

**Evaluation of the anti-inflammatory properties of a
complex homeopathic product Modul8[®] using the
BALB/c murine asthmatic animal model**

By

Hester Magdalena Oberholzer

Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy

(PhD)

In the Faculty of Health Sciences

Department of Anatomy

University of Pretoria

South Africa

Supervisor: **Prof E. Pretorius**

Department of Anatomy

Faculty of Health Sciences

2009

**Evaluation of the anti-inflammatory properties of a complex
homeopathic product Modul8® using the BALB/c murine
asthmatic animal model**

By

NANETTE OBERHOLZER

Supervisor: Prof E. Pretorius

Department: Anatomy

Degree: PhD (Anatomy)

Abstract

Modul8® is a complex homeopathic product which acts as an immunomodulator by enhancing the immunity of an individual in order to favour a particular immunological response. Asthma is a chronic inflammatory disease to which many different treatment approaches have been applied. The aim of the current study was to investigate the effect of this natural, homeopathic product on ultrastructural, immunological and haematological parameters in the treatment of asthma by using the BALB/c asthmatic animal model.

Mice have traditionally been used as experimental animals in studying human diseases and although there is no animal model that is an exact replica of the human disease, they have helped to understand some of the basic mechanisms involved as well as the development of new therapies. Since there is a correlation between allergic mice and human asthmatics, the BALB/c murine asthmatic animal model was implemented and used in the current study.

Implementation of the BALB/c murine asthmatic animal model involved the sensitization, nebulization and treatment of mice over a period of 41 days. Mice were randomly divided into four experimental groups including a control group, an asthmatic group receiving no treatment, an asthmatic group exposed to physiological comparable levels of Modul8® and lastly an asthmatic group exposed to hydrocortisone as positive control. The animals were also weighed on specific reference days throughout the study to determine if any significant changes exist between the experimental groups' weights over the experimental period.

Haematological analysis revealed that Modul8® possesses the ability to alter the asthmatic profile of the white blood cell counts and stabilize it to levels comparable with the control group. In the ultrastructural analysis of platelets and fibrin networks it seemed that Modul8® stabilized platelet and fibrin morphology to a profile similar to that of control animals. Comparison of the ultrastructure of platelets and fibrin networks of the BALB/c asthmatic animals to that of human asthmatics revealed that this animal model adequately mimics the human disease on this specific level. Modul8® was also shown to activate mice peritoneal macrophages *in vitro* and this was linked to the important role of activated macrophages in the immune response. Histological and ultrastructural analysis of the lungs showed that Modul8® has the ability to stabilize the changes and remodelling caused in the lungs by the inflammatory process involved in asthma.

It is therefore concluded that this homeopathic immunomodulator has the ability to positively influence the chosen parameters of the inflammatory response involved in asthma and therefore might successfully be used as an adjuvant therapeutic approach to the known anti-asthmatic and anti-inflammatory treatments.

Declaration

I, Hester Magdalena Oberholzer, hereby declare that this thesis entitled:

“Evaluation of the anti-inflammatory properties of a complex homeopathic product Modul8[®] using the BALB/c murine asthmatic animal model”

Which I herewith submit to the University of Pretoria for the Degree of Doctor of Philosophy in Anatomy, is my own original work and has never been submitted for any academic award to any other tertiary institution for any degree.

Date

H. M. Oberholzer

**Department of Anatomy, Faculty of Health Sciences,
University of Pretoria,
Pretoria
South Africa**

Acknowledgements

"I can do all things through Him who gives me strength"

-Philippians 4:13-

When I look back over the past two years, I realize with gratitude how fortunate I was to be granted an opportunity to do a PhD. This has been a wonderful journey and I have grown in so many fields of my life and learned so much about research. This is truly a dream come true and I will forever be grateful to Him who guided me every step of the way and who carried me through tough times. I thank God for granting me the gift of wonderful people who have made enormous contributions to my life. I truly believe that I could not have done this on my own and everyone mentioned here deserves to share my delight. I would therefore like to express my appreciation to the following people:

- ❑ My wonderful parents who have always believed in me and that this dream would one day become a reality. They laid a solid foundation for me in terms of discipline, hard work and morality; and that is what shaped me into the person I am today. I dedicate this thesis to you. My brother and sister for their love and motivation and Ruan for his ever-available words of encouragement and for his unconditional love and support.
- ❑ Professor Resia Pretorius, who is the best mentor and role model anyone could wish to have. She taught me everything I know about research. With her patience and motivation, she walked this path with me and was there whenever I needed her, with expert advice and words of encouragement.

- ❑ Marleze Rheeder at the UPBRC at Onderstepoort for her professionalism and expert handling of the animal study, and for the sacrifices she made to work long hours on this study. I am also indebted to everyone who helped with the termination of the animal study: Ilse Janse van Rensburg, Marnie Potgieter, Helena Steyn, and the Cell Biology Honours class of 2008.
- ❑ Mr. Chris van der Merwe at the Unit for Microscopy and Microanalysis for his ever-available advice and for all his help in so many different ways throughout my study. I would also like to extend a special word of thanks to the remainder of the staff at the Unit for their assistance on the various microscopes when needed.
- ❑ Professor Megan Bester for all her help and advice with my lab work, especially the ELISA.
- ❑ Warren Vieira for all his help with the statistical analysis as well as assistance in the lab. Marnie Potgieter for her help with the preparation of the samples and for just being there, offering her help on so many different occasions and Wendy van der Spuy, for assisting me in the lab whenever needed. I really appreciate it all.
- ❑ Lastly, to each and everyone who made a contribution to this project, no matter the nature thereof, I wish to convey my sincere thanks. I am infinitely grateful for the privilege granted to me to work with such wonderful people throughout this study.

Articles Published

- **Pretorius E., Oberholzer H. M.** Ultrastructural changes of platelets and fibrin networks in human asthma: a qualitative case study. *Blood coagulation and fibrinolysis* 2009; **20**: 146-149
- **Smit E., Oberholzer H. M., Pretorius E.** A Review of immunomodulators with focus on the Homeopathic product *CANOVA*. *Homeopathy* 2009; **98**: 169-176
- **Oberholzer H. M., Vieira W. A., Pretorius E.** Investigating the effect of the homeopathic immunomodulator, MODUL8[®], on blood count, bronchial lavage and fibrin ultrastructure on experimental asthmatic BALB/c mice. *International Journal of Morphology* 2009; 27(**3**): 955-963
- **Oberholzer H. M., Pretorius E.** Investigating lung remodeling in MODUL8[®] - treated BALB/c asthmatic animals. *Micron* 2009; **40**: 775-782

TABLE OF CONTENTS

CHAPTER 1.....	1
GENERAL INTRODUCTION	2
CHAPTER 2.....	6
LITERATURE REVIEW	7
2.1 Introduction	7
2.1.1 Asthma in general	8
2.1.2 Etiology.....	9
2.1.3 Immunology.....	11
2.1.3.1 Mast cells	12
2.1.3.2 Macrophages.....	13
2.1.3.3 Dendritic cells	13
2.1.3.4 Eosinophils	14
2.1.3.5 Neutrophils	15
2.1.3.6 T-lymphocytes	15
2.1.3.7 Inflammatory mediators.....	15
2.1.4. Pathology and airway remodelling	20
2.1.5 Genetics and asthma	22
2.1.6 Treatment of asthma	23
2.1.7 Alternative and complimentary medicine.....	28
2.1.8 Homeopathy.....	31
2.1.9 Animal models in asthma	43
2.2 Aims and objectives of the current study	44
2.3 Hypothesis	45
CHAPTER 3.....	46
IMPLEMENTATION OF THE BALB/c ASTHMATIC MOUSE MODEL	47
3.1 Introduction	47

3.2 Implementing the asthmatic BALB/c mouse animal model	49
3.3 Sensitization	50
3.4 Nebulization	50
3.5 Treatment	51
3.6 Conclusion	52
CHAPTER 4	53
INVESTIGATING THE EFFECT OF MODUL8® ON THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF EXPERIMENTAL ASTHMATIC BALB/c MICE	
BALB/c MICE	54
4.1 Introduction	54
4.2.1 Preparation of fibrin clots.....	56
4.2.2 Preparation of washed fibrin clot for SEM	57
4.3 Results	58
4.4 Discussion	67
4.5 Conclusion	68
CHAPTER 5	69
COMPARING THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF THE ASTHMATIC BALB/c MICE TO THAT OF HUMAN CONTROL AND ASTHMATIC SUBJECTS	
CONTROL AND ASTHMATIC SUBJECTS	70
5.1 Introduction	70
5.2 Materials and Methods.....	71
5.2.1 Samples	71
5.2.2 Preparation of fibrin clots.....	72
5.2.3 Preparation of washed fibrin clot for SEM	73
5.3 Results	74
5.4 Discussion	80
5.5 Conclusion	81

CHAPTER 6.....	82
INVESTIGATING THE EFFECT OF MODUL8® ON WEIGHT CHANGES IN THE ASTHMATIC BALB/C MOUSE MODEL	83
6.1 Introduction	83
6.2 Materials and Methods.....	85
6.2.1 Weighing analysis	85
6.3 Results	90
6.3.1 Inter- group analysis.....	90
6.3.2 Intra-group analysis.....	91
6.4 Discussion	94
6.5 Conclusion	96
CHAPTER 7.....	98
INVESTIGATING THE EFFECT OF MODUL8® ON BLOOD COUNT AND BRONCHIAL LAVAGE OF THE EXPERIMENTAL ASTHMATIC BALB/c MICE	99
7.1 Introduction	99
7.2 Materials and Methods.....	100
7.2.1 Blood smears techniques	100
7.2.2 Bronchial lavage techniques	101
7.3 Results	101
7.3.1 Bronchial lavage.....	101
7.3.2 Blood counts.....	105
7.4 Discussion	109
7.5 Conclusion	111
CHAPTER 8.....	113
LPS- AND MODUL8® - STIMULATED ACTIVATION OF PERITONEAL MACROPHAGES IN VITRO AND EVALUATION OF CYTOKINE AND NITRIC OXIDE PRODUCTION.....	113
8.1 Introduction	113

8.2 Materials and Methods	118
8.2.1 Preparation of the plastic stubs	118
8.2.2 Experimental groups	118
8.2.3 Obtaining and culturing of macrophages.....	118
8.2.4 Preparation of the macrophages for scanning electron microscopy ..	119
8.2.5 Statistical analysis	119
8.2.6 ELISA – Enzyme-linked immunosorbent assay	120
8.2.7 Griess assay for NO production	120
8.3 Results	120
8.3.1 Scanning electron microscopy	120
8.3.2 Statistical analysis.....	123
8.3.3 ELISA	125
8.3.4 Griess assay for NO production	126
8.4 Discussion	126
8.5 Conclusion	128
CHAPTER 9.....	129
INVESTIGATING CHANGES IN THE LUNGS OF CONTROL, ASTHMATIC AND	
HYDROCORTISONE-TREATED BALB/c MICE USING LIGHT AND	
TRANSMISSION ELECTRON MICROSCOPY	130
9.1 Introduction	130
9.2 Materials and Methods.....	132
9.2.1 Tissue for Histology.....	132
9.2.2 Tissue for Transmission Electron Microscopy.....	133
9.3 Results	133
9.3.1 Light microscopy	133
9.3.2 Transmission electron microscopy	137
9.3.2.1 Control.....	139
9.3.2.2 Asthma	141

9.3.2.3 Modul8®	143
9.3.2.4 Hydrocortisone	145
9.3.2.5 Caveolae	146
9.4 Discussion	147
9.4.1. Infiltration of mixed inflammatory cells in the lung parenchyma	149
9.4.1.1 Mast cells	149
9.4.1.2 Basophils	150
9.4.1.3 Eosinophils	151
9.4.1.4 Neutrophils	151
9.4.1.5 Macrophages	151
9.4.1.6 Structural cells	152
9.4.2 Function of Type I and Type II pneumocytes	153
9.4.3 The presence of caveolae in the airways	154
9.5. Conclusion	155
CHAPTER 10	156
CONCLUDING DISCUSSION	157
CHAPTER 11	162
References	162
Appendix A	
Pretorius E., Oberholzer H. M. Ultrastructural changes of platelets and fibrin networks in human asthma: a qualitative case study. Blood coagulation and fibrinolysis 2009; 20: 146-149	
Appendix B	
Smit E., Oberholzer H. M., Pretorius E. A Review of immunomodulators with focus on the Homeopathic product CANOVA. Homeopathy 2009; 98: 169-176	
Appendix C	
Oberholzer H. M., Vieira W. A., Pretorius E. Investigating the effect of the homeopathic immunomodulator, MODUL8®, on blood count, bronchial lavage and	

fibrin ultrastructure on experimental asthmatic BALB/c mice. International Journal of Morphology 2009; 27(3): 955-963.

Appendix D

Oberholzer H. M., Pretorius E. Investigating lung remodeling in MODUL8[®] -treated BALB/c asthmatic animals. Micron 2009; 40: 775-782

LIST OF FIGURES, TABLES AND DIAGRAMS

Table 2.1	Clinical trials done on HIV and cancer using an immunomodulator as treatment remedy	41
Table 3.1	The different experimental procedures conducted on specific days	50
Figure 4.1a	Control fibrin network of BALB/c mice	58
Figure 4.1b	Control platelet aggregate of BALB/c mice	59
Figure 4.1c	Fibrin network of asthmatic BALB/c mice	60
Figure 4.1d	Platelet aggregate of asthmatic BALB/c mice	61
Figure 4.1e	Fibrin network from asthmatic BALB/c mice treated with Modul8®	62
Figure 4.1f	Platelet aggregate from asthmatic BALB/c mice treated with Modul8®	63
Figure 4.1g	Fibrin network from asthmatic BALB/c mice treated with Hydrocortisone	64
Figure 4.1h	Platelet aggregate from asthmatic BALB/c mice treated with Hydrocortisone	65

Figure 5.1a	Control platelet aggregate forming dense round aggregate with pseudopodia	74
Figure 5.1b	Control fibrin network with thick, major fibers as well as thin, minor fibers	75
Figure 5.1c	Platelet aggregate and fibrin network from asthmatic mouse from the BALB/c asthmatic animal model	76
Figure 5.2	Platelet aggregate from controlled asthmatic subject with course, granular aggregate indicated with thick black arrow	77
Figure 5.3	Platelet aggregate and fibrin network from uncontrolled asthmatic individual.	78
Table 6.1	The different experimental procedures conducted on specific days	86
Table 6.2	The reference days chosen on which the various experimental procedures were conducted	87
Graph 6.1	The average mass changes of the five experimental groups over the experimental period	90
Table 6.3	A table of the relative mass changes for each of the experimental groups, relative to their baseline mass, upon specific days of the BALB/c murine models implementation	91
Table 6.4	The output derived from the various statically tests run	92
Table 7.1	The statistical comparisons preformed upon the various WBC species derived from the bronchial lavage	102

Graph 7.1	Average number of the white blood cells counted in the bronchial lavage	103
Figure 7.1	Platelet distribution in the bronchial lavage in the four different experimental groups	104
Table 7.2	The statistical comparisons performed upon the various WBC species derived from the blood	106
Graph 7.2	Average number of white blood cells counted in the blood smears	107
Figure 7.2	White blood cells in the blood smears of BALB/c mice	108
Figure 8.1	Diagram of LPS-stimulated activation of mice peritoneal macrophages	114
Figure 8.2	Scanning electron micrographs of mice peritoneal macrophages	122
Table 8.1	Average maximum macrophage diameter	124
Figure 8.3	Scanning electron micrographs of mice peritoneal macrophages to indicate measurements	124
Graph 8.1	Standard curve of increasing TNF- α concentration	125
Figure 9.1	Lung sections of the mice stained with Toluidine Blue	135

Figure 9.2	Electron micrographs of the lungs taken from the control group	139
Figure 9.3	Electron micrographs of the lungs taken from the asthmatic group	141
Figure 9.4	Electron micrographs of the lungs taken from the Modul8 [®] -treated group	143
Figure 9.5	Electron micrographs of the lungs taken from the HC-treated group	145
Figure 9.6	Presence of possible caveolae in Type pneumocyte	146

LIST OF ABBREVIATIONS AND SYMBOLS

AD	Anno Domini
AHR	Airway hyperreactivity/ hyperresponsiveness
AIP	Actin interacting protein
Al(OH)₃	Aluminum Hydroxide
ANOVA	Analysis of Variance
APC	Antigen presenting cell
ASM	Airway smooth muscle
BC	Before Christ
BMI	Body mass index
CaCO₃	Calcium carbonate
CAM	Complementary and alternative medicine
CD	Cluster of differentiation
COX	Cyclooxygenase
CRTH	Chemo attractant receptor homologous molecule expressed on TH ₂ cells
CysLTs	Cystenyl leukotrienes
CXCL	Chemokine ligand
D	Declared potencies
DPBS	Dulbecco's phosphate buffered saline
EAR	Early-phase asthmatic reaction
EEG	Electroencephalogram
EGF	Epidermal growth factor

ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FGF	Fibroblast growth factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HbsAG	Hepatitis B surface antigen
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
ICS	Inhaled corticosteroids
IES	Inhalation exposure system
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IGF	Insulin-like growth factor 1
IL	Interleukin
JNK	c-JunN-terminal kinase
LAR	Late-phase asthmatic reaction
LABA	Long acting beta agonist
LPS	Lipopolisaccharide
LTB₄	Leukotriene B ₄
LTC₄	Leukotriene C ₄
LTD₄	Leukotriene D ₄
LTRAs	Leukotriene receptor antagonists

MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MDC	Macrophage- derived chemokine
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NCSS	Number Cruncher Statistical System
NF-κB	Nuclear factor- kappa B
NHLBI	National Heart Lung and Blood Institute
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
OsO₄	Osmium tetroxide
OVA	Ovalbumin
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGD₂	Prostaglandin D ₂
PGE₂	Prostaglandin E ₂
PGI	Prostacyclin
pH	Measure of the acidity or basicity
PRP	Platelet rich plasma
RANTES	Regulated upon activation, normal T-cell expressed and released

RCF	Relative centrifugal force
ROS	Reactive oxygen species
RPM	Refs per minute
SABA	Short acting beta-agonist
SD	Standard deviation
SSE	<i>Sam so Eum</i>
SEM	Scanning electron microscope
TCD₄	Helper T lymphocyte
TEM	Transmission electron microscope
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll- like receptor
TNF-α	Tumour necrosis factor alpha
UPBRC	University of Pretoria Biomedical Research Centre
VCAM	Vascular cell adhesion molecule
%	Percentage
°C	Degrees centigrade
hr	Hour
G	Gram
mg	Milligram
ml	Millilitre
μl	Microlitre

kg

kilogram

mg/kg

Milligram per kilogram

µl/kg

Microlitre per kilogram

U/ml

Unit per millilitre

µm

Micrometer

nm

Nanometre

G

Gauge

X

Times



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 1

GENERAL INTRODUCTION

Asthma is a chronic inflammatory disease of the airways in which many different cells and cellular mediators are involved and play a role in the complex inflammatory process in asthma. Asthma is considered to be a global health problem that affects millions of people worldwide (Steinbacher and Glick, 2001).

There are many different treatment approaches to inflammatory diseases such as asthma, of which the recognized standard therapy is inhaled corticosteroids and is also currently the most effective (Sumi and Hamid, 2007). However, complementary and alternative medicine are studied extensively by many different researchers over the world and many allergies and allergic diseases such as asthma are frequently treated with alternative medication, including herbal as well as homeopathic products. Homeopathic products have been proved to exhibit biological activity in *in vivo* and *in vitro* animal studies (Piemonte and Buchi, 2002).

Modul8® is a complex homeopathic product of natural origin, composed of a mixture of plants and minerals and its effect on the immune system is investigated to determine whether this product might be an immunomodulator. Immunomodulators are defined as agents that enhance the immunity of an individual to favor a specific immunological response. In this thesis, the anti-inflammatory properties of this homeopathic product are investigated by using the BALB/c murine asthmatic animal model. The BALB/c asthmatic animal model is a known model, which has been shown to be useful in testing novel therapeutics. Since there is a correlation between the allergic response in humans and that of OVA-challenged animals, this animal model was used to investigate the anti-inflammatory properties of this homeopathic product (Epstein, 2006; Blyth *et al.*, 1996; Hogan *et al.*, 1996).



The main focus of this thesis is ultrastructure and morphology and this feature in the majority of the chapters. However, other parameters that also form an important part of this thesis include hematology and immunology. On ultrastructural level, platelets and fibrin networks, as well as tissue remodeling in the lungs of mice, were investigated by using the BALB/c asthmatic animal model and the morphology of peritoneal macrophages were investigated *in vitro*. Hematological studies involved the investigation of the white blood cell counts in the blood, as well as bronchial lavage. Platelet distribution in the bronchial lavage was also investigated. The production of the inflammatory mediators, nitric oxide and TNF-alpha, were investigated by the *in vitro* activation of peritoneal macrophages by lipopolisaccharides (LPS) and Modul8®. The different parameters investigated in this thesis will be discussed in more detail in the following paragraphs.

Platelets and fibrin networks were also investigated ultrastructurally. Both play an important role in allergic processes involved in asthma as platelets act as inflammatory cells by releasing inflammatory mediators (Camera *et al.*, 1999; Butenas and Mann, 2002; Lazarus *et al.*, 2003). The effect of alternative (herbal) products on the ultrastructure of platelets and fibrin networks has been studied previously to show that asthma has an effect on the ultrastructure of platelets and fibrin networks (Pretorius *et al.*, 2007a). Therefore, in this thesis the effect of Modul8® on the ultrastructure of platelets and fibrin networks were investigated and compared to the control and untreated asthmatic animals. Since the question always arises whether an animal model adequately mimics human disease, the results found in the above-mentioned study were compared to that of human controls and asthmatic subjects.

A wide range of inflammatory cells and mediators are involved in the inflammatory process in asthma. An influx of inflammatory cells into the airways of asthmatics is a



common feature in an asthmatic response. Therefore, this thesis also includes a chapter on the histological and ultrastructural changes that occur in the lungs of asthmatic animals. Remodeling in the lungs of untreated asthmatic animals were compared to control animals as well as asthmatic animals treated with the homeopathic product Modul8® and hydrocortisone. Hydrocortisone was used throughout the study as positive control, since it is known that hydrocortisone is used in the treatment of asthma.

White blood cells also play an important role in the inflammatory response in asthma. Eosinophil numbers in particular are increased in asthmatic subjects and these cells contribute to the airway remodeling observed in asthma (Kay *et al.*, 2004). Since it is thought that Modul8® might have a positive influence on the immune system, the number of white blood cells in the blood as well as bronchial lavage was counted and the results of the asthmatic animals were compared to the control and treatment groups.

Macrophages are important mediators of the immune response and they become activated in response to microenvironment to release a number of inflammatory mediators. Also, the morphology of macrophages changes when they become activated. It was previously found by researchers that an immunomodulator has the ability to activate macrophages *in vitro* (Piemonte and Buchi, 2002). Therefore, in this thesis mouse peritoneal macrophages were stimulated with LPS, which is a known activator of macrophages, and Modul8® to investigate whether Modul8® has the ability to activate macrophages to produce inflammatory mediators.

Therefore, the overall aim of this study was to investigate the possible effects of the homeopathic product Modul8® on ultrastructural, hematological and immunological

parameters by using the BALB/c murine asthmatic animal model. The research objectives addressed in this thesis can be summarized as follow:

1. Implementation of the BALB/c asthmatic animal model to investigate:

- ❑ Ultrastructure of platelets and fibrin networks and comparison to human subjects
- ❑ Weights changes in the animals during the experimental period
- ❑ White blood cell counts in the blood and bronchial lavage as well as platelet distribution in the bronchial lavage
- ❑ Ultrastructural changes and infiltration of inflammatory cells in the lungs

2. *In vitro* activation of mice peritoneal macrophages to investigate:

- ❑ The ability of Modul8® to activate peritoneal macrophages
- ❑ The production of inflammatory mediators by LPS-and Modul8® stimulated macrophages.

This thesis concludes with a summary of the results found in all the above-mentioned experiments as well as the possible application thereof.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this thesis the effect of the homeopathic product Modul8® will be discussed on several different parameters including weight changes, white blood cell counts in the blood and bronchial lavage, histology and ultrastructure of the lungs, ultrastructural changes in the morphology of platelets and fibrin networks and the ultrastructure of activated macrophages *in vitro*. The current chapter includes an overview on asthma in general as well as its etiology, immunology, pathophysiology, airway remodeling and treatment.

Typical treatment remedies for asthma are discussed as well as the role of complementary and alternative medicine in the treatment of asthma. Homeopathy is also frequently used in the treatment of asthma and other allergic diseases, and therefore this chapter concludes with the role of homeopathy in the treatment of asthma.

Modul8® is a modified version of the known homeopathic product Canova® (which is a potent immunomodulator and, studied extensively) and although differences exist in the composition of Canova® and Modul8®, the principles used in both these products are the same. Since no research has been done on the anti-inflammatory and immunomodulatory role of the new product, Modul8®, this chapter reviews properties of Canova®. The rationale behind using mice as experimental animals and more specifically choosing the BALB/c murine asthmatic animal model is also reviewed in this chapter.



2.1.1 Asthma in general

Asthma is considered to be a serious global health problem that has increased over the past decades and still are increasing and affecting hundreds of million people worldwide. People who are at highest risk for this disease include children, young adults and racial and ethnic minorities living in urban areas (Steinbacher and Glick, 2001). The term “asthma” is derived from a Greek word “panos” which means panting and was first used over 2000 years ago to describe shortness of breath (Rosenblatt, 1976). The Greek physician Hippocrates (460–377 BC) first described asthma and the first etiological link with bronchospasm was made by Galen (130–201 AD), who also described the association between upper and lower airways (Diamant *et al.*, 2007).

Ancient medical pioneers such as Hippocrates, Galen and Bernardino Ramazziniin, amongst others, have used this term to describe the airway condition that causes wheezing, chest tightness and an obstruction in the airways (Rosner, 1981; Marketos and Ballas, 1982).

The National Heart Lung and Blood Institute (NHLBI) states a working definition of asthma as follows: “Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyper-responsiveness to a variety of stimuli” (NHLBI, 1997).

Many years later, the term asthma is still described as a chronic inflammatory disease of the airways which involves the activation and infiltration of a large number of immune and inflammatory cells within the larger and smaller airways (Kornerup and Page, 2007). The clinical presentation of asthma includes wheezing, chest tightness, coughing and dyspnoea and the severity of the asthma symptoms may vary among patients and within individual patient episodes (Sollecito and Tino, 2001).

2.1.2 Etiology

Asthma is broadly classified into two main categories namely allergic or extrinsic asthma and idiosyncratic or intrinsic asthma (Sollecito and Tino, 2001). The first type of asthma mentioned extrinsic or allergic asthma, is the most common type and is associated with a history of atopy which includes eczema and seasonal allergies. This type of asthma typically begins in childhood (Tasota, 1996). This process involves increased serum IgE levels as well as a positive skin test for allergens. This type of asthma is associated with IgE mediated mast cell degranulation and a subsequent inflammatory response (Little *et al.*, 1997; McFadden, 1998).

Allergic asthma is also associated with reversible obstruction of airway, bronchospasm, infiltration of inflammatory cells into lung tissues, airway hyper-responsiveness (AHR), mucus overproduction and over-expression of Th2-mediated cytokines (Kon and Kay, 1999; Renauld, 2001).

Idiosyncratic or intrinsic asthma is not associated with allergy or increased IgE levels, and is more common in the elderly and middle-aged populations (Tasota, 1996). Bronchoconstriction and AHR are due to stimulation of airway parasympathetic nerves by certain allergens causing release of acetylcholine to sustain this process (Travis *et al.*, 2002). An asthmatic attack is often induced by specific triggers which

can be caused by pharmacological agents such as aspirin and beta-blockers, food preservatives, upper respiratory infections, exercise, cold, dry air and stress to mention only a few (Little *et al.*, 1997; McFadden, 1998).

Some researchers include occupational asthma and exercise-induced asthma in the classification system. Occupational asthma is caused by the exposure to biological enzymes, plastic resins, dusts, wood and metal particles in the workplace. This is often considered as a temporary form of asthma, due to the fact that the airway inflammation and bronchial hyper-responsiveness observed after the inhalation of a specific agent can be removed by removal of the causative agent (NHLBI 1997; Venables and Chan-Yeung, 1997), however, the asthmatic condition can exist even after the removal of the causative agent (Venables and Chan-Yeung, 1997).

In 1999, Jeffery described and categorized exercise-induced asthma by the narrowing of the airways after triggering by vigorous activity. The author explained that patients with exercise-induced asthma have sensitive airways, especially to changes in temperature and humidity (Jeffery, 1999). In 2004, Robison revealed that one of the major mechanisms for exercise-induced asthma is the activation of the mast cells by osmotic stimuli through the IgE receptors (Robinson, 2004).

Another classification system for chronic asthma also exists and is based on the severity of the disease according to the frequency of acute asthmatic attacks. According to this system, asthma is divided into mild intermittent, mild persistent, moderate persistent and severe persistent (Sollecito and Tino, 2001). The symptoms associated with severe persistent asthma (continual symptoms), include the following: limited physical activity, frequent exacerbations and frequent night-time symptoms. The preferred treatment for this type of asthma includes anti-inflammatory, high-dose inhaled corticosteroids, systemic corticosteroids and long-

acting bronchodilators. When daily symptoms occur even with the daily use of inhaled short-acting bronchodilators, or when exacerbations occur more than, or two times per week, and affects the activity of the individual, it is classified as moderate persistent. Typically, nocturnal symptoms in this type of asthma will occur more than once per week. The preferred treatment for moderate persistent asthma includes anti-inflammatory, medium-dose inhaled corticosteroids, low-or medium dose inhaled corticosteroids and a long-acting β_2 -agonist bronchodilator treatments.

Mild persistent asthma is classified according to the occurrence of symptoms less than two times per week and less than once per day. Exacerbations may also affect activity and night-time symptoms occur more than twice per month. Anti-inflammatory, low-dose inhaled corticosteroids, products like Cromolyn or Edocromil are the preferred treatment options for this type of asthma.

The final type of asthma, mild intermittent, is characterized by the following symptoms: symptoms occur less than, or twice per week, asymptomatic stages and normal peak expiratory flow are present between exacerbations which are brief and night-time symptoms occur less than, or twice per month. For mild intermittent asthma, no specific treatment is needed (Sollecito and Tino, 2001).

2.1.3 Immunology

Asthma is an inflammatory process in which many inflammatory cells participate and mediate a complex mixture of mediators. Cytokines in particular play an important role as mediators of chronic inflammation (Barnes, 1996). The inflammatory process in asthma involves a wide range of immune cells and immune mediators or cytokines that interact with one another in a specific way. According to Sollecito and Tino (2001), the most important cellular elements involved in asthma include mast cells, eosinophils, macrophages, epithelial cells and activated T-lymphocytes that are

associated with the Th2 response. These cells release certain mediators or cytokines, or are influenced by various mediators, cytokines and adhesion molecules (Sollecito and Tino, 2001). The role of some of these cells and mediators will be discussed individually in the following paragraphs.

2.1.3.1 Mast cells

The role that mast cells play in allergic diseases has extensively been studied over many years by a number of different researchers. Triggering of mast cells lead to the elicitation of the immediate phase of an allergic response that cause acute local responses such as edema formation, tissue swelling and bronchoconstriction. Mast cells also have an effect on the late phase responses of an allergic reaction by the release of chemotactic and pro-inflammatory mediators (Metz *et al.*, 2007; Vliagoftis and Befus, 2005).

Antigens cross-link with the IgE on mast cells and the mast cells then release mediators such as histamine, cysteinyl leukotrienes and prostaglandin D₂ (PGD₂) (Galli *et al.*, 2005) and according to Robison in 2004, airway hyperresponsiveness and increased number of mast cells in the airway smooth muscle (ASM) may be linked to one another (Robinson, 2004). Mast cells play an important role in initiating the acute responses to allergen and also to other indirect stimuli such as exercise and hyperventilation (Barnes, 1996).

In 2001, Page *et al.* also reported on the role of mast cells in the inflammatory process involved in asthma. The mast cells respond to non-IgE stimuli in the airways such as osmotic changes in the airway associated with exercise, hypertonicity and hypotonicity. Other mediators released from mast cells include prostaglandins, leukotrienes, cytokines such as IL1, IL2, IL4, IL10 and IL13, growth factors such as

platelet derived growth factor (PDGF) and transforming growth factor b (TGF), and lastly proteases such as tryptase (Page *et al.*, 2001).

In 2007, Bradding and Brightling revealed that mast cells migrate into the airway epithelium, airway mucus glands and the airway smooth muscle in human asthmatics, while virtually no mast cells are found in these regions in normal individuals. These authors also showed that the number of mast cells infiltrated into the ASM bundles; correlate significantly with the bronchial hyper-responsiveness associated with asthmatics, indicating the importance of mast cells in the pathophysiology of asthma (Bradding and Brightling, 2007).

2.1.3.2 Macrophages

Macrophages are derived from blood monocytes and they move into the airways where they may be activated by allergens via low-affinity IgE receptors (Lee and Lane, 1992). These cells produce many different products and cytokines that orchestrate the inflammatory response in asthma and therefore macrophages have the ability to initiate a particular type of inflammatory response via the release of certain cytokines (Spiteri *et al.*, 1994). According to Spiteri *et al.* (1994) macrophages may both increase and decrease inflammation, depending on the stimulus and therefore play an important anti-inflammatory role, preventing development of allergic inflammation (Spiteri *et al.*, 1994). Another characteristic of macrophages is the ability to act as antigen-presenting cells (APC) by processing allergen for presentation to T-lymphocytes, a characteristic that is less effective in alveolar macrophages than in macrophages from sites such as the peritoneum (Holt, 1993).

2.1.3.3 Dendritic cells

Dendritic cells are specialized macrophage-like cells in the airway epithelium which are very effective APC. Dendritic cells sample allergens from the airway surface and



migrate to regional lymph nodes. They interact with regulatory T cells and stimulate the production of Th2 cells from naïve T cells (Kuipers and Lambrecht, 2004). Dendritic cells may therefore play an important role in the initiation of allergen-induced responses in asthma.

2.1.3.4 Eosinophils

One of the major features of asthmatic airways is the eosinophilic infiltration into the airways. As early as 1916 the term “*chronic eosinophilic bronchitis*” was used to describe asthma (Barnes, 1996). Inhalation of allergen causes a marked increase of eosinophils on the broncho-alveolar lavage fluid in the late stage reaction that indicates that there is a close relationship between eosinophil counts in the blood and bronchial lavage and airway hyper-responsiveness (Barnes, 1996; Gleich, 1990). In 2004, Kay and co-workers also stated that eosinophil numbers are always increased in the airways and these cells are known to release basic proteins and growth factors that may damage airway epithelial cells and thereby cause airway remodeling (Kay *et al.*, 2004).

Eosinophilic infiltration is orchestrated by a number of cytokines that are released from the T- lymphocytes that are usually present in increased numbers in the airways. These cytokines include IL-4, IL-5, IL-9, and IL-13 (Larche *et al.*, 2003; Akbari *et al.*, 2006). In a study done by Oberholzer *et al.* (2008) the authors used the BALB/c murine asthmatic animal model to investigate the white blood cell count in the blood and bronchial lavage of untreated asthmatic animals and asthmatic animals treated with a plant material. The authors found an increase in the number of all white blood cells, but a significant increase in the number of eosinophils in the blood as well as bronchial lavage (Oberholzer *et al.*, 2008).



2.1.3.5 Neutrophils

Neutrophils are immune cells that play key roles in both the acute and chronic inflammatory response (Brown *et al.*, 2008). The exact pathophysiological role of neutrophils in asthma is not very clear; however neutrophils are usually increased in the sputum of patients with severe asthma, and it is stated by some researchers that this might be due to the effect of glucocorticosteroid therapy (Wenzel, 2003).

2.1.3.6 T-lymphocytes

T-lymphocytes play a very important role in the inflammatory response in asthma by releasing specific patterns of cytokines. This results in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways (Kay, 1991a). T-lymphocytes are usually present in increased numbers in the airways and they release a number of different cytokines such as IL-4, IL-5, IL-9, and IL-13 that orchestrate eosinophilic inflammation and IgE production by B-lymphocytes (Larche *et al.*, 2003; Akbari *et al.*, 2006).

2.1.3.7 Inflammatory mediators

Over the past years, a number of different mediators, which may have a variety of different effects on the airways, resulting in different pathological features, have been implicated in asthma. The role of histamine, prostaglandins, leukotrienes, cytokines and platelet activating factor (PAF) in the inflammatory response in asthma will be discussed in the following paragraphs.

- **Histamine**

Histamine is released from mast cells and contract ASM, increase microvascular leakage, increase airway mucus secretion and attract other inflammatory cells (Barnes, 1996). Histamine therefore contributes to bronchoconstriction and the

inflammatory response in asthma by causing inflammation directly (Andriopoulou *et al.*, 1999) and indirectly (Marone *et al.*, 1999).

- **Prostaglandins**

Prostaglandins are produced from arachidonic acid and are potent biologically active lipid molecules (Hata and Breyer, 2004; Funk, 2001). Cyclooxygenases (COXs)¹ are the rate-limiting enzymes for prostaglandin production and depending on subsequent isomerases and oxidoreductases distal to COX catalysis, various bioactive prostaglandins can be produced. The pattern of prostaglandin production is determined in a stimulus and cell-specific fashion and alteration of the species of prostaglandins that are produced in tissue, plays an important role in pathophysiological events of many diseases, including asthma (Peebles *et al.*, 2002; Wolff *et al.*, 1998; Lañiguez *et al.*, 1998; Burleigh *et al.*, 2002).

Prostaglandin D₂ (PGD₂) has been shown to have strong pro-inflammatory and bronchoconstrictive action in human and animal models of asthma (Hardy *et al.*, 1984) whereas prostaglandin E₂ (PGE₂), which is also detected in broncho-alveolar lavage fluid of asthmatic patients, has bronchoprotective properties and acts as an endogenous anti-inflammatory factor (Melillo *et al.*, 1994; Pavord *et al.*, 1993).

Uller *et al.* (2007) also reported on the role of PGD₂ and stated that mast cell derived PGD₂ may contribute to eosinophilic inflammation and mucus production in allergic asthma. The authors also reported that chemo-attractant receptor homologous molecule expressed on TH-2 cells (CRTH2), which is a high affinity receptor for PGD₂, mediates the trafficking of mast cells, eosinophils and Th2 cells to inflammatory sites (Uller *et al.*, 2007).



