CONNECTING ENVIRONMENTAL EXPOSURES WITH ALLERGIC AND RESPIRATORY OUTCOMES – THE ONGOING STUDY OF THE KINGSTON ALLERGY BIRTH COHORT

by

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A thesis submitted to the Department of Biomedical and Molecular Sciences

In conformity with the requirements for

the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

(August, 2019)

Abstract

Atopic diseases like allergies and asthma are mediated by several types of immune cells such as Regulatory T cells (Treg), and T helper type 1, 2, and 17 (Th1, Th2, and Th17) cells. Reductions in Treg numbers and the Th1:Th2 ratio and increases in Th17 cells have been related to atopic disease pathogenesis; however, whether these are a cause or result of disease remains unclear. Moreover, researchers have identified several early life environmental exposures that contribute to allergic disease development. However, these is a lack in understanding of how these exposures relate to changes at the cellular and genetic level, and how these exposures contribute to atopic disease progression.

We polarized T helper cells from umbilical cord blood mononuclear cell (CBMC) samples from children with high (n=3) and low (n=4) atopic risk. We gathered information on the home environment of 92 mother child pairs, determined the odds ratio (OR) of a positive SPT (SPT) at 2 years with exposure to indoor air pollutants, and compared the number of different exposures between SPT positive and negative at 2 years. Lastly, we attained prenatal and current exposure data to air pollutants of 39 mother child pairs at 6 to 7 years of age and collected a blood samples from the 24 children. We determined the relationships between exposure to air pollutants with sensitization, cough/wheeze, plasma cytokine levels, Treg and Th17 frequencies, and *FOXP3* expression.

High atopic risk CBMC samples had higher Th17 frequencies following T cell polarization. The OR of a positive SPT at 2 years was increased with exposure to candles, cats, and environmental tobacco smoke. 2 year old children with a positive SPT had more exposures at each time point than children with a negative SPT. Children with atopic disease at 6 to7 years had reductions in the regulatory/inflammatory cytokine ratios and children with exposures to cats

and high levels of indoor air pollutants had lower plasma cytokine levels. *FOXP3* expression was elevated in atopic children and in children with exposure to air pollution.

This work provides evidence supporting the importance of the early life environment to atopic disease development.

Acknowledgements

I would first like to thank my supervisor Dr. Anne K. Ellis for her guidance and support throughout this thesis. I am grateful for the opportunities to study under you in both my undergraduate and graduate careers. Thank you to my research committee members, Dr. Katrina Gee and Dr. Michael Rauh for your expertise and suggestions throughout my projects. Thank you to Wilma Hopkins and Andrew Day for your assistance with the statistical analysis

To Jenny Thiele, for your advice, kindness, and friendship along the way. I've learned a lot from you and am appreciative for your help in troubleshooting or last-minute-experiment-adjusting. Matt Rawls, we have been together on this bumpy research ride since our fourth year projects and I am thankful for the friendship we have built. Thank you to the many Ellis fourth year thesis students and volunteers; Leah, Erika, Alessandra, Carmen, Sonya, Demille, Brittany, and Lubnaa. You have all made the lab a fun place to work over the past few years.

Thank you to the clinical research team over at KGHRI; Lisa, Dan, Crystal, Sarah, Terry, Hannah, Mark, and Silvia. To Michelle Roddy and Dr. Babak Aberumand for their assistance with the 6 year visits. You both offered countless hours of your time for many "no show" visits. Thank you to the nurses and staff on Labour and Delivery, without your cooperation cord blood collection would not have happened.

To the friends I have made in grad school; Natalya Odoardi, Matt Cormier, Rhiannon Hilton, Megan Dickson, and Matteo Zago. While most of you I knew beforehand, bonding over boardgames, wine, and the woes of the research process brought us closer together. It was comforting knowing that at anytime I had band of people experiencing the same thing I was.

To my friends outside of school; Rebecca Elphick, Jessie Garcia, Andrea Howard, Susan Robinson, and Sara Villani. You have all reminded me I have important things in my life beyond

grad school and have provided several videos of baby sloths eating snacks – both of which were essential to completing this degree.

Lastly, to my amazing family. Mom, Dad, and Nic; I know most of the time you don't know exactly what I do, but I know you are proud nonetheless. I would not be where I am today without your constant love and support. You've believed in me every step of the way and made feel that I can (and will) accomplish anything I set my mind to.

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AD	Atopic Dermatitis
AF647	Alexa Fluor 647
AF750	Alexa Fluor 750
APC	Allophycocyanin
AR	Allergic Rhinitis
BV785	Brilliant Violet 785
CB	Umbilical Cord Blood
CBMC	Cord Blood Mononuclear Cell
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribose Nucleic Acid
CHILD	Canadian Healthy Infant Longitudinal Development
CI	Confidence Interval
Cy5.5	Cyanine 5.5
DEP	Diesel Exhaust Particles
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose Nucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
ETS	Environmental Tobacco Smoke
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead Box Protein 3

GATA3	GATA binding protein 3
gDNA	Genomic Deoxyribose Nucleic Acid
GUSB	Glucuronidase Beta
HR	High Atopic Risk
IAP	Indoor Air Pollution
IFNγ	Interferon gamma
IG	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
KABC	Kingston Allergy Birth Cohort
KHSC-KGH	Kingston Health Sciences Centre– Kingston General Hospital
LR	Low Atopic Risk
MIQE	Minimum Information for Publication of Quantitative RT-PCR Experiments
mRNA	Messenger Ribonucleic Acid
NAMNC	Nonadherant Mononuclear Cell
NB	Nasal brush
NP	Non Polarized
NRT	No Reverse Transcription
OAP	Outdoor Air pollution
OR	Odds Ratio
PB	Pacific Blue
PBMC	Peripheral Blood Mononuclear Cell
PC7	R Phycoerythrin Cyanin 7

PE	Phycoerythrin
PMA	Phorbol 12-Myristate 13-Acetate
RNA	Ribonucleic Acid
RORγt	Retinoic acid related orphan receptor gamma(t)
SPT	Skin prick test
STAT	Signal Transducer and Activator of Transcription
Tbet	T Box Transcription factor
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor beta
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
Th17	Type 17 T helper cell
TNFα	Tumor Necrosis Factor Alpha
Treg	Regulatory T cell
TSLP	Thymic Stromal Lymphopoietin

Chapter 1

Introduction

Atopic diseases such as allergies and asthma have increased in prevalence in the past several decades (1). In children, asthma and allergic rhinitis are often preceded by atopic dermatitis (AD) and food allergy in early life, a progression known as the atopic march (2). Research groups have identified and characterized this pattern of disease development, yet it remains unclear as to why some children progress through to allergic rhinitis (AR) and asthma, halt at AD, or never enter the “march” at all. The ability to predict at birth whether or not a child will develop atopic disorders and to identify the factors that could contribute to the onset, progression, or halting of the atopic march would be of great benefit to clinicians, parents, and children.

Currently there is no conclusive method available at birth to determine if a child will develop atopic diseases; however several factors have been identified that could promote or protect from disease (3,4). Maternal atopic status, environmental tobacco smoke (ETS), furry pets, indoor and outdoor air pollution, and mould in the home are only a few of the many factors implicated in allergic diseases (5–9).

Atopic diseases are mediated by several cell types in the immune system, such has Th1, Th2, Th17 and Tregs (10). Proper balance and function of these cells are critical for optimal health, and altered functioning of these cell types has been demonstrated in atopy (10). For example, in atopic individuals a Th2 shift has been observed in that there is an increase in the amount of Th2 cells and cytokines compared to Th1 (10). Moreover, peripheral Th17 cell numbers in allergic individuals are elevated compared to nonallergic controls (11). Tregs are responsible for maintaining proper immune cell balance, and secrete the regulatory cytokines interleukin (IL) 10 and transforming growth factor beta (TGF-β) (12). Treg numbers have been shown to be reduced in asthmatic individuals and in children with exposures to different air polluting factors (8,13,14). Forkhead Box Protein 3 (FOXP3) is the Treg transcription factor required for the differentiation and proper functioning of Tregs, and has been shown to be expressed at a lower level in children with atopic diseases (15). Given the differences observed in T helper cell frequencies, it is likely that the altered Treg frequency and expression of *FOXP3* plays a role in disease development (16).

While both these exposure-outcome relationships and differences in cellular frequencies have been established in the literature, it remains unclear as to when these cellular changes emerge, if they are a cause or result of allergy, and whether or not these environmental exposures have the capability of contributing to these changes. This presents a major research gap. Investigation into these matters would shed light onto allergy and asthma pathogenesis and could provide useful biomarkers of atopic disease or disease development.

Therefore, the overarching goal of this project is to investigate how early life environmental factors influence atopic disease progression at the genetic, cellular, and clinical level. We addressed this goal through three objectives.

1.1 Objective 1: Characterize T helper cell frequency and function in umbilical cord blood samples following T cell polarization from children of high and low atopic risk

Here we aim to investigate the T helper cell development in umbilical cord blood (CB) samples from children with high and low atopic risk as a potential early life biomarker of disease. We hypothesize that children born to mothers with allergic diseases will present with altered Th1, Th2, and Th17 cell frequencies and function following specific T cell polarization compared to children with non-atopic mothers.

1.2 Objective 2: Evaluate the influence of prenatal and early life indoor air pollution exposure on allergic sensitization at 2 years of age

Here we aim to identify the role of exposure to 7 different indoor air polluting factors on allergic sensitization at 2 years of age. We hypothesize exposure to these factors will increase the OR of a positive SPT at 2 years of age, and that SPT positive children will have more of these exposures than SPT negative children.

1.3 Objective 3: Identify connections between prenatal and current exposure to air polluting factors with cellular, genetic, and clinical outcomes at 6 to 7 years of age

Here our goal is to analyze the relationship between prenatal and current exposure to indoor and outdoor air pollution with changes in Th17 frequencies, Treg frequencies and *FOXP3* expression, cytokine profiles, and allergic and respiratory outcomes at 6 to 7 years of age. We hypothesize exposure to air polluting factors will increase the OR of these clinical outcomes, and influence Th17 frequencies, Treg frequencies, *FOXP3* expression, and the cytokine profile. We also hypothesize children with atopic outcomes will have increased Th17 frequencies, decreased Treg frequencies, reduced genetic expression of *FOXP3*, and an altered inflammatory cytokine signature.

Chapter 2

Literature Review

2.1 Atopic diseases and the atopic march

Diseases with a predisposition to hypersensitivity, such as allergies, asthma, and AD have become common in westernized countries (17). These diseases occur when there is dysregulation in the immune system and can be influenced by both genetic and environmental factors (17). In children, a phenomenon known as the "atopic march" has been observed, in that children who develop AD have a tendency to progress to AR and asthma later in childhood (2). AD is characterized by chronic pruritic skin inflammation and has an increased prevalence in children (18). AR results from inflammation of the nose caused by exposure to allergen and leads to symptoms like nasal itching, sneezing, nasal congestion, ocular itching, and cough (19). AR is estimated to affect 2 to 25 % of children, and while it is not associated with severe morbidity, it can greatly reduce the quality of life due to difficulty sleeping and decreased school productivity during peak pollen season (19). Asthma is a chronic respiratory inflammatory condition characterized by airway hyper-responsiveness, airway remodeling, and edema (20). It produces symptoms of wheeze, cough, shortness of breath, and/or chest tightness (20). In Canada, asthma is the most common chronic respiratory disease in children and it is estimated that 13% of children will be diagnosed with asthma by the age of 5 (21). Furthermore, this pediatric population is at greater risk of adverse health outcomes as primary prevention strategies are limited; partially due to the lack in understanding of early life asthma etiology (22).

2.2 Diagnosis of atopic diseases

There is not a specific diagnostic laboratory marker for AD and is instead made clinically (23). The diagnostic criteria includes: pruritus, common appearance and distribution of rash, in a chronic or reoccurring course, and atopic history (23).

Allergic rhinitis is diagnosed by medical history with symptoms of rhinorrhea, nasal obstruction, and ocular symptoms, with the production of a positive wheal following a SPT, and/or the presence of serum-specific immunoglobulin (Ig) E to a relevant aeroallergen (24).

Asthma is formally diagnosed through a combination of patient history and symptoms of recurrent dry cough, wheeze, chest tightness, and/or dyspnea, with lung function testing via spirometry and/or methacholine challenge (25). Due to the maneuvers required in spirometry, it is not accurate in preschool aged children; therefore, to diagnose this population, detailed family and medical history in conjunction with allergy testing (if suspected allergen triggered) is suggested until a formal diagnosis can take place (21,26,27).

2.3 The role of family history in allergic diseases

The connection between a family history of atopic disease and disease development in the child has been established in the literature for decades (28–30). This suggests a genetic component of disease as it is clear that maternal and paternal allergic status contributes to child atopic risk; however, evidence suggests their influence in not equal (31–33). For example, maternal allergic status is believed to have a stronger bearing on childhood atopy; where maternal asthma, AD, and rhinitis are each associated with disease development of the same type in their offspring (31–33). In contrast, paternal asthma; but not rhinitis or AD, is associated with childhood asthma (32). These parental differences are potentially results of the dynamic maternal-fetal interactions that occur during pregnancy. Additionally, the Isle of Wight Birth Cohort report differences in inheritance patterns that are dependent on the sex of the child (32). For example, maternal asthma status increased the incidence of asthma in girls but not boys 4 to 18 years of age, whereas paternal asthma increased this incidence in boys but not girls (32). Researchers hypothesize sex-specific inheritance pattern may be a result of epigenetic imprinting; wherein there is preferential expression of certain parental alleles in somatic cells of the offspring (32). Moreover, these inheritance patterns of atopy have been established in that children are more likely to develop the same atopic disease as their parents, rather than different allergic or respiratory diseases (34).

2.4 Th1, Th2, Th17, Tregs and atopy

2.4.1 Th1/Th2 paradigm

It has been accepted in the literature that there is a shift in the ratio between Th1 and Th2 cells in allergic diseases, as an increase in Th2 and decrease in Th1 cells and cytokines has been reported for those that suffer from allergies (35,36). This alteration is considered the Th2 shift. This shift has been examined in umbilical cord blood (CB) samples and results suggest a decrease in this ratio at birth in children with AD compared to healthy children (37). Furthermore, in an examination of peripheral cytokine concentrations, an increase in Th2 cytokines and chemokines, and a decrease in Th1 cytokines have been associated with atopic diseases in children (38,39).

Th1 cells are marked by their large production of interferon gamma (IFN-γ) and are best suited to defend the body against intracellular pathogens like *Leishmania major* and *Mycobacterium tuberculosis* (40,41). Th2 cells are involved in the clearing of extracellular pathogens such as helminths, they produce large quantities of IL-4, IL-5, IL-10, and IL-13, and are involved in class switching of IgG1 and IgE (35,42). Th1 and Th2 effector cytokines cross regulate each other and dysregulation of this is implicated in disease (43).

2.4.2 Th1 differentiation

The differentiation of naïve T cells into effector T helper cells requires signals from the T cell receptor (TCR), co-stimulatory molecules such as cluster of differentiation (CD) 3 and CD28, cytokines, and lineage specific transcription factors (44). IL-12 promotes IFN-γ production and induces Th1 development via activation of signal transducer and activator of transcription (STAT) 4 (43,44). T box protein expressed in T cells (T-bet) is the master regulator for Th1 differentiation and IFN-γ production, and is induced upon activation by STAT1 (45).

2.4.3 Th2 differentiation

Similarly to Th1 differentiation, the development of Th2 cells involves a shift from a naïve T cell into effector cells by TCR signaling, costimulatory molecules, and the presence of specific cytokines and transcription factors. Binding of IL-4 to the IL-4 receptor will induce STAT6 activation and begins Th2 differentiation (43). GATA binding protein 3 (GATA3) is the master regulator of Th2 differentiation and is induced by IL-4 through STAT6 (45). GATA3 allows for the opening of the IL4-IL5-IL13 locus in order for other transcription factors required for Th2 differentiation to access the locus

(45). GATA3 also prevents the expression of IFN-γ, and builds a positive feedback loop with IL-4 to enhance GATA3 expression and Th2 differentiation (45).

2.4.4 Th17 in allergies and asthma

Th17 cells were discovered in 2005 as a distinct CD4+ T helper cell population with unique cytokine production and function (46). These cells were marked by their production of IL-17 and have been implicated in clearing extracellular bacteria and fungi from the host (47). Th17 cells have been investigated in asthma, and increased levels of IL-17 in lung biopsies and bronchoalveolar lavage samples of patients with asthma has been reported (48,49). Additionally, Th17 cells have been examined in eosinophilic asthma, and findings suggest Th17 cells may first act as an initiator of the Th2 response and later as a suppressor during the effector phase of asthma (50). Th17 cells have also been implicated in AR, where serum levels of IL-17 correlate with disease severity (51).

2.4.5 Th17 differentiation

As with Th1 and Th2 differentiation, Th17 differentiation requires the presence of specific cytokines and stimulating factors to induce the shift from a naïve T cell to a mature T cell. Combinations of TGF-β, IL-6, IL-21, and IL-1β are required to produce this shift (52). Th1 cells are efficient producers of IL-21, and IL-21 in combination with TGF-β amplifies the Th17 differentiation process. Is it suggested that IL-6 induces IL-21 production in Th17 cells and self-amplifies to increase a Th17 response (44). IL-6, TGF-β1 and IL-21 act in combination to activate STAT3; an important transcription factor for IL-17 production (53). Similarly to Th1 and Th2 cells, Th17 cells have a cell specific transcription factor required for differentiation and restricted cytokine production (53).

Retinoic acid-related orphan receptor gamma(t) (RORγt), is the transcription factor unique to Th17 cells and is required for differentiation (53).

2.4.6 Treg in asthma and allergy

Tregs are a CD4+ T cell population that play a role in peripheral tolerance and control inflammation during infection, tissue repair, and hypersensitivity amongst other biological processes (16). They have the capability to upregulate immunosuppressive molecules and tissue homing receptors to regulate inflammatory responses (54). They produce of IL-10 and TGF-β, and have been shown to be reduced in number in bronchoalveolar lavage samples in asthmatic individuals (12,14). Additionally, the frequency of peripheral Tregs in allergic children and children with AD or food allergy has been demonstrated to be reduced compared to healthy children (55). This difference has also been observed at birth, where children with lower cord blood Treg frequencies had an increased risk of AD and sensitization (56). Given the regulatory properties of Tregs, the differences observed in the Th1/Th2 ratios and Th17 cells in allergy and asthma, it is likely that the altered frequency and functioning of Tregs plays a role in disease development (16).

2.4.7 FOXP3 expression in asthma and allergy

FOXP3 is the Treg specific transcription factor. Its expression is mediated by epigenetic modification of conserved non-coding sequences and is required for proper functioning and differentiation of Treg cells (16,57). Given FOXP3's crucial role in Treg development and function and the differences observed in Treg frequencies in allergic diseases, its potential involvement in disease pathogenesis has been investigated.

In children with asthma and food allergy, mRNA levels of *FOXP3* was reported to be significantly lower than in healthy children (15). Additionally, IgE levels have been investigated with *FOXP3* expression, in that high *FOXP3* expression was correlated with low IgE levels (58). These differences in expression are speculated to be a result of epigenetic alternations.

Epigenetic alterations are modifications in the in the structure of DNA via methylation or acetylation for example, and these changes can be influenced by our environment (31,59). These modifications affect the rate of transcription, and therefore gene expression, which in turn can lead to altered cellular functioning. With respect to atopy, hypermethylation of the *FOXP3* region in cord blood samples has been shown to increase the risk of atopy at 3 years of age (13). Furthermore, methylation at the *FOXP3* locus was higher in children with diesel exhaust particle (DEP) exposure and this hypermethylation increased the risk of childhood wheeze and asthma (60). When combined, these findings support that epigenetic modification is important in Treg functioning and may be influenced by environmental factors.

2.5 The use of cord blood to study predictors of atopy

As previously mentioned, cellular and genetic components in CB have been investigated under the lens of atopic disease development. The use of CB is attractive as it can provide researchers with a biological sample where the maternal-fetal interface can be studied. Moreover, difficulties of receiving ethical clearance and obtaining consent from parents/assent from children as the child ages are generally avoided, as CB can be easily collected from the placenta post-delivery in a non-invasive manner. If a reliable and predictive biomarker of atopic risk or development could be identified in CB samples, newborns could be screened at birth and their risk of developing atopy could be determined. This could allow for clinicians to suggest preventative measures to children who would benefit most from them. While the use of CB to identify biomarkers of atopy is interesting, it is important to note the source of CB is from the mother and thus the immune system cells and cytokines from this sample would be more indicative of the maternal immune status rather than the newborn's.

2.6 Developmental origins of health and disease

The field of developmental origins of health and disease; often referred to as the "Barker Hypothesis" after one of its leading investigators, describes that our environment in early life can contribute to adverse changes in metabolism or physiology and increase adult disease risk (61,62). Initial findings from this area of research suggested that small birth weight was associated with an increased risk of cardiovascular disease, dyslipidemia, hypertension, and type 2 diabetes in adulthood (63–65).

This hypothesis has additionally be described by the term "developmental plasticity", wherein environmental factors influence genomic expression in the offspring (66). These factors are speculated to operate via epigenetic mechanisms, and given their reversible nature and their sensitivities to environmental influences, a potential mechanistic link between developmental programming, environmental exposures, and later disease risk has been proposed (31). Given the increase in prevalence of allergic disease and the inability for genetics alone to explain this increase, the Barker hypothesis has been examined regarding outcomes of allergy and asthma in childhood (5,26,31).

2.7 Outdoor air pollution exposure

Outdoor air pollution (OAP) generally refers to ozone, nitrogen oxides, and particulate matter (67). Ozone is an oxidizing agent naturally formed in the troposphere, nitrogen oxides are generally produced anthropogenically, and particulate matter consists of small particles of solid, liquid, or liquid and solid suspended in the air. Individually, these compounds have toxic effects on the lung, but it is important to note that they each are potent oxidizers that can act directly on lipids and proteins, or indirectly through intracellular oxidant pathways (67,68). For example, acute ozone exposure has been demonstrated to induce airway inflammation and reduce pulmonary function in both healthy and asthmatic individuals (69,70). Ozone is capable of acting as an adjuvant with respect to allergy, and has been observed to enhance the allergic response to inhaled allergen (71). Similarly, nitrous oxide compounds can act as an adjuvant to the allergic response, due to their acidic nature and capability of causing respiratory damage (72,73). Moreover, exposure to particulate matter from DEP has been shown to increase DNA methylation in circulating mononuclear cells in regions responsible for inflammation and oxidative stress responses (74).

Exposure to OAP during the prenatal and early life windows has been linked to cellular changes as well as allergic and respiratory outcomes in children. For example, in the EDEN birth cohort, particulate matter exposure in each trimester was associated with altered immune cell profiles in cord blood (8). Researchers report that for every 10 ug/m^3 increase in particulate matter exposure 3 months before pregnancy and during each trimester there was a reduction in cord blood Treg numbers (8). In a Canadian birth cohort, the effect of ambient air pollution exposure during pregnancy on child asthma diagnosis was analyzed, and researchers reported asthmatic children had elevated mean exposures for nitrous oxides, carbon monoxide, and particulate matter compared to non-asthmatic children (75). Moreover, prenatal exposure to particulate matter was associated with an increased odds ratio for asthma diagnosis (75). This relationship has also been observed when considering current exposure to OAP. In a pooled analysis of 6 birth cohorts, there was an increased odds ratio (OR) for current asthma, ever asthma, and current wheeze in children with exposure to traffic related particulate matter (76). These findings highlight the influence OAP may have on childhood asthma symptoms and development.

2.8 Environmental tobacco smoke exposure

Similarly to OAP, environmental tobacco smoke (ETS) has been implicated in atopic diseases. This exposure has been related to altered DNA methylation patterns in cord blood, and findings from the Taiwan Birth Panel Cohort report differences in DNA methylation patterns in the thymic stromal lymphopoietin (TSLP) gene between children with and without exposure to cigarette smoke prenatally (77). This cohort study also report a connection to an increased risk of AD with prenatal smoke exposure (77). In a pooled analysis of 8 European birth cohorts, maternal prenatal smoking was associated with asthma and wheeze in children 4 to 6 years old (78). Direct and indirect cigarette smoke exposure during pregnancy have also been evaluated with childhood respiratory disease; where direct cigarette smoking during pregnancy is associated with an increased risk of wheeze and chestiness, and indirect second-hand smoke exposure increases the risk of childhood cough (6).

In addition, children with second-hand smoke exposure during early life have an elevated risk of asthma in childhood and into adolescence (79,80). Moreover, different levels of exposure to tobacco smoke have been estimated through urine cotinine levels; and a correlation between elevated cotinine with reduced pulmonary function testing, increased asthma symptoms, and frequent asthma exacerbations in children have been reported (81). This highlights that cigarette smoke exposure in early life can influence asthma development but also trigger symptoms.

2.9 Air freshener and candle exposure

The role of indoor air pollutants like air fresheners and candles on allergic disease progression has recently become a focus of research due to links between exposure to these compounds and an increased risk of allergic or respiratory outcomes (5). Given the increase in the average amount of time spent indoors there is an increased risk of adverse health outcomes related to chronic low level exposure to these compounds (70). In a cross-sectional study of in home candle use and respiratory health outcomes, researchers report lower lung function and differences in leukocyte differential counts in adults with candle use (82). The exact mechanisms as to how these exposures can induce these changes is not clear; however, the presence of phthalates in these compounds is suspected to be involved.

Phthalates are endocrine disruptors that are found in plastics, cosmetics, and scented products, amongst others (83). These compounds are capable of evaporating into the atmosphere which allow for exposure to children via breathing or through their skin (83). These compounds have not been shown to bioaccumulate, however, continuous daily exposure could create a scenario that would resemble an exposure similar to bioaccumulative compounds (83). Additionally, in a study of phthalate exposure during pregnancy in rats, transgenerational epigenetic alterations in offspring was reported (84). Furthermore, prenatal exposure to phthalate containing products was associated with an increased risk of AD at 2 years (85). When combined, these findings propose a potential link, wherein phthalate exposure induces epigenetic modifications which influences later atopic disease progression.

2.10 Mould and dampness exposure

Mould grows best in warm and damp environments like basements, bathrooms, and window frames (86). *Aspergillus* and *penicillium* are the common indoor moulds, and *Alternaria* is relevant for both indoor and outdoor settings. Mould and dampness in the home has been associated with adverse respiratory outcomes, where children living in mould infested dwellings have an increased risk of asthma (87). Moreover, it is postulated that due to their small size, fungal spores are able to penetrate into the alveolar regions to elicit their effects, although, studies describing this mechanism are lacking (70,88). Furthermore, in a systematic review of mould exposure and allergic and respiratory outcomes, visible mould in the home was associated with child asthma exacerbations (89). Mould and dampness in the home is often associated with an increase in other contaminants such as fungal growths, mycotoxins, an endotoxins; which have also been implicated in allergic diseases (89).

2.11 Dog and cat exposure

Recent evidence has indicated that pets like cats and dogs have the capability to influence the home's microbiome, and this has implications on the developing immune system of the child (3). Cats and dogs have each been shown to change the diversity and richness of microbial communities in the home as well as within this child's gut microbiome (3). For instance, fecal microbial taxa were evaluated in a subset of the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, and findings report increases in *Bifidobacteriaceae* and *Peptostreptococcaceae* in children with pets at home (90). Additionally, pet exposure to cats and dogs have been shown to have protective effects in terms of atopic disease development. For example, dog exposure in early life has been shown to reduce the risk of asthma diagnosis (91), and cat exposure has demonstrated the same effect (92). These influence are not conclusive, as conflicting findings have been reported in the literature, in that both dog and cat exposure increases the risk of allergic sensitization in childhood (93). Therefore, additional research on theses exposures and their influence on atopy is required.

2.12 House dust mite exposure

House dust mite is very common in the home and is a major source of indoor allergen. *Dermatophagoides pteronyssinus* and *Dematophagoies farinae* are two of the most common dust mite species, although their frequencies vary globally (94). These species have a life expectancy of 4 to 6 weeks, however they have a fast reproductive cycle that can colonize a home within one year (94). Given the perennial nature of this dust mite, there is a chance for chronic allergen exposure which may influence allergy and asthma outcomes (94).

Dust mite exposure during pregnancy and its implication on allergy and asthma development has been examined. For example, mice exposed to house dust mite during pregnancy had offspring with increased airway hyper-responsiveness, airway inflammation, and production of type 2 cytokines (95). Additionally, children with allergic sensitizations have significantly more dust mite exposure in the first 3 years of life compared to those without sensitizations (96).

