Mycopathologia (2018) 183:645–658
https://doi.org/10.1007/s11046-018-0254-9

ORIGINAL PAPER

RNA-Seq Profile Reveals Th-1 and Th-17-Type of Immune Responses in Mice Infected Systemically with *Aspergillus fumigatus*

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Received: 17 November 2017 / Accepted: 19 February 2018 / Published online: 2 March 2018

Abstract With the increasing numbers of immunocompromised hosts, *Aspergillus fumigatus* emerges as a lethal opportunistic fungal pathogen. Understanding innate and acquired immunity responses of the host is important for a better therapeutic strategy to deal with aspergillosis patients. To determine the transcriptome in the kidneys in aspergillosis, we employed RNA-Seq to obtain single 76-base reads of whole-genome transcripts of murine kidneys on a temporal basis (days 0; uninfected, 1, 2, 3 and 8) during invasive aspergillosis. A total of 6284 transcripts were downregulated, and 5602 were upregulated compared to baseline expression. Gene ontology enrichment analysis identified genes involved in innate and adaptive immune response, as well as iron binding and homeostasis, among others. Our results showed activation of pathogen recognition receptors, e.g., β-defensins, C-type lectins (e.g., dectin-1), Toll-like receptors (TLR-2, TLR-3, TLR-8, TLR-9 and TLR-13), as well as Ptx-3 and C-reactive protein among the soluble receptors. Upregulated transcripts encoding various differentiating cytokines and effector proinflammatory cytokines, as well as those encoding for chemokines and chemokine receptors, revealed Th-1 and Th-17-type immune responses. These studies form a basic dataset for experimental prioritization, including other target organs, to determine the global response of the host against *Aspergillus* infection.

Keywords Host–pathogen · Invasive aspergillosis · *Aspergillus fumigatus* · *Mus musculus* · RNA-Seq · Cytokines · Differential expression

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11046-018-0254-9) contains supplementary material, which is available to authorized users.

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Introduction

Humans inhale conidia from *Aspergillus* species, daily [1]. *Aspergillus fumigatus* is a ubiquitous filamentous fungus, which can cause a variety of problems ranging from allergic to invasive diseases depending on the

individual. Invasive aspergillosis is life threatening in immunocompromised patients, including those with solid organ transplants and hematologic malignancies [2, 3].

Host responses against *A. fumigatus* infection are critical for effective control of the disease [4, 5]. Studies in mouse models showed that both innate and acquired immunity of the host determine the protection against aspergillosis [5–7]. With the increasing numbers of at-risk patients, *A. fumigatus* has emerged as the most lethal mycosis worldwide [2, 8]. Established infection in these patient groups has proven very difficult to eradicate due to the lack of highly effective and curative antifungal drug treatment [2] and gaps in knowledge regarding the host immune response in normal and immunocompromised individuals.

The exploration of the host transcriptome during the course of *A. fumigatus* infection may indicate which genes play an important role in the host response and help the understanding of the development and the progression of the disease [9, 10]. Recently, RNA-Seq has been used to perform transcript profiling of in vitro cultured *A. fumigatus* [11–13], *A. orzyae* [14] and *A. flavus* [15]. However, RNA-Seq transcriptome data from in vivo or ex vivo studies are minimal [16]. Oosthuizen et al. [17] used genome-wide microarrays for fungus and human to examine the transcriptome response of bronchoepithelial cells to infection with *A. fumigatus* conidia, noting upregulation of IL-6 and innate pathways for the human cells, and upregulation of iron acquisition, and vacuolar acidification pathways, among others for the fungal cells. Similarly, Morton et al. [18] used microarrays to examine expression profiles of dendritic cells from an in vitro model of the alveolar surface and their interaction with *A. fumigatus*, noting changes in various chemokines associated with neutrophil chemotaxis. In the current studies, we used RNA-Seq technology to determine the host transcriptome in the kidneys of mice infected intravenously with *A. fumigatus* to better understand the events occurring in the most affected organ during intravenous infection and which may also be a key organ during systemic aspergillosis in humans [19]. Our study demonstrated coordinated expression of genes encoding for interleukins and interferon, interleukin receptors and receptor-associated factors, interferon regulators, interferon receptor and interferon-induced proteins, chemokines, chemokines receptors, iron homeostasis and iron binding proteins.

Materials and Methods

Infection Model

Six-week-old female CD-1 mice (Charles River Laboratories, Hollister, CA, USA) weighing approximately 25 grams each were arranged in groups of 5 and provided food and water ad libitum.

Aspergillus fumigatus strain Af293 [20] conidia for infection were prepared and infected intravenously with 1.1×10^7 viable conidia in 250 µl of saline/Tween-80 (0.05%) as described previously [21–23]. Five pre-assigned mice were euthanized on days 0, 1, 3, 5 and 8 to collect the samples. One mouse died on day 8, and thus, we considered day 8 as a last time point to collect the samples. Uninfected mice were used as normal controls to provide the baseline murine transcriptome. At each time point, kidneys were removed aseptically from each mouse in the group and pooled before being snap-frozen in liquid N_2. Samples were stored at −80 °C until used for total RNA extraction.

