CHAPTER 9

INVESTIGATING CHANGES IN THE LUNGS OF CONTROL, ASTHMATIC AND HYDROCORTISONE-TREATED BALB/c MICE USING LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

9.1 Introduction

Asthma is associated with a wide range of symptoms and effects, which include chest tightness, wheezing, coughing, increased airway obstruction and airway hyperresponsiveness. The latter part represents the classic physiology of asthma. Airway inflammation is considered to be a central feature of asthma pathogenesis and its clinical manifestations and plays a critical role in airway obstruction and hyperresponsiveness (Wenzel, 1994).

Many studies have been done by using bronchial hyperresponsiveness animal models to investigate the pathophysiology of asthma. When mice are sensitized and challenged with an allergen an influx of inflammatory cells into the airways are seen; this is a process similar to that found in humans. Because this inflammatory process are similar in humans and in mice, mice are widely used and believed to be useful as an asthmatic animal model (Evans *et al*., 2003; Hamelmann *et al*.,1999; Kumar and Foster, 2002; Leigh *et al*., 2002; Tomioka *et al*., 2002). Eosinophilia and mucus cell hyperplasia are some of the most notable histopathalogical signs of human asthmatics and is also found in mouse models of asthma (Uller *et al*., 2006; Persson *et al*., 1997).

In both humans and the mouse animal model, asthma is characterized by variable degrees of chronic inflammation and structural changes in the airways. Airway

remodeling is defined as changes in the composition, content, and organization of the cellular and molecular constituents of the airway wall and it is known to be a characteristic feature of asthma, as it has important functional implications. The structural changes that occur in the airways of asthmatics include:

- epithelial detachment,
- subepithelial fibrosis,
- increased airway smooth muscle mass,
- decrease in the distance between the epithelium and airway smooth muscle cells,
- goblet cell hyperplasia,
- mucus gland hyperplasia,
- proliferation of blood vessels,
- airway edema and
- changes in the cartilage.

Each one of these changes contributes to airway hyperreactivity (AHR), and eventually may lead to irreversible airflow obstruction with disease progression (Sumi and Hamid, 2007).

Researchers have also reported on changes in the airway blood vessels. Qualitative and quantitative changes in the airway blood vessels that contribute to the observed airway remodeling in asthma include edematous bronchial mucosa and dilated and congested blood vessels (Chetta *et al*., 2007).

Some researchers suspect that chronic inflammation is thought to initiate and perpetuate cycles of tissue injury and repair in asthma, although remodeling may also occur parallel with inflammation. Mauad and co workers in 2007 stated that the working hypothesis should be that structural alterations could lead to the

development of persistent airway hyperresponsiveness and fixed airway obstruction (Mauad *et al*., 2007).

Many researchers used animal models, *in vitro* studies, and some clinical studies to investigate the cellular and molecular pathways involved in airway remodeling and this has encouraged the development of substances or medicine aimed to target various components of airway remodeling (Mauad *et al*., 2007).

Therefore, in the current chapter the asthmatic BALB/c mouse model was used to investigate the histological as well as ultrastructural changes that occur in the lungs of asthmatic animals as well as to investigate the presence of inflammatory cells in the airway walls and spaces by using light-and transmission electron microscopy. The lungs of OVA challenged, untreated asthmatic animals were compared to animals treated with the homeopathic immunomodulator, MODUL8® as well as hydrocortisone, which was used as positive control. Several studies have shown that products of natural origin can effectively be used in treating diseases and maintaining the resistance to infections of organisms (Pereira *et al*., 2005) and are also effective against inflammatory conditions. Therefore, this product was used as treatment in the asthmatic mouse model, to investigate its possible effect on the inflammatory process involved in asthma.

9.2 Materials and Methods

9.2.1 *Tissue for Histology*

Lung tissue was collected for histological investigation. Tissue samples were fixed in 2.5% gluteraldehyde/formaldehyde solution. The tissue was removed from the fixative and rinsed three times in 0.075M sodium potassium phosphate buffer (pH 7.4) for 15 min before being serially dehydrated in 30%, 50%, 70% and 90% ethanol,

followed by three changes of absolute ethanol. The samples were then infiltrated in LR White over three days after which they were polymerized in gelatin capsules at 60°C for 48hr. 1µm sections were made with an ultramicrotome and stained with Toluidine blue. The samples were viewed with a Nikon Optiphod transmitted light microscope (Nikon Instech Co., Kanagawa, Japan).

9.2.2 *Tissue for Transmission Electron Microscopy*

Lung samples were fixed in 2.5% glutaraldehyde/formaldehyde solution for 1hr, rinsed three times in 0.075M sodium potassium phosphate buffer (pH 7.4) for 15 min before being placed in secondary fixative, 1% osmium tetraoxide solution, for 1hr. Following fixation, the tissues were rinsed again as described above. The tissues were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. The samples were then embedded in resin and ultra-thin sections (80-100nm), cut with a diamond knife using an ultramicrotome, were contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate, after which samples were allowed to dry for a few minutes before examination with the JEOL Transmission Electron Microscope (JEM 2100F).

9.3 Results

9.3.1 *Light microscopy*

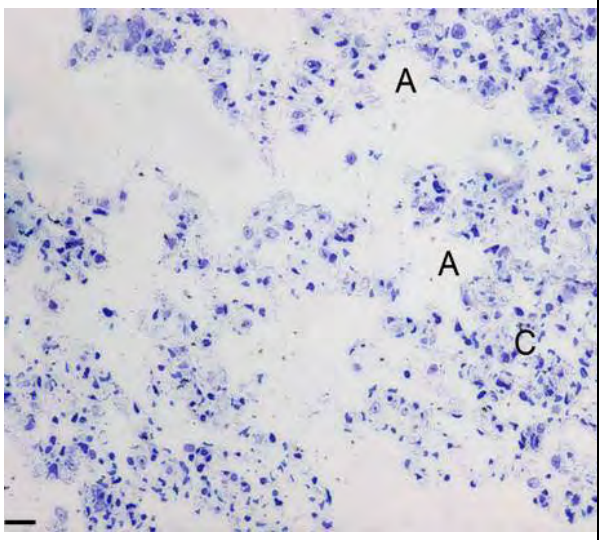
Figure 9.1A (10x magnification) and 1B (40x magnification) represent a section of the lung of a normal healthy animal where alveoli (Label A) is seen, as well as a few inflammatory cells (Label C). In the higher magnification (Figure 1B) the black arrow indicates the interalveolar area which is very small due to the lack of many inflammatory cells.

Figure 2A (10x magnification) and 2B (40x magnification) are light micrographs of the lungs of the OVA-challenged asthmatic animals where a marked influx of

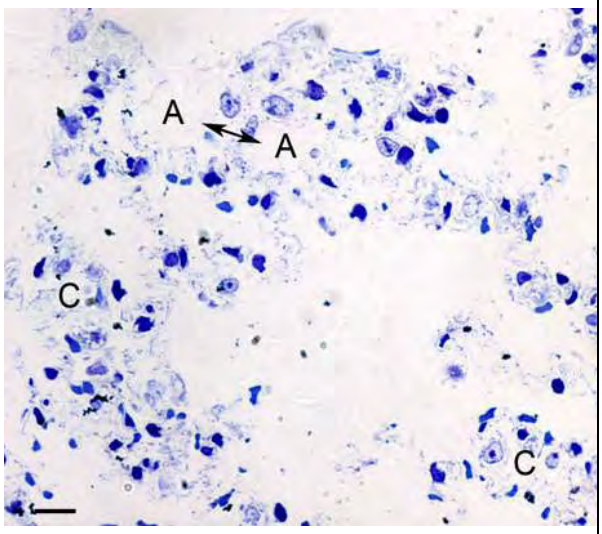
inflammatory cells (Label C) is observed. In the higher magnification (Figure 2 B) an enlarged, congested capillary is observed. The term congested is frequently used in inflammation and asthma to describe the large amount of red blood cells in the capillaries (Ross *et al*., 2003; Chetta *et al*., 2007). The black arrow indicates the interalveolar area, which seemed congested and filled with exudates, containing white blood cells, red blood cells and fibrin - characteristic of the inflammatory condition of the lungs. This congested appearance is not present in the control animals (Figure 1A and B).

Figure 3A (10x magnification) and 3B (40x magnification) shows the lung sections of asthmatic animals treated with the homeopathic immunomodulator Modul8®. Alveoli (Label A), a bronchiole (Label B) as well as inflammatory cells (Label C) are visible. The capillary (Label V) appear smaller and less congested as seen in the asthmatic animals (Figure 2B, Label V). The black arrow in Figure 3B indicates the interalveolar area that is much smaller than that seen in the asthmatic group (Figure 2B). Therefore, in this treated group congestion is reduced and much less inflammatory cells are present compared to the asthmatic group.

