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.

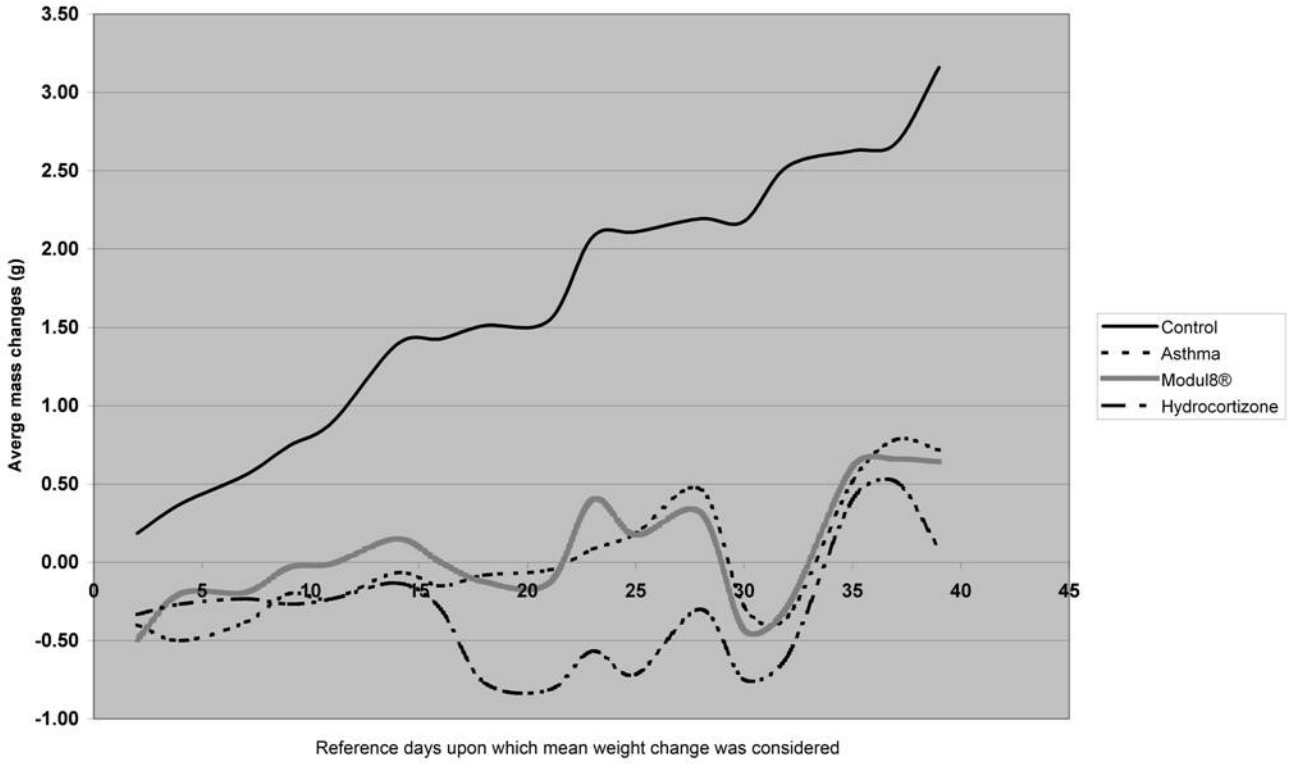
2. *Analysis was preformed in terms of absolute mass changes over time for each mouse within each of the above-specified groups*

- 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 ± std dev)			
Day	**Control* (n=6)**	**Asthma (n=6)**	**Modul8 (n=6)**	**Hydrocortisone (n=6)**
7	0.56 ± 0.13	-0.38 ± 0.54	-0.12 ± 0.54	-0.23 ± 0.46
18	1.51 ± 0.38	-0.08 ± 0.88	-0.08 ± 0.78	-0.77 ± 0.66
21	1.54 ± 0.42	-0.05 ± 0.46	-0.05 ± 0.95	-0.82 ± 0.77
28	2.19 ± 0.39	0.47 ± 0.48	0.37 ± 1.00	-0.30 ± 0.89
35	2.63 ± 0.50	0.52 ± 0.50	0.75 ± 1.07	0.40 ± 0.86
39	3.16 ± 0.72	0.72 ± 0.51	0.8 ± 1.18	0.08 ± 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 there of, 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.