- **Leukotrienes**

Leukotrienes originate from the polyunsaturated fatty acid arachidonic acid. Two classes of leukotrienes can be distinguished based on the respective synthetic pathways and their biological activities. Leukotriene B₄ (LTB₄) is a dihydroxylated derivative of arachidonic acid and has been shown to be a very potent chemotactic mediator for neutrophils and eosinophils. Leukotrienes C₄, D₄, and E₄ are synthesized from arachidonic acid by attachment of the tripeptide glutathione (yielding LTC₄) and subsequent removal of glutamic acid and glycine residues (yielding LTD₄ and LTE₄, respectively). These leukotrienes are known as cysteinyl leukotrienes. The amount of evidence available on the role of cysteinyl leukotrienes in airway inflammation is numerous (Capra *et al.*, 2007).

Cysteinyl leukotrienes are potent spasmogenic mediators and affect inflammatory cell recruitment, mucus production, airways remodeling, and pulmonary vascular leakage (Ogawa and Calhoun, 2006). Some authors have reported on the inhibition of the formation and action of cysteinyl leukotrienes and have proven amelioration in inflammatory symptoms associated with asthma and other inflammatory diseases (Engels and Nijkamp, 1998).

- **Cytokines**

Cytokines play an important role in orchestrating the inflammatory response associated in chronic inflammation. Many different inflammatory cells are capable of releasing cytokines. These include macrophages, mast cells, eosinophils and lymphocytes. These cells are capable of synthesizing and releasing these cytokines and structural cells such as epithelial and endothelial cells may also release a number of cytokines and therefore participate in the inflammatory response (Barnes, 1994).

Cytokines act as the main drivers of the inflammatory response in asthma and may also determine the severity thereof. The cytokines that are specifically important in asthma are the lymphokines that are secreted by the T-lymphocytes: Interleukin (IL)-**3** plays an important role in the survival of the mast cells in tissues, **IL-4** is critical in stimulating B-lymphocytes to produce IgE and for expression of vascular cell adhesion molecules (VCAM-1) on endothelial cells, **IL-5** plays an important role in the differentiation, survival and priming of eosinophils (Hamid *et al.*, 1991).

Other cytokines that are released from cells such as macrophages and epithelial cells and that are important in amplifying the inflammatory response include **IL-1**, **IL-6**, tumour necrosis factor- α (**TNF- α**) and granulocyte macrophage-colony stimulating factor (**GM-CSF**).

TNF- α is a pro-inflammatory cytokine that is known to play a critical role in the pathogenesis of many inflammatory diseases, including asthma (Russo and Polosa, 2005). TNF- α is an important mediator in asthma and is found in increased amounts in the airways of asthmatics. One of the biological functions of TNF- α includes the modulation of growth differentiation and proliferation of a variety of cell types and is also a well-known inducer of the inflammatory response, as well as a regulator of immunity. Other pro-inflammatory cytokines that mediates the inflammatory properties of TNF- α includes IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN- γ (interferon- γ) and TGF- β (transforming growth factor- β) (Aggarwal, 2003).

- **Platelet activating factor (PAF)**

This mediator also plays an important role in asthma as it mimics many of the features of asthma including airway hyperresponsiveness (Barnes *et al.*, 1988). PAF is a bronchoconstricting mediator secreted by activated platelets together with histamine, serotonin (5-HT) and arachidonic acid (Page, 1989). PAF is a potent

activator of platelets and neutrophils and can induce systemic anaphylaxis when given intravenously.

Ultrastructural studies have shown PAF, in addition to causing neutrophil and eosinophil recruitment into the lungs, induces intravascular platelet aggregation and degranulation in alveolar capillaries and stimulates platelet diapedesis to the alveolar lumen. The authors of this study revealed that these features are absent in guinea pigs pre-treated with prostacyclin or aspirin, and that the bronchoconstrictive response remains unchanged. With these results the authors suggested that platelet aggregation in the lungs is not essential for bronchoconstriction and that platelets release airway-constricting substances via a cyclo-oxygenasein dependent mechanism (Lellouch-Tubiana *et al.*, 1985).

Other interesting results found by authors as early as 1988 and 1990 are that platelet depletion, as well as administration of prostacyclin (PGI₂) and PAF antagonists in animal models of asthma, significantly inhibited the infiltration of eosinophils (Coyle *et al.*, 1990; Lellouch-Tubiana *et al.*, 1988).

- **Nitric oxide (NO)**

Nitric oxide is a potent vasodilator that is produced by several cells in the airways predominantly from the action of inducible nitric oxide synthase (NOS) (Ricciardolo *et al.*, 2004; Barnes, 1995). iNOS is an inducible form of the enzyme that is expressed in epithelial cells of asthmatic patients and can be induced by cytokines in airway epithelial cells (Hamid *et al.*, 1993; Robbins *et al.*, 1994). An increased concentration of exhaled NO is present in asthmatic patients (Kharitonov *et al.*, 1994) and since NO itself is a potent vasodilator it may increase plasma exudation in the airways of asthmatic patients and may also amplify the Th2 lymphocyte mediated response (Barnes and Liew, 1995).



2.1.4. Pathology and airway remodeling

In a review article on airway remodeling in asthma, Sumi and Hamid (2007) defined airway remodeling as changes in the composition, content, and organization of the cellular and molecular constituents of the airway wall. Airway remodeling is one of the characteristics of asthma, and has important functional implications. The structural changes associated with airway remodeling in asthma include epithelial detachment, subepithelial fibrosis, increased ASM mass, decreased distance between epithelium and ASM cells, goblet cell hyperplasia, and mucus gland hyperplasia, proliferation of blood vessels and airway edema and changes in the cartilage. Each of these changes can contribute to airway hyperreactivity (AHR), and may lead to irreversible airflow obstruction as the disease progresses. Remodeling is thought to be characteristic of asthma because these structural changes can be observed in asthmatics from early onset of the disease (Sumi and Hamid, 2007).

Epithelial detachment is a characteristic feature of airway remodeling and may in part be a consequence of ongoing inflammation (Trautmann *et al.*, 2002; White, 1997).

Subepithelial fibrosis is another important feature of airway remodeling in asthma and may range between simple thickening and extensive fibrosis (Sumi and Hamid, 2007). Roche *et al.* (1989) investigated the ultrastructure of subepithelial fibrosis and revealed that the basement membrane appear normal on electron microscopic level and beneath this layer there is a loose array of collagen fibrils in normal individuals whereas in asthmatic patients this layer is replaced by a dense network of collagen fibrils. This type of fibrosis is due to the deposition of extracellular matrix particularly collagen type I and III, fibronectin as well as proteoglycans (Roche *et al.*, 1989).

Activated fibroblasts cause the increased extracellular matrix and are due to the up regulation of a number of cytokines and growth factors such as tumor growth factor

beta (TGF- β) and IL-11, and they are produced by structural as well as inflamed cells, particularly eosinophils (Sumi and Hamid, 2007).

Another common observation in asthmatic airways is the increase in airway smooth muscle mass. There is a number of factors that may cause an increase in airway smooth muscle cell number, such as increased rates of division (Johnson *et al.*, 2001), decreased rates of apoptosis (Martin and Ramos-Barbon, 2003) and migration of mesenchymal cells to ASM bundles (although the latter is only speculated) (Schmidt *et al.*, 2003; Beqaj *et al.*, 2002; Hashimoto *et al.*, 2001; Vancheri *et al.*, 2005; Hirst *et al.*, 2004; Johnson *et al.*, 2004).

Stimulation of ASM cells by mitogenic or inflammatory stimuli may be one reason why ASM cells proliferate faster in asthmatic patients, compared to non-asthmatic patients. Mitogenic stimuli that have been identified include TGF- β , EGF, and IGF, PDGF isoforms, FGF-2, β -hexosaminidases, β -glucuronidase, α -thrombin, tryptase, sphingosine 1- phosphate, endothelin-1, substance P, phenylephrine, serotonin, thromboxane A₂, leukotriene D₄, mechanical stress and reactive oxygen species (ROS) (Roth *et al.*, 2004). Another explanation may be the fact that profile of the extracellular matrix protein components are altered in asthmatic cells and that this altered profile may have the potential to influence the proliferation of these cells (Ebina *et al.*, 1993).

Evidence exists that smooth muscle cells in asthma can act as inflammatory cells, because of the ability to produce chemokines and cytokines (Vermeer *et al.*, 2003).

Increased size of the mucosal glands and increased numbers of goblet cells in the airway epithelium is also features of airway remodeling in asthma that leads to excessive mucus production which eventually lead to airflow obstruction. Mucus

hypersecretion may be induced by IL-9 and IL-13 (Wills-Karp and Chiaramonte, 2003; Kuperman *et al.*, 2002; Li and Wilson, 1997; Tanaka *et al.*, 2003).

Vascular change and airway edema also forms part of the airway remodeling in asthma and changes in the airway wall can contribute to airway wall edema (Sumi and Hamid, 2007). Bronchoscopic biopsies of patients with mild asthma (Hoshino *et al.*, 2001) have showed increased airway vascularity as well as in the bronchial mucosa of stable and newly diagnosed asthma patients (Haraguchi *et al.*, 1999).

Another feature of airway remodeling in asthma is changes in the airway cartilage, which is responsible for the stiffness and integrity of the airways. Structural and biochemical changes in the cartilage of the airways will therefore have an influence on airway function (Sumi and Hamid, 2007). A decrease in the cartilage volume and fibronectin deposition has been observed in asthmatic airways, and these alterations may result on more powerful bronchoconstriction from ASM load reduction (Sumi and Hamid, 2007; Black, 2004).

Airway remodeling in established asthma is poorly responsive to inhaled corticosteroids, administration of β_2 -agonists, antileukotrienes, and theophylline, which are the currently used therapies for asthma (Sumi and Hamid, 2007). The therapeutic implications in asthma will be discussed in section 2.1.6.

2.1.5 Genetics and asthma

A number of studies have been done on the genetic aspect involved in asthma and these studies confirmed that age and sex play a role in the pathogenesis and prevalence of asthma (Lux *et al.*, 2009). The genetic influences on the pathogenesis of asthma include genetic disposition, which seems to be the primary determinant of asthmatic disease and are defined by clinical symptoms and functional tests. Melén

et al. (2004) stated that the influence of parental allergy and male sex on the development of wheezing and sensitization during childhood may possibly be determined by sex-specific hereditary components consistent with x-linked genes (Melén *et al.*, 2004).

2.1.6 Treatment of asthma

In earlier years, anti-asthmatic treatment involved the relief of symptoms or modification thereof (Hofbauer, 1931; Belcher, 1961). Nowadays there are a number of different asthma medications available that are used in many different indications. Some of these medications include inhaled β_2 -agonists (short-acting and long-lasting); oral β_2 -agonists; inhaled corticosteroids (ICS); systemic corticosteroids; mast cell stabilizers; methylxanthines; anticholinergics and leukotriene modifiers (Sollecito and Tino, 2001). The implications of some of the mentioned mediations will be discussed in the following paragraphs.

➤ Corticosteroids

ICS are recognized as standard therapy for pediatric and adult asthma (GINA, 2005; National Asthma Education and Prevention program, 2007) and are currently the most effective anti-inflammatory medication for the treatment of asthma (Sumi and Hamid, 2007). ICS are effective in most cases of asthma and some studies have shown that ICS are effective in preventing or reducing the decline of airway function in asthmatic patients over time (Selroos *et al.*, 1995).

The exact effect of corticosteroids on epithelial cells is still controversial, although it is thought that they may be beneficial by stimulating a decrease in mucus production. In an *in vitro* study done by Stewart *et al.* (1995) Dexamethasone was shown to increase 3H-thymidine incorporation in asthmatic bronchial fibroblasts, without having

any significant effect on normal fibroblast proliferation. With these findings the authors suggested that the corticosteroids could influence some aspects of the bronchial fibroblast phenotype (Stewart *et al.*, 1995).

In another study done in 2001, corticosteroids were shown to decrease smooth muscle cell proliferation (Lazaar and Panettieri, 2001), whereas other studies have shown that corticosteroids have a non-immunosuppressive effect on the ASM because no effect on the activation of nuclear factor-B transcription factor could be observed (Johnson *et al.*, 2000).

In a retrospective biopsy study done by Lundgren *et al.* (1998) partial restoration of the epithelium could be observed after the use of corticosteroids. These results showed that treating asthmatics with ICS for long terms decreased inflammation and partially improved epithelial damage.

Goblet cell metaplasia and mucus hypersecretion are some of the important features of asthma and although corticosteroids and bronchodilators are not primarily targeted to act on goblet cell activity, they do seem to have direct and indirect suppressive effects on mucus production (Rogers and Barnes, 2006). In research done by using animal models, corticosteroids have shown to be effective in reducing goblet cell metaplasia (Leung *et al.*, 2005; Kumar *et al.*, 2003).

Although pharmacotherapy is generally effective in most of the patients and higher doses of ICS were previously recommended, recent evidence suggests that the addition of a long-acting β_2 -agonist (LABA) is a better option for most. However, higher dosages of ICS are not problem-free and have been associated with a dose-related increase in risk of corticosteroid-related adverse outcomes (Cumming *et al.*, 1997; Garbe *et al.*, 1997; Greenfield and Samaras, 2006; Hubbard *et al.*, 2002).

Korsgaard and Ledet (2009) also reported on the potential side effects in patients treated with LABA and ICS, which are widely used in patients with obstructive lung disease. The authors determined the occurrence of potential side effects to ICS and LABA in an open post-marketing observational study. The authors investigated a number of potential side effects of both ICS and LABA. The pharmacological predictable side-effects of treatment with ICS include occurrence of sore throat, skin bruises and oral thrush, while the pharmacological predictable potential side effects to treatment with inhalations of LABA were, unwillingly spontaneous muscle twisting, muscle cramps in hands, toes and foot and/or in the calves in the morning. They also found occurrence of hand tremors and heart palpitations in their study.

The authors concluded that four out of five adult patients treated with ICS and LABA seemed to experience some degree of side effects and they emphasized the importance of reducing the treatment to the minimum dose needed in each specific case of asthma in patients (Korsgaard and Ledet, 2009).

In 2009, Xu *et al.* did a study on cortisol suppression as a surrogate marker for ICS-induced growth retardation in children. The authors revealed that exposure to ICS results in adrenal suppression and growth inhibition in asthmatic children (Xu *et al.*, 2009).

In another study in 2002 the authors stated that there is increasing evidence that the use of ICS is associated with a dose-related reduction in bone mineral density, and more specifically an association between the use of ICS and hip fractures (Hubbard *et al.*, 2002).

Although the clinical affectivity of ICS has been established, the prolonged treatment with high doses of ICS can neither fully reverse all chronic aspects of the airway

inflammation nor cure the disease (Dworski and Sheller, 1992; Ward and Walters, 2005).

➤ **Anticholinergics**

In the late 1970s Ipratropium, which is a synthetic anticholinergic was developed for the treatment of asthma. Tiotropium was also developed later on and both these drugs both antagonize the effect of acetylcholine at the muscarinic M_1 and M_3 receptor. The role of these drugs in asthma is still limited but evidence shows that these anticholinergics may benefit patients with genetically determined adverse responses to β_2 -agonists in up to 20% of the asthma population (Wechsler *et al.*, 2006). It was also reported that during acute exacerbation when response to short acting β_2 agonists (SABAs) is poor, addition of an anticholinergic may provide a faster-onset relief (Gross, 2006; Teale *et al.*, 1992).

➤ **β_2 Agonists**

The use of adrenal substances in the treatment of asthma dates back to 1900 and during the 1940's epinephrine or more known as adrenaline became the standard bronchodilator for the treatment of acute asthma (Solis-Cohen, 1900; Sears and Lotvall, 2005). A major breakthrough came in 1960 with the discovery of the adrenergic receptor subsets, yielding α and β receptors which are further divided into the β_1 receptors which are located in the heart and intestinal smooth muscle and the β_2 receptors which are found in the bronchial and uterus smooth muscle (Lands *et al.*, 1967; Ahlquist, 1948).

The first agonist interacting with the β adrenergic receptors was Isoprenaline, while the first agonists with a higher specificity for the β_2 adrenergic receptors were Salbutamol and Terbutaline (Bergman *et al.*, 1969; Brittain *et al.*, 1968) and today Salbutamol is the most widely used fast-acting reliever for asthma (GINA, 2005).



Other short-acting β_2 agonists include Carbuterol, Clenbuterol and Fenoterol and they have duration of action up to 6h (Engelhardt, 1976; O'Donnell, 1970). In the 1980s, LABAs such as Salmeterol were developed with an action of up to 12h (Johnson, 1995). Some researchers provided evidence that treatment with both SABAs and LABAs — in or without combination with ICS — is associated with potential masking of the airway inflammation (McIvor *et al.*, 1998; Sears *et al.*, 1990). Therapy with LABAs in combination with appropriate doses of corticosteroids, in the more severe cases, is recommended (Hasford and Virchow, 2006).

➤ Antileukotrienes

Leukotrienes have broncho-active properties and seem to mimic many features of asthma such as airway hyperresponsiveness; airway inflammation and airway remodeling (Diamant and Sampson, 1999) and research have shown that potent anti-leukotrienes effectively reduce several features of asthma in both adults and children (Bjermer and Diamant, 2004; Lane, 1998). Leukotriene receptor antagonists (LTRAs) such as Pranlukast, Zafirlukast and Montelukast also form part of anti-asthma therapy (Lane, 1998). The LTRAs combine anti-inflammatory, mainly anti-eosinophilic activity, with mild, bronchodilator properties, based on antagonism of cysteinyl leukotrienes (CysLTs) at the CysLT1- receptor within the airways and on inflammatory cells (Diamant and Sampson, 1999; Bjermer and Diamant, 2004; Lane, 1998; Ind, 1996).

2.1.7 Alternative and complimentary medicine

Alternative medicine is the term used to describe medical approaches that differ from conventional medicine and according to Passalacqua *et al.* (2006) complementary and alternative medicine (CAM) is the preferred term to use for this type of treatment (Passalacqua *et al.*, 2006). Numerous CAM techniques are known and they include physical techniques such as acupuncture, chiropractic and massage therapy,



systematic medicines such as the Chinese traditional medicines, behavioral techniques such as dissociated diets and phytotherapy such as aromatherapy and herbal medicines. Allergy and allergic diseases such as asthma are frequently treated with CAMs, where homeopathy, acupuncture, herbal medicines, and yoga are the most used techniques. (Passalacqua *et al.*, 2006).

Numerous studies have been done over many years investigating the role of alternative medicine, especially herbal products, on the allergic process in asthma. A few studies done on herbal products and asthma will be highlighted in the following paragraphs.

In 2009, Park and co-workers investigated the effect of Quercetin on the regulation of Th1/Th2 balance in a murine model of asthma. Many medicinal plants owe much of their activity to the high Quercetin content in the plant. Quercetin possesses potent anti-inflammatory properties and the authors revealed that Quercetin reduced the increased levels of IL-4, Th2 cytokine production in OVA-sensitized and –challenged mice. They also revealed that it increased IFN- γ and Th1 cytokine production in Quercetin administrated mice. It was also found that the administration of Quercetin before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions and with these finding the authors suggested that Quercetin may play a critical role in the amelioration of the pathogenetic process of asthma in mice and also these findings provide new insight into the immunopharmacological role of Quercetin in terms of its effects in a murine model of asthma (Park *et al.*, 2009).

In another study in 2008 the authors investigate an herbal medicine, *Sam So Eum* (SSE) as a remedy of allergen-induced asthma in a mouse model and the authors discussed the mechanism of restoring the immuno-modulating cytokines such as IL-10 and IFN- γ . The authors revealed that the SSE-treated mice showed reduced

levels of airway responsiveness to Methacholine, and these levels were initially elevated by the induction of asthma compared to the control group. The authors suggested the possibility for using SSE as treatment for patients with asthma and stated that its therapeutic efficacy may be involved in the restoration of the IL-10 and IFN- γ levels (Cho *et al.*, 2008).

The inhibitory effect of *Duchesnea chrysanth* (an herb with anti-oxidative, anti-inflammatory and immune-enhancing properties) extracts on OVA-induced lung inflammation in an asthmatic mouse model was investigated by authors in 2008. The results revealed that this herb significantly inhibited leukocytosis and eosinophilia on the bronchoalveolar lavage fluid and also significantly reduced the elevated level of infiltration of inflammatory cells and mucus secretion in the airways. With these results the authors concluded by suggesting the possibility that this herb can exert suppressive effects on asthma and suggested that it might be a useful agent in the treatment of allergic airway diseases (Yang *et al.*, 2008).

In another study by Lee and Kim (2008), the immunomodulatory effect of *Juglans sinensis*, *Psoralea corylifolia* and Cheong-a-hwan extract and cyclosporine A on Th1 (IFN- γ) /Th2 (IL-4) cytokine balance and eosinophil accumulation in a murine model of asthma were investigated. The results obtained from this study revealed that *J. sinensis*, *P. corylifolia* and Cheong-a-hwan have profound inhibitory effects on the accumulation of eosinophils into airways and blood. The up regulation of the production of OVA-specific Th1 cytokine (IFN- γ) and down regulation of OVA-specific Th2 cytokine (IL-4) in culture supernatant of spleen cells could also be observed. The authors also suggested that *J. sinensis*, *P. corylifolia* and Cheong-a-hwan extracts may potentially be a novel therapeutic agent for asthma by modulation of the relationship between Th1/Th2 cytokine balances (Lee and Kim, 2008).

Artemisia iwayomogi, is a perennial herb that is found around Korea and has been used as a traditional anti-inflammatory medicine in liver diseases. This plant was used by authors in 2008 to investigate the effects of actin interacting protein (AIP1) which is the water soluble carbohydrate fraction from the mentioned herb, on OVA-induced allergic asthma in BALB/c mice and to possibly determine the mechanisms of its anti-allergic action.

It was found that AIP1 significantly reduced pulmonary eosinophilia and Th2 cytokine expression in the lungs as well as serum IgE levels. Results from the flow cytometric analysis of lung-infiltrating cells revealed a decrease in surface levels of CD11c and MHC II in CD11c+MHC II+ cells and potent dendritic cells in the animals treated with AIP1. Also, expression of TNF- α was down-regulated by treatment with AIP1, and with these results the authors suggested that it could account for the suppression of pulmonary eosinophilia and Th2-type cytokine production by AIP1 (Lee *et al.*, 2008).

In 2007, Pretorius *et al.* successfully used the BALB/c murine asthmatic animal model to investigate the effect of the phytomedicine *Euphorbia hirta* on the ultrastructure of platelets and fibrin networks. The authors found that this phytomedicine does not impact on the fragility of the fibrin but it prevents the minor fibers from forming a net-like layer over the major fibers as can be seen in the untreated asthmatic animals. Platelet morphology in the *E. hirta*-treated animals appear similar to that of the control animals, and the authors therefore suggested that ultrastructural studies may lead to a better understanding of the disease and possible treatment regimes (Pretorius *et al.*, 2007a).

Numerous studies have been done on asthma by using herbal products, of which only a few are mentioned above. Homeopathy is also one of the fields of complementary and alternative medicine and is frequently used in the treatment of

asthma and other allergic diseases. Therefore, the principles of homeopathy as well as the role of homeopathic immunomodulators will be discussed in the following paragraphs.

2.1.8 Homeopathy

The term homeopathy is derived from the Greek words *homeo* which means similar and *pathos* meaning suffering or disease. Homeopathy is an alternative medical system and one of the key concepts includes stimulation of the body's defense mechanisms and processes as to prevent or treat illness. Most homeopathic substances are derived from natural substances from plants, animals and minerals and these substances are diluted in a series of steps during the preparation of the homeopathic product (National Centre for Complementary Alternative Medicine).

Jütte and Riley (2005) define a homeopathic product or medicine as a product that has been prepared in accordance with a homeopathic manufacturing procedure as defined by the European Pharmacopoeia or recognized national homeopathic pharmacopoeias (Jütte and Riley, 2005).

It is also very important to distinguish between herbal products or phytotherapy and homeopathic products or homeopathy. If a medicine is prepared by an herbalist on the basis of the available literature on the use of herbs it is known as phytotherapy rather than homeopathy and when a medicine is prescribed on the basis of a patient's symptoms in accordance with the law of similars, it is said to be homeopathy and not phytotherapy. Therefore, Jütte and Riley, (2005) stated that whether a medicinal product is homeopathic or not, is determined, from a pharmaceutical perspective, by its production in accordance with a recognized homeopathic pharmacopoeia and whether a medicinal product is homeopathic or



phytotherapeutic, from a clinical perspective, is determined by the rationale behind its use (Jütte and Riley, 2005).

An important aspect of homeopathy is the so called “*Law of Similars*” or also called “*like cures like*”. What this means is that a substance produces symptoms of illness in a well person when administered in large doses where if the same substances are administered in minute quantities, it will cure the disease in a sick person. Hahnemann, the founder of homeopathy, stated that this is because nature will not allow two similar diseases to exist in the body at the same time (Gold *et al.*, 2008). This theory might, however, sound very unscientific to some traditional researchers as well as members of the medical fraternity. Also, there are still many debates on the plausibility, theoretical principles, clinical and basic research evidence, ethical considerations and other issues surrounding homeopathy. However, there is also evidence indicating the biological activity of homeopathic products that are not seen with the placebo.

In animal studies on experimentally induced paw edema, arsenic poisoning, chemically induced liver cancer, changes in normal physiology such as EEG sleep stages, and many other research have repeatedly demonstrated the biological effect of homeopathic products (Gold *et al.*, 2008).

Homeopathic remedies exhibit biological activities across many *in vivo* and *in vitro* animal studies. The product that is focused on in the current thesis is such a homeopathic product. Therefore, the known properties of Modul8® as well as the effects of a homeopathic immunomodulator Canova®, from which Modul8® is a modified version containing some of the same components and as well as many others, will be discussed in the following paragraphs. No research has previously been done on Modul8® and therefore no literature is available on this product.

Therefore the immunomodulatory properties of the known homeopathic product Canova® will be discussed later in this chapter to highlight the effects of a homeopathic product on the immune system.

➤ **Modul8®**

Modul8® is a homeopathic product of natural origin that was developed in a laboratory in Argentina and today is produced, according to the Hanemannian homeopathic method, as a homeopathic remedy. The final product is an aqueous, odorless and colorless solution that contains: *Aconitum napellus* (D20), *Arsenicum album* (D18), *Asafoetida* (D20), *Calcarea carbonica* (D16), *Conium maculatum* (D17), *Ipecacuanha* (D13), Phosphorus (D20), *Rhus toxicodendron* (D17), Silicea (D20), Sulfur (D24), *Thuja occidentalis* (D19), Alcohol (0.2% v/v) and Purified water (44.050 ml.).

The composition per 50 ml bottle is expressed as declared potencies (D), which indicates an index of 10-fold dilution. No signs of toxicity or mutagenic effects have been reported. The properties and common uses of the most abundant components in Modul8® will be mentioned in the following paragraphs.

Aconitum napellus is commonly known as monkshood. A non-toxic, diluted extract of aconite is used in homeopathy to treat symptoms similar to that of poison. Anodyne, diuretic and diaphoretic are some of the medicinal actions of this plant. (www.botanical.com).

Arsenicum album is used in the treatment of acute ailments, chronic diseases and acute colds, bronchitis and fever and is also used in the treatment of asthma (Cummings and Ullman, 1997; Kent, 1996).

Asafoetida is also known as *Ferula* which is a genus of many species and is a perennial herb that is widely used in India as flavoring agent in food and it is also used in traditional medicine for many different conditions including indigestion, whooping cough, inflammation, epilepsy, pain, cholera, infertility and convulsions. It was reported that it possesses antioxidant and anti-carcinogenic activity in Swiss albino mice (Saleem *et al.*, 2001).

Calcareo carbonica is a component that is derived from the middle layer of shells and in chemical terms is impure calcium carbonate (CaCO_3) and is commonly used to treat acne, arthritis, vaginal discharges in women, night terrors in children and ringworm on the scalp (Cummings and Ullman, 1991).

***Conium maculatum* L.** is an umbelliferous weed known worldwide for its toxicity to many domestic animals and humans. It produces eight different alkaloids of which Coniine and γ -coniceine are the most abundant and are known to have antifungal and antimicrobial properties (Al-Barwani and Eltayeb, 2004).

Ipecacuanha is part of the family *Rubiaceae* and is a perennial medicinal plant of which the roots are the sources for an expectorant, emetic and amoebicide and this plant is cultivated for the alkaloids, emetine and cephaeline (Rout *et al.*, 2000).

Rhus toxicodendron is a traditional homeopathic name for a plant called *Toxicodendron pubescens*. This plant is used in various inflammatory conditions and in 2007 it was reported that *Rhus toxicodendron* in homeopathic dilution interfered with inflammatory mediators including histamine and other prostaglandins indicating that it may act through inflammatory mediators in the immune response when the authors investigated carrageenan-induced paw oedema in rats (Santos *et al.*, 2007).

Thuja occidentalis is a popular homeopathic remedy used to treat warts. It has also been indicated for dandruff, skin problems, torn muscles and joints and chronic urinary infections (www.botanical.com).

➤ **Homeopathic products as immunomodulators**

Modul8® is thought to have immunomodulatory properties and immunomodulators are defined as agents that enhance the immunity of an individual to favor a particular immunological response. Researchers have found that immunomodulators stimulate host defenses against several pathological states (de Oliveira *et al.*, 2006).

➤ **Effects of an immunomodulator similar to Modul8® on cellular level**

Canova® is a complex homeopathic immunomodulator that contains many of the substances found in Modul8®. Canova was developed in the Canova laboratory in Argentina and today it is produced as a homeopathic medicine in Brazil (Seligmann *et al.*, 2003), according to the Hahnemannian homeopathic method. The final product is an aqueous, odorless and colorless solution that contains *Thuja occidentalis* (Cupressaceae), *Bryonia alba* (Cucurbitaceae), *Aconitum napelius* (Ranunculaceae), *Arsenicum album* (arsenic trioxide) and *Lachesis muta* (Viperidae) (Lopes *et al.*, 2006).

It is known to be neither toxic nor mutagenic, is available as drops, and is used by researchers in a wide concentration range from 4% to 50%. The composition per 50 ml bottle is expressed as declared potencies (D), which indicates an index of 10-fold dilution. *Aconitum napelius*: D 11, *Arsenicum album*: D 19, *Bryonia alba*: D 18, *Lachesis muta*: D 18, *Thuja occidentalis* D 19, and 49.965 ml water and 0.035ml alcohol.



In 2002, Piemonte and Buchi analyzed the production of IL-2, IFN- γ and TNF- α as well as the ultrastructure of peritoneal macrophages treated with a homeopathic medicament Canova[®]. The authors revealed that 86% of the macrophages treated with Canova[®] were activated compared to the 15% of the control group. Activation was determined by certain characteristics such as enlarged nucleus, more euchromatin than heterochromatin, spreading and projections. Conduction of the ELISA revealed no difference in IL-2 and IFN- γ between the treated and control group whereas Canova[®] showed to decrease TNF- α production compared to the control group.

In another study in 2006, Abud and co-workers investigated the activation of bone marrow cells treated with Canova[®] *in vitro* (Abud *et al.*, 2006). As previous studies have found that Canova[®] induces up-regulation in a number of leukocytes and increase the number of TCD₄ lymphocytes as well as NK cells in peripheral blood, (Sato *et al.*, 2005), the authors aimed to determine whether Canova[®] can promote the differentiation, proliferation and survival of mouse bone marrow cells *in vitro* as it is the major site for blood cell formation. This study showed an increase in the number of adherent, larger and activated cells in the Canova[®]-treated group. The authors revealed that most of the adherent cells found in their experiment were macrophages (CD11b⁺) and Canova[®] is known to activate macrophages *in vivo* and *in vitro* (Piemonte and Buchi, 2002; de Oliveira *et al.*, 2006; Lopes *et al.*, 2006; Pereira *et al.*, 2005).

The authors concluded by stating that these findings are very important because Canova[®] is a non-toxic medication (Seligmann *et al.*, 2003) and therefore can possibly be used in *in vitro* cell therapy for many diseases that affect bone marrow cells (Abud *et al.*, 2006).



In 2006, Lopes *et al.* investigated the cellular aspects of macrophage activation by Canova[®]. The authors aimed to study the activation of mice peritoneal macrophages when exposed to *in vitro* and *in vivo* Canova[®] treatment as well as morphological parameters and acid phosphatase activity by using light- and electron microscopy.

The results obtained revealed a greater spreading ability in the Canova[®]-treated macrophages, a higher phagocytic activity of non-infective microorganisms as well as a tendency to lower the phagocytic activity of the infective microorganisms *T. cruzi* trypomastigotes and *Leishmania amazonensis*, comparing to the control cells. Analysis of the acid phosphatase activity showed that Canova[®] treatment stimulates an increase of the endosomal/lysosomal system. With their results, the authors concluded that Canova[®] is an effective stimulator of macrophage activity (Lopes *et al.*, 2006).

These findings correlate with those found by Pereira *et al.* in 2005 who investigated the immunomodulatory effect of Canova[®] on experimental *Leishmania amazonensis* infection. The *in vitro* results showed that at 40% Canova[®] the infection index was reduced and NO production was induced in the elicited macrophages. The *in vivo* tests also revealed reduced infection as well as decreased parasite load in the regional popliteal lymph nodes and in the spleen. From the results obtained by the authors they suggested that Canova[®] modulates experimental infection, control infection progression and limit dissemination (Pereira *et al.*, 2005).

Canova[®] was also tested using human monocytes. Canova[®] has been shown to activate macrophages *in vitro* and *in vivo*, with resultant enhanced spreading of the cells and formation of microvillus extensions.



Since monocytes are the precursor cells of macrophages and dendritic cells, the objective of the study was to investigate the effects of Canova[®] on the differentiation of human blood monocytes *in vitro*. Monocytes were isolated, grown in culture and exposed to 10% and 20% Canova[®] without the addition of cytokines. After 48 hours, monocytes were prepared for analysis by scanning electron microscopy (SEM), while cells kept in culture for 7 days and exposed to Canova[®] on days 1, 3 and 4 were analyzed by flow cytometry for alterations in the levels of expression of various markers.

SEM analysis revealed monocytes exposed to 10% Canova[®] with morphology similar to that of macrophages. Various cytoplasmic projections (pseudopodia) were observed. Flow cytometric analysis indicated high cell viability and up-regulation of CD80, compatible with differentiation into either macrophages or dendritic cells. Exposure to Canova[®] per se causes activation of monocytes with differentiation into large macrophage-like cells of indeterminate phenotype, which has increased expression of CD80. Like cytokines, Canova[®] induces differentiation of monocytes, and this may underpin the immunomodulatory activity of the product (Smit *et al.*, 2008).

➤ Homeopathy in cancer therapy

Sato and co-workers also investigated Canova[®] for its potential in cancer therapy in 2005. They studied the effect of Canova[®] in normal and sarcoma 180-bearing mice. A comparison between the Canova[®]-treated and the control groups revealed a delay in the development and reduction in size of the tumor. They also found an increase in the infiltration by lymphoid cells, granulation tissue and fibrosis surrounding the tumor in the Canova[®]-treated group. Determination of the white blood cell count showed an increase in the total number of leukocytes and lymphocytes. These results all reflect

an enhanced immune response of the host after treatment with Canova[®] (Sato *et al.*, 2005).

➤ **Homeopathy and HIV**

In 2008, Pretorius *et al.* investigated the ultrastructural changes in platelet aggregates of HIV patients. The ultrastructure of platelet aggregates of patients with HIV have shown membrane blebbing and ruptured platelet, which is indicative of apoptosis. With these observations the authors suggested that HIV patients may develop thrombocytopenia as a result of peripheral platelet destruction. The differences in platelet morphology from control, Canova[®]-treated HIV patients and non-treated HIV patients were investigated by using scanning electron microscopy.

The authors revealed slight morphological changes when comparing the control fibrin networks with Canova[®]-treated HIV and untreated HIV patients, which suggests that the HIV does not have an impact on the fragility of fibrin networks. However in the HIV patients, bleb-like bulges on the membrane of platelets as well as membrane breakages on the aggregate could be observed, where membrane blebbing was less pronounced in the Canova[®]-treated patients. From these observations, the authors suggested that Canova[®] protects the membranes of platelets and that blebbing does not appear in such great proportions as was found in the untreated HIV group. The authors concluded by stating that this study supports previous research done on Canova[®] where it was found that Canova[®] protects the immune system, by keeping the ultrastructure intact and also preventing the cyto-destructive effects of HIV (Pretorius *et al.*, 2008).

➤ **Clinical trials using a homeopathic immunomodulator**

A number of clinical trials have also been done, focusing mainly on HIV and cancer research. Table 2.1 indicates the different clinical trials done on Canova[®] as well as the findings on the studies done.



Table 2.1: Clinical trials done on HIV and cancer using an immunomodulator as treatment remedy

Year	Title	Authors	Findings
2006	Patients' recovery with HIV/AIDS in Botswana / Africa, with the use of the homeopathic Medicine Canova®.	Bernardi, R.P. <i>et al.</i>	Decrease pain; Increase appetite; Recovered capability to perform tasks; Decreased absenteeism
2005	HIV/AIDS+ patients treated with the homeopathic Medicine Canova®:Prospective Observational study in Laboratory, Clinical and of Quality of Life Indexes.	Stroparo E. <i>et al.</i>	Increase appetite and energy; Decrease pain and depression; Decrease opportunistic infections; Increase total number of lymphocytes, CD4 and erythrocytes; Increase in life quality
2004	Quality of life and handling of Cancer or Aids with Immunomodulator Canova®	Castanheira, P. <i>et al.</i>	Improvement of live quality in cancer and HIV patients
2003	Evaluation of Canova® Immunomodulator's use in patients with cancer.	Castanheira, P. <i>et al.</i>	Decrease pain complaints; Improvement of appetite and cognitive function; Decrease of tumor volumes;
2002	A Multicentric Clinical Survey of HIV/AIDS patients submitted to Canova® Immunomodulator treatment associated with Antiretrovirus Medicaments	Berbert, A.A. <i>et al.</i>	Weight gaining; Decrease viral load; Improvement of life quality
2002	Clinical study work for evaluation of Immunomodulator Canova® in the therapeutics of oncologic patient considered OTP – Out of Therapeutic Possibility	Cabral, M.P. <i>et al.</i>	Weight gain; Improved appetite; Reduction of nausea and vomits; Increase willingness for daily activities; Increase in some



			lymphocytes and monocytes
2002	Immunomodulator Canova® – Aids and quality of life.	Brito, D. J. M	Improvement of general health; around 10% weight gain and appetite improvement; decrease insomnia; decrease cramps; better perspectives; accentuated decrease of infections; Increase in total leucocytes and CD4; decrease viral load
2001	Randomized Placebo-Controlled Clinical Trial to appraise the effectiveness and safety of Immunomodulator Canova® in the therapeutics of patients who have HIV/AIDS on anti-retroviral use.	Sasaki, M.G.M. <i>et al.</i>	Reduction of viral load; Decrease occurrence of opportunistic diseases; Decrease antiretroviral toxicity; Improvement of life quality

2.1.9 Animal models in asthma

Mice are used all over the world as experimental animals in many different studies. Using mice as experimental animals offers a number of advantages. Firstly, the mouse genome is extensively characterized and exhibits a high degree of homology with its human counterpart and in 2002 it was reported that approximately 99% of genes are shared between mice and humans and around 90% of the two genomes can be portioned into regions of conserved synteny (Waterston *et al.*, 2002).

