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# Effect of macrophage depletion on asthmatic responses in a cockroach allergen induced murine model

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EFFECT OF MACROPHAGE DEPLETION ON ASTHMATIC RESPONSES IN  
A COCKROACH ALLERGEN INDUCED MURINE MODEL**

by

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B.S, University of Toronto, 2011

Submitted in partial fulfillment of the  
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Master of Arts

2014

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Boston University School of Medicine, 2014

**ABSTRACT**

Asthma is a chronic obstructive pulmonary disease (COPD) which affects 1 in every 12 Americans. Symptoms common to asthmatics include dyspnea, increased mucous production and airway hyperresponsiveness. While research over the past few decades has mostly established the immunological basis behind asthma, there have not been radical changes in the treatment modalities. It is believed that in many COPDs, alveolar macrophages play a critical role in disease progression. While evolutionarily, alveolar macrophages played a significant part in protecting the individual from harmful allergens, in asthma there may be an inappropriate activation of the alveolar macrophages to proteases such as cockroach allergen (CRA). Studies show that children living in inner cities with cockroach infestation are more likely to develop asthma than those that reside in rural areas with less exposure to cockroach allergens. In exposed individuals, when the alveolar macrophages come in contact with CRA, an immune cascade is initiated which sensitizes the child. Subsequent exposure to such an antigen will induce asthma like symptoms. One possible way of reducing such a response is to reduce the number of

alveolar macrophages thus avoiding the pathological effects. Clodronate liposomes are liposomes that are encapsulated with bisphosphonate clodronate. When a macrophage phagocytoses such a liposome, the result is cellular suicide or apoptosis. In this study, we sensitized a murine model of CRA asthma and then monitored the impact of depleted alveolar macrophages using intratracheal administration of clodronate liposomes. We then studied the effect of this depletion on the recruitment of inflammatory cells such as neutrophils and eosinophils which are primary cellular contributors to the asthmatic response. Our studies show that while clodronate liposomes are effective in alveolar macrophage depletion, the subsequent inflammation through neutrophil recruitment interferes with the study of the delicate milieu of cells in the respiratory epithelium of this murine model.

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## **ABBREVIATIONS**

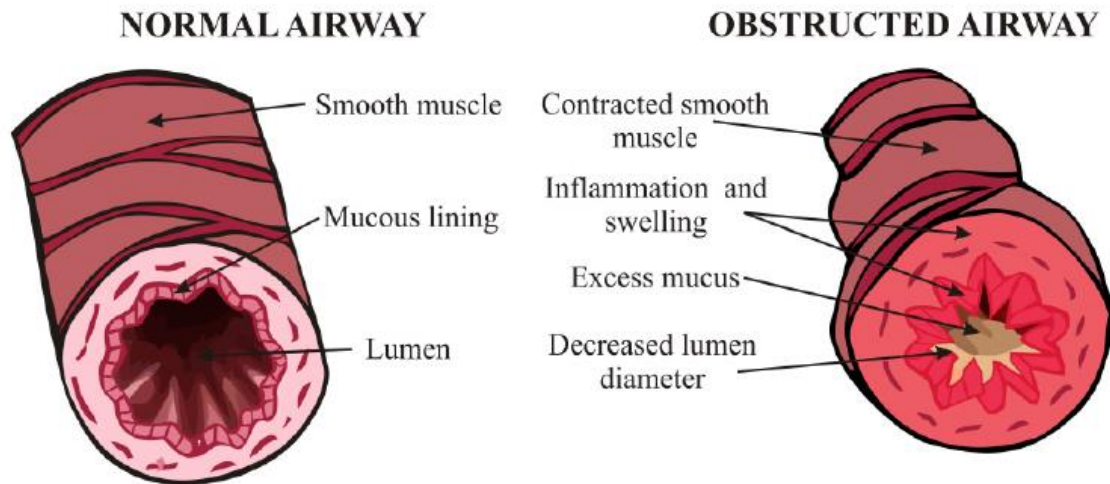
|       |   |
|-------|---|
| AAAAI | American Academy of Allergy Asthma & Immunology |
| COPD  | Chronic Obstructive Pulmonary Disease           |
| CDC   | Centre for Disease Control and Prevention       |
| BAL   | Bronchoalveolar Lavage                          |
| CRA   | Cockroach Antigen                               |
| HBSS  | Hank's Balanced Salt Solution                   |
| PBS   | Phosphate buffered solution                     |

## INTRODUCTION

Asthma is a chronic obstructive lung disease that currently affects 300 million people worldwide (Kudo, Ishigatsubo & Aoki, 2013). According to American Academy of Allergy Asthma and Immunology (AAAAI), in the United States alone, 1 in every 12 individuals are suffering from asthma which accounts for about 8% of the U.S. population (AAAAI, 2010). If the serious health problems caused by asthma are put aside for a moment, there is also an immense socioeconomic burden that accompanies the disease. In the United States, the costs in treating asthmatic patients has gone up from \$53 billion in 2002 to about \$56 billion in 2007 which accounts to a 6% rise in costs (AAAAI, 2010).

Over the past few decades, the advancement in technology and laboratory techniques has allowed scientists to better understand the pathophysiology of asthma. But the treatment modalities used to relieve the symptoms have not experienced the same level of radical advancement. The conventional treatment for asthma still involves the usage of beta-agonists in the case of mild asthma and inhaled steroid therapy in persistent or severe asthma (Busse et al, 2008). While these pharmacologic interventions are effective in relieving asthmatic symptoms and preventing a crisis, they are not a long term cure for this progressive disease. Future therapeutic advancements for the treatment of asthma requires a deeper understanding of the pathogenesis of the disease, so the emerging treatments can focus on affecting the etiology of the disease than trying to stop its progression.

