**Can epigenetics be the cause of asthma progression?**

**Layperson Summary**

Asthma is a heritable, immune-mediated disease that affects 300 million people worldwide. There are many factors and mechanisms contributing to asthma and there is still a lot that is unknown about the disease. Elucidating the mechanisms underlying asthma is necessary in order to develop therapeutic strategies that can effectively help all asthmatic patients. There are suggestions that epigenetics, the inheritance of phenotypic changes with no modifications to the DNA sequence, is a driving factor in asthma. In particular, DNA methylation differences which lead to an abnormal shift in T helper 2 (Th2) cell population seems to be an important epigenetic mechanism underlying asthma. This study seeks to address whether DNA methylation levels are different between control and an asthma model of mice, and whether these DNA methylation differences are inherited in subsequent generations of mice. The results of the experiments proposed could further establish the role of increased Th2 cell differentiation in asthma progression and could lead to innovations in therapeutic strategies to treat asthmatic patients more effectively.

**Introduction**

Asthma is a heritable, multifaceted inflammatory disease characterized by bronchial hyper-reactivity, airway remodelling, and mucus overproduction (Yang & Schwartz, 2012). As a result, patients often experience prolonged periods of chest tightness and difficulty breathing. It afflicts approximately 300 million people worldwide (Eder *et al*., 2006). The cost for asthma, in terms of medical expenses and productivity loss due to illness, is an estimated $18 billion annually in the USA (Lambrecht & Hammad, 2015). Asthma symptoms are abated in patients by using an inhaled corticosteroid (reduces inflammation) in combination with a β2-adrenergic agonist which helps relax constricting bronchial SM cells (Barnes, 2009). However, 5-10% of patients has negative reactions to the corticosteroid treatment and become hospitalized with respiratory viral infections (Lambrecht & Hammad, 2014). Elucidating the mechanisms which drive asthma is imperative for developing new therapies so that all asthma patients can be treated effectively.

There are many factors associated with the pathogenesis of asthma but genetic mechanisms only accounts for a small portion of these factors (Vercelli, 2008). There is increasing support for epigenetics to play a role in asthma. 'Epigenetics' refers to meiotically and mitotically heritable phenotypic changes (de Planell-Saguer *et al*., 2014). Epigenetic modifications result in altered gene expression without changes in the DNA sequence (Cheung & Lau, 2005; Li *et al*., 2007). These modifications include DNA methylation/acetylation at cytosine-guanine dinucleotides (CpG) regions, alteration of histone proteins, and non-coding RNA-mediated gene silencing (Koppelman & Nawjin, 2011). These epigenetic mechanisms regulate gene expression by blocking transcription factors (TFs) binding to regulatory regions, initiate chromatin remodelling by histone tail modification, and non-coding RNA binding to mRNA to prevent translation (Yang & Schwartz, 2012).

Various lines of research have made it increasingly evident that epigenetic regulation may have a role to play in asthma susceptibility. Similar to epigenetic modifications, asthma is heritable. Asthma is recognized as a strong familial condition with reports of 36-79% heritability (Vercelli, 2008). Also, in comparison to epigenetic mechanisms, heritability of asthma displays a parent-specific transmission pattern. The maternal phenotype is more likely to predict asthma and allergy in a child than the paternal phenotype. The risk of asthma transmission from an affected mother is five times more likely than transmission from an affected father for children under five years of age (Litonjua *et al*., 1998; Moffat & Cookson, 1998). In their New Zealand cohort study, Sears and his colleagues (1996) have also shown that maternal asthma is a strong risk factor for asthma in a child. These studies support the possibility of epigenetic changes as a contributing factor to asthma.

