

Mold-sensitivity in children with moderate-severe asthma is associated with HLA-DR and HLA-DQ

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Abstract

Background: Several epidemiologic studies in the United States and Europe have linked *Alternaria* sensitivity to both persistence and severity of asthma. In this study, we examined T cell responses and HLA class II alleles in children with moderate-severe asthma.

Methods: Ninety-six children with moderate-severe asthma were compared to 90 children with mild asthma. HLA class II genotyping was performed to determine HLA allelic frequencies. Th1/Th2 *Alternaria*-specific T cell cytokine responses were determined by the use of *Alternaria*-stimulated cultures. HLA class II restriction was examined by inhibition of *Alternaria*-stimulated lymphoproliferative responses with blocking anti-HLA class II monoclonal antibodies.

Results: Children with moderate-severe asthma had significantly increased sensitivities to *Aspergillus fumigatus*; sensitivities to *Alternaria* were similar in both moderate-severe and mild asthmatics. The frequency of HLA-DRB1*13 alleles were increased in mold-sensitive moderate-severe asthmatic children. HLA-DRB1*03 tended to be increased in mold-sensitive moderate-severe asthmatics. The frequency of HLA-DQB1*03 alleles was significantly decreased in mold and *Alternaria*-sensitive moderate-severe asthma. HLA class II blocking monoclonal antibodies demonstrated HLA-DR restriction. *Alternaria*-stimulated IL-5 and IL-13 synthesis was significantly increased in moderate-severe asthmatics. IL-5 and IL-13 synthesis was significantly increased in *Alternaria*-stimulated lymphocyte cultures of HLA-DQB1*03- asthmatics compared to HLA-DQB1*03+ asthmatics.

Conclusions: In children with *Alternaria*-sensitive moderate-severe asthma, there was increased Th2 sensitivity to *Alternaria* stimulation. This was associated with HLA-DR restriction and with increased frequency of HLA-DRB1*13 and HLA-DRB1*03. There was decreased frequency of HLA-DQB1*03 in *Alternaria*-sensitive moderate-severe asthma, suggesting HLA-DQB1*03 may be protective of the development of *Alternaria*-sensitive severe asthma.

Background

The severity of asthma in children varies widely from mild intermittent to severe persistent asthma. Although the risk factors associated with asthma severity are not fully understood, allergic sensitizations to house-dust mites, molds, cat dander, cockroaches, and pollens have been associated with development and/or severity of asthma (1–14). In particular, sensitization to *Alternaria alternata* has been linked to devel-

opment, persistence, and severity of asthma (15–20). In addition, high *Alternaria* spore counts have been associated with increased asthma symptoms, asthma hospital admissions, and asthma deaths (21–23). There has also been a link following thunderstorms with increased *Alternaria* spore counts and asthma attacks (24, 25).

In previous studies of allergic bronchopulmonary aspergillosis (ABPA), we reported several genetic risks, including increased IL-5 synthesis to *Aspergillus fumigatus* stimulation,

IL-4RA single nucleotide polymorphisms (SNP), HLA-DR2/HLA-DR5 restriction, and HLA-DQ2 protection in the development of ABPA compared to *A. fumigatus* sensitivity in asthmatics (26–32). In particular, we reported that HLA-DR2 (HLA-DRB1*15 and B1*16) and HLA-DR5 (HLA-DRB1*11 and HLA-DRB1*12) restriction were a risk factor for the development of ABPA compared to Aspergillus-sensitive asthmatics. Thus, the presentation of molds, e.g. *Alternaria*, may be important in the development of mold-sensitive moderate-severe asthma vs mold-sensitive. In this study, we hypothesized that similar risk factors may play a role in *Alternaria*-sensitive severe asthmatics. We examined Th1/Th2 cytokine production to *Alternaria* allergens and HLA-DR and HLA-DQ frequencies in *Alternaria*-sensitive moderate-severe asthmatic children compared to *Alternaria*-sensitive mild asthmatic children.

Methods

Study population and sample size

The study population consisted of both male and female Caucasian, African-American, and Hispanic children 5–18 years old with mild, moderate, and severe persistent asthma recruited from the Allergy and Asthma clinics at Cardinal Glennon Children's Medical Center, Saint Louis University. Classification of asthma severity was the GINA (NHBLI) criteria using day/night symptoms, pulmonary function, and medications. Patients were evaluated for allergen sensitivities by allergy prick skin testing (Multi-Test II; Lincoln Diagnostics, Decatur, IL, USA) to *A. alternata*, *Cladosporium herbarum*, *Helminthosporium sativum*, *A. fumigatus*, *Dermatophagoides pteronyssinus* and *farinae* (house-dust mites, HDM), American/German cockroach, cat hair, dog epithelium, tree pollens (oak, hickory-pecan, maple/box elder, elm, ash, sycamore, walnut, juniper, birch), grass pollens (Johnson, Bermuda, June, timothy, bahia), and weed pollens (short and giant ragweed, plantain, sorrel, mugwort, hackberry, mulberry)(reagents obtained from Greer Laboratories, Lenoir, NC, USA). Tests were regarded as positive when the mean diameter of the wheal was ≥ 3 mm. The study group consisted of *Alternaria*-sensitive moderate-severe asthmatics and control groups consisted of *Alternaria*-sensitive mild asthmatics, non-*Alternaria*-sensitive moderate-severe asthmatics, and non-*Alternaria*-sensitive mild asthmatics. The study has been fully approved by the Saint Louis University Institutional Review Board (IRB #14611, approved 9-3-2008).