RNA Extraction

Kidneys from 5 mice at each point were thawed in Trizol (Invitrogen, Carlsbad, CA, USA) and broken with 0.5-mm zirconia/silica beads (Biospec) using a Bead Beater apparatus (Biospec Products, Inc., Bartlesville, OK, USA) at high-speed setting (3 pulses of 12–15 s). Total RNA was extracted using TRIzol reagent per the manufacturer's instructions. Contaminating gDNA was removed using RNeasy mini spin column (RNeasy mini kit, Qiagen Science, MD, USA) and RNase-free DNase set (50) (Qiagen GmbH, Germany), according to the manufacturer's instructions. The quantity and quality of the extracted RNA were assessed by A260 nm/A280 nm ratio (NanoDrop 1000 spectrophotometer, Thermo Scientific), as well as by electrophoresis through a 1.5% agarose gel stained with ethidium bromide for the presence of intact 18S and 28S ribosomal RNA bands, visualized by UV transillumination at 302 nm. RNA purity was determined using A260:A280 ratios and the extinction

coefficient for RNA to quantify the amount of RNA present [24].

cDNA Library Preparation and Sequencing

Poly (A) containing mRNA was purified from 8 μg of total RNA of each sample using Sera-mag Magnetic Oligo (dT) Beads (Illumina RS-100-0801); mRNA molecules were fragmented using divalent cations under elevated temperature followed by first- and second-strand synthesis. End repair was performed and deoxyadenosine triphosphate (dATP) added to the 3′ end of the DNA fragments. Next, adapters were ligated to cDNA fragments, cDNA template purified, and finally, the cDNA libraries were amplified via PCR. All steps were performed according to manufacturer's instructions. Before sequencing, the quantity and quality of each cDNA library were assessed using a 2100 Bioanalyzer (Agilent Inc.) [14]. Libraries were sequenced as single 76-base reads using the sequencing by synthesis method (Solexa sequencing, Illumina Inc.) at the Stanford Functional Genomic Facility, Stanford University.

Differential Expression Analysis

Initially, our intention was to obtain sequence reads for *A. fumigatus* and for the mouse transcripts, to generate two profiles to better understand the host–pathogen interactions. Alignment of sequence reads to the annotated genome of *A. fumigatus* (Af293) resulted in few transcripts that could map to the genome with more than a minimum number of reads (i.e., < 10). Thus, in vivo expressed transcripts of *A. fumigatus* could be not analyzed. Transcript sequences of *Mus musculus* were downloaded from Ensembl (Build 38, GCA_000001635.2). Functional annotation and gene ontology (GO) terms were downloaded from EnsemblBiomart (GRCm38.p1). Original coding sequences (CDS) were complemented by 100 nt of flanking genomic sequence on both ends to enable alignment of short sequencing reads with untranslated regions (UTR). Short reads were aligned against the modified CDS using Bowtie [25]; only the best 20 valid alignments (-k 20) were processed. Quantification of transcript abundance and isoform-adjusted expression was done via RSEM [26]. Normalization across samples was performed using the trimmed mean of M-values method (TMM) implemented by Edge-R [27]. Differential expression was calculated between all pairs of time points using the classical method of Edge-R [28]. Only genes with false discovery rate (FDR) < 0.01 and 16-fold change between a pair of time points were reported. All the applications were executed as part of the differential expression workflow implemented by the Trinity suite [29].

Clusters of Genes with Similar Expression Profile Across Samples

Clusters with similar expression profile across time points were defined based on the mean centered logarithm on base two of the FPKM values (Fragments per Kilobase of transcript per Million of mapped reads). The mean value was based on all genes with significant differential expression in at least one pair of time points. Similarity of expression profile between genes was computed using Euclidean distance, and clusters were defined according to K-mean algorithm.

GO enrichment was defined by Fisher exact correlation test and corrected for multiple comparisons via FDR, both implemented by scripts developed at the Broad Institute. A GO node was considered overrepresented in the case of threefold difference between test and background sets and a FDR value lower or equal to 0.05. Non-informational GO terms are not shown in the main manuscript, but listed in supplemental material (Supplementary file 1: Tables 7 and 8). GO terms were summarized by Revigo using *simRel* method and 0.7 maximum similarity allowed between remaining GO terms (*small* threshold) [30].

Results

In the murine model of systemic aspergillosis, immunocompetent mice are infected intravenously with conidia to establish infection. However, quantification of the fungal burden of *A. fumigatus* is difficult [31]. The temporal burden of *A. fumigatus* in the kidneys determined by culture of homogenates shows the highest burden at day 0–1, at which time the conidia are morphologically still conidia or have germinated with short hyphae, whereas colony counts determined at later time points (i.e., day 5 and 8) are lower because the organism has developed into hyphal forms in the tissues. This can be misinterpreted as a

reduced burden, whereas the use of qPCR to quantify fungal burden demonstrates a progressive increase in the number of organisms present [32]. In addition to that, among the few reads derived from *A. fumigatus*, we observed a progressive increase in abundance of rRNA transcripts (not shown). Thus, we are confident that the model is that of progressive infection and that temporal alterations of gene expression in the kidney are a result of the rise in infectious burden.

