

Gene expression analysis in a murine model of allergic asthma reveals overlapping disease and therapy dependent pathways in the lung

MT Follettie¹, DK Ellis¹,
DD Donaldson^{2,5}, AA Hill³,
V Diesl¹, C DeClercq², JP Sypek²,
AJ Dorner¹ and M Wills-Karp⁴

¹Department of Molecular Profiling and Biomarker Discovery, Cambridge, MA, USA;

²Department of Respiratory Disease, Cambridge, MA, USA; ³Department of Bioinformatics, Wyeth Research, Cambridge, MA, USA and ⁴Division of Immunobiology, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, OH, USA

Correspondence:

Dr M Follettie, Department of Molecular Profiling and Biomarker Discovery, Wyeth Research, 35 Cambridge Park Drive, Cambridge, MA 02140, USA.
E-mail: MFollettie@Wyeth.com

Accumulating evidence in animal models and human asthma support a central role for IL-13 signaling in disease pathogenesis. In order to identify asthma and therapy associated genes, global transcriptional changes were monitored in mouse lung following antigen challenge (ovalbumin (OVA)), either alone or in the presence of a soluble IL-13 antagonist. Changes in whole lung gene expression after instillation of mIL-13 were also measured both in wild type and STAT6 deficient mice. A striking overlap in the gene expression profiles induced by either OVA challenge or mIL-13 was observed, further strengthening the relationship of IL-13 signaling to asthma. Consistent with results from functional studies, a subset of the OVA-induced gene expression was significantly inhibited by a soluble IL-13 antagonist while IL-13-modulated gene expression was completely attenuated in the absence of STAT6-mediated signaling. Results from these experiments greatly expand our understanding of asthma and provide novel molecular targets for therapy and potential biomarkers of IL-13 antagonism.

The Pharmacogenomics Journal (2006) 6, 141–152. doi:10.1038/sj.tpj.6500357; published online 10 January 2006

Keywords: asthma; expression profiling; IL-13; allergen; lung; STAT6

Introduction

Asthma is a chronic inflammatory disease of the lung characterized by recurrent episodes of reversible airway obstruction and airway hyper-responsiveness (AHR). Typical clinical manifestations include shortness of breath, wheezing, coughing and chest tightness that can become life threatening or fatal.^{1–3} Despite major advances in therapy and considerable progress in understanding the role of genetics and the environment in evoking asthma, the prevalence, morbidity and mortality of the disease continue to increase. Thus, a clear need exists for better understanding of asthma pathogenesis and identification of new approaches to its therapeutic management.^{4,5}

The mechanisms of allergic asthma have been studied in the murine model, where animals are sensitized by intraperitoneal (i.p.) injection of ovalbumin (OVA) in the presence of adjuvant, subsequently challenged by intratracheal administration of OVA and responses monitored. This procedure generates a CD4⁺ T-cell mediated T_H2 immune reaction in the lung with increased serum IgE, eosinophilia, excessive mucus secretion, and AHR similar to human asthma. We have demonstrated the inability of intratracheal OVA-challenge to elicit

⁵Current address: Millennium Pharmaceuticals Inc., 35 Landsdowne St, Cambridge, MA 02139, USA

eosinophil infiltration, mucus hypersecretion and airway hyper-reactivity in mice deficient in either CD4⁺ T-cells, IL-4 receptor or STAT6.^{4–7} The T_H2 cytokine IL-13 plays a central role in this model as administration of a soluble IL-13 antagonist (sIL-13R α 2-Fc) completely blocked OVA-challenge induced goblet cell mucus metaplasia and AHR, while intratracheal instillation of mIL-13 fully replicated asthma-related pathologies.^{8,9} These results are consistent with the fact that STAT6 activation is downstream of IL-4R α -mediated signaling and that IL-13 is required for OVA induced AHR and mucus production.^{5,10} Recent genetic studies in man have demonstrated an association between specific alleles of IL-13 and its signaling components with asthma and atopy, suggesting an important role for this pathway in the human disease.¹¹

Global transcriptional profiling using oligonucleotide microarrays¹² provides a powerful tool to elucidate novel disease-related genes and pathways within complex tissues.¹³ Because instillation of IL-13 to the lung recapitulates the asthma phenotype and administration of an IL-13 antagonist blocks OVA-challenge induced lung pathology, we investigated the expression profiles associated with disease induction and therapeutic intervention. The majority of pulmonary gene expression changes associated with the OVA-mediated response, with the exception of immunoglobulins, were also induced by IL-13 instillation. However, only a subset of disease-related genes was identified whose expression was altered by blockade of IL-13 signal transduction.

Results

Identification of asthma and therapy-associated gene expression

Expression profiles were determined from lung tissue 3 days after mice were challenged with either PBS (buffer control) or OVA and treated with either the IL-13 antagonist (sIL-13R α 2-Fc) or human IgG. To identify asthma-associated transcripts, the average lung mRNA frequencies from PBS-challenged mice (control) were compared with OVA-challenged mice (OVA). Following OVA-challenge, 113 probe sets exhibited an average fold change (AFC) of two-fold or greater and Student's *t*-test $P < 0.05$. False discovery rate (FDR) was calculated by random permutation of input data (10000 randomly reassigned groups) and determining median number of genes jointly meeting *t* test and fold-change criteria.¹⁴ The OVA modulated gene set was further filtered to remove genes with an FDR of greater than 5% resulting in an OVA-challenged set with 88 probe sets. Following sequence annotation, genes identified multiple times were removed and the unique set of significant changes in lung gene expression following OVA challenge is reported in Table 1. In total, 40 distinct probe sets identified immunoglobulin mRNA increases. Only the top five Ig sequences as defined by fold-change are reported in Table 1.

As observed at the transcriptional level, the pulmonary allergic response upregulates a diverse set of genes with

only three transcripts statistically significantly decreased (Table 1). Annotation of the allergic gene set reveals multiple members of functionally related families including immunoglobulins, Fc receptors, proteases, protease inhibitors, complement, chitinase-related proteins, and several secreted signaling proteins including chemokines and trefoil factors. The biological relevance of this transcriptional profile is supported by the observation that many of the genes and nearly all of the functional categories identified in this study have been shown to be associated with asthma, epithelial cell metaplasia, eosinophilia or airway hyper-reactivity (AHR). This analysis also identified additional genes not previously associated with the allergic response.

To determine changes in gene expression associated with resolution of AHR and mucus metaplasia in murine asthma, we compared pulmonary gene expression in OVA-challenged mice in the presence or absence of sIL-13R α 2-Fc treatment (Table 1). Administration of sIL-13R α 2-Fc completely inhibited only a subset of the OVA-induced genes. Of 32 transcripts induced following OVA challenge, 17 were reduced greater than 40% by sIL-13R α 2-Fc treatment. Many of the genes modulated by anti-IL-13 therapy have been associated with epithelial mucus production (AGR/Gob-4, CLCA3/Gob-5, trefoil factors 1 and 2 and Reg III gamma) and an immunoglobulin response. OVA-induced genes associated with the immune response, including chemokines, proteases, protease inhibitors and complement C1q, showed little or no response to blockade of IL-13 signaling. The inhibition of OVA-induced gene expression was specific to treatment with the IL-13 antagonist and not affected by the Fc-fusion moiety. To illustrate this point, gene frequency measurements for each of the experimental subgroups for 20 genes showing the highest fold-change following intratracheal OVA-challenge are shown in Figure 1.

