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Proteins and endotoxin in house dust mite extracts modulate cytokine secretion and gene expression by dermal fibroblasts

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Abstract

House dust mite extracts used for diagnostic tests and immunotherapy contain bioreactive molecules including proteins and endotoxin. These extracts can influence the cytokine secretion and adhesion molecule expression by cells in the skin and lung airways. The aim of this study was to determine the role of proteins and endotoxin in mite extracts in modulating gene expression and cytokine secretion by human dermal fibroblasts. Cultured normal human dermal fibroblasts were stimulated with whole mite extracts, mite extracts boiled to denature proteins, or mite extracts treated with polymyxin B to inactivate lipopolysaccharide. Gene expression and secretion of interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1) were determined after 6 h of stimulation. Whole *Dermatophagoides farinae*, *D. pteronyssinus* and *Euroglyphus maynei* extracts induced dose-dependent IL-6 and IL-8 secretion. In addition, *D. farinae* and *E. maynei* induced secretion of MCP-1. *D. farinae* and *E. maynei* also induced parallel cytokine gene expression. Cells stimulated with boiled *D. farinae* extract showed moderate to marked reductions in IL-6 and IL-8 secretion. In contrast, boiled *D. pteronyssinus* and *E. maynei* extracts induced equal or greater cytokine secretions than untreated extracts. The stimulating properties were reduced for all three extracts following treatment with polymyxin B. Our data suggest that both endotoxin and proteins in mite extracts modulate the secretion of cytokines by dermal fibroblasts. The biological activities of *D. farinae*, *D. pteronyssinus*, and *E. maynei* extracts are not equivalent. There appears to be a lipopolysaccharide-binding protein in some mite extracts.

Keywords

Dermatophagoides farinae; *Dermatophagoides pteronyssinus*; *Euroglyphus maynei*; house dust mite; endotoxin; immunomodulation; fibroblasts

Introduction

The house dust mites *Dermatophagoides farinae*, *D. pteronyssinus*, and *Euroglyphus maynei* are the sources of many allergenic and non-allergenic molecules. Molecules from these mites can influence the function of cells in the skin and lungs as well as cells in the blood that are directly involved in immune and inflammatory reactions. For example, Der f 1 activates eosinophils to release inflammatory mediators (Miike & Kita 2003). Der p 1 can cleave CD23 on activated B-cells and CD25 from T-cells (Hewitt et al. 1995; Schulz et al. 1998). Lung bronchial epithelial cells secrete interleukin-6 (IL-6), IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), eotaxin and prostaglandin E2 when stimulated with whole extract of *D. pteronyssinus* or specific protease mite allergens including Der p 1, Der p 3, and Der p 9 (King et al. 1998; Knight et al. 2000; Mascia et al.

2002; Sun et al. 2001; Tomee et al. 1998). Whole extract of *D. farinae* can induce human dermal fibroblasts to produce IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) (Arlian et al. 2008). Fibroblasts activated with *D. pteronyssinus* extract secrete IL-8 and M-CSF while extracts of *E. maynei* induce fibroblasts to produce IL-8 (Arlian et al. 2008). Extracts of *D. farinae*, *D. pteronyssinus*, and *E. maynei* induce human dermal endothelial cells of the microvascular to express the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin and secrete IL-6, IL-8, MCP-1, and GM-CSF (Arlian et al. 2009a).

As part of the innate protective response, many cells have toll-like-receptors (TLRs) on their surfaces that recognize sets of molecules from microbes. TLR4 on the surface of cells recognizes lipopolysaccharide endotoxin (LPS) from gram-negative bacteria. House dust mite extracts contain varied amounts of endotoxin (Arlian et al. 2009a; Avula-Poola et al. 2012; Trivedi et al. 2003; Yella et al. 2011, 2012). *D. farinae* contains greater amounts of endotoxin than *D. pteronyssinus*. *E. maynei* also contains more endotoxin than *D. pteronyssinus*. Presumably endosymbiont bacteria in the mites are the source of the LPS in extracts produced from them (Valerio et al. 2005). LPS is a proinflammatory substance and can stimulate cells via TLR2 and TLR4 to induce the secretion of cytokines during innate responses. Fibroblasts have TLR4 that can be activated by LPS to secrete prostaglandin E2, IL-6, IL-8, and MCP-1 (Wang et al. 2011). Our previous studies have shown that LPS alone can also stimulate normal human dermal fibroblasts to secrete IL-6, IL-8, and MCP-1 and M-CSF (Arlian et al. 2008).

