Evidence of an Infectious Asthma Phenotype: Chlamydia Driven Allergy and Airway Hyperresponsiveness in Pediatric Asthma

EVIDENCE OF AN INFECTIOUS ASTHMA PHENOTYPE: *CHLAMYDIA* DRIVEN ALLERGY AND AIRWAY HYPERRESPONSIVENESS IN PEDIATRIC ASTHMA

A Dissertation Presented

by

KATIR K. PATEL

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DEDICATION

To my parents, Kirit and Usha Patel, my sister Krisha Patel, and my mentor and friend Wilmore C. Webley

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I would like to thank many people who have helped me through the completion of this dissertation. The first is my advisor and friend, Wilmore C. Webley, who is captivating, honest, and the true embodiment of a mentor. In combination with the mentorship of my advisor, I am privileged to work with dynamic and intelligent committee members; Dr. John M. Lopes, Dr. Lisa M. Minter and Dr. Michele M. Klingbeil. I would also like to thank the Microbiology Department at UMass who provided me with tremendous opportunities, guidance, and memories over the years.

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My experiences and memories of UMass have been wonderful, and I will carry them with me for the rest of my life. The path to earning a Ph. D. is littered with distractions. I'd like to thank those distractions for making me the person I am…

ABSTRACT

EVIDENCE OF AN INFECTIOUS ASTHMA PHENOTYPE: *CHLAMYDIA* DRIVEN ALLERGY AND AIRWAY HYPERRESPONSIVENESS IN PEDIATRIC ASTHMA

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Asthma is the most common chronic respiratory disease affecting young children and adults all over the world. An estimated 34.1 million Americans have reported asthma in their lifetime and the disease costs ~US $56 billion dollars to treat each year. Current treatment is based on a paradigm of asthma as a non-infectious atopic condition whose root cause is inflammation. Chronically administered anti-inflammatory medications, primarily inhaled corticosteroids (ICS), ameliorate asthma symptoms in many patients. However, up to 50% of asthmatics, characterized by neutrophil infiltration, IL-17 secretion and increased risk of fatality are refractory to ICS treatment. *Chlamydia pneumoniae*, a ubiquitous, obligate intracellular pathogen with an innate propensity to persist and cause chronic infections, along with *Mycoplasma pneumoniae* have been implicated in the development of chronic, refractory asthma. *C. pneumoniae* infections are common in infants and young children, often coinciding with the development of early onset asthma in the population.

These facts lead the Webley lab to evaluate the carriage of *Chlamydia* in pediatric respiratory disease patients and the work confirmed that respiratory infections caused by *Chlamydia* is a significant risk factor in asthma development and live *Chlamydia* was isolated from the lungs of children with chronic asthma. However, the exact mechanism underlying chlamydial involvement in the disease remained unknown and we believed that a better understanding could shed important light on expanded treatment options and mechanisms of this

infectious asthma phenotype. The work presented here provides new insight into how (1) early life chlamydial infection can lead to asthma initiation and exacerbation (2) respiratory chlamydial infection induces cellular and chemical immune responses that support asthmatic inflammation (3) other respiratory pathogens (eg. *Mycoplasma*) can drive similar immunological responses resulting in significant lung pathology.

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CHAPTER 1

RATE OF *CHLAMYDIA TRACHOMATIS* AND *CHLAMYDIA PNEUMONIAE* IN PEDIATRIC RESPIRATORY INFECTIONS

Abstract

Background: An emerging body of evidence suggests that half of asthma in both children and adults is associated with chronic lung infection. The aim of this study was to determine the frequency of viable *Chlamydia pneumoniae* (Cp) and *trachomatis* (Ct) in the respiratory tracts of pediatric patients with chronic respiratory diseases.

Methods: Bronchoalveolar lavage fluid (BAL) obtained from 182 children undergoing bronchoscopy for clinical reasons, were assayed using PCR analysis, in vitro tissue culture and immunofluorescence staining for the presence of *Cp* and *Ct.*

Results: *Chlamydia*-specific DNA was detected by PCR in 124/182 (68%) patients; 79 were positive for *Cp*, 77 for *Ct* and 32 for both organisms; seventy-five patients had cultivable *Chlamydia*. *Ct* DNA prevalence decreased, whereas Cp positivity generally increased with age. Fifty-nine of 128 asthma patients and 16/54 non-asthmatics were *Chlamydia* culture-positive [p=0.048]. When the patients were divided into inflammatory versus non-inflammatory airway disease, there were 69/150 [46%] and 6/32 [18%] BAL samples with cultivable *Chlamydia* respectively [p=0.005].

Conclusions: Viable *C*p and *Ct* occur frequently in children with chronic respiratory diseases and may be more prevalent in asthma patients. To our knowledge, this is the first report of viable *Ct* in the lungs of children.

Introduction

Respiratory diseases represent a major cause of disability and mortality among all age groups and races worldwide. They are also a leading cause of hospitalization and morbidity in both adults and children especially in developing countries (1-3). Chronic respiratory diseases

which include, asthma and chronic obstructive pulmonary disease [COPD], are just as prevalent as acute forms of these disorders (2). In addition to known environmental and genetic contributors, there is growing evidence that pathogenic microorganisms may play a role in diseases such as asthma . It is often difficult, however to identify the etiologic agent(s) of these respiratory infections due to a lack of standard diagnostic tools and the need for invasive procedures such as lung aspirations or pulmonary biopsies in order to confirm diagnosis. The worldwide increase in the incidence of asthma and the impact of the disease on public health, however, has led to renewed interest and investigations into its etio-pathogenesis.

Ct and *Cp* are two of the most common members of the *Chlamydiaceae* family that infect humans. *Cp* is thought to be responsible for 10-15% of community acquired pneumonia and 5% of pharyngitis and sinusitis cases (7, 8). Approximately 50% of healthy young adults and 75% of elderly persons have serological evidence of previous *Cp* infection (9). There is increasing evidence that *Cp* may play a role in pediatric asthma onset as well as possible exacerbations of asthmatic symptoms (10, 11). On the other hand, *Ct* has been recognized as a pathogen in nongonococcal urethritis, salpingitis, endocervicitis, pelvic inflammatory disease, inclusion conjunctivitis of neonates, follicular conjunctivitis of adults, infantile pneumonia and associated diseases (12, 13). Vertical transmission of infection from mother to the infant may result in the development of conjunctivitis and pneumonia (14, 15). In the past, multiple investigations of pediatric pneumonia emphasized the importance of infections with *Ct* in infants between two weeks and four months of age (16-18). While stringent prenatal screening in the US has greatly reduced the number of cases of neonatal conjunctivitis, there have been reports that ocular prophylaxis can fail to prevent neonatal chlamydial conjunctivitis and does not prevent colonization or infection at other sites such as the lungs (3, 19). Approximately 5-22% of pregnant women are thought to have *Ct* infection of the cervix, and 30-50% of neonates born to infected mothers show culture evidence of infection. Of infected neonates, 15-25% present with clinical conjunctivitis and nasopharyngitis that in some cases develops into neonatal pneumonitis

(9). Published reports have documented that many infants infected with *Ct* at birth remain infected for months or years in the absence of specific antimicrobial therapy (20). Indeed, some reports have suggested that wheezing may be another clinical expression of *Ct* infection and that this organism should be routinely assayed for in children who wheeze but have no demonstrable allergy and do not respond to the usual anti-asthmatic medications . We recently confirmed the presence of *Chlamydia* in BAL samples from pediatric patients with various chronic respiratory diseases using PCR and tissue culture techniques (22). In the work presented here using established species specific PCR *and culture* techniques, we extend these investigations to an examination of the prevalence of both *Cp* and *Ct* in BAL samples collected from pediatric patients and show that both infectious *Cp* and *Ct* are frequently present in the lung washings from children with chronic respiratory diseases and that both organisms may contribute to *Chlamydia*-mediated pneumonitis.

Materials and Methods

Specimens

We conducted a prospective, consecutive, non-interventional, cohort analysis of patients with various pulmonary disorders undergoing elective diagnostic bronchoscopy with BAL sample collection in a group of 184 patients from a community based/academic hospital setting. Seventy of these specimens were previously studied and reported on in a prior communication (22). Two patients above the age of 20 were excluded from the study because of our inclusion criteria of 20 years old and below for this pediatric cohort based on AAP accepted criteria (23). Patients were recommended for bronchoscopy because they all met the criteria of having severe, persistent airway disease that was non-responsive to therapy. Residual BAL samples obtained from study participants were de-identified in a HIPPA compliant manner and given alpha numeric codes prior to laboratory analysis. Approval for the study was obtained from the Institutional Review Board at Baystate Medical Center. Written, informed consent was obtained from the guardian of

each patient prior to inclusion in the study and patients were not contacted during the course of the study nor were they made aware of the results of the investigation. BAL was collected as previously reported (22, 24).

PCR Analysis

Genomic DNA was isolated from BAL samples and PCR performed as previously described (22). Initially, isolated DNA was amplified using a 16S signature sequence to detect all strains of Chlamydiales as previously reported (22, 25). *Cp*-specific PCR was performed using the previously published primer pair Cpn 201 and 202 to generate a 207bp product (26), while *Ct*-specific PCR was performed using the P1 & Omp2 primer set that amplified a 1100bp segment of the omp1 gene (27). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. Photographs were taken with the Syngene GeneFlash gel documentation system [Syngene USA, Frederick, MD].

Culture

Cells from BAL samples were pelleted, rinsed with sterile phosphate buffered saline and lysed with sterile glass beads in sucrose phosphate glutamate buffer [SPG]. Cultures were performed as previously described and cells were stained with a 1:100 dilution of a rabbit anti-*Chlamydia* antibody, [BIODESIGN International, Saco, ME] and visualized with a 1:1000 dilution of Alexa-Fluor®488 goat anti-rabbit secondary antibody [Invitrogen, Molecular Probes Carlsbad, CA]. The slides were then examined and photographs taken using a Zeiss LSM 510 Meta Confocal System.

BAL Cell Counts

Upon recovery, BAL cell counts and differentials were performed according to standard techniques in the hospital's Hematology Clinical Laboratory at Baystate

Medical Center (Springfield, MA). Cell enumeration was performed manually using a cell counting chamber (hemacytometer, improved Neubauer) under phase microscopy with results expressed as "number of cells per cubic millimeter". BAL differential counts were performed using Wright stained cytospin preparations of BAL which were then examined under oil-immersion microscopy (50X or 100X). Results were expressed on the basis of a 100 cell count survey.

Statistics

Data were analyzed using Microsoft Excel® spread sheets as well as the SPSS 11.5 Graduate Pack [SPSS, Inc., Chicago, IL] statistics programs. Cross-tabs with the Fisher exact T test and Chi-Squared test were used to determine significance. Univariate and bivariate analyses as well as logistic regression were also used to establish interactions between and among variables. For all analyses, tests were two-sided, and the level of significance was $p \leq 0.05$.

Results

Patient Demographics

The cohort of pediatric respiratory disease patients in this study consisted of 100 males and 82 females. The average age of this group of patients was 8.7 years old. Patients were from four different ethnic groups as follows: 121 white patients, 45 patients were Hispanic, 15 patients Black and 1 patient of Asian descent [Table 1]. Asthma diagnosis was made by a combination of family or personal history of atopy, elevated total IgE levels, positive skin or RAST test, reversible flow limitation on spirometry, the presence of increased eosinophils, basement membrane thickening on bronchial biopsy, or positive methacholine challenge. All asthma patients met the definition of having severe-persistent disease that is uncontrolled, [Global Initiative for Asthma, GINA (28, 29)]. A diagnosis of asthma was confirmed in 128 of 182 patients [70%].

Description	Asthma Cohort	Non-asthma sub-cohort	Total cohort of patients
Number of patients with diagnosed disease	128	54	182
Average Age (yrs)	8.7	12.5	8.7
Age Range:			
0.0-2.0	11	18	29
2.1-5.0	27	13	40
5.1-10.0	41	5	46
10.1-15.0	33	13	46
15.1-17.0	16	5	21
Gender :			
M	68	32	100
F	60	22	82
Ethnicity:			
White	85	36	121
Black	13	2	15
Hispanic	30	15	45
Asian	0	1	1
Medication:			
Yes	95	31	126
No	33	23	56

Table 1: Cohort demographics

The cohort consisted of 182 patients [100 male and 82 females]. The average age of the entire cohort was 8.7 years old and 128 individuals were diagnosed with asthma. For imbalances between asthma and non-asthma for age groups, there was a significant difference in the 0-2.0 age range [Fisher's Exact Test p=0.001], and the 5.1-10.0 age range [p=0.008]. There were significantly more patients in the non-asthma cohort in the 0-2.0 years old age range than in the asthma cohort. Conversely, there were significantly more asthma patients in the 5.1-10.0 year age group than in the non-asthma cohort. No other age range, gender, ethnicity or medication use category had significant differences between the asthma and non-asthma cohorts.

Black patients were diagnosed with asthma more frequently [13/15], compared to other ethnic groups in this study [Hispanic 30/45; White 85/121]. The lone Asian patient was non-asthmatic. Non-asthmatic disorders were gastroesophageal reflux disease (GERD), aspiration, bronchitis, bacterial bronchitis, structural anomalies (tracheomalacia, large-airway bronchomalacia and minor anatomic variants - accessory bronchi, tracheal bronchus, and pinhole bronchus), chronic cough, and vascular compression – innominate artery and pulmonary artery compression of left mainstem artery cystic fibrosis and recurrent pneumonia of unknown etiology. Most of the diagnosed asthma patients also displayed GERD and bronchitis. There was no significant relationship between race or gender and BAL culture positivity for the infectious form of *Chlamydia*. One hundred and twenty six of the 182 patients were taking one or more medication(s) at the time of testing including 4 patients on antibiotics [Amoxacillin, zithromax, Trimethoprim/Sulfamethoxazole, Cefdinir and Bactrim].

Detection of *Chlamydia* in Bronchoalveolar Lavage Fluid

PCR was performed on the BAL samples using a 16S signature ribosomal DNA sequence to determine the prevalence of chlamydial DNA carriage in these samples. PCR amplification of the target sequence resulted in a 298-bp product which was identified by electrophoresis. Our data revealed that 124 patients [68%] were positive for the presence of chlamydial DNA [see Figure 1.A for representative agarose gel]. We previously reported on the frequency of *Cp* in a similar cohort but did not specifically test all the samples for the presence of *Ct* (22). This decision was mainly due to the fact that *Ct* is not routinely reported in association with lung infections or pneumonitis, rather is mainly observed in sexually transmitted infection cases as well as conjunctivitis. With strong evidence from the literature that *Ct*-mediated pneumonitis is possible, especially in neonates (3, 12), all BAL samples that tested positive for chlamydial DNA using the 16S primers we re-tested with both *Cp* and *Ct* specific primers in order to determine the frequency of lung infection with these two human pathogens.

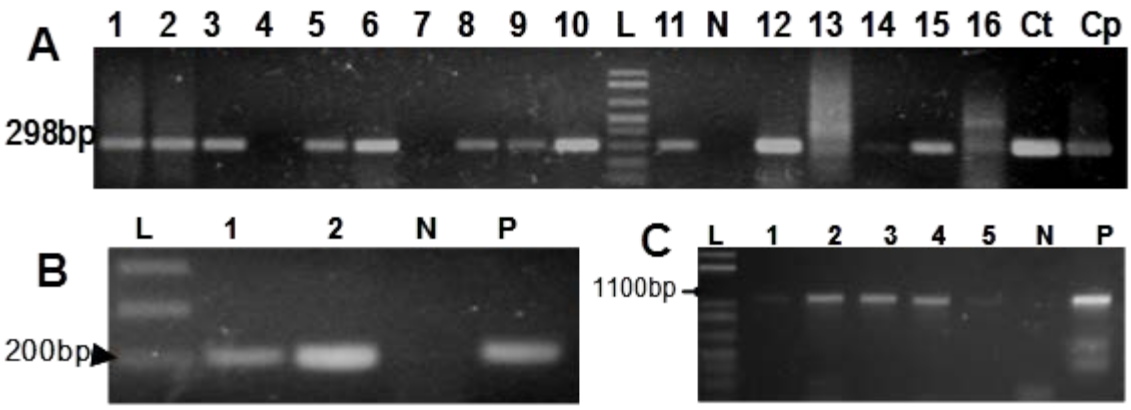


Figure 1: Representative agarose gels showing *Chlamydia* PCR products

(A) 16S amplified DNA from both *Chlamydia*-positive [1-3, 5, 6, 8-10, 11, 12, 13, 15 and 16] and negative [4, 7, 14] BAL samples compared to the positive controls [Ct and Cp] and the negative control [N]; L represents the DNA ladder. (B) *Cp* primers used to specifically amplify a 207bp product and (C) *Ct*-specific primer pair amplifies an 1100bp product from the BAL fluid of pediatric patients tested. Numbers represent individual patient samples while N and P are negative and positive controls respectively.

Seventy-nine [43.4%] of the 182 pediatric patient samples assayed were positive for the presence of *Cp*-specific DNA, while 77 [42.3%] samples were positive for *Ct*-specific DNA [Figure 1B and C]. BAL samples from 32 [17.6%] pediatric patients contained both *Cp* and *Ct* DNA.

Having recovered both *Ct* and *Cp* DNA at high rates in these BAL samples, we next attempted to determine the proportion of these organisms that were cultivable at the time of collection. All BAL samples were cultured on human or mouse macrophage cells using previously published protocols (22). The results revealed that 75 [41%] of the 182 patient samples were positive for *Chlamydia* when the BAL was cultured [Table 2]. Since all culture positive samples were also PCR positive, it is clear that 60% [75/124] of all PCR positive samples contained cultivable organisms. There was no significant difference in the finding of infectious *Chlamydia* between genders; 33 of the BAL culture–positive samples were from female patients and 42 from males. However, cultivable chlamydial organisms were found more frequently in the asthmatic population; 59 of 128 asthmatics versus 16 of 54 non-asthmatics were culture positive [P= 0.048 by Fishers Exact Test; see Table 2]. As an internal control, the patient cohort was divided into inflammatory respiratory disease [N=150] and non-inflammatory airway diseases [N=32]. The non-inflammatory respiratory disease group was categorized as such based on normal bronchial biopsy and normal BAL. Aerobic cultures were also negative for this group which consisted of structural defects, IgG and IgA deficiencies, aspirations and GERD; The inflammatory group included asthma, bronchitis, and pneumonia and were also categorized based on the bronchial biopsy, BAL and aerobic cultures. When assessed for cultivable *Chlamydia*, 69 [46%] of the inflammatory respiratory disease patients harbored cultivable *Chlamydia* versus 6 [18%] of the non-inflammatory disease group [Fisher's Exact Test (2-sided) p=0.005]. There was no significant association between these groups and the detection of chlamydial DNA [Table 3]. Evaluation of total serum IgE levels revealed no statistically significant association between patients with elevated serum IgE and the presence of *Chlamydia* DNA or cultivable organisms [Table3].

	Total patient group N=182	Asthma N=128	Non-asthma N=54	steroid treatment N=103	No steroid treatment N=79	Asthma Oral + Inhaled Steroid N=21	Asthma Oral steroid N=22	Asthma Inhaled steroid N=39
Chlamydia DNA positive, No. (% of group)	124 (68%)	86 (67%)	41 (76%)	70 (68%)	54 (68%)	17 (81%)*	7 (31%)*	24 (62%)*
Chlamydia culture positive, No. (% of group)	75 (41%)	59 (46%)*	16 (29%)*	42 (40.7%)	33 (42%)	12 (57%)	13 (59%)	13 (33%)

Table 2: Frequency of *Chlamydia* in asthma versus non-asthma patient groups and corticosteroid administration

Seventy-five of the 182 patient samples analyzed contained cultivable *Chlamydia* as determined by culture techniques. Fifty-nine [46%] were from patients diagnosed with asthma; 16 [29.6%] were from non-asthma group. * P=.048, asthma v. non-asthma (Fishers Exact test) (55). Most patients [103/182] were being treated with corticosteroids at the time of sample collection. Seventeen of the 21 [81%] *Chlamydia*-positive patients were taking a combination of inhaled and oral steroids; 7 [31%] were taking oral steroids alone. * Represents statistically significant associations

	Inflammatory Airway Disease N=150	Non-Inflammatory Airway Disease N=32	Elevated serum IgE N=72	Normal serum IgE N=110
Chlamydia Culture Positive, No (% of group)	69 (46%)*	6 (18%)*	43 (60%)	81 (74%)
Chlamydia DNA Positive, No. (% of group)	104 (69.3%)	20 (62.5%)	26 (36%)	49 (45%)

Table 3: **Prevalence of *Chlamydia* in inflammatory and non-inflammatory airway disease**

When divided into inflammatory versus non-inflammatory airway disease, the majority of patients [150] were diagnosed with various inflammation airway diseases including asthma, pneumonia and bacterial bronchitis. The remaining 32 patients had various non-inflammatory diseases [GERD, aspirations, laryngomalacia, dyspnea, airway and structural anomalies, chronic cough, and vascular compression]. Cultivable *Chlamydia* was found more frequently in inflammatory versus non-inflammatory airway disease [* Fisher's Exact Test (2-sided) p=0.005**]**. There was no significant association with PCR positive samples [p=0.531]. There was no statistically significant association between patients with elevated serum IgE and the presence of *Chlamydia* DNA or cultivable organisms.

One hundred and three of the 182 [56.6%] patients in our pediatric cohort were taking some form of steroid therapy at the time of BAL sample collection. Eighty two of the 128 [64%] asthmatics in this cohort were being prescribed corticosteroids; 23 non-asthmatics diagnosed with one or more of the other chronic respiratory diseases listed previously were also being prescribed corticosteroids at the time of sample collection. Of the 82 asthmatic patients taking corticosteroids, 52 [63%] were positive for the presence of chlamydial DNA and 38 tested positive for cultivable *Chlamydia* in culture. Eighteen of the 23 [78%] non-asthma patients being administered corticosteroids were also positive for chlamydial DNA; five of these were *Chlamydia* culture positive. Asthma patients being prescribed both oral and inhaled corticosteroids were more likely to harbor chlamydial DNA in their BAL fluid [17/21] than those taking only oral [7/22, Fishers Exact Test; p=0.0461] or inhaled corticosteroids [24/39 p=0.133, Table 2].

In addition to corticosteroids, 4 of these patients were being prescribed antibiotics at the time of sample collection. Three of these 4 patients were diagnosed with asthma [1 patient was diagnosed with both asthma and bronchitis] and one patient had bronchitis. The BAL from 2 of these patients taking bactrim and TMP/SMX harbored chlamydial DNA and one was culture positive for *Chlamydia*, Patients on amoxicillin and zithromax were both negative for *Chlamydia*.

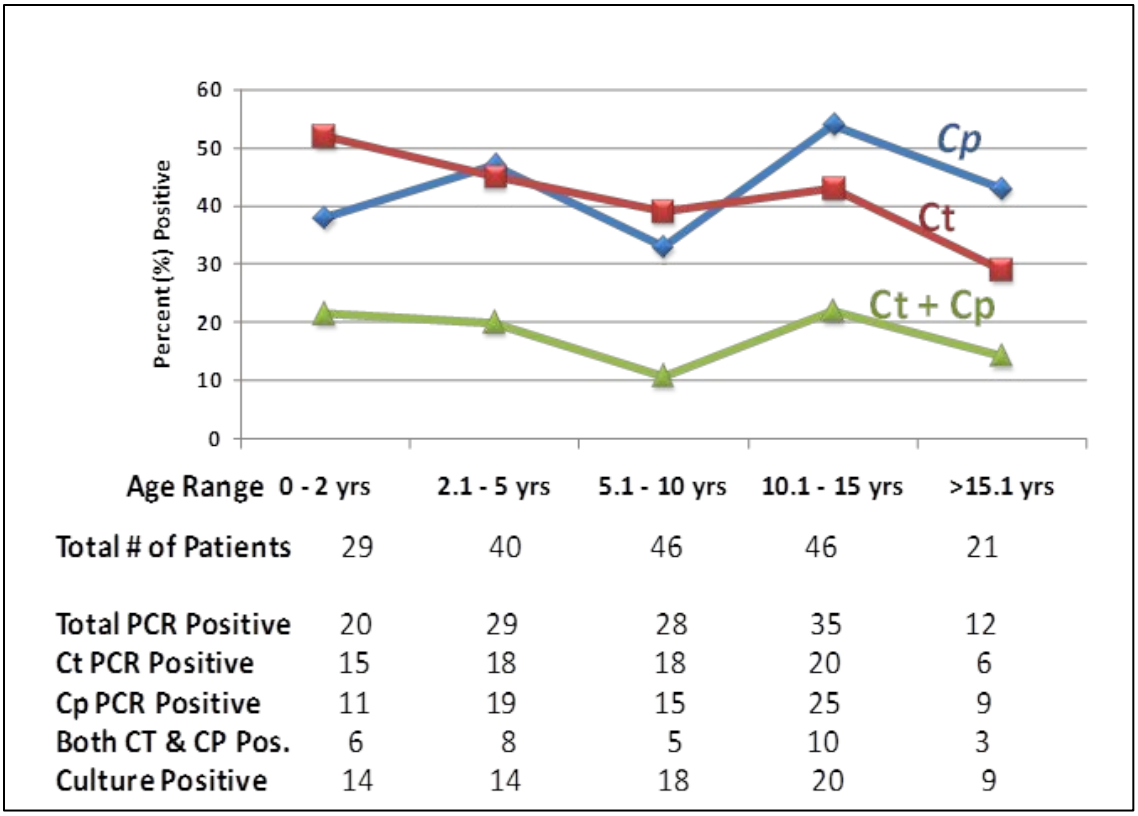
***Chlamydia*, Age and Disease Associations**

This cohort of 182 respiratory disease patients consisted of 128 [70%] diagnosed asthmatics. Chlamydial DNA was recovered by PCR amplification from 86/128 [67%] patients diagnosed with asthma [Table 2]. The remaining 41 patients were *Chlamydia* negative by PCR. Chlamydial DNA was however not exclusively found in the asthma population, with 41/54 [76%] non-asthma patient samples also PCR positive. Of the 32 patients who harbored both *Ct* and *Cp* DNA in their lungs, 20 of 128 asthmatic and 12 of 54 were non-asthmatic. Patients in the 10.1-15 year old age-range accounted for the highest prevalence [35/46, 76%] of *Chlamydia* infection

[Figure 2]. The 0-2 year old age group harbored the highest percentage of *Ct* DNA samples [15/29, 51.7%]. The percentage of patient samples testing positive for *Cp* DNA increased from 11/29 [38%] in the 0-2 age group to 19/40 [47.5%] in the 2.1-5 age range. The number of patients testing positive for the presence of *Ct* DNA decreased over time with increasing age, while the inverse was generally true for *Cp*. The prevalence of both *Cp* and *Ct* decreased after age 15 [Figure 2].

BAL Cellularity and *Chlamydia*

Bronchial lavage fluid collected from each patient was analyzed in a clinical setting for the presence of various cell types. Specifically, cell counts were performed to determine the number of lipid laden macrophages (LLM)/monocytes, eosinophils, lymphocytes and neutrophils. Forty five patient samples showed elevated levels of eosinophils, [Table 4], 149 contained lymphocytes, and monocytes were found in 89 patient samples. There was no significant difference in percentages of alveolar macrophages, lymphocytes, and monocytes in the asthma versus non-asthma group. The mean cell counts for monocytes, eosinophils, lymphocytes and neutrophils are presented for *Chlamydia* positive vs. negative samples [DNA and culture] and asthma vs. non-asthma sub-cohorts [Table 4]. The mean eosinophil count was statistically higher in asthmatics [5.5%] vs. non-asthmatics [0.06%, Fisher's Exact Test p=0.001]. The range of BAL eosinophil counts in asthmatic patients was from 1-25%. The mean neutrophil count in *Chlamydia* culture positive patients was 68.39% compared to 30.87% in culture negative subjects [Fisher's Exact Test, two-tailed; p=0.001]. Since this group of children all had severe respiratory diseases and many had GERD, it was not surprising to discover that 175 of the 182 BAL samples contained at least some LLM. The cytological evaluation did not reveal a statistically significant association between the finding of LLM and *Chlamydia* organisms. There were few LLM [1-3 LLM per field], in 14 patients, moderate [4-6 LLM per field] amounts in 66 patients and many [≥6 LLM per field] in 95 patients. Sixty eight of 75 culture-positive, versus 93 of 107 culture



Age Range	0 - 2 yrs	2.1 - 5 yrs	5.1 - 10 yrs	10.1 - 15 yrs	>15.1 yrs
Total # of Patients	29	40	46	46	21
Total PCR Positive	20	29	28	35	12
Ct PCR Positive	15	18	18	20	6
Cp PCR Positive	11	19	15	25	9
Both CT & CP Pos.	6	8	5	10	3
Culture Positive	14	14	18	20	9

Figure 2: Age-based prevalence of chlamydial DNA in BAL fluid

The correlation between age and the prevalence of *Cp* and *Ct* organisms determined by PCR analysis is shown. Note that patients in the 10.1-15 year age range accounted for the highest percentage [35%] of total *Chlamydia* organisms. The 0-2 year age group harbored the highest percentage of *Ct* organisms [15/29, 51%].

	Chlamydia culture positive N=75	Chlamydia culture negative N=107	Chlamydia DNA positive N=124	Chlamydia DNA negative N=58	Asthma N=128	Non-Asthma N=54
LLM, No. (%)						
Many	44 (58.7%)	51(48%)	60 (48%)	35 (60.3%)	73 (57%)	21 (39%)
Moderate	24 (32%)	42 (39%)	49 (40.0%)	17 (29.4%)	42 (33%)	24 (44%)
Few-None	7 (9.3%)	14 (13%)	15 (12%)	6 (10.3%)	13 (10%)	9 (17%)
Mean % cell count/ml						
Macrophage/monocytes	2.84%	2.79%	2.63%	3.26%	3.11%	2.11%
Eosinophils	1.45%	1.16 %	1.10%	1.64%	5.5%*	0.06%*
Lymphocytes	8.04%	7.83%	8.67%	6.30%	7.68%	8.48%
Neutrophils	68.39%*	30.87%*	33.85%	29.4%	29.0%	40.59%
Other	19.28%	57.35%	46.25%	59.4%	54.71%	51.24%

Table 4: BAL cellularity and the presence of *Chlamydia*

The bronchial lavage fluid of each patient was assessed for the presence of various cell types. The table outlines the presence of these cells and assesses their correlation with cultivable *Chlamydia*, chlamydial DNA and diagnosis of asthma. The mean eosinophil count was statistically higher in asthmatics compared to non-asthmatics [Fishers Exact Test p=0.001]. The mean neutrophil count was significantly increased in *Chlamydia* culture positive patients over culture negative ones [68.39% vs. 30.87% respectively; Fishers Exact Test-two-tailed p=0.001]. There was no statistically significant correlation between any of the other cell types and *Chlamydia* organism, DNA or disease association. * Represents statistically significant associations

negative patient samples displayed moderate to many LLM [Table 4].

Discussion

Chronic lung disease affects an increasingly wide cross-section of the world's population, manifesting itself mainly as asthma, COPD, pneumonia and bronchitis (2, 18). Globally, respiratory infections in childhood are a leading cause of disease and substantially contribute to school absenteeism and severe economic strain on healthcare resources (30). In the developing world, respiratory infections are also a major cause of childhood mortality (31). Respiratory diseases of infancy and childhood are predominantly infectious in nature and can be caused by either viruses, bacteria or parasites (32).

In the current observational study we demonstrate that human strains of *Chlamydia*, *Ct* and *Cp*, can be isolated from the lungs of children with chronic respiratory disease. *Cp* has long been reported as an etiologic agent of community acquired pneumonia and has been found in high prevalence in the lungs of adults (33), and also in the respiratory secretions of adult asthmatics, and lungs of COPD patients (34). Recently, we reported that *Cp* can also infect the lung tissue of children and might contribute to the pathology commonly seen in a variety of chronic respiratory diseases (22). While the number of patients with chlamydial DNA in their lower respiratory tract analyzed in this study is surprisingly high, compared to those in some recent studies (35, 36), our cohort is significantly larger. Furthermore, the type of samples collected by us differs from most published work to date which utilize samples from the upper respiratory tract. It is also noteworthy that our cohort is a select group of severely ill children with chronic respiratory disease and as such, may not be reflective of the general population. Most other studies that report a prevalence of *Cp* in the 5-30% range assess sputum, nasal aspirates or throat swab samples (35, 37, 38) as opposed to bronchial washings, a more invasive procedure with which our samples were obtained. In a 2001 study where *Cp* PCR was performed on tracheobronchial aspirate the authors reported a 51.9% prevalence (39). It should again be noted that the samples we used were residual in nature and not obtained for the purpose of research, but as a part of the diagnostic

evaluation of each patient. Importantly, most studies by others only tested for the presence of *Cp* DNA, while the presence of both *Cp* and *Ct* DNA was assayed for in this study.

The data revealed a significant association between cultivable *Chlamydia* and asthma diagnosis, consistent with our earlier findings. Importantly, the data also confirm that at earlier ages of life, *Ct* appears more prevalent than *Cp* in BAL samples. Although the organisms were also found in the neonate to two years old age group, it is not until age 5 to10 years old that an increased prevalence of *Cp* is observed. This suggests that these later infections may have been contracted through increased social interactions, possibly in pre-school or daycare settings. These findings agree with previously published data suggesting that *Ct* can be found in the lower respiratory tract of newborns and can lead to pneumonitis (12, 15, 21). Evidence for the presence of Cp in the human placental tissue also exists (40-42). This suggests a potential role for intrauterine *Ct* infection in the development of chronic respiratory disease in infants.

Increased lipid content in alveolar macrophages of bronchoalveolar lavage fluid is thought to be a useful indicator for recurrent pulmonary aspiration (43, 44). Previous studies have confirmed that *Chlamydia* has the ability to survive and even thrive in alveolar macrophages (45, 46). It has been previously reported that *Cp* induces foam cell formation by human monocyte-derived macrophages (47). Exposure of macrophages to *Cp* followed by the addition of low-density lipoprotein [LDL] in tissue culture, caused a marked increase in the number of foam cells and accumulation of cholesteryl esters (47). These data therefore suggest that an infectious agent can induce macrophage foam cell formation and implicate *Cp* as a causative factor in inflammatory diseases associated with foam cell formation, including atherosclerosis. While not statistically significant, we observed a correlation between the finding of moderate to many LLM [greater than 4 cells per high power field] and *Chlamydia*-positivity by PCR and culture. Infection of macrophages by *Chlamydia*, coupled with epithelial cell damage in the airways could increase the inflammatory response in the lungs. With increased oxidative bursts by these

phagocytes and the release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8, airway hyperreactivity and pulmonary inflammation might be significantly increased.

In the current study, asthma patients treated with a combination of oral and inhaled corticosteroids were more likely to harbor chlamydial DNA [Table 4]. Inhaled glucocorticoids are a mainstay of asthma therapy. Oral steroid treatment is the most potent therapeutic intervention available for the effective relief of symptoms in acute and chronic asthma, especially for patients with severe disease. However, corticosteroids negatively affect many aspects of cell-mediated immunity and favor the shift from a T-helper-1-type response towards a T-helper-2-type response. Corticosteroids may thus severely impact the host's ability to eradicate an intracellular pathogen, such as *Chlamydia,* which requires properly functioning cell-mediated [T-helper-1-type] immune responses for pathogen clearance. Previous in-vitro studies confirm that persistent *Chlamydia* in macrophages are reactivated when corticosteroid treatment is administered, resulting in the release of infectious elementary bodies (EB) particles into the immediate surroundings whereby new cells are infected (48, 49). Corticosteroids have also been shown to reactivate persistent *Chlamydia* carriage leading to an active growth phase, thus, increasing the production of pro-inflammatory cytokines at the site of infection and further amplifying inflammation in the airways of patients with asthma (50-53). The combination of both inhaled and oral steroid treatment might represent an increased amount of corticosteroids in the circulation leading to increased reactivation of *Chlamydia*.

Along with previously published studies, the work reported here suggests that further in depth investigations of the involvement of the *Chlamydiaceae* family of obligate intracellular pathogens in the etiology and exacerbation of asthma and other chronic respiratory diseases, particularly in pediatric populations are needed. The current data confirms the presence of both *Cp* and *Ct* organisms in the lungs of these patients. Importantly, patients with asthma and other inflammatory airway disease were more likely to harbor cultivable chlamydial organisms in their lower respiratory tract.

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CHAPTER 2

INFECTIOUS *CHLAMYDIA PNEUMONIAE* IS ASSOCIATED WITH ELEVATED IL-8 AND AIRWAY NEUTROPHILIA IN CHILDREN WITH REFRACTORY ASTHMA

Abstract

Background: Neutrophilic asthma is thought to be less responsive than eosinophilic asthma to anti-inflammatory therapies including corticosteroids. *Chlamydia pneumoniae* has been implicated in asthma, possibly by induction of IL-8. We hypothesized that IL-8 is increased in the bronchoalveolar lavage (BAL) fluid from children with asthma and *C. pneumoniae*.

