

Cathepsin B is a Negative Regulator in *Pulmonary Aspergillosis*

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Abstract—Lysosomal cysteine proteases play important roles in our immune-system by processing pathogens but their involvement in fungal mediated diseases remains unknown. In this study with the help of using various techniques such as immunoblotting, enzyme activity profile and ELISA, we observed that cysteine proteases (cathepsin B, L and C) are involved in fungal disease progression. In the mice model of pulmonary aspergillosis, cathepsin (CP)B and CPL activities were observed significantly high while the activity level for CPC was found low. Conversely, treatment of these infected mice with antifungal compounds showed statistically decreases in the activity of CPB ($p < 0.05$) without significant change in CPL and CPC activities, demonstrating CPB role in fungal infection. Notably, altered in the cytokines levels i.e., decreased in IL-4 and increased in IFN- γ in antifungal treated mice further support the resistant effect of antifungal compounds to fungal infection. Importantly, this alteration in cytokines levels is similar to CPB activity profile additionally depicts the regulatory effect of this protease in fungal infection. The involvement of lysosome in this process was confirmed by a lysosomal marker i.e., acid phosphatase. Present study reveals a novel interaction between cysteine protease and pulmonary aspergillosis and illustrates a possible negative role of CPB in fungal infection, due to that it could act as an important therapeutic target and early biomarker for this disease.

Index Terms—A. fumigatus, Cathepsins, Antifungal compounds.

I. INTRODUCTION

Aspergillus fumigatus is an opportunistic pathogen which can cause a life threatening invasive aspergillosis in immunocompromised patients. The respiratory tract is general portal for entry of *A. fumigatus* which responsible for major complication in AIDS, bone marrow transplantation and other immunocompromised patients [1, 2]. Its management is hampered by increased incidence and severity of adverse reactions to standard therapies [3]. The high incidence of aspergillosis in chronic granulomatous disease showed the importance of phagocytes in resistance to aspergilli. Cathepsin B and L are highly active enzymes and important for phagocytosis [4]. The increased cysteine

protease activities have been reported in a number of lung diseases [5]. Cysteine proteases (CP), acts as endopeptidases, are mainly involved in intracellular protein degradation. These enzymes are synthesized as inactive precursors and are regulated by several inhibitors and or by pH, for their lytic effects [6]. They are optimally active in the slightly acidic milieu found in lysosomes. On the tissue surface, they can degrade foreign proteins and the extracellular matrix. CPs also participates in proteolytic cascades which can lead to pathological damage, and facilitate the penetration of tissues by foreign cells. Additionally, cysteine proteases in lysosomes play another important role in the functional differentiation of MHC class II-restricted CD4+ T cells. Furthermore, MHC class II antigen presentation is under the control of the cytokines, especially IFN- γ [7, 8] which is known to potentiate the antifungal activity of macrophages. While, other cytokines such as IL-4 helps in proliferating fungal infection explains the beneficial or deleterious effect of cytokines on fungal infection [9]. In recent years, various approaches have been made to eradicate the aspergillosis including the identification of novel antifungal compounds from natural resources. Our group also identified and isolated 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrole-2-yl)-1-methylethyl pentanoate (DHP), a novel antifungal compound, from *Datura metel* [10] which is active at both *in vivo* and *in vitro* conditions. From the above fact and also from many published evidences it was found that cysteine protease activity not only increased abruptly very high in various pathological conditions but also has some correlation with macrophages/phagocytes. Nevertheless we didn't find any relationship between enzymes and diseases mediated by fungal infection. Therefore, we hypothesized a relationship between cathepsins and *A. fumigates* mediated diseases. In the present study, an attempt has been made to study the alterations in the activities of cysteine protease in mice model of aspergillosis and also studied their involvement in the disease progression and healing processes.

