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Cytokine milieu in renal cavities of immunocompetent mice in response to intravenous challenge of *Aspergillus flavus* leading to aspergillosis

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ABSTRACT

Investigations in mice have demonstrated that *Aspergillus flavus* is more virulent than all other *Aspergillus* species except *A. tamari*. However, there is a complete lack of information on the immune responses elicited by *A. flavus* in systemic model. This communication reports the progression of infection and cytokine profile in BALB/c mice in response to intravenous challenge *of A. flavus*. The pathogenesis of infection was evaluated morphologically and by the analysis of Colony Forming Units (CFUs) in kidney homogenates. The kinetics of regulated cytokines was determined in kidneys by cytokine-specific murine ELISA. During the initial phase of infection the rate of clearance of *A. flavus* was high, most likely through recruited neutrophils and the resident renal macrophages with concurrent significant release of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-12/IL-23p40, IL-6) indicating antifungal innate immune response to be active at the site. However, at 24 h PI there was a significant rise of IL-17 and IL-23 suggesting the activation of IL-17/IL-23 axis of inflammation resulting in rise of CFU. The lack of significant induction in the anti-inflammatory cytokines like IL-4 and IL-10 confirmed the absence of Th2 type of response. In the late phase, after 3 days post-infection, there was a rise in the number of pathogen in the kidneys as determined by histopathology and CFU counts. The *A. flavus* hyphae were evident in the renal pelvis and ureter and we propose the production of blastoconidia by metamorphosed hyphae.

1. Introduction

The mold *Aspergillus flavus* causes a broad spectrum of disease in humans, ranging from hypersensitivity to invasive infections associated with angioinvasion. After *Aspergillus fumigatus*, *A. flavus* is the second most leading cause of invasive and noninvasive aspergillosis [1,2]. Studies in non-immunocompromised murine models have reported *A. flavus* to be more virulent than almost all other *Aspergillus* species, with only *A. tamarii* having little higher virulence [3]. More recently, studies in both normal and immunocompromised mice have demonstrated that LD_{90} inocula for *A. flavus* are 100-fold lower than those required for *A. fumigatus* [4,5]. Following intravenous administration of *A. flavus* spores in non-neutropenic mice, the infection rapidly concentrate in the liver and lungs within 4 h. The fungal burden in the lungs rapidly declines by 95% over 24 h whilst the burden in the liver declines more slowly for 5 days following infection. In contrast, the burden in the kidneys and brain increases until a lethal burden develops 5–10 days post-infection [3]. The precise cause of death in mice with disseminated infection has not been characterized but tissue burdens immediately before death are much lower than that occurs in *A. fumigatus* infections [6]. It is clear that aflatoxin is not a major factor in disease development as strains that are unable to produce aflatoxin *in vitro* are equally virulent [6]. Additionally, aflatoxin-producing strains cause infections in which the toxin is undetectable in tissues [6].

Climate and geographical factors are important determinants of the local prevalence of *A. flavus* infections. In countries like Saudi Arabia and Sudan, with semi-arid and arid dry weather conditions, *A. flavus* is one of the main etiological agent of aspergillosis [7,8]. It is also one of the main pathogens responsible for pulmonary aspergillosis in Africa [9]. For unknown reasons, the frequency of infections caused by *A. flavus* is also elevated in some hospitals, in different locales.

Studies in mice have indicated that both innate [10] and acquired immunity [11–15] contribute in the protection of mice to aspergillosis and other fungal infection. In particular, in the invasive aspergillosis production of Th1 and Th2 cytokines occur differently in mice resisting or succumbing to the infection [13]. Even, mice inoculated with heat killed *Saccharomyces cerevisiae* as a vac-

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cine has been reported to induce Th1 type of immune responses in mice model [16]. Experimental models of infectious diseases have advanced our knowledge regarding the role of cytokines in developing protective immunity and immuno-pathogenesis of infections. Cytokines regulate both the initiation and maintenance of the immunological response, by orchestrating the mechanisms underlying innate and adaptive immunity to fungal pathogens [17]. In an earlier communication, we reported the cytokine profile and immune cell recruitment in response to intratracheal dose of *A. flavus* [18]. While these studies suggest a directive role of cytokines in leukocyte-mediated defense against *A. fumigatus* and *A. flavus* alveolar infections, the cytokine profile and their contribution to susceptibility in intravenous model of infection has not been reported earlier. Previous studies have depicted kidney as the primary sites of the progressive *A. flavus* infections [6]. In the current study, immunocompetent BALB/c mice were intravenously challenged to a virulent strain of *A. flavus*, and the pathogenesis of infection was evaluated morphologically and by the analysis of Colony Forming Units (CFUs) in kidney homogenates. The kinetics of cytokines was measured in kidneys by cytokine-specific murine enzyme-linked immunosorbent assays (ELISAs).

