

Effects of low molecular weight fungal compounds on inflammatory gene transcription and expression in mouse alveolar macrophages

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ABSTRACT

The inflammatory potential and molecular mechanisms underscoring inflammatory responses of lung cells to compounds from fungi that grow on damp building materials is poorly understood *in vitro*. In this study we evaluated the effect of pure fungal compounds on potentiating acute inflammatory response in primary mouse alveolar macrophages (AMs) and tested the hypothesis that AM responses to low molecular weight fungal compounds exhibit temporal and compound specificity that mimic that observed in the whole lung. Transcriptional responses of 13 inflammation/respiratory burst-associated genes (*KC=Cxcl1*, *Cxcl2*, *Cxcl5*, *Cxcl10*, *Ccl3*, *Ccl112*, *Ccl20*, *IL-1 β* , *IL-6*, *ifit27*, *Tnfa*, *iNOS* and *Blvr1b*) were evaluated in mouse AMs exposed to a 1 ml (10^{-8} mol) dose of either pure atranone C, brevianimide, cladosporin, curdlan, LPS, neoehinulin A & B, sterigmatocystin or TMC-120A for 2 h, 4 h and 12 h PE using customized reverse transcription (RT)-PCR based arrays. Multianalyte ELISA was used to measure expression of 6 pro-inflammatory cytokines common to the transcriptional assays (*Cxcl1*, *Cxcl10*, *Ccl3*, *IL1 β* , *Ifn- λ* and *Tnf- α*) to determine whether gene expression corresponded to the transcription data. Compared to controls, all of these compounds induced significant (≥ 2.5 -fold or ≤ -2.5 -fold change at $p \leq 0.05$) time- and compound-specific transcriptional gene alterations in treatment AMs. The highest number of transcribed genes were in LPS treatment AMs at 12 h PE (12/13) followed by neoehinulin B at 4 h PE (11/13). Highest fold change values (>30) were associated with *KC*, *Cxcl2*, *Cxcl5* and *IL1 β* genes in cells exposed to LPS. Compound exposures also induced significant ($p \leq 0.05$) time- and compound-specific pro-inflammatory responses manifest as differentially elevated *Cxcl1*, *Cxcl10*, *Ccl3*, *Ifn- λ* and *Tnf- α* concentrations in culture supernatant of treatment AMs. Dissimilarity in transcriptional responses in AMs and our *in vivo* model of lung disease is likely attributable to whole lung vs. isolated cell responsive and dose differences between the two studies. The results not only indicate that low molecular weight compounds from fungi that grow in damp built environments are potentially pro-inflammatory *in vitro*, it further highlights the important role AMs play in innate lung defence, and against exposure to low molecular weight fungal compounds. These observations further support our position that exposure to low molecular weight compounds from indoor-associated fungi may provoke some of the inflammatory health effects reported from humans in damp building environments. They also open up a hypothesis building process that could explain the rise of non-atopic asthma associated with fungi.

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1. Introduction

Cognizant authority reviews [1–4] articulate the effects on population health of mold in the context of building dampness. These include increased allergic and upper respiratory disease. Health Canada [5] and CDC [6] both state that there is sufficient evidence for health benefit from the remediation of mold and dampness. Fungi are associated with new onset asthma in both adults and children and with non-atopic asthma [7,8]. Although it has been

assumed that the 'exposure' in buildings was to spores, the evidence is that in moldy buildings, the exposure is mainly to fine fragments of spores and mycelium [9]; see also following] that contain allergens/antigens, triple helical glucan, and various low molecular weight compounds in high concentrations but low absolute amounts [10].

Miller et al. [11] and Rand et al. [12–14] have shown that exposure to various low molecular weight compounds (e.g. atranones A and C brevianamide C, cladosporin, mycophenolic acid, neoehinulin A & B, roquefortine C, sterigmatocystin, TMC and the β 1,3, glucan, curdlan) from these types of fungi provoke a variety of inflammatory responses in intratracheally instilled 3-week old Swiss Webster mice. These responses include significant dose,

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time and compound-dependent transcription and/or expression of inflammation-associated genes related to acute pulmonary inflammation, especially in mediating recruitment of T-cells, alveolar macrophages (AMs), and neutrophils. Interestingly, these results all provided a biological basis for some of the inflammatory health effects reported from humans in damp, moldy building environments [11,14].

A feature of our recent *in vivo* studies was the observation based on immuno-histochemistry that three cytokines associated with lung inflammation (CCL3, MIP-2 and Tnf- α) were expressed by alveolar type II cells (ATIIs), alveolar macrophages (AMs) and bronchiolar epithelium [11,14]. This result suggested that these are all immunosentinel cells and may have an important role in the modulation of inflammatory responses in lungs exposed to fungal compounds. However, the inflammatory potential of compounds from fungi that grow on damp building materials on any of these cells is still poorly understood *in vitro* and molecular mechanisms underscoring their inflammatory responses are not understood for any, as far as we are aware. This prompted us to evaluate the effect of pure fungal compounds on potentiating acute inflammatory response in primary mouse AMs and to test the hypothesis that AM responses to low molecular weight fungal compounds exhibit temporal and compound specificity that mimics those observed in the whole lung. These cells were chosen as the model immunosentinel cell in this study as they are traditionally considered to be the principal defense cells of the respiratory system and responsible for the acute phases of the inflammatory response by releasing chemokines and cytokines into the lung environment [15]. This early response in turn results in responses by other lung cells, and migration of those in systemic circulation into the inflamed lung. AMs were also chosen because there are a few other studies that describe the inflammatory and/or cytolytic effects of pure low molecular compounds (e.g. atranones, glucan, macrocyclic trichothecenes) from fungi that grow in damp buildings on AMs [10,16,17].