2.13 The use of birth cohorts to study atopic diseases

Given the multiple factors that have been investigated and implicated in atopic disease development and the lack of complete understanding as to how these exposures elicit their effects, the use of large scale birth cohorts presents a useful opportunity to study these factors (4). Research groups utilizing these cohorts have the capability to gather detailed exposure data on diverse populations and can connect the timing of exposures with the onset of disease. Unfortunately, findings from birth cohort studies have been conflicting; which may be a result of different exposure classifications, differences in outcome definitions, or missing account of confounding variables such as sex, family history, or exposure to some of the factors previously mentioned (4). However, with several birth cohorts currently underway aimed at addressing some of these pitfalls, we can only expect a deeper pool of knowledge of what factors are important for atopic diseases development. Additionally, as more information on these factors arise, research groups using birth cohort studies could adjust their dataset to include additional exposure data and create complex models to incorporate newly discovered factors that influence atopy with previously established ones.

The overarching goal of this type of research is to identify children at an increased risk of allergy and asthma, and to determine what factors can contribute to and/or slow their disease progression. These birth cohort studies have been imperative in contributing evidence to develop current policy and guidelines on preventing atopic disease progression, like the NIAID guidelines for food allergy (97).

We have established a prospective birth cohort in Kingston, Ontario known as the Kingston Allergy Birth Cohort (KABC) to study these types of influences with allergic and respiratory disease outcomes in children. We have detailed home environment data and/or umbilical cord blood samples for over 500 mother child pairs (5). Moreover we have a diverse sample population consisting of children from urban and rural environments, from diverse socioeconomic backgrounds and with unique exposure profiles (5). This diversity is beneficial as it provides a unique population to assess gene-environment interactions and atopic disease outcomes.

Chapter 3

Material and Methods

3.1 Cohort design

The KABC is a prospective birth cohort that received ethical clearance from the Queen's University Health Sciences Research Ethics Board (DMED-1161-08) and was established in 2011. Healthy pregnant women 18 years or older, in the second or third trimester of their pregnancy were recruited into the study via posters in Kingston Health Sciences Centre – Kingston General Hospital site (KHSC-KGH). If participants responded positively to a query regarding the KABC from a health care professional, they completed an informed consent session with a study worker after which they provided written informed consent to donate their CB, with the additional option to participate in any future follow-up visits. After consent was obtained but prior to delivery, mothers completed a prenatal survey which captured information on parental atopic status in addition to maternal home environment during pregnancy. A schematic breakdown of the participants involved in each KABC project is summarized in Figure 1.

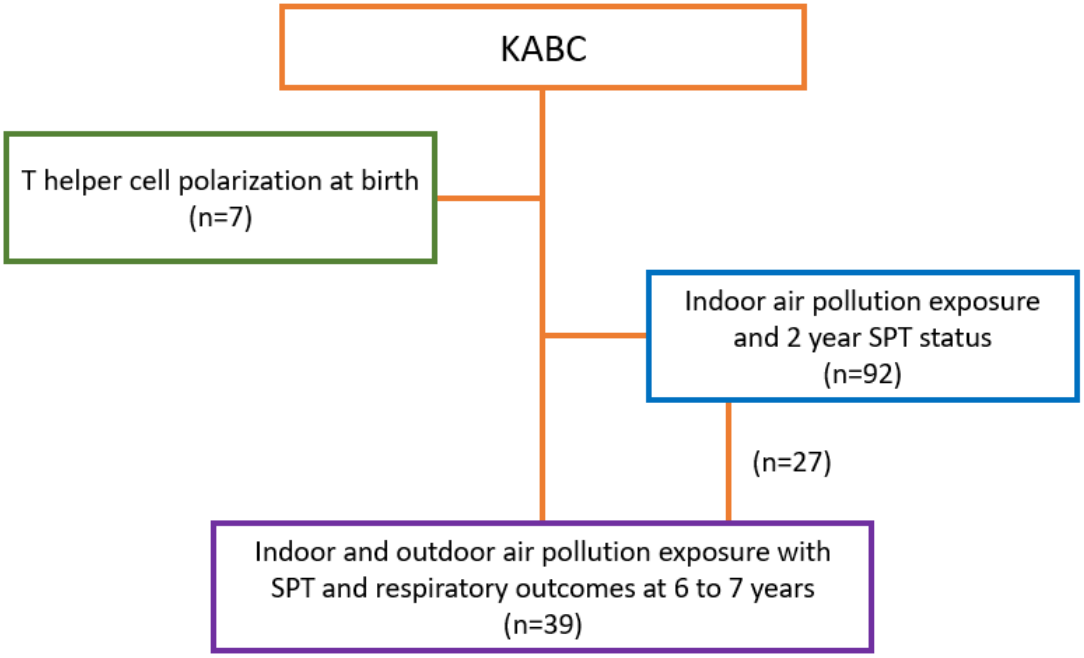

Figure 1. Overview of participant involvement in each project.

7 mother-child pairs were involved in the T cell polarization work. 92 different mother-child pairs were followed up to 2 years of age and participated in the 2 year outcome work. Of these pairs, 27 completed the 6 to 7 year follow-up visit with 12 additional mother child pairs in the KABC.

3.2 T helper cell frequency and function following polarization in high and low atopic risk CBMCs

3.2.1 Participants

A subset of 7 mother-child pairs from the KABC were included in this pilot project. Child atopic risk was assigned as either low risk (LR, n = 4) if the mother reported no allergies and/or asthma for herself or high risk (HR, n = 3) if she self-reported her own allergies and/or asthma. Information on cigarette smoking during pregnancy was collected and each woman in this pilot project reported no smoking during pregnancy.

3.2.2 Umbilical cord blood collection and processing

CB was collected from the placenta following caesarean section delivery in a 60 mL syringe containing 2 mL heparin. Samples were incubated in 1 % Dextran (Acros Organics, ThermoFisher Scientific) at 37 ° C for 2 hours to deplete red blood cells. Following this incubation CBMCs were isolated from the supernatant and layered over Ficoll-Paque PLUS density gradient media (GE Healthcare Life Sciences). CBMC samples were then obtained from the “buffy coat”, suspended in freezing media containing McCoys 5A media (ThermoFisher Scientific) supplemented with 10% dimethyl sulfoxide (DMSO) (Acros Organics, ThermoFisher Scientific) and 50 % fetal bovine serum (FBS) (Wisent), and frozen at – 80 ° C for later use.

3.2.3 Nonadherent mononuclear cell isolation

To remove any potential adherent cells like monocytes from the CBMC samples and to obtain a sample rich in nonadherent cells like T cells, a nonadherent mononuclear cell (NAMNC) isolation step was performed (adapted from (98)). Frozen CBMC samples were thawed rapidly at 37 ° C and suspended in warmed McCoy’s 3+ media containing McCoy’s 5A media supplemented with 15 % FBS, 1 % penicillin-streptomycin (Thermofisher Scientific), and 1 % 2-mercaptoethanol (Acros Organics, ThermoFisher Scientific). Cells were washed and centrifuged twice at 400 g for 10 minutes and resuspended in McCoy’s 3+ media at 1.0×10^6 cells/mL. Samples were plated on a tissue culture flask and incubated for 2 hours at 37 ° C and 5 % CO_2. After the incubation, NAMNCs were obtained and immediately cultured for T cell polarization.

3.2.4 T cell polarization

Following the NAMNC isolation, samples were washed and resuspended in warmed RPMI-1640 media (ATTC modificiation) (Gibco, ThermoFisher Scientific) supplemented with 10 % FBS, 1 % non-essential amino acids (Gibco, ThermoFisher Scientific), 0.1 % penicillin-streptomycin and 50 uM 2-mercaptoethanol. Cells were seeded at $1x10^6$ cells/well in a 24 well primeria plate in Th1 polarizing (n = 7, LR = 4, HR = 3) (100 U/mL human recombinant IL-2, 10 ng/mL human recombinant IL-12, and 2 ug/mL anti-human IL-4), Th2 polarizing (n = 7, LR = 4, HR = 3) (100 U/mL human recombinant IL-2, 10 ng/mL human recombinant IL-4, and 2 ug/mL anti-human IL-12), Th17 polarizing (n = 5, LR = 3, HR = 2) (10 ng/mL human recombinant IL-1β, IL-6, and IL-23, 5 ng/mL human recombinant TGF-β, and 2 ug/mL anti-human IL-4 and anti-human IL-12), and a non-polarizing (NP) control (n = 7, LR = 4, HR = 3) (100 U/mL human recombinant IL-2, 10 ng/mL and 2 ug/mL anti-human IL-4 and anti-human IL-12) (all from Biolegend) (Th1 and Th2 polarization conditions adapted from (99) , and Th17 adapted from (100)). All culture conditions were stimulated with ImmunoCult ™ Human αCD3/αCD28 T cell activator (StemCell Technologies) as indicated from the manufacturer. Cells were incubated for 7 days at 37 ° C and 5 % CO_2. On day 3 100 U/mL of IL-2 was added to the Th17 culture conditions and on day 4 100 U/mL of IL-2 was added to the Th1, Th2 and NP culture conditions.

3.2.5 Flow cytometric analysis

Prior to the NAMNC isolation CBMCs were stained to determine the frequencies of immature and mature T cells. CBMCs were thawed rapidly at 37 ° C, washed and centrifuged at 400 g twice in McCoy's 3+ media. $4x10^6$ cells were resuspended in McCoy's 3+ and stimulated for 5 hours at 37 ° C and 5 % CO_2 with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) containing phorbol 12-myristate 12-acetate (PMA), ionomycin, brefeldinA and monensin **(**ThermoFisher Scientific). Following stimulation, $1.2x10^6$ cells from each sample were isolated and washed twice in Dulbecco's phosphate buffered saline (DPBS) (Thermofisher Scientific) and centrifuged at 400 g. Samples were incubated for 25 minutes at room temperature in Zombie Yellow Fixable Viability Kit (BioLegend). 500,000 cells were fixed and permeabilized using the PerFix-nc (no centrifugation) kit (Beckman Coulter). Cells were washed twice in DPBS, centrifuged at 400 g for 10 minutes, and were incubated in PerFix buffer 1 (Fixative reagent) (Beckman Coulter) for 15 minutes at room temperature. Cells were washed once in DPBS and centrifuged at 400g for 5 minutes followed by an incubation for 45 minutes at room temperature with FBS, PerFix-buffer 2 (Permeabilization reagent) (Beckman Coulter) and human monoclonal antibodies for extracellular and intracellular staining. Duraclone T helper cell panel tubes (Beckman Coulter) containing dry-coated human monoclonal conjugated antibodies were used in combination with liquid human monoclonal antibodies (Biolegend). The complete antibody panel includes: Alexa-Fluor-750-(AF750)-conjugated-CD3, Allophyocyanin-(APC)-conjugated-CD4, Fluorescein-Isothiocyanate-(FITC)-conjugated-IFN-γ, R-Phycoerythrin-Cyanine-7-(PC7)-conjugated-IL-4, Pacific-Blue-(PB)-conjugated-IL-17a (Duraclone, Beckman Coulter), Brilliant-Violet-785™-(BV785)-conjugated-CD45RA and phycoerythrin-(PE)-conjugated-

CD45RO (Biolegend). Following this incubation cells were washed and resuspended in PerFix buffer 3 (Final solution) (Beckman Coulter) and immediately analyzed with the cytometer (CytoFLEX S, Beckman Coulter). An unstained cell sample and single colour compensation controls were completed for each fluorescent dye with VersaComp beads (Beckman Coulter) and ArC Amine Reactive Compensation beads (ThermoFisher Scientific). Samples were analyzed using the FlowJo Software (Flowjo).

On day 7 of the polarization culture, cells from each condition were stimulated for 5 hours at 37 ° C and 5 % CO_2 with eBioscience Cell Stimulation Cocktail. Cells were harvested and stained following the identical staining protocol for the uncultured CBMCs.

3.2.6 Cytokine supernatant analysis

Cell culture supernatants were extracted on day 7 of the polarization culture and stored at – 80 ° C for future analysis. Samples were thawed and cytokine concentrations were determined using the Milliplex Human High Sensitivity T cell kit (Millipore Sigma) as per protocol. The following analytes were measured: IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-21, IFN-γ, and Tumor necrosis factor alpha (TNF-α). IFN-γ, IL-13, and IL-8 exceeded the limit of detection for this assay and were subsequently analyzed individually using target specific human ELISA Ready-SET-go kits as per protocol (Fisher Scientific).

3.2.7 Statistical analysis

Wilcoxon tests were used to compare the frequencies of naive and mature T cells at baseline and following polarization for each culture condition. T helper cell frequency in each polarization condition were compared using Wilcoxon tests. Mann-Whitney tests were used to compare T helper cell frequencies following polarization in high and low atopic risk samples. The culture supernatant cytokine concentrations were compared between polarization condition and between the high and low atopic risk groups using the Mann-Whitney test. All statistical analyses were conducted using the GraphPad Prism 8.0 software (GraphPad).

3.3 The relationships between early life exposures to indoor air polluting factors and allergic sensitization at 2 years of age

3.3.1 Participants and exposure assessment

A subset of 92 women (108 mother-child pairs) from the KABC were included in this analysis and completed a prenatal survey capturing information on their home environment during pregnancy prior to the delivery of their child. These women completed additional surveys when their child was 6 months (n=76), 1 year (n=72), and 2 years (n=82) of age. Prenatal and postnatal exposure to dogs, cats, mould, carpet, air fresheners, candles/incense and ETS was captured for each time point. If the mother reported any of these exposures for herself during pregnancy or her child at the 6 month, 1 year, or 2 year timepoints, the child was classified as exposed.

3.3.2 Skin prick testing

At two years of age, children returned to the research site for skin prick testing. The SPT was completed by a trained doctor or nurse. A panel of 14 common food and environmental allergens were included in the SPT panel, in addition to a positive control (histamine) and a negative control (glycerinated phenol-saline). The allergens tested on the panel were as follows: dog hair, ragweed mix, cat pelt, grass mix, dust mite mix, mould mix, tree mix, peanut, soy, tree nut mix, sesame, cow's milk, wheat, and egg white. The SPTs were read 15 minutes after application and a positive SPT result was defined as a wheal size 3 mm larger than the negative control.

60 mothers completed skin pick testing 6 or more months following the delivery of their child. The test was performed by a trained doctor or study nurse. The panel of allergens for the mother's SPT included the positive (histamine) and negative (glycerinated phenol-saline) controls in addition to the following environmental allergens: ragweed (short), *Dermatophagoides pteronyssinus*, *Dermatophaoides farinae*, (dust mite species) cat pelt, dog hair, grass mix, tree mix, Russian thistle, and Alternaria mould. The SPT was performed as outlined for the child's SPT.

3.3.3 Statistical analysis

Baptista-Pike and Fisher Exact tests were used to determine the odds ratios associated with each individual exposure and any positive SPT result at 2 years of age. Mann-Whitney tests and Chi Square tests for trend were conducted to assess the relationships between the number of different exposures with any SPT positive result and the effect of multiple exposures at each time point on positive SPT outcomes. All statistical analyses were conducted using GraphPad Prism 8.0. No corrections for multiple comparisons were made due to sample size limitations.

3.4 Connecting environmental exposures with allergic and respiratory outcomes at 6 to 7 years of age

3.4.1 Participants, exposure assessment, and follow-up visit

39 mother-child pairs from the KABC were included in this project and were followed from the child's birth to 6 to 7 years of age. Mothers completed a prenatal

survey prior to delivery of their child which captured detailed information on the pregnancy as well as the maternal exposures to the following air polluting factors during pregnancy: ETS, cats, dogs, candles/incense, mould, air fresheners, carpet in the home, and OAP. OAP exposure was defined as report of the home within 100 m of any of the following: major road, factory, farm, gas station, paring lot, or construction. Mothers were classified as exposed to the factor if they reported "yes" to the exposure on their survey. Level of indoor air pollution (IAP) exposure was determined and classified as low or high based on maternal report of 0 to 2 or 3 or more respectively of the following indoor air pollutants: ETS, cats, dogs, candles/incense, mould, air fresheners, and carpet.

At 6 to 7 years of age, the children returned to the research site for a follow-up visit. Prior to any study visit related tasks, the parent and child completed an informed consent and assent session with a study team member. After this session, the parent completed a new age-specific survey which provided information on the current home environmental exposures for the child. The exposures captured at this time point were the same as the prenatal time point exposures, except for carpet exposure which was not determined. Similarly to the prenatal survey, if the parent indicated an exposure on the survey the child was classified as exposed to that factor.

3.4.2 Skin prick testing and respiratory outcome classification

Children completed an updated SPT at 6 to 7 years of age. Mothers completed a SPT 6 or more months after delivery of their child. The panel for both the mother and child SPT were identical to panels described in 3.3.2 with identical positive and negative controls. Both SPTs were performed as outlined in 3.3.2.

Children were defined as having respiratory symptoms if parents responded yes to a question regarding whether or not their child has had a cough and/or wheeze without a cold within the last year.

3.4.3 Nasal brush collection

38 mother-child pairs consented and assented to provide a nasal brush (NB) sample. A trained physician used a cytobrush to take a sample of the nasal tissue of both the left and right nasal cavity of the child. The brush was cut and stored in a cryovial. The vial was flash frozen in liquid nitrogen immediately after the sample was obtained and was stored at – 80 ° C for future use.

3.4.4 Blood collection and processing

24 mother-child pairs consented and assented to provide the child's blood sample. Peripheral blood was obtained by a trained phlebotomist and collected into heparin and ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Biosciences). EDTA coated tubes were centrifuged at 1300 g for 10 minutes. Plasma samples were isolated and stored at – 80 ° C for later analysis. Peripheral blood mononuclear cells (PBMC) were isolated from the heparin tubes using Ficoll-Paque PLUS density gradient media. PBMCs were aliquoted, stored in freezing media, and frozen at – 80 ° C for later use.

3.4.5 Treg/Th17 Flow cytometry

PBMC samples were thawed rapidly at 37 ° C and were washed and centrifuged at 400 g twice in McCoy's 3+ media. $4x10^6$ cells were resuspended in McCoy's 3+ media and stimulated with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) containing PMA, ionomycin, brefeldinA, and monensin. These cells were incubated for 5 hours at 37 ° C and 5 % CO_2. The remaining unstimulated cells were refrozen in freezing media. After the incubation period $1x10^6$ were washed twice with PBS and incubated with Zombie Yellow Fixable Viability kit (BioLegend). Cells were washed in flow cytometry buffer (BD Biosciences) and stained using a human Th17/Treg phenotype kit (BD Biosciences) as per manufacturer's instructions. The kit contained the following fluorescent human monoclonal antibodies: Peridinin-chlorophyll-protein-complex-(CY5.5)-conjugated-CD4, Alexa-Fluor®-647-conjugated-(AF647)-FOXP3, PE-conjugated-IL17A. The appropriate singe colour compensation controls were completed using VersaComp Beads and ArC Amine Reaction compensation beads. Samples were stored at 4 ° C until acquisition with the cytometer (CytoFLEX S). Samples were analyzed using the FlowJo software.

3.4.6 Plasma cytokine quantification

Previously frozen plasma samples were thawed and cytokine concentrations for IL5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-21, TNF-α, and IFN-γ were measured using the Milliplex Human High Sensitivity T cell kit as per protocol. TGF-β1, TGF-β2, and TGF-β3 plasma concentration levels were measured using the Milliplex TGF-beta 1, 2, 3 Magnetic bead kit (Millipore Sigma) as per protocol.

3.4.7 RNA extraction

PBMC samples (n=23) were rapidly thawed at 37 ° C and washed in McCoy's 3+ media. Up to $6x10^6$ cells from each sample were used for RNA isolation. RNA was isolated using the All Prep ® DNA/RNA/Protein kit (QIAGEN) as indicated in the protocol. RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA samples were stored at -80 ° C until cDNA synthesis.

Cryovials containing nasal brush samples (n=23) were removed from liquid nitrogen storage and thawed at room temperature. RLT buffer from the All Prep ® DNA/RNA/Protein kit was added to each cryovial. Samples were immediately vortexed and centrifuged to remove all tissue and lysate from the brush. Following this step, samples were processed using the All Prep ® DNA/RNA/Protein Kit as indicated in the manufacturer's protocol. RNA concentration for each sample was measured with the NanoDrop 1000 spectrophotometer and stored at – 80 ° C until cDNA synthesis.

3.4.8 Reverse transcription

Previously frozen RNA samples from PBMCs and NBs were thawed on ice and samples were diluted to 40 ng/mL for the reverse transcription reaction using nuclease free water. cDNA was synthesized using the Quantitect Reverse Transcription kit (QIAGEN) as described in the manufacturer's protocol. Genomic DNA contamination was eliminated prior to the reverse transcription reaction using the gDNA wipeout buffer (QIAGEN). Reverse transcription reactions were completed in a thermocycler (CFX Connect, Bio-Rad Laboratories, Inc) for 15 minutes at 42 ° C and 3 minutes at 95 ° C. No reverse transcription (NRT) controls were completed to ensure no gDNA contamination. cDNA samples and NRT controls were aliquoted and stored at – 20 ° C for later analysis.

3.4.9 Evaluation and selection of reference gene

A reference gene analysis was performed to determine the most stable reference gene as per the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (101). A reference gene panel of 14 candidate genes was purchased (Bio-Rad) and included the following candidate reference genes: *ACTB, B2M, G6PD, GAPDH, GUSB, HMBS, HPRT1, PGK1, RPL13A, RPLP0, RPS18, TBP, TFRC, YWHAZ*. 3 SPT positive and 3 SPT negative PBMC and NB cDNA samples diluted 1:8 with RNase free water were used for the analysis. SsoAdvanced Universal SYBR green supermix (Bio-Rad) was used for each qPCR reaction. A standard qPCR reaction using a thermocycler (CFX connect, Bio-Rad) was completed with the following protocol: 2 minute enzyme initiation at 95 ° C followed by 40 cycles of 5 seconds at 95 ° C and 30 seconds at 60 ° C. Melt curves were performed following the qPCR reactions. The stability of each gene was measured using the GeNorm software in BioRad CFX Maestro (Bio-Rad). *GUSB* was determined to be the most stable candidate gene across the samples in this panel for both NB and PBMCs (Avg M Value = 0.071, Stability = 2.638) and thus was selected for the reference gene.

3.4.10 Optimization of qPCR for FOXP3, IL-10, and GUSB

Eight-point standard curves were generated using pooled cDNA samples for NB and PBMCs for *FOXP3*, *IL-10* and *GUSB*. Two-fold dilutions in RNase free water were used for each curve to determine optimal cDNA concentration, efficiency and Cq values for amplification. The amplicon sequences for the *FOXP3*, *IL-10* and *GUSB* (BioRad, *FOXP3* unique assay ID: qHsaCID0007630, IL-10 unique assay ID: qHsaCED0044704, GUSB unique assay ID: qHsaCID0011706) are listed in Table 1. SsoAdvanced

Universal SYBR green supermix was used for each qPCR reaction. The protocol for the thermocycler was identical to that outlined in 3.4.9.

A dilution of 1:8 was determined optimal for *FOXP3* and *GUSB* in PBMC samples. The standard curves for FOXP3 in NB as well as IL-10 in both NB and PBMCs could not be established, thus no qPCR analysis for these targets and sample types were completed.

Table 1. Amplicon sequences.

Primer	**Amplicon sequence**	**Amplicon length (bp)**	**Assay design**
FOXP3	GAAGGCAAACATGCGTGTGAACCAGTGGTAG ATCTCATTGAGTGTCCGCTGCTTCTCTGGAGC CTCCAGGATGGCCCAGCGGATGAGCGTGGCG TAGGTGAAAGGGGGTCGCATGTTGTGGAACT TGAAGTAGTCCATGTTGTGGAGGAACTCTGG GAAT	129	Intron spanning
IL-10	TATCCCAGAGCCCCAGATCCGATTTTGGAGAC CTCTAATTTATGTCCTAGAGTCTATAGAGTCGC CACCCTGATGTCTCAGTTTCGTATCTTCATTGTC ATGTAGGCTTC TATGTA	86	Exonic
GUSB	ACCAAGAGTAGTAGCTGTTCAAACAGATCACAT CCACATACGGAGCCCCCTTGTCTGCTGCATAGTT AGAGTTGCTCACAAAGGTCACAGGCCGGGAGGG GTCCAAGGA	79	Intron spanning

3.4.11 Real-time qPCR evaluation of FOXP3 and GUSB in PBMC samples

cDNA samples (n=23) were thawed on ice and diluted 1:8 with RNase free water. A mastermix containing SsoAdvanced SYBR green supermix with either the *GUSB* primer or the *FOXP3* primer was used to measure specific mRNA levels. 2 plates were completed with each sample, NRT control, and no template control in duplicate for each gene. The CFX connect thermocycler was used following the same protocol outlined in 3.4.9. ΔCq values (normalized expression) and ΔΔCq values (relative normalized expression) were determined using the CFX Maestro Software.

3.4.12 Statistical analysis

Baptista-Pike and Fisher Exact tests were used to determine the odds ratios associated with maternal allergic status or each exposure with a positive SPT result at 6 years, parental report of wheeze at 6 years, or both. Mann-Whitney tests were conducted to determine the differences between cytokine concentrations, Treg frequencies, Th17 frequencies, and the lnExpression of *FOXP3* for SPT positive and negative children, children with/without wheeze at 6 years, children with no Atopy and those with a positive SPT and wheeze, and each of the exposures at the prenatal and 6 to 7 year time points. Due to sample size limitations no corrections for multiple comparisons were completed.

Chapter 4

Results

4.1 Frequency and function of freshly polarized T cells in CBMC samples of high and low atopic risk

4.1.1 Polarization culture conditions successfully polarize immature T cells into a mature T cell

To ensure the polarization culture conditions actively differentiated immature T cells into mature T helper cells, and that these cells were not a result of previously existing mature T cells in the CBMC sample, uncultured baseline CBMC samples and post cultured samples for all polarization conditions were analyzed via flow cytometry. The percent frequencies of live $CD3^+CD4^+CD45RA^+CD45RO^-$ (immature) and $CD3^+CD4^+CD45RA^-CD45RO^+$ (mature) lymphocytes were compared between all baseline and cultured samples using Wilcoxon tests (Figure 2, Appendix A Table 10). There was a significantly higher percentage of immature T cells in the baseline CBMC samples compared to the samples following the Th1, Th2, or NP culture conditions (Th1 p = 0.016, Th2 p = 0.016, NP p = 0.016). There was a greater percent frequency of immature T cells in the baseline samples than the samples cultured under Th17 polarizing conditions; however, this difference was not significant (p=0.0625). In contrast, there was a significantly lower percent frequency of mature T cells in the baseline samples compared to the post cultured Th1, Th2, and NP conditions (Th1 p = 0.016, Th2 p = 0.016, NP p = 0.016). The percent frequency of mature T cells following Th17 polarizations was greater than the baseline sample, although this increase was not statistically significant (p=0.0625).

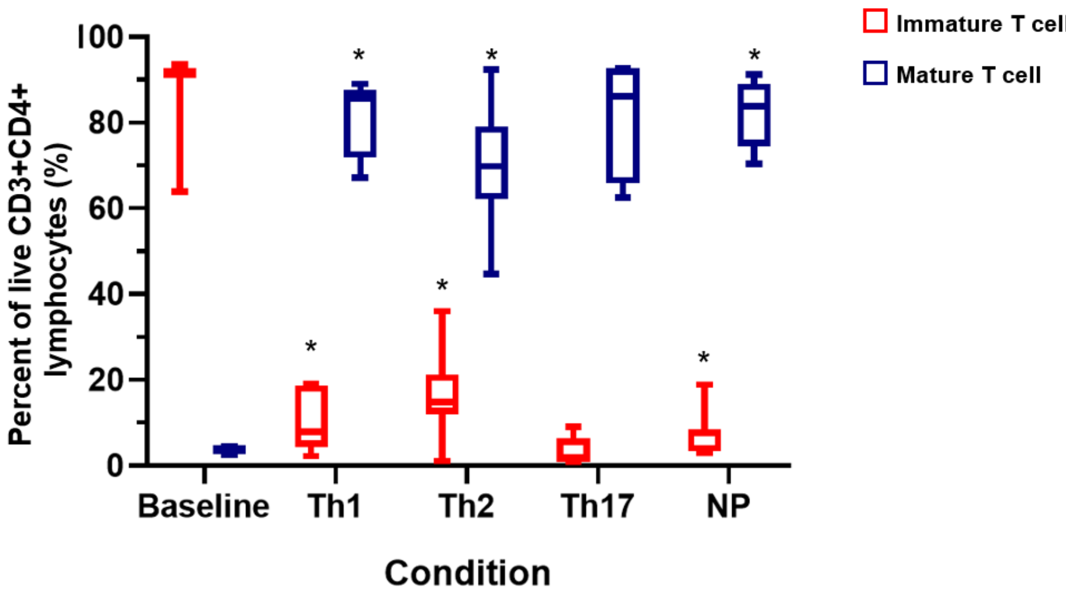

Figure 2. Percentage of immature and mature T cells following T cell polarization.