The most intriguing link for epigenetic regulation mediating asthma is the regulation of T cell differentiation. Naïve helper T cells are capable of differentiating into Th1 cells (which produce the cytokines Interferon (INF)-γ and Interleukin (IL)-12) or Th2 cells (responsible for producing pro-allergic cytokines IL-4, IL-5, and IL-13). Asthma is characterized by the skewing of naïve T cell differentiation into the Th2 subtype (Lloyd & Hessel, 2010). The irregular shift in the proportion of the Th2 cell population and its associated cytokines along with simultaneous suppression of regulatory Th1 cytokines has been demonstrated to drive airway inflammation and hyperresponsiveness (Kupperman *et al*., 2002; Calderon *et al*., 2009). Prescott and his colleagues (Prescott *et al*., 1999) demonstrated that cord blood and peripheral blood mononuclear cells (PBMCs) had increased Th2 cytokine expression when samples were cultured with an allergen, further solidifying the role of T cells in asthma. Interestingly, epigenetic mechanisms have been shown to regulate T cell differentiation into Th1 and Th2 cells (Takemoto *et al*., 2000; Fields *et al*., 2002; Webster *et al*., 2007), thus supporting the possibility of epigenetic modifications being linked to asthma occurrence. Of the epigenetic mechanisms mentioned previously, DNA methylation seems to be particularly important in the regulation of Th2 cytokine expression during Th2 cell differentiation. When naïve T cells are stimulated under Th2-polarizing conditions, the IL-4 gene promoter undergoes extensive demethylation (Janson *et al*., 2009). Webster *et al*. examined Cd4+ T cells from human cord blood samples and discovered that CpG methylation levels at the IL-13 promoter was significantly less in Th2 cells as compared to Th1 cells (Webster *et al*., 2007). These studies raise the possibility that methylation changes occurring during excessive Th2 cell differentiation in asthmatic parents are inherited, leading to higher asthmatic incidence in their children. Based on the evidence presented previously, I hypothesize that Th2 lymphocytes obtained from asthmatic mice will exhibit lower DNA methylation and higher DNA acetylation as compared to lymphocyte populations from control mice at genes necessary for the regulation of Th2 cell differentiation (IL-4, STAT6, GATA3) and Th2 associated cytokines (IL-5, IL-13). I predict that DNA methylation differences observed between asthmatic and WT mice will be inherited in subsequent generations. The results of this proposed research could further corroborate the role of irregular Th2 cell population shift in the pathogenesis of asthma and could help tailor future therapeutic strategies to treat asthma more effectively.

**Experimental Method**

I will use BALB/c mice to generate a mouse model of asthma, as described by Kumar and his colleagues (Kumar *et al*., 2011). BALB/c mice are much more hyperresponsive to allergenic stimulation than C57BL/6 mice and thus better for this experiment (Chen *et al*., 2005). I will challenge mice daily for two weeks by intranasally administering 10 μg/mL ovalbumin mixed in phosphate buffer solution (PBS). House dust mite (HDM) is also used as an allergen in mice models but the use of ovalbumin is more established. After the two week challenge, the asthmatic mice will be split into two equal groups. First, one group will be used to determine whether DNA methylation changes can be observed between control and asthmatic mice immediately following allergenic stimulation. The second group will be used to determine whether epigenetic changes, if observed from the first experiment, remain in subsequent generations of mice.

The mice in the first group will be challenged with 10 μg/mL ovalbumin while control mice will be administered with vehicle (PBS only) for two weeks. After the challenge, bronchioalveolar lavage fluid (BALF) as well as the thymus, lungs, and spleen of the control and asthmatic mice (n= 6-9 mice for both groups) will be harvested. I will use the T cell-specific magnetic cell sorting kit (Stemcell, 2015) to isolate T cells from the control and asthmatic samples. This will provide a heterogeneous mix of T cell subtypes for all the samples being tested. To further isolate Th2 cells, I will use a Fluorescence-activated cell-sorting (FACS) machine; I will use the cell surface receptor CRTH2 as a marker to identify Th2 cells (Sanguine Biosciences, 2015). Once Th2 cells have been sufficiently isolated for all samples, I will collect genomic DNA from my different Th2 populations and begin bisulfite conversion of genomic DNA as described by Brand (Kumar *et al*., 2011). Once bisulfite conversion is done, I will conduct pyrosequencing via the Pyromark ID system (Qiagen, 2015) to identify possible DNA methylation changes at the IL-4, IL-5, IL-13, STAT6, and GATA3 CpG regions between control and asthmatic mice.

The second experiment involves the second group of asthmatic mice, which will be interbred and they will give birth to a litter that will be known as the first generation. Mice from the first generation will be interbred and give birth to a litter that will be identified as the second generation. The same procedure will be applied to control mice (treated with vehicle only). BALF, lungs, thymus, and spleen will be harvested from the parents of the first generation mice (n=6-9) once the first generation mice have been weaned and are independent for both control and asthmatic mice. The same samples will be taken from first and second generation of mice (n=6-9). Th2 cell isolation and bisulfite conversion of genomic DNA followed by pyrosequencing will be the same as the first experiment. The same genes will be tested for this experiment. The pyrosequencing results for the first and second generation of mice will be compared to the results of the parents of the first generation for both control and asthmatic mice.