Asthma is a subclass of COPD which involves reversible airway obstruction due to bronchiolar constriction (figure 1). Asthmatics experience increased: smooth muscle contraction, mucous production, airway inflammation and airways hyperresponsiveness which prevent them from performing a normal exhalation. Additional symptoms include wheezing, chest tightness and difficulty breathing. While there is a genetic predisposition in asthmatics, 90% of asthma cases have an identifiable environmental agent (CDC, 2012). According to Dr. Daniel Remick, approximately 50% of asthma patients in the inner city areas are allergic to cockroach allergen (Elissa, 2009). Since asthma is a common illness among children and individuals living in urban areas with low socio-economic index, cockroach infestation has shown to be a significant causative agent for asthma. The contents of cockroach allergen which are thought to activate the innate and adaptive branches of immune system are shown in Table 1. Among these substances, Blag1 and Blag2 are most significant and are found mostly in voids of German cockroach body parts as well as in fecal droppings Field et al, 2013). Blag1 has been shown to cause asthma symptoms in sensitive individuals at concentrations as low as 8U/g. (EMSL Analytical, 2013).



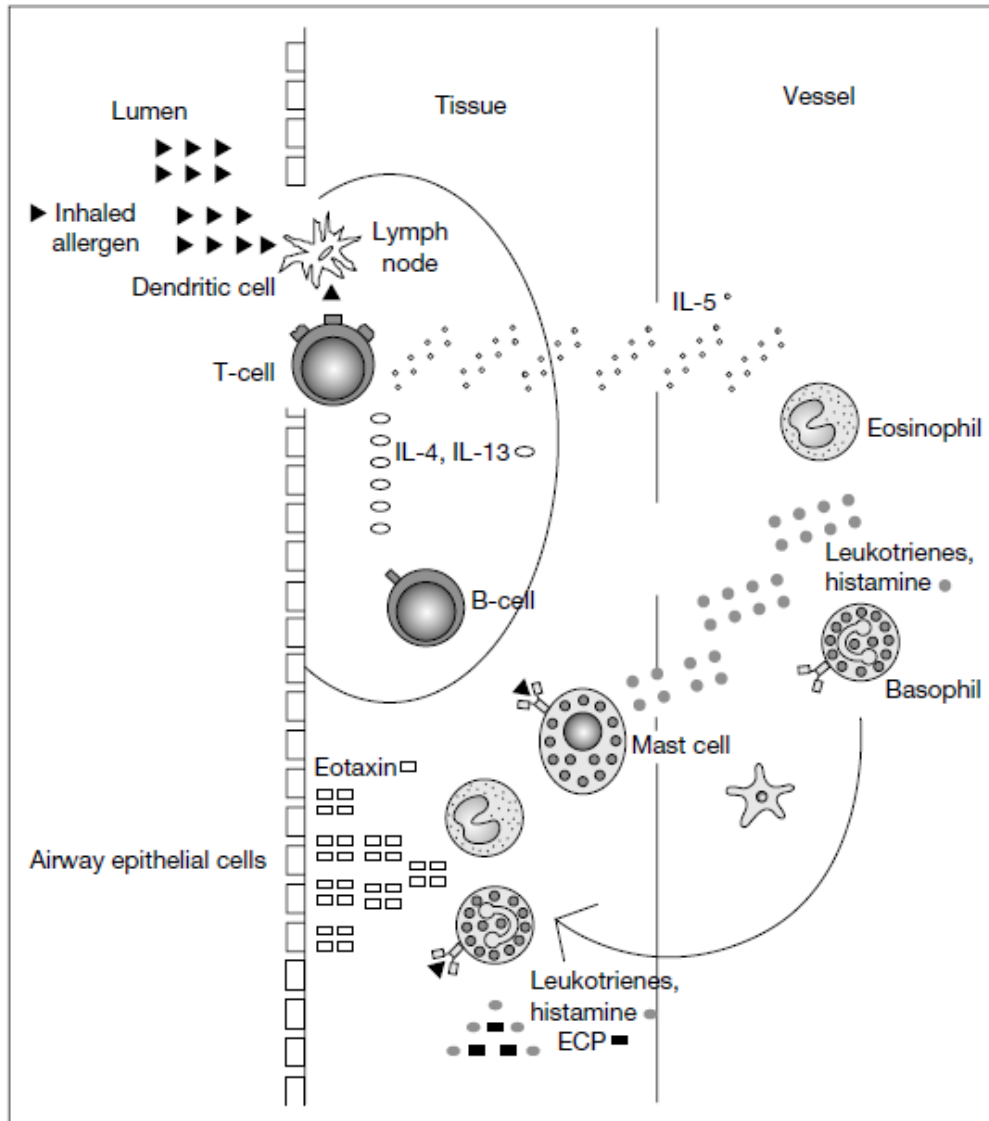
**Figure 1. Bronchiolar obstruction as seen in an acute asthmatic response.** Secondary exposure of sensitized allergen to the respiratory mast cells results in the release of histamine granules which eventually cause smooth muscle constriction in the terminal and respiratory bronchioles of an asthmatic. (Adapted from Bronchial Asthma-Emerging Therapeutic Strategies, Elizabeth Sapey, 2012)

| Component    | Biological Action   | Innate/ Adaptive |
|--------------|---|------------------|
| Blag1/ Blag2 | Protein antigen   | Adaptive         |
| LPS          | TLR4/CD14/MD2 Ligand  | Innate           |
| Chitin       | Macrophage mannose receptor, TLR2, leukotriene B4 receptor ligand | Innate           |
| Other        | Proteases   | Both             |

**Table 1. Biological composition of cockroach allergen as obtained from *Blatella germanica*.** Considering the myriad of substances present in CRA which could possibly induce an acute immune response in asthmatics, Blag1 and Blag2 are the most potent.