Gene expression changes in mouse lung induced by mIL-13 instillation

Intratracheal administration of IL-13 recapitulates the asthma phenotype induced by allergen challenge. To identify transcriptional changes associated with the pulmonary response to IL-13 and to determine the role of STAT6 signaling on this transcriptional response, mice were treated at 0, 24 and 48 h with 5 μ g of recombinant mIL-13 or PBS prior to harvesting of lungs at 72 h. Comparison of gene expression profiles from wild-type mice treated by intratracheal instillation of mIL-13 or buffer-alone identified 145 transcripts with AFC greater than two-fold ($P < 0.05$). Selection for FDR $< 5\%$ reduced the IL-13 modulated probe sets to 116 sequences. The nonredundant gene set modulated by intratracheal IL-13 instillation is reported in Table 2 sorted generally by functional annotation. All of the statistically significant changes in gene expression represent increases in transcript levels mediated by mIL-13 instillation except for a decrease in NAD⁺-dependent hydroxyprostaglandin dehydrogenase (HPGD) expression.

Many of the genes in the IL-13 induced gene set fall within and expand the same functional categories identified by the OVA-challenge allergic reaction. Additional

Table 1 Genes modulated by OVA-challenge and response to IL-13 antagonist sIL-13Ra2

<i>Published title</i>	<i>Name</i>	<i>Buffer frequency</i>	<i>OVA</i>	<i>OVA+sIL13Ra2</i>	<i>AFC OVA/ buffer</i>	<i>t-test OVA/ buffer</i>	<i>FDR</i>	<i>% Inhibition sIL13Ra2</i>
<i>Ion flux</i>								
Chloride channel calcium activated 3/Gob-5	CLCA3	7.7	703.8	10.5	91.8	0.004	<0.001	100
<i>Chitinase related</i>								
Acidic mammalian chitinase (AMCase)	CHIA	29.5	221.5	57	7.5	0.003	<0.001	86
Chitinase 3-like 3 (YM1)	CHI3L3	47.3	350.5	166.0	7.4	0.001	<0.001	61
<i>Fc receptor</i>								
Fc fragment of IgG binding protein (IgFcBP)	FCGBP	16.0	215.8	18.5	13.5	0.002	<0.001	99
Fc receptor, IgG, low-affinity IIb	FCGR2B	12.7	68.8	100	5.4	<0.001	<0.001	0
Polymeric immunoglobulin receptor	PIGR	34.7	182.8	49.5	5.3	<0.001	<0.001	90
Fc receptor, IgG, low affinity III	FCGR3	32.7	98.3	102.0	3.0	<0.001	<0.001	0
<i>Innate immunity</i>								
Serum amyloid A 3	SAA3	10.0	59.0	105.5	5.9	0.001	<0.001	0
Complement component 1, q subcomponent, alpha	C1QA	30.8	127.0	276.5	4.1	0.004	<0.001	0
Complement component 1, q subcomponent, beta	C1QB	27.7	100.0	204	3.6	0.001	<0.001	0
Complement component 1, q subcomponent, c	C1QC	44.2	131.8	217	3.0	0.012	<0.001	0
Mannose receptor, C type 1	MRC1	36.7	86.5	58.5	2.4	0.014	0.022	56
Complement component 3	C3	398.0	931.8	679.5	2.3	0.004	0.008	47
<i>Protease</i>								
Cathepsin K	CTSK	23.0	49.3	53	2.1	0.010	0.031	0
Cathepsin S	CTSS	127.8	264.3	318	2.1	0.002	0.010	0
<i>Protease inhibitor</i>								
Serine protease inhibitor 2-1/eb1	Serpina3G	19.3	94.3	94	4.9	0.020	<0.001	0
Serine protease inhibitor 2-2/eb4	Serpina3N	36.5	161.8	154.0	4.4	<0.001	<0.001	6
Tissue inhibitor of metalloproteinase	TIMP	13.2	56.5	87	4.3	0.003	<0.001	0
<i>Secreted signal transduction</i>								
Trefoil factor 1	TFF1	9.3	158.5	12.5	17.0	0.011	<0.001	98
Anterior gradient 2 homolog	AGR2	6.5	40.8	7.0	6.3	<0.001	<0.001	99
Trefoil factor 2 (spasmolytic protein 1)	TFF2	27.2	134.8	22	5.0	<0.001	<0.001	105
Small inducible cytokine A9: MRP-2; Mip-1 gamma	SCYA9	33.0	112.8	115.0	3.4	<0.001	<0.001	0
Small inducible cytokine A6; C10 chemokine	SCYA6	18.5	41.0	48.5	2.2	0.010	0.025	0
Rat generating islet-derived, homolog 3 gamma	REG3G	114.2	228.0	67.5	2.0	0.003	<0.001	141
<i>Metabolism</i>								
Arginase 1, liver	ARG1	7.2	15.8	10.0	2.2	0.003	0.012	67
Ribonucleotide reductase M2	RRM2	13.5	30.0	27.5	2.2	0.008	0.021	15
Folate binding protein 2	FOLBP2	13.8	28.5	38.5	2.1	0.005	0.027	0

Table 1 Continued

Published title	Name	Buffer frequency	OVA	OVA+sIL13R α 2	AFC OVA/ buffer	t-test OVA/ buffer	FDR	% Inhibition sIL13R α 2
Antibody	Ig germline D-J-C region	6.0	292.0	68.5	48.7	0.018	<0.001	78
	Ig heavy chain 4 (serum IgG1)	8.0	378.3	122	47.3	0.009	<0.001	69
	Ig rearranged gamma-1 chain	11.5	323.3	125	28.1	0.019	<0.001	64
	Ig heavy chain Fd region	31.7	234.3	128.5	7.4	0.016	<0.001	52
	Anti-PAH Ig heavy chain V and CH1 regions	11.7	59.8	21.5	5.1	0.014	<0.001	80
Decrease	CYP2A4	189.8	43.3	82.0	-4.4	0.003	<0.001	26
	Adrenergic receptor, beta 2	69.8	26.5	37.0	-2.6	0.002	<0.001	24
	Palate, lung, nasal epithelium carcinoma associated	448.8	174.8	150.5	-2.6	0.002	<0.001	0

Columns 1 and 2 provide title and HUGO approved name for genes modulated by allergen-challenge. Average lung mRNA frequency values (p.p.m.) for control, OVA-challenged, OVA+sIL-13R α 2-Fc treated mice are reported in columns 3, 4 and 5, respectively. Average fold change in mRNA expression, Student's t-test statistic and calculated FDR comparing OVA-challenged and controls are reported in columns 6 and 7, respectively. The percentage inhibition of OVA gene expression by sIL-13R α 2-Fc for each gene is reported in column 8 with negative values defaulted to zero. Genes increased are sorted by function and descending AFC in OVA-treated mice. The three genes whose expression was decreased (CYP2A4, ADRB2, and PLUNC) are located at the bottom of the table and the decrease designated by a negative fold-change.