Human leukocyte antigen typing

To examine HLA-DR and HLA-DQ allelic frequencies in *Alternaria*-sensitive asthmatics, HLA-DR and DQ typing was performed in the HLA Laboratory as previously described (30–32). HLA-DRB1 alleles were detected by PCR amplification of genomic DNA with sequence specific primers (PCR-SSP; Dynal, Inc, Oslo, Norway). HLA-DQ typing was performed by PCR amplification of genomic DNA by using

low-resolution HLA-DQB allele-specific primers identifying five DQ alleles (One Lambda, Canoga, Park, CA, USA).

Th1/Th2 cytokines and chemokines

To determine Th1/Th2 *Alternaria*-specific T cells, *Alternaria*-stimulated cultures were performed as previously described (26–30). In 1 ml volume of Rosewell Park Memorial Institute (RPMI) supplemented with 10% fetal calf serum (FCS), 1×10^6 peripheral blood lymphocytes (PBL) were cultured for 1 week in a humidified 5% CO₂ atmosphere at 37°C. Cultures were stimulated in media alone, 25 mcg/ml of *A. alternata* extract, 25 mcg/ml of Alt a1, 25 mcg/ml of Asp f3, and 25 mcg of Der p1. Tetanus toxoid 4 LF/ml (Connaught Laboratories, Toronto, Canada) was used as a Th1 control antigen. The culture supernatants were obtained and frozen at –70°C until analyzed. *Alternaria* extract and Alt a1 were obtained from Dr Hari Vijay, and Asp f3 was obtained from Dr Viswanath P. Kurup. Der p1 was obtained from Greer Laboratories. Measurement of Th1/Th2 culture supernatant cytokines and chemokines was performed by Flex Cytometric Bead Assay from BD Pharmingen (San Diego, CA USA) to measure Th1/Th2 cytokines, IL-4, IL-5, IL-10, IL-13, and IFN- γ synthesis, as previously described (27, 30).

Inhibition of *Alternaria*-stimulated T cells with anti-DR, -DP, and -DQ mAbs

To examine HLA class II restriction, inhibition of *Alternaria*-stimulated lymphoproliferative responses was assessed with blocking anti-HLA-DR, -DP, and -DQ monoclonal antibodies as previously described (31). In 0.2 ml volume of RPMI supplemented with 10% FCS, 2.5×10^5 PBL was cultured for 7 days in a humidified 5% CO₂ atmosphere at 37°C. Cultures were stimulated in media alone or 25 mcg/ml of *A. alternata* extract and Alt a1. In parallel cultures, blocking anti-DR, -DP-, and DQ mAbs 20 mcg/ml were added (Leinco Technologies, Inc., St Louis, MO, USA). Cultures were then pulsed for 16 h with 1.0 mCi tritiated thymidine (³H TdR). ³H TdR which was incorporated into DNA during lymphoproliferation was harvested onto glass filter paper. Proliferation was measured as ³H TdR incorporation and expressed as mean counts per minute and stimulation index.

Statistical analysis

The data for pulmonary function tests (PFT) and cytokine levels were expressed as the mean $\pm$ SD and for IgE geometric mean $\times/\div$ SD. Statistical analysis using two-tailed Mann–Whitney *U*-test was used comparing mold-sensitive moderate-severe asthma to other groups. For multiple comparisons, the Kruskal–Wallace test (nonparametric ANOVA) was performed. *Post hoc* analysis was performed using Dunn's multiple comparisons test; *P*-value <0.05 was considered significant. Two-sided Fisher's exact test analysis was used comparing mold-sensitive moderate-severe asthma to other groups. Paired Student's *t*-test was used to compare blocking anti-HLA-DR, anti-HLA-DP, and anti-HLA-DQ

lymphoproliferative studies. P -values <0.05 were considered significant, using GRAPHPAD INSTAT software package (GraphPad Software Inc., La Jolla, CA USA).

Results

Demographics

In Table 1, the demographics of moderate-severe asthma are compared to mild asthma in children. Total serum IgE levels were significantly increased in moderate-severe asthma compared to mild asthma, 353 vs 100 IU/ml ($P < 0.0001$; Table 1). Children with moderate-severe asthma had significantly increased sensitivities to *A. fumigatus*, 30% vs 15% ($P < 0.01$), to Der p and/or Der f, 52% vs 33% ($P < 0.01$), and to cockroach, 28% vs 14% ($P < 0.03$). Sensitivities to *Alternaria* and *Cladosporium* were similar in both moderate-

severe and mild asthmatics, 63% vs 54% and 32% vs 25%, respectively. Because sensitivities to *Aspergillus* and house-dust mite were increased in moderate-severe asthmatic children, in our studies of allergen-stimulated cytokine synthesis, *A. fumigatus* and Der p were also examined. There was significantly increased usage of omalizumab, high-dose inhaled corticosteroids, and long-acting beta-agonists in moderate-severe asthmatics compared to mild asthmatics. Results of pulmonary function studies performed on current medications revealed that forced vital capacity (FVC), FEV-1, FEF-25-75, and FEV-1/FVC ratio was significantly decreased in moderate-severe asthma compared to mild asthma ($P < 0.0001$). The demographics of our study population were consistent with GINA criteria for classifying asthma severity.