In this study, we compared inflammatory responses in AMs to a variety of low molecular weight compounds from fungi that are commonly recovered from damp building materials. Using primary AMs from our mouse model of lung disease, the objectives of this study were: (1) to evaluate time course (2 h, 4 h, 12 h post-exposure (PE)) inflammatory activation and/or recruitment responses associated with pro-inflammatory gene transcription in AMs exposed to 10^{-8} mols of atranone C, brevianamide, cladosporin, curdlan, neoehinulin A & B, sterigmatocystin and TMC-120A (hereafter TMC 120) using customized mouse inflammatory gene and receptor arrays as well as reverse transcription (RT)-PCR; (2) to employ multi-analyte ELISA to determine whether cytokine expression responses corresponded with gene transcription data; and (3) to evaluate whether AM transcriptional responses common to our *in vitro* studies were similar to those of the whole lung. As AMs are considered the principal lung defense cells, we hypothesized that time course and compound specific gene transcriptional responses in compound-exposed AMs will closely correspond to those in common with our whole lung experiments [11,14].

2. Materials and methods

2.1. Compound preparations

The compounds in this study come from *Alcaligenes faecalis*, (curdlan (Sigma–Aldrich C7821, lot # 89H4032 $\geq 99\%$ purity)) a widely used model to study the pulmonary effects of β glucans, and from common damp building fungi [46] associated with (1) high water activity: *S. chartarum*, *S. chlorohalonata* (atranone C), (2) intermediate water activities: *Penicillium brevicompactum* (bre-

vianamide), *Aspergillus versicolor* (sterigmatocystin Sigma–Aldrich Canada (S3255)) and *A. insuetus/A. calidoustus* = *A. ustus sensu lato* (TMC-120A) and (3) low water activity building materials: *Eurotium amstelodami*, *E. herbariorum* and *E. rubrum* (neoehinulin A & B) and cladosporin from *E. herbariorum*). The isolation and purity of these compounds has been previously described by [11,14]. These compounds were dissolved in pyrogen-free PBS to 10^{-8} mols working solution, prepared fresh before the *in vitro* experiments and administered at a dose of 1 ml of 10^{-8} mols cell culture medium. Bacterial lipopolysaccharide (LPS) from *Escherichia coli* (L2880 $\geq 99\%$ purity Sigma–Aldrich) was used as a positive control. It was dissolved in pyrogen-free PBS, and administered at a dose of 1 ml of 0.1 μ g/ml cell culture medium.

2.2. Animals

Seven to nine-week-old, random-bred, pathogen-free Carworth Farms white (CFW) Swiss Webster male mice were used in these experiments. All mice used in the experiments were housed according to the standards of the Canadian Council for Animal Care [18] and with approval from the Dalhousie University Animal Care Committee. The mice were given food and water *ad libitum* and acclimatized for one-week prior to use.

2.3. Alveolar macrophage recovery and cell culture

For cell harvesting, mice were killed using a CO₂ overdose. They were then exsanguinated by cutting the abdominal artery. Mouse lungs were then gently massaged and lavaged in 6 ml $\times$ 0.8 ml aliquots using a warm ($\approx 37^\circ\text{C}$) lavage solution prepared by dissolving 0.1 g of lidocaine (Sigma–Aldrich) in 50 ml of pyrogen-free PBS. The bronchioalveolar lavage fluid (BALF) of two mice were pooled together and placed on ice. Lavage fluid was centrifuged at $350 \times g$ for 10 min at 4°C . Pellets were resuspended in PBS and cell aliquots examined microscopically for viability (Trypan blue exclusion), purity (Diff-Quick stain) and enumeration. Viability ranged from 87% to 95%, differential cell counts from the two combined BALF samples indicated that $95 \pm 2\%$ of the 1.1×10^6 cells employed for the culture assays were AMs. Cells were then suspended in 1 ml RPMI $1 \times$ Medium 1640 (Gibco) supplemented with 5% fetal bovine serum (FBS) (Sigma–Aldrich), 1% of penicillin-G/streptomycin (Sigma–Aldrich), and 0.015 g of L-glutamine (Sigma–Aldrich). The cell suspensions and medium were transferred into 6-well culture plates and incubated overnight at 95% air/5% CO₂ and 37°C .

After 24 h incubation, AMs were exposed to 1 ml of each of the compounds for a working dose of 10^{-8} mols (or for LPS 0.1 μ g/ml) and for 2 h, 4 h, and 12 h post exposure (PE). The carrier saline solution was used as a control. All experiments were done in triplicate, at least. After each exposure time, the medium from control and treatments (with the exception of TMC 120) was removed, flash-frozen in liquid nitrogen and stored at -80°C for pro-inflammatory chemokine/cytokine activity using enzyme-linked immunosorbent assays (ELISA) (see below). The cells washed with $3 \times$ with 1 ml PBS. The PBS was then removed and RLT buffer (Qiagen) (90% RLT, 10% β -mercaptoethanol) was added. The cells were scraped into this buffer and the lysate was transferred into a microcentrifuge tube and stored at -80°C for up to 1 week before RNA extraction.

2.4. RNA extraction and reverse-transcription real-time PCR

RNA extraction was performed using the Qiagen RNeasy® micro kit according to the manufacturer's instructions. Briefly, the lysate was homogenized with a syringe and a needle, precipitated using 70% EtOH, and applied to an RNeasy® minielute spin column. The samples were centrifuged, washed with RWI buffer (Qiagen), and

treated with RNase-free DNase (Qiagen) to eliminate DNA contamination. RNA was then washed again with RWI buffer (Qiagen) and RPE Buffer (Qiagen), and then dried with 80% EtOH and centrifugation. The RNA was then resuspended in 16 µl of RNase/DNase-free water (Sigma–Aldrich). The concentration and integrity of RNA in the samples was determined using a NanoDrop® ND-1000 and RNA Nano chips (Agilent 2100 Bioanalyzer). Samples with sufficient RNA concentrations (≥ 5 µg/ml) and with 260/280 nm ratio of ≥ 2.0 were used for array analysis.

