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Mechanisms of Occupational Asthma: Not all Allergens are Equal

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Abstract

Asthma is a heterogeneous lung disorder characterized by airway obstruction, inflammation and eosinophil infiltration into the lung. Both genetics and environmental factors influence the expression of asthma, and not all asthma is the result of a specific immune response to allergen. Numerous asthma phenotypes have been described, including occupational asthma, and therapeutic strategies for asthma control are similar regardless of phenotype. We hypothesized that mechanistic pathways leading to asthma symptoms in the effector phase of the disorder differ with the inciting allergen. Since route of allergen exposure can influence mechanistic pathways, mice were sensitized by identical routes with a high molecular weight occupational allergen ovalbumin and a low molecular weight occupational allergen trimellitic anhydride (TMA). Different statistical methods with varying selection criteria resulted in identification of similar candidate genes. Array data are intended to provide candidate genes for hypothesis generation and further experimentation. Continued studies focused on genes showing minimal changes in the TMA-induced model but with clear up-regulation in the ovalbumin model. Two of these genes, arginase 1 and eotaxin 1 are the focus of continuing investigations in mouse models of asthma regarding differences in mechanistic pathways depending on the allergen. Microarray data from the ovalbumin and TMA model of asthma were also compared to previous data using *Aspergillus* as allergen to identify putative asthma 'signature genes', i.e. genes up-regulated with all 3 allergens. Array studies provide candidate genes to identify common mechanistic pathways in the effector phase, as well as mechanistic pathways unique to individual allergens.

Key words: trimellitic anhydride, asthma, microarray, eosinophils, arginase

Introduction

An estimated 300 million people are affected by asthma world-wide (1) with varying percentages of the population affected in different countries. Estimates of the prevalence of asthma range from 2% in Greece and 6% in Japan to as many as 15% in New Zealand. The economic burden of asthma is substantial and includes both direct costs (hospitalization and medication) and indirect costs (days lost from work and reduced quality of life). Numerous phenotypes of asthma have been described, and a better understanding of the pathophysiological mechanisms of different asthma phenotypes, including occupational asthma, can provide the basis for more targeted treatment of this heterogeneous lung disorder. This review will focus on occupational asthma and experiments done in animal models pointing to mechanistic differences in the effector phase of asthma depending on the inciting allergen.

What is Asthma?

Asthma is a lung disorder characterized by symptoms of cough, wheezing and chest tightness. These symptoms are attributed to the development of reversible airway obstruction, inflammation, airway hyperresponsiveness and mucus production. An operational definition of asthma developed by experts in the field (1) describes major attributes of the disorder. "Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsive-

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ness to a variety of stimuli." Clearly this definition of asthma does not include a cause. Both host factors (genetics) and environmental factors are known to influence the development and expression of asthma, and a vast array of triggers of asthma have been identified. Various asthma phenotypes have been described in attempts to better define the lung disorder. Wenzel in a recent review (2) proposed three phenotypic categories of asthma: Clinical or physiological phenotypes, phenotypes related to trigger, and inflammatory phenotypes. Clinical or physiological phenotypes are based on descriptors such as the severity of the disease (mild, moderate, severe) or the age of onset (child, adult). Phenotypes related to trigger are based on exposures that initiate the asthmatic symptoms (aspirin-induced, exercise-induced, occupational, etc.), and inflammatory phenotypes rely on a pathophysiological description (eosinophilic, neutrophilic, etc.). The variety of descriptions of asthma phenotypes indicates the heterogeneity of asthma and the uncertainty in the field regarding the cause of asthma. The heterogeneity of clinical symptoms under the umbrella of asthma has indeed prompted some experts to suggest that we abandon asthma as a disease concept (3). Debate also continues regarding the best measure of asthma control (4), inflammation or pulmonary function. Asthma therapies target both the inflammatory component (steroids) and the airway obstruction component (bronchodilators). Asthma control may also differ with the asthma phenotype. A recent study found that children with a severe asthma phenotype had less airflow obstruction compared to adults with severe asthma (5) suggesting that anti-inflammatory therapy may be more critical for asthma control in the phenotype of childhood asthma.