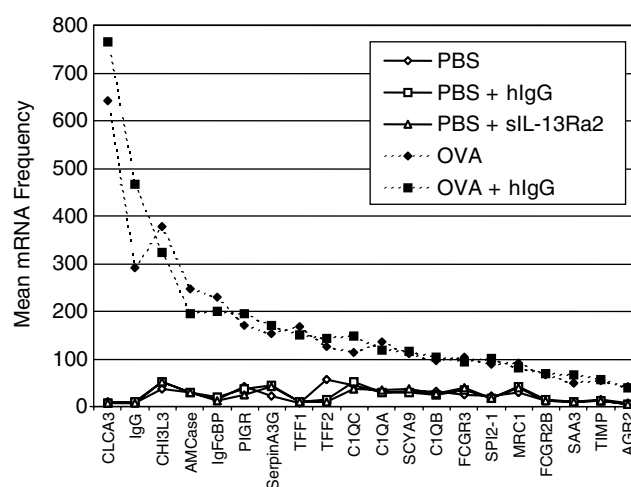


Figure 1 Pulmonary gene expression induction is responsive to OVA-challenge alone. Average lung mRNA frequency values for a single representative immunoglobulin (IgG) sequence and 19 nonimmunoglobulin genes with highest fold induction following OVA-challenge are plotted for each of the experimental and buffer-alone control subgroups. Mice received either intratracheal OVA antigen-challenge or PBS-buffer, alone or in combination with intraperitoneal injection of hIgG or sIL-13R α 2-Fc as described in text. Genes labeled in X-axis with HUGO approved name and corresponded directly to names and mRNA expression values reported in Table 1.

proteases/antiproteases including MMP-12, aspartyl aminopeptidase, and cystatin B and an expanded set of genes related to chloride ion flux are seen following IL-13 instillation. New functional categories were also identified by intratracheal IL-13 instillation including induction of extracellular matrix proteins, endoplasmic reticulum-associated proteins involved in secretion and stress response, metabolic enzymes, and coagulation-related factors. The detection of additional IL-13 modulated genes not identified in the OVA-induced gene set most likely reflects a difference in strength of signal provided by intratracheal instillation of the cytokine and as such may reflect an extended set of disease-related genes. Strikingly, IL-13 does not induce expression of immunoglobulin genes increased following OVA treatment.

A comparison of Tables 1 and 2 reveals a large degree of overlap in gene expression induced by allergen (OVA) challenge and IL-13 instillation consistent with the finding that OVA challenge and IL-13 induce similar asthma phenotypes. Of the 27 nonimmunoglobulin genes induced following OVA-challenge, 20 demonstrated statistically significant two-fold or greater induction following IL-13 instillation. Table 3 identifies the genes responsive to both OVA-challenge and IL-13 instillation with fold-changes measured for the common allergen/IL-13 induced pulmonary transcriptome depicted in Figure 2. Examining the pattern of inhibition of OVA-induction in these genes by the IL-13 antagonist (sIL-13R α 2) defines classes of transcriptional response (Table 3). For genes such as CLCA3, AGR2, PIGR and trefoil factors, IL-13 is both sufficient and required

Table 2 Genes induced by mL-13 intratracheal instillation in Balb/C mice

Published title	Name	Frequency Balb/C buffer	Frequency Balb/ C+mL-13	AFC mL- 13/buffer	t-test mL- 13/buffer	FDR
<i>Ion flux</i>						
Chloride channel calcium activated 3/Gob-5	CLCA3	10.5	658.0	62.7	<0.001	<0.001
Solute carrier family 4, member 2	SLC4A2	12.3	68.3	5.6	0.022	<0.001
Guanylate cyclase activator 2 (guanylin)	GUCA2A	14.8	39.3	3.3	0.009	0.023
FXRD domain-containing ion transport regulator 3	FXRD3	35.5	71.8	2.0	0.004	0.022
<i>Chitinase related</i>						
Chitinase 3-like 3 (YM-1)	CHI3L3	63.8	712.7	11.2	0.001	<0.001
Acidic mammalian chitinase (AMCase)	CHIA	57.8	614.5	10.6	0.002	<0.001
<i>Protease</i>						
Macrophage metalloelastase	MMEL	12.0	128.3	10.7	0.005	<0.001
Cathepsin K	CTSK	30.5	175.5	5.8	0.001	<0.001
Aspartyl aminopeptidase	DNPEP	44.3	131.5	3.0	0.045	0.026
<i>Protease inhibitor</i>						
Serine protease inhibitor 2-2/eb4	SerpinA3N	26.3	281.7	10.7	0.006	<0.001
Serine protease inhibitor 2-1/eb1	SerpinA3G	72.5	588.7	8.1	0.004	<0.001
Tissue inhibitor of metalloproteinase	TIMP	30.8	152.3	5.0	0.009	<0.001
Cystatin B	CSTB	50.5	118.5	2.3	0.015	0.023
<i>Fc receptor</i>						
Fc fragment of IgG binding protein (IgFcBP)	FCGBP	11.8	227.8	19.4	0.022	<0.001
Polymeric immunoglobulin receptor	PIGR	21.5	204.5	9.5	0.001	<0.001
Fc receptor, IgG, low-affinity IIb	FCGR2B	20.3	51.7	2.6	0.022	0.021
Fc receptor, IgG, low-affinity III	FCGR3	45.5	103.7	2.3	0.010	0.019
<i>Immune related</i>						
CD83 antigen	CD83	21.3	57.7	2.7	0.012	0.002
CD14 antigen	CD14	82.5	218.7	2.7	0.007	<0.001
CD52 antigen	CD52	61.3	160.5	2.6	0.048	0.036
<i>Innate immune</i>						
Complement component 3	C3	380.8	1059.8	2.8	0.003	<0.001
Properdin factor, complement	PFC	29.8	79.8	2.7	0.046	0.034
Complement component 1, q subcomponent, c chain	C1QC	42.5	112.3	2.6	0.029	0.023
<i>Redox</i>						
Glutathione S-transferase omega 1	GSTO1	56.5	340.0	6.0	0.018	<0.001
Glutamate cysteine ligase, catalytic	GCLC	18.8	62.3	3.3	0.032	0.013
Glutaredoxin	GLRX1	26.3	73.2	2.8	0.031	0.020
Quiescin 6/(FAD)-sulfhydryl oxidase	QSCN6	14.8	38.8	2.6	0.014	0.007
<i>Endoplasmic reticulum</i>						
KDEL receptor 3	KDEL3	30.8	110.0	3.6	0.021	<0.001
Endoplasmic reticulum protein 29	ERP29	30.8	86.0	2.8	0.030	0.019
Signal sequence receptor, delta	SSR4	59.5	154.0	2.6	0.004	<0.001
Sec61 beta subunit	Sec61B	89.3	185.2	2.1	0.003	0.018
Heat shock 70kDa protein 5	HSPA5	102.5	206.0	2.0	0.008	0.037
<i>Coagulation</i>						
von Willebrand factor	VWF	114.0	387.8	3.4	0.015	<0.001
Plasminogen activator, tissue	PLAT	24.8	78.3	3.2	0.020	<0.001
Tissue factor/coagulation factor III	F3	18.3	51.0	2.8	0.008	<0.001
Coagulation factor VII	F7	24.3	55.3	2.3	0.023	0.038

