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ORIGINAL ARTICLE **Basic Mechanisms in Allergic Disease**

Influence of *Aspergillus fumigatus* conidia viability on murine pulmonary microRNA and mRNA expression following subchronic inhalation exposure

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Clinical & Experimental Allergy

Summary

Background Personal exposure to fungal bioaerosols derived from contaminated building materials or agricultural commodities may induce or exacerbate a variety of adverse health effects. The genomic mechanisms that underlie pulmonary immune responses to fungal bioaerosols have remained unclear.

Objective The impact of fungal viability on the pulmonary microRNA and messenger RNA profiles that regulate murine immune responses was evaluated following subchronic inhalation exposure to *Aspergillus fumigatus* conidia.

Methods Three groups of naïve B6C3F1/N mice were exposed via nose-only inhalation to *A. fumigatus* viable conidia, heat-inactivated conidia (HIC), or HEPA-filtered air twice a week for 13 weeks. Total RNA was isolated from whole lung 24 and 48 h postfinal exposure and was further processed for gene expression and microRNA array analysis. The molecular network pathways between viable and HIC groups were evaluated.

Results Comparison of data sets revealed increased *Il4*, *Il13* and *Il33* expression in mice exposed to viable vs. HIC. Of 415 microRNAs detected, approximately 50% were altered in mice exposed to viable vs. HIC 48 h postexposure. Significantly down-regulated ($P \leq 0.05$) miR-29a-3p was predicted to regulate *TGF-β3* and *Clec7a*, genes involved in innate responses to viable *A. fumigatus*. Also significantly down-regulated ($P \leq 0.05$), miR-23b-3p regulates genes involved in pulmonary IL-13 and IL-33 responses and *SMAD2*, downstream of TGF-β signalling. Using Ingenuity Pathway Analysis, a novel interaction was identified between viable conidia and *SMAD2/3*.

Conclusions and Clinical Relevance Examination of the pulmonary genetic profiles revealed differentially expressed genes and microRNAs following subchronic inhalation exposure to *A. fumigatus*. MicroRNAs regulating genes involved in the pulmonary immune responses were those with the greatest fold change. Specifically, germinating *A. fumigatus* conidia were associated with *Clec7a* and were predicted to interact with *Il13* and *Il33*. Furthermore, altered microRNAs may serve as potential biomarkers to evaluate fungal exposure.

Keywords allergens and epitopes, animal models, genetics

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Introduction

Exposure to fungal bioaerosols in indoor environments has been associated with adverse respiratory health effects [1, 2]. Interest in understanding the mechanisms associated with fungal exposures has increased following recent natural disasters, including hurricanes Katrina, Rita, and more recently Sandy that have resulted in the prolific contamination of flooded indoor building materials [3]. *Aspergillus*

fumigatus is a saprophytic mould naturally found in the environment that produces respirable asexual conidia [4, 5]. In some damp indoor environments, *A. fumigatus* can be found growing on common building materials such as wood, gypsum board, and chipboard [6] and disturbance of these contaminated building materials can result in elevated personal exposure [7]. To date, the influence of conidia viability on the microRNA (miRNA) and messenger RNA (mRNA) profiles underlying pulmonary immune and toxicological responses to *A. fumigatus* exposure are not fully characterized.

Our laboratory has developed a murine inhalation model of fungal conidia exposure to reproduce exposures encountered in fungal contaminated indoor, outdoor and occupational environments [8]. Dry homogenous aerosolized fungal conidia are delivered to mice housed in a multi-animal nose-only inhalation chamber. Initially, our laboratory reported an IL-17-secreting CD8a+ cytotoxic T cell population (Tc17) dominant in the lungs of mice after 4 weeks of inhalation exposure to *A. fumigatus* that correlated to the onset of *in vivo* conidia germination [8]. By 13 weeks, inhalation of *A. fumigatus* was found to elicit a mixed Type 1 T helper (Th1) and Type 2 T helper (Th2) response in mice exposed to viable *A. fumigatus* conidia [unpublished data]. Subchronic exposure to viable conidia significantly increased $CD4^+$ T cells and associated cytokines, such as interleukin 13 (IL-13) and interferon gamma (IFN-γ) compared to heat-inactivated conidia (HIC). This study corroborated previous observations demonstrating that conidia germination was a crucial parameter in the induction of allergic inflammation in the lungs.

Compared to viable conidia, HIC were produced through heat and pressure treatment (autoclaving) that appears to degrade the proteins without changing the morphology of the conidia. The degraded proteins render conidia inactivated, ultimately inhibiting germination. Previously, we reported an accumulation of eosinophils in the airways of mice exposed to repeated instillations of HIC over a short period of time [9]. Another study demonstrated that a Th1 dominant response follows exposure to HIC, compared to a mixed Th1 and Th2 response in murine airways following exposure to viable *A. fumigatus* conidia [10]. A mixed response was also observed in mice exposed to viable *A. fumigatus* conidia for 13 weeks [11]. Heat inactivation and protein degradation may inhibit the secretion of critical allergens influencing pulmonary immune responses [12]. Inhalation of *A. fumigatus* conidia in mice is known to elicit an allergic inflammatory phenotype, with the hallmarks of allergic asthma such as the recruitment of eosinophils and lymphocytes [13]. In humans, *A. fumigatus* is known to cause allergic bronchopulmonary aspergillosis that results from a hypersensitization to *Aspergillus* antigen exposure characterized by a dominant Th2 immune response and a lower presence of Th1 cytokines, as well as eosinophilic inflammation [14, 15]. Despite extensive characterization of *A. fumigatus* induced pulmonary immune responses, few studies have evaluated the molecular processes associated with subchronic fungal exposure.