2. Materials and methods

2.1. Animals

The immuno-competent male BALB/c mice (5–6 weeks) were obtained from Indian Institute of Toxicological Research (IITR), Lucknow, India. Mice were maintained as per the guidelines of Indian National Science Academy (INSA). Experimental design was approved by Council of Scientific and Industrial Research (CSIR), Government of India. Mice were monitored throughout the study and were found to be free from the diseases as well as ecto- and endo-parasites. The animals were maintained on a standard diet (mice chow) and water *ad libitum*. Mice were acclimatized for 1 week at the facility prior to beginning of the experiment. Only 6–7 week old male mice were used throughout the study.

2.2. Isolation and preparation of A. flavus inoculums

Aspergillus flavus was isolated from the garden soil of Patna, India. The pure culture was identified and documented at Microbial Type Culture Collection and Gene Bank (MTCC) as *A. flavus* MTCC 9367. The mold was sub-cultured on Sabouraud-dextrose agar yeast (SDAY) plates and incubated for 4 days at 30 °C. Conidia were harvested gently by scraping the surface of culture with the help of a sterile wire loop and collected in vials containing 5 ml phosphate buffer saline (PBS), pH 7, and 0.01% (v/v) sterile polyoxyethylene sorbitan monooleate (Tween-20, Merck) solution. The concentration of conidia in different suspension was determined by direct count using Neubauer hemocytometer at 400× magnification. Serial dilutions were prepared in sterile PBS containing Tween-20 as above. The viability of conidia was determined by plate count technique on SDA [18] and was found to be in the range of 90–95%. Freshly prepared conidial suspensions were used throughout the study.

2.3. Animal infection

Previous report suggests that the main target of infections in the intravenous challenge model is kidney and brain (3). To find out the pathogenic potential of *A. flavus* isolate, we initially infected BALB/c mice intravenously through lateral tail vein with different concentration of conidia. The pathogenicity and the induction of cytokines, was determined by an intravenous injection (through the lateral tail vein) of 3.3×10^5 conidia/0.5 ml of *A. flavus* (MTCC 9367).

2.4. Experimental protocol

In order to determine the renal immune responses and sequential events in infection, mice were intravenously injected with the conidial dose (3.3×10^5) of *A. flavus*. Infected and uninfected control (0.5 ml of PBS + 0.01 v/v of Tween-20) animals were sacrificed by cervical dislocation at different hours post-infection (h PI). Kidneys were excised out, homogenized and 0.1 ml of the homogenate was spread over SDAY plates to determine the Colony Forming Units (CFUs). Twenty mice were also infected to follow death rate. In general ten mice per time point was used for the cytokine analysis and CFU counting from *A. flavus* infected group of mice. Cytokine profile from six vehicle treated mice was used as control.

2.4.1. Viable burdens of A. flavus in the kidneys

At specific h PI 10 mice were euthanized, their kidneys were removed and homogenized in 5 ml of sterile PBS under aseptic condition. Fungi were quantified in 0.1 ml of the homogenate by plating 10-fold dilutions onto Sabouraud-dextrose agar and incubated for 48 h at 30 °C. Three replicates per time point per mice were used for the enumeration of Colony-Forming Units (CFUs) and the average was expressed as $\log_{10}$ CFU in kidneys.

2.4.2. Histological examinations

The kidneys of three infected mice (euthanized as above) were removed at 48 and 96 h PI, fixed in 10% neutral buffered formalin (10% formalin containing 4 g sodium dihydrogen orthophosphate monohydrate and 6.5 g disodium hydrogen orthophosphate anhydrous per liter of solution), and embedded in paraffin. Ultra thin sections (5 μm thick) were either stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) counter stained with hematoxylin (Mayer's). The stained sections were viewed at different magnification under the Olympus microscope (CX-41) with photographic attachments. The cell recruitment (inflammatory) and fungal growth were investigated in particular during the microscopic examinations. Specifically recruitment of cells in the sections was determined by blinded scoring method.

2.4.3. Cytokine analysis from kidneys (whole organs)

Ten mice were humanely euthanized and the kidneys from each mice were homogenized with 5 ml of PBS. Kidneys homogenates were stored at −20 °C until used for cytokine analysis. The homogenate was subsequently centrifuged at 10,000 RPM for 5 min and the supernatant was used for the analysis of cytokines. The titers of different cytokines in control mice (treated with 0.5 ml of vehicle) were also determined in six mice at each time point post-inoculation. Kidneys were assayed for Interleukin-4 (IL-4), IL-6, IL-17, IL-23, Interferon-γ (IFN-γ), Tumor necrosis factor (TNF-α), IL-10 and IL-12/IL-23p40 at the specified h PI, using the eBioscience ELISA kits (San Diego, USA). The protocol of manufacturer was followed for the analysis of cytokines.

