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Aspergillus flavus induces granulomatous cerebral aspergillosis in mice with display of distinct cytokine profile

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ABSTRACT

Aspergillus flavus is one of the leading *Aspergillus* spp. resulting in invasive aspergillosis of central nervous system (CNS) in human beings. Immunological status in aspergillosis of central nervous system remains elusive in case of both immunocompetent and immunocompromised patients. Since cytokines are the major mediators of host response, evaluation of disease pathology along with cytokine profile in brain may provide snapshots of neuro-immunological response. An intravenous model of *A. flavus* infection was utilized to determine the pathogenicity of infection and cytokine profile in the brain of male BALB/c mice. Enumeration of colony forming units and histopathological analyses were performed on the brain tissue at distinct time periods. The kinetics of cytokines (TNF-α, IFN-γ, IL-12/IL-23p40, IL-6, IL-23, IL-17A and IL-4) was evaluated at 6, 12, 24, 48, 72 and 96 h post infection (hPI) in brain homogenates using murine cytokine specific enzyme linked immunosorbent assay. Histological analysis exhibited the hyphae with leukocyte infiltrations leading to formation of granulomata along with ischemia and pyknosis of neurons in the brain of infected mice. Diseased mice displayed increased secretion of IFN-γ, IL-12p40 and IL-6 with a concomitant reduction in the secretion of Th2 cytokine IL-4, and Th17 promoting cytokine, IL-23 during the late phase of infection. *A. flavus* induced inflammatory granulomatous cerebral aspergillosis in mice, characterized by a marked increase in the Th1 cytokines and neurons undergoing necrosis. A marked increase in necrosis of neurons with concurrent inflammatory responses might have led to the host mortality during late phase of infection.

1. Introduction

Aspergillus flavus is the second most causative agent of invasive Aspergillosis and has been reported to be more virulent than almost all other *Aspergillus* species. It causes hypersensitive reactions to invasive aspergillosis depending on the immunity of host [1–3]. LD_{90} inocula for *A. flavus* are much less than those required for *Aspergillus fumigatus* in both normal and immunocompromised mice [4–5]. Invasive aspergillosis in organs such as brain, kidneys etc. mostly results from systemic spread secondary to lung infection or may result from direct extension from the paranasal sinuses. According to a human autopsy report, CNS is among the most frequent secondary organ involved in invasive aspergillosis [6]. Cerebral aspergillosis due to *A. flavus* in immunocompetent hosts has been reported from several countries [7–9]. Most of these cases occurred as a complication of chronic granulomatous sinusitis [7–10]. In a study fatal aspergillosis infection in neonate has been reported, where invasive aspergillosis was detected in multiple organs including brain [11]. Recently 29 cases of aspergillosis were reported where about 39.3% cases of sinusitis and brain abscess were due to *A. flavus* [12]. Cerebral aspergillosis in humans leads to nonspecific symptoms that include altered mental status, fatigue, convulsions, weakness on one side of the body (hemiparesis) and motor speech disorder (dysarthria) with or without fever [13–14]. Moreover in immunocompromised patients, cerebral aspergillosis is difficult to cure by medications and the mortality rate is close to 100% [15].

Immune response initiation, amplification or suppression in the CNS depends on a number of factors such as, the activation state of microglia, level of cytokine and cytokine receptor in glial and immune cells, relative quantity of pro-inflammatory and anti-inflammatory cytokines, locations of these cytokines within the CNS, and the temporal sequence in which a particular cell is exposed to various cytokines [16]. While studies suggest a directive role of

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cytokines in leukocyte-mediated defense against *A. fumigatus* and *A. flavus* alveolar infections [17]; the cytokine profile and their contribution in CNS infection in intravenous model of *A. flavus* infection has not been reported. In the current study, immunocompetent BALB/c mice were intravenously challenged with a virulent strain of *A. flavus*. The pathogenesis of infection was evaluated histologically and by analysis of colony forming units (CFU) in brain homogenates. Further, the kinetics of cytokines production was determined.

2. Materials and methods

2.1. Animals

Male BALB/c mice aged between 5 and 6 weeks were obtained from the vivarium maintained inside the National Institute of Immunology (NII) campus. All the experiments were approved by institutional ethics committee of NII with the approval number IAEC#/297/12.

2.2. Isolation and preparation of A. flavus inoculums

The preparation of *A. flavus* (MTCC 9367) inocula was as described earlier [18].