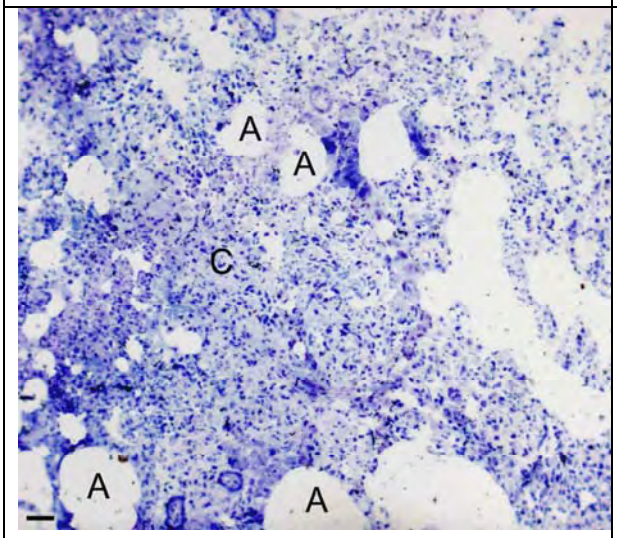
Figure 4A (10x magnification) and 4B (40x magnification) represents the micrographs of the hydrocortisone-treated animals, where even less inflammatory cells (Label C) are present. The black arrow (Figure 4B) indicates the interalveolar area which seems similar to that of the Modul8® treated group and much smaller than that of the asthmatic group; the area has more open spaces and is less congested without the presence of exudate, as are seen in the asthmatic group. This morphology is expected, as hydrocortisone is an effective anti-inflammatory treatment for asthma.



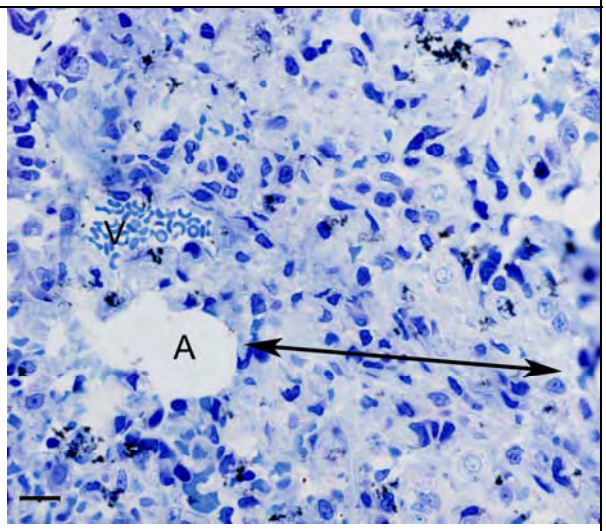
1A: 10x (scale bar = 50µm)



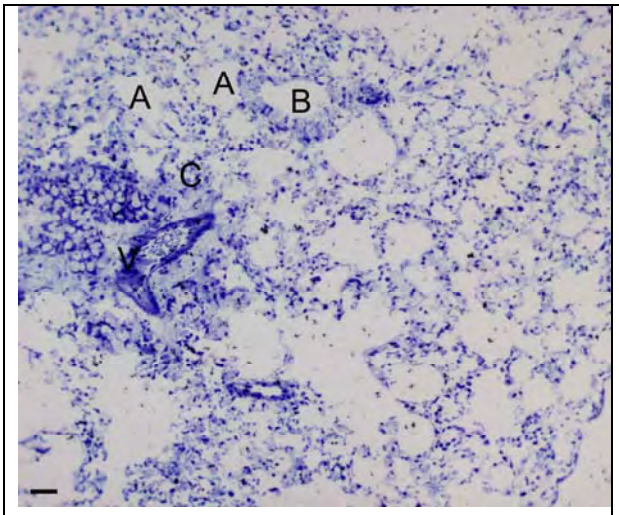
1B: 40x (scale bar = 20µm)



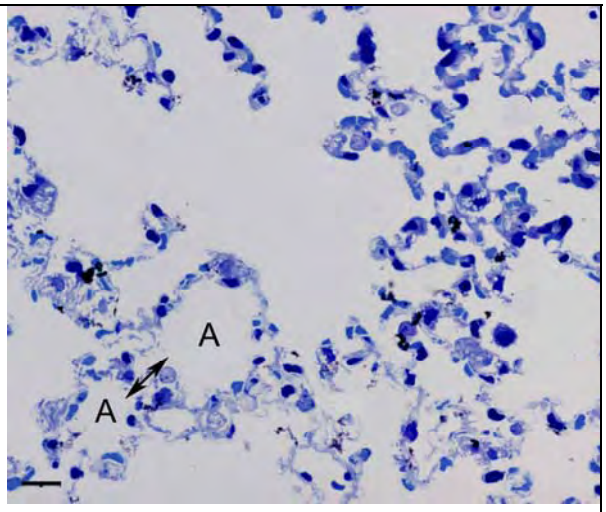
2A: 10x (scale bar = 50µm)



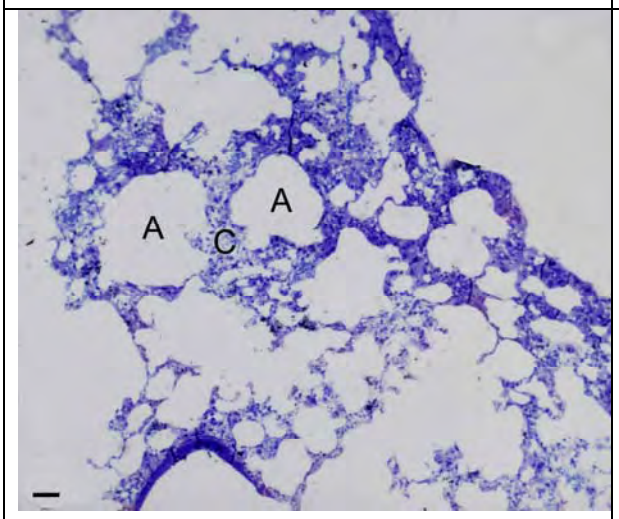
2B: 40x (scale bar = 20µm)



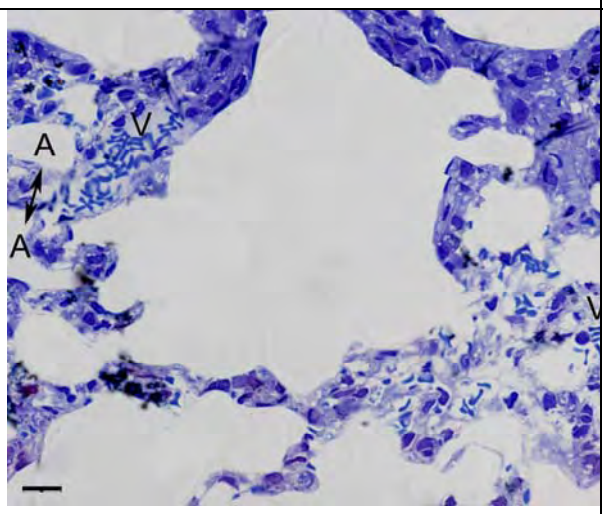
3A: 10x (scale bar = 50µm)



3B: 40x (scale bar = 20µm)



4A: 10x (scale bar = 50µm)



4B: 40x (scale bar = 20µm)

Figure 9.1: Lung sections of the mice stained with Toluidine Blue. **1A** and **B**: Control, **2A** and **B**: Asthma, **3A** and **B**: MODUL8® and **4A** and **B**: Hydrocortisone.

A Alveoli	**B** Bronchiole
C Inflammatory cells	**V** Blood vessel filled with red blood cells

9.3.2 *Transmission electron microscopy*

The results from analyzing the transmission electron microscopy micrographs support those found in analysis of the light microscopy micrographs. Figure 9.2 A-D are transmission electron micrographs of areas in the lungs of the control group where Figure A is a representation of the little inflammatory infiltrate present in the interalveolar area whereas in the asthmatic group, as can be seen in Figure 9.3 A, the interalveolar areas are filled with inflammatory cells and exudate as could also be observed in the light microscopy results (Figure 9.1 2A and 2B). Figure B shows a capillary filled with erythrocytes and figure C is a representation of the collagen deposition that could be observed which were very little compared to the asthmatic group. Transverse as well as longitudinal collagen fibrils could be seen. Figure D is an electron micrograph of a typical macrophage which could be observed in all the experimental groups.

Figure 9.3 A-F are transmission electron micrographs of areas in the lungs of the asthmatic group where Figure A shows the marked influx of inflammatory cells into the lung parenchyma and the increase in the area between the alveoli. Figure B shows a congested capillary, which could also be observed in the light microscopy results (Figure 9.1 2B). Figure C shows collagen fibrils in longitudinal section, which seems to be more densely packed as could be observed in the other experimental groups where the collagen fibrils could be clearly seen and distinguished. Figure D shows a number of cells that form part of the inflammatory infiltrate in the lungs. A lymphocyte, monocyte, mast cell and fibroblast are seen in this figure. Figure E is a representation of the air-blood barrier showing the components of the diffusion barrier between the blood and alveolar air. A type I pneumocyte, a basement membrane and the thin cytoplasm of the endothelial cells can be seen in this figure. Figure F is a micrograph of a type II pneumocyte. These cells are also known as surfactant cells and are responsible for surfactant production. Type II pneumocytes

are recognizable by the presence of lamellar bodies. Most of the cell is surrounded by a basement membrane and only a small portion is in direct contact with the alveolar space where numerous small microvilli could also be observed.