Currently, therapeutic management of asthma is the same whether an individual is allergic to dust mites, toluene diisocyanate, or cat dander. However, different allergens may contribute to asthma heterogeneity or different asthma phenotypes by initiating unique mediator pathways leading to the asthma symptoms. Improved understanding of the effector mechanisms initiated by different allergens will help determine how best to tailor asthma treatment in the individual.

What is Occupational Asthma?

Occupational asthma is asthma caused by workplace exposure (6). This is distinguished from pre-existing asthma that is exacerbated by workplace exposures. Occupational asthma is further subdivided into occupational asthma with (allergic) or without (non-allergic) a latency period. During the latency period of allergic asthma, a specific immune response to the allergen occurs, without asthma symptoms being manifested. Non-allergic asthma does not require a latency period for symptoms to be apparent and by inference does not involve a specific immune response to the offending substance. Irritant-induced asthma is considered a subset of non-allergic occupational asthma. Some occupational asthma to irritating chemicals may be a combination of allergic and non-allergic asthma. Allergic asthma is most amenable to modeling in animals so studies of occupational asthma have primarily investigated the pathophysiology of allergic occupational asthma following sensitization and challenge with occupational allergens. Occupational allergens are generally categorized as either high or low molecular weight allergens. Examples of high molecular weight occupational allergens include proteases used in the detergent industry, laboratory animal allergens, and ovalbumin (OVA) exposure in egg processing facilities (7–9). Low molecular weight allergens are generally reactive chemicals that act as haptens. Diisocyanates (10) and acid anhydrides (11) are known low molecular weight allergens that cause occupational asthma.

Use of microarrays to identify novel effector mechanisms

In allergic asthma two phases are considered: the sensitization or induction phase and the challenge or effector phase. In the sensitization phase during the latent period, a specific immune response occurs in response to allergen exposure. No asthma symptoms are manifested. After a specific immune response to allergen, whether chemical or protein, re-exposure to the allergen can result in the symptoms of asthma, the effector phase. With the advent of microarray technology, rapid determination of changes in gene expression during the effector phase became possible. This technique allows widespread screening and identification of many genes not previously implicated in the allergic response. For example, it was generally believed that the complement system was not involved in effector mechanisms in allergy because complement activation is not triggered by IgE antibody associated with allergic diseases. However, Karp et al. (12) used microarray analysis of pulmonary gene expression and single nucleotide polymorphism-based genotyping, combined with quantitative trait locus analysis to determine that the complement component C5 was associated with allergen induced airway hyperresponsiveness to OVA as the allergen. For this analysis, A/J mice that easily develop allergen induced airway hyperresponsiveness were crossed with C3H/HeJ mice to obtain new mouse strains. Zimmermann et al. (13) also capitalized on microarray techniques to identify genes that were up-regulated in the lungs of mice sensitized and challenged with either the allergen OVA or *Aspergillus*. This 'discovery science' identified arginase, trefoil factor-2, small proline rich protein 2, ADAM 8, and other novel genes as potentially important genes in the asthmatic response. Many of the candidate genes were not classically associated with allergic respiratory responses. The identification of arginase 1 from array studies in a mouse model led to identification of increased arginase in lung tissue from human asthmatics as well (13). Following initial identification by array analysis, continued studies have begun a more detailed analysis of the expression and regulation of some of these candidate genes in allergen or IL-13 induced inflammation and airway hyperresponsiveness in mouse models (14–17). Rigorous experiments to implicate these molecules as critical to the allergen induced symptoms in mouse models of asthma have not yet been done.

