

2010

# The Influence of Stored Product Mites on the Function of Skin Microvascular Endothelial Cells

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**THE INFLUENCE OF STORED-PRODUCT MITES ON THE FUNCTION OF  
SKIN MICROVASCULAR ENDOTHELIAL CELLS**

**A thesis submitted in partial fulfillment of the requirements for the degree  
of Master of Science**

**By**

**JENNIFER GRAFF CURP**

**B.S., Wittenberg University, 2008**

**2010**

**Wright State University**

WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

April 13, 2010

I HEREBY RECOMMEND THAT THE THESIS PREPARED  
UNDER MY SUPERVISION BY Jennifer Graff Curp  
ENTITLED THE INFLUENCE OF STORED-PRODUCT MITES  
ON THE FUNCTION OF SKIN MICROVASCULAR  
ENDOTHELIAL CELLS BE ACCEPTED IN PARTIAL  
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE  
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## **Abstract**

Curp, Jennifer Graff. M.S., Department of Biological Sciences, Wright State University, 2010.

### **THE INFLUENCE OF STORED-PRODUCT MITES ON THE FUNCTION OF SKIN MICROVASCULAR ENDOTHELIAL CELLS**

The prevalence of allergenic diseases and sensitization to stored product mites is increasing worldwide. Stored product mites are prevalent in homes, foods, and some work environments. Stored product mites need to be further studied so we can characterize their inflammatory properties to understand their role in skin disease, and provide proper treatment to patients with mite allergies. The purpose of the study was to show the role of stored product mites in modulating the inflammatory and immune response to human dermal endothelial cells in the skin.

The epidermis serves as the first line of defense between the body and the environment. Disturbance of the epidermal barrier can allow the penetration of allergens and other exogenous molecules into the skin. Interaction between allergens and other mite molecules, and cells in the epidermis and dermis such as keratinocytes, fibroblasts, antigen presenting cells, leukocytes and microvascular endothelial cells in the skin may trigger inflammation and immune reactions. Once inside the skin, mite allergens and other mite molecules contact endothelial cells, and those molecules may affect endothelial cell function. Endothelial cells control the movement of inflammatory cells from the blood into the dermis during allergic reactions (atopic dermatitis) and other skin diseases.

Our experiments tested the hypothesis: components of stored product mites influence functioning of normal human dermal microvascular endothelial cells.

Stored product mite extracts regulated cell adhesion molecule expression and cytokine secretion by microvascular endothelial cells. *Acarus siro* up-regulated ICAM-1. *A. siro* down-regulated the TNF- $\alpha$  induced expression of VCAM-1. *Chortoglyphus arcuatus* down-regulated the TNF- $\alpha$  induced expression of E-selectin. *Tyrophagus putrescentiae* down-regulated the TNF- $\alpha$  induced expression of ICAM-1. *A. siro* and *Lepidoglyphus destructor* down-regulated the constitutive secretion of VEG-F. *A. siro* induced secretion of GM-CSF and IL-6. *C. arcuatus* induced secretion of IL-6 and IL-8. These responses were not mediated through PARs or TLRs.

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## **ACKNOWLEDGEMENTS**

I would like to acknowledge and show my appreciation to Dr. Larry Arlian for giving me the opportunity to conduct research while pursuing an advanced degree within his laboratory. I wish to thank Dr. Marjorie Morgan for all of her support and instruction within the laboratory. I also wish to thank Dr. Laurie Elder for all of her encouragement and instruction in the laboratory. During my time at Wright State University, these individuals have shown a lot of patience and support towards my completion of this project.

I also wish to acknowledge and thank my committee members, Dr. Barbara Hull and Dr. Courtney Sulentic, for their time overseeing my thesis. I wish to thank DiAnn Vyszenski-Moher for her support and encouragement.

Financial support for this research was provided by Dr. Larry Arlian through a grant obtained from the National Institutes of Health.

## Introduction

### A. Anatomy and Physiology of Skin

The skin is responsible for a variety of functions. It serves as a protective shield against heat, light, chemicals, mechanical injury, and infectious agents. Also, the skin regulates body temperature, reduces water loss, and stores water, fat, and vitamin D. To achieve these functions, skin is composed of an outer epidermis and an inner dermis.

The thin superficial epidermal layer is composed of the stratum corneum, keratinocytes, basal layer, and Langerhan cells. The stratum corneum continually sheds dead keratinocytes. Keratin, a protein that is abundant in dead keratinocytes, is responsible for protecting the skin. The next inner layer contains living keratinocytes. Keratinocytes are the building blocks of the epidermis. The basal layer is the inner layer of the epidermis, containing basal cells which continually divide to form new keratinocytes and thus epidermis. The epidermis contains melanin pigment producing melanocytes, as well as Langerhan cells, the antigen presenting cells that serve as the frontline defense against infection (Proksch et al. 2008).

The dermis is below the epidermis. It consists of a network of collagen, fibroblasts, fat cells, and the microvascular (blood vessels). This region is responsible for conserving body heat and protecting organs from injury by acting as a shock absorber. The blood vessels are important in this layer to regulate immune and inflammatory responses, water loss, and temperature (Proksch et al. 2008).

Endothelial cells make up the inner lining of blood vessels throughout the circulatory system. They are unique multifunctional cells; besides reducing friction of the flow of blood these cells also provide a barrier between the vessel wall and the blood, and between the blood and extravascular tissues. The endothelial cells react physically and chemically to stimuli in the blood circulation to regulate homeostasis, blood clotting, vasomotor tone, and immune and inflammatory responses. The capillary and venule endothelial cells control the passage of materials and cells in and out of the bloodstream. In the skin, post-capillary venule endothelial cells regulate movement of inflammatory and immune cells between the blood and the dermis (Sumpio et al. 2002).

Endothelial cells adapt rapidly to changes in their environment and can change their function when they become activated. These cells secrete cytokines and chemokines and express cell adhesion molecules and chemokine receptors either constitutively or under stimulation. They can respond to exogenous environmental factors that penetrate the skin and to cytokines, chemokines and cell adhesion molecules from other cells in the vicinity (McLaren and Kennedy 2005).

## **B. Skin Immune System**

The human immune system mobilizes a large range of innate and adaptive immune responses to protect against pathogens that it encounters. The skin is an important part of the immune system. Apart from the physical barrier, there are specialized cells of the immune system throughout the layers of the skin. Some of these cells, such as B cells and Langerhan cells act as antigen

presenting cells and detect invasion by foreign proteins such as bacteria or viruses. Other cells, such as macrophages, neutrophils and cytotoxic T cells, have the function of destroying and removing foreign material. If a virus enters the body, it is likely that it will get into a cell. The infected cell will display MHC class I complexes with foreign protein on its surface, and the migrating cytotoxic T cell will recognize it. The cytotoxic T cell binds to the infected cells and releases lytic granules onto the surface of the infected target cell. The lytic granules enter the infected cell and initiate apoptosis (von Bubnoff et al. 2001).

Some mite proteins that cause an allergic reaction are enzymes and their enzymatic properties favor penetration through the epidermis and dermis. Disturbance of the epithelial barrier can allow the penetration of allergens and other exogenous molecules into the skin. An example of a mite molecule is the Der p 1 allergen which is a protease. This allergen increases permeability through lung epithelial cells by opening tight junctions. Altering the epithelial barrier not only promotes penetration of these mite proteins, but also of other proteins in the environment (Berard et al. 2003). Irritation is known to provoke barrier defects thereby allowing proteins to enter into direct contact with viable epidermal and dermal cells including Langerhan cells, keratinocytes, fibroblasts, and other cells of the innate immune system.

Interactions between allergens and antigen presenting cells in the skin may trigger inflammation and induce sensitization and subsequently allergic reactions. Antigen presenting cells play a role in transferring antigens from the periphery to lymphoid organs to induce an immune response. Antigen

presenting cells in the skin such as Langerhan Cells and dendritic cells are responsible for the primary immune response (von Bubnoff et al. 2001).

### **C. Role of Endothelial Cells in the Immune System**

The role of endothelial cells in regards to the inflammation and immune responses is to regulate extravasation of inflammatory and immune cells from the blood stream into the dermis. The inflammatory response functions to deliver effector cells and molecules to the site of infection to start to form a physical barrier between the healthy tissue and infected tissue and to repair the tissue. Effector cells destroy the pathogens and non-self molecules through innate and adaptive immune processes (McLaren and Kennedy 2005).

Endothelial cells control leukocyte physical interactions with the vessel wall via cell-adhesion molecules. The endothelial cells express two cell-adhesion molecules (CAMs): P-selectin and E-selectin at the start of the extravasation process. P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury. When endothelial cells are activated by molecules, such as histamine or thrombin, P-selectin moves from an internal cell location to the endothelial cell surface, aiding the inflammation response. After further stimulation, different cell-adhesion molecules from the immunoglobulin family, like ICAM and VCAM, are expressed by endothelial cells and integrins (LFA-1) are expressed on leukocytes, and the two different cells can bind. At this point cell-adhesion is irreversible and the leukocyte, bound to the endothelium, will transmigrate through the basement membrane (McLaren and Kennedy 2005).

The leukocytes are able to migrate through the blood vessel wall (between the cells) and towards the site of infection.

Regulated secretion of pro-inflammatory mediators like cytokines, chemokines, and expression of different adhesion molecules and cytokine and chemokine receptors on endothelial cells attract the leukocytes. Other cells in the vicinity, such as keratinocytes, fibroblasts, and macrophages may also secrete cytokines and chemokines that influence the function of vascular endothelial cells and the course of inflammation (McLaren and Kennedy 2005).

Vascular endothelial cells, keratinocytes and fibroblasts can come in contact with circulating endogenous pathogenic molecules and exogenous factors that penetrate the epidermis (e.g. mite molecules, bacteria, and parasites), and initiate inflammatory and immune responses (McLaren and Kennedy 2005). They secrete cytokines when they are activated which alters the homeostatic balance by bringing in effector cells to the infected tissue and starting the inflammation response (McLaren and Kennedy 2005). Endothelial cells, keratinocytes, and fibroblasts play an important part in the body's defense against pathogens or products from storage mites, house dust mites and scabies mites (Arlan et al. 2003, Arlian et al. 2007).

Toll-like receptors (TLRs) and protease-activated receptors (PARs) are present on the external and internal endothelial cell membranes. TLRs play critical roles in the innate immune system as microbial (LPS, peptidoglycans, lipoproteins) pattern-recognition receptors. Signaling activates antigen presenting cells to provoke innate immunity. Each TLR has common effects, like



up regulating an inflammatory cytokine, and has a specific function. TLR signaling can lead to the activation of several transcription factors, and induce expression of immune response genes. One example of a signaling pathway common in TLR occurs when ligand binding to TLR triggers association of MyD88 with TLR domain and assembles IRAK complexes. TRAF6 binds to the IRAK complex and activates protein kinase TAK1. TAK1 activates the NLkB pathway which activates genes to expand the inflammatory response (Kaisho and Akira 2006).

Endothelial cells express functional TLR2, TLR4, and TLR9 on their cell membrane. Others are located on internal membranes. Under proinflammatory conditions they can express all ten TLRs (Fitzner et al. 2008). There is evidence of house dust mites stimulating cells via TLRs. Previous studies have shown that house dust mites contain endotoxin (Trivedi et al. 2003, Valerio et al. 2005). House dust mite allergen triggers TLR4 on dendritic cells in the lung and causes the release of proinflammatory molecules (Hammad et al. 2009). House dust extract-activated bone marrow-derived dendritic cells produce IL-6 and IL-12 indicating that toll like signaling pathways were activated (Boasen et al. 2005). One study focused on TLR-2 and how it modified the immune response to house dust mite allergen. TLR-2 ligands inhibit allergen-specific T helper 2 cell responses in individuals (Taylor et al. 2006).

PARs were first identified as a mechanism for the interaction between blood clotting and platelet activation. Exogenous proteases from mites can react with PARs on cell surfaces to generate leukocyte infiltration and to amplify the

response to allergens. They contribute to the inflammation characteristic of allergic diseases. PARs are expressed on cells in blood vessels, connective tissue, leukocytes, and epithelium. PARs are stimulated by protease enzymes (Reed and Kita 2004). Activation is initiated when serine proteases cleave the amino acids at a specific site of the N terminus of the receptor resulting in the generation of a new tethered ligand that interacts with the receptor within the extracellular loop. PAR stimulation can cause epithelial cells to open tight junctions, degranulation of eosinophils and mast cells, and produce cytokines to upregulate inflammation (Shpacovitch et al. 2007). The proteolytic activation is irreversible and once cleaved, receptors are degraded by lysosomes. Proteases from mites might be the basis for increased prevalence of asthma (Reed and Kita 2004).

Previous studies have shown that house dust mite allergens activate lung epithelium cells and human eosinophils mediated through PARs. HDM extract, Der p 1 (cysteine protease) and Der p 5 dose-dependently increase the production of IL-6 and IL-8 (Kauffman et al. 2006, Thomas et al. 2007). Also Der p 3 and Der p 9 activate lung epithelium through PARs. These dust mite allergens are serine proteases that may use PAR-2 to activate lung epithelial cells. Der p3 and Der p9 cleave the peptide corresponding to the N terminus of PAR-2 at the activation site (Sun et al. 2001). The effects of cysteine proteases from mite allergen Der f1 on functions of human eosinophils were examined. Papain, a cysteine protease, induces human eosinophils to degranulate and release inflammatory mediators (Miike and Kita 2003). However, Mascia et al.

(2002) showed that *D. pteronyssinus* stimulates the release of cytokines and chemokines by bronchial epithelial cells, but not by keratinocytes, which argues against direct proinflammatory activity of house dust mites on the skin.

#### **D. Mite Influence on the Function of Human Cells**

Arlian et al. (2009) studied cell adhesion molecule expression and cytokine secretion from human dermal endothelial cells activated by house dust mites. Mite extracts from *Dermatophagoides farinae*, *D. pteronyssinus* and *Euroglyphus maynei* stimulates cells to express ICAM-1, VCAM-1, and E-selectin, and to secrete IL-6, IL-8, MCP-1 and GM-CSF. The scabies extract down regulates TNF- $\alpha$  induced expression of VCAM-1 and it inhibits secretion of IL-8 in human dermal endothelial cells (Elder et al. 2009).

Arlian et al. (2003, 2008) studied the cytokine secretion in normal human keratinocytes and fibroblasts in response to molecules from scabies mites and house dust mites. Keratinocytes express increased levels of IL-6, VEG-F and G-CSF in response to *Sarcoptes scabiei*. Fibroblasts also show increased expression of those cytokines as well as IL-8 in response to *Sarcoptes scabiei*, and to house dust mites in a dose dependent manner. House dust mites also induce secretion of MCP-1 and M-CSF from dermal fibroblasts (Arlian et al. 2008). In the presence of proinflammatory cytokines, scabies extracts down-regulated levels of IL-8 secretion from keratinocytes and fibroblasts. Scabies extracts also down regulate GM-CSF secretion from fibroblasts. Scabies extracts with proinflammatory cytokines induce secretions of G-CSF from fibroblasts, and CTACK, GRO $\alpha$ , and TGF $\alpha$  from keratinocytes (Mullins et al.

2009). These results suggest that many molecules in the scabies extract cause a delayed inflammatory response.

IL-6 has a diverse array of proinflammatory and anti-inflammatory functions. It probably plays proinflammatory roles in the response to mites. It can increase vascular permeability, activate Th1 cells, and activate Th2 cells to drive antibody production (Arlian et al. 2003). Fibroblasts and keratinocytes can secrete VEG-F when they are stimulated. VEG-F is usually associated with wound healing by initiating inflammation. Fibroblasts also release G-CSF in large amounts to promote the formation of neutrophils (Arlian et al. 2003). MCP-1 may play a role in the recruitment of monocytes to sites of injury and infection. GM-CSF locally activates leukocytes at the site of inflammation (Arlian et al. 2009).

IL-8 is secreted from fibroblasts stimulated with scabies mite extract (Arlian et al. 2003), and it could possibly be released from endothelial cells when stimulated with storage mite extracts. This cytokine helps regulate the inflammatory response by stimulating various cells (McLaren and Kennedy 2005). Endothelial cells store IL-8 in their Weibel-Palade bodies. IL-8 enhances inflammation, but its primary function is inducing chemotaxis of neutrophils. IL-8 gene transcription is activated by NF- $\kappa$ B. When this molecule is phosphorylated, it activates several genes including IL-8. NF- $\kappa$ B is activated by inducer of inflammation like LPS or TNF- $\alpha$  (Mukaida et al. 1994). Fibroblasts in the dermis also produce IL-8 (Arlian et al. 2007). Neutrophils migrate up a gradient of the chemoattractant to get into the dermis from the bloodstream (Bickel 1993).