2.5. Aflatoxins detection in kidneys

The *A. flavus* demonstrated a very high virulence and resulted in the death of mice at very low doses. We questioned whether the strain produces aflatoxins, a potent carcinogen during *in vivo* growth and multiplication and resulted in the death of mice. The AgraQuant total aflatoxins detection kit (Romer Labs, Inc., America) was used for the analyses of the aflatoxins in the kidney homogenates as per recommendations of the supplier.

2.6. Statistical analysis

The quantities of different cytokines in the samples were determined using the MasterPlex ReaderFit software (Hitachi Software

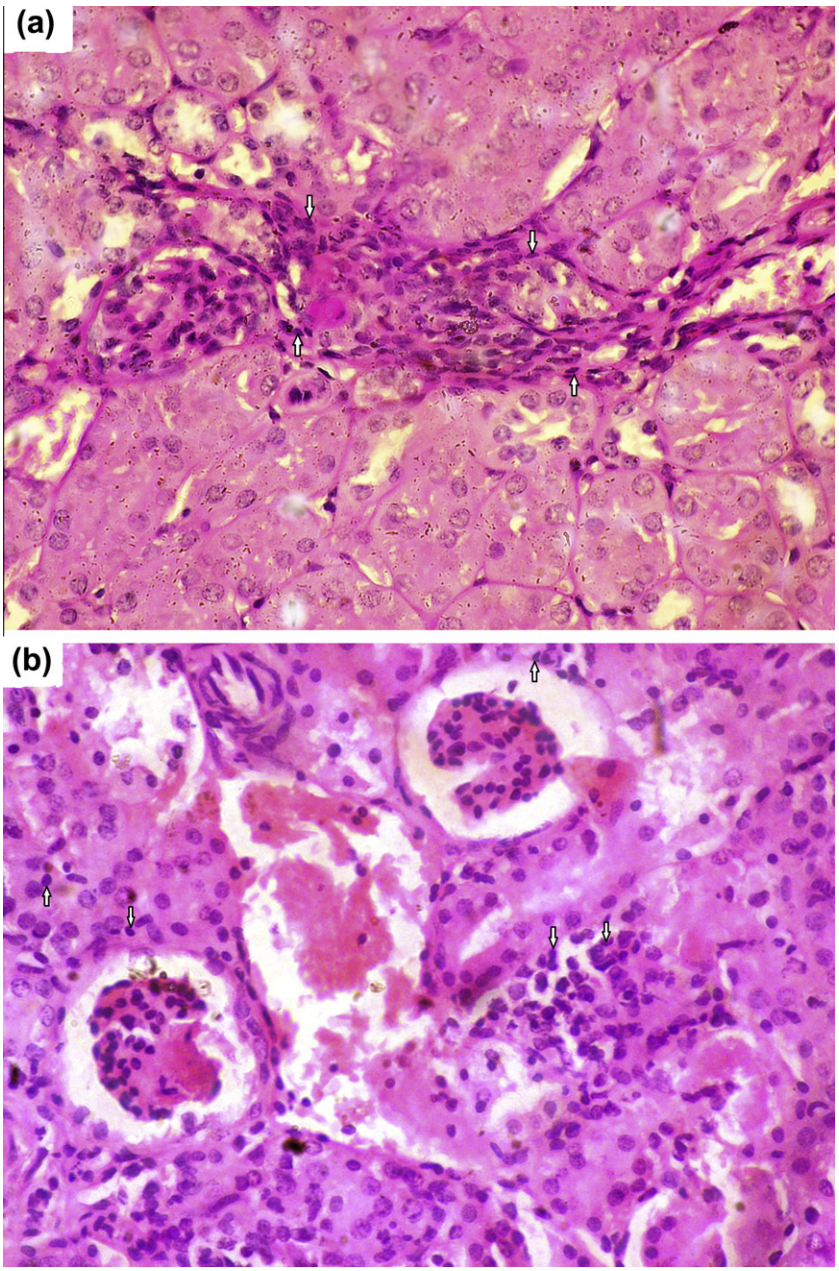

Fig. 1. Kidney tissue sections of BALB/c mice infected intravenously with 3.3×10^5 conidia of *Aspergillus flavus* (MTCC 9367). The microphotographs are (a) from 48 h PI mice and (b) from 96 h PI mice showing the leukocyte infiltrations. The sections of kidneys were stained either with hematoxylin–eosin or PAS–hematoxylin (Mayer's). The solid arrows denote the influx of neutrophils and lymphocytes. Magnification was 400×.