2.3. Experimental protocol

In order to determine the immune responses in brain and sequential events post infection, mice were intravenously injected with a conidial dose of 3.5×10^5 *A. flavus*. Infected and control [treated with vehicle (0.5 ml of PBS, 0.01 v/v of Tween 20)] animals were sacrificed by cervical dislocation at different hours post infection (hPI). On third and fourth day, the mice showing bending of head and circular movement when lifted with tail were selected for sacrifice. Brains were aseptically excised out from the sacrificed mice and subjected to the following delineated experimental procedures:

2.3.1. Determination of viable pathogen burdens in the CNS

At specific hPI five mice were euthanized, their brains 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 (SDA) and incubated for 48 h at 30 °C. For each mouse three replicates per time point were used for the enumeration of CFUs and average was expressed as $\log_{10}$ CFU in brain.

2.3.2. Histological examinations

Brains of three infected mice were removed at 48 and 96 hPI (about to die), fixed in 10% neutral buffered formalin and embedded in paraffin as described earlier [18]. Sections from two control mice treated with vehicle were sampled at 96 hPI. Sections were stained either with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) followed by counter staining with hematoxylin. Nikon (Eclipse 80*i*) with photographic attachments were used for the observation of stained sections. An estimation of pathology of neurons and the fungal growth were particularly investigated using blinded scoring method.

2.3.3. Cytokine analysis from brain

Aseptically excised brains were flash frozen in liquid nitrogen and kept at −80 °C until homogenized in 5 ml of PBS. The resultant homogenate was subsequently filtered through 0.22 μm filters (Millipore). Brain homogenates were stored at −80 °C until used for cytokine analysis. The titers of different cytokines in six treated and five control mice were determined at specified time point post inoculation. Individual brain was assayed for Interleukin–4 (IL-4), IL-6, Interferon-γ (IFN-γ), Tumor necrosis factor (TNF-α), IL-17A, IL-23 and IL-12/ IL-23p40 using the ELISA kits (eBioscience, San Diego, USA). The protocol of manufacturer was followed for the analysis of cytokines. Two replicates of each sample were assayed and the mean was used for statistical analysis.

2.4. Statistical analysis

The quantities of different cytokines in the samples were determined using the MasterPlex ReaderFit software (Hitachi Software Engineering America Ltd., USA), applying the best curve fit method. The student *t*-test for independent variables at a significance level of 0.05 was also performed using the Microcal™ Origin Software from Microcal Software, Inc. USA. The *t*-tests were performed in comparison with control. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Host responses to A. flavus CNS infection

Mice responded to the systemic infection marked by defined perturbations in the physical, histological and immunological parameters that can be defined under the following headings.

3.1.1. Rate of clearance of fungi from infected brain

Rate of *A. flavus* clearance from infected brain was determined by culture of homogenates. A value of 3.36 Log_{10} CFU of *A. flavus*, were recovered from the brain of mice at 6 hPI. The mean Log_{10} - CFU at 12 hPI was 2.85. At later time points Log_{10} CFU demonstrated an increase after each fall in the CNS fungal burden (Fig. 3a). Finally a mean fungal burden of 2.9 Log_{10} CFU was detected at 96 hPI (Fig. 3a).

3.1.2. Histological examinations

In order to gain insights to the induction of *A. flavus* mediated pathological conditions inside the brain of infected mice, we next observed the histological sections microscopically.

3.1.2.1. Inflammatory cells in CNS. Histological sections of brain showed activated microglial cells, leukocytes particularly neutrophils and few monocyte or lymphocytes in the brain tissues from the mice sacrificed at 48 hPI (Fig. 1a and b). Growing fungal hyphae surrounded by leukocytes were observed in the brain sections taken on second day (Fig. 1c and d). The brain sections sampled on second day demonstrated a number of inflammatory granulomatous foci formed by the microglia and permeated leukocytes specially neutrophils and few monocytes (Fig. 1e and f).