Human peripheral blood monocytes have been analyzed in patients exposed to scabies mite extracts (Arlan et al. 2006). Lymphocytes produce large amounts of IFN- $\gamma$  and IL-10 when stimulated with mite extract. TNF- $\alpha$  is also released by the monocytes. Peripheral monocytes and dendritic cells derived from these cells did not secrete IL-10 when stimulated with scabies mite (Arlan et al. 2004). *S. scabiei* extracts may stimulate T regulatory cells, which secrete IL-10 and IFN- $\gamma$ , and Th1 cells that secrete IL-2 and IFN- $\gamma$ . T regulatory cells suppress inflammation and immune responses by the secretion of IL-10. IL-10 suppresses the immune system by inhibiting the expression of MHC class II molecules on antigen presenting cells so T helper cells cannot be activated. IL-10 also directly down-regulates the production of most pro-inflammatory cytokines (TNF- $\alpha$ , IL-2 and IFN- $\gamma$ ) by inhibiting production primarily at the transcriptional level with the involvement of p50 and p65 nuclear factor- $\kappa$ B (Heyen et al. 2000). IL-10 prevents amplification of the inflammatory process (Arlan et al. 2006).

Lung epithelium can also be affected by mite molecules. House dust antigens Der p 3 and Der p 9 both sequentially stimulate phosphoinositide hydrolysis, transient cytosolic Ca<sup>2+</sup> mobilization, and the release of GM-CSF and eotaxin in human pulmonary epithelial cells (Sun et al. 2001). Der p 2 also activates epithelium. In BEAS-2B cells, Der p 2 induces dose-dependent up-regulation in the secretion of GM-CSF, IL-6, IL-8, monocyte chemotactic protein-1 and macrophage inflammatory protein-3 $\alpha$ . ICAM-1 expression is also up-regulated (Osterlund et al. 2009). King et al. (1998) found that der p 1 and der p

9 both increase the release of GM-CSF, IL-8, and IL-6, suggesting that proteolytic activity in allergens may stimulate the release of proinflammatory cytokines from bronchial epithelium in humans. Because lung epithelial cells and endothelial cells have some of the same receptors (TLR 2, TLR 4, G-Protein coupled receptors), we expect they follow similar pathways in inflammation (Jiang et al. 2005).

### **E. Background on biology and ecology of storage mites**

Many species of storage mites infest stored foods such as grain, flour, cereals, dried fruits and vegetables, pet foods, cheese, dried milk, sugar, paper, tobacco, molds, and animal nests (Oconner 1982). They feed on protein rich substances. *Acarus siro*, *Tyrophagus putrescentiae*, *Chortoglyphus arcuatus* and *Lepidoglyphus destructor* are common storage mites that induce allergic reactions in some people. In nature, storage mites are predominantly found in agricultural environments, but increasing numbers of these mites are contributing to the allergen content of house dust in human dwellings and in food made from stored grains. The existence of the storage mite in house dust is connected with damp housing conditions where mold would serve as their main food source for some species (Olsson and van Hage-Hamsten 2000). These mites can survive temperatures near freezing and may become more prevalent in storage facilities during colder months (van Hage-Hamsten and Johansson 1992). Stored product mites commonly occur in dense populations. Under heavy infestation, a coating of brownish "mite dust" may appear on open shelving, around the base of flour

sacks, and on the surface of cheese or in other foods. Such piles consist of dead and living mites, cast skins and feces.

The bodies of storage mites are almost colorless, but the mouthparts and legs are pale yellow to reddish-brown. The adults are 300-400  $\mu\text{m}$  long. The females are slightly larger than the males. The development of most species progresses through the egg, larva, protonymph, tritonymph and adult life stages. Some species have a non-feeding deutonymphal stage (hypopi) between the protonymph and tritonymph (van Hage-Hamsten and Johansson 1992).

Under favorable conditions of relative humidity and temperature, the life cycle of grain mites may be completed in about 14-30 days (Cunnington 1985). Under optimum conditions a female mite may lay 2-5 eggs in a day (Cunnington 1985). Duration of the larval and nymphal stage may be 19 to 20 days with a complete life cycle of two to five weeks (Cunnington 1985). Adult females live for about 45 days under ideal conditions (Cunnington 1985). Stored product mites develop best when humidity levels are in the range of 75 to 85 percent and temperatures range from 20-25°C (Arlian and Platts-Mills 2001).

The second nymphal form (deutonymph) in some species may be replaced by a special stage known as the hypopus. This stage is highly resistant to unfavorable conditions, and may exist for several months without feeding. The hypopus can move from place to place by clinging to small animal forms such as insects or mice. Air currents can also disperse hypopi. When the hypopus encounters favorable conditions, it sheds its skin and resumes normal growth and development. The peculiar adaptation through the hypopus stage makes it

very difficult to eradicate this mite in stored grains and grain products (Cunnington 1965).

## **F. Allergies to Stored Product Mites**

Stored product mite bodies and feces are the source of molecules such as carbohydrates, lipids, and proteins that may penetrate the epidermis and dermis, and cause inflammatory and immune responses in humans. Sensitization and allergic reactions to the storage mites *Acarus siro*, *Tyrophagus putrescentiae*, *Chortoglyphus arcuatus* and *Lepidoglyphus destructor* are well documented (Yadav et al. 2006, Kondreddi et al. 2006, Bravo et al. 1999, Vidal et al. 2004). Allergens from these mites can induce asthma, allergic rhinitis and possibly atopic dermatitis (Arlian and Platts-Mills 2001, Arlian et al. 2007).

The mechanism for sensitization and allergic reaction to mite allergens is similar to that for other allergens such as pollen, food, and insects. Sensitization is the first reaction where IgE antibody is made to the allergen in a predisposed person. B cells physically present the antigen to helper T cells. This interaction causes T cells to release cytokines that induce B cells to proliferate and differentiate into plasma cells that secrete IgE antibody. IgE binds strongly to mast cell's FC receptor on its surface. Thus, the mast cells now have antigen specific receptors. Subsequent exposure to the mite allergens may cause an allergic reaction. Allergic reaction occurs when an allergen binds to antigen-specific IgE (already bound to a mast cell). In this case, mite allergen binds to the IgE on mast cells. Cross-linking by allergen and membrane IgE can induce or trigger an intense reaction. The interaction between antigen and antigen-



specific IgE causes the degranulation of the mast cells and they release potent inflammatory mediators. The mediators cause the symptoms associated with an allergic reaction. Histamine is one of the chemical mediators released from mast cells during degranulation. It can bind with receptors on endothelial cells as well as other cells, and results in increased vascular permeability and inflammation (Schindler 1990).

Since the storage mites contaminate nonoccupational environments (homes) as well as occupational ones (farms) and food products throughout the world, it is not surprising that many people in urban and agricultural populations are sensitized to allergenic proteins from these mites. Bakers and farmers are occupationally exposed to storage mites and they can become sensitized to them (Olsson and Van Hage-Hamsten 2000). People ingest storage mites and/or their allergens when they consume baked products such as pancakes, donuts, and cakes.

Some storage mite allergens are shown to be enzymes. The enzyme activity may have a specific function in the sensitization process as well as the pathology of the mite allergy. Allergens may have enzymatic activity on the skin cell barrier (Olsson and Van Hage-hamstan 2000). It has been proposed that the breakdown of mite allergen proteins increases allergic reaction by breaching the lung epithelial barrier. The allergens can cross the lung epithelium, because the enzyme activity disrupts the tight junctions (Carnes et al. 2008).

Allergic reactions can occur systematically or locally. If the mite allergens are inhaled and contact mucous membranes of the nose and eyes, they can

induce rhinitis (hayfever). Contact with mite infested foods, grains and hay may produce skin atopic dermatitis (Olsson and Van Hage-Hamsten 2000). An example of this is baker's itch that occurs among bakery workers (Revsbech and Dueholm 1990). Stored product mite allergens can induce an anaphylactic reaction (Guerra Bernd et al. 2001). Anaphylaxis refers to a rapidly developing and serious allergic reaction that affects a number of different areas of the body at one time, and can be fatal. Anaphylaxis is often triggered by substances that are injected or ingested and thereby gain access into the blood stream. An explosive reaction involving the skin, lungs, nose, throat, and gastrointestinal tract can result (El-Shanawany 2008). Symptoms reported are caused by a combination of direct skin contact with mites and inhalation of mite fragments or their excretory pellets among other allergens. These mite problems can have severe effects on the health and safety of employees in grain handling industries (Halliday 2003).

Many cases of mite induced anaphylaxis are reported in the literature (Dutau 2002, Edston and van Hage-Hamsten 2002, Antunes et al. 1999, Matsumoto et al. 2001). In one case, the ingestion of corn flour contaminated with mites induced anaphylaxis. An 18-year-old woman developed nausea, abdominal cramps, cough, and dyspnea within minutes of eating cooked corn flour (Guerra Bernd et al. 2001). Later, allergenic sensitization was assessed by skin prick tests with foods and inhaled allergens. The patient showed strong reactions to mite allergens. The sample of corn flour contained *T. putrescentiae* as well as other mites.

Many studies document that sensitization to stored product mites is a worldwide problem. Studies of serum samples from random patients in southwest Ohio of the United States found IgE to allergens from *A. siro*, *L. destructor*, and *T. putrescentiae*. Thirty-two (5.3%) of the 600 serum samples had IgE to allergens from at least one mite *A. siro* or *L. destructor*. Twelve serum samples had IgE against proteins in the extract of *A. siro*, and 18 serum samples had IgE against proteins in the extracts of *L. destructor* (Yadav et al. 2006). Serum samples from these 600 people were also analyzed for IgE to allergens from *T. putrescentiae* by electrophoresis and slot-blots (Kondreddi et al. 2006). Twenty-one percent of the 600 serum samples screened had IgE against proteins in the extracts of *T. putrescentiae*, and 11.7% had IgE to the allergens from at least one of the three mite species (Kondreddi et al. 2006).

In a different study, Bravo et al. (1999) determined the sensitivity to *A. siro*, *T. putrescentiae*, *C. arcuatus* and *L. destructor* in 133 patients that were skin test positive for *Dermatophagoides* house-dust mites. This study took place in Vigo, Spain. Ninety-eight of 133 (74%) of patients allergic to *Dermatophagoides* had positive specific IgE against at least one storage mite. IgE to *L. destructor* was detected in 68% of the patients allergic to *Dermatophagoides*. Sixty-five, fifty-seven, and forty-eight percent of patients in this study that were allergic to *Dermatophagoides*, showed IgE to proteins in extracts of *T. putrescentiae*, *C. arcuatus* and *A. siro*, respectively.

Vidal et al. (2004) performed a study in Gulicia, Spain to evaluate prevalence and factors associated with sensitization of humans to storage mites.

Because of the warm and humid climate, mites are a problem, occupationally and nonoccupationally in homes, among humans living in Galicia, Spain. Skin prick tests to the storage mites *T. putrescentiae* and *L. destructor* along with pollens, molds and animal danders were performed. Twenty-eight percent of subjects had a positive skin prick test to at least one allergen tested, which means they had made IgE to that particular allergen. Positive skin prick tests revealed that in the sensitized population, 24.4% of cases were sensitized to *T. putrescentiae* and *L. destructor*, and these mites were the leading causes of allergic sensitization. Serum from those people contained IgE against proteins in the extracts of *T. putrescentiae* or *L. destructor* or both. Therefore, storage mites *T. putrescentiae* and *L. destructor* are major aeroallergens and sensitization to them is prevalent in Galicia, Spain. Surveys from the study also indicated that storage mite sensitization was not associated with sex, smoking, education level, farming profession, rural environment, presence of pets or with indoor humidity, however, it was inversely associated with age. This is interesting considering those factors could favor growth or exposure to storage mites, but since they are so widespread in the studied area, most of the population can be exposed to the mites throughout their lives (Vidal et al. 2004).

Arlian et al. (1997) studied the allergenicity of *T. putrescentiae* in farmers from Gotland (an island in the Baltic Sea). People who work with grain and hay are occupationally exposed to storage mites and sensitization to them may occur. To characterize the allergens of *T. putrescentiae*, twenty-four serum samples from occupationally exposed farmers were analyzed using crossed

immunoelectrophoresis and crossed radio immunoelectrophoresis. Five to eleven of fourteen allergens from *T. putrescentiae* bound IgE in the serum of these farmers. Farmers who were occupationally exposed to storage mites had serum IgE specific for many potent allergens from *T. putrescentiae*. Farmers are at a higher risk for sensitization and allergic reaction since they are exposed to storage mites in an occupational setting.

Puerta et al. (1992) studied the prevalence of specific IgE to the storage mites *Aleuroglyphus ovatus* and *C. arcuatus* in 77 individuals with allergic asthma in Cartagena, Colombia. All these individuals had a positive skin test to extracts of *Dermatophagoides pteronyssinis* and/or *Dermatophagoides farinae*. Seventy-six percent of the sera tested had a positive RAST to *D. pteronyssinis* and at least one of these storage mites. Among these sera, 75.8% were RAST positive to *C. arcuatus*.

Valdivieso et al. (2006) identified the mite species in house dust at three different altitudes in Ecuador. Twenty-one mite species were identified. Large populations of mites were detected above 2,500 m of altitude where humidity levels remain high all year round. All the dust samples from the homes (n=67) contained detectable levels of Der p 1 or Der f 1. Positive skin prick test reactions to *L. destructor*, *T. putrescentiae*, and *A. siro* were obtained in 19.3, 10.6 and 8.8 percent of the patients (n=435), respectively.

### **G. Allergen Nomenclature, Classification and Characteristics**

Mite allergens are divided into specific groups (1-20) based on when they were characterized and their biochemical composition, sequence homology and

molecular weight (Stewart and Robinson 2003). Purified allergens are named using the systematic nomenclature of the Allergen –Nomenclature Subcommittee of the World Health Organization and International Union of Immunological Societies (Chapman et al. 2007). Allergens from plants and animals are named using the first three letters of the genus, followed by a single letter for the species and a number indicating the chronologic order of allergen purification (Chapman et al. 2007). The nomenclature is not italicized and it has a space after each of the first two elements. The nomenclature covers different isoallergens, which are multiple molecular forms of the same allergen that share extensive IgE cross-reactivity (Chapman et al. 2007).

Many different proteins can induce IgE antibody in patients allergic to mites. These proteins are divided into groups. Major IgE binding has been reported for the group 1, 2, 3, 9, 11, 14 and 15 allergens (Thomas et al. 2007). The group 4, 5, 7 and 8 allergens have shown intermediate IgE binding and the group 10 tropomyosins are of interest because of their potential cross-reactivity with allergen from disparate species (Thomas et al. 2002).

Group 1 allergens originate from cells lining the intestinal tract of the mite. They are glycoproteins with cysteine protease activity (Thomas et al. 2007). They enhance allergenicity by cleaving specific receptors of immune cells (Shpacovitch et al. 2007). For example, Der p1 can cleave the CD23 IgE receptor on the membrane of human B cells. Group 2 allergens have an ML domain for lipid binding proteins (Thomas et al. 2007). They are 14-kd nonglycosylated proteins that have high sequence homology (Arlan and Platts-

Mills 2006). Group 3 proteins are trypsins, and are major constituents of mite feces and are in low concentrations in body extracts (Thomas et al. 2007). Group 4 proteins are amylases, and have high sequence conservation in cross reactivity tests due to the amylase specific sequences. Group 9 is another major IgE binding protein in allergens. Group 9 proteins are serine proteases with collagenolytic activity (Thomas et al. 2007). Group 11 proteins are paramyosin. Groups 13 consists of fatty acid binding proteins and are found abundantly in mite extracts. Group 14 are members of the large lipid transfer protein family. Group 15 chitinase allergens are major allergens in house dust mite allergic dogs (Thomas et al. 2007). They have not been studied extensively in humans except for Weber et al. 2003. Der f 18 bound IgE from the sera of HDM allergic patients at a lower frequency (54%) than the groups 1 and 2 allergens. So far, there has been no report on Der f 15-specific IgE binding for humans but estimating from its higher frequency of IgE binding for dogs, it could be even more allergenic for humans than Der f 18 (Weber et al. 2003).