Methods: BAL fluid was analyzed for *C. pneumoniae* and IL-8 using PCR and ELISA from 2 asthma patient populations Bronx, NY and Massachusetts with an average age of 8 and 8.7 years old, respectively. For comparison, samples were also analyzed for *C. trachomatis* and *Mycoplasma 16S DNA*.

Results: Of 18 Bronx samples analyzed, 6 (33%) were PCR-positive for *C. pneumoniae*, 10 (56%) for *C. trachomatis* and 8 (44%) for *Mycoplasma 16s DNA*. IL-8 from *C. pneumoniae*-positive samples was 3.3-fold higher compared with negative samples (p= 0.003). There was no difference between patients tested for *C. trachomatis* or *Mycoplasma*. Of 84 Massachusetts samples analyzed, 42 (50%) were PCR-positive for *C. pneumoniae*, 42 (50%) for *C. trachomatis* and 13 (16%) for *Mycoplasma*. IL-8 concentration from *C. pneumoniae*-positive samples was 10.49-fold higher compared with negative samples (p= 0.0001). As in the Bronx cohort, there were no differences between patients tested for *C. trachomatis* or *Mycoplasma*. Lastly, BAL neutrophilia predicted the presence of *C. pneumoniae* but not *Mycoplasma* or *C. trachomatis*.

Conclusions: Children with asthma who were PCR-positive for *C. pneumoniae* demonstrated elevated levels of IL-8 and neutrophils in BAL fluid compared to similar patients who were positive for *C. trachomatis* or *Mycoplasma* organisms, but PCR-negative for *C. pneumoniae*. Undiagnosed *C. pneumoniae* infection in children may therefore contribute to poorly controlled asthma via induction of IL-8.

Introduction

There is increasing evidence that inflammatory phenotype in asthma partly dictates both severity of disease and response to therapy (1, 2). For example, patients with neutrophilic asthma, as measured by induced sputum or bronchoalveolar lavage (BAL) fluid, tend to be less responsive to traditional asthma medications including inhaled and oral corticosteroids (3-5). Although the precise mechanism for these observations is currently unclear, some postulate that undiagnosed chronic bacterial infection may contribute to this phenomenon by induction of specific proinflammatory cytokines including IL-8, a potent neutrophil chemoattractant and activating factor (6). IL-8 is known to be released by monocytes (7), macrophages (8), fibroblasts (8), and airway epithelial cells (9). Increased levels of IL-8 have been described in chronic neutrophilic airway inflammation in patients with refractory asthma, cystic fibrosis and other chronic airway diseases (4, 10-12). Although acute exposures to airway irritants, including particulate matter, viruses and endotoxin have been shown to induce production of IL-8, it is unclear whether chronic, undiagnosed infections can lead to a sustained increase in this inflammatory cytokine, and thus contribute to ongoing disease (13).

Chlamydia pneumoniae, an obligate intracellular bacterium, has also been implicated in poorly controlled asthma (14, 15). In addition, past investigations have demonstrated that *C. pneumoniae* can enhance IL-8 secretion (16), and that this secretion may be initiated by adherence of the organism to human airway epithelial cells (17). Importantly, confirmation of infection can be difficult, and thus true prevalence of infection is unknown. As such, the specific role for *C. pneumoniae* in chronic respiratory diseases including asthma is also unclear (18).

In previous investigations, we utilized modified culture techniques and molecular methods to demonstrate a high prevalence of *C. pneumoniae* in BAL fluid from children with asthma (14, 15). It is unclear from these past studies whether the presence of *C. pneumoniae* alters the cytokine profile in the airway of such patients. Thus, we sought to determine whether *C.*

pneumoniae was associated with IL-8 levels in BAL fluid from children with poorly controlled asthma. Portions of this manuscript were presented as a poster presentation at the 2009 ATS International Meeting (19).

Materials & Methods

Sample Collection

We obtained banked BAL samples that were collected from patients undergoing flexible bronchoscopy with lavage under a separate IRB-approved protocol. We specifically sought to identify samples collected from patients with poorly controlled asthma and no other identifiable pathology contributing to their persistent symptoms. BAL was performed according to the normal protocol for our institution and as previously reported (14, 15). Briefly, the right middle lobe or lingula was lavaged with a total of 3cc/kg of normal saline divided into 3 equal aliquots (maximum aliquot of 20cc). If a patient required lavage in a separate lobe for clinical purposes (i.e. recurrent pneumonia in a different lobe), the sample obtained at that time was utilized. Additional lavage was not performed for investigational purposes. Up to 5cc of recovered fluid was immediately frozen and stored in –80°C until analysis.

PCR Analysis

Genomic DNA was isolated from BAL using the QIAMP DNA Blood mini kit (Qiagen). Polymerase chain reaction (PCR) was used to detect both *Chlamydia* and *Mycoplasma* DNA. We used the following primer sets to determine the presence and prevalence of *C. pneumoniae* and *C. trachomatis* respectively: Cpn201 and 202 primers (product 207 bp) and *Ct* using primers Ct sense and Ct anti-sense (504 bp). The PCR products were then separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. *Mycoplasma* 16S was performed using commercial kits (Maxim Biotech, Inc. *Mycoplasma* 16S rRNA kit-SP-10521 and

PCR Primer Amplification Kit, *Mycoplasma pneumoniae*, Attachment Protein P1; SP-10523) according to the manufacturers guidelines.

Performing IL-8 ELISA

Immediately following bronchoscopy, BAL samples were processed and stored at -80°C until analysis. Concentration of IL-8 was determined in BAL fluid using BD OptEIA Human-IL-8 ELISA Kit II (BD Biosciences, San Jose, California) according to the manufacturer's instructions. Briefly, the ELISA test used was a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay) which uses a monoclonal antibody specific for IL-8 coated on a 96-well plate. Standards and 100ul of undiluted BAL fluid samples were added to the wells. The wells were washed and streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-8 antibody was added. Wells were subsequently washed again and TMB substrate solution was added, producing a blue color which is in direct proportion to the amount of IL-8 present per sample. The Stop Solution was then added, changing the color to yellow, and the microwell absorbances were then read at 450 nm.

Cell Counts

BAL cell counts and differentials were performed according to standard techniques in the hospital's Hematology Clinical Laboratory at Baystate Medical Center (Springfield, MA) and Children's Hospital at Montefiore, Albert Einstein College of Medicine (Bronx, NY) as previously reported (14). Briefly, cell enumeration was performed manually using a cell counting chamber under phase microscopy with results expressed as "number of cells per cubic millimeter". BAL differential counts were performed using Wright stained cytospin preparations of BAL which were then examined under oil-immersion microscopy (50X or 100X). Results were expressed on the basis of a 100 cell count survey.

Statistical Analysis

All statistical analyses for IL-8 concentration among the different groups of the patient population were performed using Microsoft Excel®. Statistical associations were determined with Fisher's exact test and Chi-square with Yates correction (SPSS 15.0 software). A p-value of 0.05 was considered significant and all tests were two-tailed. Sensitivity, specificity, positive predictive value and negative predictive values were performed using the Clinical Calculator1 online program at http://faculty.vassar.edu/lowry/VassarStats.html.

Results

Bronx patients

Of 31 BAL samples collected and banked between July 2007 and July 2008, 20 were obtained from patients with poorly controlled asthma and no identifiable airway pathology contributing to persistent respiratory symptoms. Of these 20 patients, 2 were excluded from analysis because their asthma was complicated by allergic bronchopulmonary aspergillosis. Thus, a total of 18 samples were analyzed (12 males and 6 females). All patients included in the study were diagnosed with moderate to severe persistent asthma that was under poor control, as defined by the current NIH guidelines(20). All were chronically treated with high dose inhaled corticosteroids, although 2 patients self-discontinued these medications prior to bronchoscopy. With the exception of one child, all patients in this cohort were also receiving or had previously been managed with additional therapies including leukotriene receptor antagonists (LTRA) and/or long-acting beta agonists (LABA). 2 patients were receiving omalizumab (Table 5).

BRONX	C. pneumoniae Positive (%) (n=6)	C. trachomatis Positive (%) (n=10)	Mycoplasma Positive (%) (n=8)
Age (yr)	**9.0**	**10.0**	**10.3**
Gender			
Male	**4 (66.7%)**	**6 (60.0%)**	**5 (62.5%)**
Female	**2 (33.3%)**	**4 (40.0%)**	**3 (37.5%)**
Race			
Black	**1(17%)**	**6 (60%)**	**2 (25%)**
Hispanic	**5 (83%)**	**4 (40%)**	**6 (75%)**
Asthma	**6 (100.0%)**	**10 (100.0%)**	**8 (100.0%)**
Medication			
Inhaled steroids	**6 (100.0%)**	**6 (60.0%)**	**4 (50.0%)**
Oral steroids	**2 (33.3%)**	**1 (10.0%**	**1 (12.5%)**
Other*	**4 (66.7%)**	**3 (30.0%)**	**3 (37.5%)**

Table 5: Characteristics of Bronx NY, Study Population

The Bronx patient cohort consisted of 18 patients, 12males and 6 females undergoing diagnostic bronchoscopy. All patients were being prescribed high dose corticosteroid treatment at the time of sample collection. Indeed several patients in this cohort had previously been managed with long-acting beta agonists (LABA). Medications at the time of sample collection consisted mainly of inhaled corticosteroids, leukotriene receptor antagonist and long-acting beta agonists (LABA).

Massachusetts patients

We previously analyzed *C. pneumoniae, C. trachomatis and Mycoplasma* status in a large cohort of patients undergoing clinically indicated bronchoscopy in Massachusetts (14, 15). Because of the initial results obtained from our smaller Bronx cohort, we reviewed data from a select sub-cohort of these samples in order to identify similar patients who underwent bronchoscopy because of poorly controlled asthma and no alternate diagnosis for persistent symptoms. For comparison we also tested 7 samples from patients with culture-documented bacterial bronchitis and asthma. We identified 84 patients who underwent flexible bronchoscopy with lavage for poorly-controlled asthma and no other diagnosis to explain persistent respiratory symptoms. At the time of bronchoscopy, all were being prescribed high doses of inhaled corticosteroids, and all were either receiving or had not responded to adjunct therapies including LABA, LTRA, omalizumab or anti-reflux medications (Table 6).

IL-8 concentration and *C. pneumoniae* in Bronx patients

Of the 18 Bronx samples analyzed, 6 (33%) were PCR-positive for *C. pneumoniae*. IL-8 concentrations from *C. pneumoniae*-positive samples were 3.3-fold higher on average compared with *C. pneumoniae*-negative samples (Figure 1A; 167.0 ± 56 pg/ml vs. 51.0 ± 60 pg/ml, respectively; $p = 0.0003$). In comparison, 10 samples (56%) were PCR-positive for *C. trachomatis* and 8 (44%) were positive for *Mycoplasma*. When IL-8 was analyzed in relation to *C. trachomatis* and *Mycoplasma* positivity, no differences were observed between DNA positive and negative samples (79.5 ± 81 pg/ml vs. 102.4 ± 82 pg/ml, and 101.0 ± 74 pg/ml vs. 114.2 ± 88 pg/ml, respectively; Figures 3B and 3C).

Massachusetts	*C. pneumoniae* Positive (%) (n=42)	*C. pneumoniae* Negative (%) (n=42)	Total
Age (yr)	8.1	7.8	8.0
Gender			
Male	25 (59.5%)	21 (50.0%)	46
Female	17 (40.5%)	21 (50.0%)	38
Race			
Caucasian	25 (59.5%)	29 (69.1%)	54
Black	6 (14.3%)	4 (9.5%)	10
Hispanic	11 (26.2%)	9 (21.4%)	20
BMTH			
Positive	32 (76.2%)	25 (59.5%)	57
Negative	4 (9.5%)	11 (26.2%)	15
Not Done	6 (14.3%)	6 (14.3%)	12
Asthma Medication	42 (100.0%)	42 (100.0%)	84
Inhaled steroids	23 (54.8%)	20 (47.6%)	43
Oral steroids	10 (23.8%)	9 (21.4%)	19
Other*	19 (45.2%)	21 (50.0%)	40

Table 6: Characteristics of Study Population in Massachusetts Cohort

This cohort consists of patients mainly from western Massachusetts. There were 120 patients with an average age of 8 years old and like the Bronx cohort presented with chronic, severe, respiratory disease and prescribed high doses of corticosteroids. Seventy four of these patients were asthmatics and IL-8 positivity had a significant correlation with basement membrane thickening based on biopsy taken during bronchoscopy. There were no significant correlations between IL-8 positivity and age, gender or race. *This includes acid reflux and other asthma medications.

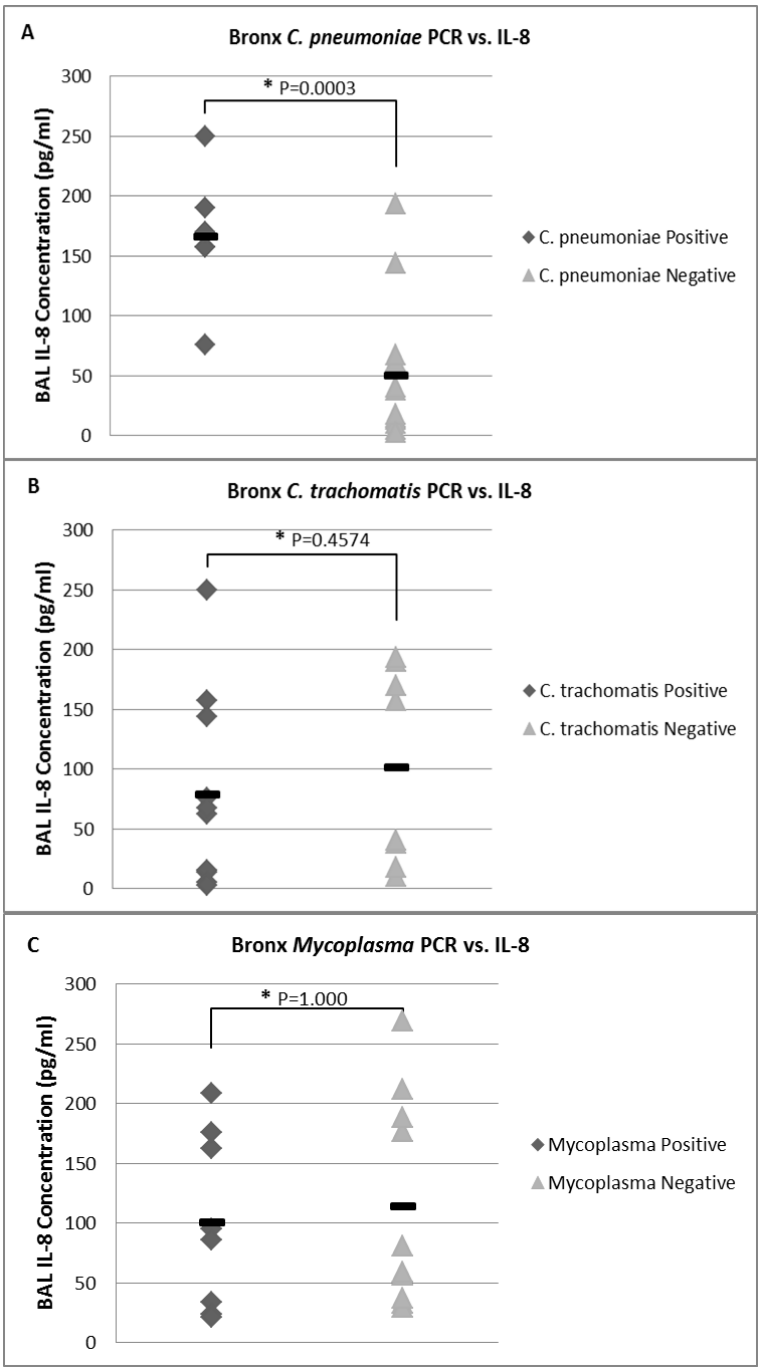

Figure 3: Elevated IL-8 in the BAL of *C. pneumoniae* positive Bronx Patients.

We analyzed18 samples in our Bronx cohort and 6 (33%) were PCR positive for *C. pneumoniae*. *C. pneumoniae*-positive patient samples had an IL-8 concentration that was 3.3 fold higher on average compared with *C. pneumoniae*-negative samples, p = 0.0003 (*graph A*). When similarly analyzed, *C. trachomatis* and *Mycoplasma*-positive samples did not demonstrate significant differences, compared to negative samples (*graphs B and C respectively*). Dark horizontal line represents the mean concentration for each group and symbols represent individual patient samples in the positive or negative column. P-values are indicated for each set of positive and negative group.

***IL-8* concentration and *C. pneumoniae* in Massachusetts patients**

Similar, but more striking results were seen in the Massachusetts patients. Of the 84 samples analyzed 42 (50%) were PCR-positive for *C. pneumoniae* (note that 21 of the 42 samples were positive for both *C. pneumoniae* and *C. trachomatis*). IL-8 concentration from *C. pneumoniae*-positive samples was 10.49-fold higher on average compared with *C. pneumoniae*-negative patient samples (Figure 2A; 129.0 ± 65 pg/ml vs. 12.3 ± 28 pg respectively, p = 0.0001). There was no difference in gender distribution, age or medications being prescribed at the time of sample collection between *C. pneumoniae* PCR-positive and negative patients (Table 2). In comparison, 42 samples (50%) were positive for *C. trachomatis* and 13 (16%) were positive for *Mycoplasma*. As in the Bronx cohort, there were no significant differences in IL-8 levels between DNA positive and negative samples patients tested for *C. trachomatis* or *Mycoplasma* (76.0 ± 79 pg/ml vs. 73.6 ± 79 pg/ml and 60.7 ± 51 pg/ml vs. 66.5 ± 73 pg/ml, respectively; Figure 4B and 4C).

Because PCR positivity may not equate with viability of organisms, we reviewed previous culture data for the Massachusetts patients (14, 15). Consistent with the PCR data from both the Bronx and Massachusetts cohorts, we found that culture-positive samples demonstrated a 9.85-fold increase in IL-8 levels compared to culture-negative samples (Figure 5; 113.3 ± 69 pg/ml vs. 11.5 ± 30 pg/ml, respectively; p = 0.0001).

Lastly, to determine whether there were differences in IL-8 levels between *C. pneumoniae* and other common bacterial pathogens, we tested 7 asthmatic patients within our cohort, that were also diagnosed as bacterial bronchitis patients, with one or more of the following microbes in their BAL fluid: *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Neisseria species* and *Streptococcus pneumoniae*. Interestingly, IL-8 levels in the asthmatic patients were 2.0 fold higher when compared to those of the bacterial

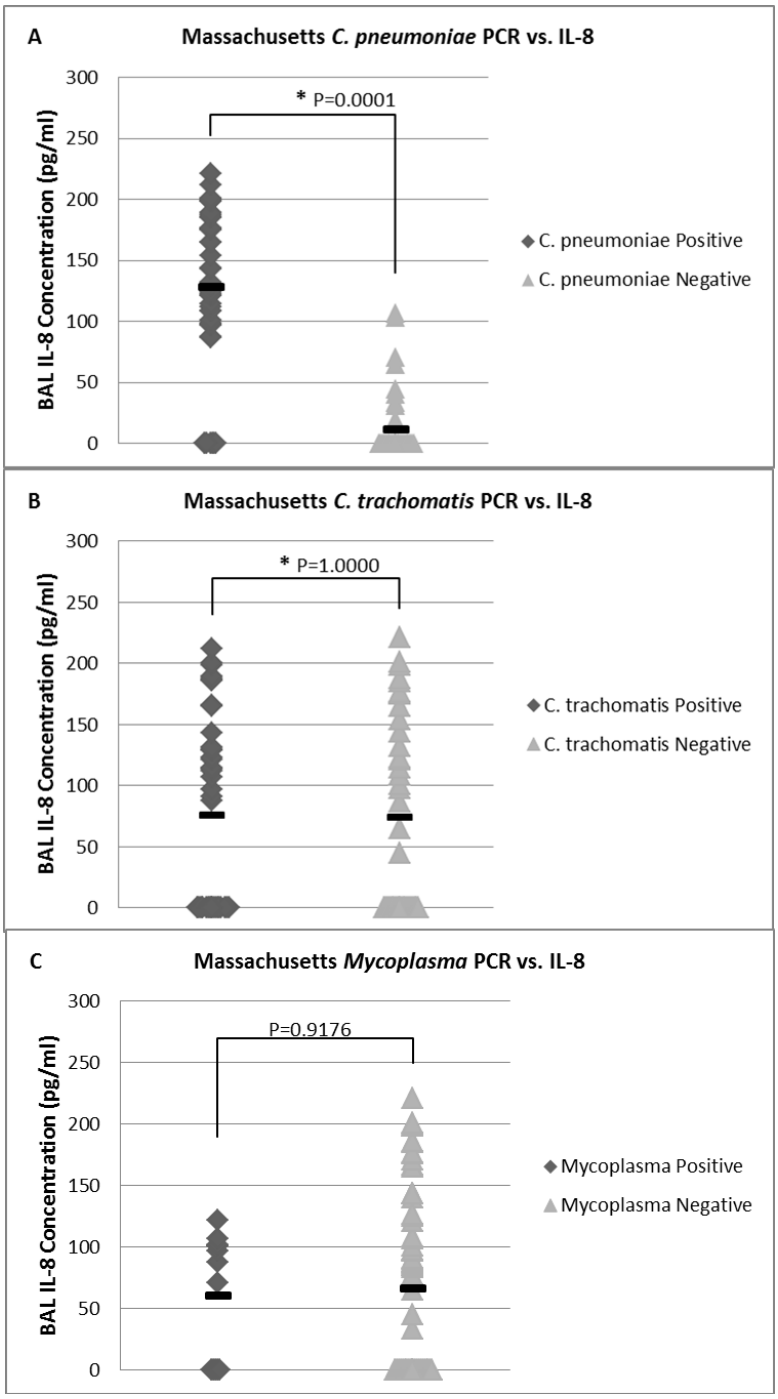

Figure 4: Elevated IL-8 in the BAL of *C. pneumoniae* positive Massachusetts Patients

There was an average of 10.75 fold higher IL-8 concentration in the BAL of *C. pneumonia* positive patients compared to *C. pneumoniae*-negative ones (129.0 ± 65 pg/ml versus 11.8 ± 28 pg/ml respectively, p = 0.0001; *graph A*). When we assessed the IL-8 concentrations for samples positive for *C. trachomatis* and M*ycoplasma* DNA there was no significant difference (77.5± 74 pg/ml vs. 75.9 ± 78 pg/ml, and 60.7 ± 51 pg/ml vs. 66.9 ± 68 pg/ml; *graph 2B and 2C*, respectively).

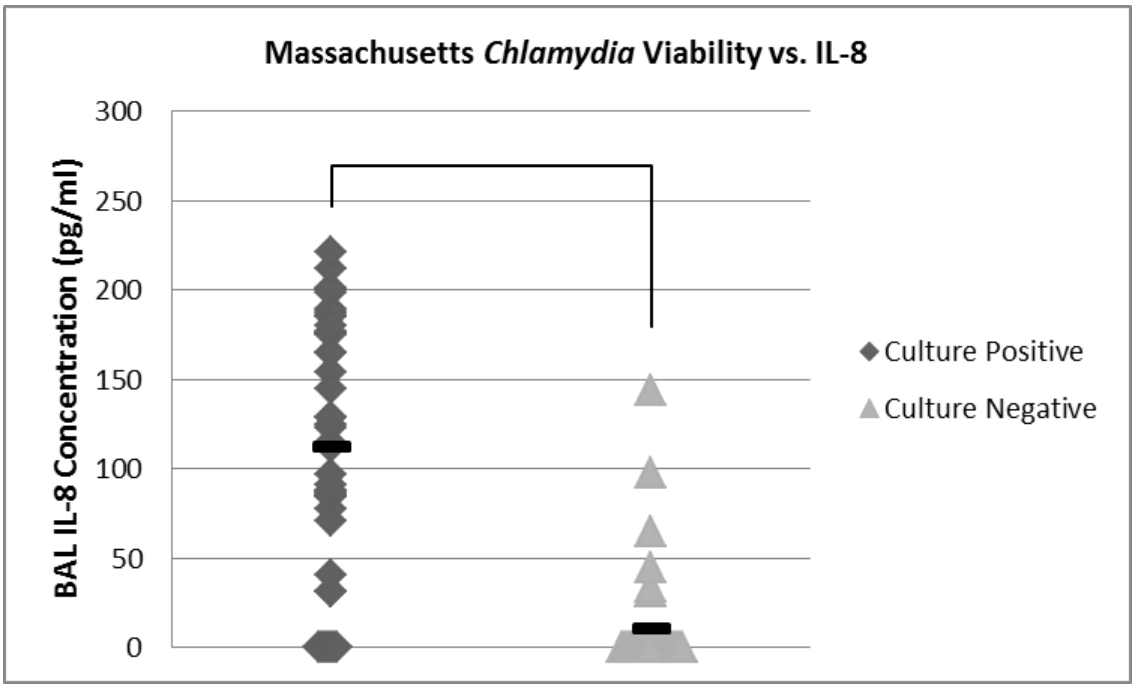

Figure 5: Elevated IL-8 is strongly associated with cultivable *Chlamydia*

Forty five of 51(88.2%) IL-8 positive samples were *Chlamydia* culture-positive confirming that there was possibly an active infection ongoing at the time of sample collection. The culture positive group had average IL-8 levels of 113.5 ± 73 pg/ml vs. 11.5 ± 30 pg/ml for culture negative samples (p=0.0001, Fisher exact T-test).

Bronchitis/asthma patient sub-cohort described above (86.1 ± 74 vs. 42.9 ± 50, p = 0.0001; Figure 6). When *C. pneumoniae*-positive asthmatic patients were compared to bacterial bronchitis/asthma patients who were also positive for *C. pneumoniae*, we found a 2.87-fold in IL-8 levels for the asthmatic patients (129.3 ± 62 vs. 45.0 ± 56, Figure 6) this difference was also statistically significant (p= 0.0001).

BAL Neutrophilia Predict *C. pneumoniae* Infection

Because we found an association between *C. pneumoniae* status and IL-8 levels in both patient populations, and since the molecular and modified culture techniques utilized in our study may not be readily available in the clinical setting, we then sought to determine whether BAL neutrophilia could predict the presence of *C. pneumoniae*. Because of the limited number of samples collected from Bronx patients, we were unable to draw any reliable conclusions in this patient population. Bronchial neutrophilia (>10% of neutrophils) in the Massachusetts patient cohort, however was significantly associated with the presence of *C. pneumoniae* DNA (Fisher's exact test**,** two-tailed P–value = 0.0001). The overall sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of >10% BAL neutrophils/ml to predict *C. pneumoniae* infection were 93%, 76%, 80% and 92%, respectively (Table 7). Neither *C. trachomatis* nor *Mycoplasma* DNA was a strong predictor of airway neutrophilia with sensitivity of 55% and 53%; specificity 38% and 40%; PPV 46% and 14%; NPV 45% and 82% respectively.

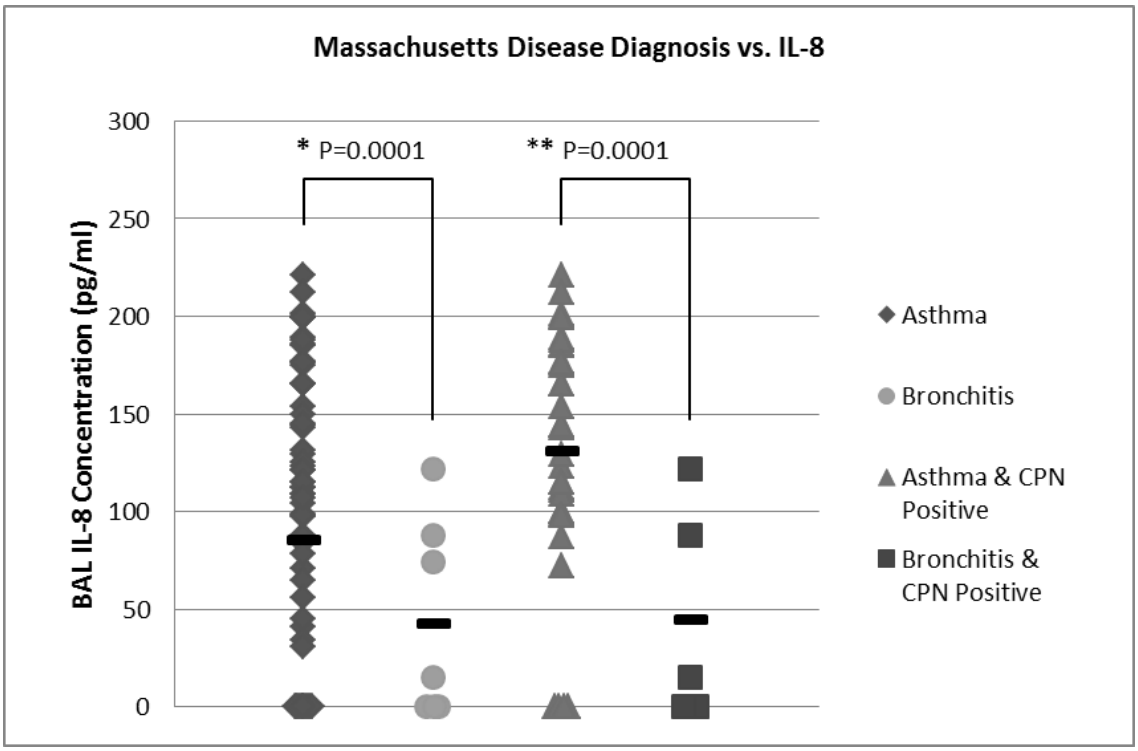

Figure 6: Asthmatic patients with *C. pneumoniae* have higher IL-8 levels than bacterial bronchitis patients

Seven patients tested were diagnosed with bacterial bronchitis. The averageIL-8 level in our asthma patients was 73.7 pg/ml while the average level in the bronchitis patients was 27.1 pg/ml (P=0.0108). The data also shows that while not statistically significant, asthma patients who were *C. pneumoniae* positive had a 3.6 fold higher IL-8 level compared to *C. pneumoniae* positive bronchitis patients.

	Cp Positive	CP Negative	Subtotals
Elevated Neutrophils	69 (30.6%)	10 (11.9%)	49
Non-Neutrophilic	18 (8%)	32 (38.1%)	35
Subtotals	42	42	

Table 7: Elevated neutrophil counts predict C. pneumoniae infection.

The presence of the C. pneumoniae DNA by PCR was assessed to determine its association with elevated BAL neutrophil counts. **C. pneumoniae* (Cp) positivity was statistically significant (Fisher's exact test**,** P= 0.0001) in patients with elevated neutrophil counts (>10%) compared to neutrophil negative patients (<10%).

Discussion

Patients with poorly-controlled asthma have persistent symptoms despite treatment with inhaled corticosteroids and other adjunct therapies including LABA and LTRA (20, 21). While the majority of patients with asthma demonstrate enhanced allergic inflammatory responses, some patients with poorly-controlled disease exhibit a non-allergic, TH1-dominant inflammatory phenotype that may be less responsive to traditional anti-inflammatory therapy (3-5). Currently, the mechanisms underlying different inflammatory phenotypes in asthma are unknown. Similarly, the clinical relevance and potential implications of a non-allergic inflammatory phenotype in asthma are not clear. In the present study, we utilized a convenience sample of patients with poorly-controlled asthma who underwent flexible bronchoscopy and found that BAL fluid from patients that were DNA positive for *C. pneumoniae* demonstrated elevated IL-8 levels compared to patients who were negative for *C. pneumoniae*. This finding was even more significant for patients with culture-positive BAL (Massachusetts cohort). Importantly, none of these patients was suspected of having *C. pneumoniae* in their lower respiratory secretions by clinical measures (i.e. active pneumonia). Because this pattern was seen in 2 distinct patient populations but did not hold true for *C. trachomatis* or *Mycoplasma*, it seems less likely that this association simply reflects a generalized response to airway colonization with atypical bacteria, but rather a response to *C. pneumoniae*. As such, it seems plausible that *C. pneumoniae* may represent a sub-phenotype of poorly-controlled asthma that is associated with a non-allergic (i.e. IL-8-associated) inflammatory response. We also demonstrated that airway neutrophilia may be an indicator for *C. pneumoniae* infection, particularly when conventional cultures fail to identify other bacterial organisms in the lower airway. Because IL-8 is a known neutrophil chemotactic factor, and since *C. pneumoniae* is known to induce IL-8 (22, 23), the association with neutrophilia may not seem overly surprising. However, because the molecular and modified culture techniques utilized to identify *C. pneumoniae* in our investigation may not be readily available in the clinical setting,

BAL fluid cell counts may become a useful screening tool during evaluation of poorly-controlled asthma.

There are important limitations to our investigation that should be noted. Because we obtained samples from a convenient population of poorly-controlled asthmatics who underwent flexible bronchoscopy, our results may not be applicable to the majority of patients whose disease is easily controlled with intermittent use of beta agonists or low dose inhaled corticosteroids. Indeed, regular utilization of an invasive procedure (bronchoscopy) seems unwarranted for such patients. Nevertheless, our findings may be important for the minority of patients whose symptoms remain poorly-controlled despite standard treatment. Current recommendations for such patients focus on escalation of existing therapy and identification of co-morbid conditions including gastroesophageal reflux, allergic disorders, sinusitis or obstructive sleep apnea (21). *C. pneumoniae* may represent an additional co-morbid condition that is under diagnosed, and importantly, one for which additional adjunct therapies could potentially be designed. An additional limitation to our study is that we were unable to analyze true control samples (for example, children with no respiratory disease who are undergoing elective surgery), which has been a common limitation in similar pediatric BAL studies (24-26). Nevertheless, an important underlying objective of our study was to characterize a potential sub-phenotype of poorly-controlled asthma, namely that associated with *C. pneumoniae*. As such, our control group consists of patients with poorly-controlled asthma who do not demonstrate evidence of *C. pneumoniae* in the lower airway. Although we acknowledge that analysis of true controls could enhance our findings, our results demonstrating different inflammatory phenotypes between *C. pneumoniae*-positive and negative patients should not be overlooked.

Our findings may have future implications in the diagnosis and treatment of children with poorly-controlled asthma. Because macrolide therapy is widely employed in the clinical setting for community acquired pneumonia and other respiratory illnesses including sinusitis, and since prolonged courses of macrolide antibiotics are increasingly utilized in chronic inflammatory

diseases such as cystic fibrosis, its efficacy and safety profiles are well documented(27, 28). From the current investigation, it is unclear whether patients with poorly-controlled asthma who are also harboring *C. pneumoniae* would benefit from the addition of macrolide antibiotics. However, a previous investigation in adults utilized clarithromycin as an adjunct treatment for refractory asthma, documenting a reduction of IL-8 levels during treatment as well as clinical benefits that were most dramatic for patients with neutrophil-predominant asthma (as opposed to allergic/eosinophilic disease) (29). In this study, the mechanism of action was presumed to involve the anti-inflammatory properties of macrolides. However, given our current work, there is a possibility that some of these patients, particularly those with neutrophil-predominant inflammation, were unknowingly infected with *C. pneumoniae.* A similar large-scale investigation in pediatric patients demonstrated no benefit of azithromycin for children with moderate-to-severe persistent asthma (30). Unlike the previous study in adults, however, these investigators did not perform analysis based on inflammatory phenotype, and only upper airway secretions were tested for the presence of *C. pneumoniae*. It has been documented that *C. pneumoniae* preferentially colonizes the lower airways (31, 32). Lastly, it should be noted that *C. pneumoniae* has a propensity to cause chronic or latent infections that may not be adequately treated with standard courses of therapy used for community-acquired pneumonia or bacterial sinusitis (33, 34). In such cases, prolonged courses of therapy may be required.

Based on our current work and the previous work of others, we propose additional investigations focusing on the evaluation and management of children with poorly-controlled asthma which include analysis of inflammatory phenotype and, based on the observed phenotype, further analysis for the presence of *C. pneumoniae* in the lower respiratory tract.

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CHAPTER 3

THE PREVALENCE AND IDENTITY OF *CHLAMYDIA*-SPECIFIC IgE IN CHILDREN WITH ASTHMA AND OTHER CHRONIC RESPIRATORY SYMPTOMS

Abstract

Background: Recent studies have confirmed the presence of viable *Chlamydia* in the bronchoalveolar lavage (BAL) fluid of pediatric patients with airway hyperresponsiveness. While specific IgG and IgM responses to *C. pneumoniae* are well described, the response and potential contribution of Ag-specific IgE are not known. The current study sought to determine if infection with *Chlamydia* triggers the production of pathogen-specific IgE in children with chronic respiratory diseases which might contribute to inflammation and pathology.

Methods: We obtained BAL fluid and serum from pediatric respiratory disease patients who were generally unresponsive to corticosteroid treatment as well as sera from age-matched control patients who saw their doctor for wellness checkups. *Chlamydia*-specific IgE was isolated from BAL and serum samples and their specificity determined by Western blot techniques. The presence of *Chlamydia* was confirmed by species-specific PCR and BAL culture assays.