Cytokine synthesis

In *Alternaria*-sensitive moderate-severe asthmatics, *Alternaria* extract and Alt a1 stimulated lymphocytes had significantly increased synthesis of IL-5 and IL-13 compared to *Alternaria*-sensitive mild asthmatics ($P < 0.008$ and <0.004 , and $P < 0.07$ and <0.007 , respectively)(Fig. 1). Thus, this would suggest that in *Alternaria*-sensitive moderate-severe asthmatics, *Alternaria* exposure results in increased Th2 allergic inflammatory responses compared to *Alternaria*-sensitive mild asthmatics. There was no difference between *Alternaria*-sensitive moderate-severe asthmatics vs *Alternaria*-sensitive mild asthmatics to Asp f3 and Der p stimulated T cell cytokine synthesis, suggesting that the increased Th2 cytokine synthesis was specific to *Alternaria* stimulation.

As sensitivity to *A. fumigatus* was increased in the moderate-severe asthmatics, we examined separately *Aspergillus*-sensitive asthmatics (Fig. 2). All of the patients who were *Aspergillus*-sensitive moderate-severe asthmatics were also *Alternaria*-sensitive, *Aspergillus/Alternaria*-sensitive asthmatics. In Asp f3 stimulated cultures, IL-5 and IL-13 synthesis was comparable in *Aspergillus/Alternaria*-sensitive and *Alternaria*-sensitive alone moderate-severe asthmatics. IL-13 synthesis was significantly increased in *Alternaria*-sensitive alone moderate-severe asthmatics to Asp f3 stimulation compared to *Aspergillus*-sensitive alone mild asthmatics ($P < 0.03$). In *Alternaria*-stimulated lymphocyte cultures, IL-5 and IL-13 synthesis was significantly increased in *Aspergillus/Alternaria*-sensitive asthmatics compared to *Alternaria*-sensitive mild asthmatics ($P < 0.004$ and <0.007 , respectively). IL-5 and IL-13 synthesis was increased similarly in both *Aspergillus*-sensitive and *Alternaria*-sensitive moderate-severe asthmatics compared to *Alternaria*-sensitive mild asthmatics.

HLA-DR and HLA-DQ typing

We subsequently examined allelic frequencies of HLA-DR HLA-DP, and HLA-DQ in mold-sensitive moderate-severe asthmatics (Table 2). The frequencies of HLA-DP alleles were not significantly different when comparing the groups (data not shown). Chi-squared test for independence was used for multiple comparisons of HLA-DRB1*13. Results

Table 1 Demographics of children with moderate-severe asthma

Study	Moderate-severe (96)	Mild (90)	P
Age, years	11 $\pm$ 4	10 $\pm$ 4	
Sex, % male/female	58/42	59/41	
White/Black/Hispanic, %	33/67/0	66/30/4	<0.0001
Atopic dermatitis, %	36	28	
Medications, %			
Omalizumab	28	0	<0.0001
ICS-H	41	4	<0.0001
ICS-M	46	24	0.003
ICS-L	12	52	<0.0001
LABA	87	36	<0.0001
LTRA	78	60	0.011
Pulmonary function*			
FVC	89 $\pm$ 15	100 $\pm$ 12	<0.0001
FEV-1	79 $\pm$ 16	95 $\pm$ 12	<0.0001
FEF-25-75	65 $\pm$ 22	90 $\pm$ 21	<0.0001
FEV-1/FVC	84 $\pm$ 14	93 $\pm$ 10	<0.0001
IgE, IU/ml*	353 $\times/\pm$ 4.16	100 $\times/\pm$ 5.28	<0.0001
Sensitivities, %			
<i>Alternaria</i>	63	54	
<i>Cladosporium</i>	32	25	
<i>Helminthosporium</i>	24	22	
<i>Aspergillus</i>	30	15	0.01
Der p and/or Der f	52	33	0.01
Cat	42	29	
CR	28	14	0.03
Trees	61	57	
Grasses	46	44	
Weeds	56	43	

For Pulmonary Function data presented as Mean $\pm$ SD, for IgE as Geometric Mean $\times/\pm$ SD, and for Sensitivities as percentage of patients.

ICS-H, inhaled corticosteroid-high dose; ICS-M, -medium dose; ICS-L, -low dose; LABA, long-acting beta-agonist; LTRA, leukotriene antagonist; FVC, forced vital capacity; FEV-1, forced expiratory volume 1 s; FEF, forced expiratory flow; CR, cockroach.

* P -value by Student's t -test; #by Fisher's exact test.