2.5. Reverse-transcription real-time PCR and array analysis

Reverse-transcription (RT) polymerase chain reaction (PCR) was carried out using a reaction-ready first strand cDNA synthesis kit (C-03 SABiosciences™) according to the manufacturer's specifications. For the AM assays, a custom-made 96-well PCR array for selected mouse inflammatory cytokines and chemokines was used (SABiosciences™). The array contained primers for 13 inflammatory focused genes, 2 housekeeping genes, and 1 no reverse transcription control (NRT) (Table 1). Nine of these genes (*KC=Cxcl1*, *Cxcl5*, *Cxcl10*, *Ccl3*, *Ccl112*, *Ccl20*, *IL-1β*, *ifl27* and *Tnfα*) were common to our *in vivo* gene array experiments and considered to have central roles in the orchestration of compound-induced pro-inflammatory lung responses [11,14]. Two genes (*Cxcl2=MIP2*) and *IL-6* have been recovered from bronchioalveolar lavage (BALF) from mice intratracheally exposed to purified fungal products and represent hallmarks of pulmonary inflammation. The remaining 2 genes have been associated with respiratory burst (iNo2) and mitochondrial perturbation (Blvrd). The real-time (RT) PCR reactions were carried out using the ABI Prism 7000 Sequence Detection System® (Applied Biosystems) as described previously [11,14]. Relative gene expression was determined according to the comparative C_t method, with the Act-β housekeeping gene and carrier control references set as the calibrators.

2.6. Pro-inflammatory cytokine analysis

To confirm gene array data that cytokine gene transcription was being translated to cytokine protein expression in treatment AMs, multianalyte ELISA was used. Concentrations of the pro-inflammatory chemokines *Cxcl1*, *Cxcl10*, *Ccl3*, *IL1β*, *Ifn-λ* and *Tnf-α* in AM culture supernatant were determined in triplicate using custom made ELISArray® multi-analyte enzyme-linked immunosorbent assays (ELISA) purchased from SABiosciences™. All standard curves for ELISA testing were generated according to the manufacturer's instructions. Final absorbencies were read

at 450 nm using a Bio-Tek Instruments EL-800 microplate reader. Cytokine concentrations were reported in pg/ml of supernatant.

2.7. Statistical analysis

Genes and treatment groups were clustered using the SuperArray web-based hierarchical clustering software package for the PCR Array System (<http://www.sabiosciences.com/pcrarraydataanalysis.php>). A Shapiro–Wilks test for normality was performed to verify if the samples were normally distributed. A two-tailed unpaired *t*-test was then performed to determine if the treatments showed significant inflammatory gene transcription in comparison with carrier controls. All data are shown as their mean fold change values. One-way ANOVA and Tukey's pair-wise analyses were also carried out using SYSTAT version 11.0. Gene transcription results were considered significant if they had ≥ 2.5 -fold or ≤ -2.5 -fold change at $p \leq 0.05$ probability level. One-way ANOVA to test for time responses and Tukey's pairwise analyses were also carried out using SYSTAT version 11.0. To determine the similarity of the gene responses to all compounds tested, hierarchical cluster analyses of standardized data were done by Ward's squared Euclidean distance method with SYSTAT v.12 software (San Jose, CA). Clusters were evaluated for significance using root mean square standard deviation and Calinski and Harabasz's pseudo F analyses. For ELISA, all data were tested for normality using a normal probability plot. Data were then tested for statistical significance using one-way ANOVA and Tukey's multiple comparison post hoc test. All results were considered significant at ≤ 0.05 probability level.

3. Results

3.1. Inflammation-associated gene modulation

Compared to controls, the number of genes significantly transcribed varied amongst the treatments. ANOVA followed by Tukey's pair-wise comparisons revealed that all of the 13 inflammation-associated genes assayed were significantly up-regulated in AMs exposed to at least one of the compounds tested and at one time point (≥ 2.5 -fold up-regulated or ≤ -2.5 -fold down-regulated change at $p \leq 0.05$) (Table 2). None of the treatments resulted in significant mRNA transcriptional changes in all genes assayed at all time points. With modest exception (e.g. cladosporin 4 h PE; sterigmatocystin 2 h PE), treatments resulted in up-regulated transcriptional changes. Of the 13 genes assayed, 3 (*Cxcl2*, *Ccl13*, and *Tnf-α*) were significantly transcribed in all treatment AMs at one time point, at least. Genes least transcribed were *Ccl20* (4/9 treat-

Table 1

Reference sequences and descriptions of inflammatory/respiratory burst focused and housekeeping genes used in customized reverse transcription (RT)-PCR based arrays.

RefSeq	Official NCBI gene symbol	Description	
NM.030845	<i>Cxcl1</i>	Inflammatory genes	Keratinocyte cell derived chemokine (KC)
NM.053647	<i>Cxcl2</i>	Inflammatory genes	Macrophage inflammatory protein 2 (MIP-2)
NM.022214	<i>Cxcl5</i>	Inflammatory genes	CXC chemokine ligand 5
NM.139089	<i>Cxcl10</i>	Inflammatory genes	CXC chemokine ligand 10
NM.013035	<i>Ccl3</i>	Inflammatory genes	Macrophage inflammatory protein 1α (MIP-1α)
XM.213425	<i>Ccl12</i>	Inflammatory genes	CC chemokine ligand 12
NM.019233	<i>Ccl20</i>	Inflammatory genes	Macrophage inflammatory protein 3 (MIP-3α)
NM.031512	<i>Il1β</i>	Inflammatory genes	Interleukin 1β
NM.012589	<i>Il6</i>	Inflammatory genes	Interleukin 6
NM.012675	<i>Tnf</i>	Inflammatory genes	Tumour necrosis factor-α
NM.130743	<i>Ifi271</i>	Inflammatory genes	Interferon λ-inducible protein 27-like
NM.012611	<i>Nos2</i>	Inflammatory genes	Nitric oxide synthase 2, inducible
XM.214823	<i>Blvrb</i>	Inflammatory genes	Biliverdin reductase B (flavin reductase (NADPH))
NM.017008	<i>Gapdh</i>	Housekeeping genes	Glyceraldehyde-3-phosphate dehydrogenase
NM.031144	<i>Act β</i>	Housekeeping genes	Actin β
	Control (Actb)	Control	No reverse transcription (NRT) control

Table 2

Inflammation-associated transcriptional gene fold changes in toxin exposed AMs at 2 h, 4 h and 12 h PE. Significant (≥ 2.5 -fold or ≤ -2.5 -fold change at $p \leq 0.05$) are highlighted in yellow. Numbers in green are up-regulated while those in red are down-regulated genes.