Another advantage of using mice is the fact that a wide range of developmental, biochemical, and physiologic features are shared between humans and mice and this resemblance that exists, especially on immunological level, holds meaning that many human functions and dysfunctions can be modeled efficiently in mice and that these models deliver important and relevant insights into human pathology (Willis-Owen and Valdar, 2009).

In 2002, Pennisi reported that mice are an established experimental tool and have been used in the laboratory since 1664 (Pennisi, 2002). Another advantage also includes the fact that mice are quick to breed and economical to house, since they have a short gestation period of just 19-21 days, an early puberty with an average of 42 days to reach fertility, and a short estrus cycle of 4-5 days and tend to produce relatively large litters usually 4-12 pups. Taking all these characteristics into account, it is said that mice have become the foremost mammalian system in which to model human disease (Willis-Owen and Valdar, 2009).

In conclusion, it is clear from the literature that many studies have been done on asthma by using different substances and investigating different parameters aiming to better understand the mechanisms involved in this disease. The present study therefore aims to contribute to the global pool of knowledge and research that exist



up to date by investigating the possible role of a homeopathic product in the treatment of asthma.

2.2 Aims and objectives of the current study

The general aims and objectives of this study include the following:

1. Implementing the asthmatic BALB/c mouse model
2. To use the BALB/c mouse model to investigate the ultrastructure of platelets and fibrin networks by using scanning electron microscopy
3. To compare the ultrastructure of platelets and fibrin networks of the asthmatic BALB/c mice to that of human control and asthmatic subjects
4. To statistically evaluate the changes in weight of each individual mouse in the different experimental groups over the 43- day experimental period
5. To use the BALB/c mouse model to determine the effect of Modul8® on white blood cell counts in blood as well as bronchial lavage
6. To investigate the ultrastructure of the LPS-activated peritoneal macrophages *in vitro* by using scanning electron microscopy and to analyse the production of TNF- α and nitric oxide (NO) in LPS-activated peritoneal mouse macrophages exposed to Modul8®
7. To use light and transmission electron microscopy to investigate the histological and ultrastructural changes in the lungs including inflammatory cell infiltration



2.3 Hypothesis

In asthma, platelets, fibrin networks and lung tissue are altered morphologically. Also, white blood cell counts are increased in the blood as well as in the bronchial lavage and the levels of TNF- α are changed. Therefore the hypothesis of this study is that Modul8[®] will stabilize the platelets, fibrin networks as well as lung tissue in asthmatic BALB/c mice, and that the ultrastructure of the mentioned tissues will appear similar to that of the control animals. We also hypothesise that the white blood cell counts in asthmatic animals will decrease and the levels of TNF- α will be stabilized due to Modul8[®] administration and that Modul8[®] will activate macrophages *in vitro*. It is also known that the experimental procedures influence the weights of the animals over the experimental period. We hypothesise that Modul8[®] will positively influence the weight changes by stabilizing it.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 3

IMPLEMENTATION OF THE BALB/c ASTHMATIC MOUSE MODEL

3.1 Introduction

Research that has been done by using animal models has provided important information over the years on many aspects of the pathophysiology of human diseases. Well-performed animal studies can be used to determine potential benefits of proposed therapeutic interventions and the results obtained from many experimental animal models on many different diseases have also served as the basis for many clinical trials (O'Neil *et al.*, 1999). Although there is no animal model that exactly reproduces the pathology of human asthma, researchers in 2003 stated that they play an important role in the development of novel therapies and in the understanding of the pathogenesis of the disease (Isenberg-Feig *et al.*, 2003).

A wide range of different animal models is available and choosing the appropriate model for a specific study is very important and sometimes difficult. Mice and rats and also guinea pigs are some of the most popular animal models used (Zosky and Sly, 2007). Murine asthmatic animal models have contributed significantly to the knowledge and understanding of airway disease, such as in delineating the role of T lymphocytes in the orchestration of inflammation and airway hyperresponsiveness and the induction of respiratory tolerance (Willis-Owen and Valdar, 2009).

According to Epstein (2006), antigen-induced mouse allergic asthma is a useful model for testing novel therapeutics (Epstein, 2006) and that these models offer excellent tools for studying the effects of allergens *in vivo* in their natural target organs (Epstein, 2004).

In vivo animal models have been used successfully during the past few years to study diseases like asthma (Epstein, 2004).

Since a correlation exists between the allergic response in sensitive mice and that of human asthmatics, asthmatic animal models have been developed and used to study the immune and inflammatory mechanisms of the disease. These models have also been used to investigate the efficiency of new drugs in the treatment of asthma. There are a number of corresponding features, with regards to airway remodeling, in humans and mice. This includes mucus cell metaplasia, shedding of the epithelium, thickening of the basement membrane and edema (Blyth *et al.*, 1996; Hogan *et al.*, 1996).

However, there are important differences in the airway development and morphology in humans and mice and thereby preventing the direct extrapolation of results between species. Differences in the number of branches, the pattern of branching, lobation and submucosal gland distribution exist (Metzger *et al.*, 2008).

The murine model in particular, is used successfully because mice allow for a variety of *in vivo* immunological applications (Bice *et al.*, 2000), however it is important to take into account that mouse models are not exact replicas of human asthma but they help to understand some of the basic mechanisms involved in the production of this disease (Roberto *et al.*, 2005).

The murine asthmatic BALB/c mouse model was used as experimental model in this study, and therefore this chapter includes the materials and methods used on implementation of the BALB/c asthmatic mouse model. The different experimental procedures that were conducted on specific days are indicated in Table 3.1. This model with its experimental procedures was also used before by researchers to

investigate the role of alternative products on asthmatic animals (Oberholzer *et al.*, 2008). Experimental procedures, including sensitization, nebulization and treatment are stated as well as the equipment, materials and methods used during each of the abovementioned procedures.

3.2 Implementing the asthmatic BALB/c mouse animal model

6-week-old (female) BALB/c mice (each of average weight 20g), maintained in the University of Pretoria Biomedical Research Centre and provided OVA-free food (Balanced EpolT mice cubes and pellets, obtained from EPOL- a division of Rainbow Farms PTY LTD, SA) and water *ad libitum*, were used. Polycarbonate type III cages were obtained from Techniplast. The animals were kept in a barrier unit with a temperature range of 20-24°C and a relative humidity of 40-60% with a 12h daylight and 12h night-time.

6 mice were housed per cage and autoclaved pinewood shavings were used as embedding material. White facial tissue paper was also added for enrichment. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee and ethical clearance was obtained from the University Animal Ethics Committee (UPBRC). Ethical clearance number: (151/2006).

Animals were monitored daily by the lab animal technologist for clinical signs and abnormalities. No clinical signs of respiration, stridor or abnormal food intake could be observed. Also, no abnormal behaviour could be seen in any of the animals.

Mice were randomly divided into the following groups:

- 6 control mice
- 6 asthmatic mice receiving no treatment
- 6 asthmatic mice exposed to physiologically comparable levels of Modul8® (10%) (referred to as the Modul8® group) and
- 6 asthmatic mice exposed to hydrocortisone (referred to as the hydrocortisone group) at a concentration of 100mg/kg body weight.

Table 3.1: The different experimental procedures conducted on specific days

DAY: 0, 5	Sensitization
DAY: 13, 14, 15	Nebulisation
DAY: 15, 16, 17, 18	Treatment
DAY: 22, 23, 24, 25	Treatment
DAY: 30, 31, 32	Nebulisation
DAY: 36, 37, 38, 39	Treatment
DAY: 42, 43	Termination

3.3 Sensitization

Sensitization of mice involved the intraperitoneal injection of 25mg ovalbumin (OVA) (Grade V; Sigma Aldrich) and 2mg Al(OH)₃ dissolved in 0.5ml of 0.9% saline solution. All mice except those in the control group were sensitized.

3.4 Nebulization

The mice were nebulized with 1% OVA dissolved in PBS. An inhalation exposure system (IES) (Glas-COL® Inhalation Exposure System, Model 099C A4212, Terre Haute, Indiana) was used for the nebulization. The protocol used for the nebulization

is standard and the times used are recommended by the manufacturer of the IES and programmed on the machine.

Nebulization involved placing the 6 mice from each experimental group except the control group, inside a stainless steel wire mesh basket, which is divided into five equal compartments. Each compartment therefore held the six animals from the same experimental group. One complete cycle in the IES included a preheat cycle of 15 minutes followed by the nebulization with the OVA for 60 minutes, a cloud decay cycle for 15 minutes and a decontamination cycle also 15 minutes. Mice were nebulized twice daily.

3.5 Treatment

Treatment involved the administration of Modul8[®] and hydrocortisone to asthmatic animals. Solu-Cortef[™] 100mg hydrocortisone [Pfizer Laboratories (Pty) Limited], dissolved in sterile PBS was injected intra-peritoneally, while Modul8[®] [Natel Healthcare SA (PTY) Ltd] was administered orally on the specific treatment days as indicated in Table 3.1.

For humans it is generally advised to take 10 drops of Modul8[®] 3 times per day. And since the average of 10 drops equals approximately 600 μ l, it can be said that humans take 600 μ l three times per day. This will equal an amount of 1800 μ l per day (1.8ml/day). When the average weight of a human adult is also considered (between 60-70kg: average 65kg): It can therefore be assumed that a normal person that weighs 65kg will take in $1800\mu\text{l} = 1800\mu\text{l} / 65\text{kg}$. This will imply that the intake is: 28 μ l/kg. The average weight of a mouse is around 20g (0.02kg). And since it was established that the intake per day is 28 μ l/kg, it can be calculated that each mouse should receive 0.56 μ l of the Modul8[®] per day in order to keep the same values as the

human. Since only one daily administration of the test compound was needed, oral gavage was used as it allows for administration of a controlled amount of compound.

The adult dosage of 100mg/kg body weight hydrocortisone was converted to the average mass of a mouse, which was taken at 20g. The hydrocortisone was administered via intraperitoneal injection, as was indicated on the product itself and since it is one of the most common routes of administration in mice.

3.6 Conclusion

The BALB/c murine asthmatic animal model was successfully implemented in this study over the 43 –day experimental period. No animals died during the course of the study nor did any of the animals suffer excessive weight loss. The results presented in the chapters to follow, indicate that this model was successfully implemented and that the animals were indeed asthmatic. It is also reported that there is a correlation between the allergic response in sensitive mice and that of human asthmatics.

Therefore the results found in this study indicate that the BALB/c asthmatic mouse model can successfully be used to study a disease like asthma and furthermore that this OVA-induced allergic mouse model is useful in testing alternative medication on asthma.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 4

INVESTIGATING THE EFFECT OF MODUL8® ON THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF EXPERIMENTAL ASTHMATIC BALB/c MICE

4.1 Introduction

Platelets and fibrin play an important physiological role in allergic processes and immunological mechanisms including those associated with asthma where platelets participate by acting as inflammatory cells as they are releasing mediators, spasmogens and interacting with other inflammatory cell types. Platelets are activated by a number of stimuli and this activation may be due to, amongst others, inflammatory processes (Camera *et al.*, 1999; Butenas and Mann, 2002; Lazarus *et al.*, 2003).

An important role for platelets in various inflammatory diseases has been reported on including atherosclerosis, rheumatoid arthritis, eczema, allergic rhinitis and asthma (Kornerup and Page, 2007). It was also found that platelets play an important role in asthma as they have been found to actively participate in most of the main features in asthma such as bronchial hyperresponsiveness, bronchoconstriction, airway inflammation and airway remodeling. Platelet-release products as well as the expression of adhesion molecules on the surface of the platelet and the ability to undergo chemotaxis are all involved in these features in the inflammatory process in asthma (Kornerup and Page, 2007).

Platelets also play an important role in bronchoconstriction and several animal studies have been done to investigate this. Activated platelets secrete various mediators that contribute to the bronchoconstriction such as histamine, serotonin (5-

HT), platelet activating factor (PAF) and arachidonic acid metabolites (Page, 1989). In a study done on guinea pigs, the intravenous administration of PAF resulted in acute bronchoconstriction and recruitment of macrophages, eosinophils and platelets into the airways (Vargaftig and Braquet, 1987).

PAF is a potent activator of platelets and neutrophils and can induce systemic anaphylaxis when given intravenously and ultrastructural studies have also shown that in addition to causing neutrophil and eosinophil recruitment into the lungs, PAF also induces intravascular platelet aggregation and degranulation in alveolar capillaries and stimulates platelet diapedesis to the alveolar lumen, features which are absent in guinea pigs pre-treated with prostacyclin or aspirin (Lellouch-Tubiana *et al.*, 1985).

Platelets also play a role in the recruitment of inflammatory cells into the airways of asthmatic individuals. It was previously reported that platelet depletion, as well as administration of prostacyclin (PGI₂) and PAF antagonists in asthmatic animal models, significantly inhibits eosinophil infiltration into the lungs (Coyle *et al.*, 1990; Lellouch-Tubiana *et al.*, 1988). It was also shown in an OVA-induced murine model of allergic lung inflammation that eosinophil recruitment into the airways is inhibited in thrombocytopenic animals (Pitchford *et al.*, 2003), suggesting that platelets are essential for leukocyte recruitment in allergic models of asthma.

Together with the role of platelets in bronchoconstriction and the recruitment of inflammatory cells into the airways, is the role of platelets in the process of airway remodeling in asthma. Platelets secrete various mediators including free radicals and cationic proteins that have been shown to contribute to the increase in vascular permeability of the airway epithelium and the stimulation of mucus secretion

(Kornerup and Page, 2007). Platelets therefore represent an interesting new target for the study of inflammatory diseases whose pathogenesis remains unclear.

In the current chapter the BALB/c mouse model was used to investigate the possible effect of Modul8® on the ultrastructure of platelets and fibrin networks by using scanning electron microscopy. Hydrocortisone is used as positive control in the study as it is the most commonly used medicine in the treatment of asthma (methodology of model described in Chapter 3).

4.2.1 Preparation of fibrin clots

Blood was collected on the day of termination via orbital puncture. 11 µl of citrate was added for every 100 µl of blood drawn. Blood was then centrifuged at 1250 rpm for 2 minutes to obtain platelet rich plasma (PRP).

Human thrombin (provided by The South African National Blood Services) was used to prepare the fibrin clots. The thrombin is 20 U/ml and is made up in biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

10 µl of mouse PRP was mixed with 10 µl of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 µm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. A washing process followed where the millipore membranes with the coagula were placed in PBS and

magnetically stirred for 1hr. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al.*, 2006).

4.2.2 Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1hr. Each fibrin clot was rinsed three times in phosphate buffer for 5 minutes before being fixed for 1hr with 1% Osmium tetroxide (OsO_4). The samples were rinsed three times with DPBS for 5 minutes and dehydrated serially in 30%, 50%, 70%, and 90% and three times with 100% ethanol.

The SEM procedures were completed by drying of the material with hexamethyldisilazane (HMDS) (Araujo *et al.*, 2003), mounting, coating with ruthenium tetroxide (SPI Supplies, West Chester USA) and examination of the tissue with a ZEISS ULTRA plus FEG scanning electron microscope.

4.3 Results

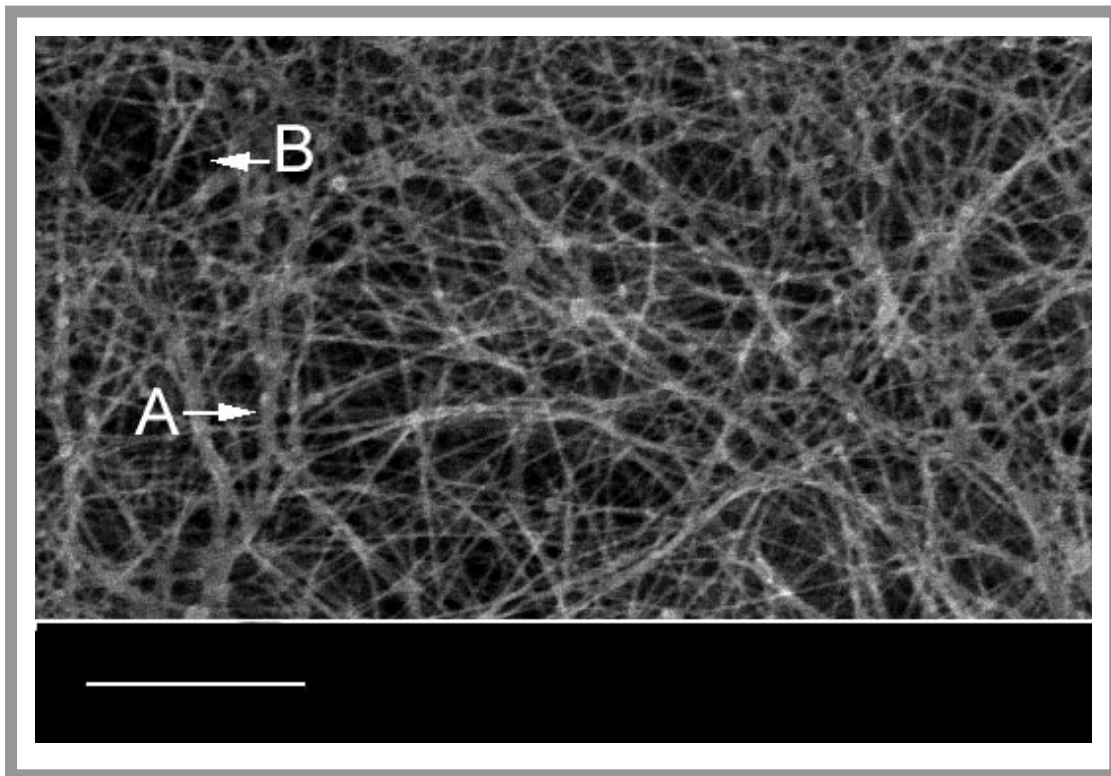


Figure 4.1a: Control fibrin network of BALB/c mice with thick, major fibers as well as thin, minor fibers. Label A = thick, major fibers; Label B = thin, minor fibers. (Scale bar = 200nm)

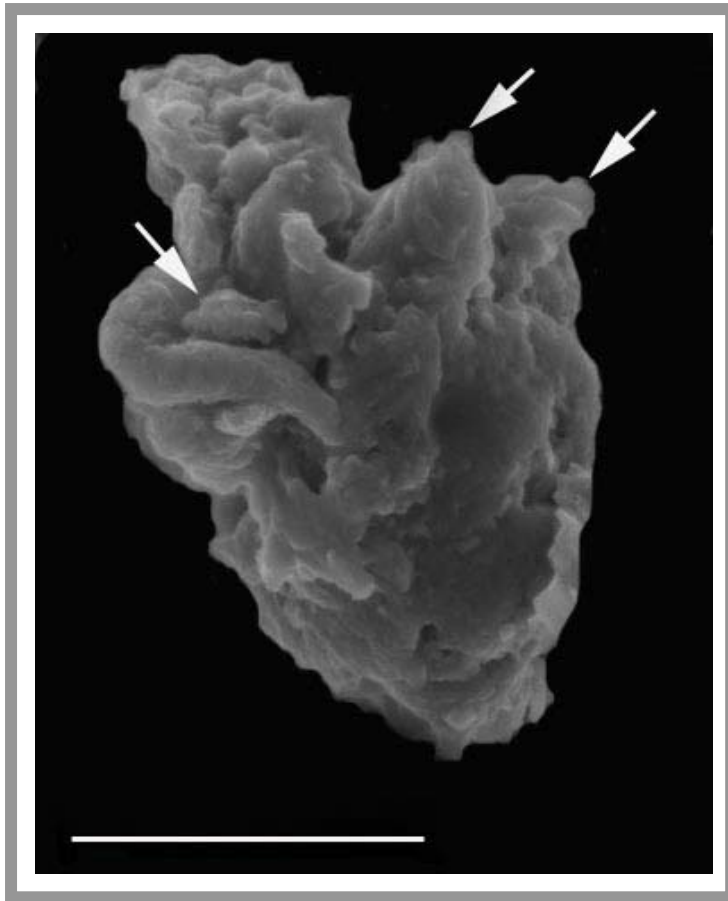


Figure 4.1b: Control platelet aggregate of BALB/c mice; white arrows – pseudopodia.
(Scale bar = 1 μ m)

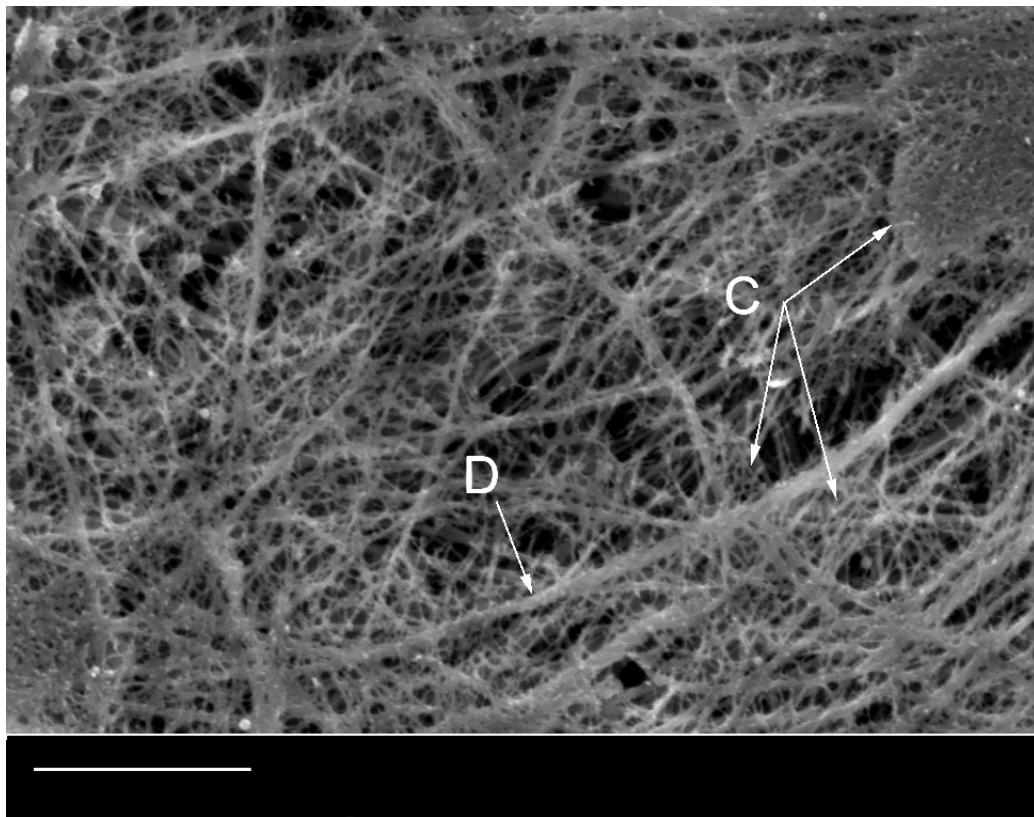


Figure 4.1c: Fibrin network of asthmatic BALB/c mice, forming flimsy fibrin network. **Label C** = matted, thin minor fibers; **Label D** = thick, major fibers.
(Scale bar = 200nm)

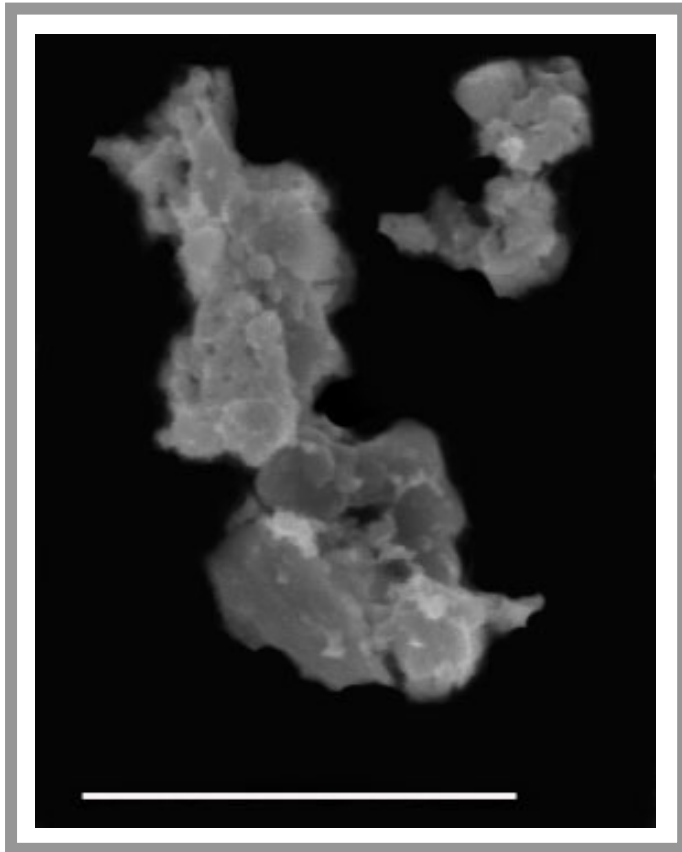


Figure 4.1d: Platelet aggregate of asthmatic BALB/c mice.
(Scale bar = 1 μ m)

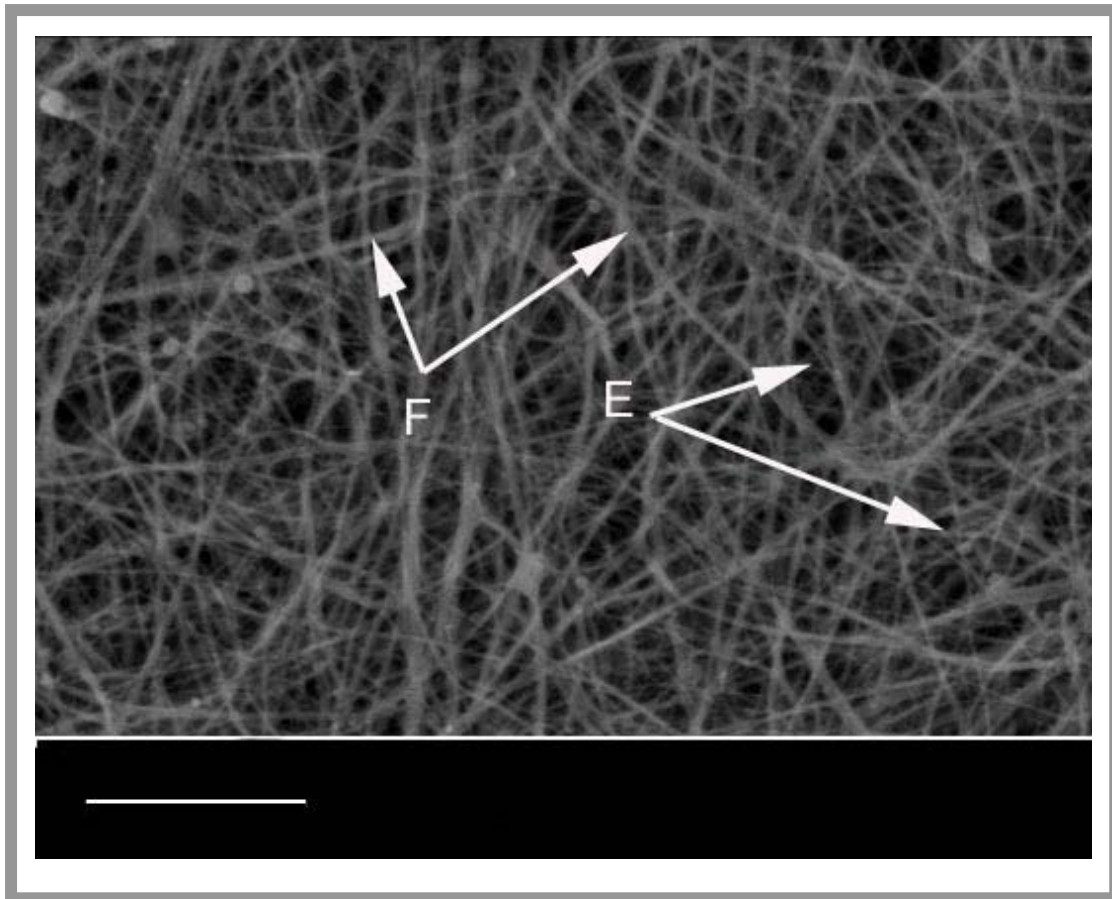


Figure 4.1e: Fibrin network from asthmatic BALB/c mice treated with Modul8®. **Label E** = Thin, minor fibers; **Label F** = Thick, major fibers
(Scale bar = 200nm)

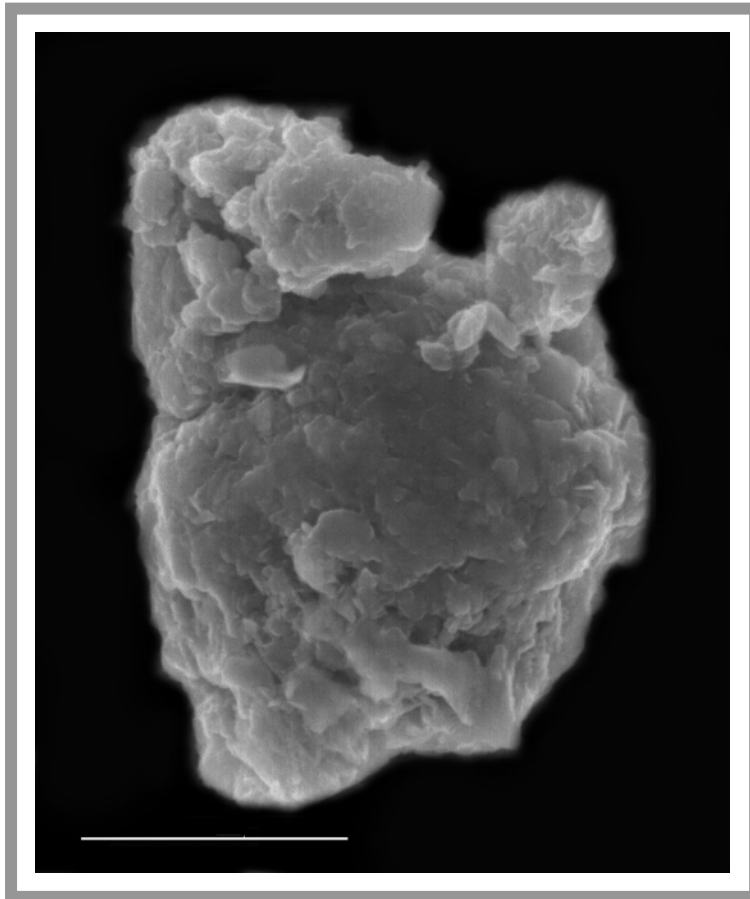


Figure 4.1f: Platelet aggregate from asthmatic BALB/c mice treated with Modul8®.
(Scale bar = 1µm)

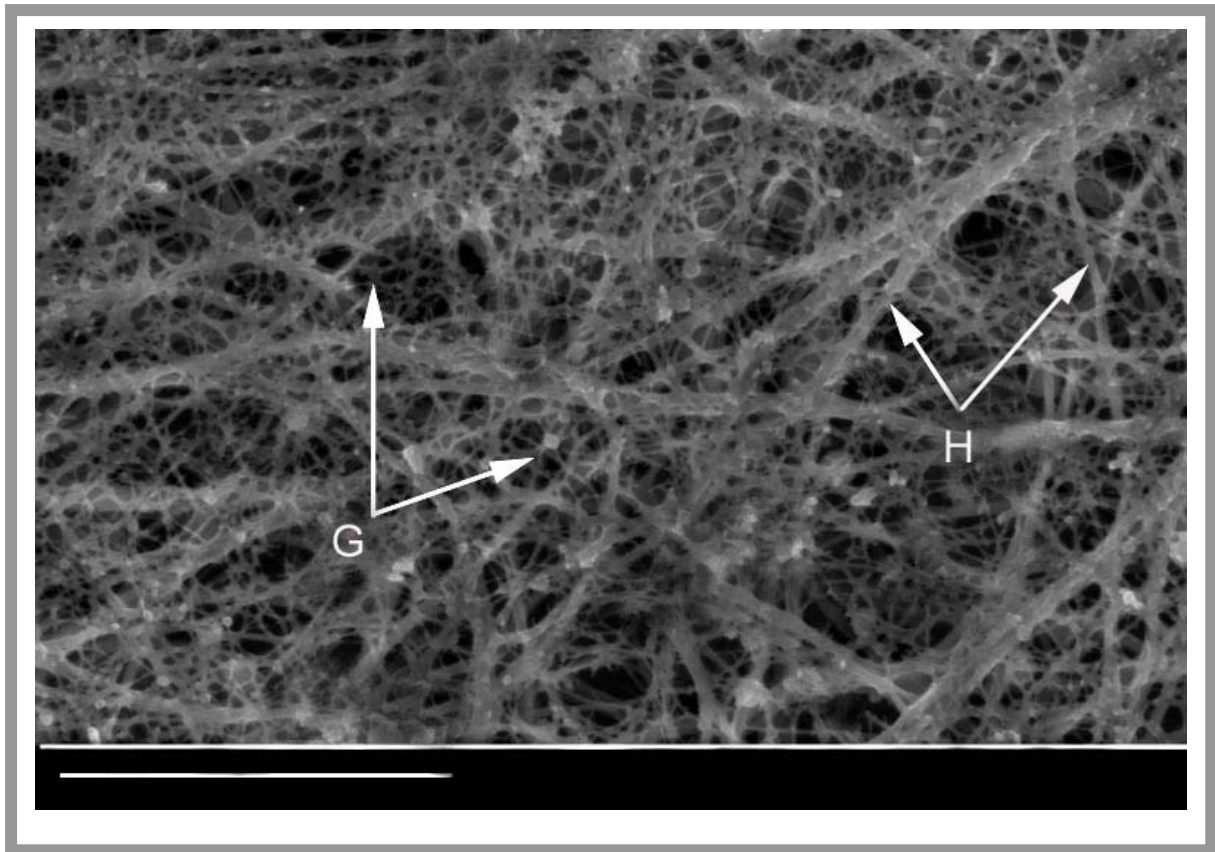


Figure 4.1g: Fibrin network from asthmatic BALB/c mice treated with hydrocortisone. **Label G** = thin, matted minor fibers; **Label H** = thick major fibers (Scale bar = 1 μ m)

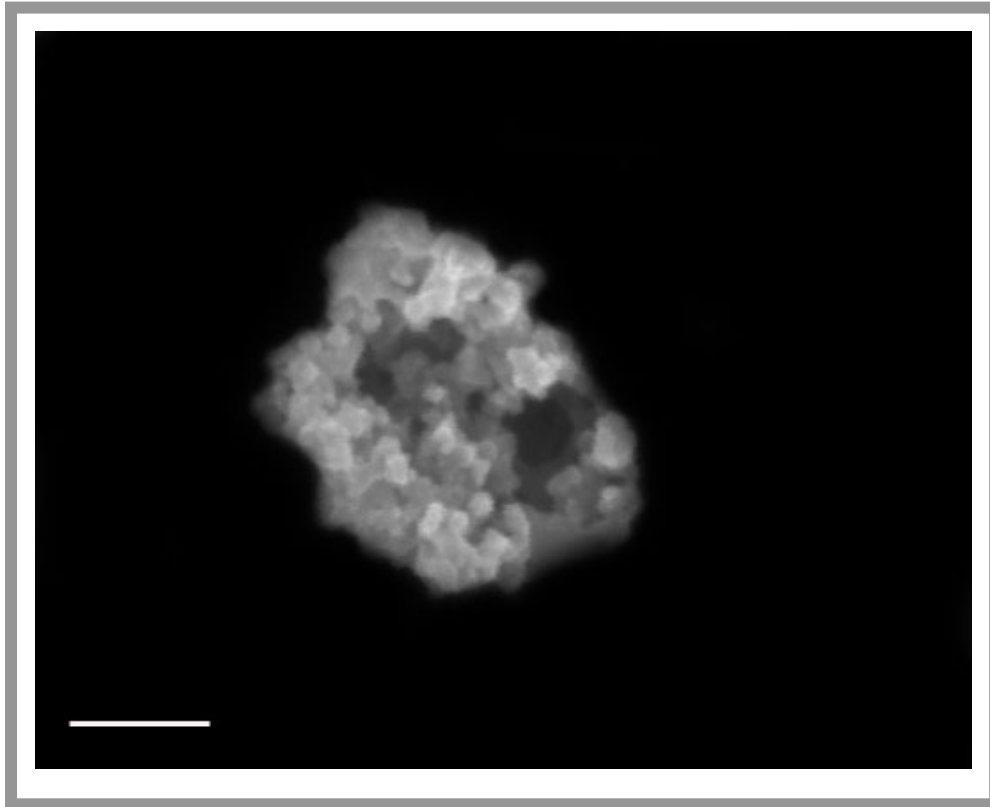


Figure 4.1h: Platelet aggregate from asthmatic BALB/c mice treated with hydrocortisone.
(Scale bar = 200nm)

Figure 4.1 a-h shows the differences in the ultrastructure of platelets and fibrin networks in the different experimental groups. Figure 4.1a and b shows control fibrin networks and a platelet aggregate. Thick major fibers are indicated with Label A and Label B shows fine, minor fibers (Figure 4.1a). Figure 4.1b shows a round compact platelet aggregate with pseudopodia (white arrows).

Figure 4.1c shows fibrin networks of asthmatic animals where the minor fibers (Label C) form a thick, matted layer over the major fibers (Label D). Figure 4.1d shows a platelet aggregate of untreated asthmatic animals, where the aggregates are loosely arranged.

Figure 4.1e and f shows the fibrin network and platelet aggregate of Modul8[®]-treated animals showing thin minor fibers (Label E) and thick, major fibers (Label F).

Figure 4.1 g and h are fibrin networks and platelet aggregate micrographs of the asthmatic animals treated with hydrocortisone. Label G and H indicate respectively the matted layer of thin fibers covering the thick, major fibers.

