table-4.2. The maximum interacting partners were observed for proteins tyrosine-protein kinase (Jak1), RIPK4, STAT1, KIF5C, PIK3C2A, MHC class II antigen (HLA-DRB1) and PIK3CD. Further, the biological pathways of major interacting proteins were also identified using Kyoto Encyclopedia of Genes and Genomes (KEGG). Major interacting proteins JAK1, STAT1 and RIPK were identified in biological pathways such as JAK/STAT signaling pathway, Th17 cell differentiation, chemokine signaling, NOD receptor, TLR, TNF and NF-kβ signaling pathways.

Table-4.2: Identified proteins used for construction of interactome network with the help of String database

Accession number	Gene name	Protein name	PLGS Score
Signaling			
H7BYL6	*AKAP9*	A-kinase anchor protein 9	225.64
A0A024R998	*PPP1R12B*	Protein phosphatase 1	215.22
F8W9P4	*PIK3CD*	Phosphatidylinositol 4,5-bisphosphate 3-kinase	190.42
Q9UQC2	*GAB2*	GRB2-associated-binding protein 2	183.25
Q04759	*PRKCQ*	Protein kinase C theta type	162.62
A0A0A0N0M2	*JAK1*	Tyrosine-protein kinase	156.49
O75628	*REM1*	GTP-binding protein REM 1	140.32
A0A087WTD7	*Akap*	A-kinase anchor protein 13	136.17
P33402	*GUCY1A2*	Guanylate cyclase soluble subunit alpha-2	125.17
Q13972	*RASGRF1*	Ras-specific guanine nucleotide-releasing factor 1	92.79
Q15283	-	Ras GTPase-activating protein 2	84.93
O00443	*PIK3C2A*	Phosphatidylinositol 4-phosphate 3-kinase C2 domain	63.14
A7KAX9	*ARHGAP32*	Rho GTPase-activating protein 32	52.28
B2RAH5		Protein phosphatase 1 regulatory subunit	53.23
P57078	*RIPK4*	Receptor-interacting serine/threonine-protein kinase 4	78.84
P42224	*STAT1*	Signal transducer and activator	70.26

		of transcription 1-alpha/beta	
P52333	*JAK3*	Tyrosine-protein kinase JAK3	80.67
Transcription			
Q9H609	*ZNF576*	Zinc finger protein 576	318.19
Q5TEC3	*ZNF697*	Zinc finger protein 697	284.36
Q96PQ6	*ZNF317*	Zinc finger protein 317	207.33
Q03938	*ZNF90*	Zinc finger protein 90	202.69
Transport			
A0A0A0MRV0	*RRBP1*	Ribosome-binding protein 1	359.44
Q9UPT6	*MAPK8IP3*	C-Jun-amino-terminal kinase-interacting protein 3	150.34
P63010	*AP2B1*	AP-2 complex subunit beta	146.31
O75843	*AP1G2*	AP-1 complex subunit gamma-like 2	122.57
Q0VD83	-	Apolipoprotein B receptor	82.03
Q14524	*SCN5A*	Sodium channel protein type 5 subunit alpha	70.56
Immunity			
P01031	-	Complement C5	50.09
A0A0K0KRG9	*HLA-DRB1*	MHC class II antigen	109.55
P48740	-	Mannan-binding lectin serine protease 1	69.57
A0A0A7E7T3	-	Endoplasmic reticulum aminopeptidase 1	69.56
Q13123	-	Protein Red	123.05
A0A087WT25	*TULP1*	Tubby-like protein	307.45

4.4 Discussion

In this objective, the global proteome profile of A549 lung epithelial cells was done upon interaction with *A. terreus* germinating conidia. At 16 hours time point *A. terreus* conidia were at germinating stage during the interaction with lung epithelial cells, thus considered for proteome analysis for the interaction study [198]. During the interaction we observed 1253 proteins showed expression in human lung epithelial cells infected with *A. terreus* conidia whereas 427 proteins were expressed in uninfected lung epithelial cells. On comparison with the control (uninfected lung epithelial cells), we observed expression of 63 proteins in both conditions (Figure-4.5 & Appendix-II-Table-4.1). Thus, a profound effect on the expression of lung epithelial cells proteins were observed upon interaction with *A. terreus* conidia. Properties of A549 epithelial cell lines are moderately distinct from Alveolar Type II cells as described by Swain *et al* [233]. Kroeker *et al* compared results from A549 cell lines with that to primary human archeobronchial airway epithelial infected with influenza virus to compare the response between primary and secondary cell lines [234]. Thus, it could be important to carry out interaction studies on primary human bronchial epithelial cell lines infected with *Aspergilli* and comparison to secondary cell line A549 could be assessed to derive relevant host response. Studies on primary cell lines have been increased over the years due to the resemblance with the *in-vivo* conditions as compared to secondary cell lines [234-235]. Using GO enrichment analysis of human lung epithelial cells (A549) expressed proteins, we observed proteins associated with internalization process such as phospholipase D and isoforms of phospholipase B. Previously, it has been reported that phospholipases expressed by A549 cells help to internalize *A. fumigatus* conidia. Further, it has been suggested that this protein interacts with dectin-1 receptor present on lung epithelial cells and increases the internalization of *A. fumigatus* conidia [83]. Furthermore, it has been observed that the fungal conidia internalization by lung epithelial cells requires cytoskeleton rearrangement, microtubule based movement and actin polymerization, and thus lung epithelial cells may perform similar mechanism to phagocytosis [83, 236]. In our study we have observed the expression of various proteins that may be involved in cytoskeleton rearrangement and internalization of *A. terreus* conidia. Cadherin-24, protocadherin gamma-A8, protocadherins, clathrin proteins and cytoskeleton rearrangement such kinesin-like protein, activity regulated cytoskeleton-associated protein, nexilin, pericentriolar material 1 protein, girdin, vinexin, and KIF6 protein were identified in lung

epithelial cells upon interaction with *A. terreus* conidia.

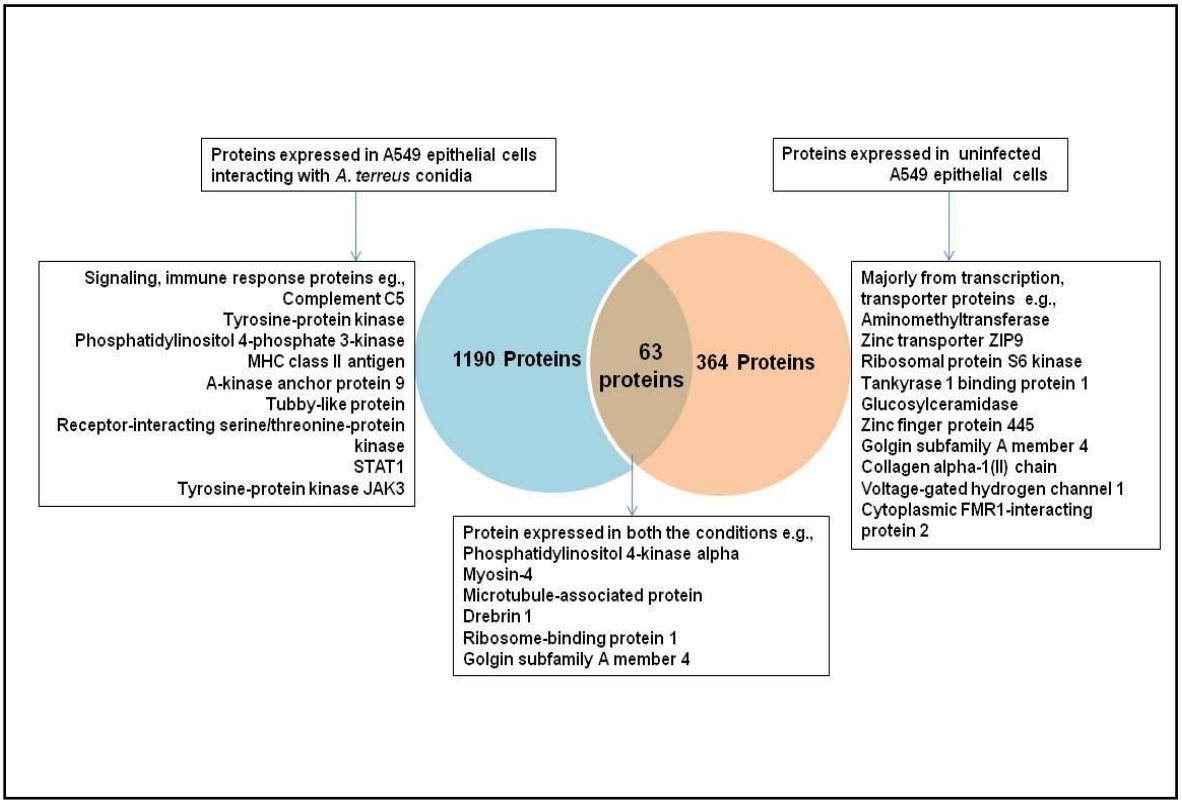


Figure-4.5: Venn diagram of expressed protein in human lung epithelial cells upon interaction with *Aspergillus terreus* and uninfected human lung epithelia cells

Further, previous studies on A549 cells suggested the role of cadherin proteins in activation of phagocytosis of *A.* fumigatus conidia upon interaction. Lung epithelial cells interacting with *A. fumigatus* suggested the role of cadherins proteins in phagocytosis of conidia by A549 cells and their silencing decreases the phagocytosis by lung epithelial cells [84, 237]. We also observed protein such as activity regulated cytoskeleton protein that suggests the rearrangement of cytoskeleton in lung epithelial cells. It has been observed that activity regulatory cytoskeleton protein is a vital constituent of Arp2/3 complex, thus, activates actin polymerization and cytoskeleton rearrangement [86]. These results suggested that the cytoskeleton rearrangement is the key step during the internalization of *Aspergilli* conidia by lung epithelial cells. Additionally, it has been observed that the epithelial cells not only act as anatomical barrier for invasive pathogens but also involved in evoking the immune response [152]. Thus, lung epithelial cells may participate in early immune response against inhaled conidia or germinating conidia of

Aspergilli [33, 57]. In current study, the proteins were identified that may involve in activation of signaling pathways that further activates immune-regulatory molecules. We have identified Tyrosine-protein kinase (Jak1), RIPK4 and STAT1 proteins in lung epithelial cells upon interaction with *A. terreus* germline. The gene ontology of these proteins suggested they were involved in various signaling pathways and string database analysis predicted their interaction with each other. Further, KEEG pathway analysis of these proteins suggest the activation of various cellular pathways like vise of Jak/Stat signaling pathway, Th17 cell differentiation, chemokine signaling, NOD signaling pathway, TLR-signaling, TNF and NF-kβ signaling pathways during interaction of lung epithelial cells (A549) with *A. terreus* germline. These findings suggested that proteins involved in activation of intracellular signaling pathways that lead to secretion of cytokines and chemokines and differentiation of other effectors cells such as T-helper cells and thus initiate a strong immune response to *A. terreus* conidia [7]. Further, the identification of RIPK protein activates NF-kβ transcription factor that lead to IL-8 expression. Balloy *et al* observed the increased expression of IL-8 activated by transcription factor NF-kβ due to *A. fumigatus* infection [238]. Similarly, the activation of innate immunity response was observed after 6 hours by lung epithelial cells exposed to *A. fumigatus* [33]. Further, Bellanger *et al* observed a stronger immune response by lung epithelial cell with germinating conidia as compared to conidia of *Aspergilli* [239]. This study also showed the germination of *A. terreus* conidia at 16 hours of incubation with lung epithelial cells. Thus, germinating conidia interaction with A549 cells lead to the activation of various intracellular signaling pathways that may involve in activation of strong immune response. Aimanianda *et al* reported that the longer incubation time help in exposure of more fungal antigen on germinating conidia. Furthermore, these germinating conidia or their antigenic molecules on cell wall recognized by immune cells (macrophages and dendritic cells) and they activate innate and adaptive immune response [240]. Further, *Aspergilli* infection leads to hyper immune response if not controlled in patients. The overproduction of key pro-inflammatory cytokines leads to the damage of infected lungs [241]. Recent study showed secretion of IL-8 in human lung epithelial cells in response to *A. fumigatus* [238]. In our data we observed proteins involved in signaling of NF-kβ which mediates the production of IL-8. In addition, inflammatory response mediated by IL-8 due to *Pseudomonas aeruginosa* and *A. fumigatus* antigens has been observed in cystic fibrosis patients [241]. Thus, studies can be extended on IL-8 as an

inflammatory marker in *A. terreus* associated infections as a target to control the inflammatory response. Thereafter, the expression of complement component C5 was also observed in epithelial cells upon interaction with *A. terreus*. Complement component C5 is important in the activation of complement cascade [242-243]. Studies in mice showed that the deficiency of C5 increased the susceptibility to *A. fumigatus* infection and also required 50% lower lethal conidia dosages of invasive infection [58]. Further, C5 trigger the release of various inflammatory cytokines such as TNF-α and IL-6, which further help to clear fungal pathogens [244]. We also observed the expression of mannan-binding lectin serine protease in lung epithelial cells upon interaction with *A. terreus*. Previously, it has been elucidated that the mannan-binding lectin serine proteases activate the lectin pathway of complement system [245]. Furthermore, it is also reported that the mannan-binding lectins binds to *A. fumigatus* conidia and initiate lectin pathway with the help of mannan-binding lectin serine proteases in lung epithelial cells, thus, modulate the innate immune response to invading fungal pathogens [246]. So, this objective concluded that, *A. terreus* germination could activate Jak/Stat signaling lectin complement pathways and NF-kβ pathway to elicit immune response in lung epithelial cells during interaction.

Structural-functional relation of deleterious nsSNPs in pentraxin-3 (a soluble receptor) and in dectin-1 (a cell bound receptor) that recognize *Aspergilli* conidia during their phenotypic transition to germinating conidia

5.1 Introduction

In the beginning of personalized genomic medicine era, the study on genetic variations in human population has showed tremendous importance to human health. With the availability of human genome and various genotyping technologies, the numerous association studies were performed on genetic variations linked to various diseases such as metabolic disorder, inherent genetic disorder, cancer, neurological disorders and susceptibility to various microbial infections [137]. The genetic changes or variations are classified as either mutations or single nucleotide variations. These single base changes in the genome of an organism are also known as SNPs that present in population with the frequency of more than 1%, where as mutations occur <1% in human population. The mutations and SNPs equally accepted as alteration in DNA sequences. The variations in human genomes are due to SNPs that together made up-to 90% genetic variations in human genome and further 1000 genome project concluded 84.7 million SNPs in 2,504 individuals among 26 populations [132]. SNPs may present in any region of genes from promoters to intronic and exonic regions. Most of the SNPs present in genes have negligible effect especially those present in intronic region of gene that encoded proteins. The SNPs present in promoters or exonic parts of coding region may lead to amino acid substitutions that may alter the proteins function and structure of encoded proteins [139, 247]. On the basis of these effect SNPs are classified as nsSNPs or synonymous SNPs. nsSNPs result in change of amino acids thus have deleterious effect on encoded proteins [22]. In last few years, the occurrence of SNPs in genes involved in recognition of various fungal pathogens and activation of immune response have gained importance [248]. The cells that encounter these pathogens act as first line of defense due to the presence of PPRs. SNPs located in domains of PRRs that recognize PAMPs may alter the ability of PRRs to bind pathogens [249,250]. Further, the SNPs in transmembrane domains or domains that interact with other components of immunity can lead to defects in intracellular signaling and effector immune response [248]. More importantly, various

studies on genes that encode receptor proteins shown profound effect on protein's structure, function, stability and their impact on binding efficiency with the ligand molecules [22, 137, 251].Thus, it is important to study the SNPs that are present in genes that encode various PRRs such as mannose binding lectin proteins, TLRs, lectin proteins (dectin-1 & 2) and various surfactant proteins (SP-A & D) that recognize fungal pathogens. The presence of nsSNPs in PRRs may affect their structure and function and the persons that having those nsSNPs may be vulnerable to *Aspergilli* infections.

