

Research

## Microarray profile of differentially expressed genes in a monkey model of allergic asthma

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### Abstract

**Background:** Inhalation of *Ascaris suum* antigen by allergic monkeys causes an immediate bronchoconstriction and delayed allergic reaction, including a pulmonary inflammatory infiltrate. To identify genes involved in this process, the gene-expression pattern of allergic monkey lungs was profiled by microarrays. Monkeys were challenged by inhalation of *A. suum* antigen or given interleukin-4 (IL-4) treatment; lung tissue was collected at 4, 18 or 24 h after antigen challenge or 24 h after IL-4. Each challenged monkey lung was compared to a pool of normal, unchallenged monkey lungs.

**Results:** Of the approximately 40,000 cDNAs represented on the microarray, expression levels of 169 changed by more than 2.5-fold in at least one of the pairwise probe comparisons; these cDNAs encoded 149 genes, of which two thirds are known genes. The largest number of regulated genes was observed 4 h after challenge. Confirmation of differential expression in the original tissue was obtained for 95% of a set of these genes using real-time PCR. Cluster analysis revealed at least five groups of genes with unique expression patterns. One cluster contained genes for several chemokine mediators including eotaxin, PARC, MCP-1 and MCP-3. Genes involved in tissue remodeling and antioxidant responses were also identified as regulated by antigen and IL-4 or by antigen only.

**Conclusion:** This study provides a large-scale profile of gene expression in the primate lung following allergen or IL-4 challenge. It shows that microarrays, with real-time PCR, are a powerful tool for identifying and validating differentially expressed genes in a disease model.

### Background

Asthma is one of the most serious allergic diseases. Characteristic features of atopic asthma are circulating specific IgE antibodies, positive skin tests to common allergens, and infiltration of the bronchial mucosa with eosinophils and Th2 cells. The resulting pulmonary inflammation leads to

bronchoconstriction, airway hyper-responsiveness, and ultimately to airway remodeling [1]. Many cellular mediators, including cytokines and chemokines, are involved in asthma; Th2-type cytokines such as interleukin-4 (IL-4), IL-5, IL-9 and IL-13 may contribute to pathophysiological changes in asthma [2]. The complexity of asthma originates

from the interaction of an unknown number of genes with environmental factors [3]. Studies of twins have shown that concordance rates for asthma are significantly higher in monozygotic twins than in dizygotic twins, and that the heritability of asthma may be as high as 75% [4]. Linkage analysis of asthma within families has revealed that there are multiple chromosomal regions, containing potential candidate genes, associated with various asthma phenotypes [3,5,6].

Microarray technology offers a new opportunity to gain insight into global gene-expression profiles in asthma, leading to the identification of asthma-associated genes. Microarrays are a 'gene-chip'-based technology in which cDNA sequences representing individual genes are spotted on glass slides at high density [7]. These sequences are then hybridized to cDNA probes derived from paired RNA samples of interest. The two sets of cDNA probes can be labeled with two different fluorescent dyes. The competitive hybridization of a fluorescently labeled probe pair to a microarray enables a comparison of the relative abundance of transcripts of that gene in each sample. In this way, microarrays can detect the differential expression levels of thousands of genes simultaneously.

Cluster analysis is often applied to the analysis of microarray data. In this method, statistical algorithms are used to arrange genes according to similarity in their pattern of expression [8]. The combination of microarray data and cluster analysis to make a genetic portrait of a disease has been used to characterize breast tumors [9], to categorize the subtype of diffuse large B-cell lymphoma [10], and to identify genes whose expression is associated with mitotic misregulation and human aging [11].

The allergic cynomolgus monkey (*Macaca fascicularis*) has been used as a non-human primate model to study inflammatory responses in the lung following allergen challenge [12,13]. This monkey has a natural hypersensitivity to the antigen of the nematode *Ascaris suum*. Inhalation of the *A. suum* extract causes IgE-mediated immediate and delayed allergic reactions and an influx of inflammatory cells into the lung [14]. The resulting lung airway inflammation, eosinophilia and hypersensitive bronchoconstriction have similarities to the symptoms and processes seen in human asthma [1]. Therefore, examination of the gene-expression profile in this non-human primate model could provide useful insights into genes regulated in asthma and the mediators produced.

In the study reported here we investigated the differential gene-expression profile in the acute phase of allergic reaction to the *Ascaris* antigen in monkey lungs. As the cytokine IL-4 is an important inflammatory mediator in asthma [2], an IL-4-challenged monkey lung was also included in this study.

## Results and discussion

### Experimental design

DNA microarrays were used to identify the gene expression pattern induced in the lung of allergic monkeys by inhalation of *A. suum* extract. Six control animals and four experimental animals were used for microarray hybridization experiments. Because of initial limitations on the availability of allergic monkeys, a strategy was used whereby microarray studies were conducted using a single animal at each of three different time points: 4, 18 and 24 h after challenge by inhalation of *A. suum* extract. The selection of time points is based on our previous study of *Ascaris*-challenged monkeys [14]. Following the identification of regulated genes, a second method - quantitative reverse transcription polymerase chain reaction (RT-PCR) using Taqman™ - was used to establish that selected, regulated genes detected by microarray could be confirmed in the same sample by RT-PCR, to compare the magnitude of regulation determined by microarray and RT-PCR and to verify the validity of selected regulated genes by examining the gene expression levels in lung RNA from multiple, individual control and challenged monkeys. Lastly, because IL-4 is implicated as an important inflammatory mediator in asthma [2], one monkey was treated with recombinant human IL-4 by inhalation and lung RNA isolated 24 h later. In summary, a total of four experimental monkey lung tissue samples were used for microarray hybridization (4, 18 and 24 h after allergen challenge, or 24 h after IL-4 challenge). The gene-expression levels in each of these samples were compared to those of an RNA pool prepared from equivalent amounts of lung RNA from each of six normal control monkeys.

Approximately 40,000 cDNA elements on the microarrays were hybridized with the four probe pairs to examine differential gene expression. Genes were chosen for further study by being up- or down-regulated by at least 2.5-fold compared to controls in at least one of the four probe pairs. The selection of these genes was empirical. The use of a higher criterion (that is, threefold) excluded some genes, such as those for MCP-1 and ICAM-1, which are known to be regulated in the acute phase of allergen challenge [15,16]; on the other hand, a lower criterion, such as twofold, increased the probability of false-positive genes and lack of confirmation by RT-PCR (see Validation of the microarray gene expression, below).