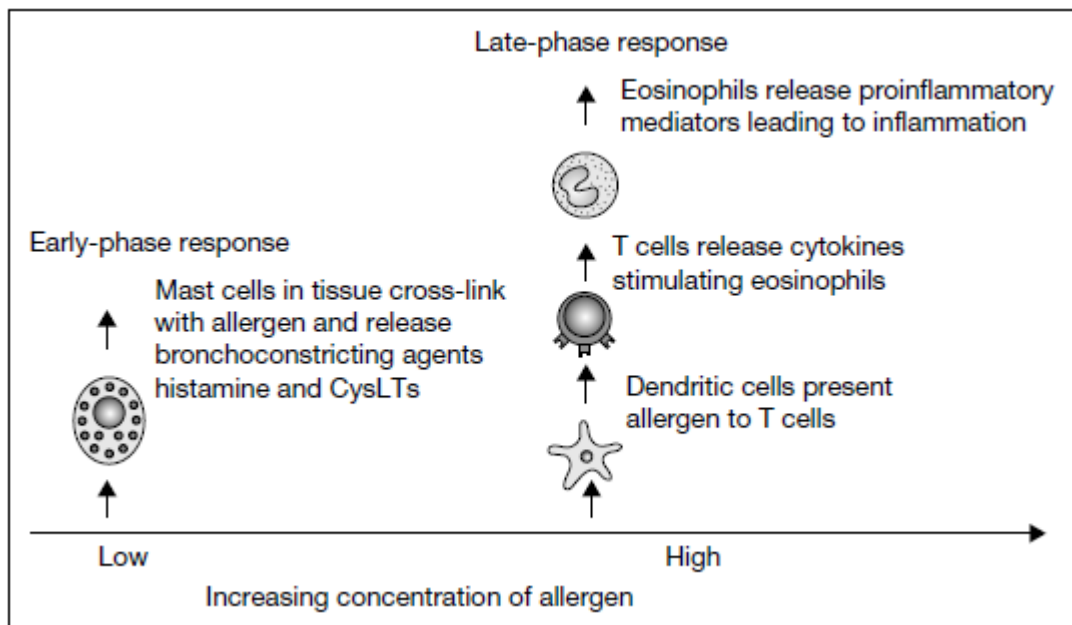
While there is locus heterogeneity in the extent and severity of asthma in genetically susceptible individuals, it has been established that the sensitization and progression of the disease follows a similar pattern of immunopathogenesis (figure 2). When a predisposed individual inhales a novel allergen such as cockroach allergen, the dendritic cells and macrophages present in the lumen of the respiratory tract interact with the allergen. These antigen presenting cells with the antigen-receptor complex can then travel to the local lymph nodes where they present the antigen to a CD4+ T lymphocyte. If the T lymphocyte recognizes the antigen as a foreign particle which could compromise the immune integrity, it undergoes clonal expansion and production of Th2 type cytokines IL-4, IL-5, IL-6, IL-9 and IL-13. Cytokines IL-4 and IL-13 activate the immature B cells in the lymph nodes to clonally expand and produce IgE antibodies specific to the inhaled allergen. The resulting IgE antibodies travel back to the respiratory lumen and bind to the receptors on the membrane of mast cells. Secondary exposure to the sensitizing antigen results in binding of the offending agent to IgE antibodies, subsequent crosslinking of IgE receptors followed by mast cell degranulation releasing histamine. Histamine causes an increase in vascular permeability at the post-capillary venules resulting in extravasation of leukocytes and proteins to engage the offending pathogen. IL-5 is another important Th2 cytokine required for eosinophilic proliferation. Eosinophils are key cells in propagating the asthmatic response. They produce monocyte chemotactic factors such as eotaxin-1 (CCL11) and eotaxin-2 (CCL24) which could then indirectly cause a rise in the alveolar macrophage population. Eosinophils also play a major role in causing airway hyperresponsiveness and mucous production during an

asthmatic response (Kay, 2005). The charcot-laden crystals commonly seen in the sputum of asthmatics consists of the eosinophil basic protein produced by eosinophils.



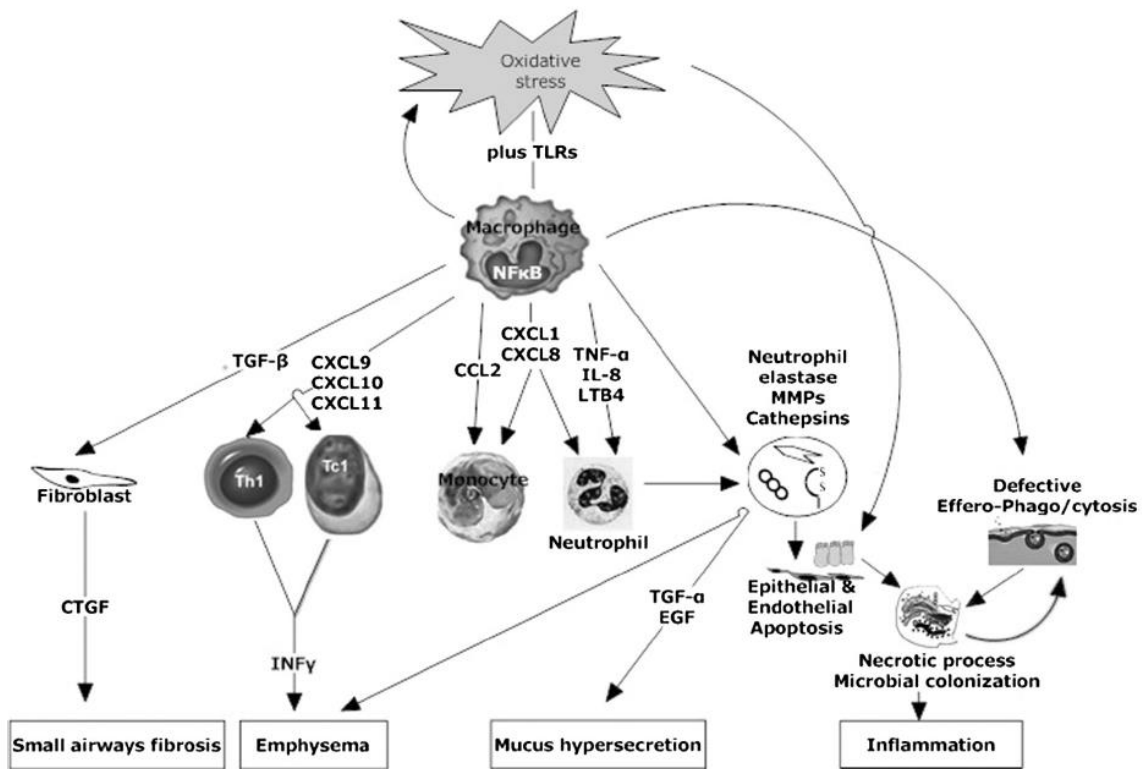
**Figure 2: Sensitization to inhaled allergen in asthmatics.** Exposure of an inhaled antigen to the innate immune cells of the respiratory tract result in clonal expansion of antigen specific B cells and production of IgE antibodies which bind to mast cells and equip the individual to future exposure to the sensitized allergen. (Adapted from Models of Exacerbations in Asthma and COPD, A.Schmidt and H.Herwald, 2007)