Approximately 179 million sequencing reads were generated (Supplementary Table S1), of which 136 million were successfully mapped to mouse transcripts. With respect to transcript coverage, of 83,070 mouse transcripts used as reference sequences, 57,007 (69%) had more than 10 reads mapped and approximately 29,000 (35%) had a normalized FPKM of one or greater. When considering each sample/day separately, at least 26% of transcripts reported a FPKM greater than or equal to one (Supplementary Table S2). Transcripts that were significantly differentially expressed in at least one pairwise comparison between time points accounted for 17% of mouse transcripts (14,397) (Supplementary Table S3). When comparing each time point to day 0 (non-infected animal, baseline), 6284 transcripts (8%) were downregulated and 5602 (7%) were upregulated. Despite approximately 2000 transcripts found to be upregulated on days 1 and 3 in comparison with day 0 (Fig. 1 and Supplementary Table S4 for the complete list of upregulated genes in comparison with day 0), there was no consistency in terms of the biological process associated with those genes, as evaluated by GO term enrichment. A concerted positive response only appeared on day 5, consisting of genes associated with leukocyte aggregation, acute inflammatory response, positive regulation of chemokines and other GO terms associated with immune response (set A in Fig. 1; Supplementary Table S7 for the list of enriched GO terms). Downregulation was maximal on day 1 with twice the number of transcripts (4295) (set D in Fig. 1 and Supplementary Table S5) than those upregulated at the same day. Genes with lower expression when compared to non-infected mice were consistently enriched for processes and functions associated with iron and heme binding, electron carrier activity and aromatase activity (Fig. 1b and Supplementary Table S7). A detailed inspection of the downregulated genes responsible for the enrichment of these processes and functions indicated that the majority were members of the cytochrome P450 family of proteins (Supplementary Table S10).

Thirty-five clusters with similar expression during the course of infection were defined (Supplementary Table 6). A set of three clusters representing positive, negative and mixed response are shown in Fig. 2a, with three of those having enrichment of GO terms. The positive response (cluster 7) was characterized by genes associated with adaptive and innate immune response, and the negative response (clusters 10 and 18) was enriched with transcripts associated with the assembly and binding of lipoproteins, including apolipoproteins H and A2, previously reported as negative regulator of neutrophils and angiogenesis [33]. The acute inflammatory response and negative regulation of cytokine production were also among the biological processes enriched in genes with lower expression after day 1, cluster 10 and 18 (Fig. 2b and Supplementary Tables 8).

A comparison focusing on the dynamics of cytokines and iNOS response between our work and a study on mice infected with *Cryptococcus neoformans* [34] indicated a similar pattern of robust response on day 5 and upregulation of TNF-alpha on day 1, in spite of the differences in infected tissue (brain), pathogen and technique (RT-PCR) between the two studies (Supplementary Table S11). A lower correlation and an earlier detection was reported by an ELISA-based evaluation of cytokines response in kidneys of mice infected with *A. flavus* [35], possibly different due to the nature of those assays.

Recruitment of Immune Cells at the Site of Infection

We found upregulated transcripts encoding for chemokines and chemokine receptors. Chemokines (*Ccl4, Ccl5, Ccl7,* and *Ccl8*) and their receptor, *Ccr1,* present on neutrophils, were upregulated (Supplementary Fig. S1). On monocytes, chemokine receptors *Ccr2* and *Ccr5* and their ligand chemokines (e.g., *Ccl2*, *Ccl7*, *Ccl8,* and *Ccl4*, *Ccl5, Ccl8*, respectively) were also upregulated (Supplementary Fig. S1). Receptors present on macrophages, *Ccr1* and *Cxcr3*, and their corresponding chemokines (i.e., *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8* and *Cxcl9*, *Cxcl10*, respectively) were also upregulated (Supplementary Fig. S1). Chemokines *Ccl2, Ccl3, Ccl4, Ccl5, Ccl7, Ccl8* and the receptors *Ccr1, Ccr2* and *Ccr5,* which are specific

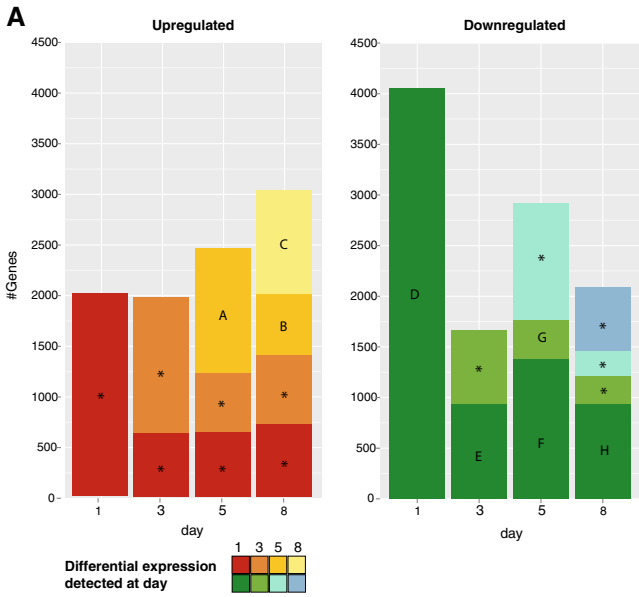


Fig. 1 Genes differentially expressed during the course of infection in comparison with non-infected mouse (day 0). **a** Colored bars indicate the number of transcripts detected on each day (*X*-axis) and the day they were first detected according to colors in the legend. Only transcripts with significant difference in expression (FDR lower than 0.01 and 16-fold change) when compared to day 0 were accounted. Bars marked with letters represent sets of transcripts with significant enrichment (Bonferroni-adjusted p value < 0.05) of at least one GO term. Gene ontology enrichment analysis is described in "Materials and methods" section. Bars marked with an asterisk indicate sets with no significant enrichment. **b** Summarized list of GO terms enriched in the sets defined on panel A. GO summarization is described in "Materials and methods" section. Gray blocks indicate the set (table header) in which the GO term has a proportionally higher frequency than expected by chance

to dendritic cells, were also upregulated in response to Af293 infection in mice (Supplementary Fig. S1).