In total, 169 cDNA elements were identified to be regulated by more than 2.5-fold in at least one of the pairwise probe comparisons. This is 0.43% of the 40,000 cDNA elements on the microarray. Factors contributing to this low frequency of regulated genes may include: the criterion used for selection (2.5-fold of the normal control level); induction of only a limited set of genes by this allergen, as its inhalation causes an acute inflammation response mainly in airways; and the use of probes from whole-lung homogenates, which may result in a dilution of signals, making it difficult to detect genes induced only in the airways. Therefore, the actual

number of genes induced by the allergen may be higher. In comparison, a large-scale gene-expression study of a complex tissue - breast tumors - demonstrated that 22% of the total genes examined were regulated by at least fourfold in at least three of the 84 probe pairs tested [9].

Lastly, the interspecies hybridization using monkey cDNA to probe human cDNA arrays could also contribute to the low number of regulated genes identified. The sequence homology between monkey and human genes is quite high, however. For example, our analysis of all available cDNA sequences of cynomolgus monkey for 139 known genes shows that the average percentage identity between monkey and human cDNA is about 95%, ranging from 88 to 99%. Only five sequences (3.5%) have identity less than 90%. A study of the sensitivity and specificity of oligonucleotide (50mer) microarray demonstrated that sequences with greater than 75-80% identity will cross-hybridize and contribute to the overall signal intensity [17]. When we use RT-PCR to validate microarray results, most of the primers and probes in RT-PCR are designed on the basis of human sequences, which in fact works well on monkey cDNAs. Therefore, most of genes on the human cDNA array should have been detected by monkey cDNA probes in this study.

A total of 149 genes were represented by the 169 cDNA elements identified as regulated in this study. Of the 149 genes, 52 were novel and 97 were known genes. Table 1 provides a summary of the numbers of differentially expressed cDNA elements in each of the four probe pairs. At 4 h after challenge, the highest number of downregulated cDNA elements (92) was observed, while the highest number of upregulated cDNA elements (51) was seen in the IL-4-challenged monkey. The largest number of differentially expressed cDNA elements (127) was seen 4 h after the allergen challenge, with fewer observed at 18 h (46 cDNA elements) or 24 h after challenge (31 cDNA elements). Interestingly, the second highest number of regulated genes (97) occurred in the IL-4-challenged monkey at 24 h. While definitive conclusions are precluded by the fact that each time point or condition is represented by only a single probe pair, it is noteworthy that the highest number of regulated genes was observed early (4 h) after challenge.

#### Cluster analysis of microarray data

A statistical cluster program was used to analyze the 169 cDNA elements identified as regulated by more than 2.5-fold in one or more probe pairs. Of the 169 cDNA elements, 161 met with the minimal requirements of the cluster program (see Materials and methods) and were subjected to hierarchical cluster analysis. This methodology [8] has been used by others to define the gene-expression profile of breast tumors [9], to subtype diffuse large B-cell lymphomas [10] and to identify genes whose expression is associated with mitotic misregulation and human aging [11]. Figure 1a illustrates the cluster-analysis results. All the genes with a

similar spatial and temporal expression pattern were clustered together on the basis of their similarity scores. To visualize the temporal expression pattern of the clusters, the gene-expression levels are displayed for a majority of genes with known functions (Figure 1b).

One of the clusters that displayed the largest number of the upregulated genes is cluster A. The genes in this cluster demonstrated a similar temporal gene-expression pattern (Figure 1b). Four hours after allergen challenge, genes in this cluster were increased by two- to fourfold of the level observed in normal controls. The elevated gene expression observed at 4 h after challenge was decreased by 18 h post-challenge and was generally near baseline levels 24 h after the challenge. Most of the genes in this cluster were also upregulated by IL-4. Cluster A is dominated by genes for chemokines and adhesion molecules, which are known to be involved in inflammatory responses.

More specifically, MCP-1 [2,16], MCP-3 [2,18], eotaxin [16,18], pulmonary and activation-related chemokine (PARC) [19], and vascular cell adhesion molecule 1 (VCAM-1) [15] have been identified as mediators of pulmonary inflammation in asthma or relevant animal models. Many of the genes in cluster A derived from the IL-4-treated monkey were generally increased above the control level. Importantly, several of these genes - those for eotaxin [20], VCAM-1 [21], MCP-1 [22] and MCP-3 [20] - were previously known to be inducible by IL-4 in cells relevant to pulmonary inflammation, and this regulation is corroborated here *in vivo* in the pulmonary tissue of a non-human primate.

Other genes in cluster A are involved in tissue remodeling (those encoding tissue inhibitor of metalloproteinase-1 (TIMP-1) [23], plasminogen activator inhibitor-1 (PAI-1) [24], and chitinase [25,26]) or in wound healing (tumor-associated antigen L6). Interestingly, in another microarray study using the Incyte UniGEM-V chip [24], PAI-1 was identified as a highly expressed inducible gene in human mast cells. This gene is known to be involved in a pathway that controls fibrosis and regulates the extracellular matrix during tissue remodeling. Studies of PAI-1 deficient or overexpressing mice indicate that PAI-1 regulates the amount of collagen deposition in the lung [27]. Another cluster A gene is alpha<sub>1</sub>-antichymotrypsin (ACT), a member of the serine proteinase inhibitor (serpin) family, which inhibits the neutrophil proteinase cathepsin G and mast cell chymases, and protects the lower respiratory tract from damage by proteolytic enzymes [28]. A recent linkage study of an Italian population of allergic asthmatics suggests that ACT or a gene in close proximity on chromosome 14 is an asthma-susceptibility gene in this population [29]. Lastly, several antioxidant genes were also identified (Mn superoxide dismutase-1 (SOD-1), SOD-2, glutathione peroxidase (GPx)). There is evidence that oxidative stress and reactive oxygen species contribute to the inflammation of asthma [30].

**Table 1**

<b>cDNA elements differentially expressed in challenged monkey lungs</b>				
Gene expression*	4 h†	18 h	24 h	IL-4
Up >1.5-fold	35	29	15	51
Down < -1.5-fold	92	17	16	46
Subtotal	127	46	31	97
Unchanged -1.4 ~ 1.4-fold	42	123	138	72
Total	169	169	169	169

\*In the challenged lungs versus the pool of normal, control lungs. †Hours after *A. suum* challenge or 24 h post IL-4 challenge.