MiRNAs are non-coding RNAs located in the 3′ untranslated region that are 19–25 nucleotides in length and capable of influencing gene expression through a variety of mechanisms [16–20]. Early studies suggested that miRNAs could only degrade the target messenger RNA or inhibit translation; however, more recent data suggests that miRNA also activate the translation of certain target mRNA [18, 19]. MiRNA regulation of gene expression is complex because not only can hundreds of genes be targeted by one miRNA, but also many miRNAs can target one individual gene. These small RNAs play a critical regulatory role in numerous diseases and cellular processes such as cancer, heart disease, cellular development, apoptosis, and immune responses [17, 20, 21].

MiR-21 is a miRNA that has been extensively studied as part of the response to inflammatory stimuli and in a variety of disease models including, cancer [22–24], allergic airway inflammation [25], psoriasis [26], as well as in viral [27, 28], bacterial and protozoan infection models [29, 30]. Expression of miR-21, miR-22, miR-27b, miR-31, miR-126, miR-155, miR-210 and miR-301a was increased after dermal exposure to toluene 2,4-diisocyanate [31]. MiR-126 and miR-145 have been shown to be up-regulated in the airways following exposure to house dust mite allergen [32, 33]. Inhibition of miR-126 in a house dust mite-induced asthma model resulted in decreased allergic inflammation [32]. Airborne pollutants are also known to alter the miRNA environment in the murine lung; for example, cigarette smoke down-regulates let-7c, let-7f, miR-34b, miR-34c and miR-222 [34–37]; diesel exhaust up-regulates miR-135b [38]; and volatile organic compounds have been shown to up-regulate miR-125a and miR-466, while down-regulating miR-125b [35, 39, 40]. Previous studies have reported the dysregulation of miRNAs following Staphylococcal enterotoxin B [41] and nicotine exposure [42].

Although other studies have examined miRNA and mRNA environments in other models and to different stimuli, this study is among the first to evaluate the pulmonary miRNA and mRNA environment following subchronic exposure to aerosolized fungal conidia. To our knowledge, this is the first study to examine the influence of viable *A. fumigatus* conidia on the miRNA and mRNA profiles and pathways involved in pulmonary immune responses.

Methods

Animals

Female B6C3F1/N mice, aged 5–6 weeks were acquired from the National Toxicology Program and mouse colony housed at Taconic (Germantown, NY, USA). Mice were acclimated for approximately 1 week prior to exposures and housed in filtered, ventilated polycarbonate cages in groups of five on autoclaved hardwood chip bedding. Mice were provided with NIH-31 modified 6% irradiated rodent chow (Harlan Teklad) and tap water *ad libitum*. The National Institute for Occupational Safety and Health (NIOSH) animal facility is an environmentally controlled barrier facility that is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All animal procedures were performed under a NIOSH Animal Care and Use Committee approved protocol.

Fungal cultures

Aspergillus fumigatus strain B5233/ATCC 13073 (ATCC, Manassas, VA, USA) conidia were inoculated on malt extract agar plates for 7–10 days at 25°C and harvested in 2 mL of filter sterilized, endotoxin-free water (Sigma Aldrich, St. Louis, MO, USA). Autoclaved Mahatma brown rice (10 g) (Riviana Foods Inc., Houston, TX, USA) was inoculated with 3 mL of 2.5×10^6 conidia/mL and incubated for 10–14 days. HIC were prepared by autoclaving the *A. fumigatus* laden rice cultures for 15 min at 121°C. This high temperature and pressure treatment did not alter the morphology of the conidia, but did reduce the viability by at least 97%. It is assumed that the potential germination of the remaining 3% or less viable conidia did not have an appreciable effect on the pulmonary immune response. Viable and HIC were transferred to a desiccator for 3 days prior to inhalation exposures.

Aspergillus fumigatus *conidia exposures*

Mice were acclimated to the nose-only exposure chamber for 1 week prior to exposures. Eighteen mice (3/exposure group) were separated into three exposure groups: (i) 1×10^5 *A. fumigatus* viable conidia; (ii) 1×10^5 heat-inactivated *A. fumigatus* conidia (HIC); or (iii) HEPA-filtered air-only control. The conidia numbers represented the estimated conidia deposited in the lungs of animals for each exposure as described previously [8]. Mice were exposed in the nose-only chamber of the acoustical generator system (AGS) twice a week for a total of 13 weeks (26 exposures) in a manner similar to Buskirk et al. [8]. The AGS automatically shut off conidia aerosolization once the desired estimated lung deposition had been reached for each exposure. At 24 and 48 h postfinal exposure, mice were sacrificed via intraperitoneal injection of 200 mg/kg sodium pentobarbital solution (Fatal-Plus; Vortech Pharmaceuticals, Ltd., Dearborn, MI, USA), and once determined to be unconscious and unresponsive, the mice were exsanguinated via cardiac puncture.

Harvesting of lung tissue

The lungs were harvested from mice at 24 and 48 h postfinal exposure. Total RNA was isolated from whole lung homogenate using Exiqon's miRCURY RNA Isolation Kit for tissue following the manufacturer's protocol (Exiqon, Woburn, MA, USA) or using Qiagen's RNeasy Plus Universal RNA Isolation Kit following the manufacturer's protocol (Qiagen, Redwood City, CA, USA).

MicroRNA array profiling

All miRNA experiments were conducted at Exiqon Services, Denmark. Total RNA (500 ng) from both sample and reference was labelled using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon Inc., Vedbaek, Denmark) following the manufacturer's instructions. The Hy3™-labelled samples and a Hy5™-labelled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon) according to the miRCURY LNA™ microRNA Array instruction manual, using a Tecan HS4800™ hybridization station (Tecan, Mannedorf, Austria). The miRCURY LNA™ microRNA array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., Santa Clara, CA, USA), and the image analysis was completed using the ImaGeneR 9 (miRCURY LNA™ microRNA Array Analysis Software; Exiqon Inc.). The quantified signals were then corrected for background noise and normalized using the global Lowess regression algorithm. The array analysis examined all miRNAs for human, mouse or rat registered in miRBASE 18.0.