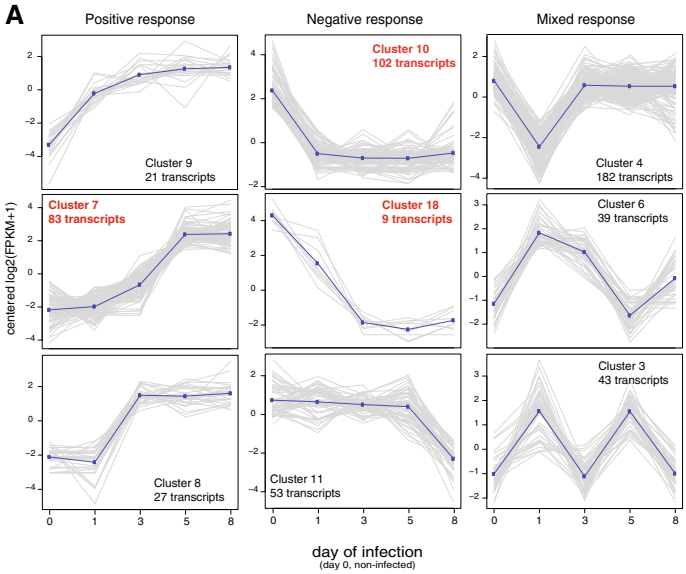
Proteins associated with the innate response that we searched included β-defensins, C-type lectins (e.g., dectin-1), TLRs, complement and adherence factors (e.g., VCAM or selectins) (Fig. 3, Supplementary Fig. S2 and S3). In general, increased duration of infection resulted in increasing number of transcripts for VCAM- and L-selectin, which would correlate with increased influx of host immune cells into the kidneys (Fig. 3). Furthermore, we noted increased expression of chemotactic complement factors, as well as some increased expression of mannose binding lectin (MBL), which can activate complement through an alternate pathway in response to binding of mannose in the fungal cell wall. Pentraxin-3 (Ptx-3) and C-reactive protein also can activate the complement pathway, and their expression was modulated postinfection (Supplementary Fig. S2). As might be expected, we noted increasing expression of C-type lectins (Fig. 3) and TLRs (Supplementary Fig. S2), which were highest at the day 8 after infection, also reflecting the influx of immune cells.

Activation of Th-1 and Th-17 Cells

Transcripts encoding chemokines, *Ccl2*, *Cxcl9*, *Cxcl10*, *Cxcl11*, *Cxcl13* and *Ccl20* (Supplementary Fig. S1), were upregulated, as were the trafficking receptors for chemokines *Ccr5* and *Cxcr3,* associated with Th-1 cells and other trafficking receptors for chemokines, *Ccr2* and *Cxcr4,* which are associated with Th-17 cells.

Th Cell-Mediated Immune Response and Interplay of Cytokines

We found activation of a Th-1 response mediated by proinflammatory cytokines. The genes encoding IFN-γ and IL-27 (IL-12 family) were observed to be steadily upregulated beginning 3 days postinfection (Supplementary Fig.S4). The effector cytokines, IL-18 and IL-24, were found to be upregulated beginning



B

Positive response (cluster 7)

Enriched GO terms
antigen processing and presentation of exogenous peptide antigen via MHC class II
chaperone mediated protein folding requiring cofactor
cell activation
positive regulation of chemokine (C-X-C motif) ligand 2 production
cellular response to interferon-beta
chronic inflammatory response
response to virus
immune effector process
positive regulation of T cell differentiation

Negative response clusters

10	18	Enriched GO terms
		acute inflammatory response
		regeneration
		phospholipid efflux
		high-density lipoprotein particle clearance
		response to peptide hormone stimulus
		positive regulation of interleukin-8 biosynthetic process
		locomotor rhythm
		diacylglycerol catabolic process
		regulation of lipase activity
		negative regulation of cytokine secretion involved in immune response
		positive regulation of cholesterol esterification
		high-density lipoprotein particle binding
		negative regulation of very-low-density lipoprotein particle remodeling
		regulation of intestinal cholesterol absorption
		apolipoprotein receptor binding
		high-density lipoprotein particle assembly
		phosphatidylcholine-sterol O-acyltransferase activator activity
		high-density lipoprotein particle receptor binding

Fig. 2 Cluster of transcripts presenting a similar profile of expression during the course of the infection. **a** Graphs depicting the normalized FPKM (*Y*-axis) of members of each cluster during the 8 days of sample collection (*X*-axis). Dark blue lines describe the approximate general pattern presented by members of each cluster. Graphs with caption in red indicate clusters with enriched GO terms. **b** Summarized list of GO terms enriched on clusters 7, 10 and 18. Gray blocks indicate the set (table header) in which the GO term has a proportionally higher frequency than expected. GO enrichment analysis and GO terms summarization are described in "Materials and methods" section

5 days postinfection in this model. IL-18 induces Th-1 response in the presence of IFN-γ and IL-12, whereas IL-24, a member of the IL-10 family, induces a Th-2-type response if differentiating cytokines IL-4 or IL-10 are present. On day 8 postinfection, we found upregulated transcripts encoding for IL-16; IL-16 promotes a Th-1 type response. We also observed activation of Th-17 pathway response genes mediated via proinflammatory cytokines IL-6 and IL-1α on day 5 postinfection. We found an increased number of transcripts encoding for interleukin IL-1β at day 5 postinfection, followed by a significant increase in transcript copies at day 8 postinfection (Supplementary Fig. S4).