Engineering America Ltd., USA), applying the best curve fit method. Standard errors and deviations were calculated using MS-Excel. The student *t*-test for independent variables at a significance level of 0.05 was also performed using the Microcal (TM) Origin Software from Microcal Software, Inc., USA. The *t*-tests were performed in comparison with control and also between the samples when specifically mentioned. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Pathogenicity of systemic infection in BALB/c mice

In order to determine the virulence of *A. flavus* isolate we administered different doses of conidia, intravenously. Even with 3.3×10^4 conidia there was 100% mortality in the mice within 10 days. The mice died earlier in about 4–7 days with a ten-fold increase in the number of conidia injected (3.3×10^5). The external signs of infections such as lethargy, restricted feeding, impaired movements, bending of head and crowding at a place were visible between 72 and 96 h PI. Further after 72 h when the mice were lifted with tail they showed a circular movement. The symptoms appeared early with the increase in the conidial doses.

3.2. Rate of growth of fungi from infected kidneys

The rate of *A. flavus* growth from infected kidneys was determined by culture of homogenates. Approximately 8.0×10^3 organisms were recovered from the kidney of mice at 6 h PI. Within the next 6 h the fungal burden in the kidney reduced about 6-fold i.e., 1.4×10^3 CFU followed by a zigzag pattern with a final detection of 4.4×10^3 *A. flavus* CFU from the kidneys at 96 h PI.