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

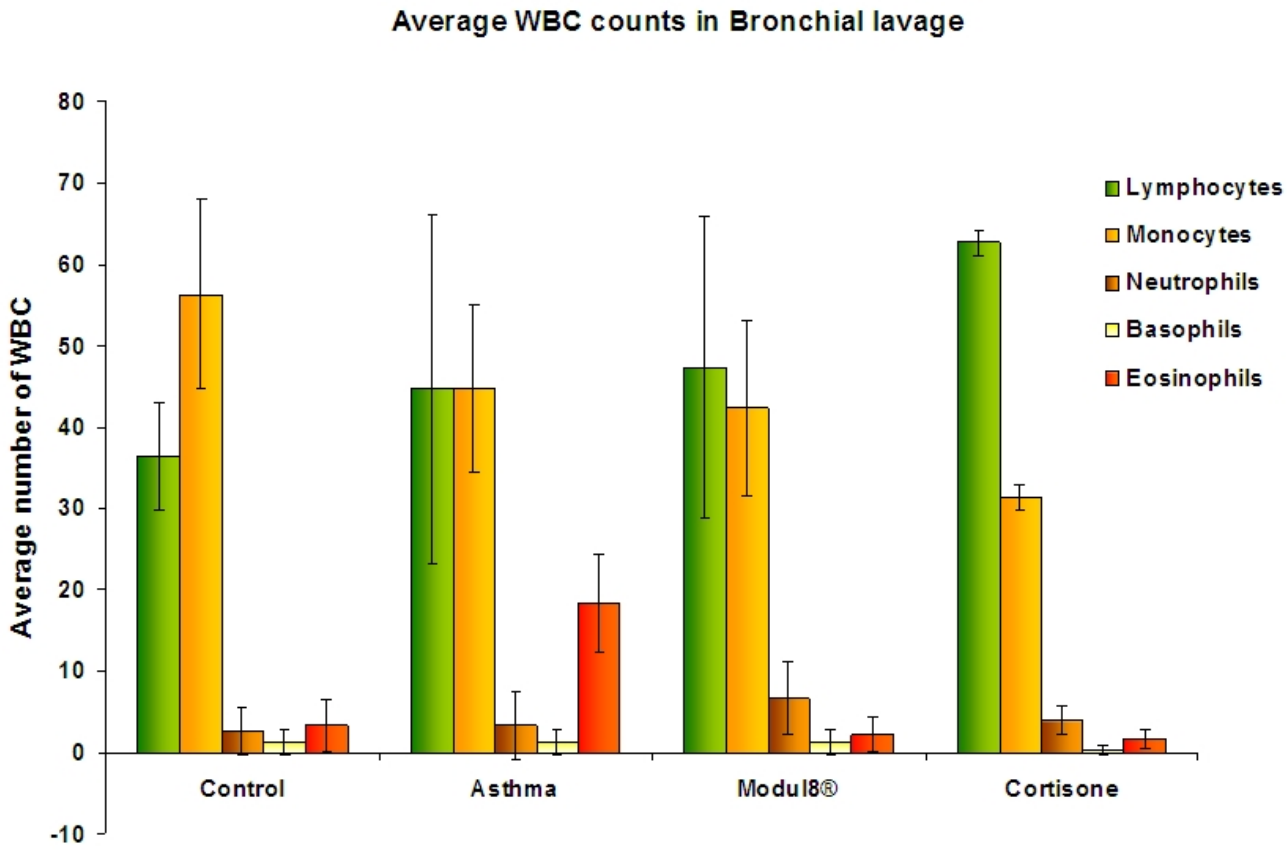
7.3.1 *Bronchial lavage*

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 preformed 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



Graph 7.1: Average number of the white blood cells counted in the bronchial lavage