Transcripts encoding IL-33, IL-34, IL-1f6 were upregulated in response to infection in kidney cells of mice. IL-34 transcripts had their highest expression at days 3 and 5 postinfection, but this was followed by a decline in transcript copies at day 8. IL-1f6 transcripts were upregulated at day 5 and were highest at day 8 postinfection (Supplementary Fig. S4).

Iron Regulation

A consistent enrichment of iron and heme binding, electron carrier activity and aromatase activity was found among downregulated genes (Fig. 1b). This was primarily a result of lower expression of members of the cytochrome P450 family of proteins during infection compared to baseline expression (Supplementary Table S10). However, a more detailed analysis revealed that key components of iron homeostasis had an increased numbers of transcripts in infected mice in comparison with uninfected mice, e.g., *Nos1* (nitric oxide synthase 1), *Nos2* (nitric oxide synthase 2), lipocalin 2(*Lcn2*), *p4ha2* (procollagen–proline), *Ogfod1* (2-oxoglutarate and iron-dependent oxygenase domain containing 1), *Ltf* (lactotransferrin), *Epb4.2* (erythrocyte protein band 4.2) and *Heph* (hephaestin) (Supplementary Fig. S5). Transcripts that showed a decreased expression compared to uninfected mice were *Tdo2* (tryptophan 2.3-dioxygenase), *Lepre1* (leprecan 1), *Hbq 1a* (hemoglobin theta 1A), *Ttc7* (tetratricopeptide repeat domain 7) and *Hamp* (hepcidin, antimicrobial peptide) (Supplementary Fig. S5).

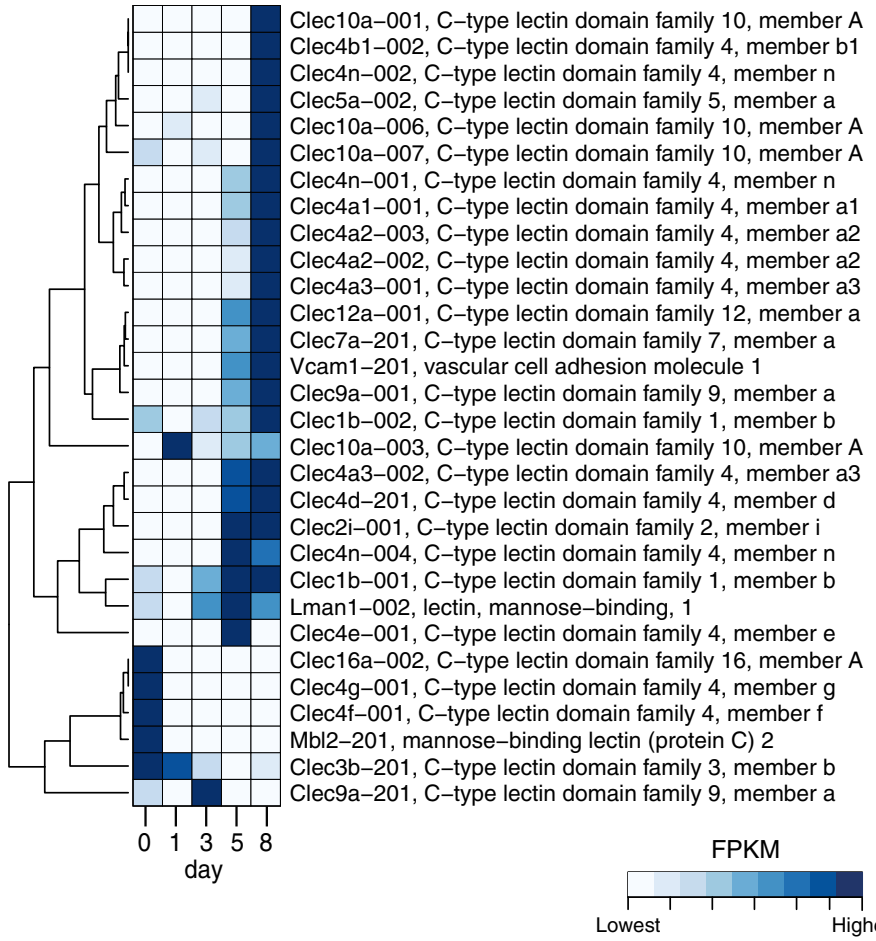


Fig. 3 Expression profile of differentially expressed C-type lectins and VCAM genes. Only genes differentially expressed in at least one pair of time points are shown. Color indicates the expression level of each transcript in terms of Fragments per Kilobase of transcript per Million of mapped reads (FPKM) on each time point: lowest FPKM values are depicted in white, and the highest values are shown in darkest blue, according to the legend. The dendrogram indicates the similarity of expression profile between transcripts

Discussion

The model of intravenous infection with *Aspergillus* was chosen to study the normal immune response to this infection in the absence of various immunosuppressive regimens that could be interposed, each of which would produce different perturbations of the immune response, and thus need each to be studied separately. Such studies can now follow, based on our data. To study the immune response in a setting absent the classical immunosuppressive risk factors also takes on topical relevance, owing to the rising incidence of aspergillosis in the non-immunocompromised [36]. The disease produced in the model also mimics disseminated infection with *Aspergillus,* where the kidney is a target organ, which is seen in one-third of cases of aspergillosis [19]. Finally, this model has been used extensively in studies of anti-*Aspergillus* therapy [21] and the assets of this model have been discussed elsewhere [7, 37, 38]. A limitation of the model used in this work is that the host is not immunocompromised, whereas most aspergillosis occurs in immunocompromised hosts. In the latter, the usual entry route is the lung, though tissue breaches can also lead to dissemination. Our original intention was to evaluate the interacting transcriptome of host and pathogen along the course of infection. Unfortunately, a very small number of sequencing reads was derived from *A. fumigatus* transcriptome, forcing us to focus our analysis on the host. For those willing to

implement similar experimental design, we would recommend the utilization of either mRNA depletion or enrichment technologies, such as hybrid selection [39].

