

B-cell isotype control in atopy and asthma assessed with cDNA array technology

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Brutsche, Martin H., Ingrid Carlen Brutsche, Peter Wood, Nesrin Mogulkoc, Adnan Custovic, Jim Egan, and Ashley Woodcock. B-cell isotype control in atopy and asthma assessed with cDNA array technology. *Am J Physiol Lung Cell Mol Physiol* 280: L627–L637, 2001.—B-cell isotype switching and the production of IgE is regulated by a variety of gene products through different mechanisms. A better understanding of these processes has the potential to identify markers of disease and new therapeutic targets. The aim of the study was to investigate human B-cell isotype control and IgE production in atopy and asthma with cDNA array technology. Eighteen atopic asthmatic, eight atopic nonasthmatic, and fourteen healthy control subjects were included. Peripheral blood mononuclear cells were separated by gradient centrifugation, mRNA was purified, and the reverse-transcribed probes were hybridized to cDNA membranes. Group differences were assessed with the Mann-Whitney *U*-test. Twenty-three of seventy-eight tested IgE-related genes had significantly altered expression in atopy and asthma compared with that in the healthy subjects. The differentially expressed genes include surface molecules involved in T- and B-cell interaction and activation, cytokines, intracellular signaling products, and transcription factors. In conclusion, both atopic nonasthmatic and atopic asthmatic individuals had activated proinflammatory pathways, a minimal requirement for B-cell isotype switching, and a clear net pro-IgE cytokine climate.

complementary deoxyribonucleic acid; bronchial asthma; immunoglobulin E; B-cell isotype switch; gene expression

ATOPY IS CHARACTERIZED by increased synthesis of IgE specific for common allergens (14). Cross-linking of IgE with an allergen results in rapid release of a variety of mediators, including histamine, leukotrienes, prostaglandins, and proteases, which account for many of the inflammatory changes underlying the symptoms of immediate hypersensitivity. In addition, cell activation involving IgE leads to a prolonged inflammatory response lasting several hours after allergen exposure (10, 22), although it is well established that an influx and activation of inflammatory cells such as neutro-

phils, eosinophils, and T cells are central to the chronicity of the response. Continuous low-grade daily allergen exposure (5, 6) thus results in an ongoing inflammatory stimulus leading to chronic inflammation such as seen in asthmatic airways and the nasal mucosa in allergic rhinitis. A better understanding of the induction and regulation of IgE synthesis in B cells is crucial for the elucidation of the pathogenesis of IgE-dependent disorders such as asthma. In addition, it enables refinement of therapies targeting IgE.

Production of IgE and other immunoglobulins results from reciprocal activation of T and B cells (28). As a key initial step in the chain of events necessary for the induction of immunoglobulin production, resting B cells bind allergens through their membrane-bound antigen-specific immunoglobulin. After internalization of the antigen receptor complex, antigens are processed and presented to T cells as peptide fragments in association with the class II major histocompatibility complex. Induction of IgE synthesis by human B cells requires two signals (25, 26): signal 1 results from cognate interaction between membrane-bound receptors and ligands expressed by activated helper T and B lymphocytes, whereas signal 2 involves the T cell-derived cytokines interleukin (IL)-4 and/or IL-13. Engagement of the B-cell antigen CD40 by the CD40 ligand (CD40L), expressed on T cells, leads to isotype switching during immunoglobulin synthesis. The CD40-CD40L interaction is well established (for a review, see Ref. 25a) as a key signal for the induction of isotype switching, whereas the elucidation of the role of other cell-cell interactions, for example, through adhesion molecules, needs further study. An important counteracting cytokine for IgE synthesis is interferon (IFN)- γ , which is produced mainly by T lymphocytes (19). Several surface molecules and cytokines and various hormones have been shown to modulate IgE synthesis in vitro, suggesting that a complex network of molecular events is involved in the production of IgE. However,