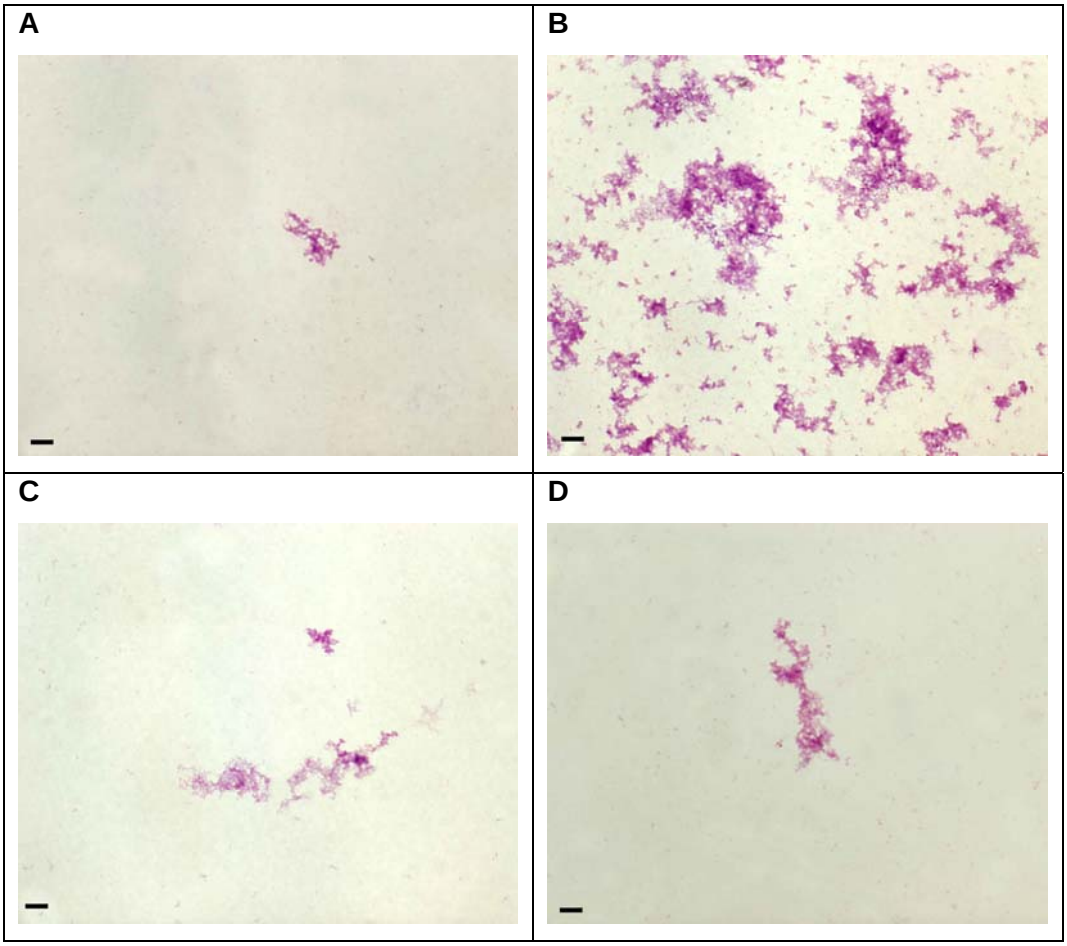


Figure 7.1: Platelet distribution in the bronchial lavage in the four different experimental groups. Control (**A**), Asthma (**B**), Modul8® (**C**), Hydrocortisone (**D**). (Scale bar = 10µm)