Uncultured CBMC samples (n=7) and samples cultured under Th1, Th2, NP (n=7), and Th17 (n=5) were evaluated via flow cytometry to determine the frequencies of live $CD3^+CD4^+CD45RA^+CD45RO^-$ lymphocytes (immature, red) and live $CD3^+CD4^+CD45RA^-CD45RO^+$ lymphocytes (mature, blue). Data displayed as Tukey plots. Comparison by Wilcoxon test, *$p < 0.05$.

4.1.2 Polarization culture conditions created the correct T helper cell type

To determine the frequencies of Th1, Th2, and Th17 cells following cell specific T cell polarization, the percent frequencies of each cell type following polarization were measured via flow cytometry. The percent frequencies of live lymphocytes expressing $CD3^+CD4^+CD45RA^-CD45RO^+IFN\gamma^+$ (Th1), $CD3^+CD4^+CD45RA^-CD45RO^+IL4^+$(Th2), and $CD3^+CD4^+CD45RA^-CD45RO^+IL17^+$ (Th17) were determined and compared between each polarization condition and the NP control using Wilcoxon tests (Figure 3).

The median percent frequency of Th1 cells following Th1 polarization was 46.40 IQR 20.90 – 75 % compared to 7.26 IQR 4.33 – 20.90 % in the NP control condition.

This elevated Th1 frequency was statistically significant ($p = 0.016$). There was a lower percent frequency of Th2 cells following Th2 polarization compared to the NP control, with median percent frequencies of 3.70 IQR 2.19 – 5.50 % for Th2 and 10.90 IQR 3.25 – 18.00 % for NP ($p = 0.047$). There were similar Th17 cell frequencies between the Th17 polarization conditions and the NP control, with median percent frequencies of 2.21 IQR 1.31 – 3.505 % and 2.38 IQR 1.02 – 3.60 % for Th7 and NP respectively ($p = 0.813$).

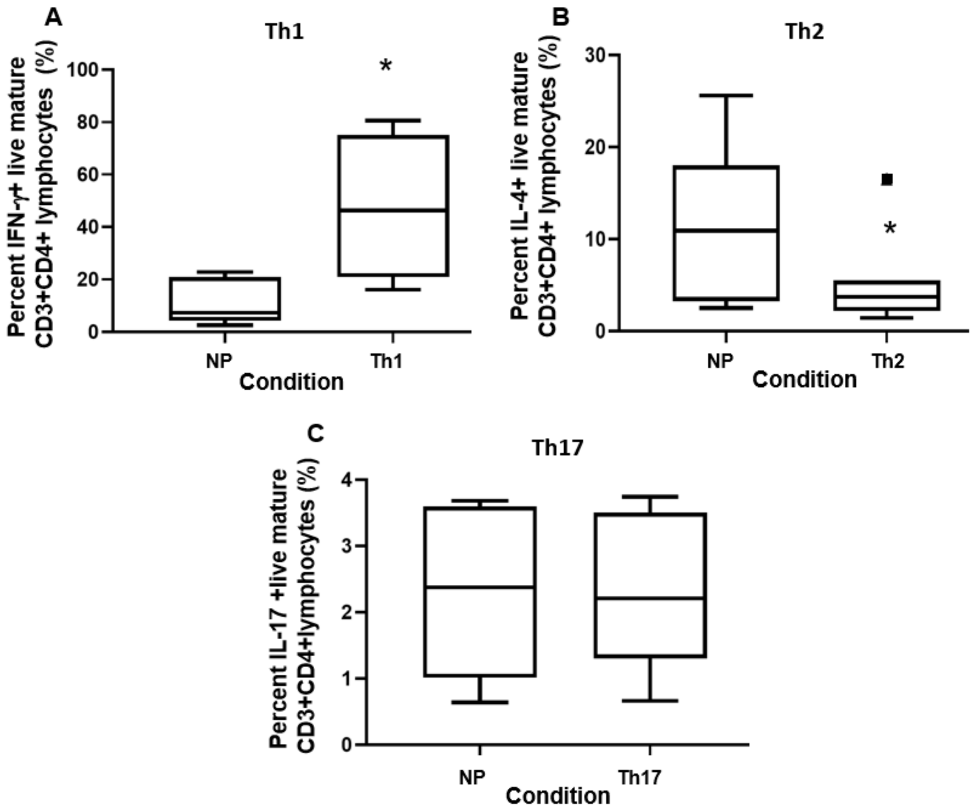

Figure 3. Successful polarization into T helper 1 cells.

(A) Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IFN\gamma^+$ lymphocytes following Th1 and NP culture conditions (n=7). (B) Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IL4^+$ lymphocytes in Th2 and NP culture conditions (n=7). (C) Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IL17^+$ following Th17 and NP culture conditions (n=5). Data is portrayed as Tukey plots, black square indicates outliers. Comparison by Wilcoxon test, *p < 0.05.

4.1.3 Polarization culture conditions create unique cytokine production profiles for each culture condition

To additionally confirm the presence of specific T helper cells, cytokine concentrations of 9 different cytokines were quantified from the supernatant of each culture condition. Luminex® technology was used to measure IL-5, IL-6, IL-10, IL-17A, IL-21, and TNF-α, and ELISAs were used for IL-8, IL-13, and IFN-γ (Figure 4, Appendix A Table 11). Mann-Whitney tests were used to compare the cytokine concentrations between the culture conditions. All cytokines were analyzed for the 7 samples cultured under the Th1, Th2, and NP conditions; with the exceptions of one NP IL-6 concentration, one Th1 IL-10 concentration, one Th1 IL-8 concentration, and one Th2 TNF-α concentration because these values were either extrapolated beyond the standard curves or outside the limit of detection. All cytokines were evaluated for the 5 samples cultured under the Th17 condition with the exception of one IL-6 value due to extrapolation.

The Th1 polarization conditions produced high levels of IL-8, IL-10, IL-21 and IFNγ. IL-8 concentration was significantly higher in the Th1 condition compared to the Th2 condition ($p = 0.001$), and IL-10 was significantly higher in the Th1 condition compared to the NP control ($p = 0.001$). The concentration of IL-21 was significantly higher in the Th1 condition compared to each of the other polarization conditions (Th1 vs Th2 $p = 0.0006$, Th1 vs Th17 $p = 0.0025$, and Th1 vs NP $p = 0.0023$). The concentration of IFNγ following polarization was significantly higher in the Th1 condition than each of the other polarization conditions (Th1 vs Th2 $p = 0.0006$, Th1 vs Th17 $p = 0.003$, and Th1 vs NP $p = 0.0006$).

The cytokines IL-5, IL-13, and IL-10 were detected at higher concentrations following Th2 polarization. IL-5 was produced at the greatest amount in the Th2 polarization condition and was significantly higher than the IL-5 concentration following Th1 polarization ($p = 0.0006$). IL-13 concentration was also significantly higher in the Th2 condition than the Th1 condition ($p = 0.0023$). The concentration of IL-10 was significantly greater in the Th2 condition than the NP control ($p = 0.004$).

IL-6, IL-17A, and TNF-α were detected at higher concentrations following Th17 polarization conditions. IL-6 was higher in the Th17 conditions than the other polarization conditions, although this difference was not significant. IL-17A was only detectable in the Th17 condition, thus no statistical analysis was completed due to insufficient comparison groups. TNF-α concentration was significantly higher in the Th17 condition compared to the NP control ($p = 0.018$).

The NP control condition had a mixture of Th1 and Th2 cytokines in the culture supernatant following the 7 day culture. IL-5 and IL-13 were significantly greater in the NP condition than the Th1 condition (IL-5 $p = 0.006$ and IL-13 $p = 0.0006$). IFNγ was produced at a significantly higher concentration in the NP control compared to the Th2 condition ($p = 0.0041$).

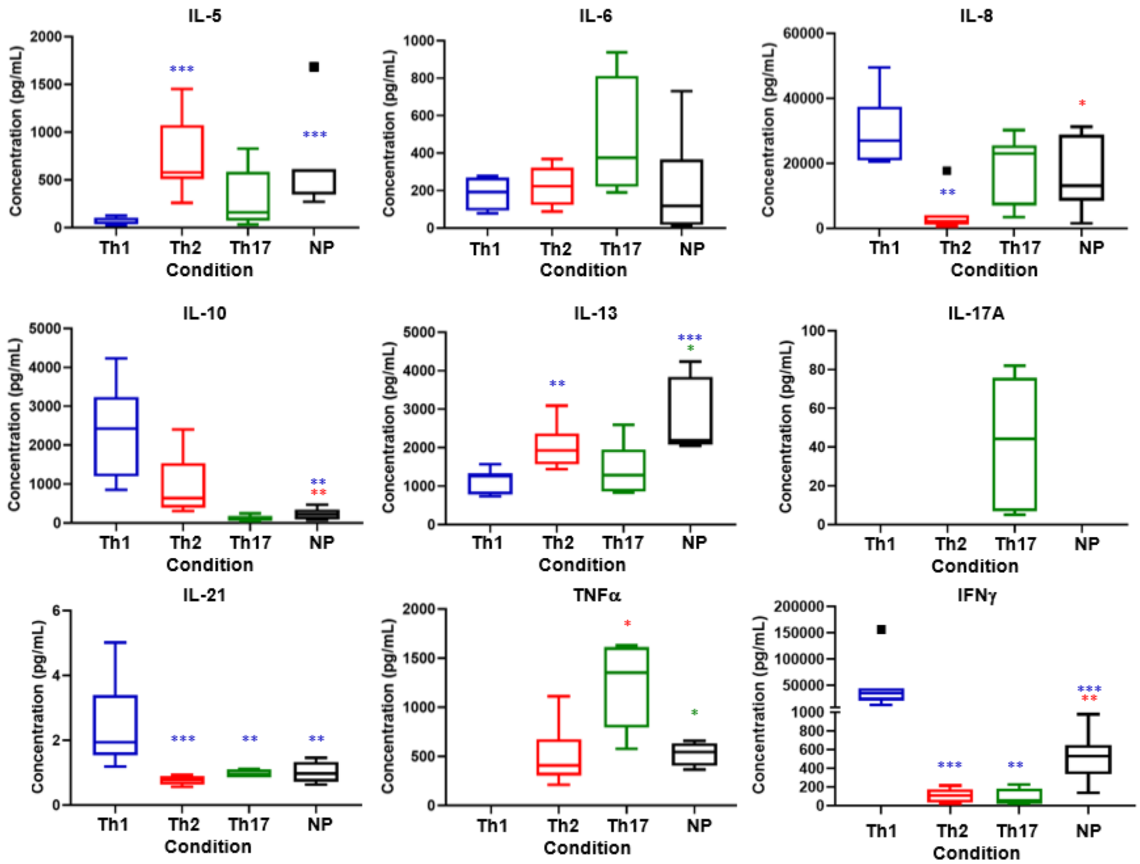

Figure 4. Cytokine profiles following T cell polarization were unique between polarization conditions.

Concentrations of IL-5, IL-6, IL-10, IL-17A, IL-21, and TNF-α in culture supernatants quantified via Luminex technology. Concentration of IL-8, IL-13, IFN-γ determined via ELISA. Th1 concentrations in blue (n=7), Th2 concentrations in red (n=7), Th17 concentrations in green (n=5), and NP concentrations in black (n=7). Data displayed as Tukey plots with black squares depicting outlier values. Comparisons by Mann-Whitney test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Blue asterisks denote comparison is made between the Th1 condition, red between the Th2 condition, and green between the Th17 condition.

4.1.4 Lower Th1 and Th2 cell frequencies but elevated Th17 frequency in HR compared to LR samples following T cell polarization

To assess the impact of maternal atopic status on the polarizability of T helper cell subtypes, Th1, Th2, and Th17 cell frequencies were compared between LR and HR

samples cultured under each of the polarizing or NP control conditions using Mann-Whitney tests (Figure 5, Appendix A

Table 12). There was a numerically lower percent frequency of Th1 cells in the post culture samples from the Th1 and NP conditions for HR CBMC samples compared to LR CBMC samples (Th1 p = 0.857 and NP p = 0.629). Similarly, Th2 percent frequencies for both the Th2 and NP conditions were numerically lower in HR samples compared to LR counterparts (Th2 p = 0.857 and NP p = 0.4). In contrast, Th17 percent frequencies were numerically elevated in HR samples compared to LR samples following the Th17 polarization and NP culture conditions (Th17 p = 0.4 and NP p = 0.2).

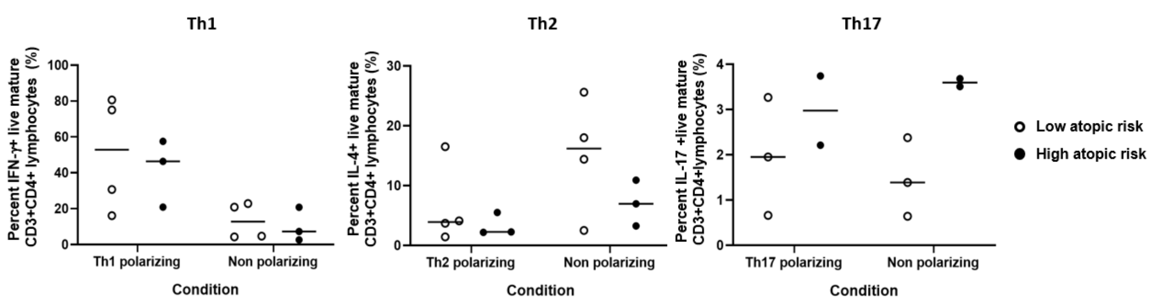

Figure 5. Lower Th1 and Th2 frequencies but elevated Th17 frequencies in HR CBMC samples following T cell polarization.

(A)Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IFN\gamma^+$ lymphocytes of LR (n=4) and HR (n=3) CBMC samples following the Th1 or NP culture. (B) Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IL4^+$ lymphocytes from LR (n=4) and HR (n=3) samples in Th2 and NP culture conditions. (C) Percent frequency of live $CD3^+CD4^+CD45RA^-CD45RO^+IL17^+$ from LR (n=3) and HR (n=2) samples following

Th17 and NP culture conditions (n=5). Black bar depicts the median. Comparisons made by Mann-Whitney, all are non-significant.

4.1.5 Numerical differences in IL-10, IL-21, TNF-α, and IFN-γ concentrations in culture supernatant between HR and LR samples following T cell polarization

To evaluate the impact of maternal atopic status on the functionality of newly polarized T cells, the supernatant concentrations of IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-21, TNF-α, and IFN-γ from each polarizing condition were compared between HR and LR samples using Mann-Whitney tests (Table 2). There were no statistically significant differences between any cytokine concentration and maternal atopic status, however some numerical differences were observed for IL-8, IL-10, IL-21, TNF-α, and IFN-γ.

IL-8 concentration was similar following Th1 polarization between HR and LR samples, but was numerically lower under Th2, Th17, and NP conditions in HR samples compared to LR samples. There was no statistically significant difference in IL-10 concentration between LR and HR samples following Th1 polarization. There was a numerical increase in IL-10 following Th2, Th17 and the NP conditions in HR compared to LR counterparts. IL-21 concentrations were numerically lower in HR samples than LR samples for Th1, Th2, and NP culture conditions. No comparison was made between LR and HR samples following Th1 polarization for TNF-α as the Th1 condition concentrations were below the limit of detection. HR samples had higher TNF-α concentrations than LR counterparts for Th2, Th17 and NP conditions. There were similar concentrations of IFN-γ between HR and LR samples in the Th1 polarizing supernatant. HR samples had elevated levels of IFN-γ in Th2, Th17, and NP conditions compared to LR counterparts. IL-17A concentration between HR and LR could only be compared for the Th17 culture condition. HR samples had numerically lower IL-17A concentration in the culture supernatant than LR counterparts.

IL-5 and IL-13 concentrations were similar between LR and HR samples for each of the polarizing and nonpolarizing control conditions. Additionally, there were no significant differences between IL-6 concentrations in the culture supernatants between LR and HR samples for each of the conditions.

Table 2. Post culture supernatant cytokine concentration in HR and LR.

	Th1 polarization			**Th2 polarization**		
	Low Risk (median IQR pg/mL)	High risk (median IQR pg/mL)	p	Low Risk (median IQR pg/mL)	High risk (median IQR pg/mL)	p
IL-5	62.49 (32.60 - 112.0)	44.86 (36.67 - 105.7)	0.857	544 (322.0 - 1234)	789.5 (527.3 - 1073)	0.629
IL-6	192.4 (109.1 - 261.6)	178.4 (78.75 - 278.1)	>0.999	223.1 (97.66 - 357.0)	223.7 (176.0 - 260.9)	>0.999
IL-8	21665 (20985 - 32356)	22388 (20669 - 49481)	0.700	3213 (1708 - 14392)	1241 (621.9 - 3854)	0.229
IL-10	2849 (854.2 - 4231)	2008 (1311 - 2912)	>0.999	574.1 (359.2 - 1276)	1534 (395.3 - 2404)	0.400
IL-13	1293 (867.7 - 1331)	1189 (784.2 - 1571)	>0.999	1924 (1558 - 2263)	1997 (1566 - 3095)	0.629
IL-17A	No data	No data	N/A	No data	No data	N/A
IL-21	2.54 (1.64 - 3.33)	1.90 (1.19 - 5.02)	0.857	0.88 (0.74 - 0.92)	0.63 (0.56 - 0.77)	0.114
TNFα	No data	No data	N/A	361.9 (211.3 - 530.1)	451.8 (338.1 - 1111)	0.700
IFNγ	34403 (23844 - 127602)	36367 (12848 - 44091)	>0.999	66.05 (24.40 - 157.3)	157.7 (107.2 - 216.6)	0.229
	Th17 polarization			**No polarization**		
	Low Risk (median IQR pg/mL)	High risk (median IQR pg/mL)	p	Low Risk (median IQR pg/mL)	High risk (median IQR pg/mL)	p
IL-5	159.6 (114.2 - 826.9)	189.2 (32.67 - 345.8)	0.800	453.8 (351.6 - 1402)	361.7 (273.3 - 611.7)	0.857
IL-6	626.2 (314.8 - 937.6)	313.1 (189.9 - 436.3)	0.667	135.8 (18.41 - 246.4)	102.4 (13.09 - 730.2)	>0.999
IL-8	24135 (22992 - 30245)	12345 (3430 - 21259)	0.200	16991 (8522 - 29810)	13124 (1516 - 28828)	0.857
IL-10	93.88 (20.16 - 129.0)	163.9 (81.69 - 245.40)	0.800	173.9 (84.7 - 418.1)	218.5 (214.8 - 348.3)	0.857
IL-13	1284 (883.9 - 2595)	1073 (836.5 - 1310)	0.800	2179 (2076 - 3725)	2747 (2082 - 3846)	0.857
IL-17A	44.25 (8.63 - 81.99)	37.36 (5.15 - 69.57)	0.800	No data	No data	N/A
IL-21	0.95 (0.86 - 1.12)	0.97 (0.86 - 1.08)	>0.999	1.16 (0.98 - 1.43)	0.72 (0.63 - 0.77)	0.057
TNFα	1007 (577.4 - 1631)	1474 (1352 - 1596)	0.800	474.6 (375.2 - 629.3)	609.9 (439.5 - 632.0)	0.629
IFNγ	25.79 (18.82 - 136.6)	142.9 (58.56 - 227.2)	0.400	511.9 (227.7 - 620.9)	625.1 (338.7 - 980.2)	0.629

4.2 The relationships between early life exposures to indoor air polluting factors and allergic sensitization at 2 years of age

4.2.1 Participant Demographics

At the 2-year time point 88 and 20 children had a negative and positive SPT result respectively. Of the mothers that completed a SPT, 24 had a negative SPT result and 36 had a positive SPT result. Percentages of parental report for each exposure was similar across time points and are summarized in Table 3.

Table 3. Environmental exposures to 7 factors from birth to two years of age.

Exposure	Prenatal (n=92)		6 Month (n=76)		1 Year (n=72)		2 Year (n=81)	
	No (%)	Yes (%)	No (%)	Yes (%)	No (%)	Yes (%)	No (%)	Yes (%)
Dogs	60 (65)	32 (35)	46 (61)	30 (39)	43 (60)	29 (40)	52 (64)	29 (36)
Cats	49 (53)	43 (47)	43 (57)	33 (43)	40 (56)	32 (44)	51 (63)	30 (37)
Air Fresheners	54 (59)	38 (41)	57 (75)	19 (25)	50 (69)	22 (31)	54 (67)	27 (33)
Candles	54 (59)	38 (41)	60 (79)	16 (21)	54 (75)	18 (25)	56 (69)	25 (31)
Mould	65 (71)	27 (29)	53 (70)	23 (30)	55 (76)	17 (24)	63 (78)	18 (22)
ETS	76 (83)	16 (17)	65 (86)	11 (14)	60 (83)	12 (17)	64 (79)	17 (21)
Carpet	34 (37)	58 (63)	43 (57)	33 (43)	43 (60)	29 (40)	45 (56)	36 (44)

4.2.2 Increased OR of a positive SPT at 2 years with exposure to indoor air pollutants

To assess the relationship between a positive SPT at 2 years of age with prenatal and postnatal exposure to dogs, cats, air fresheners, candles, mould, ETS, and carpet, the OR and 95 % confidence interval (CI) of a positive SPT at 2 years was calculated for each exposure at each time point using Fisher's exact tests (Figure 6, Appendix Table 13). Exposure to cats during the 6 month time period, candles during the prenatal window, and ETS at 2 years of age significantly increased this OR (cat OR 4.267, 95 %

CI 1.096 – 15.68, p = 0.048; candles OR 5.096, 95 % CI 1.697 – 13.86, p = 0.006; ETS OR 3.78, 95 % CI 1.189 – 11.18, p = 0.04). Exposure to cats at the prenatal, 1 year, and 2 year timepoints resulted in an increased OR of a positive SPT at 2 years, although it was not significant. Similarly to exposure during the prenatal window, exposure to candles at the 6 month, 1 year, and 2 year timepoints resulted in an increased OR; however without statistical significance. Exposure to ETS during the prenatal, 6 month, and 1 year windows slightly increased the OR of a positive SPT at 2 years; although, not significantly. There was an increased OR with exposure to air fresheners at the prenatal, 1 year, and 2 year timepoints, and a decreased OR at the 6 month window; although, none were statistically significant. Carpet exposure during the prenatal and 6 month windows neither increased nor decreased the OR of a positive SPT at 2 years; however, exposure at the 1 and 2 year timepoints decreased this OR without statistical significance. There was an increased OR with exposure to dog or mould at each time point, although these increases were not significant.

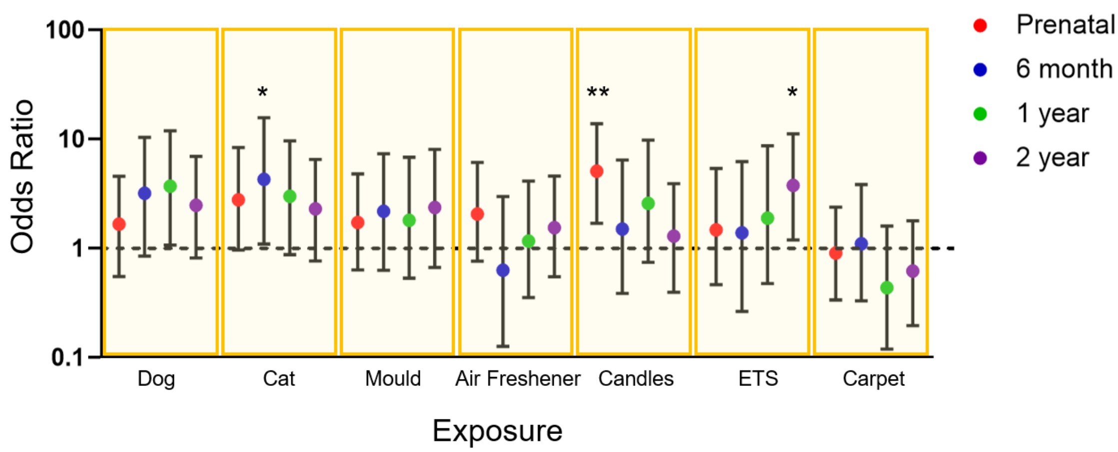

Figure 6. Exposure to cats, candles and ETS increased the OR of a positive SPT at 2. ORs of a positive SPT at 2 years with exposure prenatally (n=92, red), at 6 months (n=76, blue), 1 year (n=72, green), and 2 years (n=81, purple), to dog, cat, mould, air fresheners, candles, ETS, and carpet were determined using Fisher's exact test. Dots indicate the OR of the exposure and black bands are the 95 % CI. *$p \leq 0.05$, **$p \leq 0.001$.

4.2.3 Children with a positive SPT at 2 years had more exposures during the prenatal and early life windows compared to children with a negative SPT

To identify if children with a positive SPT have a higher number of different exposures than children with a negative SPT at 2 years, the total number of exposures (dogs, cats, mould, air fresheners, candles, ETS, or carpet) for each child at each time point was determined and compared using Mann-Whitney tests (Figure 7). Prenatal exposure data was obtained for all participants included in this analysis (n = 92, SPT negative n = 74, SPT positive n = 18). SPT positive children had a higher total number of different exposures during the prenatal time period compared to SPT negative children, with median exposure numbers of 4 and 2 respectively (p = 0.0052). Exposure data at the 6 month time point included fewer participants (n = 76, SPT negative n = 65, SPT positive n = 11); however a similar trend of more exposures at this time point for SPT positive children compared to SPT negative children was observed with median total exposure numbers of 3 and 2 respectively (p = 0.064). At the 1 year (n = 72, SPT negative n = 60, SPT positive n = 12) and 2 year (n = 81, SPT negative n = 64, SPT positive n = 17) time points, SPT negative children had fewer total exposures than SPT positive children, with SPT negative median exposure values of 2 and SPT positive median exposure values of 3 (1 year p = 0.0319 and 2 year p = 0.0075).

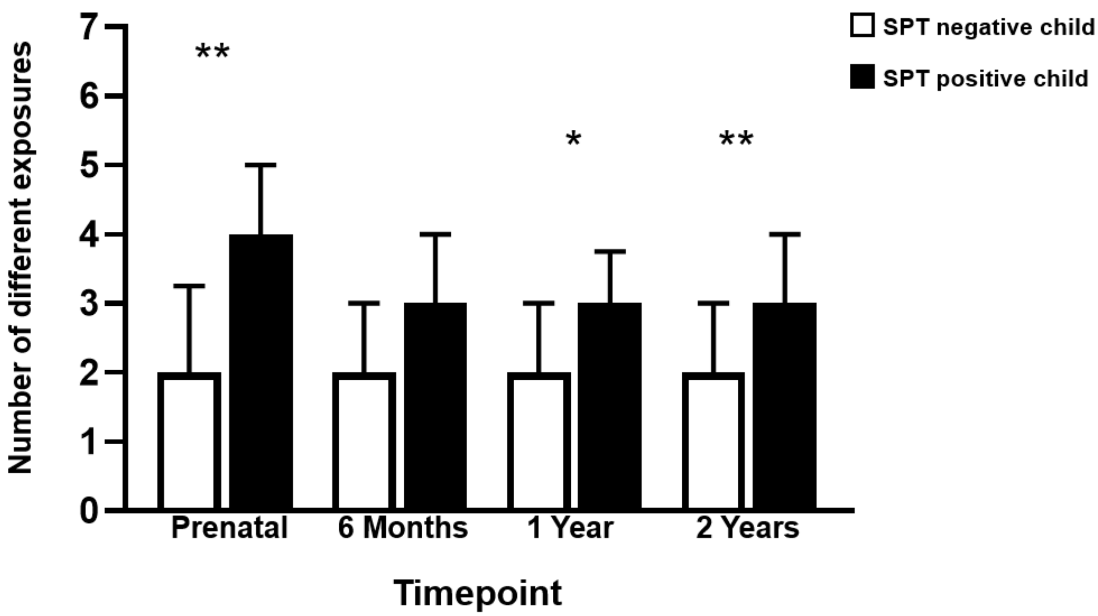

Figure 7. SPT positive children have higher total number of different exposures than SPT negative children.

