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Immunoproteomic Studies of Aspergillus: Application in the Development of Immunotherapies, Vaccines and Biomarkers

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Abstract

Filamentous *Aspergillus* fungi play a vital role as a pathogenic agent which causes superficial and invasive infections and allergy reactions in people. Invasive infections of *Aspergillus* are explicitly regarded by an unusual fatality rate owing to challenges in proper identification, characterization and inadequate antifungal treatment. The immunoproteomic techniques offer immense potential to identify new diagnosis, immunotherapies and targets for the production of vaccines associated with diseases, especially caused by *Aspergillus* species. One of the common antigenic protein identification approaches is Serological Proteome Analysis (SERPA), which integrate 2D electrophoresis with western blot method. In recent times, however, various new protein detection techniques have been introduced which either amplify antibody reactions or determine T-cell immunity targets for allergic or invasive fungal infected patients. Here, we look at the developments in immune responses against *Aspergillus* and the current methods for diagnosis and treatment of *Aspergillus* infections.

Keywords: Serological Proteome Analysis (SERPA), *Aspergillus*, T-cell, Diagnosis, Immunotherapy.

Introduction

Immunoproteomic is an extensive protein study that contributes to the identification of an organism's immune response. This is used to define proteomic approaches that categorize significant antigens which ultimately led to the production of antibodies that stimulate adaptive immune reactions. The immunoproteomic study of host-pathogenic response provides an opportunity to find antigens for the diagnostics and vaccine development (Jungblut, 2001). In the field of medical mycology, the dynamics of immunoproteomic approaches are noticeable concern for doctors and researchers. Despite of the fact that vast progress has been made in developing immunotherapies against fungal infections, their diagnosis still remain difficult and therefore, new options are severely required for antifungal therapies (Schelenz et al., 2015).

Aspergilli are of major medical importance in the category of filamentous fungi, notably *Aspergillus fumigatus*. They illustrate a typical saprophytic soil fungus. *Aspergilli* readily spread through asexually produced spores (conidia) into the air, that are relatively tiny and can easily be inhaled and penetrate the human respiratory tract (Latge & Chamilos, 2020). *Aspergillus* spores are effectively cleared from the immune system, but if the host has a weakened immunity, then spores will germinate, propagate and inhabit the respiratory tract and cause a variety of disease from non-invasive to invasive, critical and allergic (Kosmidis & Denning, 2015). Here, we discussed *Aspergilli* affected diseases, especially caused by *A. fumigatus* and immune reactions against these human-pathogenic fungi. We also reviewed

immunoproteomic techniques to identify and use pathogenic proteins for diagnosis against *Aspergillus*, and development of immunotherapy and vaccine for aspergillosis.

1. *Aspergillus* species causing infections

Invasive aspergillosis (IA) is the most serious illness caused by *Aspergillus* species. It occurs when infection is spread into the bloodstream from the pulmonary system, which may further diffuse to different body organs such as the eyes, skin, and brain. People suffering from neutropenia, chronic pulmonary diseases or on certain drug therapies, are more at risk of developing IA (Kosmidis & Denning, 2015). *A. fumigatus* is a prime reason for invasive aspergillosis in 65% patients undergone transplantation, followed by *A. flavus* and *A. niger* (Balajee et al., 2009). *Aspergilli* have major human health impacts and more than 200,000 IA cases are projected to arise worldwide every year, and out of them 30-95% are deadly. The key factor of this increased risk of death is a late or improper characterization and diagnosis which leads to ineffective antifungal treatment (Brown et al., 2012).

Aspergillus species also potentially contribute to persistent infections called chronic pulmonary aspergillosis (CPA) besides invasive fungal infections in immunocompromised individuals. A chronic cavity aspergillosis is the widespread form of CPA which is described by formation of colony of fungus in pulmonic spaces and approximately 25 percent of CPA patients suffer from aspergilloma that may advance to critical fibrotic destruction if untreated (Denning et al., 2016). The inhaled *Aspergillus* conidia also colonize into mycelium and cause allergic reactions, leads to critical forms of asthma. It is likely due to the reason that they are able to propogate and cause infection in host tissue, therefore, they have a large effect on eliciting allergic reaction (Denning et al., 2006). In patients with asthma or cystic fibrosis, there is a fungal sensitization that leads to allergic bronchopulmonary aspergillosis (ABPA). In patients with ABPA, the continuous inhalation of conidia causes fungal colonization in airway which triggers an allergic reaction along with amplified levels of total IgE and anti-*A. fumigatus* IgE (Shah & Panjabi, 2016). Approximately 4 million patients are distraught by ABPA and ultimately causing bronchiectasis in which airways are relentlessly damaged (Agarwal et al., 2013).