The gene expression pattern of cluster B resembled that of cluster A. One of the main differences between cluster A and cluster B is that most of genes in cluster B were minimally regulated by IL-4. As for cluster A, the expression of the genes in cluster B was also two- to fourfold higher at 4 h after challenge compared to control. However, the elevated gene expression was at baseline levels 18 h after challenge. Due to the low number of known genes identified by these microarray studies (149) and the few genes contained within the clusters, such as cluster B, specifying the biochemical pathway(s) defined by each cluster is difficult; however, several genes of note are included in this cluster. In contrast to the three antioxidant genes in cluster A, which are inducible by IL-4, the gene for glutathione-S-transferase (GST), a member of cluster B, is not regulated by IL-4. Additional genes involved in remodeling are contained within cluster B, such as those for MMP-7 (also known as matrilysin [31]), thrombospondin [32] and haptoglobin-alpha. Haptoglobin-alpha is highly expressed in alveolar macrophages and eosinophils in inflamed human lung tissues [33]. Lastly, the intercellular adhesion molecule, ICAM-1, a cluster B gene, was previously found to be increased on inflamed airway epithelium in a primate model of pulmonary inflammation [34].

It should be noted that genes for cytokines such as IL-4, IL-5, IL-9 and IL-13, which are known to be involved in allergic

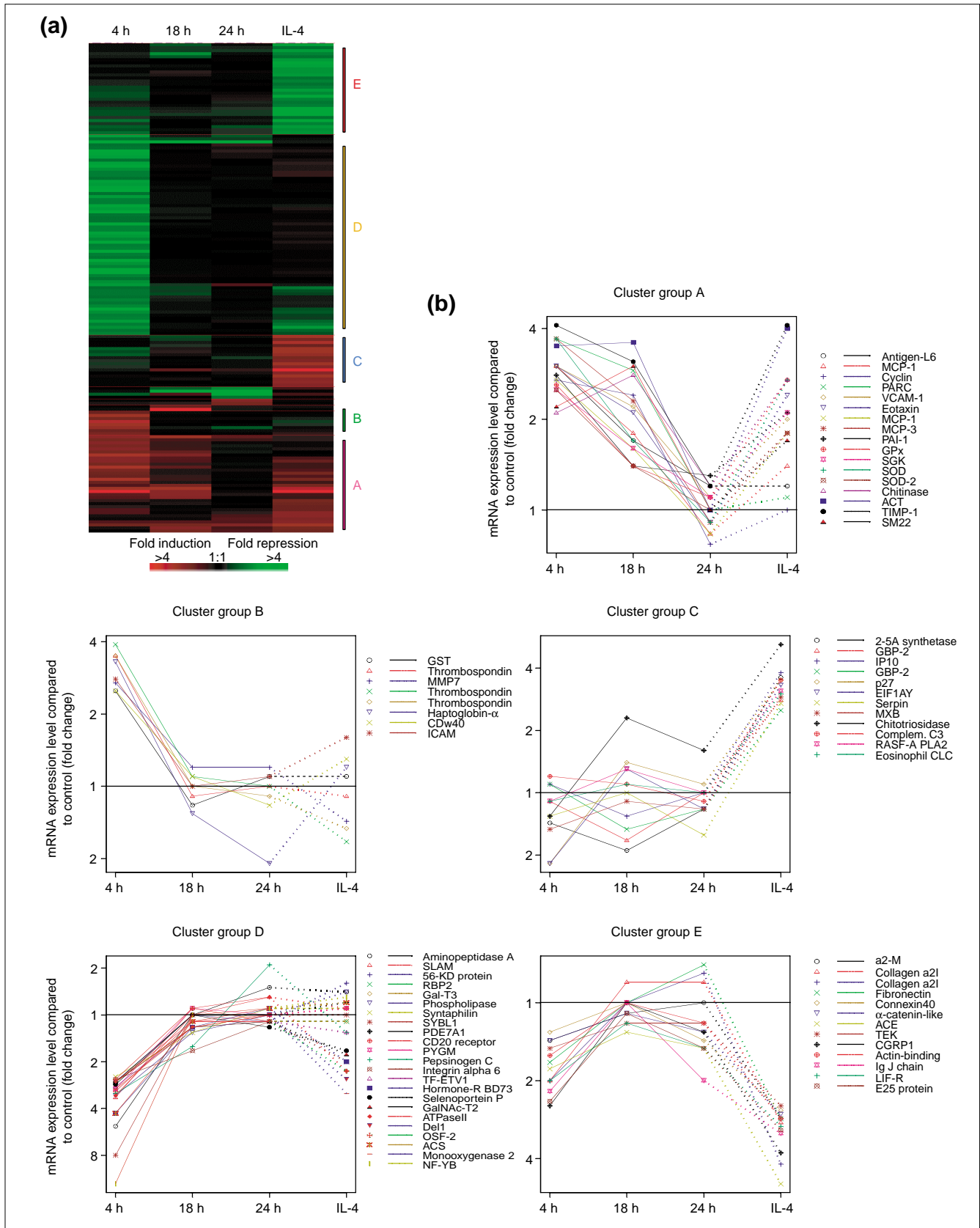
pulmonary inflammation [2] and would be expected to be observed in cluster A or B, were not identified because their cDNA sequences were not present on the microarrays used.

In contrast to clusters A and B, the genes in cluster D were downregulated in response to the allergen challenge (Figure 1b). The level of expression of genes in this cluster was decreased between 2.5-fold and 12-fold 4 h after challenge compared to the control pool, with minimal changes observed 18 h after challenge. The largest changes in gene-expression levels were observed in this cluster: the genes for CCAAT-binding transcription factor-B (NF-YB) (12-fold), SYBL1 (8-fold) and aminopeptidase A (5-fold). IL-4 treatment had a minimal effect on the genes in this cluster. They do not clearly define any one cellular pathway, but rather multiple processes. One gene in particular is interesting - SLAM, signaling lymphocytic activation molecule. *In vitro* studies in polarized human Th1 and Th2 CD4<sup>+</sup> T-cell subsets indicate that higher levels of SLAM are observed in Th1 cells [35]. The downregulation of SLAM gene expression observed here after allergen challenge, which induces a Th2 response, is consistent with this earlier observation. In addition, levels of soluble SLAM in serum were found to be lower in patients with elevated IgE than in normal controls, and low production of SLAM was associated with a Th2 response *in vivo* [36].