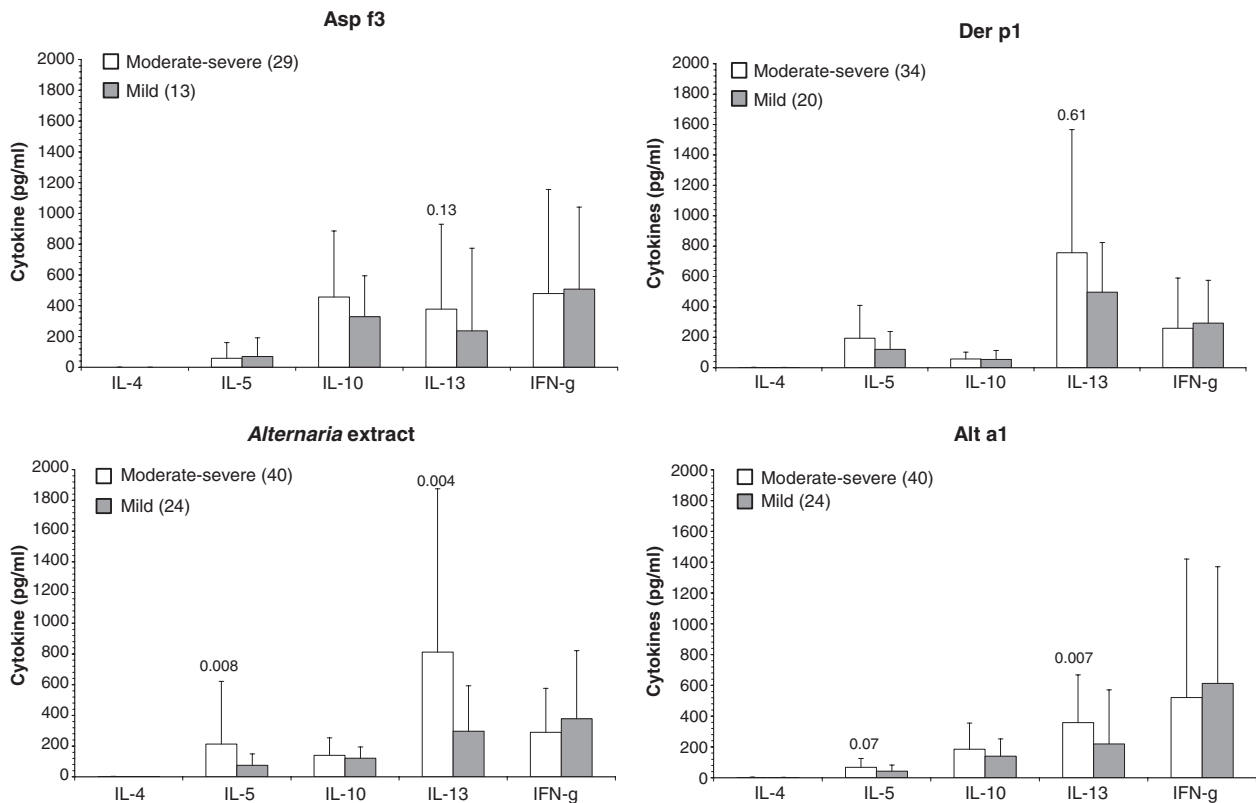


Figure 1 *Alternaria*-stimulated cytokine synthesis in *Alternaria*-sensitive moderate-severe asthma. IL-5 and IL-13 synthesis was significantly increased in both *Alternaria* extract stimulated and Alt a1 stimulated T cells by *Alternaria*-sensitive moderate-severe asthmatic patients compared to *Alternaria*-sensitive mild asthma

($P < 0.008$ and 0.004 , < 0.07 and 0.007 , respectively). In Asp f3 and Der p1 stimulated cultures, there were no significant differences of IL-13 and IL-5 synthesis. Data presented as Mean $\pm$ SD. P -value using Mann-Whitney U -test comparing *Alternaria*-sensitive moderate-severe asthma vs *Alternaria*-sensitive mild asthma.

were not significant for the percentages of patients and allele frequencies of HLA-DRB1*13 ($P > 0.05$). However, comparison of HLA-DRB1*13 was present in 38% of mold-sensitive moderate-severe asthmatics compared to 20% of mold-sensitive mild asthmatics ($P < 0.03$). Likewise, the allele frequency of HLA-DRB1*13 was increased in mold-sensitive moderate-severe asthma compared to mold-sensitive mild asthma, 0.199 vs 0.110 ($P < 0.06$). When *Alternaria*-sensitive asthmatics were examined separately, similar increase was found for HLA-DRB1*13, 37% vs 20% of patients. HLA-DRB1*13 was also significantly increased compared to individuals in the dbMHC database (<http://www.ncbi.nlm.nih.gov/projects/mhc/ihwg.cgi>). HLA-DRB1*13 ranged from 0.005 of 1330 individuals in North America ($P < 0.0001$), 0.098 of 2587 individuals in Europe ($P < 0.0001$), and to 0.139 of 976 individuals in North Africa ($P < 0.0001$). Interestingly, HLA-DRB1*03 tended to be increased in mold-sensitive moderate-severe asthmatic children compared to mold-sensitive mild asthmatics, 29% vs 20%. When *Alternaria*-sensitive asthmatics were examined separately, a similar increase was found for HLA-DRB1*03, 29% vs 20% of patients.

Chi-squared test for independence was used for multiple comparisons of HLA-DQB1*03. Results were significant

for the percentages of patients and allele frequencies of HLA-DQB1*03 ($P = 0.0084$ and $P = 0.0133$, respectively). The allele frequency of HLA-DQB1*03 was significantly decreased in mold-sensitive moderate-severe asthmatics compared to mold-sensitive mild asthmatics, 0.243 vs 0.381 ($P < 0.02$), and HLA-DQB1*03 was significantly decreased in 43% of mold-sensitive moderate-severe asthmatics compared to 61% of mold-sensitive mild asthmatics ($P < 0.03$). When *Alternaria*-sensitive asthmatics were examined, similar results were found, 39% vs 63% of moderate-severe vs mild asthmatics ($P < 0.002$). In the dbMHC database, the HLA-DQB1*03 allele frequency ranged from 0.785 of 1328 individuals in North America ($P < 0.0001$), 0.328 of 482 individuals in Europe ($P < 0.06$), and to 0.263 of 586 individuals in North Africa (NS). These preliminary results of increased frequency of HLA-DRB1*13 and decreased frequency of HLA-DQB1*03 in mold-sensitive and *Alternaria*-sensitive moderate-severe asthmatics will need to be confirmed with a larger study population.