## **H. Characterized Allergens from Stored Product Mites**

### **1. *L. destructor***

An epidemiological study was performed to investigate the IgG1, IgG4, and IgE response to *L. destructor* in Swedish farmers. Olsson and Van Hage-Hamstan (2000) found that Lep d 2 (a 15kDa protein) is the major allergen responsible and that it has a widespread distribution in the mite body and is present in the fecal pellets. A 25 kDa component, most likely Der p 1, was also present in *L. destructor*. Eriksson et al. (2001) identified 20 IgE binding

components in the whole extract of *L. destructor*. The major allergen, Lep d 2 was cloned as well as three new allergenic components: Lep d 5, 7 and 13. Lep d 5 was a 13kDa component of the whole extract. Lep d 13 was a 15kDa component. The size of Lep d 7 cannot be determined, because it is present in different sizes. Lep d 10 has been described as well (Eriksson et al. 2001).

## **2. *C. arcuatus***

Several proteins with catalytic properties have been identified in whole body extracts and fecal matter from *C. arcuatus* including mite allergen groups 1, 3, 4, 6, 8, 9, and 15. The influence of the storage mite *C. arcuatus* on the innate immune system has been studied. The break down of protein in mite feces interacts with pulmonary elastase and inactivates a human elastase inhibitor *in vivo* (Carnes et al. 2008). Because these elastase inhibitors have antimicrobial activity, the inactivation of these innate components of the lung defense system by proteolytic enzymes present in *C. arcuatus* feces may increase the susceptibility of patients with allergic inflammation to infection and this produces a possible exacerbation of allergic respiratory diseases. Chronic exposure to *C. arcuatus* is an important risk factor for the development of sensitization, allergic respiratory diseases and chronic lung inflammation (Carnes et al. 2008).

## **3. *T. putrescentiae***

Arlian et al. (1984) analyzed allergenic and antigenic properties of *T. putrescentiae*. The mite with culture medium including feces extracts contained 18 antigens. The feces contained 13 of these antigens and the antigen components were much stronger. Five antigens were derived from TP materials



(bodies and secretions). All heterologous crosses indicated a close antigenic relationship between feces and whole body TP extracts despite some antigens being specific to feces or mite bodies (Arlian et al. 1984).

Allergenic components in extracts of *T. putrescentiae* have been identified by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) and immunoblotting. *T. putrescentiae* contains at least 20 allergenic components (Johansson et al. 1994). A 16 kDa allergen was found to be the most important to *T. putrescentiae*-allergic patients, with the sera of 52% containing IgE antibodies to this allergen (Johansson et al. 1994). Considerable cross-reactivity between *T. putrescentiae* and *Dermatophagoides* species was demonstrated by ELISA. Jeong et al. (2005) found fifteen allergenic components in *T. putrescentiae*. The researchers cloned and characterized the allergen, Tyr p 2. Of the fifteen allergens identified, a 16-kD allergen was most prevalent and its IgE binding was completely inhibited by Der f 2 (Jeong et al. 2005).

#### **4. *A. siro***

*Acarus siro* is an inducer of occupational allergy among farmers, but sensitisation has also been found in non-farming populations (Eriksson et al. 1999). Fifteen and 17-kD allergens were identified in a fraction of *A. siro* extract. The cDNA of the 15-kD allergen was isolated, cloned and sequenced and the allergen was expressed as a recombinant protein. The allergen was named Aca s 13 (Eriksson et al. 1999).

## Hypothesis and Specific Aims

The prevalence of allergenic diseases is increasing worldwide and mites are a common cause of allergy. Mites have been an important cause of allergies in occupational settings such as green houses, food processing, farming, grain and hay storage and transfer facilities, fruit orchards, and in human dwellings (Arlan 2000, Jeebhay et al. 2007). One specific example of an allergenic disease in humans is atopic dermatitis, which storage mites may be a contributing factor for some people (Ado et al. 1992). “Atopic dermatitis is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers” including stored product mites (Cork et al. 2006). One cause of atopic dermatitis is the production of increased levels of specific IgE to common allergens. Mite or other mite molecules may contribute to atopic dermatitis and pathology of the skin by stimulating cells such as keratinocytes, fibroblasts, or vascular endothelial cells. Clinical symptoms of atopic dermatitis include erythema, edema, and leukocyte recruitment.

How storage mites cause inflammation in the skin needs further study. Understanding this mechanism may lead to proper treatment of patients with mite allergies, atopic dermatitis, and other skin diseases. If we can see that a mite modulates the function of keratinocytes, fibroblasts or vascular endothelial cells, it could help us determine how the mite influences the immune response and inflammatory responses in the skin of patients experiencing allergic reactions to these mites. The purpose of the study was to show how storage mites can influence the functioning of human dermal microvascular endothelial cells in

modulating the inflammatory and immune response in the skin. We tested the hypothesis that components of stored product mite extracts influence the functioning of normal human dermal microvascular endothelial cells.

We addressed the following questions:

1. Do molecules from stored product mites induce cell adhesion molecule expression or cytokine secretion from endothelial cells?

I addressed this question by growing normal human dermal endothelial cells, challenging them with four different storage mite extracts, and then determining their cytokine secretion and cell adhesion molecule expression by ELISA. Dose-response and time-response experiments were done to profile dose and time responses by the cells.

2. What mechanism are the molecules from stored product mites using to influence the function of endothelial cells?

After looking at the initial cytokine secretion and cell adhesion molecule expression, experiments were carried out to look at potential mechanisms used by the mite molecules. Toll-like receptors and protease activated receptors were blocked in two different experiments.

## Materials and Methods

### I. Summary of Approach

This research investigated previously unexplored secreted cytokines and expressed cell adhesion molecules from normal human microvascular dermal endothelial cells that were stimulated with spent culture extracts of the stored product mites, *T. putrescentiae*, *C. arcuatus*, *A. siro*, and *L. destructor*.

#### A. Influence of Mite Extract on Constitutive Secretion and Expression

Normal human microvascular endothelial cells in endothelial cell media were grown to 70% confluency in a T75 flask and plated on 96-well tissue culture plates (Arlian et al. 2007). Then, cells were challenged with four different concentrations (0, 25, 50, 100 µg/ml) of four stored product mite extracts made from spent culture material. Cells were analyzed for expression of cell adhesion molecules and cell supernatants for cytokines secreted by the cells after 6 hours, 12 hours and 24 hours of stimulation with the mite extracts by ELISA.

#### B. Influence of Mite Extract on Secretion and Expression Induced by TNF- $\alpha$

TNF- $\alpha$  is a proinflammatory cytokine that can stimulate endothelial cells (Elder et al. 2009). We investigated if stored product mites modulated (up or down) the human cytokine secretion and adhesion molecule expression by endothelial cells that were stimulated by TNF- $\alpha$ .

#### C. Influence of Mite Extract on Secretion and Expression Induced by LPS

LPS (endotoxin) can stimulate cells by acting through TLRs (Henneke et al. 2002). Mite extracts can contain varied amounts of LPS. We investigated the role of TLRs on endothelial cells from stimulation with the different mite extracts.

Polymyxin B was used to block LPS in the mite extracts. LPS can alter the normal function of endothelial cells using TLR 2 and TLR 4. If we blocked LPS in the mite extracts using Polymyxin B, and the extracts still stimulated the cells, then we could assume that LPS was not responsible for the inflammatory response seen on the endothelial cells stimulated by the mite extracts.

#### **D. Influence of Mite Extract on Secretion and Expression Induced by Protease Inhibitors**

Endothelial cells have protease-activated receptors (PARs) on their outer membrane (Reed and Kita 2004). Mite extracts contain protease enzymes. If using protease inhibitors in the mite extracts did not change the response, we can conclude that the mite extracts were not working through PARs.

## **II. Detailed Methods**

### **A. Stored-Product Mite Extracts**

Thriving cultures of *T. putrescentiae*, *C. arcuatus*, *A. siro*, and *L. destructor* are maintained in Dr. Arlian's lab at Wright State. Mites were cultured at 75% relative humidity and room temperature and fed about every three weeks. Mite extracts were prepared from spent cultures. Spent cultures contain all life stages of live and dead mites as well as feces, eggs, exoskeletons, and minimal food. Cultures in which mites have depleted the food supply are called "spent cultures".

First mites from spent cultures were washed using 45 um sieves. To do this, approximately 3.5 g of mite culture was added to 50 ml centrifuge tubes filled with saturated NaCl solution. Tubes were vortexed and then centrifuged for

15 minutes at 2400 rpm. Three layers were separated, and the top two layers (mites and wash) were poured into the sieve and washed with 180 ml of DI water. Washed mites were put into a 50 ml centrifuge tube and weighed. Endotoxin free water was added (1:3 extraction) to each tube, vortexed and stored overnight at 4° C. A 1:3 extraction means that we use one part mite material and three parts water in the extract. The next day, mites were ground in a Tenbroek homogenizer on ice and centrifuged to remove insoluble material. The aqueous extract was filtered through a 0.22 µm filter using a syringe into a sterile vial for storage at 4° C. Total protein content in the mite extracts was determined through the Bradford protein assay (Bradford, 1976) using Bovine Serum Albumin (BSA) as a comparative standard.

## **B. Normal Human Microvascular Endothelial Cells**

Cryopreserved adult human microvascular endothelial cells (HMVEC-D), all media, and growth factors were purchased from Cambrex Bio Science Walkersville (Walkersville, MD). Cells were stored in vials at -80° C, media was stored at 5° C and growth factors were stored at freezing temperature. To bring cells out of the freezer, a 37° C water bath was prepared, and the vial of frozen cells was rapidly thawed. The vial was decontaminated using 70% ethanol, and cells along with their frozen media were pipetted into a T75 cell culture flask containing 15 ml of endothelial cell basal medium-2 (EBM-2). All cells were cultured in Clonetics endothelial cell basal medium-2 supplemented with EGM-2-MV growth factors in a T75 cell culture flask at 37° C in 5-7% CO<sub>2</sub>. Prior to being used in cytokine detection assays, cells were placed into wells of a 96-well plate

with 200  $\mu$ l of media. Each well contained about 5000 cells and cells were allowed to grow for 2-3 days to 75% confluency in Costar 96-well culture plates (Corning Glassworks, Corning, NY) for subsequent cell enzyme-linked immunosorbent assay (ELISA) and cytokine secretion studies. Confluency was determined by looking at each well under the light microscope and estimating visually the area covered. All studies were performed on cells between passage 6 and 12.

### **C. HMVEC-D Challenged with Stored Product Mite Extract**

Data obtained from my own preliminary experiments and Arlian et al. (2007) helped determine the optimum dosage and time course by which to challenge HMVEC-D with stored product mites. Cytokine secretion from these cells was measured from the cell culture supernatant via ELISA.

Briefly, HMVEC-D were plated onto 96-well plates at a cell density of about 5,000 cells/well. This number was calculated by knowing how many cells were needed and how many cells were counted. Cells were allowed to grow to proper confluency (70%) in EBM-2. In preparation for challenging the HMVEC-D, each of the stored product mites was prepared at concentrations of 200, 100, and 50  $\mu$ g protein/ml in EBM-2 with and without TNF- $\alpha$  prior to the start of the experiment. To challenge the cells with different concentrations of mite extracts, one hundred microliters of medium was aspirated from all wells of 96-well plate and discarded in bleach. One hundred microliters of desired stimulants: either 0, 50, 100 or 200  $\mu$ g/ml of *T. putrescentiae*, *C. arcuatus*, *A. siro*, and *L. destructor* were added to each well as indicated on ELISA sheets to give the final

concentration of 0, 25, 50, or 100 µg/ml. Each stimulant was added to three separate wells to give replicates for data analysis. The plated cells were incubated for 6, 12, or 24 hours at 37° C and 6% CO<sub>2</sub>. Then, 180 µl of supernatant was aspirated from each well and put into bullet tubes (96 bullet tubes needed for each time period) for cytokine assays. Cell adhesion ELISA was performed on cells remaining in the wells to determine expression of adhesion molecules.

#### **D. Cell-ELISA Procedure**

The expression of cellular adhesion molecules was determined using a cellular-ELISA procedure previously described by Elder et al. (2006). The cell adhesion molecules that were monitored included: VCAM-1, ICAM-1 and E-selectin. HMVEC-D cells (3,000-6,000 cells/well) in 96-well plates were incubated in 200 µl of EGM-2 medium at 37°C in 5-7% CO<sub>2</sub> for 48-72 hours until a 70% confluent monolayer had been reached. At the beginning of the experiment, 100 µl of medium was replaced with 100 µl of fresh medium without or with storage product mite extract at specific concentrations and with or without TNF-α at 4ng/ml (final concentration). Cells were reincubated for a specific period of time up to 24 hours. At the conclusion of the incubation period, the cell supernatants were removed, collected into microcentrifuge tubes and stored at -80°C. These supernatants were later assayed for cytokine production.

The expression of cellular adhesion molecules was determined using a cellular ELISA procedure modified from Yokote et al. (1993) and described previously by Elder et al. (2006) and Arlian et al. (2009). To assay for the



presence of cell surface markers, cells were washed with Delbecco's phosphate buffered saline with 0.05% Tween 20 (PBST), and wells were briefly blocked for 30 minutes at 37°C in 5-7% CO<sub>2</sub> with 300 µl of endothelial cell basal medium containing 2% bovine serum albumin (EBM/BSA). EBM/BSA was removed, and 50 µl of desired concentration of primary antibody (diluted in EBM/BSA) was added to each well. Wells were incubated for minutes at 37°C in 5-7% CO<sub>2</sub> and then washed twice with PBST. Binding of biotinylated antibodies was detected by incubating the cells for 30 minutes at 37°C in 5-7% CO<sub>2</sub> in 50 µl of 1:2000 dilution (in EBM/BSA) of HRP-SA. After the final wash, fifty microliters of TMB ELISA substrate (Sigma) was added to each well, and the plate was incubated for up to 15 minutes before color development was stopped with the addition of 1 M sulfuric acid. Absorbance was measured at 450 nm using a Bio-Tek EL800X micropate reader (Bio-Tek Instruments, Winooski, VT).

#### **E. Cytokine-ELISA Procedure**

Culture supernatants collected at the conclusion of the stimulation of endothelial cells with and without TNF- $\alpha$  and different storage mite extracts were assayed for the presence of the selected cytokines. Secretion of the cytokines IL-6, IL-8, MCP-1, VEG-F, G-CSF, and GM-CSF was analyzed. DuoSet ELISA kits for detection of interleukin-6 (IL-6), interleukin-8 (IL-8), human monocyte chemoattractant protein-1 (MCP-1), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), and vascular endothelial cell growth factor (VEG-F) were purchased from R and D Systems (Minneapolis, MN). All assays were performed according to

manufacturer's instructions, and previous studies in this lab (Elder et al. 2006, Arlian et al. 2007 and 2009). First each ELISA plate coated with capture antibodies in PBS (100  $\mu$ l/well) is stored overnight at 4° C. The next day, plates were washed three times using PBST and blotted before the block solution (1% BSA in PBS) was added (300  $\mu$ l/well). An hour later, plates were washed with PBST and test solutions were added (100  $\mu$ l/well) to the plates for 2 hours. Then, plates were washed in PBST, and detecting antibodies in B-PBS were added (100  $\mu$ l/well). Two hours later, plates were washed with PBST and 100ul/well of streptavidin-HRP antibody in BPBS was added. After 20 minutes, plates were washed with PBST and eighty microliters of TMB ELISA substrate (Sigma) was added to each well, and the plate was incubated for up to 15 minutes before color development was stopped with the addition of 1 M sulfuric acid (40  $\mu$ l/well). TMB absorbance was read using the Bio-Tek EL800X microplate reader set to 450 nm.

#### **F. Block LPS from Active Participation via PmB**

Endothelial cells have toll like receptors 2 and 4 (TLR 2 and TLR4) on their surface. An endotoxin assay verified that the mite extracts contained LPS. LPS can alter the normal function of endothelial cells using TLR 2 and TLR 4. Polymyxin B binds to LPS so that it cannot activate TLRs (Fitzner et al. 2008). When we blocked LPS in the mite extracts using Polymyxin B, we assumed that LPS was not responsible for the response induced on the endothelial cells by the mite extracts. Two doses of LPS were used as a positive control.

HMVEC-D cells (3,000-6,000 cells/well) in 96-well plates were incubated in 200  $\mu$ l of EGM-2 medium at 37°C in 5-7% CO<sub>2</sub> for 48-72 hours until a 70% confluent monolayer had been reached. At the beginning of the experiment, 100  $\mu$ l of medium was replaced with 100  $\mu$ l of fresh medium without or with storage product mites at specific concentrations and with or without PmB at 30  $\mu$ g/ml (final concentration). Cells were reincubated for 6 hours. At the conclusion of the incubation period, the cell supernatants were removed, collected into microcentrifuge tubes and stored at -80°C. These supernatants were later assayed for cytokine production. The expression of cellular adhesion molecules was determined using a cellular-ELISA procedure as described previously.