Clusters C and E are composed of genes that are regulated 24 h after IL-4 inhalation but not significantly regulated by the antigen challenge, even at a similar time point. The clusters differ in that the genes in cluster C were upregulated and genes in cluster E were downregulated by IL-4. Three genes are noteworthy. The genes for complement C3 and IP-10 (IFN-induced protein of 10 kDa), a non-ELR CXC chemokine, were among the genes upregulated by IL-4 in the monkey lung. In support of the microarray findings, IL-4 is known to increase C3 mRNA levels and subsequent protein production in A549 cells, a pulmonary epithelial cell line [37]. In addition, IP-10 mRNA, which is induced in a variety of cell types by interferon- $\gamma$  (IFN- $\gamma$ ), is augmented by IL-4 in bone marrow stromal cells [38] and keratinocytes [39] but not in respiratory epithelial primary cells or cell lines [40]. The upregulation of IP-10 mRNA observed here in monkeys challenged by IL-4 inhalation warrants further

**Figure 1** (see figure on the next page)

Cluster analysis of 161 genes whose expression in challenged monkey lungs changed >2.5-fold in at least one of the experimental conditions. **(a)** Cluster image of microarray expression profiles of 161 genes. The genes were clustered hierarchically into groups by the program S-plus on the basis of the similarity of their expression profiles. The expression pattern of each gene is displayed as a horizontal strip. The ratio of mRNA levels in allergen-challenged versus control unchallenged monkey lungs is represented by a color, according to the color scale shown (red, overexpression; green, underexpression). **(b)** Expression profile of individual genes in each group of the cluster analysis in (a). Each treatment group (4 h, 18 h, and 24 h after *A. suum* challenge and 24 h after IL-4 challenge) is an independent point; the connecting line is only for the purpose of easy visualization of the individual genes. (The solid line connects 4 h, 18 h and 24 h; the dotted line connects 24 h and IL-4). Only representative known genes are displayed from each cluster. In some clusters, a single gene is represented by multiple cDNAs. In each plot, a horizontal line is drawn at fold value = 1, which indicates equal expression in control and allergen-challenged monkey lungs. Points above this line represent fold overexpression in corresponding allergen-challenged monkeys, and points below this line represent fold underexpression (see Materials and methods).



**Figure 1** (see legend on the previous page)

investigation to determine the cell type and mechanism of IL-4 regulation of *IP-10* *in vivo*. Lastly, the cyclin-dependent kinase inhibitor p27 was upregulated by IL-4 in the monkey lung. The p27 protein is a negative regulator of cytokine-stimulated T-cell growth [41] as revealed in p27-deficient mice. The upregulation of *p27* mRNA by IL-4, identified by microarray studies, suggests that *p27* is an IL-4-inducible gene. Further studies of IL-4-mediated regulation of this and other genes in cluster A, such as those for PAI-1 and TIMP-1, and in cluster E, such as angiotensin I-converting enzyme (ACE), tyrosine kinase receptor (TEK), and leukemia inhibitory factor receptor (LIF-R), are warranted on the basis of the microarray results presented here.

#### Validation of microarray gene-expression data

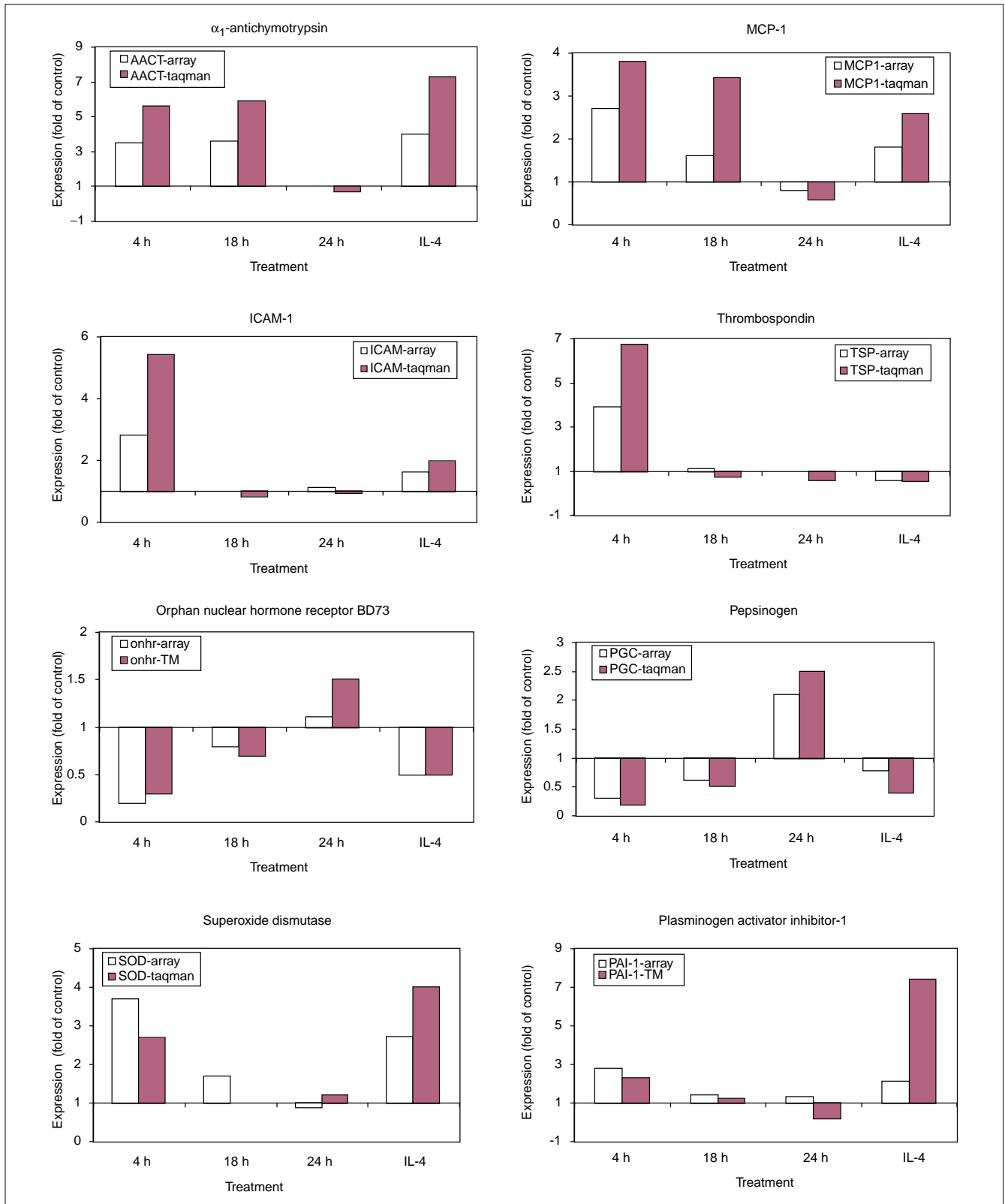
The quality of the microarray data was estimated by examining a small subset of the cDNA elements that was present in duplicate or triplicate on the microarrays. Each of these replicate cDNA elements on the microarray encodes a different region of the same gene. Therefore, where possible, the similarity of the expression levels of the replicate cDNA elements representing a given gene was examined. For example, among 149 genes encoded by the 169 cDNA elements, nine genes were represented by two cDNA elements and two genes by three cDNA elements. For seven of these eleven genes, their multiple cDNA elements were located on the different microarrays in the total set of six microarrays used. Impressively, the microarray readings between these duplicate or triplicate cDNA elements were similar to each other, such that the standard deviations were in general less than 30% of the mean expression value for the 4-, 18-, 24-h and IL-4-challenged groups. Furthermore, all the replicate cDNA elements that encoded the same gene were grouped into the same cluster and were generally in the adjacent rows in the cluster (data not shown), indicating that the replicate cDNAs on microarray produce very similar hybridization results.