It was seen that Modul8[®] stabilized both the fibrin networks and platelet aggregates and SEM analysis revealed the morphology to be the same as that of the control animals. Platelets and fibrin networks play an important role in asthma and therefore it is an important observation that Modul8[®] positively influenced the ultrastructure of these structures.



4.4 Discussion

Figure 4.1 shows scanning electron micrographs of the ultrastructure of platelets and fibrin networks in the different experimental groups. Previously it was reported that asthma has an effect on the ultrastructure of platelets and fibrin networks. Pretorius *et al.* in 2007a reported on the ultrastructure of platelets and fibrin networks of asthmatic mice compared to that of control mice, hydrocortisone-treated mice and asthmatic mice treated with the plant *Euphorbia hirta*. The results indicated that control mice possess major, thick fibers and minor thin fibers and tight round platelet aggregates could be observed showing typical pseudopodia formation. The minor fibers from the asthmatic animals formed a net covering the major fibers and the platelets appeared as loosely arranged granular aggregates compared to the tight round appearance of the control mice. Hydrocortisone seemed to influence the fibrin networks by making it more fragile and platelet morphology changed from a tight platelet aggregate to a more granular aggregate, almost similar to that of the asthmatic animals (Pretorius *et al.*, 2007a).

This was also found in the current study as shown in Figure 4.1 g and h where the minor fibers (Label G) of the fibrin network of the hydrocortisone-treated animals formed a thin, matted layer over the major fibers (Label H) and the platelet aggregate also changed from a tight aggregate as seen in the control- and Modul8®-treated animals to a granular, loosely arranged aggregate as can be seen in the asthmatic animals.

The current research showed that Modul8® stabilized fibrin networks and platelet aggregates. Fibrin networks of Modul8®-treated animals (Figure 4.1e) appeared similar to that of the controls (Figure 4.1a) while the fibrin networks of the untreated asthmatic animals (Figure 4.1c) as well as that of the hydrocortisone-treated group appeared fragile and the thick fibers had a matted covering formed by the thin, minor

fibers. Modul8® seems to also stabilize platelet aggregates (Figure 4.1f) and these aggregates looked similar to the control animals (Figure 4.1b). Platelet aggregates of the untreated asthmatic animals (Figure 4.1d) as well as that of the hydrocortisone-treated asthmatic animals (Figure 4.1h) appeared granular without the tight round appearance as can be seen in the control platelet aggregates. The ultrastructure of the platelets and fibrin networks of the Modul8® treated group are similar to that of the control group where thick major and thin minor fibers can clearly be distinguished and a tight mass of platelet aggregate could be observed.

4.5 Conclusion

From this study it is clear that platelet aggregate and fibrin network morphology are changed in the asthmatic animals. It is also well known that platelets and fibrin play a fundamental role in asthma since platelets act as inflammatory cells in the immune response. Modul8® seemed to stabilize platelet and fibrin morphology to a profile similar to that of the control animals. However, the question arose whether results from the BALB/c asthmatic animal study are indeed comparable to that of human asthmatics. In chapter 5 the control and asthma platelets and fibrin networks from the BALB/c asthmatic animal model will be compared to that of human control and asthmatic subjects.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 5

COMPARING THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF THE ASTHMATIC BALB/c MICE TO THAT OF HUMAN CONTROL AND ASTHMATIC SUBJECTS

5.1 Introduction

Platelets play an important role in asthma and there is a vast amount of clinical data that reveal that platelet activation accompanies allergen-induced bronchoconstriction in humans (Pitchford *et al.*, 2003; Pitchford *et al.*, 2008). According to Pitchford in 2007, the role of platelets in asthma are distinct from their classically known actions performed during thrombosis and haemostasis; and include the expression of adhesion molecules and contact-dependent activation of leucocytes, the release of a plethora of inflammatory mediators, activation in cells of the adaptive immune response and the ability to migrate and undergo chemotaxis (Pitchford, 2007). The author also mentions that clinical data from patients suffering from asthma, allergic rhinitis and allergic dermatitis reveal changes in platelet behaviour and function during or after allergen exposure. It is therefore useful to use platelets and fibrin to study a disease such as asthma.

Fibrinogen itself is also widely recognised as a marker for systemic inflammation as it is considered an acute phase protein.

The murine BALB/c asthmatic animal model is a widely used animal model and it has previously been used successfully for a number of *in vivo* immunological applications and for testing novel therapeutics. Pretorius and co-workers in 2007 were the first to use this model to study ultrastructure and changes in platelet ultrastructure during asthma were noted (Pretorius *et al.*, 2007a). Bronchial lavage and white blood cell

counts, additionally confirmed an asthmatic state in the animals. This model was therefore proven to be a reliable, clinically relevant facsimile of human asthma. In the study, the ultrastructure of fibrin networks and platelets of control mice were compared to asthmatic mice treated with hydrocortisone and a plant material (Pretorius *et al.*, 2007a). Results indicated that control mice possess major, thick fibers and minor thin fibers as well as tight round platelet aggregates with typical pseudopodia formation. Minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates.

The question that arises is whether platelets and fibrin networks of humans with asthma will have the same ultrastructure as seen in the BALB/c asthmatic model. In order to answer this question, ultrastructure of platelets and fibrin networks from asthmatic subjects were studied and compared to that of human controls as well as the animals from the asthmatic BALB/c mouse model.

5.2 Materials and Methods

5.2.1 Samples

Blood was collected from two subjects: a controlled asthma subject and her 13 year old son, with severe, uncontrolled asthma. The female subject with controlled asthma has been suffering from asthma since childhood. However, the disease is kept under control with a strict diet for the past 10 years. She follows a diet rich in anti-oxidants, no grains or bread in any form, and at least seven different types of fruit and vegetables per day with very little meat and additional anti-oxidant supplements. This diet has been followed for the past 10 years, resulting in controlled asthma, without medication for the same number of years. However, a bronchodilator, Ventolin [salbutamol; Glaxo-SmithKline South Africa (Pty) Ltd, Johannesburg, South Africa] is used occasionally when an asthmatic response is triggered.

The second subject is a 13 year old male with chronic, severe asthma, treated with Seratide [salmeterol; Glaxo-SmithKline South Africa (Pty) Ltd, Johannesburg, South Africa] once a day and Ventolin once or twice per day. This subject does not follow a strict diet, where possible dietary triggers are excluded and do not include more than 2 vegetables and fruit daily, also no extra anti-oxidant supplementation is taken.

5.2.2 Preparation of fibrin clots

Blood was collected from two asthmatic subjects and three control subjects (Research Ethical Committee of the University of Pretoria, South Africa; ethical clearance number 115/2006). Fresh platelet-rich plasma (PRP) was prepared by drawing 40 ml of blood which was centrifuged at 1 000 rpm (maximum RCF = $17.523 \times g$; 1 250 g) for 2 minutes. Fibrin clots were prepared in order to obtain platelet aggregates as well as fibrin fibers from both the patients as well as the control groups. Human thrombin (provided by the South African National Blood Service) was used to prepare these fibrin clots from the asthmatic patients as well as the controls. Human thrombin is prepared from a single regular donor by calcium chloride activation of a euglobulin fraction of plasma obtained by apheresis. Each individual unit is tested and has to be non-reactive for hepatitis B surface antigen (HbsAg), HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, hepatitis V virus (HCV) antibody and antibodies to *Treponema pallidum*. These tests are performed by licensed assay methods. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, e.g. transforming growth factor, platelet-derived growth factor and fibroblastic growth factor are released into the coagulum. 20 μ l of the PRP was mixed with 20 μ l of human thrombin on a 0.2 μ m millipore membrane to form the coagulum (fibrin clot). This millipore membrane was then placed in a Petri dish on

filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37 °C for 10 minutes (Pretorius *et al.*, 2007a). A washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 1hr followed this. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al.*, 2006).

5.2.3 Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1hr. Each fibrin clot was rinsed thrice in phosphate buffer for 5 minutes before being fixed for 1hr with 1% osmium tetroxide (OsO_4). The samples were rinsed thrice with distilled water for 5 minutes and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting, coating with ruthenium tetroxide (SPI Supplies, West Chester USA) and examining the tissue with a ZEISS ULTRA plus FEG scanning electron microscope. A series of micrographs were taken over the filter to ensure that the morphology seen is representative of the whole clot area.

5.3 Results

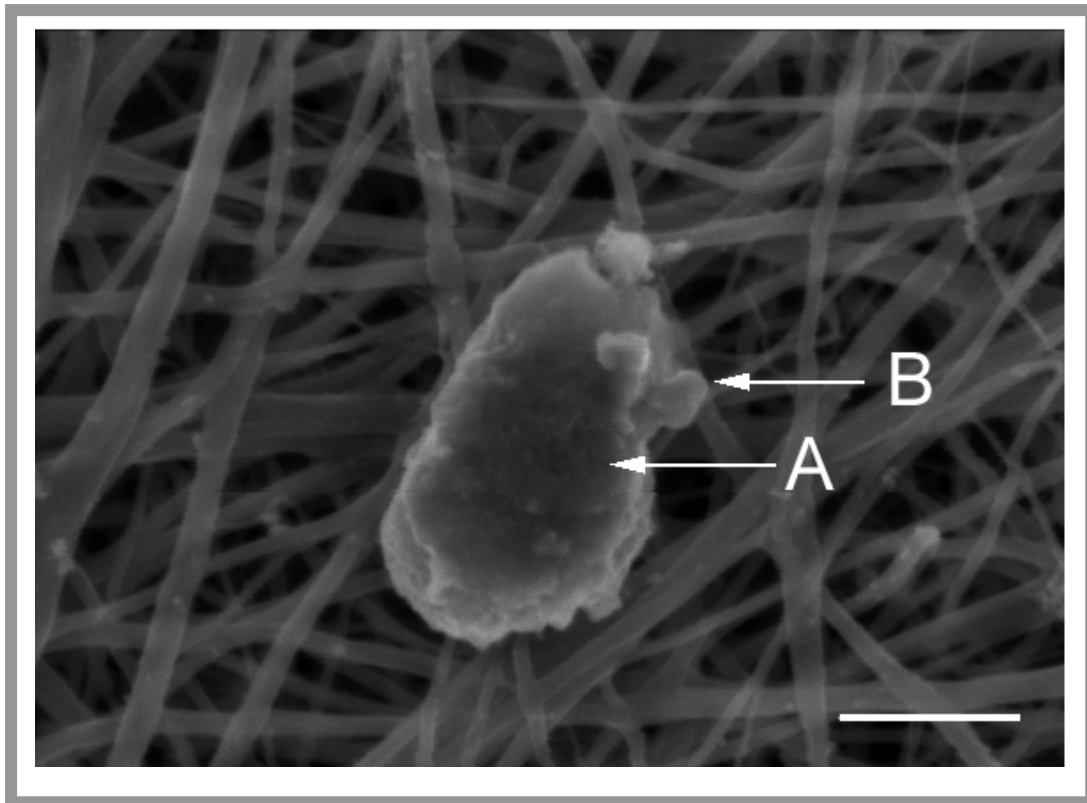


Figure 5.1a: Control platelet aggregate forming dense, round aggregate with pseudopodia.

Label A = smooth membrane of aggregate, **Label B** = pseudopodia. (Scale = 1 μm)

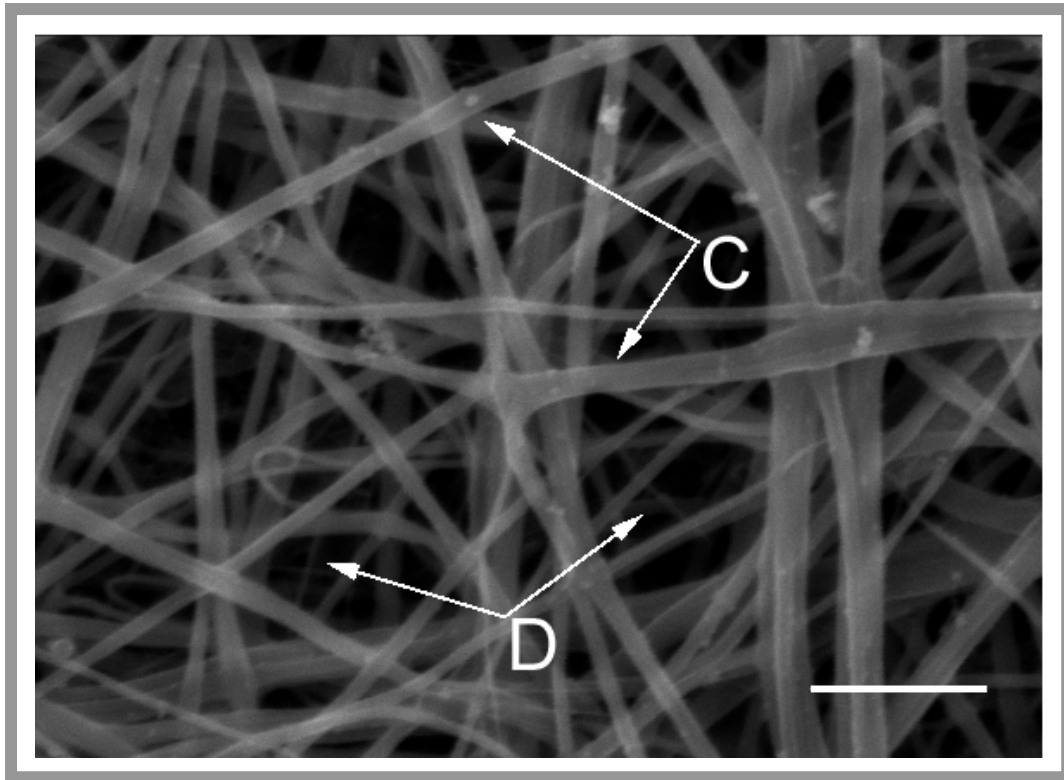


Figure 5.1b: Control fibrin network with thick, major fibers as well as thin, minor fibers. **Label C** = thick, major fibers; **Label D** = thin, minor fibers. (Scale = 1 μ m)

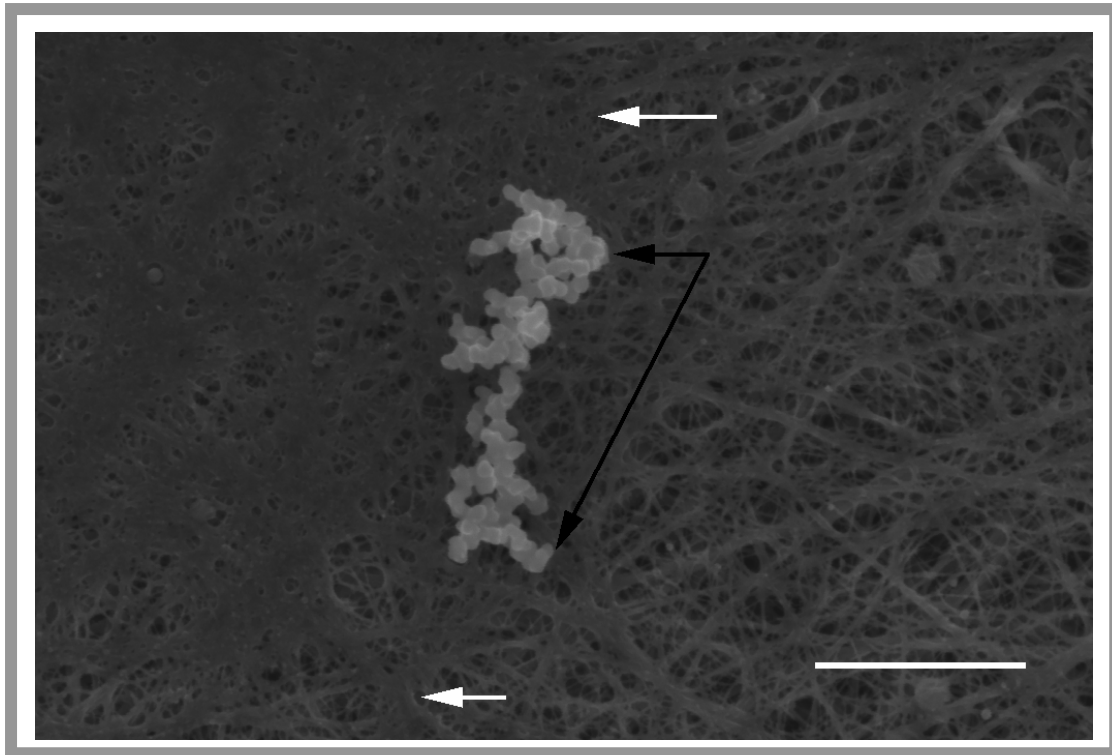


Figure 5.1c: Platelet aggregate and fibrin network from asthmatic mouse from the BALB/c asthmatic animal model. Loosely arranged platelet aggregate shown by thick black arrow; thick white arrow showing matted fibrin network. (Scale = 1 μ m)

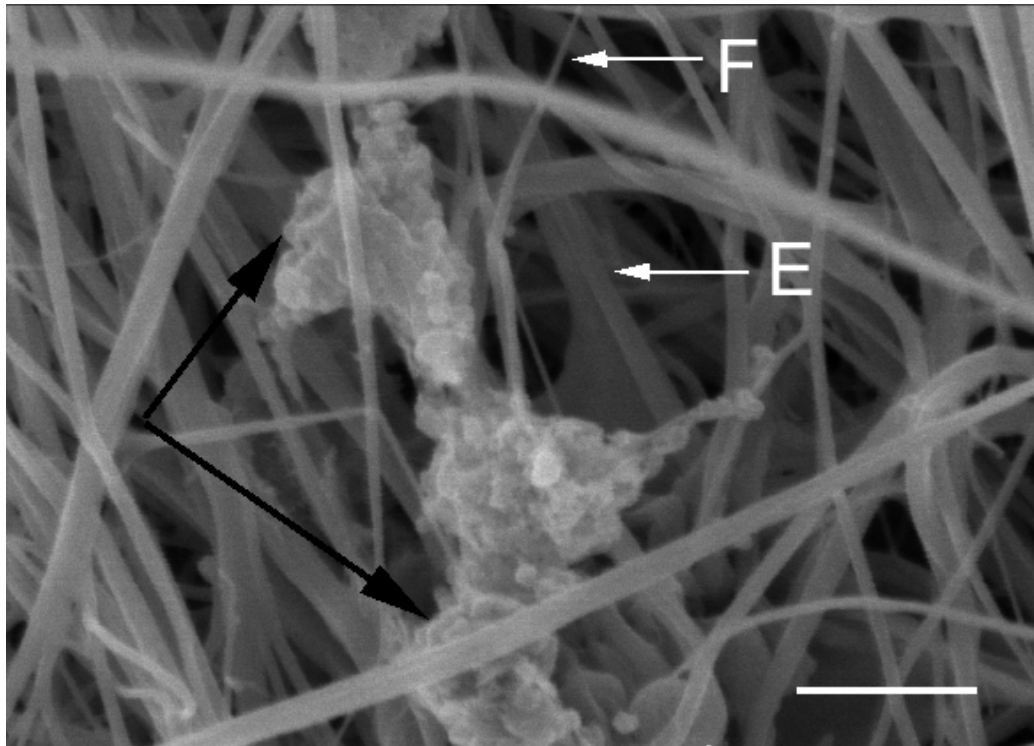


Figure 5.2: Platelet aggregate from controlled asthmatic subject, with coarse, granular aggregate indicated with thick black arrow. Fibrin network is seen with thick, major fibers as well as thin, minor fibers. **Label E** = thick, major fibers; **Label F** = thin, minor fibers. (Scale = 1 μ m)

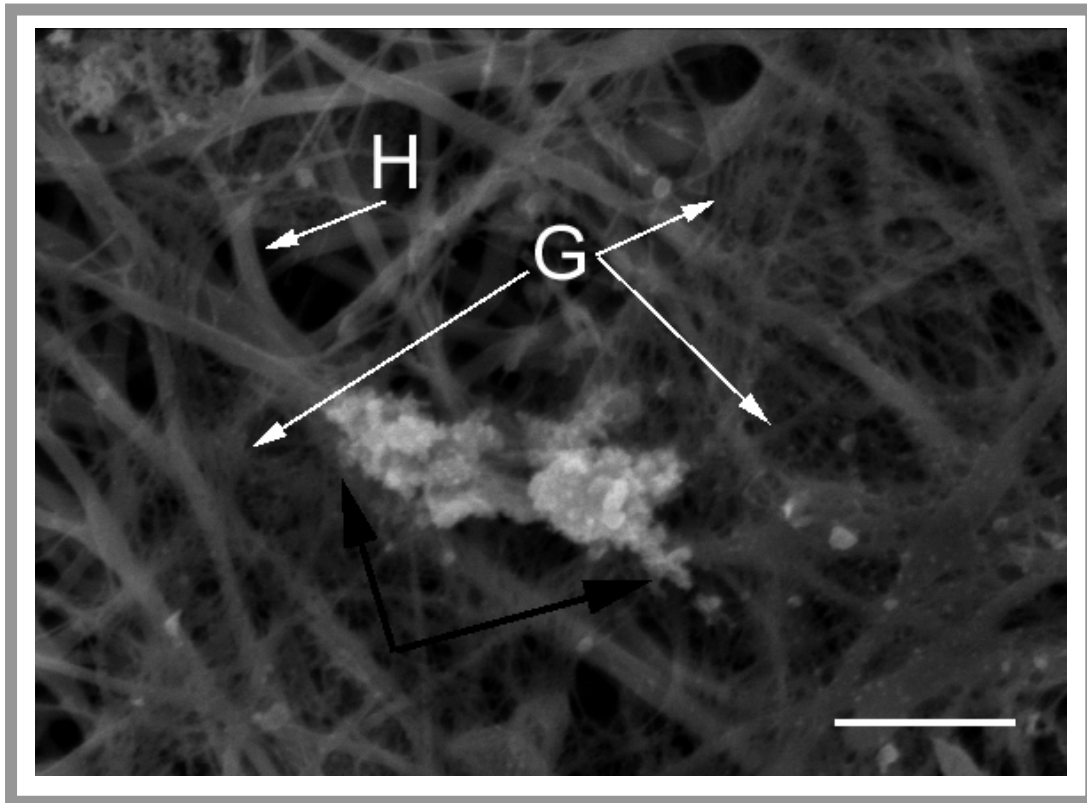


Figure 5.3: Platelet aggregate and fibrin network from uncontrolled asthmatic individual. Thick black arrow shows course, loosely arranged platelet aggregate. **Label G** = major fibers; **Label H** = minor fibers showing matted fibrin network. (Scale = 1 μ m)

In order to study the SEM results, each clot area (diameter 3mm) was studied by systematically viewing the whole clot using SEM. This was done to make sure the morphology seen in the given figures was indeed representative of the whole clot structure. Previous ultrastructural results using the BALB/c asthma model have shown that platelets and fibrin have a changed ultrastructure in asthma.

Platelets from the control BALB/c animals showed a smooth membrane surface with pseudopodia. Fibrin networks of control animals showed thick, major fibers and thin, minor fibers (Pretorius *et al.*, 2007a; Pretorius *et al.*, 2007b), as was also found in the current study as indicated in chapter 4. However, platelets from the asthmatic animals showed a tendency to form loosely connected, granular aggregates, while minor fibers of asthmatic mice have a netlike appearance covering the major fibers. This was also seen in the current study when looking at Figure 5.1c.

The question that arose was whether platelets and fibrin networks of humans with asthma would show a similar ultrastructure. The current research investigated 2 individuals with asthma; the one subject has controlled asthma, while the second subject has chronic, uncontrolled asthma.

Figure 5.1a and b show a platelet aggregate and fibrin network of a typical control subject, respectively. Typical control platelet aggregates (Figure 5.1a), possess smooth membranes (Label A) with pseudopodia (Label B) protruding from the body of the aggregate. Figure 1b shows fibrin networks from the control human subject. Fibrin fibers can be morphologically divided into major, thick fibers (Label C) and minor, thin fibers (Label D). The major, thick fibers branch off from each other and the fibers are typically seen as single fibers forming relative straight lines. Thin, minor fibers are much thinner than the major, thick fibers and are found sparsely distributed among the major fibers.

Figure 5.1c shows a platelet aggregate and fibrin network of a typical asthmatic mouse model. A loosely connected, granular aggregate is shown (thick black arrow) while minor fibers of asthmatic mice have a netlike appearance covering the major fibers (thick white arrow). These results were also found and discussed in Chapter 4.

Figure 5.2 shows a platelet aggregate and fibrin network of a controlled asthma subject. Platelet aggregates in this human subject from loosely connected, granular aggregates (thick black arrow), similar to that previously found by Pretorius *et al.*, 2007a in the asthmatic BALB/c murine model and the same results were found in the current study (Figure 5.1c, thick black arrow). However, in this subject, fibrin networks appeared as that of the controls with major, thick fibers (Label E) and minor, thin fibers (Label F).

Figure 5.3a shows a platelet aggregate and fibrin network of the subject with uncontrolled, chronic asthma. Platelet aggregates also appeared loosely connected and granular (thick white arrow), as is seen in the asthmatic mouse model (Figure 5.1c). Also, fibrin networks were similar to that found in the asthmatic BALB/c animals (Figure 5.1c). In the human subject, the thick, major fibers (Label G) were covered with a fine net of minor, thin fibers (Label H) as seen in the asthmatic animal model (Figure 5.1c, thick white arrow).

5.4 Discussion

These results suggest that the BALB/c asthmatic murine model mimics human asthma when studying the ultrastructure of platelets and fibrin networks. Additionally, these results may also perhaps be useful in planning treatment regimes for asthma. Here it shows that a subject with controlled asthma still has changed platelet morphology (Figure 5.2) similar to asthmatic animals (Figure 5.1c), however, fibrin networks seem to be stabilized if the asthma is under control, and appear similar to

that of human control fibrin networks (Figure 5.1b). The subject with uncontrolled asthma showed both changed platelet morphology and fibrin networks (Figure 5.3). It is known that patients with asthma have extra-vascular thrombin, fibrinogen, and fibrin have been found in the sputum of patients with asthma (Banach-Wawrzenczyk *et al.*, 2000; Pizzichini *et al.*, 1996; Wagers *et al.*, 2004). There might be a link between the presence of fibrinogen and fibrin in the sputum and the fibrin net covering the major fibers. However, this cannot be confirmed with the present study.

5.5 Conclusion

Therefore it can be concluded that human platelet and fibrin asthmatic ultrastructure is similar to that found in the BALB/c murine asthmatic model. The question that always arises when using animal models is whether the model adequately mimics the human disease; the current research therefore shows additional support for the use of this model in asthma.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 6

INVESTIGATING THE EFFECT OF MODUL8[®] ON WEIGHT CHANGES IN THE ASTHMATIC BALB/C MOUSE MODEL

6.1 Introduction

Weight has a fundamental influence on diseases like asthma. Numerous studies over the past few years have reported on the relationship between airway inflammation; including asthma, and excessive body weight or obesity as well as anomalous body mass index (Aaron *et al.*, 2004; Beuther *et al.*, 2006; Camargo *et al.*, 1999; Hakala *et al.*, 2000; Weiss and Shore, 2004). It is also known that obesity plays a role in asthma and often co-exists in the same patient (Castro-Rodriguez *et al.*, 2001; Sutherland *et al.*, 2008). A number of hypotheses have been reported on the relationship between obesity and asthma, but the exact nature of this association remains unclear.

One of the reasons for weight gain in asthmatic patients is the use of hydrocortisone, one of the most frequently used treatments for asthma; and its uses have been linked to weight gain, high blood pressure and puffiness of the face. However, in a review in 2006, Shore suggested that it's the disease itself that possibly could lead to obesity (Shore, 2006).

As also reported by researchers in 1999, individuals suffering from asthma may gain weight as a result of limited activity by the individuals but the exact relationship between the body mass index (BMI), (calculated as weight in kilograms divided by the square of height in meters), and the risk of developing asthma is not known (Camargo *et al.*, 1999).

Animal models of weight studies have also been reported on by many researchers, as well as the effect of alternative medication such as herbal medicines and plant extracts on the management of weight changes in experimental animals (Harris *et al.*, 1998; Retana-Ma´rquez *et al.*, 2003; Snibson *et al.*, 2005). Hausberger and Hausberger (1958) reported that 60% of male Wistar rats which received cortisone (5 mg/day) had diminished gain of body weight and total body protein just as a marked decrease in weight was observed in another study in mice receiving hydrocortisone (Borovitskaya *et al.*, 1971). Also, following administration of a high-dose of systemic dexamethasone for 3 days, Kumar *et al.* 1997, reported a marked catabolic effect with weight loss in rats (Kumar *et al.*, 1997).

In another study, Prednisolone was found to reduce body weight in mice and guinea pigs (Nagao *et al.*, 2004) while other studies with antipsychotic drugs have shown varying results (Ganguli, 1999; Goudie *et al.*, 2002).

The question that now arises is whether herbal products or immunomodulators used to alleviate the symptoms of asthma will have an effect on weight.

In the current study, the asthmatic BALB/c mouse model was used to study the effect of Modul8[®] on the weight changes and compared to the weight of control and asthmatic animals without treatment and as positive control, asthmatic animals treated with hydrocortisone. No indication of a direct effect of the individual components of Modul8[®], in such high dilutions, on body weight could be found and therefore, for the purpose of the current chapter it was assumed that the different components did not have an effect on the mass of the animals. Also, administration of substances might be stressful to the animals. In the current study the hydrocortisone was administered intraperitoneally, which is one of the most common



routes of administration, while the Modul8[®] was administered orally. Both these procedures might be stressful to the animals, however, only one treatment procedure was conducted in the specific treatment group per day and therefore it is believed that neither of the processes induced a significant change in the mass of the animals due to stress.

6.2 Materials and Methods

6.2.1 *Weighing analysis*

The mice were weighed three times per week during the 43-day period of the experimental study. The animals were weighed before any other necessary procedures were conducted on each of the three days.

Day zero of the experimental implementation correlated to the first day upon which mass measurements were acquired. The collected masses, in grams, were then used to determine if the sensitization, nebulization and or treatment administration had any statistically significant impact on the masses, a representation of general health, of the evaluated BALB/c mice.

In order to effectively determine if the various experimental procedures employed had any significant effect on the mice's masses, specific reference days were chosen for evaluation. Table 6.2 indicates the reference days chosen. These days were chosen according to the days on which the various experimental procedures were conducted and in relation to the mass collection days.

Day zero represented the baseline mass for each of the mice within the study. Although sensitization was conducted on day zero, in order to begin the process of asthma induction, the mice were weighed before this procedures implementation and thus it would have had no effect on the recorded masses. This data therefore



represented the unexposed and untreated mass of each of the BALB/c mice, all of which were at the same age and thus in turn a suitable base line for comparison to their masses upon days which various experimental procedures may have induced an effect.

Table 6.1: The different experimental procedures conducted on specific days

DAY: 0, 5	Sensitization
DAY: 13, 14, 15	Nebulisation
DAY: 15, 16, 17, 18	Treatment
DAY: 22, 23, 24, 25	Treatment
DAY: 30, 31, 32	Nebulisation
DAY: 36, 37, 38, 39	Treatment
DAY: 42, 43	Termination

Indicated in Table 6.1, sensitization of the mice was performed on day's zero and five while the first set of nebulizations started on day 13 and the first set of treatments on day 15. Day seven was therefore chosen to determine whether the sensitization procedure was responsible for any mass changes within the mice.

On day 15, when treatment began, it was assumed that the processes of sensitization and nebulization would have induced asthma within all the exposed mice which included all the treatment groups except for the control group; day 18 hence providing information on any early effects of the various treatments upon the weights of the treated asthmatic mice in comparison to the untreated asthmatic mice.

Day 21 was situated midstream of the first round of treatments while day 28 was situated after the conclusion of the first set of treatments but before the second set nebulization, both allowing for the treatments effects over time to be assessed.

Day 35 was chosen to evaluate the effects of re-nebulization, long term asthma, while day 39 provided an additional opportunity to consider if there were any effects on mass relating to the various treatments after prolonged asthma induction.

Table 6.2: The reference days chosen on which the various experimental procedures were conducted

Day of analysis	Reason
0	Baseline weight - no sensitization, nebulization and or treatment administration had yet been preformed
7	Early effects of sensitization
18	Early effects of treatment administration
21	Midstream effects of treatment administration
28	Late effects of treatment administration
35	Effects of re-nebulization before re-treatment administration
39	Effects post-nebulization after re-treatment administration

The weight analysis was conducted on two levels:

1. *Analysis was preformed in terms of mean mass change over time between the above-specified groups*

- Each of the 4 specified experimental groups could not be compared with one another in terms of absolute masses, as each mouse's baseline mass differed not only between the groups but within the groups themselves. Instead, the mass change for each mouse could be calculated over time and compared directly between the 4 experimental groups, as the change is independent of the absolute masses.
- The mass change for each mouse was determined on days 7, 18, 21, 28, 35 and 39, in relation to the baseline mass (mass at day zero), so that the variations between the mice's initial weights could be ignored.
- **Mass change = mass on specified day – mass on day zero**
- The mean mass changes for each of the 6 mice, for each of the 4 groups, for each of the specified days were then calculated. The mean weight change for each of the groups, for each of the specified days were then statistically compared with one another by means of a one-way ANOVA, with Tukey-Kramer Multiple-Comparison Test's being used in cases of significance, in order to determine which groups differed from each other. The statistical tests were preformed at a level of significance of 0.05. All the necessary assumptions were considered, tested and verified before the one-way ANOVAs were conducted. This procedure allowed any effect, induced by the

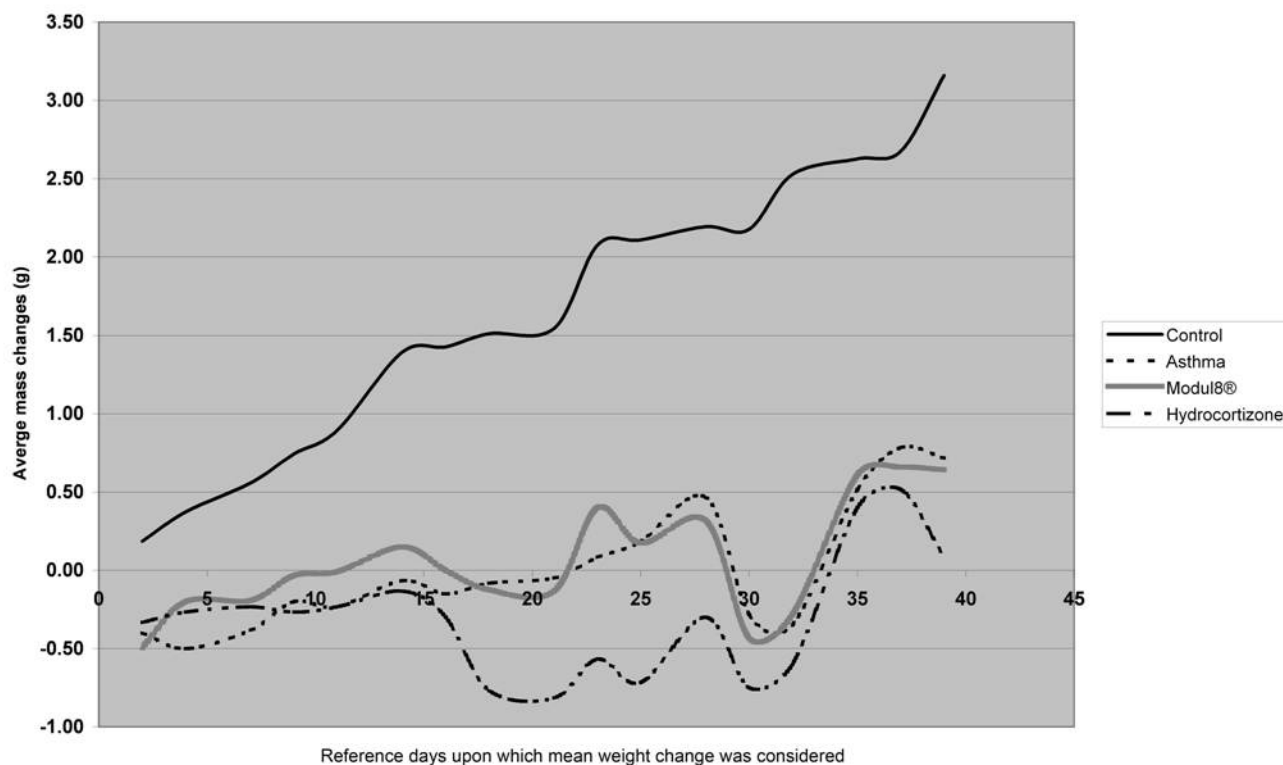
different-between-group treatments or exposure protocols, to be quantified.

2. Analysis was preformed in terms of absolute mass changes over time for each mouse within each of the above-specified groups

- Within each of the above-specified groups, one can also consider the effects of the sensitization, nebulization and the particular treatment administered by comparing the absolute mass of each mouse over time. Unlike the situation in level 1, each mouse was analyzed as its own control, and hence the variation in baseline mass between individuals was not of importance.
- The mass of each mouse on days 7, 18, 21, 28, 35 and 39 were compared separately to the baseline mass (day zero) via a repeated measure ANOVA or Friedman two-way ANOVA depending upon whether the necessary assumptions were met for the parametric test or not. All the necessary assumptions were considered, tested and verified. Tukey-Kramer Multiple-Comparison Test's were preformed in cases of significance, in order to determine which days differed from each other. The statistical tests were preformed at a level of significance of 0.05. This procedure allowed for further quantification of any effects induced by the different-group treatments or exposure protocols.

6.3 Results

6.3.1 Inter- group analysis



Graph 6.1: The average mass changes of the four experimental groups over the experimental period

Graph 6.1 shows the fluctuations of the average mass changes of the BALB/c mice for each of the four experimental groups, stated above, during the duration of the experiment. The control group showed an increase in mass change with time. The one-way ANOVA showed that for each of the six days considered the average mass change for the control group was significantly greater than all the other groups, which were not significantly different to each other. Table 6.3 states the acquired P values for each of the statistical tests run and their implications.