#### **G. Block PARs from Active Participation via protease inhibitors E-64 and Aprotinin**

Endothelial cells have protease-activated receptors (PARs) (Reed and Kita 2004). Mite extracts contain protease enzymes. We blocked the protease enzyme activity in order to determine if other components in the extract stimulated the cells by non-PAR pathways. Protease inhibitors bind to the active site of proteases, so that the protease cannot bind to the PAR. By using protease inhibitors in the mite extracts and seeing no change, we can conclude that the molecules in mite extracts were not working through PARs.

HMVEC-D cells (3,000-6,000 cells/well) in 96-well plates were incubated in 200  $\mu$ l of EGM-2 medium at 37°C in 5-7% CO<sub>2</sub> for 48-72 hours until a 70% confluent monolayer had been reached. At the beginning of the experiment, 100  $\mu$ l of medium was replaced with 100  $\mu$ l of fresh medium without or with storage

product mites at specific concentrations and with or without Aprotinin or E-64 50  $\mu\text{g/ml}$  (final concentration). Cells were reincubated for 6 hours. At the conclusion of the incubation period, the cell supernatants were removed, collected into microcentrifuge tubes and stored at  $-80^{\circ}\text{C}$ . These supernatants were later assayed for cytokine production. The expression of cellular adhesion molecules was determined using a cellular-ELISA procedure.

#### **H. Statistical Analysis**

Data are reported as mean  $\pm$  SD of the cytokine levels in the cell-free culture supernatant. Sample size (n) in each group was (n=3) unless otherwise noted. A single factor ANOVA analysis was used to compare means between different groups. Differences between groups where  $p \leq 0.05$  were considered significant.

## Results

### I. Effects of Mite Extract on Constitutive and TNF- $\alpha$ induced Expression and Secretion

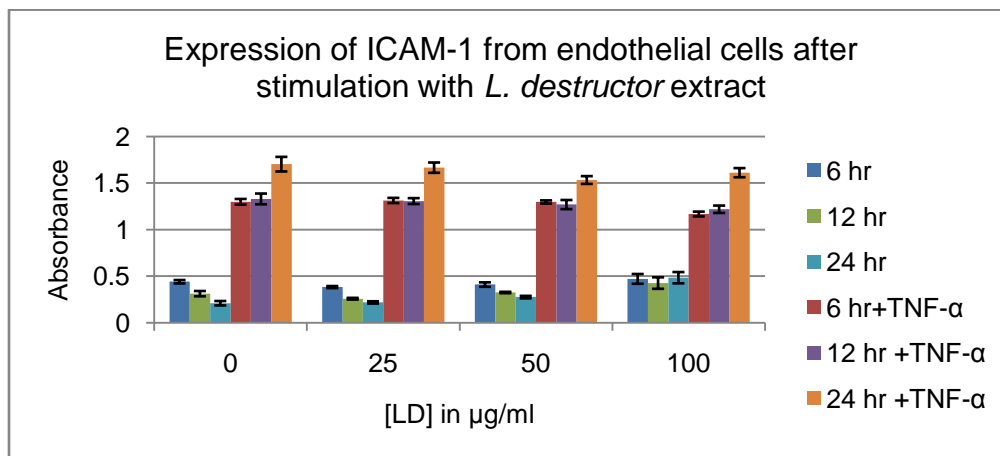
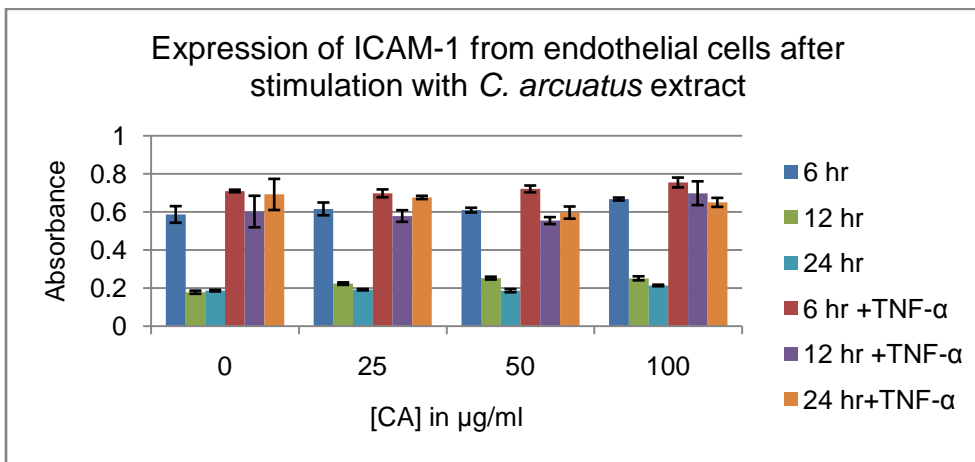
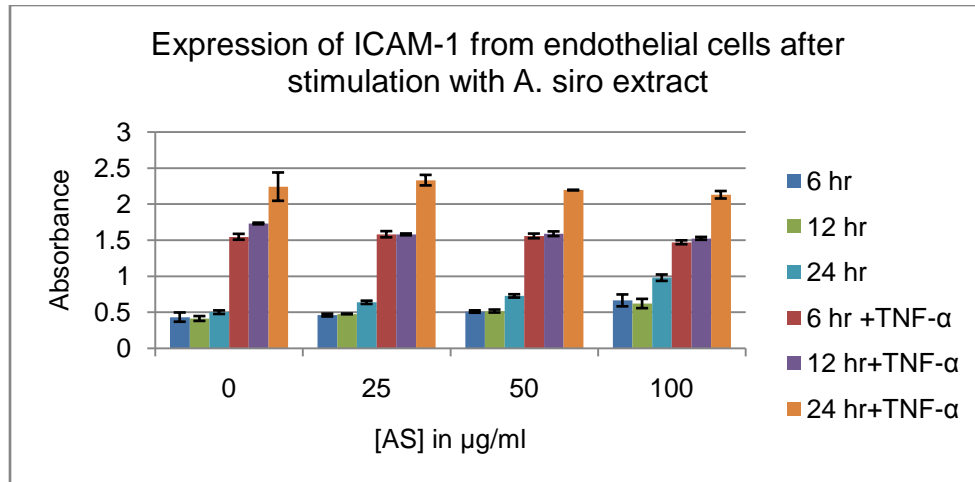
**A. Cell adhesion molecules.** HMVEC-D cells were stimulated with mite extracts from *Acarus siro*, *Chortoglyphus arcuatus*, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*, without or with TNF- $\alpha$  (4 ng/ml), and then assayed for expression of ICAM-1, VCAM-1, and E-selectin. There was constitutive expression for ICAM-1 (figure 1), but not for VCAM-1 or E-selectin (figures 2 and 3). TNF- $\alpha$  is a proinflammatory cytokine that increases cell adhesion molecule expression by endothelial cells (Elder et al. 2009). TNF- $\alpha$  (4 ng/ml) alone stimulated the expression of ICAM-1, VCAM-1, and E-selectin by HMVEC-D cells (figures 1-3). TNF- $\alpha$  was added to *A. siro*, *C. arcuatus*, *L. destructor* and *T. putrescentiae* extract in order to stimulate the cells to express ICAM-1, VCAM-1, and E-selectin so that we could determine if any down regulation was induced by the mite extracts. E-selectin is a cell adhesion molecule that appears quickly and does not last long. Therefore, only 6 hour data is shown (figure 3).

*A. siro* alone induced the dose dependent expression of ICAM-1 at 6, 12 and 24 hours above constitutive level (figure 1), but did not affect the expression of the constitutive levels of VCAM-1 (figure 2). The *A. siro* extract down-regulated the TNF- $\alpha$  induced expression of VCAM-1 at 12 and 24 hours in a dose dependent fashion (figure 2). *A. siro* did not affect the TNF- $\alpha$  induced expression of E-selectin (figure 3). *A. siro* alone did not induce expression of E-selectin (figure 3).

*C. arcuatus* did not affect the constitutive expression of the ICAM-1 (figure 1), VCAM-1 (figure 2), or E-selectin (figure 3). TNF- $\alpha$  induced expression of ICAM-1 (figure 1) and VCAM-1 (figure 2) were not affected by the *C. arcuatus* extract. Although the levels are low, the data showed that TNF- $\alpha$  induced expression of E-selectin was significantly ( $p < 0.05$  between all doses) down regulated by *C. arcuatus* at 6 hours (figure 3).

*L. destructor* did not affect the constitutive or TNF- $\alpha$  induced expression of ICAM-1 (figure 1), VCAM-1 (figure 2), or E-selectin (figure 3).

*T. putrescentiae* did not affect the expression of the constitutive levels of ICAM-1 (figure 1), VCAM-1 (figure 2), or E-selectin (figure 3), but TNF- $\alpha$  induced expression of ICAM-1 was significantly ( $p = 0.04$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$  doses) down regulated by *T. putrescentiae* at 24 hours for the 25, 50 and 100  $\mu\text{g/ml}$  doses of extract (figure 1). TNF- $\alpha$  induced expression of VCAM-1 (figure 2) or E-selectin (figure 3) was not affected by the *T. putrescentiae* extract.



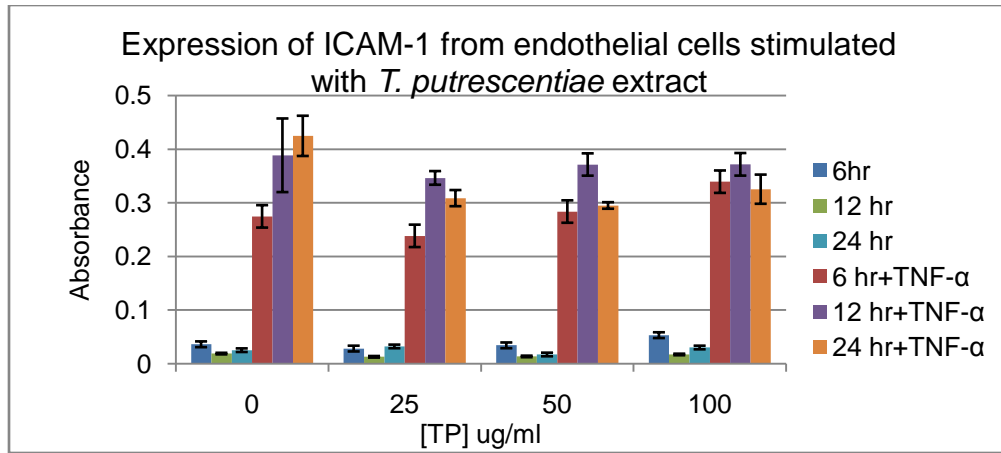
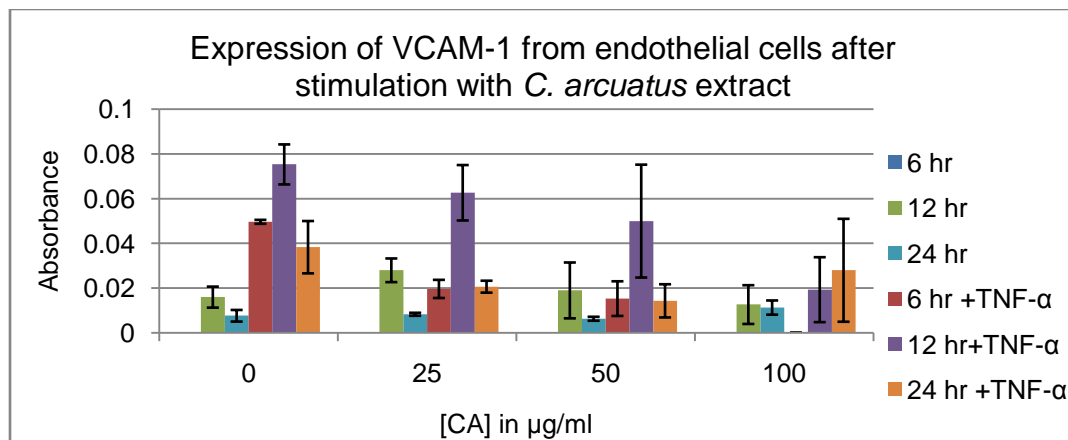
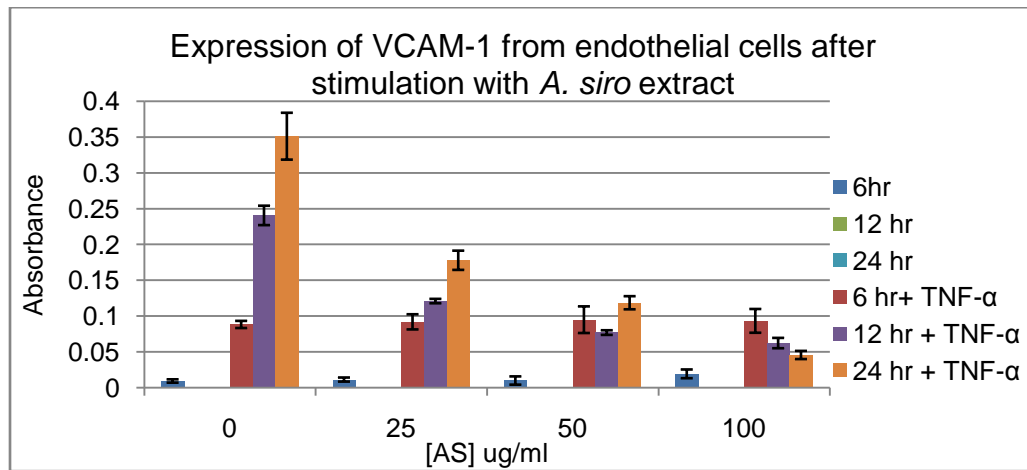


Figure 1. Expression of ICAM-1 from normal human microvascular endothelial cells stimulated with varying doses of *A. siro*, *C. arcuatus*, *L. destructor*, and *T. putrescentiae* extract with and without TNF- $\alpha$  for 6, 12, and 24 hours. Data are presented as means  $\pm$  SD of three replicate wells of cells.





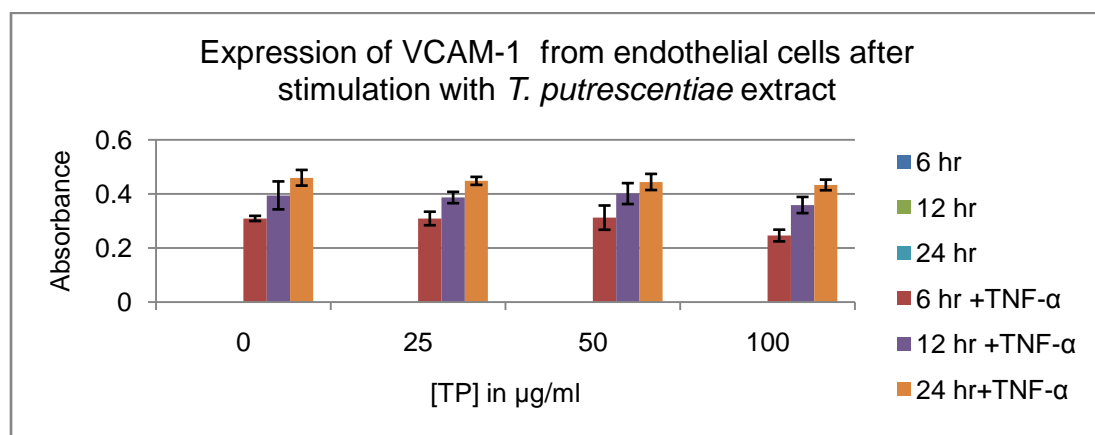
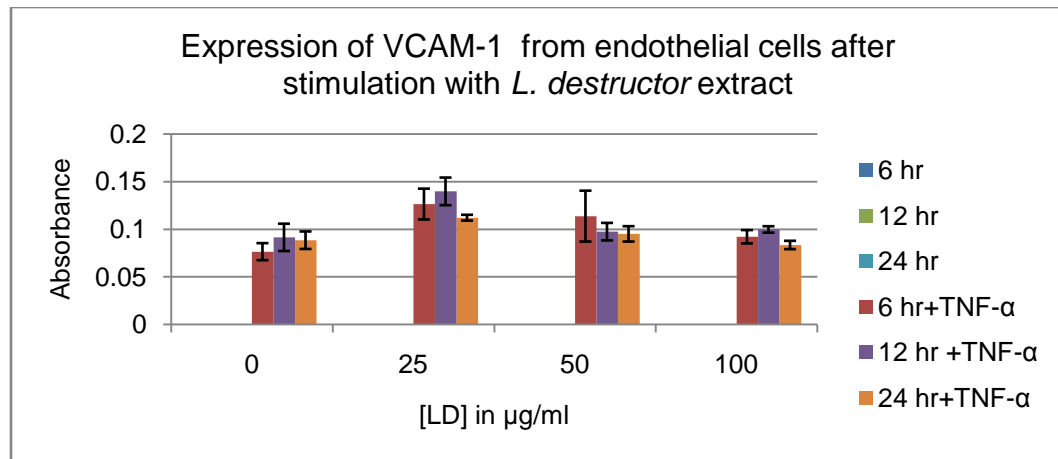
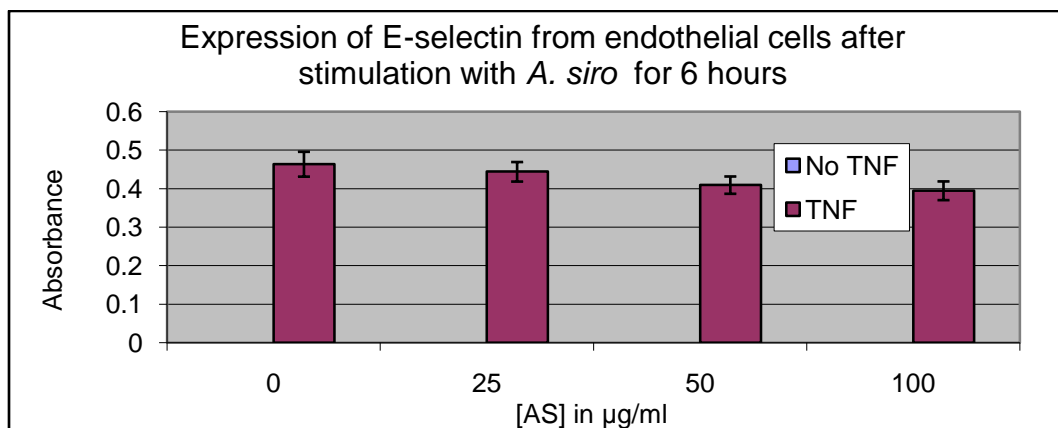


Figure 2. Expression of VCAM-1 from normal human microvascular endothelial cells stimulated with varying doses of *A. siro*, *C. arcuatus*, *L. destructor*, and *T. putrescentiae* extract with and without TNF- $\alpha$  for 6, 12, and 24 hours. Data are presented as means  $\pm$  SD of three replicate wells of cells.