Not all allergens are equal: Use of microarrays to identify differences in effector pathways

Experimental design considerations

The pathophysiology leading to asthma symptoms in the effector phase is generally assumed to be the same regardless of the inciting allergen, though this assumption is relatively unexplored. Zimmermann et al. (13, 18) used microarray analysis with OVA and *Aspergillus* as allergens primarily to suggest common mechanisms in the pathogenesis of the effector phase of asthma, regardless of the inciting allergen. However, they also identified many genes that were uniquely up-regulated by each allergen, suggesting that each allergen has a unique effector pathway. To test the hypothesis that effector pathways differ with different allergens, we examined differences in gene expression in the mouse lung in response to two different occupational allergens, OVA and trimellitic anhydride (TMA). OVA was chosen because of the extensive literature in animal models using OVA as the prototype protein allergen. OVA is also known to cause occupational asthma (7–9). The low molecular weight allergen TMA is an organic acid anhydride that is used in the paints and plastics industry and is a known cause of occupational asthma in humans (11). Zimmermann's study design employed a chronic allergen exposure (*Aspergillus*) and an acute allergen exposure (OVA). Studies in animal models using the protein allergen OVA indicate that effector mechanisms of asthma can differ depending on the route of allergen sensitization and/or challenge (19). With OVA, the role of IL-5 and IgE in mouse models of asthma differs depending on whether the initial sensitization is dermal or respiratory. Effector mechanisms also can differ depending on the mouse strain used (20). Because of Zimmermann's experimental design (13), the differences identified in the transcriptome with two different protein allergens could reflect the different sensitization and challenge regimens as well as the different allergens. Thus, to test the hypothesis that effector pathways differ with different allergens, our studies controlled for the sensitization/challenge regimen by using identical routes of exposure for both allergens. This minimizes changes in gene expression due to differences in allergen delivery.

Interpretation of changes in the transcriptome is most straightforward if a pure cell population is considered. Microarray data using mixed cell populations may reflect an increase in gene expression, or simply a change in the cell population associated with an inflammatory influx of cells (21). The decision to look at gene expression changes in whole lung stems from the definition of asthma and the realization that no one single cell type is critical to the development of expression of asthma. Asthma results from a complex interplay of numerous cells and mediator systems and cannot be modeled using a single cell type. Our study examined gene expression in whole lung using allergen doses that resulted in the same increase in eosinophil infiltration with both allergens, with minimal changes in neutrophils. Since eosinophils are the predominate cell type altered following allergen challenge in our model, differences observed in the transcriptome with these different allergens more likely reflect changes in gene expression due to mechanistic differences rather than changes in the cell population being surveyed.

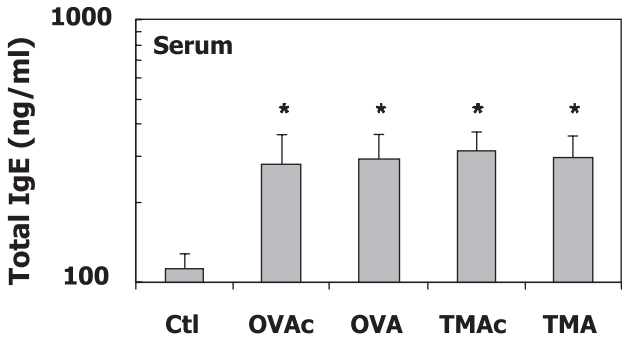

Fig. 1 Balb/c mice were unsensitized (Ctl) or sensitized with ovalbumin or trimellitic anhydride in corn oil, followed by intratracheal challenge with TMA conjugated to mouse serum albumin (TMA) or ovalbumin (OVA) to elicit the allergic phenotype. Mouse serum albumin challenge in OVA sensitized (OVAc) or TMA sensitized (TMAc) animals served as control. Experimental details of sensitization and challenge are provided in Reference 22. Total serum IgE was determined by standard ELISA methodology. * $p<0.05$ compared to Ctl.

Many chemical allergens, including TMA, are not water soluble or quickly lose their ability to react with protein if dissolved in aqueous vehicles. Thus, Balb/c mice were sensitized with OVA or TMA suspensions in corn oil, followed by intratracheal challenge with OVA alone (OVA group) or TMA conjugated to mouse serum albumin (TMA group), respectively, to elicit the allergic phenotype. Mouse serum albumin challenge in OVA sensitized (OVAc) or TMA sensitized (TMAc) animals served as control. As an indicator of the degree of sensitization with the two different allergens, we have confirmed that increases in total serum IgE are the same for both allergens compared to naïve unsensitized mice (Fig. 1). At 72 hours after allergen challenge, measurements of eosinophil peroxidase in homogenized lung indicated that both allergens caused the same significant increase in eosinophilia (22). To assess gene expression, RNA was isolated from lung lobes of 6–8 animals in the 4 treatment groups (OVA, OVAc, TMA, TMAc) and hybridized with Affymetrix U74Av2 microarrays. Cui and Churchill (23) recommend an 'n' of 6 or more to detect relevant biological changes using arrays.