Figure 9.4 A-F are electron micrographs of the lungs taken from the Modul8®-treated group. Figure A is a representation of the alveolar spaces where lots of open spaces can be observed with little inflammatory infiltrate present in the interalveolar areas. Figure B shows a capillary with an erythrocyte and Figure C shows a lymphocyte and two fibroblasts with collagen deposition, which was observed to be not as abundant as could be seen in the asthmatic group. Figure D shows the ultrastructure of a macrophage, revealing cytoplasmic projections or pseudopodia that are involved in movement and phagocytosis. Mitochondria, golgi apparatus, lysosomes and residual bodies could be identified within the activated macrophage. Figure E is a micrograph of a typical neutrophil with its lobed nucleus which was found in the Modul8® group. Figure F is a type II pneumocyte that was found in the Modul8® group. Many lamellar bodies could be identified as well as numerous mitochondria in the cytoplasm of this cell.

Micrographs from the hydrocortisone- treated group (Figure 9.5 A-D) revealed more or less the same ultrastructure as was found in the control and Modul8® -treated group. Figure A shows the alveolar spaces with very little inflammatory infiltrate present in the inter alveolar space. Figure B is a micrograph of a capillary filled with red blood cells and Figure C shows transverse and longitudinal collagen fibrils which appear similar to that found in the control and Modul8® -treated group, and were not as densely packed as could be observed in the asthmatic group. Figure D also shows a type II pneumocyte that was found in the hydrocortisone group with numerous lamellar bodies and mitochondria present in the cytoplasm.

Figure 9.6 is an electron micrograph taken from the asthmatic group where possible caveolae could be observed in the membrane indicated with the black rectangle. Figure 9.6 is a micrograph of a Type I and II pneumocyte and the caveolae could be observed in the Type I pneumocyte as indicated with the rectangle.

9.3.2.1 Control

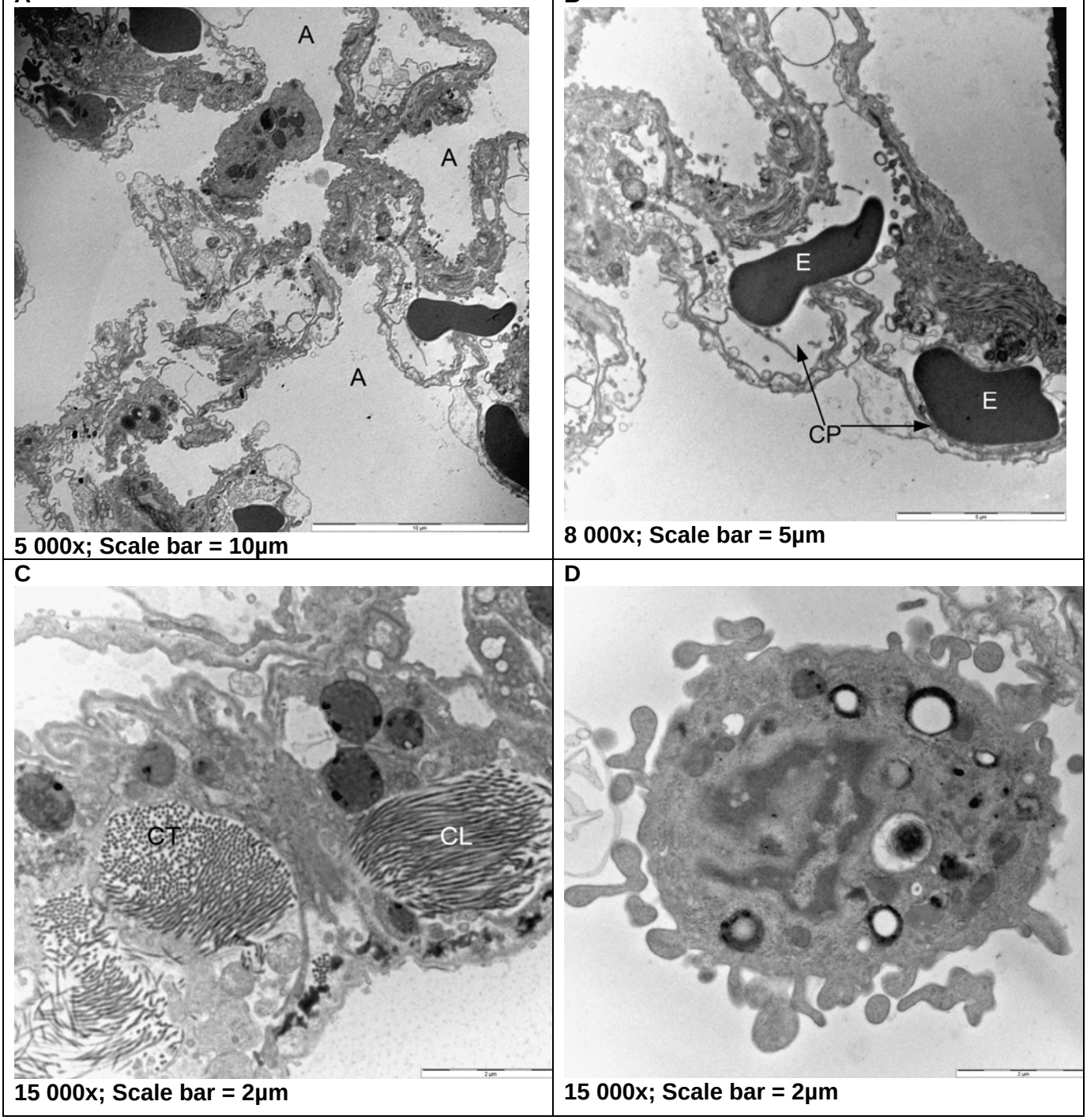
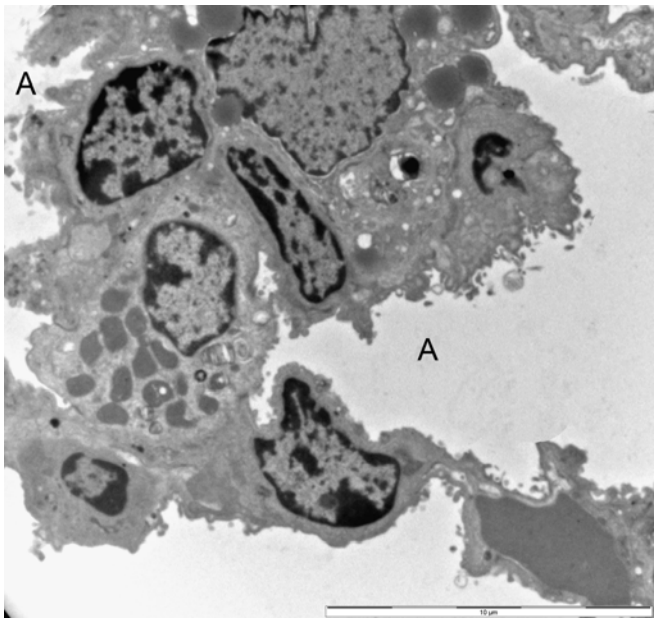


Figure 9.2 A-D Electron micrographs of the lungs taken from the control group

A Alveolus	**Nu** Nucleus
BM Basement membrane	**M** Macrophage
C Capillary	**Ma** Mast cell
Cl Collagen fibrils in longitudinal section	**Mi** Mitochondria
Ct Collagen fibrils in transverse section	**Mo** Monocyte
E Erythrocyte	**MV** Microvilli
En Endothelium	**P** Pseudopodia
F Fibroblast	**P_1** Type 1 Pneumocyte
L Lymphocyte	**P_2** Type 2 Pneumocyte
LB Lamellar bodies	**R** Residual bodies
N Neutrophil	