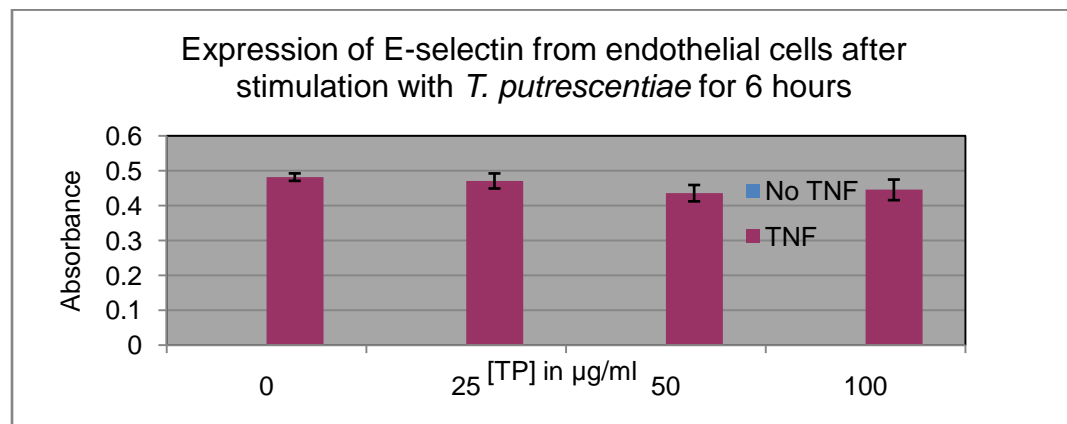
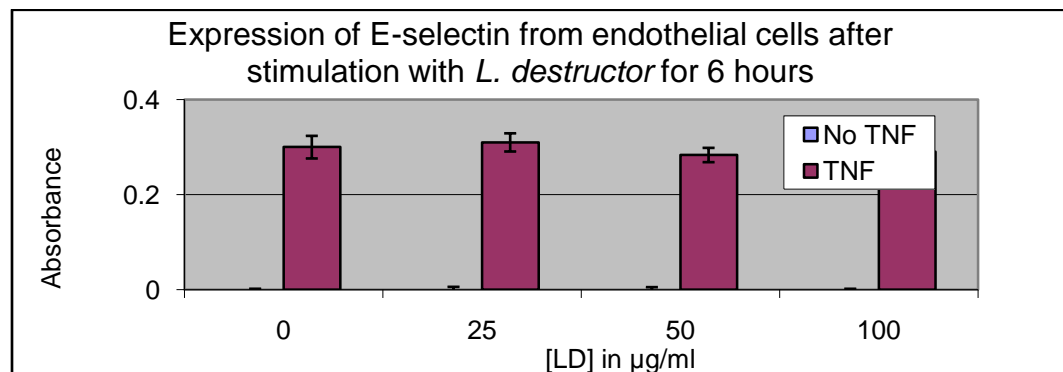
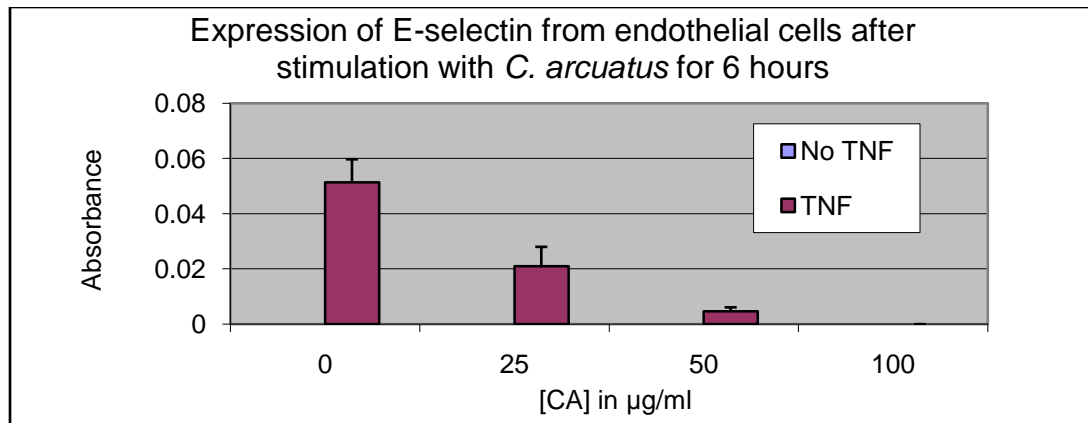


Figure 3. Expression of E-selectin from normal human microvascular endothelial cells stimulated with varying doses of *A. siro*, *C. arcuatus*, *L. destructor* and *T. putrescentiae* extract for 6 hours. Data are presented as means  $\pm$  SD of three replicate wells of cells.

**B. Cytokines.** Supernatants from cells incubated without and with extracts of storage product mites were assayed for VEGF, GM-CSF, IL-6, IL-8, G-CSF, and MCP-1. HMVEC-D constitutively secreted at least small amounts of all of the cytokines measured (figure 4-11). Cells were also incubated with TNF- $\alpha$  and assayed for the release of the same cytokines. TNF- $\alpha$  (4 ng/ml) alone stimulated the secretion of GM-CSF (figure 6), IL-6 (figure 7 and 8), IL-8 (figure 9), G-CSF (figure 10), and MCP-1 (figure 11) above constitutive levels, but not VEGF (figure 4-5).

*Acarus siro* and *Lepidoglyphus destructor* significantly ( $p=0.02$ ,  $p=0.04$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ ) down-regulated the constitutive secretion of VEGF at 6, 12, and 24 hours in the presence or absence of TNF- $\alpha$  (figure 4 and 5).

*Acarus siro* alone induced a significant ( $p=0.03$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ ) dose dependent secretion of GM-CSF and IL-6 ( $p=0.01$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ ) at 6, 12, and 24 hours above constitutive levels and enhanced the TNF- $\alpha$  induced secretion of GM-CSF and IL-6 (figure 6 and 7). *A. siro* had no effect on the secretion of other cytokines (data not shown).

*Chortoglyphus arcuatus* alone induced a significant secretion of IL-6 ( $p=0.05$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ ) and IL-8 ( $p=0.04$  between 0  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ ) in a dose dependent fashion and enhanced the TNF- $\alpha$  induced secretion of these cytokines (figure 8 and 9). *C. arcuatus* did not stimulate secretion of the other cytokines measured (data not shown).

*Tyrophagus putrescentiae* did not induce secretion of any of the cytokines that were measured. Data for G-CSF are shown in figure 10 as a representative example.

*A. siro*, *C. arcuatus*, *L. destructor* and *T. putrescentiae* extract did not affect the constitutive or TNF- $\alpha$  induced secretion of MCP-1 from endothelial cells. Data for MCP-1 secretion from endothelial cells after stimulation with *L. destructor* are shown in figure 11 as a representative example.

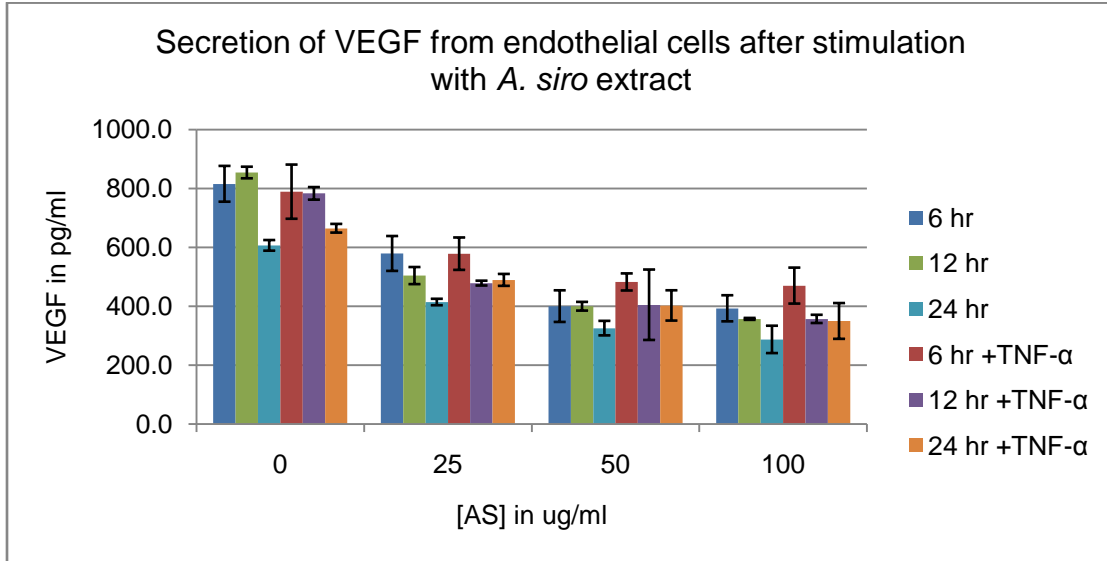


Figure 4. Dose dependent secretion of VEGF from normal human microvascular endothelial cells stimulated with varying doses of *A. siro* extract for 6, 12, and 24 hours. Data are presented as means  $\pm$  SD of three replicate wells of cells.

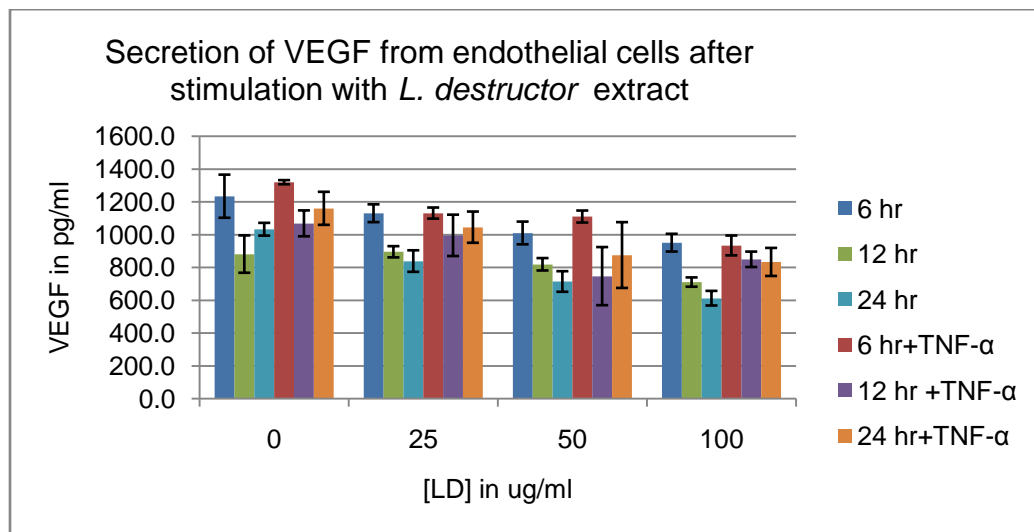


Figure 5. Secretion of VEGF from normal human microvascular endothelial cells stimulated with varying doses of *L. destructor* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

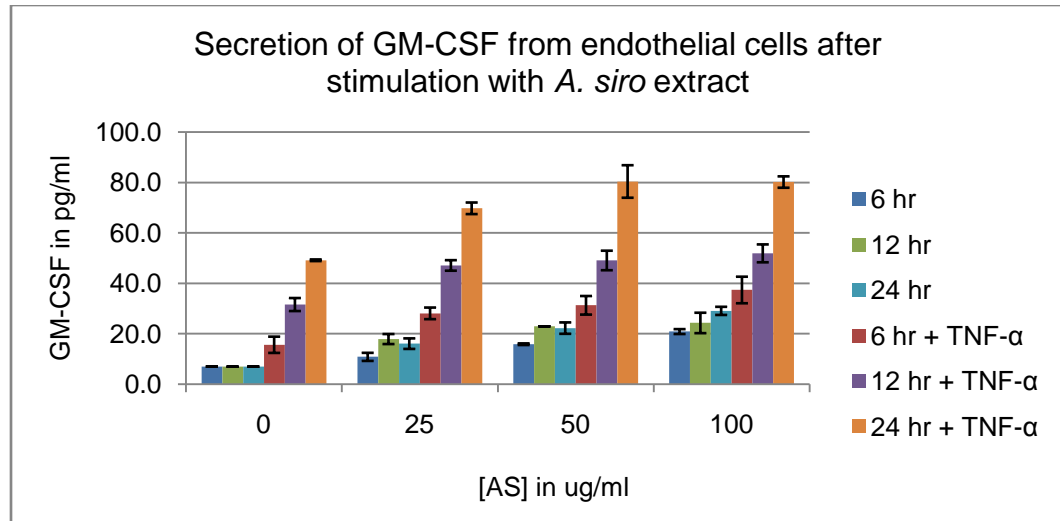


Figure 6. Dose dependent secretion of GM-CSF from normal human microvascular endothelial cells stimulated with varying doses of *A. siro* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

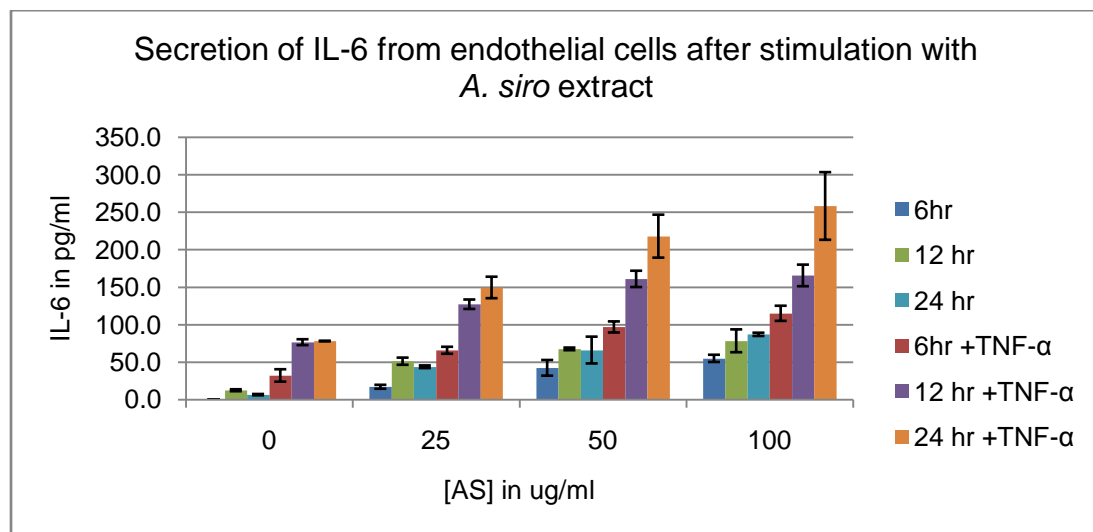


Figure 7. Dose dependent secretion of IL-6 from normal human microvascular endothelial cells stimulated with varying doses of *A. siro* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