Table 2 *Continued*

Published title	Name	Frequency Balb/C buffer	Frequency Balb/ C+mIL-13	AFC mIL- 13/buffer	t-test mIL- 13/buffer	FDR
<i>Secreted signal transduction</i>						
Resistin-like beta	RETNLB	9.3	115.7	12.5	0.009	<0.001
Anterior gradient 2 homolog	AGR2	9.8	108.8	11.2	0.000	<0.001
Rat generating islet-derived, homolog 3 gamma	REG3G	43.8	370.0	8.5	0.001	<0.001
Small inducible cytokine A9	SCYA9	31.5	251.5	8.0	0.008	<0.001
Trefoil factor 2 (spasmolytic protein 1)	TFF2	21.5	110.2	5.1	0.000	<0.001
Trefoil factor 1	TFF1	15.3	44.0	2.9	0.024	0.009
Heparin binding EGF-like growth factor	HEGFL	131.0	358.3	2.7	0.002	<0.001
Small inducible cytokine A6; C10 chemokine	SCYA6	26.5	72.2	2.7	0.007	<0.001
Stromal cell derived factor 5	SDF5	15.0	36.2	2.4	0.033	0.039
Colony-stimulating factor 1 (macrophage)	CSF1	21.5	48.2	2.2	0.016	0.032
<i>Cytoskeletal</i>						
Small proline-rich protein 2A	SPRR2A	35.0	929.2	26.5	0.001	<0.001
Actin, gamma 2, smooth muscle, enteric	ACTG2	56.5	148.8	2.6	0.003	<0.001
<i>Extracellular matrix</i>						
Procollagen, type VI, alpha 1	COL6A1	204.5	694.8	3.4	0.014	<0.001
Collagen, type 1, alpha 1	Col1A1	64.5	217.2	3.4	0.002	<0.001
Procollagen, type I, alpha 2	COLA2	25.0	74.0	3.0	0.017	0.001
Procollagen, type VI, alpha 2	COL6A2	253.8	739.7	2.9	0.013	0.001
Fibrillin 1	FBN1	66.0	147.5	2.2	0.022	0.040
Fibronectin 1	FN1	176.5	387.8	2.2	0.013	0.031
<i>Lipocalin</i>						
von Ebner minor salivary gland protein	U46068	83.5	543.2	6.5	0.005	<0.001
Lipocalin 2	LCN2	151.0	372.5	2.5	0.012	0.008
<i>Metabolism</i>						
Transketolase	TKT	53.0	221.3	4.2	0.032	0.006
Malic enzyme, supernatant	MOD1	26.0	88.3	3.4	0.003	<0.001
Creatine kinase, mitochondrial 1, ubiquitous	CKMT1	42.0	126.0	3.0	0.002	<0.001
Arginase 1, liver	ARG1	9.5	28.2	3.0	0.014	<0.001
Acid phosphatase 5, tartrate resistant	ACP5	20.5	57.8	2.8	0.006	<0.001
Adenylate kinase 2	AK2	29.8	80.2	2.7	0.019	0.012
Spermidine synthase	SRM	42.3	111.3	2.6	0.004	<0.001
Carbonic anhydrase 2	CAR2	138.0	358.5	2.6	0.005	<0.001
Prostaglandin-endoperoxide synthase 1	PTGS1	23.3	60.2	2.6	0.003	<0.001
Pyruvate carboxylase	PC	29.8	76.0	2.6	0.039	0.021
Ferredoxin 1	FDX1	67.8	164.7	2.4	0.002	0.001
Hydroxyprostaglandin dehydrogenase 15 (NAD)	HPGD	242.5	90.8	-2.7	0.040	0.031
<i>Miscellaneous</i>						
Arginine-rich mutated in early stage tumors	ARMET	126.0	416.5	3.3	0.007	<0.001
Growth arrest and DNA-damage-inducible, gamma	GADD45G	41.3	128.0	3.1	0.019	<0.001
Transcobalamin 2	TCN2	148.8	438.8	3.0	0.012	<0.001
Reduced expression 3	REX3	38.0	108.0	2.8	0.000	<0.001
Ser/Thr protein kinase PIM3	PIM3	40.0	111.8	2.8	0.012	0.002
Cytokine inducible SH2-containing protein	CISH	20.8	57.7	2.8	0.029	0.019
Viral envelope-like G7e	D17H6S56E-5	18.3	49.3	2.7	0.007	<0.001
Sialyltransferase 7	SIAT7B	17.5	45.7	2.6	0.028	0.023
Nuclear receptor corepressor 2	NCOR2	39.3	94.0	2.4	0.046	0.050
Eosinophil-associated ribonuclease 2	EAR2	132.5	298.5	2.3	0.003	0.008

Columns 1 and 2 provide title and HUGO approved name for genes modulated by pulmonary mIL-13 treatment. Average lung mRNA frequency values (p.p.m.) for Balb/C wild type following multiple intratracheal instillations of PBS-buffer alone control (column 3) or mIL-13 (column 4). Average fold change in lung mRNA expression, Student's *t*-test statistic and FDR from pair-wise comparison of wild-type Balb/C mIL-13 instillation and PBS-buffer controls are reported in columns 5, 6 and 7, respectively. Genes are sorted by function and descending AFC in IL-13 treated mice. The single gene decreased in concentration (HPGD) is designated with negative fold change value.