**Discussion**

The experiments I have proposed here should help answer whether epigenetic changes can be observed in mice following allergenic stimulation and whether these changes are inherited. The percentage of DNA methylation in control mice CpG regions will be set to one. The percentage of DNA methylation in asthmatic mice CpG regions will be expressed relative to control mice values. I expect that based on my prediction, asthmatic mice from the first experiment will exhibit between 35-40% less DNA methylation at the IL-4, IL-5, and IL-13 CpGs when compared to control mice. Assuming the asthmatic mice exhibit aberrant Th2 cell differentiation, then there is likely to be more Th2 cytokine expression in the asthmatic mice and thus less repressive DNA methylation as compared to control. It's also possible that excessive Th2 cell populations in asthmatic mice is due to transcription factors (needed for Th2 cell differentiation) such as STAT6 and GATA3 being overexpressed. If this is the case, then I expect that overexpression of these factors (due to less DNA methylation-mediated repression) leads to excessive TH2 cell differentiation in the asthmatic mice. As a result, I expect the STAT6 and GATA3 CpG regions in asthmatic mice to display significantly less DNA methylation than the control mice. These results are summarized in Table 1.

I also predict that epigenetic changes, if any observed, would be inherited in subsequent generations. I expect that I won't see the same degree of DNA demethylation in the second generation of mice as compared to first generation or the parents of the first generation. This is because it's possible that DNA methylation changes following allergenic stimulation isn't fully inherited by the subsequent generation; it could be that some DNA methylation changes are permanently inherited while others are lost. With that in mind, I expect to see the differences in DNA methylation at the CpG regions for control and asthmatic mice to decrease for every subsequent generation. These results are also summarized in Table 1.

The experimental results proposed in Table 1 are based on some assumptions. There are several scenarios that could prove the assumptions wrong and make it difficult to interpret the results obtained from the pyrosequencing experiments. First, I am assuming that my mouse model of asthma, as described by Kumar *et al*. (2011) is an accurate representation of asthma. Even if the model is accurate, there are many factors which contribute to asthmatic phenotype. It's possible that something other than increased Th2 cell differentiation is responsible for the asthmatic phenotype in my asthmatic mice; if this is the case, then I would obtain DNA methylation levels to be similar between the control and asthmatic mice for both set of experiments. I could take blood samples from my mice prior to collecting other samples to test cytokine levels in the blood. If there is abnormally high Th2 cytokine levels in the blood of asthmatic mice as compared to control, then I can reasonably assume the asthma is due to high Th2 differentiation and the experiment can be continued. Another assumption I have made is that the DNA methylation changes observed in the parents of the first generation of mice tested will be inherited in every generation thereafter. If these DNA methylation changes are acquired after allergenic stimulation but not inherited, then the first generation and second generation mice are likely to exhibit DNA methylation levels similar to that of control mice. Finally, I have made the assumption that DNA methylation marks are mostly likely to be repressive. Some methylation changes are considered to be activating marks. If this is considered, then interpreting the results become more difficult. Both active and repressive DNA methylation marks need to be identified when analyzing the pyrosequencing results to better understand the effects of inheriting epigenetic marks following allergenic stimulation.

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|  | **IL-4** | **IL-5** | **IL-13** | **STAT6** | **GATA3** |
| **1st experiment** |  |  |  |  |  |
| **Mice tested immediately following allergenic stimulation** | 40% less DNA methylation in asthmatic mice | 35% less DNA methylation in asthmatic mice | 35% less DNA methylation in asthmatic mice | 120% less DNA methylation in asthmatic mice | 110% less DNA methylation in asthmatic mice |
|  | | | | | |
| **2nd experiment** |  |  |  |  |  |
| **Parents of first generation mice** | 40% less DNA methylation in asthmatic mice | 35% less DNA methylation in asthmatic mice | 35% less DNA methylation in asthmatic mice | 120% less DNA methylation in asthmatic mice | 110% less DNA methylation in asthmatic mice |
| **First generation mice** | 37.5% less DNA methylation in asthmatic mice | 32.5% less DNA methylation in asthmatic mice | 32.5% less DNA methylation in asthmatic mice | 115% less DNA methylation in asthmatic mice | 105% less DNA methylation in asthmatic mice |
| **Second generation mice** | 35% less DNA methylation in asthmatic mice | 30% less DNA methylation in asthmatic mice | 30% less DNA methylation in asthmatic mice | 110% less DNA methylation in asthmatic mice | 100% less DNA methylation in asthmatic mice |

**Table 1**: Hypothetical experimental results. The percentage of DNA methylation at CpG regions of genes being tested is set to one for control mice. The percentage of DNA methylation values for asthmatic mice is expressed relative to the values of the control mice.

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