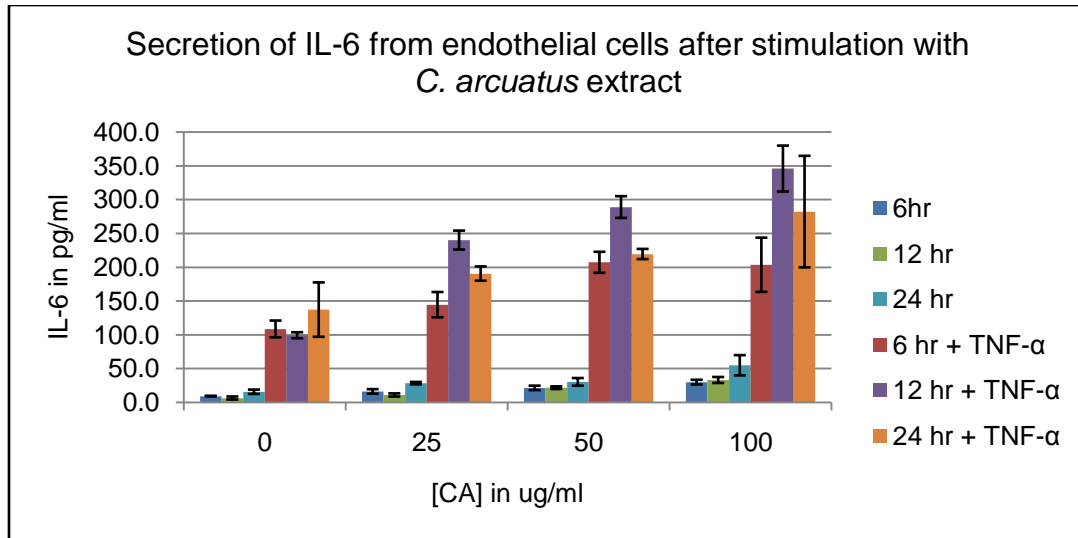


Figure 8. Dose dependent secretion of IL-6 from normal human microvascular endothelial cells stimulated with varying doses of *C. arcuatus* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

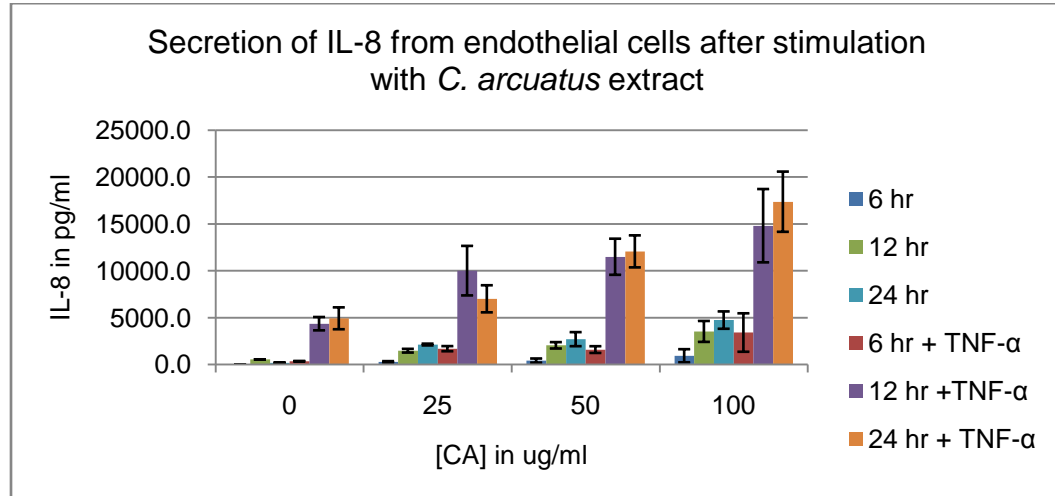


Figure 9. Dose dependent secretion of IL-8 from normal human microvascular endothelial cells stimulated with varying doses of *C. arcuatus* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

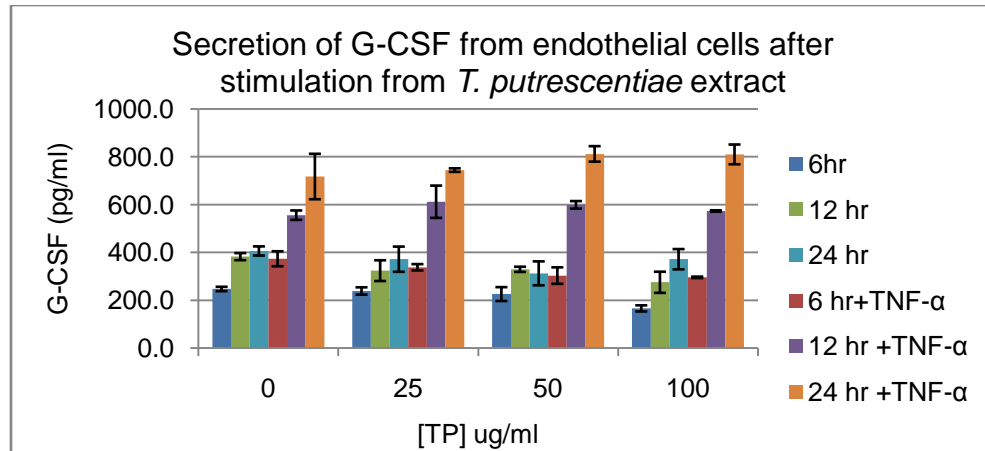


Figure 10. Secretion of G-CSF from normal human microvascular endothelial cells stimulated with varying doses of *T. putrescentiae* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.

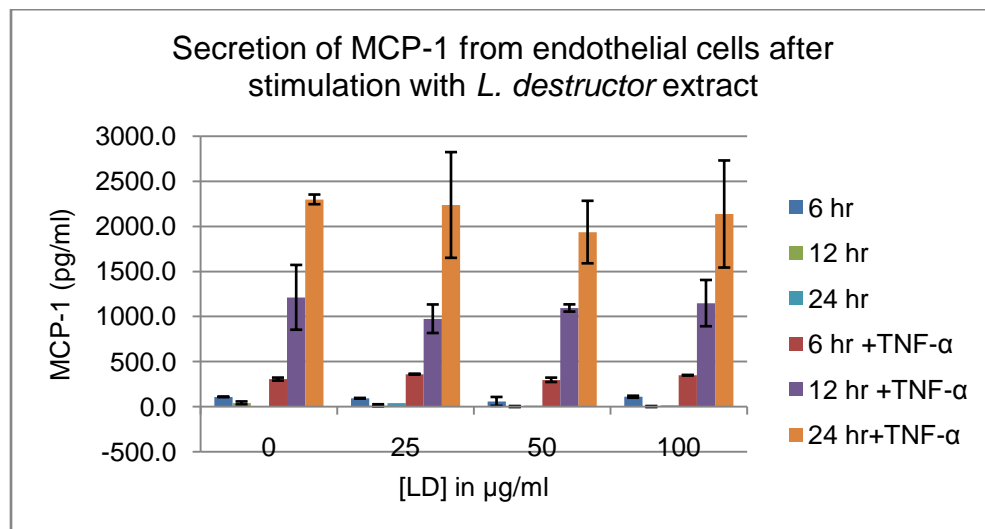


Figure 11. Secretion of MCP-1 from normal human microvascular endothelial cells stimulated with varying doses of *L. destructor* extract for 6, 12, and 24 hours with and without TNF- $\alpha$ . Data are presented as means  $\pm$  SD of three replicate wells of cells.



## II. Effects of Mite Extract on Secretion and Expression Induced by LPS

We measured the endotoxin levels in our extracts and determined that *A. siro* extracts contained > 45,000 EU/ml, and *C. arcuatus*, *L. destructor*, and *T. putrescentiae* contained <20,000 EU/ml. Therefore, it was possible that LPS in the mite extracts could induce a cellular response. Figure 12 shows the effect of LPS on ICAM-1 expression from endothelial cells. A low (80ng/ml) and high (200 ng/ml) dose were used to stimulate the cells, because the endotoxin concentration in each mite extract fell within the range of the two doses. PmB blocked the effect of LPS. With our cells, LPS induced ICAM-1 expression, but when PmB was added, the LPS induced expression of ICAM-1 decreased and confirmed that the doses of PmB were appropriate (figure 12).

There was no significant change in ICAM-1 expression by the cells when Polymyxin B was added to the mite extracts to block LPS activity (figure 13). Similar results were observed for cytokine secretion (figure 14). In the positive control, LPS was blocked when PmB was added to the plate. For any of the cytokines tested (IL-6, IL-8, and MCP-1), compared to just mite extract alone, there was no significant decrease in the secretion induced by the mite extract when PmB was added to the extract (figure 14). This indicated that the endotoxin present in the extracts was not responsible for the mite induced cytokine secretion.

Interestingly, the opposite effect was seen with the secretion of IL-8 and MCP-1 after stimulation with *A. siro*. Instead of the secretion decreasing with the addition of PmB, the PmB seemed to induce the secretion of IL-8 and MCP-1

(figure 14). This suggests that there is something else present in the mite extract affecting cytokine secretion.

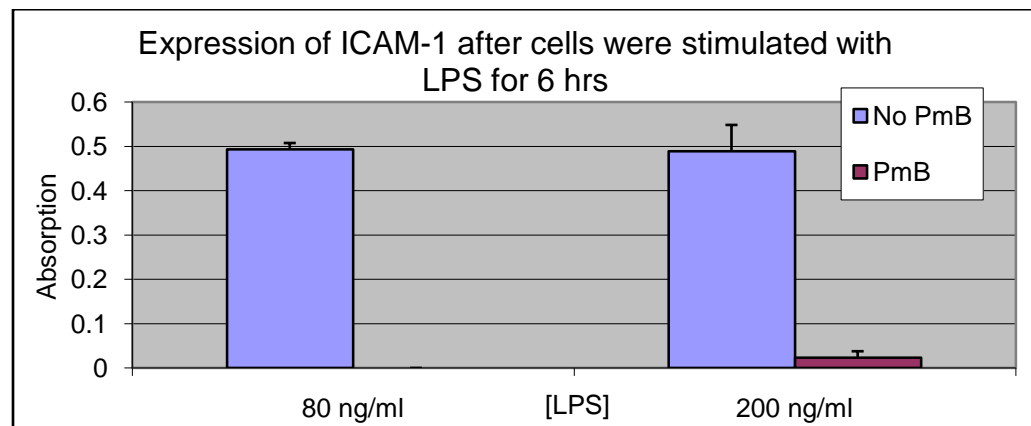


Figure 12. Effect of LPS on ICAM-1 expression from normal human microvascular endothelial cells. Cells were incubated with two doses (80 and 200 ng/ml) of LPS for 6 hours. Data are means  $\pm$  SD (n=3).

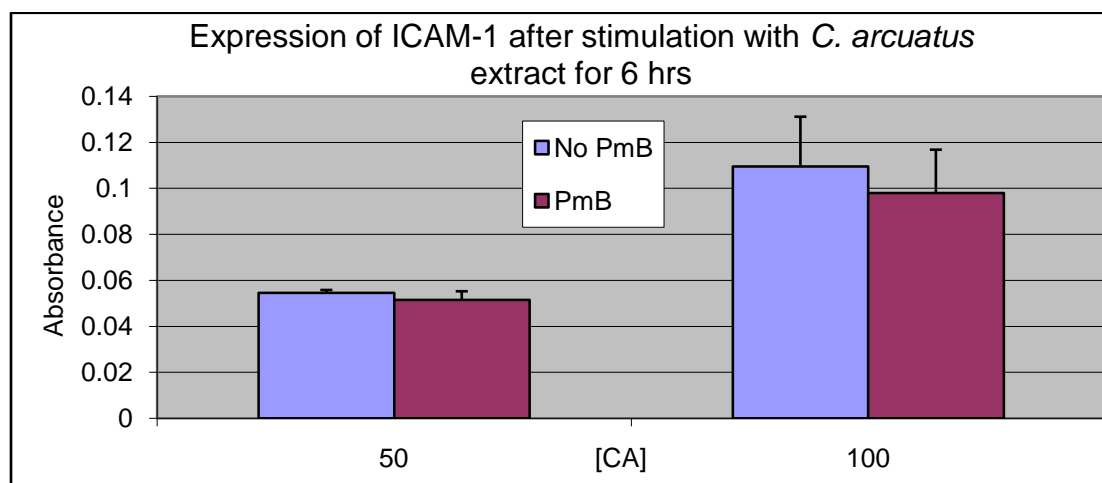


Figure 13. Effect of PmB on ICAM-1 expression from normal human microvascular endothelial cells. Cells were incubated with 50 and 100  $\mu$ g/ml of *C. arcuatus* with and without PmB for 6 hours. Data are means  $\pm$  SD (n=3).

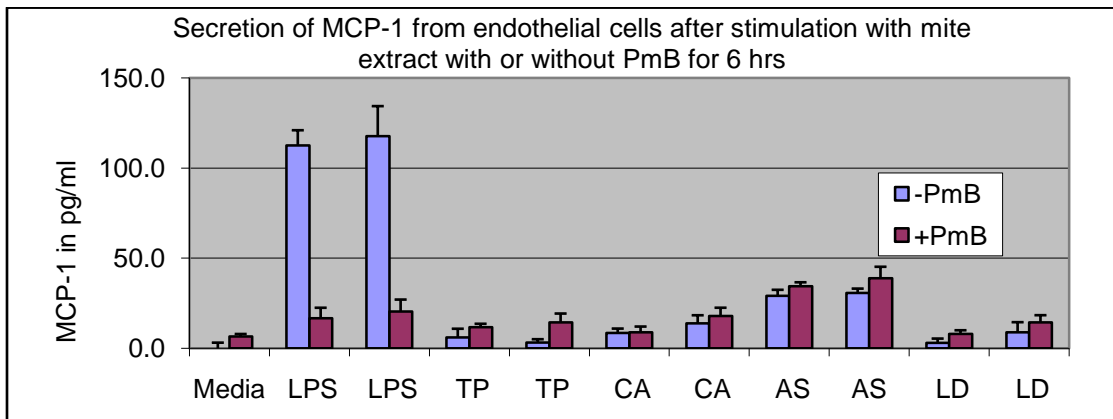
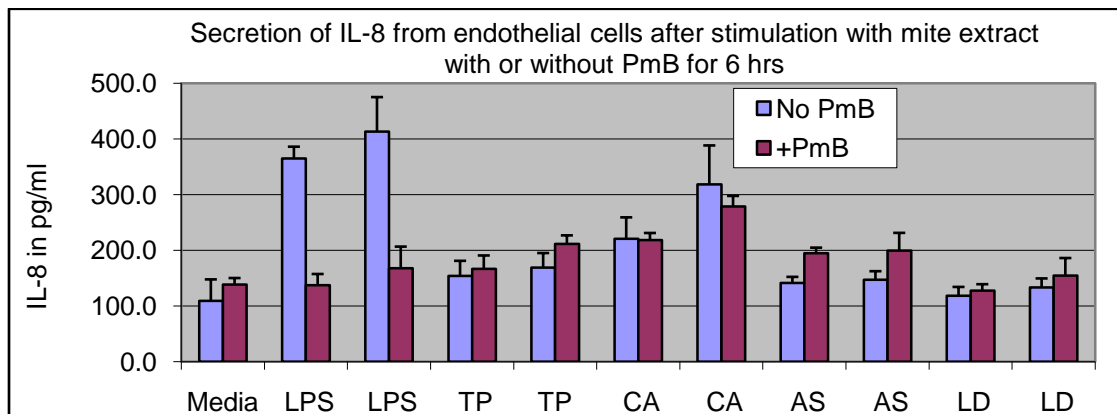
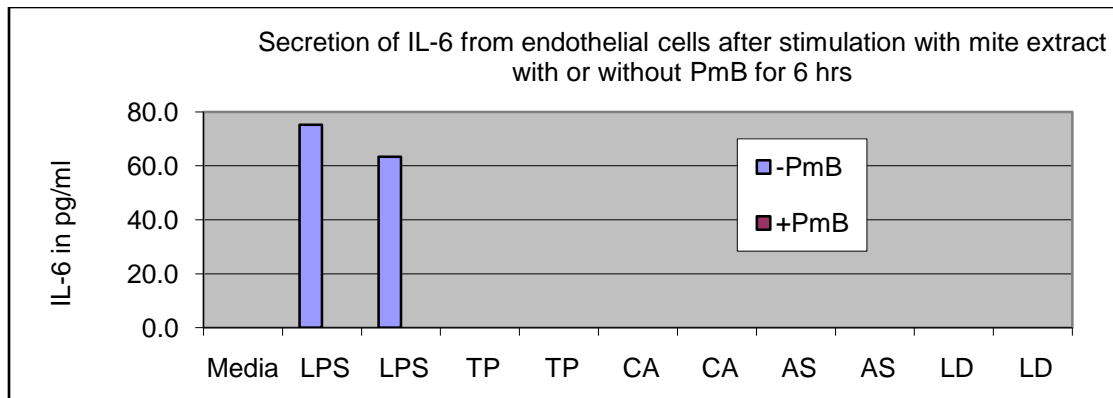


Figure 14. Effect of PmB on IL-6, IL-8 and MCP-1 secretion from normal human microvascular endothelial cells. Cells were incubated at two concentrations (50 and 100  $\mu\text{g/ml}$ ) of each mite extract with and without PmB for 6 hours. Data are means  $\pm$  SD (n=3).

