Global Transcriptomic Profiling of Innate and Adaptive Immunity During *Aspergillus flavus* Endophthalmitis in a Murine Model

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PURPOSE. Fungal endophthalmitis is characterized by chronic inflammation leading to the partial or complete vision loss. Herein, we analyzed the transcriptomic landscape of *Aspergillus flavus* (*A. flavus*) endophthalmitis in C57BL/6 mice to understand the host-pathogen interactions.

METHODS. Endophthalmitis was induced by intravitreal injection of *A. flavus* spores in C57BL/6 mice and monitored for disease progression up to 72 hours. The enucleated eyeballs were subjected to histopathological analysis and mRNA sequencing using the Illumina Nextseq 2000. Pathway enrichment analysis was performed to further annotate the functions of differentially expressed genes (DEGs) and validation of cytokines was performed in vitreous of patients with fungal endophthalmitis using multiplex ELISA.

RESULTS. Transcriptomic landscape of *A. flavus* endophthalmitis revealed upregulated T-cell receptor signaling, PI3K-AKT, MAPK, NF-κB, JAK-STAT, and NOD like receptor signaling pathways. We observed significant increase in the T-cells during infection especially at 72 hours infection along with elevated expression levels of IL-6, IL-10, IL-12, IL-18, IL-19, IL-23, CCR3, and CCR7. Furthermore, host-immune response associated genes, such as T-cell interacting activating receptor, TNF receptor-associated factor 1, TLR1, TLR9, and bradykinin receptor beta 1, were enriched. Histopathological assessment validated the significant increase in inflammatory cells, especially T-cells at 72 hours post-infection along with increased disruption in the retinal architecture. Additionally, IL-6, IL-8, IL-17, TNF-α, and IL-1β were also significantly elevated, whereas IL-10 was downregulated in vitreous of patients with Aspergillus endophthalmitis.

CONCLUSIONS. Regulating T-cell influx could be a potential strategy to modulate the excessive inflammation in the retina and potentially aid in better vision recovery in fungal endophthalmitis.

Keywords: endophthalmitis, *Aspergillus flavus* (*A. flavus*), transcriptomics, RNA-seq, adaptive immunity

Fungal endophthalmitis is a sight threatening infection in the posterior segment of the eye, characterized by chronic inflammation due to the excessive host-immune response.[1,2] It commonly exhibits a subacute onset, with symptoms worsening within days to few weeks due to limited ocular space, delayed presentation of patients to the clinic, and misdiagnosis.[3,4] Whereas *Candida* species predominates the West, filamentous fungi*,* especially *Aspergillus flavus (A. flavus)* is reported to be one of the leading fungal pathogens in the tropical countries, like India, Middle East, and Africa, and is also associated with higher rates of evisceration and poor visual outcomes.[5,6] Treatment of this opportunistic fungus remains a challenge, primarily due to the limited potential of antifungal drugs to tackle the aggravated inflammation associated with these pathogens.[7–9] Fungal infections have been gaining increased attention across the globe, because they use complex mechanisms to evade the host immune system and establish rampant infection, inducing broad spectrum of host responses which includes increased uncontrolled inflammation.[10,11] Although inflammation is responsible for tackling the pathogens, in endophthalmitis, however, it can act as a double-edged sword. The inflammatory cascade activated by the specific toxic effects of the pathogen ultimately determines the final anatomic and functional visual outcome leading to partial or complete loss of vision because the influx of inflammatory cells could disrupt the retinal architecture.[12]

The initial treatment for suspected fungal endophthalmitis includes intravitreal injection of amphotericin B and voriconazole and these injections may be repeated after 24 hours, 48 hours, or 72 hours depending on the clinical evolution and antifungals.[13,14] For cases where the pathogen

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is resistant to these common antifungals, caspofungin, isavuconazole, or micafungin is also administered.[15,16] The eyes with *Aspergillus* endophthalmitis present in a larger proportion with visual acuities inferior to hand movement (compared to eyes with other fungal etiologies) and this trend was maintained until the end of the follow-up despite the prompt treatment initiation. In a retrospective *Aspergillus* endophthalmitis study reported by Dave et al. (2020), 34% eyes had a final visual acuity greater than or equal to counting fingers. The prime factors associated with better visual outcomes were: presenting vision greater than hand motions, absence of corneal infiltrate, early vitrectomy, and the use of intravitreal voriconazole (as compared to intravitreal amphotericin B). Additionally, for recalcitrant cases, the repeated intravitreal injections of antifungals further exacerbates the inflammation.[17] The use of intravitreal corticosteroids in fungal endophthalmitis is controversial because they are presumed to impair the efficacy of antifungals and interfere with the immunogenic response and further exacerbate the infection.[18] The number of studies and experimental evidences reporting on the influence of the corticosteroids in the infectious endophthalmitis is not adequate and requires extensive investigation. Considering the limited effectiveness of current antifungal treatments, there is a pressing need to explore targeted immunotherapeutic interventions as a safer alternative to ameliorate immunopathology during fungal endophthalmitis to enhance vision recovery.[19,20] Strategies aimed at regulating the immune system or host-directed therapeutics to direct the host immune system and regulating established infections, represent a promising avenue for exploration.[21] Tramsen et al. (2014)[22] highlighted the potential use of antigen-stimulated T-cells during *Aspergillus* infection.[23] Monoclonal antibodies, such as Sulfasalazine,[24] IgG,[25] Mycograb,[26] β-glucan specific IgG2b,[27,28] and several other antibodies, have been tested against *Aspergillus* and *Candida* species which have proven to invoke immunomodulation. Additionally, Sionov and Segal (2003)[29] reported an adjuvant therapy of enhancing phagocytic function and myelopoiesis by colony stimulating factors (CSFs) to treat invasive aspergillosis, which significantly improved the treatment outcome.[30] The majority of these therapies, however, are targeted to elevate the host-immune response in systemic fungal infections, whereas fungal endophthalmitis requires targeted and regulated immune suppression to aid in better vision recovery following fungal clearance.

In this study, transcriptomic and immunohistochemical profiling of *A. flavus* endophthalmitis in C57BL/6 mice model, provided insights into the host-immune response against fungal infection and the pathways associated with inflammation was assessed to understand disease pathobiology which may provide the foundation for developing an alternative or adjuvant treatment strategy for fungal endophthalmitis.

MATERIALS AND METHODS

Ethics Statement

A common inbred laboratory of C57BL/6J mice of both sexes, aged 6 to 8 weeks, were included in the study. The mice were treated following the guidelines outlined by Association for Research in Vision and Ophthalmology (ARVO) statement for the ethical use of animals in ophthalmic and vision research. The experiments were approved by the Institutional Animal Ethics Committee (IAEC), Vyas Labs, Hyderabad, India – (Protocol approval number IAEC/VL/08/2022-23).

Fungal Culture Preparation

A. flavus (L-416/2018) strain was isolated from vitreous of patients diagnosed with fungal endophthalmitis. The strain was cultured on a Potato dextrose agar (PDA) containing 5% chloramphenicol and maintained at 25°C for 5 days until the plate was covered with conidia. The fresh spores were gently scraped from the surface of the media and suspension was prepared in a 5 mL sterile phosphate buffer saline (PBS), which was passed through muslin cloth to separate the Conidia from hyphae. Following the centrifugation at 7100 X g for 5 minutes, the pellet was suspended in sterile PBS. The spores were quantified using a hemocytometer and serially diluted to obtain a final concentration of 15,000 spores/µL.

Establishment of Mouse Model of *Aspergillus flavus* Endophthalmitis

Prior to the experiment, the mice were screened for the presence of the basic abnormalities, such as cataract, retinal damage, or any other visual impairment, by an on-site veterinarian. All the mice which passed the screening were included in the study. The *A. flavus* endophthalmitis mice model was developed as reported previously.[31] Briefly, C57BL/6 mice were anesthetized by administering ketamine (35 mg/Kg) and xylazine (5 mg/Kg) along with topical anesthetic (0.5% proparacaine hydrochloride). Eyes were wiped clean with a topical antiseptic 10% betadine (Win-Medicare Pvt Ltd), and the pupils were dilated with 1% tropicamide (Ophtha Pharma) to allow visualization of the injected needle inside the eye. Then, 1 µL of fungal spore suspension containing 15,000 spores were intravitreally injected into the mid vitreous using a modified needle injector[31] under a surgical microscope (ZEISS Stemi 508 Stereo Microscope; Zeiss, Jena, Germany). The contralateral eye was injected with sterile 1 × PBS which served as the control. Eyes were clinically examined throughout the course of infection development through a slit lamp (PSLAIA-11, Appasamy Associates). Eyes enucleated at 72 hours post-infection were subjected to whole transcriptomic analysis and histopathological analysis was carried out at both 24 hours and 72 hours post-infection.

RNA Sample Preparation and Sequencing

Total RNA was extracted from mice eye ball ($n = 3$) using RNeasy mini kits (Qiagen) following the manufacture's instructions followed by DNase treatment. The concentration of RNA was checked using the NanoDrop Spectrophotometer (Biorad), NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and Qubit 4 Fluorometer (Thermo Fisher Scientific, Indianapolis, IN, USA), whereas RNA quality was assessed with the Tapestation 4200 (Agilent Technologies, Santa Clara, CA, USA).

The cDNA was synthesized using reverse transcriptase and random hexamers in a first strand synthesis reaction. Prepared libraries were sequenced on Illumina Nextseq 2K to generate 40 M, 2 × 150 bp reads /sample. Up to 75% of

the sequenced bases were of Q30 value > 90. Sequenced data were processed to generate FASTQ files and proceeded with data analysis.