Results: Chlamydial DNA was detected in the BAL fluid of 134/197 (68%) patients. Total IgE increased with age until 15 years old and then decreased. *Chlamydia*-specific IgE was detected in the serum and/or BAL of 107/197 (54%) patients suffering from chronic respiratory disease, but in none of the 35 healthy control sera ($p<0.0001$). Of the 74 BAL culture-positive patients, 68 (91.9%, $p=0.0001$) tested positive for *Chlamydia*-specific IgE. Asthmatic patients had significantly higher IgE levels compared to non-asthmatics ($p=0.0001$). Patients who were positive for *Chlamydia* DNA or culture had significantly higher levels of serum IgE compared to negative patients ($p=0.0071$ and $p=0.0001$ respectively). Only 6 chlamydial antigens induced *Chlamydia*-specific IgE and patients with *C. pneumoniae*-specific IgE had significantly greater levels of total IgE compared to *C. pneumoniae*-specific IgE negative ones ($p=0.0001$).

Conclusions: IgE antibodies play a central role in allergic inflammation; therefore production of

Chlamydia-specific IgE may prove significant in the exacerbation of chronic, allergic airway diseases, thus highlighting a direct role for *Chlamydia* in asthma pathogenesis.

Introduction

Inflammation of the airways is the most common finding in all asthma patients and today, most asthma experts consider airway inflammation a central feature of asthma pathogenesis (1, 2). The genetic predisposition to asthma development has been well recognized and the IgE-mediated response to common aero-allergens represents the most common form of the disease in childhood and early adulthood (3). Published evidence strongly suggests a relationship between microbes and asthma (4). It has long been known that the most potent triggers of wheezing are mediated by viruses, however, we do not understand the mechanisms involved in how they contribute to disease onset and progression. Even less is known about the relationship between asthma and bacteria. Recent studies confirm that bacterial respiratory infections are frequently associated with increased airway obstruction in patients with bronchial asthma (3). While the hygiene hypothesis predicts that infections in early life by non-pathogenic microbes should protect against asthma and atopy (5), there is increasing evidence that certain chronic pathogenic infections might also promote airway hyperresponsiveness and asthma development or exacerbation (6-8).

Approximately 20% of wheezing children have serological evidence of an immune response to *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarralis* (9, 10). More recently, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* have been identified in 5–25% of children with asthma exacerbations (11-13). Hahn et al also reported a significant improvement in overall asthma symptoms at treatment completion which persisted for 3 months despite withdrawal of azithromycin in an adult population (14, 15).

Earlier studies by Welliver *et al.* (16) reported IgE responses to RSV in children with bronchiolitis and showed that this IgE response was related to recurrent wheeze, but not to lung

function and allergic sensitization at an early age. This suggests that IgE antibodies to RSV occur independently of atopy and may be indicative of an ongoing asthmatic process. Specific IgE to bacterial colonizers has also been reported. *H. influenzae* and *S. pneumoniae* specific IgE antibodies have been found in the serum of approximately a third of atopic children and asthmatic adults, however, they were all related to a subject's atopic status (17). The implication here is that IgE has a complex relationship with asthma that might not be dependent on the specific allergens that are routinely assayed for (18).

In the current study we examined the BAL fluid and serum of a large cohort of children with chronic respiratory disease for the presence of *Chlamydia*-specific IgE antibodies. We hypothesize here that the presence of *Chlamydia*-specific IgE antibodies explains, at least in part, the mechanism of chlamydial involvement in either initiating or exacerbating chronic allergic airway disease.

Materials & Methods

Patients and Samples

Serum and BAL samples were analyzed from 197 patients between the ages of 0-20 who were patients at Baystate Medical Center. BAL samples were originally obtained from patients for diagnostic or treatment purposes. Children were considered for bronchoscopy only after a thorough, non-invasive evaluation did not yield a definitive diagnosis; symptoms were not improving with time; aggressive medical management had not been successful and the child was truly compromised i.e., was missing school, could not participate in physical activity and had frequent symptoms. Airway reactivity was monitored by spirometry in children capable of performing the test as well as clinically, by frequency and severity of symptoms and response to therapy. In children not capable of performing spirometry, clinical characteristics were assessed.

We also obtained 35 age and gender-matched control patient sera as residual samples from patients undergoing wellness check for school records or general checkup at the University

of Massachusetts Health Services complex over a similar period of time as the patient samples. We obtained IRB approval and patient consent to collect and use these residual samples for research from the Baystate Medical Center and University of Massachusetts Amherst IRB. In addition, a subset of 20 (of the 197) respiratory disease patients presented with physical airway anomalies including most commonly, tracheal bronchus, obstruction by a foreign object, laryngomalacia, tracheomalacia, and bronchomalacia with abnormal cough. These were considered non-inflammatory controls when compared with asthmatics and patients with pneumonia and bronchitis.

Chlamydia detection in patient samples

Genomic DNA was isolated from BAL samples and PCR detection of chlamydial DNA was performed in the same manner as previously described for all samples in this cohort (19). All BAL samples were also analyzed by tissue culture techniques to determine *Chlamydia* viability as previously reported (12, 19). DNA was also isolated from control serum samples in a similar manner as the BAL and evaluated using the same primers.

Total IgE Evaluation

Total IgE was evaluated using the Elecsys IgE kit (Roche Diagnostics, Indianapolis, IN), with the electrochemiluminescence immunoassay according to the manufacturer's instructions. Plates were read on the Roche Elecsys 1020 analyzer which automatically calculated the IgE concentration of each sample based on a standard curve. Elevated IgE levels were determined based on the manufacturer's recommended threshold by age range.

Isolating Serum and BAL IgE Antibodies

Because the normal concentration for IgE in serum is approximately 0.0005 mg/ml, and is even less in BAL fluid, we utilized affinity beads in a similar manner as Kadooka et al (20) to isolate

the IgE antibodies in order to ensure effective reaction of these antibodies with chlamydial antigens on our blots. We used recombinant Protein G sepharose gel (Sigma-Aldrich, St. Louis, MO) to adsorb IgG antibodies from the serum samples. Since recombinant protein G does not bind IgE antibodies (21, 22), individual patient serum samples were added to the beads and allowed to bind with slow stirring overnight. The supernatant that was now 'enriched' for IgE antibodies was then removed and analyzed for the presence of *Chlamydia*-specific IgE antibodies.

To test for IgE in BAL samples, recombinant protein A beads (Sigma-Aldrich, St. Louis, MO) were utilized in the same manner as the protein G beads above. In this case the protein A beads were used to adsorb any antibodies detectable in the BAL fluid. IgE antibodies present in the BAL fluid were bound by the protein A beads and the bound antibodies were eluted using a pH 2.5 glycine solution followed by centrifugation to obtain the supernatant containing IgE and other antibodies if present. The eluted antibodies were pH balanced using Tris-HCL and then used as the primary antibody in the Western blot procedure. Because of the limited volume of some BAL fluid samples available and the low concentration of IgE in each sample, BAL samples were initially assessed in batches of 4 to 7 samples each.

Western Blot Procedure

As previously reported, Western blot is a highly sensitive and efficient technique in detecting specific IgE (23-25). *C. pneumoniae* (TW183) and *C. trachomatis* (serovar E) elementary bodies were purified by 20%-50% (vol/vol) Renografin gradient centrifugation as previously described (26) and normalized for protein content using the Bradford protein assay. Proteins were separated by electrophoresis on NuPage 4-12% Bis Tris gels (Invitrogen, Carlsbad, CA). Following electrophoresis the separated proteins were transferred to PVDF membranes, blocked and each well was cut into individual strips. Each strip was incubated with patient sample that had been processed with protein A or G beads overnight. After incubation, the strips were washed and a 1:500 dilution of AP-conjugated anti-human IgE, epsilon chain specific antibody (KPL,

Gaithersburg, Maryland) was added to each strip for 2 hours. Strips were again washed and developed with a BCIP/NBT alkaline phosphatase substrate and reactions were stopped after several washes with ultrapure distilled water. Blot strips were analyzed for the presence and identity of *Chlamydia*-specific IgE bands. All samples were assayed for the presence of specific IgE using identical conditions including sample dilution.

Cell Counts

BAL cell counts and differentials were performed according to standard techniques in the hospital's Hematology Clinical Laboratory at Baystate Medical Center (Springfield, MA) as previously reported (19, 27). Briefly, cell enumeration was performed manually using a cell counting chamber under phase microscopy with results expressed as "number of cell/mm^3. BAL differential counts were performed using Wright stained cytospin preparations of BAL and examination was performed under oil-immersion microscopy (50X or 100X). Results were expressed on the basis of a 100 cell count survey. Inflammatory asthma sub-phenotypes were determined based on BAL neutrophil and eosinophil cells counts, as previously reported (28)

Statistical Analysis

All statistical analyses among the different groups of the patient population were performed using Microsoft Excel®. Statistical correlations were determined with Fisher's exact test and Chi-square with Yates correction (SPSS 15.0 software). A p-value of 0.05 was considered significant and all tests were two-tailed.

Results

Characteristics of Study Patients

We obtained both BAL fluid and serum from 197 pediatric respiratory disease patients, average age 7.9 years old, who presented to a pediatric pulmonary practice in Springfield, MA with chronic respiratory disease and were generally unresponsive to corticosteroid treatment. The patient demographic (Table 8), shows that 143/197 (73%) were diagnosed with asthma and that these asthmatics were predominantly between the ages of 2 to 10 years old. We utilized a healthy control group of 35 patients, average age 5.8 years, 21 males/14 females who saw their doctors for wellness checks. Total serum IgE generally increased with age up to 15.0 years old (40 IU/ml to 250 IU/ml; see Figure 7). The levels decreased significantly in the 15.1 to 20.0 year old age group.

Prevalence of *Chlamydia* in patient cohort

Polymerase chain reaction (PCR) was utilized to determine if *C. trachomatis* and/or *C. pneumoniae* organisms were present in patient BAL samples. A total of 134/197 (68%) patient samples were positive for chlamydial DNA. Species-specific PCR revealed that 65 samples were positive for *C. pneumoniae* DNA only, 34 were positive for *C. trachomatis* DNA only and 35 patients harbored both *C. trachomatis* and *C. pneumoniae* DNA. In order to determine if the organisms detected by PCR were viable, all BAL samples were subjected to a modified tissue

Description	Asthma (*percent within asthma)	Non-asthma (*percent within non-asthma)	Total
Diagnosis	143 (72.6%)	54 (27.4%)	**197**
Average age	8.3	7.1	**7.9**
Age range			
0-2.0	14 (9.8%)	16 (29.6%)	**30**
2.1-5.0	38 (26.6%)	13 (24.1%)	**51**
5.1-10.0	41 (28.7%)	8 (14.8%)	**49**
10.1-15.0	29 (20.3%)	9 (16.7%)	**38**
15.1-20.0	21 (14.6%)	8 (14.8%)	**29**
Gender			
Female	65 (45.5%)	25 (46.3%)	**90**
Male	78 (54.5%)	29 (53.7%)	**107**
Ethnicity			
White	107 (74.8%)	43 (79.6%)	**150**
Black	7 (4.9%)	4 (7.4%)	**11**
Hispanic	29 (20.3%)	6 (11.1%)	**35**
Asian	0 (0.0%)	1 (1.9%)	**1**
BMTH			
Yes	118 (82.5%)	17 (31.5%)	**135**
No	10 (7.0%)	18 (33.3%)	**28**
Not Done	15 (10.5%)	19 (35.2%)	**34**

Table 8: Patient demographics of the pediatric patient cohort

Most of the patients in this cohort were asthmatics (72.6%) with an average age of 8.3 years old (7.9 years old for entire cohort). The majority of asthma patients were between ages 2 to 10 years old and basement membrane thickening (BMTH) was significantly correlated with asthma diagnosis.

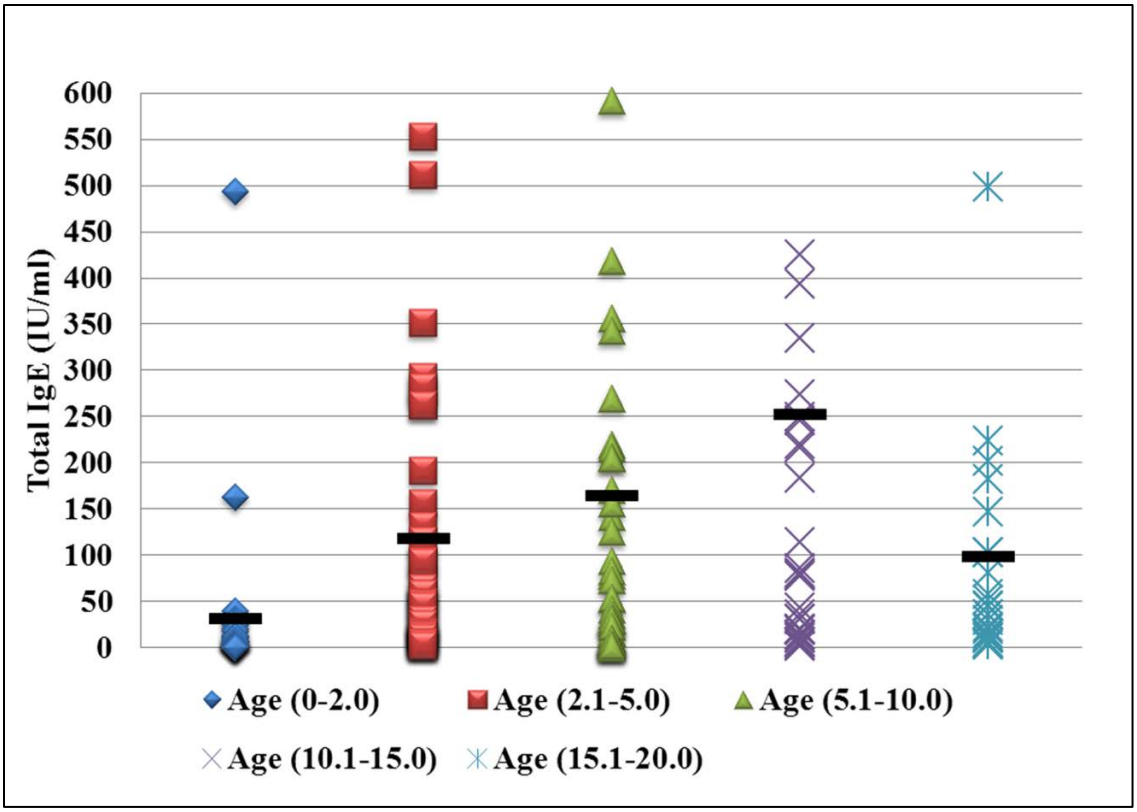

Figure 7: Total Serum IgE levels generally increased with age

Assessment of total serum IgE levels showed a general increase with age up to age 15.0 years old (40 IU/ml to 250 IU/ml). The levels then decreased to 100IU/ml. Dark lines represent the average IgE level for each age range

culture technique as previously described. The data showed that 74/197 (38%) patients assayed were culture positive for *Chlamydia*. Importantly, 62/74 (84%, (Fisher's exact test $p<0.0001$) culture positive patients were diagnosed with chronic, severe asthma according to GINA guidelines (29). Analyses of the control patient sera revealed that 7/35 (20%) were positive for the presence of chlamydial DNA.

Total IgE antibody serum levels

Data analysis revealed that 65/197 (33%) patients had elevated serum IgE levels, while 132/197 (67%) had normal IgE levels (Fisher's exact T-test $p < 0.0001$). Patients who were positive for *Chlamydia* by DNA or culture had significantly higher levels of total serum IgE compared to IgE negative patients ($p=0.0071$ and $p= 0.0001$ respectively; Figure 7). Patients who tested positive for *C. pneumoniae* specific IgE antibodies also had an average IgE level that was higher than the specific IgE negative cohort. Total serum IgE generally increased with age up to 15.0 years old (40 IU/ml to 250 IU/ml; see Figure 8). The levels decreased significantly in the 15.1 to 20.0 year old age group.

***Chlamydia*-specific IgE Antibody is Present in the BAL and Serum of Infected Patients**

In light of the increased prevalence of chlamydial organisms in the BAL fluid of patients in this cohort, patient serum and BAL samples were analyzed to determine the response of the host to the organism in each case and to determine if there is pathology associated with its presence. We therefore developed a Western blot assay to determine the presence, prevalence and identity of *Chlamydia*-specific IgE in both the serum and BAL fluid from each patient. Since BAL samples were assessed in batches of 4 to 7 samples each (because of limited sample volume), we cannot ascertain the exact percentage of patients with *Chlamydia*-specific IgE in

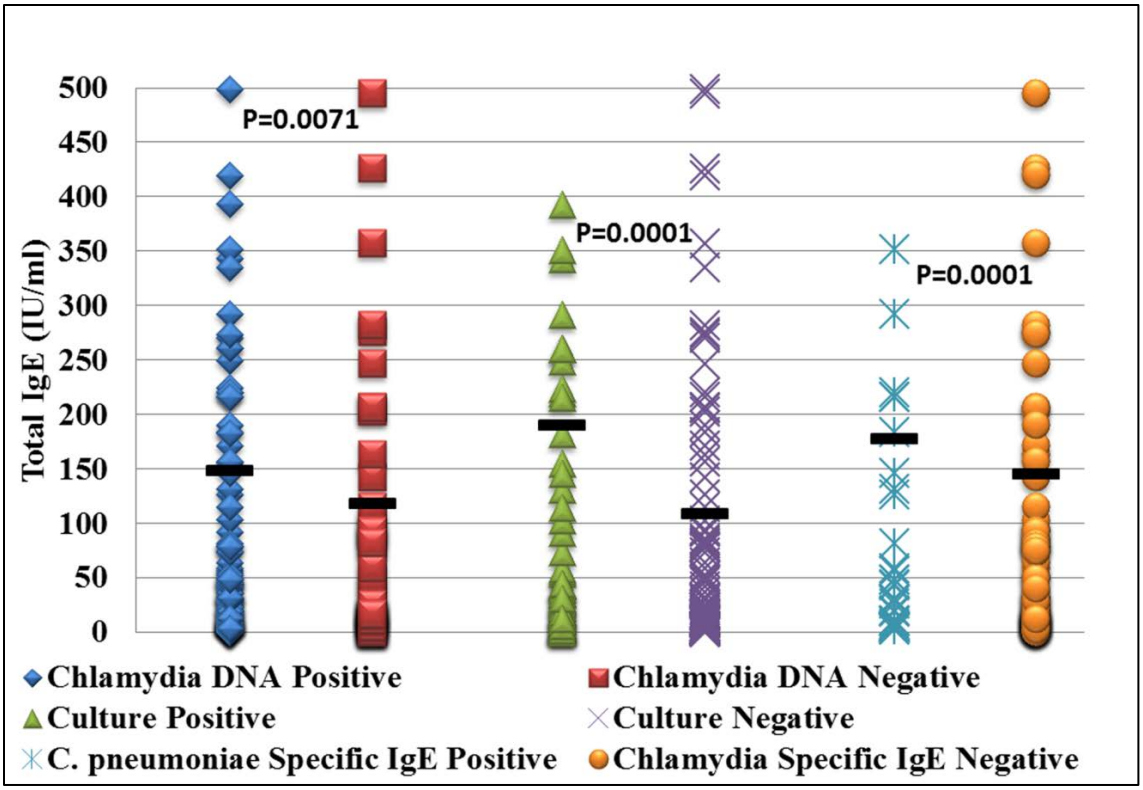

Figure 8: Presence of Chlamydia organisms is associated with higher levels of serum IgE

While the average total serum IgE levels in this patient cohort was not significantly elevated (138 IU/ml), IgE levels were significantly more elevated in patients who were either Chlamydia DNA positive or who had viable organisms as determined by culture. Patients with C. pneumoniae-specific IgE also had an average total IgE level that was higher than their C. pneumoniae-negative counterparts. Horizontal dark bars represent the average IgE level and each shape in the respective columns represents a single patient.

their BAL fluid. However, qualitatively, BAL fluid samples from this patient cohort contained *Chlamydia*-specific IgE antibodies (Figure 9). To our knowledge, this is the first report of pathogen-specific IgE in human BAL fluid.

Chlamydia-specific IgE was also detected in the sera of 107/197 (54%) patients suffering from chronic respiratory disease but none of the healthy control patient sera (0/35, $p < 0.0001$). In addition, a subset of 20 patient samples from our respiratory disease cohort who were diagnosed with structural anomalies rather than inflammatory airway disease, were also assessed for the prevalence of *Chlamydia*-specific IgE. The data shows that 1/20 (5%) had *Chlamydia*-specific IgE in their sera compared to the inflammatory patient cohort (Table 9). Assessment to determine the prevalence of *Chlamydia*-specific IgE antibodies in the BAL culture-positive patient cohort showed that 68/74 (91.9%, P=0.0001) tested positive for *Chlamydia*-specific IgE.

Both *C. trachomatis* and *C. pneumoniae*-specific IgE antibodies were found in the BAL fluid (Figure 10). Importantly, although *Chlamydia* has 800 – 1000 coding genes, only 6 chlamydial antigens appear to induce pathogen-specific IgE responses in this patient cohort and the frequency of these bands differ significantly between *C. trachomatis* and *C. pneumoniae* (Figure 10b and 10c). In fact, most of the patient samples contained IgE antibodies to a single protein band on the blots (see Figure 9).

Overall, 61% of the *Chlamydia*-specific IgE antibodies in BAL fluid recognized the chlamydial major outer membrane protein (MOMP). This was generally true however, for *C. pneumoniae* IgE antibodies, where 81% of the positive patients had IgE that recognized MOMP, compared to only 17% for *C. trachomatis* MOMP. Thirty three percent of BAL fluid positive patient samples displayed IgE antibodies against an un-annotated 250kDa *C. trachomatis* protein.

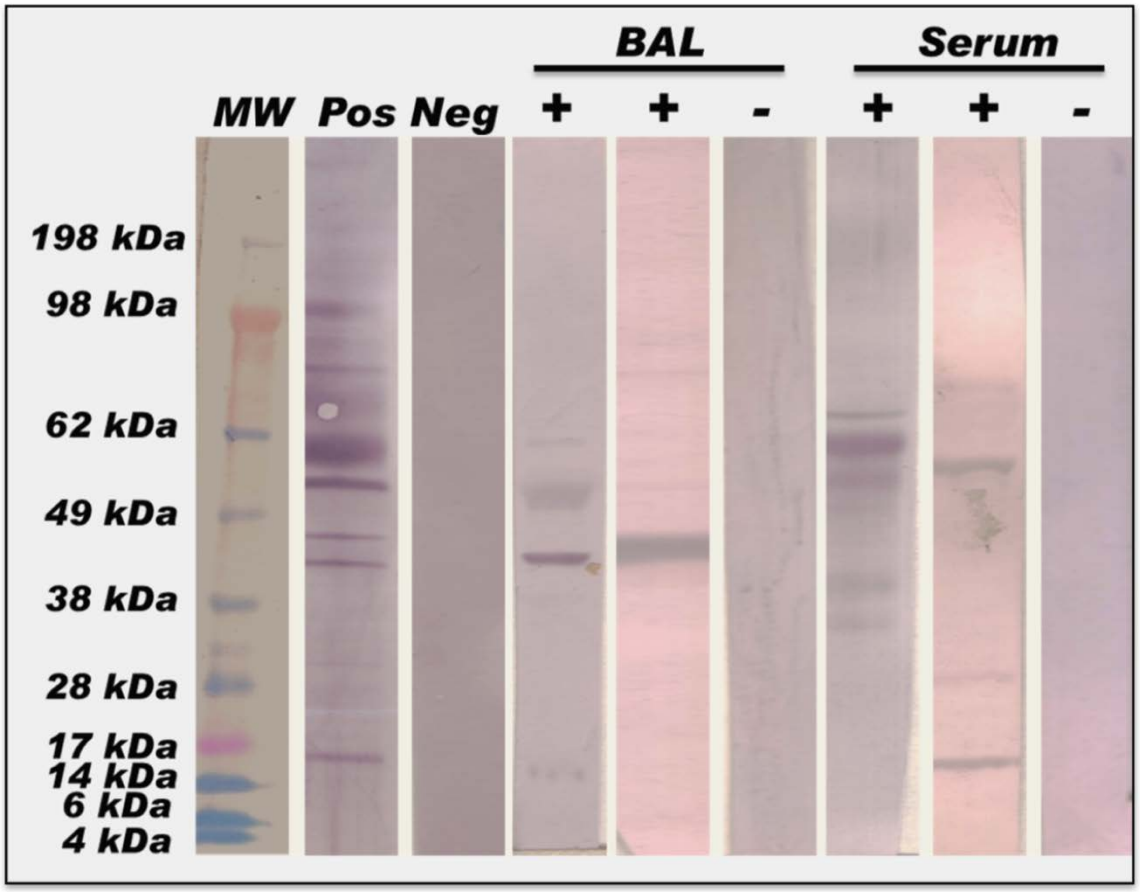

Figure 9: Western blot of *Chlamydia*-specific IgE

The blots depict representative patient BAL and serum samples that were positive for the presence of *Chlamydia*-specific IgE. Band specificities were determined by a *Chlamydia*-positive control and the *Chlamydia* protein data-base was used to confirm the corresponding protein. Note that of all the chlamydial proteins seen on the control strip stained with a polyclonal antibody solution, only a finite number are recognized by specific IgE antibodies.

	Respiratory Disease	Non-Inflammatory Disease	P-values	Healthy Controls	P-values
Number of Patients	177	20		35	
Serum IgE, No. (%)					
Positive	97 (55%)	1 (5%)	0.0030*	0 (0%)	0.0001*
Negative	80 (45%)	19 (95%)		35 (100%)	
Chlamydial DNA, No. (%)					
Positive	124 (70%)	10 (50%)	0.4417	7 (20%)	0.0208*
Negative	53 (30%)	10 (50%)		28 (80%)	

Table 9: The Prevalence of *Chlamydia*-specific IgE in Patient Sera

Fifty five percent of patients with inflammatory respiratory disease had *Chlamydia*-specific IgE antibodies in their sera compared to 5% of the 20 patients with structural anomalies and 0% of healthy controls ($p = 0.0030$ and 0.0001 respectively. 70% of the patients diagnosed with respiratory disease also tested positive for chlamydial DNA, whereas only 20% of healthy controls tested positive for chlamydial DNA ($p=0.0208$).

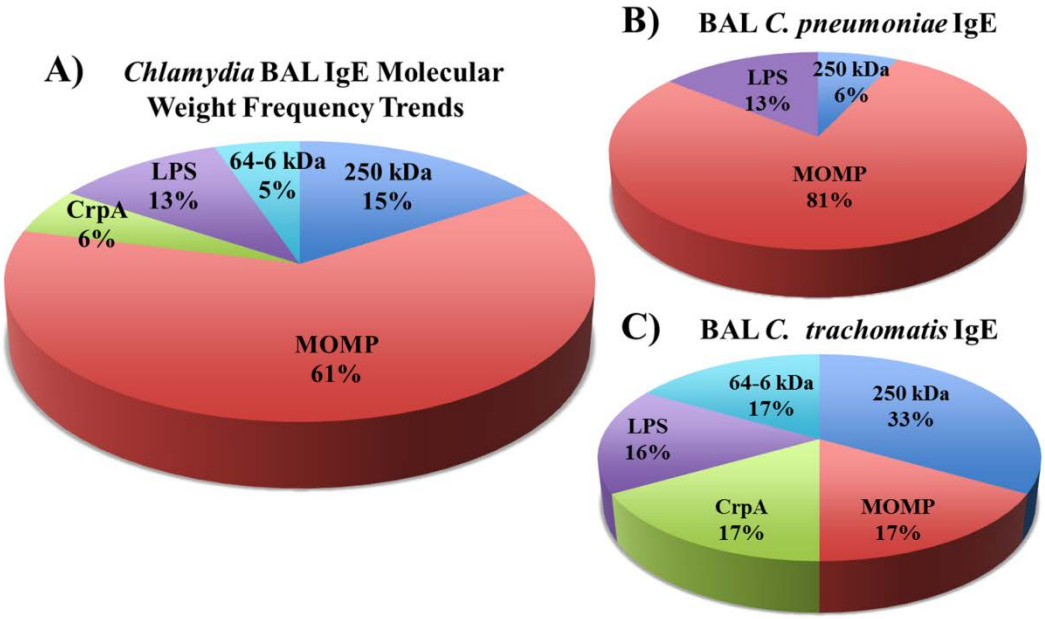

Figure 10: *Chlamydia* protein molecular weight frequency trends in BAL fluid

Chart A shows the overall molecular weight of proteins recognized by *Chlamydia*-specific IgE antibodies from patient BAL samples. These are for *C. trachomatis* and *C. pneumoniae* specific antibodies combined. The major outer membrane protein (MOMP) is recognized 61% of the time by these positive patient sera. However, chart B clearly shows that the MOMP from *C. pneumoniae* organisms were more likely to induce IgE antibodies than *C. trachomatis* MOMP (Chart C). The hypothetical 250KDa protein was the most frequently recognized *C. trachomatis* protein in BAL.

Assessment of individual serum samples for the presence of *Chlamydia*-specific IgE antibodies revealed bands on Western blot ranging in molecular weight from 6kDa to 250kDa (Figure 11A). As was true for the IgE antibodies in the BAL fluid, the identity of serum IgE antibodies produced in response to *C. trachomatis* were significantly different from *C. pneumoniae* – specific antibodies (Figure 11B and C). Antigen frequency analysis revealed that 42% of IgE positive serum samples contained IgE antibodies that recognized *C. pneumoniae* chlamydial lipopolysaccharide (cLPS). *C. trachomatis* - induced cLPS IgE antibodies were not detected in any serum sample. *C. pneumoniae* did not induce MOMP-specific IgE in serum, while 8% of IgE positive-patients had antibodies that recognized *C. trachomatis* MOMP (Figure 11C). There were also significant differences in the production of *Chlamydia*-specific IgE in the serum versus BAL fluid. Chlamydial heat shock protein 60 (Hsp 60 or GroES) and the polymorphic outer membrane protein (POMP) did not induce IgE antibodies in the BAL fluid, however, they each accounted for the *Chlamydia*-specific IgE found in 20% of positive patient sera assayed. The frequency of MOMP-induced IgE in the BAL fluid was 61%, while the frequency in serum was only 5%, demonstrating possible site-specific differential cytokine and lymphocyte responses or antibody release (see figures 10A and 10A).

Inflammatory asthma phenotype and serum IgE concentrations

Eosinophilic inflammation, characterized by high levels of eosinophils, atopy and elevated serum IgE concentration, has long been considered one of the most distinctive pathological hallmarks of asthma (30). However, it has recently been recognized that airway eosinophilia is not a universal finding and a significant subset of patients with refractory asthma display a non-eosinophilic phenotype. BAL cell counts revealed that 115/143 (80%) asthma patients in this cohort presented with a non-eosinophilic inflammatory asthma phenotype. Overall, the majority of patients in this cohort 166/197 (84%) had normal BAL eosinophil counts. Asthmatic patients had a significantly higher total IgE level compared to non-asthmatic patients

and patients with an eosinophilic asthma phenotype had significantly higher total serum IgE levels than other inflammatory asthma groups ($p = 0.0001$; see Figure 12).

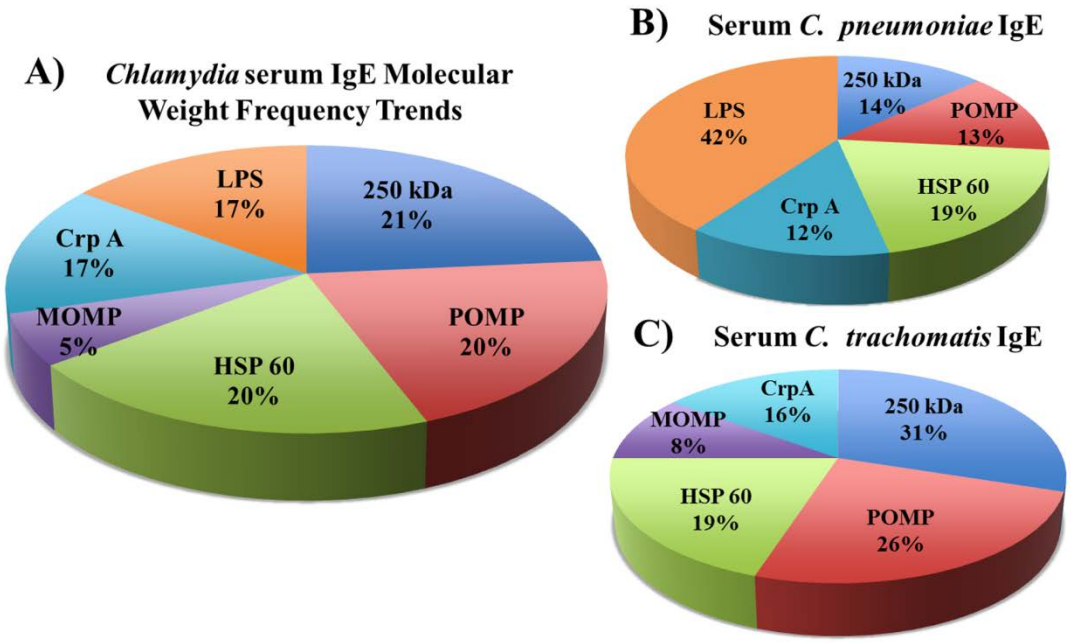

Figure 11: *Chlamydia* protein molecular weight frequency trends in serum

Unlike the BAL fluid proteins in figure 2, serum IgE antibodies against MOMP only appeared in 5% of the patients tested, while the other 5 antigens appeared at similar frequency to that of the BAL (Chart A). Closer analysis shows that while serum IgE antibodies recognized *C. pneumoniae* LPS in 42% of positive samples (Chart B), no *C. trachomatis* LPS was recognized by these samples (Chart C). Although MOMP is the major antigen on the chlamydial EB surface, *C. pneumoniae* MOMP did not induce serum IgE production in this study.

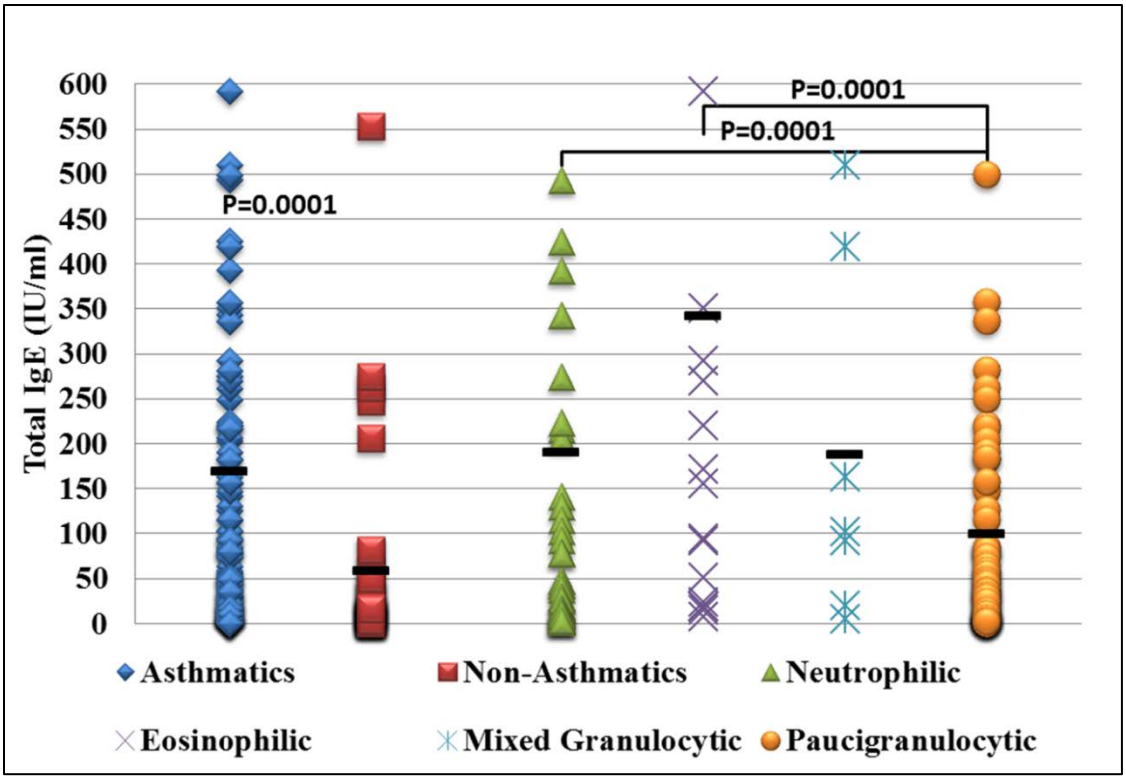

Figure 12: Asthma phenotypes and total serum IgE levels

Asthmatic patients had significantly higher total IgE levels compared to non-asthmatic patients. Inflammatory asthma phenotypes determined by BAL cell counts revealed that eosinophilic asthmatics had higher IgE levels compared to non-eosinophilic phenotypes (neutrophilic, paucigranulocytic and mixed granulocytic patients).