Table 3 Genes common to both mL-13 and OVA-challenge induction in mouse lung

Published title	Name	AFC OVA/ buffer	t-test OVA/ buffer	FDR	AFC mL-13/ buffer	t-test mL- 13/buffer	FDR	% inhibition sIL13Ra2
<i>Ion flux</i>								
Chloride channel calcium activated 3	CLCA3	91.8	0.004	<0.001	62.7	<0.001	<0.001	100
<i>Chitinase related</i>								
Acidic mammalian chitinase (AMCase)	CHIA	7.5	0.003	<0.001	10.6	0.002	<0.001	86
Chitinase 3-like 3 (YM-1)	CHI3L3	7.4	0.001	<0.001	11.2	0.001	<0.001	61
<i>Secreted signal transduction</i>								
Trefoil factor 1	TFF1	17.0	0.011	<0.001	2.9	0.024	0.009	98
Trefoil factor 2 (spasmolytic protein 1)	TFF2	5.0	<0.001	<0.001	5.1	0.000	<0.001	105
Small inducible cytokine A9: MRP-2; Mip-1 gamma	SCYA9	3.4	<0.001	<0.001	8.0	0.008	<0.001	0
Small inducible cytokine A6; C10 chemokine	SCYA6	2.2	0.010	0.025	2.7	0.007	<0.001	0
Anterior gradient 2 homolog	AGR2	6.3	<0.001	<0.001	11.2	<0.001	<0.001	99
Rat generating islet-derived, homolog 3 gamma	REG3G	2.0	0.003	<0.001	8.5	0.001	<0.001	141
<i>Fc receptor</i>								
Fc receptor, IgG, low-affinity IIb	FCGR2B	5.4	<0.001	<0.001	2.6	0.022	0.021	0
Polymeric immunoglobulin receptor	PIGR	5.3	<0.001	<0.001	9.5	0.001	<0.001	90
Fc fragment of IgG binding protein (IgFcBP)	FCGBP	13.5	0.002	<0.001	19.4	0.022	<0.001	99
Fc receptor, IgG, low-affinity III	FCGR3	3.0	<0.001	<0.001	2.3	0.010	0.019	0
<i>Protease related</i>								
Cathepsin K	CTSK	2.1	0.010	0.031	5.8	0.001	<0.001	0
Serine protease inhibitor 2-1/eb1	Serpina3G	4.9	0.020	<0.001	8.1	0.004	<0.001	0
Serine protease inhibitor 2-2/eb4	Serpina3N	4.4	<0.001	<0.001	10.7	0.006	<0.001	6
Tissue inhibitor of metalloproteinase	TIMP	4.3	0.003	<0.001	5.0	0.009	<0.001	0
<i>Inate immune</i>								
Complement component 1, q subcomponent, c chain	C1QC	3.0	0.012	<0.001	2.6	0.029	0.023	0
Complement component 3	C3	2.3	0.004	0.008	2.8	0.003	<0.001	47
<i>Metabolism</i>								
Arginase 1, liver	ARG1	2.2	0.003	0.012	3.0	0.014	<0.001	67

Columns 1 and 2 provide title and HUGO approved name for genes commonly modulated by pulmonary OVA-challenge or mL-13 instillation. Average fold change in mRNA concentration, Student's *t*-test and calculated FDR is reported for OVA induction (columns 3, 4 and 5) and mL-13 instillation (columns 6, 7 and 8).

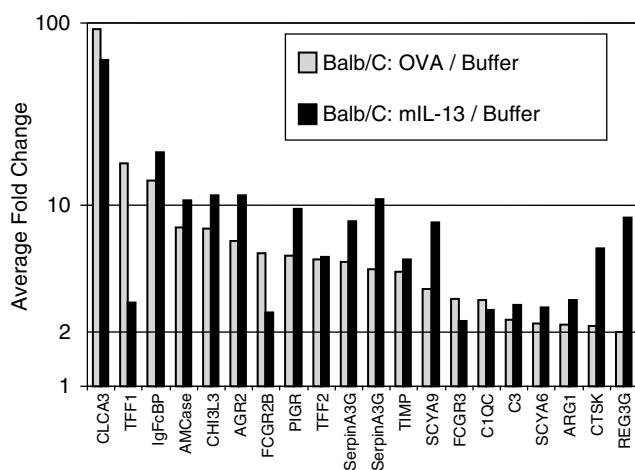


Figure 2 Intratracheal allergen-challenge or direct IL-13 instillation induces an overlapping transcriptome in the lung. The average fold change values for 20 overlapping genes induced by both pulmonary OVA-challenge (gray bars) or mIL-13 instillation (black bar). Genes labeled in X-axis with HUGO approved name and corresponded to names and mRNA expression values reported in Table 3.

for transcriptional induction. For other genes commonly modulated, however, including the chemokines (SCYA6, SCYA9) and protease inhibitors, IL-13 is sufficient for induction but not required in the context of allergen-challenge.

STAT-6 dependent IL-13-induced gene expression was identified using STAT-6 deficient knockout mice. Physiological studies demonstrate an inhibition of pulmonary eosinophil infiltration, mucus overproduction and AHR elicited by OVA challenge in mice harboring the STAT6^{-/-} null allele.^{6,15} Expression profiling of the lung tissue of STAT-6 deficient following multiple dose mIL-13 lung instillation did not reveal any genes that met the dual fold change and statistical criteria. Specifically, none of the IL-13 modulated genes in wild-type Balb/C mice were statistically different ($P < 0.05$) from vehicle in the absence of STAT6 function. The differential response of Balb/C wild type and STAT6^{-/-} mice to multidose, intratracheal instillation of mIL13 is illustrated in Figure 3. The IL-13 induced genes in wild-type mice were sorted by expression in OVA-challenge murine lung tissue and compared to frequency values obtained for similarly treated mice harboring the STAT6^{-/-} allele. Pulmonary gene expression from both buffer and IL-13 treated STAT6^{-/-} cohorts mirrors the expression of buffer-treated wild-type mice. These data demonstrate the requirement for STAT6 function for all of the measured mIL-13 mediated gene induction in the Balb/C wild type, consistent with the lack of physiological response to allergen challenge in the STAT6^{-/-} null mice.

Discussion

We previously demonstrated that administration of a soluble IL-13 antagonist to OVA challenged mice inhibited

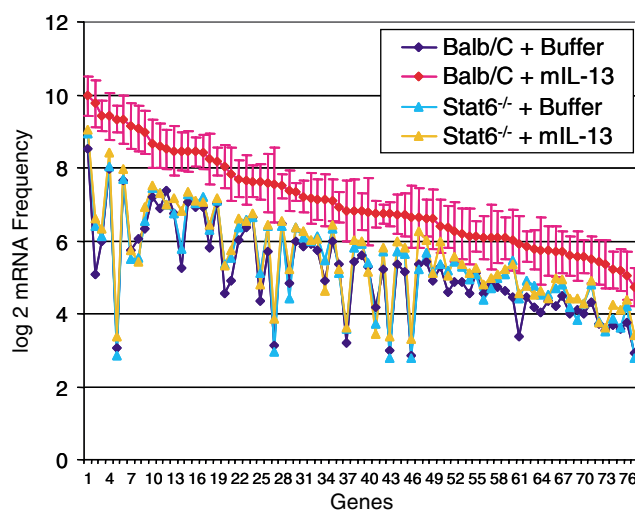


Figure 3 IL-13-mediated mRNA induction is dependent on STAT6 function. Genes and expression values correspond directly to all genes identified as mIL-13 responsive in Balb/C mice and reported in Table 3. Average mRNA frequency expressed in mouse lung following buffer (dark blue diamond) or mIL-13 (red diamond) instillation. Similar buffer (light-blue triangle) or mIL-13 (gold triangle) instillation does not demonstrate similar induction in STAT6 deficient mice.

two critical aspects of the asthma phenotype, AHR and mucus production.^{8,9} Similarly, we have shown that intratracheal instillation of mIL-13 induced IgE, pulmonary eosinophilia, mucus production and AHR.^{8,9,16} In this study, we have extended our analysis of this model using microarray expression profiling and have identified genes uniquely associated with IL-13 activity and antagonism. In addition, we have demonstrated a substantial overlap in gene expression changes following OVA challenge and intratracheal instillation of IL-13.