RNA-Seq Alignment and Analysis

Paired-end raw sequence reads quality report was generated by using FastQC to estimate base quality and contamination by sequencing artifacts. Adaptors and poor quality sequences were trimmed using Trim Galore and mapped to the reference transcriptome with STAR splice aware alignment tool. The Rsubread R package was used to obtain feature specific expression counts and low count features across samples were detected and removed with the NOISeq R package. Expression counts normalization was performed with TMM method from NOISeq R package. Heatmap was generated using the heatplot function from made4 R package for top upregulated and downregulated features based on log2 fold change (FC). The intersection plot has been created using the upset function from UpSetR R package. The volcano plot was generated with the Enhanced Volcano R package. The criteria for screening differentially expressed genes (DEGs) were selected as log2 fold change $\geq$ 1.5 and $P \leq 0.05$.

Pathway Enrichment Analysis

The DAVID online tool (http://david.abcc.ncifcrf.gov) was used to perform Gene Ontology (GO) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed for the genes to identify enriched pathways using KEGG mapper tool. The DEGs with a $P < 0.05$ were considered to be significantly enriched. Enrichment analysis was performed on a significantly enriched set of DEGs from our analysis. Official gene symbols were used as identifiers for the *Mus musculus* species, resulting in a functional annotation chart that highlights the enriched pathways found in the KEGG database.

Histopathology and Immunohistochemistry Validation

Mice eyeballs were enucleated at 24 hours and 72 hours post-infection, rinsed in sterile $1\times$ PBS and fixed in 10% neutral buffered formalin, embedded on paraffin blocks, and sections were cut through the pupillary-optic nerve axis. These sections were stained with hematoxylin and eosin (H&E) stain to assess the vitreo-retinal changes during the infection and associated inflammation. To confirm the presence of fungal filaments, the sections were stained with Grocott's Methenamine Silver (GMS). Additionally, inflammatory markers, such as CD3 (T cell marker), CD20 (B cell marker), CD45 (leukocyte marker), CD68 (macrophage marker), MPO (neutrophil marker), and GFAP (astrocyte and muller glia marker), were examined and quantified. Table 1 lists the antibodies and the catalog numbers.

The eyeballs were scored by a pathologist who was blinded to the type of infection. The procedure and histologic scoring were performed as detailed in our previous study.[32,33] Briefly, deparaffinization of all 4 μm thick paraffin sections of mice eyeballs were done with xylene and rehydrated through graded alcohol of 100%, 90%, and 80% dilution. They were then incubated with primary antibodies, for isotype control, IgG antibody (Vitro Master) was used, and

TABLE 1. List of Antibodies Used

Antibodies	Catalog Number	Supplier
CD45	M0701	Dako
MPO	A0398	Dako
CD3	M7254	Dako
CD20	M0755	Dako
GFAP	Z0334	Dako
IgG	E20-V	Vitro Master

all the sections were further incubated with secondary antibodies (Dako, Agilent, Santa Clara, CA, USA) They were then washed thrice with PBS, incubated for 5 minutes with DAB substrate (catalogue no-HK124-9K; BioGenex, Fremont, CA, USA), washed with distilled water, and counter stained with Harris's hematoxylin. Finally, the slides were mounted and analyzed by counting the total positive cells in the posterior chamber by taking the average of 5 random microscope fields per case at $\times$ 400. Histopathological evaluation was done by a pathologist who was blinded to the study and type of infection. Scoring was given on a 4-point scale with 0 as the least disease severity and 4 grade as the highest disease severity. The parameters considered for scoring were inflammation in the posterior segment and retinal architecture and score 0 was attributed to no infiltrating inflammatory cells and intact retinal architecture, whereas a score of 4 was attributed to 100+ inflammatory cells and indistinguishable retinal layers. Table 2 describes the parameters assessed by the pathologist to score the retinal architecture integrity.

TUNEL Assay

Magnitude of DNA fragmentation within the retina, a reliable indicator of apoptosis, was assessed by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay performed according to the manufacturer's instructions (Click-iT TUNEL Alexa Fluor 488; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the nuclei were stained with DAPI and DNase I enzyme was used to induce strand breaks in the DNA, resulting in a positive TUNEL reaction. A total of 100 μL of the reaction mixture was added to the coverslip and allowed to incubate at room temperature for 30 minutes, followed by a wash with deionized water and subjected to TdT reaction. Images were obtained using an AXIO Imager. M2 Zeiss fluorescence microscope under a $\times$ 20/0.8 lens. For each of the three biological replicates, three serial sections were taken and the TUNEL$^+$ cells were counted by a pathologist blinded to the type of tissue and slide identity. TUNEL$^+$ cell density was calculated as the ratio of TUNEL$^+$ cells/mm^2 of the length of the region of interest using an automated ImageJ macro, as described in our previous study.[34]

Vitreous Validation of Inflammatory Mediators

Vitreous samples were collected from patients seen at the LV Prasad Eye Institute between 2019 and 2021. The study was performed in accordance with the guidelines and regulations of the Declaration of Helsinki, and prior consent was obtained. Appropriate institutional review board approval was taken before starting the study. The study population included five patients who were diagnosed with culture proven fungal endophthalmitis and five patients who underwent vitrectomy for other non-infectious retinal disorders

TABLE 2. Histologic Assessment of Mice Model of Endophthalmitis

Score	Inflammation (Posterior Chamber)	Retinal Architecture
0	No infiltrating inflammatory cells	Retina completely intact
1+	1–10 infiltrating cells per field	No fibrin retinal folds in < 25% of the retina
2+	10–50 infiltrating cells per field	Mild fibrin reaction retinal folds/detachment in 25–50% of the retina
3+	50–100 infiltrating cells per field	Significant fibrin reaction retinal folds/detachment in 50–75% of the retina
4+	Both segments filled with fibrin and infiltrating cells Complete detachment	Retinal layers indistinguishable

around the same time which served as the control. For microbiology workup, vitreous samples were investigated according to institutional protocol, as described earlier.[35] Fungal species were identified based on the sporulation and their growth characteristics and upon lacto phenol cotton blue wet mount.

Multiplex Immunoassay

Briefly, protein concentration of each sample was determined using bicinchoninic acid (BCA; G-Biosciences, St. Louis, MO, USA) and equal concentration of protein was used for the assay. Then, 25 µL of each sample and cytokine standards of different concentration were added to a 96-well plate. Further, samples were checked for the concentrations of the cytokines (IL-6, IL-8, IL-10, IL-17, IL-1β, and TNF-α) by MILLIPLEX enzyme-linked immunosorbent assay (ELISA) using MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A (HCYTA-60K-08; Millipore Sigma, Burlington, MA, USA). The experiment was conducted according to manufacturer's instructions. After incubation with primary and secondary antibodies, the plate was washed rigorously with wash buffer and the plate was resuspended in drive fluid. The plate was read and analyzed in the Luminex MAGPIX Multiplex System (Merck Millipore). Standard curves of known concentrations of recombinant human cytokines (R&D Systems, Minneapolis, MN, USA) were used to convert fluorescence units to cytokine concentration (pg/mL).[34]

Statistical Analysis

Statistical analysis and graphical representation were performed using Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Any $P < 0.05$ was considered to be significant. R scripts were used to construct volcano plots and the heatmap. All the experiments were conducted with at least three biological and three technical replicates. Differences in the means were calculated using the two-tailed unpaired t-test: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, and ****, $P \leq 0.0001$.

RESULTS

Clinical and Microbiological Assessment of *Aspergillus flavus* Infected Murine Model Demonstrated the Gradual Progression of Infection

C57BL/6 mice infected with 15,000 spores of *A. flavus* was monitored everyday using a hand-held slit-lamp. Clinical assessment at 24 hours and 72 hours post-infection, demonstrated the gradual disease progression and the hallmarks

of which were progressive increase in corneal opacity, the presence of hypopyon, angiogenesis, and intraocular inflammation over time. Control eyes showed no visible signs of inflammation (Figs. 1A, 1B). Following mouse euthanasia and enucleation at 24 hours and 72 hours post-infection, eyeballs were homogenized and its viability and purity of the infection was assessed by plating the 10^{-2} dilution onto a PDA media. Then, 48 hours post-inoculation, the colonies of *A. flavus* were formed on the media and had a whitish cottony appearance confirming the presence and purity of the infection. The total number of *A. flavus* colonies per eye, was significantly ($P = 0.0061$) higher at 72 hours post-infection (1733 ± 88.19), when compared to 24 hours (1233 ± 33.33; Fig. 1C). This confirmed the establishment of the mouse model of *A. flavus* endophthalmitis.

Transcriptomic Profiling at 74 Hours Post-Infection Reveals Distinct Clusters of Gene Signatures in *A. flavus* Endophthalmitis

To understand the host-immune response and identify the transcriptional signatures associated with fungal endophthalmitis, we performed the comparative transcriptomics during *A. flavus* endophthalmitis at 72 hours post-infection with a vehicle control in a C57BL/6 mice eyeball. The RNA quality was assessed and the samples with RIN ≥ 7 were selected for transcriptomics. The transcriptomic profiling revealed a total of 17,112 DEGs, of which 568 genes with FC ≥ 1.5 and adjusted P value ≤ 0.05 were considered for further analysis. To obtain a simplified visualization of the DEGs, their relative abundance was plotted as Heatmap, Principal Component Analysis (PCA) plot, and volcano plot. The PCA plot illustrates the differential clustering of the genes in the infection and control, where PC1 and PC2 represented 48% and 19% variance. This clustering was also evident in heatmap, for which the gene abundance was log2 transformed and are displayed in colors ranging from yellow to red with downregulated genes in yellow and overexpressed genes in red. Deeper the color, the greater the fold change of expression. Heatmap and PCA Plot evidently demonstrates the distinctive pattern of gene expression in the control and infected groups and the gene clustering among the biological replicates is also strikingly similar (Fig. 2, Fig. 3A). Additionally, the volcano plot represents the overall DEGs with fold change (log2FC) > 1 and false discovery rate (FDR) value of 0.05 (Fig. 3B).