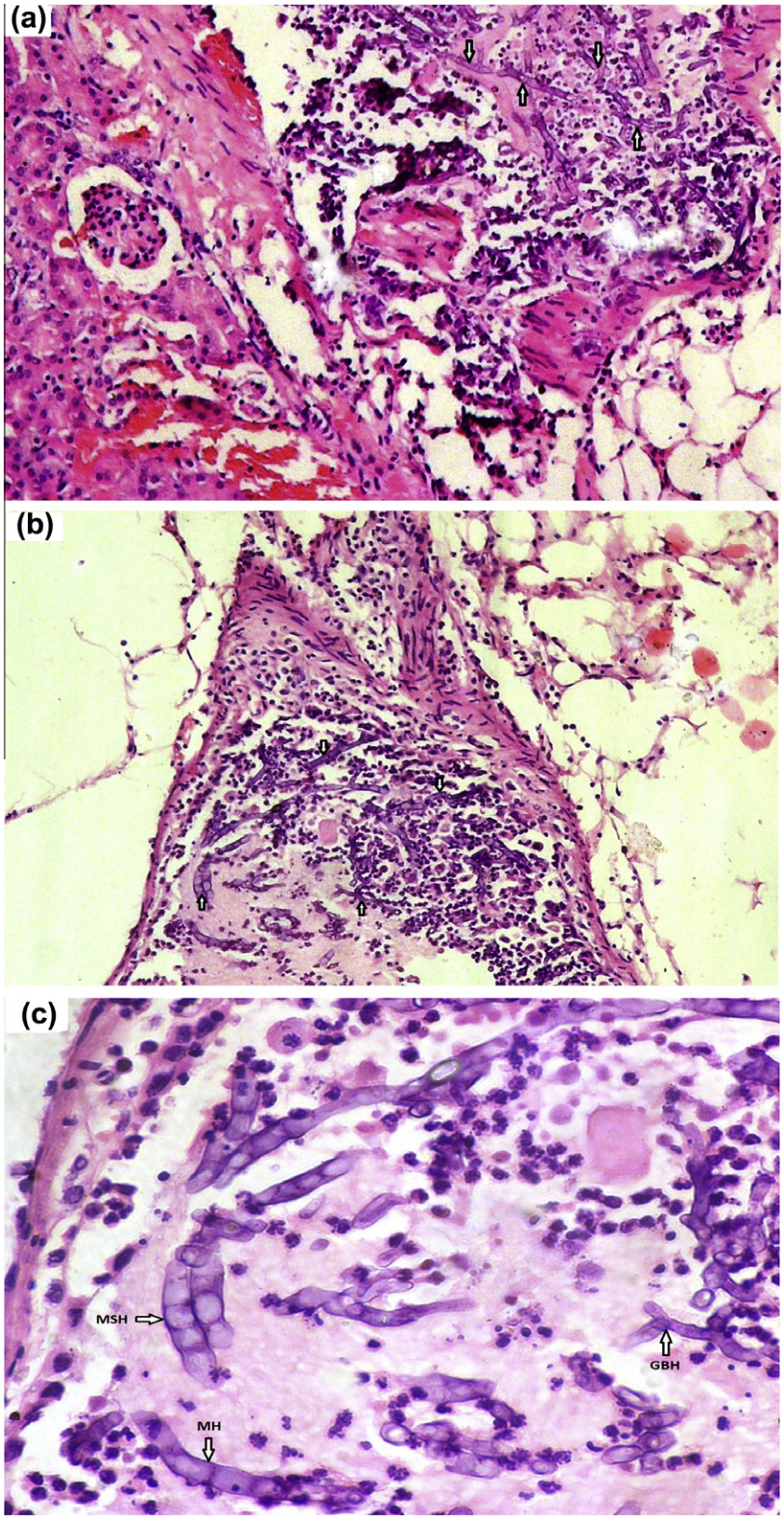

Fig. 2. The BALB/c mice infected intravenously with 3.3×10^5 conidia of *Aspergillus flavus* (MTCC 9367) were humanely euthanized and the kidneys were histologically analyzed. The section showing the presence of fungal growth in ureter and pelvis of kidneys sacrificed at fourth days post-infection (a and b). The fungal hyphae were septate, branched and both growing and mature hyphae were present (c). In some sections, the blastoconidia coming out of the transformed hyphae were also evident (d and e). The sections of kidneys were stained either with hematoxylin–eosin or PAS–hematoxylin (Mayer's). Magnification was 400× (a and b) and 1000× (c, d, and e). The solid arrow in (a and b) denotes fungal hyphae. In (c–e) the solid arrows denotes, MSH: mature and septate hyphae, MH: mature hyphae, GBH: growing and branched hyphae, MHPB: metamorphosed hyphae producing blastoconidia, B: blastoconidia.

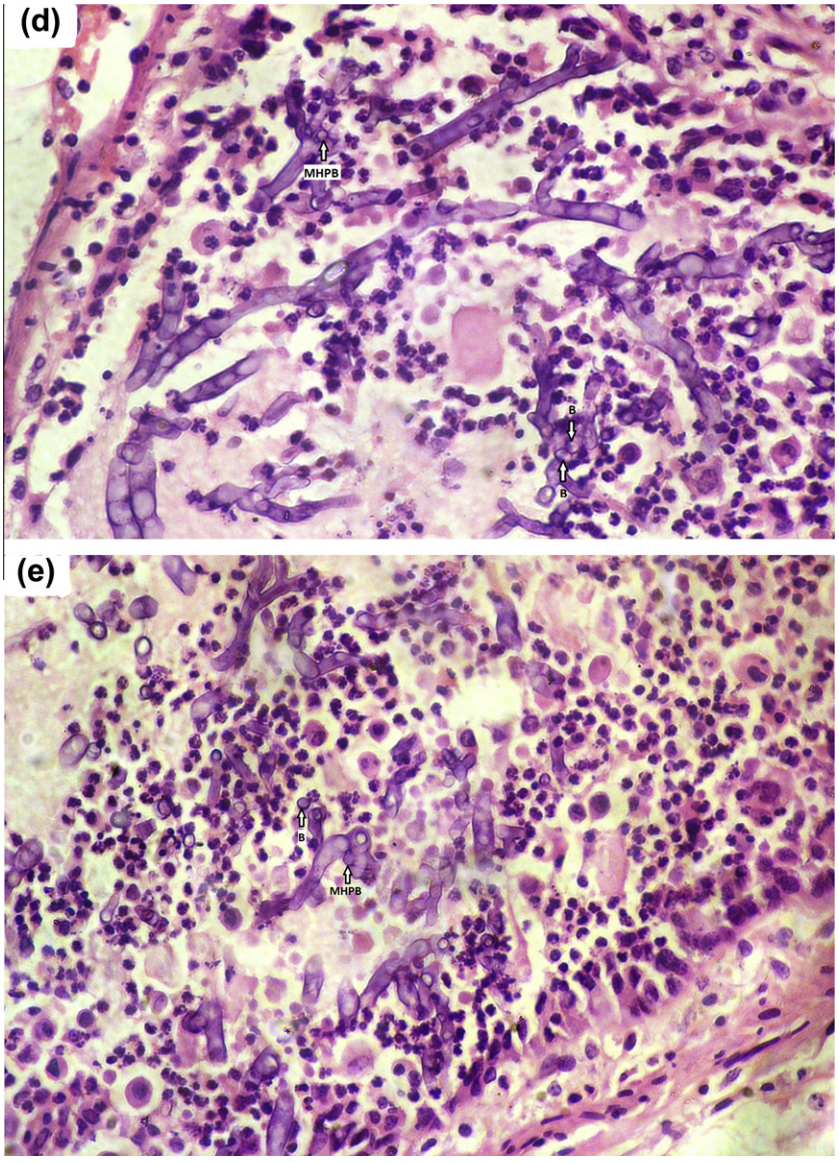

Fig. 2. (*continued*)