2. Adaptive immune system role in *Aspergillus* infections

The first line of defense mechanism against *Aspergillus* is the innate immunity that flush 90% inhaled conidia of *Aspergillus* only in 24-48 hours with the help of epithelial cells and phagocytes such as macrophages, neutrophils and dendritic cells (Brakhage et al., 2010; Park & Mehrad, 2009). Therefore, neutropenia is supposed to be the major factor that affects IA in immunocompromised patients (Kosmidis & Denning, 2015). However, supplementary data informs that $CD4^+$ T cells also take part as defensive and preventive role against IA which is illustrated by an association of T cells specific for *Aspergillus* with the transplant patients infected with *Aspergillus* (Jolink et al., 2014). Therefore, it was suggested that specific T cells might be a possible therapeutic or prophylactic treatment (Bacher et al., 2015; Deo et al., 2016; Papadopoulou et al., 2016). The immunocompetent patients affected by non-invasive *Aspergillus*-related medical difficulties and with the impaired lung activity are also required T cells as defensive mechanism. However, an elusive balance has to be maintained by the adaptive immune responses between defense from invading infection and sufferance to prevent inflammation against *Aspergillus*.

Various understandings on adaptive immune reactions has been considered by examining

serum antibodies that are specific for fungus and also produced by B cells specific for fungus (Singh et al., 2014; Singh et al., 2010a; Singh et al., 2010b). A few studies suggested that a certain quantity of antibodies might influence the path of fungal infection by either being defensive or increasing illness such as the lung clearing of *A. fumigatus* was demonstrated to make B-cell deficient mice more efficient (Montagnoli et al., 2003). Specifically, $CD4^+$ T helper cells are the core regulators in aacquired immune system which can be separated by functional divisions that play a pleiotropic role and cooperate with B cells to produce antibodies. In fact, the subdivisions of T helper cells participate actively not only in mediating tolerance, but also synchronize preventive immune responses against invasive or allergic infections. Hence, T helper cells are considered as unambiguous detectors which react and vigorously control various phases of host and fungus interactions, and consequently demonstrate that T cell based diagnostic methods and therapies might measure T helper cells as their major target (Ito, 2011).

Since the fungal proteome is complex in nature, a straight investigation of fungus-specific T cells is impenetrable as they are low in frequencies (usually below 0.1%) as compared to peripheral blood T cells. Mostly, examination of T cells based methods rely on indirect assays such as production of effective cytokines like IFN-γ or *in vitro* antigen worsening for long-term (Chaudhary et al., 2010; Hetty Jolink et al., 2014; Potenza et al., 2013; Stuehler et al., 2015). These methods has some limitations as they do not allow identification of functional subdivisions and may undervalue accurate incidences since only few of the reactive T cells produce cytokines. Moreover, efficiency of phenotypical physiognomies of the specific T cells are moderated by determined stimulation (Bacher & Scheffold, 2013).

In addition to crude fungal lysates, various *Aspergillus* proteins were examined to trigger human $CD4^+$ T cell responses, largely from antibody-binding protein (Bacher et al., 2014; Jolink et al., 2015; Jolink et al., 2014). Through these studies, it was suggested that the most of the healthy individuals have T cell reaction to these proteins, displaying overwhelming immune reaction against *Aspergillus*. Thus, quantitative aspects of classifications together with qualitative analysis of T cells, specific for fungus, are requisite for diagnosis in T cell-based therapy or vaccine development.

3. Methods for identification of *Aspergillus* antigens

Numerous immunoproteomic techniques have been developed and deployed in the last decade for the detection of new potential biomarkers for their use in diagnosis in aspergillosis or identification of antigens present in *Aspergillus* and other pathogenic microbes having application in vaccine development. Identifying probable antigens enables emerging diagnostic or prognostic tests for fungal infections. Therefore, numerous high performance immunoassays have been identified and developed such as immunoassays with magneto-nanosensor and ELISA (Kim & Wang, 2012; Sulahian et al., 2001).