To gain insights into the functional relevance of the host-response gene signatures, KEGG was performed which revealed major dysregulated, specifically upregulated pathways, such as cytokine-cytokine receptor interaction, Neutrophil extracellular trap formation, Natural killer cell mediated cytotoxicity, metabolic pathways, C-type lectin receptor signaling pathway, JAK-STAT signaling pathway,
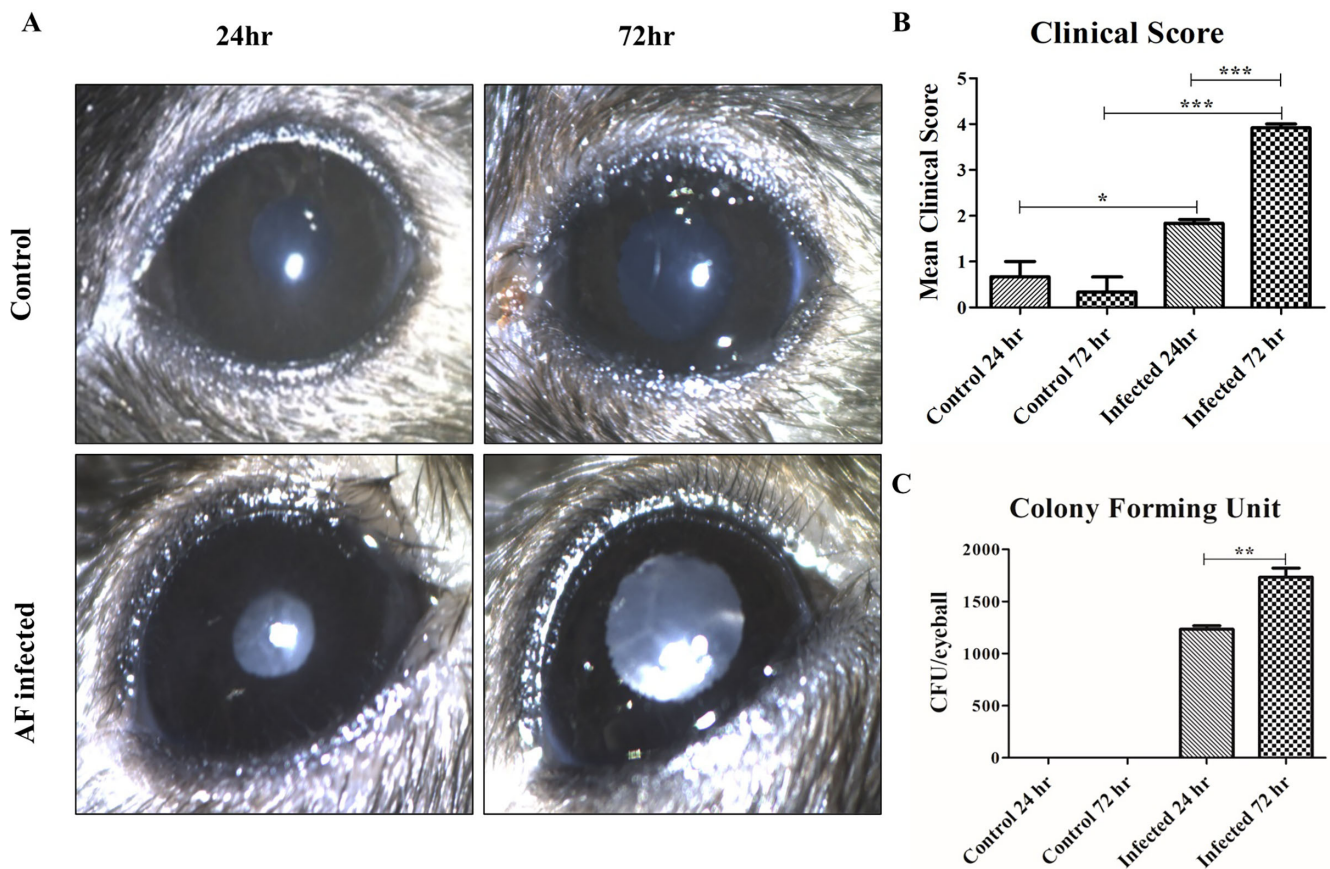
FIGURE 1. Clinical assessment of the disease progression and intraocular fungal growth sequelae following *A. flavus* infection. (**A**) C57BL/6 mouse eyes were injected with 15,000 spores of clinical isolate of *A. flavus* for 24 hours and 72 hours and were clinically scored. (**B**) Clinical scores were plotted as bar graphs representing mean ± SEM (two-tailed unpaired *t*-test), $n = 3$ eyes/group. (**C**) Viability of fungus was assessed at 24 hpi and 72 hpi and the mean ± SEM values were plotted as bar graph. Data provided is the representative of at least three similar experiments with at least three mice/group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

T-cell signaling pathway, phagosome, TNF signaling pathway, PI3K-Akt signaling pathway, Toll-like receptor signaling pathway, Chemokine signaling pathway, NOD-like receptor signaling pathway, Th17 cell differentiation, Th1 and Th2 cell differentiation, and B-cell signaling pathway (Fig. 4A). Downregulated pathways also included metabolic pathways, neuroactive ligand-receptor interaction, ECM-receptor interaction, cAMP signaling pathway, calcium signaling pathway, miRNAs in cancer, Focal adhesion, Rap1 signaling pathway, and wnt signaling (Fig. 4B). Although activation of these pathways is crucial to limit the pathogen localization inside the host, it is also to optimally regulate them to avoid immune-mediate host damage especially to the retina.

Transcriptional Changes Orchestrate the Induction of Signaling Pathways That Regulate Adaptive Immune Responses

During *A. flavus* infection, host-immune response associated genes manifest as pre-ordained transcriptional changes. For instance, C-type lectin signaling pathway associated genes, such as C-type lectin domain family 4 member D (FC = 2.1), C-type lectin domain family 4 member E (FC = 2.2), C-type lectin domain family 7 member A (FC = 2.1),

C-type lectin domain family 6 member A (FC = 2.0), early growth response protein 2 (FC = 2.0), early growth response protein 3 (FC = 2.2), phosphoinositide-3-kinase regulatory subunit alpha/beta/delta, polo-like kinase 3 (FC = 1.7), IL-6 (FC = 2.5), IL12A (FC = 2.5), spleen tyrosine kinase (FC – 1.5), caspase recruitment domain family, member 9 (FC = 1.6), and B-cell lymphoma 3 (FC = 1.5). Interestingly, however, we did not find Dectin-1 in our study which is an important innate immune pattern recognition receptor involved in the recognition of fungi.[36] C-type lectin receptors are observed to trigger distinct signaling pathways, such as Toll-like receptor signaling pathway and cytokines signaling pathway that determines T cell polarization fates.[37] In our study, we observed several genes associated with Toll-like receptor signaling pathway, such as toll-like receptor 1 (FC = 1.6), toll-like receptor 6 (FC = 1.9), C-X-C motif chemokine 11 (FC = 2.0), secreted phosphoprotein 1 (FC = 2.0), CD80 antigen (FC = 2.0), and CD86 antigen (FC =1.6). Furthermore, T cell signaling associated genes, such as inducible T-cell co-stimulator (FC =1.9), T-cell interacting activating receptor (FC = 2.4), lymphocyte cytosolic protein 2 (FC = 2.3), T-cell surface glycoprotein CD8 alpha chain (FC = 2.0), T-cell surface glycoprotein CD8 beta chain (FC = 1.6), and cytotoxic T-lymphocyte-associated protein 4 (FC = 2.1) were also elevated. These genes were observed to directly or indirectly induce T-cell activation.[38] Few cytokines, such as inter-
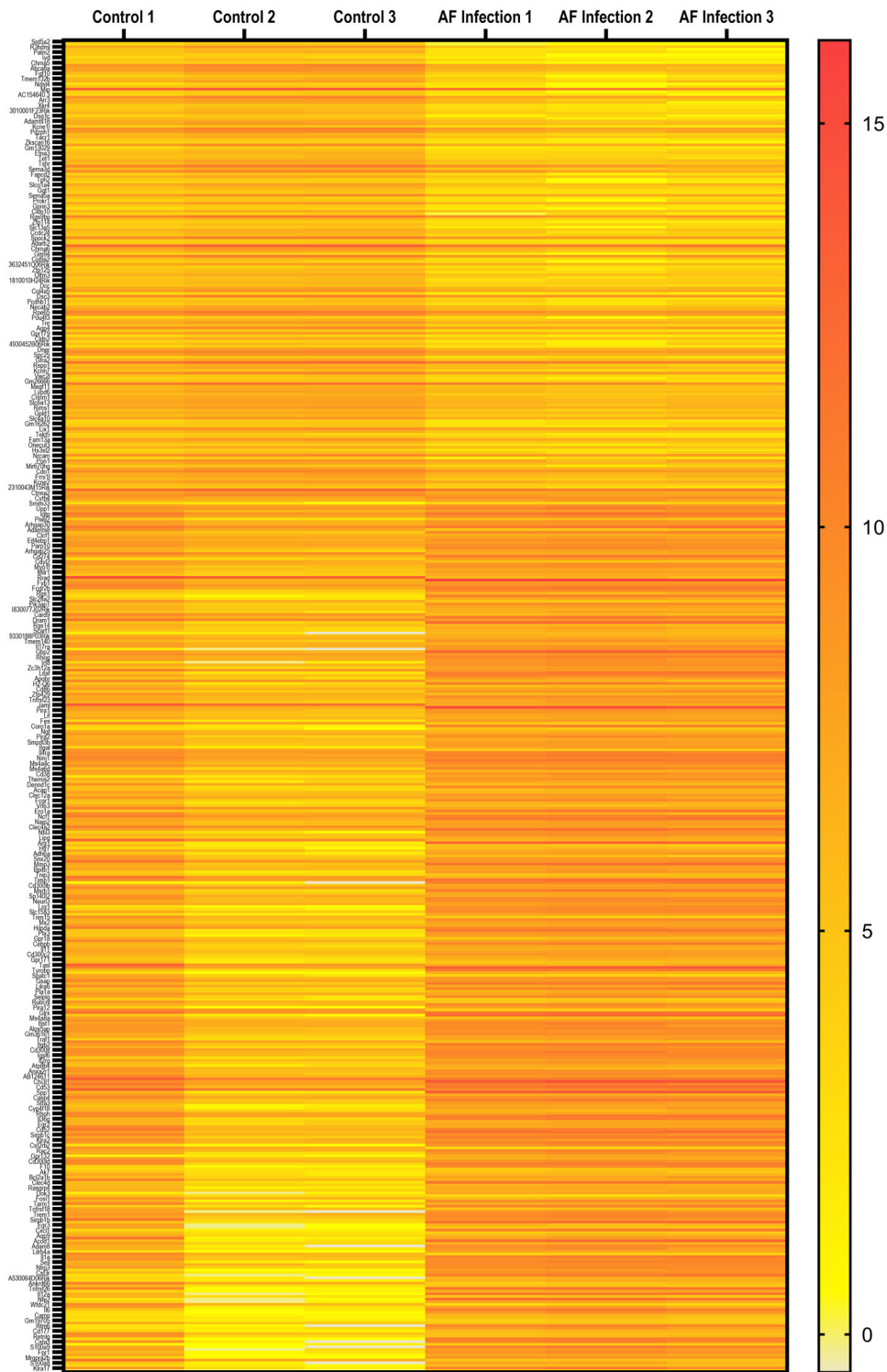
FIGURE 2. Transcription profiling identifies important host-associated dysregulated genes in *A. flavus* endophthalmitis. Heatmap representing the expression of genes differentially expressed (adjusted *P* < 0.05, fold change ≥ 1.5, and FDR ≤ 0.1) in *A. flavus* infected mice eyeballs both at 72 hpi. Normalized expression levels, arranged by unsupervised hierarchical clustering, reflect overexpression (*red*) or underexpression (*yellow*) of genes (*rows*) for the biological replicates (*columns*).