II. MATERIALS AND METHODS

A. Chemicals

The β -naphthylamide, -4-methoxy- β -naphthylamide substrates like Z-Phe-Arg-4m β NA, Z-Arg-Arg-4m β NA, Gly-Arg--4m β NA, Leu-4m β NA and β -naphthylamine and 4-methoxy- β -naphthylamine were purchased from Bachem Feinchemikalein AG, Switzerland. Fast Garnet GBC and Amphotericin B (AmpB) were procured from Sigma-Aldrich, USA. DHP was purified from *Datura metel* [11] and used as an antifungal compound AmpB. Cathepsin B

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(G60) and cathepsin L antibodies were purchased from Cell Signaling Tech (USA) and Lifespan Biosciences, Inc (USA) respectively.

B. Pathogen cultivation and Inoculum preparation

Clinical isolate of *A. fumigatus* (190/96) obtained from Vallabhbhai Patel Chest Institute, Delhi, India, was used along with standard strain (ITCC 4517, obtained from IARI, Delhi, India). Organism was grown on Sabouraud dextrose agar (Merck) plated at 37 °C for 4 days. The conidia were collected from the culture plates using PBS (pH 7.2) containing 0.05% Tween 80 (Sigma) and the suspension was filtered through sterile glass wool. The conidia were pelleted by centrifugation at 2000 rpm and re-suspended in PBS (pH 7.2). The number of conidia was counted and adjusted to 1×10^8 conidia/ml using hemocytometer. The viability of the conidia was determined by plating the dilutions of suspensions on Sabouraud dextrose agar.

C. Groups of animals and treatment

Ethical clearance was obtained from institutional ethics committee for the use of animals. Animals were divided into five groups of 9 animals in each group. The groups were designated as 1-5 (Table 1). Three days prior to infection with conidia, mice (group no 2 to 5) were injected subcutaneously with 3 doses (250.0 mg/kg/day) of cortisone acetate in 400.0 μ l of PBS. On the infection day, each mouse received approximately 2×10^7 conidia by nasal instillation of a single droplet of conidial suspension. Animals of groups no 3-5 were infected with *A. fumigatus* conidia [12]. Groups 4 and 5 were treated with six doses of antifungal compounds i.e. DHP and AmpB of 250 and 3.0 mg/kg body weight/day respectively [10]. The mice of group 1 received only PBS and acted as wild-type control group (WT). Group 2 animal were treated with cortisone only and used as a negative control (NC) while group 3 animals initially treated with cortisone and then infected with *A. fumigatus*, were used as positive control (also called infected group, I).

D. Tissue preparation

Animals were sacrificed on 7th day and lungs were isolated in aseptic conditions. Lysosomal fraction was prepared (per gram wet tissue weight) in 0.1 M sodium acetate buffer, pH 5.5 containing 0.2 M NaCl and 1mM EDTA, as described by Cohen *et al.* 2005 [13].

E. Assay for CPB, CPL and CBC

The activity for enzymes cathepsin B, L and C were carried out using their specific substrates i.e. Z-Arg-Arg-4m β NA (12.9 μ mole/ml DMSO) [14], Z-Phe-Arg-4m β NA (9.27 μ mole/ml DMSO) [15, 16] and Gly-Arg-4m β NA (2.17 μ mole/ml DMSO) [17] respectively. The activity units have been expressed as number of picomoles of 4-methoxy- β -naphthylamine or β -naphthylamine liberated per min per ml enzyme solution at 37°C.

F. Cytokines profile

Levels of IL-4 and IFN- γ in the blood samples of each animal group were determined by performing enzyme linked immune sorbent assay (ELISA) in immunoplates (Nunc, Maxisorb). The kit for the estimation of IL-4 and

IFN- γ were purchased from Pharmingen and ELISA (enzyme linked immunosorbent assay) was performed as per the manufacturer's instructions. The OD was read at 490 nm in an automated ELISA reader (Molecular Devices, Spectra Max 190).

G. Assay of Acid Phosphates

Acid phosphatase assay was performed according to the method of Shiloko and Tappel [18]. Briefly, lung lysosomal fraction was incubated with 32 mM p-nitrophenyl phosphate (PNPP, disodium salt) in 0.2 M sodium acetate buffer (pH 5.5) for 15 min at 37°C. The reaction was stopped with 0.16 M Tris-HCl (pH 8.5) containing 0.06 M K₂HPO₄. The reaction product was measured at 420 nm.