The total number of exposures (0 to 7 of cat, dog, mould, air fresheners, candles, ETS, and carpet) at the prenatal (n=92, SPT negative n=74, SPT positive n=18), 6 month (n=76, SPT negative n=65, SPT positive n=11), 1 year (n=76, SPT negative n=65, SPT positive n=11), and 2 year (n=81, SPT negative n=64, SPT positive n=17) time points were determined and compared between SPT negative and SPT positive children. Data displayed as the median with the IQR. Mann-Whitney tests were used for comparison. *p ≤ 0.05, **p≤0.001.

4.2.4 As total number of exposures increase the percentage of SPT positive children increases and percentage of SPT negative decreases

To assess the relationship between multiple exposures and SPT outcomes at 2 years, children were grouped based on their number of exposures and the percentages of SPT positive and negative children in each group were measured at each time point (Table 4, Figure 8). Chi square tests for trend were completed for each timepoint. A positive trend between exposure number and positive SPT result was observed for all timepoints. This trend was statistically significant for exposures at the prenatal ($p = 0.005$), 1 year ($p = 0.031$), and 2 year ($p = 0.013$) time points, and trending towards significance at the 6 month timepoint ($p = 0.067$).

Table 4. Summary of exposure number and SPT status for each time point.

Number of exposures	Prenatal (n=92)		6 Month (n=76)		1 Year (n=72)		2 Year (n=81)	
	SPT negative	SPT positive	SPT negative	SPT positive	SPT negative	SPT positive	SPT negative	SPT positive
0	5	0	7	0	7	0	6	0
1	14	0	21	2	13	1	16	2
2	19	5	16	3	17	3	21	4
3	18	3	10	1	17	5	14	5
4	10	5	5	4	4	2	4	5
5	7	4	6	1	2	1	2	1
6	1	1	0	0	0	0	1	0
7	0	0	0	0	0	0	0	0
Total	74	18	65	11	60	12	64	17

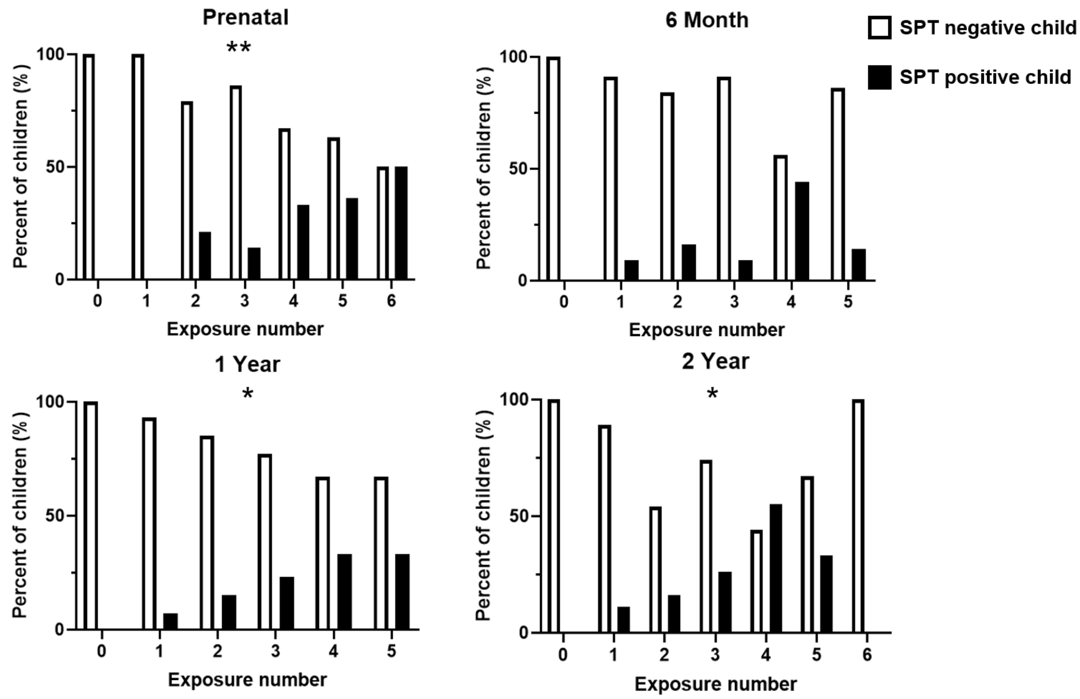

Figure 8. Proportion of children with a positive SPT at 2 years increases with total exposures.

Children were grouped by the number of exposures (0 to 7 of cat, dog, mould, air fresheners, candles, ETS, and carpet) they had at each time point. The percentage of SPT positive and SPT negative children in each exposure group is displayed and a Chi square test for trend was conducted, *$p \leq 0.05$, **$p \leq 0.001$.

4.3 Connecting environmental exposures with allergic and respiratory outcomes at 6 years of age

4.3.1 Participant demographics

39 mother child pairs returned for a follow-up visit at 6 to 7 years of age. Of these children, 18 were male and 21 were female. 15 children and 23 mothers had a positive SPT, and 9 mothers reported cough and/or wheeze without a cold for their child. Of the

SPT positive children, 10 were male and 5 were female, and of the children with respiratory symptoms, 5 were male and 4 were female. There was an increased OR of a positive SPT and respiratory symptoms for males compared to females, although these were not significant (SPT OR 4.00, 95 % CI 1.033 – 16.13, p = 0.055 and OR 1.635, 95 % CI 0.356 – 6.12, p = 0.706) (Table 5). Prenatal exposure to OAP, IAP, air fresheners, candles, mould, cats, dogs, and carpet was captured for 33 mother child pairs, prenatal exposure to ETS was obtained for 39 mother child pairs, and 6 to 7 year exposure to OAP, IAP, air fresheners, candles, mould, cats, dogs and ETS was gathered for 39 mother child pairs (Table 6).

Table 5. Summary of clinical outcomes.

	SPT status		Cough/Wheeze without a cold	
	Negative (%)	Positive (%)	No report (%)	Report (%)
Mother	13 (36)	23 (64)	N/A	N/A
OR (95 % CI)	3.06 (0.63 - 12.11)		Undefined	
p	0.175		**0.01**	
Child	24 (62)	15 (38)	30 (77)	9 (23)
Male (n=18)	8 (44)	10 (56)	13 (72)	5 (28)
Female (n=21)	16 (76)	5 (24)	17 (81)	4 (19)
OR (95 % CI)	4.00 (1.03 - 16.13)		1.64 (0.36 - 6.12)	
p	0.055		0.706	

Table 6. Summary of prenatal and 6 to 7 year exposures.

Exposure	Prenatal exposure		6 to 7 year exposure	
	No (%)	Yes (%)	No (%)	Yes (%)
OAP	19 (58)	14 (42)	16 (41)	23 (59)
IAP (low/high)	15 (45)	18 (55)	23 (59)	16 (41)
Air fresheners	20 (60)	13 (40)	25 (64)	14 (36))
Candles/	20 (60)	13 (40)	24 (61)	15 (39)
Mould	25 (75)	8 (25)	23 (59)	16 (41)
Cats	16 (54)	15 (46)	24 (61)	15 (39)
Dogs	18 (54)	15 (46)	24 (61	15 (39)
ETS	27 (69)	12 (31)	27 (69)	12 (31)
Carpet	17 (51)	16 (49)	No data	No data

4.3.2 Maternal allergic status increased the odds ratio of a positive SPT and cough and/or wheeze without a cold

To determine the influence of maternal allergic status on child SPT status and respiratory symptoms, ORs were determined using Fisher's exact test (Figure 9). There was an increased OR of a positive SPT for children with mothers with a positive SPT, although this was not significant (OR 3.06, 95 % CI 0.63 – 12.11, p = 0.175). When considering maternal allergic status and child respiratory symptoms, all children with cough and/or wheeze without a cold had a mother with a positive SPT (OR undefined, p = 0.01).

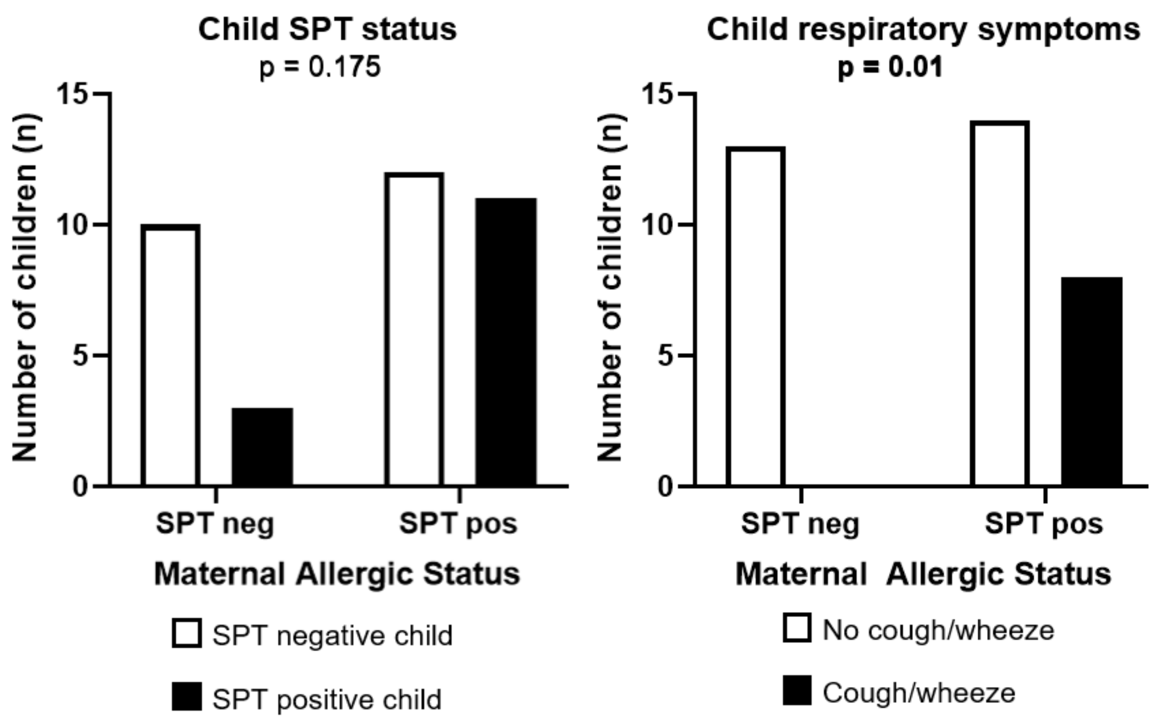

Figure 9. Maternal SPT status increased the OR of child positive SPT at 6 to 7 years of age.

Fisher exact tests were conducted to determine the OR related to the effect of maternal allergic status on child SPT status and report of cough and/or wheeze without a cold.

4.3.3 Prenatal exposure to carpet increases the OR of a positive SPT at 6 to 7 years

To evaluate the relationships between prenatal and 6 to 7 years exposure to various indoor and outdoor air pollutants with childhood allergic status, the OR of a positive SPT was determined for prenatal and 6 to 7 year exposure to air fresheners, candles, cats, dogs, mould, ETS, carpet, OAP, and IAP level (Figure 10, Appendix A Table 14). Carpet exposure in the prenatal window was the only exposure to significantly increase the OR of a positive SPT (OR 5.417, 95 % CI 1.109 – 19.96, p = 0.037).

Exposure to OAP during the prenatal window reduced the OR of a positive SPT and exposure at 6 to 7 years increased this OR. High level of exposure to indoor air pollutants during the prenatal window increased the OR of a positive SPT but did not affect this OR at the 6 to 7 year time point. Additionally, air freshener exposure during both the prenatal and 6 to 7 year window decreased the OR of a positive SPT at 6 to 7 years, although not significantly Exposure to candles during the prenatal window increased the OR of a positive SPT; however, exposure at 6 to 7 years reduced it. Cat exposure during the prenatal window increased the OR but did not affect it at the 6 to 7 year time point. Additionally, the OR was increased for children with dogs in the home during the prenatal and 6 to 7 year timepoints. Exposure to ETS during the prenatal and 6 to 7 year time points slightly decreased the OR of a positive SPT, however not significantly. Exposure to mould during both the prenatal and 6 to 7 year timepoints neither increased nor decreased the OR of a positive SPT.

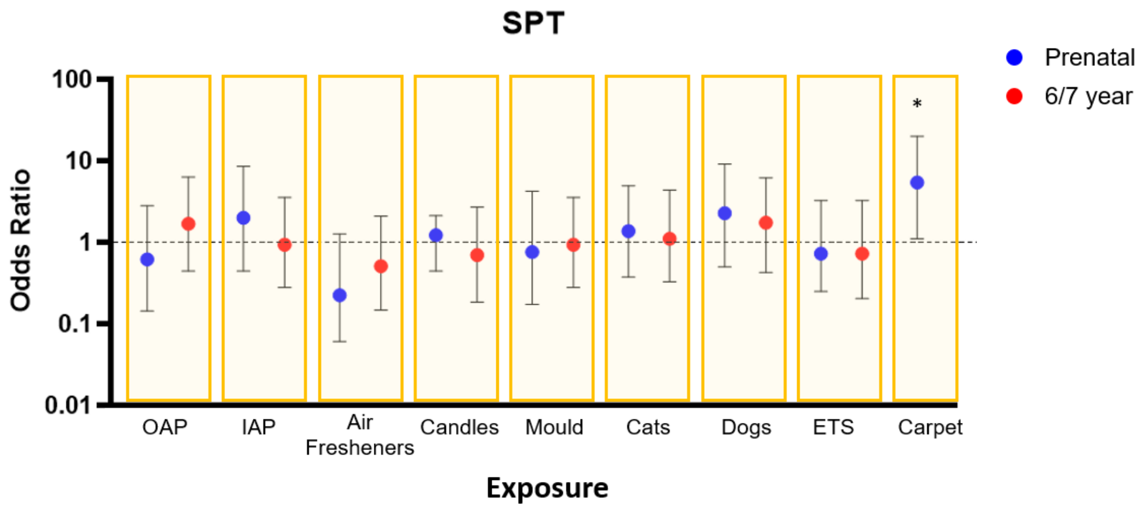

Figure 10. The OR of a positive SPT at 6 to 7 years of age with exposure to different indoor and outdoor air pollutants.

ORs of a positive SPT at 6 to 7 years with exposures to OAP, IAP, air fresheners, candles, mould, cats, dogs, ETS, and carpet prenatally (n=33, blue) and at 6 to 7 years (n=39, red) were determined using Fisher's exact test. Dots indicate the OR of the exposure and black bands are the 95 % CI, $^{*}p \leq 0.05$.

4.3.4 Prenatal and 6 to 7 year exposure to several indoor and outdoor air pollutants increased the OR of cough and/or wheeze without a cold

To evaluate any relationships between prenatal and 6 to 7 year exposure to different air pollutants with report of child respiratory symptoms, ORs were determined for prenatal and current exposure to air fresheners, candles, cats, dogs, mould, ETS, carpet, OAP, and IAP level (Figure 11, Appendix A Table 14). Several exposures during both the prenatal and 6 to 7 year window increased this OR, although none were significant. 6 to 7 year exposure to OAP increased the OR of respiratory symptoms but prenatal exposure did not. Exposures to high levels of IAP during the prenatal window increased the OR of cough and/or wheeze without a cold but did not affect the ratio at the

6 to 7 year timepoint. Air freshener exposure increased the OR of respiratory symptoms for both the prenatal and 6 to 7 year exposure windows. Prenatal exposure to candles neither increased nor decreased the OR of respiratory symptoms, but 6 to 7 year exposure did increase this OR. Alternatively, cat exposure during the prenatal window increased this OR, but 6 to 7 year exposure did not affect it. During the prenatal window, dog exposure reduced the OR of respiratory symptoms and 6 to 7 year exposure did not affect this OR. ETS exposure during the prenatal and 6 to 7 year timepoints increased the OR of child respiratory symptoms, although not significantly. Prenatal exposure to carpet did not affect the OR of respiratory symptoms at 6 to 7 years. During the prenatal window mould exposure increased the OR of respiratory symptoms but decreased this OR during the 6 to 7 year window.

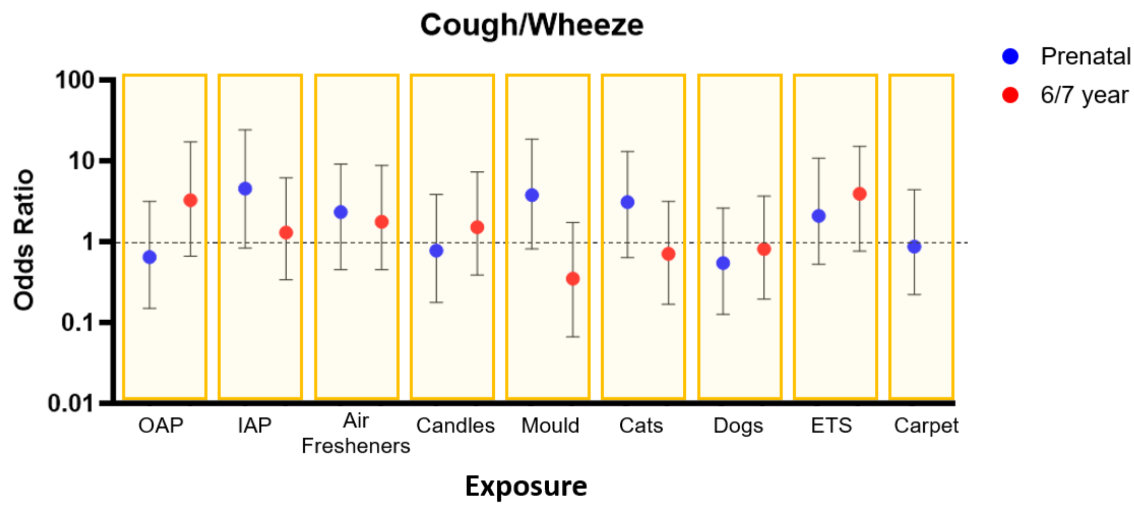

Figure 11. Exposure to different indoor and outdoor air pollutants increased the OR of respiratory symptoms at 6 to 7 years.

ORs of a positive SPT at 6 to 7 years with exposures to OAP, IAP, air fresheners, candles, mould, cats, dogs, ETS, and carpet prenatally (n=33, blue) and at 6 to 7 years (n=39, red) were determined using Fisher's exact test. Dots indicate the OR of the exposure and black bands are the 95 % CI, all ORs are non-significant.

4.3.5 Peripheral blood frequency of Treg and Th17 cells compared between clinical outcome groups

To determine if there are any differences in the frequencies of Tregs and Th17 cells in PBMC samples of SPT negative and positive children (SPT neg n = 16 SPT pos n = 8), children without and with respiratory symptoms (respiratory negative n = 18 respiratory positive n = 6), and children with no atopy vs those with both a positive SPT and respiratory symptoms (no atopy n = 14 SPT positive and respiratory positive n = 4), PBMC samples were analyzed using flow cytometry and percent frequencies of live CD4+FOXP3+ lymphocytes (Treg) and live CD4+IL17+ lymphocytes (Th17) were determined. These cellular frequencies were compared within clinical outcome groups using Mann-Whitney tests (Figure 12 and Figure 13).

PBMC Treg frequencies were numerically higher in children with a positive SPT compared to negative SPT counterparts, with median percent frequencies of 6.43 IQR 5.97 – 7.32 % and 7.37 IQR 7.06 – 7.85 % for SPT negative and positive children respectively ($p = 0.172$). There was no difference in the Th17 percent frequencies when comparing SPT status, as the median percent frequency for SPT negative children was 1.47 IQR 1.01 – 1.67 and 1.4 IQR 1.07 – 1.90 % for SPT positive children ($p = 0.44$).

In children with respiratory symptoms, the median percent frequency of Tregs and Th17 were higher than children without these symptoms, although these differences were not significant. Median percent frequency of Tregs in children with respiratory symptoms was 7.39 IQR 7.07 – 7.60 % compared to 6.45 IQR 6.00 – 7.37 for children without respiratory symptoms ($p = 0.343$). Additionally, median percent frequency of Th17 cells in children with and without respiratory symptoms were 1.55 IQR 1.23 – 2.41 % and 1.27 IQR 1.02 – 1.62 % respectively ($p = 0.256$).

The Th17 percent frequencies were similar between children with no atopy and children with both a positive SPT and respiratory symptoms, with median percent frequencies of 1.35 IQR 1.00 – 1.62 % and 1.34 IQR 1.11 – 1.90 % for non-atopic children and SPT positive with respiratory symptoms children respectively ($p = 0.44$). Alternatively, the Treg frequency was numerically higher in children with a positive SPT and respiratory symptoms than non-atopic children. The median percent frequency for non-atopic children and children with a positive SPT and respiratory symptoms were 6.30 IQR 5.94 – 7.06 % and 7.45 IQR 6.25 – 8.27 % ($p = 0.442$).

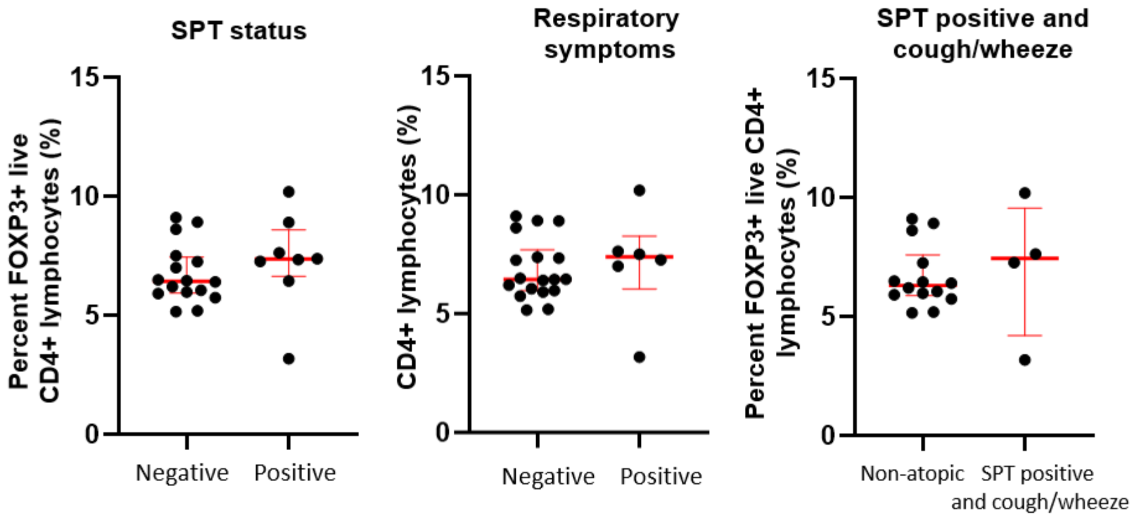

Figure 12. Slightly higher Treg frequency in all clinical outcome groups.

The percent frequency of live CD4+ lymphocytes expressing FOXP3 in PBMC samples was determined and compared between children with a negative (n=16) and positive (n=8) SPT, without (n=18) and with (n=6) cough and/or wheeze without a cold, and between children with a positive SPT and cough/wheeze (n=4) and non-atopic children (n=14). Data displayed as individual percent frequencies with red bars as the median and red bands displaying the IQR. Comparisons made by Mann-Whitney tests, no significant differences.

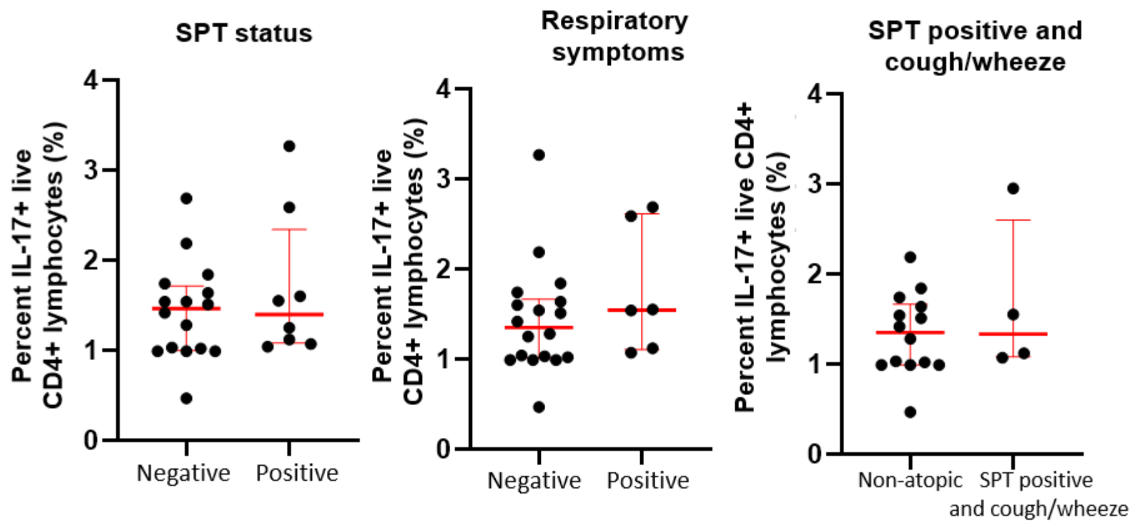

Figure 13. Similar Th17 frequencies between clinical outcome groups.

The percent frequency of live CD4+ lymphocytes expressing IL-17 in PBMC samples was determined and compared between children with a negative (n=16) and positive (n=8) SPT, without (n=18) and with (n=6) cough and/or wheeze without a cold, and between children with a positive SPT and cough/wheeze (n=4) and non-atopic children (n=14). Data displayed as individual percent frequencies with red bars as the median and red bands displaying the IQR. Comparisons made by Mann-Whitney tests, no significant differences.

4.3.6 Peripheral blood Treg frequencies higher in children with prenatal exposure to OAP and air fresheners

To identify any differences in Treg frequencies for children with prenatal exposure to different indoor and outdoor air pollutants, peripheral Treg frequencies were compared using Mann-Whitney tests between children with and without prenatal exposures to the following air polluting factors: OAP (no exposure n = 10 exposure n = 10), IAP (low = 8 high n = 12), Air fresheners (no exposure n = 15 exposure n = 5), candles (no exposure n = 12 exposure n = 8), mould (no exposure n = 14 exposure n = 6), cats (no exposure n = 8 exposure n = 12), dogs (no exposure n = 11 exposure n = 9), carpet (no exposure n = 13 exposure n = 7), and ETS (no exposure n = 14 exposure n = 10) (Figure 14, Appendix A Table 15). There were no significant differences within the exposure groups; however, numerical differences were observed between children with exposures to OAP and air fresheners.

Children with prenatal exposures to OAP and air fresheners had numerically higher percent frequencies of Tregs compared to those without these exposures. The median percent Treg frequencies were 7.43 IQR 6.48 – 8.97 % and 6.72 IQR 5.183 – 7.44 % for children with and without OAP exposure respectively ($p = 0.075$). The Treg median percent frequency for children exposed to air fresheners was 7.51 IQR 6.27 – 9.56 % and 7.00 IQR 6.40 – 7.63 for those not exposed ($p = 0.23$).