Based on the amount of allergen exposure, the sensitized individual can experience acute symptoms followed by a late-phase (figure 3). The early-phase of asthmatic response involves cross-linking of IgE receptors on mast cells resulting in mast cell degranulation and histamine release resulting in acute bronchoconstriction. This early phase of response could then be followed by a late-phase where-in the resident macrophages and dendritic cells can then recruit pro-inflammatory mediators and cells such as eosinophils and neutrophils to the affected area.



**Figure 3. Phases of asthmatic response.** Exposure to sensitized allergen in low concentrations induces the early-phase response which consists of mast cell degranulation and resulting bronchoconstriction. Chronic exposure to high concentrations of the allergen initiates a late-phase response where the resident dendritic cells and macrophages present the antigen to CD4+ T helper cells which recruit eosinophils resulting in prolonged inflammation. (Adapted from Models of Exacerbations in Asthma and COPD, A.Schmidt, H.Herwald, 2007)

In most cases of COPDs such as asthma, emphysema and chronic bronchitis, there is increasing evidence that the alveolar macrophages are the driving cells in inflammation through the release of inflammatory cytokines which attract monocytes, neutrophils and T cells to the affected area (figure 4). Evolutionarily, the alveolar macrophages do have a protective role against agents invading the respiratory epithelium, in asthma there is an inappropriate activation of these alveolar macrophages against antigens like the cockroach allergen. This unwarranted activation of the immune system is no longer protective in nature but is harmful for the survival of the affected individual. Given this critical role of macrophages in immune related diseases, if the macrophage population involved in immune related diseases is effectively reduced, it is possible to limit the pathology. One such compound used to induce various tissue macrophages to commit suicide is bisphosphonate clodronate. Studies have shown that liposomes encapsulating bisphosphonate clodronate are capable of causing apoptosis of tissue specific macrophages such as Kupfer cells in the liver, splenic macrophages in the spleen and alveolar macrophages in the lungs (Rooijen & van Kesteren-Hendrikx, 2003).



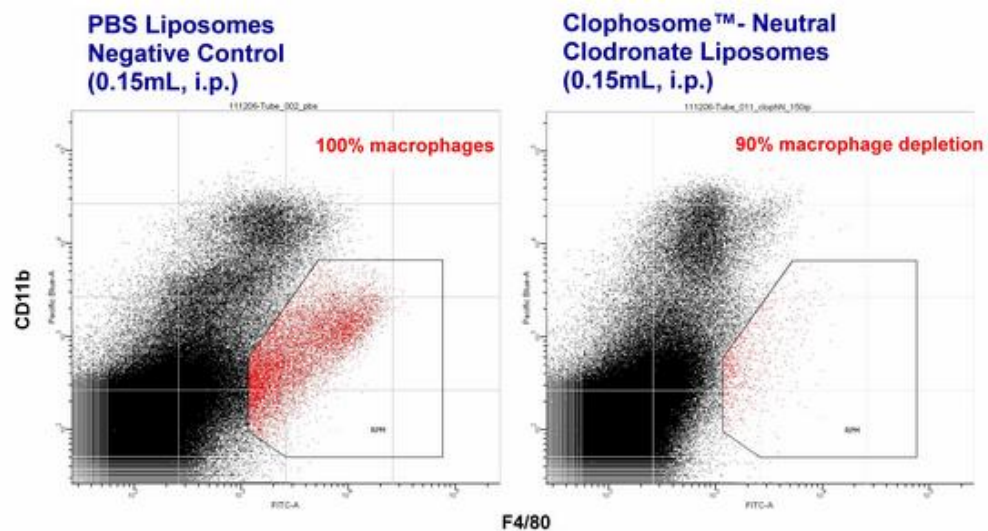
**Figure 4. Immunological role of macrophages in inducing an asthmatic response.**

This figure shows an overview of the critical role that macrophages play in asthma. Through the release of cytokines and chemokines, macrophages activate a myriad of potent immune cells which propagate the asthmatic response. The resulting phenotype of such an activation includes bronchoconstriction, airway fibrosis, inflammation and mucus hypersecretion. (Adapted from Pappas et al, 2013)

## OBJECTIVES

Considering the critical role that alveolar macrophages play in the propagation and progression of asthma symptoms, being able to study the immunological response in the absence of alveolar macrophages will contribute to our understanding of the pathways involved. With the advent of clodronate liposomes (Rooijen, 1989) it is now possible to

understand the role of alveolar macrophages in the recruitment of inflammatory mediators and other role players in the asthmatic response. Following the depletion of alveolar macrophages using clodronate liposomes, we can monitor the bronchoalveolar milieu for changing cell types and numbers (figure 5). Considering the role of macrophages in the recruitment of eosinophils and neutrophils, we expect that depleting the alveolar macrophages should reduce the BAL levels of these two cell types. Which should in turn should reduce inflammatory responses and mucous production. While the affect that the dying macrophages will have on the surrounding alveolar environment is still unknown, we expect an overall decrease in asthmatic response using clodronate liposomes compared to control.