Statistical considerations

Since our experimental design included more than two treatment groups, the initial statistical analysis we reported (22) employed an ANOVA F test assuming unequal variances to test for any differences across the 4 treatment groups (OVA, OVAc, TMA, TMAc). A q-value, a type of False Discovery Rate (24), was computed to guard against false positives when a large number of comparisons are being done simultaneously. Using a q-value cutoff of 0.1, 853 probe sets were identified as different across the 4 treatment groups (Table 1, ANOVA F test). Expression ratios of OVA/OVAc and TMA/TMAc were calculated and used as a secondary cutoff for selection of our relevant candidate gene set. An expression ratio of 1.2 or greater (or less than 1/1.2) was chosen as an indicator of biological significance. This expression ratio cutoff was based on a review of the literature and the fact that we used a whole tissue where important changes in gene expression in a single

Table 1 Statistical analysis of array data: comparison of methods

	Number of probe sets	
Criteria	Anova F test[a]	Empirical Bayes Method[b]
Statistical significance	853	764
Statistical significance and expression ratio cutoff (>1.2 or <1/1.2)	376	566
Up-regulated by both OVA and TMA	112	133
Uniquely up-regulated by OVA	89	157
Uniquely up-regulated by TMA	86	113

[a] From Reference 22.
[b] From Figure 2A.

cell type may be diluted. Adding an expression ratio cutoff narrowed our candidate gene list to 376 probe sets (Table 1). This included 112 probe sets up-regulated in common with both allergens, with 86 uniquely up-regulated with TMA and 89 uniquely up-regulated with OVA. The remaining probe sets were down-regulated with either or both allergens. In our study (22), we found 201 probe sets up-regulated with OVA (112+89; Table 1). Zimmermann's study (13) identified 496 probe sets up-regulated with OVA. This difference in the number of OVA up-regulated genes in the two studies could be related to numerous factors, including the routes of allergen exposure, number of allergen challenges and the use of alum adjuvant for OVA in Zimmermann's study. Zimmermann et al. (13) also used less stringent statistical cutoffs and did not adjust for multiple comparisons. Both of these factors likely increased the candidate data set in Zimmermann's study compared to ours. However, Zimmermann's use of 2 or 3 chips per treatment group would tend to reduce the size of their candidate gene list.

Statistical analysis of array data is a rapidly advancing area of investigation. In a follow-up analysis, Zimmermann et al. used the same raw data but a more sophisticated bioinformatics processing that corrected for multiple comparisons and found the number of genes in common with OVA and *Aspergillus* as 242 vs 291 in the previous analysis (13, 18). Our initial data analysis with OVA and TMA as the allergens employed Robust Multichip Analysis (RMA) to accomplish background correction, normalization and calculation of expression levels from chip intensities (25). This method increases the power to detect effects for genes with low expression and uses only Perfect Match values from Affymetrix U74Av2 chips, not Mismatches. As statistical methods have improved, we have also re-analyzed our raw data using an Empirical Bayes method that generates a Moderated F statistic (26). A Moderated F statistic guards against assigning small changes as being significant due to misleadingly small variances. Using this Empirical Bayes method on our previously published data with similar statistical cutoffs, 764 probe sets are considered statistically significant (Table 1). These probe sets are also plotted in Figure 2A with two primary differences noted from the original analysis (22). As expected, with the Empirical Bayes methodology many genes with a small change in expression ratio are no longer significantly different. In addition, many genes with larger variances and larger expression ratios attain significance. Statistical analysis of array data is complex, and there is more than one valid statistical analysis that can be applied to data sets.