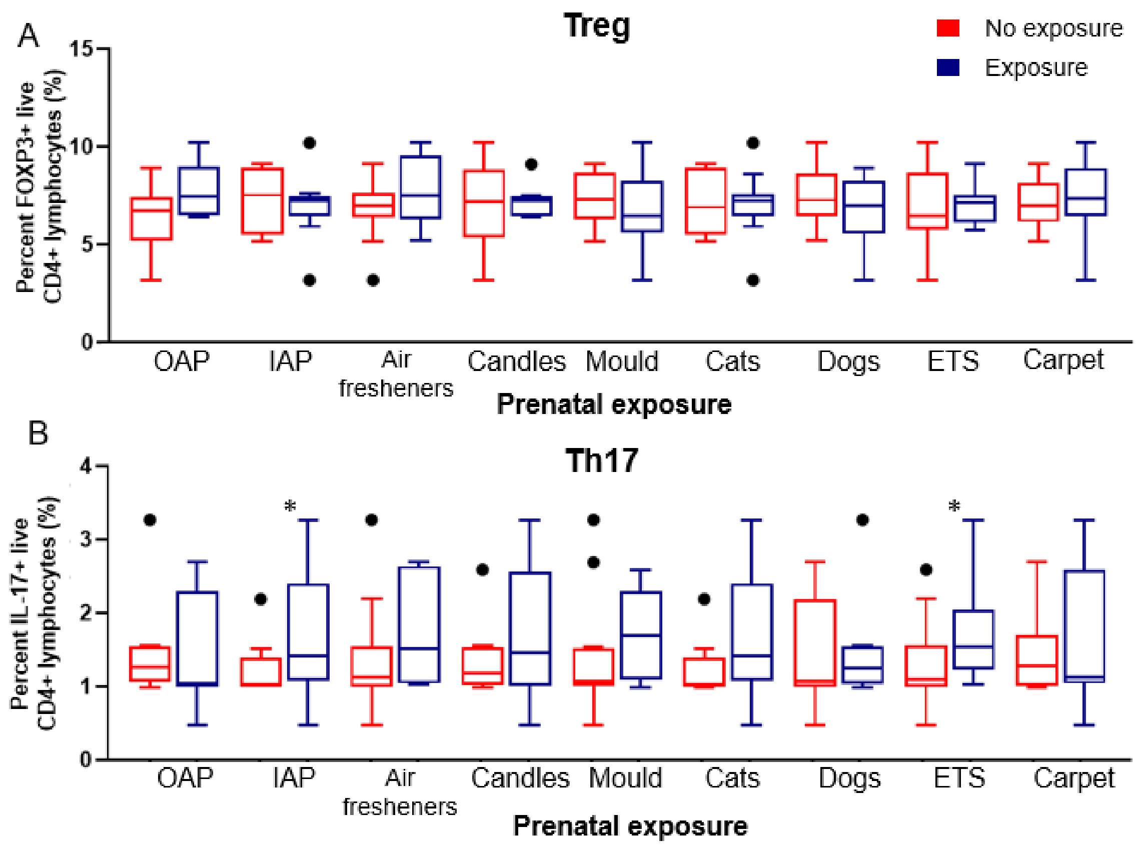

Figure 14. Treg and Th17 PBMC frequencies in PBMC samples in children with prenatal exposures to different indoor and outdoor air pollutants.

(A) Percent frequencies of live CD4+ lymphocytes expressing FOXP3 in PBMC samples from children with (red) or without (blue) prenatal exposure to different indoor and outdoor air pollutant factors were determined and compared using Mann-Whitney tests. (B) Percent frequencies of live CD4+ lymphocytes expressing IL-17 in PBMC samples from children with (red) or without (blue) prenatal exposure to different indoor and outdoor air pollutant factors were determined and compared using Mann-Whitney tests. Data is displayed as the Tukey plots, black circles indicate outliers, $*p \leq 0.05$.

4.3.7 Elevated peripheral blood Th17 frequency at 6 to 7 years in children exposed prenatally to several indoor air pollutants

To identify any differences in Th17 cell frequencies between children with and without exposure to air pollutants, Th17 percent frequencies were compared using Mann-Whitney tests (Figure 14, Appendix A Table 15). Children with prenatal exposure to high levels of IAP, air fresheners, candles, mould, cats, dogs, and ETS had greater Th17 frequencies than children without these exposures; however only IAP level and ETS exposures were significant. Children with exposures to OAP and carpet had similar Th17 frequencies as children without the exposures.

The median Th17 percent frequency was 1.41 IQR 1.08 – 2.40 % for children exposed to high levels of IAP compared to 1.03 IQR 0.99 – 1.39 % for low level IAP exposed children ($p = 0.038$). Additionally, children with prenatal air freshener exposure had a median Th17 cell frequency of 1.51 IQR 1.04 – 2.64 % compared to 1.12 IQR 0.99 – 1.55 % for those without air freshener exposure ($p = 0.395$). For children with and without ETS exposure, the median Th17 percent frequencies were 1.55 IQR 1.23 – 2.05 % and 1.09 IQR 0.99 – 1.57 % respectively ($p = 0.05$).. For children with and without prenatal exposure to cats, median Th17 percent frequencies were 1.41 IQR 1.08 – 2.40 % and 1.04 IQR 0.99 – 1.40 % respectively ($p = 0.097$).

4.3.8 Peripheral blood Treg frequencies higher in children exposed to OAP, high levels of IAP, candles, and cats at 6 to 7 years

To determine if there are any differences in Treg frequencies with current exposure to OAP (no exposure n = 9 exposure n = 15), IAP (IAP low n = 13 IAP high n = 11), air fresheners (no exposure n = 16 exposure n = 8), candles (no exposure n = 13 exposure n = 11), mould (no exposure n = 16 exposure n = 8), cats (no exposure n = 13 exposure n = 11), dogs (no exposure n = 14 exposure n = 10), and ETS (no exposure n = 16 exposure n = 8), peripheral Treg frequencies were compared between exposed and non-exposed children using Mann-Whitney tests (Figure 15, Appendix A Table 15). Children with exposure at 6 to 7 years to OAP, high levels of IAP, candles, and cats had greater Treg frequencies than children without these exposures; however none were statistically significant. Furthermore, children with exposure to air fresheners, dogs, and ETS had similar Treg frequencies as children without these exposures. Lastly, children with exposure to mould at 6 to 7 years had lower Treg frequencies than those without mould exposure. None of the differences observed were statistically significant.

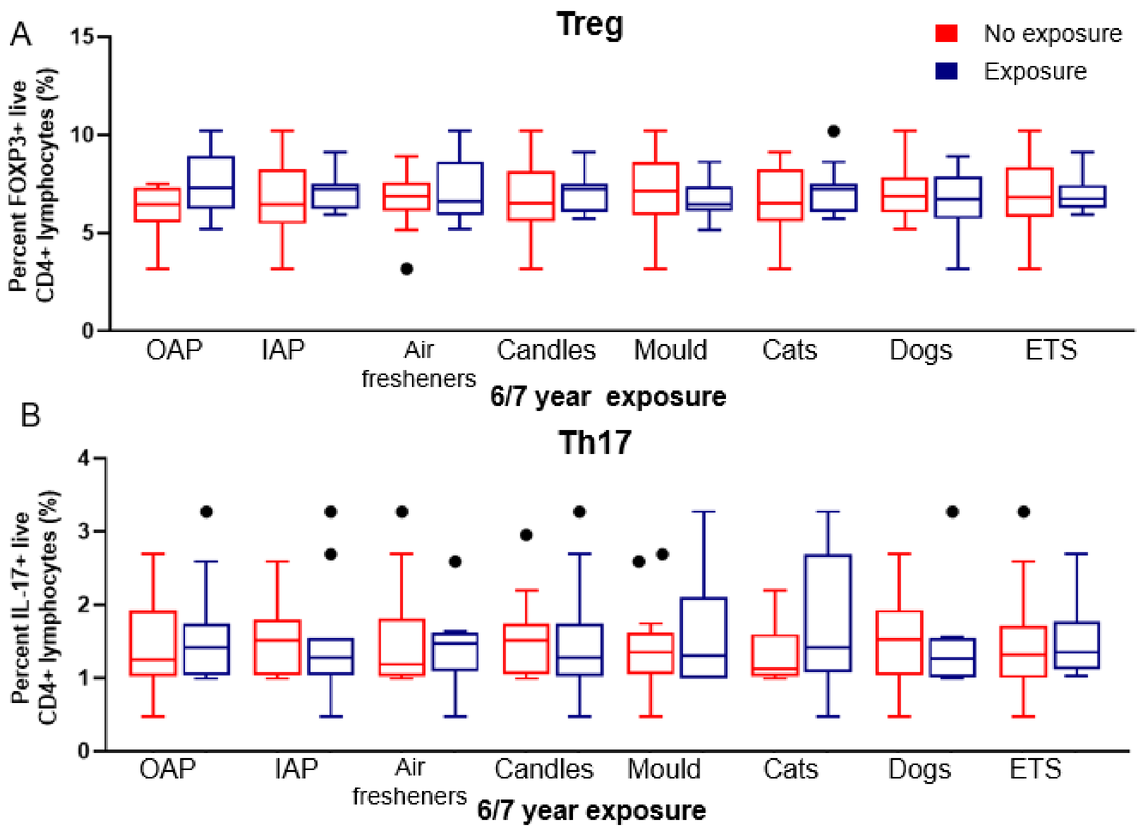

Figure 15. Treg and Th17 PBMC frequencies in PBMC samples in children with current exposures to different indoor and outdoor air pollutants

(A) Percent frequencies of live CD4+ lymphocytes expressing FOXP3 in PBMC samples from children with (red) or without (blue) current 6 to 7 year exposure to different indoor and outdoor air pollutant factors were determined and compared using Mann-Whitney tests. (B) Percent frequencies of live CD4+ lymphocytes expressing IL-17 in PBMC samples from children with (red) or without (blue) 6 to 7 year exposure to different indoor and outdoor air pollutant factors were determined and compared using Mann-Whitney tests. Data is displayed as the median with the IQR, no differences were statisically signifiant.

4.3.9 No significant differences in Th17 percent frequencies between children with or without current exposures to different indoor and outdoor air pollutants

To determine if there are any differences in peripheral Th17 cells levels between children with or without current exposure to different air pollutants, Th17 cell frequencies were determined and compared using Mann-Whitney tests (Figure 15, Appendix A Table 15). There were no statistically significant differences between children exposed or not exposed to any of the air polluting factor. Children with OAP, air freshener, and cat exposure had slightly higher Th17 frequencies. In contrast, children with exposure to high levels of IAP, dogs, and candles had lower Th17 frequencies than unexposed children. There were similar Th17 frequencies between children with and without exposure to ETS and mould.

4.3.10 SPT positive children have similar plasma cytokine concentrations as SPT negative children, but a reduced IL-10:IL-8 ratio

To investigate any differences in the inflammatory profile of children with or without a positive SPT, plasma cytokine concentration values were determined for the following cytokines: IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-21, TNF-α, IFN-γ, TGF-β1, TGF-β2, and TGF-β3. TGF-β3 was below the limit of detection in plasma samples and could not be analyzed. Raw cytokine concentration values were compared between SPT positive and negative children using Mann-Whitney tests. To investigate into the regulatory and inflammatory axes, as well as the Th1 to Th2 paradigm, the following cytokine ratios were compared between SPT positive and negative children: IL-10:IL-8, IL-10:IFN-γ, IL-10:IL-13, IL-10:IL-17, IL-13:IFN-γ, IFN-γ:IL-17, TGF-β1:IL-10, TGF-β1:IL-17, and TGF-β1:IL-21 (Table 7). The comparison of cytokine ratios allows for the normalization of plasma cytokine levels to the individual, where the balance between proinflammatory (IL-8, IL-13, IFNγ, IL-17) and regulatory (IL-10, IL-21, TGF-β) cytokines can be directly evaluated.

There were no significant differences in the plasma cytokine concentration between SPT positive and SPT negative children; however numerical increases in IL-5, IL-13, and IL-17 was observed in children with a positive SPT compared to SPT negative children (IL-5 $p = 0.423$, IL-13 $p = 0.752$, IL-17 $p = 0.776$). Children with a positive SPT had reduced IL-10:IL-8, IL-10:IL-13, and IL-10: IFN-γ ratios compared to SPT negative children; however, only the IL-10:IL-8 ratio was significantly lower (IL-10:IL-8 $p = 0.045$ IL10:IL-13 $p = 0.417$, and IL-10:IFN-γ $p = 0.073$).

Table 7. Plasma cytokine concentrations and ratios for clinical outcomes at 6 to 7 years of age.

	SPT negative (median IQR pg/mL)	SPT positive (median IQR pg/mL)	p	Respiratory negative (median IQR pg/mL)	Respiratory positive (median IQR pg/mL)	p	No atopy (median IQR pg/mL)	SPT positive and wheeze (median IQR pg/mL)	p
IL-5	2.15 (1.83 - 2.75)	2.45 (1.55 - 3.58)	0.423	2.27 (1.73 - 2.88)	2.27 (1.67 - 2.86)	0.935	2.13 (1.73 - 2.79)	2.27 (1.06 - 3.39)	0.948
IL-6	1.00 (0.80 - 1.30)	0.95 (0.55 - 2.58)	0.822	1.05 (0.74 - 1.34)	0.9 (0.59 - 1.63)	0.545	0.96 (0.74 - 1.33)	0.74 (0.33 - 2.43)	0.393
IL-8	1.75 (1.50 - 2.18)	2.05 (1.28 - 4.30)	0.374	1.90 (1.51 - 2.45)	1.74 (1.52 - 3.38)	0.974	1.76 (1.51 - 2.29)	1.74 (1.43 - 5.89)	0.878
IL-10	7.00 (6.33 - 7.90)	7.25 (3.90 - 12.60)	0.869	7.03 (6.17 - 10.38)	6.19 (6.17 - 10.38)	0.334	6.92 (6.17 - 8.25)	4.46 (1.91 - 9.13)	0.150
IL-13	1.90 (1.50 - 2.60)	2.35 (1.00 - 4.30)	0.752	2.06 (1.43 - 2.84)	2.44 (1.20 - 3.15)	0.858	1.94 (1.43 - 2.84)	2.44 (0.89 - 4.27)	0.778
IL-17A	7.60 (5.18 - 7.60)	8.05 (4.08 - 17.45)	0.776	8.33 (5.53 - 9.49)	7.17 (3.63 - 15.67)	0.638	8.33 (5.53 - 9.49)	8.33 (2.88 - 28.58)	0.878
IL-21	1.00 (0.53 - 1.68)	1.00 (0.35 - 1.63)	0.684	1.05 (0.52 - 1.77)	0.58 (0.27 - 1.18)	0.181	1.04 (0.52 - 1.80)	0.61 (0.20 - 1.71)	0.338
TNFα	2.35 (1.03 - 2.95)	2.30 (1.95 - 3.18)	0.797	2.40 (1.94 - 3.05)	2.22 (2.08 = 2.69)	0.661	2.29 (1.94 - 2.96)	2.16 (1.99 - 2.66)	0.703
IFNγ	7.55 (5.85 - 9.55)	7.90 (5.50 - 19.73)	0.686	7.92 (6.20 - 11.40)	6.27 (4.32 - 12.46)	0.280	7.92 (6.20 - 9.88)	7.56 (2.77 - 20.55)	0.878
TGFβ1	4160 (3454 - 7734)	4812 (3840 - 7921)	0.569	4165 (3531 - 7595)	5638 (3736 - 9996)	0.343	4160 (331 - 7998)	6984 (4115 - 12497)	0.328
TGFβ2	448.7 (389.7 - 700.8)	472.6 (387.2 - 643.8)	0.9781	431.6 (388.5 - 690.0)	541.5 (383.3 - 753.9)	0.626	448.7 (407.6 - 745.9)	592.7 (417.9 - 918.5)	0.561
	SPT negative (median IQR)	**SPT positive (median IQR)**	**p**	**Respiratory negative (median IQR)**	**Respiratory positive (median IQR)**	**p**	**No atopy (median IQR)**	**SPT positive and wheeze (median IQR)**	**p**
IL-10:IL-8	4.40 (3.150 - 4.70)	2.92 (1.65 - 3.89)	**0.045**	4.21 (3.12 - 4.85)	2.56 (1.32 - 3.86)	**0.033**	4.40 (3.12 - 4.85)	1.85 (1.08 - 2.71)	**0.003**
IL-10:IFNγ	0.98 (0.78 - 1.10)	0.78 (0.57 - 0.87)	0.073	0.89 (0.76 - 1.06)	0.75 (0.53 - 1.29)	0.569	0.96 (0.76 - 1.06)	0.59 (0.46 - 0.81)	**0.024**
IL-10:IL-13	3.81 (2.85 - 5.10)	2.77 (1.68 - 5.43)	0.417	4.14 (2.91 - 5.47)	2.48 (1.98 - 3.83)	0.137	3.81 (2.91 - 5.09)	2.14 (1.68 - 3.08)	0.061
IL-10:IL-17A	1.01 (0.79 - 1.27)	0.90 (0.53 - 1.37)	0.481	1.08 (0.82 - 1.32)	0.76 (0.46 - 1.22)	0.246	0.97 (0.73 - 1.26)	0.54 (0.35 - 0.85)	**0.033**
IL-13:IFNγ	0.26 (0.21 - 0.35)	0.28 (0.15 - 0.35)	0.964	0.25 (0.17 - 0.34)	0.29 (0.25 - 0.39)	0.271	0.25 (0.20 - 0.34)	0.275 (0.22 - 0.35)	0.591
IFNγ:IL-17A	1.10 (0.92 - 1.25)	1.01 (0.90 - 1.54)	0.892	1.10 (0.96 - 1.36)	0.91 (0.81 - 1.10)	0.079	1.10 (0.94 - 1.28)	0.91 (0.75 - 1.03)	0.106
TGFβ1:IL-10	624.7 (507.5 - 913.7)	1181 (417.5 - 1622)	0.417	591 (390.0 - 942.6)	1350 (729 - 2002	**0.047**	642.7 (461.4 - 942.6)	1543 (1332 - 2603)	**0.024**
TGFβ1:IL-17A	764.1 (391.7 - 953.1)	664.7 (435.1 - 1334)	0.788	656.3 (346.9 - 908.8)	825.2 (500.9 - 1723)	0.31	764.1 (346.9 - 908.8)	825.2 (4.69.8 - 2303)	0.442
TGFβ1:IL-21	5388 (2178 - 10554)	6358 (4194- 20003)	0.500	4042 (2094 - 10013)	8533 (6398 - 24611)	0.056	4029 (2094 - 12294)	15194 (6049 - 27011)	0.101

4.3.11 Children with respiratory symptoms have lower plasma IL-10 and IL-21 concentrations, a lower IL-10:IL-8 ratio, but an increased TGF-β1:IL-10 ratio

Plasma cytokine concentrations and ratios were determined and compared between children with and without parental report of respiratory conditions using Mann-Whitney tests (Table 7). A reduction in IL-10 and IL-21 was observed in children with parental report of cough and/or wheeze without a cold compared to children without these symptoms; however, these differences were not significant (IL-10 $p = 0.334$ and IL-21 $p = 0.181$). The IL-10:IL-8 ratio was significantly lower in children with respiratory symptoms compared to those without ($p = 0.033$), and the TGF-β1:IL-10 ratio was significantly higher ($p = 0.047$).

4.3.12 Children with a positive SPT and cough/wheeze have similar cytokine concentrations as non-atopic children, but an altered regulatory profile

Cytokine concentrations and ratios were determined for children with a positive SPT and wheeze/cough and compared to non-atopic children using Mann-Whitney tests (Table 7). Plasma cytokine concentrations were similar between the groups; with numerical reductions in IL-10 and IL-21 in children with a positive SPT and cough/wheeze (IL-10 $p = 0.150$ and IL-21 $p = 0.338$). There were reductions in the IL-10:IL-8, IL-10:IFN-γ, IL-10:IL-13, IL-10:IL-17A, and IFN-γ:IL-17A ratios in children with a positive SPT and cough/wheeze compared to non-atopic children (IL-10:IL-8 $p = 0.003$, IL-10:IFN-γ $p = 0.024$, IL-10:IL-13 $p = 0.061$, IL-10:IL-17A $p = 0.033$, and IFN-γ:IL-17A $p = 0.106$). Additionally, children with a positive SPT and wheeze/cough had higher TGF-β1:IL-10, TGF-β1:IL-17A, and TGF-β1:IL-21 ratios than non atopic children (TGF-β1:IL-10 $p = 0.024$, TGF-β1:IL-17A $p = 0.442$, and TGF-β1:IL-21 $p = 0.101$).

4.3.13 Children with prenatal exposure to high levels of indoor air pollutants and cats have reduced plasma cytokine concentrations and altered regulatory profiles

Prenatal exposure to OAP, IAP level, air fresheners, candles, mould, cats, dogs, ETS, and carpet were related to plasma cytokine concentration and ratios at 6 to 7 years. Cytokine concentrations and ratios were compared between children exposed or not exposed to each of the factors using Mann-Whitney test (Table 8).

Children with a high level of exposure to IAP prenatally had generally lower plasma concentrations of the measured cytokines compared to children with low levels of IAP exposure, with significant reductions in IL-5, IL-10, IL-21, and IFN-γ concentrations (IL-5 $p = 0.048$, IL-10 $p = 0.009$, IL-21 $p = 0.01$, and IFN-γ $p = 0.016$). Moreover, the IL-10:IL-8 ratio was significantly reduced in high IAP exposure children ($p = 0.004$).

Children with prenatal exposures to cats had a global reduction in plasma cytokine concentrations compared to children without cat exposure. Of the cytokines measured, IL-5, IL-10, IL-21, TNF-α, and IFN-γ were either significantly reduced or trending towards significance in these children (IL-5 $p = 0.06$, IL-10 $p = 0.004$, IL-21 $p = 0.008$, TNF-α $p = 0.085$, and IFN-γ $p = 0.025$). Additionally, children with these exposures had altered regulatory and inflammatory signatures, with the IL-10:IL-8 and IFN-γ:IL-17A significantly reduced, and the TGF-β1:IL:10 and TGF-β1:IL-21 ratios significantly increased (IL-10:IL-8 $p = 0.016$, IFN-γ:IL-17A $p = 0.041$, TGF-β1:IL:10 $p = 0.031$, and TGF-β1:IL-21 $p = 0.025$).

Children with and without prenatal exposure to mould had similar plasma concentrations of the measured cytokines. There was a numerical reduction in plasma IL-10 concentration and a slight increase in IL-17A concentration for children exposed to mould compared to those not exposed, although this was not significant (IL-10 $p = 0.283$ and IL-17A $p = 0.614$). However, these IL-10 and IL-17A differences were apparent when considering the IL-10:IL-17A ratios, as mould exposed children had a significantly lower IL-10:IL-17A ratio compared to children not exposed ($p = 0.026$). There were no significant differences in the other ratios for children exposed or not exposed to mould.

There were no significant differences in the plasma cytokine concentrations between children that were exposed during pregnancy to ETS and those that were not exposed to ETS. The cytokine ratios were similar between these children; however, the IFN-γ:IL-17A ratio was significantly lower in children with ETS exposure compared to those without ($p = 0.040$).

There were similar cytokine concentrations between children with and without prenatal exposure to carpet. Children with prenatal carpet exposure had significantly lower IL-21 plasma concentrations than children without carpet exposure ($p = 0.040$). There were no significant differences in any of the cytokine ratios between these children. There were no significant differences in the plasma cytokine concentrations or ratios for children with or without prenatal exposures to OAP, air fresheners, candles, or dogs.

Table 8. Plasma cytokine concentrations and ratios for prenatal exposures.

	OAP 0 (median IQR pg/mL)	OAP 1+ (median IQR pg/mL)	p	IAP low (median IQR pg/mL)	IAP high (median IQR pg/mL)	p	No Air fresheners (median IQR pg/mL)	Air fresheners (median IQR pg/mL)	p
IL-5	2.41 (1.83 - 4.34)	2.15 (1.86 - 2.79)	0.516	2.76 (2.13 - 4.56)	2.01 (1.20 - 2.56)	**0.048**	2.13 (1.65 - 2.67)	2.66 (2.22 - 3.69)	0.135
IL-6	1.23 (0.69 - 3.02)	0.85 (0.72 - 1.08)	0.118	1.07 (0.77 - 2.18)	1.02 (0.65 - 1.29)	0.381	0.96 (0.73 - 1.34)	1.06 (0.74 - 1.66)	0.890
IL-8	2.39 (1.53 - 4.68)	1.79 (1.52 - 2.13)	0.315	2.23 (1.63 - 3.54)	1.80 (1.52 - 2.33)	0.456	1.76 (1.51 - 2.48)	2.17 (1.74 - 3.02)	0.353
IL-10	8.33 (4.09 - 14.40)	7.13 (6.59 - 8.26)	0.726	9.11 (7.13 - 14.40)	6.19 (4.20 - 7.55)	**0.009**	7.03 (5.62 - 9.68)	7.40 (6.63 - 13.55)	0.481
IL-13	2.61 (1.77 - 4.87)	1.94 (1.61 - 2.41)	0.065	2.41 (1.80 - 3.76)	2.17 (1.47 - 2.58)	0.401	2.06 (1.41 - 2.89)	2.50 (2.19 - 3.15)	0.430
IL-17A	8.77 (5.93 - 16.16)	8.33 (5.53 - 9.18)	0.424	8.68 (7.59 - 13.20)	7.32 (4.39 - 9.22)	0.169	8.33 (6.83 - 10.47)	7.79 (4.84 - 10.62)	0.614
IL-21	1.17 (0.53 - 1.82)	1.00 (0.52 - 1.14)	0.446	1.29 (1.04 - 1.87)	0.56 (0.45 - 1.05)	**0.010**	1.04 (0.52 - 1.77)	0.85 (0.50 - 1.58	0.984
TNFα	2.35 (1.94 - 3.27)	2.29 (2.00 - 2.69)	0.402	2.64 (2.10 - 3.13)	2.18 (1.94 - 2.64)	0.181	2.29 (1.94 - 2.85)	2.34 (2.18 - 2.81)	0.587
IFNγ	8.56 (5.66 - 16.07)	7.92 (6.96 - 8.97)	0.911	10.41 (7.92 - 16.35)	6.70 (5.27 - 8.42)	**0.016**	7.92 (6.52 - 11.47)	7.85 (5.70 - 15.30)	0.841
TGFβ1	3932 (3605 - 6453)	5811 (4184 - 8623)	0.166	5446 (3844 - 9364)	4401 (3706 - 6053)	0.603	4262 (3605 - 7790)	5750 (4082 - 9096)	0.353
TGFβ2	424.0 (383.3 - 628.6)	564.7 (457.3 - 745.9)	0.143	560.2 (424.0 - 919.0)	431.6 (385.9 - 569.3)	0.260	448.7 (389.8 - 745.9)	564.6 (417.5 - 747.0)	0.546
	OAP 0 (median IQR)	**OAP 1+ (median IQR)**	**p**	**IAP low (median IQR)**	**IAP high (median IQR)**	**p**	**No Air fresheners (median IQR)**	**Air fresheners (median IQR)**	**p**
IL-10:IL-8	3.13 (1.90 - 4.33)	4.48 (3.12 - 4.98)	0.136	4.64 (3.30 - 5.29)	2.98 (2.26 - 4.04)	**0.004**	3.73 (2.34 - 4.66)	3.40 (3.07 - 4.79)	0.779
IL-10:IFNγ	0.88 (0.73 - 1.12)	0.83 (0.76 - 1.04)	0.682	0.94 (0.80 - 0.99)	0.83 (0.61 - 0.83)	0.809	0.83 (74 - 1.01)	0.99 (0.71 - 1.29)	0.3634
IL-10:IL-13	2.96 (1.92 - 4.90)	3.8 (2.98 - 5.66)	0.340	3.68 (2.86 - 5.66)	3.19 (2.13 - 4.34)	0.331	3.43 (2.03 - 5.47)	2.89 (2.60 - 5.30)	0.718
IL-10:IL-17A	0.97 (0.72 - 1.27)	0.92 (0.72 - 1.35)	0.931	0.97 (0.87 - 1.35)	0.95 (0.56 - 1.29)	0.370	0.905 (0.66 - 1.25)	1.23 (0.87 - 1.58)	0.109
IL-13:IFNγ	0.20 (0.19 - 0.41)	0.26 (0.17 - 0.29)	0.307	0.23 (0.16 - 0.35)	0.29 (0.26 - 0.37)	0.302	0.26 (0.20 - 0.31)	0.32 (0.25 - 0.38)	0.263
IFNγ:IL-17A	1.00 (0.91 - 1.17)	1.05 (0.91 - 1.33)	0.778	1.15 (0.96 0 1.37)	0.96 (0.85 - 1.07)	0.107	1.02 (0.91 - 1.63)	1.09 (0.90 - 1.62)	0.533
TGFβ1:IL-10	649.1 (382.5 - 1332)	815.2 (535.4 - 1134)	0.739	461.8 (424.4 - 1031)	828.6 (511.9 1385)	0.230	885.4 (410.2 - 1332)	753.2 (512.4 - 1097)	0.841
TGFβ1:IL-17A	550.1 (335.9 - 892.0)	907.8 (599.6 - 1230)	0.190	626.0 (495.7 - 871.6)	701.9 (392.4 - 1379)	0.824	632.6 (335.9 - 1260)	764.1 (549.7 - 1295)	0.602
TGFβ1:IL-21	4948 (2327 - 7679)	7962 (3741 - 13090)	0.248	4293 (2913 - 7890)	6978 (3926 - 10709)	0.131	6358 (2338 - 10013)	7123 (3365 - 16105)	0.602