DISCUSSION

The nature of the clinical association that exists between *Chlamydia* airway infection and subsequent development of asthma in young children remains controversial. In an effort to find discriminative serological or biological markers to differentiate between patients with infection-mediated airway responsiveness and those with other aeroallergen-specific disease, we utilized Western blot techniques to determine and compare the *Chlamydia*-specific IgE response in the sera of 197 pediatric patients with chronic, severe respiratory disease.

The development of pathogen-specific IgE antibodies is not unique to *Chlamydia* infection. Numerous reports have demonstrated production of specific IgE antibodies in response to infection by pathogens, including a variety of viruses (31-33) and Mycoplasma (34). Emre *et al*, previously reported the presence of *C. pneumoniae*-specific IgE in a cohort of children with chronic respiratory disease, and suggested that production of specific IgE may be an underlying mechanism leading to reactive airway disease in some patients with *C. pneumoniae* infection (35). With our larger cohort, our data is in line with this previous report and is strongly predictive of severe reactive airway disease and poor asthma outcome. To this end, while the role of pathogen-specific IgE antibodies has still not been fully determined, when studied, in most cases, the responses were associated with a poorer prognosis (33).

Approximately 68% of the patients analyzed in the current study harbored chlamydial DNA and 54% produced detectable levels of specific IgE antibodies in response to chlamydial infection. Age matched, healthy controls had no detectable *Chlamydia*-specific IgE in their serum. There was also a significant association between the presence of *Chlamydia*-specific IgE antibodies in the serum and cultivable *Chlamydia* from BAL fluid, suggesting that the presence of viable, actively replicating organisms are more likely to induce pathogen-specific IgE production and could therefore lead to greater allergic type pathology. A limitation of the current study however, is that we were not able to assess individual BAL samples for the presence of *Chlamydia*-specific IgE because of limited sample volume and therefore have only qualitative

data on BAL IgE from pools of 4 – 6 samples. However, we assessed individual serum samples that showed similar banding patterns as the pooled BAL samples. A second limitation is that our control cohort consisted of only 35 patients and we did not obtain BAL samples from these patients since this is a highly invasive procedure. These patients were not assessed clinically for any respiratory disease since they appeared healthy upon physical examination as part of a wellness checkup by a primary care physician. However, compared to the respiratory disease population, these healthy controls did not have *Chlamydia*-specific IgE antibodies in their sera, suggesting that this phenomenon is specific to a chronic, severe, respiratory disease.

Chlamydial antigens recognized by specific IgE antibodies were significantly different in the BAL and serum for *C. trachomatis* versus *C. pneumoniae*. It is not clear at the present time why these dramatic differences in antigen recognition exist. However, only 6 chlamydial antigens were recognized by either BAL or serum specific IgE in this patient cohort. The only obvious unique quality about these antigens is that they are surface exposed or secreted by the organism. We have assessed these proteins by utilizing reducing and non-reducing SDS-PAGE conditions with the same results. We confirmed the identity of the MOMP protein and the cLPS antigen using recombinant proteins but used the *Chlamydia* protein data base to match the most likely molecular weight for the other antigens. Further assessment of the patient cohort for the presence and level of total IgE confirmed that only 31% of patients who had *Chlamydia*-specific IgE had an overall elevated IgE level. This strongly suggests that patients who are assessed as non-atopic may nonetheless produce *Chlamydia*-specific IgE, which we hypothesize, could lead to airway pathology.

The primary biological function of IgE is to provide immunity against multicellular parasitic pathogens (36). However, in developed countries with good sanitation where parasitic diseases are rare, IgE responses are most often directed against innocuous allergens resulting in type I allergic disease. Mouse-model studies have consistently demonstrated that inhalation of innocuous aerosolized protein antigens typically does not induce antigen-specific Th2 responses

leading to IgE production (37). However, it has been demonstrated that ongoing Th2 responses produced either by antigen challenge or infection during antigen inhalation can prevent the establishment of aerosol-induced IgE tolerance and lead instead to Th2 priming (38). It is possible as suggested by Hollams *et al* (39), that upon bacterial infection Th2 cytokines may just be suppressing other pro-inflammatory cytokines, such as tumor necrosis factor-α and IL-1, as well as mediators such as prostaglandin, hence, counter-regulating the increased Th1 immune responses. However, since *C. pneumoniae* surface antigens have to be processed and presented by antigen presenting cells (APC) to activate T-cells and, thus, may not directly induce a Th2 response, this theory might not hold true in this case.

C. pneumoniae has long been implicated in the inflammatory response experienced by chronic asthmatics; however, a specific mechanism of its involvement has remained elusive. While the role and regulation of IgE antibodies towards microbial antigens is far from being elucidated, results from the current study strongly suggest that this obligate intracellular pathogen can induce pathogen-specific IgE production and could therefore lead to mast cell degranulation and release of vasoactive agents. The presence of *Chlamydia*-specific IgE in the serum would suggest that the organisms play a direct role in asthma pathogenesis by continuous induction of IgE, since unlike most aeroallergens that a patient can avoid, the chlamydial organisms reside in the lower airways and are continuously secreting bacterial antigens. In addition, even if the organism is cleared by antibiotic use or natural immune responses, *Chlamydia* – specific IgE antibodies might still play an important role in the development of exaggerated airway responsiveness during subsequent subclinical or even asymptomatic infection.

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CHAPTER 4

COLONIZATION OF THE PAEDIATRIC LOWER RESPIRATORY TRACT WITH GENITAL *MYCOPLASMA* SPECIES

Abstract

Background and Objectives: Recently, much attention has been given to the possible role played by pathogens that colonize neonatal or pediatric airway and their potential involvement in chronic respiratory disease. While *Mycoplasma pneumoniae* has been implicated in various acute and chronic respiratory diseases, the genital *Mycoplasma* species have not been studied. The goal of the current study was to evaluate the prevalence of urogenital *Mycoplasma* organisms in the bronchial lavage (BAL) fluid of pediatric patients suffering from a variety of chronic respiratory diseases to determine if there was any clear disease association.

Methods: We examined 319 pediatric BAL samples for the presence of *M. genitalium, M. hominis, U. urealyticum, U. parvum* and *M. pneumoniae* DNA via PCR using species-specific primers.

Results: *Mycoplasma* DNA was found in 104/319 (32.6%) patient samples; 32/104 (30.8%) were positive for *M. pneumoniae*; 28/104 (26.9%) for *U. parvum*; 99/104 (8.6%) for *U. urealyticum*; 15/104 (14.4%) for *M. hominis* and 29/104 (27.9%) for *M. genitalium*. There were ten 16S DNA-positive samples that remained unidentified and 16 patient samples contained two or more species.

Conclusions: Our data indicate that in addition to *M. pneumoniae*, urogenital *Mycoplasma* species may colonize the airway of patients with chronic respiratory diseases. There was however, no association between chronic asthma and *Mycoplasma* colonization in this study.

Introduction

Chronic respiratory diseases, airway inflammation, chronic pneumonia and chronic obstructive pulmonary disease, are known to be associated with chronic infections. *Mycoplasma*

pneumoniae is a common cause of upper and lower respiratory tract infections in persons of all ages and according to many reports, may be responsible for up to 40% of community-acquired pneumonias (1). *Mycoplasma pneumoniae* can cause pharyngitis, otitis, and tracheobronchitis, but may also remain asymptomatic (2). Recent research indicates that like infections due to *Chlamydia pneumoniae* or to viruses, acute *M. pneumoniae* infection may not only promote the exacerbation of asthmatic symptoms but also may be accompanied by wheezing in non-asthmatic children (3-6). However, after years of research and much debate, the significance of these organisms in chronic respiratory disease is still under investigation and remains unclear. Despite the experimental evidence for an association between lower airway infection and chronic asthma, testing patients for such infections in the clinical setting is problematic. In recent studies, subjects seronegative for *M. pneumoniae* tested PCR-positive for the bacterial DNA, clearly suggesting that seropositivity is not a reliable marker for *Mycoplasma* infection of the airways and hints at the fact that other species might be the source of the DNA (7). The urogenital mycoplasmas represent a complex and unique group of microorganisms that have been associated with a wide array of infectious diseases in adults and infants (8). *Mycoplasma hominis, Mycoplasma genitalium* and *Ureaplasma* species, known collectively as the genital mycoplasmal organisms, are generally considered opportunists that cause invasive infections in susceptible populations. These genital strains of *Mycoplasma* have been detected in more than 12% of females who presented at gynecological services, and have been associated with acute and nonspecific, non-gonococcal urethritis (9), with *M. hominis* and *M. genitalium* assuming increasing importance as neonatal pathogens (10). *Ureaplasmas* have been isolated from the endotracheal secretions in up to 40% of newborn infants within 30 min to 24 h after birth (11). In 1999 the *Ureaplasmas* were divided into two biovars on the basis of phylogenetic analysis. Strains classified as biovar 1 were designated as a separate species, *Ureaplasma parvum*, whereas strains with biovar 2 traits retained the *U. urealyticum* designation (12, 13). Recently, it has been reported that intrauterine infection of *U. parvum* can cause congenital pneumonia and sepsis even in term newborns (14).

New studies suggest that *U. urealyticum and U. parvum* may be an important cause of lung injury in newborns and neonates through a number of mechanisms including the inhibition of pulmonary surfactant as well as the production of interleukins and soluble intercellular adhesion molecules (15). In the current study, we examined BAL from children with a variety of chronic respiratory conditions to determine the prevalence of both genital and respiratory associated Mycoplasmas as well as to establish if there is an association between DNA evidence of the bacteria and specific diseases in this pediatric cohort.

Materials and Methods

Patients and Specimens

Residual BAL collected for clinical reasons from patients with an array of chronic respiratory diseases undergoing diagnostic bronchoscopy procedures with lavage under an IRB-approved protocol at Baystate Medical Center (BMC), a community/academic hospital setting in Springfield MA, over the period from July 2003 to July 2009 were used for this study. Children were referred for bronchoscopy only after thorough evaluation did not yield a definitive diagnosis or after medical management did not produce an adequate improvement in their clinical condition. Specifically, the children in this study were considered for bronchoscopy because 1) their symptoms were not improving with treatment over time, 2) non-invasive methods did not generate a definitive diagnosis, 3) aggressive medical management had not been successful and 4) the child was truly compromised, missing multiple school days, excluded from physical activity and presenting with recurring symptoms. Many patients presented with severe persistent asthma which was poorly responsive to maximal medical therapy or infection was suspected. The criteria for bronchoscopy followed in this study has been previously described (16). This study was approved by the BMC Institutional Review Board and complied with the ethical guidelines of BMC and the University of Massachusetts. Informed consent was obtained from all patients or their guardians prior to specimen collection.

PCR Analysis

Upon arrival to the research lab, genomic DNA was isolated from each BAL sample using the QIAMP DNA Blood mini kit (Qiagen). All original samples and isolated DNA were stored in a -80°C freezer until time of testing. Polymerase chain reaction (PCR) was used to detect *Mycoplasma* DNA. All samples were processed using best practices to reduce DNA contamination including wearing clean lab coats and fresh gloves, maintaining separate areas and dedicated equipment and supplies for sample preparation, setup, amplification and analysis of PCR products. Lab benches and equipment are periodically cleaned with a 10% bleach solution and positive-displacement pipets along with pipet tips containing hydrophobic filters are a standard part of DNA work in the lab. *Mycoplasma* 16S and *M. pneumoniae* specific PCR was performed using commercial kits (Maxim Biotech, Inc. *Mycoplasma* 16S rRNA kit-SP-10521 and PCR Primer Amplification Kit, *Mycoplasma pneumoniae*, Attachment Protein P1; SP-10523) according to the manufacturers guidelines. *M. hominis*, *M. genitalium*, and *U. urealyticum* were specifically detected using previously published primers and protocols: *Mh* fwd and rev (334 bp), *Mg* fwd and rev (425 bp), and *UMS* 170 and *UMA* 263r (476 bp) respectively (13, 17, 18). U. parvum positive samples were further tested using previously published *U. parvum* serovars 1, 3, 6, and 14 specific, previously published primers (13). All serovars of *U. parvum* were initially detected using UMS 57 and UMA 222 primers (327bp). Subsequent amplicon sizes of 398 bp, 442 bp, or 369 bp were considered to be positive for serovar 1, serovars 3/14, and serovar 6 of *U. parvum*, respectively (13). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. Photographs were taken with the Syngene GeneFlash gel documentation system [Syngene USA, Frederick, MD].

BAL Cell Counts

BAL cell counts were performed as previously reported (19). Briefly, cell enumeration was performed manually using a cell counting chamber with a phase contrast microscope. The results

are expressed as “number of cells per cubic millimeter.” Differential counts were performed with Wright stained cytospin preparations of BAL. Results were expressed on the basis of a 100 cell count survey. Inflammatory asthma sub-phenotypes were determined based on BAL neutrophil and eosinophil cells counts, as previously reported (20, 21).

Endobronchial Biopsy Eosinophil counts

Where performed, the endobronchial specimens were preserved in formalin, processed, and embedded in paraffin. Routine histological sections were cut at 5μm in thickness and stained with hematoxylin-eosin. The presence of eosinophils was determined by their characteristic bilobed nucleus and eosin-staining granules within the cytoplasm.

Evaluation of Total IgE

Total IgE was performed using the Elecsys IgE kit (Roche Diagnostics, Indianapolis, IN). This kit utilized the electrochemiluminescence immunoassay and tests were performed according to the manufacturer’s instructions. Plates were read on the Roche Elecsys 1020 analyzer which automatically calculated the IgE concentration of each sample based on a standard curve. Elevated IgE levels were determined based on the manufacturer’s recommended threshold by age range (neonates, 3·6 ng/ml; infants in first year of life, 36 ng/ml; children aged 1–5 years, 144 ng/ml; children aged 6–9 years, 216 ng/ml; children aged 10–15 years, 480 ng/ml; adults, 240 ng/ml).

Statistical Analysis

Data were analyzed using Microsoft Excel® spread sheets as well as the SPSS 15.0 Graduate Pack [SPSS, Inc., Chicago, IL] statistics programs. Cross-tabs with the Fisher exact T test and Chi-Squared test were used to determine significance. T tests were two-sided, and the level of significance was $p \leq 0.05$.

Results

Characteristics of study participants

The study population consisted of 319 pediatric patients and was made up of 145 females and 174 males from White (228), Black (24), Hispanic (66) and Asian (1) ethnicity. The average age of this cohort was 7.9 years old and 225 were diagnosed with chronic asthma (Table 10). The asthmatics fulfilled criteria for asthma diagnosis and classification of asthma severity in accordance with GINA guidelines and Recommendations of the National Asthma Education and Prevention Program guidelines (22, 23). Through biopsy evaluation, basement membrane thickening (BMTH) was observed in 77.8% of asthma patients compared to non-asthmatics ($P < 0.0001$). Respiratory disorders in the non-asthmatic group included aspiration pneumonia, cystic fibrosis, bronchitis, immune deficiency, gastroesophageal reflux disease, structural and airway anomalies, and recurrent pneumonia of unknown etiology. There was no correlation between *Mycoplasma* DNA positivity and gender (51 females and 53 males). A total of 17 patients were being prescribed antibiotics at the time of sample collection (11 of these were taking macrolide antibiotics).

BAL cellularity was assessed to determine if there was an association with the presence of *Mycoplasma* DNA in the lungs of patients and the presence of cellular pathology. As shown in table 1, there was no statistically significant association between the presence of *Mycoplasma* organisms and changes in the numbers of immune cells in the BAL fluid analyzed.

Diagnosis/BAL Cell Types/Age	Mycoplasma DNA Positive N=104	Mycoplasma DNA Negative N=215
Average Age	7.6	8.2
Sex		
Female	51 (49.0%)	94 (43.7%)
Male	53 (51.0%)	121 (56.3%)
Asthma	69 (66.3%)	156 (72.6%)
Inflammatory Phenotype		
Neutrophilic	32 (30.8%)	57 (26.5%)
Eosinophilic	11 (10.6%)	30 (14.0%)
Neutro/Eosin(both)	2 (1.9%)	9 (4.2%)
Paucigranulocytic	59 (56.7%)	119 (55.3%)
Biopsy Eosinophils		
Positive	37 (35.6%)	116 (54.0%)
Negative	37 (35.6%)	68 (31.6%)
Not Done	30 (28.8%)	31 (14.4%)
Mean % Cell Count		
Mac/Mono	3.0	3.4
Eosinophils	1.0	1.4
Lymphocytes	8.6	8.2
Neutrophils	40.3	38.5
Other*	47.1	48.5
Serum IgE Levels		
Normal IgE	80 (77.0%)	130 (60.5%)
Elevated IgE	24 (23.0%)	85 (39.5%)

Table 10: Characteristics of Patient Cohort.

Cohort demographics of 319 pediatric patients with an average age of 7·9 years old. There was no significant link between cell types found in the BAL fluid and the presence of *Mycoplasma* DNA. Inflammatory asthma phenotype was based in eosinophil and neutrophil counts in the BAL fluid and biopsy eosinophils were assessed in fixed H&E stained sections. *Other was mostly epithelial cells in this study.

Analysis of serum IgE in this cohort revealed that these antibodies were elevated in a total of 109 (34% of all patients tested). Elevated levels of IgE were found more often in *Mycoplasma* negative patients than patients with bacterial DNA, 85/109 vs. 24/109 respectively, Fisher exact T test, $P < 0.0001$, see Table10. While the majority of patients displayed a non-eosinophilic asthma phenotype, there was no significant correlation between the finding of *Mycoplasma* organisms and inflammatory asthma phenotypes.

Frequency of *Mycoplasma* DNA in BAL fluid

We previously reported on the prevalence of *C. trachomatis* and *C. pneumoniae* in a subset of this cohort and demonstrated that genital strains of *Chlamydia* were just as prevalent as the respiratory strains (19). The current study utilized a PCR assay that amplified the 16S DNA of all *Mycoplasma* species in the BAL of our patient cohort in order to determine the frequency of these organisms in the lungs. The data revealed that 104/319 (32.6%) patient samples contained *Mycoplasma* DNA. Assessment of disease association based on asthma diagnosis confirmed that *Mycoplasma* 16S DNA was more frequently associated with an asthma diagnosis (69/104 vs. 35/104; p=0.007 Fisher's exact test, two-tailed). However, there was no statistical association between any of the *Mycoplasma* species tested and an asthma diagnosis (see Table 11).

Genital *Mycoplasma* by patient age range and asthma diagnosis

While *M. pneumoniae* is routinely assayed for in respiratory disease and we have epidemiological evidence of its frequency, very little is known about the frequency of genital *Mycoplasmas* in respiratory disease. Species-specific primers were therefore used to assess the frequency of five relevant species based on previous reports. Of the 104 patient samples positive for *Mycoplasma* DNA, 32 (30%) were *M. pneumoniae* positive, 29 (28%) were positive for *M. genitalium*, 15 (14%) were positive for *M. hominis*, 28 (27%) were positive for *U. parvum*, and 9 (9%) were positive for *U. urealyticum* (Table 11). *U. parvum* positive samples were further

tested using serovar-specific primers and revealed that 4/28 (14.3%) were *U. parvum* serovar 1; 5/28 (17.9%) were *U. parvum* serovar 6, and 22/28 (78.6%) were *U. parvum* serovars 3 and/or 14. Three samples were positive for more than one serovars. Ten of the *Mycoplasma* 16S DNA positive samples were not identified by any of our species-specific primers and 19 samples contained combinations of two or three different species. Figure 13 shows representative agarose gels with PCR products for each of the species tested.

We previously reported the frequency of *C. trachomatis* in the BAL of pediatric patients with chronic respiratory disease and presented evidence confirming that the organisms were acquired as the neonates passed through the birth canal (19). Here, the patient cohort was assessed to determine if there were trends in the prevalence of *Mycoplasma* DNA according to patient age range which would indicate if younger children were more likely to harbor the organisms. *M. genitalium* was just as prevalent in the 0-5 year old age group as *M. pneumoniae* and generally decreased with an increase in age (Figure 14). *M. hominis* and *U. urealyticum* were also most prevalent in the 0-5 age group but declined dramatically in prevalence in older age groups. Unlike *M. hominis* and *U. urealyticum*, *U. parvum* generally remained constant throughout the pediatric age range and peaked in the 15-20 year old age group. Importantly, *M. pneumoniae* was responsible for only 30% of all *Mycoplasmas* found in the lungs of this pediatric population.

Mycoplasma Species	Asthma (N=225)	Non-Asthma (N=94)	P Value
Mycoplasma 16s (N=104)	69 (30.6%)	35 (37.2%)	P=0.4621
M. pneumoniae (N=32)	18 (8%)	14 (14.9%)	P=0.1090
M. genitalium (N=29)	18 (8%)	11 (11.7%)	P=0.3982
M. hominis (N=15)	9 (4%)	6 (6.4%)	P=0.3951
U. parvum (N=28)	19 (8.4%)	9 (9.6%)	P=0.8297
U. urealyticum (N=9)	6 (2.6%)	3 (3.2%)	P=0.7271
Mycoplasma Negative (N=215)	156 (69.3%)	59 (62.8%)	P=0.6270

Table 11: Prevalence of *Mycoplasma* Species in BAL Fluid by Disease Diagnosis

Of the 104 patient samples harboring *Mycoplasma* DNA in their lower airways, 69 (66.3%) were diagnosed asthmatics (p= 0.007). There was no significant difference between the number of asthmatic and non-asthmatic patients with *M. pneumoniae*, *U. urealyticum*, *M. hominis*, *M. genitalium*, or *U. parvum* in their lungs.

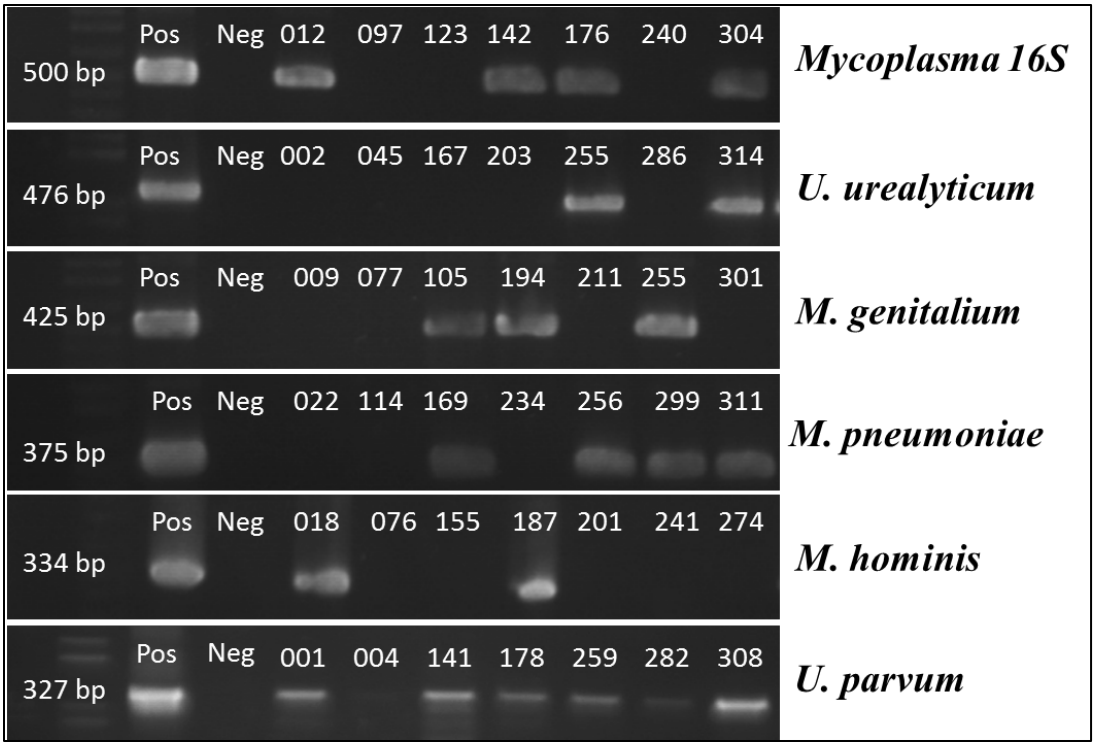

Figure 13: Representative agarose gel showing PCR products of Mycoplasma DNA

Each lane represents an individual patient BAL sample with sample code number. Pos and neg. represent the positive and negative controls respectively and the bp # represents the product size.

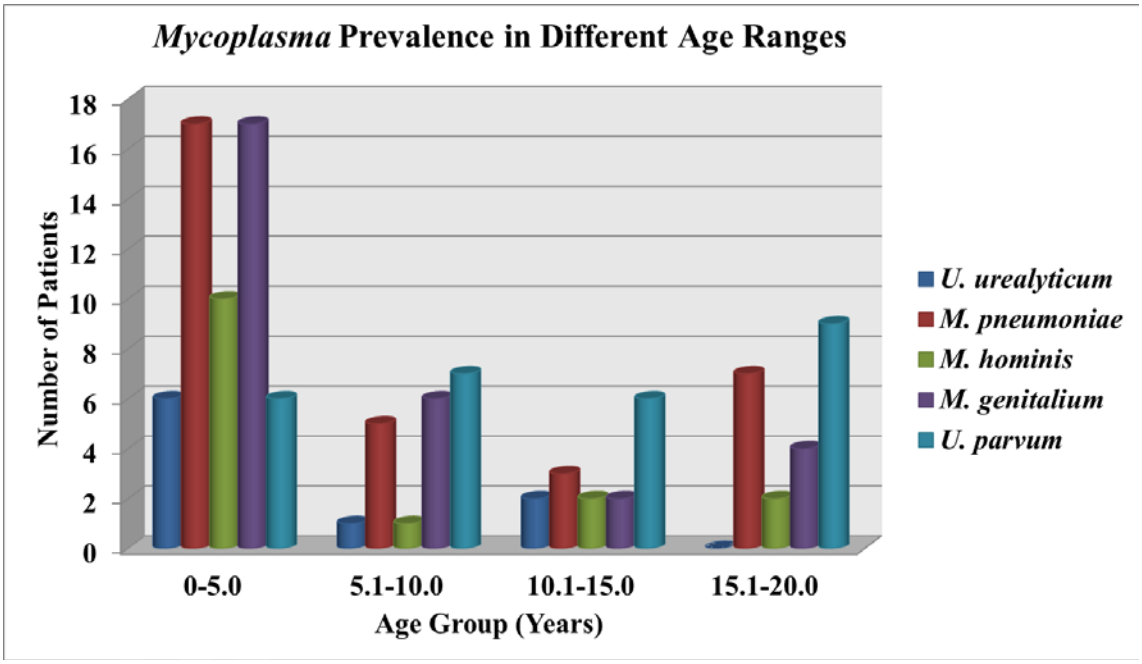

Figure 14: ***Mycoplasma*** **prevalence according to age**

Mycoplasma DNA was more prevalent in the 0-5 year old age range with *M. genitalium* accounting for the same number as *M. pneumoniae* in this age group. All species except *U. parvum*, which remained relatively constant, generally decreased over the age range from 0 to 20 years old.

Seasonal Variation of *Mycoplasma* Infections

Previous reports have described a seasonal variation in *Mycoplasma* infections, highlighting its prevalence in the midsummer and winter months (24, 25). The peak incidence of *Mycoplasma* 16S DNA was in the mid-summer month of July (Figure 15A). However, elevated numbers of patients harboring *Mycoplasma* DNA in their lungs were also seen during the late fall to winter months, with numbers higher in the months of November, December, January and February. Genital strains of *Mycoplasma* (*U. Urealyticum, U. parvum, M. hominis,* and *M. genitalium* combined) and *M. pneumoniae* also peaked in prevalence in July, and to a lesser extent increased once again during fall and winter months. We determined that the increase in *Mycoplasma* incidence in the month of July was not due to increased patient sample collection in that month. The data was expressed relative to the total number of BAL samples for each month and demonstrated that the months of November to March had an overall greater number of BAL procedures performed without similar increases in *Mycoplasma*-positive outcomes (figure 16). With respect to the peak prevalence of *M. pneumoniae*, these patient samples were collected year round over a 6 year period, thereby negating the probability that this trend is the result of a single outbreak in a particular year. There was no indication of a *Mycoplasma* epidemic over this time period with each year showing similar prevalence rates (Figure 15B).

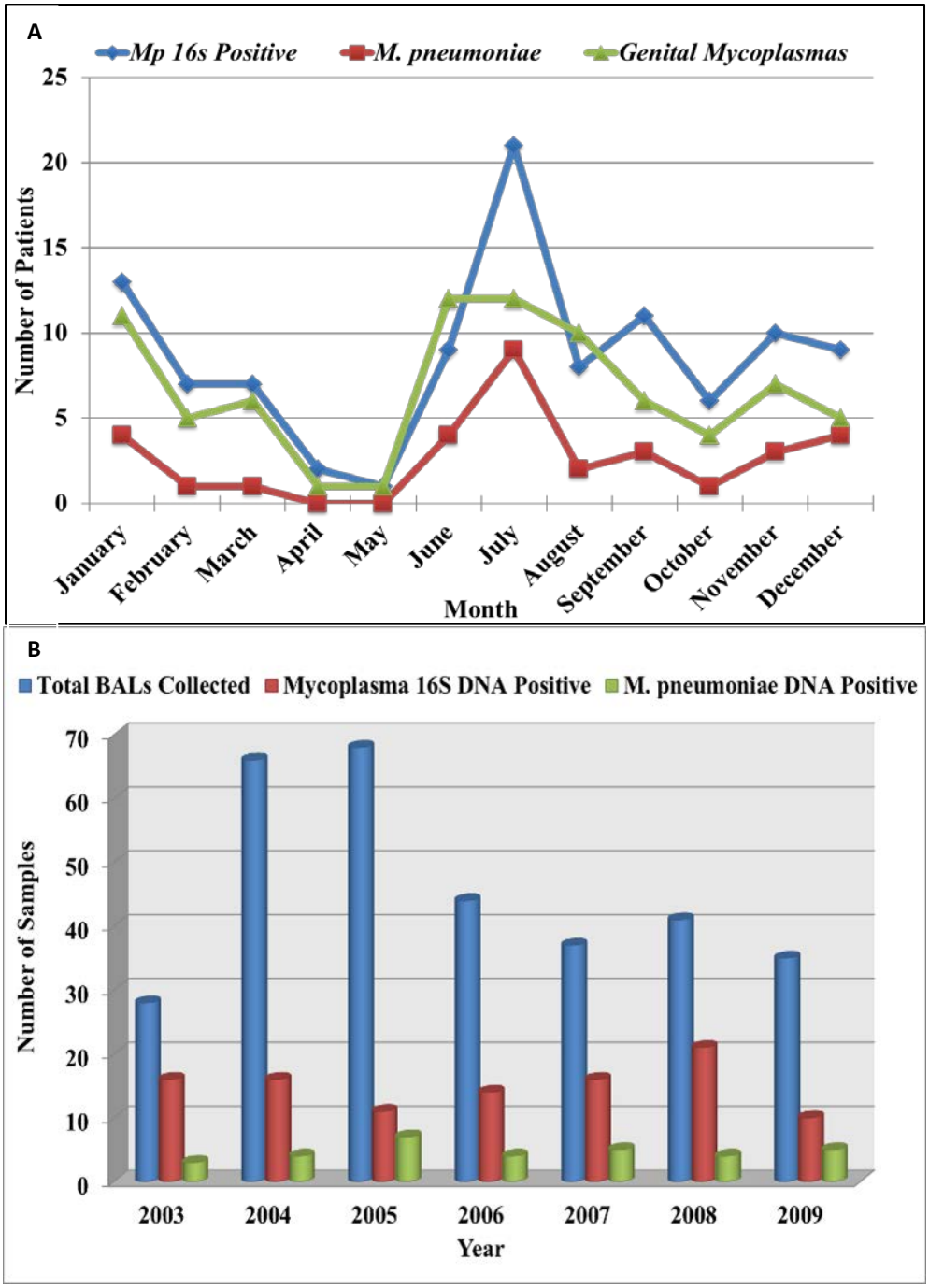

Figure 15: Seasonal variations of *Mycoplasma* species incidence in pediatric lung disease

BAL samples were collected over the course of 6 years and tested by PCR for the presence of *Mycoplasma* DNA in a species-specific manner. The highest prevalence of *Mycoplasmas* in these patient samples was in samples collected in the month of July. *Mycoplasma* prevalence was also generally higher in the late fall to early winter months. Mycoplasma prevalence was highest in 2007-2008.

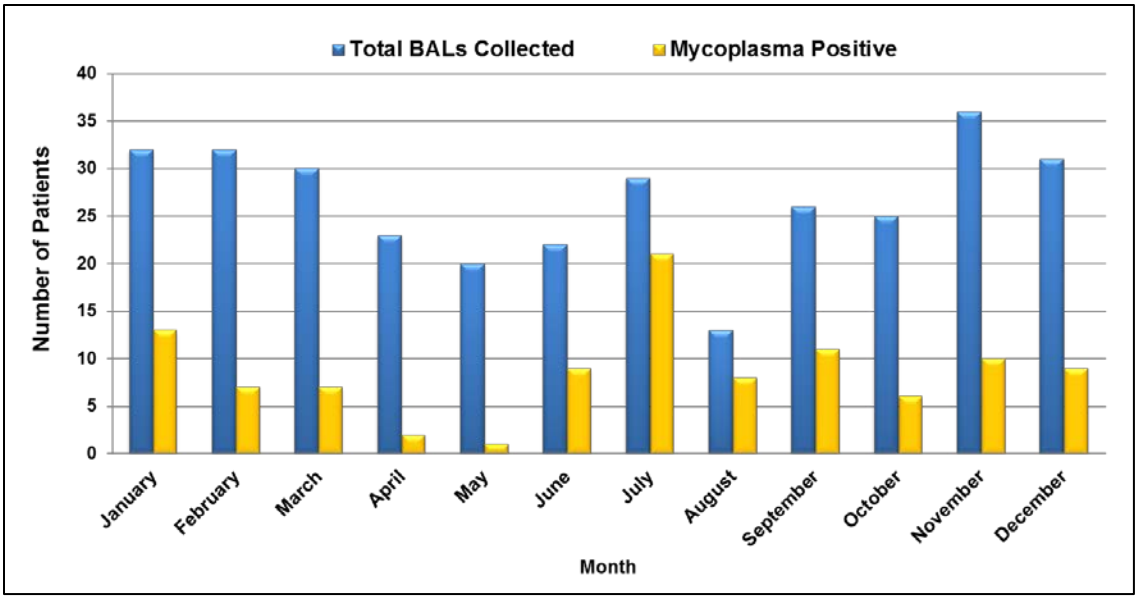

Figure 16: Monthly prevalence of patient BAL samples and *Mycoplasma* positivity

Analysis of the distribution of total patient samples collected per month was compared to the total number of *Mycoplasma*-positive samples to determine if the increased positivity seen in the month of July was influenced by the total number of samples tested for that month. The data revealed that *Mycoplasma* positivity is not a reflection of increased monthly prevalence.

Discussion

Mycoplasma and *Ureaplasma* organisms can be isolated with considerable frequency from the female urogenital tract and are thought to cause a wide range of syndromes including nongonococcal urethritis, cervicitis, amnionitis, pyelonephritis, post-partum septicemia, neonatal pneumonia, neonatal conjunctivitis, pelvic inflammatory disease, infertility, low birth weight infants, and premature rupture of membranes (26). *Mycoplasma hominis* and *Ureaplasma urealyticum* are the most common isolates from the genital tract and may be present as normal flora in up to 40% of asymptomatic males and females. The urogenital *Mycoplasmas* have previously been associated with respiratory distress in newborns (26) and neonatal meningitis (27), while the *Ureaplasmas* have been linked with preterm labor (28), intrauterine lung disease (29), and neonatal pneumonia (30).

The data presented here from analysis of 319 pediatric respiratory disease patients demonstrated no association between an asthma diagnosis and the presence of respiratory or genital *Mycoplasma* species. While it has been recognized as a common respiratory pathogen, *M. pneumoniae* accounted for only 30% of *Mycoplasma* organisms in the lungs of this pediatric cohort. It is not clear what role, if any, the urogenital *Mycoplasmas* and *Ureaplasma* play in acute or chronic respiratory disease, and clinical labs do not routinely test for these urogenital strains in respiratory samples. These data suggest that a negative *M. pneumoniae* PCR test does not necessarily mean that *Mycoplasma* is absent. Increasing evidence suggests that urogenital *Mycoplasmas* can be the cause of serious extra-genital infections, mainly in immunocompromised patients although extragenital infections are uncommon in healthy patients and cases of pneumonia are exceptional (31). In the current study, *M. genitalium* was the most frequent urogenital strain isolated from BAL fluid. Species identification of the *Ureaplasma* biovars revealed that *U. parvum* was more commonly isolated than *U. urealyticum* as reported by other studies (32), with *U. parvum* serovars 3/14 accounting for the majority of cases (78.6% of all *U. parvum* isolated).

