Epigenetic Changes in Foxp3 in Treg of Identical Twins Discordant for Asthma

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Asthma is characterized by airway inflammation, wheezing, coughing, and breathlessness due to dysfunctional regulatory T cells (Treg) that are normally regulated by the Foxp3 transcription factor. In this study, we investigated the role of ambient air pollution exposure in the development of asthma and increased severity of symptoms via epigenetic mechanisms. We found that identical twins had different disease outcomes, in which the asthmatic twin had lower Treg function and Foxp3 expression. Our study shows the influence of hazardous environmental exposures on health and highlights the importance of further exploration into epigenetics.

Materials and Methods

The study has over 54 fraternal and identical twins enrolled for a series of biological tests and verbal questionnaires. We enrolled any pairs of twins (up to eighty years of age) regardless of discordance or previous medical conditions. The focus is to have 25 controlled pairs (in which both twins have similar health conditions and no disease) and 25 discordant pairs (in which one twin has asthma and/or allergies and the other does not) by the end of July 2011. Each patient is first asked to sign a consent form in which our lab and clinic acknowledge confidentiality and is reimbursed with $50 for participating as well as $0.50 per mile traveled, round trip.

To confirm diagnosis of asthma, each enrolled twin participated in spirometry testing, in which forced vital capacity (FVC) was measured. Spirometry is a diagnostic tool which measures amount of air blown into the device over time—less air produced through the tube implies a degree of lung inflammation that can indicate asthma. In addition, a universal asthma control test was conducted: a series of questions addressing the severity of asthma and impact on daily life. For example: “In the past four weeks, how much of the time did your asthma keep you from getting as much done at work, school, or at home?” All results and confirmation of diagnosis would be interpreted by Dr. Kari Nadeau.

To test for zygosity (whether the twins were fraternal or identical) and allergies, blood draws of 30-100mL per patient were taken for analysis. Flow sorting techniques, Treg purification, and Sanger genomic DNA sequencing were performed on the cells derived from the blood samples. More specifically, blood plasma and leukocytes were purified from purified blood cells as well as from bronchoalveolar lavage. Zygosity as well as genetic make-up were also analyzed through buccal swab tests, in which a swab was rubbed against the inner cheeks and gums of the patient to collect cells.

Aside from biological diagnostic testing, a detailed interview of the twins...
was conducted to determine medical history of disease as well as various environmental exposures, such as whether parents smoked or not as well as duration of smoking history, both in years and number of packs smoked per day. Other questions included locations of all homes the patients have ever lived in for at least six months or more. Follow-up research will be done to determine ambient air pollution levels in those areas during those periods.

**Results**

Previous studies determined that increased methylation of the CpG region in the Foxp3 locus was correlated with decreased function of Treg, thus resulting in increased asthma severity (Bromberg et al., 2009), a sign of epigenetic occurrence. In other words, Foxp3 expression—altered by CpG island methylation—is required for the suppressive effects of Treg that prevent inflammation and asthma. Thus, it was expected that the asthmatic twin would have higher methylation levels than their non-asthmatic, identical counterpart. Healthy twins would be expected to have similar levels of methylation. We will look at pairs in which one twin has asthma and the other one does not, comparing the methylation levels and regions of residence, along with other social and environmental variables.

In the twelve initial twins enrolled, it was found that Foxp3 gene CpG methylation differences exist in monozygotic twins discordant in asthma.

The data demonstrated that Foxp3 CpG island (21 total islands were sequenced) methylation in peripheral blood (PB) and bronchoalveolar lavage (BAL)-

**Fig. 1** Higher levels of methylation in asthmatics compared to non-asthmatics. PB Treg and BAL Treg (CD4+CD25hiCD127l) were purified via flow sorting techniques, genomic DNA sequenced as described by the Nadeau laboratory. (A) MZT-D twin pairs (n=8 pairs) (A vs NA) (B) MZT-C twin pairs (concordant in asthma, n=4 pairs). Mann Whitney Test (*significant if p < 0.05, NS=non significant).