	No Candles (median IQR pg/mL)	Candles (median IQR pg/mL)	p	No Mould (median IQR pg/mL)	Mould (median IQR pg/mL)	p	No Cats (median IQR pg/mL)	Cats (median IQR pg/mL)	p
IL-5	2.44 (1.07 - 3.53)	2.01 (1.80 - 2.48)	0.150	2.29 (2.01 - 2.79)	2.09 (1.09 - 3.19)	0.480	2.82 (2.07 - 5.34)	2.13 (1.42 - 2.50)	0.060
IL-6	1.06 (0.72 - 1.62)	0.91 (0.75 - 1.16)	0.610	1.06 (0.74 - 1.34)	0.91 (0.50 - 1.58)	0.506	1.09 (0.76 - 2.64)	0.96 (0.67 - 1.26)	0.315
IL-8	2.34 (1.51 - 4.09)	1.76 (1.56 - 2.07)	0.305	2.17 (1.56 - 2.51)	1.79 (1.47 - 3.33)	0.547	2.34 (1.75 - 4.07)	1.74 (1.50 - 2.27)	0.181
IL-10	7.19 (5.38 - 14.12)	7.34 (6.32 - 8.83)	0.692	7.40 (6.56 - 13.55)	6.60 (4.18 - 8.59)	0.283	11.19 (7.34 - 14.40)	6.46 (4.43 - 7.47)	**0.004**
IL-13	2.58 (1.09 - 4.56)	1.94 (1.67 - 2.35)	0.119	2.38 (1.61 - 2.89)	2.18 (1.55 - 3.03)	0.674	2.50 (2.03 - 4.31)	2.06 (0.89 - 2.55)	0.151
IL-17A	9.25 (5.47 - 14.00)	7.38 (6.08 - 8.50)	0.188	7.38 (5.53 - 10.47)	8.92 (6.32 - 15.67)	0.614	8.68 (7.39 - 14.00)	7.38 (4.72 - 9.27)	0.296
IL-21	1.190 (0.478 - 1.77)	0.95 (0.54 - 1.05)	0.484	1.04 (0.59 - 1.41)	0.79 (0.27 - 1.97)	0.587	1.29 (1.06 - 1.92)	0.58 (0.48 - 1.04)	**0.008**
TNFα	2.22 (1.96 - 2.78)	2.52 (1.99 - 2.92)	0.865	2.34 (2.00 - 2.93)	2.26 (1.86 - 2.85)	0.560	2.73 (2.24 - 3.22)	2.16 (1.94 - 2.58)	0.085
IFNγ	9.10 (6.20 - 17.20)	7.46 (6.27 - 8.10)	0.208	7.85 (5.95 - 14.45)	7.99 (5.98 - 13.96)	0.779	12.40 (7.74 - 17.20)	7.06 (5.41 - 8.47)	**0.025**
TGFβ1	4304 (3638 - 9796)	5395 (4116 - 7157)	0.970	4812 (3683 - 6685)	5819 (3316 - 9996)	0.904	4827 (3710 - 8318)	4812 (3716 - 8073)	0.91
TGFβ2	431.6 (393.6 - 897.8)	506.6 (409.8 - 672.0)	0.910	465.5 (410.2 - 603.5)	568.8 (364.4 - 898.8	0.841	513 (420.3 - 825.1)	465.5 (387.2 - 646.1)	0.678
	No Candles (median IQR)	**Candles (median IQR)**	**p**	**No Mould (median pg/mL)**	**Mould (median pg/mL)**	**p**	**No Cats (median IQR)**	**Cats (median IQR)**	**p**
IL-10:IL-8	3.16 (2.49 - 4.63)	4.23 (3.20 - 4.66)	0.427	3.83 (3.10 - 4.79)	2.96 (1.32 - 4.54)	0.179	4.60 (3.24 - 5.31)	3.02 (2.29 - 4.33)	**0.016**
IL-10:IFNγ	0.83 (0.66 - 0.98)	1.02 (0.79 - 1.23)	0.188	0.98 (0.80 - 1.15)	0.80 (0.57 - 0.90)	0.095	0.96 (0.79 - 1.00)	0.83 (0.62 - 1.21)	0.637
IL-10:IL-13	2.89 (2.14 - 5.71)	3.81 (3.22 - 4.94)	0.571	4.14 (2.49 - 5.59)	3.24 (2.15 - 3.46)	0.353	3.50 (2.81 - 5.50)	3.29 (2.14 - 4.94)	0.792
IL-10:IL-17A	0.96 (0.64 - 1.36)	1.05 (0.74 - 1.29)	0.792	1.21 (0.82 - 1.41)	0.79 (0.50 - 0.93)	**0.026**	1.11 (0.90 - 1.39)	0.85 (0.57 - 1.26)	0.208
IL-13:IFNγ	0.28 (0.14 - 0.37)	0. 27 (0.24 - 0.29)	0.955	0.29 (0.17 0 0.37)	0.26 (0.22 - 0.27)	0.407	0.25 (0.19 - 0.35)	0.29 (0.22 - 0.35)	0.748
IFNγ:IL-17A	0.99 (0.91 - 1.41)	1.03 (0.91 - 1.19)	0.836	1.03 (0.95 - 1.32)	0.93 (0.79 - 1.10)	0.124	1.18 (0.98 - 1.41)	0.94 (0.86 - 1.07)	**0.041**
TGFβ1:IL-10	649.1 (436 - 1605)	815.2 (472.5 - 943.7)	0.910	649.1 (430.9 - 968.5)	1129 (514.6 - 2002)	0.274	583.5 (265.4 - 882.7)	938 (625.5 - 1367)	**0.031**
TGFβ1:IL-17A	656.3 (366.7 - 1334)	784.5 (527.9 - 1138)	0.792	693.5 (492.3 - 1239)	691.5 (349.7 - 1400)	0.841	656.3 (331.3 - 861.0)	838.6 (394.8 - 1441)	0.305
TGFβ1:IL-21	5807 (2631 - 17621)	6453 (3907 - 9756)	0.910	5368 (3051 - 9858)	7073 (3447 - 24611)	0.444	3710 (3171 - 6624)	9935 (4379 - 14702)	**0.025**

	No Dogs (median IQR pg/mL)	Dogs (median IQR pg/mL)	p	No ETS (median IQR pg/mL)	ETS (median IQR pg/mL)	p	No Carpet (median IQR pg/mL)	Carpet (median IQR pg/mL)	p
IL-5	2.27 (1.92 - 2.80)	2.41 (1.48 - 3.50)	0.836	2.31 (1.73 - 2.91)	2.13 (1.81 - 2.56)	0.462	2.25 (2.01 - 2.94)	2.43 (1.42 - 2.80)	0.895
IL-6	0.96 (0.75 - 1.10)	1.27 (0.54 - 2.62)	0.485	0.86 (0.70 - 1.26)	1.11 (0.76 - 1.50)	0.395	1.07 (0.77 - 1.43)	0.88 (0.52 - 1.24)	0.279
IL-8	1.76 (1.53 - 2.07)	2.39 (1.62 - 4.34)	0.208	1.79 (1.47 - 2.45)	1.88 (1.58 - 2.39)	0.667	2.06 (1.69 - 2.55)	1.69 (1.44 - 2.45)	0.427
IL-10	7.19 (6.32 - 8.83)	8.28 (4.67 - 13.42)	0.777	7.03 (5.54 - 13.55)	7.08 (5.51 - 9.19)	0.763	7.34 (6.37 - 10.12)	6.98 (4.44 - 12.30)	0.664
IL-13	2.14 (1.67 - 2.45)	2.61 (0.97 - 4.56)	0.295	2.35 (1.14 - 2.84)	2.06 (1.47 - 2.28)	0.943	2.44 (1.72 - 3.38)	2.23 (0.86 - 2.54)	0.460
IL-17A	8.33 (6.98 - 9.21)	8.71 (3.51 - 18.05)	0.836	8.33 (4.87 - 9.86)	7.38 (6.27 - 9.40)	0.989	8.92 (7.26 - 11.21)	1.22 (3.69 - 9.09)	0.188
IL-21	0.95 (0.53 - 1.08)	1.29 (0.60 - 1.77)	0.372	1.07 (0.42 - 1.80)	0.77 (0.55 - 1.23)	0.677	1.17 (0.93 - 1.92)	0.54 (0.35 - 1.08)	**0.040**
TNFα	2.33 (2.05 - 2.76)	2.41 (1.94 - 3.14)	0.806	2.18 (1.94 - 2.96)	2.52 (2.12 - 2.92)	0.594	2.52 (2.06 - 2.82)	2.20 (1.94 - 3.04)	0.610
IFNγ	7.92 (6.17 - 9.93)	8.56 (5.66 - 18.89)	0.792	8.46 (5.85 - 14.45)	7.06 (5.72 - 8.64)	0.371	8.39 (7.33 - 13.39)	6.71 (5.30 - 15.64)	0.270
TGFβ1	5811 (4391 - 8705)	3934 (3585 - 4345)	0.083	4304 (3703 - 7831)	4672 (3219 - 7998)	0.796	5638 (3740 - 9755)	4264 (3716 - 5538)	0.473
TGFβ2	564.7 (474.3 - 825.1)	424.0 (387.2 - 431.6)	0.069	448.7 (408.9 - 719.9)	472.6 (356.6 - 690)	0.752	541.5 (431.6 - 946.0)	411.3 (378.1 - 545.0)	0.082
	No Dogs (median IQR)	Dogs (median IQR)	p	No ETS (median IQR)	ETS (median IQR)	p	No Carpet (median IQR)	Carpet (median IQR)	p
IL-10:IL-8	4.45 (3.08 - 4.70)	3.08 (1.67 - 3.89)	0.135	3.92 (3.10 - 4.87)	3.34 (2.34 - 4.43)	0.213	3.40 (2.54 - 4.70)	3.73 (2.89 - 4.56)	0.851
IL-10:IFNγ	0.89 (0.75 - 1.12)	0.86 (0.68 - 0.98)	0.720	0.86 (0.76 - 0.99)	0.97 (0.58 - 1.25)	0.763	0.89 (0.67 - 1.00)	0.86 (0.76 - 1.21)	0.665
IL-10:IL-13	3.50 (2.85 - 5.43)	3.18 (1.80 - 5.17)	0.521	3.53 (2.60 - 5.54)	3.77 (2.03 - 5.01)	0.752	3.25 (2.30 - 5.37)	3.66 (2.30 - 5.43)	0.851
IL-10:IL-17A	0.95 (0.70 - 1.25)	1.10 (0.66 - 1.37)	0.851	0.97 (0.88 - 1.30)	0.87 (0.47 - 1.38)	0.575	0.91 (0.62 - 1.26)	1.12 (0.87 - 1.34)	0.343
IL-13:IFNγ	0.27 (0.21 - 0.33)	0.28 (0.19 - 0.37)	0.835	0.26 (0.17 - 0.34)	0.27 (0.20 - 0.37)	0.763	0.26 (0.20 - 0.35)	0.29 (0.16 - 0.40)	0.557
IFNγ:IL-17A	0.96 (0.90 - 1.19)	1.04 (0.97 - 1.41)	0.333	1.14 (0.97 - 1.36)	0.93 (0.79 - 1.16)	**0.040**	0.97 (0.91 - 1.20)	1.03 (0.93 - 1.54)	0.484
TGFβ1:IL-10	885.4 (608.8 - 1171)	591.9 (272.1 - 1248)	0.427	698.1 (482.2 - 1211)	697.7 (410.2 - 1332)	0.841	753.2 (580.2 - 1222)	938.3 (319.0 - 1171)	>0.999
TGFβ1:IL-17A	784.5 (534.7 - 1249)	477.8 (291.8 - 1307)	0.343	855.1 (510.3 - 1097)	550.1 (346.9 - 963.0)	0.312	693.5 (366.6 - 1111)	783.0 (461.0 - 1414)	0.571
TGFβ1:IL-21	7962 *3965 - 14390)	3594 (2137 - 9080)	0.157	6082 (2338 - 17847)	6178 (3449 - 9090)	0.752	4948 (2631 - 8360)	10245 (3532 - 17080)	0.238

4.3.14 Children with current exposure to cats and high levels of indoor air pollutants had reduced plasma cytokine concentrations

To investigate the relationships between current exposure to indoor and outdoor air pollutants with inflammatory and regulatory cytokines in children, the plasma concentration of 11 cytokines and 9 cytokine ratios were compared between children with different exposures using Mann-Whitney tests (Table 9).

When comparing children with and without cat exposure, children with current exposure to cats had lower plasma cytokine concentrations for all measured cytokines except for TGFb1 and TGFb2, which were elevated. Of the measured cytokines, IL-5, IL-8, IL-10, IL-13, IL-21, TNF-α, and IFN-γ were significantly lower or trending towards significance (IL-5 $p = 0.06$, IL-8 $p = 0.072$, IL-10 $p = 0.011$, IL-13 $p = 0.027$, IL-21 $p = 0.019$, TNF-α $p = 0.084$, and IFN-γ $p = 0.022$). There were not any significant differences in the cytokine ratios between children with or without cat exposure; however, the TGF-β1:IL-17A and TGF-β1:IL-21 ratios were numerically higher and trending towards significance (TGF-β1:IL-17A $p = 0.093$ and TGF-β1:IL-21 $p = 0.055$).

Children with high levels of IAP exposure had lower plasma cytokine concentrations than children with low IAP exposure levels, with the exceptions of TGF-β1 and TGF-β2; which were elevated. IL-5, IL-7, IL-13, IL-21, TNF-α, and IFN-γ were either significantly reduced or trending towards significance (IL-5 $p = 0.048$, IL-8 $p = 0.047$, IL-13 $p = 0.053$, IL-21 $p = 0.056$, TNF-α $p = 0.090$, and IFN-γ $p = 0.013$). Of the cytokine ratios measured, all were similar between low and high IAP exposure groups with the exceptions of TGF-β1:IL-10, TGF-β1:IL-17A, and TGF-β1:IL-21; all of which were elevated (TGF-β1:IL-10 $p = 0.106$, TGF-β1:IL-17A $p = 0.030$, and TGF-β1:IL-21 $p = 0.063$).

Children with any exposure to OAP at 6 years had elevated cytokine plasma concentrations for IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-21, TNF-α, and IFN-γ; although only IL-17A was significantly increased ($p = 0.036$). When considering the cytokine ratios, there was a general reduction in each ratio, with IL-10:IL-13, IL-10:IL-17A, and IFN-γ:IL-17A either significantly reduced or trending towards significance in children with OAP exposure (IL-10:IL-13 $p = 0.096$, IL-10:IL-17A $p = 0.036$, and IFN-γ:IL-17A $p =0.001$).

Children with in-home candle exposure at the 6 to 7 year time point had significantly lower IFN-γ plasma concentration than children not exposed to candles ($p = 0.041$). Of the remaining measured cytokines, there were numerical decreases in the plasma cytokine concentration in children with candle exposure compared to children without this exposure; with the exception of TGF-β2, which was slightly higher. The cytokine ratios were similar between exposure and non exposure groups.

When comparing the plasma cytokine levels of children with and without ETS exposure, there was an overall decrease in IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-21, TNF-α, and IFN-γ levels and no difference between the TGF-β1 and TGF-β2 levels. Of these decreases, IL-5, IL-10, and IFN-γ were either significantly decreased or trending towards significance (IL-5 $p = 0.026$, IL-10 $p = 0.1$, and IFN-γ $p = 0.052$). The cytokine ratios were similar between exposure groups with no significant differences. No significant differences were observed in the plasma cytokine concentrations or cytokine ratios between children with or without current exposure to air fresheners, mould, or dogs.

Table 9. Plasma cytokine concentrations and ratios for current exposures.

	OAP 0 (median IQR pg/mL)	OAP 1+ (median IQR pg/mL)	p	IAP low (median IQR pg/mL)	IAP high (median IQR pg/mL)	p	No Air fresheners (median IQR pg/mL)	Air fresheners (median IQR pg/mL)	p
IL-5	1.89 (1.46 - 2.57)	2.32 (2.01 - 3.00)	0.075	2.76 (1.07 - 3.38)	2.01 (1.61 - 2.32)	**0.048**	2.27 (1.68 - 2.85)	2.15 (1.83 - 2.80)	0.940
IL-6	0.81 (0.57 - 1.22)	1.04 (0.75 - 1.51)	0.233	1.11 (0.76 - 2.25)	0.81 (0.65 - 1.18)	0.163	1.04 (0.76 - 1.28)	0.90 (0.67 - 1.43)	0.663
IL-8	1.780 (1.38 - 2.27)	1.80 (1.57 - 2.58)	0.379	2.06 (1.68 - 3.54)	1.57 (1.41 - 2.09)	**0.047**	1.90 (1.50 - 2.41)	1.77 (1.53 - 2.45)	0.976
IL-10	6.72 (4.94 - 10.62)	7.13 (6.09 - 9.42)	0.737	7.13 (6.46 - 13.83)	6.72 (4.20 - 7.55)	0.130	7.13 (5.80 - 10.20)	6.73 (5.38 - 8.64)	0.499
IL-13	1.71 (0.93 - 2.53)	2.41 (1.65 - 3.62)	0.188	2.41 (1.94 - 4.16)	1.65 (0.69 - 2.46)	0.053	2.06 (1.34 - 2.55)	2.50 (1.67 - 3.58)	0.358
IL-17A	5.71 (3.70 - 8.68)	8.80 (7.20 - 10.10)	**0.036**	8.68 (5.95 - 13.20)	7.20 (4.51 - 8.80)	0.191	7.71 (4.42 - 9.25)	8.00 (6.90 - 9.88)	0.558
IL-21	0.60 (0.34 - 1.19)	1.02 (0.56 - 1.77)	0.232	1.20 (0.58 - 1.94)	0.60 (0.45 -1.02)	0.056	1.04 (0.54 - 1.29)	0.77 (0.47 - 1.87)	0.94
TNFα	2.18 (1.92 - 2.64)	2.39 (2.13 - 3.20)	0.188	2.64 (2.16 - 3.10)	2.18 (1.94 - 2.64)	0.090	2.40 (1.06 - 2.92)	2.20 (1.93 - 2.96)	0.538
IFNγ	5.99 (5.20 - 12.54)	7.56 (6.0 - 9.70)	0.379	9.13 (7.39 - 16.35)	5.99 (5.34 - 8.28)	**0.013**	7.46 (5.63 - 13.60)	8.35 (5.88 - 9.56)	0.976
TGFβ1	4401 (3573 - 5811)	4207 (3616 - 8746)	0.599	4113 (3262 - 8136)	5222 (3922 - 7803)	0.303	4165 (3618 - 5901)	6686 (3638 - 8705)	0.383
TGFβ2	431.6 (401.2 - 564.7)	465.9 (375.5 - 706.2)	0.770	431.6 (370.2 - 768.2)	499.4 (391.1 - 684.7)	0.608	431.6 (382 - 567.0)	585.6 (427.8 - 700.8)	0.319
	OAP 0 (median IQR)	**OAP 1+ (median IQR)**	**p**	**IAP low (median IQR)**	**IAP high (median IQR)**	**p**	**No Air fresheners (median IQR)**	**Air fresheners (median IQR)**	**p**
IL-10:IL-8	4.42 (3.12 - 5.62)	3.18 (2.52 - 4.61)	0.215	3.26 (2.96 - 4.60)	4.04 (2.52 - 4.64)	0.733	3.83 (3.00 - 4.70)	3.20 (2.61 - 4.56)	0.569
IL-10:IFNγ	0.88 (0.76 - 1.09)	0.83 (0.61 - 1.10)	0.588	0.83 (0.74 - 0.98)	1.05 (0.63 - 1.24)	0.283	0.86 (0.78 - 1.03)	0.87 (0.62 - 1.12)	0.916
IL-10:IL-13	4.40 (3.35 - 5.62)	2.96 (2.13 - 4.71)	0.096	3.19 (2.14 - 4.20)	4.4 (2.81 - 5.52)	0.150	4.01 (2.87 - 5.37)	2.89 (2.00 - 4.71)	0.291
IL-10:IL-17A	1.24 (0.96 - 1.47)	0.84 (0.56 - 1.27)	**0.036**	0.95 (0.76 - 1.34)	1.18 (0.60 - 1.29)	0.722	1.10 (0.77 - 1.42)	0.97 (0.57 - 1.15)	0.313
IL-13:IFNγ	0.24 (0.16 - 0.29)	0.29 (0.20 - 0.37)	0.268	0.26 (0.22 - 0.35)	0.28 (0.12 - 0.37)	0.786	0.25 (0.19 - 0.29)	0.32 (0.22 - 0.38)	0.139
IFNγ:IL-17A	1.20 (1.14 - 1.51)	0.95 (0.85 - 1.01)	**0.001**	1.07 (0.93 - 1.39)	1.01 (0.90 - 1.20)	0.400	1.14 (0.92 - 1.41)	0.96 (0.87 - 1.13)	0.197
TGFβ1:IL-10	801.7 (458.4 - 996.1)	593.7 (437.7 - 1385)	0.953	576.9 (272.3 - 1129)	828.6 (593.7 - 1385	0.106	640.4 (348.4 - 1248)	766.6 (532.3 - 1512)	0.452
TGFβ1:IL-17A	869.6 (560.1 - 1421)	563.2 (358.0 - 886.7)	0.138	516.1 (291.7 - 855.1)	886.7 (563.2 - 1379)	**0.030**	664.7 (333.4 - 1252)	785.9 (503.9 - 968.6)	0.697
TGFβ1:IL-21	9781 (3709 - 13880)	4158 (2096 - 8757)	0.318	3900 (2092 - 7073)	9781 (5739 - 17052)	0.063	5368 (2631 - 10012)	7687 (2572 - 15682)	0.697

	No Candles (median IQR pg/mL)	Candles (median IQR pg/mL)	p	No Mould (median IQR pg/mL)	Mould (median IQR pg/mL)	p	No Cats (median IQR pg/mL)	Cats (median IQR pg/mL)	p
IL-5	2.32 (1.60 - 3.32)	2.01 (1.77 - 2.56)	0.338	2.29 (1.83 - 2.88)	2.25 (1.68 - 2.50)	0.578	2.76 (1.95 - 3.38)	2.01 (1.61 - 2.29)	0.060
IL-6	1.04 (0.76 - 2.13)	0.81 (0.72 - 1.29)	0.579	1.06 (0.67 - 1.51)	0.96 (0.80 - 1.22)	0.965	1.07 (0.77 - 2.13)	0.81 (0.65 - 1.29)	0.310
IL-8	2.02 (1.53 - 3.54)	1.67 (1.51 - 2.24)	0.277	1.92 (1.53 - 2.56)	1.73 (1.50 - 2.25)	0.383	2.06 (1.66 - 3.54)	1.58 (1.41 - 2.09)	0.072
IL-10	7.24 (5.67 - 13.83)	6.92 (5.67 - 7.55)	0.296	7.03 (5.38 - 10.12)	7.03 (5.80 -9.058)	0.988	7.91 (6.93 - 13.83)	6.09 (4.20 - 6.92)	**0.011**
IL-13	2.41 (1.32 - 4.16)	1.71 (1.47 - 2.46)	0.201	2.35 (1.47 - 3.48)	1.06 (1.45 - 2.45)	0.621	2.58 (1.94 - 4.16)	1.47 (0.69 - 2.41)	**0.027**
IL-17A	9.16 (6.90 - 13.20)	7.20 (4.51 - 7.97)	0.079	7.05 (4.54 - 9.88)	8.33 (7.35 - 9.25)	0.461	8.68 (6.90 - 13.20)	7.32 (4.39 - 9.22)	0.246
IL-21	1.05 (0.49 - 1.84)	0.91 (0.52 - 1.29)	0.638	0.81 (0.47 - 1.77)	1.04 (0.62 - 1.23)	0.940	1.29 (0.77 - 1.94)	0.6 (0.45 - 1.02)	**0.019**
TNFα	2.39 (1.98 - 2.91)	2.18 (1.94 - 2.95)	0.853	2.34 (1.94 - 2.96)	2.29 (2.06 - 2.87)	0.940	2.64 (2.22 - 2.98)	2.13 (1.94 - 2.64)	0.084
IFNγ	9.13 (6.67 - 16.35)	6.70 (5.41 - 7.56)	**0.041**	7.78 (5.47 - 13.21)	7.53 (6.88 - 9.93)	0.742	9.13 (7.39 - 16.35)	6.27 (5.34 - 8.42)	**0.022**
TGFβ1	4401 (3488 - 9446)	4207 (3922 - 6053)	0.910	4924 (3638 - 8387)	4118 (3323 - 6950)	0.610	4113 (3488 - 8054)	5222 (3922 - 7803)	0.459
TGFβ2	431.6 (380.7 - 919.0)	465.9 (391.1 - 569.3)	0.820	465.5 (396.1 - 681.4)	441.2 (383.3 - 777.0	0.787	431.6 (380.7 - 785.5)	499.4 (391.1 - 671.7)	0.820
	No Candles (median IQR)	**Candles (median IQR)**	**p**	**No Mould (median pg/mL)**	**Mould (median pg/mL)**	**p**	**No Cats (median IQR)**	**Cats (median IQR)**	**p**
IL-10:IL-8	3.26 (2.92 - 4.63)	4.04 (2.52 - 4.72)	0.691	3.22 (2.61 - 4.41)	4.56 (3.31 - 5.61)	0.093	3.42 (3.10 - 4.98)	3.61 (2.52 - 4.42)	0.531
IL-10:IFNγ	0.82 (0.74 - 0.93)	1.05 (0.63 - 1.12)	0.122	0.86 (0.66 - 1.10)	0.89 (0.78 - 1.03)	0.940	0.88 (0.76 - 0.99)	0.82 (0.61 - 1.12)	0.966
IL-10:IL-13	3.19 (2.14 - 5.62)	4.34 (2.76 - 4.97)	0.691	3.18 (2.14 - 5.10)	4.01 (3.22 - 5.19)	0.528	3.19 (2.46 - 4.57)	4.40 (2.13 - 5.14)	0.303
IL-10:IL-17A	0.95 (0.72 - 1.15)	1.26 (0.60 - 1.43)	0.296	1.01 (0.66 - 1.42)	0.91 (0.70 - 1.26(	0.444	0.97 (0.87 - 1.26)	1.18 (0.56 - 1.40)	0.943
IL-13:IFNγ	0.26 (0.16 - 0.36)	0.28 (0.23 - 0.35)	0.638	0.29 (0.20 - 0.37)	0.25 (0.19 - 0.28)	0.373	0.26 (0.20 - 0.37)	0.28 (0.12 - 0.29)	0.578
IFNγ:IL-17A	1.00 (0.88 - 1.30)	1.05 (0.95 - 1.20)	0.897	1.06 (0.92 - 1.43)	0.98 (0.90 - 1.20)	0.461	1.07 (0.90 - 1.30)	1.01 (0.91 - 1.20)	0.764
TGFβ1:IL-10	944.2 (303.6 - 1261)	691.7 (576.9 - 942.1)	0.691	753.2 (507.5 - 1248)	634.3 (355.2 - 1275)	0.788	5901.1 (303.6 - 943.1)	828.6 (593.7 - 1701)	0.167
TGFβ1:IL-17A	590.7 (331.1 - 1365)	843.1 (516.1 - 975.2)	0.649	764.1 (417.5 - 1138)	632.6 (331.3 - 869.0)	0.569	516.1 (331.1 - 855.1)	886.7 (563.2 - 1379)	0.093
TGFβ1:IL-21	6978 (2911 - 16593)	5739 (2096 - 10709)	0.776	6798 (2388 - 10554)	4833 (2791 - 14581)	0.834	3900 (2255 - 7962)	10089 (3926 - 17340)	0.055

	No Dogs (median IQR pg/mL)	Dogs (median IQR pg/mL)	p	No ETS (median IQR pg/mL)	ETS (median IQR pg/mL)	p
IL-5	2.27 (1.86 - 2.91)	2.285 (1.16 - 2.61)	0.594	2.44 (1.98 - 2.97)	2.01 (1.24 - 2.19)	**0.026**
IL-6	1.05 (0.76 - 1.33)	0.97 (0.44 - 1.34)	0.518	1.09 (0.74 - 1.46)	0.89 (0.55 - 1.15)	0.258
IL-8	1.79 (1.57 - 2.19)	1.91 (1.40 - 2.48)	0.841	1.93 (1.51 - 2.48)	1.71 (1.45 - 2.07)	0.417
IL-10	7.03 (6.06 - 10.15)	7.03 3.93 - 9.68)	0.595	7.13 (6.72 - 12.56)	6.14 (3.88 - 7.47)	0.102
IL-13	2.12 (1.61 - 2.71(	2.38 (0.62 - 2.89)	0.763	2.35 (1.53 - 3.24)	1.80 (0.84 - 2.55)	0.358
IL-17A	7.71 (5.41 - 9.18)	8.06 (4.50 - 10.47)	0.852	8.33 (5.17 - 11.01)	7.32 (5.02 - 9.07)	0.538
IL-21	0.95 (0.52 - 1.80)	1.03 (0.45 - 1.41)	0.897	1.07 (0.55 - 1.87)	0.58 (0.47 - 0.96)	0.131
TNFα	2.53 (2.17 - 3.05)	2.10 (1.94 - 2.69)	0.149	2.53 (2.05 - 3.15)	2.22 (1.94 - 2.58)	0.208
IFNγ	7.92 (6.20 - 11.40)	7.35 (5.29 - 10.94)	0.508	8.46 (6.52 - 14.59)	6.27 (5.29 - 8.08)	0.052
TGFβ1	5335 (3531 - 7790)	4165 (3683 - 9289)	0.977	4165 (3618 - 8441)	4812 (3638 - 7365)	0.928
TGFβ2	506.6 (374.2 - 680.3)	431.6 (389.8 - 763.7	0.977	448.7 (387.2 - 816.5)	472.6 (389.5 - 655.8)	0.976
	No Dogs (median IQR)	**Dogs (median IQR)**	**p**	**No ETS (median IQR)**	**ETS (median IQR)**	**p**
IL-10:IL-8	4.00 (3.12 - 4.85)	3.08 (2.13 - 4.62_	0.213	4.21 (3.15 - 4.70)	3.02 (2.40 - 4.36)	0.214
IL-10:IFNγ	0.89 (0.74 - 1.10)	0.85 (0.62 - 1.05)	0.656	0.91 (0.75 - 1.04)	0.83 (0.60 -1.21)	0.846
IL-10:IL-13	3.50 (2.09 - 4.82)	3.87 (2.61 - 5.93)	0.546	3.54 (2.30 - 4.63)	4.08 (1.96 - 5.43)	0.834
IL-10:IL-17A	0.97 (0.80 - 1.34)	1.00 (0.54 1.32)	0.615	0.97 (0.85 - 1.28)	0.87 (0.53 - 1.37)	0.643
IL-13:IFNγ	0.27 (0.23 - 0.35)	0.23 (0.12 - 0.36)	0.332	0.26 (0.21 - 0.33)	0.27 (0.14 - 0.37)	0.940
IFNγ:IL-17A	1.10 (0.94 - 1.23)	0.99 (0.81 - 1.32)	0.445	1.06 (0.96 - 1.31)	0.93 (0.83 - 1.19)	0.167
TGFβ1:IL-10	698.1 (390.0 - 942.6)	820.8 (493.3 - 2596)	0.285	640.9 (417.5 - 1222)	871.9 (523.3 - 1301)	0.452
TGFβ1:IL-17A	693.5 (448 - 955.2)	703.2 (383.8 - 1653)	0.667	656.3 (417.5 - 928.8)	794.3 (402.8 - 1332)	0.610
TGFβ1:IL-21	5368 (2094 - 10244)	6798 (3051 - 18063)	0.537	4577 (2178 - 15466)	7687 (4360 - 7687)	0.653

4.3.15 qPCR standard curve was established for FOXP3 in PBMCs but not for nasal brushes

To determine the optimal cDNA starting concentration and to prove efficiency of the qPCR reaction for *FOXP3* genetic expression, 2 eight-point two-fold diluted standard curves were generated using pooled cDNA from PBMC and NB samples.