Mucus secretion and airway plugging have been shown to be consistent features of human asthma, asthma associated mortality¹⁷ and of the OVA challenge murine model. In our study, we identified multiple IL-13 induced genes previously implicated in mucus expression and function, including AGR2/Gob-4, RegIII gamma, and IgGBP, trefoil factors (TFF1 and 2). Expression of these genes was completely dependent on IL-13 and STAT6 signaling. TFF2 and CLCA3/Gob-5, expressed by epithelial cells and associated with the presence of goblet cells and mucus production, were recently implicated in preclinical lung inflammation models and in genetic studies of human asthma.^{18,19} However, the induction of other mucus associated genes including AGR2/Gob-4, RegIII gamma and FCGBP, following IL-13 administration and their dependence on IL-13 and STAT6 signaling have not been previously reported.

In mouse lung, CLCA3/Gob-5 mRNA expression is induced by STAT6-dependent IL-13 stimulation and allergen mediated induction of the CLCA3 transcript is completely dependent on IL-13 signaling. In addition to CLCA3, intratracheal instillation of IL-13 induced ion channel associated genes SLCA4a2, FXDY3, Guanylin and carbonic

anhydrase II. Expression of these genes was not observed in direct OVA-challenge profiles. The murine guanylin gene, encodes a 15 amino-acid endogenous ligand regulator of the intestinal guanylate cyclase-C regulator, STaR.²⁰ Activation of intestinal STaR induces a chloride secretion generating an accumulation of water in the gut and subsequent diarrhea. Typically, guanylin expression is restricted to intestinal epithelium; however, guanylin has been shown to activate outwardly rectifying chloride current in normal human bronchial epithelial cells.²¹ The IL-13 mediated induction of guanylin and its activity in airway epithelial cells may be significant to the mucosal edema observed in asthmatic airways and its role in airway obstruction. These results are consistent with an emerging awareness of the involvement of chloride flux in the asthma pathology.

Consistent with the results of Zimmermann and co-workers,^{18,22} we showed that two members of the trefoil family, P-domain protein (TFF1/pS2) and spasmolytic protein (TFF2/mSP), were induced by OVA challenge. We now show that trefoil expression was completely dependent on IL-13 signaling. Trefoil proteins, small secretory proteins, are characteristic constituents of mucus gels and associated with cellular sites of mucin production.^{23,24} The upregulation of TFF-proteins by allergic-challenge or IL-13 thus may have dual roles of exacerbating the airway mucus obstruction while mediating a protective role in repair of the inflammatory damage to the epithelium.

OVA challenge and IL-13 exposure induced the expression of several members of the complement cascade, including C1q, C3 and properdin. We demonstrate that the IL-13 induction of C3 is dependent on STAT6 signaling. Furthermore, for the first time show that C3 gene expression is partially inhibited (50% of Ova-induced expression) by administration of sIL-13R α 2-Fc to OVA challenged mice. These findings are of particular interest in the light of the recent demonstration that C3 is critical for the development of allergen induced AHR in guinea pig and murine asthma models and pulmonary viral infection.^{25–28} Furthermore, recent studies have shown that the levels of the complement anaphylatoxin peptides are elevated in the lungs of asthmatic patients.²⁶ Although further studies are clearly needed to elucidate the exact sequence of events and site of C3 expression in our model, and the involvement of C3 in induction of Th2 cytokines and AHR,²⁹ our studies suggest that IL-13 induced AHR may be mediated via the local induction of complement C3 gene expression.

Our studies also identified a limited number of additional functional categories following IL-13 administration not detected with OVA challenge. For example, four genes were identified in the category of coagulation regulation, one of which, tissue plasminogen activator, was recently identified in a murine model of lung inflammation and studied in a murine model of asthma. The initiators of the extrinsic coagulation cascade, tissue factor and Factor VII were also induced by IL-13 consistent with results from a murine asthma model showing fibrin deposition in airways and BAL and demonstration of thrombin in the airways of asthmatic

patients.³⁰ Together these results provide further evidence for abnormalities in procoagulant and fibrinolytic pathways in asthma and support further study in this area.

In combination, these findings support the reliability of this approach by demonstrating that many genes or gene families identified in this study, including small proline-rich proteins, chloride channels, complement proteins, have been shown to be associated with either asthma, epithelial cell metaplasia, or AHR. Furthermore, instillation of mIL-13 recapitulated all the gene expression increases observed with OVA-challenge except the elevation of Ig mRNA levels. The lack of increase in Ig mRNA concentration following pulmonary mIL-13 administration may reflect timing or more likely lack of antigen-specific signaling supporting B-cell expansion and trafficking.³¹ Pulmonary administration of mIL-13 also resulted in identification of additional genes within the OVA challenge functional categories and additional functional categories. We view the expanded gene set measured in IL-13-treated lungs as a reflection of an increased strength of signal mediated by intratracheal cytokine instillation. We cannot, however, rule out temporal differences in expression or potential factors induced in the OVA-challenge allergic reaction that act to moderate or attenuate certain aspects of the IL-13 specific pathway.

The experiments described here demonstrate the power of gene expression profiles to provide a global view of disease-related transcription and response to therapy. Understanding gene expression profiles associated with asthma and the clinical features of AHR and increased mucus production may elucidate key players in the molecular pathway of the disease. IL-13 has been suggested to play a key role in the pathogenesis of bronchial asthma and is critical in inducing an allergic asthma response in animal models.^{32,33} Using an animal model of asthma, we have identified genes regulated by IL-13 whose functions are consistent with disease pathology induced by OVA challenge. In particular, the induction of genes associated with AHR and mucus production suggests a molecular pathway for the observed IL-13 effects that are consistent with a role in human asthma.

Therapeutic intervention with a soluble IL-13 antagonist confirms the direct role of IL-13 in the induction of these genes. These preclinical data also suggest that a comparison of gene expression changes in asthma and following therapeutic intervention be applied in clinical studies to monitor patient response to drug at a molecular level. This work has identified potential new targets for drug development as well as candidate markers of drug response in the treatment of asthma. Taken together these results further support the central role of IL-13 in asthmatic pathophysiology and the therapeutic value of antagonism of IL-13 in human asthma.

Methods

Experimental design

Balb/C wild-type mice (6–8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). STAT6

deficient and wild-type controls were generated and bred on a Balb/C background as previously described.³⁴ All animals used in this study were housed in an environmentally controlled, pathogen-free facility under laminar flow hoods. All experiments conformed to the principals for laboratory animal research as outlined in the Animal Welfare Act and the Department of Health, Education and Welfare (NIH) guidelines for the experimental use of animals.

To identify transcriptional changes in genes associated with OVA challenge or therapy Balb/C mice (six per group) were immunized by an i.p. injection of 10 μ g of OVA (Sigma, St Louis, MO, USA) in 200 μ l of PBS on day 0. On days 14 and 25, mice were anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg) and challenged intratracheally with 50 μ l of a 1.5% solution of OVA or an equivalent volume of PBS. Two animals from each group were injected i.p. either with 100 μ l of PBS, hIgG (400 μ g/ml) or sIL-13R α 2-Fc (400 μ g/ml) on days 24, 25 and 27. Purification of hIgG, sIL-13R α 2-Fc and murine IL-13 was as described previously.^{35,36} Lungs were collected and snap frozen for RNA isolation on day 28.