	Gene Symbol												
	Cxcl1	Cxcl2	Cxcl5	Cxcl10	Ccl3	Ccl12	Ccl20	IL-1	Il6	ifi27	Tnf	Nos2	Blvrb
Treatment													
Atra2h	-7.17	6.15	-1.93	2.51	2.69	1.76	1.43	1.75	1.17	4.42	3.13	3.87	2.78
Atra4h	2.42	5.01	2.14	-1.06	3.92	1.14	1.09	1.97	6.34	-1.31	5.04	3.13	3.69
Atra12h	2.41	4.78	1.53	1.02	3.8	4.44	3.97	-1.44	1.94	-1.47	7.94	2.98	4.8
Brevi2 h	1.11	4.45	1.25	3.42	5.79	1.65	3.42	1.16	1.47	3.73	4.53	7.18	2.85
Brevi4h	2.76	3.38	6.12	2.1	1.1	1.3	1.39	6.12	-1.69	1.93	1.14	7.46	1.92
Brevi12h	5.07	1.01	-3.07	1.97	-1.89	1.82	3.63	-2.13	3.26	-1.53	4.44	-1.26	3.17
Clad2h	-3.43	-2.63	-1.08	-2.25	-1.76	3.97	-1.13	-2.27	-4.09	-3.17	1.14	3.01	1.38
Clad4h	-1.38	-2.28	-2.86	1.18	-6.64	3.6	3.55	-7.3	-4.62	-6.43	4.21	1.84	3.33
Clad12h	8.2	2.75	1.12	1.02	-1.28	1.18	1.12	-1.95	7.3	-1.73	6.63	-1.93	1.78
Curdlan2h	4.06	4.29	7.43	2.18	1.42	1.44	-1.38	16.53	6.54	1.47	1.83	1.3	1.31
Curdlan4h	5.83	2.88	5.63	6.37	1.75	-1.05	1.23	4.27	1.13	-1.05	2.69	3.47	1.07
Curdlan12h	6.88	11.79	12.04	7.25	16.13	-1.46	1.12	21.95	1.57	1.82	15.93	3.41	2.53
LPS2h	1.14	15.45	1.024	1.845	1.53	1.25	2.529	34.14	9.16	11.35	1.11	1.54	-1.53
LPS4h	60.55	56.87	4.67	11.79	22.52	-1.04	18.93	2.97	2.59	18.44	11.06	2.89	1.08
LPS12h	36.17	17.67	34.05	17.55	23.32	1.52	31.78	6.51	11.31	2.9	20.12	6.67	2.79
NeoA2h	2.9	4.9	1.24	1.27	3.06	5.9	2.25	-1.14	1.78	1.83	2.89	1.41	1.62
NeoA4h	3.66	5.68	-1.44	-3.85	4.43	1.25	2.34	1.81	1.31	1.44	1.08	3.66	1.24
NeoA12h	10	2.87	4.4	-1.23	5.86	1.99	1.97	3.13	3.52	4.02	5.51	3.18	9.95
NeoB2h	-1.12	4.92	-3.96	1.52	3.51	6.89	5.52	1.43	1.17	1.05	1.35	1.95	1.05
NeoB4h	7.4	7.17	4.72	1.58	10	-1.38	4.28	3.52	7.12	3.95	5.68	8.56	3.08
NeoB12h	1.19	1.36	-2.15	5.23	-2.17	5.02	1	-3.38	-3.83	-1.51	3.03	4	6.42
Ster12h	-4.36	1.68	-6.09	-1.04	-3.11	1.09	1.38	-1.14	-1.06	1.21	2	2.14	2.01
Steri4h	7.88	1.47	-1.22	1.35	3.98	-1.18	1	2.91	-1.36	1.25	5.46	3.71	3.47
Steri12h	4.54	2.95	2.31	1.25	4.54	1.51	1.68	2.98	2.42	2.1	4.45	1.15	8.2
TMC2h	-1.01	4.12	-2.17	1.06	-4.45	1.33	1.39	-1.91	1.12	1.01	4.66	2.23	1.15
TMC4h	1.06	1.38	-1.56	-1.16	2.63	4.42	1.93	-2.53	-3.45	-2.46	6.44	-1.59	4.5
TMC12h	2.71	-1.54	4.48	1.24	3.57	1.35	1.52	2.74	5.12	3.33	4.58	2.13	7.71

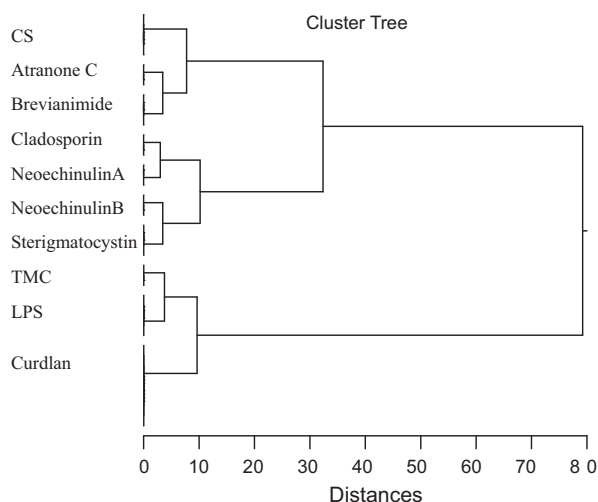


Fig. 1. Hierarchical cluster analyses of standardized transcriptional gene fold data for 12 h PE compound treated AMs using Ward's squared Euclidean distance method. Three clusters were formed. Atranone C and brevianimide cluster nearest to carrier saline (CS), cladosporin, neoechinulin A & B, and sterigmatocystin form the second cluster; TMC-120, LPS and curdlan form the third cluster.

ments), *Cxcl10* (5/9 treatments) and *Ccl12* (5/9). Highest number of transcribed genes were in LPS treatment AMs at 12 h PE (12/13) followed by neoechinulin B at 4 h PE (11/13). Highest fold change values (>30) were associated with *Cxcl1* (=KC), *Cxcl2*, *Cxcl5* and *Il1 β* genes in cells exposed to LPS.