Table 6.3: A table of the relative mass changes (in grams) for each of the experimental groups, relative to their baseline mass, on specific days of the BALB/c animal model

	Average mass change relative to day 0 (baseline mass) (mean $\pm$ std dev)			
Day	Control* (n=6)	Asthma (n=6)	Modul8 (n=6)	Hydrocortisone (n=6)
7	0.56 $\pm$ 0.13	-0.38 $\pm$ 0.54	-0.12 $\pm$ 0.54	-0.23 $\pm$ 0.46
18	1.51 $\pm$ 0.38	-0.08 $\pm$ 0.88	-0.08 $\pm$ 0.78	-0.77 $\pm$ 0.66
21	1.54 $\pm$ 0.42	-0.05 $\pm$ 0.46	-0.05 $\pm$ 0.95	-0.82 $\pm$ 0.77
28	2.19 $\pm$ 0.39	0.47 $\pm$ 0.48	0.37 $\pm$ 1.00	-0.30 $\pm$ 0.89
35	2.63 $\pm$ 0.50	0.52 $\pm$ 0.50	0.75 $\pm$ 1.07	0.40 $\pm$ 0.86
39	3.16 $\pm$ 0.72	0.72 $\pm$ 0.51	0.8 $\pm$ 1.18	0.08 $\pm$ 0.96

(* indicates significance at a level of 0.05 for the control group on each examination day to all the other groups, except for the Modul8 group on day 7 where no significant difference existed between the two).

6.3.2 Intra-group analysis

Within the control group itself, on each of the reference days, each mouse could be seen to have significantly increased in its mass in comparison to its baseline mass.

The asthmatic group showed no significant increase in their baseline masses and their masses upon the chosen days of analysis, except for day 39. The Friedman 2-way ANOVA revealed that the asthmatic mice increased significantly in their mass in relation to day zero. This increase in mass was significantly lower than that seen in the control group, see the between group analysis, on this day.

The induced asthma can thus be correlated to the lower increase in mass seen within these mice. The mice treated with Modul8[®] showed no increase between their

baseline masses and their masses on days 7, 21 and 28. The Modul8® group on days 35 and 39 were significantly greater than that of their baseline.

The mice treated with hydrocortisone showed no increase between their baseline masses and any of the chosen days analyzed - not even on day 39 did the mice show the expected increase in mass, expected due to the relationship seen in the asthmatic mice. Table 6.4 reveals the statistical tests run and the acquired results, by which the situations of significance or lack thereof, explained above, could be deduced. The days are compared to day 0.

Table 6.4: The output derived from the various statically tests run in order to evaluate if there was any statistically significant difference in the absolute mass changes of the mice, within each specified group, during the 43-day period. (*Significance was set at a level of 0.05)

Group	Day	Test utilized	P value*	Difference between compared days
Control	7	Repeat-measures ANOVA	0.0001	Pattern of increasing mass from day 0 to day 7
	18	Repeat-measures ANOVA	0.0002	Pattern of increasing mass from day 0 to day 18
	21	Repeat-measures ANOVA	Less than	Pattern of increasing mass from day 0 to day 21
	28	Repeat-measures ANOVA	Less than	Pattern of increasing mass from day 0 to day 28
	35	Repeat-measures ANOVA	Less than	Pattern of increasing mass from day 0 to day 35
	39	Repeat-measures ANOVA	0.0001	Pattern of increasing mass from day 0 to day 39
Asthma	7	Friedman two-way ANOVA	0.4142	No association between the baseline and the considered day's masses
	18	Friedman two-way ANOVA	0.6547	No association between the baseline and the considered day's masses
	21	Repeat-measures ANOVA	0.8004	No association between the baseline and the considered day's masses



	28	Repeat-measures ANOVA	0.0615	No association between the baseline and the considered day's masses
	35	Repeat-measures ANOVA	0.0511	No association between the baseline and the considered day's masses
	39	Friedman two-way ANOVA	0.0143	Pattern of increasing mass from day 0 to day 39
Modul8®	7	Repeat-measures ANOVA	0.1752	No association between the baseline and the considered day's masses
	18	Repeat-measures ANOVA	0.478	No association between the baseline and the considered day's masses
	21	Repeat-measures ANOVA	0.5555	No association between the baseline and the considered day's masses
	28	Repeat-measures ANOVA	0.1402	No association between the baseline and the considered day's masses
	35	Repeat-measures ANOVA	0.0213	Pattern of increasing mass from day 0 to day 35
	39	Repeat-measures ANOVA	0.0304	Pattern of increasing mass from day 0 to day 39
Hydrocortisone	7	Repeat-measures ANOVA	0.2682	No association between the baseline and the considered day's masses
	18	Friedman two-way ANOVA	0.1025	No association between the baseline and the considered day's masses
	21	Friedman two-way ANOVA	0.1025	No association between the baseline and the considered day's masses
	28	Friedman two-way ANOVA	0.3173	No association between the baseline and the considered day's masses
	35	Repeat-measures ANOVA	0.3063	No association between the baseline and the considered day's masses
	39	Repeat-measures ANOVA	0.8407	No association between the baseline and the considered day's masses



6.4 Discussion

Asthma is an inflammatory disease of the airways and involves a wide range of cell types and cellular mediators. Inflammation in asthma is a consistent feature and the pattern of inflammation in the airways appears to be similar in all clinical forms of asthma. However, according to Cohn *et al.* (2004) and Bousquet *et al.* (2004), the relationship between the intensity of the inflammation in asthma and the severity of asthma is not clearly understood. Tulic *et al.*, (2001) then also suggested that the inflammatory process on pathological and physiological level extends beyond the central airways to the peripheral airways and lung parenchyma.

In this thesis, as indicated in chapter 7, asthmatic mice showed a significant increase in their number of eosinophils counted in the blood smears as well as bronchial lavage. In contrast, both the treatment groups (Modul8[®] and hydrocortisone), showed a significant decrease in their number of eosinophils counted, based upon the derived P values as can be seen in chapter 7. As stated earlier the number of eosinophils in the blood and bronchial lavage is a distinctive feature in the inflammatory response in asthma. Since it is known that Modul8[®] has an effect on asthma by decreasing the circulating and infiltrating eosinophil counts in a similar manner to hydrocortisone, and since asthma has an effect on weight, the current chapter investigated the possible downstream effects of this product on asthma-induced weight gain.

Weight comparisons are seen in Graph 6.1 and the average mass changes of the four experimental groups over the experimental period. The asthma, hydrocortisone and Modul8[®] group showed alterations in average mass change over time. The two treatment groups – hydrocortisone and Modul8[®] – followed similar fluctuation patterns, which visually differs from the fluctuation pattern followed by the asthmatic

mice. However, it seems as if Modul8[®] aided stability of the asthmatic animals, and their weights were more than that of the hydrocortisone group at the given dates.

Table 6.3 shows a table of the relative mass changes for each of the experimental groups, relative to their baseline mass, upon specific reference days. Significance was found for the control group on each examination day to all the other groups, except for the Modul8[®] group on day 7 where no significant difference existed between the two. The results expressed in Table 6.3 may imply that the induction of asthma has a negative impact on the mass changes of the mice over time, as this was the only constant factor between the asthma, Modul8[®] and hydrocortisone groups and the constant absent factor within the control group in comparison to these four groups.

Intra-group analysis showed that within the control group itself, on each of the reference days, each mouse has significantly increased in its mass in comparison to its baseline mass. A possible explanation for these results may be that the control animals were not under stress from the disease itself or its associated induction procedures. Good health and the unrestricted food supply may also be a reason.

The asthmatic group showed no significant increase in weight between their baseline masses and their masses upon the chosen days of analysis, except for day 39.

The mice treated with Modul8[®] showed no increase in their baseline masses and their masses on days 7, 21 and 28. The Modul8[®] group on days 35 and 39 were significantly greater than that of their baseline. The results seen on day 39 were expected due to this response being seen within the asthmatic mice, and thus expected to be the normal bodily response of the BALB/c mice to the long term effects of asthma and its associated induction procedures.

The significance associated with day 35 was not seen in the asthmatic group and must thus have been associated with the utilized Modul8[®] treatment. At this date the mice had been re-nebulized but not exposed to the homeopathic treatment again. Thus the early utilization of Modul8[®] could be showing a long-term activity, taking affect in the mice by this date and allowing them to respond as closely to healthy control mice as possible. The between group analysis revealed that their increases in mass, seen on the 35th and 39th day, were still significantly lower than that of the controls.

Thus the homeopathic product, Modul8[®], was not able to completely compensate against the devastating effects of asthma upon the general health of the animals as indicated through their body mass.

The mice treated with hydrocortisone could be seen to show no increase in their baseline masses and any of the chosen days analyzed -not even on day 39 did the mice show the expected increase in mass, expected due to the relationship seen in the asthmatic mice.

6.5 Conclusion

The data obtained in the current study revealed that the control group increased in mass change throughout the duration of the study. This can be based on the assumption that the control animals were uninterrupted with normal physiological states throughout the 43-day period. The asthma group on the other hand revealed a negative change in mass when compared to the control group.

The asthma, Modul8[®] and hydrocortisone groups revealed more or less the same fluctuation in pattern, although, it seems as if Modul8[®], although only slight, in comparison to the baseline weights, could possibly show a slight stabilizing effect on

asthmatic animals, when comparing results seen in Graph 6.1. If comparing the results of weight between the asthmatic animals treated with Modul8[®] and the hydrocortisone, hydrocortisone seem to have a greater impact on weight loss than Modul8[®].

It could be hypothesized that although the asthmatic mice showed an increase in body weight at the end of the study which the Modul8[®]-treated group exhibit as well, Modul8[®] was able to facilitate the body's quicker adaptation to the disease than the body could have on its own.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 7

INVESTIGATING THE EFFECT OF MODUL8[®] ON BLOOD COUNT AND BRONCHIAL LAVAGE OF THE EXPERIMENTAL ASTHMATIC BALB/c MICE

7.1 Introduction

Asthma is a complex clinical disease characterized by airway obstruction, airway inflammation and airway hyperresponsiveness to a variety of stimuli and involves a wide range of cells that each play an important role in the allergic process involved in asthma. The development of the allergic process in asthma exists of three phases. The first is the induction phase where antigen-presenting cells play a major role. This phase is followed by the early-phase asthmatic reaction (EAR) where the most important cells are mast cells and then the late-phase asthmatic reaction (LAR) where various cell types, such as eosinophils, neutrophils, T cells, macrophages, dendritic cells, and cells that endow structure are involved (Verstraelen *et al.*, 2008a).

The most important pathological feature of allergic asthma is caused by complex interactions between different immunological mediators, which are produced by inflammatory cells. These cells include T lymphocytes, mast cells, macrophages, eosinophils, basophils, neutrophils, dendritic cells and structural cells (Bharadwaj and Agrawal, 2004).

White blood cells, especially eosinophils have a major contribution to the airway remodelling associated with chronic asthma. Airway hyperresponsiveness, inflammatory infiltrates and structural changes in the airways are all features of asthma (Humbles *et al.*, 2004).

Platelets also play an important physiological role in allergic processes and immunological mechanisms including those associated with asthma where platelets participate by acting as inflammatory cells as they are releasing mediators, spasmogens and interacting with other inflammatory cell types. Platelets are activated by a number of stimuli and this activation may be due to, amongst others, inflammatory processes (Butenas and Mann, 2002; Camera *et al.*, 1999; Lazarus *et al.*, 2003).

Inhalation of an antigen starts off the process of airway hyperresponsiveness and bronchial inflammation in asthma. Most of the antigens are then cleared by the mucociliary escalator and the ones that escapes this process are then taken up by antigen-presenting cells (APC,s), such as dendritic cells, macrophages and B cells (Holt, 2000) of which dendritic cells are the most effective APC,s since they can activate naïve Th cells by expressing a high level of class II major histocompatibility complex (MHC) and co stimulatory molecules.

Because it is thought that Modul8[®] might have a modulation effect on the immune system, and it has been suggested that it might be useful in the treatment of asthma, the question arose whether it has the ability to reduce white blood cell counts in bronchial lavage and blood counts. Therefore, in the current chapter the effect of the immunomodulator Modul8[®], on white blood cell counts in the blood and bronchial lavage will be investigated by using the murine asthmatic BALB/c mouse model.

7.2 Materials and Methods

7.2.1 Blood smears techniques

Blood samples of mice from each group were collected via orbital puncture on the day of termination. Histological blood smears were prepared and stained with Rapid

Heamatological stain. A total of hundred leucocytes were counted in each blood smear.

7.2.2 Bronchial lavage Techniques

After termination, a small skin incision was made in the skin of each mouse in the ventral of the trachea. The trachea was exposed by blunt dissection and a small transversal incision was made below the larynx. Through the sheath of a 21G venous catheter 0.3 ml of saline was injected into the trachea and aspirated with a syringe. The bronchial lavage fluids collected for the individual groups were pooled, centrifuged for 2 minutes at 1000rpm and smears were made. The smears were stained with Rapid Heamatological Stain. White blood cells were counted under a 100x magnification and up to a hundred cells were counted per slide.

7.3 Results

7.3.1 Bronchial lavage

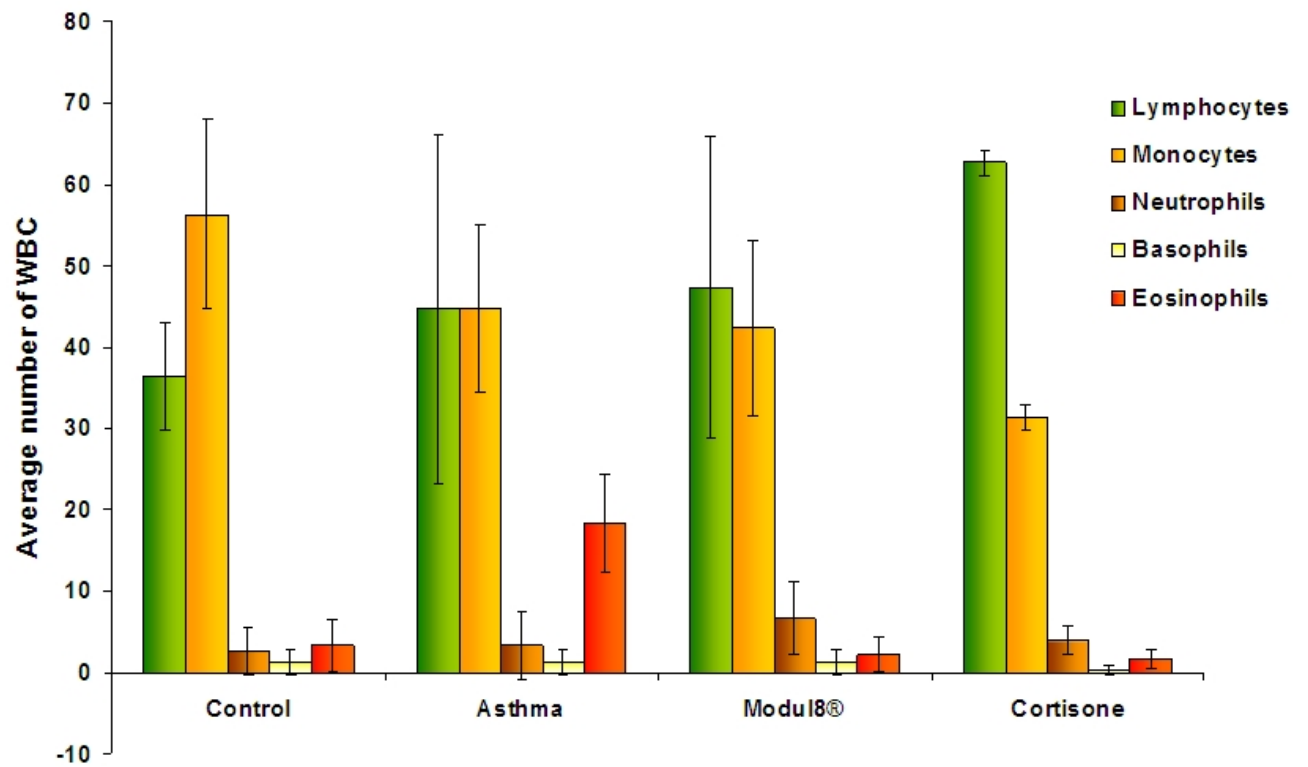
Three bronchial lavage smears were chosen at random, from each of the four experimental groups, for microscopic evaluation. The numbers of each white blood cell type – monocyte, lymphocyte, eosinophil, basophil and neutrophil – were quantified. Statistical analysis of the recorded counts was performed with the aid of NCSS in order to determine if any difference amongst the group's specific white blood cell counts existed. Each white blood cell species was therefore considered separately via a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending upon whether the necessary assumptions for the parametric test were met or not.

One-way ANOVA's were utilized for the between group comparisons for each type of white blood cell. This was facilitated due to the observations meeting all the necessary assumptions for this parametric tests utilization - the use of equal sample sizes allowed one to ignore the assumption of equal variance. Table 7.1 expresses the statistical tests run for each white blood cell species and the outcomes thereof.



Table 7.1: The statistical comparisons performed on the various white blood cells derived from the bronchial lavage of the assessed BALB/c mice (* Significance was set at a level of 0.05)

White blood cell	Test utilized	P value*	Differences between compared groups
Monocyte	One-way ANOVA	0.0389	The control group possessed a significantly greater monocyte count in comparison to the hydrocortisone group
Lymphocyte	One-way ANOVA	0.244	No significant difference existed between any groups lymphocyte counts
Eosinophil	One-way ANOVA	0.00132	The asthmatic group possessed a significantly greater eosinophil count in comparison to all other assessed groups
Neutrophil	One-way ANOVA	0.551	No significant difference existed between any groups neutrophil counts
Basophil	One-way ANOVA	0.751	No significant difference existed between any groups basophil counts

Average WBC counts in Bronchial lavage**Graph 7.1:** Average number of the white blood cells counted in the bronchial lavage

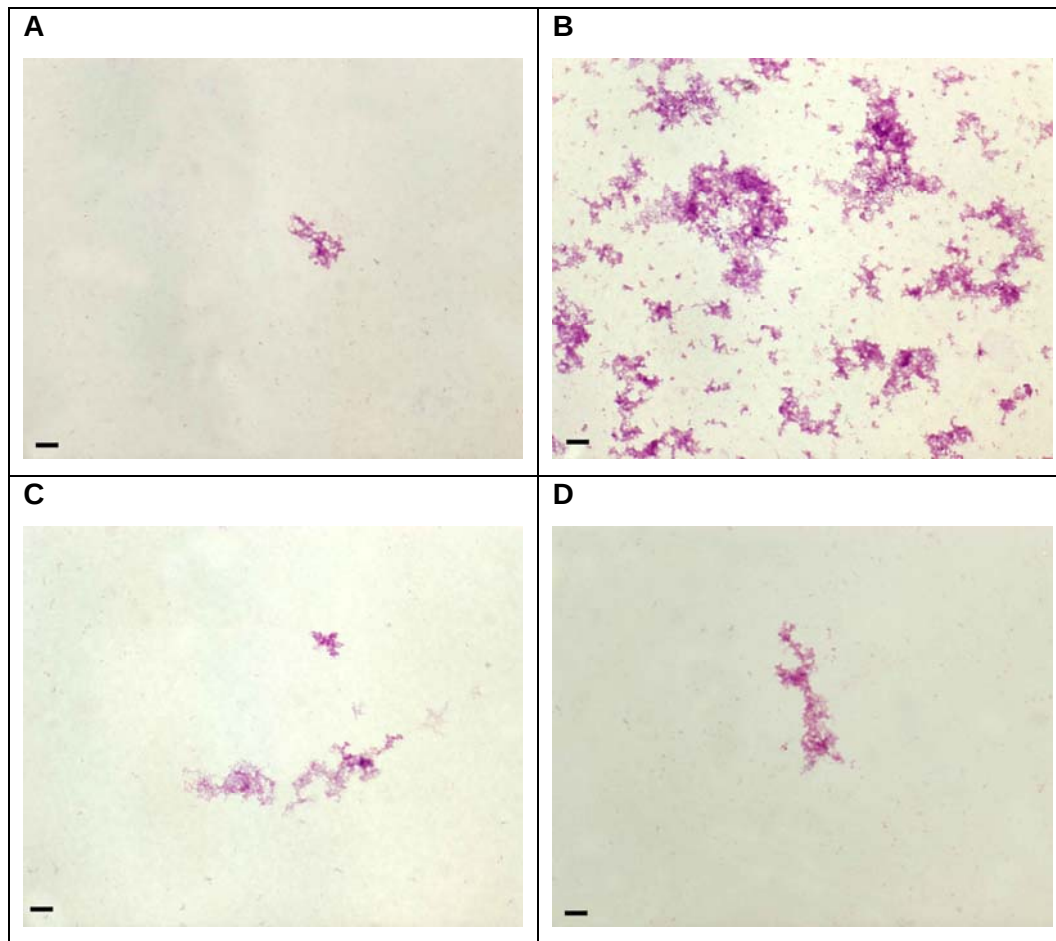


Figure 7.1: Platelet distribution in the bronchial lavage in the four different experimental groups. Control (A), Asthma (B), Modul8[®] (C), Hydrocortisone (D). (Scale bar = 10 μ m)

7.3.2 Blood counts

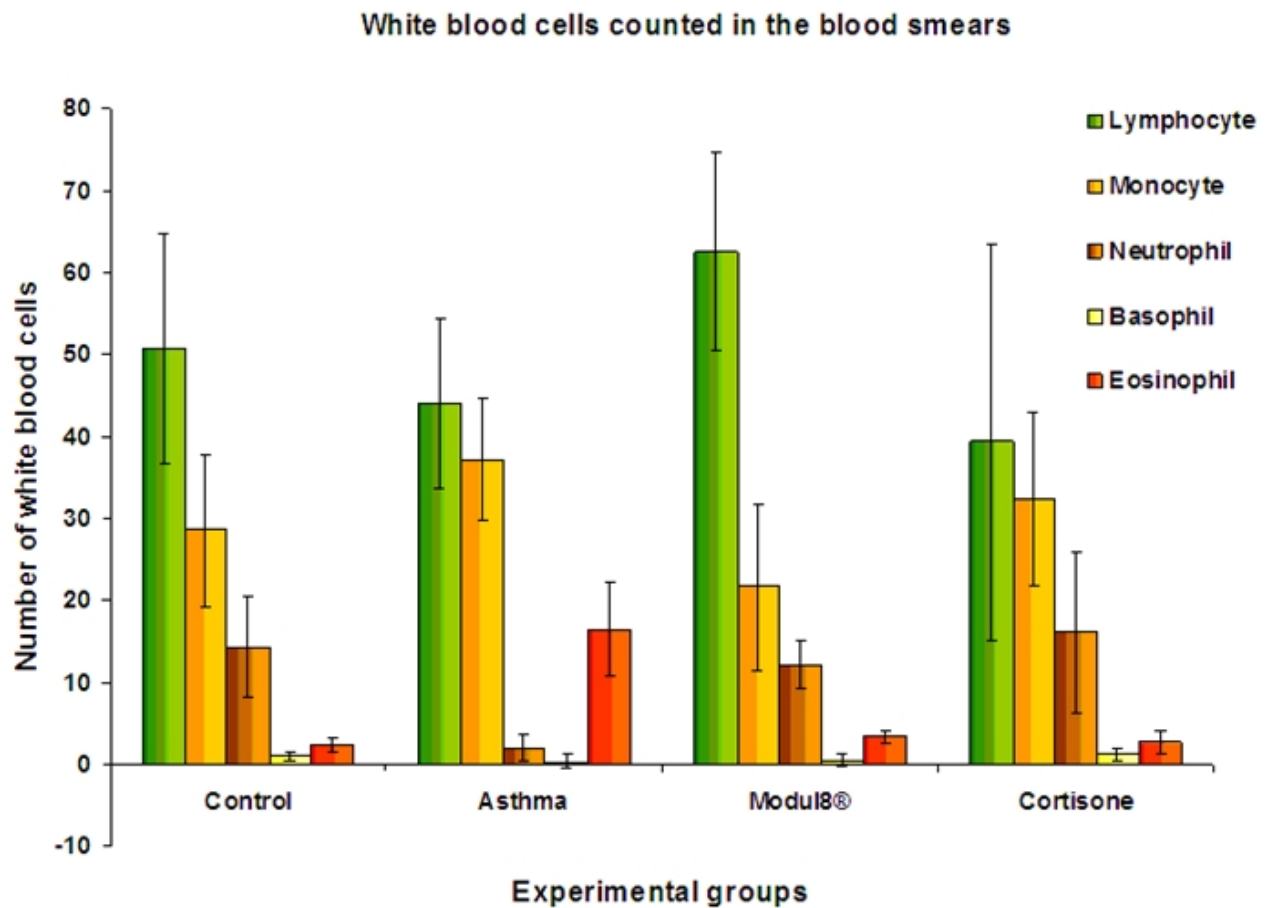
Six blood smears were chosen at random, from each of the four experimental groups, for microscopic evaluation. With the use of a light microscope the numbers of each white blood cell species – monocyte, lymphocyte, eosinophil, basophil and neutrophil – were quantified. Statistical analysis of the recorded counts was preformed with the aid of NCSS in order to determine if any difference amongst the group's specific white blood cell counts existed. Each white blood cell species was therefore considered separately via a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending upon whether the necessary assumptions for the parametric test were met or not.

One-way ANOVA's were utilized for the between group comparisons for the assessment of monocytes, lymphocytes, neutrophils and basophils. This was facilitated due to the observations meeting all the necessary assumptions for this parametric tests utilization - the use of equal sample sizes allowed one to ignore the assumption of equal variance. The eosinophil comparison was conducted with the aid of the non-parametric Kruskal-Wallis one-way ANOVA, as the data did not assume a normal distribution. Table 7.2 expresses the statistical tests run for each white blood cell species and the outcomes thereof.



Table 7.2: The statistical comparisons performed upon the various white blood cell species derived from the blood of the assessed BALB/c mice (* Significance was set at a level of 0.05)

White blood cell	Test utilized	P value*	Differences between compared groups
Monocyte	One-way ANOVA	0.062	No significant difference existed between any groups monocyte counts
Lymphocyte	One-way ANOVA	0.111	No significant difference existed between any groups lymphocyte counts
Eosinophil	Kruskal-Wallis one-way ANOVA	0.00100	The asthmatic group possessed a significantly greater eosinophil count in comparison to all other assessed groups
Neutrophil	One-way ANOVA	0.00302	The asthmatic group possessed a significantly lower neutrophil count in comparison to all other assessed groups
Basophil	One-way ANOVA	0.217	No significant difference existed between any groups basophil counts



Graph 7.2: Average number of white blood cells counted in the blood smears

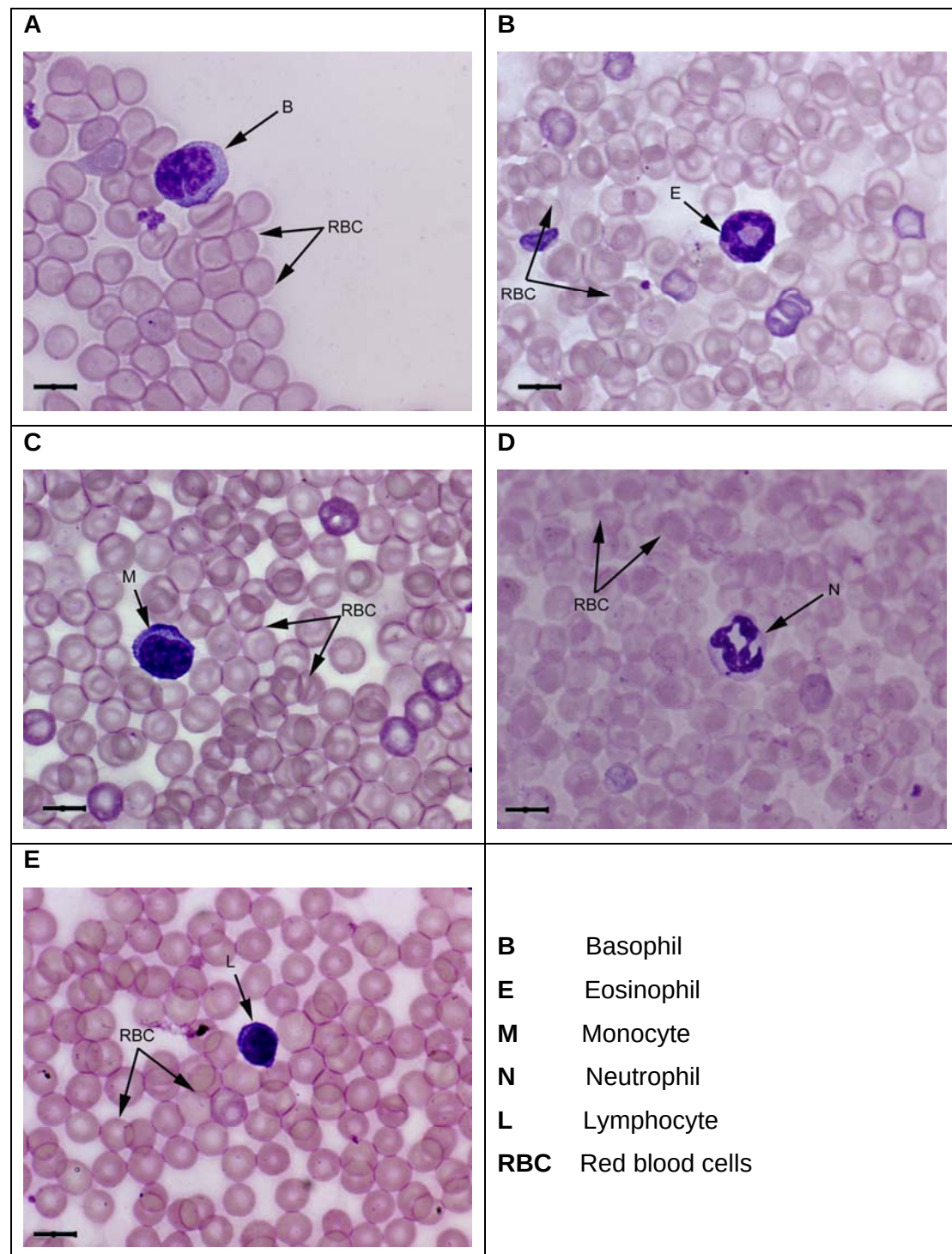


Figure 7.2: White blood cells in the blood smears of BALB/c mice. Basophil (A), Eosinophil (B), Monocyte (C), Neutrophil (D), Lymphocyte (E). (Scale bar = 20µm)



7.4 Discussion

Asthma is a chronic condition characterized by airflow obstruction, airway hyper-responsiveness (AHR) and chronic airway inflammation that usually has an eosinophilic component. Other characteristics associated with this disease include accumulation of eosinophils and CD4 lymphocytes, activation of the epithelium and smooth muscle, mucus hyper-secretion, thickening of the sub-epithelial collagen layer, mast cell degranulation, and smooth muscle hypertrophy and hyperplasia (Kay, 1996). One of the characteristics of asthma in humans or animals is the pulmonary eosinophilia and the progressive tissue damage that is caused by the eosinophilic inflammation (Hogan *et al.*, 2003).

Other characteristics include airway hyper-responsiveness, mucus secretion, and the production of IgE, infiltration of inflammatory cells as well as the release of a number of different mediators (Boulet *et al.*, 1998). Mast cells, eosinophils, T lymphocytes, dendritic cells, macrophages and neutrophils are some of the cells present in the blood smears and bronchial lavage of asthmatic mice. In 2004, Kay revealed that eosinophil numbers are always increased in the airways and that these cells are responsible for the release of proteins and growth factors that cause damage to the airway epithelial cells and thereby cause airway remodelling (Kay *et al.*, 2004).

Increased eosinophil and T lymphocyte numbers in the broncho-alveolar lavage fluid are also characteristic of the inflammatory response in patients with asthma (Caramori *et al.*, 2005; Tillie-Leblond *et al.*, 2005; Walker *et al.*, 1991). Up to 90% of the cells in the bronchial lavage of control mice are macrophages.

In 2007, Larsen and co-workers also used the BALB/c mouse model and reported that with inflammation the total number of white blood cells increased, but more

specifically, the distribution of cells is changed. An increase in eosinophils, neutrophils and sometimes lymphocytes could be observed (Larsen *et al.*, 2007).

Table 7.1 reveals the statistical comparisons performed upon the various white blood cell species derived from the bronchial lavage of BALB/c mice and Graph 7.1 shows the average number of white blood cells counted in the three slides of the bronchial lavage smears. Figure 7.1 shows the platelet distribution in the bronchial lavage between the different experimental groups. Table 7.2 is the statistical comparisons performed upon the various white blood cell species derived from the blood of the BALB/c mice and Graph 7.2 is a representation of the average number of white blood cells counted in the blood in the different experimental groups. Figure 7.2 shows micrographs of the different white blood cells found in the blood smears of the animals.

Results from the bronchial lavage revealed a significantly higher monocyte count in the control group compared to the other groups whereas the asthmatic group revealed a significantly higher eosinophil count compared to the other experimental groups. No significant difference could be observed when comparing the average number of lymphocyte, neutrophil and basophil counts of the different groups (Table 7.1 and Graph 7.1).

Results from the blood smears revealed more or less the same pattern with a significantly higher eosinophil count in the asthmatic group as well as a significantly lower neutrophil count when compared to the other assessed groups. No significant differences could be observed when comparing the lymphocyte, monocyte and basophil counts in the other experimental groups (Table 7.2 and Graph 7.2).

Figure 7.1 A-D indicates the change in platelet distribution amongst the different experimental groups. Platelet distribution changed significantly in the asthmatic group when compared to the treatment groups, as numerous platelet clumps were found distributed in the bronchial lavage of the asthmatic animals and the absence, or less prominence thereof in the treatment groups could be observed.

7.5 Conclusion

During asthma white blood cell counts in bronchial lavage and blood smears are significantly altered due to the inflammatory response associated with the disease. Also, platelet distribution is changed. The eosinophils in particular, are elevated during the inflammatory response in asthma.

In the current research, the same trends are noted. Here it is seen that Modul8[®] alters the asthmatic profile of the white blood cell counts and these cell numbers appear similar to that of the controls. Also, platelet distribution in the treated asthmatic groups changed to a profile similar to that of the control animals. It is therefore concluded that Modul8[®] may be successfully used in the treatment of the inflammatory asthmatic condition.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 8

LPS- AND MODUL8[®] - STIMULATED ACTIVATION OF PERITONEAL MACROPHAGES *IN VITRO* AND EVALUATION OF CYTOKINE AND NITRIC OXIDE PRODUCTION

8.1 Introduction

Macrophages are major mediators of the immune response and the role of macrophages in the innate and adaptive immune response is crucial as macrophages are the first line of defense of the organism against pathogens. They become activated in response to microenvironment (Varin and Gordon, 2009). Macrophages are potent phagocytic cells and are involved in the presentation of antigens, the secretion of cytokines and they have the ability to kill microorganisms and tumor cells (Fock *et al.*, 2007). When peritoneal macrophages are stimulated with LPS, they release a number of mediators including:

- Interleukins (IL-1 β ; IL-6; IL-8; IL-12)
- Prostaglandins (PGE₂)
- Nitric oxide (NO) and
- Tumor necrosis factor alpha (TNF- α)

A diagram of LPS-stimulated activation of macrophages can be seen in Figure 8.1.

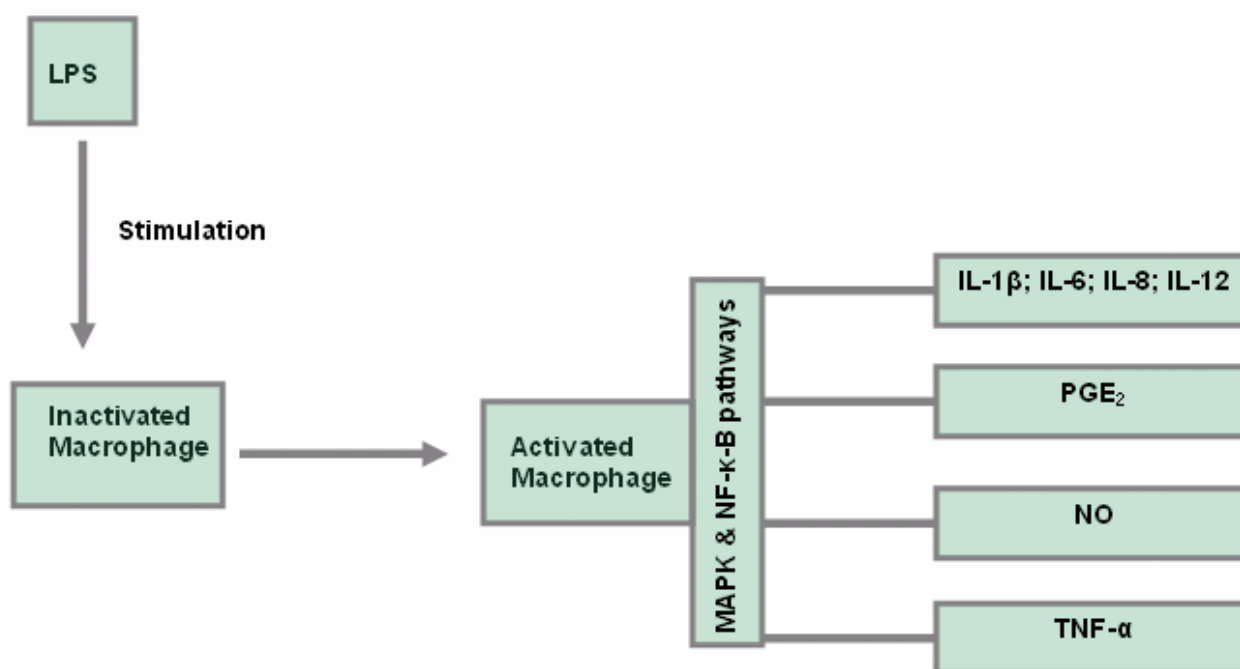


Figure 8.1: Diagram of LPS-stimulated activation of peritoneal macrophages indicating the pathways involved as well as the production of specific mediators (Adams and Hamiltonm, 1984; Kim *et al.*, 2004; An *et al.*, 2006; Fu *et al.*, 2001; Jung *et al.*, 2007; Zhang *et al.*, 2000; Chao *et al.*, 2009).

The focus of the current chapter is to investigate whether mouse peritoneal macrophages will be activated in the presence of Modul8[®], and whether this can be seen with scanning electron microscopy (SEM). Although the main focus of this chapter is morphology, activation was investigated in cell culture to detect the presence of some of the above-mentioned mediators (Figure 8.1). Two of the mentioned parameters, NO and TNF- α were chosen to determine whether they were released by the activated macrophages through the stated signaling pathways (Figure 8.1). It is known that LPS can induce macrophage activation; therefore the four main focus areas of this chapter can be outlined as follows:

1. To stimulate macrophages with LPS

LPS is a major constituent of the outer membrane of Gram-negative bacteria and cause a strong stimulation of the host immune responses (Fock *et al.*, 2007). When LPS induces macrophage activation, it leads to the secretion of a number of cytokines including TNF- α , IL-1 and IL-6 and the up-regulation of these cytokines play an important role in the host defense mechanisms. The LPS-induced synthesis of pro-inflammatory cytokines occurs by means of a complex of receptors and proteins including the CD14 receptor, a glycosyl-phosphatidylinositol-anchored membrane protein a TLR-4 (toll-like receptor 4) and the MD2 protein (Fock *et al.*, 2007; Fujihara *et al.*, 2003). It is also known that LPS affects macrophages on other levels including the cellular functioning and cell morphology (Fock *et al.*, 2007).