3.1.2.2. Inflammation and neuropathogenesis. During the experiment two kinds of pathology was observed in infected mice. Initially during the first two days major pathology in the form of ischemic events in neurons characterized by the eosinophilic cytoplasm and necrosis were observed (Fig. 2a and b). Additionally to a lesser extent necrosis by forming pyknotic nuclei were also observed in the sections taken at 48 hPI (Fig. 2a and b). Compared to control there was a significant increase in ischemic necrotic neurons and pyknotic neurons at 48 hPI and at 96 hPI (Fig. 2c and d, Table 1). Sections also demonstrated significant increase in ischemic necrotic neurons and pyknotic neurons in the 96 h infected brain sections compared to 48 h infected samples (Table 1). CNS structures like dentate gyrus, cerebellum etc., where neurons are densely packed, necrotic degeneration of neurons mostly

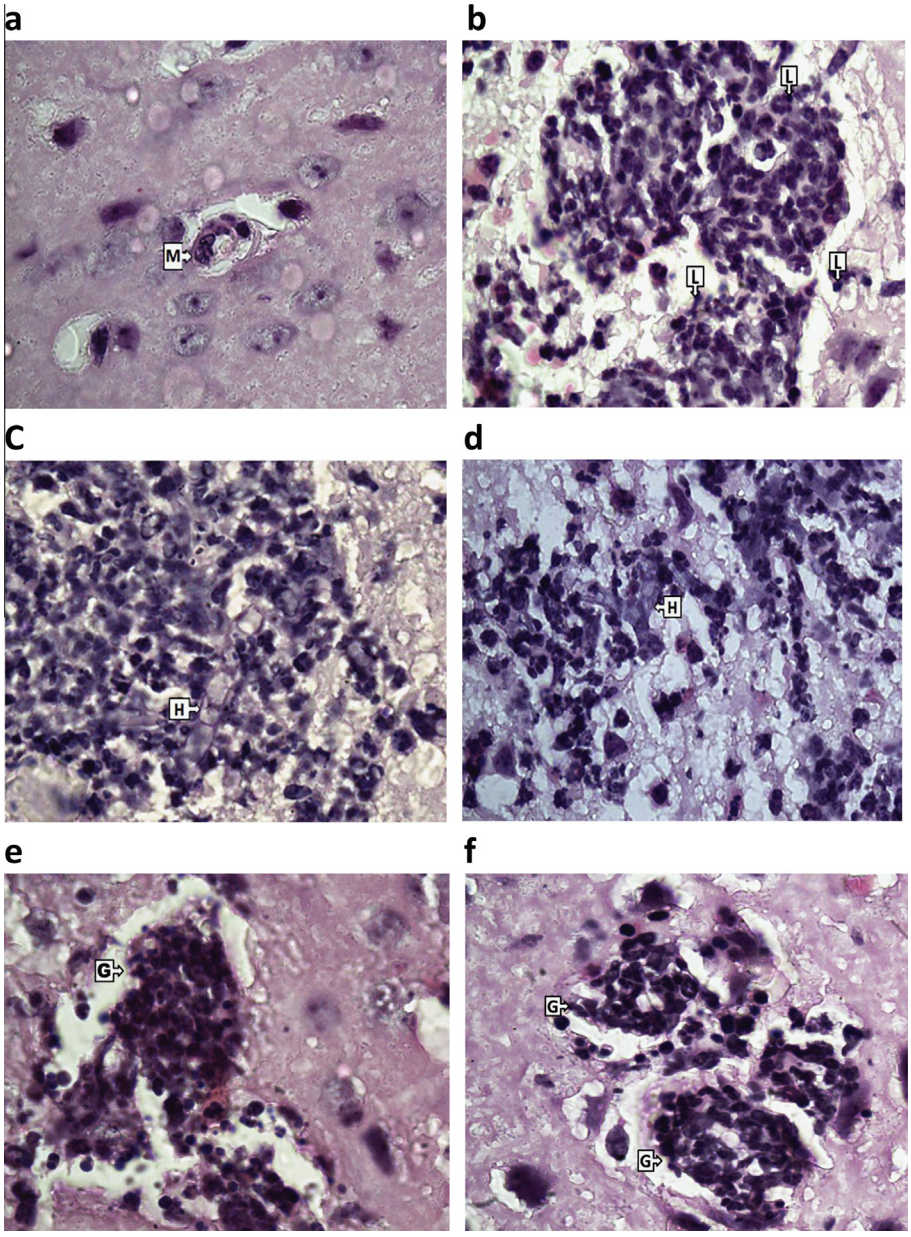

Fig. 1. Brain tissue sections of BALB/c mice displaying leukocyte infiltrations, granuloma and hyphae. BALB/c mice were infected intravenously with 3.5×10^5 conidia of *Aspergillus flavus* (MTCC 9367) and brain tissue sections were prepared for histological studies. The microphotographs displaying activated microglia (a) and the leukocyte infiltrations (b) in the sections from the mice sacrificed at 48 hPI. Hyphae were also visible in the sections of mice on second day after infection (c and d). Further a number of granulomatous lesions/foci were also observed in the mice at 48 hPI (e and f). The sections of brain were stained either with hematoxylin–eosin or PAS–hematoxylin (Mayer's). Microglia denoted by M, leukocytes by L, Hypha by H and Granuloma by G in figure. The magnification was 100×.