Mite extracts also contain proteolytic enzymes (Thomas et al. 2007). The group 1 (cysteine proteases) and groups 3, 6, and 9 (serine proteases) mite allergens may act through protease-activated receptors (PARs) on the surface of cells (Reed & Kita 2004). PARs are present on many cell types including fibroblasts. Dermal fibroblasts activated via PAR-1 and PAR-2 produce vascular endothelial growth factor (VEGF), IL-8, MCP-1 and platelet-derived growth factor (Reed & Kita 2004).

These heterogeneous stimulating properties of the extracts from the three different species of house dust mites suggests that the extracts from them contain many different molecules and/or different concentrations of the same molecules that can stimulate dermal fibroblasts. These molecules may include endotoxins, proteins, hormones, neurotransmitters, biogenic amines, lipids, and carbohydrates. The aim of this study was to determine the role of endotoxins and proteins in the mite extracts in modulating the gene expression and secretion of cytokines by normal human dermal fibroblasts.

Materials and Methods

Mite material

The house dust mites *Dermatophagoides farinae* (DF), *D. pteronyssinus* (DP) and *Euroglyphus maynei* (EM) were grown at room temperature and 75% relative humidity (Avula-Poola et al. 2012). Mites were collected as they migrated from thriving cultures, killed by freezing and used to prepare soluble extracts in endotoxin-free water (Lonza, Walkersville, MD) as previously described (Arlian et al. 2009a). Protein contents were measured (Bradford 1976) and endotoxin levels were determined using the *Limulus* Amebocyte Lysate (LAL) QCL-1000 assay kit from Lonza (Arlian et al. 2009a).

Normal Human Dermal Fibroblasts

Normal human dermal fibroblasts of adult origin (NHDF) were purchased from Clontech (Mt. View, CA) and were maintained at 37°C, 6% CO₂ in the supplier's medium as previously described (Arlian et al. 2008). All experiments were carried out using cells that

were between passages 6 and 14. For secretion studies, cells were grown for 48 h in 96-well plates (Costar 3997, Corning Inc., Corning, NY) with an initial seeding of 5.0×10^3 cells/mL. Gene expression studies used cells grown for 48 h in T-25 flasks (BD Falcon, Franklin Lakes, NJ) that were seeded with 1.5×10^5 cells/mL.

Cell challenges

For cell challenge studies, mite extracts were diluted in medium to protein concentrations of 0, 12.5, 25, 50 and 100 $\mu\text{g/mL}$. At the beginning of the experiment, the growth medium was removed from the wells and replaced with the challenge solution (extract in medium). Plates were incubated at 37°C, 6% CO_2 for 6 h, after which the media was collected and frozen. IL-6, IL-8, MCP-1, and M-CSF cytokine secretion into the media was measured by enzyme-linked immunosorbent assay (DuoSet ELISA kits, R&D Systems, Minneapolis, MN). Challenges were conducted several times. Data here are from a representative experiment and are presented as the mean $\pm$ standard error of the mean of 3 replicate wells for each test condition.

Polymyxin B (PmB) isolated from fermentation of *Bacillus polymyxa* (Sigma-Aldrich Co., St. Louis, MO) was used to bind and inactivate the endotoxin present in the extracts. For PmB pre-treatment, extracts were prepared at 2X concentration and combined with an equal volume of 2X PmB (final conc. 50 $\mu\text{g/mL}$). Samples were incubated overnight at 4°C and then used for cell challenge.

Protein denaturation was carried out by incubating 1.5 mL microfuge tubes containing whole mite extract in a boiling water bath for 5 or 30 min. Aggregated protein was removed by centrifugation at $14k \times g$ for 10 min and the supernatants were collected and diluted in parallel with the untreated mite extracts.

Gene expression studies

Gene expression studies were conducted on cells challenged for 6 h. Mite extracts (untreated, or boiled for 5 or 30 min) were tested at 50 μg protein/mL. When endotoxin was included as a control, *Escherichia coli* lipopolysaccharide (LPS; Sigma) was used at a concentration approximately matched to that present in 50 $\mu\text{g/mL}$ of the mite extract being tested.