Gene expression arrays

Total RNA (5 μg) was converted to cDNA by the RT^2 First Strand Kit (Qiagen). Equal amounts of cDNA were then subjected to real-time PCR according to manufacturer's instructions (Qiagen). The genes examined were grouped into Th1-Th2 response and Th17 response pathways. The primers were included in prefabricated PCR plates (RT^2 Profiler™ PCR Array Mouse Th1 and Th2 Responses and RT^2 Profiler™ PCR Array Mouse

Th17 Response). RT^2 SYBR Green ROX qPCR Mastermix was used for quantification of cDNA (Qiagen). Data were normalized by gene expression relative to the levels of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) for the Th1-Th2 response pathway and beta-glucuronidase (*Gusb*) for the Th17 response pathway. If specific genes included in the response pathways resulted in undetermined expression levels, the genes were removed from the heat maps (generated using R software [44]) that are reported in Fig. 1a and b.

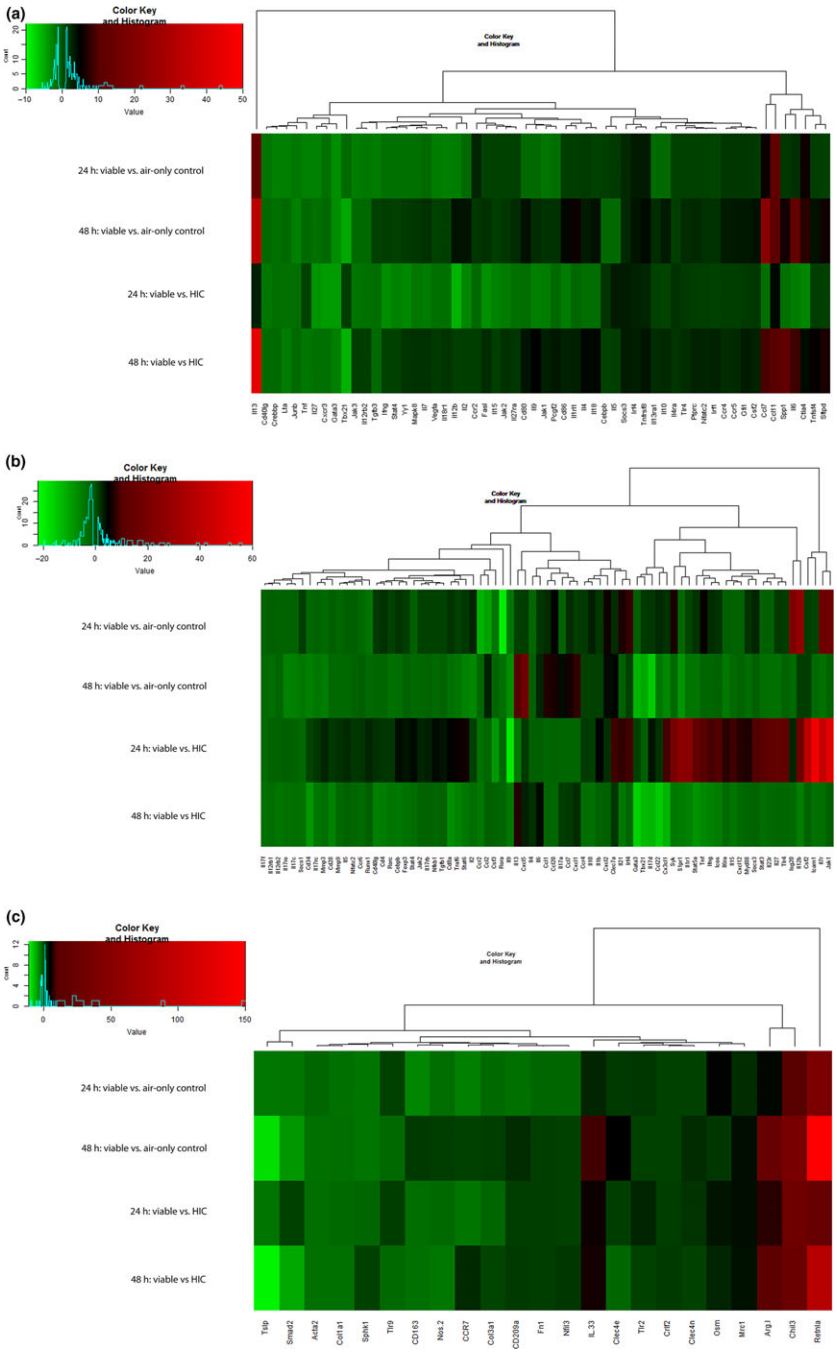

Fig. 1. (a) Heat map of genes involved in the Th1 and Th2 immune response pathways. Genes are colour-coded (red or green for up- and down-regulation, respectively). (b) Heat map of genes involved in the Th17 immune response pathways. Genes are colour-coded (red or green for up- and down-regulation, respectively). (c) Heat map of additional genes involved in the immune response. Genes are colour-coded (red or green for up- and down-regulation, respectively).

Real-time polymerase chain reaction: validation and additional genes

Total RNA (1 μg) was isolated and converted to cDNA by the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Carlsbad, CA, USA). Equal amounts of cDNA were subjected to real-time PCR. Murine genes and associated assay identifiers (Life Technologies) that were validated are listed in Table S3, while the additional genes and associated assay identifiers (Life Technologies) are reported in Table S4. TaqMan® Fast Universal PCR Master Mix (2×) and no AmpErase® UNG (Life Technologies) were used for quantification of cDNA. Data were normalized by gene expression relative to the levels of *Gapdh*. Heat maps were generated using R software [43] and Bimax Biclustering ('BCBimax' clustering algorithm from Package 'biclust' version 1.0.2) as the clustering algorithm.

Ingenuity pathway analysis

After miRNA identification and mRNA and miRNA quantification was assessed, the fold changes and false discovery rates of the differentially expressed genes were imported into Ingenuity Pathway Analysis software (IPA; Qiagen) for core, comparative and miRNA target filter analysis. The software was utilized for the construction of interacting mRNA and miRNA networks identified within the viable exposure group and compared to HIC and HEPA-filtered air control groups.