manifested pyknosis from the mice about to die on fourth day (Fig. 2c,). However, in a few neuron rich area major pathology observed was ischemic necrotic neurons (Fig. 2d). Microscopic examinations of sections from fourth day control mice demonstrated very few ischemic events and negligible number of neurons showing pyknosis (Fig. 2e and f). The number of inflammatory granulomata was higher at second day compared to that of fourth day (Table 1) post infection suggesting active machinery that resolved the inflammatory granulomatous foci once the fungal components were killed.

3.2. Cytokines in brain

Microscopy of histological sections revealed infiltrations of leukocytes in the brain resulting in the formation of inflammatory granulomatous foci. Therefore, in order to gain insight into the potential role of different cytokines in pathogenesis of *A. flavus* infection, temporal induction of cytokines were assessed in whole brain of infected and control mice using murine cytokine-specific ELISAs. Analysis of cytokines from brain homogenates allowed the quantification of total cytokines present in the brain. The pro-inflammatory cytokine TNF-α did not show any considerable difference during the study (Fig. 3a). Amount of IL-12/IL-23p40 subunit increased remarkably from second to fourth day (Fig. 3b). Likewise a distinct increase in the levels of the pleiotropic cytokine IL-6 from second to fourth day was observed (Fig. 3c). However the pro-inflammatory cytokine, IFN-γ levels enhanced significantly only on fourth day post infection (Fig. 3d). Another pro-inflammatory cytokine IL-17A did not show any marked variation throughout the study period (Fig. 3e). Surprisingly the