3.1 Serological Proteome Analysis (SERPA)

The 2DE immunoblotting methodology, commonly known as SERPA, is the most commonly used immunoproteomic method for detecting antigens. Proteins are separated by first dimensional isoelectric focusing (IEF) method according to their respective isoelectric point (pI), while second dimension is SDS-PAGE separates proteins based on their molecular size. The proteins are subsequently transported from 2D gel to the PVDF or nitrocellulose membrane via western blotting procedures. The membrane is then evaluated by probing with patient's serum, and the visualized by reacting it with the desired

secondary antibody using fluorescence, chemiluminescence, etc. The desired protein spots are cut from 2D gels, allowed to proteolytic digestion, and then determined by using LC-MS/MS or MALDI-TOF/TOF practices. This serological screening approach is very challenging as it perceives only linear epitopes, but post-translational modifications of proteins are generally retained. Since this approach has a high resolution while separating proteins, but the identification of antigens is still difficult due to the solubility of hydrophobic proteins, sensitivity constraints and identification of western blot signals (Dutoit-Lefèvre et al., 2015). Nevertheless, SERPA is considered a potent method and broadly used to identify antigenic molecules in *Aspergillus* and other pathogens.

3.2 Protein array

Protein selection is a highly effective technique for the screening of proteins for their antibody targets. Proteins articulated by recombination or separated from proteomic samples and fractionated by liquid chromatography, can be marked and immobilized on a chip through microarray printing techniques (Barbulovic-Nad et al., 2006; Hueber & Robinson, 2006). Afterwards, sera from patients are probed on protein array chips, allowed to incubate with secondary antibodies, led to detection and visualization of reactive antigens. Further fractionation, immunoprecipitation or detection through mass spectrometry can be performed to recognize each antigen spot. The serum required in such method is significantly lower in volume (2 µl) as compared to SERPA (50-100 µl). Also, this method allows a fast screening of a large amount of sera and high-throughput competency. The chip-based serum screening technique can also be applied to examine protein glycosylation and epitopes to identify antigen reactivity (Blixt et al., 2010; Kracun et al., 2010; Reis et al., 2011). Since this approach has only been reported for *Candida albicans*, but has not yet been taken into consideration for *Aspergillus* species (Mochon et al., 2010).

3.3 Immunocapture mass spectrometry (icMS)

Sera of patients are fixed on to protein A-coupled columns or G-coupled columns in immunocapture mass spectrometry. The immunogenic proteins are secreted in culture media from fungal cell lysates are detained and supplemented on the protein coupled columns. These immunocaptured antigens are eluted from the column, submitted to proteolytic digestion and recognized by tandem mass spectrometry. This technique is highly efficient and makes it possible to simultaneously examine huge statistics of sera of patients against solubilized antigens. This approach can even sensitively detect antigens with lower molecular weight (<20 kDa) (Tjalsma et al., 2008). Since this technique has successfully applied for the detection of antigens of *Streptococcus bovis*, responsible for colon cancer, but not yet been applied for fungal antigens (Tjalsma et al., 2006).

3.4 Serological analysis of recombinant cDNA expression libraries (SEREX)

This is a process to identify antigens by appearance of arrays but has not yet been taken into account for *Aspergillus*. SEREX is called reverse proteomics where cDNA libraries are utilized to recognize novel antigens (Lee & Jeoung, 2007). Fungal mRNA may be used for the preparation of cDNA libraries evaluated for patient serum detection.