Statistics

All miRNA analytical calculations were performed by Exiqon Inc. using R/BIOCONDUCTOR software, which included moderated *t*-tests, accounting for the variance of the entire data set. MiRNAs with fold changes ≤ -2 (down-regulated) or ≥ 2 (up-regulated) were considered altered. The adjusted *P*-values ≤ 0.05 were considered significant.

For the RT^2 Profiler PCR array results, which included genes grouped into Th1, Th2 and Th17 response pathways, the C_t values and subsequent statistical analysis were processed using Qiagen's GeneGlobe Data Analysis Center. For both the RT^2 Profiler PCR arrays, the additional genes measured and the validation of genes measured by qRT-PCR, statistics were completed using the same methods described above. Briefly, the fold change was the normalized gene expression in the test sample divided by the normalized gene expression in the control sample. Genes with fold changes ≤ -2 (down-regulated) or ≥ 2 (up-regulated) were considered altered. The *P*-values were calculated based on a Student's *t*-test of the replicate values for each gene in the control group and treatment groups, and *P*-values ≤ 0.05 were considered significant.

Results

MicroRNA analysis

After threshold filtering, 415 murine miRNAs were detected in the Exiqon analysis (Table S1). Comparing the viable exposure group to HIC (viable vs. HIC) and air-only control (viable vs. air-only control) groups 24 h postexposure, 117 and 115 miRNAs, respectively, were shown to be differentially expressed (Table S2). At 48 h postexposure, 217 and 210 miRNAs were altered, respectively (Table S2). Interestingly, all but 4 of the miRNAs that were differentially expressed at 24 h were further dysregulated at 48 h. There were no significantly dysregulated miRNAs when comparing HIC to air-only control for either time point.

The miRNAs with the largest fold change at 24 and 48 h following exposure were the same miRNAs when comparing viable vs. air-only control and viable vs. HIC (Table 1). These miRNAs included miR-23b-3p, miR-29a-3p and miR-30c-5p, as well as miR-2137, miR-677-3p and miR-1947-3p. Although the miRNAs were not significantly altered at 24 h postexposure, the fold changes of these miRNAs reached statistical significance at the 48-h time point. IPA identified that these dysregulated miRNAs targeted specific genes that participate in the immune response and were associated with different diseases and functions. IPA revealed the involvement of miR-2137 and miR-1947-3p in chemokine- and T cell receptor signalling, respectively, while miR-23b-3p and miR-29a-3p regulated different genes involved in the inflammatory response.

mRNA array analysis

mRNA array analysis demonstrated the expression of 96 genes associated with either the Th1 and Th2 or Th17 response pathways. Figure 1a depicts the heat map that shows the fold changes of genes involved in the Th1 and Th2 response pathways. A greater number of genes were down-regulated (indicated by green bars in Fig. 1a) in both exposure groups at 24 h, compared to 48-h time points. After 48 h, only three genes were down-regulated, whereas the majority of genes were up-regulated in both the viable vs. air-only control, and in the viable vs. HIC group (indicated by black and red bars in Fig. 1a). While as many as 46 genes involved in the Th17 response pathway were up-regulated in both groups 24 h postexposure, no more than 12 were up-regulated after 48 h following exposure, whereas the majority of genes were down-regulated (Fig. 1b). Analysis of the genes involved in the Th1 and

Table 1. Top five largest dysregulated miRNAs

	Viable/Air-only control			Viable/HIC	
	Fold change	FDR		Fold change	FDR
	24 h			24 h	
mmu-miR-23b-3p	−16.47	0.11	mmu-miR-23b-3p	−12.08	0.16
mmu-miR-30c-5p	−10.58	0.11	mmu-miR-24-3p	−9.51	0.16
mmu-miR-24-3p	−10.44	0.12	mmu-miR-30c-5p	−9.06	0.16
mmu-miR-30b-5p	−7.65	0.11	mmu-miR-30b-5p	−6.83	0.16
mmu-miR-23a-3p	−6.95	0.11	mmu-miR-29a-3p	−6.60	0.21
mmu-miR-2137	10.23	0.11	mmu-miR-2137	7.11	0.16
mmu-miR-592-3p	9.11	0.02*	mmu-miR-677-3p	6.43	0.16
mmu-miR-677-3p	8.84	0.11	mmu-miR-1843b-3p	6.23	0.16
mmu-miR-1947-3p	6.44	0.11	mmu-miR-1947-3p	5.91	0.16
mmu-miR-1843b-3p	6.40	0.11	mmu-miR-592-3p	5.67	0.12
	48 h			48 h	
mmu-miR-23b-3p	−62.23	0.00*	mmu-miR-23b-3p	−52.84	0.00*
mmu-miR-24-3p	−34.34	0.00*	mmu-miR-24-3p	−32.66	0.00*
mmu-miR-23a-3p	−25.30	0.01*	mmu-miR-29a-3p	−22.03	0.01*
mmu-miR-30c-5p	−24.90	0.00*	mmu-miR-23a-3p	−21.05	0.01*
mmu-miR-29a-3p	−20.97	0.01*	mmu-miR-30c-5p	−21.05	0.00*
mmu-miR-677-3p	15.98	0.00*	mmu-miR-2137	13.14	0.00*
mmu-miR-2137	15.35	0.00*	mmu-miR-677-3p	12.42	0.00*
mmu-miR-1947-3p	13.52	0.01*	mmu-miR-1947-3p	11.39	0.01*
mmu-miR-1843b-3p	13.30	0.00*	mmu-miR-1843b-3p	10.64	0.00*
mmu-miR-3103-3p	12.69	0.00*	mmu-miR-3103-3p	10.22	0.00*

FDR, false discovery rate; HIC, heat-inactivated conidia.
*P-value ≤ 0.05 is considered significant.

Th2 response pathway revealed no significantly dysregulated genes at either time point. In contrast, of the 96 measured genes involved in the Th17 response pathway, 10 genes were dysregulated in the HIC compared to air-only control at 24 h postfinal exposure, whereas only one gene was dysregulated at 48 h following the final exposure.