### III. Effects of Mite Extract on Secretion and Expression Induced by Protease Inhibitors

House dust and stored product mites are the source of many molecules including proteases. Proteases may activate cells via PARs, and endothelial cells have PARs on their cell membranes (Reed and Kita 2004). Endothelial cells stimulated with the mite extracts in the presence of either aprotinin (serine protease inhibitor) or E-64 (cysteine protease inhibitor) did not decrease expression of ICAM-1, VCAM-1 or E-selectin, or IL-8, MCP-1, or VEG-F cytokine secretion. We would expect a decrease in expression or secretion if the protease enzymes were stimulating the PARs on endothelial cells when the inhibitors were added. ICAM-1 was the only cell adhesion molecule expressed in the absence of TNF- $\alpha$  (figure 15, 16). There was not a significant decrease in ICAM-1 expression when a protease inhibitor was added to the mite extracts (figure 15, 16). ICAM-1 expression was greater for cells co-stimulated with *C. arcuatus* and aprotinin together than for cells co-stimulated with *C. arcuatus* alone (figure 15). Likewise, expression of ICAM-1 was greater for cells co-stimulated with *C. arcuatus* and E-64 together, and *L. destructor* and E-64 together than with either *C. arcuatus* or *L. destructor* alone (figure 16).

There was no significant decrease of VEGF, IL-8 or MCP-1 cytokine secretion from endothelial cells induced by the mite extracts when aprotinin or E-64 was added (figure 17). However, aprotinin with *T. putrescentiae* and aprotinin with *C. arcuatus* induced greater secretion of MCP-1 than did *T. putrescentiae* or *C. arcuatus* extracts alone. These results suggest that proteolytic cleavage of

PARs was not responsible for the induction of any cytokine secretion or ICAM-1 expression.

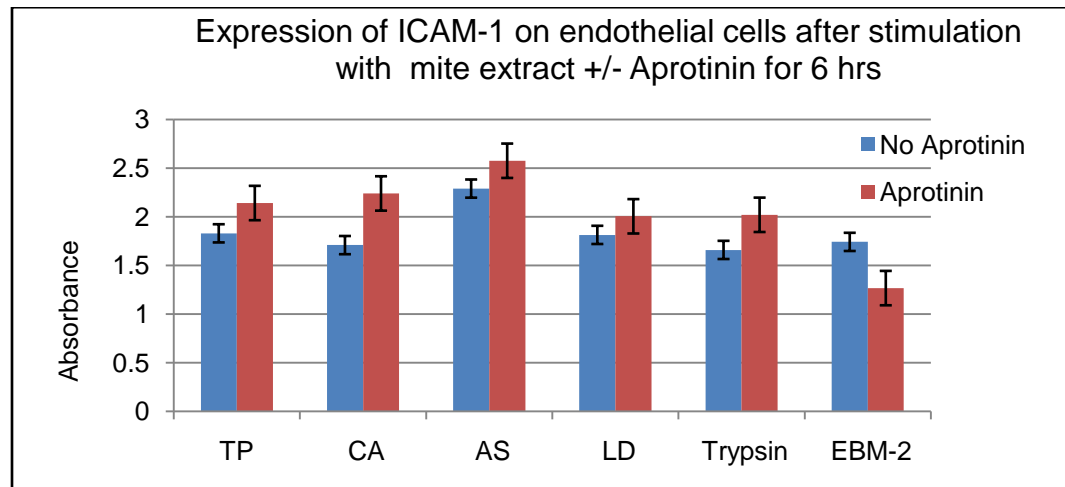


Figure 15. Effect of aprotinin (protease inhibitor) on mite induced ICAM-1 expression from normal human microvascular endothelial cells. Cells were incubated with and without aprotinin and mite extracts for 6 hours. Data are means +/- SD (n=3).

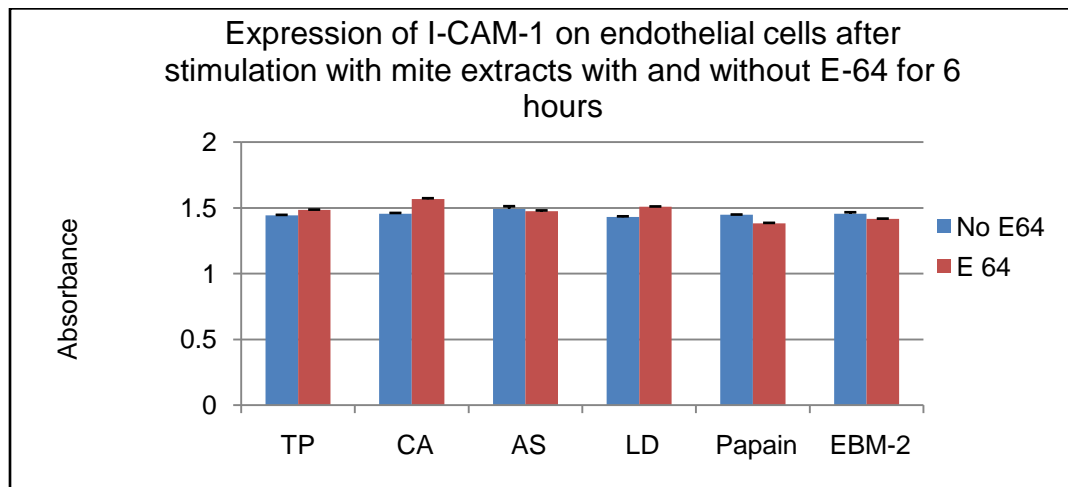
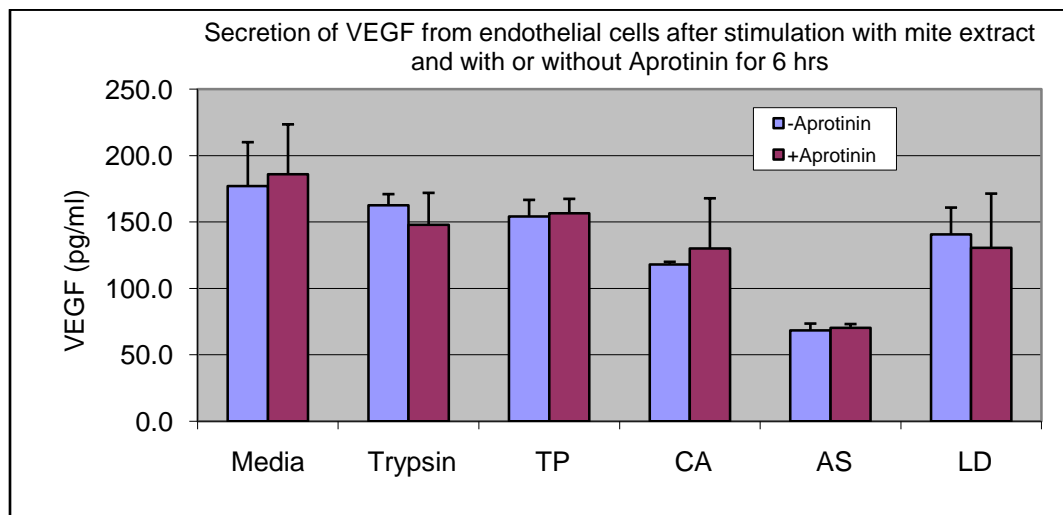
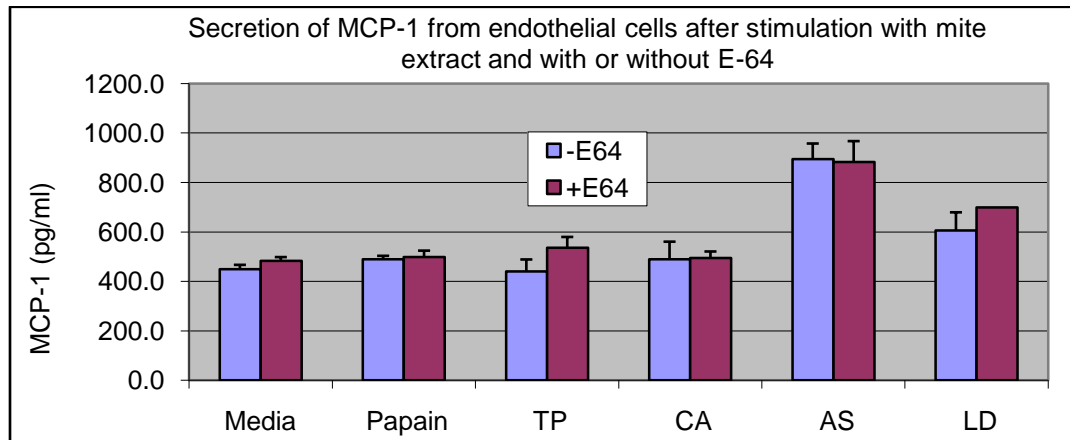
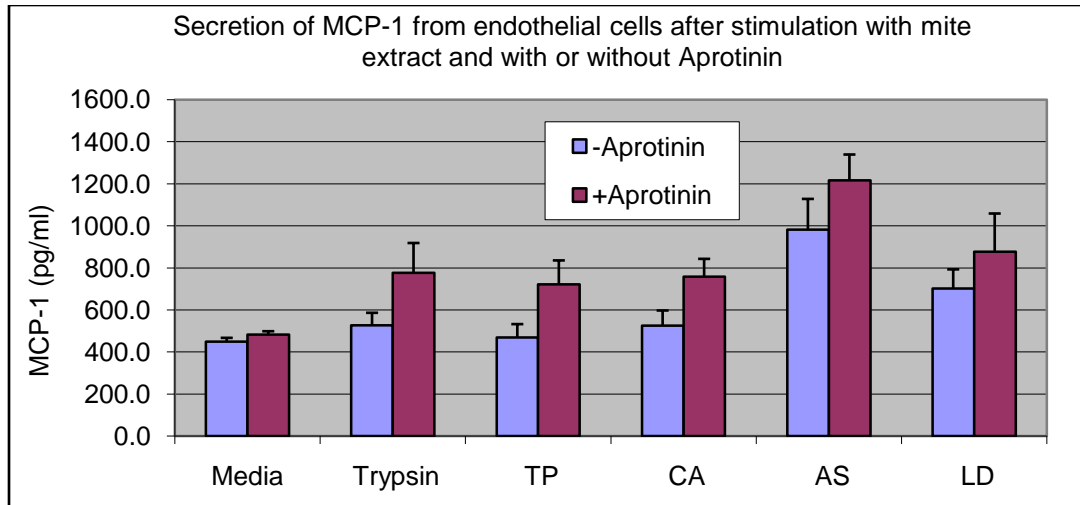


Figure 16. Effect of E-64 (protease inhibitor) on mite induced ICAM-1 expression from normal human microvascular endothelial cells. Cells were incubated with and without E-64 for 6 hours. Data are means +/- SD (n=3).



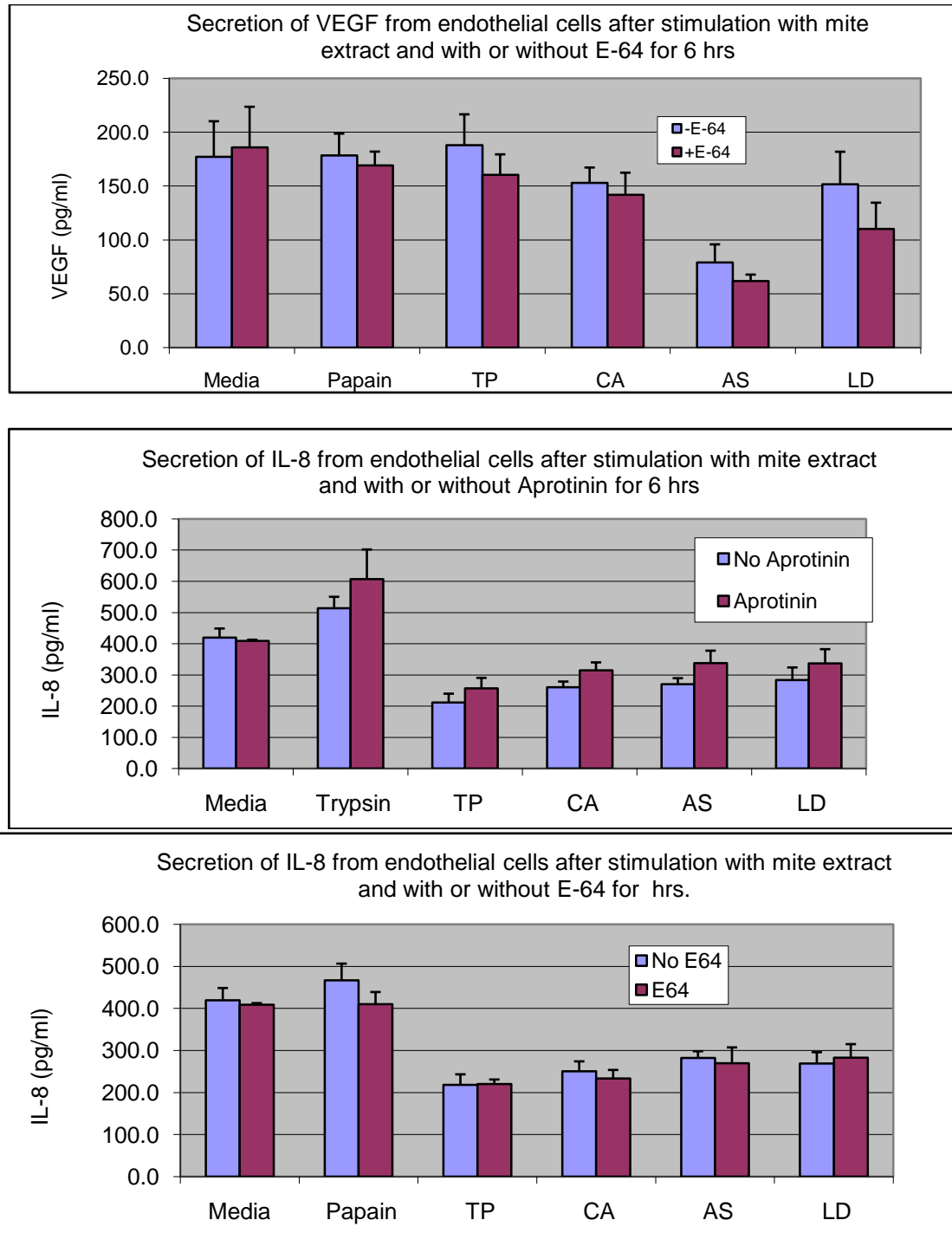


Figure 17. Effect of aprotinin or E-64 (protease inhibitors) on MCP-1, VEGF, and IL-8 secretion from normal human microvascular endothelial cells. Cells were

incubated with and without aprotinin or E-64 for 6 hours. Data are means +/- SD (n=3).



## Discussion

### General/Introduction

The stored product mites *A. siro*, *C. arcuatus*, *L. destructor*, and *T. putrescentiae* are important sources of allergens throughout the world. Stored product mites and molecules from stored product mites can be found in house dust in human dwellings and in food made from stored grains. Stored product mites are the source of molecules that can disturb the epidermis and dermis (Arlan et al. 2003, 2008, Morgan and Arlian 2006). Disturbance of the epithelial barrier can allow the penetration of allergens and other exogenous molecules into the skin and then these molecules may contact resident cells (keratinocytes, fibroblasts, and endothelial cells of the microvasculature), and influence their function (Bernard et al. 2003).