H. Western blotting

Lung tissue was homogenized in lysis buffer A (50 mM Tris-Cl, pH 8.0, 200 μ M NaCl, 50 mM NaF, 0.3% Nonidet P-40, 2 mg/ml leupeptin, 2 mg/ml aprotinin, phenylmethyl sulfonyl fluoride (PMSF), 0.5 mg/ml benzamidine, 1 mM dithiothreitol, and 1 mM sodium orthovanadate) and clarified by centrifugation as described by Goyal *et al* with slight modifications [19]. Lung extracts was normalized based on the amount of protein using the *Lowry's method* [20]. To check levels of cathepsin B and L, 100 μ g protein extracts from each sample was separated via 12% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblot with the cathepsin specific antibody. A 1:500 dilution was used for both primary antibodies (cathepsin B and L) and 1:1000 dilution was used for secondary antibodies. The same blots were stripped and blotted for total b-actin (1:1000, Cell Signaling Technology, USA). The bands were detected with enhanced chemiluminescence using a ChemiImager (Chemidoc, BioRad). Results was analysed and quantified by using Image J software (NIH, USA).

I. Statistical Analysis

Results are expressed as mean \pm S.D. The Student's *t*-test or ANOVA was used to compare quantitative data populations with normal distributions and equal variance. A value of *p* < 0.05 was considered statistically significant unless otherwise specified.

III. RESULTS

A. Optimum level of CPL in infected mice

To examine any correlation between cathepsins and pulmonary aspergillosis, *A. fumigates* infected mice were sacrificed every day after infection and lung tissue was collected to study the activity level of cathepsins. Interestingly, as shown in fig 1, a regular increase in the CPL activity was observed till 6th day and after then it starts decreasing gradually. To prove further this correlation between cathepsins and fungal infection, we made five mice groups in presence or absence of antifungal compounds (Table 1, Fig 2-3) and studied the cathepsins and cytokines profiles. Since CPL activity was observed optimum at 6th day therefore, sixth day was selected to sacrifice all the groups of mice and thereafter all experiments were performed.

B. Effect of *A. fumigatus* infection on the lysosomal cysteine proteases activities

After fungal infection, the activity level of CPL observed significantly very high (~2 fold) in infected group (I) while its level found decreased in animal treated with antifungal compounds (DHP and AmpB) compared to wild-type and negative control animals (Fig 2a). Similarly the activity level of CPB increased very high after infection and found decreased in their activity (more than 3 folds) in antifungal compounds treatment (Fig 2c). Surprisingly only in the case of CPB, we also observed elevation in the activity level of CPB in negative control (treated with cortisone only, an immune-suppressant) animals which is almost equally high as in infected group (I) when compared with wild-type control. Though it's not clear why the level of CPB got altered after use of immune-suppressant but on the basis of published reports [21] we predict that after infection, fungus also release some toxins that suppress the immune system which may responsible to maintain the CPB level, and results appearance of same activity level in both groups (2 and 3). To answered the question whether this change in activity profile of these enzymes due to fungal infection is only at activity level or it also effect the protein level, we also observed the protein expressions of these enzymes (cathepsin B and L) in all treated and non-treated lung samples by using western blots. Like the activity profiles of these enzymes, protein expression levels were also found very much similar for both enzymes i.e. for cathepsin B and L (Fig 2b and 2d) in all groups of mice. In other words, immunoblotting further confirms our activity findings. Furthermore, as shown in fig 2e, we observed slightly decreased in the activity level of CPC in infected group compared to wild-type and negative control. Moreover after antifungal treatment the activity level of CPC was not showing any significant difference from the level of infected group. Similarly we also didn't observe any alteration in CPH activity level in the lung lysosomal fractions of all five groups of the animals (data not shown). We used acid phosphatase as a marker for confirmation of lysosomal fractions and also tried to omit any chance of impurity with fungal secretory proteases. Fig 2f showed the activity profile of acid phosphatase which depicts that our work was done only with lysosomal fractions of the lung tissue.