leukin 6 (FC = 2.5), interleukin 4 receptor (FC = 1.7), and interleukin 2 receptor gamma (FC = 2.0), are linked to Th17 cell differentiation and interleukin 12A (FC = 2.5) draws a connection to Th1 and Th2 cell differentiation.

Although some of the crucial genes observed in our study were associated with the T-cell signaling pathway, there were few genes such as phosphoinositide 3-kinase adapter protein 1 (FC = 1.5), tyrosine-protein phosphatase non-
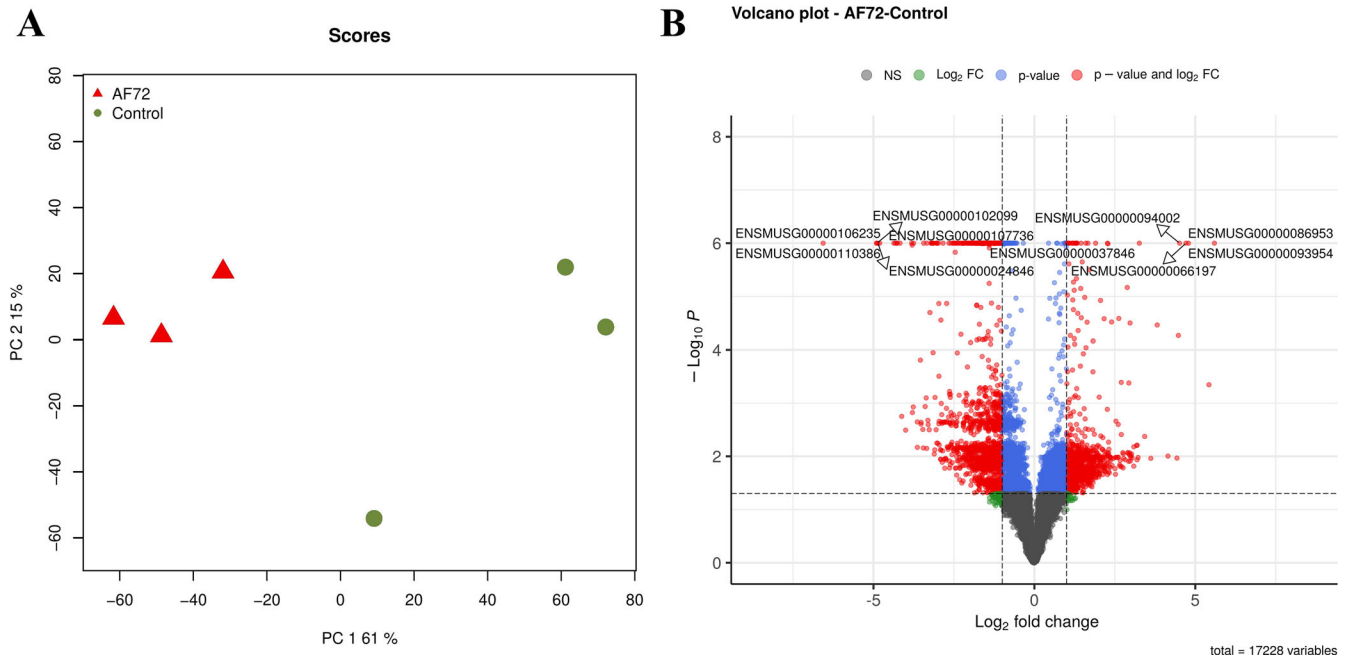
FIGURE 3. (**A**) PCA plot represents the distinct clusters of controls (*green dots*) and *A. flavus* infection (*red triangles*). (**B**) *Red dots* in the volcano plot represents the significantly upregulated (*right*) and downregulated (*right*) genes.
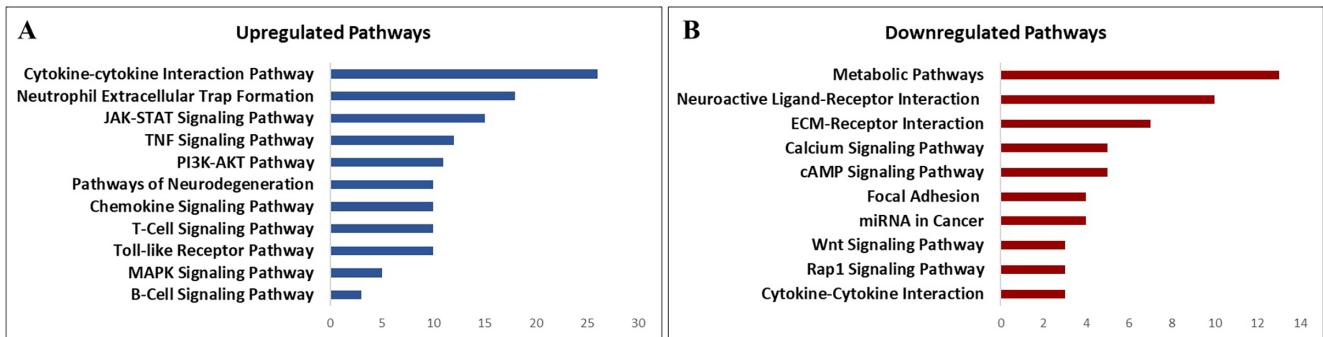

FIGURE 4. Pathway enrichment analysis of differentially upregulated host genes induced by *A. flavus* endophthalmitis. For the pathway enrichment analysis, differentially expressed genes were with adjusted $P < 0.05$, fold change ≥ 1.5, and FDR ≤ 0.1 were considered. (**A**) Upregulated pathways (**B**) downregulated pathways. The x-axis shows the number of genes, and the y-axis shows the KEGG pathway terms.

receptor type 6 (FC = 1.5), and leukocyte immunoglobulin-like receptor (FC = 1.6) linked to the B-cell receptor signaling pathway. Although several studies report an involvement of the PI3K-Akt signaling pathway in B cell activation and differentiation,[39] genes associated directly with the B-cell signaling pathway were not observed in our study. Neutrophil extracellular trap formation pathway associated crucial genes were formyl peptide receptor 1 (FC = 3.0), C5a anaphylatoxin chemotactic receptor 1 (FC = 1.8), integrin alpha (FC = 1.7), cathelicidin antimicrobial peptide (FC = 2.6), phosphoinositide-3-kinase regulatory subunit alpha/beta/delta (FC = 1.7), aquaporin-9 (FC = 2.3), and integrin beta 2 (FC = 2.0).