Endothelial cells secrete pro-inflammatory mediators like cytokines, chemokines, and express different adhesion molecules and cytokine and chemokine receptors on their surface when they are activated by exogenous molecules. These are key factors that attract the leukocytes to specific sites in the skin. Other cells in the vicinity, such as keratinocytes, fibroblasts, Langerhan cells and macrophages may also secrete cytokines and chemokines that influence the function of vascular endothelial cells and the course of inflammation (McLaren and Kennedy 2005).

When any exogenous molecules including allergens penetrate the dermis, microvascular endothelial cells may be stimulated. The purpose of this research was to investigate if and how components of stored product mite extracts cause

an immune and inflammatory response by modulating cytokine secretion and cell adhesion molecule expression on human microvascular endothelial cells in the dermis. The important finding is that components from different stored product mite extracts modulate the expression of cell adhesion molecules and secretion of cytokines from microvascular endothelial cells by either inducing the secretion of cytokines and cell adhesion molecules that were not constitutively secreted, increasing the production of cytokines and cell adhesion molecules above the levels that were constitutively secreted or reducing the level of secretion or expression of constitutively or stimulated secreted cytokines and expressed cell adhesion molecules.

### Cell Adhesion Molecules

The role of endothelial cells in regards to the inflammation and immune responses is to regulate extravasation of inflammatory and immune cells from the blood stream into the dermis. ICAM-1, VCAM-1 and E-selectin are cell adhesion molecules that can be expressed on endothelial cells. Endothelial cells control leukocyte physical interactions with the vessel wall via cell-adhesion molecules. The endothelial cells express two cell-adhesion molecules (CAMs): P-selectin and E-selectin at the start of the extravasation process. P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury. When endothelial cells are activated by molecules, such as histamine or thrombin, P-selectin moves from an internal cell location to the endothelial cell surface, aiding the inflammation response. After further stimulation, different cell-adhesion molecules from the immunoglobulin family, like ICAM and VCAM, are expressed

by endothelial cells and integrins (LFA-1) are expressed on leukocytes, and the two different cells can bind. At this point cell-adhesion is irreversible and the leukocyte, bound to the endothelium, will transmigrate through the basement membrane (McLaren and Kennedy 2005). The leukocytes are able to migrate through the blood vessel wall (between the cells) and towards the site of infection.

We found that some stored product mite extracts influenced expression of selected surface cell adhesion molecules in cultured human microvascular endothelial cells.

**Table 1.** Summary of the modulatory effects of stored product mite extracts on cell adhesion molecule expression by normal human microvascular endothelial cells

	AS	AS+TNF- $\alpha$	CA	CA + TNF- $\alpha$	LD	LD TNF- $\alpha$	TP	TP+TNF- $\alpha$
ICAM-1	↑	—	—	—	—	—	—	↓
VCAM-1	—	↓	—	—	—	—	—	—
E-selectin	—	—	—	↓	—	—	—	—

We measured expression of ICAM-1, VCAM-1 and E-selectin, because they are expressed on the surface of activated endothelial cells. ICAM-1 was constitutively expressed by microvascular endothelial cells, and *A. siro* up-regulated ICAM-1. This may promote inflammation, because leukocytes and endothelial cells are more likely to bind and extravasation is able to occur. VCAM-1 and E-selectin were not constitutively expressed. However, after

VCAM-1 was stimulated with TNF- $\alpha$ . *A. siro* down regulated expression of VCAM-1. *A. siro* contains molecules that influence the function of human microvascular endothelial cells. *C. arcuatus* did not affect the expression of ICAM-1, VCAM-1 or E-selectin. TNF- $\alpha$  induced expression of ICAM-1 and VCAM-1 were not affected by the *C. arcuatus* extract. Although the levels are low, the data showed that TNF- $\alpha$  induced expression of E-selectin was down regulated by *C. arcuatus* at 6 hours. *L. destructor* did not affect the constitutive or TNF- $\alpha$  induced expression of ICAM-1, VCAM-1, or E-selectin. *T. putrescentiae* did not affect the expression of the constitutive levels of ICAM-1, VCAM-1, or E-selectin, but TNF- $\alpha$  induced expression of ICAM-1 was down regulated by *T. putrescentiae* at 24 hours. TNF- $\alpha$  induced expression of VCAM-1 or E-selectin was not affected by the *T. putrescentiae* extract. The different stored product mites gave different results. The reason that different stored product mites gave different results may be due to the fact that they are different species, so they may contain different molecules.

Previous studies found that house dust mites and scabies mites also influence the expression of cell adhesion molecules from endothelial cells. Similar to our study, Elder et al. 2006 found that ICAM-1 was produced constitutively by endothelial cells; however, scabies mite extract did not affect the constitutive expression of ICAM-1. However, it was observed that scabies mite extract inhibited endothelial cell from expressing E-selectin and VCAM-1 (Elder et al. 2006). A primary infestation with scabies burrowing in human skin does not induce clinical symptoms for 4-8 weeks. Scabies mites are parasites and contain

molecules that delay the early initiation of host inflammation and immune responses (Arlian et al. 2003, 2004, 2006, and Mullins et al. 2009). Stored product mites are not parasites. Therefore, they may not contain the molecules that suppress inflammatory and immune responses, because there is no need for such molecules in free living mites. Molecules from stored product mites do cause an immediate (6-24 hour) inflammatory and immune response from endothelial cells. Likewise, the house dust mites *D. farina*, *D. pteronyssinus* and *E. maynei* all stimulate endothelial cells to express ICAM-1, VCAM-1 and E-selectin (Arlian et al. 2009). Interestingly, parasites like scabies mites, and nonparasites like house dust mites and stored product mites cause the endothelial cells to modulate the inflammation and immune responses, but in different ways.

### Cytokines

The following cytokines were measured: VEG-F, GM-CSF, IL-6, IL-8, G-CSF, and MCP-1. These cytokines are all secreted from endothelial cells and they promote inflammation and immune responses. Our study found that different mite extracts modulated the secretion of these cytokines in different ways.

**Table 2.** Summary of the modulatory effects of stored product mite extracts on cytokine secretion by normal human microvascular endothelial cells

	AS	AS+TNF- $\alpha$	CA	CA+TNF- $\alpha$	LD	LD+TNF- $\alpha$	TP	TP+TNF- $\alpha$
IL-6	↑	↑	↑	↑	—	—	—	—
IL-8	—	—	↑	↑	—	—	—	—
MCP-1	—	—	—	—	—	—	—	—
G-CSF	—	—	—	—	—	—	—	—
GM-CSF	↑	↑	—	—	—	—	—	—
VEG-F	↓	↓	—	—	↓	↓	—	—

Different mite extracts up-regulated secretion of some cytokines (IL-6, IL-8, and GM-CSF) and down-regulated secretion of others (VEG-F). Components of *A. siro* and *L. destructor* extracts down-regulated the high constitutive secretion of VEG-F. *Acarus siro* up-regulated the low secretion of IL-6 and GM-CSF. *Chortoglyphus arcuatus* up-regulated the secretion of IL-6 and IL-8 in a dose dependent fashion. These results suggest that the mite extracts contain different molecules that have different effects on endothelial cells. Different mites induce different cytokines to be secreted. This is significant because all of the mites have different molecules that induce inflammation and immune responses. If the mites were crushed on the skin and the molecules penetrated, it would make the skin itchy, red and swollen. When mite molecules from stored product mites penetrate, they stimulate cells such as keratinocytes, fibroblasts or endothelial cells. In sensitized individuals, some of these molecules may be

allergens that bind to IgE on mast cells and induce atopic dermatitis. Atopic dermatitis is a skin condition that occurs when mast cells are stimulated and chemical mediators from these cells are released and induce clinical symptoms like erythema, edema, pruritis and leukocyte recruitment.

Our results are not unexpected because other mites can modulate functions of cells in the skin. Arlian et al. (2003, 2008) studied the cytokine secretion in normal human keratinocytes and fibroblasts in response to molecules from scabies mites, house dust mites and stored product mites. Keratinocytes express increased levels of IL-6, VEG-F and G-CSF in response to *Sarcoptes scabiei* (Arlian et al. 2003). Fibroblasts also show increased expression of those cytokines as well as IL-8 in response to *Sarcoptes scabiei*, and to house dust mites in a dose dependent manner (Arlian et al. 2003). House dust mites also induce secretion of MCP-1 and M-CSF from dermal fibroblasts (Arlian et al. 2008). In the presence of proinflammatory cytokines, scabies extracts down-regulated levels of IL-8 secretion from keratinocytes and fibroblasts (Arlian et al. 2003). Scabies extracts also down regulate GM-CSF secretion from fibroblasts. Scabies extracts with proinflammatory cytokines induce secretions of G-CSF from fibroblasts, and CTACK, GRO $\alpha$ , and TGF $\alpha$  from keratinocytes (Mullins et al. 2009). These results suggest that many molecules in the scabies extract cause a delayed inflammatory response and there are different molecules in house dust mites and stored product mites that do not delay the immune response and inflammation. It is interesting that *A. siro* and *L. destructor* extracts down-regulated secretion of VEG-F by endothelial cells while the parasitic mite

*S. scabiei* up-regulated VEG-F secretion by keratinocytes. The significance of this is unclear.

### IL-6

IL-6 has a diverse array of proinflammatory and anti-inflammatory functions. It probably plays proinflammatory roles in the response to mites if their molecules penetrate the skin. It can increase vascular permeability (Arlian et al. 2003) and induces maturation of B cells and T cells into immunoglobulin secreting plasma cells and T helper cells (Turksen et al. 1992). In this study, secretion of IL-6 was slightly upregulated by *A. siro* and *C. arcuatus*, but not by *L. destructor* or *T. putrescentiae*. In contrast, Arlian et al. (2003, 2008) studied the IL-6 secretion in normal human keratinocytes and fibroblasts in response to molecules from scabies mites, stored product mites and house dust mites and scabies. The stored product mites *A. siro*, *C. arcuatus*, and *L. destructor* and house dust mite, *D. farinae* upregulated IL-6 in both types of cells. However, IL-6 was upregulated to a greater extent from fibroblasts compared to endothelial cells in this study when induced by *A. siro* and *C. arcuatus*. Since secretion of IL-6 was only slightly upregulated with *A. siro* and *C. arcuatus* extracts, it suggests that endothelial cells in the skin may not be the primary cell producing IL-6. IL-6 production may come mostly from fibroblasts.

### IL-8

Recruitment of leukocytes and lymphocytes is important to start an effective innate and adaptive immune response in areas of tissue injury and inflammation. IL-8 enhances inflammation as its primary function by inducing



chemotaxis of neutrophils (Arlan et al. 2003). Neutrophils spend most of their lives circulating in the blood stream, until inflammation is initiated. IL-8 gene transcription is activated by NF- $\kappa$ B. When this molecule is phosphorylated, it activates several genes including IL-8. NF- $\kappa$ B is activated by inducer of inflammation like LPS or TNF- $\alpha$  (Mukaida et al. 1994). IL-8 binds to endothelial cell surfaces and triggers neutrophils to increase the affinity of the neutrophil integrin receptor for coupling with adhesion molecules. The principle function of neutrophils is phagocytosis, where it then internalizes and destroys the pathogen. In this study, endothelial cells constitutively produce large amounts of IL-8 and this secretion of IL-8 was up-regulated in cells stimulated with *C. arcuatus* extract. In contrast to these results, a previous study showed that scabies mites down regulated IL-8 secretion by microvascular endothelial cells (Elder et al. 2006). This helps confirm that stored product mites may not contain the molecules that scabies contain that suppress inflammatory and immune responses

Interleukin-8 is known to be produced by fibroblasts and mononuclear cells (Arlan et al 2008). Scabies mite extract up-regulated secretion by peripheral blood mononuclear cells. The house dust mites *D. farina*, *D. pteronyssinus* and *E. maynei* and the stored product mites mites *A. siro*, *C. arcuatus*, and *L. destructor* up-regulated secretion of IL-8 in fibroblasts as well (Arlan et al. 2008). Since secretion of IL-8 is constitutively high from microvascular endothelial cells and is upregulated by stored product mite extracts *A. siro*, *C. arcuatus*, and *L. destructor*, it may be an important player in

starting the immune and inflammatory response in humans. It may be the primary initiator of inflammation by attracting neutrophils.

### GM-CSF

GM-CSF locally may activate leukocytes at sites of inflammation by stimulating stem cells to produce granulocytes (neutrophils). *A. siro* up-regulated the low constitutive secretion of GM-CSF by microvascular endothelial cells. This research is different than previous research that showed that GM-CSF is not constitutively expressed from dermal fibroblasts or keratinocytes, and extracts from stored product mites and house dust mites do not stimulate secretion of GM-CSF (Arlian et al. 2003, 2008). It is important that microvascular endothelial cells secrete GM-CSF in order to promote growth and differentiation of granulocytes (mostly neutrophils) and monocytes (macrophages). GM-CSF is thus part of the immune and inflammatory cascade, where activation of a small number of macrophages and neutrophils can rapidly lead to an increase in their numbers, and fight the pathogens better.

### Toll-like Receptors

Toll-like receptors (TLRs) are present on the external and internal endothelial cell membranes. TLRs play critical roles in the innate immune system as microbial (LPS, peptidoglycans, lipoproteins) pattern-recognition receptors (Kaisho and Akira 2006). Endothelial cells have toll like receptors 2 and 4 (TLR 2 and TLR4) on their surface (Fitzner et al. 2008) that recognize endotoxin (LPS). An endotoxin assay verified that the stored product mite extracts that we used to stimulate endothelial cells contained LPS. LPS can alter

the normal function of endothelial cells using TLR 2 and TLR 4 (Arlan et al. 2007). Polymyxin B binds to LPS so that it cannot activate TLRs. When we blocked LPS in the mite extracts using Polymyxin B, we assumed that LPS was unavailable to stimulate TLR's and thus not responsible for the response induced on the endothelial cells by the mite extracts. LPS without polymyxin B was used to stimulate the cells so that we knew endothelial cells expressed TLRs. We observed that there was no decrease in ICAM-1 expression or IL-6, IL-8, or MCP-1 secretion when Polymyxin B was added to the mite extracts. Therefore, the endotoxin present in our extracts was not responsible for the mite extract induced expression of cell adhesion molecules and secretion of cytokines that were observed.

In contrast to our results, there is evidence of house dust mites stimulating cells via TLRs. Previous studies have shown that house dust mites contain endotoxin (Trivedi et al. 2003, Valerio et al. 2005). House dust mite allergen triggers TLR4 on dendritic cells in the lung and causes the release of proinflammatory molecules (Hammad et al. 2009). House dust extract-activated bone marrow-derived dendritic cells produce IL-6 and IL-12 indicating that toll like signaling pathways were activated (Boasen et al. 2005). One study focused on TLR-2 and how it modified the immune response to house dust mite allergen. TLR-2 ligands inhibit allergen-specific T helper 2 cell responses in individuals (Taylor et al. 2006).

### Protease Activated Receptors

Protease-activated receptors (PARs) are present on endothelial cell surfaces (Shpacovitch et al. 2007). These receptors are activated by protease enzymes and can increase leukocyte infiltration and amplify the immune response to allergens (Reed and Kita 2004). Mite extracts contain protease enzymes (Thomas et al. 2007). We blocked the protease enzyme activity in our extracts in order to determine if other components in the extract stimulated the cells by non-PAR pathways, and thus were responsible for the cell's response to the extract. Protease inhibitors bind to the active site of proteases, so that the protease cannot bind to the PAR. By adding protease inhibitors into the mite extracts and seeing no change in the expression of cell adhesion molecules or secretion of cytokines from endothelial cells, we can conclude that the molecules in mite extracts were not working through PARs. ICAM-1 was the only cell adhesion molecule expressed by the endothelial cells that we detected. There was not a significant decrease in ICAM-1 expression when a protease inhibitor was added to the mite extracts. There was no significant decrease of VEG-F, IL-8, or MCP-1 cytokine secretion from endothelial cells induced by mite extracts when protease inhibitors were added to the extracts. These results suggest that proteolytic cleavage of PARs was not responsible for the stored product mite induction of any cytokine secretion or ICAM-1 expression.

### Conclusion

In conclusion, this study suggests that human microvascular endothelial cells have significant roles in the immune/inflammatory response to stored product mite allergens. Molecules from stored product mites can modulate the

immune and inflammatory response from endothelial cells. These responses were not mediated through protease-activated or toll-like receptors (PARs or TLRs).

### Future Studies

The conclusions found in this investigation lead to new scientific questions. What mechanisms do the stored product mite molecules use to affect the endothelial cells? Do all parts of the extracts give the same effects? Can we separate the extract into parts and see different effects? How would the effects be different in vivo?

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