Regardless of the analysis, array data are intended to provide candidate genes for hypothesis generation and further experimentation. In our study, selected significant genes with varying expression ratios were verified by quantitative RT-PCR methods (22). The list of candidate genes was then examined to focus our further experimentation on differences in mechanistic pathways with the two different occupational allergens.

Differences in arginase 1 in the effector mechanism

Since the initial aim of our study was to determine differences in gene expression in whole lung of mice sensitized and challenged with two different occupational allergens, OVA and TMA, our initial focus was on genes that were uniquely up-regulated by OVA, with minimal changes seen with TMA sensitization and challenge. Genes up-regulated in common with OVA and TMA, as well as unique to OVA and TMA are depicted by a Venn diagram in Figure 2B. Confirming previous studies of Zimmermann et al. (13), gene expression for arginase 1 was significantly increased in OVA induced asthma (Arg 1 in Fig. 2A and 2B). Quantitative RT-PCR analysis confirmed the microarray findings for gene expression in the lung tissue and found that arginase 1 gene expression was significantly less in TMA induced asthma compared to OVA induced asthma (22). In addition, evaluation of arginase enzyme activity in lung homogenates also confirmed that protein activity was significantly less in TMA induced asthma compared to OVA induced asthma (22). Differences in arginase 1 expression with the two different allergens could reflect mechanistic differences leading to a common set of asthma symptoms or different mechanistic pathways leading to a distinct profile of asthma symptoms unique to the individual allergen. Thus, we speculated that in the OVA sensitized and challenged animals increased arginase minimizes nitric oxide production by iNOS via competition for the arginine substrate, favoring airway hyperresponsiveness (13, 22). In the TMA sensitized and challenged animals, reduced arginase activity would favor arginine metabolism by iNOS leading to increased nitric oxide, bronchodilation, and minimal airway hyperresponsiveness. In fact, our unpublished studies using unrestrained barometric plethysmography and a dimensionless variable called enhanced pause as an indicator of airway hyperresponsiveness demonstrate that increased responsiveness to methacholine is evident in OVA sensitized and challenged animals but not in TMA sensitized and challenged animals. Thus, these data suggest that the differences in gene expression reflect different mechanistic pathways leading to a distinct profile of asthma symptoms unique to the individual allergen.

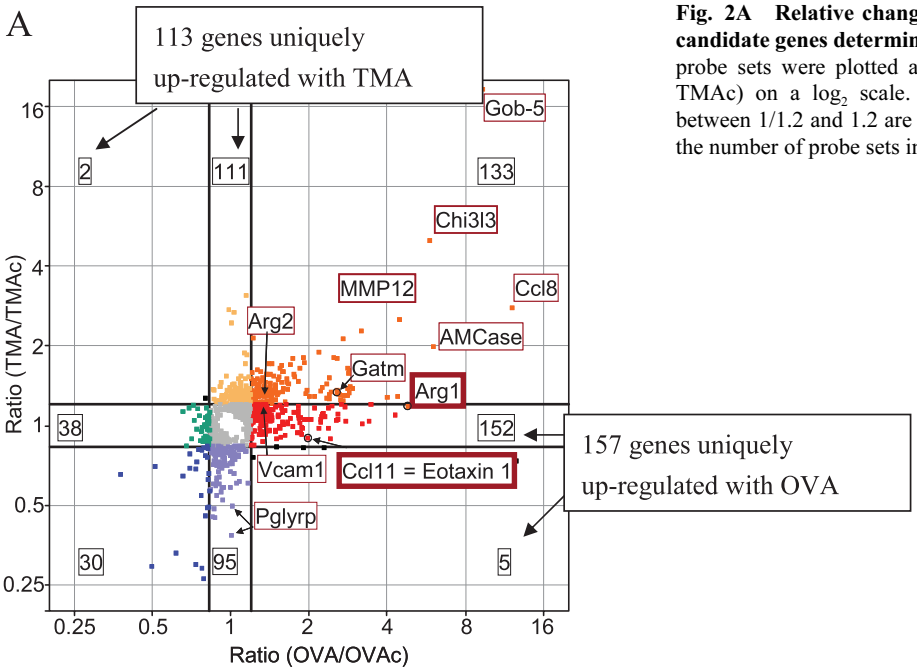

Fig. 2A Relative changes in gene expression signal intensity for candidate genes determined by Empirical Bayes analysis. Candidate probe sets were plotted as the ratio of (OVA/OVAc) versus (TMA/TMAc) on a $\log_2$ scale. Experimental to control expression ratios between 1/1.2 and 1.2 are lined in bold. The boxed numbers designate the number of probe sets in the given region of the graph.