The *Aspergillus* species produces thousand of conidia or 1-100 conidia per m^2. The hundreds of these conidia are inhaled by humans daily. Some of these inhaled conidia entrapped by upper respiratory tract and clear them. Despite of upper respiratory tract function they reached to lower respiratory tract due to their small size and entrapped in lungs alveoli [1]. Not only various factors in respiratory system like mucous, upper epithelium ciliated layer that trap these inhaled conidia but they also recognize by human immune system through PRRs of innate immune cells (macrophages, Neutrophils etc.) or by soluble pattern recognition receptors that function as opsonin [8]. The recognition of ingested conidia initiates various downstream signaling pathways that result in an efficient immune response [252]. The transition of conidia to germinating conidia begins various morphological changes. The loss of hydrophobic or protein layers on the *Aspergillus* conidia surface exposes inner part of cell wall during germination. Germination of inhaled conidia is very critical for successful establishment of the pathogen in the host tissue [174]. Carbohydrate contents of conidia or germinating conidia allow these carbohydrate moieties to interact with host immune cells, these moieties called PAMPs. The main polysaccharides that fungal cell wall consist are galactomannan, chitin, mannan and β-glucan [7]. Thus, the transition of conidia to germinating conidia is important because it initiates the recognition of inhaled conidia that further leads to their ingestion by various immune cells or start various downstream immune pathways such as those involved in production of various chemokines and cytokines. Hence these receptors also help to bridge the innate and adaptive immune response to efficiently clear fungal pathogens [68, 174].

There are various soluble and cell bound receptors that help to recognize *Aspergilli* [8]. Among soluble receptors, pentraxins, and pulmonary collectins are one of the most important soluble receptors present in lungs and help to clear *Aspergilli* conidia

from lungs. Whereas, among cell bound receptors dectin-1 and TLRs have gained importance [61,140]. Pentraxin proteins are evolutionary conserved proteins [156]. These proteins are further classified into long and short pentraxins on bases of their size. Pentraxin-3, pentraxin-4, neuronal pentraxin-1 & 2 are long pentraxin whereas serum amyloid protein and-reactive protein are short pentraxins [126]. In recent years PTX-3 has been recognized as a key soluble pathogen recognition receptor in lung infections. This protein is produced in response to fungal, bacterial and viral pathogens that gain entry to respiratory system [126, 159, 253]. Pentraxin-3 protein inhibits the invasion of respiratory system by various fungal pathogens most importantly *Aspergilli* and *Candida* species. It recognizes various moieties of fungal pathogens such as galactomannan of conidia of *Aspergilli* and help to clear them by opsonization to various effector cells of immune system [248]. Further, it interacts with complement pathway protein C1q and helps to activate classical pathway of complement system in response to pathogen recognition [254]. Thus, pentraxin-3 is an important fraction of innate immunity against fungal pathogens [255]. Being an important component of innate immunity, the gene which encodes pentraxin-3 protein is highly polymorphic in nature and further, it is not clear how nsSNPs present in this gene affects its function as a receptor against fungal and other microbial pathogens. On the other side, cell bound receptor such as dectin-1 has emerged as important fungal recognition receptor that required for the clearance of invasive fungal infections [146]. Being a type-II transmembrane receptor, it is present on innate immune cells or lung epithelial cells [152]. It contains different domains such as extracellular CTLD-domain and a carboxyl-terminal domain. Apart from these domains, it also represents by a stalk, transmembrane domain and an intracellular immune-receptor tyrosine based activated motif (ITAM) [146]. CTLD-domain functions as a PRR involved in the recognition of conserved carbohydrate moieties present on the microbial cell wall called PAMPs [147]. Dectin-1 receptor is an important fungal pathogen recognition receptor of innate immune cells, which interacts with β-glucan (a major constituent of the filamentous fungal cell wall) of *Aspergillus species* and *Candida* species [146]. During cell wall remolding in germinating conidia of fungi or at mycelia stage of fungi, β-glucan becomes exposed to extracellular environment. Phosphorylation of ITAM motif of dectin-1 receptor occurs during the interaction of dectin-1 receptor with that of β-glucan [63]. The dectin-1 receptor also interacts to other proteins such as TLR-2 and SyK. SyK kinase initiates the downstream signaling and activates NF-kβ, which further regulates effector

function of innate immunity by activating various cytokines and chemokines [152]. Thus, efficient functioning of pentraxin-3 and dectin-1 receptors present on immune cells is important in the clearance of *Aspergilli* or other fungi. In recent years SNPs in genes encoding various proteins/ receptors have gained importance due to their impact on structure and function of these proteins [22,139,162]. Further, recently more efforts have been made to disclose the effect of nsSNPs on protein's structure and functions [161]. There is a large number of SNPs dataset that is not yet annotated with respect to SNPs/nsSNPs associated with infectious agents that may have impact on proteins structure and function. An effective alternative to these hurdles are various *in-silico* or molecular dynamic approaches to elucidate biochemical severity of the amino acid substitution, as well as the protein sequences and/or structural information, thus provide a more feasible approach for phenotype prediction. Thus, various *in-silico* approaches were used to illustrate the severity of nsSNP in pentraxin-3 & dectin-1 receptors.

5.2 Material and Methods

5.2.1 SNP data retrieval for dectin-1 and pentraxin-3

Single nucleotide polymorphisms (SNPs) for dectin-1 and pentraxin-3 genes were taken from dbSNP database of NCBI and also from Ensembl genome browser [256,257]. Further, the corresponding protein sequences of proteins encoded by these genes were obtained from UniProt data base having following UniProt identification numbers UniProt KB IDQ9BXN2 and P26022.

5.2.2 Functional consequences prediction of non-synonymous-SNPs using different algorithms

Non-synonymous SNPs (nsSNPs) of dectin-1 and pentraxin-3 genes were obtained from the total SNPs that were retrieved from databases. Thereafter, the functional consequences of these SNPs were analyzed by using five *in-silico* algorithms: SIFT, polyphen version 2, SNAP, PhD-SNP and MAPP algorithms were used for prediction of deleterious or neutral nsSNPs of dectin-1 gene. The SIFT algorithm is defined as Sorting Intolerant from Tolerant that uses the protein sequence homology and align the sequences of orthologous and paralogous proteins that having natural occurring SNPs with input sequence to predict the effect of nsSNPs as a deleterious or neutral. The less than 0.05

SIFT score indicates that the nsSNPs have deleterious impact on protein's functions. Non-synonymous-SNPs having SIFT scoreless then 0.05 indicated as deleterious nsSNPs.

Polyphen-2 algorithm uses variation in amino acids in protein sequence to find out deleterious effects of nSNPs on proteins function. It indicates an amino acids change or mutation to protein sequence as a benign, a probably damaging and as a possibly damaging. Polyphen also tells about PISC value for substituted amino acids in protein sequence and predict the effect of non-synonymous-SNPs on protein's functions. In additional to these algorithms, MAPP, PhD-SNP and SNAP were used to predict the deleterious effect of non-synonymous-SNPs. Furthermore, the non-synonymous-SNPs were considered as high-risk, if they were predicted as deleterious or damaging using these different in-silico-algorithms. Then, their locations were predicted to functional domains of dectin-1 and pentraxin-3 proteins.

5.2.3 Conservation profile of dectin-1 and pentraxin-3 protein receptors

Evolutionary conservation profile of dectin-1 and pentraxin-3 proteins were analyzed by a web based tool called as ConSurf web server. ConSurf estimates the evolutionary conserve nucleic acids and amino acids in DNA/ proteins on the basis of phylogenetic relationship between homologous protein or DNA sequences [258]. The PSI-BLAST and BLST algorithms are used by ConSurf web server to search the close homologous of submitted protein or DNA sequences. We submitted the amino acid sequence of dectin-1 and pentraxin-3 receptors to ConSurf server to predict evolutionary conserved amino acid residues.

5.2.4 Non-synonymous SNPs effect on stability of dectin-1 and pentraxin-3 proteins

The effect of amino acids change on protein's stability was assessed using I-Mutant version-2 tool [259]. This tool predicts Gibbs- free energy changes (DDG) by subtracting the unfold Gibbs free energies of mutant proteins from native or wild type proteins's energies. Further, it represents the change in Gibbs free energy by the sign of increase (Inc) or decrease (Dec) with a reliability index score of altered amino acid. The reliability index score ranges from 0 to 10 where 10 indicate the highest reliability and 0 indicates lowest reliability for an altered amino acid. The value of DDG less than 0 shows the decreases in stability of proteins, whereas score above 0 signifies increased proteins

stabilities. During prediction of change in Gibbs free energy for all substituted amino acids in protein's sequence, the pH and temperature were set at 7.0 and 25°C respectively [259].

5.2.5 Identification of non-synonymous SNPs locations in domains of dectin-1 and pentraxin-3 proteins

To deduce the locations of non-synonymous SNPs in various domains of dectin -1 and pentraxin-3 protein, A Conserved Domain Search tool from NCBI database was used [260]. This tool uses protein sequences or their IDs as a query to predict domains in proteins. We submitted the protein sequences of dectin-1 and pentraxin-3 proteins to NCBI Conserved Domain Search tool for the prediction of different dectin-1 and pentraxin-3 domains [260].

5.2.6 Homology modeling of domains of dectin-1 and pentraxin-3 proteins and structure stability determination

The 3-dimensional structure of wild-type CTLD of dectin-1 and pentraxin domain of pentraxin-3 proteins were modeled using Phyre2 version-2, a web-based server [261]. For homology modeling, the protein sequence of dectin-1 and pentraxin-3 proteins were retrieved from UniProt database (UniProt ID: Q9BXN2 and P26022). PDB and PFAM databases were used by Phyre2 to generate 3d structures of submitted protein sequences based on similarity of recognized structure. For the generation of 3D-structure of c-type lectin and pentraxin domains, FASTA protein sequences of the domains were submitted to Phyre2 server and intensive mode was used to generate 3D structure. The PDB template c2bpdB (% identity: 61, Murine Dectin-1 protein) was used by Phyre2 tool for building the structure of CTLD and whereas in case of pentraxin domain structure construction, following PDB templates have been used: PDB ID (% identity); [1sac (24%), 1b09 (24%), 4pbo (23%), and 3flp (22%)]. Further, mutant structures of CTLD and pentraxin domains with high-risk SNPs were generated by Swiss-PDB mutation tool by replacing wild type or coresponding amino acids with that to mutated amino acids. ModRefiner tool was used for structure refinement by providing PDB files of wild-type and mutant structure of C-type lectin and pentraxin domains [262]. After that YASARA force field minimization tool was used to minimize energy [263]. Modeled structures validations such as stereo-chemical properties were carried out by ProCheck. Further, RMSD values and TM-Scores were estimated by using TM-align tool [264].

5.2.7 Protein –protein interaction analysis for dectin-1 and pentraxin-3 proteins

Protein interaction studies are important for all functional interaction of proteins with other cellular proteins. Dectin-1 and pentraxin-3 proteins interaction study was carried out by using online STRING database (STRING version 10.0). STRING database (string.db.org) provides the experimental and theoretical evidence of protein interactions. Currently, STRING database version 10.0 has data for 2031 organisms, 9.2 million proteins and 184 million interactions [204]. Dectin-1 and pentraxin-3 protein sequences were given as input in STRING database to deduce the interaction of dectin-1 and pentraxin-3 with other cellular proteins. The interaction studies were carried out in different modes such as action view mode, interactive view mode, confidence and evidence view modes to reveal the significant interactions of dectin-1 and pentraxin-3 proteins with other proteins.

5.2.8 Molecular docking of native CTLD-domain and its variant protein structures with β-glucan

The molecular docking study for CTLD-domain of dectin-1 protein (native and mutant type) with β-glucan was carried out with AutoDock 4 [265]. PubChem database was used to retrive β-(1-3) glucan structure. The structure of β- glucan was docked with native and mutant CTLD-domain of dectin-1 to determine the difference in the free binding energies between native and mutant type structure. In AutoDock 4, we have used lamarckian genetic algorithm with default constraints to perform molecular docking. The docking run was set to 50 in number and the lowest binding energy conformation was selected for LIGPLOT analysis [266].

5.3 Results

5.3.1 SNP Datasets

SNP datasets were evaluated for deleterious or nsSNPs in dectin-1 and PTX-3 genes were retrieved from dbSNP and Ensembl genome browser. We observed 91 and 65 missense variants in dectin-1 and PTX-3 genes respectively, which were further used to predict potential deleterious SNPs. Other then missense variant, dectin-1 and PTX-3 genes contained, 5&4 SNPS in 5’prime UTR, 32 & 42 SNPs in 3’prime and 582 & 170 SNPS

in intronic region of these genes. Protein sequences of dectin-1 and PTX-3 genes were retrieved from UniProt database for further analysis.

5.3.2 Identification of deleterious nsSNPs in dectin-1 and PTX-3

Various improved *in-silico* algorithms are available to predict potential deleterious nsSNPs from large datasets of SNPs. Five algorithms likewise; Sorting Intolerant from Tolerant (SIFT), Polyphen-2, Prediction of Human Disease-SNP (PhD-SNP), Screening of Non-Acceptable Polymorphisms (SNAP) and MAPP were utilized to analyze 91 & 65 nsSNPs of dectin-1 and PTX-3 genes and to determine the structural and functional impact on proteins encoded by these genes. Table-5.1& 5.2 showed the distribution of various neutral and deleterious nsSNPs of dectin-1and PTX-3genes predicted by five *in-silico* algorithms.

Table-5.1: Number of predicted deleterious or neutral non-synonymous-SNPs in PTX-3 using five algorithms

Prediction	**nsSNPs (%)**				
	SIFT	**Polyphen2**	**MAPP**	**PhD-SNP**	**SNAP**
Deleterious	25(38)	27(41)	37(57)	18(28)	29(45)
Neutral	40(62)	38(59)	28(43)	47(72)	36(55)

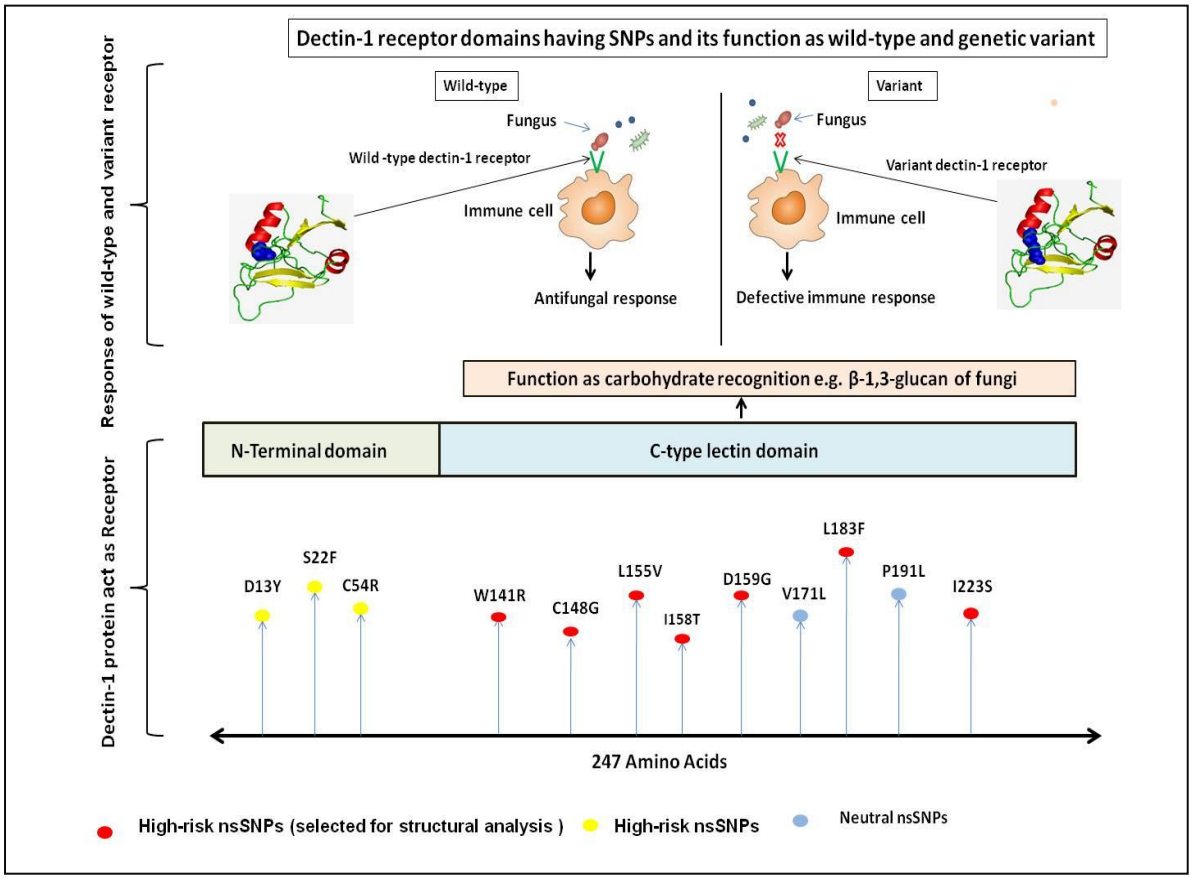
Table-5.2: Number of predicted deleterious or neutral non-synonymous-SNPs in dectin-1 using five algorithms