The first line of defense against *Aspergillus* infections is the macrophages, neutrophils and dendritic cells that are recruited to the site of infection. These cells recognize pathogen-associated molecular patterns (e.g., β-glucan, mannans) that are exposed on the surface of germinating conidia or hyphae through pathogen recognition receptors (PRRs) including soluble receptors and cell bound receptors [5, 40, 41]. Among cell bound receptors, our data showed increased transcripts for genes encoding for C-type lectins (e.g., dectin-1), Toll-like receptors (TLR-2, TLR-3, TLR-8, TLR-9 and TLR-13), Ptx-3 and C-reactive protein were among the soluble receptors (Fig. 3 and Supplementary Fig. S2). In addition, we noted the increase in transcripts for β-defensins, which are cationic proteins found in the lysosomal contents of PMNs and contribute to the killing of microorganisms (Supplementary Fig. S2).

During kidney infection, *A. fumigatus* cells are recognized by antigen-presenting cells (APCs) and resident renal macrophages via PRRs. Furthermore, these immune cells induce the production of differentiating cytokines that act on naive $CD4^+$ Th cells [42]. For instance, we observed upregulation of the differentiating cytokine IL-27 (IL-12 family) 3 days postinfection, which induces Th-1 cells, and the cytokine IL-6, 5 days postinfection, which induces Th-17 cells (Supplementary Fig. S4). Subsequently, release of various proinflammatory effector cytokines, like IL-1β, IL-18, IFN-γ, IL-1α, IL-34 and IL-6, suggests the possible role of Th-1 and Th-17 cell responses (Fig. 4) [43]. Similar to our observation at 24 h postinfection, Armstrong-James et al. did not observe a significant increase in the level of IFN-γ in pulmonary invasive aspergillosis [44]. In a similar study of invasive renal aspergillosis induced by *A. flavus*, Anand et al. [35] observed significant induction of IFN-γ in kidney homogenates after 12 h of infection and levels of IFN-γ were higher along with IL-6, after 12, 72 and 96 h in infected mice compared to uninfected mice. Furthermore, in *A. flavus*-mediated cerebral aspergillosis mouse model studies, brain homogenates showed an increased level of IFN-γ, IL-12p40 and IL-6 at 24 h postinfection, with a decline in IL-4 and IL-23 cytokines [45]. Interestingly, we did not find signature transcripts encoding for the cytokines IL-4 and IL-10 suggesting the lack of an early Th-2-type of response. However, we noted the upregulated transcripts encoding for Ccl11 and IL-33 at later time point during infection (Supplementary Figs. S1 and S4); both contribute to Th-2-type response [46, 47]. It should be noted that the effect of IL-33 can be either Th-1 or Th-2 depending on the disease and model [48, 49]. We observed later during infection (i.e., day 5 postinfection) more transcripts for genes encoding for IL-1β and IL-1α indicated the possible role of Th-17 pathway involvement and suppression of Th-2 immune-responsive cytokines (Supplementary Fig. S4). Similar to our observations, Caffrey [50] et al. also observed upregulation of IL-1β and IL-1α during the initial days of a pulmonary *A. fumigatus* murine model. In addition, Cortez [51] et al. observed upregulated transcripts of IL-1β during the initial 2–6 h of interaction of *A. fumigatus* conidia with human monocytes. IL-1β also helps in activation of immune system and phagocytosis to decrease the load of *Aspergillus* at the infection site [52]. On the other hand, increased numbers of transcripts of the gene encoding for IFN-γ by day 5 postinfection (Supplementary Fig. S4) indicate earlier activation of the Th-1 immune response and suggest a possible coevolution of Th-1 and Th-17-type immune responses during kidney infection. Interestingly, expression of IL-34 throughout infection suggests survival of monocytes and their differentiation into immunosuppressive macrophages at the site of infection [53]. We also observed increased transcripts of genes encoding for the chemokine Cxcl10 and its receptor Cxcr3 (Supplementary Fig. S1), which activates immune cells responsible for IFN-γ secretion and inhibition of Th17 regulating cytokines [54]. The current data showed a notable profile of proinflammatory cytokines (e.g., IL-27, IFN-γ, IL-6, IL-34, IL-16, IL-1α and IL-1β). Measurement of selected cytokines in the blood samples of aspergillosis patients may be promising as a tool for the monitoring of treatment responses [55, 56].

Transcripts encoding numerous chemokines were upregulated. The trafficking receptor for chemokines Cxcl9, Cxcl10 and Cxcl11 is *Cxcr3,* and for chemokine Cxcl13, it is *Cxcr5*; we found transcripts for both these receptors were upregulated. These receptors are reported to be associated with Th-1 cells [57]. Similarly, we observed upregulation of transcripts encoding for