3.3. External morphology and histological examinations

As the mice died with progressive infection in kidneys, we next focused our efforts on the morphological and histological analysis. The external symptoms of kidneys infection like white-pinkish patches were visible in the mice sacrificed at 96 h PI. Out of five mice sacrificed at the last time point, we observed unilateral hydronephrosis (one kidney filled with serum like fluid) in two mice. Further the size of kidney filled with fluid also increased several fold. Since the fungal CFUs first decreased and further increased in the kidneys, we next tried to find out the real picture about what was happening inside the kidneys by examining the kidney tissues histologically under microscope. The histological sections of kidneys showed infiltration of leukocytes in the kidney tissues from the mice sacrificed at second day (Fig. 1a). The kidneys sections of 96 h PI showed highest recruitment of neutrophils followed by macrophages/monocytes and lymphocytes (Fig. 1b). We invariably isolated organisms from the homogenates of kidneys; however, our search for the fungal hyphae in the kidneys sections was successful only in the ureter and pelvis of mice sacrificed at third and fourth days post-infection (Fig. 2a and b). The fungal hyphae were septate, branched and both growing and mature hyphae were present (Fig. 2c). Although the conidia were observed, all the attempts to locate the fruiting body of *A. flavus* in the kidneys were unsuccessful. Therefore, we hypothesize that the conidia to be the blastoconidia and in several sections, the blastoconidia coming out of the transformed hyphae were also evident (Fig. 2d and e).

3.4. Renal inflammation

3.4.1. Inflammatory cells in kidney

Initially the resident macrophages and renocytes were responsible for the inflammation and phagocytosis. At the end of second day there was massive leukocyte influx (Average leukocytes in diapediasis: 10×10^3/section and average monocytes/macrophage: 81/section). At later time points there was large influx of neutrophils in ureter area where the fungal growth was evident (Fig. 2c and d). The high neutrophil influx (6.7×10^3 neutrophils/section) and the concurrent rise in monocytes/macrophage (4.3×10^3 monocytes/macrophage) and lymphocytes (1.3×10^3 lymphocytes/kidney section) demonstrated fungal pathogen specific renal inflammation in the mice at the end of fourth day. The growing