Other crucial upregulated genes that could be play a major role in host-immune response or pathogen killing were TNF receptor-associated factor 1 (FC = 2.0), matrix metalloproteinase-3 (FC = 1.8), matrix metalloproteinase-9 (FC = 2.2), selectin (FC = 1.8), tumor necrosis factor receptor superfamily member 1B (FC = 2.0), NLRP3 (FC = 2.4), neutrophil cytosolic factor 1 (FC = 1.7), tyrosine-protein kinase Fgr (FC = 2.05), and cannabinoid receptor 2 (FC = 1.7). Additionally, genes such as diacylglycerol kinase (FC = −1.6), cysteine dioxygenase (FC = −1.5), gamma-glutamyltranspeptidase (FC = −2.0), retinoid isomerohydrolase (FC = −1.7), glutamate carboxypeptidase II (FC = −1.7), sphingosine-1-phosphate phosphatase 2 (FC = −1.9), and glycosylphosphatidylinositol phospholipase D (FC = −1.5), associated with metabolic pathways were downregulated. Other downregulated genes include, CD44 (FC = −1.7), proenkephalin B (FC = −1.9), ephrin-A (FC = −2.1), tenascin (FC = −1.7), claudin (FC = −1.7), and thyrotropin receptor (FC = −2.1). STRING analysis demonstrates the interaction between crucial pathways which could be directly
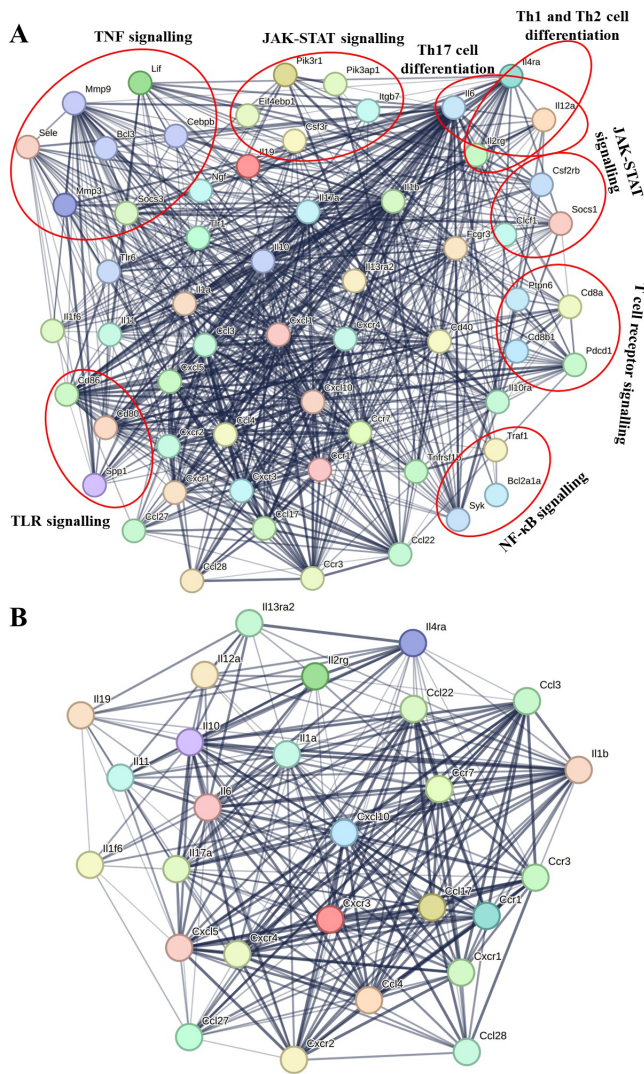
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FIGURE 5. STRING network analysis of the (**A**) top inflammation-associated pathways and their interactions, (**B**) interactions of cytokines. Each gene is represented as *small circles* and the *black lines* depict their interaction with other genes and the darker the line, the stronger is the interaction. *Red circles* represent the genes associated with the specified pathway indicating the interaction of the pathways via cytokine regulation.

or indirectly involved in activation of T-cell activation (Fig. 5A).

A. flavus Endophthalmitis Manifests Hypercytokinemia Indicating the Excessive Inflammation Burden in the Eye

The immunological counteraction is an immediate response against the pathogen leading to the orchestrated release of cytokines which play a major role as inflammatory mediators.[40] In our study, cytokine-cytokine receptor interaction pathway was the top dysregulated pathway which comprised a total of 64 genes inclusive of chemokines, such as CXCR1 (FC = 2.5), CXCR2 (FC = 2.3), CXCR4 (FC = 2.0), CCR1 (FC = 2.1), CCR3 (FC = 2.3), CCR7 (FC = 3.1), CCL3 (FC = 2.7), CXCL5 (FC = 2.8), CCL7 (FC = 2.0), CCL28 (FC = 2.1), CCL17 (FC = 2.3), CXCL10 (FC = 3.2), CXCL11 (FC

= 2.1), CCL4 (FC = 3.0), CCL22 (FC = 3.0), and CCL27 (FC = 2.1). Elevated interleukins were IL-1α (FC = 2.4), IL-1β (FC = 2.3), IL-6 (FC = 2.5), IL-10 (FC = 2.4), IL-11 (FC = 1.9), IL-19 (FC = 2.2), and IL-36α (FC = 1.6). Similarly, IL-31 (FC = −2.8) was downregulated. Additionally, complement factors such as coagulation factor X (FC = 2.1), C5a anaphylatoxin chemotactic receptor 1 (FC = 1.8), coagulation factor VII (FC = 2.0), plasminogen activator inhibitor 1 (FC = 1.8), alpha-2-antiplasmin (FC = 2.3), kininogen (FC = 3.6), bradykinin receptor B1 (FC = 2.5), and bradykinin receptor B2 (FC = 1.8) were upregulated. This unregulated and augmented inflammatory response of the immune system to fungal endophthalmitis indicates the manifestation of hypercytokinemia which could potentially lead to disruption in the retinal architecture resulting in irreversible vision loss. STRING analysis shows the interaction of different cytokines (Fig. 5B).

Histopathological Assessment Confirms the Presence of Fungal Filaments and Validates the Heightened Inflammatory Cells Influx and Retinal Stress

Gross histopathological examination of the 24 hours and 72 hours *A. flavus* infected eyeballs was performed to assess the influx of inflammatory cells, structural changes in the retina, and detect fungal filaments within the retina. The severity of the infection was assessed by different parameters, such as inflammation in the posterior segment, and integrity of the retinal architecture, and score 0, was attributed to no infiltrating inflammatory cells and intact retinal architecture, whereas a score of 4 was attributed to 100+ inflammatory cells and indistinguishable retinal layers. H&E staining revealed mild inflammatory influx with minimal retinal folds at 24 hpi ($P = 0.1012$), which increased to a significant accumulation of the inflammatory cells and retinal layers were indistinguishable at 72 hpi ($P = 0.0021$) in comparison to healthy controls (Fig. 6A). Additionally, GMS stain revealed the intraocular fungal filaments localized within the vitreous cavity and along the margins of the retina both at 24 hpi and 72 hpi (Fig. 6B). Glial fibrillary acidic protein (GFAP) staining is an indicator of retinal, especially astrocytes and muller glia stress and is associated with the retinal degeneration.[41] At 24 hpi, a minor stress is indicated in the retina ($P = 0.1012$), however, at 72 hpi, there was a significant ($P = 0.0132$) increase in the intensity of the immunoreactivity when compared to the healthy controls. On the contrary, examinations of the contralateral control eyes were consistent with lowest histological scores with no signs of inflammation and intact retinal integrity (Fig. 6C). The IgG isotype showed minimal or no staining. This control showed no background staining indicating no non-specific binding of the primary antibody to Fc receptors (Fig. 6D).

A. flavus Induces Exacerbated Immune Response (CD45 and MPO), Emphasizes the Involvement of Adaptive Immunity (CD3 and CD20)