There are, however, limitations to our investigation. All samples were obtained from a convenient population of patients with poorly-controlled, chronic respiratory disease who underwent diagnostic bronchoscopy. Therefore, our results may not be broadly applicable to the majority of patients whose respiratory disease is readily controlled and are seen in a regular primary care setting. In addition, we did not have a healthy control population, leaving open the possibility that the prevalence of both genital and respiratory *Mycoplasma*s in our test cohort is reflective of the carriage in the healthy population.

It has been estimated that more than 2 million cases of *M. pneumoniae* infections occur annually, accounting for approximately 20% of community-acquired pneumonias requiring hospitalization and probably an even greater proportion of those that do not(33). While *M. pneumoniae* disease tends to be non-seasonal, there are generally increases in late summer and early fall months (33). This was indeed the case with our sample set, demonstrating highest prevalence in July. However, there was also an increase in *M. pneumoniae* prevalence in early winter months. It is probably valuable to note that late summer to early fall are generally times when children head back to school, and communal attendance in class may spur a *Mycoplasma* infection. In fact all of the *Mycoplasma* and *Ureaplasma* organisms assayed in this study showed seasonal variations. Although limited by the fact that only one sample was collected from each patient and we therefore cannot comment on the status of infection in individual patients over time, data from vaginal versus C-section birth as previously reported (19), suggest that the genital strains of the organisms were acquired for the most parts at the time of birth through the birth canal. Moreover, further investigation did not reveal a *Mycoplasma* epidemic over the period of the study.

Ongoing research into the importance of atypical pathogens in chronic respiratory diseases, including asthma will further elucidate whether these infections are important in disease development or whether their prevalence is increased in subjects due to chronic airway inflammation or other, yet unidentified, predisposing factors. Prolonged proinflammatory

responses initiated by *Mycoplasma* infections could contribute to altered developmental signaling in the immature lung and may lead to long-term respiratory problems (34). Increasingly, basic and clinical research suggest that genital strains of *Mycoplasma* can cause respiratory disease not just in immunocompromised, but even healthy adults and children and are often not easy to identify or treat (31). While we did not find a definitive link between the presence of individual organisms and chronic lung disease in this study cohort, *Mycoplasma* 16S DNA was found more often in asthmatic patients. Therefore, the presence of urogenital *Mycoplasma* species should not be underestimated, but rather evaluated in patients with chronic respiratory diseases, especially in those whose symptoms have not improved over time with standard therapy.

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CHAPTER 5

EVIDENCE OF INFECTIOUS ASTHMA PHENOTYPE: *CHLAMYDIA*-INDUCED ALLERGY AND PATHOGEN-SPECIFIC IgE IN A NEONATAL MOUSE MODEL

Abstract

Rationale: Asthma is a chronic respiratory disease whose etiology is poorly understood. Recent studies suggest that early-life respiratory infections with atypical bacteria may play an important role in the induction/exacerbation of chronic respiratory disease.

Purpose: The current study utilized a neonatal mouse ovalbumin (OVA) sensitization model of asthma to determine the course of early-life respiratory tract infection by *Chlamydia*.

Methods: Neonatal (day 1) and adult (6 wks) BALB/c mice were infected intranasally with *Chlamydia (MoPn)* and 7 weeks later were sensitized and challenged with ovalbumin. Allergic airway disease was characterized by examination of serum and bronchoalveolar lavage fluid (BAL) cellularity, cytokine production and antibody response. The presence of *Chlamydia* was determined by PCR and culture. Ova-specific IgE was quantified by ELISA and *Chlamydia*-specific IgE was determined via Western blot analysis.

Results: Chlamydial infection in neonatal mice induced increased production of Th_2 cytokines (IL-4, 5, 10, and 13) in both BAL and serum, while infected adult mice produced increased Th_1 cytokines (IL-2, IFN-γ). The BAL from infected neonates contained significantly elevated levels of eosinophils compared to infected adult mice. Although adult mice cleared the infection ~30 days post infection (pi), neonates were still infected 66 days after initial infection. *Chlamydia*-specific IgE was detected in both BAL and serum of neonatal mice beginning 28 days post infection, however, infected adult mice did not produce *Chlamydia*-specific IgE antibodies over the course of the study. When allergic airway was induced using Ova albumin, infected neonatal mice increased their production of IL-4, IL-5 and IL-13 by >2 fold compared to uninfected controls and infected adult groups.

Conclusions: Our findings demonstrate that early-life *Chlamydia* infection induces a Th_2-dominant cytokine response in the airways of neonatal mice, leading to chronic infection. More significantly, early life respiratory colonization with *Chlamydia* elicits pathogen-specific IgE production, which provides further evidence of an infectious asthma phenotype.

Introduction

According to the most recent available data, the total incremental cost of asthma to society was $56 billion, with productivity losses due to morbidity accounting for $3.8 billion and productivity losses due to mortality accounting for $2.1 billion (1). Over the last 2 to 3 decades there has been a significant increase in asthma prevalence in Western countries and recent data suggests that while these levels might be peaking, many low and middle income countries are now beginning to experience increases in prevalence (2). Until recently it was widely believed that asthma was an atopic disease caused by allergen exposure and many that the global increases in asthma prevalence are due to increases in exposure to aeroallergens which lead to eosinophilic infiltration, mast cell degranulation, hyper-responsiveness and airflow obstruction; and was fundamentally linked to a patient's genetic inheritance (2, 3). However, it is becoming increasingly evident that this allergen-mediated, eosinophilic airways inflammation model is an overly simplified explanation of this very complex disease and that no single etiology can be defined to date (4). While it is indisputable that there are many clear cases of allergen exposure leading to asthma development in adults, overall there is little evidence that allergen exposure is a major primary cause of asthma in children, and even some evidence that allergen exposure early in life may have a protective effect (3). Moreover, recent studies support the conclusion that non-allergic or non-eosinophilic airways inflammation may account for over half of all asthma cases (5). Eosinophilic asthma is now classified as a distinct asthma phenotype that is characterized pathologically by significant basement membrane thickening and pharmacologically by corticosteroid responsiveness. In contrast, non-eosinophilic asthma, that includes most patients

with severe disease, has very little basement membrane thickening of the basement appears to be relatively corticosteroid resistant (6). These published reports strongly suggest that despite clinically similar features, not all asthma is the same and patients may therefore benefit from personalized treatment. Moreover, surveys have consistently shown that many patients with asthma do not have their disease well controlled. A recent CHOICE survey study concluded that of all asthma patients on controllers, only 14.3% were well controlled (7). However, before these patients can be effectively treated, a better understanding of non-allergic asthma etiology is necessary.

Since allergic asthma seems to be a Th2-disease, immunomodulating factors such as early childhood infections, LPS-exposure or other factors influencing gene-environment interaction and individual susceptibility might be relevant for the development of childhood asthma (8). The hygiene hypothesis suggests that early-life infections are crucial in shaping and developing dominant immune responses; it also suggests that exposure to Th1-inducing pathogens is essential so that neonates can mount protective Th1 responses in later life (9). However, recent findings are clearly suggesting that while exposure to certain infectious microbes can protect from atopy, other infections appear to promote allergic diseases. It now appears that the timing of exposure to infection, the virulence properties of the infectious agent, and the genetic susceptibility of the host, all play an important role in the future development of allergic disease (9). It has been shown that neonatal immunity is limited in its ability to generate strong Th1 responses to infection. Although chlamydial infections induce and are ultimately cleared by T_H1-mediated immune responses, clinical studies link chlamydial lung infection with the development of asthma in children (10, 11). Indeed, a recent study from our lab showed that over 68% of children with asthma harbored viable *Chlamydia* in their lungs and that atopy was strongly associated with infection (12). These data suggest that only in some predisposed individuals does infection induce Th2 responses (12). To this end Hansbro et al have proposed two hypotheses to explain the association between T_H1-inducing infections and asthma (13). The

first is that neonatal responses to infection are highly polarized towards T_H2 immunity and thus early life chlamydial infection in neonates reinforces rather than suppresses this response, which leads to atypical T_H2 responses to the infection (13). This has the potential to drive the immune system to develop an allergic phenotype, which can ultimately lead to persistent infection and increasing the severity of T_H2-type inflammatory responses to environmental antigens, and drive asthma disease. The second hypothesis is that T_H1-inducing infections may cause a generalized inflammation of the airways that leads to the exacerbation of allergen-induced inflammation and asthma later in life (13).

Previously published data from our lab demonstrates strong evidence linking *Chlamydia pneumoniae* infection with the development and exacerbation of asthma in pediatric patients. *C. pneumoniae* has, therefore, been associated with both protective (Th1) as well as pro-asthmatic (Th2) immune responses. As a corollary, and in an attempt to better understand the mechanisms involved, we utilized a mouse model to investigate whether chlamydial infection in early life stimulates protective immunity or drives Th2 responses leading to the development and/or exacerbation of asthma later in life. We utilized a murine ovalbumin (Ova)-induced allergic airway disease (AAD) model that was previously developed and refined by Horvat, et al (14) to determine the impact of early life chlamydial infection on the subsequent development of AAD in adulthood.

Materials & Methods

Mouse Models

For neonatal experiments, pregnant BALB/c mice were purchased from Jackson Labs (Bar Harbor, ME) and used with the approval of the IACUC, University of Massachusetts Amherst. Within 1 hour of birth (Day 0) mice were infected intranasally (IN) with *C. muridarum,* formerly known as the mouse pneumonitis biovar of *C. trachomatis* (approximately 200 inclusion-forming units (IFU) in 5µl sucrose phosphate-glutamate buffer, SPG).

For adult experiments, 5 week old virgin female BALB/c mice were utilized. The groups of adult mice were treated in a similar manner as the newborns. Adult mice were anaesthetized by isoflurane inhalation, held in the upright position and the inoculum, 200 IFU in sucrose phosphate glutamate buffer (SPG, 50ul final volume), was pipetted onto the nares until the whole inoculum was inhaled. Control mice for both groups received equivalent volumes of SPG via a similar route.

Seven weeks post infection/placebo treatment (Day 50) mice were sensitized to Ova by intra-peritoneal (IP) injection of 50µg Ova in 200µl 0.9% sterile saline. Twelve days after sensitization (day 62) mice were challenged IN with Ova (10µg, 50µl PBS, for 4 consecutive days). One day later (day 5 post Ova challenge), mice were euthanized by sodium pentobarbital overdose and features of acute airway disease (AAD) were characterized.

Chlamydial Infection

All mice were weighed initially and then daily. The rate of weight gain/loss was calculated (g/d) over the entire course of the study as an indicator of health status. Chlamydial lung infection was initially assessed by PCR and organism numbers in the lungs, liver and spleen were determined tissue culture. Briefly, lung tissues was cut and weighed followed by homogenization in sterile SPG. Homogenates were centrifuged and the supernatant used for culture and DNA isolation.

Chlamydial organisms were cultured utilizing 24-well plates with 12 mm coverslips seeded with a semi-confluent monolayer of mouse macrophages (J774A.1) for 36 hours. Following incubation, cells were fixed and immunostained using Pathfinder antibody (Bio-Rad, Hercules, CA) according to manufacturer's instructions and inclusion forming unit counts (IFUs) were done.

Genomic DNA was isolated from BAL & lung tissue using the QIAMP DNA Blood mini kit (Qiagen) PCR was used to detect chlamydial DNA. Previously published *Ct* primers P1 and Omp2 were used to amplify a 1,130bp fragment (12).

Bronchoalveolar lavage fluid Lavage (BAL) and Cellularity Analysis

Bronchoalveolar lavage fluid (BAL) was obtained by cannulation of the trachea and lavaging the airways with 2x 1 ml sterile saline. BAL cell counts were performed with a cell counting chamber (hemacytometer, improved Neubauer) under phase microscopy with results expressed as "number of cells per cubic millimeter". BAL differential counts were performed using Wright stained cytospin preparations of BAL. At least 200 cells were counted per slide to obtain statistically significant counts.

Serum and BAL Antibodies

Serum antibody titer to *Chlamydia* as well as Ova was evaluated from tail bleeds taken on a weekly basis and at time of euthanasia. *Chlamydia* antibody titers were evaluated by enzyme linked ImmunoSorbent assay (ELISA). The ELISA wells were coated with purified chlamydial EBs (300 ug/ml) and serial dilutions of each mouse serum added. Following the required washes, bound primary antibodies were detected by an AP-conjugated goat anti-mouse secondary antibody. Quantitative assessment of OVA-IgE in the BAL fluid and serum was conducted using OVA-specific mouse-IgE ELISA kit from BD Biosciences, according to the manufacturer's instructions. Chlamydia specific IgE antibodies were detected using Western blot assay as previously described (15, 16)

Cytokine Production in BAL

Total cytokines secreted in the lung milieu was determined through analysis of BAL fluid. Concentrations of IL-2, IL-4, IL-5, IL-12, IL-13, and IFN-γ were determined using OptEIA Mouse ELISA kits (BD Biosciences) according to manufacturer's instructions.

Cytokine Production in WBCs from Lymph Nodes

Upon euthanasia, mediastinal lymph nodes were collected from each mouse, pooled in pairs and homogenized by forcing them through cell sieves. The cells were pelleted and resuspended in RBC lysing buffer, followed by another centrifuge step and resuspension in DMEM culture media containing 5% FBS. Cell numbers were determined by counting on a hemocytometer and approximately 2 x 10^6 cells/ml were used to seed 24-well plates and were then stimulated with Ova, formalin killed *Chlamydia*, J774A.1 cell lysates, or PBS. Supernatant was removed at 12h intervals over a 72h time period and frozen until time of testing. These supernatants were evaluated for production of IL-2, IL-4, IL-5, IL-12, IL-13 and IFN-γ using the BD Bioscience kit described in the previous section.

Statistics: Mice were evaluated in groups of 4. Results are presented as mean± SEM from each test and control group of mice. All analyses were performed using the SPSS Graduate Pack 11 for Windows and significant associations or differences are based on two tailed tests with p values <0.05.

RT PCR

Messenger RNA was isolated from lung tissue using Trizol extraction. RT-PCR was used to determine expression of mRNA for CD23, IgE heavy chain, histidine decarboxylase (HDC), CD40L, and FcεRI alpha, beta, and gamma using previously published primers (7).

Results

Chlamydial Lung Infection and Associated Pathology

The OVA sensitization model of asthma is widely used and well characterized displaying features very similar to those seen in human allergic asthma. We performed intranasal inoculation of the chlamydial organism or control in each of our mouse groups as described in the methods

section and assessed the course of infection-related pathology as well as changes associated specifically with the induction of bronchial inflammation and airway hyperresponsiveness following Ova sensitization and challenge. Animals were weighted each day and the weight trend shows significant weight loss in *Chlamydia*-infected vs. uninfected adult mice starting at four days post infection. The most significant differences in weight gain were observed in neonates between days 32 and 52, where on average infected neonatal mice were 28% (6.1g) smaller than uninfected neonates (Figure 17B). In adults the most significant weight loss was between days 8 and 28, where on average infected adults were 26% (5.9g) smaller than their uninfected counter parts (Figure 17A). However, the rate of weight gain between infected and uninfected adult groups was not significantly different during the later parts of the time course (Figure 17A). Infected neonatal mice, however, demonstrated retarded weight gain throughout the course of the study compared to uninfected neonates (Figure 17B) at an IFU of 200.

In an effort to assess the humoral immune response to intranasal chlamydial challenge, we collected sera from each animal on a weekly basis and assayed for anti-*Chlamydia* antibody response. The data revealed strong antibody responses to *Chlamydia* infection in the adult mice. Neonatal animals whose immune system was not yet fully developed had an attenuated antibody response initially, but the response continued to increase for the duration of the study (Figure 18). Titers began to decrease in infected adult animals around the time of infection clearance; however infected neonatal mice did not clear the infection, explaining why the titers consistently increased over the course of the study.

The concentration of chlamydial organism in the lungs (IFU/mg) of neonatal animals increased significantly with time and there was almost a 2 fold increase from day 14to day 66 (Figure 19). In contrast, infected adults never had significant increases in chlamydial IFU/mg during the course of the study, and ultimately cleared the infection 28 days pi. By day 7, chlamydial organisms could be found in the liver, spleen and peripheral blood (tail bleeds), demonstrating dissemination from the respiratory mucosa and leading to systemic infection.

WBC and Cytokine Response to Respiratory Chlamydial Infection

It is well established that genital chlamydia infection induces a Th1 immune response characterized by IFN-γ secretion and that this type of response is essential for clearance of this intracellular pathogen. It is also well documented that AAD is a Th2-driven disease characterized by the influx of Th2 cytokines, eosinophils and allergic hyperresponsiveness. We therefore sought to determine what would characterize lung infection with *Chlamydia*. BAL fluid was analyzed for the presence of Th1 and Th2 cytokines using BD Biosciences cytokine kits. The data confirmed that infected adults secreted significantly elevated amounts of Th1 cytokines (IFN-γ and IL-2) compared to uninfected animals (Figure 21A and B). However, infected neonates responded to chlamydial infection with a robust Th2 cytokine response (IL-4, IL-10, IL-5, and IL-13) compared to their compared to their uninfected and adult infected counterparts (Figure 21C-F). Infected neonatal mice had significantly elevated IL-4 and IL-10 production compared to infected adults 28 days post infection (P=0.0248, 0.0434 respectively). Mediastinal lymph node T cells from infected adult and neonatal mice were also isolated, cultured and stimulated with

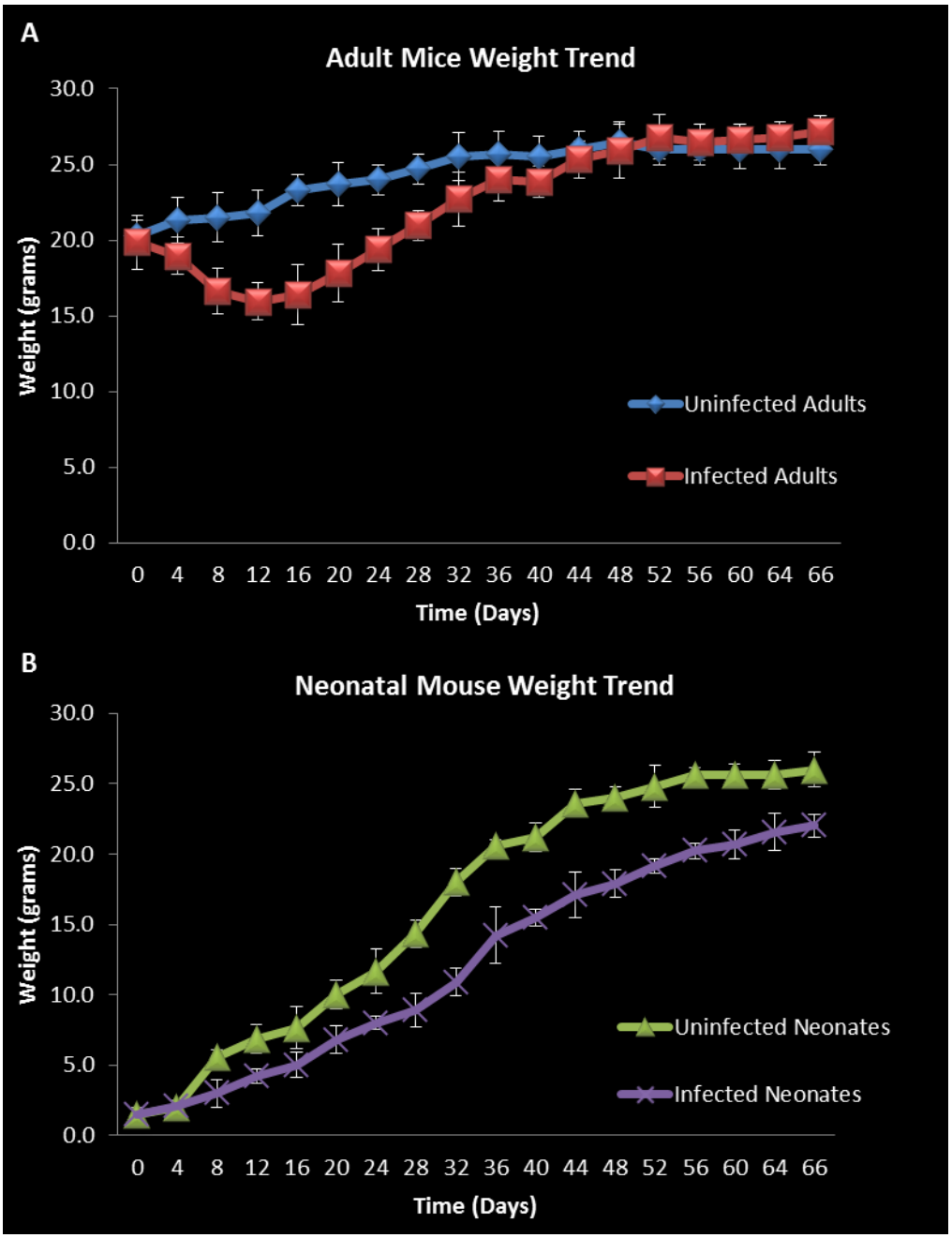

Figure 17: Adult and neonatal weight trends

Weight trend graphs show significant weight loss in Chlamydia infected adults vs. uninfected adults between days 8 and 28 post infection (on average 5.9g smaller (26%)). However, infected adults recovered lost weight by day 44 (A). Infected neonatal mice showed retarded weight gain throughout the course of the study compared to uninfected neonates but the most significant differences could be seen between days 32 and 52 (on average 6.1g smaller (28%)) (B).

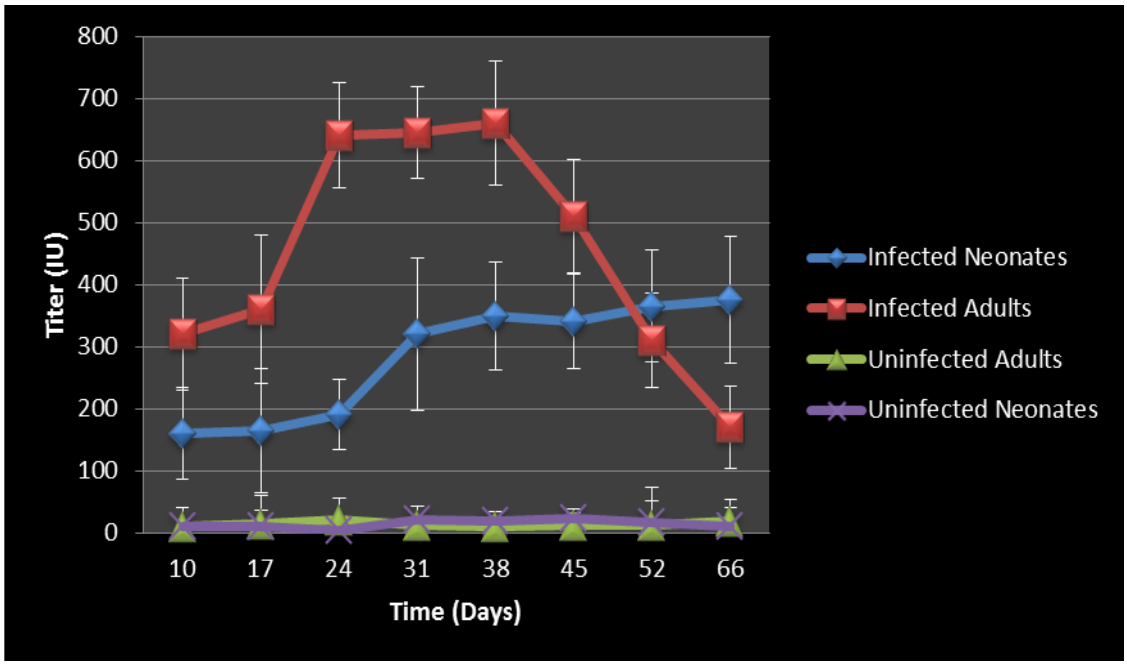

Figure 18: Chlamydia antibody titers over time

Antibody titer graph shows strong antibody responses to Chlamydia from infected adult mice but relatively decreased and /or retarded antibody titers in the infected neonatal mouse groups. Infected adult titers dropped once infections cleared, however infected neonatal mice did not clear the infection and their titers consistently increased over the course of the study.

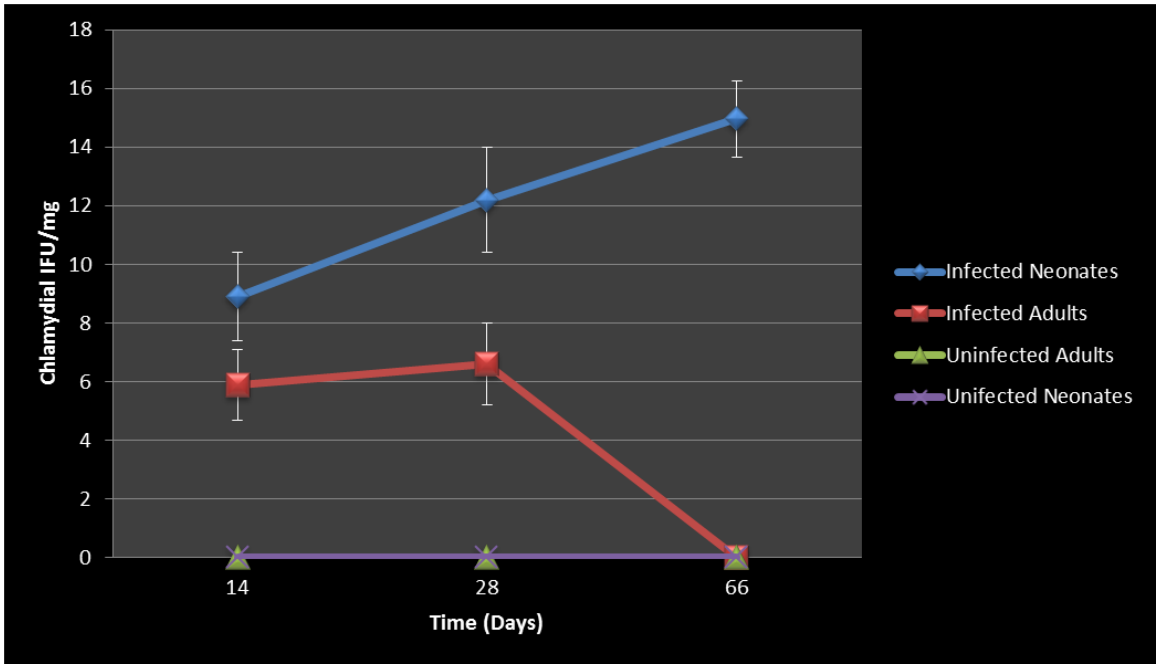

Figure 19: Cultured chlamydial carriage/concentration in the lungs

Infected adult mice were able to clear the chlamydial infection after day 28. Infected neonates were unable to clear the chlamydial infection during the course of this study and chlamydial concentration per/mg of tissue as well as total chlamydial carriage in lung continued increase over time.

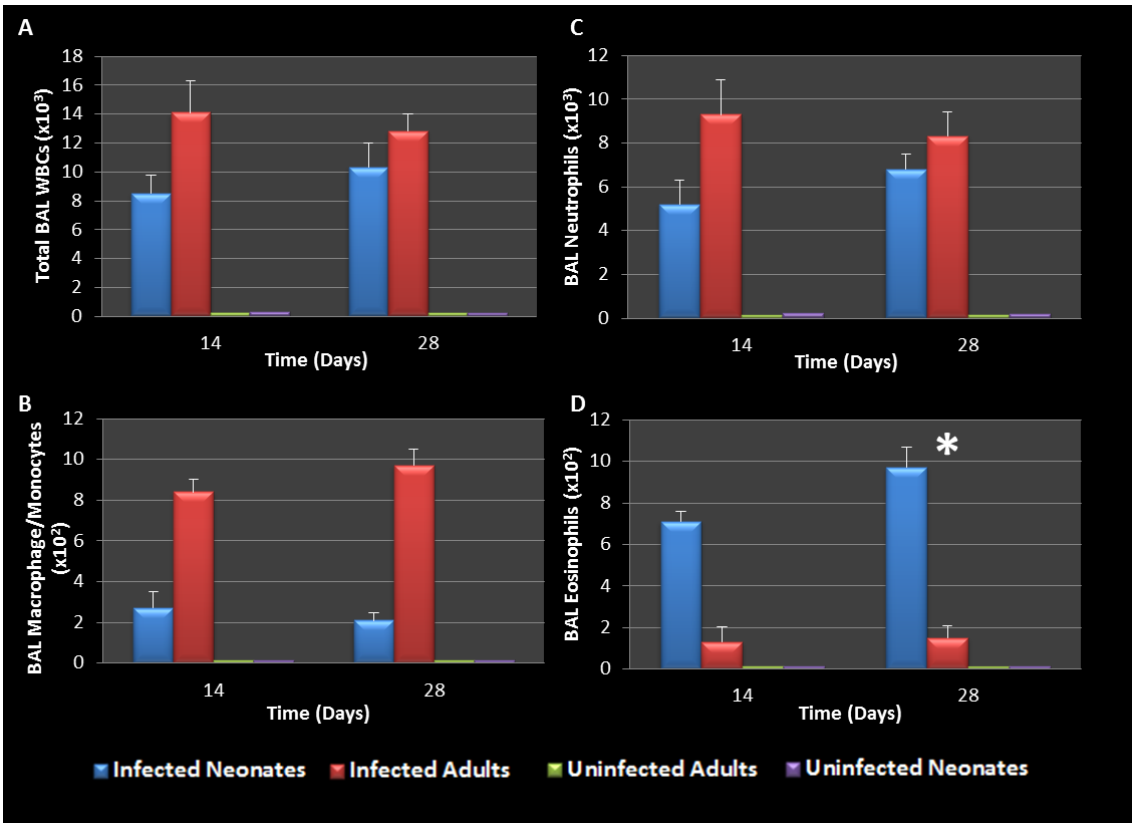

Figure 20: BAL cellularity during chlamydial infection

Elevated total WBCs and neutrophil levels were seen in infected neonatal and adult groups (A, C). Infected adults had elevated macrophage/monocytes, whereas infected neonates were characterized by significantly elevated eosinophil levels on day 28 pi compared to uninfected adults (P=0.0449, panel D).

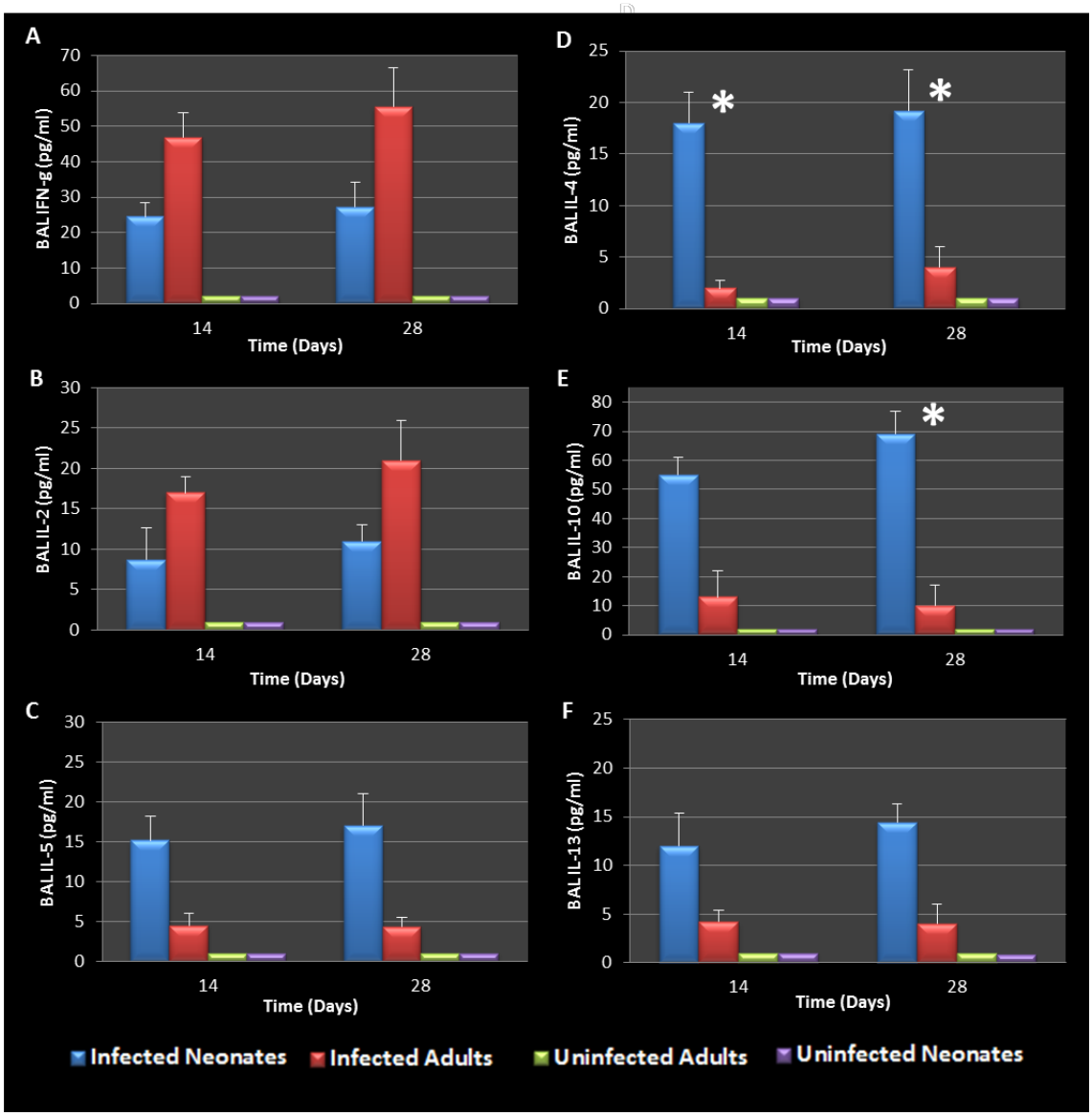

Figure 21: BAL cytokines during chlamydial infection

Infected adults displayed a more pronounced Th1 cytokine response (IFN-γ and IL-2) than infected neonates (A, B). Infected neonates responded to chlamydial infection with a robust Th2 cytokine response (IL-4, IL-10, IL-5, and IL-13) compared to their adult counterparts (C-F). Specifically, neonatal mice produced significantly elevated levels of IL-4 compared to infected adults 14 and 28 days post infection (P=0.0312, 0.0248 respectively, D) as well as IL-10 28 days post infection (P=0.0434 E).

purified chlamydial elementary bodies to assess T- cell specific cytokine production. The cytokine profiles were identical to that seen in the BAL fluid, clearly highlighting the contrasting Th1 cytokine production seen in adult animals compared to the Th2 cytokine profile elicited by neonatal respiratory chlamydial infection (Figure 6). Specifically, neonatal mice had significantly more IL-5 and IL-4 cytokine production in response to chlamydial antigen (P=0.0456, 0.0444 respectively (Figure 22C, D). The magnitude of cytokine response in the tissue culture fluid from draining lymph node T cells was significantly greater than that seen in the BAL fluid.

The robust cytokine response in the airway of *Chlamydia*-infected adults and neonatal mice was complemented by the accumulation of predominantly macrophages ($9.7x10^2$) and neutrophils ($8.3x10^3$) in the BAL fluid of adult mice 28 days post infection, along with markedly less eosinophils ($1.5x10^2$). In contrast, 28 days pi infected neonatal mice presented with significantly elevated eosinophils ($9.7x10^2$, P=0.0449) compared to infected adults. There was no significant difference in infected neonatal BAL neutrophils ($6.8x10^3$), or macrophages ($2.1x10^2$) compared to infected adults (Figure 20 A-D), however infected groups had significantly elevated levels of all cell types when compared to uninfected controls. This highlights a major difference in the early life response of mice to chlamydial infection compared to adult counterparts.