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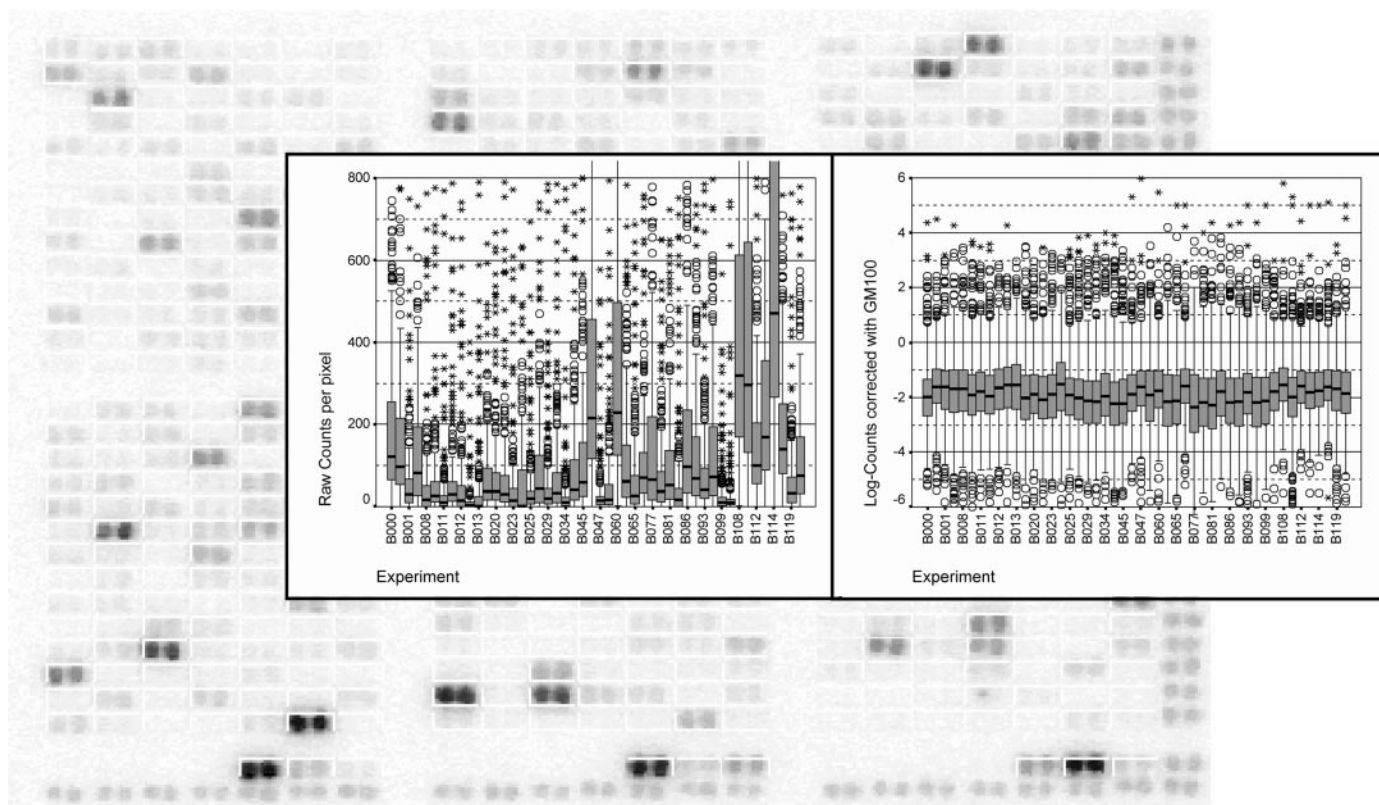


Fig. 1. Example of a cDNA array after hybridization. With this technique, mRNA can be copied into radiolabeled cDNA with reverse transcriptase so that the relative abundance of individual mRNAs is reflected in the cDNA product. Thus the intensity of the hybridization signal for a given gene product is a result of its relative abundance in the target sample. This method has been proven to provide excellent specificity and reproducibility (7, 20, 21). mRNA species comprising 1:10,000–100,000 of the mass of the target poly(A)⁺ RNA, which corresponds to ~1 transcript/100,000, could readily be detected (7, 9). Single-strand DNAs with a length of 200–600 bases of known genes can be fixed to a membrane and probed with the RNA-derived cDNA probe. The intensity of the hybridization signal for a given gene is a result of its relative abundance in the RNA-derived DNA probe. Each gene product, including positive and negative controls, is represented in duplicate. *Insets:* effect of the standardization procedure against the 100 most expressed genes (GM₁₀₀) on the hybridization results of 48 different experiments. Box plots show the distribution of the results in each experiment before (*left*) and after (*right*) this procedure, which enables direct comparison of results. Each experiment is represented by 1 box. The shaded boxes cover 25–75% of data, with the median value shown as a thick solid line. Error bars indicate 10 and 90%. ○, Outliers; *, extreme values. Over the range of 609 different gene products, the assumption has been made that peripheral blood mononuclear cells (PBMCs) express similar amounts of mRNA with comparable overall mean hybridization intensities. It can therefore be expected that the distribution of the signals resulting from the different experiments must be similar. Ideally, a standardization method would be able to produce a unique distribution for all experiments. Shown is the efficacy of the GM₁₀₀ method for this standardization process; e.g., experiments B000, B060, B108, and B114 had tendencies for higher values before the application of GM₁₀₀, which was corrected to a large extent after standardization.

the relevance of these factors for IgE production *in vivo* requires further elucidation.

In the present study, we sought to develop a novel approach to study the regulation of human B-cell isotype control resulting in IgE synthesis. In this first study of its kind, we restricted our investigation to peripheral blood of atopic individuals, although it is likely that additional important information will be obtained from studying the lymphoid tissue where IgE is produced. In addition to seeking evidence for a broad range of genes being differentially expressed in atopic disease, we have sought to identify differences between asthmatic individuals with the disease ranging from mild to severe in an attempt to provide insight into the determinants of disease severity. Gene expression was

studied with cDNA array technology to standardize array data for comparison purposes, focusing the analysis and modeling to genes known to be involved in B-cell isotype control and the production of IgE.