Verification of differential gene expression in the lungs of challenged monkeys was examined on a small set of known genes. First, the accuracy of microarray technology was tested using quantitative real-time RT-PCR (Taqman). The gene-expression levels obtained by RT-PCR were normalized to that of gene for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT) and expressed as the fold increase or decrease relative to that of the normal control pool. Twenty known genes were chosen from among the five clusters for this analysis; the majority were selected from cluster A, which contained the largest number of the most highly upregulated genes. Microarray gene-expression levels were compared to those obtained by RT-PCR, using the same monkey lung samples used in the microarray hybridization. Figure 2 displays the results of 8 of 20 genes tested for confirmation and these are among the 10 genes that were significantly regulated ( $p < 0.05$ ) when subsequently tested by RT-PCR on multiple 4-h-challenged monkeys (see below). The pattern of gene expression (up- or

down-regulation) was similar for the genes when the two methods of measurement were compared. The differences between these two methods become prominent at lower levels of expression. Importantly, the direction and degree of differential expression of 19 of 20 genes initially identified by microarray were confirmed by quantitative RT-PCR (Figure 2, Table 2).

Table 3 summarizes the ratio of expression levels derived from the two methods for 20 genes (listed in Table 2). Among 80 pairs of results compared, 55% of data pairs are almost identical (ratio of microarray to RT-PCR, or *vice versa*, is 1-1.4-fold expression relative to normal control). In the remaining 45% of the data pairs, which differed by 1.5-fold or more between the two methods, 35% of the paired comparisons were higher by RT-PCR than by microarray, whereas only 10% were higher by microarray than by quantitative PCR. This suggests a smaller dynamic range for microarray technology compared to that of RT-PCR.

For the second level of validation, we sought to establish that genes identified by microarray technology represented genes truly regulated by inhalation antigen challenge. This was done by determining that the regulated expression levels identified by microarray technology from a single animal could be confirmed by RT-PCR using multiple similarly treated animals. The timepoint chosen for these studies was 4 h after challenge, the timepoint representing the largest number of regulated genes (Table 1). Twenty genes were tested by quantitative RT-PCR in each of four challenged animals (each was 4 h after challenge, of which one was the original monkey) as well as in the six individual, normal unchallenged monkey lungs, which previously constituted the normal control pool. Representative genes are shown in Figure 3 and all 20 genes are summarized in Table 2. Ten of the 20 genes showed significant differences ( $p < 0.05$ ) between the challenged and normal control lungs (Table 2); 8 of these 10 genes were initially identified by microarray as being expressed  $\geq 2.7$ -fold above control. Thus the genes most highly induced in multiple monkeys 4 h after allergen challenge were those for: MCP-3 (400-fold), MCP-1 (14-fold), eotaxin (13-fold), thrombospondin (8-fold), PAI-1 (5-fold), and  $\alpha_1$ -antichymotrypsin (4-fold). The remaining two significantly regulated genes (for orphan nuclear hormone receptor BD73 and pepsinogen C) were initially identified as downregulated by greater than fourfold. For the ten other genes that did not show a significant difference between normal and challenged monkeys, highly concordant results for most of them were observed between the original microarray fold-expression level and that obtained by RT-PCR in multiple challenged monkeys. It is noteworthy that among the genes analyzed that were not found to be significantly regulated when tested in multiple monkeys 4 h after antigen challenge, there were two genes from cluster E: for fibronectin and calcitonin gene-related peptide receptor-1 (CGRP-R1). It is perhaps not surprising that these genes



**Figure 2** Comparison of gene-expression level by microarray and RT-PCR (Taqman). The genes displayed and the cluster they belong to are as follows: cluster A: alpha<sub>1</sub>-antichymotrypsin, MCP-1, superoxide dismutase, plasminogen activator inhibitor-1; cluster B: ICAM-1, thrombospondin; cluster D: orphan nuclear hormone receptor BD73, pepsinogen C. The expression levels are displayed as the fold of the control unchallenged level, which is set to a value of one.

**Table 2****Validation of microarray expression levels by RT-PCR in multiple 4-h challenged monkeys**

Gene	Cluster	Single monkey*		Multiple monkeys†	
		Array	RT-PCR	Fold ± SE	p-value‡
TIMP-1	A	4.10	8.50	3.44 ± 1.74	0.0600
Thrombospondin	B	3.90	6.70	7.60 ± 1.40	0.0008
MCP-3	A	3.70	92.00	400 ± 244	0.0001
Mn superoxide dismutase	A	3.70	2.70	2.70 ± 0.06	0.0010
Pulmonary and activation-regulated chemokines	A	3.70	3.50	2.26 ± 0.60	0.0900
α <sub>1</sub> -Antichymotrypsin	A	3.50	5.60	4.20 ± 1.20	0.0118
Eotaxin	A	3.00	15.00	13.40 ± 7.60	0.0264
ICAM-1	B	2.80	5.40	3.50 ± 0.63	0.0123
Plasminogen activator inhibitor-1	A	2.80	2.30	5.04 ± 1.06	0.0024
MCP-1	A	2.70	3.80	13.60 ± 4.00	0.0009
SM22	A	2.20	2.30	1.40 ± 0.38	0.3700
Chitotriosidase	C	0.77	0.67	1.10 ± 0.58	0.7700
Fibronectin	E	0.59	0.50	0.61 ± 0.04	0.6400
Calcitonin gene-related peptide receptor-1	E	0.40	0.50	1.08 ± 0.34	0.6500
Integrin alpha 6	D	0.38	0.50	0.64 ± 0.05	0.3700
N-acetylgalactosaminyl-transferase-2	D	0.37	1.90	1.26 ± 0.27	0.5000
Selenoprotein-P	D	0.36	0.20	0.55 ± 0.16	0.1300
Orphan nuclear hormone receptor BD73	D	0.23	0.27	0.29 ± 0.12	0.0279
Pepsinogen C	D	0.20	0.31	0.50 ± 0.30	0.0427
DMBT1	D	0.20	0.22	0.26 ± 0.04	0.1900

\*Fold expression level determined by microarray or RT-PCR in original 4-h challenged monkey. †Mean fold expression (± SE) determined by RT-PCR in 4-h challenged monkeys (*n* = 4) compared to normal, control monkey (*n* = 6). ‡p-values were determined by *t*-test (unpaired).