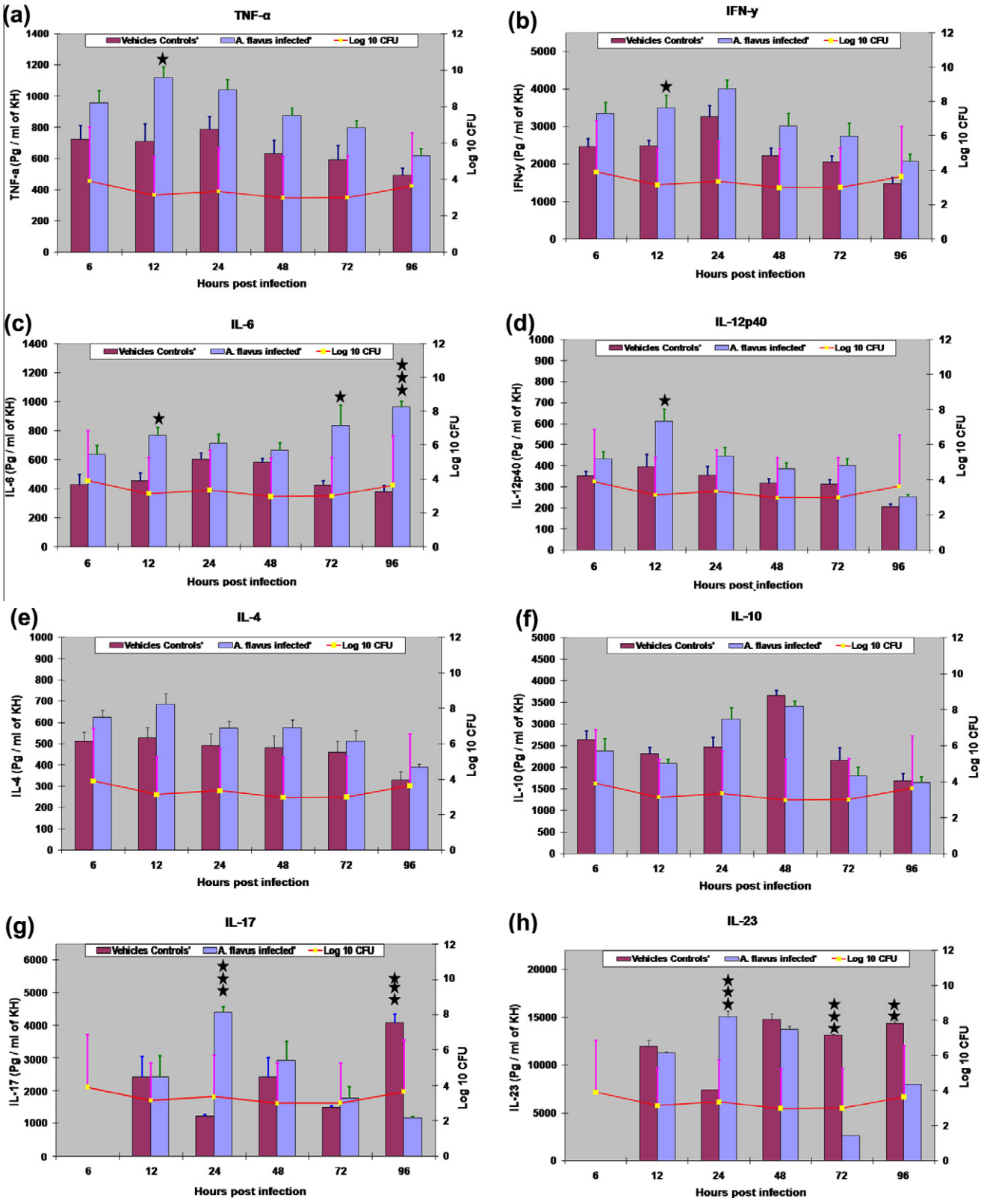

Fig. 3. The profile of pro-inflammatory cytokines; TNF-α (a), IFN-γ (b), IL-6 (c), IL-12p40 (d) and anti-inflammatory/Th2 cytokines IL-4 (e), IL-10 (f), IL-17 (g) and IL-23 (h) at different hours post-infection in the kidneys homogenates (KH) of BALB/c mice infected intravenously with 3.3×10^5 conidia of *Aspergillus flavus* (MTCC 9367). The control treated with vehicle has been marked as vehicle controls in the figure legend. Results of the experiment are depicted and reflect the mean ± standard error of mean of 10 and 6 animals per time point for treated and vehicles control groups respectively. Two replicates of each sample were assayed and the average of the two was used for further statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}P < 0.001$.

hyphae attracted more neutrophils than the old or matured hyphae. Further both the blastoconidia and transformed hyphae producing blastoconidia were relatively free of neutrophils or macrophage attack in the ureter (Fig. 2d and e).

3.5. Cytokines in kidneys of mice systemically infected with A. flavus

In the histological sections we observed fungal growth and multiplication in the kidneys, therefore, in order to gain insight into the potential role of different cytokines in the pathogenesis of renal *A. flavus* infection, temporal induction of cytokines were assessed in whole kidneys of infected and control mice using murine cytokine-specific ELISA. Analysis of cytokines from kidneys homogenates allowed the quantification of total cytokines present in the kidneys. Results demonstrated that the pro-inflammatory cytokines TNF-α and IFN-γ increased significantly at 12 h PI followed by a steady decline with no significant change further during investigations (Fig. 3a and b). The pro-inflammatory cytokine IL-6, like the other pro-inflammatory cytokines enhanced significantly at 12 h PI followed by a steady decline. However, its production in the kidneys again increased significantly at late phase of infection as observed at third and fourth day (Fig. 3c). The IL-12/IL-23p40 subunit like other pro-inflammatory cytokines increased significantly at 12 h PI followed by a steady decline with no significant changes (Fig. 3d). The inflammatory axis due to IL-17 and IL-23 got activated at 24 h PI in the infected group of mice (Fig. 3e). There was no significant increase in the anti-inflammatory cytokine IL-4 at all the experimental h PI ($p > 0.05$), however, the cytokine demonstrated declining trend just like pro-inflammatory cytokines (Fig. 3e). The level of anti-inflammatory cytokine IL-10 showed no significant rise in comparison to control at all the time points during investigation. However, the titer of IL-10 was highest at 48 h PI (Fig. 3f).

3.5.1. T helper cell response

We observed a significant rise in pro-inflammatory cytokines (TNF-α, IFN-γ and IL-12/IL-23p40) at 12 h PI followed by a steady decline for cytokines TNF-α and IFN-γ (Fig. 3). On the other hand, the level of Th2 cytokines (IL-4 and IL-10) demonstrated no significant change during the current investigations confirming the absence of Th2 type response. At later time points on third and fourth days of infection there was a significant rise in IL-6 however, in the absence of rise of IL-17 and IL-23 we can presume that the activation of Th17 pathways at later time points did not occur in our experiment (Fig. 3).

3.6. Aflatoxin screening in kidneys

Since our *A. flavus* strain produced aflatoxins in *vitro* assay, we performed the aflatoxin detection assay in kidneys homogenates. However, no detectable aflatoxins were observed in the infected kidneys homogenates.

4. Discussion

To the best of our knowledge there is no report on immunopathogenesis caused by *A. flavus* in the intravenous challenge model. In this report we describe the host response to *A. flavus* in intravenous infection model in BALB/c mice. By studying the sequential events during the developments of infection in kidneys, we evaluated the chronology of events that characterize inflammatory cell recruitment, fungal growth, release of cytokines and blastoconidia production.