No of predicted nsSNPs (%)					
	SIFT	**PhD-SNP**	**Polyphen2**	**SNAP**	**MAPP**
Deleterious	42 (46)	31(34)	36(40)	20(22)	54(59)
Neutral	49(54)	60(66)	55(60)	71(78)	37(41)

SIFT algorithm uses sequence homology and constructs multiple sequence alignment of protein sequences to recognize the effect of amino acid substitution. A total 42 deleterious or 49 neutral nsSNPs has been predicted by SIFT algorithm in dectin-1 whereas in PTX-3, 25 (deleterious) & 40 (neutral) nsSNPs were predicted. Only nsSNPs having SIFT score <0.05 are considered as deleterious nsSNPs**.** Polyphen-2 algorithm uses a structural and

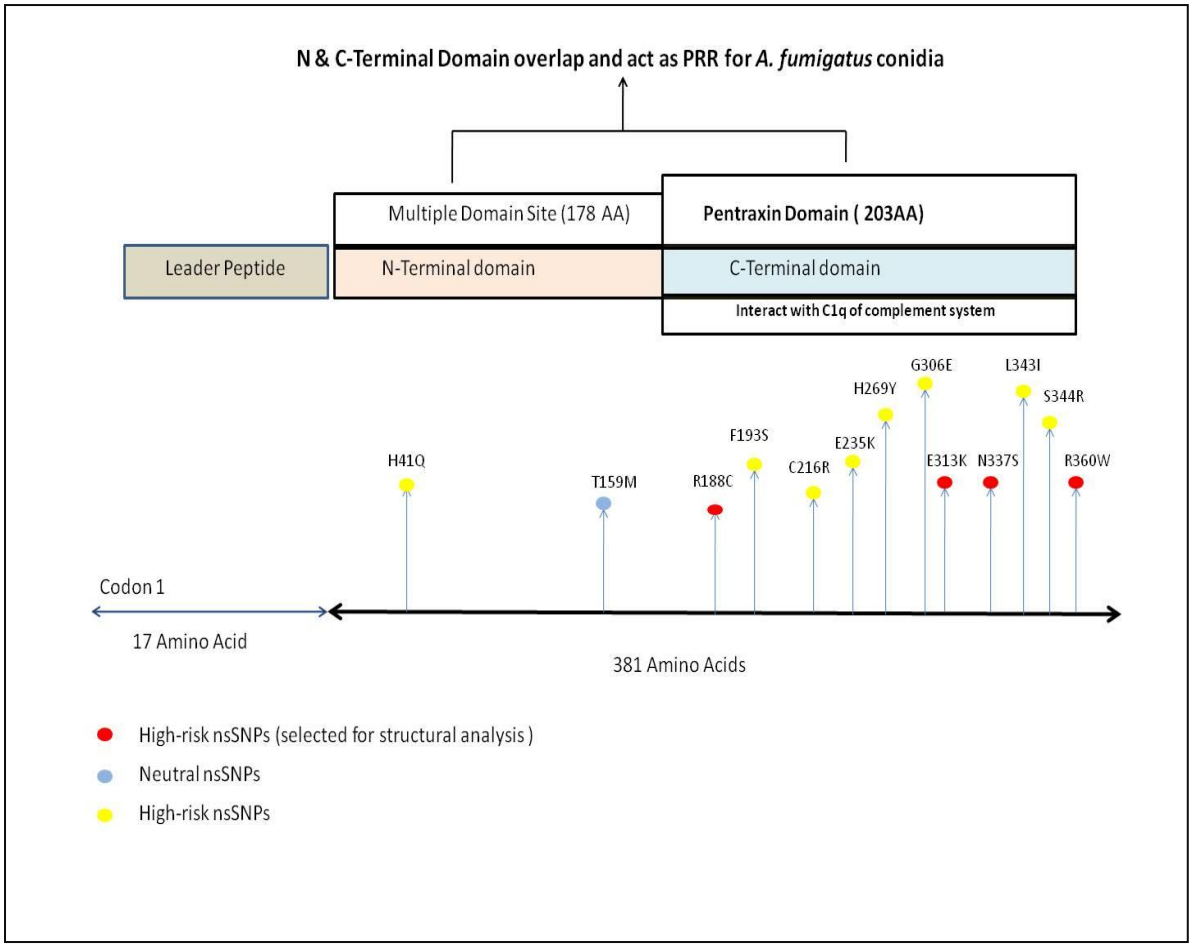
sequence based approach to predict the effects of amino acid changes. This tool uses various structural features to predict the deleterious or damaging nsSNPs likewise solvent accessibility of burial amino acid residues in protein structure and crystallographic B-factor. Out of 91 nsSNPs, polyphen-2 predicted 36 deleterious or damaging nsSNPs in dectin-1 and in PTX-3, only 27. Each of the algorithms used different parameters to predict deleterious nsSNPs. Further, deleterious nsSNPs were classified as highly or potential deleterious nsSNPs, when four or more than four different *in-silico* algorithms predicted highly deleterious nsSNPs. A total of 22 (dectin-1) and 10 (PTX-3) deleterious nsSNPs were classified as highly deleterious nsSNPs (Appendix-II-Table-5.1). Further, to predict the locations of these highly deleterious nsSNPs in different domains of these proteins, conserved domains were identified using conserved domain database (CDD) from NCBI. Dectin-1 protein contains a conserved extracellular domain called c-type lectin domain, which help in recognize carbohydrate moieties of fungal pathogens. C-type lectin domain contained 14 highly deleterious nsSNPs (Appendix-II-Table-5.1 & Figure-5.1).

Figure-5.1: Over all representation of dectin-1 receptor protein and its function



Whereas PTX-3 contains a pentraxin domain at C-terminal end of protein which overlaps the N-terminal domain and they act as pathogen recognition receptor. PTX-3 protein contains 11 highly deleterious nsSNPs in Pentraxin domain that recognizes fungal pathogens such as *Aspergilli* (Figure-5.2 and Appendix-II- Table-5.2).

Figure-5.2: Over all representation of pentraxin-3 receptor protein and its functions



5.3.3 Prediction of conserved amino acid residues among high-risk nsSNPs

The evolutionary conserved amino acids of dectin-1 and PTX-3 proteins were predicted from online tool ConSurf web browser. Using ConSurf, we have predicted the functional, structural and evolutionary conserved amino acids of dectin-1 and PTX-3 proteins (Figure-5.3 & 5.4). Out of 22 amino acids that coincide in highly deleterious nsSNP location in dectin-1 protein (Appendix Table-5.1), only 13 amino acid residues were predicted to be functional, structural and highly evolutionary conserved (ConSurf scale >8) (Appendix Table-5.2). Among the 13 evolutionary conserved amino acid residues, 4 were located in N-terminal domain of dectin-1 protein and rests of the amino acid were

located in extracellular or c-type lectin domain. Also among evolutionary conserved amino acid of c-type lectin domain, 4 amino acids (C148G, I158T, L183F, and C233Y) were structural residues and 2 amino acids (W141R, D159G) were functional residues. Whereas pentraxin domain contained four highly conserved amino acids. Also these amino acids were structural and functional residues of PTX-3 protein (Appendix-II-Tables-5.3). Further, selected high-risk nsSNPs from CTLD and pentraxin domain were used for structural analysis (Figure-5.1&5.2 and Tables-5.3 &5.4).

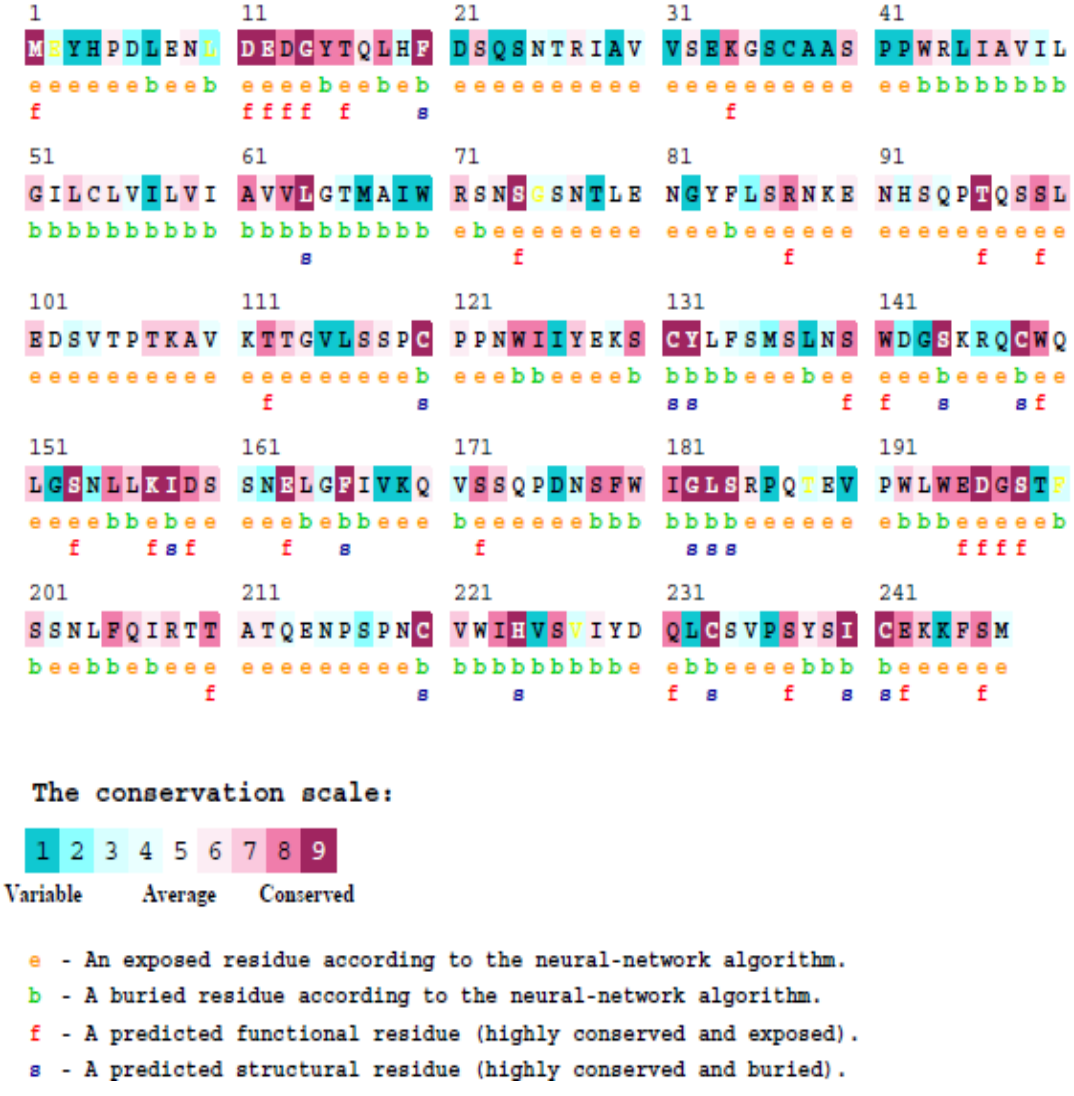


Figure-5.3: ConSurf results of dectin-1 receptor protein

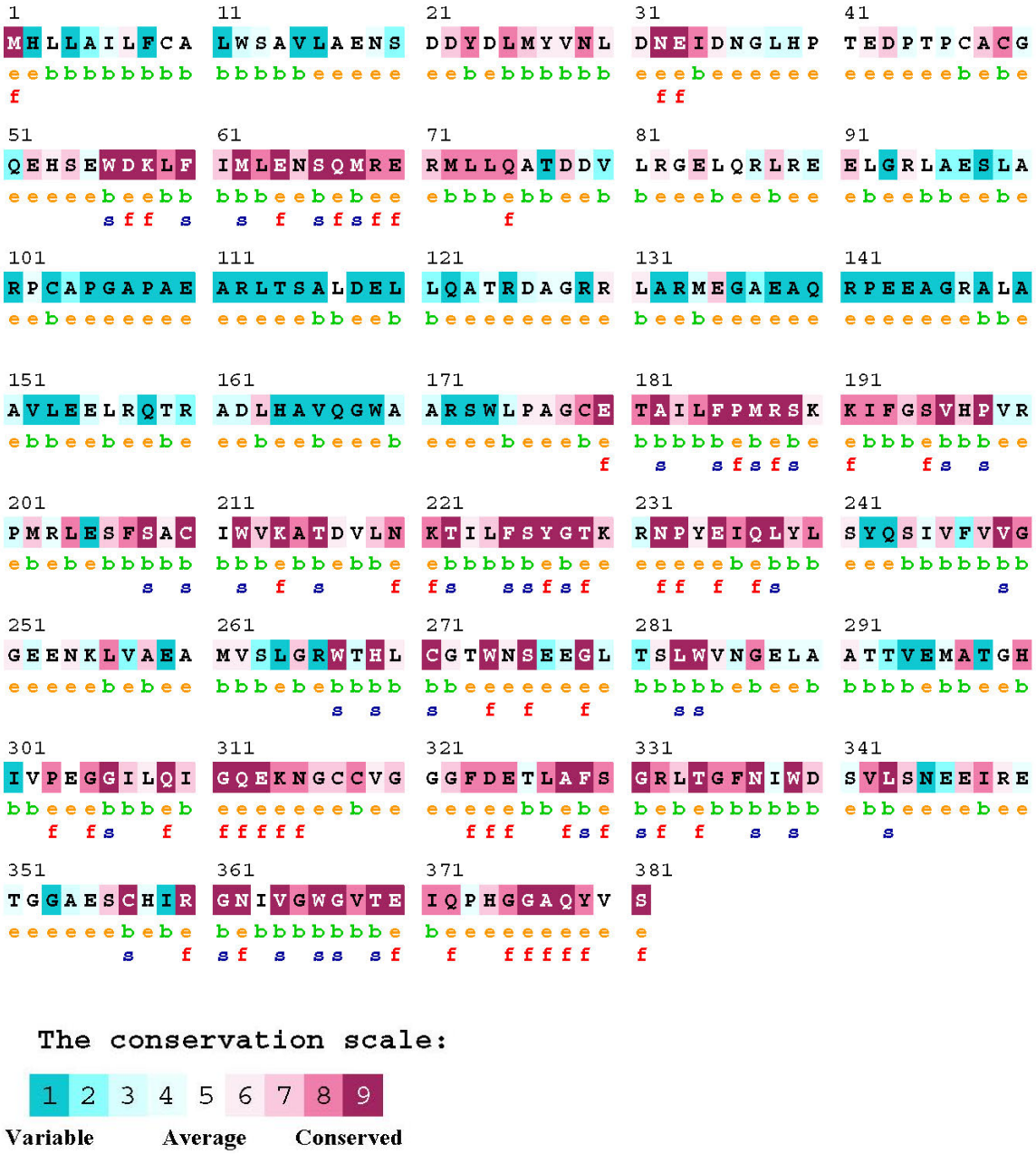


Figure-5.4: ConSurf results of pentraxin-3 receptor protein

Table-5.3: Deleterious non-synonymous-SNPs of dectin-1 used for Structural analysis

nsSNPs ID	Mutation position	ConSurf Score	Deleterious Predictions
rs369482852	W141R	8	5
rs746386372	C148G	9	5
rs747442135	L155V	8	5
rs138005591	I158T	9	5
rs758623997	D159G	8	5
rs140318683	L183F	9	5
rs16910527	I223S	8	4

Table: 5.4: Deleterious non-synonymous-SNPs of pentraxin-3 used for Structural analysis

nsSNP ID	Mutation Position	ConSurf Score	Deleterious prediction
rs4478039	E313K	9	6
rs1536891	R188C	9	6
rs76994524	N337S	9	5
rs138818541	R360W	9	6

5.3.4 Homology modeling of C-type lectin (CTLD) and pentraxin domains of dectin-1 and PTX-3 proteins and their structure stability

Homology modeling of c-type lectin and pentraxin domains of dectin-1 and pentraxin-3 proteins were carried out by using phyre2 web based server. Phyre2 tool aligned submitted sequences with proteins having known three dimensions structure by using Hidden Markov method. Then, these alignments are used by this tool to predict homology based three dimension models of query sequences. Ab-initio folding simulation also known as poing was used by Phyre2 to model a region of a query sequence. The PDB template c2bpdB (Murine Dectin-1 protein) and 1b09 (human C-reactive protein) were used by Phyre2 tool for the building of 3-dimensional structure of CTLD and pentraxin domains of the dectin-1 and pentraxin-3 proteins. Further, Ramachandran plot analysis showed that the most favored region represented by 91.7 % amino acid residues, while 6.4 % residues were present in allowed region followed by 1.8 % residues in liberally

allowed region and disallowed region represent 0% amino acids for CTLD Where as in case of pentraxin domain, 98% amino acids were present in most favored region, followed by 1.5 % amino acids in allowed area and outlier part of the Ramachandran plot contained 0.5 % amino acids. Further, selected amino acid coincides in high-risk nsSNPs of C-type lectin and Pentraxin domains were used to generate variant or mutant 3D structures by substituting corresponding amino acid residues using Swiss PDB viewer (Figures-5.4 & 5.6).