METHODS

Subjects. Fourteen nonatopic control subjects (12 women; mean age 42 yr, range 27–65 yr), eight atopic nonasthmatic (AN) subjects (1 woman; mean age 36 yr, range 23–49 yr), and fifteen atopic asthmatic (AA) subjects (12 women; mean age 41 yr, range 18–66 yr) gave informed consent and participated in the study. Atopy was defined as a wheal 3 mm or larger in diameter than the negative control wheal on skin prick testing to a range of four common aeroallergens in the United Kingdom (*Dermatophagoides pteronyssinus*, cat, dog,

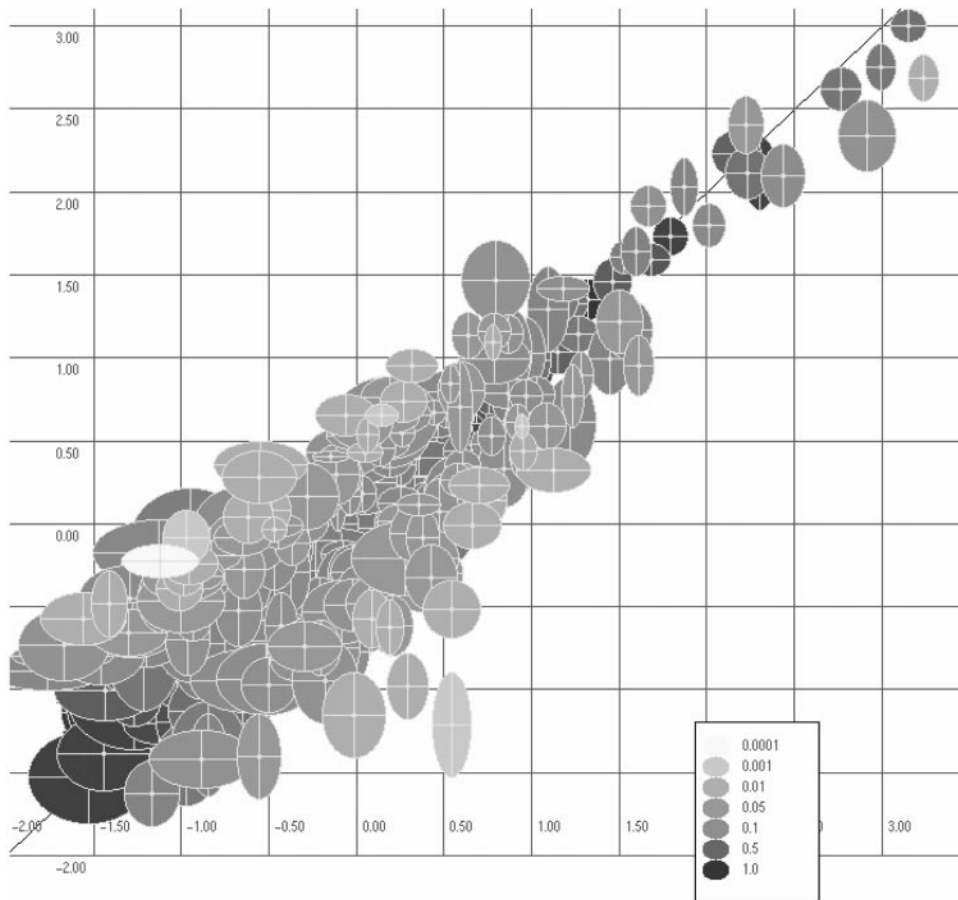


Fig. 2. Dot plot comparing the gene expression in healthy control subjects (x-axis) with the expression in atopic asthmatic subjects (y-axis). Both axes give log-transformed gene expression values standardized with GM₁₀₀ method. Each gene is represented by its mean expression together with its confidence limit (30%) in both axes designed as an oval. Significance levels of Mann-Whitney *U*-tests are color-coded. The brighter the color, the more significant the difference in gene expression between the 2 groups. The more distant from the line of identity, the greater the difference in gene expression. The smaller the oval, the lower the variability and the greater the chance of significance. This visual approach is useful for rapid screening of candidate genes and integrates the individual spread of data in the 2 axes in addition to the group means.

and mixed grasses). Atopic asthma was defined as symptomatic bronchial hyperreactivity or reversibility in a sensitized individual. All patients were diagnosed and treated for asthma before being enrolled in the study, and no attempt was made to change their medication. The asthmatic subjects were scored according to the Aas (1) asthma severity score. The Aas score is a five-step scale clinical score that takes into account events occurring during the previous year. Patients with atopic asthma had a range of disease severity (I, $n = 2$; II, $n = 1$; III, $n = 4$; IV, $n = 9$; V, $n = 2$). They were taking inhaled corticosteroids (beclomethasone equivalent dose: none, $n = 2$; 400 $\mu\text{g/day}$, $n = 2$; 800 $\mu\text{g/day}$, $n = 1$; 1,000 $\mu\text{g/day}$, $n = 3$; 1,500 $\mu\text{g/day}$, $n = 3$; 2,000 $\mu\text{g/day}$, $n = 6$). None had received oral or parenteral corticosteroids for at least 2 mo.

Isolation of peripheral blood mononuclear cells and cDNA hybridization. Peripheral blood was drawn, and peripheral blood mononuclear cells (PBMCs) were separated immediately by gradient centrifugation followed by washing in AIM-V serum-free culture medium. Purified mRNA (TRIzol Reagent, Life Technologies, Paisley, UK; Oligotex, QIAGEN, Crawley, UK) was reverse transcribed with an oligo(dT) primer mix and labeled with [³²P]dATP (Amersham Life Sciences). After an overnight hybridization onto membranes with an immobilized probe cDNA for 609 gene products in duplicate (Atlas, Clontech, Palo Alto, CA; the complete list of genes with accession numbers is published at <http://www.clontech.com>), quantification was performed by autoradiography and phosphorimaging.