4. Allergens/antigens detected in ABPA

Aspergillus fumigatus is the main causative fungus for ABPA among the *Aspergillus* species. The fungus develops a hefty number of allergens in sensitive individuals which cause

serological IgE or IgG antibody reactions. So far, 98 IgE and IgG reactive molecules have been reported from *A. fumigatus* (Singh et al., 2014). Singh et al. investigated various allergens from secreted proteins and germinating conidia of *A. fumigatus* from pooled sera of ABPA patients and scanned their reaction with IgE and IgG antibodies (Singh et al., 2010a; Singh et al., 2010b). Similarly, 5, 12 and more than 12 alleregns have been identified from *A. flavus, A. niger* and *A. versicolor* respectively (Benndorf et al., 2008; Vermani et al., 2015). There are many studies that majorly focused on cell wall, cytosol, cellular fractions or the secretome of *A. fumigatus*. Arabinase and chitosanase extracellular enzymes are shown to be important allergens with IgE immunological activities in ABPA patients (Gautam et al., 2007). There is one more major allergen Asp f 34 has been found from *A. fumigatus* while screening cDNA reference library for cell wall constituents (Glaser et al., 2009). It is a cell wall protein found in conidiophores of *A. fumigatus* and is similar to protein PhiA of *A. nidulans*, required in conidia development.

5. Detection of antigens in IA

IA is occurred in immunocompromised patients but also in immunocompetent individuals by *Aspergilli*. The serological reactions in IA patients have been studied through preparation of immunoblots using sera of patients (Matthews et al., 1985). At that time, researchers could not recognize and characterize antigenic constituents due to limited techniques and sources. The human sera were tested for antigen reactivity and different animal models were developed for IA which was used for the detection of allergens during the past decades. In a study, *A. fumigatus* conidia were used to infect rabbits where 59 varied soluble proteins were detected demonstrating immunoreactivity with rabbit serum (Asif et al., 2010). These antigenic proteins have found to be involved in biological processes such as glycolysis, other metabolic pathways, oxidative stress, and provided assistance in immune responses generated in an experimental aspergillosis. SERPA technique has also been used with sera of patients who were supposed to be infected with IA (Virginio et al., 2014), and they acknowledged 40 antigens during germination of *A. fumigatus*. In a study of *A. fumigatus* secretome performed by Kumar et al. where they recognized 15 secreted immunogenic proteins and might be considered as potential biomarkers to diagnose aspergillosis (Kumar et al., 2011). In a conclusion, 17 different immunogenic proteins were investigated and among them, thioredoxin reductase GliT identified to have the highest immunoreactivity and thus, considered as a probable biomarker (Kumar et al., 2011; Shi et al., 2012; Singh et al., 2010a).A recent research acknowledged 49 antigenic molecules and showed a great diversity of IgG antibodies specific to *A. fumigatus* during serological examination in IA patients (Teutschbein et al., 2016).

6. Clinical applications of detection of *Aspergillus* infections by antibodies and antigens

The diagnosis of IA largely depends on detection and identification of specific antigens or antibodies. The patients have asthma, aspergilloma, or ABPA are able to generate an antibody response which ultimately helps in the detection of *Aspergillus*-specific antibodies. On the other hand, the immunocompromised patients, with speculation of emerging IA, are unable to generate considerable humoral immune (Asif et al., 2010; Matthews et al., 1985), and in such cases diagnosis generally depend on the recognition of antigenic molecules of fungus.

6.1 *Aspergillus*-specific antibody response detection

There are many commercial assays available which detect *Aspergillus*-specific antibodies in clinical samples, and these generally depend on fungal extracts and recombinant antigens. In COPD and ABPA patients, an important diagnostic measure is the detection of *Aspergillus*-specific IgE and IgE antibodies (Fukutomi et al., 2016; Jhun et al., 2013). Up till now, 21 *Aspergillus* antigenic proteins have been identified and are well-defined based on corresponding allergens from other fungi. Crameri et al. first evaluated the diagnostic use of recombinant *Aspergillus* antigens and demonstrated Asp f 1, 3, 4 and 6 in a commercial diagnostic measure to discriminate ABPA from *Aspergillus* sensitization (Crameri, 1998). In another investigation, 8 recombinant proteins were used along with galactomannan for the analysis of antibody reactions in ABPA, IA or aspergilloma patients (Sarfati et al., 2006). In patients with aspergilloma and ABPA, antibody reaction to 18kDa ribotoxin, catalase CAT1 and 88kDa dipeptidylpeptidase V were also detected, but no antibody response was shown by IA patients. In a study by Beck et al, antibody reaction was measured in response to recombinant 18-kDa ribotoxin and chitosanase B in immunocompromised individuals (Beck et al., 2014). These studies revealed that *Aspergillus*-specific antibodies are possible to be spotted but cannot be found suitable for diagnostic methods in case of IA patients. Studies suggest that the assays based on recombinant antigens are worthwhile tools for the detection of *Aspergillus* invasive or non-invasive infections (Guitard et al., 2012).