Figure-5.5: **Wild type and Mutated structures of CTLD-domain of dectin-1 protein**

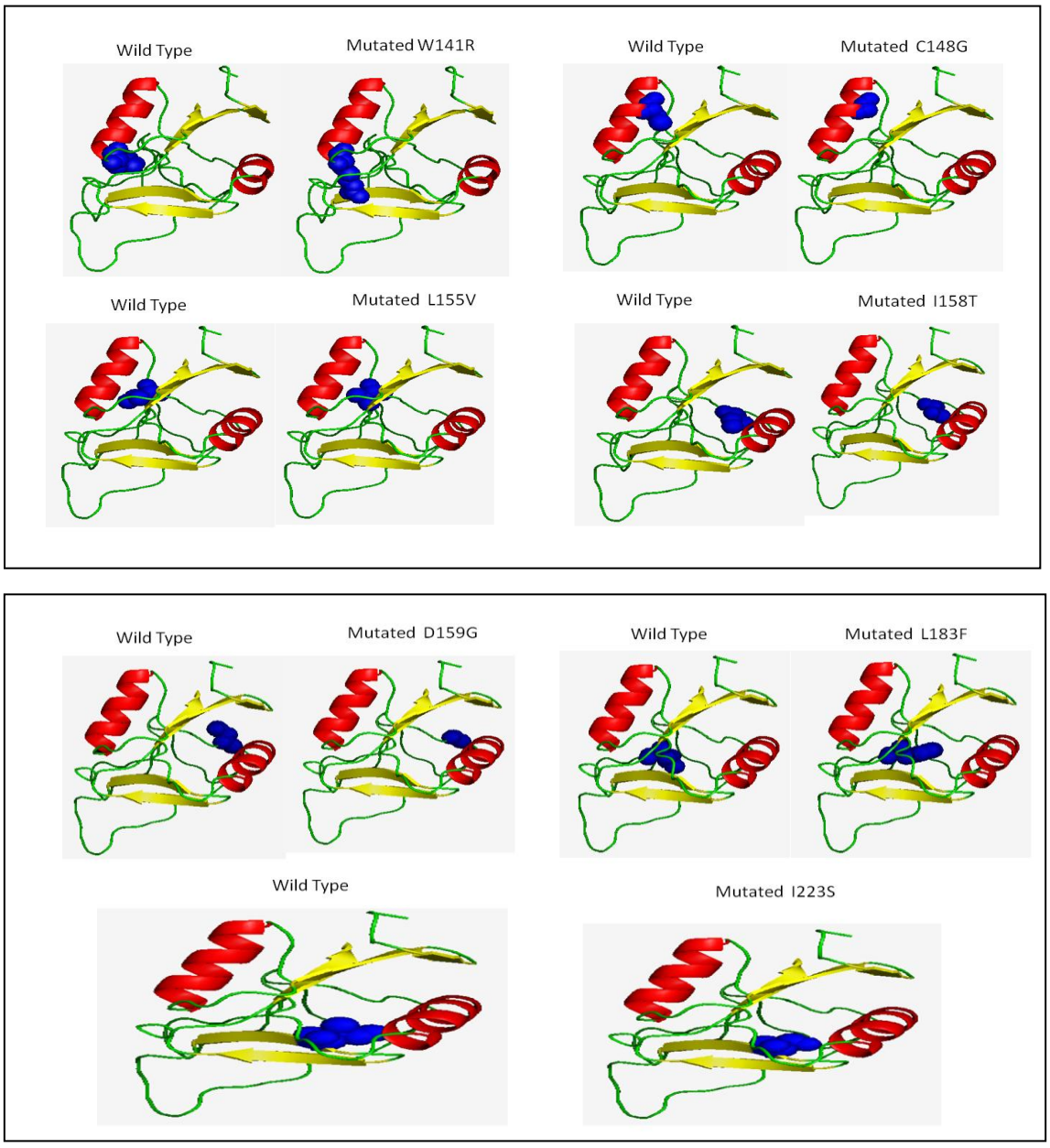


Figure-5.5: These figures show the 3D modeled structure of native C-type lectin domain of dectin-1 receptor generated by Phyre-2 tool and mutated structure models of high-risk nsSNPs

having corresponding amino acid residues. The sites of every wild-type and mutated amino acids in CTLD represented with blue color.

Further, protein structure stability was checked using I mutant 2.0 server. This server is based on a neural network that predicts the change in protein stability. It measures the free energy change scores using FOLD-X web based tool. High risk nsSNPs in C-type lectin and pentraxin domains were predicted to be provide less stability to protein structures in comparison to wild type protein structure and free energy change ranged from -1.02 to -2.37 (Table-5.5 & 5.6). Furthermore, RMSD and TM score was also calculated to see the effect of deleterious SNPs on CTLD of dectin-1 and pentraxin domain of PTX-3 proteins (Table- 5.7&5.8).

Figure-5.6: **Wild type and Mutated structures of pentraxin domain of PTX-3 protein**

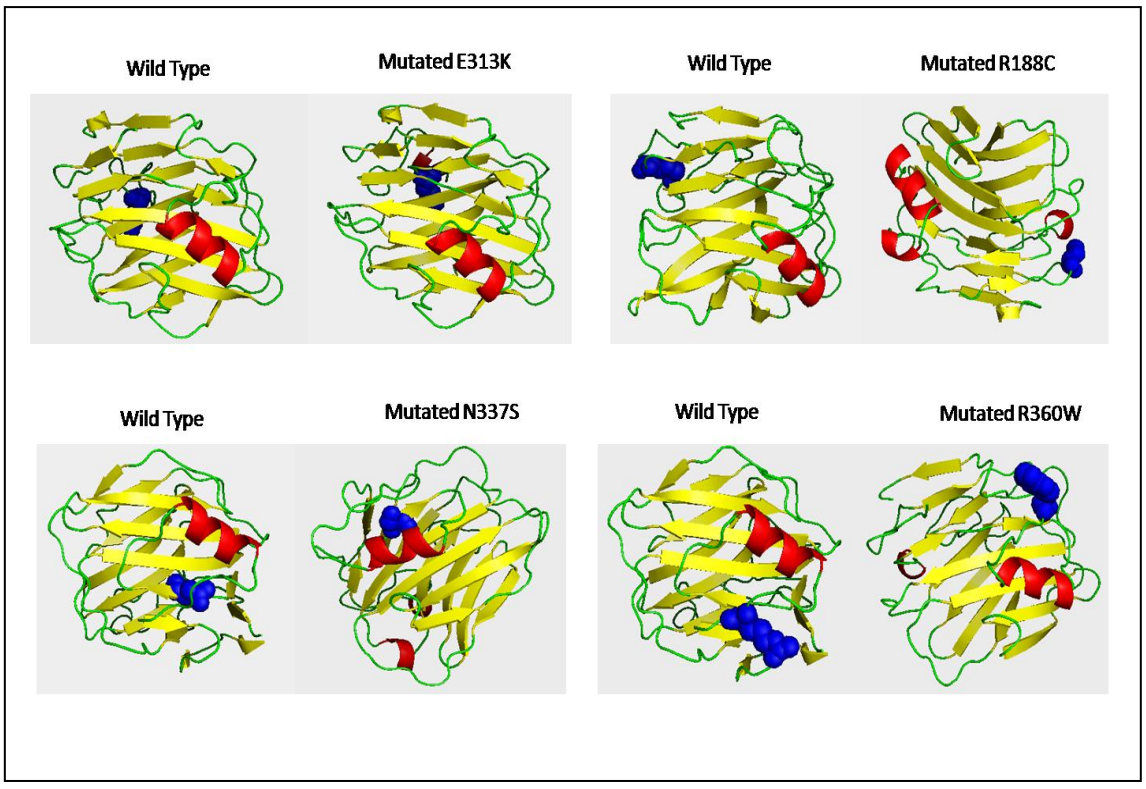


Figure-5.6: This figure shows the 3D modeled structure of native pentraxin domain of PTX-3 receptor generated by Phyre-2 tool and mutated structure models of high-risk nsSNPs having corresponding amino acid residues. The sites of every wild-type and mutated amino acids in pentraxin domain represented with blue color.

5.3.5 Protein-Protein Interactions of dectin-1 and pentraxin-3 protein receptors

Protein- protein interaction network of dectin-1 and pentraxin-3 protein receptors were predicted by using STRING database. In interactive mode STRING predicted interaction of dectin-1 with toll like receptor 2 (TLR2), CD37 (leukocyte-specific tetraspanin), SyK-spleen tyrosine kinase that mediate signal transduction and regulates innate and adaptive immune response, KTN1 (A receptor for Kinesin), TMPRSS4 (transmembrane protease, serine 4), ZNF444 (Zinc finger protein 444), BNIP3 (BCL3/ adenovirus E1B 19kDa interacting protein). Whereas PTX-3 protein showed interactions with complement components (C1qA, C1qB, complement factor H), fibroblast factor 2 (FGF2) and potassium voltage-gated channels. Other interacting proteins with dectin-1 and PTX-3 were showed in appendix-I-Figure-5.1 & 5.2.

Table 5.5: Free energy change prediction using I-mutant tool for deleterious nsSNPs in dectin-1

nsSNP ID	Amino acid change	Free energy change(DDG)	Sign of change in energy	Reliability index
rs562749381	Y3D	-1.08	dec	6
rs780024327	E12D	-0.72	dec	4
rs756166982	D13Y	0.07	inc	5
rs759032825	S22F	0.27	inc	4
rs775715931	C54R	-0.21	dec	2
rs781427660	L64P	-1.52	dec	7
rs771094993	G65D	-1.04	dec	7
rs112345533	S117F	0.00	inc	2
rs753757159	L133P	-1.42	dec	4
rs760554156	F134Y	-0.90	inc	2
rs761503556	W141R	-1.02	dec	8
rs369482852	W141S	-1.56	dec	9
rs746386372	C148G	-1.02	dec	7
rs747442135	L155V	-1.31	dec	6
rs138005591	I158T	-2.15	dec	7
rs758623997	D159G	-1.50	dec	7
rs70583536	F179L	-0.90	dec	5
rs140318683	L183F	-1.03	dec	5
rs548792483	R185Q	-1.00	dec	9
rs371994700	W222R	-1.05	dec	8
rs16910527	I223S	-2.37	dec	9
rs141153031	C233Y	-0.43	dec	3

5.3.6 Docking analysis of CTLD domain of dectin-1 with β-glucan

CTLD of dectin-1 receptor recognizes β-glucan a carbohydrate moiety of filamentous fungi cell wall, and thus initiates a intracellular signaling that active inborn and acquired

immunity response to clear fungal pathogens. The substitution of deleterious amino acid in (CTLD may affect the binding capability of CTLD with β-glucan. This binding affect may lead to altered immune response and thus fungal pathogen can escape mediation via dectin-1 receptor present on innate immune cells. Hence we analyzed the binding ability of C-type lectin domain with wild type and mutant modeled structures using AutoDock 4. We have studied the interaction of CTLD-domain with β-glucan on the base of free binding energy, intermolecular energy, RMSD and inhibition constant. The non-covalent interaction such as vander waal forces, hydrogen bonding and electrostatic forces are involved in protein and ligand interaction. Thus, we also analyzed the interaction of β-glucan with CTLD by using LIGPLOT (Appendix-I-Figure-5.3). Wild type C-type lectin domain showed -11.03 Kcal\mol binding energy. Lowest binding energy correlates with strong binding of ligand with their receptor. Among all mutated structure, L183F showed lowest binding energy (-12.04 Kcal\mol) where as variants I223S and L155V showed high-binding energy (-10.83 and -10.58 Kcal\mol respectively).

Table 5.6: Free energy change prediction using I-mutant tool for deleterious nsSNPs in pentraxin-3

nsSNP ID	Mutation position	DDG	Sign of DDG	RI
rs34655398	H39Q	-1.97	Dec	8
rs143387231	P40S	-1.41	Dec	9
rs148384694	T41I	-0.56	Dec	7
rs3816527	A48D	0.50	Dec	1
rs367899909	V80A	-1.68	Dec	6
rs557539937	A148T	-1.32	Dec	8
rs572907291	A151T	-1.12	Dec	8
rs112277608	T159M	0.43	Dec	0
rs370211025	L184S	-2.21	Dec	9
rs564774580	R188C	-1.15	Dec	4
rs529759691	K190E	0.64	Dec	3
rs529759691	F193S	-2.58	Dec	8
rs532972316	E235K	-0.87	Dec	6
rS190837481	H269Y	0.51	Dec	1
rs56265729	V246M	-1.03	Dec	8
rs144979346	G306E	0.14	Inc	2
rs4478039	E313K	-0.15	Dec	2
rs76994524	N337S	-0.81	Dec	8
rs146705881	L343I	-0.93	Dec	9
rs140073706	S344R	-0.99	Dec	2
rs373203093	I348V	-1.48	Dec	8
rs138818541	R360W	-0.34	Dec	5

Table 5.7: RMSD value and TM-score of high risk nsSNPs in C-type lectin domain of dectin-1 protein receptor calculated using TM-align

nsSNP ID	Mutation position	Tm-Score	RMSD (Å)
rs369482852	W141R	0.85	2.64
rs746386372	C148G	0.74	2.67
rs747442135	L155V	0.83	2.50
rs138005591	I158T	0.68	2.84
rs758623997	D159G	0.70	2.80
rs140318683	L183F	0.80	2.60
rs16910527	I223S	0.50	2.90

Table 5.8: RMSD value and TM-score of high risk nsSNPs in pentraxin domain of PTX-3 protein receptor calculated using TM-align

nsSNP ID	Mutation position	TM-Score	RMSD(Å)
rs4478039	E313K	0.82	2.62
rs1536891	R188C	0.84	2.19
rs76994524	N337S	0.84	2.13
rs138818541	R360W	0.84	2.17

5.4 Discussion

In the personalized medicine era affordable and accessible next generation sequencing technology not only help to sequence human genome but also provide insight into human genome such as single nucleotide polymorphism. The central objective of the personalized medicine, population genetics and molecular biology is to characterize nsSNPs in genes associated with various irregular biological functions. Further, The United States has initiated the "Precision medicine initiative" to sequence 1 million individuals' genome and United Kingdom also proposed to sequence 100,000 human genomes [137]. Thus, the data available from these projects would be revealed individual

persons variability in terms of Single Nucleotide Polymorphism that has to be correlated to disease susceptibility and drug or vaccine responses**.** Furthermore, the understanding of nsSNPs will not only provide the insight into disease susceptibility but also highlight the drug targets and biomarkers. The presence of nsSNPs in exonic regions of genes may lead to alter their protein structure and functions. Further, alteration in proteins structure and function can account an individual susceptible to disease.

Dectin-1 and pentraxin-3 proteins are important receptors of innate immunity. Dectin-1 recognizes not only fungal pathogens via cell wall carbohydrate moiety, β-glucan, but also bacterial pathogens such as *Mycobacterium tuberculosis* and non-typeable *Haemophilus influenzae* by unknown mechanisms [151, 152, 267]. Whereas pentraxin-3 is a soluble receptor that recognizes various moieties of fungal pathogens such as galactomannan of conidia of *Aspergilli* and help to clear them by opsonization to various effector cells of immune system. After recognition of fungal pathogens, they initiate effector immune functions by activating various cytokines and chemokines [126]. Various experimental studies have been conducted to determine the relationship of nsSNPs to disease susceptibility [247, 248, 268]. Further, the increases in SNPs in database submitted by different laboratories make it impossible to study their biological significance at laboratory scale. Thus various *in-silico* algorithms or tools have been developed to analyze highly deleterious nsSNPs among various SNPs present in databases.