2. To evaluate the production of TNF- α in cell culture supernatant

TNF- α is a pro-inflammatory cytokine that is known to play a critical role in the pathogenesis of many inflammatory diseases, including asthma (Russo and Polosa, 2005). TNF- α is a non-glycosylated protein of 17 kDa consisting of 157 amino acids and this pro-inflammatory cytokine belongs to a family of peptide ligands that activate a corresponding set of structurally related receptors (Russo and Polosa, 2005; Bazzoni and Beutler, 1996; Idriss and Naismith, 2000). TNF- α is produced by several pro-inflammatory cells, including macrophages, monocytes, dendritic cells, B-cells, CD4⁺ cells, neutrophils, mast cells and eosinophils and also structural cells such as fibroblasts, epithelial cells and smooth muscle cells (Russo and Polosa, 2005; Coward *et al.*, 2002). A number of stimuli including physical, chemical and immunological stimuli, can lead to the rapid production and release of TNF- α , and can also be generated in response to a wide range of pro-inflammatory cytokines including TNF- α itself (Russo and Polosa, 2005; Coward *et al.*,

2002). One of the biological functions of TNF- α includes the modulation of growth differentiation and proliferation of a variety of cell types and is also a well-known inducer of the inflammatory response as well as a regulator of immunity (Aggarwal, 2003). Other pro-inflammatory cytokines that mediate the inflammatory properties of TNF- α includes IL (interleukin) - 1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN- γ (interferon- γ) and TGF- β (transforming growth factor- β) (Aggarwal, 2003). Macrophages also play an important role in the release of TNF- α and are major sites of TNF- α synthesis.

3. To evaluate NO production in the cell culture supernatant

NO is a signaling molecule involved in important physiological processes including vasodilation and neurotransmission and is synthesized in various cells from L-arginine and molecular oxygen by the nitric oxide synthase (NOS) enzymes (Pacher *et al.*, 2007; Udenigwe *et al.*, 2009). NO is produced in large amounts in macrophages and neutrophils by calcium/calmodulin (CaM)-independent inducible NOS (iNOS) in response to pro-inflammatory cytokines and endotoxins (Udenigwe *et al.*, 2009). NO also reacts with O_2^- to form $ONOO^-$, which is a more reactive DNA-damaging molecule that can also be converted to $^{\cdot}OH$ (Pacher *et al.*, 2007). These highly reactive intermediates could also lead to the inflammation-related tissue damage that is observed in inflammatory disorders (Ames, 1983; Ames *et al.*, 1993; Liu *et al.*, 2002; Pacher *et al.*, 2007; Torre *et al.*, 2002).

4. To investigate the ultrastructure of macrophages to statistically compare the inactivated macrophages from the control group to that of the LPS- and Modul8[®]- stimulated groups

During a typical immune response, macrophages act as coordinators, by presenting antigens and secreting cytokines (Sato *et al.*, 2005). Macrophage

activation involves an increase in the metabolic state, mobility and phagocytic activity of the cell (Piemonte and Buchi, 2002). Typical morphology of activated stage macrophages, such as enhanced spreading and numerous cellular projections was observed in isolated macrophages treated with an immunomodulator (Lopes *et al.*, 2006). A large nucleus, with spreading and many microvilli or projections were regarded as morphological characteristics of activated macrophages when evaluated by SEM and confocal microscopy (Piemonte and Buchi, 2002). Activation of macrophages can be determined by various characteristics including: enlarged nucleus; more euchromatin than heterochromatin; spreading of the cell and projections from the cell. Studies on macrophage activation and an immunomodulator have been done in 2002 where the authors revealed that 86% of the macrophages treated with the immunomodulator were activated in comparison to the 15% of the control (Piemonte and Buchi, 2002). In another study on the same immunomodulator in 2006, the authors investigated the *in vitro* and *in vivo* effect of the product on many different cellular aspects and they reported that the immunomodulator-treated group revealed a greater spreading ability, a higher phagocytic activity of non-infective microorganisms and a tendency to lower the phagocytic activity of an infective microorganism (Lopes *et al.*, 2006).

Therefore, in the current chapter, mouse peritoneal macrophages were derived from male CD1 mice and challenged with LPS to mimic a state of infection or inflammation in order to delineate the macrophage-activation properties of Modul8® *in vitro* by using scanning electron microscopy. The effect of the immunomodulator, Modul8® on the release of TNF- α and production of NO from activated macrophages were also investigated by using *Enzyme-linked immunosorbent assay* (ELISA) and the Griess assay.

8.2 MATERIALS AND METHODS

8.2.1 *Preparation of the plastic stubs*

Plastic stubs from the bottom of 24 well plates were used to fix the cells on. The stubs were placed in 100% EtOH for 30 minutes. They were removed from the EtOH and covered with 200 µl Poly-L-lysine for 1hr. The Poly-L-lysine was removed and the stubs were allowed to dry during the collection of the peritoneal macrophages.

8.2.2 *Experimental groups*

- Control macrophages
- Macrophages stimulated with LPS
- Macrophages stimulated with Modul8®
- Macrophages stimulated with LPS plus addition of Modul8®

8.2.3 *Obtaining and culturing of macrophages*

6 male CD1 mice were obtained from the University of Pretoria Biomedical Research Centre (UPBRC). Resident macrophages were harvested from the peritoneal cavities of the mice. Immediately after killing the animals via cervical dislocation, the skin was removed to expose the peritoneum. PBS (+/- 7ml) was injected into the peritoneum with an insulin needle. While injecting, the peritoneum was rubbed slightly for approximately 30 seconds to loosen the cells. The peritoneal fluid was collected with a 21g needle and all the fluid from each animal containing the peritoneal cells were pooled in a centrifuge tube. The pooled cells were centrifuged for 5 minutes at 1250rpm. The supernatant was drawn off and frozen at -70°C for future studies. 3 ml of medium (DMEM) containing 2% antibiotics and 10% fetal calf serum was added to the cells. This mixture was vortexed for 10 seconds after which 200µl of the mixture as well as 1µg/ml LPS was placed on the plastic stubs in the appropriate wells. The stubs were incubated for 2hr. 20% of Modul8® was added to the cells. The cells were

incubated for another 24hr after which another 1% of the product was added. The cells were incubated for another 24hr after which the experiment was terminated.

8.2.4 Preparation of the macrophages for scanning electron microscopy

After the last 24hr incubation period the macrophages on the stubs were fixed in 2.5% gluteraldehyde (in 0.075M NaPO₄ buffer) for 1hr. After the fixation, the macrophages were washed three times in phosphate buffer for 5 minutes each before fixing in osmium tetroxide for 1hr. After the fixation the macrophages were again washed in phosphate buffer for three times of 5 minutes before being dehydrated in a series of 30%, 50%, 70%, 90% and three times 100% EtOH. The macrophages were then dried using hexamethyldisilazane (HMDS) (Araujo *et al.*, 2003) (Electron Microscopy Sciences, FT. Washington. USA) after which they were mounted and coated with ruthenium tetroxide (SPI Supplies, West Chester, USA) and viewed with a ZEISS ULTRA plus FEG scanning electron microscope.

8.2.5 Statistical analysis

Twenty macrophages from each of the experimental groups (Control, LPS, LPS/Modul8[®] and Modul8[®] alone), viewed with the ZEISS ULTRA plus FEG scanning electron microscope, where quantified in terms of their maximum diameter, with aid of iTEM Soft Imaging Systems for each of the studied mice. The twenty measured diameters of each experimental groups 6 mice where then grouped together. Statistical analysis was preformed to determine if any statistically significant difference existed between the different experimental groups in terms of their macrophage diameters with the aid of a Kruskal-Wallis one-way ANOVA, utilizing the statistical program NCSS and a level of significance was set at 0.05.

8.2.6 ELISA – *Enzyme-linked immunosorbent assay*

A mouse TNF- α (Tumor Necrosis Factor alpha) ELISA Ready-SET-GO Kit with pre-coated plates was obtained from BIOCOM biotech and the experimental procedures were conducted according to the supplied protocol. For the ELISA the macrophages were cultured for 2hr before adding 20% of the products. The cells were then incubated for 24hr after which only 1% of the products were added. The cells were left for another 24hr after which the supernatant was collected and frozen at - 70°C until conduction of the ELISA.

8.2.7 Griess assay for NO production

Nitric oxide production was measured in the cell culture supernatant by using Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). A standard curve was prepared for the assay to ensure accuracy. A dilution series of sodium nitrite starting from 100 μ l, in medium, of the same composition that will be used to culture the experimental cells was performed. 50 μ l of the supernatant of each group were transferred to a 96 well plate. A volume of 50 μ l of a solution of 1% sulfanilamide in 5% phosphoric acid was added to each well, and incubated at room temperature for 5-10 minutes in a dark environment. A volume of 50 μ l of 0.1% N-1-naphthylethylenediamine dihydrochloride was then be added to each well, and incubated for 5-10 minutes at room temperature in the dark. Absorbance was measured within 30 minutes at a wavelength between 540 and 550nm.

8.3 Results

8.3.1 Scanning electron microscopy

Figure 8.2 shows scanning electron micrographs of the macrophages isolated from the peritoneal cavities of mice. Figure A is a representation of typical control cells where no activation could be seen in terms of spreading and pseudopodia formation.



The average diameter of the control cells is also much smaller than the diameter of the activated cells as can be seen in Figure 8.3. Figure B, C and D are micrographs of macrophages stimulated with LPS alone, LPS and Modul8® and Modul8® alone. These macrophages are spread out and the cells appear elongated with projections protruding from the cells. These are typical characteristics of activated macrophages, which could not be observed in the control cells.

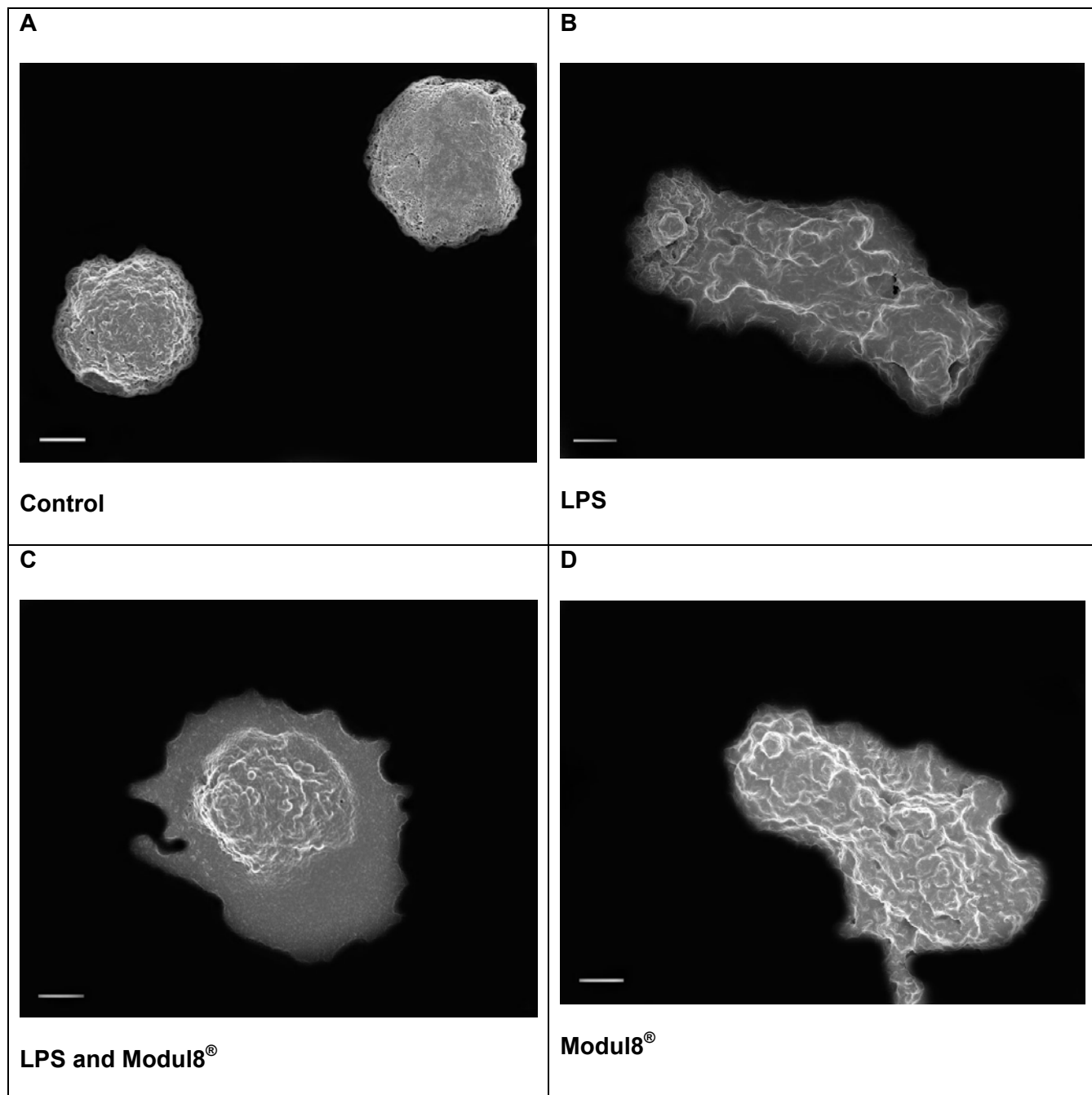


Figure 8.2 Peritoneal macrophages. **A** Control, inactivated macrophage; **B** LPS-stimulated macrophage; **C** Macrophage stimulated with both LPS and Modul8® ; **D** Macrophage stimulated with Modul8® (Scale bar = 2µm)



8.3.2 Statistical analysis

The average macrophage diameter, for each experimental group is expressed in Table 8.1. As the data acquired for statistical assessment failed to meet a normal distribution pattern, the parametric means of comparison could not be employed. The non-parametric Kruskal-Wallis One-way ANOVA subsequently reported a significant result ($P < 0.000001$) of two or more of the experimental groups differing from each other. The use of Tukey-Kramer Multiple-Comparison tests revealed that the control group's macrophage diameters were significantly smaller (implying statistically lower levels of activation) than all the other assessed groups. However, the Modul8[®] exposure group's macrophage diameters were significantly larger than all the other experimental groups (implying that treatment with Modul8[®] alone results in the greatest level of macrophage activation). The LPS and LPS/ Modul8[®] exposure groups did not differ significantly from each other in terms of their macrophage diameters.

Figure 8.3 depicts scanning electron micrographs of control and activated macrophages to demonstrate how the measurements were taken. In each case the widest area of the cell was measured and documented for statistical analysis. Figure A represents two inactivated control macrophages with diameters of 6.52 μm and 7.46 μm respectively. Figure B represents a LPS-stimulated macrophage with a diameter of 17.07 μm . In Figure C a diameter of 12.54 μm was measured in the LPS/Modul8[®] treated macrophages and the Modul8[®] activated macrophage with a diameter of 16.11 can be seen in Figure D.

Table 8.1: Average maximum macrophage diameter derived from various murine macrophage cell cultures under various experimental exposures

Experimental groups	Maximum macrophage diameter $\pm$ SD (μm)
Control	5.75 ± 1.11
LPS	15.27 ± 4.60
LPS/Modul8 [®]	15.50 ± 3.88
Modul8 [®]	17.19 ± 4.87

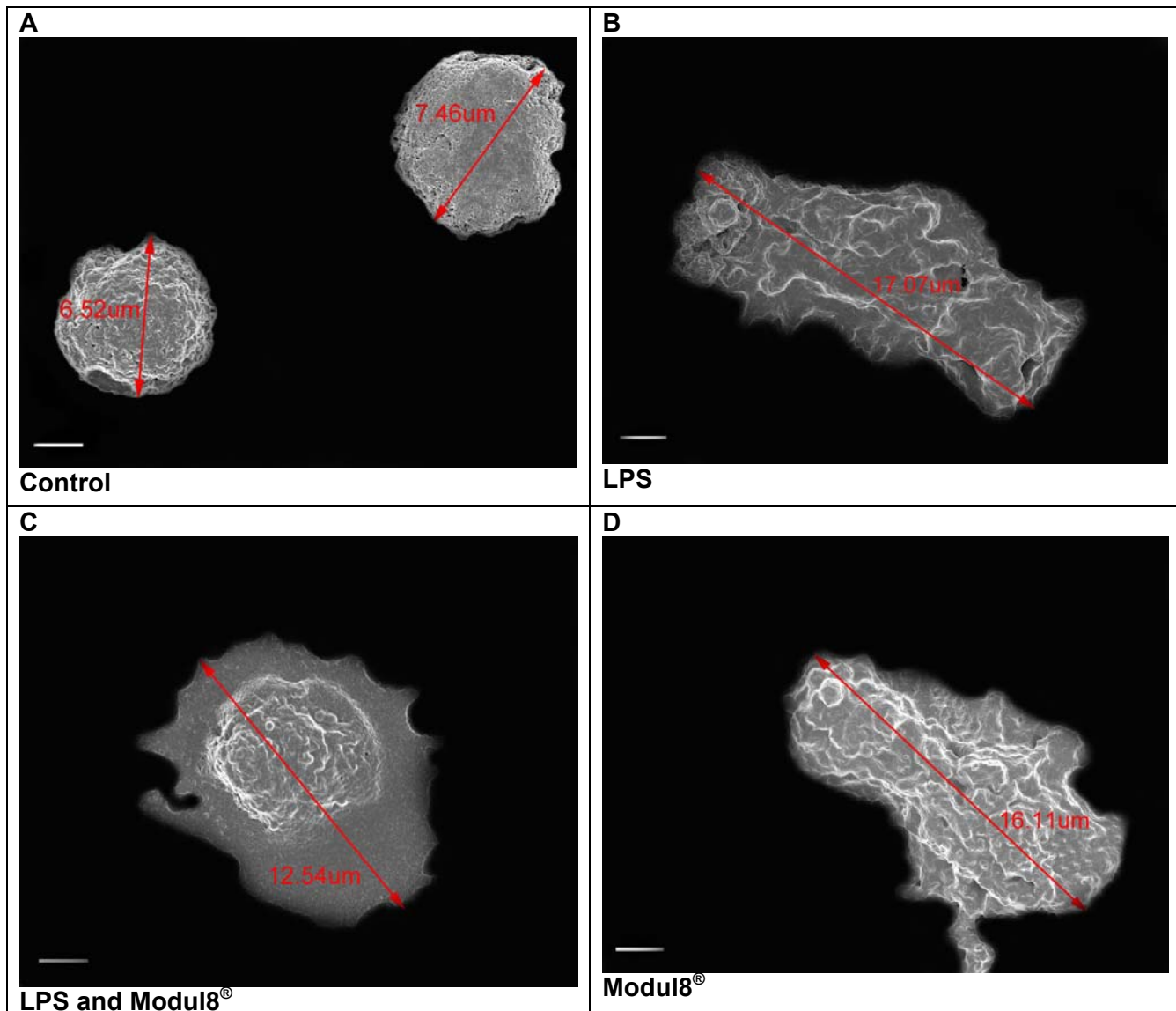
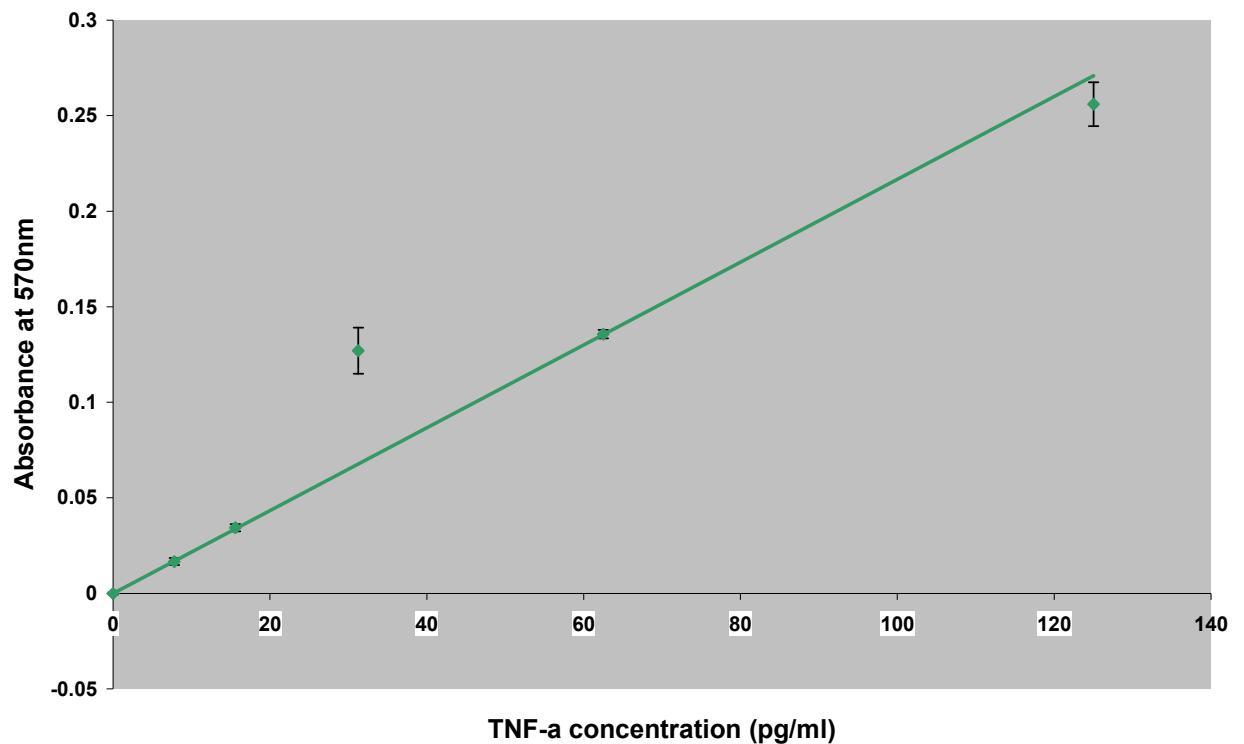


Figure 8.3 Micrographs of macrophages to show how measurements were taken (Scale bar = 2 μm)

8.3.3 ELISA

Standard curve of increasing TNF-alpha concentrations



Graph 8.1 Standard curve of increasing TNF-a concentration determined at 570nm. Equation of line $y = 0.0022x$; $r^2 = 0.9213$; y-error bars represent standard error

No detectable level of TNF- α was found in the assayed macrophage cell supernatants, concluded after multiple analyses at various dilutions. A linear standard curve, with an r^2 value of 0.91, was obtained with the standards provided by the manufacturers of the kit (Graph 8.1) implying that the method was correctly executed.

8.3.4 Griess assay for NO production

The results obtained in the ELISA were confirmed by the measurements of the NO levels in the cell culture, which were found not to be raised.

8.4 Discussion

Peritoneal macrophages are important cells in the immunological defense mechanisms of an organism by removing foreign particles by phagocytosis. Macrophages are capable of movement, phagocytosis of pathogenic or other foreign particles and present antigens to T cells (De and Chattopadhyay, 2009; Harrison and Grinstein, 2002). Macrophages become bigger, metabolically active and show increase in adherence when they are activated (Yayoshi-Yamamoto *et al.*, 2000). Macrophages have the ability to increase or decrease inflammation, depending on the stimulus and this also emphasizes the important anti-inflammatory role of macrophages as they can prevent the development of allergic inflammation (Spiteri *et al.*, 1994). A number of studies have described the effects of an immunomodulator on macrophage activation and it has also been suggested that macrophages need to be activated in order to achieve efficiency (de Oliveira *et al.*, 2006).

LPS is one of the most prominent activators of resting macrophages via binding to the CD14/toll-like receptor 4 (TLR4)/MD2 receptor complex. This leads to the overproduction of pro-inflammatory mediators as well as promotion of phagocytosis. LPS also induces the differentiation and maturation of monocytes or macrophages (Wright *et al.*, 1990; Zhang *et al.*, 2005; Drozina *et al.*, 2006; Crume *et al.*, 2007). Several extracellular signaling pathways are activated when LPS stimulate macrophages. These pathways include the I κ B kinase nuclear factor-kappa B [(IKK)-NF- κ B] pathway and three mitogen-activated protein kinase (MAPK) pathways, extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK (Kim *et al.*, 2005).

TNF- α serve as an immunostimulant and mediator of the inflammatory response and also plays an important role in the in normal host resistance to infections and to growth of malignant tumors (Peppelenbosch *et al.*, 1999). TNF- α is the most widely studied pleiotropic cytokine of the TNF superfamily and is an important cytokine in the innate immune response (Medzhitov and Janeway, 2000). TNF- α is mainly produced by macrophages but can also be produced by other pro-inflammatory cells such as monocytes, dendritic cells, B cells, CD41 cells, neutrophils, mast cells and eosinophils. Structural cells such as fibroblasts, epithelial cells, and smooth muscle cells also play a role in the production of TNF- α (Cazzola and Polosa, 2006).

Although TNF- α plays a critical role in the immune response, an overproduction of this cytokine has been implicated to play a role in some pathological conditions such as cachexia, septic shock and other autoimmune disorders (Peppelenbosch *et al.*, 1999).

Part of the aim of this study was to determine the activation effect of Modul8[®] on mouse peritoneal macrophages to investigate whether the studied immunomodulator can activate macrophages to the same or greater extend as LPS that is known to be an activator of macrophages. The pronounced spreading of the macrophages as seen in Figure 8.1 B, C and D is a typical morphological characteristic of activated macrophages. There are a number of cellular processes associated with the cell spreading observed such as phagocytosis, endocytosis and cell motility. The spreading of the macrophages provides morphological evidence for the cellular alterations that take place within the cell. These alterations include reorganization of the cytoskeleton and changes in the activity and expression of membrane proteins (Wojciak-Stothard *et al.*, 1997; Berton and Lowell, 1999).



In the current study, the release of inflammatory mediators, NO and TNF- α , could not be detected in cell culture. In 2006, researchers investigated the effect of an herbal medicine on inflammatory mediator release in IFN- γ and LPS-stimulated mouse peritoneal macrophages. They found a reduction in NO production and related it to the inhibition of iNOS expression. They evaluated the levels of IL-12, TNF- α and IL-6, which correlate with activation of macrophages. The researchers found that the herbal medicine inhibited LPS-induced TNF- α and IL-12 production and did not affect IL-6 production. With these results the authors suggested that the production of TNF- α , IL-12 and NO occur through different signaling pathways than other cytokines, in their case, IL-6 (An *et al.*, 2006).

8.5 Conclusion

In conclusion, focusing on the ultrastructure of macrophages, this chapter demonstrates that Modul8[®] has the ability to activate macrophages to possibly enhance immune functions. Macrophages produce many different products and cytokines that orchestrate the inflammatory response in asthma and therefore macrophages have the ability to initiate a particular type of inflammatory response via the release of certain cytokines (Spiteri *et al.*, 1994). Since a large body of evidence suggests that macrophages must be activated in order to achieve efficiency and exert a certain function in the immune system, Modul8[®] may possibly be considered as a new adjuvant therapeutic approach to known anti-inflammatory treatment. Since neither TNF- α , nor NO could be detected in the cell culture, it can be hypothesized that an alternative pathway of activation and cytokine production might have taken place as was also found previously by An *et al.*, 2006. Further, more extensive research in the specific pathways involved, might shed light on this matter.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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 histopathological 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% glutaraldehyde/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 tetroxide 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

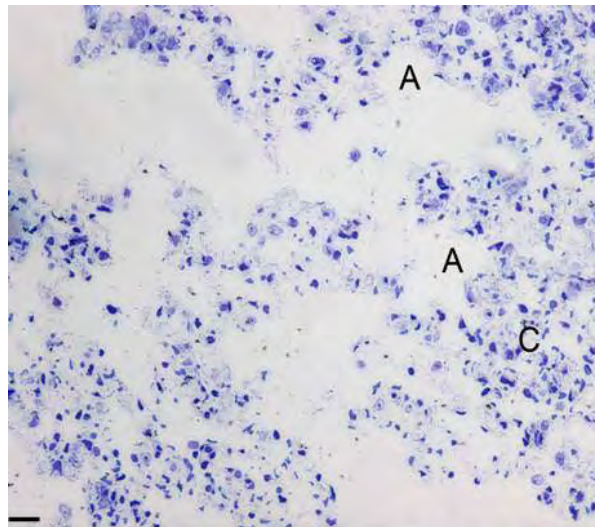
Figure 9.1A (10x magnification) and 1B (40x magnification) represent a section of the lung of a normal healthy animal where alveoli (Label A) is seen, as well as a few inflammatory cells (Label C). In the higher magnification (Figure 1B) the black arrow indicates the interalveolar area which is very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

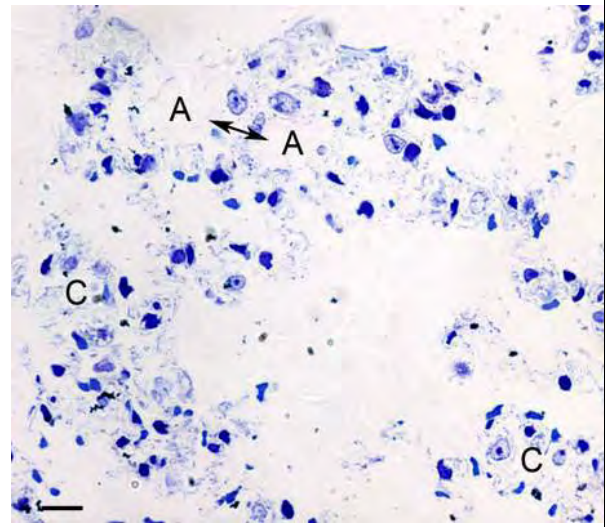
inflammatory cells (Label C) is observed. In the higher magnification (Figure 2 B) an enlarged, congested capillary is observed. The term congested is frequently used in inflammation and asthma to describe the large amount of red blood cells in the capillaries (Ross *et al.*, 2003; Chetta *et al.*, 2007). 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.

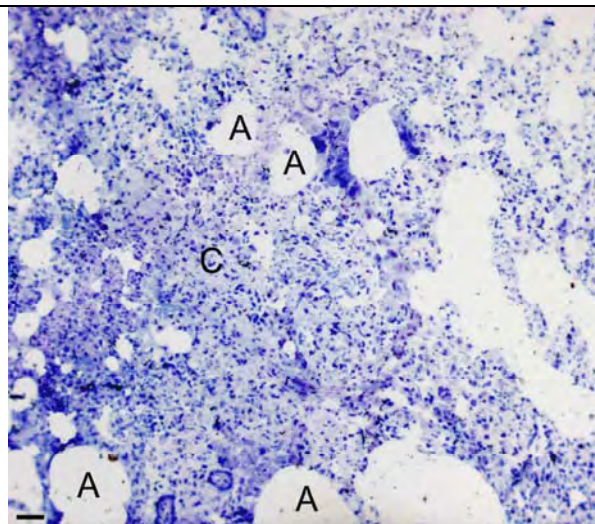
Figure 4A (10x magnification) and 4B (40x magnification) represents the micrographs of the hydrocortisone-treated animals, where even less inflammatory cells (Label C) are present. The black arrow (Figure 4B) indicates the interalveolar area which seems similar to that of the Modul8® treated group and 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.



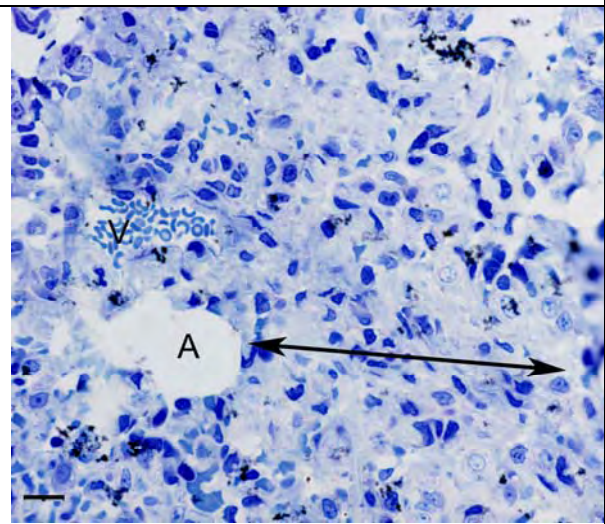
1A: 10x (scale bar = 50µm)



1B: 40x (scale bar = 20µm)



2A: 10x (scale bar = 50µm)



2B: 40x (scale bar = 20µm)

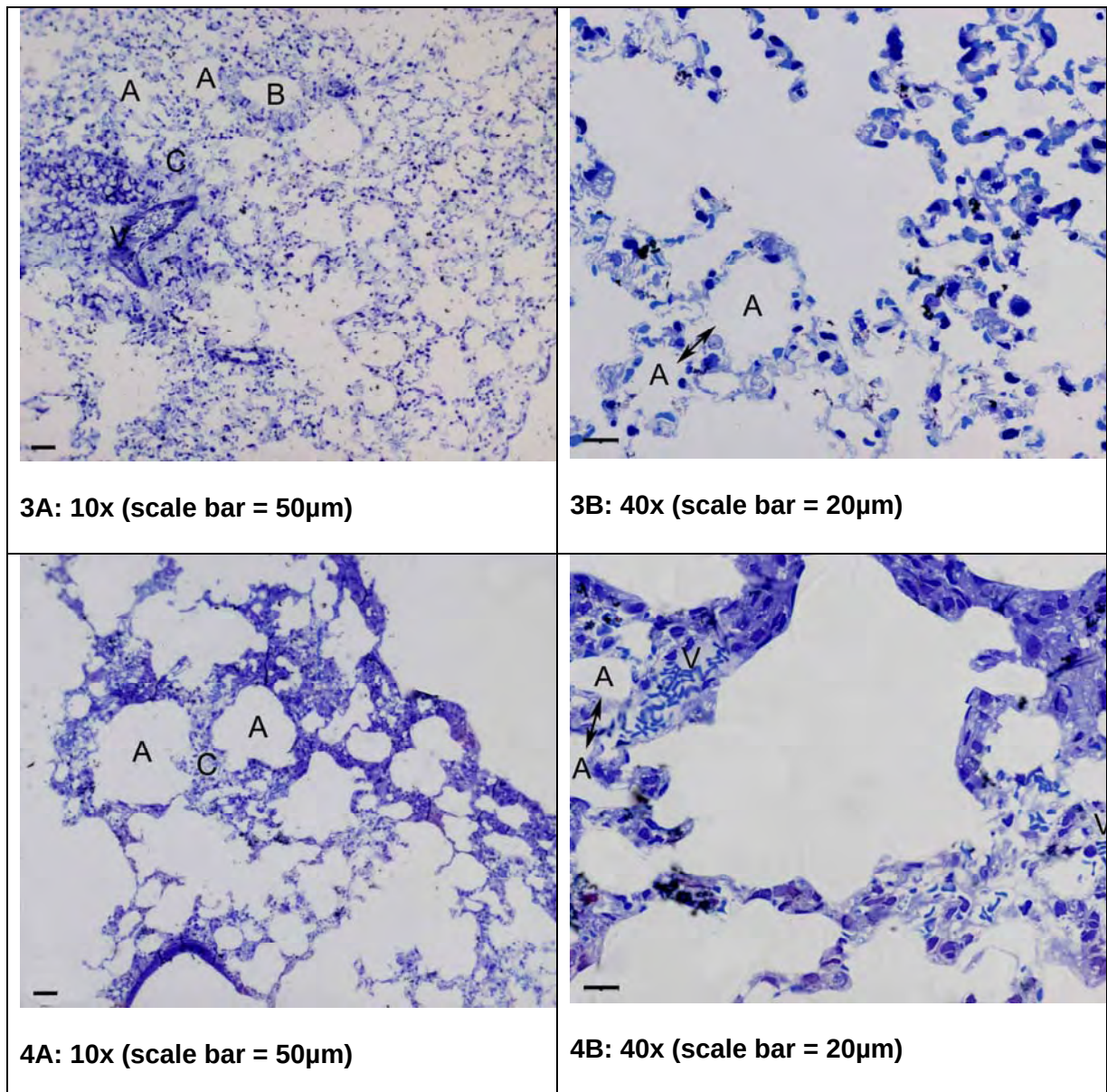


Figure 9.1: Lung sections of the mice stained with Toluidine Blue. **1A** and **B**: Control, **2A** and **B**: Asthma, **3A** and **B**: MODUL8[®] and **4A** and **B**: Hydrocortisone.