To quantify the inflammatory cell infiltration, the CD45+, MPO+, CD3+, and CD20+ cells were counted in the vitreous and retinal section and the mean was plotted. CD45 is a leukocyte marker and we observed a significant increase at 24 hpi ($P = 0.0003$) and 72 hpi ($P < 0.0001$) with the
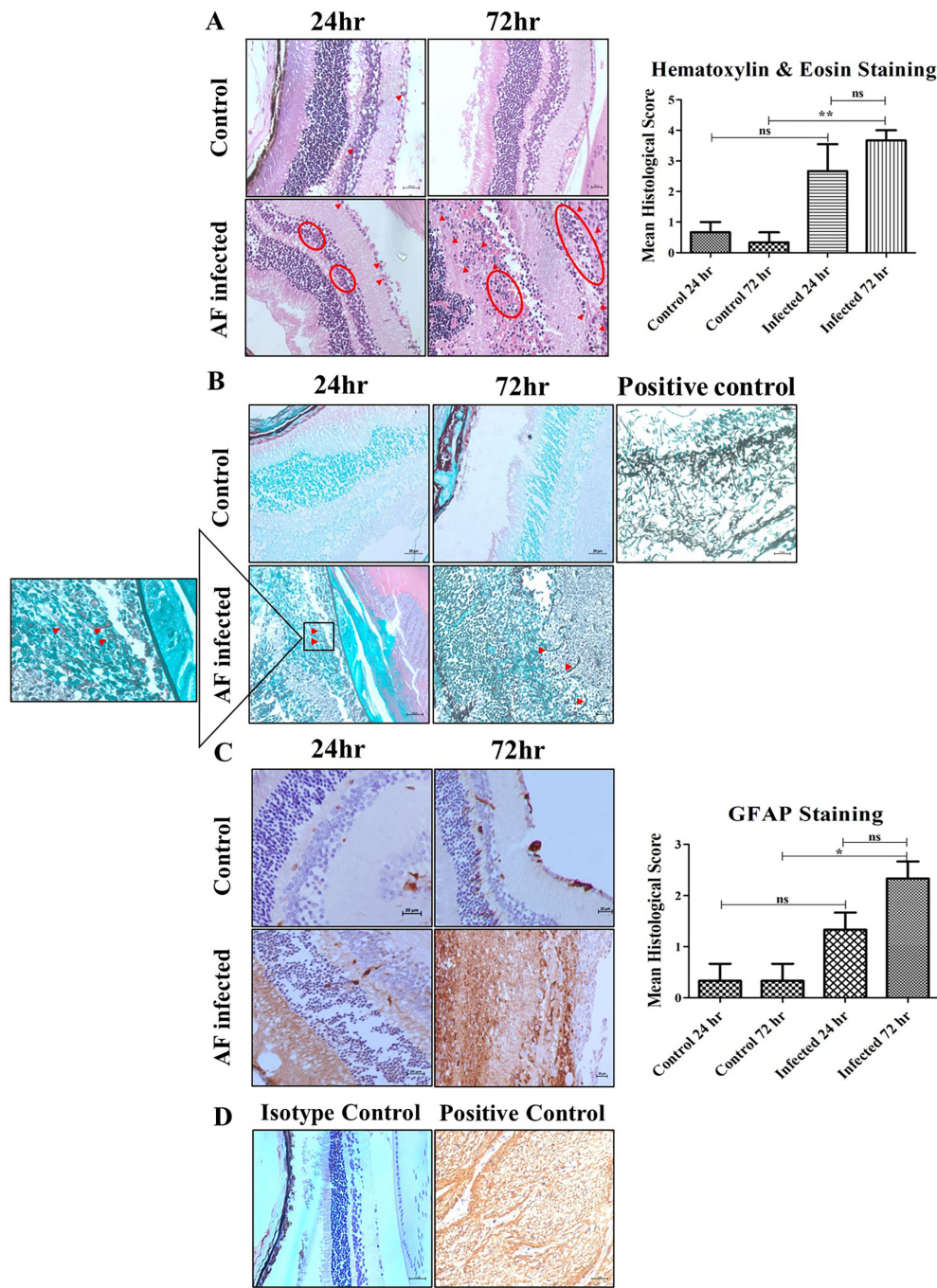
FIGURE 6. Immunohistochemical (H&E, GMS, and GFAP) examination of the mice eyeball. C57BL/6 mice eyes were injected with 15,000 spores of *A. flavus*. Infected eyes along with vehicle control were collected for histological assessment. The tissue sections were stained with (**A**) H&E, (**B**) GMS, and (**C**) GFAP, (**D**) isotype control (IgG), and positive control (Pleomorphic adenoma). In H&E, positive inflammatory cells (indicated by *red circles*) were counted and scored by a pathologist, the values were plotted in the bar graph as mean ± SEM. The presence of fungal filaments (*red arrows*) in the vitreoretinal region was confirmed and the intensity of retinal stress was scored in GFAP stained section. Pictures are the representation of one of the biological replicates and graphs represents the data combined from three independent experiments where there were three in each group. Original magnification = × 400. ns – non significant; *$P < 0.05$; **$P < 0.01$.

number of CD45+ infiltrates 14 ± 1.155 and 24.33 ± 1.453, respectively (Fig. 7B). MPO+ cells, however, showed significant increase at 24 hpi ($P = 0.0036$) when compared to the healthy controls. Additionally, at 72 hpi, although the mean value of MPO+ cells were higher in comparison to healthy controls and 24 hpi, the values were not significant ($P =$

0.052; Fig. 7C). To further confirm the presence of T cells, CD3 staining was performed and, similarly, we observed the significant elevated levels of CD3+ cells at both 24 hpi ($P = 0.0029$) and 72 hpi ($P < 0.0001$) indicating the prominent role of T-cells in *A. flavus* endophthalmitis (see Fig. 6C). CD20 is a marker for B cells, and, in our study, although the
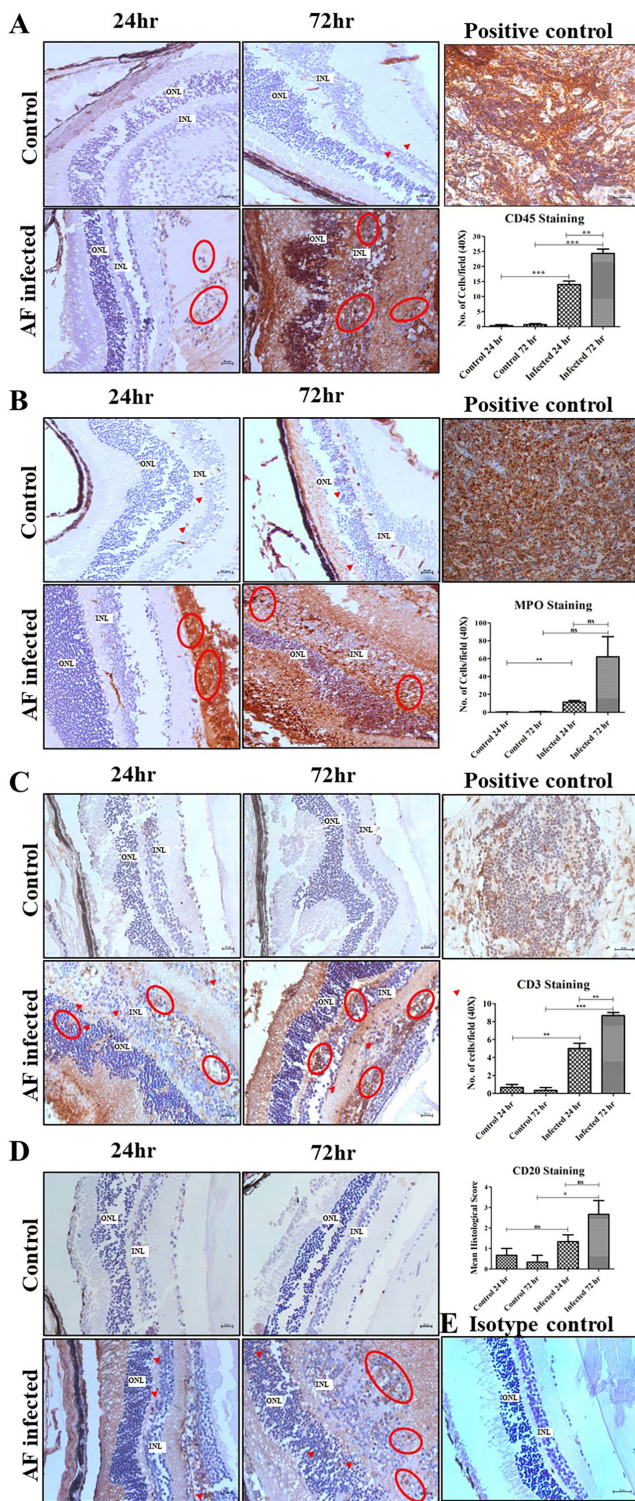
FIGURE 7. Immunohistochemical examination of the mice eyeball. C57BL/6 mice eyes were injected with 15,000 spores of *A. flavus*. Infected eyes along with vehicle control were collected for histological assessment. The tissue sections were stained with (**A**) CD45 with positive control (tonsil), (**B**) MPO with positive control (myeloid sarcoma), (**C**) CD3 with positive control (lymphoid tissue), (**D**) CD20, and (**E**) isotype control (IgG). The positive cells were counted and the mean ± SEM values were represented as bar graphs. Pictures are the representation of one of the biological replicates and graphs represents the data combined from three independent experiments where there were three in each group. Original magnification = × 400. ns – non significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

results showed a significant increase of CD20 cells at 72 hpi ($P = 0.0352$), the number of B cells were relatively lower in comparison to T cells (CD3+; Fig. 7D).

A. flavus Endophthalmitis Induces Retinal Cell Death at 72 Hours Post-Infection

To correlate the clinical and immunohistochemistry findings, TUNEL assay was performed. We observed the significant increase ($P < 0.0001$) in the retinal cell death at 72 hpi (107.7 ± 1.856 apoptotic cells) when compared to 24 hpi (63 ± 1.528 apoptotic cells) which, in turn, is comparable with the increased disease severity and fungal burden observed in the clinical and histopathological examination. The majority of the cell deaths was observed in the inner nuclear layer and the outer nuclear layer of the retina. Furthermore, several studies have shown that increased retinal cell death is associated with declined visual function.[42,43] Retinal cell death accompanied by robust inflammatory cells influx could potentially contribute to a larger extent of vision loss (Fig. 8).

Validation of Inflammatory Mediators by ELISA in Vitreous of Patients With Fungal Endophthalmitis

Demographic, Clinical Features, and Microbiology of the Study Population. The study population included a total of five patients with culture proven fungal endophthalmitis. The mean age of the cohort was 61.4 ± 9.5 years (range = 46-70) and included 4 men and 1 woman. The etiology of the study group included three patients who were clinically diagnosed with exogenous endophthalmitis (postoperative and traumatic) and two endogenous endophthalmitis cases. Of the five patients, three were identified to have *Aspergillus spp* endophthalmitis and two patients had *Fusarium spp* endophthalmitis. The healthy control group included 5 patients with mean age 53 ± 17 years (range = 21–77 years) who were undergoing vitrectomy for other eye disorders like retinal detachment, vitreous hemorrhage, etc. A summary of the demographics, clinical characteristics, and microbiology data of the patients are included in Table 3.