Standardization of quantitative hybridization signals. To compare the results of different hybridization experiments,

the data had to be standardized for nonspecific variation. This was done by expressing the results relative to the geometric mean of the 100 genes with the greatest expression (GM₁₀₀; the assumption was made that PBMCs express similar amounts of mRNA with comparable overall mean hybridization intensities over the range of 609 gene products; Fig. 1), which proved to be superior to other methods of standardization.

Verification of cDNA array data: immunofluorescent staining and flow cytometry. Several identified differentially expressed genes have been validated on a mRNA level by RT-PCR [for example, IL-4, IL-6, IFN- γ , vascular endothelial growth factor, and transcription factor CP2 (TFCP2)], on a protein level by ELISA [for example, transforming growth factor (TGF)- β 1 to - β 3], and on a receptor level by double-stained fluorescence-activated cell sorter (FACS) analysis [for example, integrin family of genes (ITG) B4 and α -subunit of IL-2 receptor (IL-2R α)].

For FACS analysis, another 20 control, 22 AN, and 25 AA individuals with the same inclusion and exclusion criteria as above were recruited. Five milliliters of venous whole blood was centrifuged for 5 min at 750 g at 4°C, the plasma was discarded, and the erythrocytes were lysed in two rounds of 4:1 Boyles' medium for 4 min at 37°C. The cells were then washed in phosphate-buffered saline. At the end of this procedure, the blood leukocytes were incubated with a panel of directly conjugated monoclonal antibodies (10 μl each) for 45 min at 4°C. Each of the fluorescein isothiocyanate (FITC)-conjugated CD4, CD8, and CD19 monoclonal antibodies was

Table 1. *Relative gene expression in atopic individuals with and without asthma compared with that in healthy control subjects*