As there were a greater percentage of African-Americans in the mold-sensitive moderate-severe asthma group, we analyzed this group compared to African-American mold-sensitive mild asthmatics. HLA-DRB1*13 was significantly increased in 46% of African-American mold-sensitive moderate-severe asthmatics

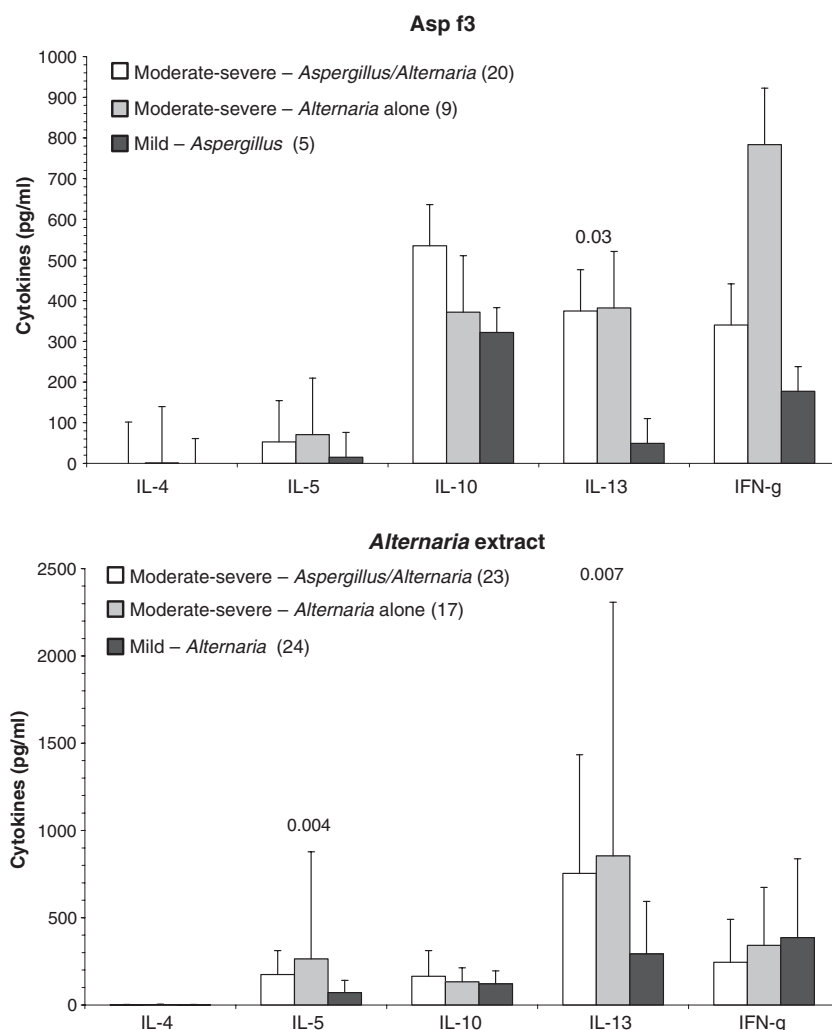


Figure 2 Asp f3 and *Alternaria*-stimulated cytokine synthesis in *Aspergillus/Alternaria*-sensitive and *Alternaria*-sensitive moderate-severe asthma. All of the *Aspergillus*-sensitive moderate-severe asthmatics were also *Alternaria*-sensitive, *Aspergillus/Alternaria*-sensitive asthmatics. In Asp f3 stimulated cultures, IL-5 and IL-13 synthesis was comparable in *Aspergillus/Alternaria*-sensitive and *Alternaria*-sensitive alone moderate-severe asthmatics. IL-13 synthesis was significantly increased in *Alternaria*-sensitive alone moderate-severe asthmatics to Asp f3 stimulation compared to

Aspergillus-sensitive alone mild asthmatics ($P < 0.03$). In *Alternaria*-stimulated lymphocyte cultures, IL-5 and IL-13 synthesis was comparable in *Aspergillus/Alternaria*-sensitive and *Alternaria*-sensitive moderate-severe asthmatics. IL-5 and IL-13 synthesis was significantly increased in *Aspergillus/Alternaria*-sensitive asthmatics compared to *Alternaria*-sensitive mild asthmatics ($P < 0.004$ and < 0.007 , respectively). Data presented as Mean $\pm$ SD. Kruskal-Wallis test (nonparametric anova) was performed. *Post hoc* analysis was performed using Dunn's multiple comparisons test.

ics compared to 21% of African-American mold-sensitive mild asthmatics ($P < 0.03$). HLA-DRB1*13 allele frequency was also increased in African-American mold-sensitive moderate-severe asthmatics compared to African-American mold-sensitive mild asthmatics, 0.480 vs 0.241 ($P < 0.055$). Likewise, HLA-DQB1*03 was significantly decreased in 38% of African-American mold-sensitive moderate-severe asthmatics compared to 66% of African-American mold-sensitive mild asthmatics ($P < 0.02$) with significantly decreased allele frequency 0.420 vs 0.793 ($P < 0.002$).