**Figure 5: Macrophage depletion experiment using clodronate liposomes.** Clodronate liposomes are effective in inducing macrophage apoptosis. The above flow cytometry results show that compared to control, clodronate liposomes deplete 90% of macrophage pool in a mouse spleen 24 hrs following an intraperitoneal injection. (Adapted from FormuMax Scientific Inc, 2013)

## METHODS

### Experimental model:

For this experiment, female HSD-ICR mice were used (Harlan Sprague Dawley Inc.) By using outbred mice, we tried to replicate the genetic variability seen in individuals susceptible to asthma. The stock clodronate and empty liposomes were obtained from Dr. Nico Van Rooijen (1007 MB, Amsterdam, Netherlands). The data obtained represents the combination of 2 replicates for all conditions. All experiments were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

### Allergen sensitization:

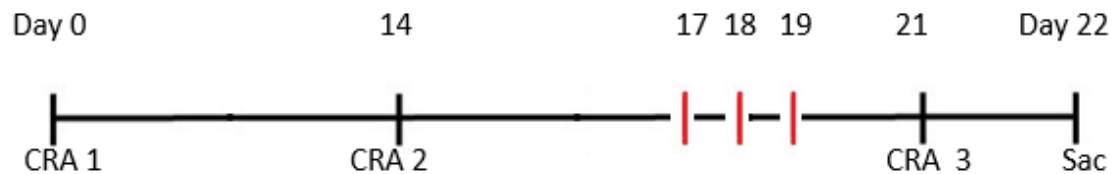
CRA extract from German cockroach *Blattella germanica* was purchased from Greer Laboratories (Lenoir, NC, Item# 46). The CRA was then reconstituted in sterile PBS so that 50 ul of PBS solution containing 8 ug of combined Blag1 and Blag2. To start the procedure, the mice are first anesthetized using 250 ul of isofluorine until the pulse drops to around 60 bpm. Then 2x 20 ul (total 40 ul) of freshly made CRA is then instilled intratracheally (figure 6). A successful instillation is consistent with observing foamy bubbles coming out of the nasal passages. The anesthetized mouse is then allowed to recover in an incubator at 37°C and then returned to its cage. Identical steps are taken with the control mice, except that CRA is replaced with the same quantity of PBS. Figures 7 and 8 show variations in experimental timeline in order to determine the effect of acute macrophage depletion on asthmatic response (figure 7) compared to prolonged macrophage depletion (figure 8)



**Figure 6: Intratracheal instillation of CRA and PBS.** Anesthetized mouse is suspended by their dentition and 2 doses of 20 ul of CRA or PBS is then pipetted into the trachea. A proper installation can be confirmed visually by the foamy bubbles near the nostrils.

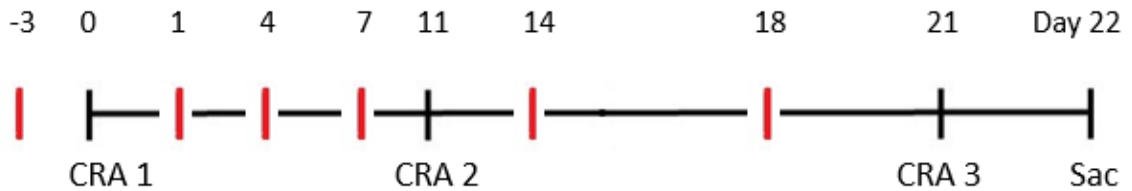
Experimental timeline:

Timeline 1:



**Figure 7: Acute macrophage depletion following sensitization.** From the timeline above, we can see that the first day when the mice are exposed to either CRA or PBS is denoted as day 0. On day 14, the mice receive a second dose of either CRA or PBS. On days 17, 18 and 19, the mice receive 2x 20 ul of either clodronate liposomes or empty liposomes by the same technique as seen in figure 6. On day 21, the mice receive their 3<sup>rd</sup> and final CRA dose and are sacrificed using approved protocols on day 22.

Timeline 2:



**Figure 8: Prolonged macrophage depletion prior to and following CRA sensitization.** 3 days prior to the mice receiving their first dose of either CRA or PBS, the mice are exposed to either clodronate liposomes or control in order to deplete macrophages prior to sensitization. The mice are then exposed to CRA or PBS on days 0, 11 and 21. In between CRA treatments, the mice receive either clodronate liposomes or empty liposomes on days 1, 4, 7, 14 and 18. The mice are then sacrificed on day 22 using approved protocols.

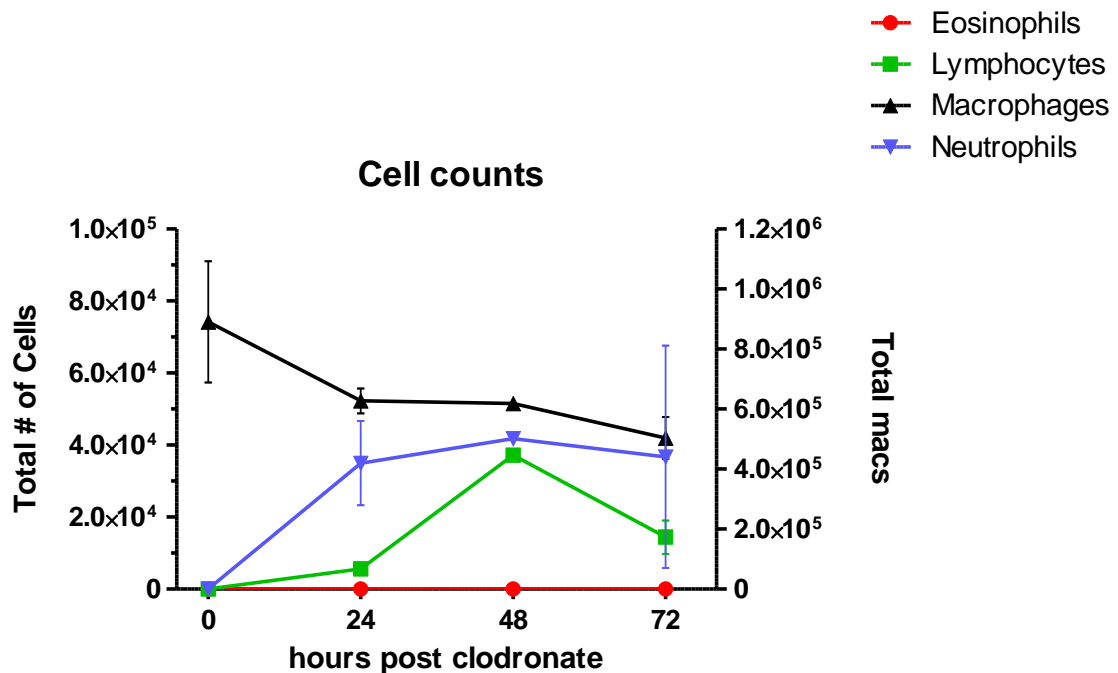
Sacrifice and data collection:

On day 22, the mice are sacrificed by exposure to isofluorine followed by cervical dislocation. In order to obtain the immune cells, bronchoalveolar lavage (BAL) was performed using 5 ml of HBSS. The first ml of solution obtained through the BAL is used for cytokine detection whereas the next 18 ml are used to perform a differential BAL cell count. The left lung is then removed and placed in 70% ethanol for histology. The BAL cells are then centrifuged and stained with trypan blue in order to count the number of viable macrophages.

## RESULTS

Macrophage depletion in naïve mice:

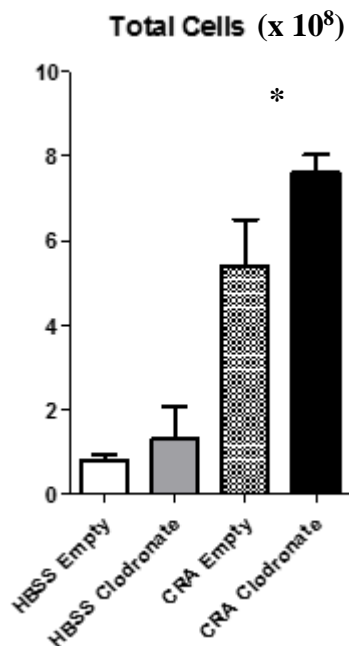
This initial experiment was performed to determine the effect of clodronate liposomes on macrophage depletion and differential count (figure 9). In this experiment, the liposomes were instilled intratracheally on day 0 and mice were then sacrificed at various time periods to determine the effectiveness of clodronate liposomes over a prolonged period. We can see from figure 9 that over a period of 72 hours after instillation of clodronate liposomes, the macrophage count decreased from an initial count of  $9.0 \times 10^5$  to  $5.0 \times 10^5$ . Meanwhile, the neutrophils which are not present in a naïve mice increase in number to  $4.2 \times 10^5$  about 48 hours post clodronate liposome instillation.



**Figure 9. Differential BAL count following clodronate liposomes in naïve mice.** 72 hours post clodronate liposome treatment, there is significant decrease in BAL macrophage count whereas a significant increase in BAL neutrophil count post 48 hours.

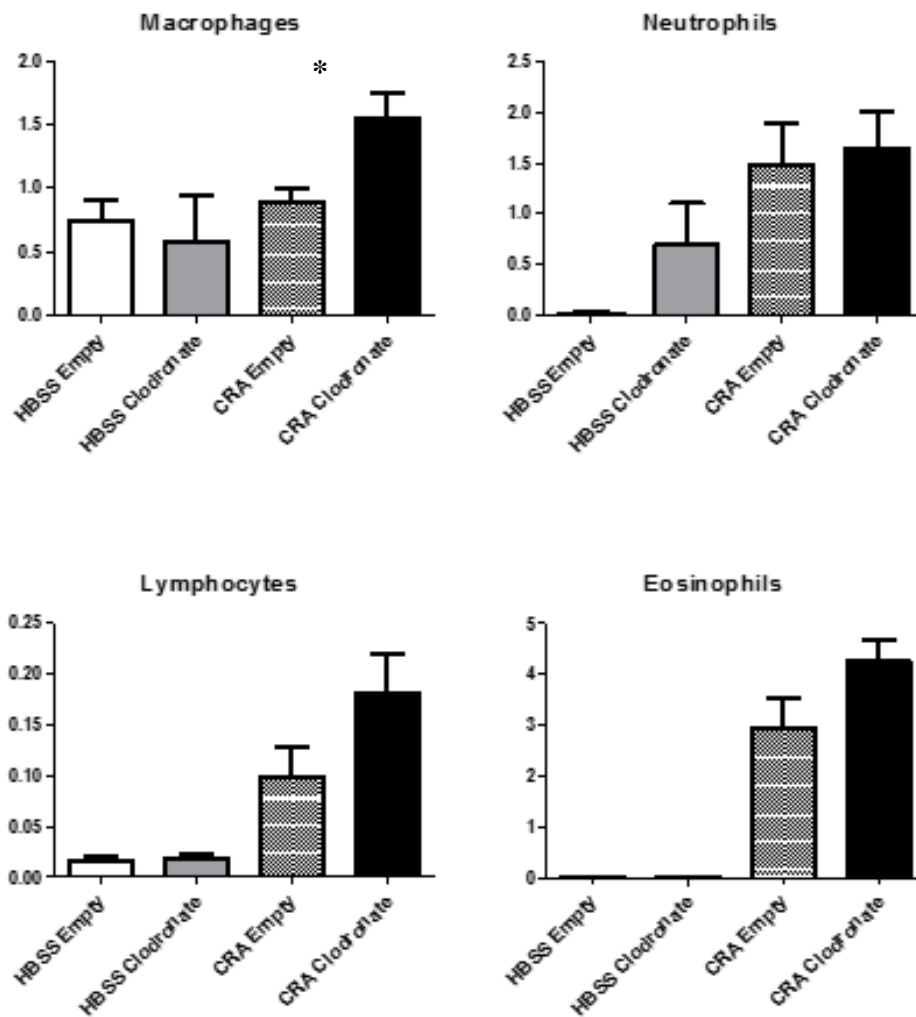
Acute macrophage depletion:

As indicated in figure 7, following 2 doses of CRA challenge, on days 17, 18 and 19, the mice were exposed to either clodronate or empty PBS liposomes. Following timeline 1 on day 22, the total BAL cell count shows a significant increase in the mice which received CRA and clodronate liposomes ( $8 \times 10^8$ ) compared to mice which received CRA and empty liposomes ( $5.8 \times 10^8$ ).