At the conclusion of the 6-h cell challenge, cells were harvested, 1×10^5 cells were lysed and a “Cells to cDNA” Kit (Life Technologies Corp, Grand Island, NY) was used to prepare cDNA according to the manufacturer's directions. Briefly, the cell lysate underwent reverse transcription using random decamers provided by the kit to generate cDNA. Gene expression was measured using cytokine-specific gene expression assays and carried out in triplicate in a 96-well optical reaction plate (Life Technologies). IL-6, IL-8, MCP-1 and GAPDH gene expression was quantified by the Comparative C_T Assay using an ABI 7900HT Fast System. Relative quantification was determined following standard thermal cycling conditions for the Fast Protocol. Briefly, step 1 consisted of initial polymerase activation at 50°C for 2 min then 95°C for 10 min. This step was followed by 40 polymerase chain reaction (PCR) cycles that each melted the template cDNA for 15 sec at 95°C and annealed and extended PCR primers at 60°C for 1 min. For each cytokine tested, gene expression was quantified and compared to constitutive amounts of expression by normal human dermal fibroblast cells grown in media alone. GAPDH was used as a housekeeping gene.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was carried out using Criterion Any kD gels with Tris/Glycine/SDS running buffer (Bio-Rad, Hercules, CA) at 200 V (constant) for 42 min. Gel lanes were loaded with 10 µg of whole mite extract protein or with an equivalent amount of boiled extract supernatant. Protein profiles were visualized by silver staining. As previously described (Arlian et al. 2009b), proteins on a parallel gel were transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA) at 50 V (constant) for 75 V* h in 10 mM CAPS/10% methanol. Following blocking, blots were probed overnight with rabbit anti-*D. farinae*, anti-*D. pteronyssinus*, anti-*E. maynei* polyclonal antiserum (all 1:1000) or biotinylated-mouse monoclonal anti-Der 2 (7A1 at 1:2500; Indoor Biotechnologies, Charlottesville, VA). Rabbit antibody binding was detected by 1 h incubation in alkaline phosphatase-labeled goat anti-rabbit Ig followed by AP-Blue membrane substrate (Sigma). Anti-Der 2 binding was developed with peroxidase-labeled streptavidin and the chromogenic substrate of Young (1989).

Results

Polymyxin B dose

The mite extracts used in this study contained varying amounts of endotoxin (Table 1). *E. maynei* extract contained the highest concentration of endotoxin and *D. pteronyssinus* the lowest. As a control, we first determined the effect of endotoxin on normal human dermal fibroblast cells by examining the effect on cytokine secretion of an amount of LPS equivalent to that present in the *E. maynei* extract. Then, this LPS sample was treated with polymyxin B (PmB) to determine the dose of PmB that would inactivate the endotoxin. We assumed that this dose of PmB would inactivate the lower doses of endotoxin present in the *D. farinae* and *D. pteronyssinus* mite extracts. Also, regardless of the mite extract used to stimulate the cells, all the cells would be exposed to a standard amount of PmB.

Fibroblasts constitutively secreted small amounts of IL-6, IL-8, and MCP-1 (Fig. 1). Cells stimulated with a dose of LPS that was similar to the endotoxin load present in *E. maynei* extract at 50 µg protein/mL secreted increased amounts of IL-6, IL-8, and MCP-1. Cells that were stimulated with LPS that had been treated with PmB to bind the endotoxin exhibited an 80% reduction in IL-8 secretion and complete elimination of IL-6 and MCP-1 cytokine secretions compared to cells challenged with the untreated LPS. This confirmed that the PmB dose selected for use (50 µg/mL) was adequate to eliminate most or all of the cytokine secretion induced by the endogenous endotoxin present in the mite extracts and was not lethal to the cells.

Dermatophagoides farinae—Fibroblasts stimulated with *D. farinae* extract exhibited a dose-dependent increase in secretion of IL-6, IL-8, and MCP-1 and increased expression of mRNA for these cytokines (Fig. 2). No M-CSF cytokine secretion was detected following a 6-h stimulation with *D. farinae* extract (data not shown). The *D. farinae* extract was also pre-treated with PmB to inactivate LPS prior to cell challenge. At an extract dose of 50 µg/mL, this treatment eliminated all of the IL-6 secretion, 91% of the extract-induced IL-8 secretion, and 84% of induced MCP-1 secretion (Fig. 2). Parallel reductions were observed in gene expression for these cytokines suggesting that these effects were exerted at the level of gene expression.