We utilized five *in-silico* algorithms to predict deleterious and neutral nsSNPs in dectin-1 and pentraxin-3 protein receptors, specifically SIFT, Polyphen2, PhD-SNP, SNAP and MAPP. SIFT predicted 42(46%) & 25(38%) deleterious and 49 (54%) & 40(62%) neutral nsSNP, PhD-SNP identified 31(34%) & 18(28%) deleterious and 60 (66%)& 47(72%) neutral nsSNPs, PolyPhen predicted 36(40%) & 27(41%) deleterious and 55(60%) & 38 (59%) neutral nsSNPs, where as SNAP and MAPP determined 20(22%), 54(59%) & 29(45%), 37(57%) deleterious and 71(78%), 37(41%) & 36(55%), 28(43%) neutral nsSNPs for dectin-1 and pentraxin-3 respectively. The difference in prediction by these tools might be the algorithms or features used by these tools. Previously various studies used these *in-silico* tools for prediction of deleterious and neutral nsSNPs. By comparing all five in-silico algorithms and conserve domain analysis, 7 nsSNPs (W141R, C148G, L155V, I158T, D159G, L183F, and I223S) were observed as high-risk or significant deleterious nsSNPs that were located in CTLD-domain and 4

nsSNPs (E313K, R188C, N337S and R360W) were there in pentraxin domain. Further, structure and functional analysis of high-risk nsSNPs were done. The murine dectin-1 protein c2bpdB (% identity: 61) and 1b09 (human C-reactive protein) were used as templates by Phyre2 software to construct 3D structure of C-type lectin and pentraxin domains. Previously Yadav *et al* also used murine dectin-1 protein structure to modeled 3D protein structure of *Bulbalus bulbalis* with 58.91 % sequence similarity [269]. Furthermore, selected amino acids coincide in high-risk nsSNPs of C-type lectin and pentraxin domains were used to generate variants or mutant 3D structures by substituting corresponding amino acid residues using Swiss PDB viewer (Figure-5.5&5.6). All the high-risk nsSNPs were found to be altered the 3-D structures of CTLD and pentraxin domains of dectin-1 and PTX-3. Further, to elaborate results, the RMSD value and Template modeling-score were calculated for each high-risk nsSNP by using Tm-Align software. RMSD is used to measure the typical space among α-carbon backbone of native and mutated models of any protein structures, whereas, the topological resemblance between wild and mutant models of protein structures is determined by TM-score (Table-5.7&5.8). If RMSD values become high, it denotes the change in mutant structure in comparison to native protein structure. The highest RMSD values were determined in E313K (2.62), R188C (2.19) and R360W (2.17) high-risk nsSNPs of pentraxin domain. Whereas, I223S substitution in CTLD-domain showed TM-score (0.50) and RMSD value of (2.90). Thus, the predicted results determined that these nsSNPs significantly modify the structure of CTLD and pentraxin domains of dectin-1 and PTX-3 receptors. Along with these, I-mutant analysis showed that, there is change in protein stability or protein stability decreased after substituting amino acid in wild type dectin-1 receptor protein. The high change in energy or stability of protein (-2.37) was observed in high-risk nsSNP (rs16910527) by substitution of isoleucine by serine at 223 amino acid position followed by I158T (-2.15) and D159G (-1.50) in dectin-1 protein receptor. Thus, the overall analysis showed that the predicted foremost nsSNPs can affect the protein structure stability and function of this receptor. Previously wolf *et al* showed that the two SNPs in coding region of dectin-1protein receptor lead to loss of phagocytic function. They demonstrate that the amino acid 223 plays an essential structural role in dectin-1 protein receptor and substitution of this amino acid may affect receptor's function [270]. Further, various studies showed that the presence of Y238X polymorphism in dectin-1 protein receptor impaired its function and has been associated with invasive infection of

aspergillosis in high-risk patients such as those undergo bone marrow transplant or having impaired immunity [268, 271, 272]. *In-vitro* and *in-vivo* studies on Y238X polymorphism suggested that it impaired production of cytokines such as interferon-γ and interleukine-10. Thus, modulate the immune response of susceptible individuals to fungal pathogens [248]. Further, dectin-1 interaction with other proteins was annotated by using STRING database. This database predicts the binding interaction of dectin-1 receptor with TLR-2, CD37 and other proteins. Ferwerda *et al* showed that TLR-2 and dectin-1 receptors work synergistically in response to fungal infection and initiate cross talk signaling and cytokines productions [267]. Recently, Fischer *et al* showed that the polymorphisms associated with dectin-1 and TLR-2 receptors are the predisposing factors for invasive fungal infections in acute myeloid leukemia patients [272]. Further, it has been reported that the dectin-1 acts together with CD37 leukocyte specific tetraspanin that co-localized with this receptor on antigen presenting cells. It has been suggested that their interaction is important for dectin-1 stabilization in antigen presenting cell membrane and cytokine production [273]. Thus, the high-risk nsSNPs present in dectin-1 receptor or in C-type lectin domain may affect the interaction between TLR-2, CD37 and other proteins and may abolish the downstream signaling that could affect cytokine production and immune response against fungal pathogens. Furthermore, I-mutant analysis of nsSNPs in pentraxin domain suggested that damaging non synonymous-SNPs which substitutes amino acid N337S and R360W showed less stability as compared to native protein having Gibbs free energy changes of -0.81 and -0.34. The clinical studies on PTX-3 gene polymorphism showed that the SNP (rs3816527) which substitute A48D change in pentraxin-3 protein is associated with invasive aspergillosis in individuals that go through hematopoietic stem cell transplantation [248]. Further, interaction network of pentraxin-3 protein showed that it interacts with various components or factors that involve in innate immunity [274]. In interaction network, it has been predicted that the pentraxin protein interacts with complement component 1qA, complement component1qC and complement component factor H. These components of complement pathway help to initiate classical complement system that eradicates microbial pathogens [274]. Further, it has been elucidated that these components of complement system interact through pentraxin domain with PTX-3 protein receptor [254]. If any of the high-risk nsSNPs will encode in pentraxin domain it may alter its structure and function. Due to alteration in structure, the interaction of complement components may alter. Therefore, the individuals those harboring these

nsSNPs may be vulnerable to diverse group of pathogens ranging from bacteria (*Pseudomonas spp*. and *Streptococcus spp*.) to fungi and including viruses such as *influenza virus* [157,158,248,275]. These nsSNPs may also compromise the function of PTX-3 as PRR and thus amend the function of phagocytic cells.

Dectin-1 protein receptor recognizes fungal pathogens through β-glucan, a carbohydrate moiety exposed to extracellular environment during cell wall modification [41,63,276,277]. Thus docking study has been performed to analyze the effect mutations on binding capacity of CTLD-domain of dectin-1 receptor. The high-risk mutations (W141R, C148G, L155V, I158T, D159G, and I223S) observed to be unfavorable for β-glucan binding with CTLD-domain of receptor. The lowest binding energies (-11.03 Kcal\mol and -12.04 Kcal\mol) were observed in native or wild type or a mutant (L183F) CTLD-domain of dectin-1 receptor. The lowest binding energies of these complexes suggested their good interaction and compatibility with β-glucan. The higher binding energies were observed in I223S and L155V variants (-10.83 Kcal\mol and -10.58 Kcal\mol) respectively, which indicates their defective binding with C-type lectin domain of dectin-1 receptor. Previously, it has been explored that the nsSNPs influence the binding of ligand molecules with their receptors [137, 162]. Further, to gain insight into ligplot analysis was done, which suggested that the change in hydrogen bond distance with interacting amino acid residues and ligand molecules, could affect the thermodynamic stability of ligand-receptor complex. Thus it may be feasible that these major nsSNPs may impair the functions of dectin-1 and pentraxin-3 receptors as pathogen recognition receptor and may further affect their phagocytosis and effector function of phagocytic cells.

Summary and Future Prospects

Current work has provided the global proteome profile of germinating conidia of *A. terreus,* as well as of lung epithelial cells (A549) interacting with *A. terreus* conidia. Further, deleterious effect of nsSNPs on innate immune receptors those recognize galactomannan, β-glucan was assessed. The results from germination of *A. terreus* conidia suggested that the morphogenesis from conidia to germ tube required proteins synthesis, carbohydrate metabolism, transport and respiratory functions. Further, various proteins involve in biosynthesis pathways of secondary metabolites (Terretonin & Geodin) were also observed at germinating time. Furthermore, the high-expression of terrelysin gene which encodes proteins specific to *A. terreus* was observed and could be investigated as a biomarker for *A. terreus* infections. A549 cells respond to *A. terreus* conidia by activating various cellular pathways upon interaction. The proteins involved in internalization of conidia, cytoskeleton rearrangement, signaling pathways and those function in immunity were observed. Most significantly proteins or factors that activate NF-kβ signaling pathway were observed. This pathway initiates the production of IL-8. Thus, IL-8 could be targeted to control inflammation and to avoid organ damages in infected hosts. Further, during interaction study, the homologous of C-type lectin domain protein was identified. So, dectin-1 receptor (containing lectin-domain) present on epithelial cells, which acts as PRR for *Aspergilli* cell wall component β-glucan. Therefore, if, non-synonymous SNPs are present in CTLD-domain, they can affect binding efficiency with PAMPs. In current work, 7 high-risk nsSNPs were identified which substitute different amino acid in CTLD-domain. Among them, I223S & L155V considered as high-risk due to their effects on binding efficacy with β-glucan cell wall moiety of cell wall of *Aspergilli*. In future prospects, the interaction studies on the functions of secondary metabolites of *Aspergilli* interacting with respiratory epithelium will help to understand dynamic interplay between host and pathogens. An improved understanding of these interactions could lead to more effective treatment outcomes and new diagnostic markers. Further, more studies on affect of nsSNPs on innate immune receptors will help to predict the deleterious effect on structures and subsequently to the functions of dectin-1(C-type lectin) and pentraxin-3 receptors. Due to presence of high risk nsSNPs, it could be possible that the domains of these proteins may not be interacting properly and humans with these nsSNPs in their genomes could be susceptible to

infections for selected pathogens. For future prospective, to establish new early diagnostic markers against invasive aspergillosis, it is necessary to carry out the *in-vitro* studies using mice model of invasive aspergillosis and clinical studies (patients having invasive aspergillosis) for detection of secondary metabolites or study of their kinetics in infected patient sera. Furthermore, future population based studies on high risk nsSNPs will pave way for the early biomarker or individuals susceptibility to fungal infections.

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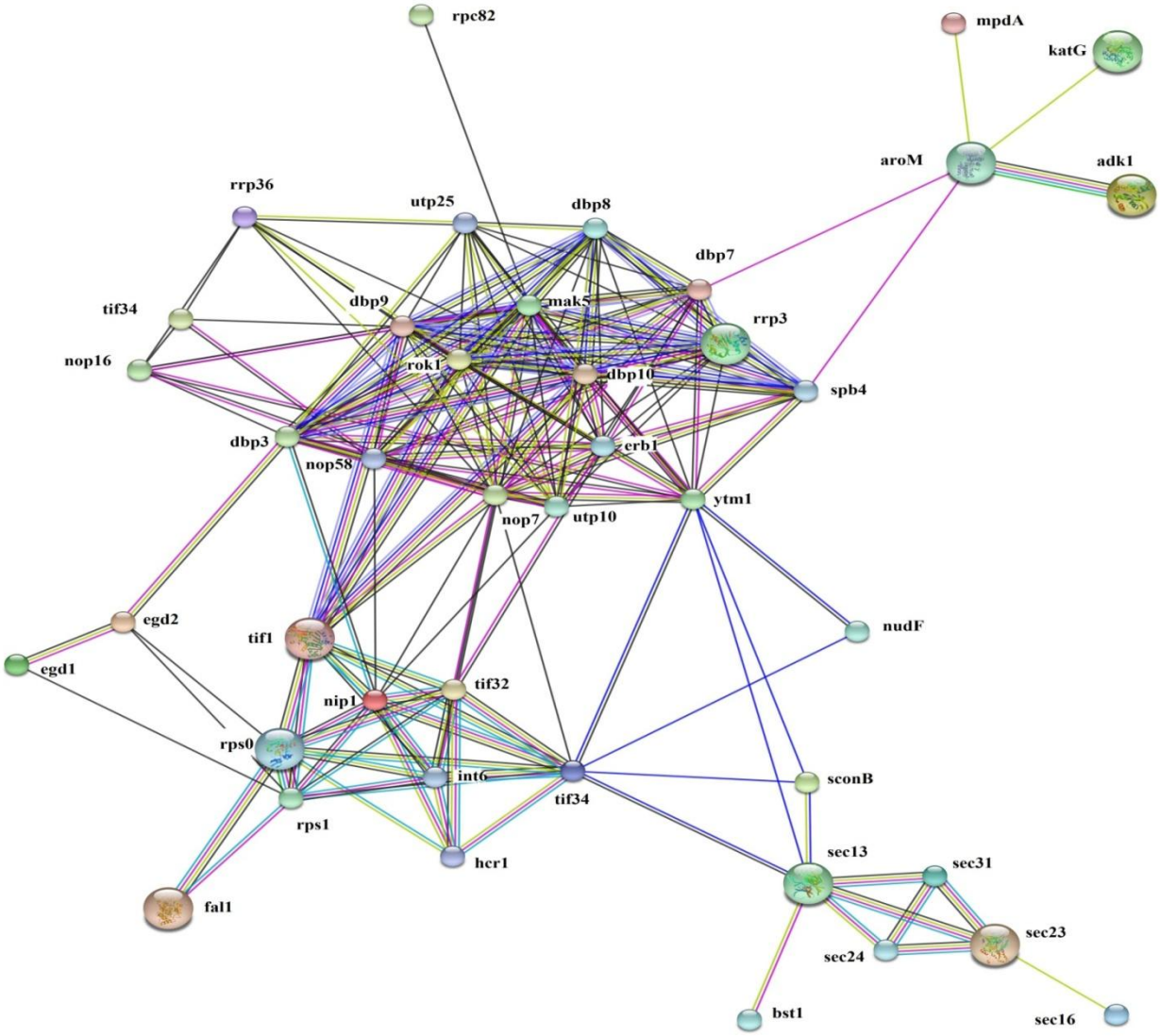
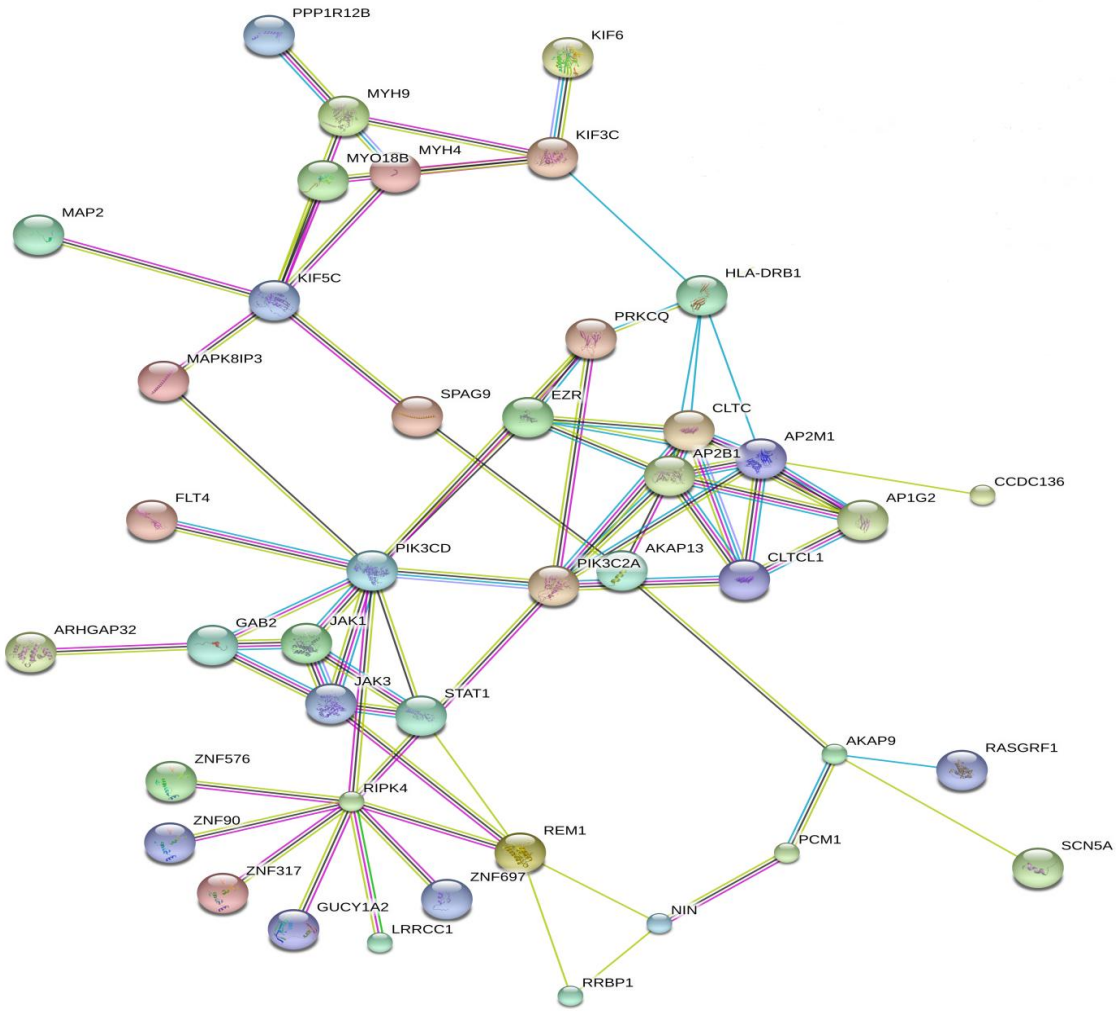
APPENDIX-I