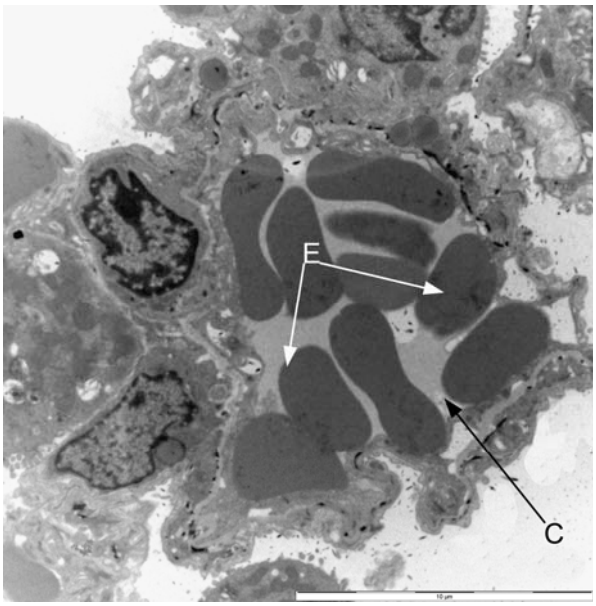
9.3.2.2 Asthma

A



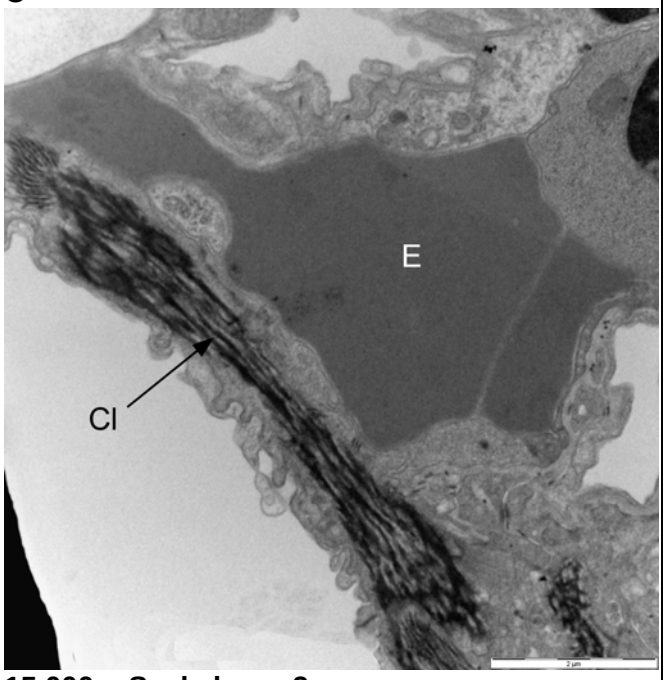
6 000x; Scale bar = 10µm

B



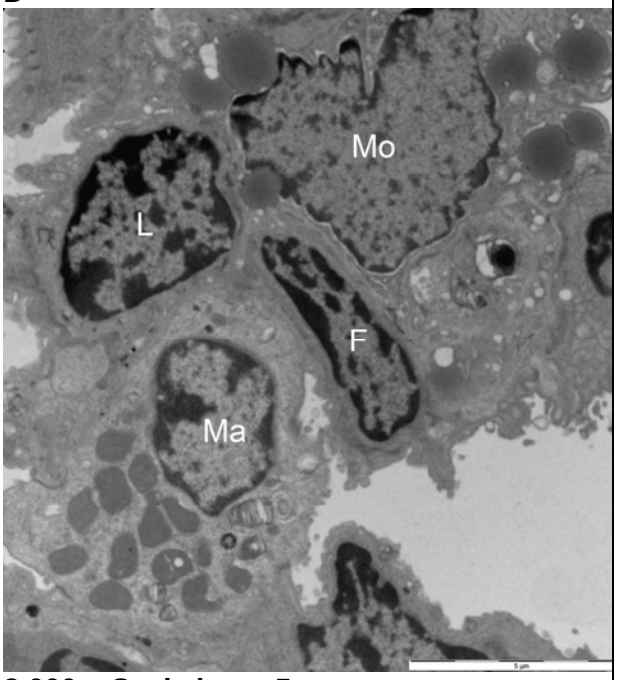
6 000x; Scale bar = 10µm

C

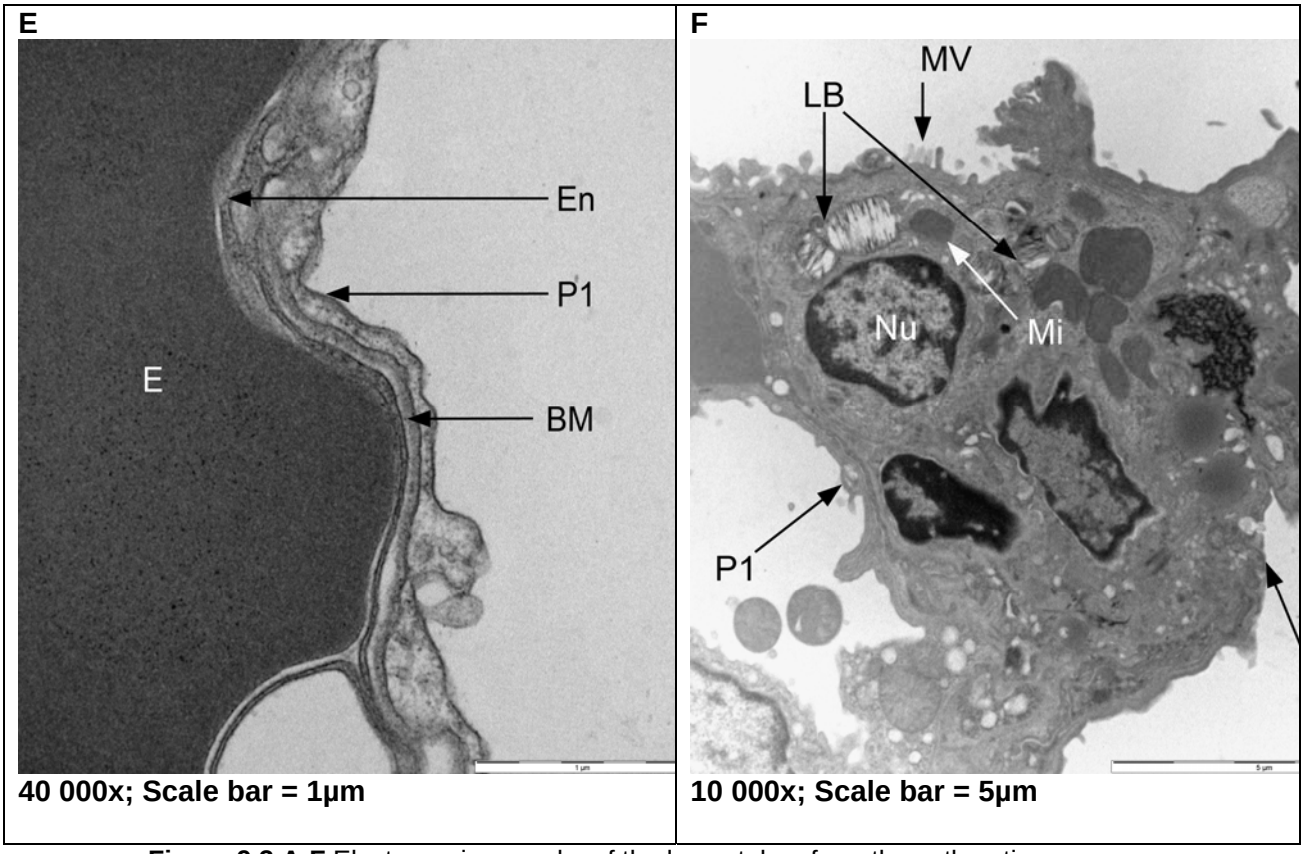


15 000x; Scale bar = 2µm

D



8 000x; Scale bar = 5µm



40 000x; Scale bar = 1µm

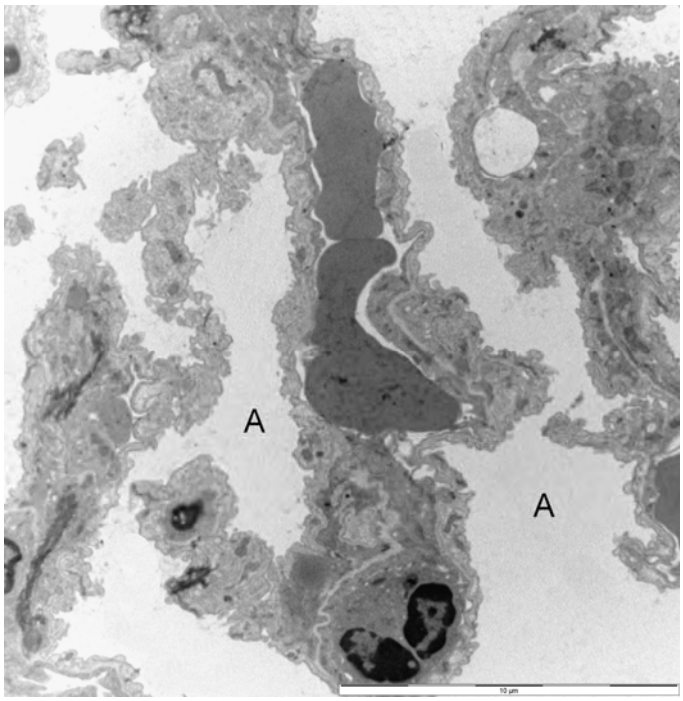
10 000x; Scale bar = 5µm

Figure 9.3 A-F Electron micrographs of the lungs taken from the asthmatic group

A Alveolus	**Nu** Nucleus
BM Basement membrane	**M** Macrophage
C Capillary	**Ma** Mast cell
Cl Collagen fibrils in longitudinal section	**Mi** Mitochondria
Ct Collagen fibrils in transverse section	**Mo** Monocyte
E Erythrocyte	**MV** Microvilli
En Endothelium	**P** Pseudopodia
F Fibroblast	**P_1** Type 1 Pneumocyte
L Lymphocyte	**P_2** Type 2 Pneumocyte
LB Lamellar bodies	**R** Residual bodies
N Neutrophil	