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

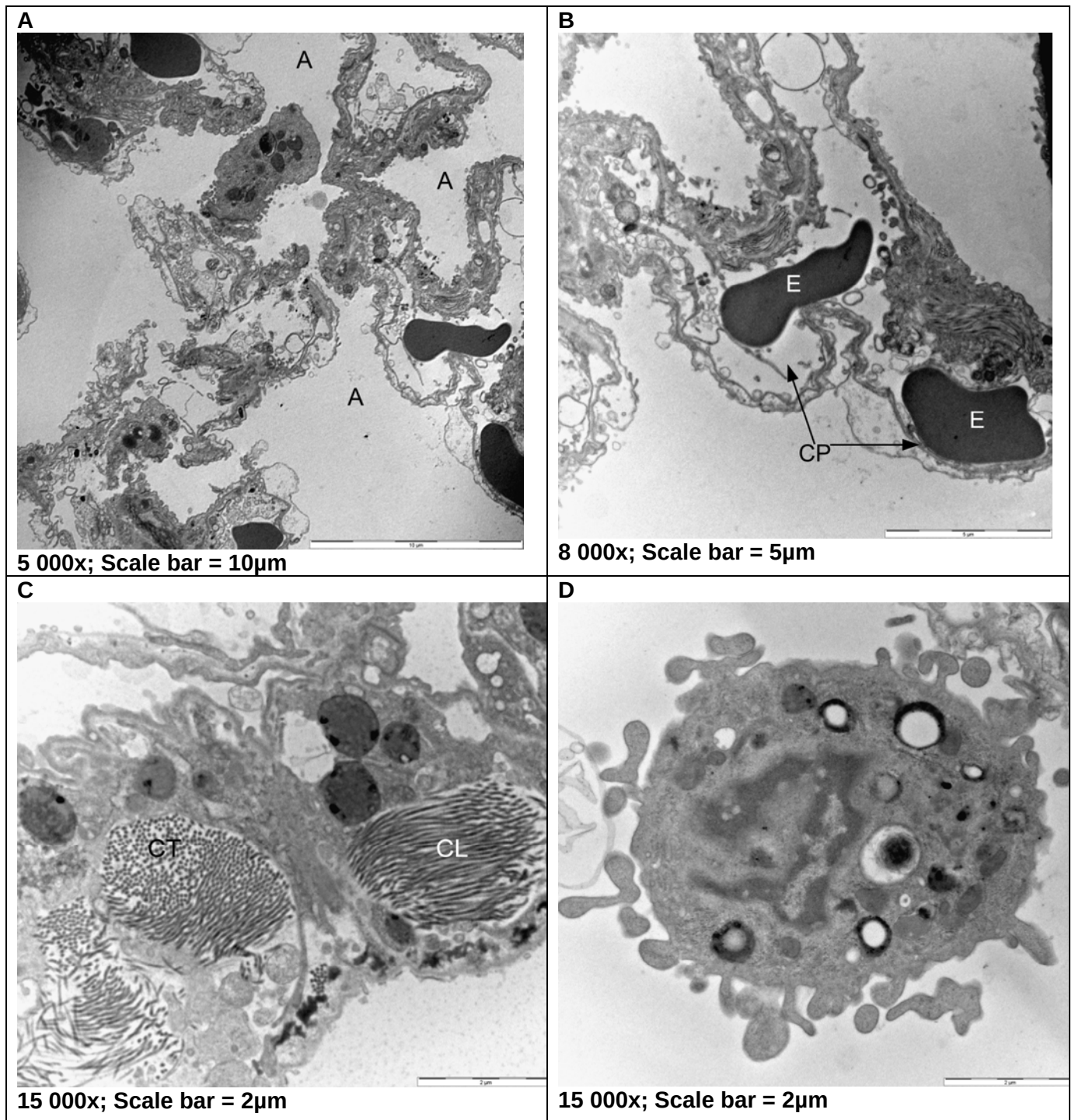
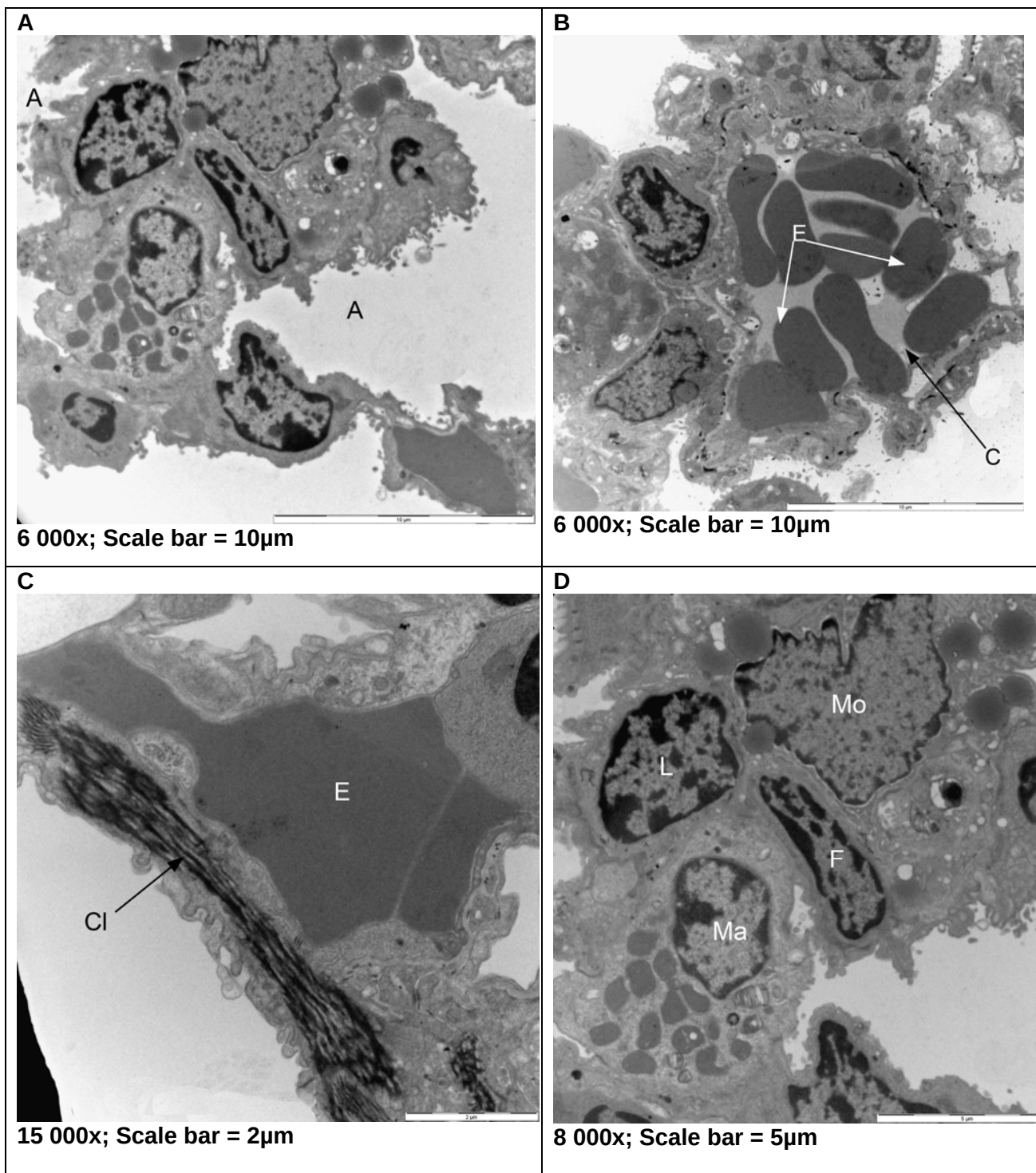


Figure 9.2 A-D Electron micrographs of the lungs taken from the control group



A Alveolus	Nu Nucleus
BM Basement membrane	M Macrophage
C Capillary	Ma Mast cell
CI 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	P₁ Type 1 Pneumocyte
L Lymphocyte	P₂ Type 2 Pneumocyte
LB Lamellar bodies	R Residual bodies
N Neutrophil	

9.3.2.2 Asthma



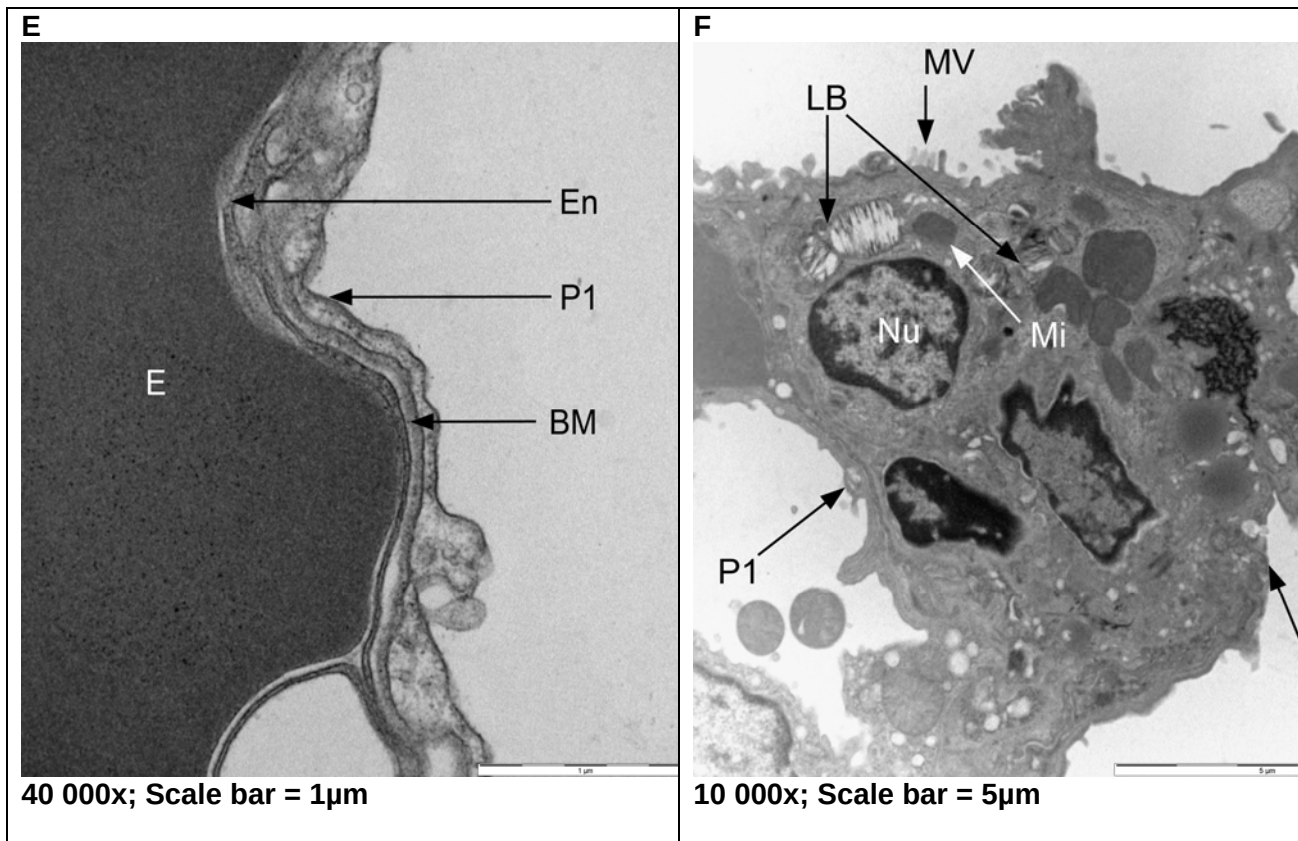
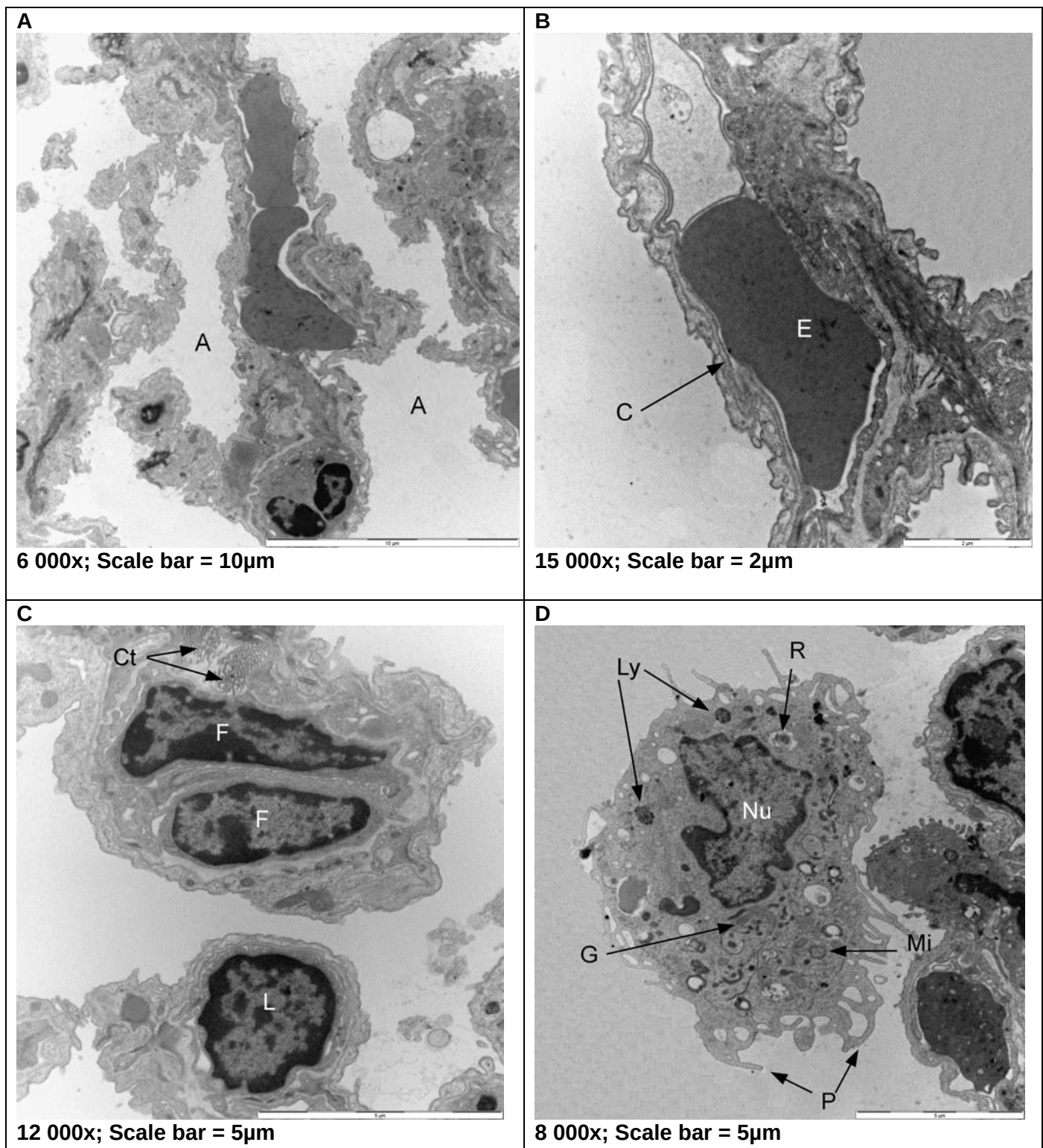


Figure 9.3 A-F Electron micrographs of the lungs taken from the asthmatic group

A Alveolus	Nu Nucleus
BM Basement membrane	M Macrophage
C Capillary	Ma Mast cell
CI 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	P₁ Type 1 Pneumocyte
L Lymphocyte	P₂ Type 2 Pneumocyte
LB Lamellar bodies	R Residual bodies
N Neutrophil	

9.3.2.3 Modul8®



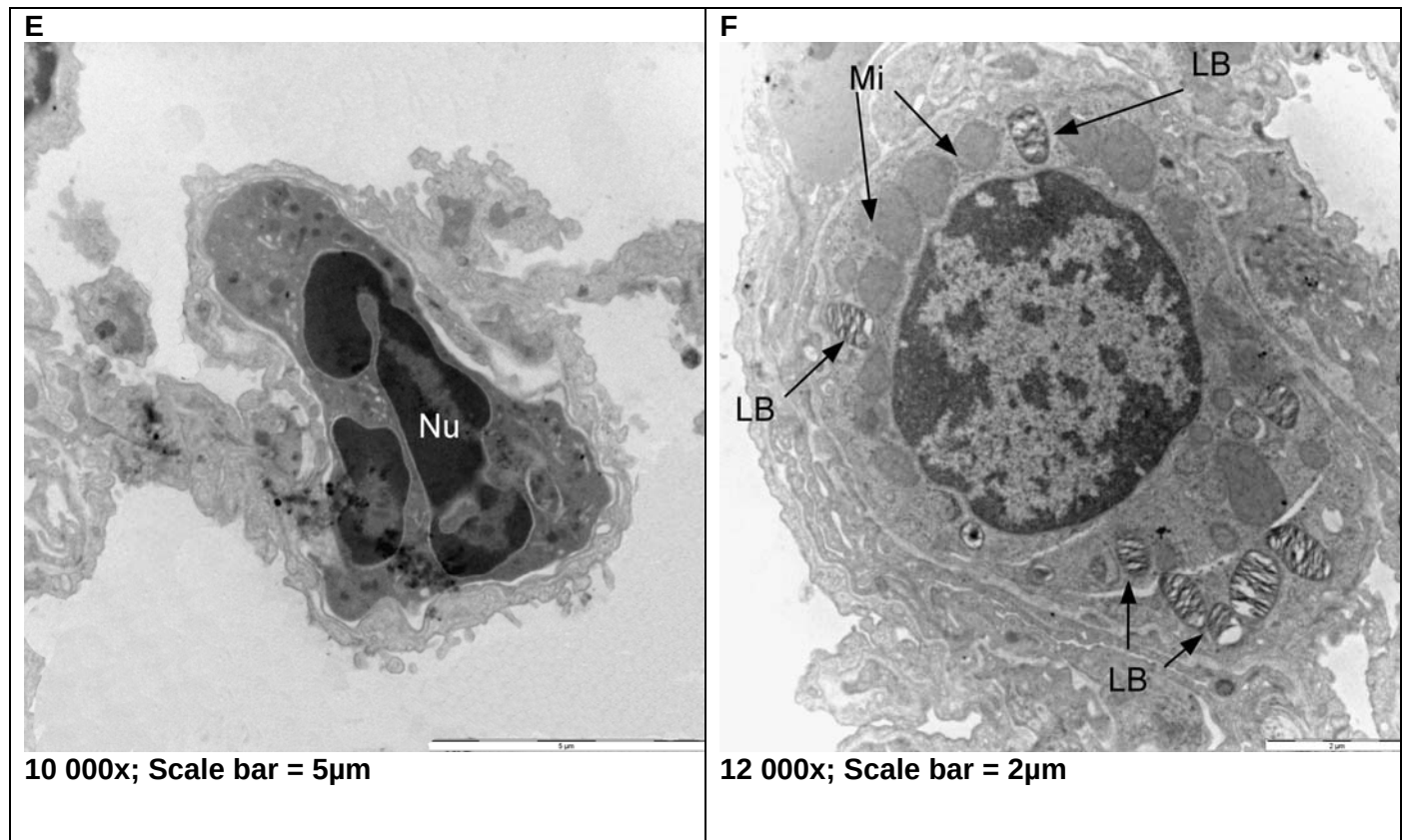


Figure 9.4 A-F Electron micrographs of the lungs taken from the Modul8®-treated group

A Alveolus	Nu Nucleus
BM Basement membrane	M Macrophage
C Capillary	Ma Mast cell
CI 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	P₁ Type 1 Pneumocyte
L Lymphocyte	P₂ Type 2 Pneumocyte
LB Lamellar bodies	R Residual bodies
N Neutrophil	

9.3.2.4 Hydrocortisone

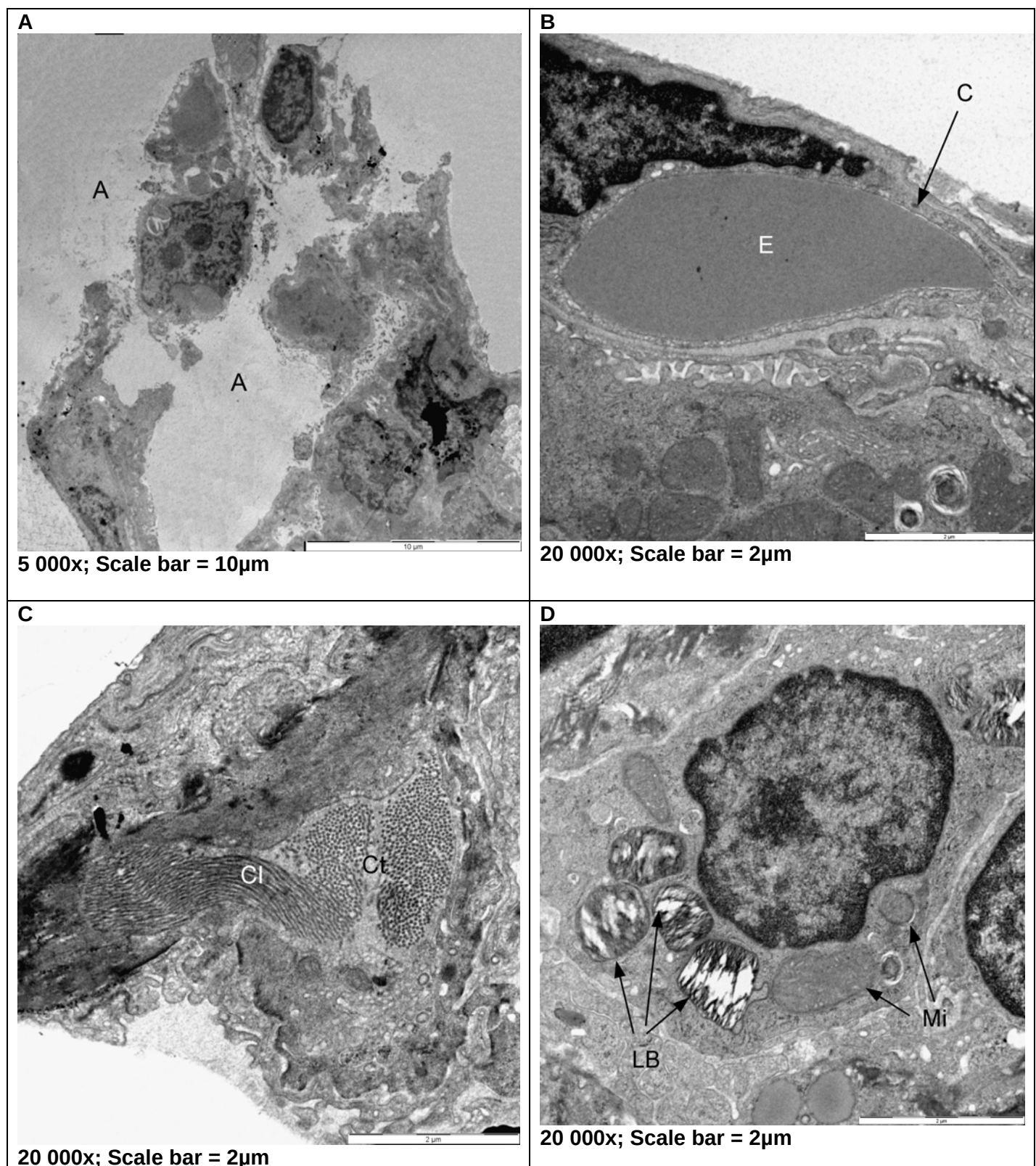


Figure 9.5 A-D Electron micrographs of the lungs taken from the Hydrocortisone-treated group

9.3.2.5 Caveolae

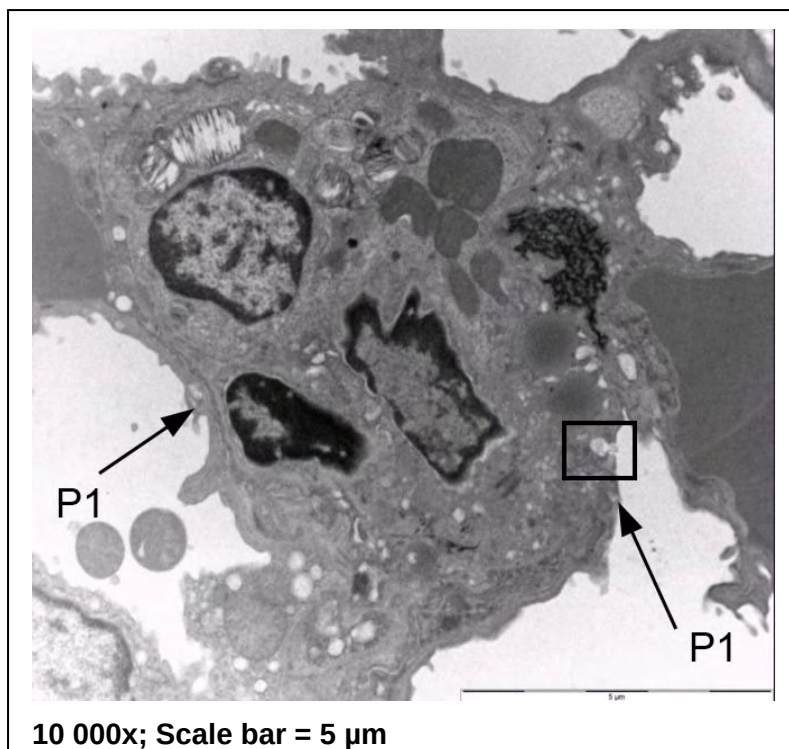


Figure 9.6. Presence of possible caveolae in Type I pneumocyte

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	P₁ Type 1 Pneumocyte
L Lymphocyte	P₂ 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-1 α (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 al.*, 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.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 11

REFERENCES

- Aaron S. D., Fergusson D., Dent R *et al.* Effect of weight reduction on respiratory function and airway reactivity in obese women. *Chest* 2004; **125**: 2046–2052
- Abud A. P. R., Cesar B., Cavazzani L. F. M *et al.* Activation of bone marrow cells treated with Canova *in vitro*. *Cell Biology International* 2006; **31**: 1-9
- Adams D. O., Hamiltonm T. A. The cell biology of macrophage activation. *Annual Reviews in Immunology* 1984; **2**: 283–318
- Aggarwal B. B. Signalling pathways of the TNF superfamily: a double-edged sword. *Nature Reviews. Immunology* 2003; **3**: 745–756
- Ahlquist R. P. A study of the adrenotropic receptors. *American Journal of Physiology* 1948; 153(3): 586–600
- Akbari O. J., Faul L., Hoyte E. G *et al.* CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *The New England Journal of Medicine* 2006; **11**: 17-29
- Al-Barwani F. M., Eltayeb E. A. Antifungal compounds from induced *Conium maculatum* L. plants. *Biochemical Systematics and Ecology* 2004; **32**: 1097-110

- Ames B. N. Dietary carcinogens and anticarcinogens: Oxygen radicals and degenerative diseases. *Science* 1983; **221**:1256–1264
- Ames B. N., Shigena M. K., Hegen T. M. Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Science USA* 1993; **90**: 7915–7922
- An H., Jeong H., Um J *et al.* *Glechoma hederacea* inhibits inflammatory mediator release in IFN- and LPS-stimulated mouse peritoneal macrophages. *Journal of Ethnopharmacology* 2006; **106**: 418-424
- Andriopoulou P., Navarro P., Zanetti A *et al.* Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adheren junctions. *Arteriosclerosis Thrombosis and Vascular Biology* 1999; 19(**10**): 2286-2297
- Araujo J. C., Téran F. C., Oliveira R. A *et al.* Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. *Journal of Electron Microscopy* 2003; 52(**4**): 429-433
- Banach-Wawrzenczyk E., Dziedziczko A., Rosc D. Fibrinolytic system in bronchial asthma after prednisone treatment. *Pol Mercuriusz Lek* 2000; **7**: 9-11
- Baraniuk J.N. Pathogenesis of allergic rhinitis. *Journal of Allergy and Clinical Immunology* 1997; **99**: 763-772
- Barnes P. J., Chung K. F., Page C. P. Platelet-activating factor as a mediator of allergic disease. *Journal of Allergy and Clinical Immunology* 1988; **81**: 919–934

- Barnes P. J. Cytokines as mediators of chronic asthma. *American Journal of Respiratory and Critical Care Medicine* 1994; **150**: 42- 49
- Barnes P. J. Nitric oxide and airway disease. *Annals of Medicine* 1995; **27**: 91-97
- Barnes P. J., Liew F. Y. Nitric oxide and asthmatic inflammation. *Immunology Today* 1995; **16**: 128–130
- Barnes P. J. Pathophysiology of asthma. *British Journal of Clinical Pharmacology* 1996; **42**: 3–10
- Bazzoni F., Beutler B. The tumor necrosis factor ligand and receptor families. *The New England Journal of Medicine* 1996; **334**: 1717–1725
- Belcher J. R. The surgical treatment of asthma. *British Journal of Diseases of the Chest* 1961; 55(2): 77–85
- Beqaj S., Jakkaraju S., Mattingly R. R *et al.* High RhoA activity maintains the undifferentiated mesenchymal cell phenotype, whereas RhoA down-regulation by laminin-2 induces smooth muscle myogenesis. *The Journal of Cell Biology* 2002; **156**: 893-903
- Berbert A. A., Castanheira P. T., Feliu D. G. G *et al.* A multicentric clinical survey HIV/Aids patients submitted to Canova immunomodulator treatment associated with antiretroviral medicaments. 2002, Available at: www.canovadoBrasil.com [Accessed: August 2008]

- Bergeron C., Boulet L.P. Structural changes in airway diseases: characteristics, mechanisms, consequences, and pharmacologic modulation. *Chest* 2006; **129**: 1068-1087
- Bergman J., Persson H., Wetterlin K. Two new groups of selective stimulants of adrenergic beta-receptors. *Experientia* 1969; 25(9): 899–901
- Bernardi R. P. D., 2006. Patients recovery with HIV/Aids in Botswana/ Africa, with the use of the homeopathic medicine Canova. PhD. Federal University of Paraná.
- Berton G., Lowell C. A. Integrin signaling in neutrophils and macrophages. *Cell signaling* 1999; 11(9): 621-635
- Beuther D. A., Weiss S. T., Sutherland E. R. Pulmonary Perspective: Obesity and Asthma. *American Journal of Respiratory and Critical Care Medicine* 2006; **174**: 112–119
- Bharadwaj A., Agrawal D. K. Immunomodulation in asthma: a distant dream or a close reality? *International Immunopharmacology* 2004; 4: 495–511
- Bice D. E., Seagrave J., Green F. H. Y. Animal models of Asthma: Potential usefulness for studying Health effects of Inhaled Particles. *Inhalation Toxicology* 2000; 12(9): 829-862
- Bjermer L., Diamant Z. Current and emerging nonsteroidal anti-inflammatory therapies targeting specific mechanisms in asthma and allergy. *Treatments in Respiratory Medicine* 2004; 3(4): 235–246

- Black J.L., Johnson P.R. Factors controlling smooth muscle proliferation and airway remodelling. *Current Opinion in Allergy and Clinical Immunology* 2002; **2**: 47-51
- Black J. L. Asthma-More muscle cells or more muscular cells? *American Journal of Respiratory and Critical Care Medicine* 2004; **169**: 980-981
- Blyth D. I., Pedrick M. S., Savage T. J *et al.* Lung inflammation and epithelial changes in a murine model of atopic asthma. *American Journal of Respiratory, Cell and Molecular Biology* 1996; **14**: 425-438
- Borovitskaya M. P., Volodchenko N. I., Vysokoostrovskaya I. B *et al.* Side effects of hydrocortisone and prednisolone on mice of various age groups. *Bulletin of Experimental Biology and Medicine* 1971; **72** (6): 22–24
- Boulet L. P., Chakir J., Dube J *et al.* Airway inflammation and structural changes in airway hyper-responsiveness and asthma: an overview. *Canadian Respiratory Journal* 1998; **5**: 16-21
- Bousquet J. P., Chanaz J. Y., Lacoste G *et al.* Eosinophilic inflammation in asthma. *The New England Journal of Medicine* 1990; **323**: 1033–1039
- Bousquet I. J., Ansotegui R., van Ree P. G *et al.* European Union meets the challenge of the growing importance of allergy and asthma in Europe. *Allergy* 2004; **59**:1-4

- Bradding P., Walls A. F., Holgate S. T. The role of the mast cell in the pathophysiology of asthma. *Journal of Allergy and Clinical Immunology* 2006; 117(6): 1277-1284
- Bradding P., Brightling C. Mast cell infiltration of airway smooth muscle in asthma. *Respiratory Medicine* 2007; **101**: 1045
- Brewster C.E., Howarth P.H., Djukanovic R *et al.* Myofibroblasts and subepithelial fibrosis in bronchial asthma. *American Journal of Respiratory Cell and Molecular Biology* 1990; **3**: 507–511
- Brito D. J. M. Immunomodulator Canova-Aids and quality of life. 2002. Available at: www.canovadoBrasil.com [Accessed: August 2008]
- Brittain R. T., Farmer J. B., Jack D *et al.* Alpha- [(t Butylamino)methyl]-4-hydroxy-m-xylene-alpha 1,alpha 3-diol (AH.3365): a selective beta-adrenergic stimulant. *Nature* 1968; 219(**156**): 862–863
- Brown A. S., Levine J. D., Green P. G. Sexual dimorphism in the effect of sound stress on neutrophil function. *Journal of neuroimmunology* 2008; **205**: 25-31
- Burgess J.K., Carlin S., Pack R.A *et al.* Detection and characterization of OX40 ligand expression in human airway smooth muscle cells: a possible role in asthma? *Journal of Allergy and Clinical Immunology* 2004; **113**: 683-689
- Burleigh M. E., Babaev V. R., Oates J. A., Harris R. C., Gautam S., Riendeau D., Marnett L. J., Morrow J. D., Fazio S., Linton M. F. Cyclooxygenase-2

promotes early atherosclerotic lesion formation in LDL receptor-deficient mice. *Circulation* 2002; **105**: 1816–1823

Butenas S., Mann K. G. Blood coagulation. *Biochemistry* 2002; **671**: 3-12

Cabral M. P., Soffritti E. M., Nader J. R. Clinical study work for evaluation of immunomodulator Canova in the therapeutics of oncologic patients considered OTP – Out of therapeutic possibility. 2002. Available at: www.canovadoBrasil.com [Accessed: August 2008]

Camargo C. A., Weiss S. T., Zhang S *et al.* Prospective study of body mass index, weight change, and risk of adult-onset asthma in women. *Archives of Internal Medicine* 1999; **159**:2582–8

Camera M., Giesen P. L., Fallon J *et al.* Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. *Arteriosclerosis, Thrombosis and Vascular Biology* 1999; **19**: 531-537

Capra V., Thompson M. D., Sala A *et al.* Cysteinylleukotrienes and their receptors in asthma and other inflammatory diseases: critical update and emerging trends. *Medicinal Research Reviews* 2007; **27**: 469–527

Caramori G., Pandit A., Papi A. Is there a difference between chronic airway inflammation in chronic severe asthma and chronic obstructive pulmonary disease? *Current Opinion in Allergy and Clinical Immunology* 2005; **5**: 77-83

- Castanheira P.T., Oliveira A. P., Rajao M. C. S *et al.* Evaluation of Canova Immunomodulator's use in patients with cancer. 2003. Available at: www.canovadoBrasil.com [Accessed: August 2008]
- Castanheira P.T., Oliveira A. P., Rajao M. C. S *et al.* Evaluation of Canova Immunomodulator's use in patients with cancer. 2004. Available at: www.canovadoBrasil.com [Accessed: August 2008]
- Castro-Rodriguez J. A., Holberg C. J., Morgan W. J *et al.* Increased incidence of asthma like symptoms in girls who become overweight or obese during the school years. *American Journal of Respiratory and Critical Care Medicine* 2001; **163**:1344-1349
- Cazzola M., Polosa R. Anti-TNF-alpha and Th1 cytokine-directed therapies for the treatment of asthma. *Current Opinion in Allergy and Clinical Immunology* 2006; **6**: 43-50
- Chao W., Kuo Y., Li W *et al.* The production of nitric oxide and prostaglandin E₂ in peritoneal macrophages is inhibited by *Andrographis paniculata*, *Angelica sinensis* and *Morus alba* ethyl acetate fractions. *Journal of Ethnopharmacology* 2009; **122**: 68-75
- Chetta A., Zanini A., Olivieri D. Therapeutic approach to vascular remodeling in asthma. *Pulmonary Pharmacology and Therapeutics* 2007; **20**: 1-8

- Cho S. J., Kim H. W., Kim B *et al.* *Sam So Eum*, an herb extract, as the remedy for allergen-induced asthma in mice. *Pulmonary Pharmacology and Therapeutics* 2008; **21**: 578–583
- Cohn L., Elis J. A., Chupp G. L. Asthma: mechanisms of disease persistence and progression. *Annual review of immunology* 2004; **22**:789–815
- Corrigan C. J., Kay A. B. T cells and eosinophils in the pathogenesis of asthma. *Immunology Today* 1992; **13**: 501–507
- Coward W. R., Okayama Y., Sagara H *et al.* NF- κ B and TNF- α : a positive autocrine loop in human lung mast cells? *Journal of Immunology* 2002; **169**: 5287–5293
- Coyle A. J., Spina D., Page C. P. PAF-induced bronchial hyperresponsiveness in the rabbit: Contribution of platelets and airway smooth muscle. *British Journal of Pharmacology* 1990; **101**: 31–38
- Crume K. P., Miller J. H., La Flamme A. C *et al.* An antimitotic agent specifically decreases tumor necrosis factor- α production by lipopolysaccharide-stimulated murine macrophages. *Experimental Biology and Medicine* 2007; **232**: 607–613
- Cummings S., Ullman D. 1991. Every-body's Guide to Homeopathic Medicines. 1st ed. New York: G. P. Putnam's Sons

- Cummings S., Ullman D. 1997. Everybody's Guide to Homeopathic Medicines. 3rd ed. New York: G. P. Putnam's Sons.
- Cumming R. G., Mitchell P., Leeder S. R. Use of inhaled corticosteroids and the risk of cataracts. *The New England Journal of Medicine* 1997; **337**: 8-14
- De A., Chattopadhyay S. The variation in cytoplasmic distribution of mouse peritoneal macrophage during phagocytosis modulated by mangiferin, an immunomodulator. *Immunobiology* 2009; **214**: 367-376
- de Oliveira C. C., De Oliveira S. M., Godoy L. M. F Et al. Canova, A Brazilian medical formulation, alters oxidative metabolism of mice macrophages. *Journal of Infection* 2006; **52**: 420-432
- Diamant Z., Boot J. D., Virchow J. C. Summing up 100 years of asthma. *Respiratory Medicine* 2007; **101**: 378-388
- Diamant Z., Sampson A. P. Anti-inflammatory mechanisms of leukotriene modulators. *Clinical and Experimental Allergy* 1999; 29(**11**): 1449–1453
- Drozina G., Kohoutek J., Nishiya T et al. Sequential modifications in class II transactivator isoform 1 induced by lipopolysaccharide stimulate major histocompatibility complex class II transcription in macrophages. *The Journal of Biological Chemistry* 2006; **281**: 39963–39970
- Dworski R., Sheller J. Inhaled steroids in asthma. *Compehensiver Therapy* 1992; 18(**3**): 3–5

- Ebina M., Takahashi T., Chiba T *et al.* Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma: a 3-D morphometric study. *The American review of respiratory disease* 1993; **148**:720-726
- Engelhardt G. Profile of pharmacological actions of NAB 365 (clenbuterol), a novel broncholytic agent with selective activity on adrenergic beta2-receptors (author's transl). *Arzneimittel-Forschung* 1976; 26(7):1404–1420
- Engels F., Nijkamp F. P. Pharmacological inhibition of leukotriene actions. *Pharmacy World and Science* 1998; **20**: 60–65
- Epstein M. M. Do mouse models of allergic asthma mimic clinical disease? *International archives of Allergy and Immunology* 2004; **133**: 84-100
- Epstein M. M. Are mouse models of allergic asthma useful for testing novel therapeutics? *Experimental and Toxicologic Pathology* 2006; 57(2): 41-44
- Evans K. L. J., Bond R. A., Corry D. B *et al.* Frequency dependence of respiratory system mechanics during induced constriction in a murine model of asthma. *Journal of Applied Physiology* 2003; **94**: 245–252
- Fock R. A., Vinolo M. A. R., Rocha V. S. M. S *et al.* Protein-energy malnutrition decreases the expression of TLR-4/MD-2 and CD14 receptors in peritoneal macrophages and reduces the synthesis of TNF- α in response to lipopolisaccharide (LPS) in mice. *Cytokine* 2007; **40**: 105-114