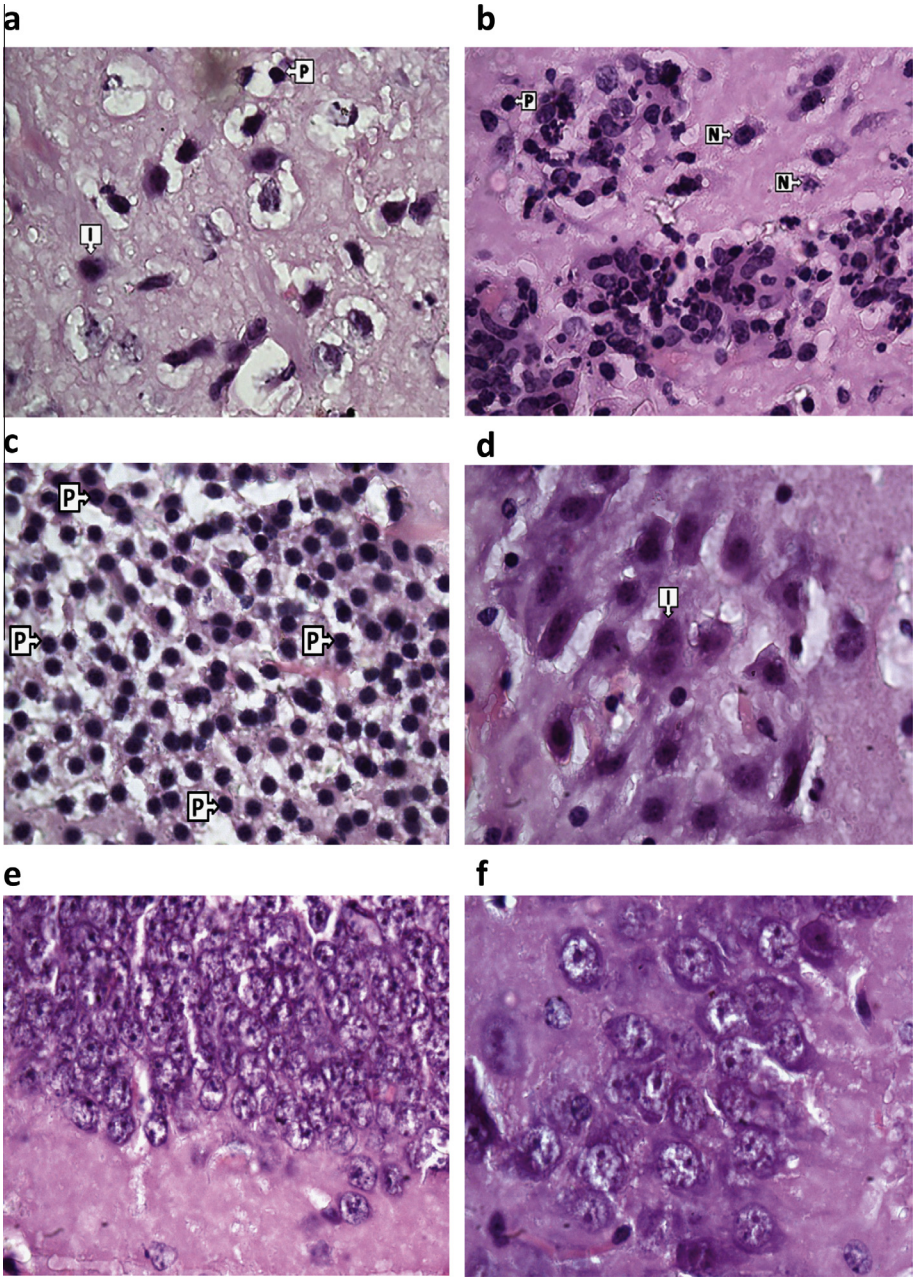

Fig. 2. Sections of brain tissue from *A. flavus* infected BALB/c mice showing pathology of neurons. BALB/c mice were infected intravenously with 3.5×10^5 conidia of *Aspergillus flavus* (MTCC 9367). Ischemic and pyknotic neurons as observed in the sections taken at 48 hPI (a and b). The pyknotic neurons displayed in the sections from the mice that was about to die on fourth day (c). CNS structures where neurons are densely packed, necrotic degeneration of neurons mostly manifested pyknosis from the mice about to die on fourth day (c). At few places in neuron dense area sections sampled at 96 hPI demonstrated ischemic neurons as a major event (d). Histological examination of brain from control mice sampled at 96 hPI suggested a few ischemic events and a negligible pyknosis of the nucleus of neurons (e and f). The sections of brain were stained either with hematoxylin–eosin or PAS–hematoxylin (Mayer's). Ischemic neurons denoted by I, pyknotic neurons by P, and Necrotic neurons by N. The magnification was 100×.

cytokine IL-23 that contains the IL-12p40 subunit decreased sharply at 72 hPI (Fig. 3f). Similarly, there was a noticeable decrease in the anti-inflammatory cytokine IL-4 at 72 hPI during the experiment (Fig. 3g).

3.2.1. T helper cell response

The cytokine milieu comprising of IFN-γ, IL-12p70 (difference between the amount of IL-12p40 and Interleukin 23) and IL-6 during late phase of infection and a pronounced reduction in the Th2 signature cytokine IL-4, a Th17 promoting cytokine IL-23 during late phase (third day) along with no markable variation in IL-17, a Th17 signature cytokine led us to propose that the CNS responded to infection by promoting inflammatory Th1 response.

4. Discussions

Sequential events during progression of *A. flavus* mediated granulomatous aspergillosis in CNS that characterize activation of microglia, recruitment of leukocytes, inflammation and sequential release of cytokines leading to neuronal mortality were evaluated.

In the histological examinations, we observed a rise in the number of activated microglia and leukocyte leading to the formation