9.3.2.3 Modul8®

A



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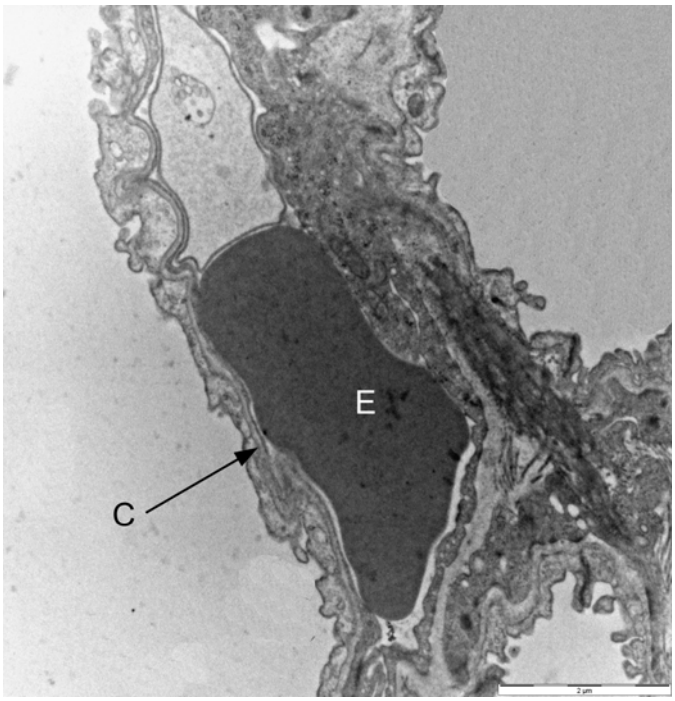
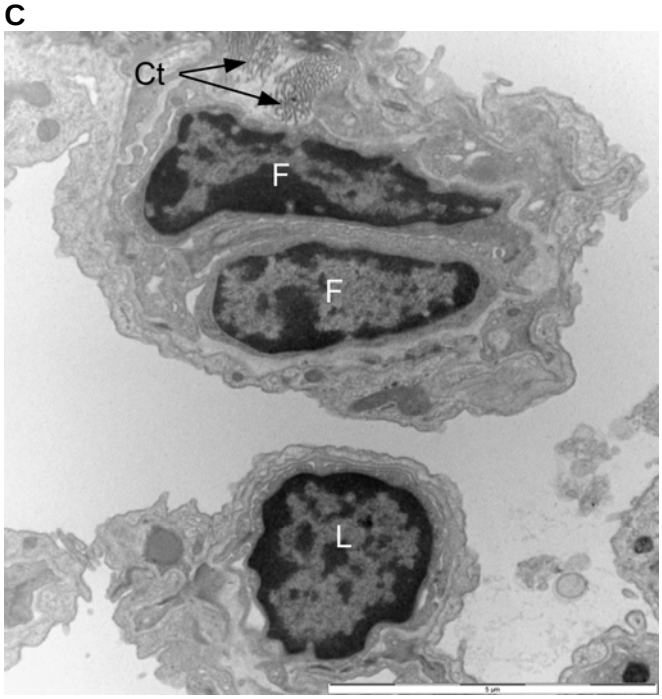
B



15 000x; Scale bar = 2µm

C



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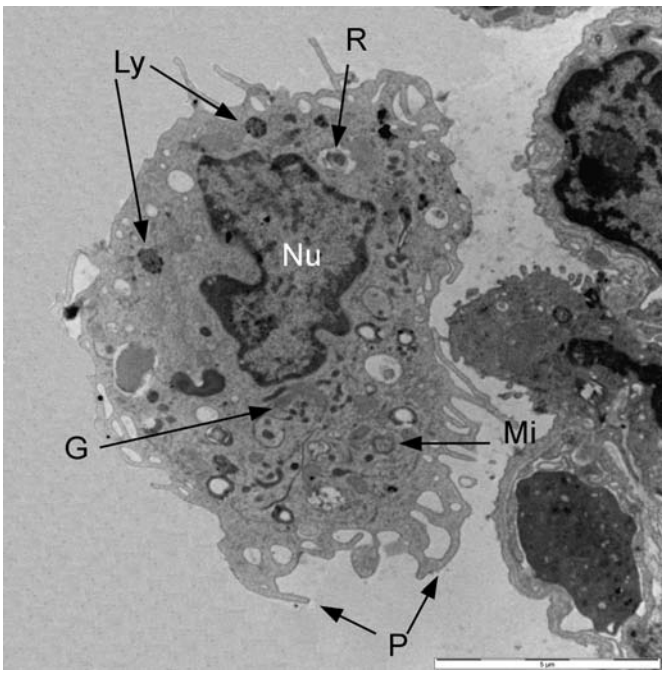
D



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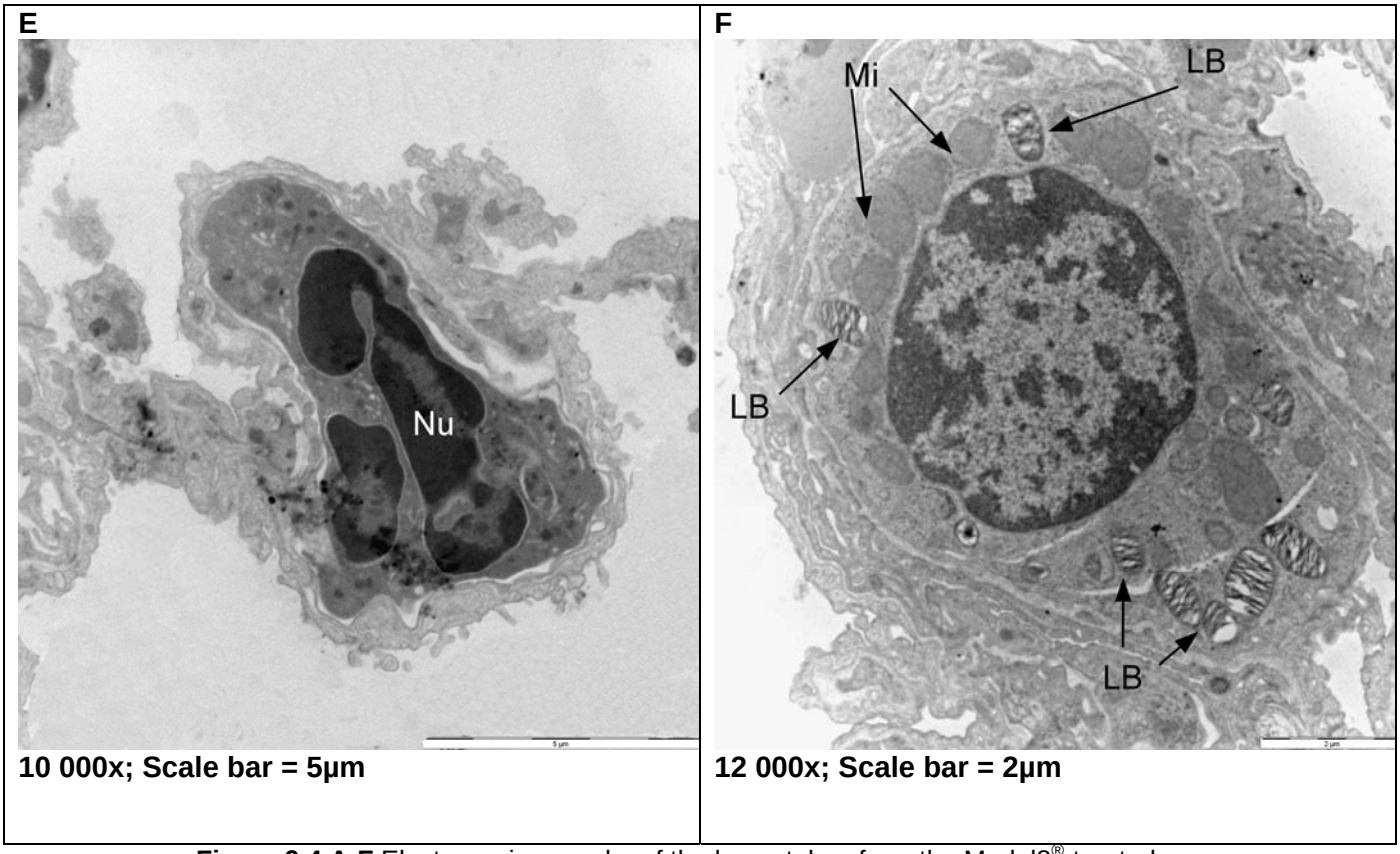
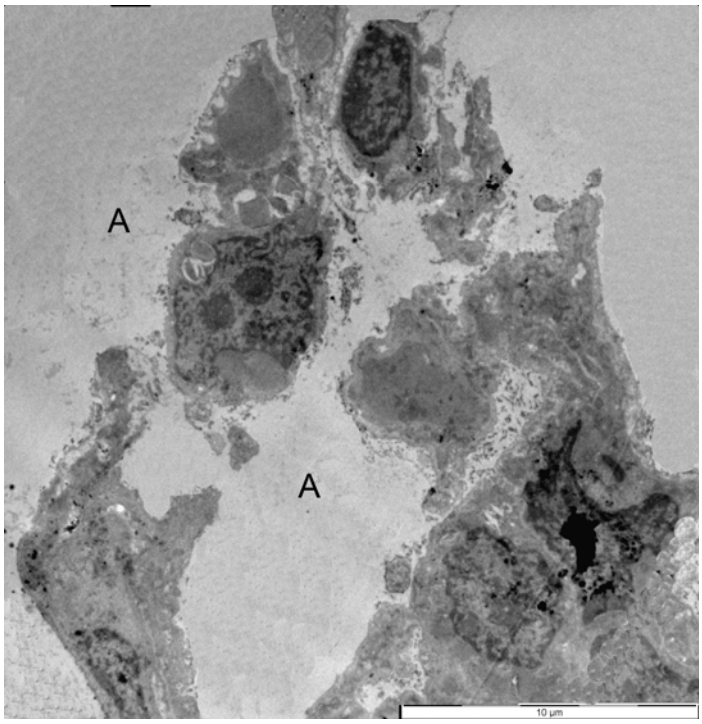


Figure 9.4 A-F Electron micrographs of the lungs taken from the Modul8®-treated group