We next boiled the extract for 5 min to investigate the contributions of the protein components to extract-induced cytokine gene expression and secretion. Boiling *D. farinae* extract for 5 min reduced IL-6 secretion by 80 - 100% for all doses (Fig. 2). Likewise,

boiling the extract resulted in reductions of > 85% of the IL-8 and MCP-1 secretion induced by low doses (12.5 µg and 25 µg) and 52 – 85% of IL-8 and MCP-1 secretion at higher doses (50 µg and 100 µg) of *D. farinae* extract. IL-8 gene expression in the presence of 50 µg boiled *D. farinae* extract was reduced by 40% while MCP-1 gene expression was essentially unchanged. Thus, boiling the extract alone did not eliminate extract-induced cytokine secretion nor did it reduce the secretion of these cytokines as much as treating the unboiled extract with PmB. However, addition of PmB to the 5 min boiled extracts further reduced cytokine gene expression and reduced secretion of all three cytokines to near constitutive levels. Extract boiled for 30 min (to both denature protein and destroy the LPS) and extract boiled for 30 min and then treated with PmB (to bind LPS) did not induce IL-6 gene expression or secretion by the challenged fibroblasts and most IL-8 expression and secretion were also reduced. These treatments also inhibited MCP-1 cytokine secretion while 30-min boiling alone had no effect on MCP-1 gene expression.

Using SDS-PAGE, we assessed the effect of boiling on the protein components of the extracts in order to determine if heat stable proteins remained in the extracts and could possibly contribute to stimulation of the fibroblasts. SDS-PAGE profiles showed that the supernatants collected following centrifugation to remove proteins that were denatured/aggregated during boiling, contained small amounts of proteins over the molecular weight range 10 – 150 kDa with one prominent band at about 14 kDa that was identified as Der f 2 by immunoblotting (Fig. 3). Supernatant of extract boiled for 30 min showed fewer protein bands than were present in extract boiled for 5 min.

Dermatophagoides pteronyssinus—*D. pteronyssinus* extract at 50 and 100 µg/mL induced a small increase in the secretion of IL-6 and IL-8 by fibroblasts that was abrogated by pretreating the extract with PmB before the cells were stimulated (Fig. 4). *D. pteronyssinus* challenge for 6 h did not induce secretion of MCP-1 or M-CSF by fibroblasts. *D. pteronyssinus* extract that was boiled for 5 min to denature proteins induced IL-6 and IL-8 secretion that was equal to or greater than the secretion induced by the native extract but this effect was abrogated when the boiled extract was treated with PmB before challenging the cells. Boiling the extract for 30 min to denature proteins and destroy the LPS in the extract reduced nearly all of the IL-6 secretion and about 35 – 60% of the IL-8 secretion induced by untreated extract. PmB treatment of boiled *D. pteronyssinus* extract reduced IL-8 secretion to constitutive levels.

Similar to *D. farinae* extracts, SDS-PAGE showed that the supernatant from *D. pteronyssinus* extracts boiled for 5 minutes still contained some heat stable soluble proteins (10 – 150 kDa) but many fewer and lesser quantities than in the untreated extract (data not shown). Most of the soluble protein including Der p 2 was absent in the supernatants from the extract after it was boiled for 30 min.

Euroglyphus maynei—*E. maynei* extract also induced a dose-dependent increased secretion of IL-6, IL-8, and MCP-1 that was mostly abrogated by pre-treating the extract with PmB (Fig. 5). The effects on cytokine gene expression were largely parallel. Similar to the *Dermatophagoides* extracts, no M-CSF was detected in response to a 6-h stimulation with *E. maynei* extract. Extract that was boiled for 5 min induced about the same or more IL-6, IL-8, and MCP-1 secretion as the unboiled extract for most doses of extract. This effect on IL-6 secretion was completely abrogated by treating the boiled extract with PmB and mostly abrogated for IL-8 (50%) and MCP-1 (30%) secretion. Interestingly, *E. maynei* extracts boiled for 30 min had greater stimulating ability than did the untreated extract and most of this additional stimulating ability was reduced after the boiled extract was treated with PmB prior to challenge. As for the *Dermatophagoides* spp, SDS-PAGE showed that extracts boiled for 5 or 30 min still contained some heat stable proteins (data not shown).