For the PBMC sample, the 1:2 dilution and the 1:256 values were excluded from the standard curve analysis due to the presence of inhibitors or the lack of proper amplification. The resulting standard curve had an efficiency of 110.1 % and an $R^2 = 0.93$ and the melt curves did not suggest the presence of primer dimers (Appendix A Figure 18).

For the NB sample, one of the 1:32 and both duplicates for the 1:64, 1:128, and 1:256 dilutions were excluded from the analysis as they did not amplify. The resulting standard curve generated from the remaining dilutions produced an efficiency of 217.1 % and an $R^2 = 0.599$ (Appendix A Figure 18). Similar values were produced in an additional attempt to establish an acceptable standard curve. Therefore, *FOXP3* genetic expression in NB was not evaluated as a reliable standard curve could not be established.

4.3.16 qPCR standard curves could not be generated for *IL-10* in PBMCs or NB

To determine the optimal cDNA starting concentration and to prove efficiency of the qPCR reaction for *IL-10* genetic expression, 2 8 point 1:2 fold diluted standard curves were generated using pooled cDNA from PBMC and NB samples.

For the PBMC samples only the 1:2, 1:4, and 1:16 conditions amplified; however, they amplified at a similar cycle number as the NRT controls (Appendix A Figure 19). The resulting 3 points were not sufficient to generate an appropriate standard curve. An additional attempt was made to establish the standard curve; however, similar results of no amplification or amplification at the same cycle as the NRT was observed.

In the NB samples the 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions amplified; however, similarly to the PBMC samples, amplified at the same cycle number as the NRT controls (Appendix A Figure 19). An attempt was made to generate a cleaner standard curve; however, similar results were observed.

An appropriate standard curve could not be established for *IL-10* genetic expression in PBMCs or NB; therefore, *IL-10* genetic expression could not be evaluated.

4.3.17 *GUSB* was the most stable candidate gene in PBMC samples

To determine a stable reference gene for normalization of *FOXP3* expression, the expression of 14 candidate genes were measured in 3 SPT negative children and 3 SPT positive children PBMC samples. The stability of each gene was assessed using the GeNorm software. *ACTB, YWHAZ,* and *TRFC* were excluded from the stability analysis as they did not amplify in all the samples. Of the 11 candidate genes include in the analysis, *GUSB* was determined to be most stable with an Avg M value of 0.072 and a stability value of 2.638 (Appendix A Table 16).

4.3.18 *GUSB* standard curve could be established for PBMC samples

To determine the appropriate starting concentration of cDNA and to approve efficiency in the *GUSB* qPCR reaction, an 8-point two-fold dilution standard curve was generated using pooled PBMC samples. The 1:2 and 1:256 diluted samples were excluded from the standard cure analysis as there were inhibitors in the samples or the sample did not amplify. The remaining dilutions produced a standard curve with an efficiency of 100 % and an R^2 = 0.977, and the melt curves suggest no presence of primer dimers (Appendix A Figure 20).

4.3.19 Increased *FOXP3* genetic expression in children with a positive SPT, respiratory symptoms, and positive SPT with cough/wheeze

To identify any differences in Treg functionality via genetic expression in children with allergic and respiratory outcomes, *FOXP3* genetic expression normalized to *GUSB* was measured and relative expression was determined for SPT positive children, children with respiratory symptoms, and children with a positive SPT and wheeze/cough without a cold. Relative expression was determined by comparing normalized expression between the clinical outcome and the control outcomes (SPT negative, respiratory symptom negative, and non-atopic children) (Figure 16).

Children with a positive SPT had a relative normalized *FOXP3* expression of 1.20 95 % CI 0.89 – 1.60 ($p = 0.368$). Children with cough and/or wheeze without a cold had a relative normalized *FOXP3* expression of 1.13 95 % CI 0.76 – 1.66 ($p = 0.590$). Children with a positive SPT and respiratory symptoms had a relative normalized *FOXP3* expression of 1.20 95 % CI 0.59 – 2.42 ($p = 0.549$).

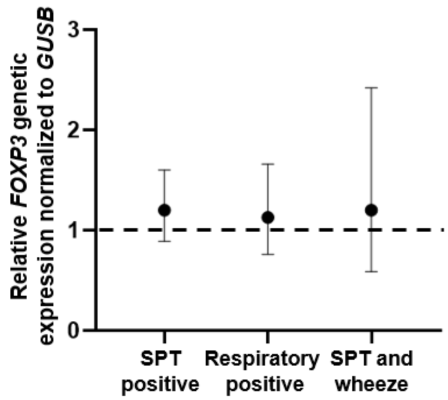

Figure 16. FOXP3 genetic expression higher in SPT positive children, children with respiratory symptoms, and children with a positive SPT and wheeze.

FOXP3 genetic expression normalized to GUSB and relative to either SPT negative children, children with no respiratory symptoms, or non-atopic children was determined for SPT positive children, children with cough/wheeze, and children with a positive SPT and wheeze. Dots indicate the geometric mean with bands representing the 95 % CI. Comparisons made by Mann-Whiney tests, no differences significant.

4.3.20 Increased *FOXP3* PBMC genetic expression at 6 to 7 years in children with prenatal exposure to several indoor and outdoor air pollutants.

To identify any relationships between prenatal exposure to indoor and outdoor air pollution and *FOXP3* genetic expression, *FOXP3* genetic expression normalized to *GUSB* was measured and relative expression was determined for children exposed to OAP, IAP, air fresheners, candles, mould, cats, dogs, ETS, and carpet prenatally. Relative expression was determined by comparing normalized expression between children with each exposure to children without each exposure (Figure 17).

Children with exposure to OAP, high levels of IAP, candles, cats, ETS and carpet had higher expressions of *FOXP3* compared to children without these exposures. The relative normalized *FOXP3* expression for OAP, IAP, candles, cats, dogs, ETS, and carpet were 1.13 95 % CI 0.83 – 2.54 (p = 0.633), 1.51 95 % CI 1.12 – 2.05 (p = 0.034), 1.30 95 % CI 0.89 – 1.90 (p = 0.237), 1.35 95 % CI 1.03 – 1.76 (p = 0.104), 1.25 95 % CI 0.89 – 1.74 (p = 0.269), 1.15 95 % CI 0.78 – 1.69 (p = 0.358), and 1.30 95 % CI 0.93 – 1.81 (p = 0.211) respectively. Of these exposures, only high IAP exposure was significant. There was no difference in the normalized genetic expression of *FOXP3* between children exposed or not exposed to air fresheners with a relative normalized *FOXP3* expression of 1.01 95 % CI 0.77 – 1.32 (p = 0.963). Children with prenatal exposure to mould had reduced normalized *FOXP3* genetic expression compared to children not exposed, with relative normalized genetic *FOXP3* expression of 0.909 95 % CI 0.55 – 1.5 (p = 0.612).

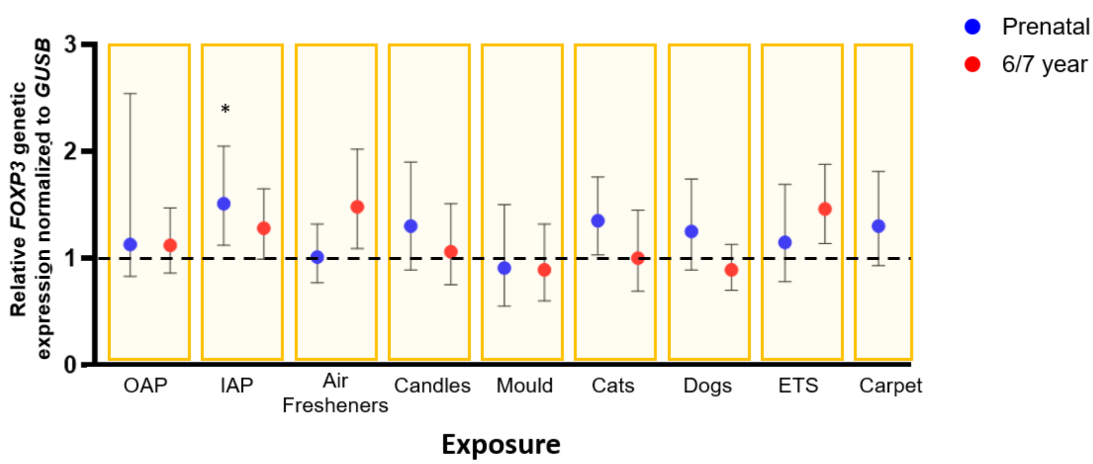

Figure 17. FOXP3 genetic expression higher in children with prenatal and current exposure to different indoor and outdoor air pollutants.

FOXP3 genetic expression normalized to GUSB and relative to children without the exposure was determined in PBMC samples of children with prenatal (blue) and current (red) exposure to various air polluting factors. Dots indicate the geometric mean with bands representing the 95 % CI. Comparisons made by Mann-Whitney tests, *p ≤ 0.05.

4.3.21 Children with current exposure to OAP, high levels of IAP, ETS, air fresheners had elevated *FOXP3* genetic expression.

To determine if there are any relationships between *FOXP3* genetic expression and current exposure to different indoor and outdoor air pollutants, *FOXP3* expression normalized to *GUSB* was measured and relative expression was determined for exposure to OAP, IAP, air fresheners, candles, mould, cats, dogs, and ETS (Figure 17).

Children with exposure to OAP, high levels of IAP, ETS, and air fresheners had elevated *FOXP3* expression compared to children without the exposures. The relative normalized *FOXP3* expression for children with exposure to OAP, IAP, ETS, and air fresheners were 1.12 95 % CI 0.86 – 1.47 ($p = 0.471$), 1.28 95 % CI 0.99 – 1.65 ($p = 0.228$), 1.46 95 % CI 1.14 – 1.88 ($p = 0.066$), and 1.48 95 % CI 1.09 – 2.02 ($p = 0.056$). None of these increases were statistically significant; however, both ETS and air freshener exposure were trending towards significance. There was no difference in *FOXP3* genetic expression between children with or without current exposure to candles or cats. The relative normalized expression of *FOXP3* were 1.06 95 % CI 0.75 – 1.51 ($p = 0.722$) for candles and 1.00 95 % CI 0.69 – 1.45 for cats (0.771). There was a lower normalized relative expression for children with current exposure to mould or dogs, with a relative normalized *FOXP3* expression of 0.89 95 % CI 0.60 – 1.32 ($p = 0.488$) and 0.89 95 % CI 0.70 – 1.13 ($p = 0.431$) for mould and dogs respectively; although these decreases were not statistically significant.

Chapter 5

Discussion

5.1 Frequency and function of freshly polarized T cells in CBMC samples of high and low atopic risk

In this study we evaluated the frequency and function of freshly polarized T helper cells from CBMC samples of high and low atopic risk as a potential biomarker for early-life atopic status. We aimed to polarize Th1, Th2, and Th17 cells and compare their frequency and function via cytokine secretion between HR and LR samples. Our results suggested successful polarization into the Th1, Th2, and Th17 lineages with unique cytokine production profiles; however, no significant differences were observed when considering atopic status. We believe this may be due our low sample size and lack of confirmed atopic status.

There was a high proportion of naïve T cells and low proportion of mature T cells in the uncultured CBMC samples. This phenotype switched to a low frequency of naïve T cells and a high frequency of mature T cell following the 7 day culture conditions. This shift is indicative of the polarization process, as the immature T cells have lost their naïve T cell marker CD45RA and gained the mature T cell marker CD45RO (102). This result supports that any difference observed in the Th1, Th2, and Th17 cell frequencies are a result of the polarization and differentiation process; rather than any pre-existing T helper cells.

The Th1 polarization conditions produced a significantly higher percentage of Th1 cells compared to the NP conditions. The culture supernatant had elevated levels of IL-8, IL-10, IL-21 and IFN-γ compared to the NP and Th2 conditions. The supernatant also had significantly lower levels of the Th2 cytokines IL-5 and IL-13, and there was no detection of the Th17 cytokine IL-17A. When combined, these findings suggest the successful polarization of Th1 cells in the Th1 conditions. The highest concentration of IL-10 was observed in the Th1 conditions which was unexpected, as IL-10 is thought to be a regulatory cytokine produced in most abundance by Tregs; however, IL-10 is produced by T helper cell subsets, macrophages, dendritic cells and other immune cells (103). Furthermore, it is hypothesized that Th1 cells produce IL-10 in a negative feedback fashion during infection to prevent excessive inflammation that may harm the host (104). Given the high concentration of IFN-γ produced under this condition, it is hypothesized this negative feedback loop is responsible for the elevated IL-10 production. IL-8 was also observed at the highest concentration in the Th1 condition, and we hypothesize this may be due to the presence of IL-18. IL-18 has been described as an IFN-γ inducing cytokine, and in combination with IL-12 upregulates IFN-γ production in Th1 cells (105,106). It is expressed initially as an inactive precursor in cells, and exists in substantial quantities inside cells (106). IL-18 in combination with anti-CD3 have been demonstrated to induce Th1 cell production of IL-8 (107). This pathway may potentially explain the observed elevated IL-8 concentration. While we are unable to confirm IL-18 presence in our culture supernatants at this moment, additional work to do so could strengthen this finding.

When considering atopic status following Th1 polarization, there were lower percent frequencies of Th1 cells in both the Th1 and NP control conditions in HR samples compared to LR samples. This may suggest a reduced response to the Th1 polarizing cytokine IL-12 or a reduced ability to generate Th1 cells under a Th1 inflammatory environment and a neutral environment. This is important when considering the reduced Th1 to Th2 cell ratio observed in atopic individuals (10). Given the Th2 dominant environment during pregnancy (108), it has been hypothesized the Th2 shift in allergic individuals may be a result of a reduced ability to revert to a balanced Th1/Th2 environment (109). Our observation of lower Th1 cell frequencies following Th1 polarization at birth in CBMC samples supports this hypothesis. Of the cytokines measured, there were no significant differences in cytokine concentration in Th1 conditions between HR and LR samples; however, there was a slight reduction in IL-10 and IL-21 production. Given the immunoregulatory properties of IL-10 and IL-21, this finding may suggest a reduced regulatory ability of the Th1 cells in HR individuals at birth.

The NP conditions produced significantly more Th2 cells than the Th2 conditions. Of the Th2 cytokines IL-5 and IL-13, the Th2 condition produced numerically higher concentrations of IL-5 but numerically lower IL-13 compared to the NP condition. Both the Th2 and NP conditions produced higher concentrations of these cytokines than the Th1 and Th17 conditions. IL-10 is also secreted by Th2 cells (110) and was significantly higher in the Th2 condition compared to the NP condition. Furthermore, IL-10 production is enhanced by IL-4 and can contribute to the modulation of the Th1/Th2 paradigm (111). When combined, these findings suggest the successful polarization of Th2 cells. The lower Th2 cell frequency in the Th2 condition may be a result of higher IL-10 in the Th2 condition; as IL-10 is immunoregulatory and can dampen Th2 responses and prevent T cell expansion and proliferation (112). The low percentage of Th2 cells may also be a result of the use of IL-4 as the intracellular marker. IL-4 could be secreted from the cells prior to stimulation for flow cytometry, or additional stimulation time may be needed to yield adequate IL-4 production for detection. Measuring IL-4 concentration in the culture supernatant may shed light on whether or not the cells are releasing IL-4; however, given that it is used to polarize the naïve cells and is present initially in the culture supernatants, an accurate measure of IL-4 production would be unlikely. The use of an extracellular surface marker such as CRTH2 or CCR4 in conjunction with the other surface markers used in our panel could be useful to identify Th2 cells (99,102,113).

Our results indicated a reduced ability to generate Th2 cells following T cell polarization in samples of high atopic risk compared to low atopic risk. IL-13 was elevated in HR samples following both Th2 and NP conditions, and IL-5 and IL-10 were elevated in HR samples following Th2 polarization. These findings suggest there may be an increased functionality in Th2 cells in children with a high atopic risk. There were no significant differences observed between HR and LR samples which may be a result of our low sample size and unconfirmed atopic status of both the mother and the child.

Similar percentages of Th17 cells were observed between Th17 polarization and NP polarization; however, the Th17 cytokine IL-17 was only detected in Th17 conditions. Furthermore, Th1 cytokines and Th2 cytokines were detected at low levels in the Th17 culture supernatant. When considered together, these findings suggest successful polarization of Th17 cells. The percent frequencies of Th17 cells were fairly low, however comparable to findings in the literature using similar Th17 polarization techniques (114). Evidence suggests Th17 cell differentiation is dependent on aryl hydrocarbon agonism, in addition to the presence of IL-6, TGF-β1, IL-21 and IL-1β (114). Aryl hydrocarbon agonists are generally found in very low concentrations in

RMPI medias, but is at a higher concentration in Iscove's modified Dulbecco's media (114). It would be worthwhile to alter the Th17 polarization condition to include a media with greater amounts of aryl hydrocarbon agonists in efforts to yield greater Th17 cell frequencies.

We observed greater Th17 percent frequencies in HR samples compared to LR samples following Th17 and NP conditions. These findings suggest an increased ability to generate Th17 cells in both a Th17 stimulating and neutral environment. It is to the best of our knowledge that direct Th17 differentiation in cord blood as a potential predictor of atopy has not been investigated in the literature; however, findings of elevated circulating Th17 cells in PBMC samples of individuals with atopic disorders have been well established (44,115,116). Our finding is promising in terms of identifying an early-life biomarker of atopy; although, more work is needed to confirm this. Increasing our sample size and confirming atopic status of the mother and future atopic status of the child will strengthen these findings.

As previously mentioned, atopic risk was assigned by maternal report of atopy. The lack of confirmed maternal atopic status presents a major limitation to this work. Furthermore, the intent of this study is to relate early-life immune function to later atopic status and not knowing the allergic status of the children limits the work. Lastly, with a sample size of 7 it is difficult to make any strong conclusions regarding the polarizability or functionality of T helper cells as they relate to atopic status.

5.2 The relationships between early life exposures to indoor air polluting factors and allergic sensitization at 2 years of age

In this study we evaluated the impact of prenatal and early life exposure to common indoor air pollutants on allergic sensitization at 2 years of age. We examined exposure to dogs, cats, air fresheners, candles, mould, ETS, and carpet; all of which have been associated with childhood allergies and atopy (5,87,117,118). Several of the exposures we examined increased the OR of a positive SPT at 2 years, and there was a significant increase with prenatal exposure to candles, 6 month exposure to cats, and 2 year exposure to ETS.

Exposure to furry pets such as cats and dogs during the prenatal and early life windows have been previously investigated through epidemiological models. Several findings suggest dog exposure in the first years of life decreases the risk of allergic sensitization, atopic dermatitis, and wheeze in childhood (119). It has been proposed that this observation is a result of an increase in the diversity in the house microbiome (91). This diversity has been associated with dogs but not with cats (120). Additionally, cat exposure in early life has also been shown to reduce the risk of childhood asthma and atopic dermatitis (92); but in a manner that proposes either very low level or very high level of cat exposure provides the greatest protection from allergic sensitization (121). In contrast, one study examined dog and cat exposure in early life and specific dog or cat allergy at 4 years of age and determined the exposures increased the OR of these outcomes (93). Our findings suggest increased risk of allergic outcomes with pet exposure, which may be due to differences in age of the children included in the analysis; as these children are 2 years old and could potentially grow out of their sensitizations.

Additionally, it is possible the cat exposure levels of the children in our cohort are in the middle of the “exposure range”, an area that could potentially confer the greatest risk (121). Lastly, it is important to consider findings that suggest dog and cat exposure are protective could be a result of bias; as families that do not have pets may do so because of family members with pre-existing animal allergies.

Air freshener and candle exposure are of great interest regarding their impact on childhood allergy and asthma. These compounds contain phthalates; which on their own have been associated with allergic and respiratory disease in children (83). In previous work with the KABC, we have established that indoor air freshener exposure is associated with respiratory symptoms at 2 years of age (5). Our findings from this current study provides additional evidence to support the impact of these compounds on allergic sensitization.

Mould in the home or ETS exposure each increased the OR of a positive SPT at 2 years, and exposure to ETS at 2 years significantly increased the OR. Living in mould contaminated homes has been associated with an increase in childhood asthma risk, and it is postulated that due to their small size, fungal spores are able to penetrate into the alveolar region to elicit damaging effects on lung health (70,88). Furthermore, studies have indicated smoking during pregnancy and exposure to second hand smoke in early life is associated with an increased risk of childhood atopy (78,122).

In our analysis, carpet exposure neither increased nor deceased the OR of a positive SPT at 2 years of age. Recent findings suggest carpet can alter the microbiome of the home and increase the amount of fungal species (123). This may have implications on mould exposure and increase the risk of allergic sensitization. Additionally, carpet is a major source of dust mites and in our analysis was used as a proxy for dust mite exposure. In an analysis of in home allergen exposure and allergic sensitization, a positive association between dust mite exposure and the probability of sensitization was observed (124). The lack of an association between carpet exposure and allergic outcomes at 2 years of age in our study may be due to our sampling method. We obtained carpet exposure via survey which may not be the most accurate method; visiting the homes to determine the square footage of carpet or obtaining dust samples from the home would have provided more accurate exposure data.

A secondary aim of this study was to evaluate the effect of multiple exposures on allergic outcomes at 2 years of age. Children with a positive SPT at 2 years of age had significantly more exposures prenatally, at the 1 year, and at the 2 year time points compared to children with a negative SPT. Moreover, a dose-response trend was observed in that as the number of indoor air polluting exposures increased the percentage of children with a positive SPT increased. When considered together these findings suggest that the effect of multiple exposures may contribute more to allergy development than one single exposure. In the literature, ORs have been evaluated for several of the exposures we examined and are often adjusted for confounding exposures that can impact the OR. This modeling paints an accurate picture of the risk associated with one exposure; however, this does not depict everyday life, as exposures rarely exist in singularity. We believe by considering the effect of simultaneous exposure to multiple factors on allergy outcomes we can better portray real life scenarios.

A major limitation of this work is our small sample size. This prevented any correction for multiple comparisons or confounding variables. Additionally, each exposure at each timepoint was evaluated in a univariate approach. Assessing repeat exposures or the changing of exposure levels within children would have provided more information on how these exposures work in combination to influence atopic disease.

5.3 Connecting environmental exposures with allergic and respiratory outcomes at 6 to 7 years of age

In this study we evaluated the impact of prenatal and current exposure to different indoor and outdoor air polluting factors on cellular changes, genetic changes, allergic sensitization, and cough/wheeze in children at 6 to 7 years. We observed some exposures to air polluting factors increased the OR of a positive SPT or respiratory symptoms at 6 to 7 years. We also detected an elevation in Treg and Th17 levels in children with a positive SPT, respiratory symptoms, and several prenatal and early life exposures. We identified several differences in the inflammatory and immune regulatory profile of children with allergic and respiratory outcomes, and children with exposures to high levels of IAP and cats. We observed slightly higher genetic expression of *FOXP3* in children with a positive SPT, children with respiratory symptoms, and children with different early life exposures to air polluting factors.

Our results indicate maternal allergic status increased the OR of both respiratory and allergic outcomes in children 6 to 7 years of age. This is in alignment with findings in the literature (32,34,125). We only considered the influence of maternal allergic status as we do not have confirmed allergic status for the father. Obtaining this information could provide more detailed information on the role of family history and child allergic outcomes.

When considering the impact of prenatal and current exposure to the different air polluting factors on SPT status and wheeze, only carpet exposure significantly increased the OR of a positive SPT; however, it had no effect on cough/wheeze outcomes. In general, the exposures had mixed effects on the ORs, with some showing slight harmful or protective effects; although without any significance. We believe this heterogeneity is a result of our sample size. With only 39 participants in this analysis it is difficult to accurately access these exposures with clinical outcomes. However, studies like the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort study have exposure data on over 3600 mother child pairs and have the capability to detect these relationships (126). Validating our observations against findings from birth cohort studies such as this will bolster our findings.

We observed an increase in peripheral Treg levels in children with a positive SPT and with respiratory symptoms, although without significance. When considering prenatal and current exposures to air polluting factors we observed similar frequencies of Tregs between exposed and unexposed children, with slightly higher levels for current exposure to cats, OAP, and high levels of IAP. We believe that this finding could suggest a compensatory mechanism, whereby the Tregs are increased in number in effort to dampen an inflammatory environment created from allergic and respiratory diseases or from the environmental exposures. Current findings in the literature report reduced Treg frequencies for children with allergic sensitizations, atopic dermatitis and food allergy (55). This reduction is also reported for asthmatic individuals (127). The differences observed between our results and the literature results may be a result of differences in Treg markers and gating strategies. Incorporating CD25, CD127, and CD3 into our cell panel would increase the likelihood we are measuring Tregs (128). Additionally, our respiratory symptoms are defined by parental report of cough and/or wheeze without a cold. This is not physician diagnosed asthma, as asthma cannot be formally diagnosed without spirometry testing (25). If our children were older and asthma status could be confirmed these differences could potentially be eliminated.

Our results suggested a slight elevation in Th17 cell frequency in children with respiratory symptoms and children with prenatal and exposure to high levels of IAP and ETS. This finding aligns with findings in the literature, as associations of increased peripheral Th17 cell frequency with asthma and asthma severity have been established (129). The effect of prenatal and postnatal exposures to air polluting factors on Th17 frequency have not been extensively studied in children; however, mouse models have demonstrated exposure to cigarette smoke augments Th17 cell responses (130). Combining our observations of increased Th17 frequency in children with respiratory symptoms and in children with prenatal ETS smoke exposure, suggests a potential mechanism for the connection between cigarette smoke exposure and asthma outcomes in children.