In both response pathways, GATA binding factor 3 (*Gata3*) and T-box transcription factor T-Bet (*Tbx21*) were the two genes with the greatest down-regulation in both the viable vs. HIC group and the viable vs. air-only control at 48 h. The decrease in *Gata3* expression was uncharacteristic of an allergy model; therefore, it was verified by a separate qRT-PCR analysis using TaqMan® and *Gata3* primers from a different vendor (data not shown). In contrast, interleukin 13 (*Il13*) had the greatest degree of up-regulation in both groups at 48 h following fungal exposure in both response pathways.

Previous analyses reported from our laboratory indicated the importance of fungal conidia germination on the resulting pulmonary immune response following subchronic *A. fumigatus* exposure [8]. C-type lectin domain family 7 member A (*Clec7a*), also known as Dectin-1, is a surface receptor that recognizes β-glucan on the cell wall of germinating conidia and fungal hyphae. At the 24-h time point, the expression of *Clec7a*, measured by both the Th17 response pathway and through validation, showed an 8.9-fold increase in the viable vs. HIC group (Fig. 1b). Although *Clec7a* did not show increased expression in the viable vs. HIC group in the Th17 response pathway 48 h postfinal exposure, it was significantly increased by 4.5-fold when validated ($P \leq 0.05$; data not shown). This difference could be explained by the different technologies used to obtain the measurement of *Clec7a* expression. Overall, the increase in *Clec7a* further demonstrates the importance of *A. fumigatus* conidia germination on ensuing pulmonary immunological responses.

Molecular network analysis

Utilizing the microRNA target filter in Qiagen's IPA software, the interactions between the miRNA data and the mRNA data were further examined. One of the greatest down-regulated miRNAs at both time points and in both exposure groups was miR-23b-3p. IPA identified miR-23b-3p as a regulator of genes involved in IL-13 and interleukin 33 (IL-33) responses, such as an up-regulation of arginase 1 (*Arg1*) and mannose receptor c, type 1 (*Mrc1*). Along with validating several genes in the Th1 and Th2, and Th17, response pathways, a third smaller set of genes involved in a combination of Th1, Th2 and Th17 immune responses were

also measured (Fig. 1c) and included the expression of *Il33*, which was also increased at both time points in the viable compared to HIC group.

MiR-29a-3p was also decreased in the viable vs. HIC group at 24 h and was significantly decreased ($P \leq 0.05$) at 48 h. In IPA, the miRNA target filter identified an interaction between *Clec7a* and miR-29a-3p. As previously mentioned, *Clec7a* expression was increased in the viable vs. HIC group at both time points and was associated with *in vivo A. fumigatus* conidia germination. IPA also identified miR-29a-3p to regulate transforming growth factor beta 3 (*TGF-β3*). *TGF-β3* was decreased in the viable vs. air-only control at 24 h postexposure (Fig. 1a), while *TGF-β1* was increased in the viable vs. HIC group (Fig. 1b), but neither were altered at 48 h.

The TGF-β signalling pathway is mediated by SMAD proteins that transduce the signal produced by TGF-β to the nucleus for downstream gene transcription regulation. As one of the greatest down-regulated miRNAs, miR-30c-5p was identified as a predicted regulator of *SMAD2*. To date, SMAD signalling has not been identified as having a mechanistic role in the immune response to subchronic fungal exposures. Generated in IPA, comparative analyses between the Th1 and Th2 pathway mRNA data set with miRNA data set at 48 h in the viable vs. HIC group, and filtering for inflammatory disease and either cell-mediated or humoral immune response, not only identified *SMAD2/3*, but also illustrated that most of the miRNAs regulated by *SMAD2/3* further regulate a number of different genes that are involved in the immune response (Fig. 2a and b). This comparison also highlighted the uncharacterized involvement of interleukin 1 receptor-like 1 protein (*IL1RL1*) on the immune response elicited by *A. fumigatus* exposure (Fig. 2a). Comparative analyses between the Th17 pathway mRNA data set with miRNA data set at 48 h in the viable vs. HIC group, and filtering for inflammatory disease, illustrated associations between *Clec7a* and genes involved in the inflammatory response, such as IL-13 (Fig. 3). Overall, IPA highlighted the importance of viability as a mechanism underlying the elicited immune response and revealed novel associations between genes and miRNAs involved, following subchronic viable *A. fumigatus* fungal exposure.

Discussion

The goal of this study was to evaluate the role of fungal viability on the murine pulmonary miRNA and mRNA profiles that regulate immune responses following a subchronic *A. fumigatus* exposure. Using a previously described AGS, mice were exposed via inhalation to either viable or HIC *A. fumigatus* conidia, which allowed for the influence of viability to be assessed. Utilizing genomic microarray and RT-PCR technologies, the effects of fungal exposure on the expression of specific miRNAs and genes involved in the Th1, Th2 and Th17 response pathways were evaluated. Qiagen's IPA software was then used to identify associations between these miRNAs and targeted genes that play a role in the immune response following subchronic fungal exposure.

After examining miRNAs that had demonstrated the most significant changes following subchronic *A. fumigatus* exposure, IPA identified miR-29a-3p which targets two critical genes in the inflammatory response, *Clec7a* and *TGF-β*. *Clec7A* is important relative to conidia germination and align with previous studies that have reported *A. fumigatus* germination to elicit an inflammatory response due to the recognition of β-glucan on the hyphal cell wall [44–49]. Previous studies from our laboratory showed germination of *A. fumigatus* conidia in the lungs of mice exposed to viable *A. fumigatus* conidia [8, 12]. These results are further supported by increased *Clec7A* expression in the viable vs. HIC group at both time points. The decrease in miR-29a-3p is one potential mechanism that could contribute to the increase in *Clec7A*.