TABLE 1: VARIOUS GROUPS OF ANIMALS AND THEIR TREATMENTS

Group of Animals	Treatment	Symbol Used in Figures
Group 1	Wild-type control (Normal)	WT
Group 2	Mice treated with Cortisone, Negative Control	NC
Group 3	Mice infected with <i>A. fumigates</i> (Aspergillosis Model), Infected group, Positive Control	I
Group 4	Aspergillosis model treated with DHP	DHP
Group 5	Aspergillosis model treated with AmpB	AmpB

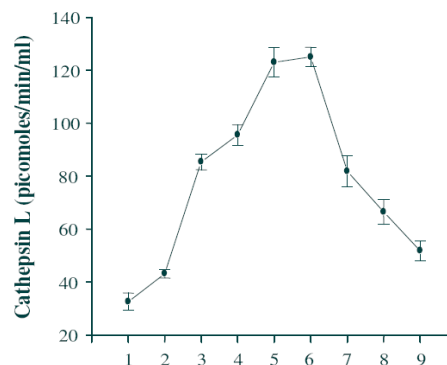
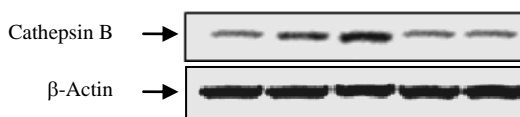
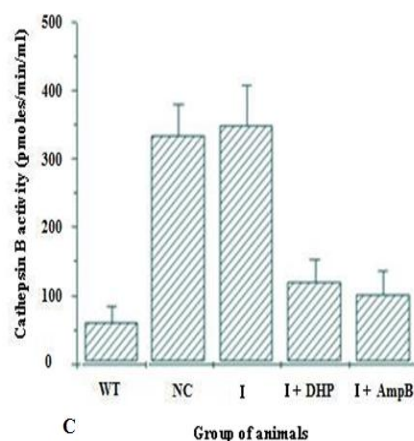
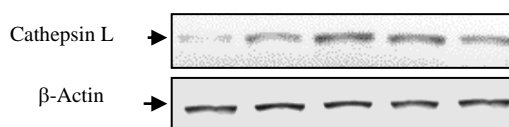
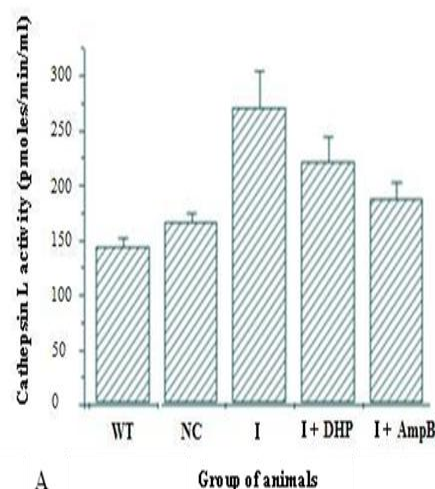


Fig.1. Activity levels of CPL in the *A. fumigatus* infected mouse lung. The expression of cathepsin L was optimum on 6th day. Mice (n=6) were sacrificed every day to measure the CPL activity level in lungs.



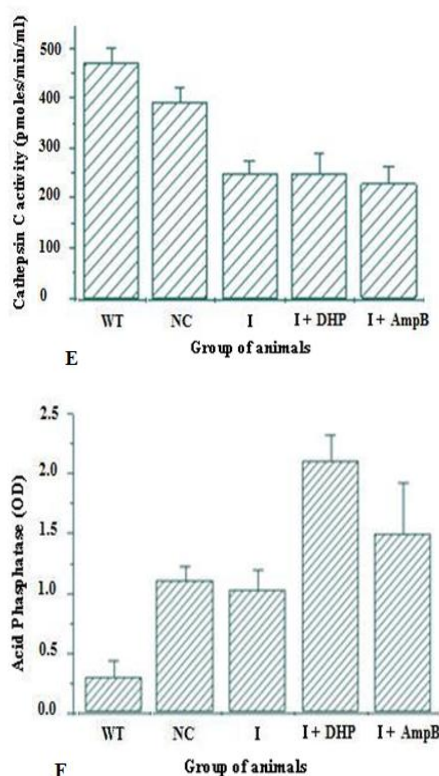


Fig 2. The effect of *A. fumigatus* and antifungal compounds on cysteine proteases activities in each experimental group (n=9) (WT, wild-type control; NC, negative control; I, infected group, I+DHP & I+AmpB, after fungal infection treatment with antifungal compounds) were measured and expressed in pmoles/min/ml. (A & C) Represent the activity levels for CPL & CPB in each group of mice respectively; (B & D) Represents the CPL & CPB protein expression in all groups of mice (as explained in Table1). Results were analyzed by western immunoblot by using CPL & CPB antibodies respectively; (E) Represents the activity level for CPC and; (F) acid phosphatase activity (a lysosomal marker).