Temporal patterns of gene transcription varied amongst compound treatments. With two exceptions (atranone C, brevianimide), highest numbers of genes were significantly transcribed in treatments at either 4 h (cladosporin and neoechinulin B) or 12 h (curdlan, LPS, neoechinulin A & TMC-120) than at 2 h PE. An equal number of genes (6/13) was transcribed at both 4 h and 12 h PE in sterigmatocystin treated cells, while in atranone C and brevianimide A treatments, highest numbers of genes were significantly transcribed (up- or down-regulated) at 2 h PE ((6/13 and 7/13, respectively) Table 2). Similarity of the gene responses to each compound evaluated using hierarchical cluster analyses of standardized data and by Ward's squared Euclidean distance method revealed time dependent variation in the patterns of gene response with secure outcome after 12 h PE. At this time, LPS and curdlan clustered by secure upper level clusters but separate out. All the compounds separated from each other in essentially two groups but with some branching that may or may not be relevant. Atranone and brevianimide clustered closest while the rest, especially LPS and curdlan clustered farthest from the control standard (Fig. 1).

3.2. Pro-inflammatory cytokine analysis

None of the treatment exposures resulted in the significant expression of all six genes assayed. The highest numbers of significantly expressed genes were in curdlan and LPS (*Tnf- α* , *Ccl3*, *KC*, *Cxcl10*, *Ifn- λ*), LPS (*Tnf- α* , *Ccl3*, *KC*, *Cxcl10*) brevianimide (*Tnf- α* , *Ccl3*, *KC*, *Ifn- λ*) and sterigmatocystin (*Ccl3*, *KC*, *Cxcl10*, *Ifn- λ*) treatment AM's. Compared to controls, genes most significantly expressed in response to treatment compounds at one time point at least were *Cxcl10* (all compounds), *Ccl3* (brevianimide, curdlan, LPS, neoechinulin A, sterigmatocystin), *KC* (brevianimide, curdlan, LPS, sterigmatocystin) and *Ifn- λ* (atranone C, brevianimide, curdlan, LPS). *Il1 β* was not significantly expressed in any of the treatment cells. Significant, time-sensitive effects were also observed (Fig. 2A–F). With modest exception (e.g. *Cxcl10* expression in neoechinulin A and sterigmatocystin 2 h treatments, and *Ifn- λ*

expression in atranone C 2 h PE treatment AMs), expression of cytokines was significantly higher in treatment AMs at 4 h and/or 12 h PE compared to controls.

4. Discussion

The results of the present study support and expand on our previous work [11,14,19]. Of the 13 genes employed in the transcriptional assays, 11 were associated with acute pulmonary inflammation (*KC*, *Cxcl2*, *Cxcl5*, *Cxcl10*, *Ccl3*, *Ccl12*, *Ccl20*, *Il1 β* , *Il6*, *Ifi27*, *Tnf- α*), and orchestrating acute inflammatory recruitment responses of eosinophils (*Ccl3*), monocytes (*Ccl3*, *Ccl20*), neutrophils (*Il1 β* , *KC*, *Cxcl2*, *Tnf- α*), T-cells (*Cxcl10*, *Ccl12*, *Ccl20*, *Il6*, *Ifi27*), and bronchiolar epithelium remodeling (*Il1 β* , *Tnf- α*), amongst others [15,20–30]. The rapid (within 12 h PE), largely time dependent transcriptional changes of these genes in treatment AMs not only indicates that low molecular weight compounds from fungi that grow in damp built environments are potentially pro-inflammatory, it further highlights the important role AMs play in innate lung defence [15], and against exposure to low molecular weight fungal compounds [11,14,31]. The 2 other genes featured in the transcriptional array set (*iNos* and *Blvrb*) and up-regulated in AMs exposed to all compounds are associated with oxidative stress response. While *iNos* has been reported to be associated with acute AM (RAW264.7 cells) response to curdlan and LPS exposures *in vitro* [32], the role of *Blvrb* in cellular responses to fungal compound exposures has been unexplored. This gene is associated with oxidative stress and activated through the Keap1-Nrf2-ARE signalling pathways [33]. The up-regulation of both *iNos* and *Blvrb* in the present study suggests that in addition to pro-inflammatory changes, oxidative stress is also an important AM response to fungal compound exposures.

The results also demonstrated that patterns of exposure outcome in the AMs were compound specific. For example, response to neoechinulin A exposure were different from those of neoechinulin B even though the polarity and structural activity of the two compounds differ only slightly [11]. That compound-dependent response patterns were observed is not unusual. The neoechinulins exhibited differential pro-inflammatory potential in our *in vivo* mouse lung studies [11] and parallel those of the atranones (produced by *Stachybotrys chartarum sensu lato*), which have also been shown to have different immunotoxicological properties *in vitro* [34], and *in vivo* [35].