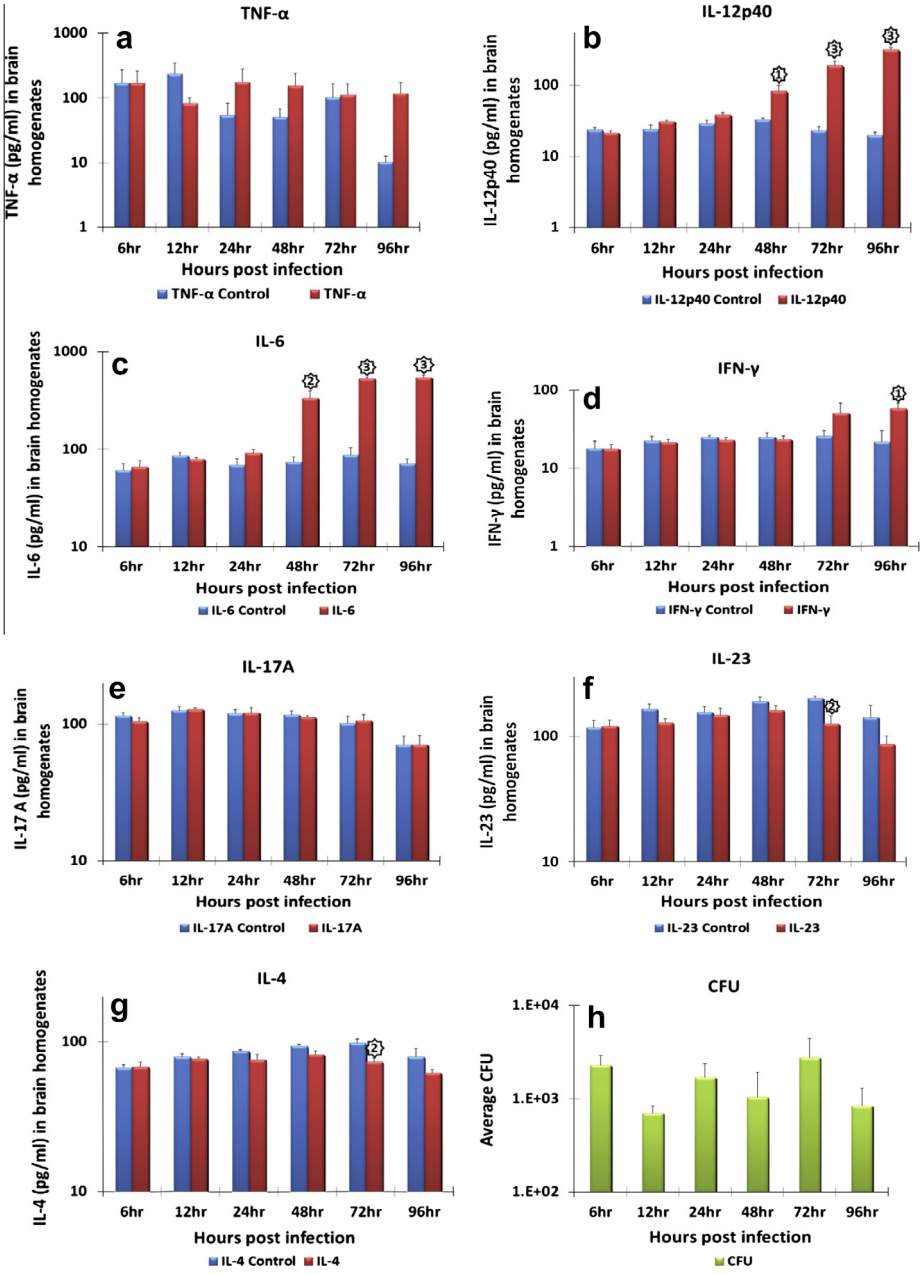

Fig. 3. Proinflammatory and anti-inflammatory cytokine profile from the infected and control mice. Profile of the pro-inflammatory cytokines; TNF-α (a), IL-12p40 (b), IFN-γ (c), IL-6 (d), and anti-inflammatory cytokines IL-4 (e), IL-17A (f), IL-23 (g) at different hours post infection in the brain homogenates of BALB/c mice infected intravenously with 3.5×10^5 conidia of *Aspergillus flavus* (MTCC 9367). Control values have been denoted by placing "Control" after respective cytokines in the legend. Results of the experiment are depicted and reflect the mean ± standard deviation of mean of 6 animals per time point and 5 animals per time point for control. ⟨1⟩ stands for a p value between 0.05 and 0.01, ⟨2⟩ for a p between 0.01 to 0.001, and similarly ⟨3⟩ symbolizes $P < 0.001$. Further the values of CFUs obtained at each time points from brain homogenates of five infected mice has been demonstrated as Log 10 CFU in figure (h).

of granuloma in *A. flavus* infected mice. Similarly formation of CNS lesion by *Aspergillus* spp. [19] and granuloma by *Cryptococcus* spp. [20] has been reported earlier. The inflammatory environment inside the CNS resulted in ischemic events in neurons followed by direct necrosis and necrosis after pyknotic nuclei formations in the cortex and other less neuron dense area. Intriguingly, the infection predominantly resulted in necrotic degeneration of neurons through the pyknosis route in structures like dentate gyrus, cerebellum (where neurons are densely packed). Based on the observations, it can be hypothesized that the neurons from the

Table 1
The average number of neurons with ischemic condition and pyknotic nuclei from the mice at second and fourth day (mice about to die at 96 h PI). The average number of granuloma/slide was also recorded. Control mice received vehicle and brain sections were sampled at day 4.