C. Alteration in the level of IL-4 and IFN- γ in antifungal compound treated group

Some cytokines (IL-4 and IFN- γ) are well reported to be involved in fungal mediated infections and play the essential role in immune-functional differentiation. To see the effect of *A. fumigatus* on these cytokines levels in our experimental model, we also studied the profiles of these cytokines in all five groups. As shown in fig 3a and fig 3b, the animals infected with *A. fumigatus* conidia and then treated with antifungal compounds (AmpB and DHP) were found decreased in the levels of IL-4 significantly i.e. approximately 4 folds while observed elevation in IFN- γ compared with infected group (group 3).

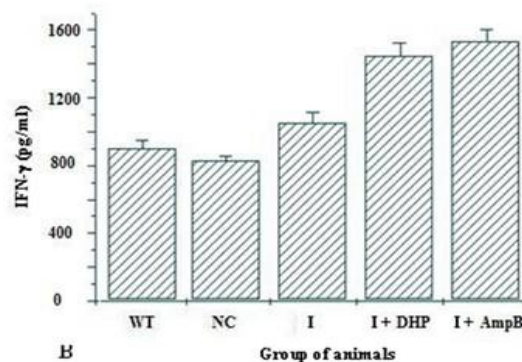
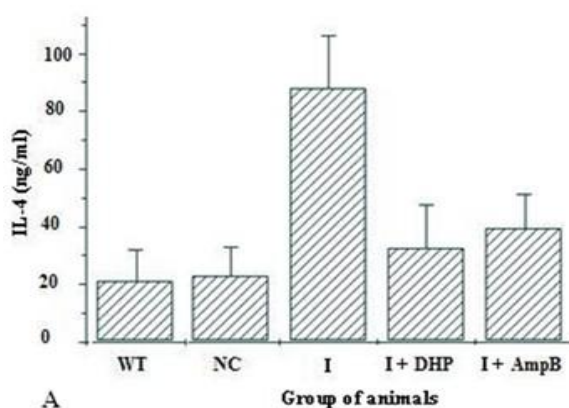


Fig. 3. The expression levels of IL-4 and IFN- γ in various groups were measured and represented in ng/ml and pg/ml respectively. (A) Represents the IL-4 levels and (B) Represents the IFN- γ level in the blood samples from each experimental mice group.

IV. DISCUSSION

There are many reports on the immune system impaired by *A. fumigatus* but scarcity of data is available related to its interaction with tissue lysosomal cysteine proteases. Cysteine proteases are very well reported in lung disorders [22, 23]. Therefore, the data of the present study provides strong evidence for relationship between lung tissue lysosomal cysteine protease and Aspergillosis.

A. *Fumigates* induces Alteration in the Cysteine Protease Activity

As reported in various disorders, we also found an increased in the CPL and CPB activities in the lung tissues of *A. fumigatus* infected mice compared to control. However after treatment with antifungal compounds i.e. DPH and AmpB, we observed 3-fold significant (~3 folds) reduction in CPB activity (Fig 2c). While, at the same time both compounds were found to stabilize the increased activity of CPL as compared to the negative controls (Fig 2a). In other words, slightly decreased in CPL activity was observed after antifungal compound treatment. This decrease in the activities of CPB and CPL indicates the importance of these enzymes and more specifically for CPB in host defense system against *A. fumigates* mediated disease. These results were also confirmed by western blot (Fig 2b and d) which further explain the regulatory effect of cathepsins in fungal infections. Furthermore, the activity level of other cysteine protease i.e. CPC, was found low in all groups of infected mice (treated as well) as compared to wild-type and negative control groups (Fig 2e). Through this CPC data, it's very hard to explore any direct relation between fungal disease and CPC but decreased in the activity level of CPC illustrates the possibility of its involvement as an essential component of immune system. It is also possible that fungal sp may secrete some irreversible inhibitors for this type of proteases which inactive the enzymes even in presence of antifungal compounds. Similar to our study, other research groups also explained that CPC deficiency results in considerably milder immune deficiency [24]. Study on another cysteine protease i.e. cathepsin H activity level was found normal in all groups of mice (Data not shown). This present available cathepsins data explored the possibility of a relation between lysosomal cysteine proteases and fungal infection.