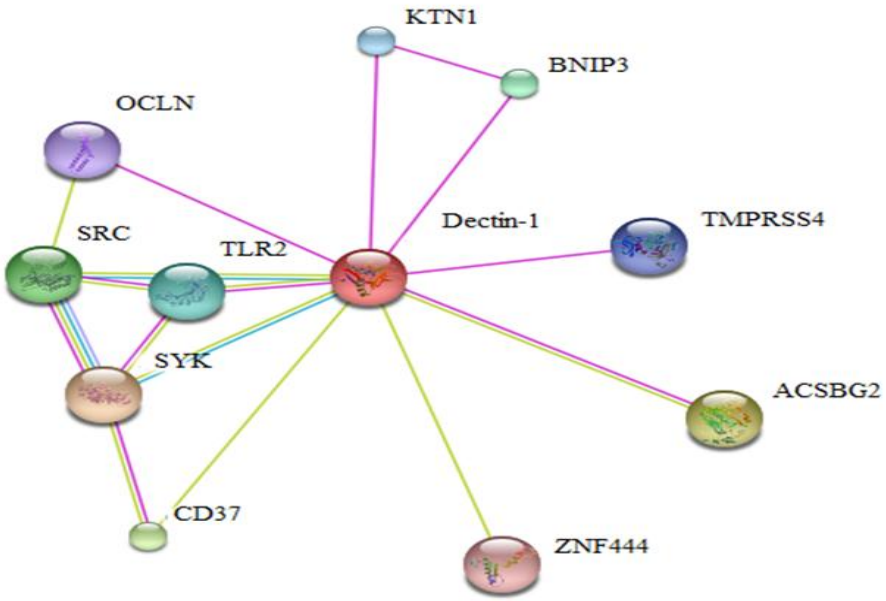
Appendix-1- Figure-3.1: Protein-protein interaction network of expressed proteins from germinating conidia: Most of these interacting proteins were associated with cell wall organization/ remolding, protein biosynthesis and transport, cell cycle, RNA processing and carbohydrate metabolism. The catalogue of interacting proteins is given in Appendix-II-Table-3.5



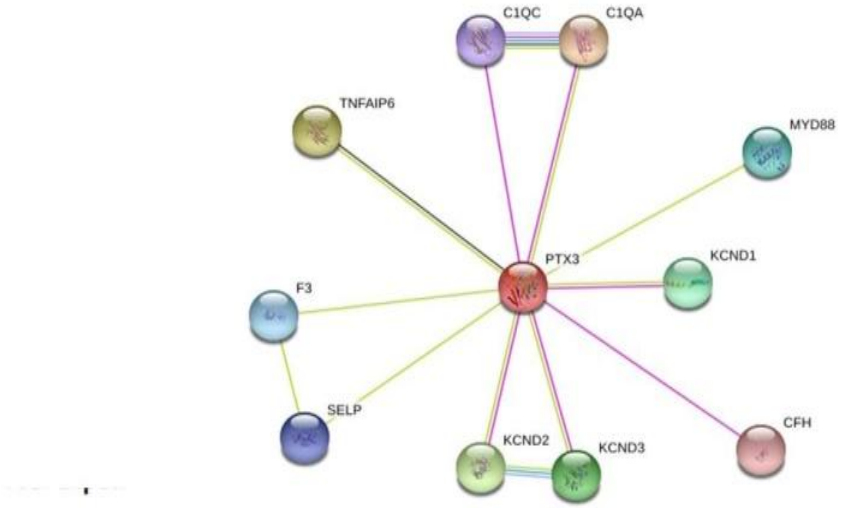
Appendix-I- Figure-4.1: Protein-protein interaction network of identified proteins involve in signaling, transcription, immune response and transport in A549 cells infected with *A. terreus*. The maximum interacting partners were observed for proteins tyrosine-protein kinase (Jak1), RIPK4, STAT1, KIF5C, PIK3C2A, MHC class II antigen (HLA-DRB1) and PIK3CD.



Appendix-I-Figure-5.1: Protein-protein interaction network of dectin-1 protein. STRING predicted interaction of dectin-1 with toll like receptor 2 (TLR2), CD37 (leukocyte-specific tetraspanin), SyK- spleen tyrosine kinase that mediate signal transduction and regulates innate and adaptive immune response, KTN1 (A receptor for Kinesin), TMPRSS4 (transmembrane protease, serine 4), ZNF444 (Zinc finger protein 444), BNIP3 (BCL3/ adenovirus E1B 19kDa interacting protein)



Appendix-I-Figure-5.2: Protein-protein interaction network of pentraxin-3 protein. PTX-3 protein showed interactions with complement components (C1qA, C1qB, complement factor H), fibroblast factor 2 (FGF2) and potassium voltage-gated channels

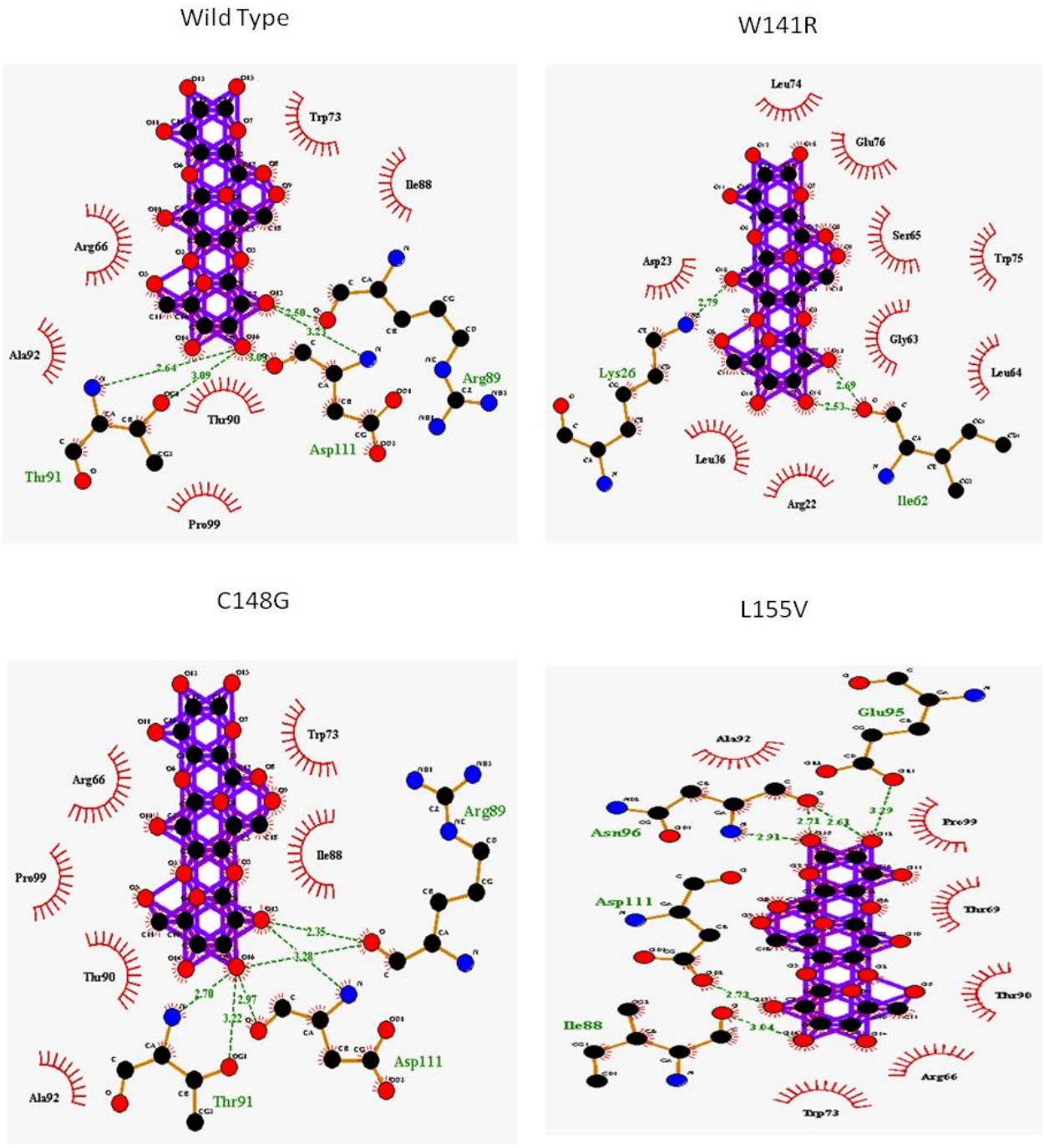


PTX3 pentraxin 3, long; Plays a role in the regulation of innate resistance to pathogens, inflammatory reactions, possibly clearance of self- components and female fertility (By similarity) (381 aa) *(Homo sapiens)*

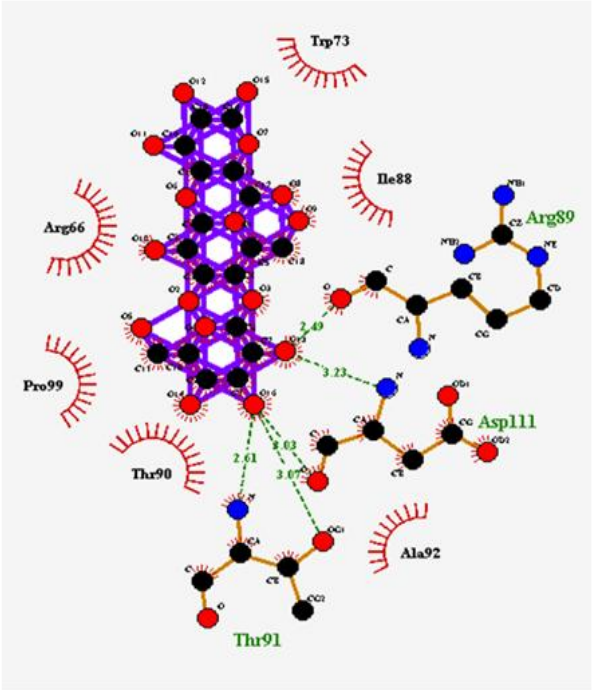
Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	
C1QA	complement component 1, q subcomponent, A chain; C1q associates with the proenzymes C1r and C1s [...] (245 aa)				•	•		0.912
TNFAIP6	tumor necrosis factor, alpha-induced protein 6; Possibly involved in cell-cell and cell-matrix [...] (277 aa)					•		0.865
KCND2	potassium voltage-gated channel, Shal-related subfamily, member 2; Pore-forming (alpha) subunit [...] (630 aa)				•	•		0.837
KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3; Pore-forming (alpha) subunit [...] (655 aa)				•	•		0.837
KCND1	potassium voltage-gated channel, Shal-related subfamily, member 1; Pore-forming (alpha) subunit [...] (647 aa)				•	•		0.837
MYD88	myeloid differentiation primary response 88; Adapter protein involved in the Toll-like receptor [...] (317 aa)					•		0.835
F3	coagulation factor III (thromboplastin, tissue factor); Initiates blood coagulation by forming [...] (295 aa)					•		0.818
SELP	selectin P (granule membrane protein 140kDa, antigen CD62); Ca(2+)-dependent receptor for myelo [...] (830 aa)					•		0.792
C1QC	complement component 1, q subcomponent, C chain; C1q associates with the proenzymes C1r and C1s [...] (245 aa)				•			0.576
CFH	complement factor H (1231 aa)				•			0.576

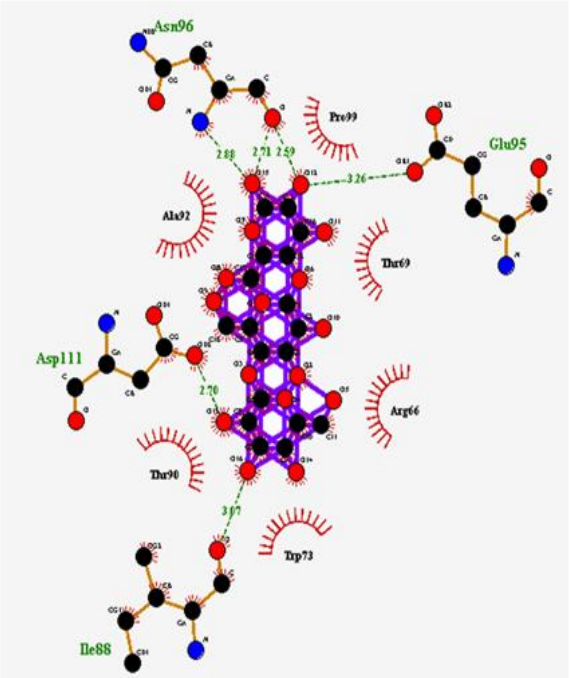
Appendix-I-Figure-5.2: LIGPLOT analysis of docked C-type lectin domain (Wild type and Mutated) with β- glucan, a carbohydrate moiety of fungal Pathogen: This figure shows the corresponding change in the hydrogen bond length between the amino acid residues of c-type lectin domain of wild type or mutated structure. The change in the length of H-bond affects the binding between receptor and ligand molecule



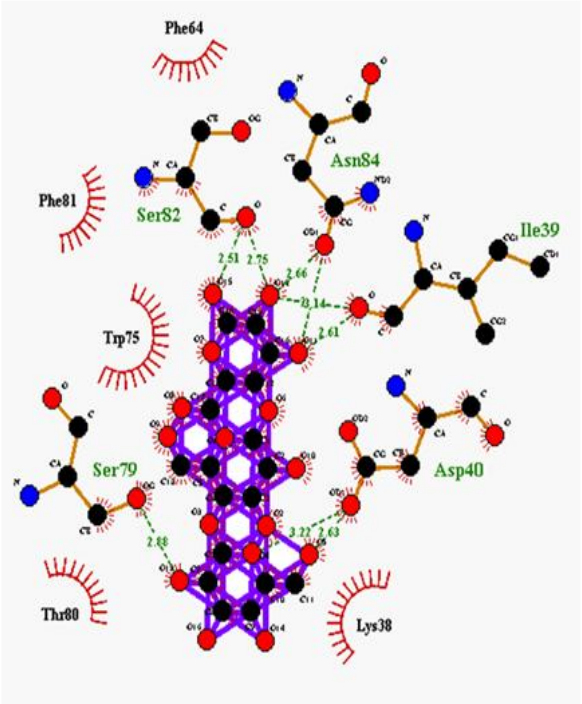
I158T



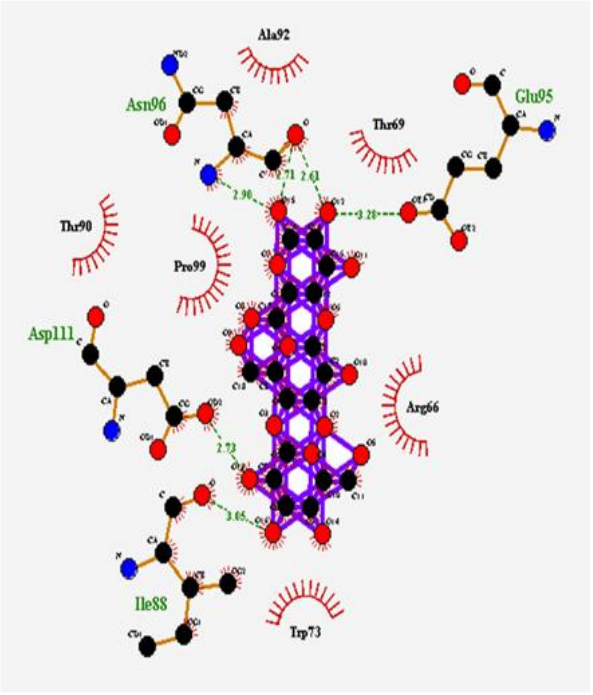
D159G



L183F



I223S



APPENDIX-II

Appendix-II-Table-3.1: Identified protein of *Aspergillus terreus* in germinating conidia and their general function annotated from UniProt data base.