TGF-β plays a critical role in immunity by maintaining tolerance to self- or non-infectious antigens and by exerting both stimulatory and inhibitory effects during an immune response [50]. *TGF-β1* and *TGF-β3* are two isoforms of TGF-β that had opposing altered expression levels at 24 h following *A. fumigatus* exposure. This result could be explained by the differing functions between the two isoforms, as well as the environment of cytokines present at each time point. One cytokine that counters the activity of TGF-β is IFN-γ, which was increased in both groups at 24 h, then decreased at 48 h. Identified by the miRNA target filter in IPA, *IFN-γ* is also regulated by miR-29a-3p. Similar to the reported increased *IFN-γ* expression following subchronic fungal exposure in our studies, Ma et al. [51] demonstrated that decreased miR-29 participated in Th1 responses to intracellular pathogens by up-regulating *IFN-γ* expression. Specifically, T cells from *Mycobacterium bovis* Bacillus Calmette–Guérin-infected or *Listeria monocytogenes*-infected mice presented lower amounts of miR-29 that in turn, assisted in the production of IFN-γ. In the current study, a possible mechanism underlying the lack of change in *TGF-β1* and *TGF-β3* expression 48 h postfungal exposure could be the increase of *IFN-γ* at 24 h.

Downstream of the TGF-β signalling pathway are transcription factors known as SMADs. Analysis of the 48-h miRNA data set revealed a network that contained a large number of the miRNAs, along with other prominent genes associated with the inflammatory disease and response pathway. This specific network reported a

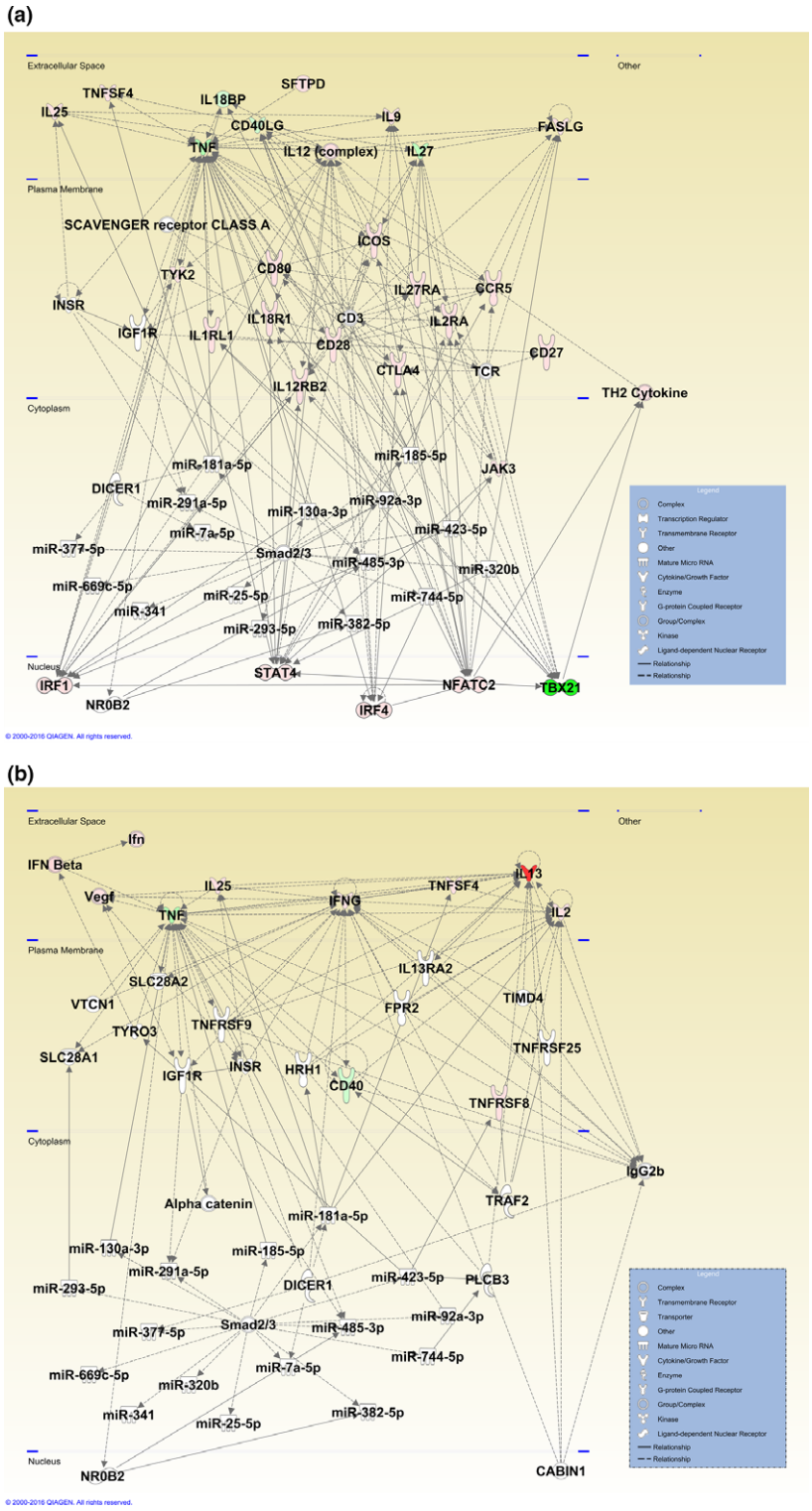

Fig. 2. (a) Network map generated by Ingenuity Pathway Analysis (IPA) depicting genes involved in the Th1 and Th2 immune response and associated miRNA involved in inflammatory and cell-mediated immune response, shaped by gene function and positioned by subcellular location. Genes are colour-coded (red or green for up- and down-regulation, respectively) for the expression of Th1 and Th2 genes in the viable vs. heat-inactivated conidia (HIC) group 48 h postexposure. (b) Network map generated by IPA depicting genes involved in the Th1 and Th2 immune response and associated miRNA involved in inflammatory and humoral immune response, shaped by gene function and positioned by subcellular location. Genes are colour-coded (red or green for up- and down-regulation, respectively) for the expression of Th1 and Th2 genes in the viable vs. HIC group 48 h postexposure.