CPB was found more effective in *A. fumigatus* mediated disease progression and regulation of activity of CPB could be effective in fungal treatment. This existence of lysosomes enzyme was further confirmed by the level of acid phosphatase (Fig 2f) which suggest that lysosomal activity is playing an essential role in fungal infection.

B. *Fumigates* induces Alteration in the Cytokines Profiles

Acute invasive aspergillosis is challenging disease because of involvement of various confounding factors including cytokines. Some cytokines may have beneficial or deleterious effect on fungal infection. Specifically, Th1 and Th2 pathways directly relate to the severity of infection. Th1-produces cytokines (IFN- γ) activate macrophages, the key effectors in invasive aspergillosis whereas Th2 cytokine, IL-4 has worse outcome [9]. According to available evidence, IL-4 knock-out mice show deficient Th2 response and found more resistant to infection [9]. In continuation of this, we further confirm these findings in our experimental models by observing the profile of cytokines (IFN- γ and IL-4) in presence of *A. fumigates* and antifungal compounds. As shown in figure 3a, after infection, the level of IL-4 was increased very significantly while in presence of antifungal compounds it comes to the level of control. However, the level of IFN γ (Figure 3b) is behaving opposite to IL-4 under both conditions. Importantly, these alterations in the pattern of cytokines levels correspond to the change in the CPB activity profile (Figure 2c). Furthermore, lysosomal cysteine proteases are also known to be involved in the antigen processing and in differentiation of functional CD4⁺ T cell subsets [25]. By using various cysteine protease specific inhibitors, the roles of CPB and CPL are well established in immune system. CPB inhibitor CAO74 was reported to change the digestion pattern of lysosomal enzyme and modulate the immune response from Th2 type to Th1 type [26]. On the other hand inhibitor of CPL potentiates Th2 type immune response [27]. Overall, to our present findings, we observed decrease in the lysosomal CPB activity and IL-4 level while increased in the IFN- γ level in presence of antifungal compounds (AmpB and DHP) which indicates the possibility in modulation of immune response from Th2 type to Th1 type. Additionally, from cancer research [28, 29] it's always observed decreased immunity of the patient and result increase in the chance of fungal infection and also found elevation in the CP and IL-4 levels without any correlation among them. From our present study, it is possible to predict about these changes. We are hypothesizing that after fungal infection, alteration in the cathepsins levels occur which may involve in tissue remodeling and result affect the surfactant proteins such as IL-4 that further alter the person immunity. Therefore, use of cancer medicines and antifungal drugs along with antithiol protease may be a beneficial combinatorial therapy approach for cancer patients.

V. CONCLUSIONS

In recent years, various approaches have been made to eradicate the Aspergillosis. Despite significance advancement in antifungal therapies, overall mortality rate is still very high. Importantly, early diagnosis is difficult

because of lack of desired specificity and sensitivity approaches. In our study, we found CPB specificity towards *A. fumigatus* mediated infection which increased with fungal infection and decreased with antifungal treatment vary significantly. By studying the activity as well as immunoblotting profiles of CPB, we could use this enzyme for early diagnosis purpose and possibly it can be used as a therapeutic target for aspergillosis. In summary, a correlation between cysteine protease and fungal infection was observed which opens a new paradigm for tissue lysosomal enzymes in fungal mediated diseases. Further studies need to be done to characterize other specific cysteine protease and also needs to elaborate the characterization of CPB in fungal infections.

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