S.No	UniProt ID	Description	Function	PLGS score
1	Q0CPF9	Eukaryotic translation initiation factor 3 subunit G (Etif)	protein biosynthesis	505.15
2	Q0CN46	Etif3-B	protein biosynthesis	492.65
3	Q0CZL5	Mitochondrial distribution and morphology protein 12	protein biosynthesis	371.46
4	Q0CXX1	54S ribosomal protein L4	protein biosynthesis	361.84
5	Q0CXD0	ATP-dependent RNA helicase eIF4A	protein biosynthesis	326.01
6	Q0CNR3	Etif3	protein biosynthesis	308.73
7	Q0CTT3	Etif3-J	protein biosynthesis	300.29
8	Q0CPA8	Etif3-L	protein biosynthesis	297.42
9	Q0CUP6	Etif3-A	protein biosynthesis	272.06
10	Q0CVT0	Etif3-C	protein biosynthesis	226.67
11	Q0D1J4	Etif3-H	protein biosynthesis	224.58
12	Q0CXH9	Etif3-I	protein biosynthesis	185.53
13	Q0CLJ6	ATP-dependent RNA helicase ded1	protein biosynthesis	176.87
14	Q0CLP3	Elongation factor G	protein biosynthesis	166.57
15	Q0CLP3	Elongation factor G	protein biosynthesis	166.57
16	Q0CCM5	Etif3-F	protein biosynthesis	140.14
17	Q0CPV5	Etif3-M	protein biosynthesis	99.32
18	Q0CCM5	Etif3-F	protein biosynthesis	140.14
19	Q0CPV5	Etif3	protein biosynthesis	99.32
20	Q0D0F3	Pentafunctional AROM polypeptide	amino acid biosynthesis	399.27
21	P0C2P2	Ornithine carbamoyltransferase	amino acid biosynthesis	361.00
22	Q0CFY3	Methylthioribose-1-phosphate isomerase	amino acid biosynthesis	224.11
23	Q0CT19	Methylthioribulose-1-phosphate dehydratase	amino acid biosynthesis	213.77
24	Q0CY06	Amino-acid acetyltransferase	amino acid biosynthesis	207.33
25	Q0CDB9	ArgJ	amino acid biosynthesis	161.34
26	Q0CRI5	Kynurenine 3-monooxygenase	amino acid metabolism	263.13

27	Q0CL94	Methionine aminopeptidase	amino acid metabolism	253.31
28	Q0CFZ0	Probable Xaa-Pro aminopeptidase pepP	amino acid metabolism	212.88
29	P56862	Sulfate adenylyltransferase	amino acid metabolism	193.82
30	Q0CGL5	Nascent polypeptide-associated complex subunit beta	Protein transport	2380.82
31	Q0CJY1	Vacuolar fusion protein mon1	Protein transport	370.87
32	Q0CSR3	Conserved oligomeric Golgi complex subunit 6	Protein transport	353.60
33	Q0CYG9	Sec31	Protein transport	271.3
34	Q0CJV3	Vacuolar protein sorting-associated protein 27	Protein transport	266.23
35	Q0CJU8	Class E vacuolar protein-sorting machinery protein hse1	Protein transport	253.28
36	Q0C7E3	Vacuolar protein sorting/targeting protein 10	Protein transport	230.56
37	Q0CUU1	Sec23	Protein transport	200.53
38	Q0CHM0	Sec13	Protein transport	139.83
39	Q0CSL7	Sec24	Protein transport	127.11
40	Q0CGY7	COPII coat assembly protein sec16	Protein transport	124.77
41	Q0C8J3	mannan endo-1,4-beta-mannosidase C	Carbohydrate metabolism/catabolism	348.91
42	Q0CUL0	Xyl1	Carbohydrate metabolism/catabolism	325.10
43	Q0CEU4	endo-beta-1,4-glucanase D	Carbohydrate metabolism/catabolism	317.63
44	Q0CBM8	endo-1,4-beta-xylanase C	Carbohydrate metabolism/catabolism	316.36
45	Q0CI96	glucan endo-1,3-beta-glucosidase btgC	Carbohydrate metabolism/catabolism	271.67
46	Q0CHR0	Transcription activator of gluconeogenesis acuK	Carbohydrate metabolism/catabolism	271.33
47	Q0CVX4	alpha-galactosidase D	Carbohydrate metabolism/catabolism	236.21
48	Q0CNV1	Pyruvate decarboxylase	Carbohydrate metabolism/catabolism	240.90
49	O93918	Pyruvate carboxylase	Carbohydrate metabolism/catabolism	233.46
50	Q0CYP4	Mannitol 2-dehydrogenase	Carbohydrate metabolism/catabolism	187.31
51	Q0CA27	GDP-mannose transporter	Carbohydrate metabolism/catabolism	184.04
52	Q0C8Z0	glucan endo-1,6-beta-glucosidase B	Carbohydrate metabolism/catabolism	178.19
53	Q0CI48	Beta-mannosidase A	Carbohydrate metabolism/catabolism	138.19
54	D0VKF5	beta-glucosidase A	Carbohydrate metabolism/catabolism	138.09
55	P0C2C8	ATP synthase subunit d	Energy production and	631.33

			conversion	
56	Q0CC00	Ubiquinone biosynthesis protein coq4	Energy production and conversion	212.36
57	Q0CGN5	Assembly factor cbp4	Energy production and conversion	202.83
58	Q0C7Y1	Adenylate kinase	Energy production and conversion	201.38
59	Q0C9L8	ATP synthase subunit beta	Energy production and conversion	144.28
60	Q0CUP0	Patatin-like phospholipase domain-containing protein ATEG_02594	Lipid biosynthesis, metabolism and catabolism	449.93
61	Q0CFY9	Leukotriene A-4 hydrolase homolog	Lipid biosynthesis, metabolism and catabolism	279.98
62	Q0CKU4	Sterol 3-beta-glucosyltransferase	Lipid biosynthesis, metabolism and catabolism	273.15
63	Q0CE43	Phosphatidylinositol transfer protein sfh5	Lipid biosynthesis, metabolism and catabolism	235.70
64	Q0CXU6	Putative lipase atg15	Lipid biosynthesis, metabolism and catabolism	221.75
65	Q0CY11	Very-long-chain 3-oxoacyl-CoA reductase	Lipid biosynthesis, metabolism and catabolism	199.30
66	Q0CMM0	NADPH--cytochrome P450 reductase	Lipid biosynthesis, metabolism and catabolism	191.01
67	Q0CNC7	Lysophospholipase nte1	Lipid biosynthesis, metabolism and catabolism	179.76
68	Q0C8L9	3-methylglutaryl-coenzyme A reductase	Lipid biosynthesis, metabolism and catabolism	123.20
69	Q0CVD7	Phosphatidylethanolamine N-methyltransferase	Lipid biosynthesis, metabolism and catabolism	114.06
70	Q9Y7D2	3-methylglutaryl-coenzyme A reductase	Lipid biosynthesis, metabolism and catabolism	103.82
71	Q0CY33	Autophagy-related protein 17	Post translation modification and chaperone	390.62
72	Q0CH70	Hsp70 nucleotide exchange factor fes1	Post translation modification and chaperone	348.04
73	Q0CVW0	Mitochondrial zinc maintenance protein 1	Post translation modification and chaperone	297.39
74	Q0CY32	E3 ubiquitin ligase complex SCF subunit sconB	Post translation modification and chaperone	286.15
75	Q0CCL1	E3 ubiquitin-protein ligase hula	Post translation modification and chaperone	279.44
76	Q0CGN5	Assembly factor cbp4	Post translation modification and chaperone	202.83
77	Q0CJ57	Cytochrome P450 monooxygenase atE	Secondary metabolite synthesis	465.89
78	Q0CCY4	Atrochrysone carboxyl ACP thioesterase	Secondary metabolite synthesis	371.26
79	Q0D1P3	Multicopper oxidase terE	Secondary metabolite synthesis	329.15
80	Q0CJ58	Terreic acid biosynthesis cluster protein D	Secondary metabolite synthesis	307.26
81	Q0CCX4	Sulochrin halogenase	Secondary metabolite synthesis	291.27
82	Q0CJ62	6-methylsalicylic acid decarboxylase atA	Secondary metabolite synthesis	277.49
83	Q9UR08	Aristolochene synthase	Secondary metabolite synthesis	276.70
84	Q0D1P1	FAD-dependent monooxygenase	Secondary metabolite	257.74

		terC	synthesis	
85	Q0CCY5	O-methyltransferase gedA	Secondary metabolite synthesis	255.05
86	Q0C8M2	Esterase LovG	Secondary metabolite synthesis	252.19
87	Q0C8A3	Methyltransferase trt5	Secondary metabolite synthesis	249.04
89	Q0D1N9	Non-reducing polyketide synthase terA	Secondary metabolite synthesis	239.20
88	Q0CCX6	Dihydrogeodin oxidase	Secondary metabolite synthesis	217.07
89	Q0D1P2	FAD-dependent monooxygenase terD	Secondary metabolite synthesis	214.77
90	Q0CJ59	6-methylsalicylic acid synthase	Secondary metabolite synthesis	204.17
91	Q0D1P0	6-hydroxymellein synthase terB	Secondary metabolite synthesis	192.61
92	Q9Y7D1	Acyltransferase LovD	Secondary metabolite synthesis	187.95
93	Q0C9L4	FAD-dependent monooxygenase ctvC	Secondary metabolite synthesis	175.02
94	Q0C9L6	Methyltransferase ctvB	Secondary metabolite synthesis	174.67
95	P0DOB3	Probable decarboxylase gedI	Secondary metabolite synthesis	148.02
96	Q0C8A5	FAD-binding monooxygenase trt3	Secondary metabolite synthesis	143.18
97	Q9Y7D3	Probable transcriptional regulator LovE	Secondary metabolite synthesis	139.56
98	Q0CCY3	Atrochrysone carboxylic acid synthase	Secondary metabolite synthesis	133.79
99	Q0C8A2	Isomerase trt14	Secondary metabolite synthesis	122.00
100	Q0C8A1	Cytochrome P450 monooxygenase trt6	Secondary metabolite synthesis	121.76
101	Q0C9L5	Citreoviridin biosynthesis protein D	Secondary metabolite synthesis	121.30
102	Q9Y7C8	Dihydromonacolin L monooxygenase LovA	Secondary metabolite synthesis	107.37
103	P0DOB2	Anthrone oxygenase gedH	Secondary metabolite synthesis	102.60
104	Q0C898	Short chain dehydrogenase trt9	Secondary metabolite synthesis	102.16
105	Q0D1P4	Kelch-like protein terF	Secondary metabolite synthesis	89.80
106	Q0C8A0	Dioxygenase trt7	Secondary metabolite synthesis	71.96
107	Q0CCX9	Monoogygenase gedF	Secondary metabolite synthesis	69.60
108	Q0CCX5	Questin oxidase	Secondary metabolite synthesis	36.54

Appendix-II-Table-3.2: Identified conidial proteins of *Aspergillus terreus* and their function annotated from UniProt database

S.No	UniProt ID	Protein name	Function	PLGS Score
1	Q0C8A2	Terpene cyclase trt1	mediates the biosynthesis of terretonin	240.09
2	Q0C7Y1	Adenylate kinase	cellular energy homeostasis and in adenine nucleotide metabolism	70.43
3	Q0CY48	ATP-dependent RNA Helicase	biogenesis of 60S ribosomal subunits	64.28
4	Q0C9E8	ATP-dependent RNA helicase mrh4,	involved in mitochondrial RNA metabolism	49.31
5	Q0CR95	Polyadenylate-binding protein	multiple roles of the poly(A) tail in mRNA biogenesis	47.71
6	Q0CNX5	Clustered mitochondria protein homolog	involved in proper cytoplasmic distribution of mitochondria	47.59
7	Q0CEX5	Myosin-1	organization of the actin cytoskeleton	46.42
8	Q0CUU1	Sec23	formation of transport vesicles	44.22
9	Q0CLW8	Molybdenum cofactor sulfurase	Sulfurates the molybdenum cofactor	43.65
10	Q0CCL1	Probable E3 ubiquitin-protein ligase hula	involved in the regulatory network controlling carbon source utilization	41.05
11	Q0CLP3	Elongation factor G	involved in the pathway polypeptide chain elongation	39.96
12	Q0CH52	rRNA biogenesis protein rrp36	involved in the maturation of rRNAs	38.12
13	Q0CKU4	Sterol 3-beta-glucosyltransferase	Involved in the biosynthesis of sterol glucoside	37.24
14	Q0CCY3	Atrochrysone carboxylic acid synthase	mediates the biosynthesis of geodin	34.54
15	Q0CC84	Probable endo-beta-1,4-glucanase celB	Carbohydrate metabolism	32.58
16	Q0CBX9	transcription of RNA polymerase II	Transcription regulation	32.38
17	Q0CJV3	Vacuolar protein sorting-protein 27	Protein transport	25.19
18	Q0CI67	Probable beta-glucosidase F	involved in the degradation of cellulosic biomass	24.57
19	Q0CA78	Putative DNA helicase ino80	involved in DNA repair	23.61
20	Q0CNC7	Lysophospholipase nte1	Lipid metabolism	22.04
21	Q0CVD7	Phosphatidylethanolamine N-methyltransferase	involved in the pathway phosphatidylcholine biosynthesis lipid metabolism	16.49
22	Q0CSI0	Autophagy-related protein 2	Autophagy, Protein transport	15.52
23	Q0CEN9	Mitochondrial thiamine pyrophosphate carrier	mediates uptake of thiamine pyrophosphate (ThPP) into mitochondria	15.34
24	Q0D1N9	Non-reducing polyketide synthase terA	involved in Secondary metabolite biosynthesis	15.17
25	Q0CHV5	Vacuolar membrane-associated protein iml1	intracellular signal transduction	12.15

Appendix-II-Table-3.3: Expressed proteins of *Aspergillus germinating* conidia participate in protein synthesis

S.No	UniProt ID	Description	Function	PLGS score
1	Q0CPF9	Eukaryotic translation initiation factor 3 subunit G	protein biosynthesis	505.15
2	Q0CN46	Eukaryotic translation initiation factor 3 subunit B	protein biosynthesis	492.65
3	Q0CZL5	Mitochondrial distribution and morphology protein 12	protein biosynthesis	371.46
4	Q0CXX1	54S ribosomal protein L4	protein biosynthesis	361.84
5	Q0CXD0	ATP-dependent RNA helicase eIF4A	protein biosynthesis	326.01
6	Q0CNR3	Eukaryotic translation initiation factor 3	protein biosynthesis	308.73
7	Q0CTT3	Eukaryotic translation initiation factor 3 subunit J	protein biosynthesis	300.29
8	Q0CPA8	Eukaryotic translation initiation factor 3 subunit L	protein biosynthesis	297.42
9	Q0CUP6	Eukaryotic translation initiation factor 3 subunit A	protein biosynthesis	272.06
10	Q0CVT0	Eukaryotic translation initiation factor 3 subunit C OS	protein biosynthesis	226.67
11	Q0D1J4	Eukaryotic translation initiation factor 3 subunit H	protein biosynthesis	224.58
12	Q0CXH9	Eukaryotic translation initiation factor 3 subunit I	protein biosynthesis	185.53
13	Q0CLJ6	ATP-dependent RNA helicase ded1	protein biosynthesis	176.87
14	Q0CLP3	Elongation factor G	protein biosynthesis	166.57
15	Q0CLP3	Elongation factor G	protein biosynthesis	166.57
16	Q0CCM5	Eukaryotic translation initiation factor 3 subunit F	protein biosynthesis	140.14
17	Q0CPV5	Eukaryotic translation initiation factor 3 subunit M	protein biosynthesis	99.32
18	Q0CCM5	Eukaryotic translation initiation factor 3 subunit F	protein biosynthesis	140.14
19	Q0CPV5	Eukaryotic translation initiation factor 3	protein biosynthesis	99.32
20	Q0D0F3	Pentafunctional AROM polypeptide	amino acid biosynthesis	399.27
21	P0C2P2	Ornithine carbamoyltransferase	amino acid biosynthesis	361.00
22	Q0CFY3	Methylthioribose-1-	amino acid	224.11

		phosphate isomerase	biosynthesis	
23	Q0CT19	Methylthioribulose-1-phosphate dehydratase	amino acid biosynthesis	213.77
24	Q0CY06	Amino-acid acetyltransferase	amino acid biosynthesis	207.33
25	Q0CDB9	Arginine biosynthesis bifunctional protein ArgJ	amino acid biosynthesis	161.34
26	Q0CRI5	Kynurenine 3-monooxygenase	amino acid metabolism	263.13
27	Q0CL94	Methionine aminopeptidase	amino acid metabolism	253.31
28	Q0CFZ0	Probable Xaa-Pro aminopeptidase pepP	amino acid metabolism	212.88
29	P56862	Sulfate adenylyltransferase	amino acid metabolism	193.82