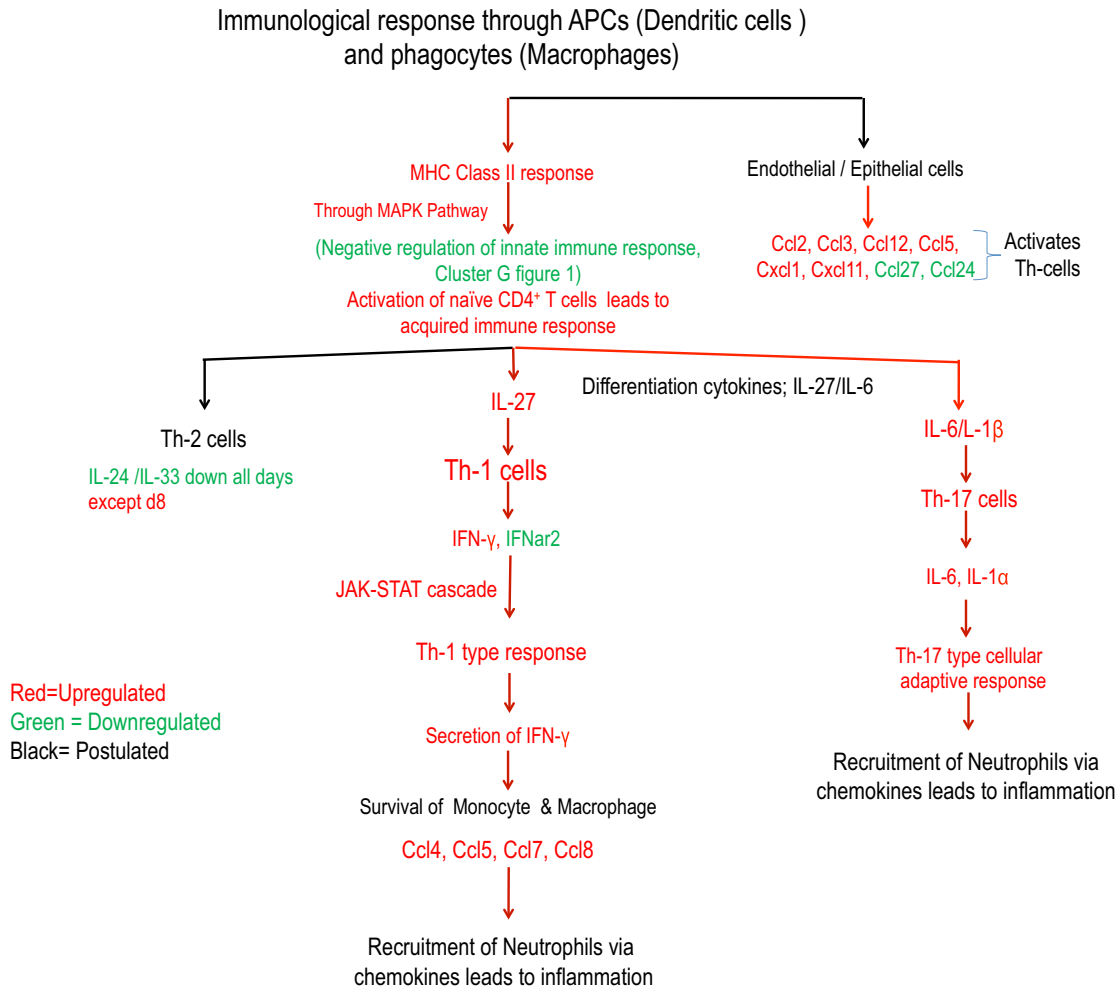


Fig. 4 Interplay of cytokines in mouse model of invasive aspergillosis. The diagram shows known and postulated immune pathways that play a role in the hosts' response to *A. fumigatus* infection. These include upregulated (shown in red) and downregulated (shown in green) gene transcripts of mice, after intravenous inoculation of Af293. The interaction of conidia with kidney cells leads to the release of cytokines and chemokines at the site of infection, which recruit and activate inflammatory and immune cells (e.g., phagocytes either circulating or resident macrophages), antigen-presenting cells (APCs; e.g., dendritic cells) and T cells. Phagocytic cells engulf the circulating conidia and destroy them via phagolysosome activity and also release of cytokines. APCs migrate and present *Aspergillus* antigens in the lymph nodes, to activate naïve $CD4^+$ T cells via MHC class II molecules. Consequently, activated T cells differentiate into Th-1 cells type via IFN-γ and IL-24 or a Th-17 cells via IL-6 and IL-1α/IL-1β

trafficking receptors *Ccr2* and *Cxcr4*. These receptors are present on Th-17 cells and respond to different chemokines (i.e., Ccl2, Cxcl10, Cxcl13, Ccl20) [58]. Consistent with our observation, Cortez [51] et al. also observed upregulation of transcripts encoding for the chemokine *Cxcl2*, *Ccl3*, *Ccl4* and *Ccl20* in human monocytes in response to *A. fumigatus*. Chemokines are released at the site of infection by epithelial cells, macrophages and neutrophils and attract an influx of more neutrophils, monocytes and macrophages, thus causing the inflammation at the site of infection [59].

Members of cytochrome P450 family were down-regulated on most days postinfection (Supplementary Table S10), recapitulating reports of transcription

modulation of those enzymes by cytokines and interferons during hepatic and renal infections [60–63]. The biological underpinnings of this phenomenon are unclear. It has been proposed that the downregulation of cytochrome P450 genes during infection may be an attempt in reducing the damaging effects of oxidizing species derived from uncoupled catalytic turnover of those enzymes when in the presence of either iron or *Nos2* (upregulated, Supplementary Fig. S5) [64]. Changes in the expression of host cytochrome P450 genes are especially relevant in the context of aspergillosis treatment and disease management. Cytochrome P450 enzymes have key roles in the activation and clearance of many drugs, including those used in the treatment of AIDS patients [65], one of the groups vulnerable to invasive aspergillosis [66].