No.	Expression Ratio		Significance level (p)			Acc.No		Name	HUGO
	AN	AA	N vs. AN	N vs. AA	AA grade				
Co-stimulation									
459	Neg	Neg	Neg	Neg	Neg	M21097	CD19 B-LYMPHOCYTE ANTIGEN [Differentiation antigen (CD19)] E5k		CD19
210	Neg	Neg	Neg	Neg	Neg	L07414	CD40 LIGAND C1n		CD40L
256	2.33	1.86	0.005	0.095	0.035	U21092	CD40 RECEPTOR ASSOCIATED FACTOR (CRAF1) [TNF RECEPTOR ASSOCIATED FACTOR 2] C5d		TRAF3
416	1.45	1.65	0.06	0.06	0.39	X60592	CD40L RECEPTOR [Cdw40 nerve growth factor receptor-related B-lymphocyte activation molecule] E2j		CD40
602	Neg	Neg	Neg	Neg	Neg	M11886	HLA CLASS I HISTOCOMPATIBILITY ANTIGEN, C-4 ALPHA CHAIN [MHC] G14		
487	0.55	0.58	0.008	0.007	0.094	Y00796	INTEGRIN ALPHA L [LEUKOCYTE ADHESION GLYCOPROTEIN LFA-1 ALPHA CHAIN; antigen CD11A (p180)] E7k		ITGAL
133	2.58	2.13	0.02	0.08	0.03	L25259	T LYMPHOCYTE ACTIVATION ANTIGEN CD86 [CD28 antigen ligand 2, B7-2 antigen] B3g		CD86
Cytokines									
427	0.40	0.73	0.004	0.040	0.298	D11086	CYTOKINE RECEPTOR COMMON GAMMA CHAIN [Interleukin 2 receptor gamma chain] E3g		IL2RG
499	Neg	Neg	Neg	Neg	Neg	X72755	GAMMA INTERFERON INDUCED MONOKINE [Humig] F1i		MIG
421	Neg	Neg	Neg	Neg	Neg	J03171	HuIFN- ALPHA -REC [INTERFERON ALPHA-BETA RECEPTOR ALPHA CHAIN] E3a		IFNAR1
426	0.32	0.46	0.001	0.001	0.684	X77722	INTERFERON ALPHA-BETA RECEPTOR BETA CHAIN E3f		IFNAR2
565	4.26	1.81	0.024	0.524	0.437	J00209	INTERFERON ALPHA-C LEUKOCYTE [IFN-ALPHA] F6e		IFNA10
564	4.70	1.71	0.064	0.772	0.243	M28622	INTERFERON BETA F6d		IFNB1
304	Neg	Neg	Neg	Neg	Neg	M91196	INTERFERON CONSENSUS SEQUENCE BINDING PROTEIN [DNA-binding protein] D1j		
555	Neg	Neg	Neg	Neg	Neg	X01992	INTERFERON GAMMA F5i		IFNG
178	0.65	0.94	0.088	0.621	0.390	X14454	INTERFERON REGULATORY FACTOR [Interferon regulatory factor 1] B6j		IRF1
541	Del	Del	Del	Del	Del	X02530	INTERFERON-GAMMA INDUCED PROTEIN F4i		INP10
431	2.14	1.01	0.151	0.675	0.964	A09781	INTERFERON-GAMMA RECEPTOR E3k		
428	0.37	0.94	0.003	0.382	0.684	J03143	INTERFERON-GAMMA RECEPTOR ALPHA CHAIN E3h		IFNGR1
150	Neg	Neg	Neg	Neg	Neg	U05875	INTERFERON-GAMMA RECEPTOR BETA CHAIN [Interferon gamma receptor accessory factor-1 (AF-1)] B4j		IFNGR2
566	1.98	1.47	0.040	0.239	0.618	M57627	INTERLEUKIN-10 F6f		IL10
430	Neg	Neg	Neg	Neg	Neg	U00672	INTERLEUKIN-10 RECEPTOR E3j		IL10R
571	Neg	Neg	Neg	Neg	Neg	M65291	INTERLEUKIN-12 ALPHA CHAIN (Natural killer cell stimulatory factor, p35) F6k		IL12A
570	Neg	Neg	Neg	Neg	Neg	M65290	INTERLEUKIN-12 BETA CHAIN (Natural killer cell stimulatory factor, p40) F6j		IL12B
434	4.08	6.95	0.001	0.001	0.385	U03187	INTERLEUKIN-12 RECEPTOR E3n		IL12RB1
567	Neg	Neg	Neg	Neg	Neg	L06801	INTERLEUKIN-13 F6g		IL13
557	Del	Del	Del	Del	Del	A14844	INTERLEUKIN-2 F5k		IL12
419	1.14	1.40	0.495	0.011	0.751	X01057	INTERLEUKIN-2 RECEPTOR ALPHA CHAIN E2m		IL2RA
422	1.20	0.70	0.375	0.028	0.258	M26062	INTERLEUKIN-2 RECEPTOR BETA CHAIN E3b		IL2RB
561	2.69	1.63	0.114	0.223	0.497	M13982	INTERLEUKIN-4 F6a		IL4
424	1.71	1.79	0.056	0.030	0.892	X52425	INTERLEUKIN-4 RECEPTOR ALPHA CHAIN E3d		IL4R
563	Neg	Neg	Neg	Neg	Neg	X04688	INTERLEUKIN-5 [T-cell replacing factor] F6c		IL5
425	Neg	Neg	Neg	Neg	Neg	M75914	INTERLEUKIN-5 RECEPTOR ALPHA CHAIN E3e		IL5RA
562	2.46	2.02	0.005	0.009	0.964	X04602	INTERLEUKIN-6 [B-cell differentiation factor] F6b		IL6
420	0.57	0.65	0.785	0.494	0.751	M20566	INTERLEUKIN-6 RECEPTOR ALPHA CHAIN E2n		IL6R
120	1.48	1.90	0.113	0.085	0.389	M57230	INTERLEUKIN-6 RECEPTOR BETA CHAIN [membrane glycoprotein gp130] B2h		IL6ST
552	0.72	0.84	0.152	0.138	0.556	Y00787	INTERLEUKIN-8 [monocyte-derived neutrophil chemotactic factor MDNCF] F5f		IL8
407	Neg	Neg	Neg	Neg	Neg	M68932	INTERLEUKIN-8 RECEPTOR (ALFA, HIGH AFFINITY) E2a		IL8RA
573	Neg	Neg	Neg	Neg	Neg	X17543	INTERLEUKIN-9 F6m		IL9
429	Neg	Neg	Neg	Neg	Neg	M84747	INTERLEUKIN-9 RECEPTOR E3i		IL9R
202	2.28	1.08	0.056	0.889	0.277	D12614	LYMPHOTOXIN-ALPHA [formerly tumor necrosis factor beta (TNF-beta)] C1f		LTA
111	1.46	2.38	0.160	0.023	0.556	L28175	PROSTAGLANDIN E2 RECEPTOR EP4 SUBTYPE B1m		PTGER4
514	0.80	0.97	0.838	0.879	0.441	M21121	RANTES PROTEIN T-CELL SPECIFIC F2j		SCYA5
5	0.84	2.03	0.785	0.407	0.556	L07594	TGF-BETA RECEPTOR TYPE III A1e		TGFB3
19	Neg	Neg	Neg	Neg	Neg	J03241	TRANSFORMING GROWTH FACTOR (BETA 3) A2e		TGFB3
495	Neg	Neg	Neg	Neg	Neg	X02812	TRANSFORMING GROWTH FACTOR BETA [1] F1e		TGFB1
497	Neg	Neg	Neg	Neg	Neg	M19154	TRANSFORMING GROWTH FACTOR BETA [2] F1g		TGFB2
201	1.76	0.78	0.041	0.500	0.242	X01394	TUMOR NECROSIS FACTOR [TNFa] C1e		TNF
209	Neg	Neg	Neg	Neg	Neg	M33294	TUMOR NECROSIS FACTOR RECEPTOR [Tumor necrosis factor receptor 1 (55kD)] C1m		TNFR1
199	0.92	1.56	0.539	0.024	0.922	M32315	TUMOR NECROSIS FACTOR RECEPTOR [Tumor necrosis factor receptor 2] C1c		TNFR2
404	0.58	0.67	0.891	0.595	0.033	M31165	TUMOR NECROSIS FACTOR-INDUCIBLE PROTEIN TSG-6 E1i		TNFAIP6
Heat Shock Group									
191	0.89	1.76	0.682	0.033	0.016	D49547	HEAT SHOCK [DNAJ PROTEIN HOMOLOG 1; heat-shock protein 40] B7i		HSPF1
194	2.07	2.11	0.004	0.000	0.135	X54079	HEAT SHOCK [Heat shock 27kD protein 1] B7j		HSPB1
192	1.33	1.87	0.585	0.023	0.221	M34664	HEAT SHOCK [Heat shock 60 kDa protein 1 (chaperonin); MITOCHONDRIAL MATRIX PROTEIN P1] B7j		HSPD1
195	2.04	1.92	0.020	0.014	0.556	M11717	HEAT SHOCK [HEAT SHOCK 70 kDa PROTEIN 1] B7m		HSPA1A
193	1.44	1.43	0.339	0.210	0.063	X07270	HEAT SHOCK [Heat shock protein 86] B7k		
317	Neg	Neg	Neg	Neg	Neg	M64673	TCF5 [HEAT SHOCK FACTOR PROTEIN 1] D2i		
Markers of T cell differentiation									
122	1.59	1.38	0.020	0.129	0.341	D21878	BST-1 [LYMPHOCYTE DIFFERENTIATION ANTIGEN CD38] B2j		CD38
453	0.99	1.01	0.891	0.939	0.113	M63928	CD27L RECEPTOR [T cell activation antigen (CD27)] E5e		CD27
454	0.85	0.70	0.633	0.087	0.030	M83554	CD30L RECEPTOR [Lymphocyte activation antigen CD30; Ki-1 antigen] E5f		CD30
466	1.29	1.06	0.339	0.939	0.389	M59040	CD44 ANTIGEN HEMATOPOIETIC FORM [Cell adhesion molecule (CD44)] E6d		CD44
465	Neg	Neg	Neg	Neg	Neg	M54992	CD72 B-CELL DIFFERENTIATION ANTIGEN E6c		CD72
54	1.81	0.99	0.174	1.000	0.174	U26710	PROTO-ONCOGENE C-CBL A4i		CBLB
527	Del	Del	Del	Del	Del	M57502	T LYMPHOCYTE-SECRETED PROTEIN I-309 F3i		SCYA1
451	Neg	Neg	Neg	Neg	Neg	M12807	T-CELL SURFACE GLYCOPROTEIN CD4 E5c		CD4
100	Neg	Neg	Neg	Neg	Neg	M15800	T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN [MAL protein] B1b		MAL