Histological and CFUs studies demonstrated two phase of host defenses in renal cavities of mice most likely by the resident macrophages and renocytes leading to intense clearing of fungi only up to first half day followed by increase in the viable fungal burden. The rise in the viable fungal burdens between 12 and 24 h PI resulted in the secretion of IL-17 and IL-23 by the immune system of host. The IL-17 and IL-23 axis of inflammation is known to recruit the leukocytes mainly neutrophils [19]. We also observed the influx of leukocytes (leukocytes in diapediasis) in our histological examinations at 48 h PI. The influx of leukocytes led to the decrease in the viable fungal count at the end of second day. Further at 72 h PI the fungal burden in kidneys was almost stable (950–1000). However the fungus established itself and led to the increase in the viable fungal burden at 96 h PI. The increase in conidia after 12 h PI may possibly be due to rise in the number of conidia coming from the body fluid during the filtration process. At the end of fourth day post-infection histological sections demonstrated hyphae and germinating conidia in the ureter and renal pelvis. Although, we found the conidia in the histological sections, attempts to locate fruiting bodies of the fungi were unsuccessful. The microscopic examination of histological slides revealed the production of conidia directly from metamorphosed hyphae, which led us to hypothesize that the *A. flavus* grows *in vivo* by producing blastoconidia. This is perhaps first report demonstrating the *in vivo* production of blastoconidia by *A. flavus*. Furthermore, we observed a greater number of neutrophils attached to young and growing hyphae than the older ones and a very less PMNL attached to blastoconidia and the metamorphosed hyphae producing blastoconidia. This may be advantageous to fungi for growth and infection. The cell walls of blastoconidia might be containing components like hydrophobins as found in conidia of *A. fumigatus* [20], which do not allow its recognition by neutrophils and other phagocytic cells. The matured hyphae and the metamorphosed hyphae producing blastoconidia might be secreting metabolites leading to the less attachment of phagocytic cells. This hypothesis is supported by the study based on Δlae mutant of *A. fumigatus* which lacks the production of some secondary metabolites resulted in the enhanced killing [21].

The increased levels of pro-inflammatory cytokines, IL-6, TNF-α and IFN-γ at early time points demonstrated an inflammatory state in kidneys. The inflammatory state continued further due to rise in the IL-17 and IL-23 at 24 h PI. The pro-inflammatory cytokine IL-6, however, unlike the other pro-inflammatory significantly increased at 72 and 96 h PI (Fig. 3d). As there was no significant difference observed in anti-inflammatory cytokines IL-4 and IL-10, we speculate that the kidneys of mice never experienced a complete anti-inflammatory state in response to the fungal infection.

A time dependent significant increase in the levels of pro-inflammatory cytokines was observed in mice during *A. flavus* infection. Early at 12 h PI a significant release of TNF-α was observed in kidney of infected mice followed by a decline at later time point of study. The pro-inflammatory cytokine TNF-α has been shown to stimulate the antifungal effector functions of neutrophils and macrophages against *A. fumigatus* and *Cryptococcus neoformans* [22,23]. We also observed the highest rate of decrease of CFU concurrently with the significant rise of TNF-α. A significantly increased level of IFN-γ at 12 h PI has been observed during this study. The pro-inflammatory cytokine IFN-γ has been shown to stimulate the migration, adherence and antifungal activity of neutrophil and macrophage against *Candida albicans*, *A. fumigatus*, *F. solani* [24]. We too observed the highest rate of phagocytosis between 6 and 12 h PI, when the IFN-γ level was significantly increased. The pro-inflammatory cytokine IL-6 increased at 12 h PI and showed a decline in the level with no significant change up to second day during investigations. Expression of IL-6 has been previously associated with recruitment of neutrophils to the site of *C. albicans* infection in mice [25,26]. Keeping the fact in mind that TNF-α, IFN-γ and IL-6 increased significantly at 12 h PI, we presume antifungal effector mechanism to be active at the site. The high rate of decrease in viable fungal count observed between 6 and 12 h PI also supports our contention.

IL-6 significantly increased at third and fourth day post-infection, with the concurrent rise in the viable fungal load due

to formation of hyphae and blastoconidia. In experimental infections, high levels of IL-6 have been correlated with susceptibility to the pathogen and severity of associated diseases [27]. Further the production of IL-6 also leads to increased survival of PMNLs at the infection sites [28,29], which leads to tissue damage by producing MPOs and ROS. This activity of IL-6 dramatically alters the infection direction leading to the susceptibility of host.

The increased levels of IL-17 and IL-23 at 24 h PI may possibly be from the innate immune cells like γδ T or iNKT cell or epithelial cells [19] resulting in the induction of epithelial cells and other immune cells during the infection [19]. Thus at 24 h PI there was the induction of IL-17 signaling pathway. How this IL-17 and IL-23 axis of inflammation led to the deteriorating immune response in infected mice will be an active area of research. It will also be interesting to find out how the IL-17/IL23 inflammatory axis is induced and what are the factors from fungus leading to this activation.

Finally, we realize that the present findings are strictly applicable only to infections in immunologically intact BALB/c mice. Our study serves as a basis for future expansion of research into different mouse strains, including mice with gene knockouts specific for the cytokines, and mice immunocompromised by other experimental approaches. The determination of factors from fungi regulating the immune response of mammalian host may also be very rewarding area of research that may lead to more specific antifungal strategy.

Acknowledgments

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