Cellular and Cytokine Response to Induction of Allergic Airway Disease in Neonatal and Adult Mice

Having established that early life chlamydial infection induces different responses in neonatal compared to adult mice, we wanted to further determine if induction of allergic airway disease had different cytokine and cellular profiles as well. In this model, following respiratory infection with Chlamydia, mice were sensitized to Ova and some groups were challenged over several days while others remained unchallenged for comparison(Table 12).Our infected neonatal group (group 3 neonate) who still had active infections, and no AAD presented with significantly elevated numbers of total white blood cells, neutrophils and eosinophils, but only background

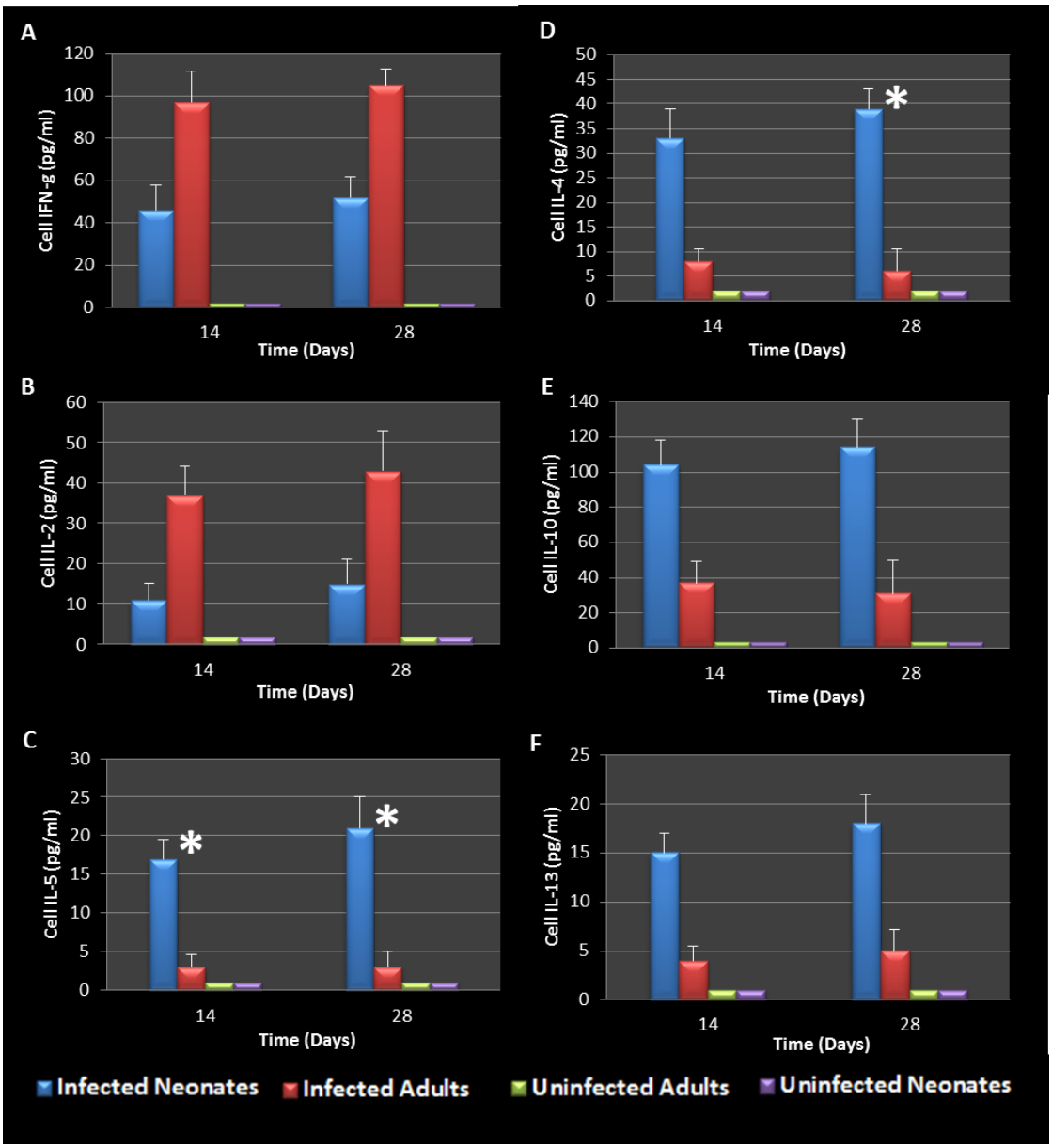

Figure 22: Mediastinal lymph node cell stimulation during chlamydial Infection.

WBCs harvested from mediastinal lymph nodes were stimulated with heat killed chlamydial antigen and resulting cytokine secretions revealed that cells from infected adult mice secreted elevated levels of IFN-g and IL-2 when compared to infected neonates and controls (A, B). Cells harvested from infected neonatal mice secreted predominantly Th2 (IL-5, 4, 10, and 13) cytokines in response to chlamydial antigen compared to infected adults and control groups (C-F). WBCs from infected neonatal mice 14 days and 28 days post infection produced significantly elevated levels of IL-5 (P=0.0353, 0.0456 respectively, C) and IL-4 (P=0.0444, D) when compared to WBCs from infected adults. Cells were also stimulated with PBS and J774A.1 proteins as controls (data not shown).

Neonatal & Adult Groups	Description of Treatment
Group 1	Infected, Sensitized with Ova & Challenged
Group 2	Uninfected, Sensitized with Ova & Challenged
Group 3	Infected, No Sensitization & No Challenge

Table 12: Animal grouping and treatments

Sensitization and subsequent challenge with ovalbumin is required to induce allergic airway disease.

numbers of monocyte/macrophage cells when compared to their infected adult counterparts (group 3 adults) who did not have active infections (P=0.0001, 0.0003, and 0.0004 respectively, Figure 23A-D). Indeed all groups which had allergic airway disease induced (see table 12) had airways characterized by elevated levels of BAL eosinophils; however, infected neonates who did not have AAD induced, had comparable levels of eosinophils in their BAL fluid to the AAD induced groups, suggesting that chlamydial infection plus challenge with Ova results in elevated eosinophils. The most significant elevation in eosinophils, however, was seen in neonatal animals that had active infections and AAD induced (Figure 23D). There was no significant increase in BAL macrophage/monocytes in any treatment group.

BAL and mediastinal lymph node cytokine production in AAD induced animals was also assessed. Infected neonatal animals demonstrated a significant increase in the production of BAL Th2 cytokines (IL-4, -5, -10 and -13) in response to Ova challenge and sensitization, but also responded with the production Th1 cytokines (IFN-γ and IL-2) compared to their adult or uninfected counterparts (Figure 24). Indeed, there was no statistical difference in the level of cytokines produced by infected neonatal mice that were not sensitized but later challenged with Ova (Group 3 Neonates vs. Group 1 Neonates in graphs, see table 1) when compared to the infected, sensitized and challenged group, suggesting that these animals might have become hyperresponsive as a result of early life chlamydial infection. In contrast, Group 3 neonates with active infections and no AAD produced significantly elevated levels of IFN-g, IL-5, and IL-4 when compared to group 3 adults which had cleared infections and no AAD(P=0.0092, 0.0362, and 0.0092 respectively, Figure 24A, C, D). Though not significant, these neonates also presented with elevated IL-2, IL-10, and IL-13 when compared to adult mice, showing that chlamydial infection alone was able to elicit a Th1/Th2 cytokine response.

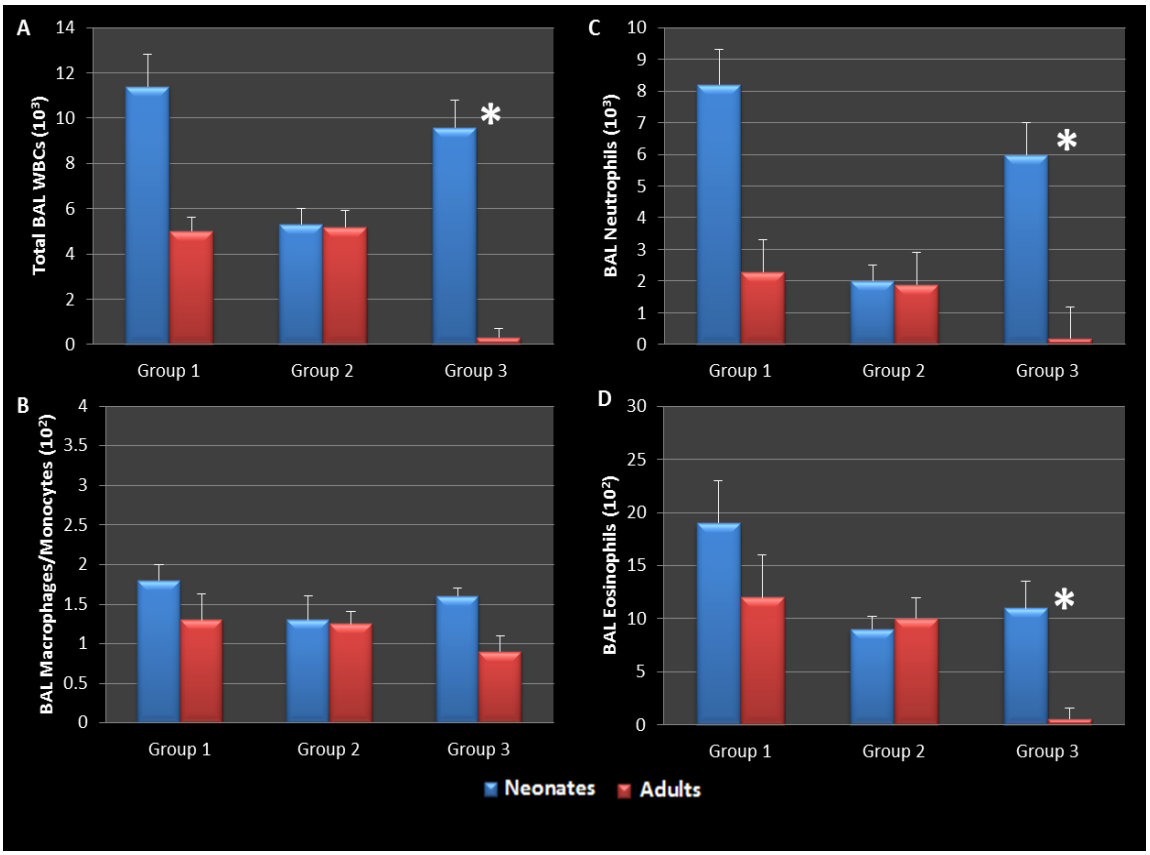

Figure 23: BAL cellularity during allergic airway disease

Infected neonatal groups with active infections had elevated levels of total WBCs, neutrophils, and eosinophils compared to all other groups (A, C, D). All groups which had allergic airway induced (AAD) with Ova had elevated levels of BAL eosinophils; however group 3 neonates who had active infections but did not have AAD induced had significantly elevated levels of BAL WBCs, neutrophils, and eosinophils when compared to their adult counter parts (P=0.0001, 0.0003, and 0.0004 respectively); group 1 neonates who had active infections and AAD induced had enhanced levels of BAL WBCs, neutrophils, and eosinophils when compared to all other groups (A, C, D). There was no significant increase in BAL macrophage/monocytes in any group (B).

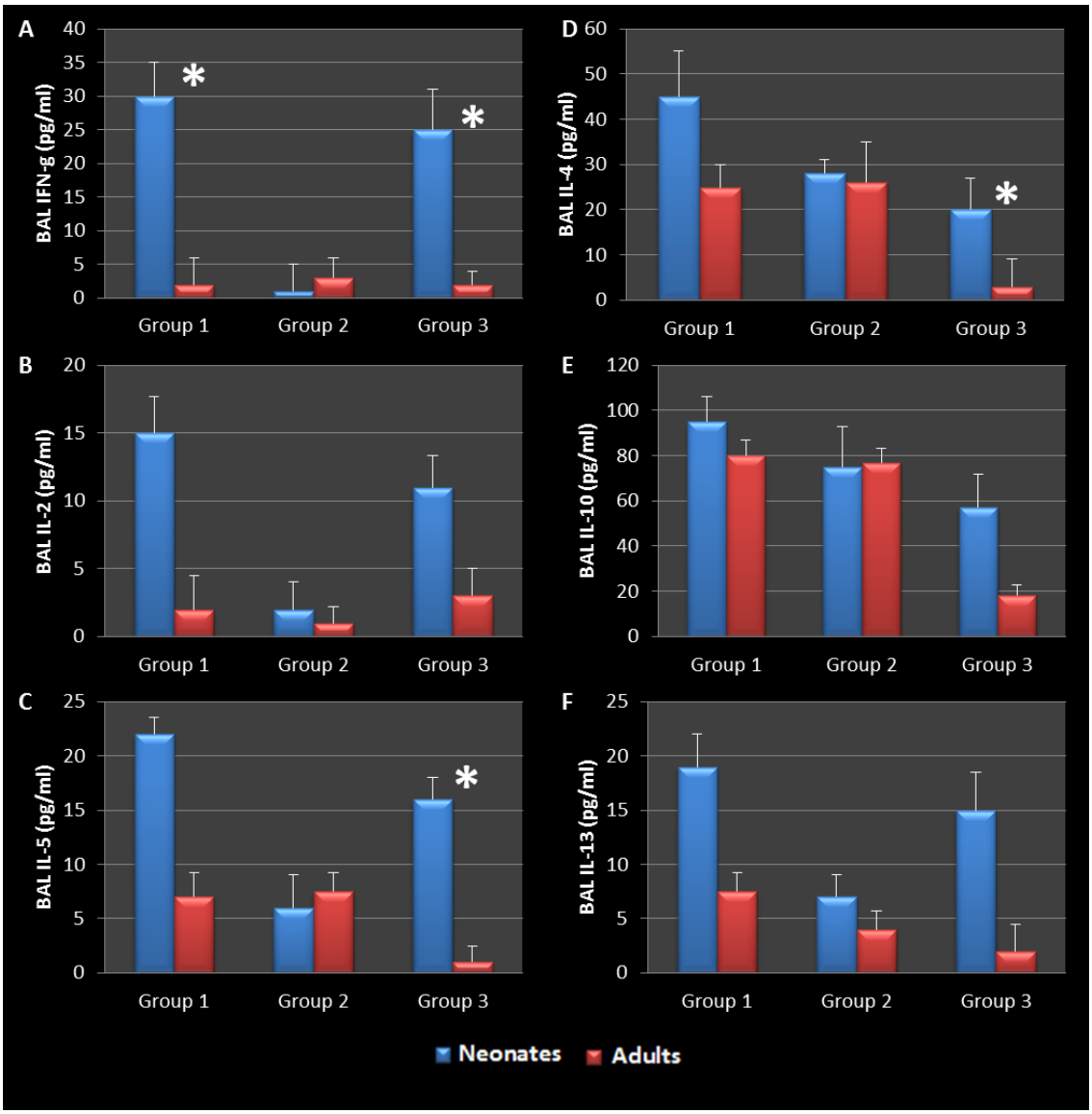

Figure 24: BAL cytokines during allergic airway disease

Infected adults, who cleared infection, developed a mixed Th1/Th2 immune response during allergic airway induction (C-F). Group 1 and 3 infected neonates responded with a significant increase in IFN-g (P=0.0049, 0.0092 respectively, A) and elevated levels of IL-2 when compared to corresponding adult groups (B). Th2 cytokines that were produced in all groups that had AAD induced (C-F). Infected neonates hyper reacted to ovalbumin compared to their adult and uninfected counterparts. Importantly to note, group 3 neonates who had active infections but no AAD produced significantly elevated amounts of IL-5 and IL-4 (P=0.0152, 0.0362 respectively C, D) and elevated levels of IL-10, and 13 compared to all other groups (E, F). Collectively, the data therefore suggests that early life chlamydial infection induces an allergic airway response upon allergen challenge that is typical of asthma pathogenesis and supports a chronic infection model airway hyperresponsiveness**.**

Mediastinal Lymph Node T cell-specific Cytokine Response to *Chlamydia* and Ova Stimulation

To assess and confirm the specific immunological response to Ova stimulation, mediastinal lymph node T cells were cultured as previously described for assessment of *Chlamydia* and Ova antigen specific cytokine. Ova-specific T cells from all Ova sensitized and challenged adult and neonatal animals produced significant levels of IL-4, -5, -10 and -13 in response to Ova stimulation in vitro (Figure 26 C-F). However, there was no production of the Th1- specific cytokines, IL-2 and IFN-γ (Figure 26 A & B). This was accompanied by a significant increase in the production of Ova-specific IgE antibodies in both the serum and BAL fluid of all sensitized and challenged groups (Figure 27). Mediastinal lymph node T cells from all *Chlamydia*-infected neonatal groups released significantly elevated levels of IL-2, -4, -5, -10, -13, and IFN-γ in response to chlamydial antigen when compared to controls (Figure 25). By contrast, mediastinal lymph node T cells from similarly treated adult mice produced no significant levels of Th2 cytokines (IL-4, -5, -10 and -13), and much lower levels of IL-2 or IFN-γ when compared to similarly treated neonatal animals. As controls, cells were also stimulated with cell lysate and sterile saline, resulting in no cytokine production. In addition, only infected neonatal mice produced *Chlamydia*-specific IgE antibodies when compared to all other groups. The level and antigen specificity of *Chlamydia*-specific IgE antibodies increased significantly over time as demonstrated by the band intensity and diversity on the Western blot image (Figure 28). Moreover, only approximately 6 chlamydial antigens appear to induce IgE production in this model, including the 40kDa major outer membrane protein (Momp), chlamydial lipopolysaccharide (cLPS at 4-6kDa), the *Chlamydia* lectin binding proteins (LBP, 18 and 27kDa) and the cysteine rich protein A (CrpA, 15kDa). Infected adult animals demonstrated no *Chlamydi*a-specific IgE in their sera, even at the time of sacrifice.

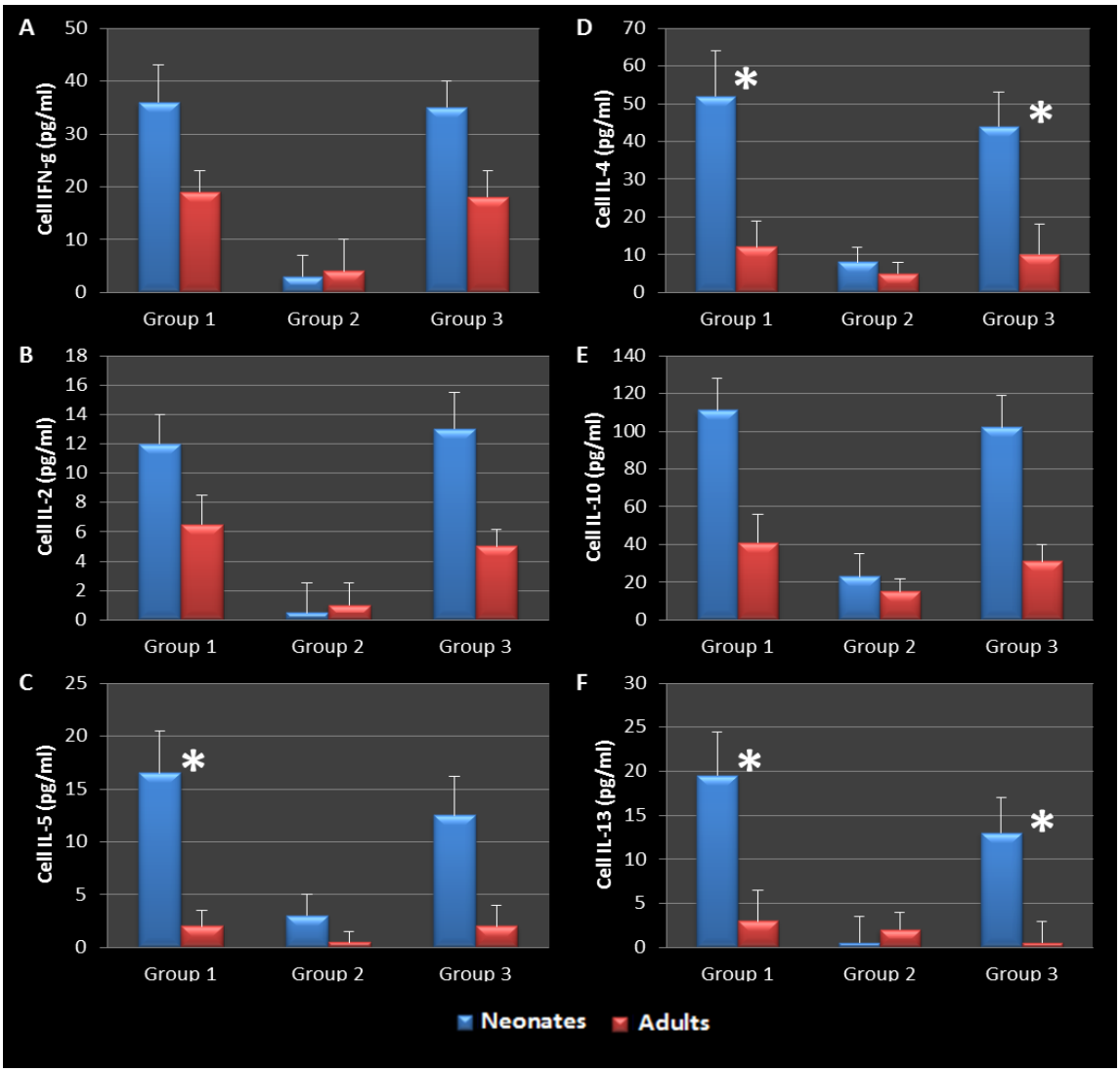

Figure 25: Mediastinal lymph node cell stimulation during allergic airway disease

WBCs harvested from mediastinal lymph nodes were stimulated as described previously described (Figure 5). Stimulation revealed that cells from infected neonatal mice with active infections (Groups 1 and 3) secreted elevated levels of IFN-g and IL-2 when compared to infected adults and controls (A, B). Cells harvested from infected neonatal mice secreted predominantly Th2 (IL-5, 4, 10, and 13) cytokines in response to chlamydial antigen compared to infected adults and control groups (C-F). Infected neonates with AAD (group 1) produced significantly more IL-5, IL-4, and IL-13 compared to corresponding adult groups (P=0.0302, 0.0437, and 0.0362 respectively C, D, F). Infected neonates which did not have AAD were also able to produce significantly elevated levels of IL-4 and IL-13 compared to adults groups as well (P=0.0456, 0.0075 respectively C, F). Cells were also stimulated with PBS and J774A.1 proteins as controls (data not shown).

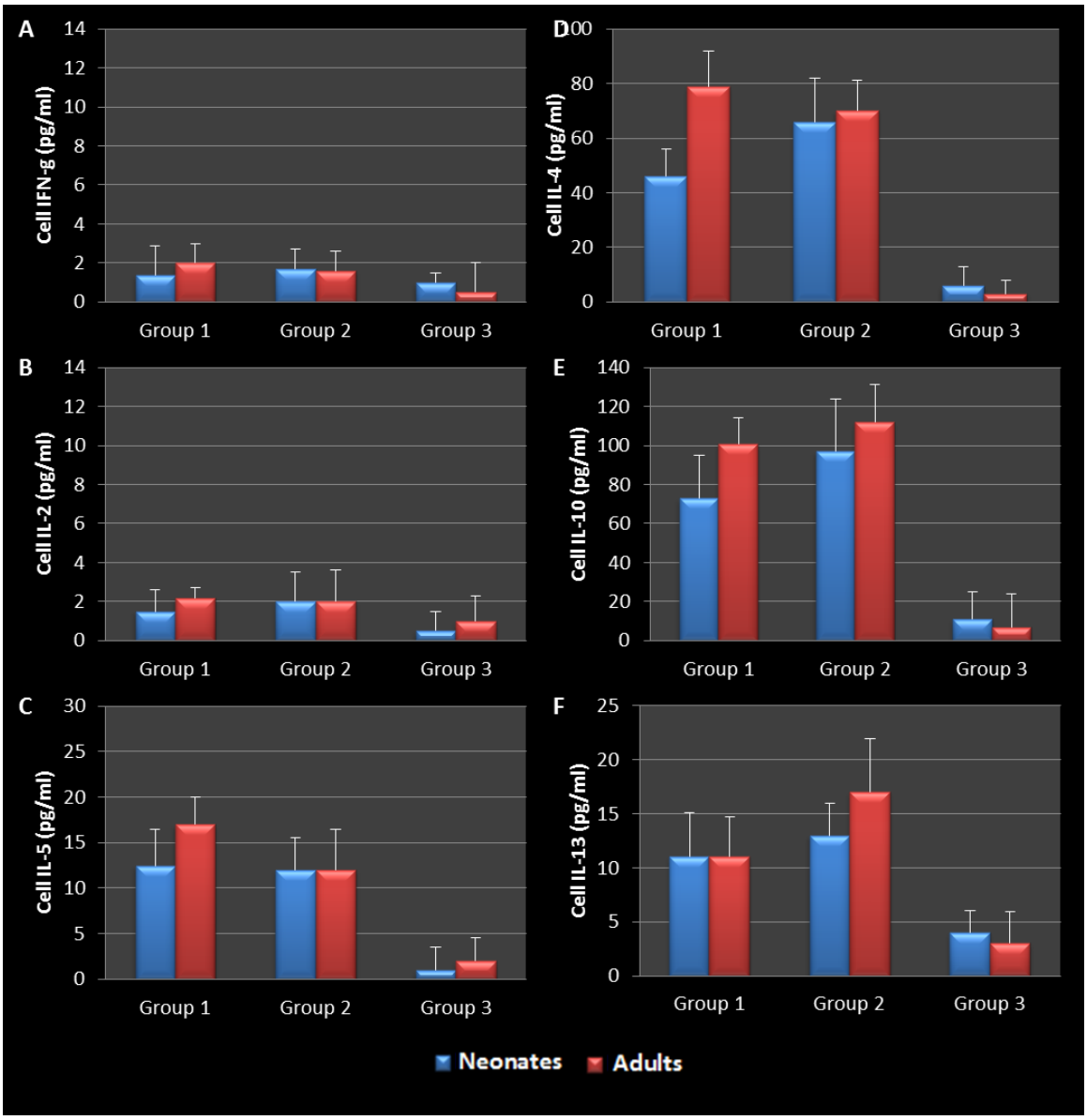

Figure 26: Mediastinal lymph node cell stimulation with OVA

WBCs harvested from mediastinal lymph nodes were stimulated as described previously described but ovalbumin was used instead of heat killed chlamydial organisms. Stimulation revealed that cells from groups 1 and 2 that were sensitized and challenged with ova secreted predominantly Th2 (IL-5, 4, 10, and 13) cytokines in response to ova antigen compared to unsensitized/challenged control groups (C-F). Th1 cytokines (IFN-g, IL-2) were produced in negligible amounts in response to ova stimulation (A, B).

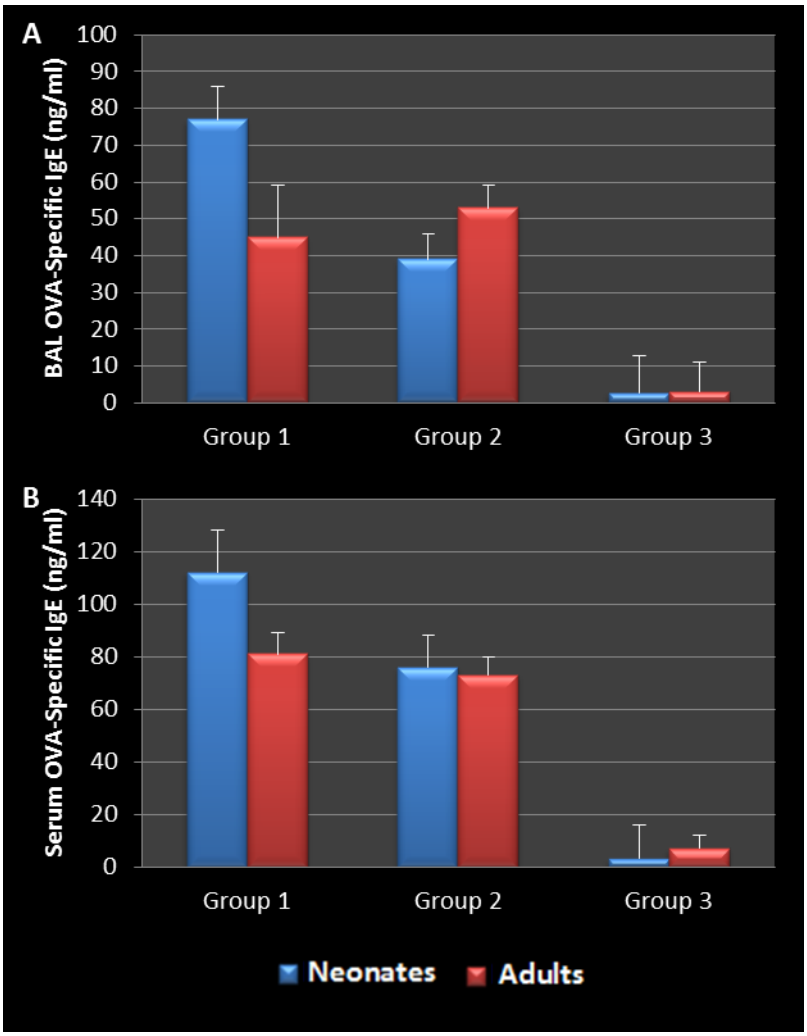

Figure 27: BAL Ova-Specific IgE during Allergic Airway Induction

Allergic airways were induced using ovalbumin. Groups which were both sensitized and challenged with ova had significantly elevated levels of ova-specific IgE antibodies present in BAL fluid (A) and serum (B), demonstrating successful allergic airway induction. Although not significant, infected neonatal group produced more ova-IgE than comparable adult group (Group 1).

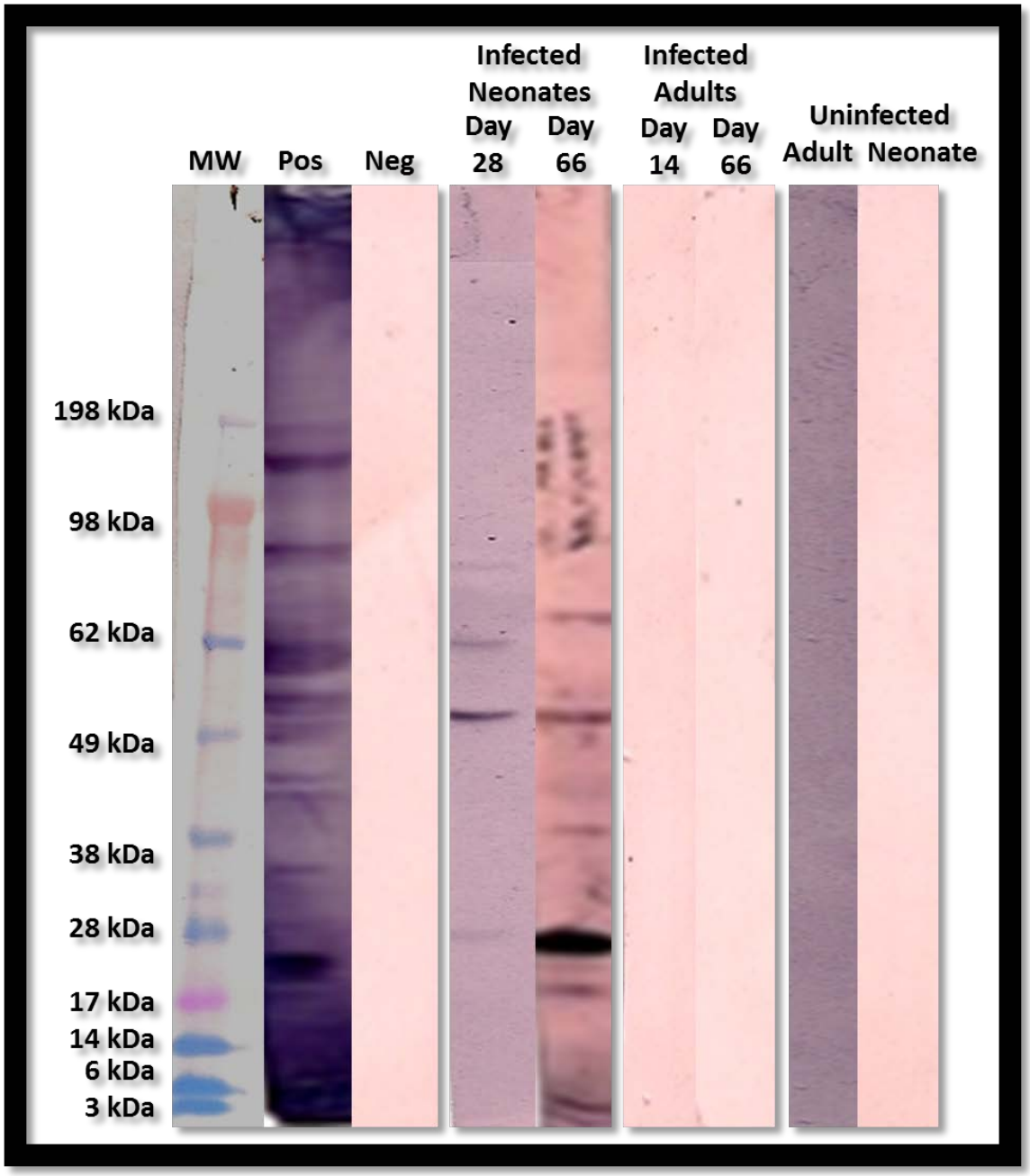

Figure 28: Detection of *Chlamydia* Specific IgE

Western Blot analysis revealed the presence of *Chlamydia*-specific IgE antibodies in the BAL fluid of infected neonatal groups only. Antibodies appeared as early as 28 days post infection and were still detectable 66 days post infection. *Chlamydia*-specific IgE antibodies appear to have been generated against a variety of chlamydial antigen representing a total of approximately 6 proteins. All proteins that induced IgE were either secreted or surface exposed.

Duration of Effects of Infection on AAD

Neonatal respiratory infection with *Chlamydia* resulted in chronic infection of lungs with increased infiltration over time. *Chlamydia* became systemic with time leading to increased bacteria load in the liver and spleen by day 14. While infected adult animals cleared the infection after day 42, neonatal mice remained infected for the duration of the study. However, these infected neonatal animals did not demonstrate overt physiologic signs of this infection and respiratory distress was not increased over time, although levels of both Th1 and Th2 cytokines remained elevated at 5 days after the last Ova challenge.

mRNA Expression of Histidine decarboxylase, IgE and its High – and Low – Affinity Receptors

Neonatal lung infection with *Chlamydia* resulted in significant induction of mRNA expression for IgE and its high- (FcεR1α,β,γ) and low- (CD23) affinity receptors, while infected adult controls did not. There was a concomitant increase in Th2 regulatory cytokine secretion (IL-4, -5, -10, and -13) in BAL and serum of neonatal mice, while adult mice secreted Th1 cytokines (IL-2, IFN-γ) in response to airway infection (Figure 29). mRNA expression of HDC, the only rate-limiting enzyme that catalyzes the formation of histamine from L-histidine, along with CD40L, were also significantly increased in infected neonates compared to controls. While infected adult mice cleared the infection by day 30, neonates never cleared the infection and secreted *Chlamydia*-specific IgE in both serum and BAL. Ova sensitization and subsequent challenge significantly increased mRNA expression of these allergic markers along with cytokine secretion in the mouse airway (Figure 30).

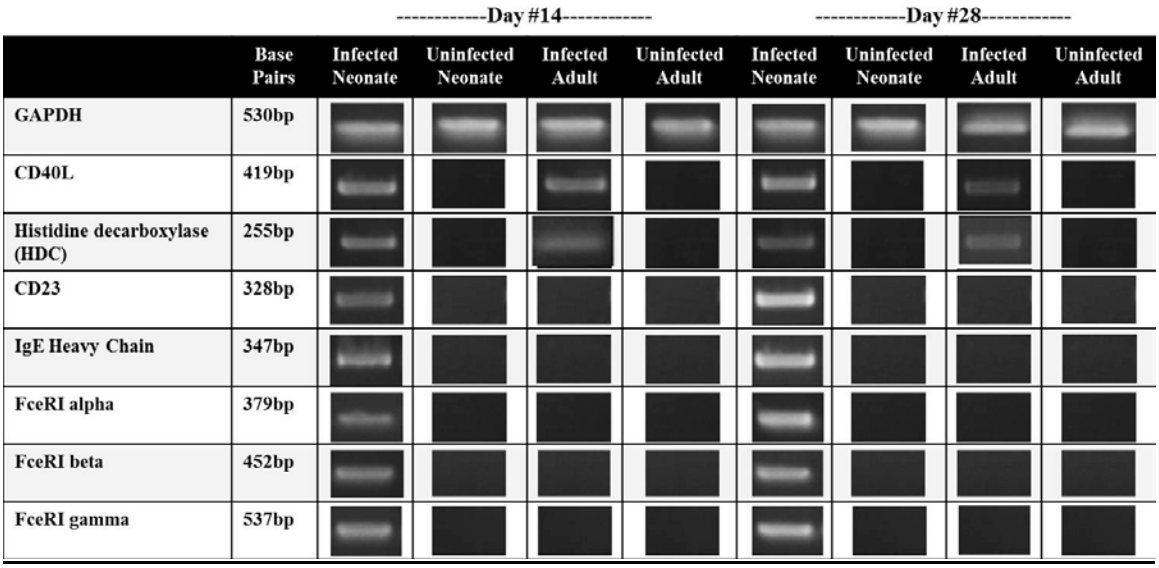

Figure 29: Characterization of chlamydial infection in neonatal and adult mice

Neonatal lung infection with *Chlamydia* resulted in significant induction of mRNA expression for IgE and its high- (FcεR1α, β and γ) and low- (CD23) affinity receptors, while infected adult controls did not. mRNA expression of HDC, the only rate-limiting enzyme that catalyzes the formation of histamine from L-histidine, along with CD40L (primarily expressed on the surface of activated T-cells leading to B-cell proliferation), were also significantly increased in infected neonates compared to uninfected controls.