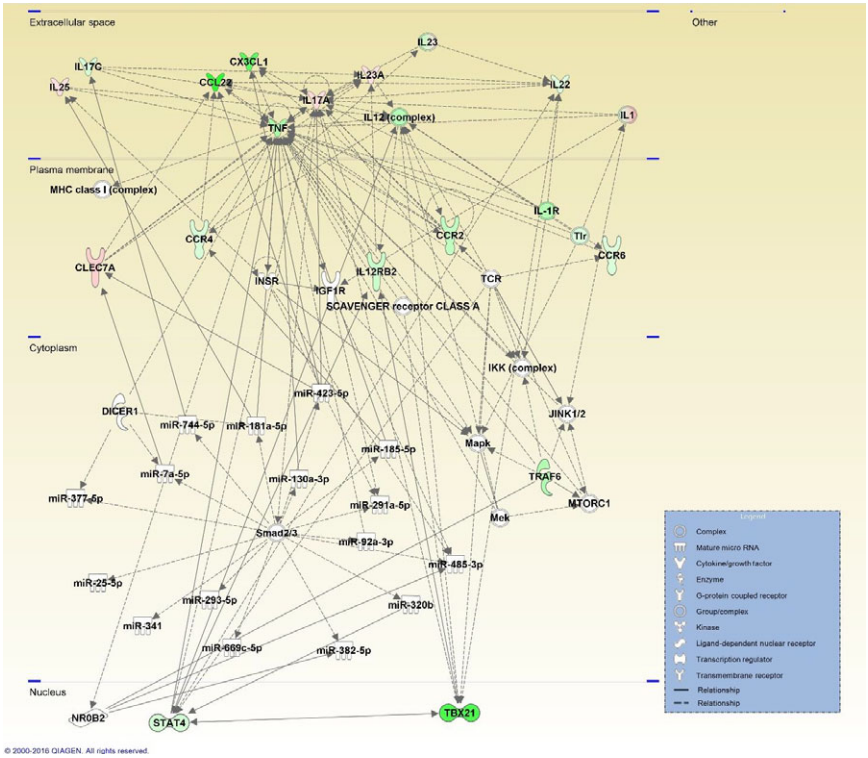

Fig. 3. Network map generated by Ingenuity Pathway Analysis (IPA) depicting genes involved in the Th17 immune response and associated miRNA involved in the inflammatory response, shaped by gene function and positioned by subcellular location. Genes are colour-coded (red or green for up- and down-regulation, respectively) for the expression of Th17 genes in the viable vs. heat-inactivated conidia (HIC) group 48 h postexposure.

predicted association with *SMAD2/3*, and to our knowledge, this is the first time SMADs have been implicated in a fungal exposure-elicited immune response. There are several isoforms of SMAD and the two identified included SMAD2 and SMAD3. Phosphorylation of these receptor-activated SMADs results in a complex formation that consists of both SMAD2 and SMAD3, as well as SMAD4. Together, these act to transduce the signal from TGF-β in the cytoplasm into the nucleus for further gene transcription regulation. The expression levels of *SMAD2* revealed a decrease 48 h postexposure in the viable vs. HIC group and the viable vs. air-only control. Upon further examination, the miRNA target filter identified several miRNAs that targeted *SMAD2*. Although miR-30c-5p was one of the greatest downregulated miRNAs and was predicted by IPA to interact with *SMAD2*, the expression of both the miRNA and the gene was decreased. MiR-302a-3p was the only miRNA increased at 48 h in the viable vs. HIC group and viable vs. air-only control and was also predicted to interact with *SMAD2*. This suggests that miR-302a-3p may serve as the more likely regulator of *SMAD2* during fungal exposures. Taken together, this study demonstrates the potential for a functional role of *SMAD2/3* in the inflammatory response and miR-302a-3p as a potential therapeutic target to reduce inflammation elicited from subchronic *A. fumigatus* exposure.

IL-13 is a hallmark cytokine of allergic response. Mice exposed to viable *A. fumigatus* conidia (1×10^6) intratracheally for 10 days showed that β-glucan recognition of dectin-1 resulted in the production of IL-4, IL-13, IFN-γ and IL-33 [52]. In the present study, increased expression of *Il13* and *Il33* in the viable vs. HIC group was observed at 48 h. Increased *Clec7a* in the viable vs. HIC group was also observed at both time points, as well as conidia germination in the lungs of mice exposed to viable *A. fumigatus* conidia [9]. These results further highlight the influence of fungal conidia germination on allergy-induced immune responses that have been previously shown using *in vitro* approaches [12].

An unexpected finding of this study was the decrease in *Gata3* expression. *Gata3* is a transcription factor expressed during the Th2 immune response to allergens [53]. This gene is also known to have a critical role in the expression of interleukin 4 (IL-4), interleukin 5 (IL-5) and IL-13; all cytokines that are characteristic of a Th2 response. Although *Il5* was increased in the viable vs. HIC at 24 h and not 48 h postexposure, both *Il4* and *Il13* were increased at 48 h in viable vs. HIC group.