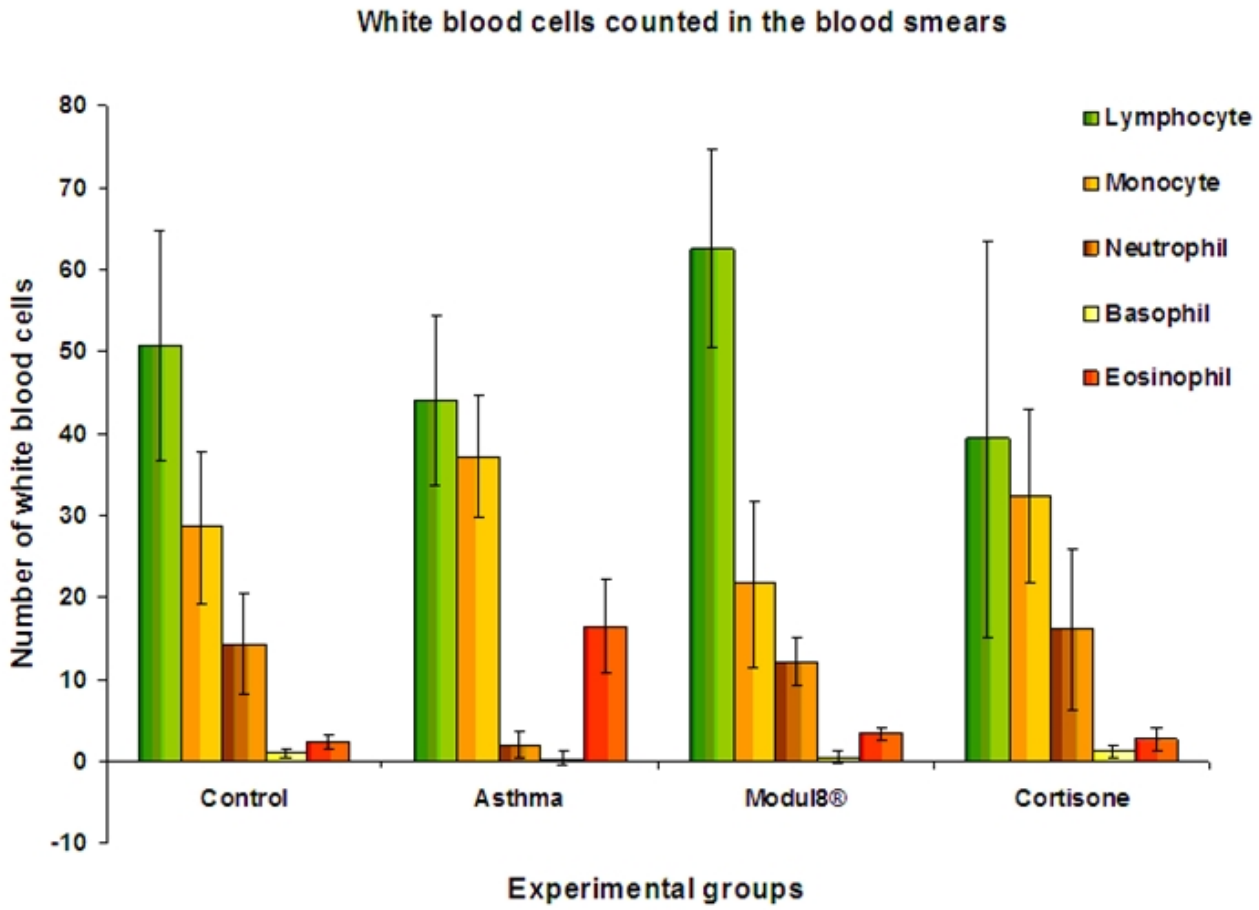
7.3.2 Blood counts

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 preformed 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