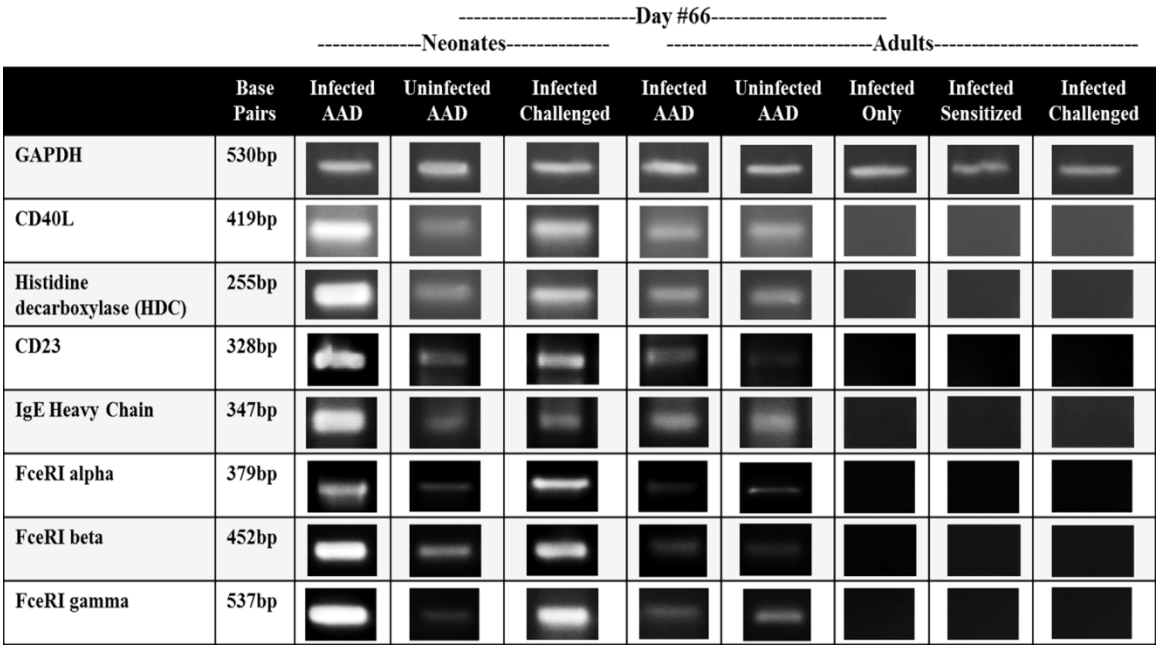

Figure 30: Characterization of chlamydial infection in neonatal and adult mice during allergic airway disease

Ova sensitization and subsequent challenge significantly increased mRNA expression of allergic markers along with cytokine secretion in the airways of mice that were sensitized and challenged with Ova. Infected neonatal mice that had not cleared infections 66 days p.i. expressed the highest levels of allergic mRNA markers. Ova sensitization and challenge enhanced expression of these markers in infected neonatal but not adult mice.

Discussion

Asthma is a major public health problem and remains a significant burden on families and society in general. The most recent asthma surveillance data from the Centers for Disease Control and Prevention (CDC) report that asthma cost our nation $56 billion each year and that 1 in 11 American children have the disease (17). Asthma is the most common chronic disease of childhood and the numbers of young people and children with asthma continues to rise. While many cases of asthma are successfully managed and patients lead a relatively normal life, there is no cure for the disease and a growing number of severe, persistent asthmatics present with a phenotype that is refractive to oral or inhaled corticosteroid treatment. New reports confirm that patients with severe refractory asthma have the greatest unmet treatment needs to improve asthma control and reduce exacerbation risk (18). In order to effectively treat, especially children with refractory asthma, it is necessary to better understand the etiology and or mechanisms involved in the development childhood asthma. Previous studies demonstrated that early-life respiratory viral infections, specifically with respiratory syncytial virus (RSV), increased the risk of subsequent development of childhood asthma (19). In the model reported in the that study, allergic airway inflammation, characterized by eosinophil recruitment and Th2 cytokine infiltration was prominent only in animals that had recovered from neonatal infection with pneumonia virus of mice and then later sensitized and chronically challenged with antigen (19).

In the current study we sought to better understand the underlying immunological mechanisms involved in the development of childhood asthma. We have demonstrated that while chlamydial organisms typically induce a Th1 type cytokine response with corresponding macrophage and neutrophil infiltration, neonatal respiratory infection led to production of Th2 dominant cytokines and infiltration of neutrophils and eosinophils in the airway. These results are similar to those previously reported by Horvat et al (14) using a similar Ova mouse model. In the current study however, we also demonstrated prolonged infection in neonatal animals that never cleared the bacteria from their lungs over the entire course of the study. While we utilized twice

the inoculum (200 IFU) used by Horvet et al, this inoculum still represents less than what would typically be found in a single chlamydial inclusion, that can hold between 500 to 1000 EBs (20).

Mediastinal lymph node T cells and BAL fluid revealed a significant increase in Th1 and Th2 cytokines upon Ova sensitization and challenge in neonatal animals, but to a significantly less extent in adult animals who produced no Th2 cytokines upon Ova sensitization and challenge. Indeed the levels of Th2 cytokines seen in neonatal animals that were infected and challenged without Ova sensitization were essentially similar to those of infected neonates who were both sensitized and later challenged with Ova. These data suggest that adult animals infected with *Chlamydia* in early life, display a hyperresponsive airway and a predisposition to allergic inflammatory diseases later in life. This hypothesis is supported by studies done by You, et al, demonstrating that severe RSV infection during neonatal development significantly altered lung structure and the pulmonary immune micro-environment, which could potentially increase the risk of airway hyperresponsiveness later in life (21).

Neonatal mice never cleared the chlamydial organisms from their lungs and the bacteria disseminated to the liver and spleen and could also be isolated from the peripheral blood starting at 7days post infection and at the time of euthanasia. This study also confirmed that mice infected as neonates but not adults, produced and released *Chlamydia*-specific IgE antibodies in both the serum and BAL fluid, while the adult mice did not.

The data have important implications in asthma development and potential treatment or intervention for early-life respiratory infection. In a recent report by Seigle et al, the authors utilized a murine model of childhood asthma to demonstrate the utility of administration of anti-IL-4 or anti-IL-25 (22). These antibodies prevented development of some key features of asthma, suggesting that suppression of a Th2 response during the neonatal period or later in childhood could be effective for preventing later chronic severe disease(22).

We therefore conclude that chlamydial airway infection early in development, can lead to significant alteration in lung structure and the immune architecture of the respiratory micro-

environment. Early life *Chlamydia* infection was not cleared in this model, suggesting that this type of structural airway damage and immune modulation through continued secretion of Th2 cytokines continued unabated. Upon allergen exposure, the interaction between early-life chlamydial infection which results in chronic pulmonary inflammation and allergen sensitization exacerbates allergic airway disease through increased cytokine secretion and cellular infiltration. This combination has the potential to lead to more severe asthma that is refractory to inhaled steroids, since the root of the initial inflammation is a pathogen and could still be lurking in the lung tissue.

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CHAPTER 6

THE ROLE OF HEPOXILINS AND HISTAMINE PRODUCING NEUTROPHILS IN INFECTIOUS ASTHMA

Abstract

Rationale: Hepoxilins are biologically active metabolites of arachidonic acid that are formed through the 12-lipoxygenase pathway. Hepoxilins were recently identified as potent neutrophil chemoattractants in the intestinal mucosa, playing an even greater role than IL-8. Our goals in this study is to determine if *C. pneumoniae* infection of the airways of asthmatics induces the release of hepoxilins, to determine if hepoxilin is a neutrophil chemoattractant in the airway, and finally, to determine if these neutrophils produce histamine in response to *C. pneumoniae* infection.

Purpose: To determine if neutrophils, provoked by *C. pneumoniae*-induced lung infection, undergo transmigration through an IL-8/hepoxilin gradient and greatly expand their capacity to synthesize histamine, thereby contributing to airway inflammation and pathology.

Methods: Adult (4 wks) BALB/c mice were infected intranasally with *Chlamydia (MoPn)* and 4 weeks later were sensitized and challenged with ovalbumin. Allergic airway disease (AAD) was characterized by examination of serum and bronchoalveolar lavage fluid (BAL) cellularity, cytokine production and antibody response. The presence of *Chlamydia* was determined by PCR and culture. We further assessed AAD by evaluating the expression of mRNA for histidine decarboxylase (HDC) in lung tissue and in purified BAL neutrophils. Hepoxilin production was assessed via Western blot using antibodies specific to 12-lipoxygenase precursor (12-LO).

Results: Chlamydial infection in mice induced increased production of Th_1 cytokines (IL-2, IL-12, TNF-a, IFN-γ) in BAL compared to uninfected mice. Infected mice responded to chlamydial infection with robust neutrophil infiltration. When allergic airway was induced using ovalbumin, AAD induced mice increased their production of IL-4, IL-5 and IL-13 by >3 fold compared to unsensitized groups. In addition, 12-LO mRNA is up regulated in mice infected with *Chlamydia*,

but not in uninfected AAD mice. Histidine Decarboxylase (HDC) mRNA expression is increased in BAL neutrophils of *Chlamydia* infected mice, but was not in AAD induced only or uninfected mice.

Conclusions: Our data indicates histamine is produced by BAL neutrophils in response to chlamydial infection and *Chlamydia* elicits 12-LO production which can induce neutrophil infiltration.

Introduction

Asthma is a multifactorial disease characterized by recurrent cough, wheezing, chest tightness or shortness of breath, leading to airway inflammation. Approximately 34.1 million Americans have been diagnosed with asthma by a health professional during their lifetime (1). Several types of cells are involved in the pathophysiology of asthma. The contribution of mast cells, lymphocytes, and eosinophils has been well established. Although eosinophilic airway inflammation is recognized as an important feature of some patients with chronic, stable asthma, evidence supports an important role for neutrophils in asthma (2). Neutrophils are polymorphonuclear leukocytes that play an essential role in the immune system, acting as the first line of defense against bacterial and fungal infections. Their role in the inflammatory process was once thought to be restricted to phagocytosis and the release of enzymes and other cytotoxic agents, but it is now known that these are the first cells recruited to the site of allergic reaction and can release diverse mediators that have profound effects on the airways of asthmatic individuals (3). Their presence may therefore influence clinical presentation and has been linked to the development of severe chronic asthma and sudden severe attacks (3). Moreover, a review of the literature found that only around 50% of asthma cases were associated with eosinophilic inflammation, and that in most other cases asthma was accompanied by an increase in airway neutrophils (3). Bacterial infections at epithelial surfaces, such as those that line the gut and the lung, stimulate the migration of neutrophils through the coordinated actions of chemoattractants

secreted from pathogen-stimulated epithelial cells (4-6). IL-8 has been shown to stimulate neutrophils to leave the bloodstream at a local site of mucosal inflammation. However, basolateral IL-8 release is insufficient to induce the migration of neutrophils across the intestinal epithelium, suggesting that the production of other inflammatory mediators, whose release would probably be polarized apically, are important for the execution of this step in the inflammatory pathway (7, 8). The chemical gradient used by neutrophils to move between adjacent epithelial cells and traverse the tight junction at the apical neck of these mucosal barriers has eluded identification for decades (9). One such factor involved in attracting polymorphonuclear leukocytes across the epithelium was identified in 2004 by the McCormick group at UMass Worcester as the eicosanoid, hepoxilin A3 (7).

HepA3 was shown to be selectively secreted from the apical surface of human intestinal epithelial stimulated with *Salmonella enterica* serotype Typhimurium (7). Hepoxilins are formed by a variety of tissues and possess a diversity of actions based on their ability to modulate ion fluxes, including calcium and potassium ions in the cell (10). More recent studies confirmed that the hepA3 chemoattractant is a key factor promoting the final step in neutrophil recruitment to sites of mucosal inflammation (9, 11). The data confirmed that hepA3 is synthesized by epithelial cells and secreted from their apical surface in response to conditions that stimulate inflammatory events (9). Once induced, hepA3 acts to draw neutrophils, via the establishment of a gradient across the epithelial tight junction complex. Disruption of the 12-lipoxygenase pathway (required for hepA3 production) dramatically reduces neutrophil-mediated tissue damage (7). However, it has not been definitively determined if hepoxilins and more specifically, hepA3, plays a role in neutrophil transmigration in the asthmatic lungs or if these cells contribute to AHA in addition to general inflammation. More importantly, it is not yet clear if specific bacterial and/or viral pathogens like *C. pneumoniae* can A) induce the increased release of hepoxilins into the airway or B) induce the release of histamine from the neutrophils that have recently arrived from the circulation due to hepoxilin or IL-8 chemotaxis.

Materials & Methods

Mouse model treatment and sample collection

IACUC approval was obtained through the University of Massachusetts to utilize 4 groups (15 each) of 2 week old mice for this study. Mouse asthma models were generated by sensitizing Balb/C animals with the 45 kDa egg-white glycoprotein, ovalbumin (OVA) intraperitoneally followed by several challenges via aerosolization. **Group 1** was infected via the airways with *C. muridarum* for 14 days and then sensitized and challenged; **group 2** was mock infected, challenged and sensitized with OVA on the same schedule as group 1. This treatment resulted in a Th-2 response in the lungs, with varying levels of airway responsiveness. **Group 3** was infected, but challenged and sensitized using saline instead of Ova; **group 4** was mock-infected and mock challenged with saline. Animals then had BAL fluid collected and the extent of hepoxilin release evaluated via measurement of hepA3 at days 7, 14, 28, and 42 when animals were sacrificed. Five animals from each group were sacrificed at each time point to obtain BAL fluid, cells and tissue for histology. Lung tissue to be used for RNA extraction was flash frozen in liquid nitrogen or on dry ice.

Analysis of mouse BAL fluid cellularity and the indirect presence of hepoxilin

Bronchoalveolar lavage fluid (BAL) was obtained by cannulation of the trachea and lavaging the airways with 2x 1 ml sterile saline. BAL cell counts were performed with a cell counting chamber (hemacytometer, improved Neubauer) under phase microscopy with results expressed as "number of cells per cubic millimeter". BAL differential counts were performed using Wright stained cytospin preparations of BAL. At least 200 cells were counted per slide to obtain statistically significant counts.

In order to determine the presence and quantity of hepoxilin in the lung tissue from the mouse model, we will utilize Western Blotting which is used routinely used by groups that work

with hepoxilins (9). With this method, we detected two lipoxygenase (LO) enzymes that selectively place epoxide structures from a hydroxyl residue positioned at the 5, or 12 carbon of arachidonic acid or its metabolites, the 5 or 12 carbon have demonstrated any capacity to stimulate hepA3 production. 12-LO is the precursor for hepoxilin and its presence directly indicates the presence of hepoxilin. Antibodies used to detect 5-LO and 12-LO were commercially produced from purchased from Santa Cruz Biotech (www.scbt.com). We further confirmed the production of hepA3 by RT-PCR for 5-LO and 12-LO as previously described utilizing published primers (9).

Isolation of BAL neutrophils and detection of histidine decarboxylase (HDC) mRNA

Cells were isolated from mice as described above and utilized for measurement of overall white blood cell infiltration and neutrophil population. Neutrophil isolation from BAL was done using a Mouse Neutrophil Negative Selection Kit from STEMCELL Technologies according to manufacturer's instructions. We utilized molecular genetics to determine if neutrophils in *C. pneumoniae* infected airways produce and release histamine. Neutrophils were also collected from mice that have not previously been exposed to *Chlamydia*. Following isolation RNA extraction was done using Trizol extraction. Expression of HDC mRNA was determined by RT-PCR using previously published primers (1).

Chlamydial infection

All mice were weighed initially and then daily. The rate of weight gain/loss was calculated (g/d) over the entire course of the study as an indicator of health status. Chlamydial lung infection was initially assessed by PCR and organism numbers in the lungs, liver and spleen were determined tissue culture. Briefly, lung tissues was cut and weighed followed by homogenization in sterile SPG. Homogenates were centrifuged and the supernatant used for culture and DNA isolation.

Chlamydial organisms were cultured utilizing 24-well plates with 12 mm coverslips

seeded with a semi-confluent monolayer of mouse macrophages (J774A.1) for 36 hours. Following incubation, cells were fixed and immunostained using Pathfinder antibody (Bio-Rad, Hercules, CA) according to manufacturer's instructions and inclusion forming unit counts (IFUs) were done.

Genomic DNA was isolated from BAL & lung tissue using the QIAMP DNA Blood mini kit (Qiagen) PCR was used to detect chlamydial DNA. Previously published *Ct* primers P1 and Omp2 were used to amplify a 1,130bp fragment (12).

Serum and BAL antibodies

Serum antibody titer to *Chlamydia* as well as Ova was evaluated from tail bleeds taken on a weekly basis and at time of euthanasia. *Chlamydia* antibody titers were evaluated by enzyme linked ImmunoSorbent assay (ELISA). The ELISA wells were coated with purified chlamydial EBs (300 ug/ml) and serial dilutions of each mouse serum added. Following the required washes, bound primary antibodies were detected by an AP-conjugated goat anti-mouse secondary antibody. Quantitative assessment of OVA-IgE in the BAL fluid and serum was conducted using OVA-specific mouse-IgE ELISA kit from BD Biosciences, according to the manufacturer's instructions**.** *Chlamydia* specific IgE antibodies were detected using Western blot assay as previously described.

Cytokine production in BAL

Total cytokines secreted in the lung milieu was determined through analysis of BAL fluid. Concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, TNF-a, TGFB1, and IFN-γ were determined using Multi-Analyte ELISArray Kits (Mouse Th1/Th2/Th17 Cytokines) (SABiosciences) according to manufacturer's instructions.

Statistical Analysis

Mice were evaluated in groups of 5. Results are presented as mean± SEM from each test and control group of mice. Analyses were performed using the SPSS Graduate Pack 11 for Windows and associations or differences are based on two tailed tests with p values <0.05.

Results

Chlamydial Lung Infection and Associated Pathology

Animals were weighed each day and the weight trend shows significant weight loss in *Chlamydia*-infected vs. uninfected mice starting at four days post infection. The most significant differences in weight gain were observed in adults between days 16 and 32, where on average infected mice were 30% (5.7g) smaller than uninfected mice. However, the rate of weight gain between infected and uninfected adult groups was not significantly different during the later parts of the time course (Figure 31).

To assess the humoral immune response to intranasal chlamydial challenge, sera weekly collected from each animal was assayed for anti-*Chlamydia* antibody titers. The data revealed strong antibody responses to *Chlamydia* infection in infected mice (Figure 32). Titers began to decrease in infected animals around the time of infection clearance (28-42 days pi).

The concentration of chlamydial organism in the lungs (IFU/mg) of infected animals increased significantly with time and there was almost a 2 fold increase from day 7 to 14 (Figure 33). However, total carriage began to drop after day 14 and infected animals ultimately cleared the infection by 42 pi.

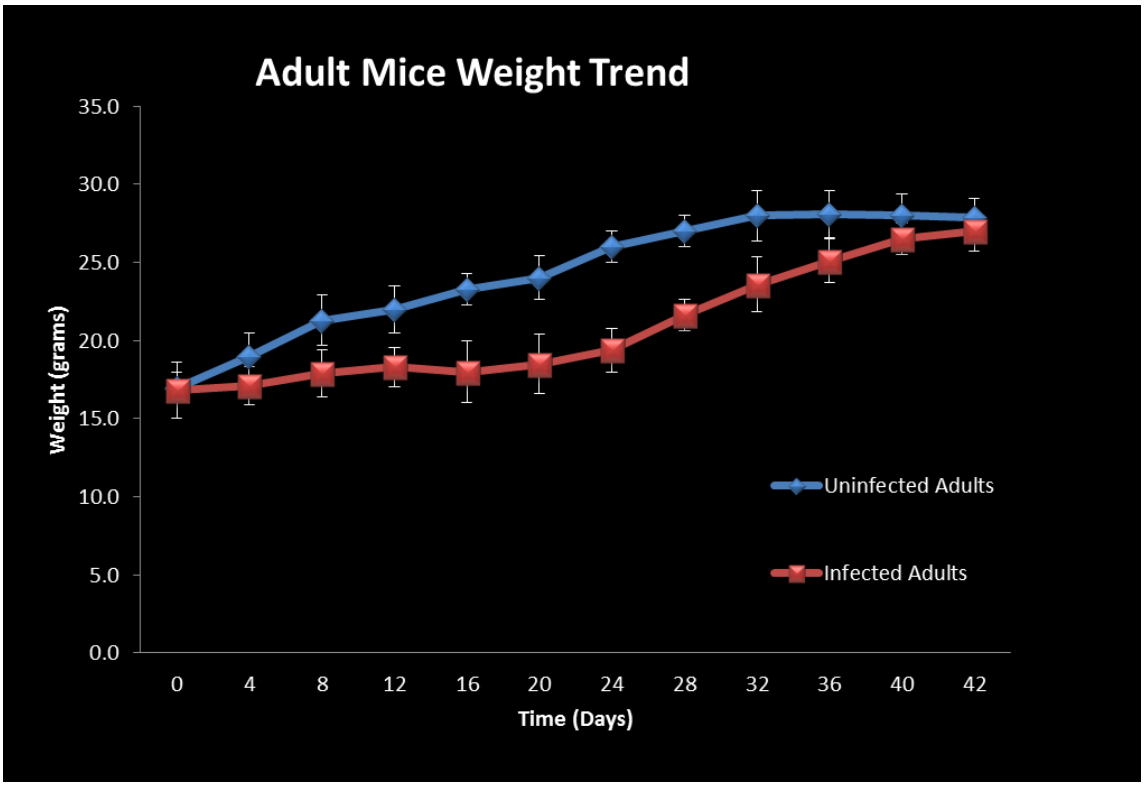

Figure 31: Adult weight trends

Weight trend graphs show weight loss in *Chlamydia* infected animals vs. uninfected animals between days 6 and 32 post infection (on average 5.7g smaller (30%). However, infected adults recovered lost weight by day 42. Error bars represent variation in animal weights within each group.

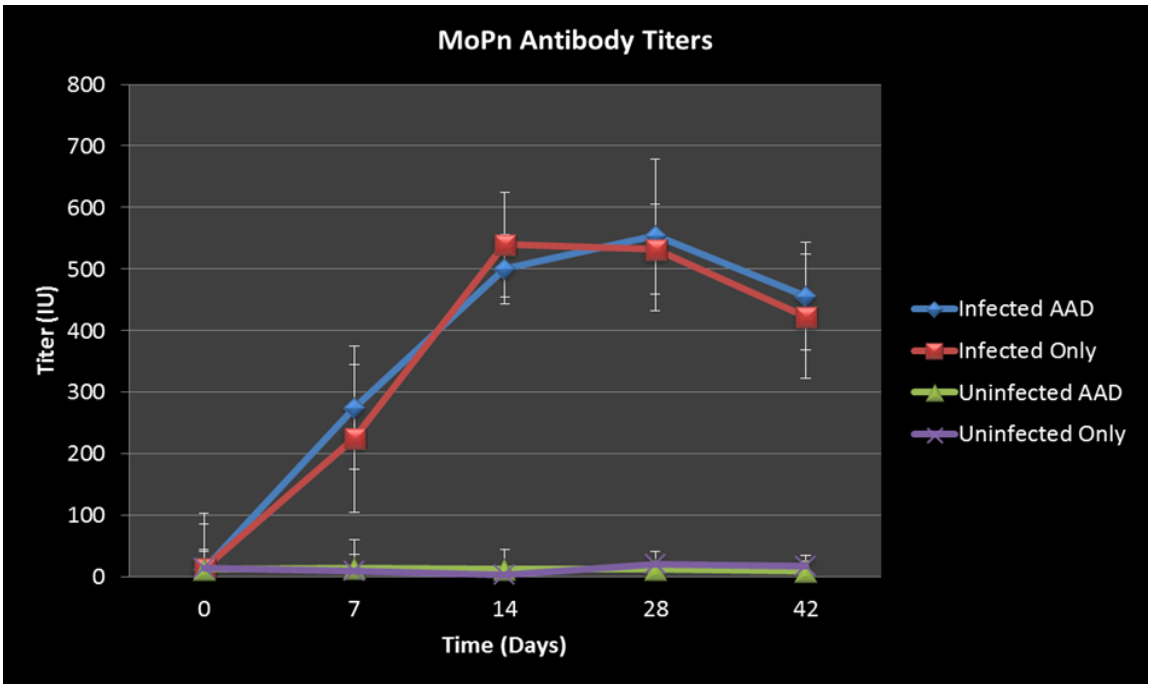

Figure 32: *Chlamydia* antibody titers over time

Antibody titer graph shows strong antibody responses to *Chlamydia* from infected animals but little to no antibody production in uninfected groups. Infected animals titers to *Chlamydia* began to drop once infections cleared.

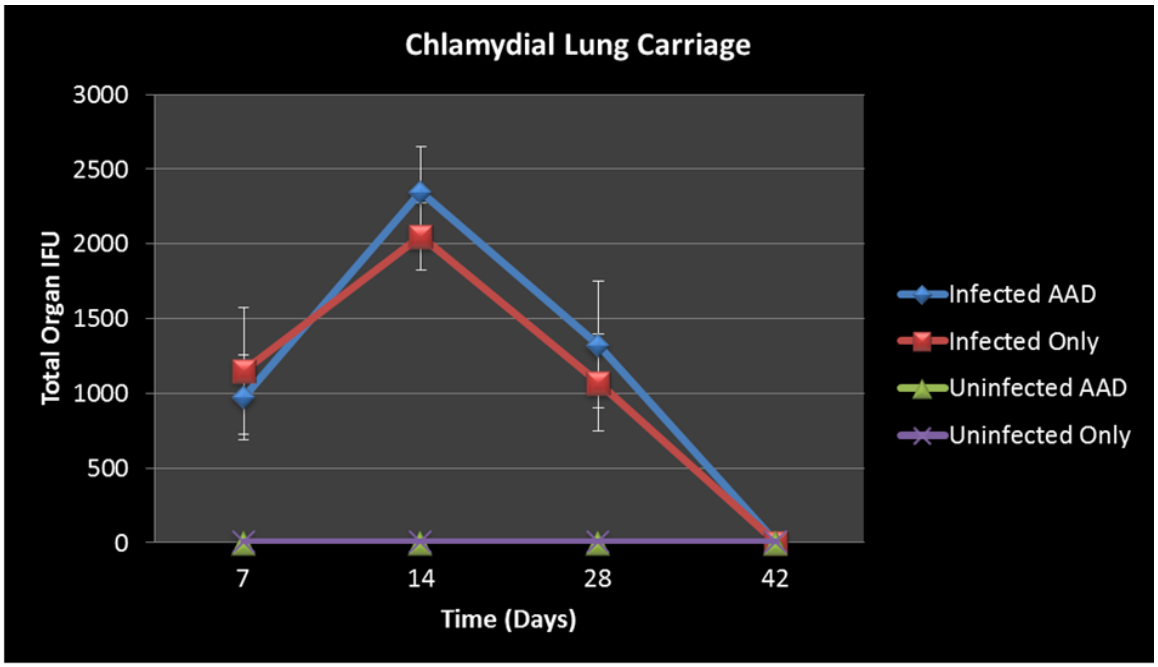

Figure 33: Cultured chlamydial carriage/concentration in the lungs

Chlamydial carriage in the lung peaked 14 days pi. Chlamydial organisms could not be detected in the lung 42 days pi. Uninfected animals had no carriage of chlamydial organisms.

WBC and Cytokine Response to Respiratory Chlamydial Infection and AAD

Typically *Chlamydia* infection induces a Th1 immune response characterized by IFN-γ secretion and that this type of response is necessary for clearance. In addition AAD is a Th2-driven disease characterized by the influx of Th2 cytokines, eosinophils and allergic hyperresponsiveness. In this study we characterized lung infection with *Chlamydia*. BAL fluid was analyzed for the presence of Th1 and Th2 cytokines using SABiosciences cytokine kits. In this model, following respiratory infection with *Chlamydia*, mice were sensitized to Ova and some groups were challenged over several days while others remained unchallenged for comparison (Table 13). The data confirmed that infected animals secreted significantly elevated amounts of Th1 cytokines (IFN-γ, TNF-a, IL-2, and IL-12) compared to uninfected animals (Figure 34). Th1 cytokine levels peaked on days 14 and 28 and dropped off by 42 once infections had cleared. Animals which were AAD induced responded to ovalbumin with a robust Th2 cytokine response (IL-4, IL-5, and IL-13) compared to their compared to their uninduced counterparts (Figure 35). Th17 cytokines (IL-17A, IL-23) were also elevated as well and peaked at days 14 and 28 in infected animals (Figure 36). Uninfected AAD induced animals had negligible levels of Th1 and Th17 cytokine production. IL-10 levels increased in infected and AAD induced animals, TGF-B1 and IL-6 were not significantly produced in any group (Figure 37).

The robust cytokine response in the airway of *Chlamydia*-infected only mice (Group 2) was complemented by the accumulation of predominantly macrophages ($9.0x10^2$) and neutrophils ($9.9x10^3$) in the BAL fluid of infected mice 28 days pi, along with markedly less eosinophils ($1.9x10^2$) (Figure 38). In contrast, 28 days pi AAD induced animals presented with significantly elevated eosinophils ($3.6x10^2$) compared to uninduced mice (Figure 37A-D). Mice that were both AAD induced and infected (Group 1) had slightly elevated levels of all cell types 28 days pi compared to AAD only groups (Group 3), but was not significant.

Neonatal & Adult Groups	Description of Treatment
Group 1	Infected, Allergic Airway Disease (Day 23)
Group 2	Infected Only
Group 3	Uninfected, Allergic Airway Disease (Day 23)
Group 4	Uninfected Only

Table 13: Animal grouping and treatments

Sensitization and subsequent challenge with ovalbumin is required to induce allergic airway disease.

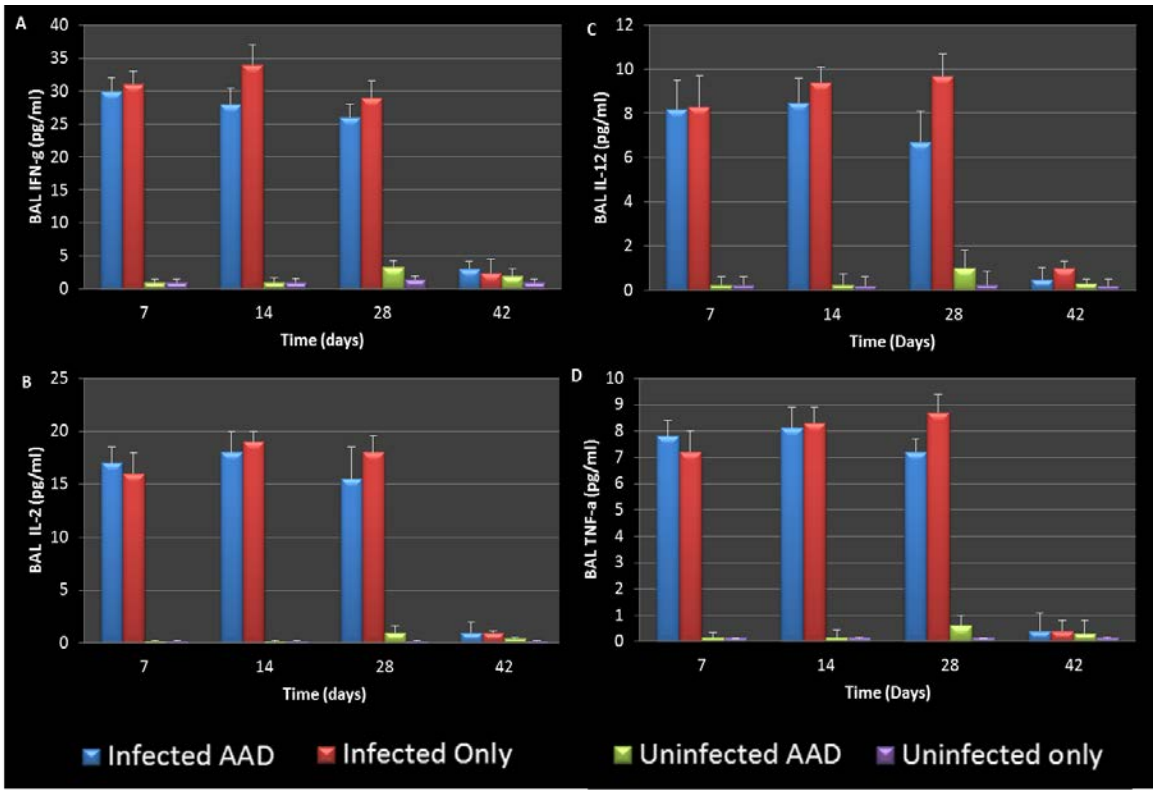

Figure 34: Th1 cytokine levels during chlamydial infection and AAD induction

Infected animals displayed a robust Th1 cytokine response (IFN-γ, IL-2, IL-12, TNF-a, A-D respecitively) in response to chlamydial infection. Infected mice cleared the infection after 28 days. Uninfected and AAD induced only animals produced little to no Th1 cytokines. Th1 cytokine production dropped off by day 42, at the same time infections began to clear in infected animals.

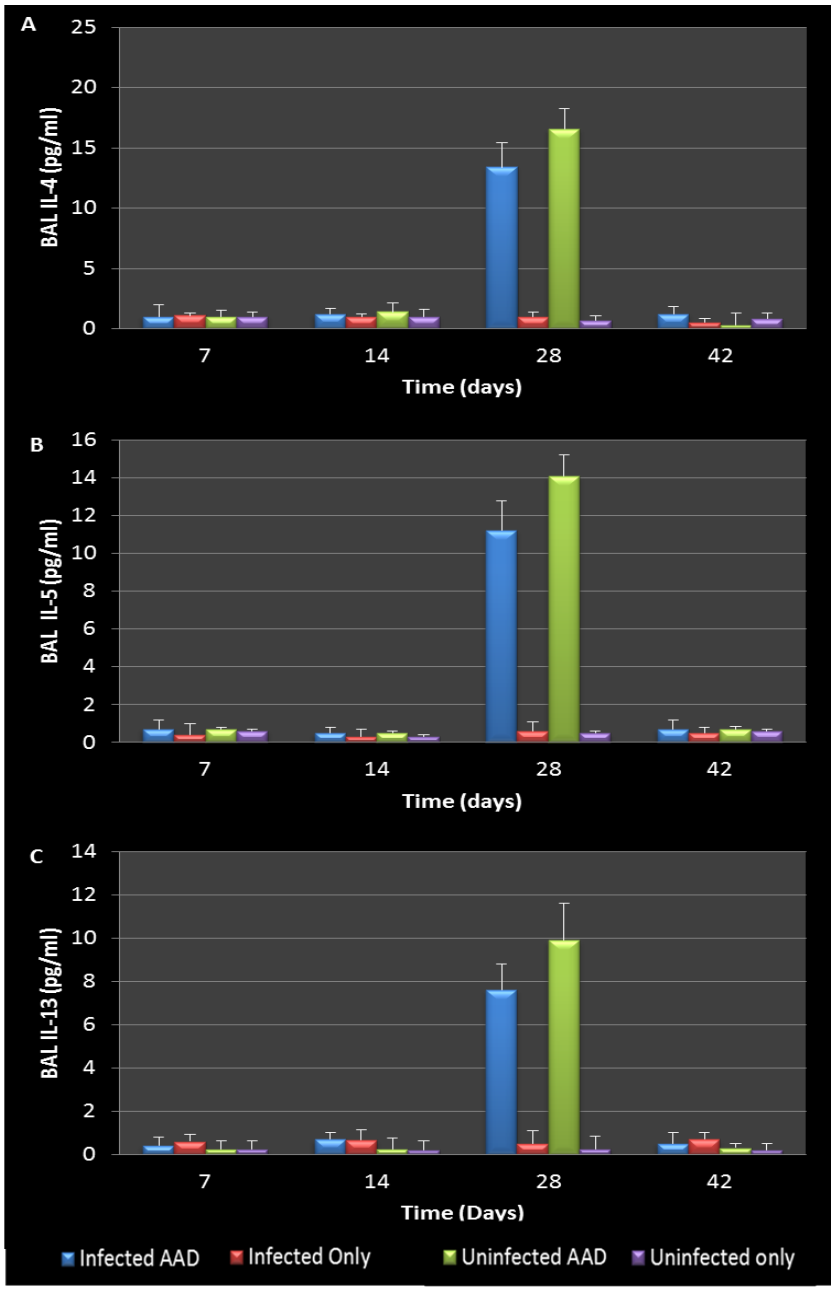

Figure 35: Th2 cytokine levels during chlamydial infection and AAD induction

All AAD induced animals developed a Th2 immune response during allergic airway induction with ovalbumin (A-C). AAD induced mice with active infections produced slightly less Th2 cytokines than their uninfected AAD induced counter parts. Animals that were not AAD induced did not produce any significant amounts of Th2 cytokines. Th2 cytokine production dropped off by day 42 once AAD induction was removed.