These results using primary AMs thus provide further support for our previous studies by demonstrating the important relationship between chemical properties of low molecular weight fungal compounds and the temporal- and compound-specific inflammatory and/or cytotoxic response patterns in animal and cell models of lung disease. Recently completed work in our lab (Robbins and Rand, unpublished data) has demonstrated that these chemical properties are important determinants of response pattern outcome by differentially activating and modulating signal transduction pathways. This new work has revealed that murine RAW264.7 macrophages exposed to 10^{-8} mols neoechinulin A showed significant ($p \leq 0.05$) time dependent, up-regulation of 5 transduction genes (*Bmp2*, *Hspb1*, *Icam1*, *Vegfa* and *Cdkn1b*). These are key genes in the hedgehog, stress, phospholipase c/NFkB, Wnt, and TGF- β /p53 pathways, respectively. Cells exposed to an equimolar neoechinulin B concentration showed significant ($p \leq 0.05$) time dependent up-regulation of only 2 genes, *Bmp2* and *Cd5*, which represent the hedgehog pathway and the NFAT pathway, respectively. These results revealing differences in both similarity and temporality of the activation signal transduction pathway genes in the two treatments not only identifies an important mechanism that helps explain fungal compound specific response patterns, it also

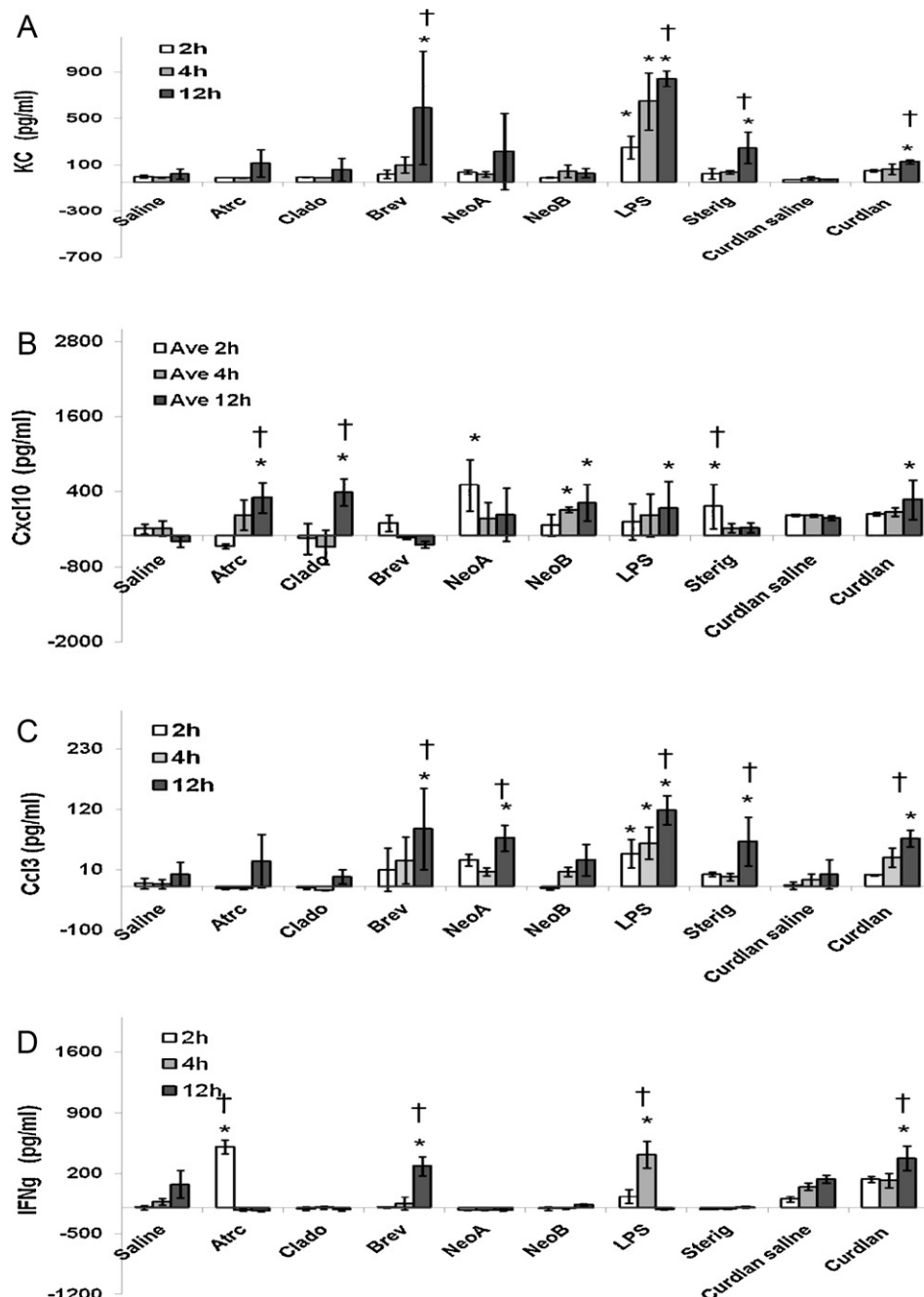


Fig. 2. Temporal changes (2 h, 4 h, 12 h PE) in alveolar macrophage cytokine expression (pg/ml) stimulated by low molecular weight fungal compounds and lipopolysaccharide exposure. (A) KC expression, (B) Cxcl10 expression, (C) Ccl3 expression, (D) IFN- γ expression, (E) IL-1 β expression and (F) Tnf- α expression. Atrc = atranone C, Brev = brevianimide, Clad = cladosporin, curdlan, LPS = lipopolysaccharide, NeoA = neoechinulin A, Neo B = neoechinulin B, Sterig = sterigmatocystin. Results for compound treatments were compared to saline; results for curdlan were compared to curdlan saline. An asterisk (*) indicates a significant increase versus the saline control level; the dagger (†) indicates significant time dependent response ($p < 0.05$).

provides a mechanism by which the epistatic modulation (i.e. a signal up- or down-regulates one gene which in turn sequentially modulates other genes [39]) of inflammatory mediators, including pro-inflammatory modulators [11,36] can be explained. This is interesting as it is now known that for asthma, gene products including some included in the present study are modulated in an epistatic fashion [37–39].