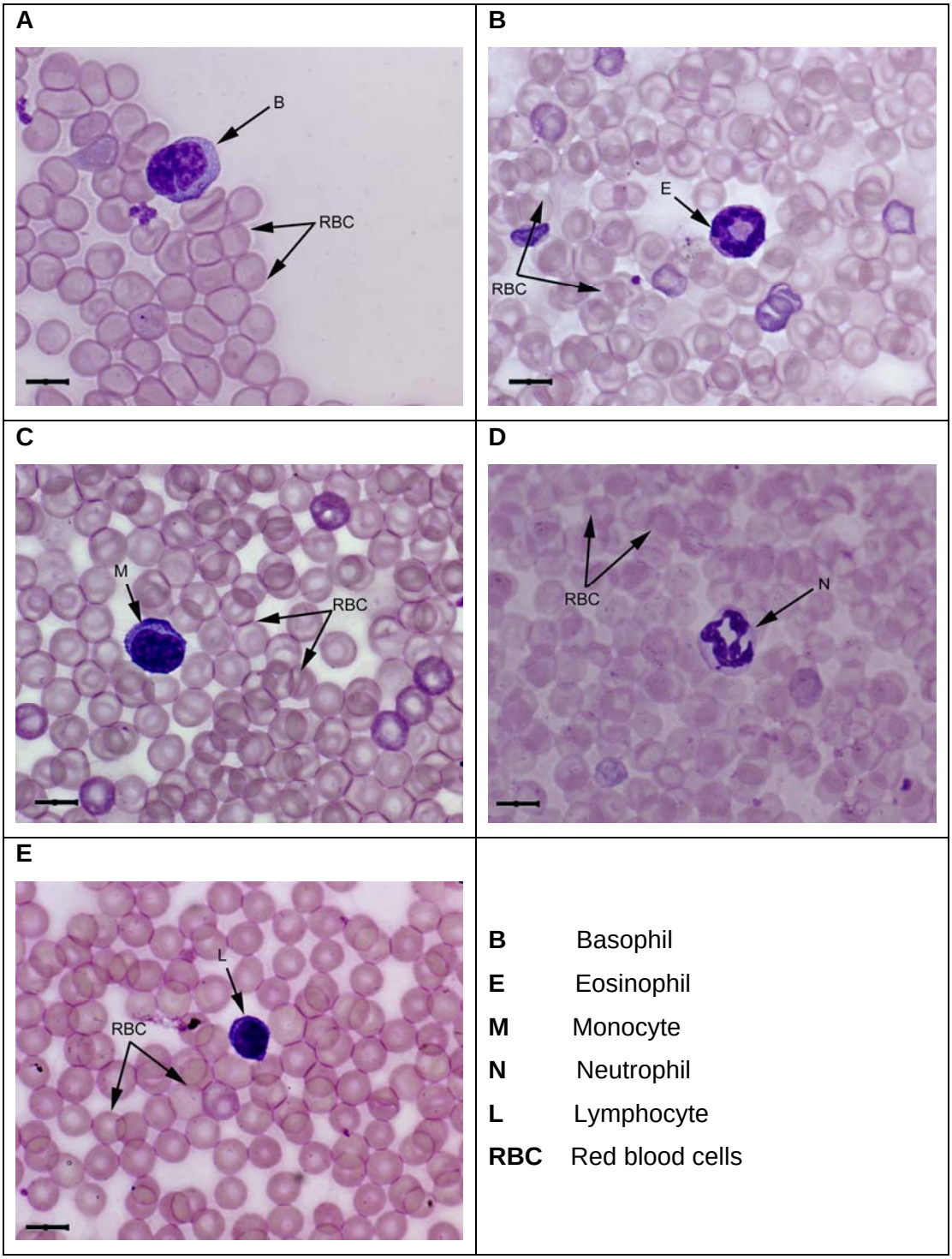


Figure 7.2: White blood cells in the blood smears of BALB/c mice. Basophil (**A**), Eosinophil (**B**), Monocyte (**C**), Neutrophil (**D**), Lymphocyte (**E**). (Scale bar = 20µm)

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 preformed 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 preformed 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 where 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.

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-1β; IL-6; IL-8; IL-12)
- Prostaglandins (PGE_2)
- 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.