We next sought to identify changes in the systemic inflammatory environment via cytokine profiling. We evaluated both raw plasma cytokine concentration and cytokine ratios. It is to the best of our knowledge that cytokine ratios are not commonly studied in the literature; however, they can offer an interesting perspective to immune status. Firstly, these ratios allow for the normalization of cytokine levels to the individual which can help control for variability in baseline cytokine productions. Additionally, these cytokine ratios create an opportunity to simultaneously examine multiple arms of the immune system, where either pro, anti-inflammatory, or regulatory functionality can be assessed. This ultimately creates a more dynamic and accurate picture of immune status, as the immune response is not governed by one cytokine or one cell type.

We did not observe any statistically significant differences in the plasma concentrations between SPT positive and negative children, although there was a numerical increase in the IL-5, IL-13, and IL-17 concentrations. These findings were expected as Th2 and Th17 cytokine concentrations have been demonstrated to be elevated in atopic diseases (131,132). We detected reductions in the IL-10:IL-8, IL-10:IL-13, and IL-10: IFN-γ ratios in SPT positive children compared to SPT negative children. This reduction suggests a weakened regulatory pathway as IL-10 is a regulatory cytokine and IL-8, IL-13, and IFN-γ are pro-inflammatory cytokines.

In children with respiratory symptoms we did not observe any significant differences in plasma cytokine concentrations, but there was a numerical reduction in IL-10 and IL-21. This finding suggests a reduction in regulatory function and has also been reported in the literature (133). Similarly to the SPT positive children, we also identified a reduction in the IL-10:IL-8 ratio, with an additional increase in the TGF-β1:IL-10 ratio in children with cough/wheeze without a cold. This also supports a reduced regulatory functionality in children with respiratory symptoms. Given that TGF-β1 and IL-10 are two main cytokines of Tregs (12), differences in this ratio suggests a difference in the regulation and secretion of Treg effector cytokines.

Children with a positive SPT and wheeze/cough without a cold have a high likelihood of developing physician diagnosed asthma later in life (21). We investigated the cytokine profile of this patient group, and did not identify any significant differences in plasma cytokine concentration; but we did observe reductions in the IL-10:IL-8, IL-10:IFN-γ, IL-10:IL-13 and IL-10:IL-17 ratios. These reductions suggest a decrease in the regulatory pathways with an increase in the inflammatory cascades. Additionally, we detected increased TGF-β1:IL-10, TGF-β1:IL-17A, and TGF-β1:IL-21 ratios in these children. These differences suggest a change in regulatory cytokine productions as well as differences in the Treg/Th17 cytokine axis. While these differences are exciting in terms of their potential as biomarkers of disease, due to our small sample size, any strong conclusions regarding their appropriateness as biomarkers cannot be made at this time.

After establishing differences in the cytokine profile of children with and without clinical outcomes, we next examined these profiles under the consideration of prenatal and postnatal exposures. We found several differences in the plasma concentrations with prenatal and current exposure to different indoor and outdoor air pollutants; however, cat exposure and the level of IAP exposure appeared to have the greatest influence. Children with cat exposure had generally lower plasma cytokines but similar cytokine ratios. Interestingly, there was no difference in the cytokine concentrations or ratios between children with or without dog exposure. When considered together, these findings suggest cats in the home but not dogs may potentially modify inflammatory processes. Additionally, children with high levels of IAP exposure had reduced cytokine concentrations but similar cytokine ratios. If these observations are combined with the clinical outcome results, these findings could suggest that these exposures initially alter the immune profile on an individual cytokine basis and atopic disease emerges when the regulatory functionality is greatly reduced. This finding could be strengthened by considering allergic and respiratory status within the exposure analysis; however, given our small sample size this type of analysis could not be completed.

After establishing the Treg frequency and cytokine profiles in our clinical outcomes and exposure groups, we aimed to investigate Treg functionality via genetic expression of *FOXP3* in PBMC samples. We initially aimed to evaluate *FOXP3* expression in nasal tissue as well; however, expression levels were too low to establish a standard curve for qPCR analysis. We also intended to evaluate IL-10 in both PBMC and NB samples, but genetic expression was also too low. We hypothesized that stimulation of PBMCs prior to RNA extraction would allow for IL-10 genetic analysis; however, this work has yet to be completed. We did not observe any significant differences in *FOXP3* genetic expression normalized to *GUSB* for any of the clinical outcome groups, but it was numerically elevated. We believe this potentially could be due to the slight increase in Treg frequency observed in these children. It has been established in the literature that children with allergic and respiratory disease have reduced *FOXP3* expression. For example, a study examining children with food allergy report *FOXP3* genetic expression was lower in children with heated-allergen tolerance and even lower in children with heated-allergen intolerance compared to children with complete food tolerance (58). Additionally, in a study of children with atopic asthma and food allergy, children with allergy had significantly lower *FOXP3* expression than children without and an negative association between asthma severity and *FOXP3* expression was reported (15). Both studies use *ACTB* as a reference gene; however, there is no discussion of the validation of *ACTB* as a reference gene or its stability, nor the use of proper controls such as NRTs, standard curves to determine optimal efficiency, or melt curves as per MIQE guidelines

(101). These guidelines are imperative for the production of reliable qPCR data and without adherence to them questions in the validity of the findings can arise (134). We believe the difference in the findings in the literature and our work may be a result of errors in the validation of *ACTB* as a stable reference gene, as this gene was determined unstable in our PBMC samples.

When considering the exposure groups, we observed an increase in *FOXP3* genetic expression with prenatal and current exposure to several of the indoor and outdoor air pollutants. When this finding is combined with the decrease in plasma cytokine concentrations observed with these exposures, we believe it suggests that Tregs may be over functioning in efforts to rebalance the widespread inflammatory changes brought on from these exposures. Improper rebalancing may promote allergic or respiratory disease and could provide an explanation for the connections seen in the literature regarding the influence of the environment on disease progression (117). This may suggest a lack of efficiency, rather than lack of function for the role of Tregs in allergy and asthma.

This study has its limitations. Firstly, a sample size of 39 to study environmental exposures with diseases is quite small. We were unable to reach the appropriate power to detect significant associations between the home environment and allergic or respiratory outcomes. Additionally, we were only able to obtain blood samples from 24 children; leaving 8 children with a positive SPT, 6 with respiratory symptoms, and 4 with both. This creates fairly small comparison groups and prevents a more robust analysis. Moreover, all exposure data was classified by parental report which may over or underestimate current exposure levels. Lastly, we measured *FOXP3* genetic expression in the entire PBMC samples, and while it is a marker specific to Tregs, specifically isolating Tregs and measuring *FOXP3* genetic expression could provide more precise information on Treg functionality.

Chapter 6

Summary

In summary, we successfully polarized T helper cells from umbilical cord blood samples of children with low and high atopic risk. We observed differences in the cytokine profiles following polarization which additionally supports this finding. We did not observe any significant differences in frequency or function of polarized T helper cells between low and high atopic risk children; although we did observe a slight increase in Th17 cell frequency in Th17 conditions as well as the NP conditions. To strengthen the findings of this pilot project, we aim to increase our sample size and confirm allergic status of the mother. It would also be of interest to follow this subset of children as they age to identify symptoms of atopy as well as allergic status once they reach 2 years of age. This would allow for a comparison of how these cells initially develop in children who do or do not go on to develop atopic diseases.

Additionally, we observed that several exposures during the prenatal and early life windows increased the OR of a positive SPT at 2 years of age. Furthermore, children with a positive SPT at 2 years of age had a higher number of different exposures during the prenatal, 6 month, 1 year, and 2 year time points than SPT negative children. We also observed a "dose response" relationship, in that as the number of exposures increases, the percentage of children with a positive SPT increases. When combined our findings support the role of the environment in atopic disease progression and suggest that the cumulative effect of multiple exposures may have a greater influence on allergic disease outcomes than single exposures. We have visited the homes of several children that participated in this 2 year follow-up visit and we have obtained dust samples of their homes. Connecting these samples with home exposure data and the SPT outcomes at 2 years would enhance this study. This would allow the confirmation of these exposures via concentrations of chemical and allergen in the dust samples. Additionally, increasing our sample size for this study would enhance the OR analysis by allowing correction of multiple comparisons and confounding variables.

Lastly, carpet was the only exposure to significantly increased the OR of a positive SPT at 6 to 7 years of age. Several of the other exposures increased the OR of a positive SPT or cough/wheeze; however not significantly. We attribute this to our small sample size. There were no significant differences in Treg or Th17 frequency when considering the clinical outcome groups, however there was elevated Th17 frequency with prenatal exposure to ETS and high levels of IAP. We observed differences in several cytokine ratios in children with or without a positive SPT, respiratory symptoms, or both. We detected a reduction in plasma cytokine concentrations for children exposed to high levels of IAP and cats. When combined these findings could suggest a mechanism for the link between exposures and disease development, wherein a global reduction in cytokine production occurs following exposure and this reduction alters the regulatory responses which in turn contributes to allergic disease progression. We measured *FOXP3* genetic expression and determined it was elevated in our clinical outcome groups and with exposure to several air polluting factors. This finding has not been demonstrated in the literature; however, we suggest this may be due to the use of unstable reference genes. If this finding is combined with the peripheral Treg frequencies and cytokine data, it may suggest that Treg efficiency is important in atopy. This study is limited by its small sample size and inability to correct for multiple comparisons. Additionally, *FOXP3* expression was measured in PBMC samples not isolated Treg samples. Isolating Tregs from these PBMB samples and measuring *FOXP3* expression would confirm these differences are a result of differences within the Treg populations. Furthermore, IL-10 could not be evaluated due to low mRNA levels. Stimulating Tregs isolated from PBMC samples prior to RNA extraction and qPCR analysis to detect IL-10 mRNA would provide more information on the functionality of these cells. Lastly, we aim to increase our sample size to allow for a richer analysis that would include exposure between the prenatal and 6 to 7 year time points, adjustments for confounding variables, and corrections for multiple comparisons.

Appendix A

Table 10. Frequency of immature and mature T cells before and after polarization.

	Immature T cell (median IQR) (%)	Mature T cell (median IQR) (%)
Baseline	91.2 (90.7 - 92.6)	4.07 (2.73 - 4.33)
Th1 polarization	7.87 (4.39 - 18.7)	85.6 (71.80 - 87.70)
Th2 polarization	14.90 (12.0 - 21.2)	69.80 (62.10 - 79.10)
Th17 polarization	0.86 (0.73 - 4.12)	86.10 (65.75 - 92.70)
NP polarization	7.51 (3.37 - 8.36)	83.8 (74.50 - 89.10)

Table 11. Cytokine concentrations following polarization.

	Th1 (median IQR pg/ml)	Th2 (median IQR pg/ml)	Th17 (median IQR pg/ml)	NP (median IQR pg/ml)
IL-5	52.85 (36.76 - 105.7)	578.3 (509.7 - 1073)***	159.6 (73.42 - 586.3)	361.7 (349.6 - 611.7)***
IL-6	192.4 (93.94 - 269.0)	223.7 (124.0 - 322.1)	376.6 (221.1 - 812.2)	119.1 (17.08 - 376.0)
IL-8	27011 (20906 - 37486)	2257 (1241 - 4169)*	22992 (12345 - 27190)	12124 (8508 - 28828)†
IL-10	2429 (1197 - 3241)	635.4 (395 - 2534)	93.88 (50.93 - 187.2)	218 (95.58 - 348.3)** ††
IL-13	1265 (784.2 - 1334)	1929 (1566 - 2375)**	1284 (860.2 - 1952)	2191 (2082 - 3846)**‡
IL-17A	below detection limit	below detection limit	44.25 (6.89 - 75.78)	below detection limit
IL-21	1.94 (1.54 - 3.39)	0.77 (0.63 - 0.90)**	0.95 (0.86 - 1.10)**	0.97 (0.72 - 1.33)**
TNFα	below detection limit	406.9 (306.4 - 657.4)	1352 (792.2 - 1613) †	544.7 (404.6 - 632.0)‡
IFNγ	35399 (20.656 - 44091)	107.2 (33.39 - 176.8)***	58.56 (22.30 - 181.9)**	531.1 (338.7 - 650.9)*** ††

* comparison between Th1 condition *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$
† comparison between Th2 condition †$p \leq 0.05$, ††$p \leq 0.01$
‡ comparison between Th17 condition ‡$p \leq 0.05$

Table 12. T helper cell frequencies in LR and HR samples following polarization.

	Low Risk (median IQR) (%)	High risk (median IQR) (%)	p
Th1 cells			
Th1 polarization	52.85 (19.75 - 79.20)	46.40 (20.90 - 57.50)	0.857
NP control	12.82 (4.43 - 22.40)	7.26 (2.65 - 7.26)	0.629
Th2 cells			
	Low Risk (median IQR) (%)	High risk (median IQR) (%)	p
Th2 polarization	3.91 (2.01 - 13.41)	2.25 (2.19 - 5.50)	0.857
NP control	16.20 (5.49 - 23.70)	6.95 (3.25 - 10.90)	0.400
Th17 cells			
	Low Risk (median IQR) (%)	High risk (median IQR) (%)	p
Th17 polarization	1.95 (0.66 - 3.27)	2.98 (2.21 - 3.74)	0.400
NP control	1.39 (0.64 - 2.38)	3.0 (3.51 - 3.68)	0.200

Table 13. OR of positive SPT at 2 years with each exposure at each time point.

Exposure	Prenatal (OR 95 % CI)	p	6 Month (OR 95 % CI)	p	1 Year (OR 95 % CI)	p	2 Year (OR 95 % CI)	p
Dog	1.67 (0.55-4.55)	0.410	3.2 (0.86-10.41)	0.100	3.72 (1.07-11.9)	0.055	2.475 (0.81-6.9)	0.152
Cat	2.77 (0.96-8.41)	0.069	4.27 (1.096-15.7)	**0.048**	3.00 (0.87-9.61)	0.117	2.30 (0.76-6.52)	0.150
Air Freshener	2.05 (0.78-6.12)	0.191	0.63 (0.13-2.97)	0.720	1.167 (1.1-11.9)	0.999	1.54 (0.55-4.6)	0.560
Candle	5.09 (1.7-13.9)	**0.006**	1.5 (0.39-6.4)	0.690	2.58 (0.74-9.8)	0.160	1.292 (0.39-3.9)	0.770
Mould	1.72 (0.63-4.8)	0.389	2.18 (0.62-7.35)	0.292	1.81 (0.53-6.82)	0.460	2.36 (0.67-8.08)	0.190
ETS	1.48 (0.46-5.38)	0.510	1.38 (0.26-6.2)	0.656	1.89 (0.47-8.7)	0.408	3.78 (1.19-11.2)	**0.039**
Carpet	0.902 (0.34-2.4)	0.999	1.1 (0.33-3.84)	0.999	0.43 (0.12-1.6)	0.340	0.618 (0.2-1.8)	0.430

Table 14. OR of clinical outcomes at 6 to 7 years with prenatal and current exposure to air polluting factors.

	Positive SPT				**Report of cough/wheeze**			
Exposure	**Prenatal exposure**		**6/7 year exposure**		**Prenatal exposure**		**6/7 year exposure**	
	OR (95 % CI)	p	OR (95 % CI)	p	OR (95 % CI)	p	OR (95 % CI)	p
OAP	0.62 (0.14 - 2.84)	0.722	1.69 (0.44 - 6.34)	0.516	0.65 (0.51 - 3.16)	0.999	3.27 (0.66 - 17.14)	0.250
IAP (low/high)	2.00 (0.44 - 8.62)	0.482	0.93 (0.28 - 3.56)	0.999	4.55 (0.84 - 24.21)	0.122	1.30 (0.34 - 6.20)	0.999
Air fresheners	0.22 (0.06 0 1.28)	0.087	0.51 (0.15 - 2.09)	0.500	2.34 (0.45 - 9.17)	0.427	1.78 (0.45 - 8.81)	0.690
Candles	1.23 (0.44 - 2.14)	0.310	0.700 (0.19 - 2.72)	0.740	0.78 (0.18 - 3.86)	0.999	1.52 (0.39 - 7.34)	0.699
Mould	0.77 (0.17 - 4.26)	0.999	0.93 (0.28 - 3.56)	0.999	3.80 (0.81 - 18.76)	0.176	0.35 (0.067 - 1.74)	0.273
Cats	1.378 (0.37 - 4.94)	0.733	1.11 (0.33 - 4.39)	0.999	3.11 (0.64 - 13.17)	0.243	0.71 (0.17 - 3.15)	0.999
Dogs	2.29 (0.50 - 9.12)	0.300	1.75 (0.43 - 6.18)	0.510	0.55 (0.13 - 2.63)	0.694	0.81 (0.20 - 3.69)	0.999
ETS	0.73 (0.21 0 3.27)	0.730	0.73 (0.21 0 3.27)	0.730	2.1 (0.53 - 10.82)	0.423	3.93 (0.77 - 15.23)	0.108
Carpet	5.42 (1.11 - 20.0)	**0.037**	no data		0.87 (0.22 - 4.25)	0.999	no data	

Table 15. PBMC and Treg frequencies for prenatal and current exposure to air pollutants.

Exposure	Treg frequency					
	Prenatal exposure			6/7 year exposure		
	No exposure median IQR (%)	Exposure median IQR (%)	p	No exposure median IQR (%)	Exposure median IQR (%)	p
OAP	6.72 (5.18 - 7.44)	7.43 (6.48 - 8.97)	0.075	6.44 (5.57 - 7.30)	7.27 (6.20 - 8.91)	0.109
IAP (low/high)	7.54 (5.49 - 8.92)	7.26 (6.45 - 7.48)	0.851	6.45 (5.47 - 8.27)	7.25 (6.30 - 7.51)	0.490
Air fresheners	7.99 (6.40 - 7.63)	7.51 (6.27 - 9.56(	0.230	6.88 (6.15 - 7.60)	6.60 (5.94 - 8.65)	0.834
Candles	7.18 (5.37 - 8.84)	7.26 (6.46 - 7.47)	0.851	6.49 (5.59 - 8.13)	7.25 (6.06 - 7.25)	0.820
Mould	7.31 (6.31 - 8.69)	6.47 (5.60 - 8.27)	0.602	7.13 (5.94 - 8.59)	6.67 (6.15 - 7.35)	0.697
Cats	6.90 (5.49 - 8.92)	7.26 (6.45 - 7.60)	0.970	6.49 (5.59 - 8.27)	7.25 (6.06 - 7.51)	0.820
Dogs	7.27 (6.45 - 8.62)	7.00 (5.54 - 8.27)	0.503	6.87 (6.01 - 7.86)	6.72 (5.73 - 7.89)	0.667
ETS	6.45 (5.78 - 8.69)	7.14 (6.13 - 7.54)	0.625	6.85 (5.80 - 8.37)	6.75 (6.26 - 7.45)	0.834
Carpet	7.00 (6.16 - 8.13)	7.35 (6.44 - 8.91)	0.699		no data	

Exposure	Th17 frequency					
	Prenatal exposure			6/7 year exposure		
	No exposure median IQR (%)	Exposure median IQR (%)	p	No exposure median IQR (%)	Exposure median IQR (%)	p
OAP	1.27 (1.06 - 1.54)	1.05 (0.99 - 2.29)	0.528	1.25 (1.03 - 1.92)	1.42 (1.03 - 1.74)	0.918
IAP (low/high)	1.03 (0.99 - 1.39)	1.41 (1.08 - 2.40)	**0.038**	1.51 (1.03 - 1.79)	1.28 (1.03 - 1.54)	0.808
Air fresheners	1.12 (0.99 - 1.55)	1.51 (1.04 - 2.64)	0.395	1.19 (1.03 - 1.82)	1.47 (1.09 - 1.62)	0.940
Candles	1.19 (1.03 - 1.53)	1.46 (1.00 - 2.57)	0.623	1.51 (1.05 - 1.74)	1.28 (102 - 1.74)	0.660
Mould	1.07 (1.01 - 1.52)	1.70 (1.09 - 2.29)	0.207	1.35 (1.05 - 1.62)	1.31 (0.99 - 2.10)	0.940
Cats	1.04 (0.99 - 1.40)	1.41 (1.08 -2.40)	0.097	1.12 (1.03 - 1.60)	1.42 (1.07 - 2.69)	0.400
Dogs	1.07 (0.99 - 2.19)	1.25 (1.03 - 1.55)	0.880	1.53 (1.04 - 1.93)	1.27 (1.01 - 1.54)	0.520
ETS	1.09 (0.99 - 1.57)	1.55 (1.23 - 2.05)	**0.050**	1.32 (1.00 - 1.72)	1.35 (1.12 - 1.77)	0.580
Carpet	1.28 (1.01 - 1.70)	1.20 (1.04 - 2.59	0.938		no data	

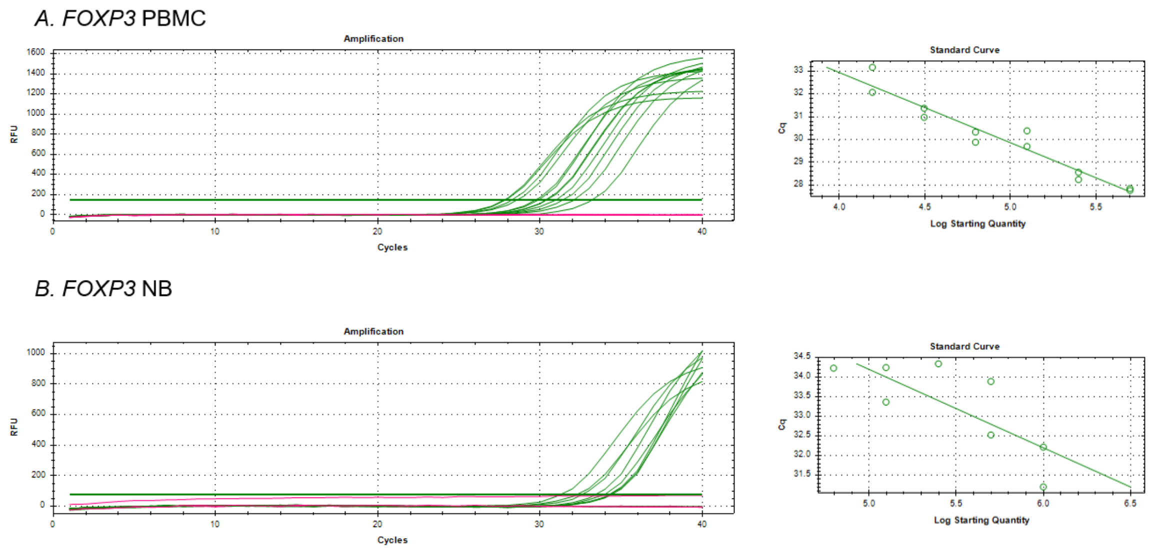

Figure 18. FOXP3 amplification and standard curve for PBMCs and NB.

Pooled samples of cDNA from PBMC samples or NB samples were diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 and 8 point standard curves were generated. A. PBMC *FOXP3* standard curve was successfully established with an efficiency of 110 % and an R^2 of 0.93. B NB FOXP3 standard curve could not be established with an efficiency of 217 % and R^2 of 0.599.

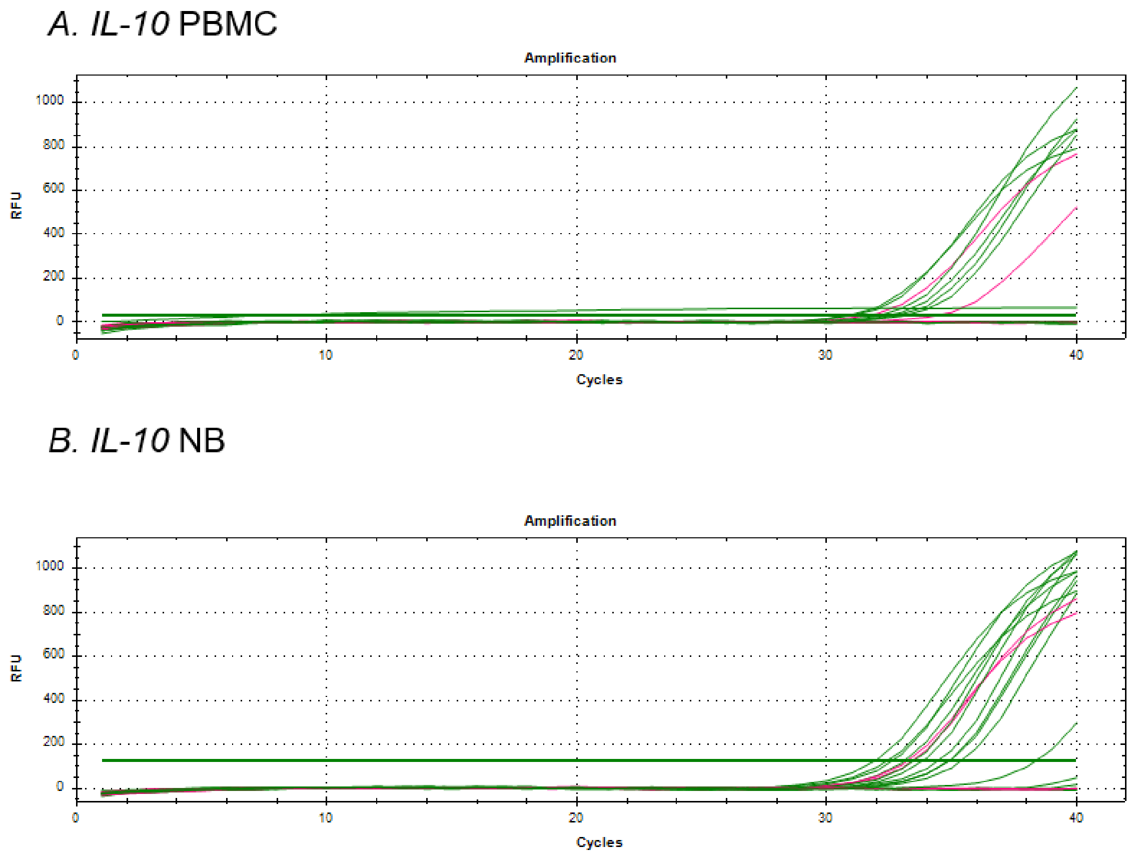

Figure 19. IL-10 amplification for PBMCs and NB.

Pooled samples of cDNA from PBMC samples or NB samples were diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 and 8 point standard curves were generated. A. PBMC IL-10 standard curve was not established as samples (Green) amplified at similar cycles as the NRT controls (pink). B. NB IL-10 standard curve was not established as samples (Green) amplified at similar cycles as the NRT controls (pink).

Table 16. Ranking of candidate genes.

Rank	Gene Name	Evaluation	Avg M Value	Stability (Ln(1/AvgM))
1	*GUSB*	Ideal	0.071	2.638
2	*RPL13A*	Ideal	0.071	2.638
3	*GADPH*	Ideal	0.073	2.622
4	*B2M*	Ideal	0.105	2.255
5	*RPS18*	Ideal	0.127	2.064
6	*G6PD*	Ideal	0.199	1.612
7	*PGK1*	Ideal	0.238	1.435
8	*RPLP0*	Ideal	0.266	1.325
9	*TBP*	Ideal	0.287	1.249
10	*HPRT1*	Ideal	0.309	1.174
11	*HMBS*	Ideal	0.342	1.074

GUSB PBMC

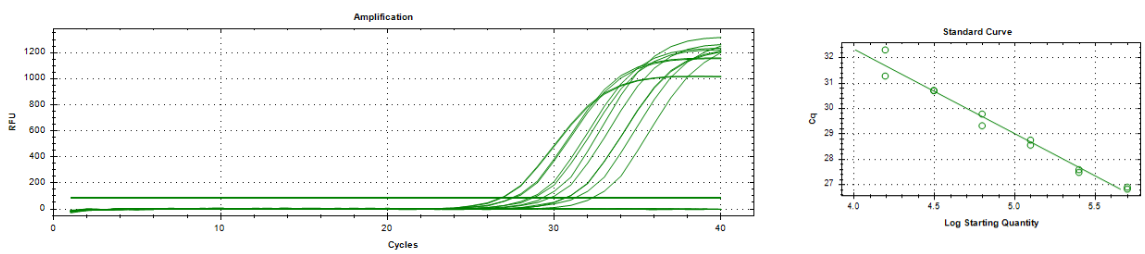

Figure 20. GUSB amplification and standard curve for PBMCs.

Pooled samples of cDNA from PBMC samples were diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 and an 8-point standard curve was generated with an efficiency value of 100 % and R^2 of 0.977.

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