Continued

combined with phycoerythrin-conjugated CD25, resulting in a panel of three probes per individual completed with a negative control. During FACS acquisition with a FACScan flow cytometer and CellQuest software (Becton Dickinson, Oxford, UK), lymphocytes, monocytes, and polynuclear cells were identified by their forward- and side-scatter characteristics and electronically gated. Acquisition was carried out until 10,000 events within the lymphocyte gate were acquired independent of the total cell number.

Statistical analysis. Data were analyzed with SPSS for Windows 9.0 (SPSS, Chicago, IL) statistical package. Multiple Mann-Whitney *U*-tests and Kruskal-Wallis tests were used to screen for differential gene expression after standardization with the GM₁₀₀ method as appropriate. Expression ratios (ERs) were calculated by dividing the geometric mean of the expression of a particular gene in AN or AA subjects by the geometric mean of the expression in control subjects. A graphical approach was taken in addition to statistical sig-

Table 1.—Continued

No.	Expression Ratio		Significance level (p)			Acc.No	Name	HUGO
	AN	AA	N vs. AN	N vs. AA	AA grade			
Intracellular signaling products								
172	1.51	1.13	0.088	0.874	0.011	X60188	ERK1 [EXTRACELLULAR SIGNAL-REGULATED KINASE 1] B6d	PRKM3
168	Neg	Neg	Neg	Neg	Neg	M84489	ERK2 [EXTRACELLULAR SIGNAL-REGULATED KINASE 2] B5n	PRKM1
173	Neg	Neg	Neg	Neg	Neg	X80692	ERK3 [EXTRACELLULAR SIGNAL-REGULATED KINASE 3] B6e	PRKM6
124	3.29	1.62	0.003	0.196	0.052	L05624	MAP KINASE [DUAL SPECIFICITY MITOGEN-ACTIVATED PROTEIN KINASE KINASE 1] B2l	PRKMK1
161	0.98	1.33	0.946	0.057	0.556	L36719	MAP KINASE [DUAL SPECIFICITY MITOGEN-ACTIVATED PROTEIN KINASE KINASE 3; MAP kinase kinase 3 (MKK3)] B5h	PRKMK3
162	Neg	Neg	Neg	Neg	Neg	L36870	MAP KINASE [JNK ACTIVATING KINASE 1; MAP kinase kinase 4 (MKK4)] B5h	SERK1
186	3.11	0.84	0.029	0.224	0.553	U39657	MAP KINASE [MAP kinase kinase 6 (MKK6)] B7d	PRKMK6
160	Neg	Neg	Neg	Neg	Neg	L35253	MAP KINASE [p38 mitogen activated protein (MAP) kinase] B5f	CSBP1
181	2.51	1.62	0.020	0.569	0.033	U09578	MAP KINASE-ACTIVATED PROTEIN KINASE [MAPKAP kinase (3pK)] B6m	..
174	Neg	Neg	Neg	Neg	Neg	Z29090	PHOSPHATIDYLINOSITOL 3-KINASE CATALYTIC SUBUNIT ALPHA ISOFORM B6f	PIK3CA
179	Neg	Neg	Neg	Neg	Neg	D42108	PHOSPHOLIPASE C B6k	PLCE
164	Neg	Neg	Neg	Neg	Neg	X06318	PROTEIN KINASE C BETA-II TYPE B5j	PRKCB1
175	Neg	Neg	Neg	Neg	Neg	L31951	PROTEIN KINASE JNK2 STRESS-ACTIVATED B6g	JNK2
177	Neg	Neg	Neg	Neg	Neg	U34819	PROTEIN KINASE JNK3 STRESS-ACTIVATED B6i	JNK3
488	0.43	0.71	0.001	0.037	0.441	U43522	PROTEIN TYROSINE KINASE PYK2 [FOCAL ADHESION KINASE; Cell adhesion kinase beta (CAKbeta)] E7l	FAK2
213	Neg	Neg	Neg	Neg	Neg	M93426	PROTEIN-TYROSINE PHOSPHATASE ZETA C2c	PTPRZ1
43	0.87	1.77	0.275	0.518	0.258	M19722	PROTO-ONCOGENE TYROSINE-PROTEIN KINASE FGR A4a	FGR
151	1.13	2.13	0.946	0.005	0.242	U07236	PROTO-ONCOGENE TYROSINE-PROTEIN KINASE LCK [Lymphocyte-specific protein tyrosine kinase] B4k	LCK
35	2.76	2.01	0.017	0.065	0.495	K03218	PROTO-ONCOGENE TYROSINE-PROTEIN KINASE SRC [c-src-1 proto-oncogene] A3g	SRC
140	Neg	Neg	Neg	Neg	Neg	M28213	RAS-RELATED PROTEIN RAB-2 B3n	RAB2
137	Neg	Neg	Neg	Neg	Neg	M28210	RAS-RELATED PROTEIN RAB-3A B3k	RAB3A
141	Neg	Neg	Neg	Neg	Neg	M28214	RAS-RELATED PROTEIN RAB-3B B4a	RAB3B
138	Del	Del	Del	Del	Del	M28211	RAS-RELATED PROTEIN RAB-4A B3l	RAB4
142	Neg	Neg	Neg	Neg	Neg	M28215	RAS-RELATED PROTEIN RAB-5A B4b	RAB5A
139	Neg	Neg	Neg	Neg	Neg	M28212	RAS-RELATED PROTEIN RAB-6 B3m	RAB6
159	Neg	Neg	Neg	Neg	Neg	L26318	STRESS-ACTIVATED PROTEIN KINASE JNK1 B5e	PRKM8
176	Neg	Neg	Neg	Neg	Neg	L34583	TYROSINE PHOSPHATASE (clone HFAP10) B6h	PTPN13
163	0.80	0.82	0.413	0.494	0.964	M16038	TYROSINE-PROTEIN KINASE LYN [V-src-1 Yamaguchi sarcoma viral related oncogene homolog] B5i	LYN