- Foster P.S., Mould A.W., Yang M *et al.* Elemental signals regulating eosinophil accumulation in the lung. *Immunological Reviews* 2001; **179**: 173-181
- Fu Y., McCormick C. C., Roneker C *et al.* Lipopolisaccharide and interferon-g-induced nitric oxide production and protein oxidation in mouse peritoneal macrophages are affected by glutathione peroxidase-1 gene knockout. *Free Radical Biology and Medicine* 2001; 31 (4): 450-459
- Fujihara M., Muroi M., Tanamoto K *et al.* Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacology and Therapeutics* 2003; **100**: 171–194
- Funk C. D. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* 2001; **294**: 1871–1875
- Galli S. J., Kalesnikoff J., Grimbaldston M. A *et al.* Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annual Review of Immunology* 2005; **23**: 749-786
- Galli S.J., Metcalfe D.D., Dvorak A.M. Basophils and Mast cells and their disorders. In: Beutler E., Lichtman M.A., Coller B.S., Kipps T.J., Seligsohn U. 2001. Williams Hematology. 6th ed. New York: McGraw-Hill
- Ganguli R. Weight gain associated with antipsychotic drugs. *Journal of clinical psychology* 1999; 60(21): 20–24
- Garbe E., LeLorier J., Boivin J. F. Inhaled and nasal glucocorticoids and the risk of

- ocular hypertension or open-angled glaucoma. *The Journal of the American Medical Association* 1997; **277**: 722-727
- Geba G. P., Askenase P. W. In: Kay A. B. Editor. 1997. Allergy and Allergic Diseases. Oxford: Blackwell
- Gleich G. J. The eosinophil and brochial asthma: current understanding. *Journal of Allergy and Clinical Immunology* 1990; **85**: 422-436
- Gleich G. J., Kita H. Bronchial asthma: Lessons from murine models. *Proceedings of the National Acadamy of Science* 1997; **94**: 2101–2102
- Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Bethersda/Martyland: NHLBI/ WHO workshop report. Last updated: 2005. 1- 1995
- Gold P. W., Novella S, Roy R *et al.* Homeopathy- quackery or a key to the future of medicine? *Homeopathy* 2008; **97**: 28-33
- Gosen R., Mutawe M., Martin S *et al.* Caveolae and caveolins in the respiratory system. *Current molecular medicine* 2008; **8**: 741-753
- Goudie A. J., Smith J., Halford J. Characterization of olanzapine-induced weight gain in rats. *Journal of Psychopharmacology* 2002; **16**: 291–296
- Greenfield J. R., Samaras K. Suppression of HPA axis in adults taking inhaled corticosteroids. *Thorax* 2006; **61**: 272-273

- Gross N. J. Anticholinergic agents in asthma and COPD. *European Journal of Pharmacology* 2006; 533(1–3): 36–9
- Gumbleton M. Caveolae as potential macromolecule trafficking compartments within alveolar epithelium. *Advanced Drug Delivery Reviews* 2001; **49**: 281–300
- Hakala K., Stenius-Aarniala B., Sovijarvi A. Effects of weight loss on peak flow variability, airways obstruction, and lung volumes in obese patients with asthma. *Chest* 2000; **118**: 1315–1321
- Hamelmann E., Takeda K., Schwarze J *et al.* Development of Eosinophilic Airway Inflammation and Airway Hyperresponsiveness Requires Interleukin-5 but Not Immunoglobulin E or B Lymphocytes. *American Journal of Respiratory Cell and Molecular Biology* 1999; **21**: 480–489
- Hamid Q., Azzawi M., Sun Y, *et al.* Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *The Journal of Clinical Investigation* 1991; **87**:1541–1549
- Hamid Q., Springall D. R., Riveros-Moreno V, *et al.* Induction of nitric oxide synthase in asthma. *Lancet* 1993; **342**:1510–1513
- Haraguchi M., Shimura S., Shirato K. Morphometric analysis of bronchial cartilage in chronic obstructive pulmonary disease and bronchial asthma. *American Journal of Respiratory and Critical Care Medicine* 1999; **159**: 1005-1013

- Hardy C., Robinson C., Tattersfield A. T *et al.* The bronchoconstrictor effect of inhaled prostaglandin D₂ in normal and asthmatic men. *The New England Journal of Medicine* 1984; **311**: 209–213
- Harris R. B., Zhou J., Youngblood B. D *et al.* Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets. *The American Journal of Physiology* 1998; **275**: 1928–1938
- Harrison R.E., Grinstein S. Phagocytosis and the microtubule cytoskeleton. *Biochemistry and Cell Biology* 2002; **80**: 509–515
- Hart P.H. Regulation of the inflammatory response in asthma by mast cell products. *Immunology and Cell Biology* 2001; **79**: 149-153
- Hasford J., Virchow J. C. Excess mortality in patients with asthma on long-acting β_2 -agonists. *European Respiratory Journal* 2006; 28(5): 900–902
- Hashimoto S., Gon Y., Takeshita I *et al.* Transforming growth factor- β_1 induces phenotypic modulation of human lung fibroblasts to myofibroblast through a c-Jun-NH₂-terminal kinase-dependent pathway. *American Journal of Respiratory and Critical Care Medicine* 2001; **163**:152-157
- Hata A. N., Breyer R. M. Pharmacology and signaling of prostaglandin receptors: Multiple roles in inflammation and immune modulation. *Pharmacology and Therapies* 2004; **103**:147–166

- Hausberger F. X., Hausberger B. C. Effect of insulin and cortisone on weight gain, protein and fat content of rats. *The American Journal of Physiology* 1958; **193**: 455 - 460
- Hirst S. J., Martin J. G., Bonacci J. V *et al.* Proliferative aspects of airway smooth muscle. *Journal of Allergy and Clinical Immunology* 2004; **114**: 2-17
- Hofbauer L. Asthma and tuberculosis. *British Journal of Tuberculosis* 1931; **25**(2): 77–79
- Hogan M. B., Weissman D. N., Hubbs A. F *et al.* Regulation of eosinophilopoiesis in a murine model of asthma. *The Journal of Immunology* 2003; **171**: 2644-2651
- Hogan S. P., Koskinen A., Foster P. S. Interleukin 5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in Balb/c mice. *Immunology and Cell Biology* 1996; **75**: 284-288
- Holgate S. T., Roche W. R., Church M. K. The role of the eosinophil in asthma. *The American Review of Respiratory Disease* 1991; **143**: 66–70
- Holsapple M.P., Jones D., Kawabata T.T *et al.* Assessing the potential to induce respiratory hypersensitivity. *Toxicological Sciences* 2006; **91**: 4–13
- Holt P. G. Regulation of antigen-presenting cell function(s) in lung and airway tissues. *The European Respiratory Journal* 1993; **6**: 120-129
- Holt P.G. Antigen presentation in the lung. *American Journal of Respiratory and Critical Care Med* 2000; **162**: 151–156

- Hoshino M., Takahashi M., Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *Journal of Allergy and Clinical Immunology* 2001; **107**: 295-301
- Huang J., Olivenstein R., Taha R *et al.* Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *American Journal of Respiratory and Critical Care Medicine* 1999; **160**: 725–729
- Hubbard R. B, Smith C. J. P., Smeeth L *et al.* Inhaled corticosteroids and hip fracture: a population-based case–control study. *American Journal of Respiratory and Critical Care Medicine* 2002; **166**: 1563-1566
- Humbles A. A., Lloyd C. M., McMillan S. J *et al.* A critical role for eosinophils in allergic airways remodelling. *Science* 2004; **305**: 1776-1779
- Idriss H. T., Naismith J. H. TNF α and the TNF receptor superfamily: structure–function relationship(s). *Microscopy Research and Technique* 2000; **50**: 184–195
- Ind P. W. Anti-leukotriene intervention: is there adequate information for clinical use in asthma? *Respiratory Medicine* 1996; 90(**10**): 575–586
- Isenberg-Feig H., Justice J. P., Keane-Myers A. Animal models of allergic asthma. *Current Allergy and Asthma Reports* 2003; 3(**1**): 70-78
- Jeffery P. K. Differences and similarities between chronic obstructive pulmonary disease and asthma. *Clinical and Experimental Allergy*. 1999; 29 (**2**): 14-26

Johnson M. Salmeterol. *Medicinal Research Reviews* 1995; 15(3): 225–57

Johnson P. R., Black J. L., Carlin S *et al.* The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. *American Journal of Respiratory and Critical Care Medicine* 2000; **162**: 2145-2151

Johnson P. R., Burgess J. K., Underwood PA *et al.* Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. *Journal of Allergy and Clinical Immunology* 2004; **113**: 690-696

Johnson P. R., Roth M., Tamm M *et al.* Airway smooth muscle cell proliferation is increased in asthma. *American Journal of Respiratory and Critical Care Medicine* 2001; **164**: 474-477

Jung H. W., Yoon C., Kim Y. H *et al.* Wen-Pi-Tang-Hab-Wu-Ling-San extract inhibits the release of inflammatory mediators from LPS-stimulated mouse macrophages. *Journal of Ethnopharmacology* 2007; **114**: 439-445

Jütte R., Riley D. A review of the use of the use and role of low potencies in homeopathy. *Complementary Therapies in Medicine* 2005; **13**: 291-296

Kay A. B. Asthma and inflammation. *Journal of Allergy and Clinical Immunology* 1991a; **87**: 893–913

Kay A. B. Eosinophils and asthma. *The New England Journal of Medicine* 1991b; **324**: 1514–1515

- Kay A. B. Pathology of mild, severe, and fatal asthma. *American Journal of Respiratory and Critical Care Medicine* 1996; **154**: 66-69
- Kay A. B., Phipps S., Robinson D. S. A role for eosinophils in airway remodeling in asthma. *Trends in Immunology* 2004; **9**: 477-482
- Kent J. T. 1996. Lectures on Materia Medica. New Delhi: B. Jain Publishers
- Kharitonov S. A., Yates D., Robbins R. A *et al.* Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994; **343**: 133–135
- Kim S. J., Jeong H. J., Moon P. D *et al.* Anti-inflammatory activity of gumiganghwaltang through the inhibition of nuclear factor-kappa B activation in peritoneal macrophages. *Biological and Pharmaceutical Bulletin* 2005; **28**: 233–237
- Kim Y., Moon J., Lee K. S *et al.* Ca²⁺/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF- κ B activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. *Biochemical and Biophysical Research Communications* 2004; **314**: 695-703
- Kon O. M., Kay A. B. T cells and chronic asthma. *International Archives of Allergy and Immunology* 1999; **118**: 133-135
- Kornerup K. N., Page C. P. The role of platelets in the pathophysiology of asthma. *Platelets* 2007; **18**(5): 319–328

- Korsgaard J., Ledet M. Potential side effects in patients treated with inhaled corticosteroids and longacting b2-agonists. *Respiratory Medicine* 2009; 103 (4) (In Press)
- Kuipers H., Lambrecht B. N. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Current Opinion in Immunology* 2004; 16(6): 702-708
- Kumar A., Sorkness R. L., Kaplan M. R *et al.* Chronic, episodic, reversible airway obstruction after viral bronchiolitis in rats. *American Journal of Respiratory and Critical Care Medicine* 1997; **155**:130-134
- Kumar R. K., Foster P. S. Modeling allergic asthma in mice: pitfalls and Opportunities. *American Journal of Respiratory Cell and Molecular Biology* 2002; **27**: 267–272
- Kumar R. K., Herbert C., Thomas P. S *et al.* Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *The Journal of pharmacology and experimental therapeutics* 2003; **307**:349-55
- Kuperman D. A., Huang X., Koth L. L *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nature Medicine* 2002; **8**: 885-889
- Lampinen M., Carlson M., Hakansson L. D *et al.* Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* 2004; **59**: 793–805

- Lands A. M., Luduena F. P., Buzzo H. J. Differentiation of receptors responsive to isoproterenol. *Life Science* 1967; 6(21): 2241–2249
- Lane S. J. Leukotriene antagonism in asthma and rhinitis. *Respiratory Medicine* 1998; 92(6): 795–809
- La'niguez M. A., Pablos J. L., Carreira, P. E *et al.* Detection of COX-1 and COX-2 isoforms in synovial fluid cells from inflammatory joint diseases. *British Journal of Rheumatology* 1998; 37: 773–778
- Larche M. D., Robinson S., Kay A. B. The role of T lymphocytes in the pathogenesis of asthma. *Journal of Allergy and Clinical Immunology* 2003; 3: 450-463
- Larsen S. T., Hansen J. S., Hansen E. W *et al.* Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl) phthalate and ovalbumin in BALB/c mice. *Toxicology* 2007; 3:119-29
- Lazaar A. L., Panettieri R. A. Airway smooth muscle as an immunomodulatory cell: a new target for pharmacotherapy? *Current Opinion in Pharmacology* 2001; 1: 259-264
- Lazarus A. H., Song S., Crow A. R. Understanding platelet functions through signal transduction. *Transfusion Medicine Reviews* 2003; 171: 45-56
- Lee J., Sung H., Jeon C *et al.* A carbohydrate fraction, AIP1 from *Artemisia iwayomogi* suppresses pulmonary eosinophilia and Th2-type cytokine production in an ovalbumin-induced allergic asthma. Down-regulation of

TNF- α expression in the lung. *International Immunopharmacology* 2008; **8**: 117–125

Lee T. M., Lane S. J. The role of macrophages in the mechanisms of airway inflammation in asthma. *The American Review of Respiratory Disease* 1992; **145**: 27–30

Lee Y., Kim S. Immunomodulatory effect of *Juglans sinensis*, *Psoralea corylifolia*, Cheong-a-hwan extract and cyclosporine A on Th1 (IFN- γ)/Th2(IL-4) cytokine balance, eosinophil accumulation in a murine model of asthma. *Phytochemistry Letters* 2008; **1**: 6–10

Leigh R., Ellis R., Wattie J *et al.* Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *American Journal of Respiratory Cell and Molecular Biology* 2002; **27**: 526–535

Lellouch-Tubiana A., Lefort J., Pirotzky E *et al.* Ultrastructural evidence for extravascular platelet recruitment in the lung upon intravenous injection of platelet activating factor (PAF-acether) to guinea-pigs. *British Journal of Experimental Pathology* 1985; **66**: 345–355

Lellouch-Tubiana A., Lefort J., Simon M. T *et al.* Eosinophil recruitment in guinea pig lungs after PAF-acether and allergen administration. Modulation by prostacyclin, platelet depletion, and selective antagonists. *The American Review of Respiratory Disease* 1988; **137**: 948–954

- Leung S. Y., Eynott P., Nath P *et al.* Effects of ciclesonide and fluticasone propionate on allergen-induced airway inflammation and remodeling features. *Journal of Allergy and Clinical Immunology* 2005; **115**: 989-96
- Li X., Wilson J. W. Increased vascularity of the bronchial mucosa in mild asthma. *American Journal of Respiratory and Critical Care Medicine* 1997; **156**: 229-233
- Little J. W., Falace D. A., Miller C. S *et al.* editors: Pulmonary disease in dental management of the medically compromised patient. 5th ed. St Louis: CV Mosby; 1997; 245-251
- Liu B., Gao H. M., Wang J. Y *et al.* Role of nitric oxide in inflammation-mediated neurodegeneration. *Annals of the New York Academy of Sciences* 2002; **962**: 318–331
- Lopes L., Godoy L. M. F., de Oliveira C. C *et al.* Phagocytosis, endosomal/lysosomal system and other cellular aspects of macrophage activation by Canova medication. *Micron* 2006; **37**: 277-287
- Lundgren R., Soderberg M., Horstedt P *et al.* Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *European Respiratory Journal* 1998; **1**: 883-889
- Lux R., Awa W., Walter U. An interdisciplinary analysis of sex and gender in relation to the pathogenesis of bronchial asthma. *Respiratory medicine* 2009; **103**: 637-649

- Marketos S. G., Ballas C. N. Bronchial asthma in the medical literature of Greek antiquity. *The Journal of Asthma* 1982; 19(4): 263-9
- Marone G., Granata F., Spadaro G *et al.* Anti-inflammatory effects of oxatomide. *Journal of Investigational Allergology and Clinical Immunology* 1999; 9(4): 207-214
- Marone G., Triggiani M., de Paulis A. Mast cells and basophils: friends as well as foes in bronchial asthma? *Trends in Immunology* 2005; **26**: 25-31
- Martin J. G., Ramos-Barbon D. Airway smooth muscle growth from the perspective of animal models. *Respiratory Physiology and Neurobiology* 2003; **137**: 251-261
- Mauad T., Bel E. H., Sterk P. J. Asthma therapy and airway remodeling. *Journal of Allergy and Clinical Immunology* 2007; 120(5): 997-1009
- McFadden E. R. Asthma. In: Fauci AS, Braunwaldt E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, *et al.*, editors. Harrison's principles of internal medicine. 14th ed. New York: McGraw-Hill; 1998; 1419-1426
- McIvor R., Pizzichini E., Turner M. O *et al.* Potential masking effects of salmeterol on airway inflammation in asthma. *American Journal of Respiratory and Critical Care Medicine* 1998; 158(3): 924–930
- McKay S., Bromhaar M.M., de Jongste J.C *et al.* Pro-inflammatory cytokines induce c-fos expression followed by IL-6 release in human airway smooth muscle cells. *Mediators of Inflammation* 2001; **10**: 135 -142

Medzhitov R, Janeway C. Innate immunity. *The New England Journal of Medicine* 2000; **343**: 338-344

Melén E., Kere J., Pershagen G *et al.* Influence of male sex and parental allergic disease on childhood wheezing: role of interactions. *Clinical and Experimental Allergy* 2004; 34(6): 839-844

Melillo E., Woolley K. L., Manning P. J *et al.* Effect of inhaled PGE₂ on exercise-induced bronchoconstriction in asthmatic subjects. *American Journal of Respiratory and Critical Care Medicine* 1994; **149**:1138–1141

Metz M., Grimaldeston M. A., Nakae S *et al.* Mast cells in the promotion and limitation of chronic inflammation. *Immunological Reviews* 2007; **217**:0020304–328

Metzger R. J., Klein O. D., Martin G. R *et al.* The branching programme of mouse lung development. *Nature* 2008; **453**: 745-751

Nagao K., Akabane H., Masuda T *et al.* Effect of MX-68 on airway inflammation and hyper-responsiveness in mice and guinea pigs. *Journal of Pharmacy and Pharmacology* 2004; 56(2): 187-96

National Asthma Education and Prevention Program. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. *Journal of Allergy and Clinical Immunology* 120: 94–138. *National Centre for Complementary Alternative Medicine*: www.nccam.nih.gov [Accessed: March 2008]

National Heart, Lung and Blood Institute (NHLBI) National Asthma Education and Prevention Program, Expert Panel Report 2. (1997). *Guidelines for the diagnosis and management of asthma*. NIH Publication No. 97-4051. Bethesda, MD: US Department of Health and Human Services

Oberholzer H. M., Pretorius E., Smit E *et al.* Investigating the effect of *Withania somnifera*, Selenium and Hydrocortisone on blood count and bronchial lavage of experimental asthmatic BALB/c mice. *Scandinavian Journal for Laboratory Animal Science* 2008; 35(4): 239-248

O'Donnell S. R. A selective [beta]-adrenoreceptor stimulant (Th1165a) related to orciprenaline. *European Journal of Pharmacology* 1970; 12(1): 35–43

Ogawa Y., Calhoun W. J. The role of leukotrienes in airway inflammation. *Journal of Allergy and Clinical Immunology* 2006; **118**: 789–798

O'Neil B. J., Kline J. A., Burkhart K *et al.* The use of Laboratory Animal Models in Research. *Academic Emergency Medicine* 1999; 6(1): 75-82

Pacher P., Beckman J. S., Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews* 2007; **87**: 315–424

Page C. P. Platelets and asthma. *Agents and Actions. Supplements* 1989; **28**: 75–84

Page S., Ammit A. J., Black J. L *et al.* Human mast cell and airway smooth muscle cell interactions: implications for asthma. *America Journal of Physiology. Lung cellular and molecular physiology* 2001; **281**: 1313-1323

- Palade G. Fine structure of blood capillaries. *Journal of Applied Physiology* 1953 **24**: 1424-1436
- Panettieri R.A. Airway smooth muscle: an immunomodulatory cell. *Journal of Allergy and Clinical Immunology* 2002; **110**: 269-274
- Park H., Lee C., Jung I *et al.* Quercetin regulates Th1/Th2 balance in a murine model of asthma. *International Immunopharmacology* 2009; 9(3): 261-267
- Passalacqua G., Bousquet P. J., Carlsen K *et al.* Systematic review of complementary and alternative medicine for rhinitis and asthma. *Journal of Allergy and Clinical Immunology* 2006; **117**(5): 1054-1062
- Patel H.H., Murray F., Insel, P.A. Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annual Review of Pharmacology and Toxicology* 2008; **48**: 359-391
- Pavord I. D., Wong C. S., Williams J *et al.* Effect of inhaled prostaglandin E2 on allergen-induced asthma. *The American Review of Respiratory Disease* 1993; **148**: 87–90
- Peebles R. S., Hashimoto K., Morrow J. D *et al.* Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. *American Journal of Respiratory and Critical Care Medicine*. 2002; **165**: 1154–1160
- Pennisi E. A mouse chronology. *Science* 2002; **288**: 248-57

- Peppelenbosch M., Boone E., Jones G. E *et al.* Multiple signal transduction pathways regulate TNF-induced actin reorganization in macrophages: inhibition of Cdc42-mediated filopodium formation by TNF. *Journal of Immunology* 1999; **162**: 837-845
- Pereira W. K. V., Lonardoni M. V. C., Grespan R *et al.* Immunomodulatory effect of Canova medication on experimental *Leishmania amazonensis* infection. *Journal of Infection* 2005; **51**: 157-168
- Persson C. G., Erjefalt J. S., Korsgren M *et al.* The mouse trap. *Trends in Pharmacological Sciences* 1997; **18**(12): 465-467
- Peters-Golden M. The alveolar macrophage: the forgotten cell in asthma. *American Journal of Respiratory Cell and Molecular Biology* 2004; **31**: 3-7
- Piemonte M., Buchi D. Analysis of IL-2, IFN- γ and TNF- α production, $\alpha 5\beta 1$ integrins and actin filaments distribution in peritoneal mouse macrophages treated with homeopathic medicament. *Journal of Submicroscopic Cytology and Pathology* 2002; **34**: 255-263
- Pignatti P., Moscato G., Casarini S *et al.* Downmodulation of CXCL8/IL-8 receptors on neutrophils after recruitment in the airways. *Journal of Allergy and Clinical Immunology* 2005; **115**: 88
- Pitchford S. C. Defining a role for platelets in allergic inflammation. *Biochemical Society Transactions* 2007; **35**(5): 1104 - 1108

- Pitchford S. C., Momi S., Baglioni S *et al.* Allergen induces the migration of platelets to lung tissue in allergic asthma. *American Journal of Respiratory and Critical Care Medicine* 2008; **77**: 604 – 612
- Pitchford S. C., Yano H., Lever R *et al.* Platelets are essential for leukocyte recruitment in allergic inflammation. *Journal of Allergy and Clinical Immunology* 2003; **112**: 109–118
- Pizzichini E., Pizzichini M.M.M., Efthimiadis A *et al.* Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize contamination. *European Respiratory Journal* 1996; **9**:1174-1180
- Pretorius E., Briedenhann S., Marx J *et al.* Structural changes in the fibrin network of a Pretoria family with dysfibrinogenaemia: a scanning electron microscopical study. *Ultrastructural Pathology* 2006; **30**:167-76
- Pretorius E., Ekpo O. E., Smit E. Comparative ultrastructural analysis of platelets and fibrin networks using the murine model of asthma. *Experimental and Toxicologic Pathology* 2007a; **59**: 105-114
- Pretorius E., Humphries P., Ekpo O.E *et al.* Comparative Ultrastructural Analyses of Mouse, Rabbit and Human Platelets and Fibrin Networks. *Microscopy Research and Technique* 2007b. **70**: 823 - 827
- Pretorius E., Oberholzer H. M., Smit, E *et al.* Ultrastructural changes in platelet aggregates of HIV patients: A scanning electron microscopy study. *Ultrastructural pathology* 2008; **32**: 1-5

- Prussin C., Metcalfe D. D. IgE, mast cells, basophils, and eosinophils. *Journal of Allergy and Clinical Immunology* 2006; **117**: 450-456
- Renauld J. C. New insights into the role of cytokines in asthma. *Journal of Clinical Pathology* 2001; **54**: 577-589
- Retana-Ma´rquez S., Bonilla-Jaime H., Va´zquez-Palacios G *et al.* Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats. *Psychoneuroendocrinology* 2003; **28**: 207–227
- Ricciardolo F. L., Sterk P. J., Gaston B *et al.* Nitric oxide in health and disease of the respiratory system. *Physiological Reviews* 2004; **84**(3): 731-765
- Roberto J., Barrios M. D., Farrah Kheradmand M. D *et al.* Asthma: Pathology and Pathophysiology. *Archives of Pathology and Laboratory Medicine* 2005; **130** (4): 447–451
- Robbins R. A., Barnes P. J., Springall D. R *et al.* Expression of inducible nitric oxide synthase in human bronchial epithelial cells. *Biochemical and Biophysical Research Communications* 1994; **203**: 209–218
- Robinson D. S. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *Journal of Allergy and Clinical Immunology* 2004; **114**(1): 58-65
- Roche W. R., Beasley R., Williams J. H *et al.* Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; **1**: 520-524

- Rogers D. F., Barnes P. J. Treatment of airway mucus hypersecretion. *Annals of Medicine* 2006; **38**: 116-25
- Rosenblatt M. B. History of bronchial asthma. In: E.B.Weiss, M.S. Segal, & M. Stein, Eds. *Bronchial asthma: Mechanisms and therapeutics*, 2nd ed. Boston, MA: Little Brown, 1976; 5-17
- Rosner F. Moses Maimonides' treatise on asthma. *Thorax* 1981; **36**: 245-251
- Ross M. H., Kaye G. I., Pawlina W. Histology: A text and atlas. Lippincott Williams and Wilkins 2003. Fourth Edition; 583
- Roth M., Johnson P. R., Borger P *et al.* Dysfunctional interaction of C_εEBP α and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *The New England Journal of Medicine* 2004; **351**: 560-574
- Rout G. R., Samantaray S., Das P. *In vitro* somatic embryogenesis from callus cultures of *Cephaelis ipecacuanha* A. Richard. *Scientia horticultrae* 2000; **86**: 71-79
- Russo C., Polosa R. TNF- α as a promising therapeutic target in chronic asthma: a lesson from rheumatoid arthritis. *Clinical Science* 2005; **109**: 135-142
- Saleem M., Alam A., Sultana S. Asafoetida inhibits early events of carcinogenesis, a chemopreventive study. *Life Science* 2001; **68**: 1913-1921
- Sampson A.P. The role of eosinophils and neutrophils in inflammation. *Clinical and Experimental Allergy* 2000; **30**: 22–27

- Santos A. L., Perazzo F. F., Cardoso L. G. V *et al.* *In vivo* study of the anti-inflammatory effect of *Rhus toxicodendron*. *Homeopathy* 2007; **96**: 95-101
- Sasaki M. 2001. Randomised Placebo- controlled clinical trial to appraise the effectiveness and safety of immunomodulator Canova in the therapeutics of patients who have HIV/Aids on anti-retroviral use. PhD. Federal University of Paraná
- Sato D., Wal R., de Oliveira C. C., *et al.* Histopathological and immunophenotyping studies on normal and sarcoma 180-bearing mice treated with a complex homeopathic medication. *Homeopathy* 2005; **94**: 26-32
- Schmidt M., Sun G., Stacey M. A *et al.* Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *Journal of Immunology* 2003; **171**: 380-389
- Sears M. R., Lotvall J. Past, present and future—[beta]2- adrenoceptor agonists in asthma management. *Respiratory Medicine* 2005; 99(2): 152–170
- Sears M. R., Taylor D. R., Print C. G *et al.* Regular inhaled beta-agonist treatment in bronchial asthma. *Lancet* 1990; 336(**8728**): 1391–1396
- Seligmann I. C., Lima P. D. L., Cardoso P. C. S *et al.* The Canova Method homeopathic compound is not genotoxic to the *in vitro* human lymphocytes. *Genetics and Molecular Research* 2003; **2**: 223-228
- Selroos O., Pietinalho A., Lofroos A. B *et al.* Effect of early vs late intervention with inhaled corticosteroids in asthma. *Chest* 1995; **108**: 1228-1234

- Shore S. A. Obesity and asthma: cause for concern. *Current Opinion in Pharmacology* 2006; 6(3): 230-236
- Smit E., Pretorius E., Anderson R., Oomen J., Potjo M. Differentiation of human monocytes in vitro following exposure to Canova in the absence of cytokines. *Ultrastructural Pathology* 2008; **32**: 147–152
- Smit J. J., Lukacs N. W. A closer look at chemokines and their role in asthmatic responses. *European Journal of Pharmacology* 2006; **533**: 277-288
- Snibson K. J., Bischof R. J., Slocombe R. F *et al.* Airway remodelling and inflammation in sheep lungs after chronic airway challenge with house dust mite. *Clinical and Experimental Allergy* 2005; **35**: 146-152
- Sollecito T. P., Tino G. Asthma. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* 2001; **92**: 485-490
- Solis-Cohen S. The use of adrenal substances in the treatment of asthma. *Journal of the American Medical Association* 1900; **34**: 1164–6
- Spiteri M. A., Knight R. A., Jeremy J. Y *et al.* Alveolar macrophage-induced suppression of peripheral blood mononuclear cell responsiveness is reversed by *in vitro* allergen exposure in bronchial asthma. *European Respiratory Journal* 1994; **7**: 1431–1438

- Steinbacher D. M., Glick M. The dental patient with asthma: An update and oral health considerations. *Journal of the American Dental Association* 2001; **132**: 1229-1239
- Stewart A. G., Fernandes D., Tomlinson P. R. The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. *British Journal of Pharmacology* 1995; **116**: 3219-3226
- Stroparo E. 2005. HIV/Aids patients treated with the homeopathic medicine Canova: Prospective observational study in laboratory, clinical and of quality of life indexes. MSc. Ferderal University of Paraná
- Sumi Y., Hamid Q. Airway remodelling in asthma. *Allergology International* 2007; **56**: 341-348
- Sutherland T. J. T., Cowan J. O., Young S *et al.* The association between obesity and asthma: interactions between systemic and airway inflammation. *American Journal of Respiratory and Critical Care Medicine* 2008; **178**: 469-475
- Tanaka H., Yamada G., Saikai T *et al.* Increased airway vascularity in newly diagnosed asthma using a highmagnification bronchovideoscope. *American Journal of Respiratory and Critical Care Medicine* 2003; **168**: 1495-1499
- Tasota F. J. Patients with chronic obstructive pulmonary diseases: In: Breu C., Cardin S., Clochesy J. M *et al.* 1996. *Critical care nursing*. 2nd ed. Boston MA: Little Brown

- Teale C., Morrison J. F., Muers M. F *et al.* Response to nebulized ipratropium bromide and terbutaline in acute severe asthma. *Respiratory Medicine* 1992; 86(3): 215–8
- Tillie-Leblond I., Gosset P., Tonnel A. B. Inflammatory events in severe acute asthma. *Allergy* 2005; 1: 23–29
- Tomioka S., Bates J. H., Irvin C. G. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *Journal of Applied Physiology* 2002; 93: 263–270
- Torre D., Pugliese A., Speranza F. Role of nitric oxide in HIV-1 infection: Friend or foe? *Lancet Infectious Diseases* 2002; 2: 273–280
- Trautmann A., Schmid-Grendelmeier P., Kruger K *et al.* Tcells and eosinophils cooperate in the induction of bronchial cell apoptosis in asthma. *Journal of Allergy and Clinical Immunology* 2002; 109: 329-337
- Travis W. D., Rush W., Flieder D. B *et al.* Non-neoplastic disorders of the lower respiratory tract. *American Registry of Pathology* Washington DC 2002; 101-111
- Tulic M. K., Christodouloupoulos P., Hamid Q. Small airway inflammation in asthma. *Respiratory Research* 2001; 2: 333–339
- Udenigwe C. C., Lin Y. S., Hou W. C *et al.* Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods* 2009; (In Press)

- Uller L., Lloyd C. M., Rydell-Tormanen K., *et al.* Effects of steroid treatment on lung CC chemokines, apoptosis and transepithelial cell clearance during development and resolution of allergic airway inflammation. *Clinical and Experimental Allergy* 2006; 36(1): 111-121
- Uller L., Mathiesen J. M., Alenmyr L *et al.* Antagonism of the prostaglandin D2 receptor CRTH2 attenuates asthma pathology in mouse eosinophilic airway inflammation. *Respiratory Research* 2007; 8: 16
- Vancheri C., Gili E., Failla M *et al.* Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor. *Journal of Allergy and Clinical Immunology* 2005; **116**: 1242-1248
- Vargaftig B. B., Braquet P. G. PAF-acether today—relevance for acute experimental anaphylaxis. *British Medical Bulletin* 1987; **43**: 312–335
- Varin A., Gordon S. Alternative activation of macrophages: Immune function and cellular biology. *Immunobiology* 2009; 214 (7): 630-641
- Venables M. K, Chan-Yeung M. Occupational asthma. *Lancet* 1997; **349**:1465–1469
- Venkatesha R.T., Berla Thangam E., Zaidi A.K *et al.* Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase. *Molecular Immunology* 2005; **42**: 581-587

- Vermeer P. D., Harson R., Einwalter L. A *et al.* Interleukin-9 induces goblet cell hyperplasia during repair of human airway epithelia. *American journal of respiratory cell and molecular biology* 2003; **28**: 286-295
- Verstraelen S., Bloemen K., Nelissen I *et al.* Cell types involved in allergic asthma and their use in *in vitro* models to assess respiratory sensitization. *Toxicology in Vitro* 2008a; **22**: 1419–1431
- Verstraelen S., Wens B., Hooyberghs J *et al.* Gene expression profiling of *in vitro* cultured macrophages after exposure to the respiratory sensitizer hexamethylene diisocyanate. *Toxicology In Vitro* 2008b; **22**: 1107–1114
- Vliagoftis H., Befus A. D. Mast cells at mucosal frontiers. *Current Molecular Medicine* 2005; **5**: 573–589
- Wagers S. S., Norton R. J., Rinaldi L. M *et al.* Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *Journal of Clinical Investigation* 2004; **114**(1): 104–111
- Walker C., Kaegi M. K., Braun P *et al.* Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *Journal of Allergy and Clinical Immunology* 1991; **88**: 935–942
- Ward C., Walters H. Airway wall remodelling: the influence of corticosteroids. *Current Opinion in Allergy and Clinical Immunology* 2005; **5**(1): 43–48

- Waterston R. H., Lindblad-Toh K., Birney E *et al.* Initial sequencing and comparative analysis of the mouse genome. *Nature* 2002; **420**: 520-562
- Wechsler M. E., Lehman E., Lazarus S. C *et al.* beta-Adrenergic receptor polymorphisms and response to salmeterol. *American Journal of Respiratory and Critical Care Medicine* 2006; 173(5): 519–526
- Weiss S. T., Shore S. Obesity and asthma: directions for research. *American Journal of Respiratory and Critical Care Medicine* 2004; **169**: 963–968
- Wenzel S. E. Asthma as an inflammatory disease. *Annals of Allergy* 1994; **72**: 261-271
- Wenzel S. Mechanisms of severe asthma. *Clinical and Experimental Allergy* 2003; **33**(12): 1622-1628
- White S. R. Epithelium as a target. In: Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ (eds). *Asthma*. Philadelphia: Lippencott-Raven, 1997; 875-900
- Willis-Owen S. A. G., Valdar W. Deciphering gene-environment interactions through mouse models of allergic asthma. *American Academy for Allergy Asthma and Immunology* 2009; **1**: 14-23
- Wills-Karp M., Chiaramonte M. Interleukin-13 in asthma. *Current opinion in pulmonary medicine* 2003; **9**: 21-27

- Wilson J.P., Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clinical and Experimental Allergy* 1997; **27**: 363–371
- Wojciak-Stothard B., Deneyer M., Mishra M *et al.* Adhesion orientation and movement of cells cultured on ultrathin fibronectin fibers in vitro. *Cellular and developmental biology* 1997; **33**: 110-117
- Wolff H., Saukkonen K., Anttila S *et al.* Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Research* 1998; **58**: 4997–5001
- Wright S. D., Ramos R. A., Tobias P. S *et al.* CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; **249**:1431–1433
- Xu J., Sabarinath S. N., Derendorf H. Cortisol suppression as a surrogate marker for inhaled corticosteroid-induced growth retardation in children. *European journal of pharmaceutical sciences* 2009; **36**: 110–121
- Yamada E. The fine structure of the gallbladder epithelium of the mouse. *The Journal of biophysical and biochemical cytology* 1955; **1**: 445-458
- Yang E. J., Lee J., Yun C *et al.* Inhibitory effects of *Duchesnea chrysanth*a extract on ovalbumin-induced lung inflammation in a mouse model of asthma. *Journal of Ethnopharmacology* 2008; **118**: 102–107

Yayoshi-Yamamoto S., Taniuchi I., Watanabe T. FRL, anovelformin-relatedprotein,binds to Rac and regulates cell motility and survival of macrophages. *Molecular and Cellular Biology* 2000; **20**: 6872–6881

Zhang J., Feng W., Li C *et al.* NF-kB regulates the LPS-Induced expression of interleukin 12 p40 in murine peritoneal macrophages: Roles of PKC, PKA, ERK, p38 MAPK, and proteosome. *Cellular Immunology* 2000: 204: 38-45

Zhang X. J., Li Y., Tai G. X *et al.* Effects of activin A on the activities of mouse peritoneal macrophages. *Cellular and Molecular Immunology* 2005; 2: 263–67

Zosky G. R., Sly P. D. Animal models of asthma. *Clinical and Experimental Allergy* 2007; 37(7): 973-988.

www.botanical.com [Accessed: March 2008]