We then examined antigen presentation of *Alternaria* antigens by HLA-DR, HLA-DP, and HLA-DQ in 22 children

with *Alternaria*-sensitive moderate-severe asthma and in 10 children with *Alternaria*-sensitive mild asthma. When blocking mAbs of HLA-DR, HLA-DP, and HLA-DQ were examined in *Alternaria* extract and Alt a1 stimulated lymphoproliferative studies, anti-HLA-DR mAb inhibited *Alternaria* extract and Alt a1 lymphoproliferative responses, 72% and 50%, respectively ($P < 0.001$ and < 0.03 , respectively) (Fig. 3). There was no significant inhibition observed with blocking anti-HLA-DP and anti-HLA-DQ mAbs. These studies indicate that when *Alternaria*-sensitivity to *Alternaria* extract and Alt a1 occurs in moderate-severe asthmatic children, *Alternaria* is HLA-DR restricted, perhaps by

Table 2 HLA-DR and HLA-DQ allele frequencies in children with mold-sensitive moderate-severe asthma

Study	M-S <i>Mold</i> -sensitive (68)	M-S Not mold-sensitive (28)	M <i>Mold</i> -sensitive (59)	M Not mold-sensitive (31)	<i>P</i> M-S Mold vs M Mold
HLA-DRB1					
*01	10 (0.051)	14 (0.071)	10 (0.059)	19 (0.097)	
*03	29 (0.154)	25 (0.125)	20 (0.102)	19 (0.097)	
*04	16 (0.088)	18 (0.089)	31 (0.161)	32 (0.161)	
*07	21 (0.110)	18 (0.089)	25 (0.136)	16 (0.097)	
*08	6 (0.029)	11 (0.054)	12 (0.068)	10 (0.048)	
*09	6 (0.029)	4 (0.018)	3 (0.017)	0 (0.000)	
*10	2 (0.007)	4 (0.018)	3 (0.017)	0 (0.000)	
*11	22 (0.118)	14 (0.089)	31 (0.169)	26 (0.129)	
*12	6 (0.029)	14 (0.071)	2 (0.008)	10 (0.048)	
*13	38 (0.199)	25 (0.125)	20 (0.110)	36 (0.177)	
*14	3 (0.015)	0 (0.000)	2 (0.008)	0 (0.000)	
*15	25 (0.147)	39 (0.214)	24 (0.136)	23 (0.113)	
*16	4 (0.022)	7 (0.036)	2 (0.008)	7 (0.032)	
HLA-DQB1					
*02	41 (0.243)	39 (0.196)	32 (0.178)	32 (0.177)	
*03	43 (0.243)	32 (0.179)	61 (0.381)	68 (0.355)	0.0084 (0.0133)
*04	10 (0.059)	11 (0.054)	10 (0.051)	7 (0.032)	
*05	31 (0.169)	39 (0.250)	22 (0.119)	32 (0.161)	
*06	44 (0.287)	54 (0.321)	49 (0.271)	48 (0.274)	

Data presented as percentage of patients with allele and in parentheses allele frequency.

Chi-squared test for independence was used for multiple comparisons of HLA-DR and HLA-DQ.

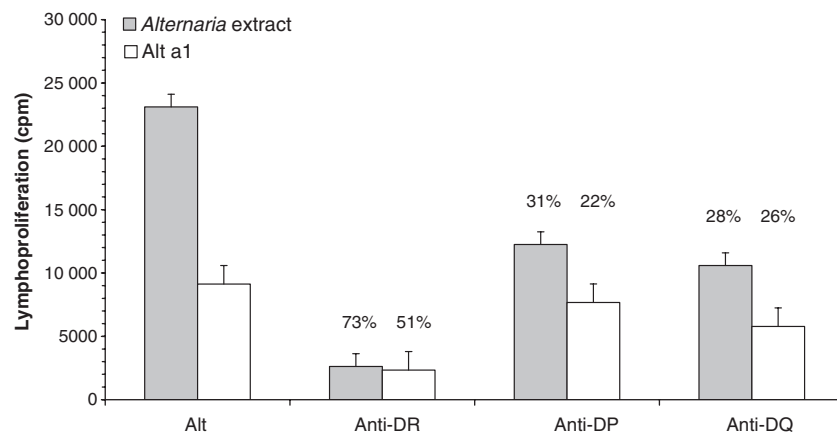


Figure 3 Inhibition of *Alternaria* stimulation with anti-DR, -DP, and -DQ mAbs. Lymphoproliferation to *Alternaria* extract and Alt a1 was significantly inhibited by blocking antibody to HLA-DR, 73% and 51%, respectively ($P < 0.001$ and < 0.03 , respectively). Data

presented as Mean $\pm$ SD. P value using paired Student's t -test comparing anti-HLA-DR, anti-HLA-DP, and anti-HLA-DQ monoclonal blocking antibodies compared to *Alternaria* extract and Alt a1 alone.

HLA-DRB1*13. Further studies are needed to confirm specific HLA-DR types.