**Table 3****Comparison of microarray versus RT-PCR**

	Ratio*	Pairs†	Percentage‡
Microarray:Taqman	1:1	44	55
	1.5:1	4	5
	2:1	3	4
	> 3:1	1	1
	1:1.5	12	15
	1:2	6	8
	> 1:3	10	12
Total		80	100

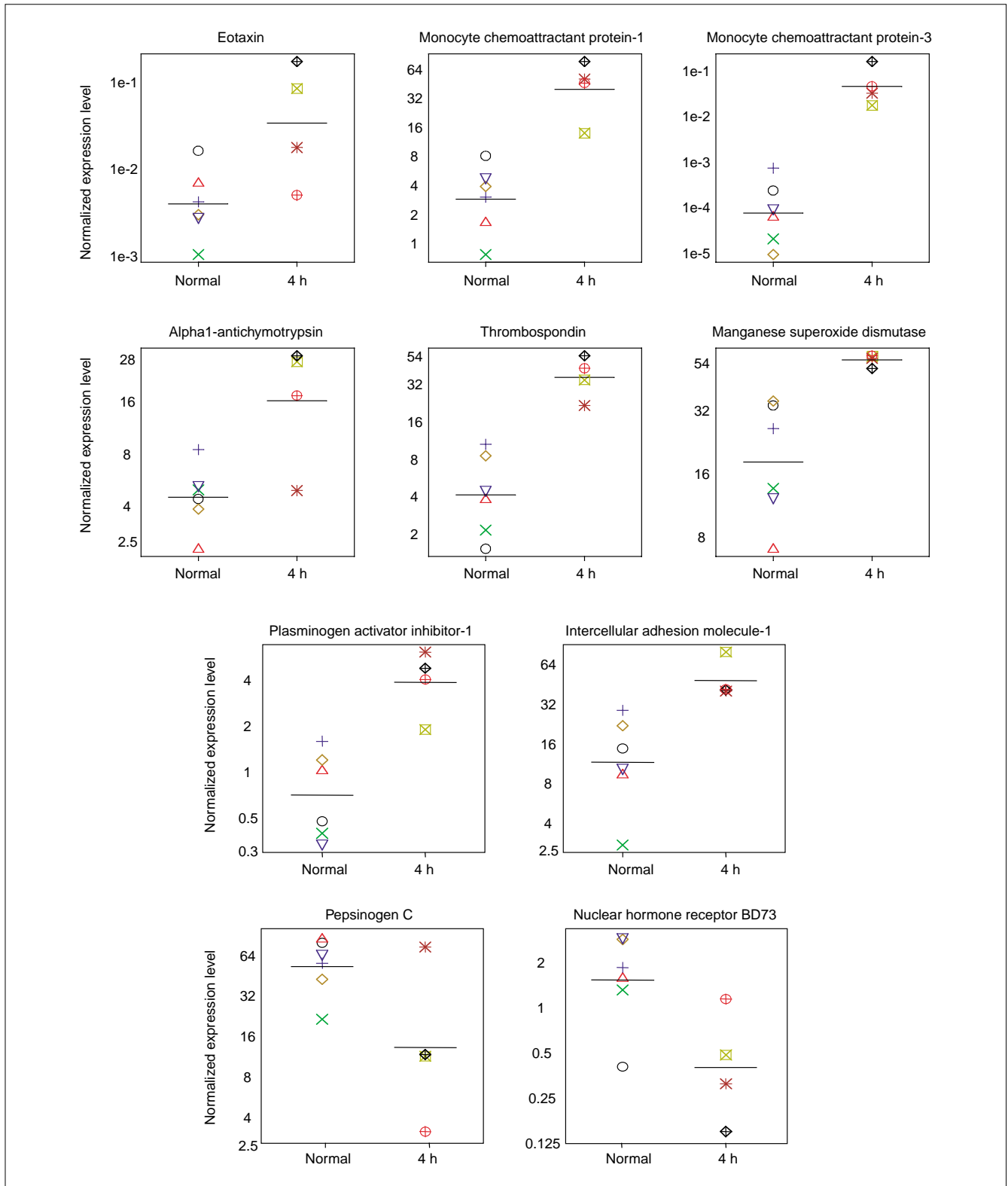
\*The ratio of expression levels (relative to unchallenged controls) determined by microarray compared to RT-PCR. The ratio of 1:1 is defined as 1:1-1.4. The genes analyzed are listed in Table 2. †Number of microarray:RT-PCR data pairs. Twenty genes were analyzed at four time points, equaling 80 data pairs. ‡Percentage of each category in the total number of data pairs.

were not found to be significantly regulated when tested in multiple 4-h antigen-challenged monkeys, as the expression signal driving the grouping of genes in cluster E originated from IL-4, as opposed to antigen challenge.

In summary, the validation tests, though limited in scope, indicate that differential expression by microarray is highly predictive (> 80% confirmation) of differential expression determined by RT-PCR. This suggests a strategy whereby with the use of a confirmatory RT-PCR step, even in the absence of replicate initial tissues and microarrays, microarray experiments can be used as a high-throughput screen to identify genes regulated in a complex *in vivo* disease model.

It is important to note, however, that no animal disease model is identical to human disease. Although there have been many efforts to develop animal models that approximate human asthma or allergy, each of the models has its own advantages and limitations. In this study, the cynomolgus monkey, which has a naturally occurring respiratory





**Figure 3**  
 Gene-expression levels in multiple individual monkey lungs determined by RT-PCR. Expression levels in control unchallenged monkeys (normal,  $n = 6$ ) and 4-h post-challenge monkey lungs (4 h,  $n = 4$ ) are shown. Genes shown are those that are significantly different between these two groups ( $p < 0.1$ , unpaired t-test, Table 2). Expression levels in each animal were calculated as described in Materials and methods and normalized to HPRT, and are on a log scale. The 4-h challenged monkey used in the microarray analysis is designated by the green boxed cross (⊠).

hypersensitivity to *A. suum* extract, was used. Inhalation of the antigen results in an IgE-mediated bronchoconstriction and inflammatory response in monkey lung characterized by an influx of eosinophils [14]. This model was used to investigate the mechanisms underlying the acute inflammatory response and hypersensitivity reaction in monkey airways. Thus, the gene-expression profile in this non-human primate model could provide useful insights into genes regulated in asthma and the mediators produced, although there is no evidence that the antigen used in this study would produce the same allergic reaction in humans. The gene-expression profiles of monkey lung are also limited to the airway responses within 4-24 h after antigen challenge, which may not be relevant to the asthmatic lung or reflect the chronic aspect of asthma. Nevertheless, this is the first attempt to produce an allergen-induced gene-expression profile in the lung of a non-human primate using genomic tools such as microarray and RT-PCR.