Elevated Levels of Cytokines in Human Vitreous of Patients With Fungal Endophthalmitis

Multiplex immunoassay was performed to estimate the expression levels of inflammatory cytokines in the vitreous of patients with filamentous fungus endophthalmitis. Unpaired student *t*-test revealed a significant difference in the levels of six inflammatory mediators (IL-6, IL-8, TNF-α, IL-1β, IL-17, and IL-10) in the vitreous of patient with fungal endophthalmitis and vitreous of uninfected healthy control. The pro-inflammatory cytokines tested, such as IL-6 (16578 ± 3874 vs. 18.25 ± 8.879 pg/mL; $P = 0.0129$), IL-17 (432.2 ± 143.4 vs. 1.512 ± 0.69 pg/mL; $P = 0.017$), TNF-α (18.49 ± 4.93 vs. 4.5 ± 0.6 pg/mL; $P = 0.0302$), IL-1β (261.5 ± 101.2 vs. 0.48 ± 0.07 pg/mL; $P = 0.032$) were found to be significantly elevated in fungal infected vitreous when compared to healthy control patients, even chemokine such as IL-8 was also found to be significantly elevated (7300 ± 2653 pg/mL) in comparison to healthy controls (29.37 ± 18.51 pg/mL; $P = 0.025$). Interestingly, the concentration of IL-10, a cytokine with potent anti-
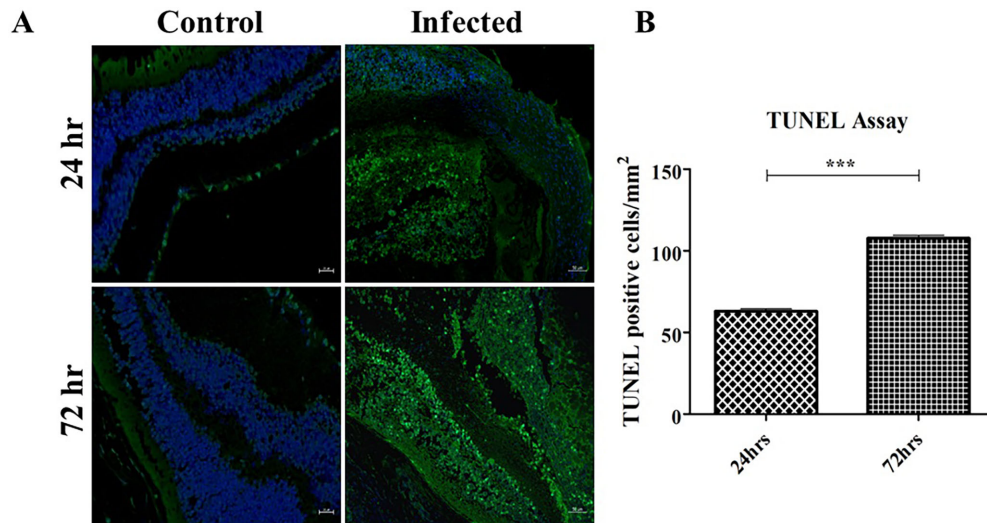
FIGURE 8. TUNEL assay indicative of apoptotic retinal cells. Apoptosis within the retina was determined by a TUNEL-based assay and counterstaining was performed with 4′-6-diamino-2-phenylindole (DAPI) after 24 hours and 72 hours post-infection with *A. flavus* (**A**) DAPI (*blue*), TUNEL (*green*). (**B**) Graph indicating the number of TUNEL-positive cells in the sections of *A. flavus* infected mice eyeballs. Significance was calculated using unpaired *t*-test test. Data were combined from three independent experiments where there were three in each group; ***$P < 0.001$.

TABLE 3. Microbiology and Demographic Details of all the Patients Included in the Study

Demographic Characteristics	Controls ($n = 5$)	Culture Positive ($n = 5$)
Age in years mean ± SD (range)	53 ± 17 (21–77)	61.4 ± 9.5 (46–70)
Male:female ratio	03:02	4:1
Diagnosis		
Postoperative endophthalmitis		2
Traumatic endophthalmitis		1
Endogenous endophthalmitis		2
Vitreous hemorrhage	3	
Retinal detachment	2	
Presenting visual acuity		
<20/200 to light perception	5	5
Final visual acuity		
≥ 20/200	3	1
< 20/200 to light perception	2	4
Microbiology		
Aspergillus flavus		1
Aspergillus versicolor		1
Fusarium solani		1
Fusarium sp.		1
Aspergillus niger		1

inflammatory property, was found to be downregulated in the infected group (81.04 ± 64.91 pg/mL) when compared to the control group (305 ± 108.2 pg/mL, $p = 0.113$; Fig. 9).

DISCUSSION

Fungal endophthalmitis is a sight threatening infection in the posterior segment of the eye, characterized by chronic inflammation due to the excessive host-immune response.[44–46] Although inflammation is necessary to keep the pathogen in check and clear the infection, inflammation could act as a double-edged sword especially in the eye because excessive influx of inflammatory cells and mediators could lead to disruption of the retinal architecture and irreversible vision loss.[47] To develop a targeted therapy and improve visual recovery, understanding the host-mediated response for fungal endophthalmitis is imperative. In this study, we present the in-depth transcriptional topology to understand the fungal pathogenesis and identify gene expression profiles specifically in a mice model of *A. flavus* endophthalmitis. Whereas several studies on fungal infection, especially in the eyes, focus on the involvement of innate immune system and overall elevation of inflammatory mediators,[4,44,48] we report the detailed transcriptomic profiling extending to host adaptive immune responses and the gene signatures. We further highlight the underlying mechanisms of interactions and their impact on the mouse retina.

Comparative transcriptional profiling reveals the striking elevation in the transcriptomic magnitude exclusively of inflammatory mediators and inflammation associated pathways such as cytokine-cytokine receptor interaction, neutrophil extracellular trap formation, natural killer cell mediated cytotoxicity, metabolic pathways, C-type lectin receptor signaling pathway, JAK-STAT signaling pathway, T-cell signaling pathway, phagosome, TNF signaling pathway, PI3K-Akt signaling pathway, Toll-like receptor signaling pathway, chemokine signaling pathway, NOD-like receptor signaling pathway, Th17 cell differentiation, Th1 and Th2 cell differentiation, and B-cell signaling pathway. Several genes associated with these pathways interact, triggering the whole range of inflammatory responses and the genes with the largest discrepancies in the fold-change were the key components of T-cell immune response to *A. flavus*. C-type lectin receptors play a major role in regulating antifungal immunity signaling cascades and induce adaptive immunity response to pathogens[49] and C-type lectin receptor signal-
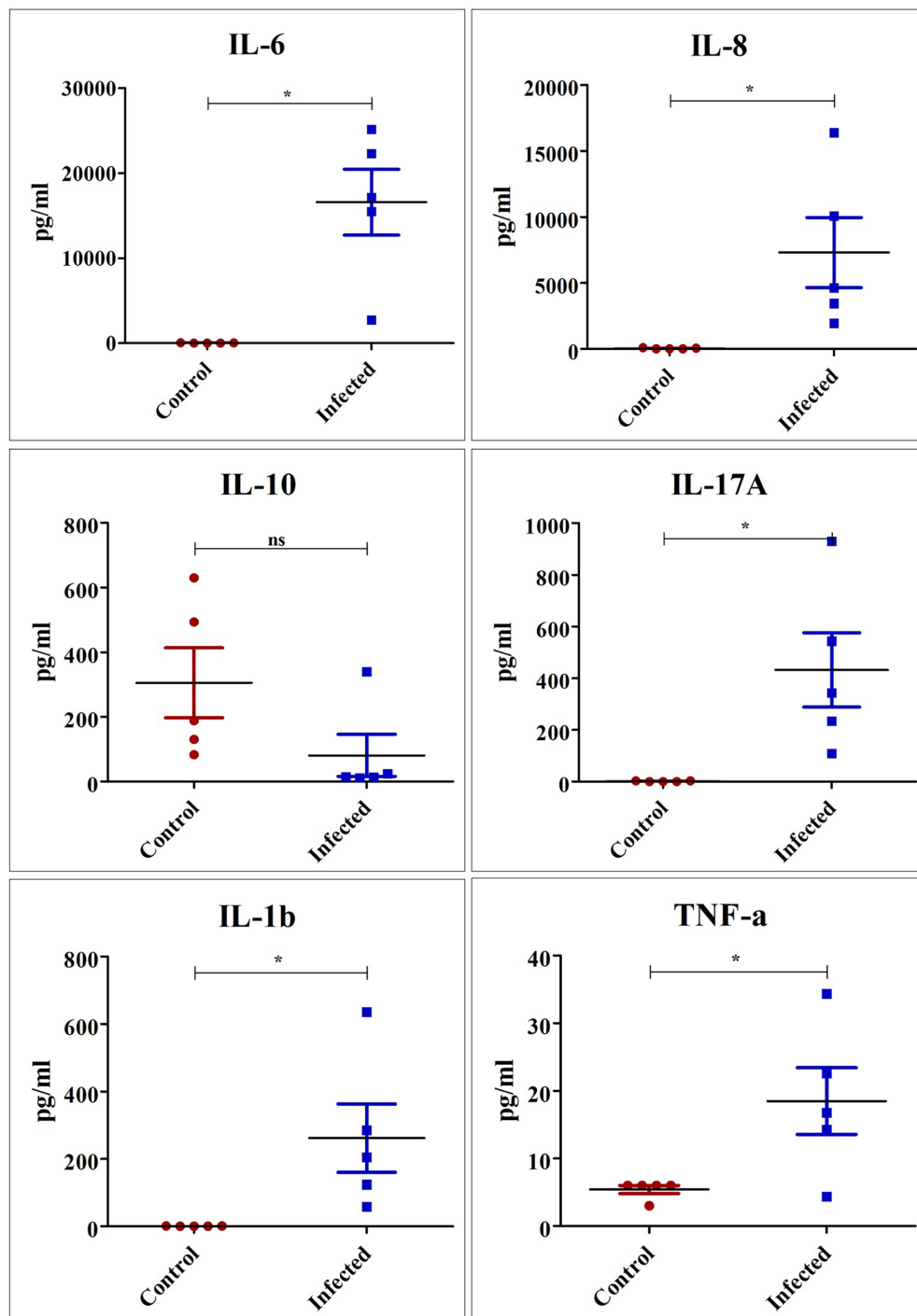
FIGURE 9. Assessment of cytokine expression in the vitreous. Scatter plot demonstrates the elevated expression levels of IL-6, IL-8, IL-10, IL-17A, IL-1β, and TNF-α in patients with fungal endophthalmitis when compared to the control. The concentrations of inflammatory mediators were compared among the above-mentioned two groups by Student's *t*-test. ns – non significant; *$P < 0.05$.