A Alveolus	**Nu** Nucleus
BM Basement membrane	**M** Macrophage
C Capillary	**Ma** Mast cell
Cl Collagen fibrils in longitudinal section	**Mi** Mitochondria
Ct Collagen fibrils in transverse section	**Mo** Monocyte
E Erythrocyte	**MV** Microvilli
En Endothelium	**P** Pseudopodia
F Fibroblast	**P_1** Type 1 Pneumocyte
L Lymphocyte	**P_2** Type 2 Pneumocyte
LB Lamellar bodies	**R** Residual bodies
N Neutrophil	

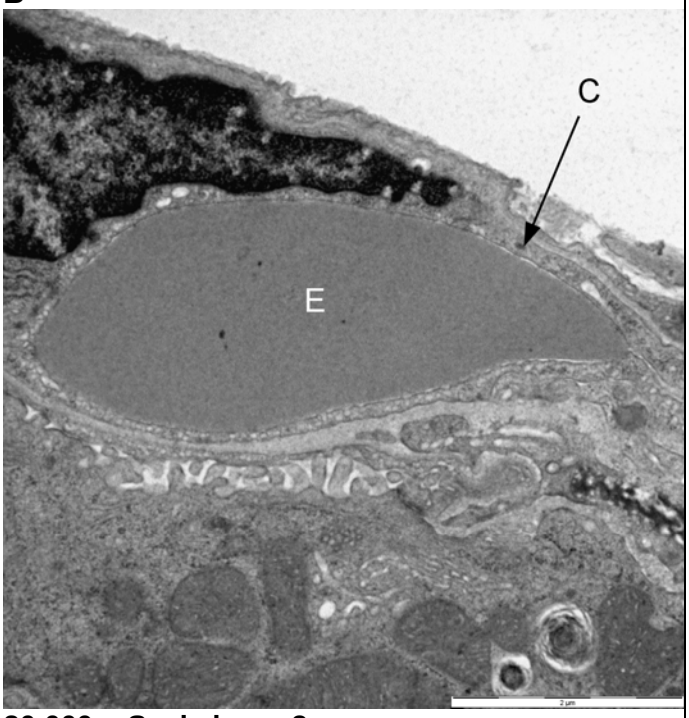
9.3.2.4 Hydrocortisone

A



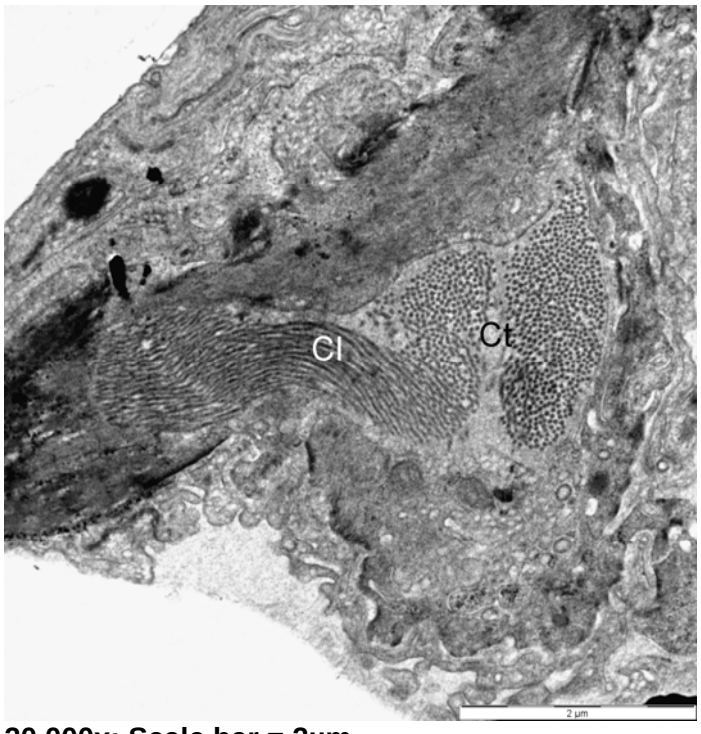
5 000x; Scale bar = 10µm

B



20 000x; Scale bar = 2µm

C



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D

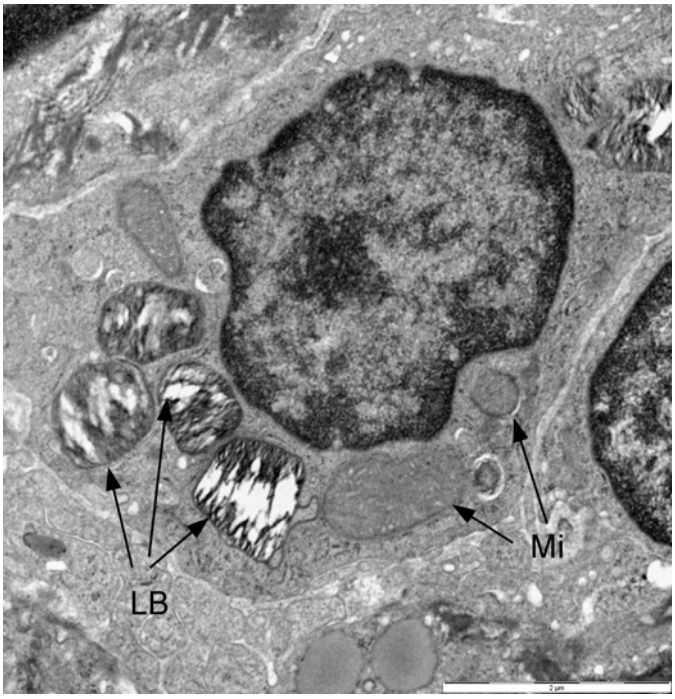

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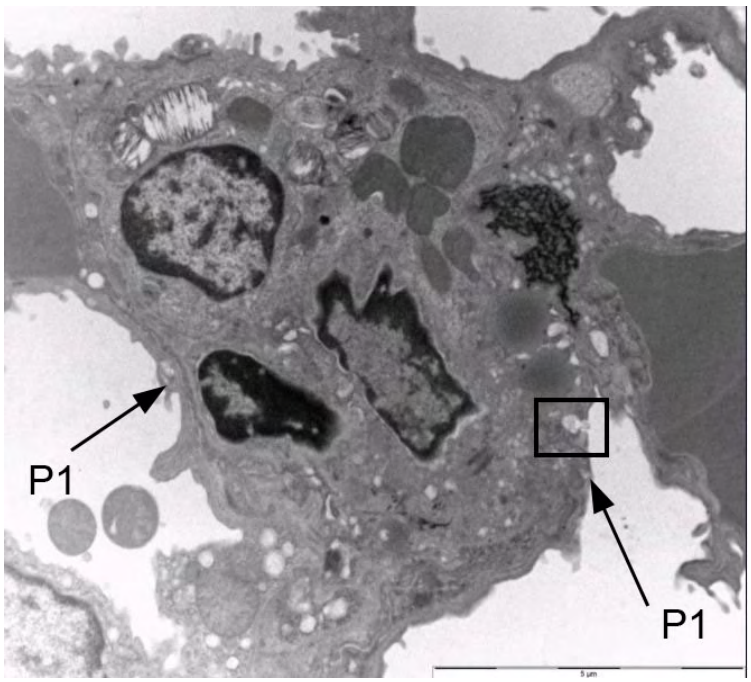
Figure 9.5 A-D Electron micrographs of the lungs taken from the Hydrocortisone-treated group

9.3.2.5 Caveolae



10 000x; Scale bar = 5 µm

Figure 9.6. Presence of possible caveolae in Type I pneumocyte

A Alveolus	**Nu** Nucleus
BM Basement membrane	**M** Macrophage
C Capillary	**Ma** Mast cell
Cl Collagen fibrils in longitudinal section	**Mi** Mitochondria
Ct Collagen fibrils in transverse section	**Mo** Monocyte
E Erythrocyte	**MV** Microvilli
En Endothelium	**P** Pseudopodia
F Fibroblast	**P_1** Type 1 Pneumocyte
L Lymphocyte	**P_2** Type 2 Pneumocyte
LB Lamellar bodies	**R** Residual bodies
N Neutrophil	

9.4 Discussion

Inflammatory conditions such as asthma involve a wide range of cell types and cellular mediators. The inflammatory cascade is a model that was developed to explain the complex process of inflammation. This model suggests that the inflammation associated with the asthmatic response occurs in seven different phases (Wenzel, 1994).

The process starts off with the *sensitization phase*, or the *antigen presenting phase*, which occurs as a result of antigen presentation to a T-lymphocyte. This phase involves a wide range of cells such as dendritic cells, monocytes and B lymphocytes. Upon delivering of the antigen to a T-lymphocyte, the latter responds by changing into an allergic T-Helper 2 or TH-2 cell and emits signals through a cytokine network. The interaction between the cytokines and the B-lymphocytes causes the B-lymphocytes to become plasma cells that produce IgE. This IgE binds to mast cells in order to bind the allergen, thereby completing the sensitization phase of the inflammatory cascade (Wenzel, 1994).

This phase is followed by the *stimulation phase* where a number of factors play a role in stimulating an exacerbation of the disease. Factors, including allergens and environmental agents, act through the triggering of mast cells and IgE together with the triggered mast cells can cause long-term asthmatic inflammation whereas blocking of IgE can limit reaction to allergen and thereby decrease the associated inflammation (Wenzel, 1994).