Discussion

A number of studies have shown that house dust mite extracts contain molecules that can induce responses from cells of the skin and bronchial epithelium of the lungs and these may influence the manifestation of inflammation and the course of allergic reactions in the skin and bronchial airway (Arlian et al. 2008, 2009a; Arlian & Morgan 2011; Boasen et al. 2005; Chen et al. 2003; King et al. 1998; Lee et al. 2008; Machado et al. 1996; Sohn et al. 2007; Tomee et al. 1998; Wong et al. 2006). Most of the lung studies have challenged epithelial cells with specific allergens (mostly group 1) or whole extract of *D. pteronyssinus*. A few have used *D. farinae* and none investigated *E. maynei*. Extracts of these mites contain many allergens as well as other molecules including endotoxins (Avula-Poola et al. 2012; Thomas et al. 2007; Trivedi et al. 2003; Valerio et al. 2005; Yella et al. 2011, 2012). Interestingly, the concentration of endotoxin found in house dust mite extracts varies considerably between species and the concentration for a species may be influenced by the culturing conditions of the mites used to make the extracts (Avula-Poola et al. 2012; Yella et al. 2011, 2012). Endotoxin has long been known to be a pro-inflammatory substance and to induce cytokine secretion from cells.

Prior to this study, little was known about the role that endotoxin, proteins and other molecules in these extracts play in influencing cell function in the skin. Our study found that both endotoxin and proteins in these mite extracts can modulate cytokine secretion and gene expression in normal human dermal fibroblasts. However, the stimulating properties of extracts from the three different species were varied and unique. For example, *D. farinae* and *E. maynei* extracts could stimulate MCP-1 secretion but *D. pteronyssinus* extract could not. Conversely, boiling *D. farinae* extract reduced or eliminated its stimulating capacity while this treatment enhanced stimulation by *D. pteronyssinus* and *E. maynei* extracts. These varied results indicate that in general, extracts of the different mite species should not be viewed as equivalent in terms of their biological activity and the responses they induce in cells involved in normal function of the skin and lungs.

Our study found that whole extract of *D. farinae*, *D. pteronyssinus* and *E. maynei* could stimulate the secretion of IL-6 and IL-8 by fibroblasts. In addition, *D. farinae* and *E. maynei* could also induce secretion of MCP-1. This property was mostly abrogated after the extracts were treated with PmB to bind the LPS in the extract. These findings suggest that endotoxin present in the extracts contribute to their stimulating properties. However, our data also show that proteins along with the LPS in the extracts contributes to their stimulating properties since *D. farinae* extracts that were boiled for 5 min to denature proteins had reduced stimulating activity.

Endotoxin is generally considered to be heat stable (Gao et al. 2006; Majde 1993). However, a more recent study suggests that boiling LPS for 15, 30, and 60 min is sufficient to inactivate ~90% of the LPS-induced tumor-necrosis factor- secretion by macrophages even in the presence of protein (Gao et al. 2006). The boiled LPS also retained its ability to be bound by polymyxin B. It is not clear if our 5-min boil inactivated some of the LPS in the *D. farinae* extract. However, *D. pteronyssinus* and *E. maynei* extracts that were boiled for 5 min retained their stimulating ability. Taken together these data suggest that the LPS in the extract was not inactivated by a 5-min boil.

We previously found that boiled extract of the storage mite *Acarus siro* exhibited similar properties on human endothelial cells of the microvasculature and proposed that this extract contained a LPS-binding protein that was denatured during boiling (Elder et al. 2012). In the present study, it appears that *D. pteronyssinus* and *E. maynei* extracts also contain a heat-labile LPS-binding protein and when it is denatured by boiling the extract, it releases LPS

that can then resume its stimulating activity (activate TLR4) in the extract. The evidence for this is that *D. pteronyssinus* and *E. maynei* extracts that were boiled for 5 min exhibited greater stimulating ability than the unboiled extracts. However, restored LPS activity in the boiled extract can be abrogated by treating the boiled extract with PmB that binds the now freed LPS, thus again inhibiting/reducing its stimulating activity. The concentration of this LPS binding protein in the extracts may vary among species just as the amount of LPS in the house dust mite extracts varies among species. It is interesting that this protein occurs in *D. pteronyssinus* extract but not *D. farinae*, two closely related species.