## Conclusion

This study provides a novel large-scale profile of gene expression in the lung in allergen-induced pulmonary inflammation in non-human primates. Not only are many novel genes differentially expressed in this disease model identified by microarray, but also many known genes which include many previously observed as regulated in asthma (for example those for eotaxin and VCAM-1) or regulated by relevant stimuli, such as IL-4, in cellular systems (for example, VCAM-1, C3). The results show that microarray analysis in combination with quantitative real-time PCR is a powerful tool for identifying mediators of allergic asthmatic disease through a genomic-based strategy using non-human primates.

## Materials and methods

The experiments were carried out on mature male cynomolgus monkeys. Animals were housed in accordance with National Institutes of Health guidelines at the Schering-Plough Research Institute, a facility approved by the American Association for Accreditation of Laboratory Animal Care. The experimental protocols were approved by the Animal Care and Use Committee of the Schering-Plough Research Institute. A total of 13 monkeys were used in this study (microarray and Taqman), and were divided into the following groups: (1) six untreated control animals, referred to as normal controls; (2) one animal challenged by inhalation with recombinant human IL-4 (see below); (3) six animals challenged with *A. suum* extract by inhalation (see below). Lung tissue was collected 4 h (four animals), 18 h (one animal) or 24 h (one animal) after inhalation challenge. Inhalation of *A. suum* extract in these animals produced a typical IgE-mediated hypersensitivity reaction, with mast-cell degranulation and bronchoconstriction [34].

## Normal control animals

The animals were lightly anesthetized with 10 mg/kg intramuscular ketamine (Ketaject; Phoenix Pharmaceuticals, St Joseph, MO) and euthanized with an overdose of intravenous pentobarbital (Euthanasia-V; Henry Schein, Port Washington, NY). Lung tissue was collected, snap frozen in liquid nitrogen and maintained at -80°C pending analysis.

## IL-4 treatment

The monkey was lightly anesthetized with 10 mg/kg intramuscular ketamine and anesthesia was maintained with intravenous propofol (Diprivan; Zeneca Pharmaceuticals, Wilmington, DE). A fiber-optic bronchoscope (BF type 3C20; Olympus America, Melville, NY) was passed into the right mainstem bronchus. A thin polyethylene catheter was advanced through the biopsy channel of the bronchoscope until the tip was clear of the bronchoscope. Recombinant human IL-4 (0.5 mg; Schering-Plough, Kenilworth, NJ) was dissolved in 1 ml PBS and injected into the bronchus through the catheter. The procedure was repeated on the left side of the lung and the monkey was allowed to recover from anesthesia. After 24 h, the monkey was re-anesthetized with intramuscular ketamine and euthanized with an overdose of intravenous pentobarbital. Lung tissue was collected and frozen as described above.

## Pulmonary antigen challenge of monkeys with *A. suum* extract

The monkeys were anesthetized with ketamine and propofol as before. The trachea was intubated and the animals ventilated with 100% oxygen. Respiratory airflow and transpulmonary pressure were measured and pulmonary resistance and compliance were calculated online (Model 6; Buxco Electronics, Sharon, CT) from the pressure and flow signals using the isovolume technique [42]. The animals were then challenged with 15 breaths of ultrasonically nebulized (Ultra-Neb 99; DeVilbiss, Somerset, PA) *A. suum* extract (Greer Laboratories, Lenoir, NC) diluted in saline. The dilution used was titrated for each animal to produce at least a 100% increase in pulmonary resistance. Animals in the 4 h group were kept anesthetized with an intravenous infusion of either propofol or pentobarbital (Nembutal sodium; Abbott Laboratories, North Chicago, IL) for 4 h. During this time they were ventilated with air, body temperature was maintained with a heated blanket and physiological parameters (blood pressure, heart rate, arterial oxygen saturation) were monitored. At the end of the 4 h time period the animals were euthanized with an overdose of intravenous pentobarbital and lung tissue collected. The two animals designated for 18 h or 24 h post-*A. suum* challenge were recovered from anesthesia after the antigen challenge. They were re-anesthetized with intramuscular ketamine either 18 or 24 h later and euthanized.

## Gene-expression profiling using DNA microarrays

The microarray experiments were conducted at Incyte (Fremont, CA) using their proprietary technology. Briefly,