The next phase in the inflammatory cascade is called the *cell signaling phase* in which the recruitment of inflammatory cells into the airways takes place. In this phase a number of cells, cytokines and markers play a role. These include T-lymphocytes, macrophages and monocytes, IL-4 and IL-13 and TNF-alpha. The cell-signaling

phase is followed by the *migration phase* where leukocyte migration into the airways takes place. Eosinophils, neutrophils, lymphocytes and monocytes are some of the cells involved, and this process is possibly mediated by the release of chemo-attractant mediators by the signaling cells.

Activation of the inflammatory cells follow in order to produce the physiological changes associated with asthma. IL-1, IL-5, TNF-alpha, eotaxin and IL-8 play a role in the activation of the inflammatory cells. Eosinophils are activated in the lungs of asthmatic patients (Wenzel, 1994). An increased number of eosinophils in bronchial lavage fluid of asthmatic animals have also been reported which emphasizes the important role of eosinophils in the inflammatory process associated with asthma (Oberholzer *et al*., 2008). This was seen in chapter seven where the effect of Modul8® was investigated on blood count and bronchial lavage and a significant higher eosinophil count was observed in the asthmatic group.

One of the characteristics of asthma is the *tissue alteration*. This takes place in the epithelium, basement membrane, smooth muscles and nerves. The changes that occur in the epithelium of asthmatics include abnormal epithelium with the presence of increased mucus-secreting cells. The sub-epithelial basement membrane appears thickened due the action of the eosinophils. The connective tissue associated has an increased collagen deposition. The smooth muscle may appear hyperplastic as well as hypertrophic. The last phase in the inflammatory cascade includes the resolution phase where the hypothesis exists that abnormal or incomplete resolution of the inflammation may play a role in the disease and its severity (Wenzel, 1994).

In 2003, Evans *et al.* reported that murine models of asthma exhibit a TH2 lymphocyte-driven eosinophilic pulmonary inflammation, as well as airway hyperresponsiveness. Both are very important features of the inflammatory process

involved in human asthmatics (Evans *et al*., 2003). One of the most prominent features of asthma is eosinophil infiltration of the peribronchial regions; one of the characteristics of airway inflammation (Bousquet *et al*., 1990; Holgate *et al*., 1991; Kay, 1991b). It is also known that eosinophil numbers correlate with the severity of the disease (Corrigan and Kay, 1992).

The presence of eosinophils in the lungs of allergic mice is an indication and confirmation of asthma-like eosinophilic inflammation (Gleich and Kita, 1997; Geba and Askenase, 1997). In the current study this could also be observed in the lungs of the asthmatic animals (Figure 2A and B) where it appeared congested with very little open spaces between the alveoli. However, the lungs of the treated animals (both Modul8® and Hydrocortisone) (Figure 3A and B, Figure 4A and B) appeared similar to the control animals (Figure 1A and B) with less severe influx of inflammatory cells and less congested with larger open spaces between the alveoli.

It is therefore clear that the infiltration of inflammatory cells into the airways play a fundamental role in the pathogenesis of the disease and it is therefore important to mention the specific inflammatory cells that are involved as well as their specific contributory function in the inflammatory process in asthma. This will be discussed in the following paragraphs.

9.4.1. Infiltration of mixed inflammatory cells in the lung parenchyma

9.4.1.1 *Mast cells*

Mast cells play an important role in the pathogenesis of asthma as they are thought to be the mail link between IgE and AHR (Venkatesha *et al*., 2005; Renauld, 2001). In the lungs, the mast cells are situated in the bronchial airway connective tissue as well as in peripheral intra-alveolar spaces (Prussin and Metcalfe, 2006). Mast cells have different profiles of chemical release and the number of mast cells increases

after allergen exposure (Baraniuk, 1997). In asthma, however, mast cells are found in the bronchial smooth muscle bundles and the bronchial epithelium and they infiltrate in the airway mucus glands (Bradding *et al*., 2006). A number of mast cells could be identified in the asthmatic group and Figure 9.3 D is a representation thereof.

Cross-linking of antigen by mast cell IgE antibodies follows on the re-exposure to allergen and this cross-linking triggers activation of signaling cascades which cause mast cell degranulation and synthesis of pro-inflammatory molecules (Hart, 2001; Holsapple *et al*., 2006). The mediators that are produced by mast cells are categorized into three groups namely preformed mediators, newly synthesized lipid mediators and cytokines. The preformed mediators include histamine, whereas the newly synthesized lipid mediators include prostaglandins and leukotrienes and the cytokines include TNF-α (Galli *et al*., 2001). Cough, bronchospasm, smooth muscle constriction, edema, mucus secretion and inflammatory infiltrate are the immediate symptoms in the respiratory tract that are caused by the released mediators of the mast cells (Holsapple *et al*., 2006). In the current study a mast cell was identified in the asthmatic group as can be seen in Figure 9.3 D; label Ma.

9.4.1.2 *Basophils*

In 2005, Marone *et al.* reported that basophils were found in increased numbers in the airways of people who died from asthma (Marone *et al*., 2005). Basophils are also an important source of histamine and the CC chemokines are potent chemo attractants for basophils as well as eosinophils, monocytes and T lymphocytes (Verstraelen *et al*., 2008a). In the current study, basophils were not as prevalent as the other inflammatory cells as is also stated in Chapter 7 with the results of the blood smears. No basophils could be identified during the TEM analysis of the lung sections.

9.4.1.3 *Eosinophils*

IL-5 is known to be the major eosinophil-activating cytokine that plays a role in the differentiation, maturation, recruitment, activation and degranulation of the eosinophils at the sight of inflammation. Other cytokines and chemokines that play an important role in eosinophil differentiation, proliferation and recruitment include GM-CSF (granulocyte macrophage-colony stimulating factor), TNF-α, IL-3 and 4, eotaxin-1, RANTES (regulated upon activation, normal T-cell expressed and released), MIP-1α (macrophage inflammatory protein), MCP-3 (monocyte chemotactic protein) and MDC (macrophage- derived chemokine) (Foster *et al*., 2001; Lampinen *et al*., 2004). In the current study, as stated in chapter seven, the asthmatic group possessed a significantly higher eosinophil count. However, no eosinophils could be found during the TEM analysis of the lungs.

9.4.1.4 *Neutrophils*

The activation of neutrophils in peripheral blood results in the migration to the site of inflammation and adhesion to the endothelium (Sampson, 2000). The most significant chemotactic and activating factor for neutrophils include CXC chemokine ligand (CXCL)8 (IL-8) (Pignatti *et al*., 2005). In the current study, neutrophils could be identified in all the experimental groups when using TEM (Fig 9.4 E) and although the number of cells was not quantified, it could be linked to the findings obtained from the white blood cell counts in chapter 7.

9.1.4.5 *Macrophages*

Alveolar macrophages are one of the first cells to encounter inhaled compounds and they act by producing many different mediators that may play an important role in asthma (Verstraelen *et al*., 2008b). Experimental evidence has shown that alveolar macrophages have the potential to inhibit the immune activation and inflammatory cellular influx into the airways after exposure to inhaled respiratory allergens (Peters-

Golden, 2004; Verstraelen *et al*., 2008b). In the current study, macrophages could be identified in all the experimental groups and Figures 9.1 D and 9.4 D are a representation of macrophages from the control and Modul8®-treated groups.

9.1.4.6 *Structural cells*

There is also evidence pointing to a pro-inflammatory role of structural cells including fibroblasts, epithelial and endothelial cells as well as airway smooth muscle cells (ASM). The role of each of these structural cells will be described briefly.

- Fibroblasts

It has been stated previously that fibroblasts contribute to airway remodeling by playing a role in the maintenance of tissue integrity as well as the repair process in response to inflammation (Verstraelen *et al*., 2008a). Myofibroblasts are stated to be the main source of collagen in asthma as their numbers are increased in asthmatic individuals and therefore correlate with the extent of collagen deposition (Brewster *et al*., 1990). As early as 1989, Roche *et al*. reported that subepithelial fibrosis at the level of the reticular lamina, is an early and fundamental change within the airways and that this can also be observed in newly diagnosed asthma (Roche *et al*., 1989). It is the result from an increased deposition of collagens I, II and V, the glycoproteins fibronectin and tenascin, and the proteoglycans lumican, versican and biglycan (Roche *et al*., 1989; Wilson and Li, 1997; Huang *et al*., 1999). In 2006, Bergeron and Boulet also reported that this increase in the deposition of macromolecules may also form a compartment where adhesion molecules, cytokines, and other inflammatory mediators are stored, and where they participate in the inflammatory response (Bergeron and Boulet, 2006). In the current study, numerous fibroblasts were found in the asthmatic group when analyzing the TEM results, which could contribute to the increased collagen deposition that could also be identified in the asthmatic group (Figure 9.3 D label F). Fibroblasts were present in the other experimental groups as

well but were not as prevalent as in the asthmatic group. Fibroblasts from the Modul8® -treated group can be seen in figure 9.4 C, label F.

- Endothelial cells

Airway endothelial cells also play a role in the recruitment and activation of white blood cells such as basophils, eosinophils and lymphocytes into the airways, which is mediated by the expression of adhesion molecules and the production of chemo attractants on their luminal side (Smit and Lukacs, 2006). In the current study the endothelial cells appeared to be in tact and no pathology of the endothelial cells could be observed. Figure 9.3 E shows the perfectly in tact components of the air-blood barrier including the endothelium.