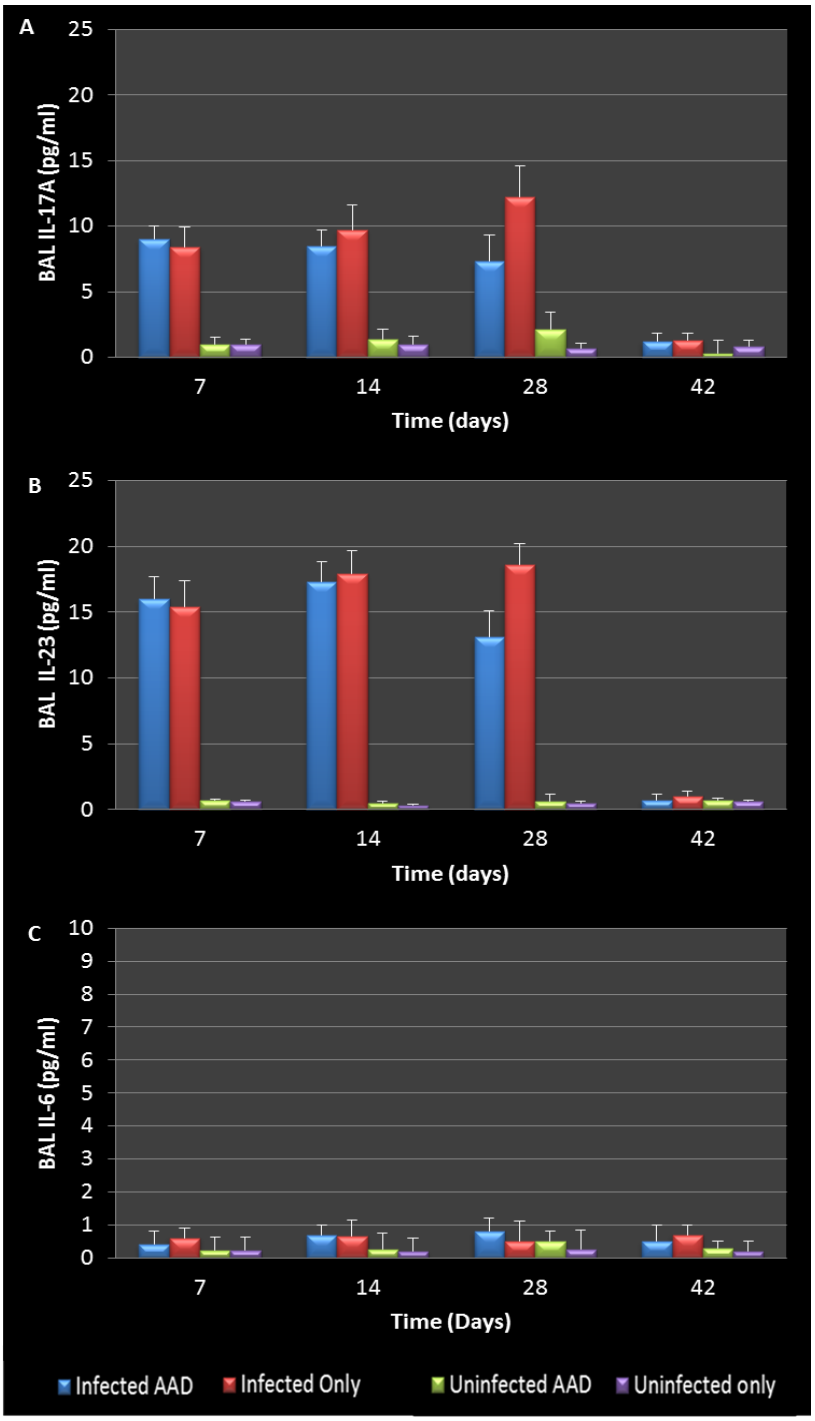

Figure 36: Th17 cytokine levels during chlamydial infection and AAD induction

Infected animals displayed a moderate Th17 cytokine response (IL-17A and IL-23) in response to chlamydial infection (A, B). However, little to no IL-6 was seen in any animal group (C). IL-17A and IL-23 levels diminished once chlamydial infections were cleared. Uninfected and AAD induced only animals produced little to no Th17 cytokines.

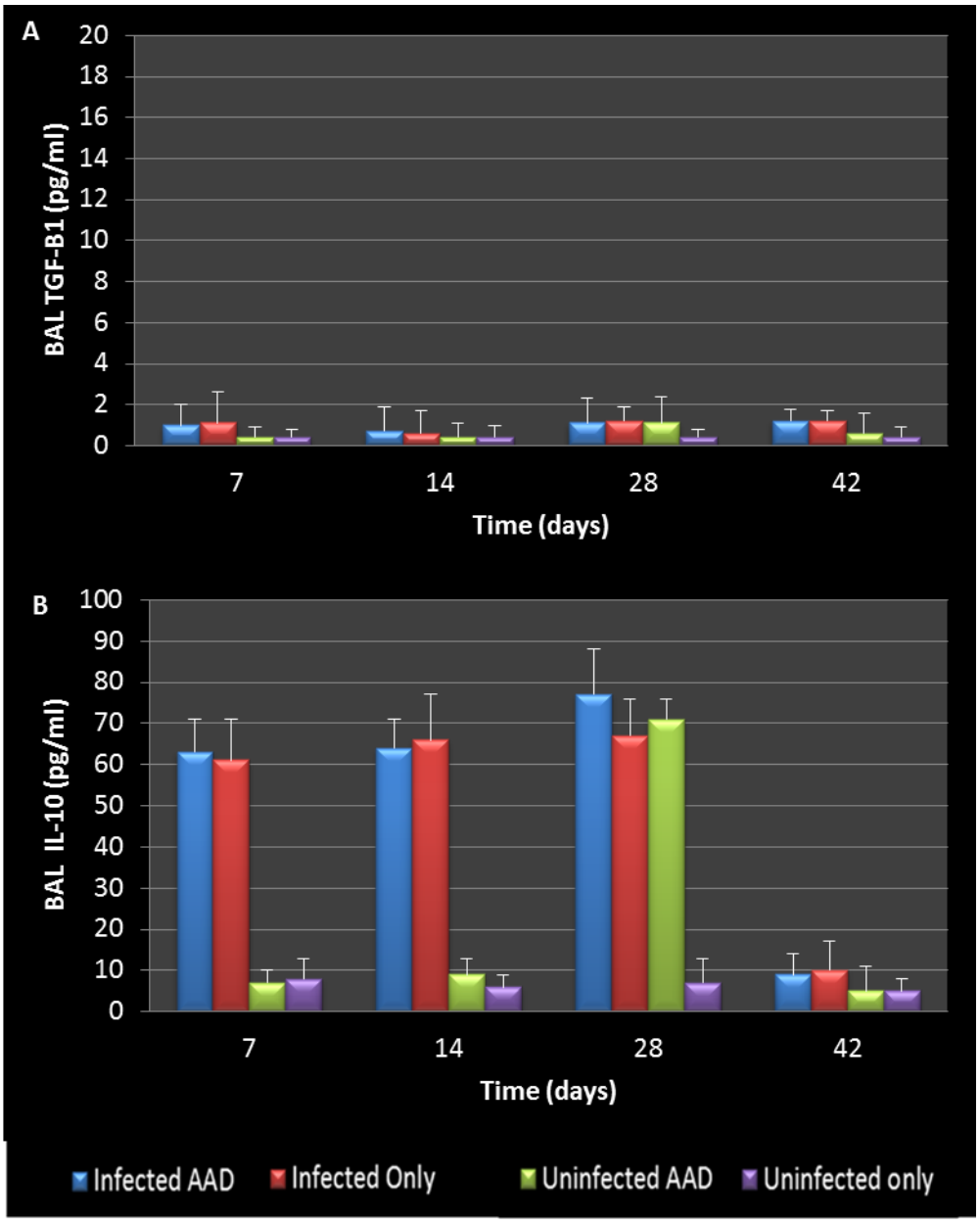

Figure 37: Regulatory T-cell cytokine levels during chlamydial infection and AAD induction

No significant amounts of TGF-B1 were produced in any animal group (A). IL-10 was produced at significant levels at days 7 and 14 in infected animals. IL-10 continued to be produced at high levels in infected animals on day 28 but was also produced in AAD induced animals as well. When AAD induction was removed and infections were cleared IL-10 levels dropped off (B).

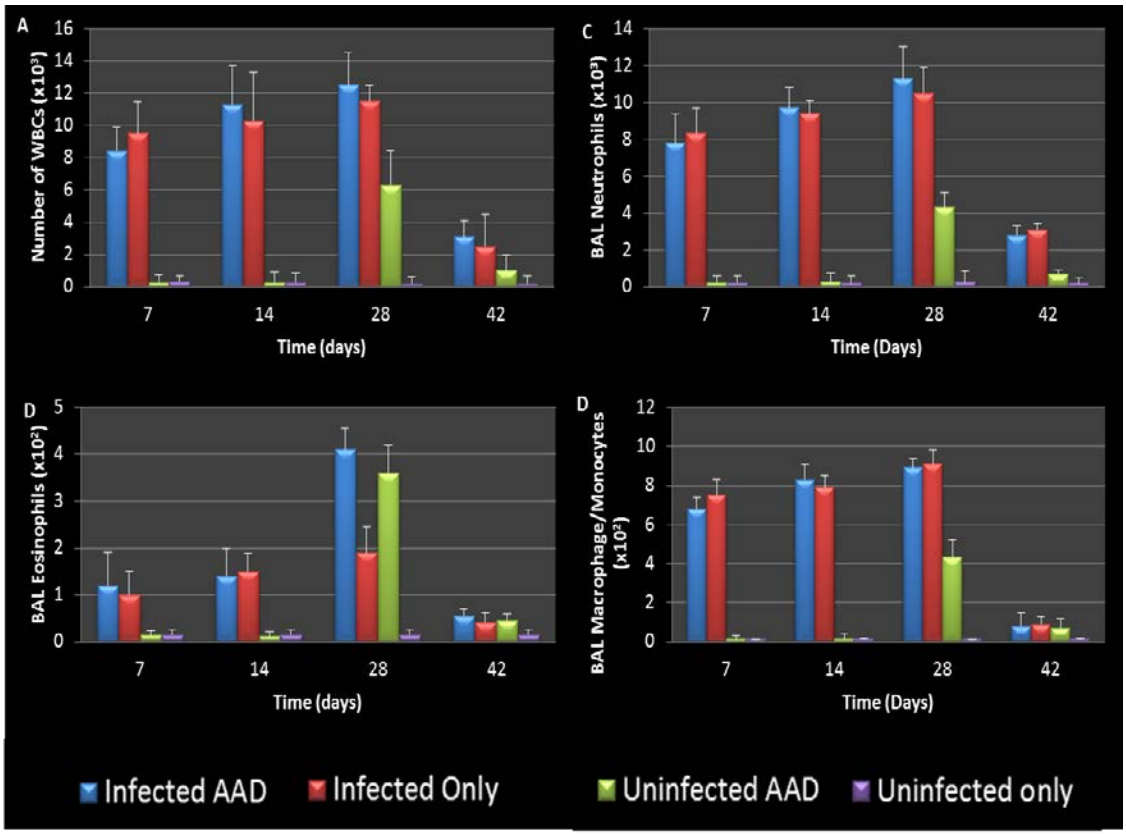

Figure 38: BAL cellularity during allergic airway disease

Infected groups with active infections had elevated levels of total WBCs, neutrophils, and macrophages compared to all other groups, which peaked on day 28 pi (A, C, D). All groups which had allergic airway induced (AAD) with Ova had elevated levels of BAL eosinophils on day 28 (when AAD was fully induced). All cell counts diminished after infections were cleared and AAD subsided.

Assessment of hepoxilin production in lung tissue

In order to determine the presence of hepoxilin in the lung tissue from the mouse model, we utilized Western blotting with specific antibodies in order to detect a hepA3 precursor, 12 lipoxygenase; we also sought to detect 5 lipoxygenase as well, which is a leukotriene precursor. With this method, we were able to indirectly detect low volumes of hepoxilin; hepoxilin itself has an extremely short half-life so direct detection is not possible. Western blotting revealed 12-LO was only detected in infected mouse groups as early as day 7 and until day 28 (Figure 39A). 12-LO was not detected in uninfected animals or AAD only groups. 5-LO, the leukotriene precursor, was detected in infected groups as well as AAD induced groups, as expected (Figure 39B).

In addition, we examined the lung tissue for HDC, 5-LO and 12-LO mRNA expression using RT-PCR. The data corresponded with our Western Blotting results. 12-LO mRNA was upregulated only in infected animals, and undetectable in AAD only or uninfected animals (Figure 40A). 5-LO expression was upregulated in infected animals and AAD induced groups (Figure 40A). Interestingly, HDC mRNA was upregulated as expected in AAD induced groups, but was also detected in infected animals which were not AAD induced (Figure 40A). Expression of 5-LO and 12-LO was seen as early as day 7 until 28, HDC was seen in infected groups from days 7-28 but was not seen in AAD only groups until day 28 (AAD was established). By day 42 no expression of 5-LO, 12-LO, or HDC was detected as a result of bacterial clearance and alleviation of AAD (Figure 40B). Our results indicate that chlamydial infection alone is able to induce 12-LO expression (ultimately hepoxilin) as well as induce HDC expression (histamine) in lung tissue.

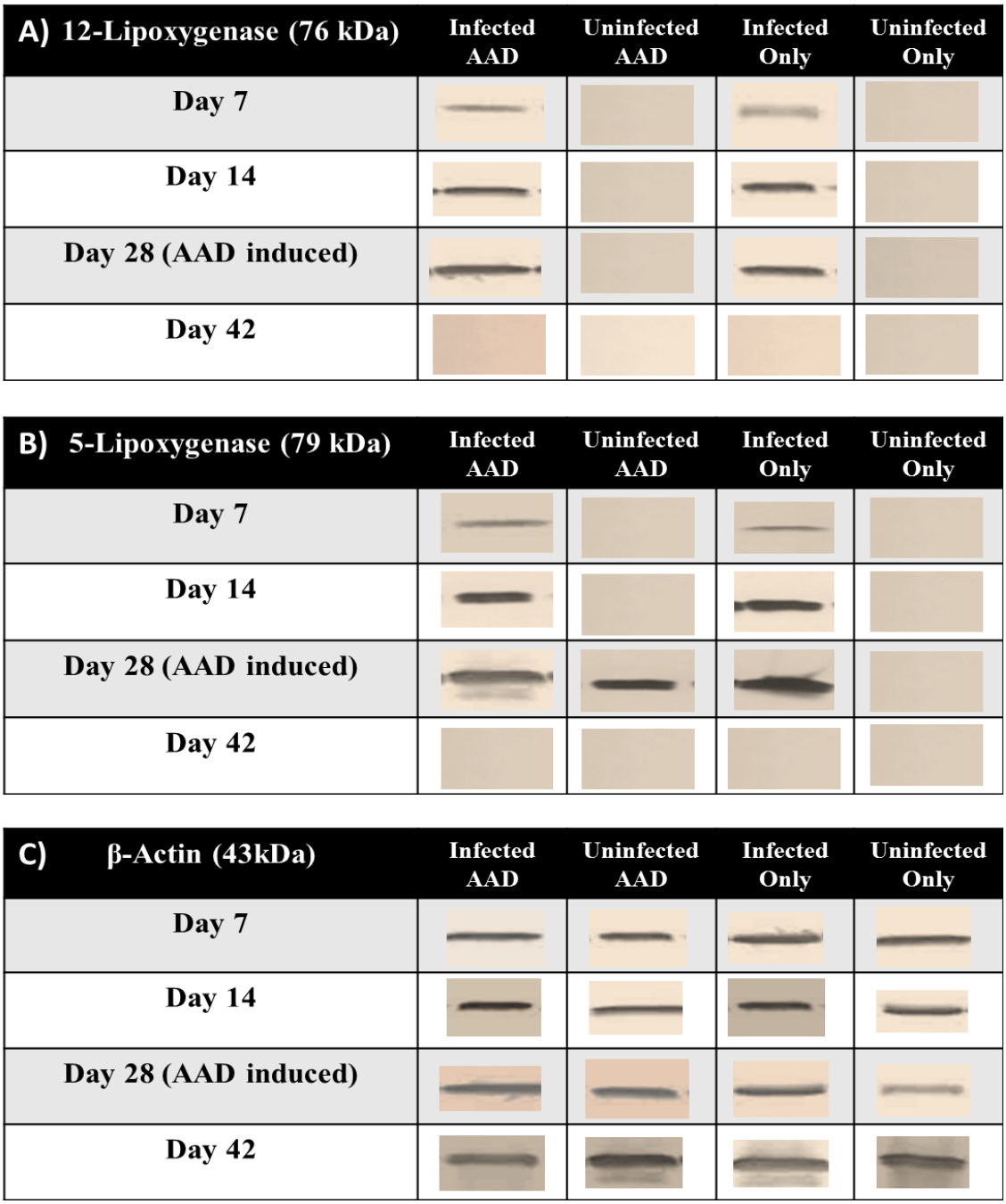

Figure 39: 5-LO and 12-LO Western Blot of mouse lung tissue

12-LO (76kDa) protein could be detected using Western Blot only in infected animals from days 7-28, it was not detected in the AAD only or in uninfected animals (A). 12-LO protein was undetectable on day 42 once the infections had cleared. 5-LO (79kDa) protein was detected in infected animals on days 7-28, it was also detected in AAD induced animals only on day 28 (day of induction) (B). 5-LO protein was no longer expressed once AAD and bacterial infections had cleared on day 42.

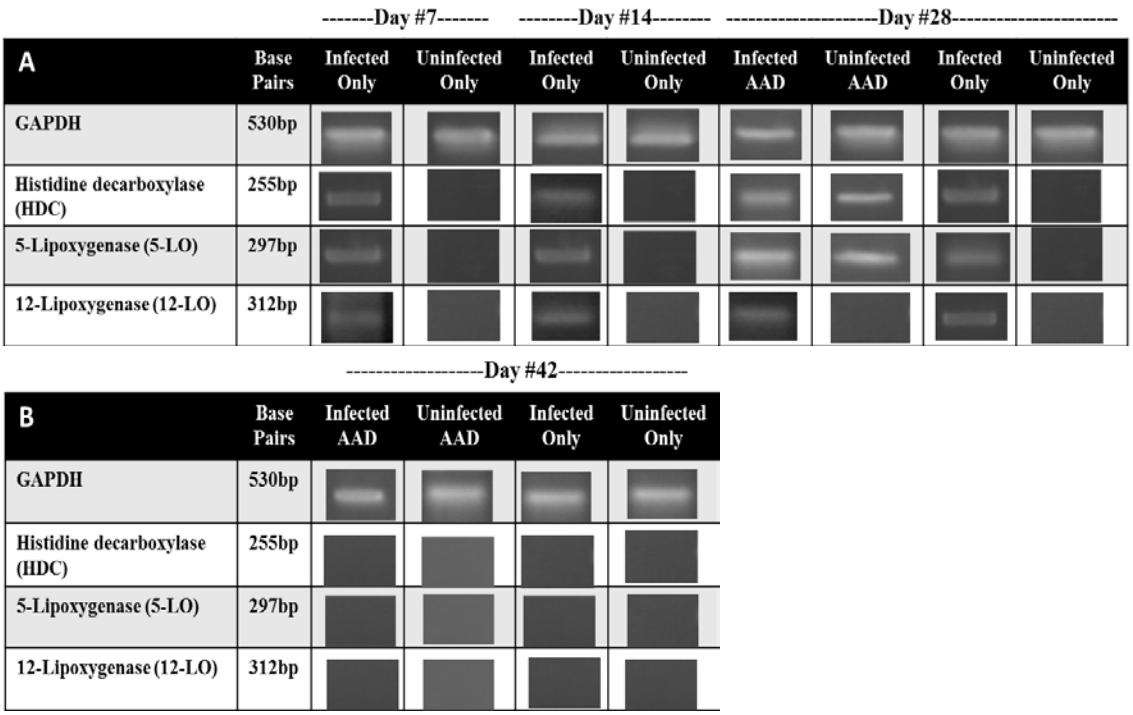

Figure 40: 5-LO, 12-LO, and HDC mRNA expression in mouse lung tissue

Analysis of mouse lung tissue for the mRNA expression of 5-LO, 12-LO, and HDC revealed that infected groups were the only one to express 12-LO from days 7-28 (A). 5-LO was expressed in both infected groups on days 7-28 as well as AAD induced groups only on day 28 (A). By day 42 there was no expression of any of these mRNAs as a result of clearance of infection and removal of AAD (B).

Assessment of histamine production in BAL neutrophils

In order to determine if chlamydial infection could induce histamine production in neutrophils, BAL neutrophils were purified from other cell types and then assessed for mRNA expression of HDC using RT-PCR. Our data demonstrated that neutrophils isolated from infected groups, regardless of AAD induction, had significant expression of HDC from day 7-28 (Figure 41A). Neutrophils from AAD induced only animals and uninfected animals had no expression of HDC, even at day 28 when AAD was fully induced. By day 42 HDC was no longer detected as a result of clearance of infection and alleviation of AAD (Figure 41B).

In addition to examining mRNA expression of HDC, purified BAL neutrophils were stimulated *in vitro* with heat killed chlamydial antigens and were then assessed to see if this elicited the release of histamine into the supernatant. Neutrophils from animals which had been infected with *Chlamydia* released histamine in response to stimulation with chlamydial antigen (Figure 42A). Histamine levels increased over time in response to antigen stimulation, and peaked 72 mins post exposure. Stimulation of BAL neutrophils with SPG or J-cell macrophage protein did not induce histamine release in any group (Figure 42B-C). Our data demonstrates that chlamydial infection induces histamine production in BAL neutrophils, furthermore, direct stimulation of these cells with chlamydial antigen causes subsequent histamine release.

Discussion

Besides well-defined environmental causes, accumulating evidence suggests that respiratory tract infections play an important role in the pathogenesis of asthma. Among these, *Chlamydia pneumoniae* infection has been discussed as a possible initiator in the development of chronic asthma. Clinical studies have shown that early life *C. pneumoniae* infection is associated with increased incidence of childhood asthma. Furthermore, this asthmatic phenotype is characterized by infiltration of neutrophils, a phenomenon not seen in typical allergen-induced airway hyperactivity which is characterized predominantly by eosinophils and basophils.

Identifying host response factors that may contribute to the pathogenesis of airway hyperreactivity (AHA) is critical for establishing a functional association between *Chlamydia*-induced airway inflammation and the subsequent development of persistent asthma. Surprisingly, recent reports have found that almost 50% of persistent asthma cases are accompanied by an increase in airway neutrophils and these patients were not only more refractory to corticosteroid treatment, but represented the subset of asthmatics at greatest risk for asthma-related mortality. A recent study carried out by Xu, et al demonstrated that *Mycoplasma pneumoniae*, a prevalent respiratory pathogen that is commonly co-isolated with *C. pneumoniae,* provokes histamine release from neutrophils in the murine airways (1). Indeed, neutrophils from infected murine airways were highly enriched in histamine compared with naive neutrophils, and significantly upregulated mRNA encoding histidine decarboxylase, the rate-limiting enzyme in histamine synthesis (1). However, it is not clear which metabolite(s) represent the major neutrophil chemoattractants in the infected airways of asthmatics.

Hepoxilins are biologically active metabolites of arachidonic acid that are formed through the 12-lipoxygenase pathway. Hepoxilins were recently identified as potent neutrophil chemoattractants in the intestinal mucosa, playing an even greater role than IL-8. Our goal in this study was to determine if *C. pneumoniae* infection of the airways induce the release of hepoxilins, to determine if hepoxilin is a neutrophil chemoattractant in the airway, and finally, to determine if these neutrophils produce histamine in response to *C. pneumoniae* infection. We therefore hypothesized that neutrophils, provoked by *C. pneumoniae*-induced lung infection, undergo transmigration through an IL-8/hepoxilin gradient and greatly expand their capacity to synthesize histamine, thereby contributing to airway inflammation and pathology.

Our data has demonstrated that chlamydial infection alone generates a Th1 type cytokine response with corresponding macrophage and neutrophil infiltration. When AAD was induced a mixed Th1/Th2 cytokine and cellular response was seen in infected animals. Uninfected AAD controls responded with robust Th2 cytokine responses. These results are similar to those

previously reported by Horvat et al (14) using a similar Ova mouse model. In addition, our current study we found that infected animals also produced moderate levels of Th17 cytokines (IL-17A, IL-23). The presentation of these cytokines along with TNF-a are mediators of neutrophil infiltration.

When we examined the lung tissue of our animals for the presence of hepoxilin (through the detection of 12-lipoxygenase), we discovered that only *Chlamydia* infected mice expressed 12-LO protein and mRNA. Animals only induced for AAD or were uninfected did not produce any 12-LO whatsoever. These data suggest that chlamydial infection is able to induce the production of 12-LO as early as 7 days pi and strongly indicates that hepoxilin is a significant chemoattractant for neutrophil infiltration. In addition, we also found that chlamydial infection alone was also able to elicit mRNA expression of HDC indicating the production of histamine production within the lung, providing potential for airway hyperresponsiveness.

In this study we also examined BAL neutrophils for the ability to produce histamine. We found AAD induction alone, did elicit some neutrophil infiltration to the lung, but these cells were not expressing HDC. Our infected animals regardless of AAD were characterized by significant neutrophil infiltration and more interestingly, that these neutrophils were in fact producing histamine in response to chlamydial infection. HDC upregulation was seen in BAL neutrophils as early as 7 days pi, and was sustained until the infection had been resolved. Furthermore upon stimulation of BAL neutrophils with chlamydial antigen caused histamine degranulation from these cells. This data provides a mechanism as to how neutrophils can mediate sudden and severe asthma airway hyperresponsiveness. Together this provides us insight into how neutrophils can cause airway hyperresponsiveness as seen in asthma.

Ultimately this data demonstrates that chlamydial infection alone is able to induce a slightly mixed Th1/Th17 cytokine response, marked by significant neutrophil infiltration. Our data also indicates that neutrophil infiltration is mediated through the production of hepoxilin. This alone is not indicative of a creating the typical Th2 driven allergic airway seen in asthmatics.

However, our data has shown that the population of neutrophils infiltrating the lung in response to chlamydial infection are expressing HDC and producing histamine. This unique population of neutrophils therein have the ability to create airway hyperresponsiveness by histamine degranulation in response to chlamydial infection. We have provided an explanation as to how neutrophilic asthma caused by a bacterial pathogen, can create an atopic airway and elicit sudden, severe airway hyperresponsiveness in an asthmatic airway.

It is clear that neutrophils are the first cells recruited to the site of an allergic reaction; they may influence clinical presentation and play a role in the development of severe chronic asthma and the onset of sudden severe attacks. Our data has shown that if hepoxilin is released as a consequence of infection, pharmacologic regulation of airway hepoxilin generation might provide a novel and appropriate therapeutic strategy for a significant percentage of asthmatics refractory to maximal doses of inhaled corticosteroid and are at increased risk for asthma mortality. The data presented here has the potential to lead to new therapeutics (12-LO and 5-LO antagonists) for hard to control asthma and/or for dampening lung inflammation.

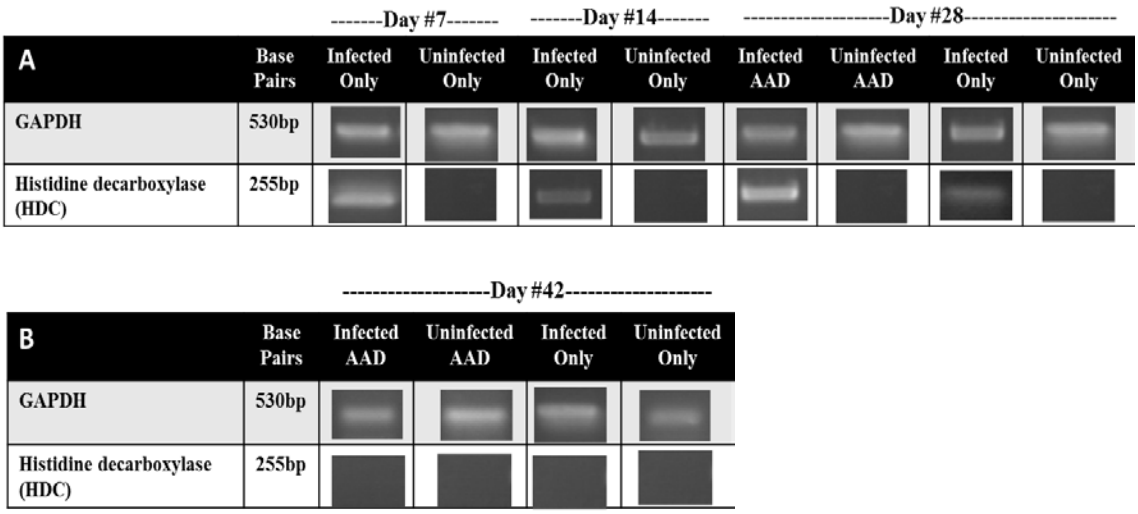

Figure 41: HDC mRNA expression in BAL neutrophils

Analysis purified BAL neutrophils for the mRNA expression of HDC demonstrated that infected groups were the only one to express HDC from days 7-28 (A). HDC was not present in BAL neutrophils in AAD only or uninfected groups. By day 42 there was no expression of any of HDC a result of bacterial clearance (B).

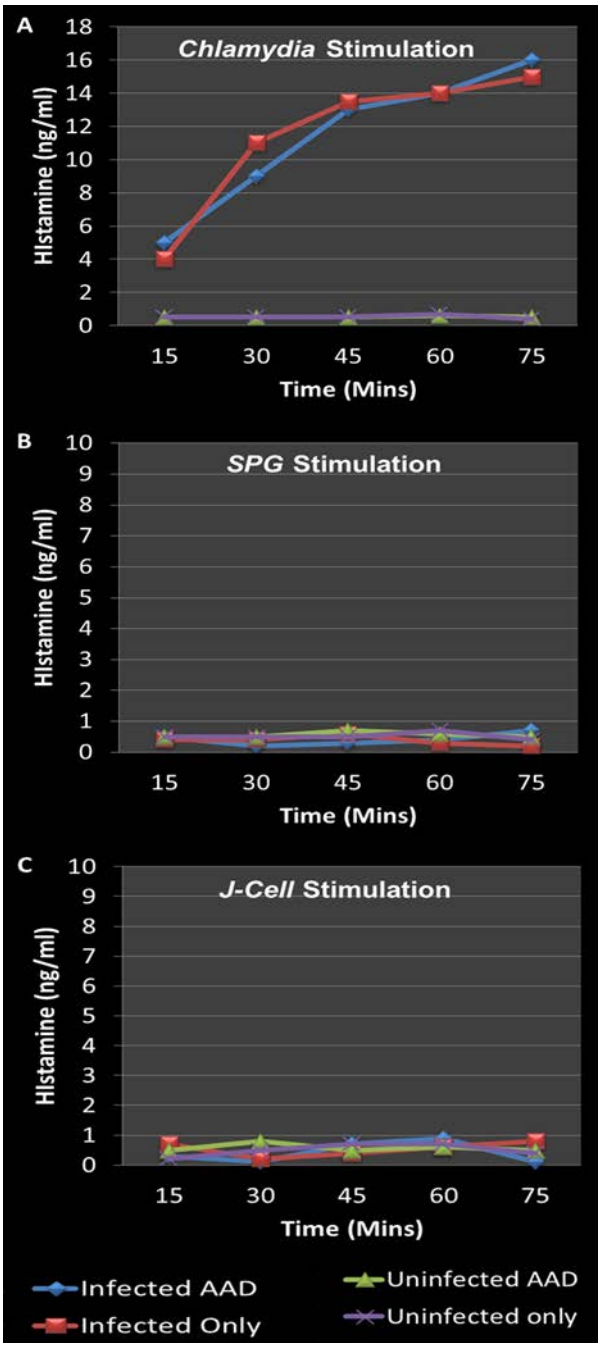

Figure 42: Histamine release from BAL neutrophils in response to chlamydial antigen

BAL neutrophils isolated from day 28 pi were challenged with chlamydial antigen *in vitro* and subsequent histamine release into the supernatant was assessed using ELISA. BAL neutrophils from infected animals released significant amounts of histamine in response to chlamydial challenge which peaked 75 minutes post stimulation (A). Neutrophils from uninfected animals had no release of histamine in response to chlamydial stimulation (A). Histamine release was not seen in any group in response to stimulation with SPG or J-Cell protein (B, C).

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CHAPTER 7

DISCUSSION & CONCLUSIONS

Most clinical researchers agree that asthma is a serious multifactorial disease, and that infectious agents play an important role in disease exacerbation. To date however, no specific mechanism describing how infection of the airway might lead to asthma initiation or exacerbation has been proposed. The data presented here initiates the first step in identifying the initial factors by evaluating the host response to chlamydial infection in early life. This data, generated here, serves multiple important purposes. First, we have established an effective animal model for studying the initiation of allergic airway disease, opening up the way for studying other respiratory pathogens such as RSV. Second, *Chlamydia* induces the production of cytokines that lead to atopy in early life, thereby increasing the risk of developing asthma later in life, antibiotic treatment to eliminate the bacteria would be highly desired early in life. This fact has the potential to revolutionize the way pediatric patients with asthma are cared for. In addition to this, we have observed that neutrophilic asthma phenotypes are becoming more prevalent and because of the higher mortality in this patient group, it is necessary to better understand the mechanisms driving inflammation. The data presented here demonstrates *Chlamydia* leads to histamine production and release by neutrophils, this highlights a distinct mechanism of allergic asthma that does not require mast cells and IgE antibodies typically seen in classical asthma (Figure 43). This means that these patients would not benefit from popular treatments such as *Omalizumab* (Xolair) which works by blocking IgE. Hepoxilins are indeed involved in neutrophil accumulation and inflammation, this is could make a great therapeutic target that is worth further exploration. Specifically we would determine the source of hepoxilin formation and release in the lungs. We will also use mouse models to determine the exact contribution of neutrophils to histamine release in the presence of *Chlamydia* by utilizing mast cell–deficient mice. As well as examine potential therapeutics that specially target hepoxilin production.

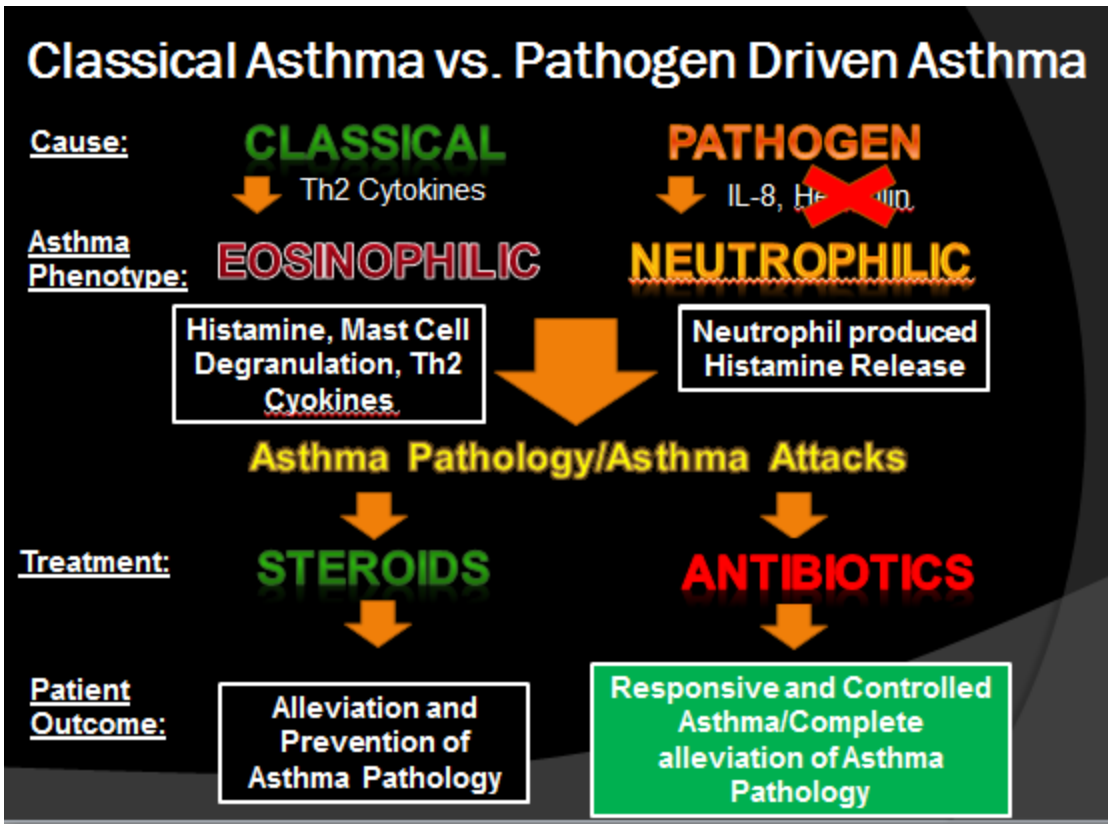

Figure 43: Classical vs. pathogen driven asthma

Classical asthma is based upon typical allergy to common allergens, which initiates asthma symptomology through histamine production from mast cells and eosinophils. Which is typically managed with cortico steroid treatment. Neutrophilic asthma is pathogen mediated and histamine is produced in neutrophils. Effective treatement of neutrophilic asthma would be accomplished via high dose antibiotics.

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