To identify transcriptional changes associated with pulmonary IL-13 expression, wild-type Balb/C mice were treated with intratracheal instillation of recombinant IL-13 ($n=6$) or PBS-buffer control. To elucidate the role of STAT6 signaling, the STAT6^{-/-} null allele was backcrossed into the Balb/C genetic background and treated with intratracheal instillation of either mIL-13 ($n=5$) or PBS buffer control ($n=4$) using an identical protocol and schedule as for the Balb/C wild-type mice. Recombinant murine IL-13 (5 μ g in 50 μ l) was administered daily for 3 days by intratracheal instillation to naïve Balb/c mice or STAT6 deficient mice that had been anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg, respectively). Lungs were collected and snap frozen in dry ice at 72 h after the initial mIL-13 administration.

RNA isolation and hybridization to oligonucleotide arrays

Snap frozen mouse lung tissue was pulverized using liquid nitrogen chilled mortar and pestle, suspended in 6 ml 4 M guanidinium isothiocyanate/0.7% 2-mercaptoethanol (GTC/ME) and pulse sonicated for 2 min. The tissue suspension was extracted twice with acid equilibrated phenol (Promega Total RNA Kit) and nucleic acid precipitated with an equal volume of isopropanol. The pellet was resuspended in 0.8 ml GTC/ME, re-extracted twice with an equal volume of acid phenol and once with chloroform. RNA was ethanol precipitated, suspended in DEPC treated H₂O and quantified by OD₂₈₀.

cDNA was synthesized from 10 μ g of total RNA using the Superscript Kit (BRL) with modifications described in detail previously.³⁷ First strand synthesis was carried out at 50°C to prevent mispriming from ribosomal RNA and utilized a T7 RNA polymerase promoter containing poly-T primer (T7T24) for subsequent *in vitro* antisense RNA (cRNA) amplification and biotin labeling. cDNA was purified using BioMag Carboxyterminated beads (Polysciences), according

to the manufacturer's instructions, and eluted in 48 μ l of 10 mM Na acetate pH 7.8.

In vitro T7 polymerase driven transcription reactions for synthesis and biotin labeling of antisense cRNA, Qiagen RNeasy spin column purification and cRNA fragmentation were performed as described.¹² GeneChip[®] hybridization mixtures contained 10 μ g fragmented cRNA, 0.5 mg/ml acetylated BSA, 0.1 mg/ml herring sperm DNA, in 1 $\times$ MES buffer in a total volume of 200 μ l, as per the manufacturer's instructions. An 11 member standard curve was added to each hybridization mixture at concentrations ranging from 0.5 to 150 pM representing RNA frequencies of approximately 3.3 to 1000 parts per million (p.p.m.), assuming an average transcript size of 2 kb. The biotinylated standard curve fragments were synthesized by T7-polymerase driven IVT reactions from plasmid-based templates.³⁶ The biotinylated RNA fragments serve both as an internal standard to assess chip sensitivity and as standard curve to convert measured fluorescent difference averages from individual genes into RNA frequencies in p.p.m.^{37,38}

Reaction mixtures were hybridized for 18 h at 45°C to Affymetrix Mu11KsubA and Mu11KsubB oligonucleotide arrays that interrogate over 13000 murine sequences. The hybridization mixtures were removed and the arrays were washed and stained with Streptavidin R-phycoerythrin (Molecular Probes) using the GeneChip[®] Fluidics Station 400 and scanned with a Hewlett-Packard GeneArray Scanner following the manufacturer's instructions. Fluorescent data were collected and converted to gene specific difference averages using MicroArray Suite 4.0 software. Technical parameters used to confirm gene chip sensitivity and overall quality are described in Supplemental Material (Table 1).

Oligonucleotide array data analysis

Average fluorescence difference between perfect match and single mismatch probe sets containing gene-specific oligonucleotides were used to determine frequency values with respect to spiked standard curve. In addition, a second set of algorithms based primarily on the fraction of individual positive or negative responding probe pairs, is used to assess the absolute presence or absence of the gene product.¹² The sensitivity of the individual microarray chip was set at one-half the minimum concentration at which two of any three adjacent standard curve spike-in templates are called present. The standard curve linear regression is forced through zero and the minimum reported gene frequency was set to the sensitivity of the individual GeneChip[®].

Multiple independent replicas for each of the treatment or control experimental conditions were measured and the expression data was initially filtered to remove probe sets that were not called present in minimally four-files (minimum cohort size). Mean mRNA frequency values computed for the 5361 genes that passed the present-call filter were compared for treatment and control animals to obtain AFC and two-tailed Student's *t*-tests assuming unequal covariance of frequency.

FDR was calculated by randomization of the input data as described by Reiner *et al.*¹⁴ Sample labels were randomly

reassigned 10 000 times and the gene filtering and statistical analysis that had been executed on the observed data was repeated on each randomly permuted dataset. The median number of genes jointly meeting *t*-test *P*-value and absolute value of fold change criteria over the 10 000 random permutations was determined and the FDR is reported as this value divided by the number of genes meeting the criteria in the observed data. Because FDR was computed using the joint probability of *t*-test *P*-value and fold change, the reported FDR is not purely proportional to either *P*-value or fold change alone.

Duality of interest

Wyeth Research has commercial interest in the therapeutic application of IL-13 antagonism.

Acknowledgments

We thank Sandy Goldman and our colleagues in Molecular Profiling and Biomarker Discovery and Respiratory Disease for support during the course of this work. We are grateful to Maryann Whitley, William Mounts and the Wyeth Bioinformatics Department for continuing support in data analysis and software development. This work was supported by grants to Marsha Wills-Karp including HL58527, U01HL66623, HL10342 from the National Institute of Health.