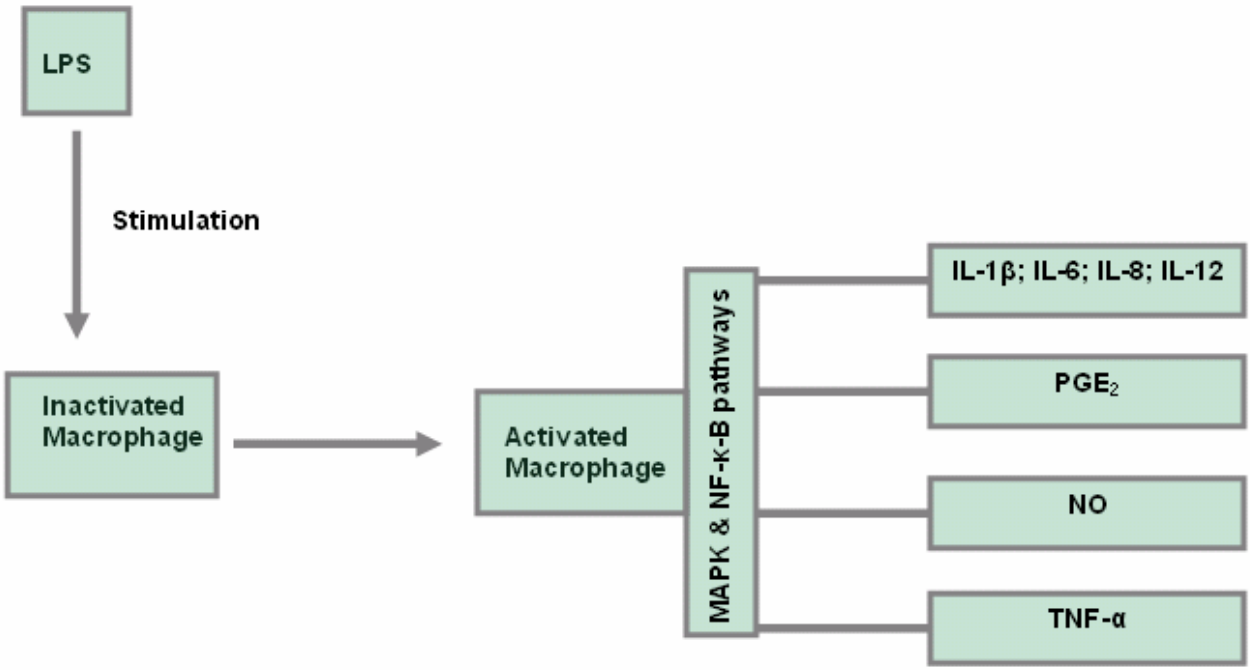


Figure 8.1: Diagram of LPS-stimulated activation of peritoneal macrophages indicating the pathways involved as well as the production of specific mediators (Adams and Hamiltonm, 1984; Kim *et al*., 2004; An *et al*., 2006; Fu *et al*., 2001; Jung *et al*., 2007; Zhang *et al*., 2000; Chao *et al*., 2009).

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 mediates 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 calsium/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 ^-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 that 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 $NaPO_4$ buffer) for 1hr. After the fixation, the macrophages were washed three times in phosphate buffer for 5 minutes each before fixing in osmium tetraoxide 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 tetraoxide (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-napthylethylenediamine dihydrochloride in 5% phosphoric acid). A standard curve was prepared for the assay to ensure accuracy. A dilution series of sodium nitrite starting from 100µl, in medium, of the same composition that will be used to culture the experimental cells was performed. 50µl of the supernatant of each group were transferred to a 96 well plate. A volume of 50µl 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 50µl of 0.1% N-1-napthylethylenediamine 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.