126	1.18	1.89	0.633	0.003	1.000	L13738	TYROSINE-PROTEIN KINASE SYK [activated p21cdc42Hs kinase (ack)] B2n	..
171	0.58	1.04	0.008	0.287	0.556	X54637	TYROSINE-PROTEIN KINASE TYK2 NON-RECEPTOR B6c	TYK2
Transcription factors								
300	0.83	1.15	0.332	0.494	0.618	M95809	BASIC TRANSCRIPTION FACTOR (62 KD SUBUNIT) [BTf2] D1f	GTF2H1
143	5.10	1.99	0.008	0.732	0.441	M31630	CAMP RESPONSE ELEMENT BINDING PROTEIN CRE-BP1 [CAMP responsive element binding protein 1] B4c	CREB2
315	0.81	0.95	0.409	0.939	0.221	L05515	CAMP RESPONSE ELEMENT BINDING PROTEIN CRE-BP1 D2g	CREB1
297	1.14	1.06	0.453	0.621	0.135	M59079	CCAAT-BINDING TRANSCRIPTION FACTOR SUBUNIT B [CCAAT-binding protein (Hap2)] D1c	NFYA
144	1.73	1.74	0.009	0.732	0.441	M34356	CREB [ACTIVE TRANSCRIPTION FACTOR] B4d	CREB1
314	0.63	0.83	0.041	0.239	0.497	D90209	CYCLOC-AMP-DEPENDENT TRANSCRIPTION FACTOR ATF-4 [DNA-binding protein TAXREB67] D2f	ATF4
311	Neg	Neg	Neg	Neg	Neg	L29277	DNA- BINDING PROTEIN APRF [SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3] D2c	STAT3
296	2.73	1.51	0.014	0.154	0.221	M68891	ENDOTHELIAL TRANSCRIPTION FACTOR GATA-2 D1b	GATA2
367	0.82	1.42	0.195	0.122	0.688	U08015	NF-ATc [Transcription factor (NFATc.b)] D6c	NFATC1
295	3.66	0.95	0.020	0.704	0.298	M58603	NUCLEAR FACTOR NF-KAPPA-B P105 SUBUNIT D1a	NFKB1
357	1.15	1.18	0.785	0.407	0.157	M96944	PAIRED BOX PROTEIN PAX-5 [B-cell specific transcription factor (BSAP)] D5g	PAX5
368	1.02	1.79	0.881	0.028	0.233	U08191	R KAPPA B D6d	NRFB
12	1.30	0.87	0.246	0.790	0.441	J04111	TRANSCRIPTION FACTOR AP-1 [c-jun proto oncogene] A1l	JUN
339	2.77	2.62	0.016	0.011	0.159	M36711	TRANSCRIPTION FACTOR AP-2 [Sequence-specific DNA-binding protein (AP-2)] D4c	TFAP2A
390	6.21	1.78	0.014	0.620	0.649	U43188	TRANSCRIPTION FACTOR ELF-1 ETS-RELATED [Ets transcription factor (NERF-2)] D7l	..
343	0.55	0.71	0.048	0.119	0.258	M62831	TRANSCRIPTION FACTOR ETR101 [T-LYMPHOCYTE ACTIVATED PROTEIN] D4g	..
342	0.67	0.59	0.152	0.068	0.821	M62829	TRANSCRIPTION FACTOR ETR103 [EARLY GROWTH RESPONSE PROTEIN 1] D4f	EGR1
379	1.31	1.02	0.194	0.648	0.094	X55122	TRANSCRIPTION FACTOR GATA-3 TRANS-ACTING T-CELL SPECIFIC D7a	GATA3
337	0.92	0.78	0.031	0.005	0.278	M34960	TRANSCRIPTION FACTOR IID D4a	TBP
155	1.08	1.26	0.551	0.210	0.298	U16031	TRANSCRIPTION FACTOR IL-4 STAT [SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 6] B5a	STAT6
50	1.15	1.24	0.709	0.383	0.828	X56681	TRANSCRIPTION FACTOR JUN-D A4h	JUND
369	0.44	1.11	0.456	0.158	0.515	U08853	TRANSCRIPTION FACTOR LCR-F1 [NRF1 protein; Transcription factor 11 (basic leucine zipper type)] D6e	TCF11
364	3.46	3.24	0.001	0.001	0.751	U03494	TRANSCRIPTION FACTOR LSF [alpha-globin transcription factor CP2] D5n	TFCP2
338	4.15	0.84	0.002	0.017	0.035	M36542	TRANSCRIPTION FACTOR LYMPHOID-SPECIFIC [TRANSCRIPTION FACTOR 2 OCTAMER-BINDING] D4b	POU2F2
127	0.79	1.47	0.219	0.139	0.390	L19067	TRANSCRIPTION FACTOR P65 B3a	RELA
384	1.15	1.36	0.413	0.102	0.892	M97190	TRANSCRIPTION FACTOR SP2 D7f	SP2
322	1.18	1.01	0.785	0.884	0.023	D26121	TRANSCRIPTION FACTOR ZFM1 (alternatively spliced product) D2n	ZNF162
321	0.29	0.62	0.005	0.068	0.102	D26120	TRANSCRIPTION FACTOR ZFM1 D2m	ZNF162
389	0.81	0.72	0.076	0.015	0.044	U30504	TRANSCRIPTION INITIATION FACTOR TFIID SUBUNIT TAFII31 D7k	TAF2G
323	1.78	1.51	0.190	0.195	0.298	D26155	TRANSCRIPTIONAL ACTIVATOR hSNF2a D3a	SMARCA2
312	1.31	1.30	0.056	0.053	0.892	D26156	TRANSCRIPTIONAL ACTIVATOR hSNF2b [estrogen receptor hSNF2b] D2d	SMARCA4
352	1.87	1.80	0.131	0.002	0.751	M87503	TRANSCRIPTIONAL REGULATOR ISGF3 GAMMA SUBUNIT [IFN-responsive transcription factor subunit] D5b	..
354	0.62	1.34	0.088	0.081	0.077	M92843	TRISTETRAPROLINE [Zinc finger transcriptional regulator] D5d	ZFP36
380	1.35	0.75	0.076	0.128	0.008	X59738	ZINC FINGER X-CHROMOSOMAL PROTEIN [ZFX put. transcription activator, isoform 1] D7b	ZFX