- **Airway smooth muscle cells**

Hyperplasia and hypertrophy of the ASM cells contribute to airway hyperresponsiveness and airway obstruction and therefore ASM cells are also involved in the pathogenesis of asthma (McKay *et al*., 2001). ASM cells have surface receptors, which can be activated to release inflammatory mediators that also contribute to airway dysfunction and bronchial inflammation (Burgess *et al*., 2004; Panettieri, 2002). Other than the above-mentioned properties of ASM cells they also secrete a large number of matrix proteins and a number of these proteins are specifically increased when cells are exposed to allergic stimuli (Black and Johnson, 2002). In the current study no ASM cell were identified in the TEM analysis of the lungs.

9.4.2 *Function of Type I and Type II pneumocytes*

Type I pneumocytes are squamous cells that line about 95% of the alveoli, and are also known as type I alveolar cells. Occluding junctions connect these type I pneumocytes to one another and to other cells of the alveolar epithelium. These cells

form part of the diffusion barrier for gas exchange. Type I pneumocytes line the lateral cell margins of type II pneumocytes and are joined to the type II pneumocytes via occluding junctions (Ross *et al*., 2003).

Type II pneumocytes are secretory cells and constitute about 5% of the alveolar air surface and are interspersed among the type I alveolar cells. Lamellar bodies are characteristic of these cells. These cells are responsible for the secretion of surfactant since they are rich in phospholipids, neutral lipids and proteins. These products are secreted by exocytosis and form an alveolar-lining surface-active agent, which is the surfactant. Among their secretory function, type II pneumocytes are also progenitor cells for type I alveolar cells. Typical ultrastructure of type II pneumocytes reveals a dome-shaped apical surface with a number of short microvilli present at the periphery (Ross *et al*., 2003). In the current study, type I and II pneumocytes could be identified in the TEM analysis as can be seen in Figure 9.3 F, 9.4 F and 9.5 D.

9.4.3 *The presence of caveolae in the airways*

Caveolae are 50-100nm in diameter and are characterized as omega-shaped invaginations of the plasma membrane of which caveolin- an integral membrane protein- is the principal component of caveolae membranes *in vivo* (Gosen *et al*., 2008). Caveolae were first discovered in 1953 when they were identified as plasmalemmal vesicles and in 1955 these vesicles were named caveolae (Palade, 1953; Yamada, 1955). Caveolae are thought to play an important role in membrane trafficking and cell signaling, endocytosis and intracellular cholesterol transport and therefore caveolae and caveolins are likely to play an important role in human physiology and pharmacology (Patel *et al*., 2008). The prominent morphological features of caveolae are recognized in many different cell types including adipocytes, muscle cells, fibroblasts, capillary endothelium and type I alveolar epithelial cells

(Gumbleton, 2001). In the current study a possible caveolae could be identified in the asthmatic group and are present in the type I pneumocyte as indicated by figure 9.6.

9.5. Conclusion

From the findings in the current chapter it can therefore be concluded that Modul8®, histologically and ultrastructurally has the ability to stabilize the changes and remodeling caused in the lungs by the inflammatory process involved in asthma. Modul8® also significantly decreased the eosinophil counts in the bronchial lavage of the asthmatic animals as was shown in chapter 7. It is therefore suggested that Modul8® possesses anti-inflammatory properties and might therefore successfully be used in the treatment of asthma.

CHAPTER 10

CONCLUDING DISCUSSION

Complementary and alternative medicine (CAM) is a fast developing discipline that became widely studied over the past few years. Currently there is an explosion in this field as more and more researchers focus on investigating alternative ways of healing and finding alternative treatment approaches to conventional medicine. Although this approach can be quite challenging, knowledge gained through this research can lead to a better understanding of the interactions and synergistic effects of alternative medicine on human diseases.

One of the emerging and most successful tools in studying the effects of CAM on many different diseases is an animal model. Animal models are widely used to study diseases and to gain important information on the pathophysiology of human disease and this can be seen by the numerous different animal models available. The BALB/c murine asthmatic animal model was the model of choice in this study, as it has been proved to offer excellent tools in studying allergic diseases (Epstein, 2004; Epstein, 2006). The immune and inflammatory mechanisms of asthma have also been studied since there is a correlation between the allergic response in sensitive mice and that of human asthmatics (Blyth *et a*l., 1996; Hogan *et al*., 1996).

Although it is known that some immunomodulators have an effect on the immune system, as was proven by previous researchers, the anti-inflammatory and immune-modulatory effects of Modul8® have never been investigated before. Therefore, the current study reported on the effect of Modul8® on ultrastructural, hematological and immunological parameters in the treatment of asthma by using the BALB/c murine asthmatic animal model. This model was successfully implemented over a 43-day period during which the animals were sensitized, nebulized and treated.

Ultrastructure of platelets and fibrin networks were investigated since platelets and fibrin play an important physiological role in the inflammatory process involved in asthma as they actively participate in most of the main features that are seen in asthmatic patients. Researchers have previously reported on the effect of asthma on the ultrastructure of platelets and fibrin networks (Pretorius *et al*,. 2007a). The current study, as can be seen in chapter 4, revealed that Modul8® has the ability to stabilize platelet aggregates and fibrin networks to a profile similar to that of control animals. Ultrastructure of the platelet aggregates and fibrin networks of the asthmatic animals appeared similar to what was previously found (Pretorius *et al*,. 2007a) with the minor fibers forming a thick, matted layer over the major fibers and the platelet aggregated appeared loosely-arranged in comparison to the control animals.

To address the question whether these results, found in the asthmatic BALB/c mice, are comparable to that of human asthmatics, chapter 5 compared control and asthmatic platelets and fibrin networks from the BALB/c animal model to that of human controls and asthmatics. The results obtained indicated that the fibrin and platelet ultrastructure of the BALB/c asthmatic animals are indeed comparable to that found in the analysis of the platelet and fibrin ultrastructure in human asthmatics and therefore supports the use of this model in asthma.

Weight plays a role in diseases like asthma and a number of animal studies have reported on the weight fluctuation and effect of alternative medication on asthma (Harris *et al*., 1998; Retana-Ma´rquez *et al*., 2003; Snibson *et al*., 2005).

In the current study the weight pattern of the animals were documented and reported on over the 43-day experimental period to determine whether any fluctuation in their weight patterns occurred. From the results obtained it is hypothesized, that Modul8®

might have a slight stabilizing effect on the asthmatic animals when their baseline weights are taken into consideration.

Immunomodulators are known to have an effect on the immune system and to enhance the immunity of an individual to have a positive effect on a particular immunological response. It is also known that white blood cell counts as well as platelet distribution are altered in asthmatic patients (Larsen *et al*., 2007). Therefore, the effect of Modul8® on white blood cell count in the blood as well as bronchial lavage were investigated in chapter 7 to determine whether Modul8® has the ability to lower the white blood cell counts in the asthmatic animals to a profile similar to that of the control animals. The results found indicated that the asthmatic animals possessed a significantly higher eosinophil count in both the blood and bronchial lavage in comparison to the other experimental groups. Modul8® positively altered the white blood cell counts in the blood and bronchial lavage as well platelet distribution to a profile similar to that found in the control animals, indicating that this product might successfully be used in the treatment of this disease.

It was previously found that immunomodulators have the ability to activate macrophages to produce many different inflammatory mediators (Piemonte and Buchi, 2002). In the current study mice peritoneal macrophages were stimulated with LPS, which is known to activate macrophages, as well as with Modul8® to investigate the ultrastructure of the activated macrophages as well to evaluate the production of two inflammatory mediators TNF-α and nitric oxide from the activated macrophages.

The results indicated that Modul8® has the ability to activate macrophages to possibly enhance immune function since it is suggested that macrophages have to be activated to exert a certain immunological function. Evaluation of the production of inflammatory mediators TNF-α and NO produced rather interesting results since

neither of the two mentioned mediators could be detected in cell culture. With these results it can be hypothesized that the release of these two mediators might take place via an alternative pathway as was also previously found by researchers (An *et al*., 2006).

Histological and ultrastructural changes in the lungs of asthmatic animals and asthmatic animals treated with Modul8® and hydrocortisone were investigated and compared to the lungs of control animals. The results found revealed that Modul8® has the ability to stabilize the changes and remodeling found in the airways of the asthmatic animals and restored it to a profile similar to that of the control animals.

The results found in this thesis clearly indicate that the homeopathic immunomodulator, Modul8®, plays an important role in the inflammatory response in asthma. This homeopathic product has the ability to stabilize the alterations in the asthmatic animals on ultrastructural and hematological level. Ultrastructural alterations include the morphology of platelet aggregates, fibrin networks and peritoneal macrophages as well as the remodeling in the lungs with the infiltration of inflammatory infiltrate. Hematological changes include the restoration of white blood cell counts and platelet distribution in the blood as well as bronchial lavage.

Therefore, it can be concluded that this product might successfully be used as an adjuvant to conventional medicine in the treatment of asthma and possibly also in other inflammatory conditions. Since no side effects of this product are known up to date and since all the components are of natural origin, this product might be useful in the treatment of inflammatory diseases without any of the side effects of some of the known asthma medication.

The research reported in this thesis on an alternative way of treating asthma, might also add on to the pool of knowledge on CAM and its role in human diseases. However, since the main focus of this thesis was ultrastructure and morphology, more extensive research on the exact mechanism of action of the active substances in this product and the immunological pathways involved might be of importance in future research.