Furthermore, we hypothesize that HLA-DQB1*03 may be protective in the development of *Alternaria*-sensitive moderate-severe asthma. To examine this hypothesis, HLA-DQB1*03- *Alternaria*-sensitive asthma was compared to HLA-DQB1*03+ *Alternaria*-sensitive asthma. As seen in Fig. 4, IL-5 and IL-13 synthesis was significantly increased in

Alternaria-stimulated lymphocyte cultures of HLA-DQB1*03- *Alternaria*-sensitive asthmatics compared to HLA-DQB1*03+ *Alternaria*-sensitive asthmatics ($P < 0.004$, < 0.001 , respectively). There was no difference in Asp f3 stimulated cultures. These results are similar to the studies in ABPA, in which the presence of HLA-DQ2 was found to be protective in patients who were *Aspergillus*-sensitive asthmatic and patients with cystic fibrosis (CF) in the develop-

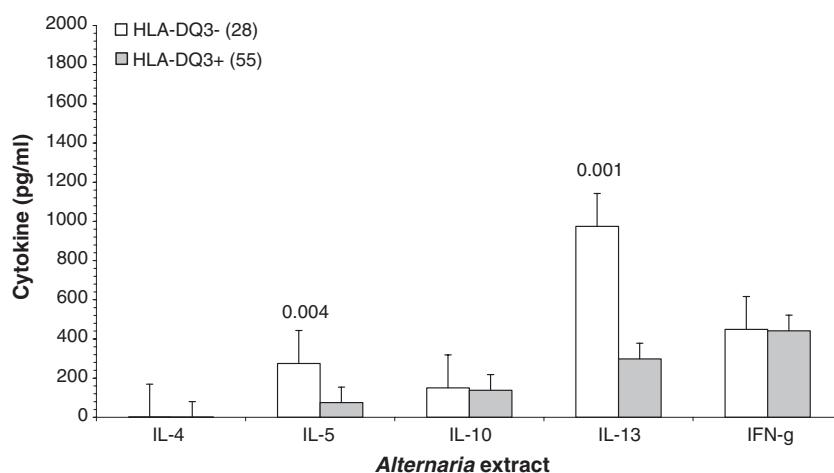


Figure 4 *Alternaria*-stimulated cytokine synthesis in HLA-DQB1*03- and HLA-DQB1*03+ *Alternaria*-sensitive asthma. IL-5 and IL-13 synthesis was significantly increased in *Alternaria*-stimulated T cells by HLA-DQB1*03- *Alternaria*-sensitive asthmatic patients compared

to HLA-DQB1*03+ *Alternaria*-sensitive asthma ($P < 0.004$, 0.001, respectively). Data presented as Mean $\pm$ SD. P -value using Mann-Whitney U -test.

ment of ABPA. In addition, TGF- β synthesis tended to be increased in HLA-DQB1*03+ asthmatics (data not shown). This might suggest that *Alternaria*-specific T regulatory cells may be increased in HLA-DQB1*03+ asthmatics. Therefore, further studies are needed to examine for *Alternaria* HLA-DR restriction and possible HLA-DQB1*03 resistance in *Alternaria*-sensitive moderate-severe asthmatics.

Discussion

Asthma afflicts approximately 6% to 8% of the United States population, and severe asthma represents approximately 10% of patients with asthma (33). This subset of severe asthmatic patients have significant morbidity and utilize health care support disproportionately more compared to asthmatic patients with less severe disease. The current medications of inhaled corticosteroids, leukotriene antagonists, and long-acting beta-2 agonists may not adequately control their asthma. Thus, it becomes important to understand the mechanism(s) as to why these patients have pulmonary inflammation that is not adequately controlled by current treatment regimens.

In previous studies of the immunopathogenesis of ABPA, we identified that the presence of HLA-DR2 and/or HLA-DR and absence of HLA-DQ2, and presence of IL-4 receptor alpha (IL-4RA) 75val SNP predisposed patients who were *Aspergillus*-sensitive asthmatic and patients with CF to develop ABPA (26–32). Using ABPA as a model, we hypothesized that in patients with *Alternaria*-sensitive moderate-severe asthma, there may be similar genetic risk factors that are operative in the development of moderate-severe asthmatic children.

Several epidemiologic studies in the United States and Europe have linked *Alternaria* sensitivity to both persistence and severity of asthma (5, 5, 15–20). *Alternaria* is a common airborne mold and is especially prevalent in the

grain-growing areas of the Midwest. Recently, Denning's group coined the term severe asthma associated with fungal sensitization in adult patients with asthma in the United Kingdom (6, 13, 34). In their studies, sensitivity to *A. fumigatus* was the most prevalent (66%), followed by sensitivities to *Cladosporium* (52%) and to *Alternaria* (34%). Furthermore, treatment of these patients with itraconazole in a 32-week trial resulted in improved asthma quality of life asthma quality of life questionnaire (AQLQ), decreased IgE levels, and increased peak flow. In these preliminary studies, we identified that IgE sensitivity to *A. fumigatus* significantly increased in 31% of children with moderate-severe asthma compared to mild asthma; whereas sensitivity to *A. alternata* was comparable in both moderate-severe and mild asthma, 63% and 57%, respectively. In our study, sensitivity to *Alternaria* was the most frequent mold sensitivity which was similar in both severe and mild asthmatics. However, sensitivity to *A. fumigatus* was significantly increased in 30% of moderate-severe asthmatics compared to 15% of mild asthmatics.