As expected, *D. farinae* and *D. pteronyssinus* extracts that were boiled for 30 min or that were boiled for 30 min and then PmB-treated had little or no stimulating ability presumably because all proteins were denatured and LPS was inactivated. The interesting observation was that *E. maynei* extract that had been boiled for 30 min had greater stimulating ability than untreated extracts and all other treatments (PmB-treated, 5-min boiled, 5 min boil plus PmB and 30 min boil plus PmB). This suggests that the LPS binding protein in the *E. maynei* extract 1) is more heat stable than for *D. pteronyssinus*, 2) is present in higher amounts, or 3) is possibly a different protein.

These findings also shed new light on our previous fibroblast study (Arlian et al. 2008). In that study, fibroblasts stimulated for 24 h with a constant dose of LPS in combination with increasing doses of *A. siro*, *D. pteronyssinus* and *E. maynei* extracts secreted decreasing amounts of IL-6 and MCP-1 – presumably due to the presence of an LPS-binding protein in these extracts which increased with extract dose. This effect was not observed for *D. farinae*, the mite extract that appears to lack an LPS-binding protein.

Relevant to our current study using dermal fibroblasts, is a study that found that *D. farinae*, *D. pteronyssinus* and *E. maynei* stimulated dermal endothelial cells of the microvascular to secrete the cytokines IL-6, IL-8, MCP-1, G-CSF and GM-CSF and express intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) and E-selectin (Arlian et al. 2009a). Extracts of these mites treated with PmB were unable to stimulate IL-6, G-CSF and GM-CSF but did still induce secretion of moderate amounts of IL-8 and small amounts of MCP-1. These results suggested that some of the stimulation was due to LPS in the extracts but some was not. PmB-treated *D. farinae* and *D. pteronyssinus* extracts did not induce expression of ICAM-1, VCAM-1, and E-selectin that was induced by untreated extracts and this suggested that LPS in these extracts was responsible. In contrast, PmB treated *E. maynei* extract still induced expression of ICAM-1, VCAM-1 and E-selectin. These findings using dermal endothelial cells coupled with our current results using skin fibroblasts further illustrates that dust mite extracts contain a diverse and complex mixture of bioactive molecules, different skin cell types respond differently to the same extract, and different mite species may induce different responses in the same cells. Furthermore, these extracts contain molecules other than LPS, proteases and other proteins that can influence cell function.

Our findings are not surprising and are consistent with other studies of the biochemical and functional properties of mite allergens. Group 2 and 7 allergens are lipid-binding proteins. Der p 2 and Der f 2 are lipid binding proteins that share sequence homology with MD-2, the LPS binding protein of the TLR4 complex (Inohara & Nunez 2002). Der p 2 also has structural homology with MD-2. Der p 2 and Der f 2 can bind LPS (Ichikawa et al. 2009; Mueller et al. 2010a). MD-2 acts by binding LPS to the TLR4 receptor and this results in activation of the TLR4 complex. Der p 2 and Der f 2 also can bind directly to the TLR4 and activate it in the absence of MD-2 (Trompette et al. 2009). Our immunoblot analysis confirmed the presence of Group 2 allergen in our extracts that could be responsible for our observed LPS binding effects. In contrast to Der 2 allergens, Der p 7 binds to the bacterial

lipopeptide polymyxin B from gram-positive bacteria and does not appear to bind LPS (Mueller et al. 2010a). It does not appear that this allergen in our extracts impacted the PmB activity in our experiments. Additionally, the Der p 5 allergen appears to be capable of binding hydrophobic lipid-like ligands (Mueller et al. 2010b). Our studies clearly show that there can be an interaction between the endotoxin and lipid-binding proteins of a mite extract that influences how cells can respond to these mites.