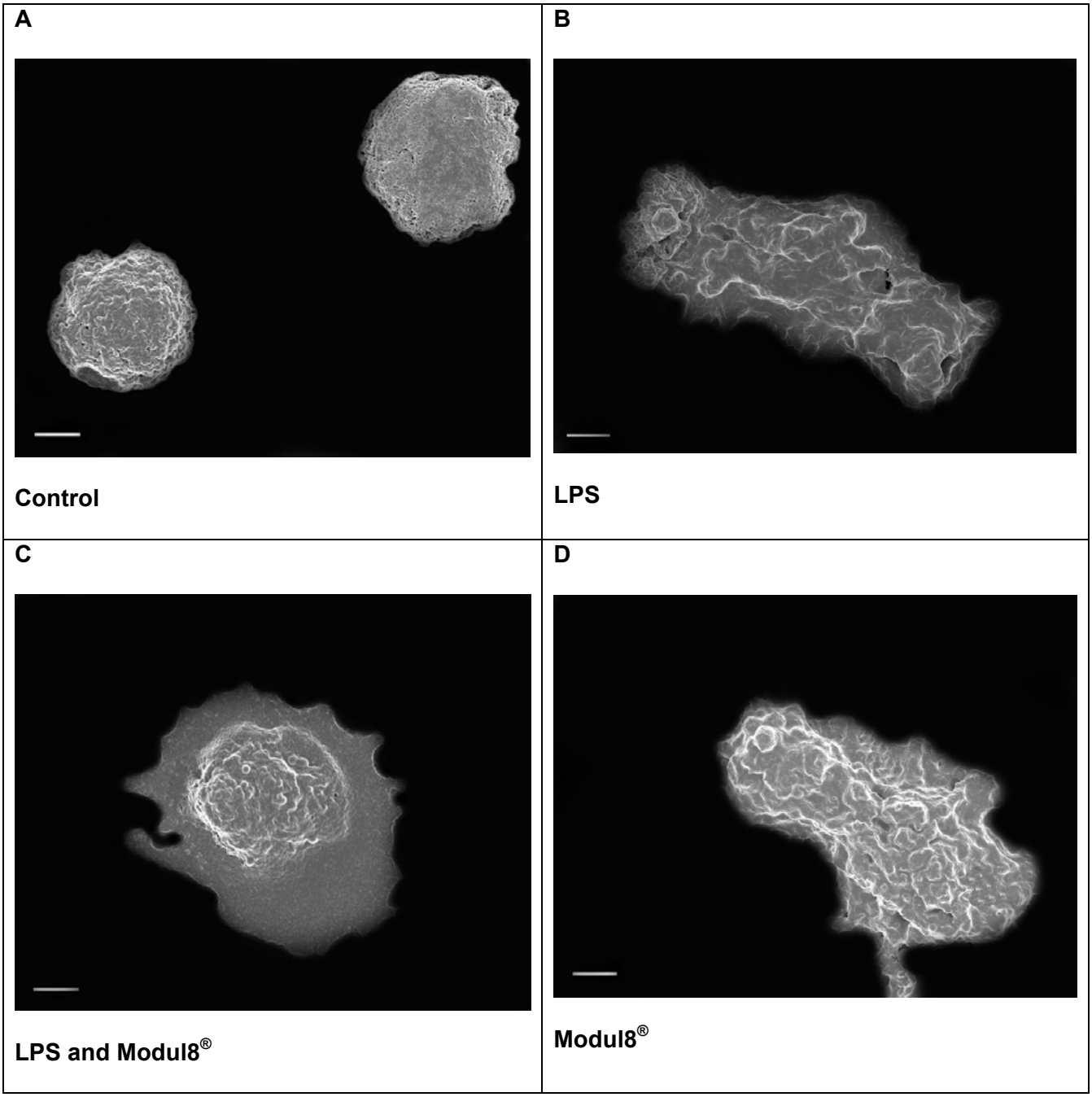


Figure 8.2 Peritoneal macrophages. **A** Control, inactivated macrophage; **B** LPS-stimulated macrophage; **C** Macrophage stimulated with both LPS and Modul8® ; **D** Macrophage stimulated with Modul8® (Scale bar = 2µm)

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 ± SD (µm)
Control	5.75 ± 1.11
LPS	15.27 ± 4.60
LPS/Modul8®	15.50 ± 3.88
Modul8®	17.19 ± 4.87