The consistent downregulation of cytochrome P450 genes postinfection and the association of those genes with iron binding and aromatase activity, according to gene ontology, caused an enrichment of those pathways in the downregulated set (Fig. 1b). This may lead to the false interpretation that iron sequestration by the host is downregulated during *A. fumigatus* infection. Actually, the key components of iron homeostasis and regulation were upregulated after infection (e.g., lipocalin 2, lactotransferrin) (Supplementary Fig. S5).

In response to the use of siderophores by pathogens to acquire iron from host iron binding proteins, the host produces proteins that sequester ferric ion–siderophore complexes away from pathogen siderophore receptors [67]. A member of this functional group, lipocalin 2, is secreted by the host in response to infection and inflammation and binds not only to the metabolites of the host but also iron–siderophore complexes, thus sequestering iron from the pathogen [68]. Lactoferrin or lactotransferrin is a glycoprotein that is secreted by exocrine glands and neutrophils. Lactoferrin has various biological functions, including roles in iron metabolism, cell proliferation and differentiation [69]. The iron binding capacity of lactoferrin is twice that of transferrin (downregulated, Supplementary Fig. S5) [70]. Because of its iron binding properties, it reduces iron availability to any pathogens and also influences the immune system and cells involved in the inflammatory response [71].

Our study indicates downregulation of genes associated with aromatase activity (Fig. 1). Aromatase catalyzes estrogen synthesis from androgens (e.g., testosterone) [72]. Estrogens have both proinflammatory and anti-inflammatory properties [73]. The estrogen 17β-estradiol, as well as IL-6, IL1β and TNF-α, stimulates aromatase activity, possibly in vascular endothelial cells or tissue macrophages [74–76]. Thus, it is possible that downregulated genes encoding for aromatase enzyme may be involved in downregulation of proinflammatory [74] or anti-inflammatory [77] cytokine production.

When an invading microbe interacts with host cells, tissue damage resulting from inflammation, thrombosis and necrosis decreases available oxygen concentrations at the site of infection. The production of the non-ribosomal peptide gliotoxin, and possibly other secondary metabolites, by *A. fumigatus* contributes to the inhibition of angiogenesis in the lung [78, 79]. However, during invasive infection, *A. fumigatus* hyphae invade the blood vessels, which lead to hypoxia at the site of infection and activates angiogenesis [78]. Angiogenesis is an important physiological response to tissue hypoxia and proinflammatory cytokines. At the site of infection, endothelial cells produce proinflammatory cytokines and leukocyte adhesion molecule chemokines, which recruit neutrophils and release H_2O_2 [78]. Among the earliest cues for initiation of the wound repair response is the release of "growth factor"-related cytokines [80]. We observed upregulation of transcripts encoding for *Vegfa* (vascular endothelial growth factor) by day 3 of infection and *bFGF* (a fibroblast growth factor) by day 1 infection. We also observed upregulation of transcripts encoding for chemokines *Cxcl1, Cxcl2* and *Cxcl3*, all of which have been reported to be stimulatory factors for angiogenesis [57, 81, 82].

Overall, our results show that infection in the kidneys with *A. fumigatus* triggers a host response in the mouse that includes differential expression of 14,000 or less genes in the mouse. Our results show that genes such as C-type lectins, Toll-like receptors, C' proteins and iron sequestration come under regulation during infection as part of the innate response. Furthermore, adaptive cellular immune response includes cytokine and chemokines and their receptors are regulated during the first week of infection. Surprisingly, we did not find evidence of a clear Th-2 cellular response, with the exception of upregulation of Ccl11 and IL-33 at later time points. We postulate that a robust response of this type may only occur later

in infections that are less severe or more chronic, evidence for that would require supplementary work. Our study is an entry to understanding host responses to aspergillosis in tissue. Target organs more frequently involved in immunocompromised patients, such as the lung and brain need to be studied for similarities and differences. These studies form a basic dataset on which to build as more work, including other target organs, is performed in determining the global response of the host to infection with *Aspergillus.*

Acknowledgements We thank Marife Martinez, Vicky Chen and Ana Claudia Maretti-Mira for their assistance in this work.

Authors' Contributions KVC and DAS conceived and designed the experiments. JS performed the experiments. GCC and JS analyzed the data. DAS, KVC and JRW contributed reagents/materials/analysis tools. GCC, JS, KVC and DAS contributed to the writing of the manuscript.

Funding These studies were funded in part by National Institute of Allergy and Infectious Diseases and National Institutes of Health (URL: nih.gov) Grant 1R21AI85566-01 to DAS.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Availability of Data and Material Sequencing runs were deposited at Short Read Archive (SRA-NCBI) under the following accession identifiers: SRX1201397, SRX1201396, SRX1201395, SRX1201394, SRX1201392.

Ethics Approval and Consent to Participate All animal studies were using a protocol approved by the Institutional Animal Care and Use Committee of the California Institute for Medical Research (CIMR) under protocol numbers 09-01:3 and 12-01:1. Animal Care and Use protocols followed the Animal Welfare Act (7 U.S.C. 2131 *et seq.*) and the Office of Laboratory Animal Welfare—Public Health Service Policy on Humane Care and Use of Laboratory Animals. CIMR protocols were approved and performed under OLAW assurance number, A3652-01. The NRC Guidelines for the Care and Use of Laboratory Animals, 8th edition, were followed for all care and use of mice. Mice were euthanized using CO_2 narcosis per the 2007 version of the American Veterinary Medical Association (AVMA) guidelines, which was the time the animal studies were performed.

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