References

- 1 Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998; **339**: 1194–1200.
- 2 Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999; **104**: 1001–1006.
- 3 Mannino DM, Homa DM, Pertowski CA, Ashizawa A, Nixon LL, Johnson CA et al. Surveillance for asthma – United States, 1960–1995. *MMWR CDC Surveill Summ* 1998; **47**: 1–27.
- 4 Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway deactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994; **10**: 587–593.
- 5 Wills-Karp M. Immunologic basis of antigen-induced airway hyper-responsiveness. *Annu Rev Immunol* 1999; **17**: 255–281.
- 6 Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med* 1998; **187**: 939–948.
- 7 Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD et al. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 1997; **272**(Part 1): L253–L261.
- 8 Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998; **282**: 2258–2261.
- 9 Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; **282**: 2261–2263.
- 10 Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J Allergy Clin Immunol* 2001; **107**: 9–18.
- 11 Wills-Karp M. The gene encoding interleukin-13: a susceptibility locus for asthma and related traits. *Respir Res* 2000; **1**: 19–23.
- 12 Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 1996; **14**: 1675–1680.
- 13 Liu Z, Kim J, Sypek JP, Wang IM, Horton H, Oppenheim FG et al. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. *J Allergy Clin Immunol* 2004; **114**: 783–790.
- 14 Reiner AD, Yekutieli D, Benjamini Y. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 2003; **19**: 368–375.
- 15 Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T et al. Abrogation of bronchial eosinophilic inflammation and airway hyper-reactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J Exp Med* 1998; **187**: 1537–1542.
- 16 Morse B, Sypek JP, Donaldson DD, Haley KJ, Lilly CM. Effects of IL-13 on airway responses in the guinea pig. *Am J Physiol Lung Cell Mol Physiol* 2002; **282**: L44–L49.
- 17 Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992; **101**: 916–921.
- 18 Nikolaidis NM, Zimmermann N, King NE, Mishra A, Pope SM, Finkelman FD et al. Trefoil factor-2 is an allergen-induced gene regulated by Th2 cytokines and STAT6 in the lung. *Am J Respir Cell Mol Biol* 2003; **29**: 458–464.
- 19 Kamada F, Suzuki Y, Shao C, Tamari M, Hasegawa K, Hirota T et al. Association of the hCLCA1 gene with childhood and adult asthma. *Genes Immun* 2004; **5**: 540–547.
- 20 Beltowski J. Guanylin and related peptides. *J Physiol Pharmacol* 2001; **52**: 351–375.
- 21 Zhang ZH, Jow F, Numann R, Hinson J. The airway-epithelium: a novel site of action by guanylin. *Biochem Biophys Res Commun* 1998; **244**: 50–56.
- 22 Zimmermann N, Mishra A, King NE, Fulkerson PC, Doecker MP, Nikolaidis NM et al. Transcript signatures in experimental asthma: identification of STAT6-dependent and -independent pathways. *J Immunol* 2004; **172**: 1815–1824.
- 23 Longman RJ, Douthwaite J, Sylvester PA, Poulosom R, Corfield AP, Thomas MG et al. Coordinated localisation of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut* 2000; **47**: 792–800.
- 24 Lee SH, Oh BH, Lee HM, Choi JO, Jung KY. Expression of mRNA of trefoil factor peptides in human nasal mucosa. *Acta Otolaryngol* 2001; **121**: 849–853.
- 25 Bautsch W, Hoymann HG, Zhang Q, Meier-Wiedenbach I, Raschke U, Ames RS et al. Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *J Immunol* 2000; **165**: 5401–5405.
- 26 Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y et al. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 2000; **406**: 998–1001.
- 27 Drouin SM, Corry DB, Kildsgaard J, Wetzel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol* 2001; **167**: 4141–4145.
- 28 Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H et al. A role for immune complexes in enhanced respiratory syncytial virus disease. *J Exp Med* 2002; **196**: 859–865.
- 29 Hawlisch H, Wills-Karp M, Karp CL, Kohl J. The anaphylatoxins bridge innate and adaptive immune responses in allergic asthma. *Mol Immunol* 2004; **41**: 123–131.
- 30 abazza EC, Taguchi O, Tamaki S, Takeya H, Kobayashi H, Yasui H et al. Thrombin in the airways of asthmatic patients. *Lung* 1999; **177**: 253–262.
- 31 Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH et al. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 2001; **167**: 4668–4675.
- 32 Donaldson DD, Elias JA, Wills-Karp M. Interleukin-13 Antagonism. In: Hansel TT, Barnes PJ (eds). *New Drugs for Asthma, Allergy, COPD*. Karger: Farmington, CT, 2001, pp 260–264.
- 33 Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003; **21**: 425–456.
- 34 Kaplan MH, Whitfield JR, Boros DL, Grusby MJ. Th2 cells are required for the *Schistosoma mansoni* egg-induced granulomatous response. *J Immunol* 1998; **160**: 1850–1856.

- 35 Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL *et al*. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. *J Immunol* 1998; **161**: 2317–2324.
- 36 Urban Jr JF, Noben-Trauth N, Donaldson DD, Madden KB, Morris SC, Collins M *et al*. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* 1998; **8**: 255–264.
- 37 Byrne MC, Whitley MZ, Follettie MT. Preparation of mRNA for expression monitoring. In: Ausubel *et al*. (eds). *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.: New York, 2000.
- 38 Hill AA, Brown EL, Whitley MZ, Tucker-Kellogg G, Hunter CP, Slonim DK. Evaluation of normalization procedures for oligonucleotide array data based on spiked cRNA controls. *Genome Biol* 2001; **2**: RESEARCH0055.

Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (<http://www.nature.com/tpj>).

Supplemental Material

Technical Parameters of Microarrays.

The oligonucleotide arrays responded with an average sensitivity of 13 ppm and 12 ppm for the Mu11KsubA and Mu11KsubB arrays, respectively. The quality of the purified RNA and derived cDNA product was monitored by comparing the ratio of frequencies calculated for actin and glyceraldehyde-3-phosphate dehydrogenase derived from independent probe sets representing the 5'-end versus those from the 3'-end of the respective genes. The measured 5'/3' ratio for RNA isolated from the different sets of control and treated animals were balanced, averaging 0.81 with a range of 0.77 to 0.90 as reported in Table 1. Of the total 13,179 tiled sequences on the combined Mu11K subA and subB GeneChips[®], an average of 5294 (+/- 533) genes were called present in the individual analyzed files (Table 1) with an overall coefficient of variance of 10.1%. Additionally, the sum of computed frequencies for all genes called present in at least one file are reported in Table 1 for each of the subgroups, averaging 485 thousand with an overall coefficient of variance of 20.9%. The similarity of chip sensitivity and RNA quality of the individual GeneChips[®] experiments are reflected in an overall balance in measured gene expression providing support for the use of a common spiked standard curve to normalize the individual files (18). The overall gene expression measured for each treatment was similar with respect to mRNA integrity; number of genes called present and total mRNA frequency computed across the various control and treatment files (Table 2).

Supplemental Table 1. Summary of Quality Control Parameters

	Balb/C 72hr (IL-13)		STAT6 72hr (IL-13)		Balb/C 72hr (OVA)		
	Control	IL-13	Control	IL-13	Control	OVA	OVA + sFc13Ra2
	N=5	N=6	N=4	N=5	N=6**	N=4**	N=2
Avg. RNA 5'/3'Ratio [*]	0.90	0.77	0.88	0.80	0.76	0.78	0.80
# Genes Present [†]	5396 +/- 813	4984 +/- 568	5553 +/- 475	5422 +/- 628	5143 +/- 285	5352 +/- 568	5210 +/-394
Frequency Total [‡]	450 +/-101	581 +/-131	482 +/- 80	539 +/- 174	447 +/- 83	414 +/-52	480 +/- 89

^{*} Average 5'/3' ratio determined from mRNA frequencies measured from independent oligonucleotide sets representing the 5' and 3' regions for both actin and Gapdh.

[†] Total number of mRNA transcripts called present of the 13,179 tile sequences.

[‡] Average total frequency of all genes called Present in at least 1 file ($\times 10^{-3}$)

****** The gene expression profile measured for PBS challenged mice was not altered significantly by administration of human IgG or sIL-13R α 2-Fc, and thus frequency values from these mice were combined as a single control set. Similarly, OVA-challenged mice treated either with buffer or hIgG were combined as a single OVA set.