	Ischemic (mean/slide)	Pyknotic (mean/slide)	Mean granuloma/slide
48 hPI	30,226	5004.6	172
96 hPI	18,8226.8	101,886	86
Control	87	43	0

cortex, and other less neuron dense area closer to the fungal hyphae induced granulomatous foci and are more prone to ischemic events in the CNS infection.

The immune response of CNS depends upon a number of factors, like activation of microglia, variations in cytokine and cytokine receptor levels in glial and immune cells, relative abundance of pro-inflammatory and anti-inflammatory cytokines, locations of these cytokines, and temporal sequence in which a particular cell is exposed to cytokines [16].

The pro-inflammatory cytokines like TNF-α demonstrated no significant change at the tissue level in the brain at different time points during our study. However, it is likely that growing hyphae stimulated the microglia to the activated form leading to secretion of TNF-α locally for the recruitment of antifungal innate immune effector cells especially neutrophils at the site that was conspicuous in the histological findings. Microglias are known to engulf microbes including bacteria, fungi and protists that migrate to brain [21].

The amount of IL-12p40 in brain of infected mice increased notably from second to fourth day. Activated microglial cells, monocytes and B-cells in CNS have been shown to secrete IL-12 [22]. Similarly a constitutive expression of IL-12p40 mRNA in brain has been reported in infected mice [23].

A significant increase of IFN-γ at 96 h PI was observed in CNS following the significant rise of IL-12p70 (IL-12p40 minus IL-23) at second and third day. IL-12 secreted by the activated microglial cells, monocytes and B cells has been reported to induce the production and secretion of IFN- γ by T-cells leading to the development of a Th1 type of cellular immune response against the ongoing infection [22,24]

The pleiotropic cytokine IL-6 increased significantly at second day and remained elevated substantially till the mortality of host. IL-6 being a multifunctional cytokine can lead to discrete responses that include modulation of inflammation, drafting the acute phase response. Further increased cerebral expression of IL-6 has been demonstrated in many CNS diseases, such as HIV encephalopathy, multiple sclerosis, Alzheimer's disease, bacterial, viral meningitis and cerebral malaria [25–31]. IL-6 in central nervous system has been proposed to be up regulated whenever neuroinflammation is expected, such as following CNS infection, injury or in many CNS diseases [32]. Further IL-6 has been designated as a crucial cytokine controlling the transition from innate to acquired immunity, which is indispensable when CNS is combating with infections [33].

Taken together cytokine profile demonstrated a sequential release of cytokines IL-12 (here a difference between IL-12p40 and IL-23), and IFN-γ and decrease in the secretion of IL-23 and IL-4 in the latter half of infection period suggesting the activation of Th1 type of adaptive response in microglia and brain. Previously similar cytokine profiles have been suggested to induce Th1 type of adaptive response [22,24]. The decrease in the fungal burden at 96 hPI further suggested the containment of fungal infection to some extent by the induction of Th1 type of cytokine response.

Based on the overall findings it can be hypothesized that initially the mice brain particularly the microglia sensed the threat in the form of conidia or growing hyphae. This threat resulted in the production of inflammatory cytokines locally at the site leading to the infiltration of leukocytes. Concurrently a sequential accumulation of Th1 promoting cytokines IL-12, IFN-γ and a pleiotropic cytokine IL-6 contributed to the advanced inflammatory state. As a result of inflammation, ischemia and pyknosis of neurons intensified that resulted in the necrosis of neurons. The leukocyte infiltrations around the fungal component along with inflammation resulted in the formation of the granulomata. Overall, *A. flavus* induced inflammatory and granulomatous cerebral aspergillosis in the mice that culminated into the sequential increase in the number of neurons undergoing necrosis either through ischemic events or through pyknosis leading to the mortality of host.

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