In the present study we hypothesized that there would be similar gene transcriptional patterns in both *in vitro* and *in vivo* [14] studies. Of the 13 inflammation-associated genes used in the AM assays, 9 were common to those employed in the whole mouse lung assays (KC (=Cxcl1), Cxcl5, Cxcl10, Ccl3, Ccl12, Ccl20, IL-1 β ,

Ilf127 and Tnf- α) [11,14]. However, apart from the curdlan and LPS exposed AMs which responded similarly to whole lungs exposed to these compounds [14; Roy and Rand, unpublished data], transcriptional responses to the remaining treatments generally differed in their direction (viz. up/down regulation) from those of the *in vivo* assays and did not support the hypothesis. Differences in response between this *in vitro* and the *in vivo* study [11] were also evident as dissimilarity between the compound clustering patterns observed in the two studies. In the *in vivo* study, the compounds clustered into three distinct patterns of gene transcription at 12 h PE: (1) brevianamide and neoechinulin B; (2) cladosporin, atranone C and TMC 120A; and (3) neoechinulin A and sterigmatocystin (Fig. 3). At

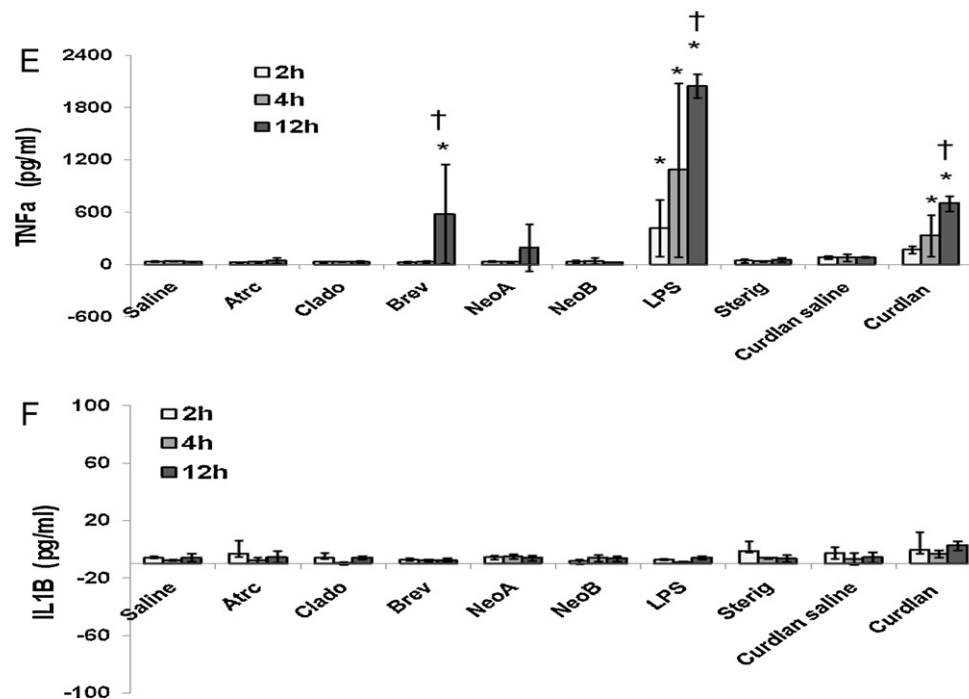


Fig. 3. Hierarchical cluster analyses of standardized transcriptional gene fold data for 12 h PE compound treated mouse lungs using Ward's squared Euclidean distance method. Three clusters were formed. Brevianimide, mycophenolic acid and neoechinulin B formed the nearest cluster to carrier saline, neoechinulin A and sterigmatocystin form the second cluster while cladosporin, atranone C and TMC 120 form the third cluster.

12 h PE, AM gene transcriptional clusters were: (1) atranone C and brevianimide; (2) cladosporin, neoechinulins A & B and sterigmatocystin; and (3) TMC 120. These differences between the *in vitro* and *in vivo* models may be a reflection of diminished interactive responsiveness, especially related to ligand reception and subsequent signaling events of isolated AMs compared to those within the whole lung [40]. Additionally, they may also be due to dose differences between the studies. The dose in our *in vivo* experiments was calculated to be within the range of potential human exposures [11–14]. In the present *in vitro* study, the dose used, as with all cell-based studies where toxicokinetic responses are largely absent, was based on best estimates of potency from available data. Based on the data from the *in vivo* studies [11–14], as well as cell data for sterigmatocystin [41], the doses used were $10\times$ less (10^{-8} mols) than those used in the *in vivo* studies ($\approx 10^{-7}$ mols) and they may

have been slightly too low to potentiate responses similar to those observed in our *in vivo* model. Moreover, for approximately 1/3rd of the genes for which there were data from cell and animal data [11] responses differed, mostly where the genes as transcribed in cells were up-regulated but down-regulated *in vivo*. For the remaining cases, there was no positive signal to compare. Therefore, the mismatch may not have been due to a lack of complementarity but rather because most (2/3rds) were not the result of positive but negative data from which conclusions cannot be drawn.

Results of the multianalyte ELISA for expression of 6 pro-inflammatory cytokines employed in the transcriptional assays (KC, Cxcl10, Ccl3, IL1 β , Ifn- λ and Tnf- α) showed temporal and compound-specific changes that mostly corresponded with the transcriptional results, with one exception. Compared to controls, IL1 β expression was unaltered by all treatments even though tran-

Table 3

Statistically-significant changes in gene transcription of IL1 β , IL1r1, IL4 and IL10 at 4 h and 12 h post exposure in response *in vivo* to doses of various fungal metabolites^a, and LPS^b to mouse lungs. Statistically-significant (≥ 1.5 -fold or ≤ -1.5 -fold change at $p \leq 0.05$ probability level) IL1 β , IL1r1, IL4 and IL10 gene transcriptional changes in fungal compounds^a and LPS^b exposed mouse lungs at 4 h and 12 h PE. Empty cells represent non-significant data.