6.2 Antigen detection

The responses generated by antibodies are commonly measured using serum, whereas plasma, bronchoalveolar lavage, CSF fluid and urine can be used to detect antigens. It is primarily important that they should be steady and sufficient in concentration so that can be measured explicitly for their detection. Antigens circulate freely in the body or accumulate in blood cells or any other location. Therefore, ideal techniques for the fulfillment and exemption of samples for each unique antigen are necessary. The EORTC/MSG consensus group is used to assess IFIs, and at present, only approved technique available to obtain indirect microbiological assays are GM and β-1, 3-glucan (Pauw et al., 2008). Therefore, at present, the most imperative biomarkers are supposed to be β- 1,3-glucan and GM of cell wall in case of *Aspergillus* infection which is strengthened by a difference shown by glycolipid structures of fungus (Latgé, 2007; Nucci et al., 2014; Odabasi et al., 2004). In order to identify and characterize complementary host-response biomarkers, scientists studied protein pooled source from plasma of patients that underwent transplantation or chemotherapy, using bio fluid analysis platform (Brasier et al., 2015). They have recognized 9 proteins from plasma and 4 host-acquired peptides that resulted into the earlier diagnosis of *Aspergillus* infections along with GM. Also in recent times, non-proteinogenic biomarkers like secondary metabolites bis(methyl)gliotoxin and gliotoxin, and unstable molecules like monoterpenes and sesquiterpenes have also captivated consideration for *Aspergillus* infections (Domingo et al., 2012).

An assay specific for *Aspergillus* antigens directed to the development of a monoclonal antibody JF5 based *Aspergillus* lateral flow assay that is used in medical setups for *Aspergillus* causing diseases detection (Thornton, 2008). JF5 is unambiguous for *Aspergilli* without any cross-reactivity with other fungi that makes this assay fast and consistent. Hyphal secretion of antigen proteins during the progression of infection can be sensed in body fluids, and glycoprotein of cell wall is recognized by JF5. This assay might be assessed by EORTC/MSG consensus group that reflects an approach towards identification of protein antigens and diagnosis of *Aspergillus* infections. Also, JF5 based positron emission

tomography (PET) using magnetic resonance and PET guided by antibody to perceive lung infection by *A. fumigatus* was established (Rolle et al., 2016). In an investigation, cell wall proteins were identified in fugal pathogens displayed restricted sequence conservation seemed appropriate for IA diagnosis (Champer et al., 2016). In an IA infected mouse model, analysis of bronchoalveolar (BAL) by a proteomic DIGE was achieved and resulted into detection of major allergen Asp f 2 of *A. fumigatus* in sera of patients which is further used for the early diagnosis of IA (Fekkar et al., 2012).

6.3 Diagnosis based on T-cell

Diagnosis based on T cell has been developed as substitute detection techniques for antigen in the last few decades. In case of patients with IA, T cells specific for *Aspergillus* produced IFN-γ heftily that has been detected and correlated with the disease improvisation (Fekkar et al., 2012; Stuehler et al., 2015). On the contrary, IL-10 amplification was found to be stated with invasive diseases advancement (Hebart et al., 2002). Furthermore, patients at risk of developing IA possessed to have huge number of $CD4^+$ T cells in blood due to which clinical facilities do not use IFN-□ producing T-cells for prophylactic methods. In a study, IA infection has been recognized and determined in patients using flow-cytometer of fungal reactive T cells based on reactive expression of CD154 (Bacher et al., 2015). This diagnostic approach based on T cell is effective but also suggests the requirement of precise analysis of antigen-specific T cell reactions.