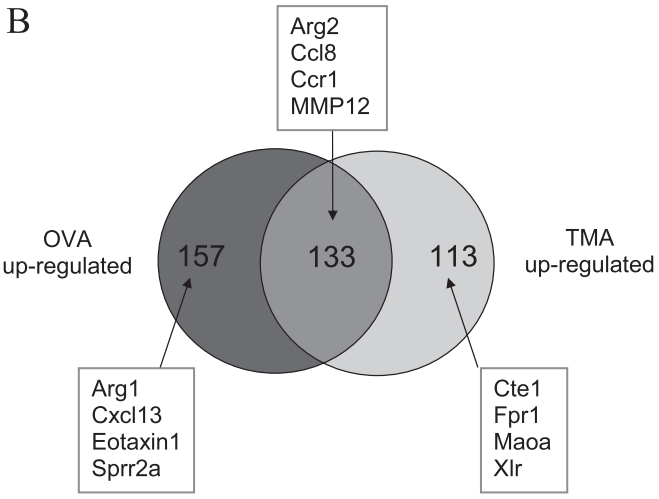

Fig. 2B Venn diagram depicting the probe sets uniquely up-regulated by either OVA or TMA as well as the probe sets commonly up-regulated by both allergens. Data from Figure 2A.

Differences in eotaxin 1 in the effector mechanism

Another differentially expressed gene of interest was eotaxin 1 (CCL11) that increased in response to OVA with minimal changes after TMA sensitization and challenge (Fig. 2A and 2B). Quantitative RT-PCR and ELISA assays confirmed these differences in eotaxin 1 in the OVA and TMA model at both the mRNA levels (22) and protein levels (unpublished data). Eotaxin 1 functions as a chemoattractant for eosinophils and is regulated by IL-5 and IL-13. A large body of literature suggests that eotaxin 1, IL-13, and IL-5 are important mediators of lung eosinophilia. Although we observed similar increases in lung eosinophilia with both allergen models, increases in eotaxin 1 and IL-13 protein are significantly less in the TMA model than the OVA model of allergic inflammation (unpublished studies). For these reasons, we propose alternative eosinophil chemoattractants and perhaps alternative pathophysiological mechanisms may be involved in the TMA model of asthma. These hypotheses are the subject of continued investigation.

Asthma signature genes

Asthma is a lung disorder with a complex genetic component. Linkage analysis has defined both chromosomal regions associated with asthma as well as specific genes involved in the asthmatic response (Reviewed in 27). Review of microarray analysis on human bronchial biopsies, airway epithelial cells and peripheral blood cells have identified genes with altered expression levels in asthmatics, providing clues to mechanisms of asthma pathogenesis (21, 27–29). Use of human tissue is complicated by the heterogeneity of biopsy samples that can be used as well as by the complicating effects of ongoing therapy for asthma and the difficulty in controlling experimental manipulations. Thus animal models benefit from more easily controlled environmental exposures and experimental manipulations to provide clues to asthma pathogenesis. Using a mouse model, Zimmermann et al. (13) suggested that some 291 genes were up-regulated in common with OVA and *Aspergillus* and