A similar study by Lee et al. (2008) showed that *D. pteronyssinus* extract induced elevated MCP-1, IL-6 and IL-8 gene expression and secretion by human THP-1 monocytes. In contrast, we found that dermal fibroblasts did not secrete increased amounts of MCP-1 at any extract dose and only increased secretion of IL-6 and IL-8 at the highest *D. pteronyssinus* extract doses tested. In parallel, at 6 h of stimulation, we did not see increased gene expression for these cytokines. The contrasting results between these studies may reflect that two different cell types were used and that the extracts were prepared differently and are probably not comparable. The Lee paper reports using extract prepared from “crude *D. pteronyssinus* culture”, presumably containing eggs, exoskeletons and fecal material in addition to active, quiescent and dead mites. In contrast, our extract was prepared from live mites as they migrated from thriving cultures and so was composed of active mites with only the eggs and fecal material contained within the bodies.

Our study also revealed that there was not always a direct association between induced gene expression and cytokine secretion. *D. farinae* and *E. maynei* induced gene expression for IL-6, IL-8, and MCP-1 in parallel with the secretion of the associated cytokines. However, *D. pteronyssinus* extract did not induce measurable gene expression at 6 h following continuous stimulation with the extract. This would suggest that in some cases the extracts up-regulated both gene expression (cytokine synthesis) and cytokine secretion but in others the extracts only induced release of the cytokine from the cell and not its synthesis. Furthermore, the IL-6 and MCP-1 gene expression induced by the *D. farinae* extract was completely abrogated when the extract was treated with PmB (bound LPS) which suggested that LPS alone in the extract could induce gene expression for synthesis of IL-6 and MCP-1.

Conclusion

Few studies have examined the biological effects of specific allergens and non-allergens in whole extracts on the biological function of specific cells in the skin and lungs that are involved in innate and adaptive immune responses. While structural and biochemical characterization of specific allergens provides insight into potential biological functions they do not elucidate the effects of the complex mixture of molecules in natural mite material (whole mite extracts) on cell function. Knowing the effects of whole mite extracts helps understand the role of these mites in promoting inflammation and allergic reactions. Our results clearly show that endotoxin, proteins and a LPS-binding protein in mite extracts can influence fibroblast function and thus the innate and adaptive cell responses and house dust mite induced allergic reactions. Furthermore, independent of an IgE-mediated allergic reaction, these extracts induce fibroblasts and other cells to secrete pro-inflammatory mediators. However, the stimulating property of house dust mite extracts varies between species. Thus, the biological activities of *D. farinae*, *D. pteronyssinus*, and *E. maynei* are not equivalent.

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Fig. 1.
Cytokine secretion by normal human dermal fibroblasts induced by 6 h stimulation with endotoxin (LPS at 120,000 EU/mL) and LPS pre-treated with Polymyxin B (PmB at 50 µg/mL) to inactivate endotoxin.

Fig. 2. Cytokine secretion and gene expression by normal human dermal fibroblasts induced by 6 h stimulation with to *D. farinae* (DF) mite extract or extract boiled for 5 or 30 min without or with Polymyxin B (PmB) pre-treatment to inactivate endotoxin. Gene expression was measured using 50 μg protein/mL.

Fig. 3. SDS-PAGE and immunoblot analysis of *D. farinae* extract, untreated (DF) or extract boiled for 5 (5) or 30 min (30). Molecular weight (MW) markers are shown at the left. The silver stained gel is shown in the right panel. Immunoblots were probed with a mouse monoclonal antibody directed against mite group 2 allergen (anti-Der f 2; left panel) or polyclonal rabbit antiserum (anti-*D. farinae*; center panel) and developed as described in the Methods section.

Fig. 4. Cytokine secretion by normal human dermal fibroblasts induced by 6 h stimulation with *D. pteronyssinus* (DP) mite extract or extract boiled for 5 or 30 min without or with Polymyxin B (PmB) pre-treatment to inactivate endotoxin. Gene expression was measured using 50 μg protein/mL.

Fig. 5. Cytokine secretion and gene expression by normal human dermal fibroblasts induced by 6 h stimulation with *E. maynei* (EM) mite extract or extract boiled for 5 or 30 min without or with Polymyxin B (PmB) pre-treatment to inactivate endotoxin. Gene expression was measured using 50 µg protein/mL.

Table 1

Endotoxin content of the house dust mite extracts.

Mite extract	EU per 50 µg protein
<i>Dermatophagoides farinae</i>	52,115
<i>Dermatophagoides pteronyssinus</i>	8,725
<i>Euroglyphus maynei</i>	132,570