**Fig. 2** Reduced Treg function and Foxp3 expression in asthmatics compared to non-asthmatics. (A) % PB Treg function from MZT-D pairs (n=8 pairs) as determined by quantization of suppressive activities of Treg. Bars show degree of suppression at a 1:1 ratio of conventional CD4+ T cells (effector T cells or Teff (purified for CD4+CD25)) to Treg (purified for CD4+CD25hiCD127lo) (% suppression of proliferation =((Tresp proliferation without Tregs – Tresp proliferation with Tregs)/(Tresp proliferation without Tregs)) x 100) (B) % Foxp3 expression in Treg as determined by presence of fluorescently labeled intracellular Foxp3 antibody in purified Treg.
purified Treg was higher in the asthmatic vs. non-asthmatic twin of the tested MZT-D pair. As a control, methylation of MZT-C twins was tested, and was found to be at similar levels for both twins (Figure 1A and B). Both PB and BAL-purified Treg were tested because in some cases of disease, Treg can have different functionality, in which PB-purified Treg could be normal and BAL-purified Treg could not, and vice-versa. By testing the areas within the airways (BAL) and away from the airways (PB), it was ensured that consistent Treg functionality was observed across different areas of the body.

Evidence was also found for methylation as a predictor of $\text{Foxp3}$ expression and thus Treg function in order to further link epigenetics to differences in twin disease development. It was determined that peripheral blood Treg function as well as Foxp3 protein expression in PB Treg was lowered in the asthmatic vs. non-asthmatic twin of the tested MZT-D pair (Figure 2).

Overall, methylation was higher in asthmatics than their matched identical discordant twin. As expected, asthmatics showed reduced Treg function as well as Foxp3 protein expression when compared to their matched identical twin without asthma.

**Discussion**

The results suggest that the environmental exposures may play a key role in the outcome of disease development due to the varying levels of methylation, $\text{Foxp3}$ expression, and Treg function in identical twins. Discordant identical twins had different levels of methylation, whereas methylation was similar in concordant twins. Our findings show that although identical twins have the same genetic makeup, their differences in health history may be attributed to varied environmental exposures that resulted in altered gene expression. Possible exposures include years of smoking (the asthmatic twin smoked while the other did not), location of residence (one twin lived in Palo Alto, whereas the asthmatic twin lived in Fresno), and so on. Our results suggest that exposure to ambient air pollutants may have led to increased methylation of the CpG island located in the $\text{Foxp3}$ locus, thereby reducing $\text{Foxp3}$ expression. Lowered $\text{Foxp3}$ expression could lead to Treg dysfunction and insufficient inflammation, possibly resulting in the development of asthma or an increase in its severity.

It is important to recognize that external environmental exposures may indeed result in genetic alterations of individuals, based on the fact that there are identical twin pairs in which one twin has developed a disease while the other one has not. However, there is insufficient information on the mechanisms in which these epigenetic processes can occur, despite the fact that climate change and chronic disease development are occurring at much faster rates in the world today. Our results imply a significant effect of methylation of the CpG locus on the outcome of disease development, especially with regards to the upstream control of gene expression. At the clinical level, more research must be done linking ambient air exposure to chronic disease prevalence and severity, particularly with asthma. And at the molecular level, we need to further elucidate the genes and pathways that regulate Treg function. With a better understanding of the factors that can lead to asthma, we will be able to advance its pharmacological treatment as well as develop preventative measures for susceptible individuals.

**References**


**Leslie Cachola** is a senior majoring in Human Biology with a concentration in Global Health and Infectious Disease and a minor in Creative Writing. A former Artistic Director of Dv8 Dance Troupe, music has always played a big role in her life, aside from reading books and eating ice cream. Leslie plans to spend the next two years pursuing some sort of music career and volunteering abroad with a public health focus before attending medical school.