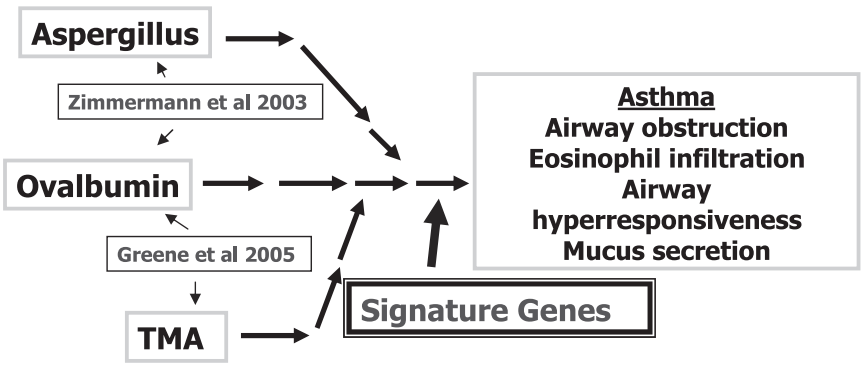

Fig. 3 Hypothetical effector pathways leading to the asthma symptoms for ovalbumin, *Aspergillus*, and trimellitic anhydride (TMA). Data from reference 13 and the Empirical Bayes analysis of raw data from reference 22 were combined. All 3 allergens are known to cause asthma symptoms. However, the effector pathways leading to those symptoms have both unique and common components. The common components can be represented by the 67 signature genes depicted in Figure 4.

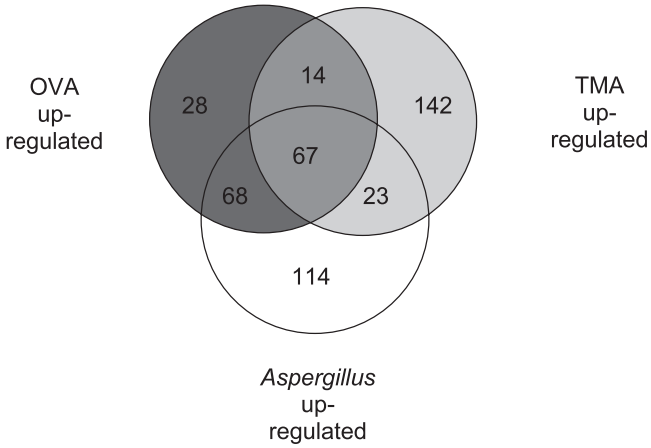

Fig. 4 Venn diagram depicting the genes uniquely up-regulated by either OVA, TMA or *Aspergillus*, as well as the genes commonly up-regulated by all 3 allergens—67 asthma signature genes.

represented asthma 'signature genes'. These signature genes could represent common mechanistic pathways to the asthma phenotype regardless of the inciting allergen. Since both Zimmermann's study and ours had used the Balb/c mouse and Affymetrix arrays of whole mouse lung, we combined our array data from OVA sensitized and challenged mice after Empirical Bayes analysis (Fig. 2A) with Zimmermann's data (13) and found that 177 genes were up-regulated by OVA in both experimental systems. The genes up-regulated by OVA were then compared with those up-regulated by *Aspergillus* and by TMA to arrive at a set of signature genes representing a common mechanistic pathway for all 3 allergens as depicted in Figure 3. The results of that analysis are depicted by a Venn diagram in Figure 4. In all, 67 genes were up-regulated in common with all 3 allergens, suggesting a group of common asthma 'signature genes' from the two microarray studies.

Our analysis combined microarray results from three different occupational allergens: acute exposure to low molecular weight TMA and high molecular weight OVA and chronic exposure to *Aspergillus*. These allergens have been extensively used in experimental models of asthma in mice. Lists of occupational allergens are available and can easily contain hundreds of molecules with new allergenic workplace hazards continually being identified (6). Whether the 67 signature genes identified by our analysis will be common to the broad range of occupational allergens encountered in the workplace requires continued experimentation and validation. However, these 67 signature genes importantly point to common mechanistic pathways that may be considered as therapeutic targets for treatment of allergic occupational asthma. Besides common mechanistic pathways, these studies also point to potential unique mechanistic pathways that lead to the asthma phenotype for each allergen. Gene expression profiling studies such as these can help focus studies in human tissue and provide the basis for generation of specific hypotheses and further experimentation to determine similarities and differences in the pathophysiological mechanisms in the effector phase of asthma.

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