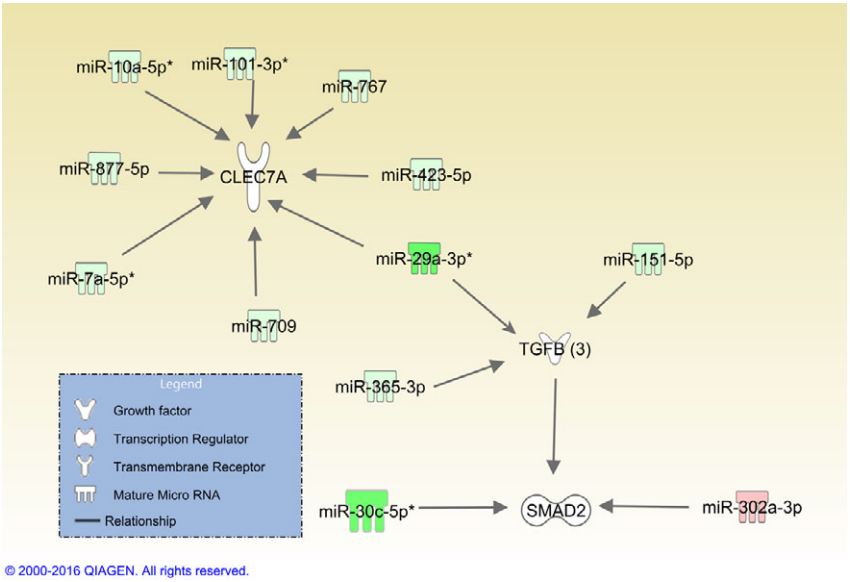

Fig. 4. Summary of network map depicting the three differentially expressed genes of interest and their associated miRNAs involved in the inflammatory immune responses. Genes and miRNAs are colour-coded (red or green for up- and down-regulation, respectively) for the expression of *Clec7a*, *TGF-β3*, *SMAD2/3* and miRNAs in the viable vs. heat-inactivated conidia (HIC) group 48 h postexposure.

Gata3 expression has been shown to be regulated by *Stat6*, whose expression was significantly down-regulated in the viable vs. HIC group at 48 h postexposure [54]. Also significantly down-regulated in the viable vs. HIC group at 48 h postexposure was the expression of Stat3, which has been shown to regulate the binding of STAT6 with GATA3 [55]. Corn et al. [56] showed that Bcl-3-deficient cells exhibited decreased Gata3, consistent with evidence that Bcl-3 can transactivate a *Gata3* promoter; however, *Bcl3* expression was not measured in the present study. *In vitro*, TGF-β has been reported to inhibit Th2-mediated immune responses through the inhibition of *Gata3* expression [57]. Although possible mechanisms exist that may negatively impact *Gata3* expression, the increase in the pro-inflammatory cytokines regulated by *Gata3*, along with previously reported *Gata3* induction during inflammation, warrants *Gata3* expression to be further examined in future fungal exposure studies.

MiRNA profiles have also been examined in cell lines and activated cell populations following exposure to different pathogens. Along with targeting *IFN-γ* and subsequently regulating the immune response following a fungal or bacterial exposure, miR-29 has also been reported to regulate the production and infectivity of human immunodeficiency virus type 1 [58]. MiR-29a is up-regulated in the serum and sputum of *Mycobacterium tuberculosis*-infected humans [59], but in contrast, miR-29 is down-regulated in murine T cells infected with *M. bovis* [51], which is in agreement with the current study. Presently, miR-155-5p decreased 48 h postfungal exposure; however, this miRNA was up-regulated in different cell types following infections from bacterial pathogens, including *Salmonella enterica*, *Helicobacter pylori*, *L. monocytogenes*, *Mycobacterium avium* and *Mycobacterium smegmatis* [29, 60–68]. In contrast to the present study, miR-23b, miR-30b, miR-30c, miR-125b, miR-15b, miR-16, miR-27b, miR-24 and miR-21 were up-regulated in *Cryptospordium parvum*-infected human host biliary epithelial cells [30]. In that same study, both miR-98 and miR-214 were decreased, a finding that is in agreement with the currently reported miRNAs following *A. fumigatus* exposure. Another study reported decreased miR-16 and miR-451 in the plasma of human patients infected with *Plasmodium vivax* [69], which was also observed in the murine lung homogenates derived from the group of mice exposed to viable *A. fumigatus* conidia. Taken together, the results from the present study suggest that subchronic *A. fumigatus* exposures result in similar miRNA profiles that include the same highly dysregulated miRNAs that have also been observed in bacterial and protozoan pathogen models.

This study offers preliminary insight into the miRNA environment and identified miRNA-mRNA interactions following subchronic exposure to aerosolized *A. fumigatus* conidia; however, there are a number of limitations associated with this study that need to be taken into consideration. In this study, the pulmonary exposure consisted of 26 exposures over a 13-week interval and data was collected only at 24- and 48-h time points following the final exposure. As a result, the miRNA environment was not evaluated at shorter time points, nor after time points beyond 48 h, postfinal

exposure. It is important to note that IPA predicts interactions between miRNAs and mRNA, even if the interactions have not been scientifically observed. In the current study, all of the interactions between the described miRNAs in the present study and *SMAD2/3* were predicted based on interactions that have only been observed in human cell culture and not in a murine inhalation exposure study. Lastly, the genetic environment was assessed using whole lung homogenate and not a specific cell population or cell line; however, specific cell populations from the same study were examined by flow cytometry and reported in a separate manuscript (unpublished data). The current manuscript is focused on the miRNA and mRNA environments following a subchronic fungal exposure, but further characterization of the immune response by flow cytometric analyses, gene and protein expression from the same study is reported separately (unpublished data). Critical genes identified in the current manuscript to be involved in the immune responses were also confirmed through proteomic analyses reported in the separate manuscript (unpublished data). Although not all fungal species with comparable sized conidia may behave in a similar manner [70], the findings in the current study provide unique insight into the molecular environment within the murine lung following a subchronic *A. fumigatus* exposure.

In conclusion, mice exposed to viable *A. fumigatus* conidia highlighted the influence of conidia germination on gene expression controlling the ensuing pulmonary immune responses. This was specifically supported by data that showed increased *Clec7a* expression and its association with IL-13 and IL-33. Down-regulated miRNAs predicted to target *Il13*, *Il33* and *Clec7a* (Fig. 4) were identified, suggesting a possible mechanism that contributes in part to an immune response following subchronic exposures to *A. fumigatus*. Furthermore, using IPA, novel interactions between *SMAD2/3* and other genes and miRNAs involved in the inflammatory response were able to be resolved for the first time. These altered miRNAs may serve as potential biomarkers to evaluate fungal exposure.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. All detected miRNAs.
Table S2. All altered miRNAs.
Table S3. Validated genes.
Table S4. Additional genes.