ing pathway associated genes, such as C-type lectin domain family 4, C-type lectin domain family 6, and C-type lectin domain family 7, observed in our study are reported to be involved in antigen uptake at the site of infection either for clearance of the antigen, or for processing and further presentation to T-cells.[50,51] Additionally, they also trigger genes such as caspase recruitment domain family member 9 and spleen tyrosine kinase which in turn triggers NF-

κB pathway consequently driving maturation of antigen-presenting cells and shaping antigen-specific priming of T-cells toward effector Th1 and Th17 cell subtypes.[51,52] Interestingly, Dectin-1, which is an important C-type binding receptor, was not identified in our study, although its consecutive signaling genes, such as SYK and CARD9, were upregulated.[36] B-cell lymphoma 3 is also reported to regulate the activation of NF-κB pathway.[53] Previously, in our in vitro
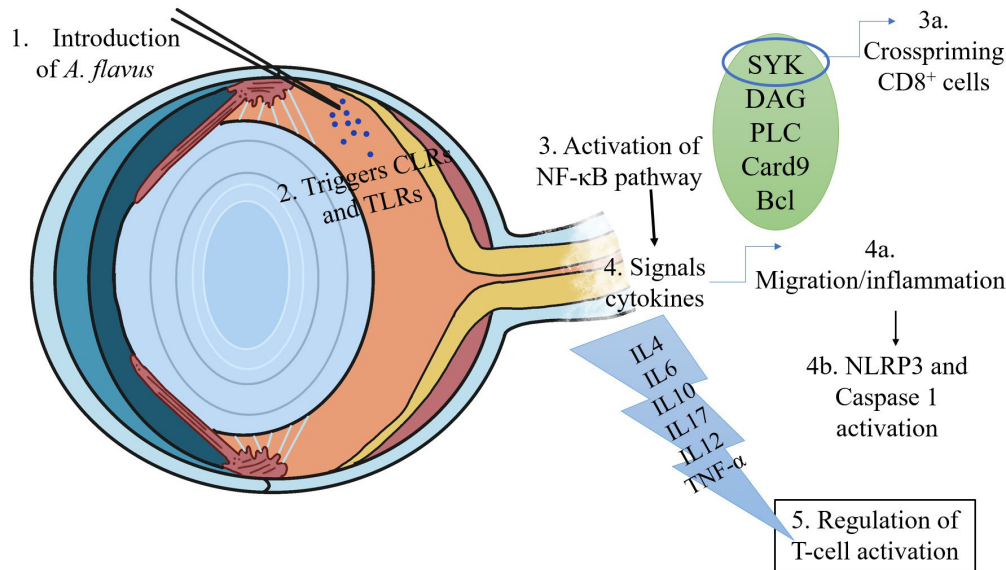
FIGURE 10. Pathogenesis of fungal endophthalmitis. The image represents the possible flow of pathogenesis of *A. flavus* endophthalmitis following the introduction of the pathogen into the mid-vitreous by intravitreal injection. The pathogen triggers the activation of CLRs and TLRs which consequently triggers genes, such as SYK, DAG, PLC, CARD9, and BCL associated with NF-κB pathway as observed in our transcriptomic analysis. This eventually triggers the cytokines and regulates T-cell activation.

studies, we reported the significant upregulation of TLR1, TLR2, and TLR6 upon *A. flavus* infection of retinal cells, which corroborated the transcriptomic results.[46] Furthermore, we also observed the upregulation of matrix metalloproteinases, such as MMP3 and MMP9 in our study, which also correlated with the in vitro results.[46] Several studies reported the role of TNF signaling pathway associated genes, such as TNF receptor-associated factor 1 and tumor necrosis factor receptor 2, are linked to the initiation of Th differentiation.[54,55] Genes such as neutrophil cytosolic factor 1 is reported to promote autoreactive CD8+ T-cell activation[55] and tyrosine-protein kinase which are involved in signals mediated by various cytokines or antigen receptors also play a prominent role in T lymphocyte activation and differentiation.[56,57] Interestingly, in addition to the inflammation-associated pathways, genes associated to metabolic pathways were also linked to regulation of immune response and T-cell activation. For instance, diacylglycerol kinase is reported to be the key molecule responsible for immune cell activation and differentiation. They are known to inhibit DAG-mediated signaling in T-cells preventing T-cell hyperactivation. Its downregulation in our study suggests the provision for T-cell activation.[58–60]

The majority of the pathways identified in our study directly or indirectly contribute to activation of cell-mediated immunity, especially CD8+ T cells activation during fungal endophthalmitis. We also observed several T-cell pathway mediated genes, such as T-cell co-stimulator, T-cell interacting activating receptor, lymphocyte cytosolic protein 2, T-cell surface glycoprotein CD8 alpha chain, T-cell surface glycoprotein CD8 beta chain, and cytotoxic T-lymphocyte-associated protein 4. Their significant elevation in our study suggests their pivotal role in *A. flavus* endophthalmitis.[61–63] Additionally, we further observed the heightened expression of few cytokines, such as IL-12A, IL-2RG and IL-4R are involved in the JAK2/STAT4 signaling pathway, promoting the proliferation of T-cells as well as NK cells. It also plays a role in Th1 cell differentiation and is essential for generating Th1-type cytokine responses and protection against infectious diseases.[64] Although there were genes, such as spleen tyrosine kinase, phosphoinositide 3-kinase adapter protein 1, and phosphoinositide-3-kinase regulatory subunit alpha/beta/delta, associated with the B-cell signaling pathway, we did not observe any major genes directly linked to the activation of B-cells which indicates that the message conveyance of humoral immunity could either be time variant or may not be playing a major role in *A. flavus* endophthalmitis. Our results further correlated with the histopathological findings which demonstrated the nonsignificant increase in the B-cells, whereas there was a significant increase in the T-cells during both 24 hpi and 72 hpi of *A. flavus*. Additionally, cytokine-cytokine receptor interaction was the most unregulated pathway in the transcriptomic analysis. An overall flow of fungal endophthalmitis based on our transcriptomic study is demonstrated in Figure 10. Our study demonstrated the consistent immune responses, supporting the previously reported pivotal role of several interleukins like IL-6, IL-8, IL-10, TNF-α, IL-1β, and IL-17A. The clinical validation of the cytokines in vitreous of patients with fungal (*Aspergillus spp* and *Fusarium spp*) endophthalmitis revealed the similar expression profile except for IL-10. Whereas IL-10 is an anti-inflammatory cytokine, it is reported to augment the expression of CD8+ T cells.[65] Although, in the transcriptomic profiling, we observed the upregulation of IL-10, which also was similar to our in vitro results,[46] we observed the decrease in the IL-10 expression in the vitreous of patients with fungal endophthalmitis when compared to healthy controls. Nonetheless, it is worth noting that the fungal endophthalmitis triggers cascade of inflammatory mediators and T-cells resulting in their rapid influx into the immune privileged site of the eye leading to retinal deterioration and vision loss.[66,67] Improving visual recovery is a crucial element in the management of endophthalmitis and as per our comprehensive transcriptomic analysis, diacylglycerol kinase could be a possible immunotherapeu-

tic target and the elevation of which could implement a restraint towards the immunogenic profile.[68] Additionally, cyclosporine A, an immunosuppressive agent, is reported to selectively inhibit the T cell activation with minimal effect on phagocytic cells which perhaps will control the excessive inflammation. Interestingly, it is also reported to have antifungal activity especially on *Aspergillus spp* rendering it as a favorable therapeutic option.[69]

Whereas the involvement of adaptive immunity 72 hours post-infection is accredited, the involvement of innate immunity is not inconsequential. Pathways like NOD-like receptor signaling pathway[70] and PI3K-Akt signaling pathways are reported to play a major role in immune cell activation especially neutrophils and mast cells suggesting that the innate immunity is still a significant part in the host-immune response.[71] Several genes, such as neutrophil cytosolic factor 1, neutrophil cytosolic factor 2, formyl peptide receptor 1, and cathelicidin antimicrobial peptide, involved in innate immunity were upregulated.[72] Additionally, higher CD45+ and MPO+ cell count per field also confirmed the heightened influx of inflammatory cells especially neutrophils associated with innate immunity. Because neutrophils are considered the cross-roads of innate and adaptive immunity, they could also potentially regulate the adaptive immune response in fungal endophthalmitis. Furthermore, there could also be a possible involvement of resident immune cells, such as hyalocytes and microglia, which could also potentially play a role in host-immune response.

Overall, our transcriptomics analysis of fungal endophthalmitis provided important insights into immunogenic profile of the host-mediated response to *A. flavus*. We identified important signal transduction pathways and their associated targets which collectively orchestrated the activation of the T-cell receptor signaling pathway suggested the prominent role of T-cell mediated immunity in *A. flavus* endophthalmitis. Furthermore, the top upregulated pathway, cytokine-cytokine receptor interaction, consisted of 46 inflammatory mediators inclusive of interleukins, chemokines, tumor necrosis factors, and interferons which is suggestive of exorbitant inflammation in the retina, which inadvertently leads to vision loss. Targeting these genes and their associated pathways may further help us in developing innovative adjuvants to suppress prophylactic immune responses against fungal endophthalmitis.

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