**Figure 10: Total BAL cell count, post-acute macrophage depletion.** The total number of BAL cells is significantly greater in mice group which received CRA and clodronate liposomes compared to those which received CRA with control ( $p < 0.05$ )

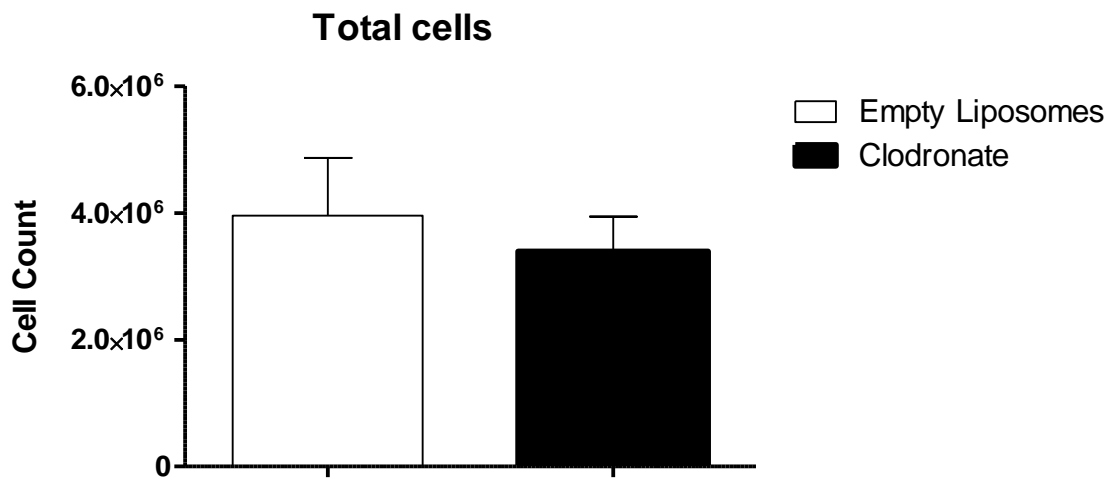
The cells obtained in the BAL were then stained and differentially counted. The results as seen in figure 11 show that the group which received CRA and clodronate had a significantly greater number of macrophages compared to the group which received CRA and empty liposomes.



**Figure 11: BAL differential count (in millions), post-acute macrophage depletion.** The group which received acute doses on clodronate liposomes on days 17, 18 and 19 had significantly greater number of macrophages in the BAL compared to the control group which received CRA and empty liposomes ( $p < 0.05$ )

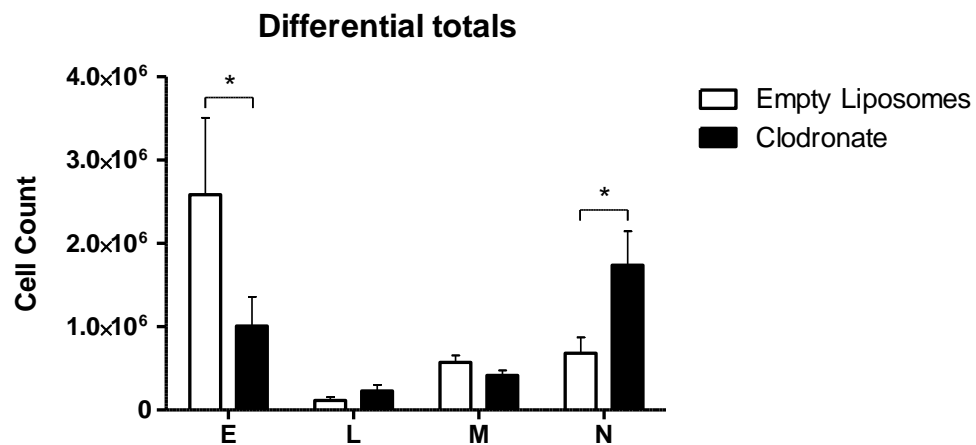
Prolonged macrophage depletion experiment:

Following timeline 2 the mice received intermittent doses of either clodronate or empty liposomes approximately every 3 days. The BAL counts show that there is no significant difference in the total cell count between the groups which received CRA with clodronate liposomes and CRA with empty liposomes.



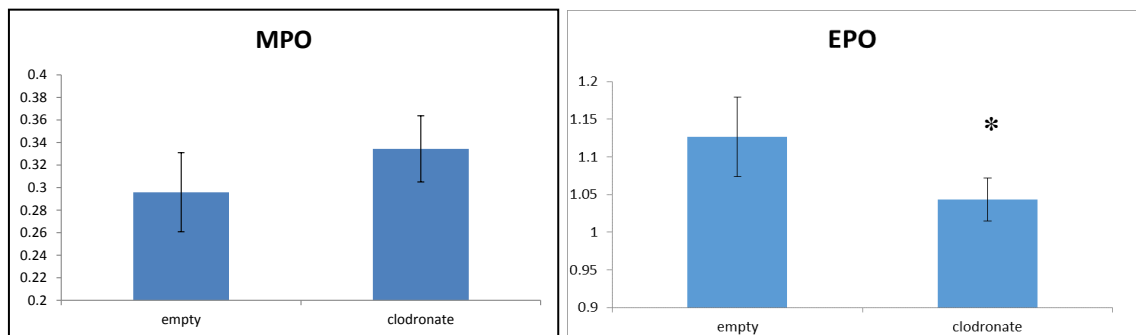
**Figure 12: Total BAL count in mice that receive CRA and either clodronate or empty liposomes.** No significant difference in BAL cell count is seen in mice groups which received empty or clodronate liposomes with CRA ( $p > 0.05$ )

Figure 13 shows the differential cell count from the BAL. We can see from this figure that sensitized mice groups which received intermittent doses of clodronate liposomes have a significant decrease in the number of eosinophils. On the other hand, consistent with figure 9, there is a significant increase in the total neutrophil infiltrate in group which received clodronate liposomes compared to control ( $p < 0.05$ )



**Figure 13: BAL differential count in mice which received CRA with either clodronate or empty liposomes.** There is a significant decrease in the total number of eosinophils in sensitized mice which received clodronate liposomes compared to the sensitized control group which received empty liposomes. There is also an increase in the total neutrophil count in the clodronate group compared to the empty liposome group.