An antigen-reactive T cell enrichment (ARTE) method is based on antigen stimulation and assembling of reactive $CD4^+$ T cell, therefore it characterizes T cells specific for antigens with great precision with no *in vitro* operation. With the help of activated marker expression, CD154+ T cells and Treg (regulatory T cell) CD137+ is identified and discrimination is established between them (Bacher et al., 2013, 2014). Only a small percent of specific T cells distinguish each protein from all the recombinant proteins on screening that confirms that healthy individuals lack immunodominat proteins. A significant prospect of effector T cell reactions in individuals is that they might progress against antigenic molecules, showing not all proteins are secured by Treg cells suggesting only few proteins are the targets of Treg. Apart from Treg, T-cells differentiate into additional beneficial (Th1) or possibly detrimental T-cell subsets such as Th2-induced allergies (Werner et al., 2009). Therefore, link between antigen reaction and T cells in *Aspergillus*-associated infections is imperative to demonstrate immunity status of individuals for treatment strategies and diagnosis. An association amid T cells and antigen reaction would momentously enhance the probable opportunities for the development of antigen-specific immunotherapy.

7. Approach for vaccination in *Aspergillus* infections

Vaccines boost the humoral immunity of a host provides protection against pathogens that depends on the identification of specific antigens. The induction of memory generating immune elements as well as persisting antibodies are the most important considerations for long-term efficacy of vaccines. In an investigation, mice were infected with *A. fumigatus* prior the experiment and attained general immunity against total fungal infections (Lehmann & White, 1976). This study was confirmed by Ito et al. in a murine model, prior infection with less lethal dose of viable conidia administered through nasal structures was done and established pulmonary aspergillosis (Ito & Lyons, 2002) One of the earliest immunization study of turkey poults has been described. The first ever vaccine was developed from germlings of turkey poults by giving different growth of *A. fumigatus* (Richard et al., 1984).

Also, extracts of *A. fumigatus* mycelia are given hypodermically or syringing in venous for the treatment against disastrous IA.

The macrophages from an immunized or boosted mice transfer immunity induced by lethal infection with *A. fumigatus* to naïve mice, but not by serum (De Repentigny et al., 1993). In incompetent mice infected intravenously with systemic aspergillosis, antibodies are not involved in sustained mice as they are B cell deficient. In a study by Cenci et al., cell-mediated immunity showed that the defense response generated by vaccination through sinus that was prepared from either conidia of *A. fumigatus* or crude lysate could be transmitted from immune to harmless animal via antigen-specific $CD4^+$ T cells (Cenci et al., 2000). In addition, continuance of recombinant Asp f 3 (rAsp f 3) boosted mice diminished by depletion of $CD4^+$ T cells and transferred antifungal protection to non-immunized animals (Diaz-Arevalo et al., 2011). Studies performed until now have suggested cellular immunity based vaccine-mediated protection.

A vaccine based on fungal organism or crude extract is unlikely to be considered for human use, especially for immunocompromised individuals. The goal should be to generate antigens prepared in line with industry standards, GMP (good manufacturing practice) guidelines and regulations. Several studies have evaluated the vaccination potential of various antigenic preparations in recent years, such as proteins, polysaccharides, and combination vaccines, yet no licensed vaccines are available to provide protection to humans against aspergillosis and other fungal diseases (Bozza et al., 2002; Torosantucci et al., 2005).

Conclusions

Progress in proteomics, fast development of the understanding of fungal infection immunology and MS technology are expanding, with the focus on the creation of new diagnostic tools and immunotherapy for fungal allergies and diseases. A foremost concern is related to identification of carefully defined patients, distinctly IA patients because it obstructs the diagnostic methods and therapeutic applications in medical settings. Since there are many challenges in treating and managing aspergillosis patients with antifungal drugs, but immunotherapy showed potential in the improvement of antifungal therapy that eventually decline rates of mortality. In allogenic hematopoietic transplant recipients with IA, immunotherapy validated a great opportunity for intensification of T cells making it safer and efficient. Immunotherapy techniques used to treat *Aspergillus* infections remain exploratory and costly, with significant side-effects, and involve genetic and cellular operations. Numerous immunogenic antigen molecules have been acknowledged at the molecular level which might be possible antigen candidates in developing human vaccines. Vaccination is among the most important triumphs in public health history. However, effective fungal vaccines in clinics are indefinite despite the increasing numbers of persons with aspergillosis and other life-threatening fungal diseases. Regardless of remarkable development, ample work still needs to be done if vaccines against aspergillosis are expected to become a reality. With the considerable horridness and mortality associated with aspergillosis, it is strictly an affair of firm study without procrastination.

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