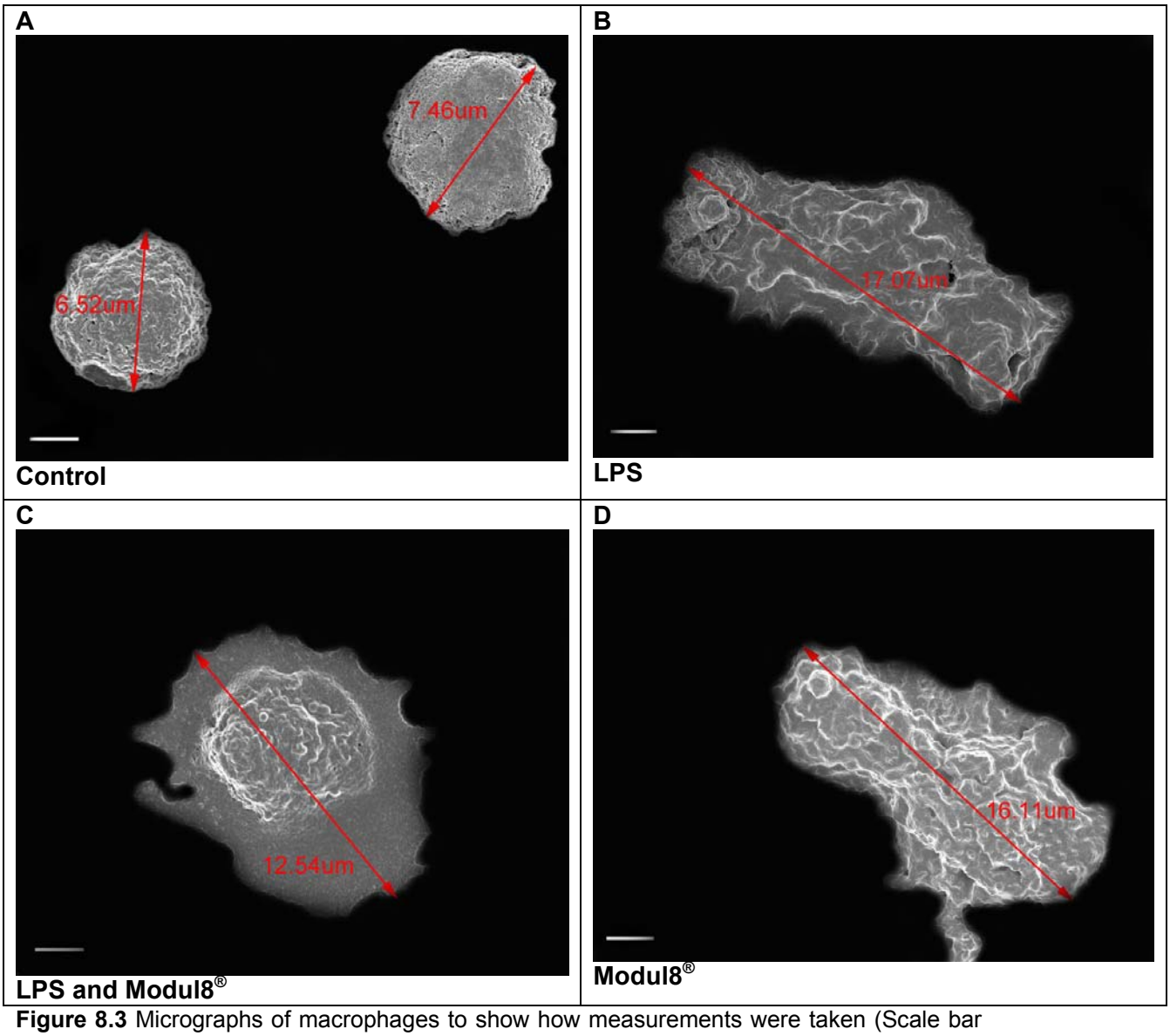
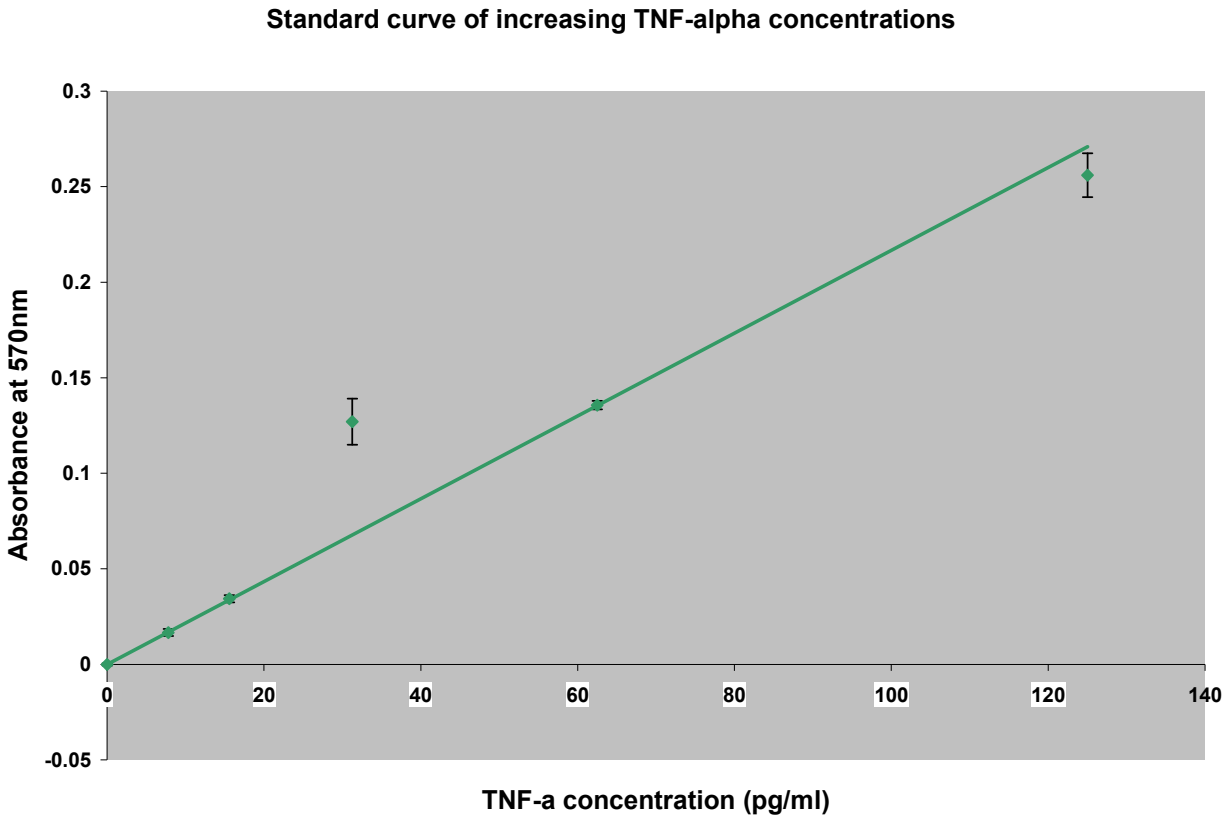


Figure 8.3 Micrographs of macrophages to show how measurements were taken (Scale bar = 2µm)

8.3.3 ELISA



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 IκB kinase nuclear factor-kappa B [(IKK)-NF-κB] 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.