We next examined whether there was a quantitative difference in sensitivity to *Alternaria* antigens in *Alternaria*-sensitive severe compared to mild asthmatics. Indeed, Th2 IL-5 and IL-13 synthesis was significantly increased to both *Alternaria* extract and Alt a1 stimulation in *Alternaria*-sensitive moderate-severe asthmatic children compared to *Alternaria*-sensitive mild asthmatics. As sensitivity to *A. fumigatus* was significantly increased in moderate-severe asthmatics, we separated the groups into *Aspergillus/Alternaria*-sensitive severe asthmatics, *Alternaria* alone severe asthmatics compared to mold sensitive mild asthmatics. Th2 cytokine synthesis was increased to *Aspergillus* stimulation in *Aspergillus/Alternaria*-sensitive severe asthmatics; however, it was also increased in *Alternaria* only sensitive moderate-severe asthmatics. This suggested cross-reactivity of Asp 3 with *Alternaria* antigens in the *Alternaria*-sensitive moderate-severe asthmatics. However, IL-5 and IL-13 synthesis was increased even more to *Alternaria*

ria stimulation in the *Aspergillus*-sensitive asthmatics comparable to the *Alternaria*-sensitive severe asthmatics. These studies suggest that in mold-sensitive asthmatics with moderate-severe asthma, there may be cross-reactivity between *Aspergillus* and *Alternaria* antigens that stimulates Th2 responses upon exposure of *Alternaria* and/or *Aspergillus*.

We next evaluated HLA class II antigens. Blocking studies of HLA class II antigens determined that *Alternaria* allergens were predominantly HLA-DR restricted. HLA-DR antigens on dendritic cells interacting with Th0 cells may determine either a Th1 or a Th2 response. In ABPA, we previously reported that HLA-DR2 (HLA-DRB1*15 and B1*16)/DR5 (HLA-DRB1*11 and HLA-DRB1*12) genotypes as a risk factor for the development of ABPA (31, 32). Previously, Juhn et al. (35) reported in a stratified sample of 340 children from the Rochester Family Measles study that the cumulative incidence of asthma by 12 years old was 33% in children who carried the HLA-DRB1*03 genotype compared to 24.2% of children who did not. Our study extends this finding. In this study, HLA-DRB1*13 genotype was significantly associated and HLA-DRB1*03 tended to be associated with development of mold-sensitive moderate-severe asthma in children. Furthermore, in *Alternaria*-sensitive asthmatics, *Alternaria*-stimulated IL-5 and IL-13 synthesis was decreased in HLA-DQ3+ asthmatics compared to HLA-DQ3- asthmatics. There were more African-Americans in both the mold-sensitive and nonmold-sensitive moderate-severe groups compared to mild asthmatics. However, analysis of African-Americans separately demonstrated increased frequency of HLA-DRB1*13 and decreased frequency of HLA-DQB1*03 in mold-sensitive asthmatics compared to mold-sensitive mild asthmatics. Interestingly, HLA-DQB1*03 was also decreased in nonmold-sensitive moderate-severe asthmatics compared to mild asthmatics. In addition, we evaluated for four molds, and other important mold sensitivities such as *Penicillium* were not identified. Thus, HLA-DQ3+ appears to be protective of development of mold and *Alternaria*-sensitive severe asthma. Interestingly, this is similar to that found in patients with ABPA compared to *Aspergillus*-sensitive asthmatics in which the presence of HLA-DQ2 even in the presence of HLA-DR2/DR5 contributed to resistance of the development of ABPA (31, 32). In addition, TGF- β synthesis tended to be increased in HLA-DQB1*03+ asthmatics (data not shown). This might suggest that *Alternaria*-specific T regulatory cells may be increased in HLA-DQB1*03+ asthmatics. Therefore, further studies are needed to examine for *Alternaria* HLA-

DR restriction and possible HLA-DQB1*03 resistance in *Alternaria*-sensitive moderate-severe asthmatics. These preliminary results of decreased frequency of HLA-DQB1*03 in *Alternaria*-sensitive moderate-severe asthma in children is similar to that found in ABPA where HLA-DQB1*02 was decreased in patients with ABPA. It was determined that HLA-DQB1*02 was protective of the development of patients who were *Aspergillus*-sensitive asthmatics and patients with CF in the development of ABPA.

In summary, using ABPA as a model, we hypothesized that in children with *Alternaria*-sensitive moderate-severe asthma, there may be similar genetic risk factors that are operative in the development of moderate-severe asthma. The results of this study indicate that there is increased Th2 cytokine synthesis to *Alternaria* stimulation in *Alternaria*-sensitive children with moderate-severe asthma. HLA-DRB1*13 and HLA-DRB1*03 may also be associated with development of mold-sensitive moderate-severe asthma. Furthermore, lack of protection by HLA-DQ3 also appeared to be a risk factor. Problems with the study include the numbers of patients examined were small and the lack of replication of the genetic associations. As this study is the initial report of HLA-DR and HLA-DQ genetic associations with mold-sensitive asthma, we did not perform an exhaustive population association study but to report our initial data. The second major reason for the lack of replication of the reported association is that a similar well-defined data set does not exist. However, the study included functional studies of blocking HLA antibodies to provide inferential *in vitro* evidence HLA-DR restriction and decreased IL-15 and IL-13 cytokine synthesis of HLA-DQB1*03 asthmatics. Future replicative studies will need to be performed for validation of our initial genetic associations. In addition, this is an on-going study in which additional asthmatics will be evaluated. Furthermore, EBV-B cell lines of known HLA-DR and HLA-DQ genotypes will be generated to confirm HLA-DRB1*13 restriction and that HLA-DQ3 is protective to *Alternaria* antigens.

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