**Table 4**

**Primer and probe sequences used for RT-PCR (Taqman)**

Gene	Forward primer	Reverse primer	Probe	Species	Accession number
$\alpha_1$ -Antichymotrypsin	GACAGGTTACGGAGGATGC	TGAGTCCTGAAAGTCAGTGGCA	AAGAGGCTGTATGGCTCCGAGGCC	Human	K01500
Calcitonin gene-related peptide receptor-1	GATTCCATGGCGACCTGAAG	AGAGACCAAAAGACCCTGGAAGT	CAGAGGAGGTATATGACTACATCATGCACATCCTTATG	Human	L76380
Chitotriosidase	GACCCTGTTAGCCATCGGAG	GTTGGCCGTGGCTACCATA	CTGGAATTTTCGGCACTCAGAAGTTCACAG	Human	U29615
DMBT1	TTGAAGGTGGCTGCAACTATGATTAT	CAAACCTCGAGCAATGAGAGGG	TTTCGATGGCCCTACCGCAGTT	Monkey	AJ000342
Fibronectin	AATGTTACAGCTCACTGGATATCGA	AGTCCTGATACAACCACGGATGA	AGAAGACCCGGACCAATGAAAGAAATCAACCT	Human	XM_055254
ICAM-1	GGAGCTTCGTGTCCTGTATGG	GAATTTTCTGGCCACGTCCA	CCCCGACTGGACGAGAGGGATTGT	Human	NM_000201
Integrin alpha 6	TGATCGAAATTCCTACCTGATG	TAATCACAGCCGGGATCTG	TGCTGTTGGTTCCTCTCAGATTCAGTAACTATTC	Human	X59512
MCP-1	GCTGTGATCTCAAGACCATTGTG	TGGAATCCTGAACCACCTTCTG	CCAAGGAGATCTGTGCTGACCCCAA	Monkey	AF276081
MCP-3	GAGCTACAGAAGGACCACCAGT	AAGTCCTGGACCCACTTCTG	TCCCCGGGAAGCTGTAATCTTCAAGA	Human	X71087
Mn superoxide dismutase	GGCCTACGTGAACAACCTGAAC	CTGTAAATCTCCCTTGGCCA	TCACCAGGAGAAGTACCAGGAGCGC	Human	Y00497
N-acetylgalactosaminyl-transferase-2	TGGCAGTGGCACTGTCTTTG	TTTGTATTTCATCCATCCAGACCTC	CGAAACACCCGCCGGGCAGC	Human	NM_004481
Orphan nuclear hormone receptor BD73	GAGGTGTGATGCCTATATCAGTTCTT	TCTCAGAACCCTCACTGTGACAA	CAGCTCAGCCTCAAGCCCTGCCT	Human	L31785
PARC	TCTGTGCTGACCCCAATAAGAA	TCCAGGCCCTCAGGC	TGGGTCCAGAAATACATCAGCGACCTG	Human	AB000221
Pepsinogen C	AAGAGTTTCTCATTGGCGGC	CCTGTATCCACGATAGCCTGG	CCTCCGGCTGGTGTCTGAGGGTT	Monkey	X59754
Plasminogen activator inhibitor-1	TCTTCGTCCAGCGGGATC	GTGCTCCGGAACAGCCTG	AAGCTGGTCCAGGGCTTCATGCC	Human	M14083
Selenoprotein-P	CAGTGACTGTGGTTGCTCTT	CAGTTTTACTCGCAGGTCTTC	CTTCAAGCCAGCTGATACCTGTG	Human	Z11793
SM22	GGTGAAGGTGCCCGAGAA	AGGAACTGAGCCACCTGCTC	CCACCCTCCATGGTCTTCAAGCAGA	Human	AF013711
Thrombospondin	CATCCGCAAAGTGACTGAAGAG	CTGTACTGAACTCCGTTGTGATAGC	CCAATGAGCTGAGGCGGCCTCC	Human	X14787
TIMPI	CACCCACAGACGGCCTTCT	AGGCTTGGAACCTTTATACATCTT	TCAACCAGACCACCTTATACCAGCGTTATGA	Human	M12670
Eotaxin	CAT#: 4312354 (PE Biosystems, Foster City, CA.)			Human	

total RNA was isolated from lung tissue by standard guanidinium isothiocyanate/cesium chloride gradients [43]. For each probe pair, poly(A) mRNA was isolated from approximately 200 µg of total RNA using oligotex beads (Qiagen, Chatsworth, CA). Labeled cDNA was prepared by reverse transcription with fluorescent dyes: Cy3 for probe 1 (normal controls) and Cy5 for probe 2 (treated animals). The labeled probe pairs were used for microarray hybridization to Gene Album Arrays 1-6, containing more than 40,000 human cDNA elements (Incyte Genomics, Palo Alto, CA) as described ([44] and Incyte, unpublished data). The specifically bound probes on the microarray were scanned for the two individual fluorescent colors and the signals were corrected for the differences between the Cy3 and Cy5 channels: each probe 2 (Cy5) signal value was multiplied by a balance coefficient in order to normalize the gene-expression data relative to probe 1 (Cy3). The coefficient equals the sum of probe 1 signals divided by the sum of probe 2 signals for the entire microarray chip. The ratio of the two normalized probe signals provided a quantitative measurement of the relative gene-expression level for the challenged lungs compared to the normal controls.

#### Cluster analysis

Cluster analysis was carried out on the gene-expression data from microarray experiments. An average linkage hierarchical clustering algorithm was used to group cDNAs according to the similarity of their expression patterns. These computations were carried out using the S-PLUS software package (Mathsoft, Seattle, WA). Valid microarray signals had a ratio of signal to background > 2.5. Genes were selected for cluster analysis on the following criteria: the signal intensity was at least 100 units above the background; among all probe pairs for any given gene, at least one pair had the probe 2 versus probe 1 ratio larger than 2.5-fold; and no more than one missing value among all probe pairs was allowed for a given gene. The expression levels of those known genes in individual cluster are also displayed in Figure 1b. The log(fold expression) values are plotted on these graphs. However, the *y*-axis represents the corresponding anti-log values, that is, original fold expression values.

#### Quantitative RT-PCR (Taqman™)

The gene-specific probes and primers for the quantitative detection of the target mRNAs were designed using Primer Express (PE Biosystems, Foster City, CA). Table 4 summarizes all the Taqman primers and probe information and sequences. Three of the 20 sets of primers and probes were designed from monkey cDNA sequences that were available from GenBank. The remaining gene-specific primers and probes were designed using conserved regions of human ortholog cDNA sequences based on the alignment of cDNA sequences from several species. Total RNA was isolated from monkey lungs with a Qiagen RNA/DNA kit (Qiagen, Valencia, CA), followed by DNase treatment (Ambion, CA) to remove genomic DNA contamination. The RNA was then

used for the first-strand cDNA synthesis. Two micrograms of total RNA were primed with oligo(dT)<sub>15</sub> and oligo(dN)<sub>6</sub> and were reverse transcribed into cDNA (Superscript II; Gibco/BRL) in a total volume of 20 µl. Aliquots of cDNA target template were diluted serially and mixed with 300 nM of primers and 200 nM of fluorogenic probe (Perkin-Elmer, Foster City, CA). The reactions were carried out in the Universal PCR master mix, containing MgCl<sub>2</sub>, dNTP and DNA polymerase AmpliTaq Gold™ (Perkin-Elmer) in a total volume of 50 µl. The PCR reactions were in duplicate on an ABI PRISM 7700 Sequence detection system (Taqman™). The reaction program was as follows: the initial step of 2 min at 50°C; denaturation at 95°C for 10 min, followed by 45 thermal cycles of denaturation (15 sec at 95°C) and elongation (1 min at 60°C). The relative quantitation of the target mRNAs was carried out using the comparative method according to [45]. The mRNA expression levels for all samples were normalized to the levels for the housekeeping gene *HPRT*.

#### Statistical analysis

One-way analyses of variance were carried out on the expression data obtained from quantitative RT-PCR. The results for each gene were analyzed separately using log(normalized expression level) as the dependent variable and the challenge status of the monkeys as the group variable.

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