Gene	Atranone C		Brevianimide		Cladosporin		Neoechinulin A		Neoechinulin B		Sterigmatocystin		TMC-120	
	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h
Il1b		3.18				2.63				-5.55				11.67
Il1r1	3.31		23.74		12.45		19.37				16.78		17.81	4.57
Il1a							-3.25		-12.32					-2.22
Il4						2.6							5.99	2.36
Il10		11.47				6.07			-2.91	4.06	13.38	2.95	3.49	-2.61
Gene	Curdlan								LPS					
	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h	4h	12h
Il1b			1.87				5.3		4.59				8.38	
Il1r1			1.61						2.35					
Il1a			19.85				31.54		3.67				17.89	
Il4									2.87					
Il10			1.9				7.38						5.5	

^a After 11, 14.

^b Unpublished data.

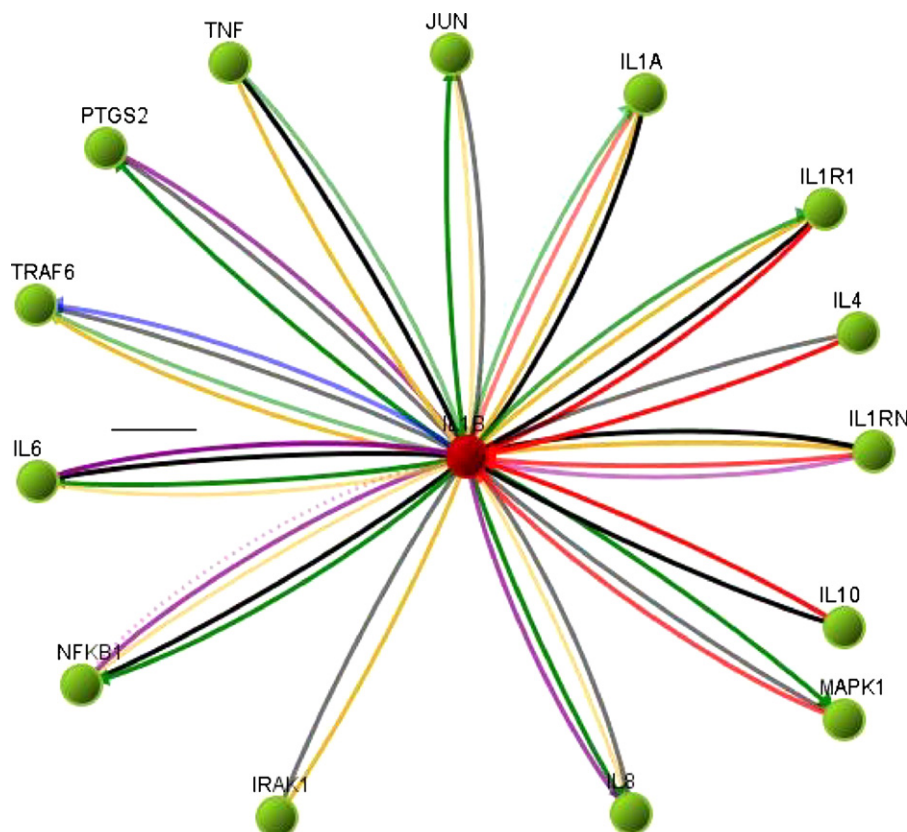


Fig. 4. Network representation of genes (green circles) partnered with IL1 β (red circle). Partnerships are based on PubMed abstracts, high-throughput proteomic screening, and microarray profiling [<http://www.sabiosciences.com/genenetwork/genenetworkcentral.php>]. (—) down-regulation; (—) up-regulation; (—) regulation; (—) coexpression; (—) chemical modification; (—) physical interaction; (---) predicted protein interaction; (---) predicted T factor regulation; (—) other. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

scriptional data revealed that it was up regulated in AMs exposed to all compounds except atranone C and neoechinulin A. This suggests transcriptional and expression patterns of this gene in AMs exposed to most compounds used in the assays were dysregulated. IL1 β dysregulation has been observed in other studies involving use of mouse *in vitro* AM and *in vivo* models of lung disease [11,14,42,43] (Fig. 4). For example, IL1 β down-regulation has been linked to up-regulated expression of IL1 α and IL1r1, a specific competitive inhibitor of IL1 β [42]. IL1 β repression has also been linked to up-regulated IL4 [44] and IL10 [45] expression. These two genes are members of the Th1 and Th2 cytokine family and key chemokines that control phenotypic response in asthma and inflammatory lung disease [38]. While AM data for IL1r1, IL1 α , IL4 and IL10 transcription are lacking, our *in vivo* results [11,14] provide some insight into the association between IL1 β and these four genes. For IL1r1 they revealed up-regulated transcription in most treatment lungs (except neoechinulin B) either before IL-1 β was transcribed or in some in which IL-1 β signal is lacking (brevianimide, neoechinulin A, sterigmatocystin) (Table 3). The relationship between the remaining 3 genes and IL-1 β is less apparent, although there appears to be IL-1 β /IL10 co-transcription. The importance of these associations in the onset and progression of fungal-induced asthma and inflammatory lung disease remains unclear and deserves attention in subsequent studies.

In summary, dose and time-dependent transcription and expression of inflammation-associated genes was observed in mouse AMs exposed to fungal compounds all at a dose of 10^{-8} M. Interestingly, this dose is within the range of concentrations in which pulmonary inflammatory changes in mice intratracheally exposed to these compounds have been reported [11,14]. These

observations further support our position that exposure to low molecular weight compounds from indoor-associated fungi may provoke some of the inflammatory health effects reported from humans in damp building environments. Additionally, the epistatic control of key chemokines that are controlled by exposures to low molecular weight fungal compounds also control phenotypic response in asthma and lung disease [38]. These data may also open up a hypothesis building process that could explain the rise of non-atopic asthma associated with fungi.

Conflicts of interest

The authors declare no conflict of interest.

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