The neutrophils and eosinophils obtained from the BAL fluid were then analyzed with MPO and EPO assay to measure the activity of myeloperoxidase and eosinophil peroxidase respectively. The results as seen in figure 14 show that there is a significant decrease in the EPO activity in mice which received CRA with clodronate liposomes compared to the group that received CRA with empty liposomes. The activity of MPO in each of the 2 BAL fluids is similar



**Figure 14: MPO and EPO assay of BAL cells from timeline 2.** A significant difference in EPO activity is observed in the experimental group which received clodronate liposomes compared to the control group which received empty liposomes ( $p < 0.05$ ). MPO activity between the 2 groups is similar ( $p > 0.05$ ).

## DISCUSSION

In this study, we determined that while it is possible to deplete the alveolar macrophages using clodronate liposomes, thus interfering with the macrophage recruitment of inflammatory cells, the ensuing cellular milieu hinders the study of CRA induced asthmatic model. Some studies show that depletion of alveolar macrophages using clodronate liposomes following sensitization in fact increases the recruitment of eosinophils and lymphocytes and causes production of Th2 cytokines IL-4, IL-5 and GM-CSF (Bang et al, 2011). In our initial study of clodronate liposomes (figure 9), we see that over 72 hours, although there is a decline in the number of alveolar macrophages, there is a reciprocal increase in the number of neutrophils which is consistent with published studies. A study by Mircescu et al (2009) shows that in mice treated with

clodronate liposomes, the neutrophil counts in the BAL fluid were marginally higher compared to mice that are treated with PBS liposomes or no liposomes. In a sense, the dying macrophages are being replaced by neutrophils. Another way to look at this is that while clodronate liposomes supposedly causes macrophage death through apoptosis, there is some form of an inflammatory response taking place which is resulting in an acute neutrophil infiltration. Stains of macrophage depleted BALs show a great extent of debris surrounded by neutrophilic infiltrate compared to BALs of mice which received PBS liposomes which consist of intact macrophages and limited number of neutrophils (images not shown). Figure 11 shows that during an acute clodronate liposome administration, there is a significant increase in macrophage recruitment even following macrophage depletion. These results contradict previous studies as to the effectiveness of clodronate liposomes in macrophage depletion and inhibition of macrophage recruitment. In figure 10 we see that following acute clodronate liposome administration, there is an increase in the total number of cells compared to mice which received empty liposomes. This increase in cell number could be attributed to the above mentioned increase in neutrophil and monocyte recruitment. Overall, through experiment 1 (figures 10 and 11), we can interpret that although clodronate liposomes might be effective in alveolar macrophage depletion in naïve mice, in the CRA sensitized mice, the corresponding recruitment of inflammatory cells which include neutrophils but also macrophages interferes with the immunological study of CRA induced asthmatic model. In such circumstances, it is difficult to establish the contribution of macrophage depletion in inhibiting the asthmatic response.

In order to study the effect of acute versus prolonged administration of clodronate liposomes, experiment 2 was performed (figures 12 and 13). Theoretically, by spacing out the administration of clodronate liposomes, we are giving the inflammatory cells such as neutrophils time to clean up the debris left from dead macrophages thus preventing further recruitment of monocytes. In this modified experiment, we see that frequent but intermittent doses of clodronate liposomes throughout the model depletes macrophages without causing a massive amount of inflammation. Compared to experiment 1, in experiment 2 we can see that there is no significant rise in the total number of cells following administration of clodronate liposomes (figure 12). Once again, as in experiment 1, there is a significant rise in the total number of neutrophils in the clodronate liposome group compared to the control (figure 13). But a difference in this model is the total number of eosinophils. Compared to the control group which received CRA along with PBS liposomes, the experimental group which received CRA with clodronate liposomes had a significant decrease in the total number of eosinophils and eotaxin production (figure 13 and 14).

A recently published study by Beal et al (2013) shows that autologous transfer of peritoneal macrophages into the respiratory epithelium of a CRA sensitized mice shows reduced recruitment of eosinophils and production of eotaxin. This study brings into question the type of macrophage that is being depleted using clodronate liposomes as not all macrophages induce inflammatory responses. While the macrophages that are most commonly associated with asthmatic response are those that are classically activated

(M1), destruction of alternatively activated macrophages (M2) by clodronate liposomes can exacerbate the asthmatic response rather than inhibiting it.

Considering the interrelationship between the various immune cells in asthmatic response, in order to study the immunology, it is important to be able to interfere with just a single factor in the disease process while holding all others constant. Clodronate liposomes while effective in depleting alveolar macrophages in a naïve lung, are also capable of interfering with the cellular environment established in an asthmatic lung. We recommend that future studies implement a more efficient way of macrophage depletion which does not interfere with too many factors in asthmatic response which could possibly sidetrack the study. In addition, following such macrophage depletion, it is important to measure its effect on other asthmatic parameters such as mucous production, airway hyperresponsiveness and cytokine production. Such experiments could possibly paint a better picture of the role of alveolar macrophages on asthmatic response.

## **LIST OF JOURNAL ABBREVIATIONS**

|      |  |
|------|--|
| AJP  | American Journal of Physiology                 |
| EMM  | Experimental and Molecular Medicine            |
| FM   | Frontiers of Microbiology                      |
| JACM | The Journal of Allergy and Clinical Immunology |
| JIM  | Journal of Immunological Medicine              |
| ME   | Methods in Enzymology                          |
| TMM  | Trends in Molecular Medicine                   |

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**VITA**