Appendix-II-Table-3.4: Terrein and lovastatin biosynthesis pathway proteins

UniProt ID	**Gene name**	**Description**	**PLGS score**
Q0D1P3	***terE***	Multicopper oxidase terE	329.15
Q0D1P1	***terC***	FAD-dependent monooxygenase terC	257.74
Q0D1N9	***terA***	Non-reducing polyketide synthase terA	239.20
Q0D1P5	***terR***	Terrein cluster-specific transcription factor terR	229.47
Q0D1P2	***terD***	FAD-dependent monooxygenase terD	214.77
Q0D1P0	***terB***	6-hydroxymellein synthase terB	192.61
Q0D1P4	***terF***	Kelch-like protein terF	89.80
Q0D1P7	***terH***	NAD-dependent epimerase/dehydratase terH	86.03
Q0D1P9	***terJ***	Efflux pump terJ	11.48
UniProt ID	**Gene name**	**Description**	**PLGS score**
Q0C8M2	***lovG***	Esterase LovG	252.19
Q9Y7D1	***lovD***	Acyltransferase LovD	187.95
Q9Y7D3	***lovE***	transcriptional regulator LovE	139.56
Q9Y7C8	***lovA***	Dihydromonacolin L monooxygenase LovA	107.37

Appendix-II-Table-3.5: List of proteins used for construction of protein-protein interaction network

S.NO	UniProt ID	String ID	Functional characterization	Gene Name
1	Q0CYG9	ATEG_01265	Protein transport	*sec31*
2	Q0CD12	ATEG_08422	Protein transport	*katG*
3	Q0CUU1	ATEG_02543	Protein transport	*sec23*
4	Q0CIV4	ATEG_06380	Protein transport	*bst1*
5	Q0CHM0	ATEG_06814	Protein transport	*sec13*
6	Q0CSL7	ATEG_03317	Protein transport	*sec24*
7	Q0CGY7	ATEG_07055	Protein transport	*sec16*
8	P0C2C7	ATEG_07080	Protein transport	*egd2*
9	Q0CGL5	ATEG_07177	Protein transport	*egd1*
10	Q0CMB0	ATEG_05174	rRNA processing	*rok1*
11	Q0CQH1	ATEG_04063	rRNA processing	*nop58*
12	Q0CCM6	ATEG_08558	rRNA processing	*utp25*
13	Q0CY48	ATEG_01386	rRNA processing	*dbp9*
14	Q0CDP3	ATEG_08191	rRNA processing	*nop16*
15	Q0CLJ4	ATEG_05440	rRNA processing	*ytm1*
16	Q0CSG1	ATEG_03373	rRNA processing	*utp10*
17	Q0CF43	ATEG_07691	rRNA processing	*dbp7*
18	Q0CH52	ATEG_06990	rRNA processing	*rrp36*
19	Q0CQF3	ATEG_04081	rRNA processing	*spb4*
20	Q0CLP9	ATEG_05385	rRNA processing	*nop7*
21	Q0CAS8	ATEG_09206	rRNA processing	*fal1*
22	Q0CMM8	ATEG_05056	rRNA processing	*dbp10*
23	Q0CNX1	ATEG_04613	rRNA processing	*dbp8*
24	Q0CCP0	ATEG_08544	rRNA processing	*erb1*
25	Q0CL13	ATEG_05621	rRNA processing	*dbp3*
26	Q0CIQ3	ATEG_06431	rRNA processing	*rrp3*
27	Q0CI35	ATEG_06649	rRNA processing	*mak5*
28	Q0CUP6	ATEG_02588	Protein biosynthesis	*tif32*
29	Q0CVT0	ATEG_02204	Protein biosynthesis	*nip1*
30	Q0CXH9	ATEG_01605	Protein biosynthesis	*tif34*
31	Q0CXD0	ATEG_01654	Protein biosynthesis	*tif1*
32	Q0CNR3	ATEG_04671	Protein biosynthesis	*int6*
33	Q0CTT3	ATEG_02901	Protein biosynthesis	*hcr1*
34	Q0CA00	ATEG_09484	Protein biosynthesis	*rps1*
35	Q0CQF6	ATEG_04078	Protein biosynthesis	*rps0*
36	Q0CE91	ATEG_07993	Transcription/ regulation	*rpc82*
37	Q0CY32	ATEG_01402	Transcription/ regulation	*sconB*
38	Q0CXS6	ATEG_01508	Carbohydrate metabolism/catabolism	*mpdA*
39	Q0D0F3	ATEG_00581	Amino acid metabolism/catabolism	*aroM*
40	Q0C7Y1	ATEG_10203	DNA replication/repair	*adk1*
41	Q0D0X6	ATEG_00408	Cell cycle	*nudF*

Appendix-II-Table- 4.1: List of proteins that were common between control cells and interacting cells during infection

Accession	Description	PLGS Score
Q59EW7	Splicing factor, arginine/serine	492.61
A0A0A0MRV0	Ribosome-binding protein 1	359.44
M1V490	KIF5B-RET(NM_020630)_K22R12 fusion protein	350.70
H0Y6I0	Golgin subfamily A member 4	310.96
A8K3D5	cDNA FLJ77065, highly similar to Homo sapiens golgi autoantigen	291.24
E7EVA0	Microtubule-associated protein	256.65
B3KSQ7	Drebrin 1, isoform CRA_d	247.49
Q5T200	Zinc finger CCCH domain-containing protein 13	242.12
Q9HCM1	Uncharacterized protein KIAA1551	212.89
B2RAG5	cDNA, FLJ94903, highly similar to Homo sapiens glyceronephosphate O-acyltransferase (GNPAT),	190.39
B3KY47	cDNA FLJ46841 fis, clone UTERU3000670	179.08
V9HWC1	Epididymis luminal protein 71	178.53
A0A024R8K8	Helicase with zinc finger, isoform CRA	161.85
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	160.07
Q86US8	Telomerase-binding protein EST1A	156.39
A0A024R542	Tankyrase 1 binding protein 1	151.74
P78332	RNA-binding protein 6	149.38
B4DYG5	cDNA FLJ60960, highly similar to Phosphatidylinositol 4-kinase alpha	145.22
Q15643	Thyroid receptor-interacting protein	144.71
Q49AJ0	Protein FAM135B	135.20
P49750	YLP motif-containing protein 1	106.85
Q702N8	Xin actin-binding repeat-containing protein 1	104.43
A7E2D5	SYNE1 protein (Fragment)	103.15
D6RJB7	Putative ankyrin repeat domain-containing protein 31	102.34
Q9UK61	Protein TASOR	101.41
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	101.00
E9PNZ4	Microtubule-actin cross-linking factor 1	99.72
B3KT58	cDNA FLJ37685 fis, clone BRHIP2013972	98.27
Q6AWA4	Putative uncharacterized protein DKFZp686O0870	93.66
H0YFN7	RAB6A-GEF complex partner protein 1	90.59
Q9Y623	Myosin-4	89.86
Q15326	Zinc finger MYND domain-containing protein 11	88.50
Q13464	Rho-associated protein kinase	86.64
Q9C0D5	Protein TANC1	86.23
A0A024R930	Proteoglycan 4, isoform CRA	84.73
A6NM11	Leucine-rich repeat-containing protein 37A2	82.50
Q9HAU0	Pleckstrin homology domain-containing family A member 5	81.36
O95785	Protein Wiz OS=Homo sapiens	77.96
Q9P2E3	NFX1-type zinc finger-containing protein 1	76.69
Q8ND23	Leucine-rich repeat-containing protein 16B	75.77
Q59F25	Alpha 3 type VI collagen isoform 5	75.27
Q86XA9	HEAT repeat-containing protein 5A	71.10
P42892	Endothelin-converting enzyme	69.59
Q5JZY3	Ephrin type-A receptor 10	69.46
A6H8W8	Intersectin 2	69.35
Q6UB98	Ankyrin repeat domain-containing protein 12	68.83
B2R5R9	cDNA, FLJ92591, highly similar to Homo sapiens regulatory factor X	68.53

Q9UKX2	Myosin-2 OS	65.63
Q96BY7	Autophagy-related protein 2 homolog B	63.70
O00443	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha	63.14
Q8TF72	Protein Shroom3 OS=Homo sapiens GN=SHROOM3 PE=1 SV=2	59.94
Q149M9	NACHT domain- and WD repeat-containing protein 1	59.86
Q5JU85	IQ motif and SEC7 domain-containing protein 2	57.34
Q9UM73	ALK tyrosine kinase receptor	56.80
P51812	Ribosomal protein S6 kinase alpha-3	56.61
Q5SW79	Centrosomal protein of 170 kDa	53.41
Q9UQE7	Structural maintenance of chromosomes protein 3	53.01
A0A087WVZ7	Zinc finger protein 229	52.78
Q8IWY7	Tau-tubulin kinase	52.65
P12883	Myosin-7	50.94
A0A087WY08	Thrombospondin type-1 domain-containing protein 7A	50.75
Q4ADV7	RAB6A-GEF complex partner protein	50.21
Q8TEW8	Partitioning defective 3 homolog B	50.19

Appendix-1I- Table-5.1: Highly deleterious nsSNPs in different domains of Dectin-1receptor protein.

nsSNP ID	Amino acid change	Deleterious prediction	Domain
rs562749381	Y3D	4	N-terminal Domain
rs780024327	E12D	4	N-terminal Domain
rs756166982	D13Y	5	N-terminal Domain
rs759032825	S22F	5	N-terminal Domain
rs775715931	C54R	5	N-terminal Domain
rs781427660	L64P	5	N-terminal Domain
rs771094993	G65D	5	N-terminal Domain
rs112345533	S117F	5	N-terminal Domain
rs753757159	L133P	5	CTLD
rs760554156	F134Y	4	CTLD
rs761503556	W141R	5	CTLD
rs369482852	W141S	5	CTLD
rs746386372	C148G	5	CTLD
rs747442135	L155V	5	CTLD
rs138005591	I158T	5	CTLD
rs758623997	D159G	5	CTLD
rs70583536	F179L	5	CTLD
rs140318683	L183F	5	CTLD
rs548792483	R185Q	4	CTLD
rs371994700	W222R	4	CTLD
rs16910527	I223S	4	CTLD
rs141153031	C233Y	5	CTLD

F= Functional amino acid residue, S= Structural amino acid residue

Appendix-II-Table-5.2: Highly deleterious nsSNPs in PTX-3 receptor protein.

nsSNP ID	Mutation position	Domain	Deleterious prediction
rs148384694	T41I	Multi domain site	5
rs564774580	R188C	PTX	6
rs529759691	F193S	PTX	6
rs532972316	E235K	PTX	5
rs190837481	H269Y	PTX	6
rs144979346	G306E	PTX	6
rs4478039	E313K	PTX	6
rs532972316	E235K	PTX	5
rs76994524	N337S	PTX	5
rs146705881	L343I	PTX	5
rs140073706	S344R	PTX	6
rs138818541	R360W	PTX	6

Appendix-1I-Ttable-5.3: Conserved amino acids in PTX-3 receptor protein that coincide in location with high-risk nsSNPs

nsSNPs ID	Amino acid position	CS	ConSurf prediction
rs148384694	T41I	5	Exposed
rs564774580	R188C	9	Highly conserved and Exposed (F)
rs529759691	F193S	8	Highly conserved and Buried
rs532972316	E235K	9	Highly conserved and Exposed (F)
rs190837481	H269Y	9	Buried (S)
rs144979346	G306E	9	Highly conserved and Buried (S)
rs4478039	E313K	9	Highly conserved and Exposed (F)
rs532972316	E235K	5	conserved and Exposed (F)
rs76994524	N337S	9	Highly conserved and Buried (S)
rs146705881	L343I	9	Highly conserved and Buried (S)
rs140073706	S344R	7	Exposed
rs138818541	R360W	9	Highly conserved and Exposed (F)

F = Functional residue, S = Structural residue

LIST OF PUBLICATIONS

Research Articles

1. **Raman Thakur** and Jata Shankar. (2017). Proteome profile of *Aspergillus terreus* conidia at germinating stage; Identification of probable virulent factors and enzymes from mycotoxin pathways. **Mycopathologia**.182 (9-10) 771-784 doi: 10.1007/s11046-017-0161-5.

2. **Raman Thakur** and Jata Shankar. (2016). *In silico* Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens. **Front. Microbiol**. 7:192. doi: 10.3389/fmicb.2016.00192

3. **Raman Thakur** and Jata Shankar. (2016). In silico identification of potential peptides or allergen shot candidates against *Aspergillus fumigatus*, **BioResearch** 5:1, 330–341, DOI: 10.1089/biores.2016.0035

4. **Raman Thakur** and Jata Shankar. Proteome analysis revealed Jak / Stat signaling and cytoskeleton rearrangement proteins in human lung epithelial cells during interaction with *Aspergillus terreus* (Communicated).

5. **Raman Thakur** and Jata Shankar. Comprehensive in-silico Analysis of High-risk Non-synonymous SNPs in Dectin-1 Gene of Human and their Impact on Protein Structure, **Current Pharmacogenomics and Personalized Medicine**, 16**,**1-12, 2018. doi: http://dx.doi.org/10.2174/1875692116666180115142706.

Review Articles

1. **Raman Thakur**, Rajesh Anand, Shraddha Tiwari, Agam P Singh, Bhupinder N Tiwary and Jata Shankar. (2015). Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells? ***Front. Microbiol***.6:429. doi: 10.3389/fmicb.2015.00429

2. **Raman Thakur** and Jata Shankar. (2017). New treatment regime for *Aspergillus* associated infections. **Virology and Mycology.** 6: 162. doi: 10.4172/2161-0517.1000162 **(I.F 0.7)**

Book Chapter

1. **Raman Thakur** and Jata Shankar. Strategies for gene expression in Prokaryotic and Eukaryotic system. In kalia, V.C, Saini, A.K, Saini, R.V, Sharma, D.K, Strategies for metabolic engineering in bioactive compounds and processes. **Springer** ISBN 978-981-10-5511-9

CONFERENCES/WORKSHOPS

1. **Raman Thakur** and Jata Shankar. (2015) presented poster entitled "Heat shock proteins influence Aflatoxin biosynthesis in *Aspergillus flavus"* in National conference on 'Emerging Trends in Host-Microbe Interaction' organized by DAV University, Jalandhar, Punjab

2. **Raman Thakur** and Jata Shankar. (2015) Attended National symposium on computational system biology organized by dept. of biotechnology and bioinformatics JUIT, March- 1^{8th}- 2^{0th} 2016, Waknaghat, Solan, H.P., India and NNMCB (Mohali Node), SERB and presented poster entitled "In silico analysis of single nucleotide polymorphisms in human dectin-1 gene and their impact on fungal infections.

3. **Raman Thakur** and Jata Shankar. (2015) Participated in conference on Recent Trends in Biomedical Engineering, Cancer Biology and Bioinformatics organized by "Krishi Sanskriti" at JNU, 28^{th} Nov., 2015, New Delhi and presented poster entitled "In-silico T-cell epitope mapping from antigens of *Aspergillus fumigatus* for potential candidate for *Aspergillus*-specific T cells"

4. 8^{th} Advances against Aspergillosis **Lisboa Congress Centre, Lisbon, Portugal, 1-3 February 2018.** Proteome profile of A549 lung epithelial cells interacting with *Aspergillus terreus* conidia.