Genes are listed in alphabetical order and grouped according to function. The results are given in the form of a ratio of expression in atopic nonasthmatic (AN) or atopic asthmatic (AA) subjects to expression in healthy volunteers (N). Shaded boxes, upregulation in gene expression (arbitrary cutoff value of 1.3); hatched boxes, downregulation in gene expression (arbitrary cutoff value of 0.7). Acc.No, GenBank accession number; HUGO, human genome nomenclature short name; Neg, negative; Del, deleted. *P* values of significance testing with Mann-Whitney *U*-test are given for the comparison of N vs. AN, N vs. AA, and AA with low-grade asthma [Aas (1) asthma severity scores ≤ 2] vs. AA with higher-grade asthma (AA grade; Aas asthma severity scores > 2).

nificance testing to identify group differences between the phenotypes. Figure 2 shows a dot plot comparing log-transformed and standardized gene expression in healthy control and AA subjects. For FACS analysis, absolute values of the staining intensities were found to be nonparametric and were compared with the Mann-Whitney *U*-test for two independent groups or Kruskal-Wallis test to compare all three groups (control, AN, and AA). ANOVA was used to compare

the data of the quadrant analysis between the groups. A conventional significance level of 0.05 was taken.

RESULTS

Expression of genes involved in B-cell isotype control and IgE production: comparison between control, AN, and AA subjects. Twenty-three of seventy-eight genes

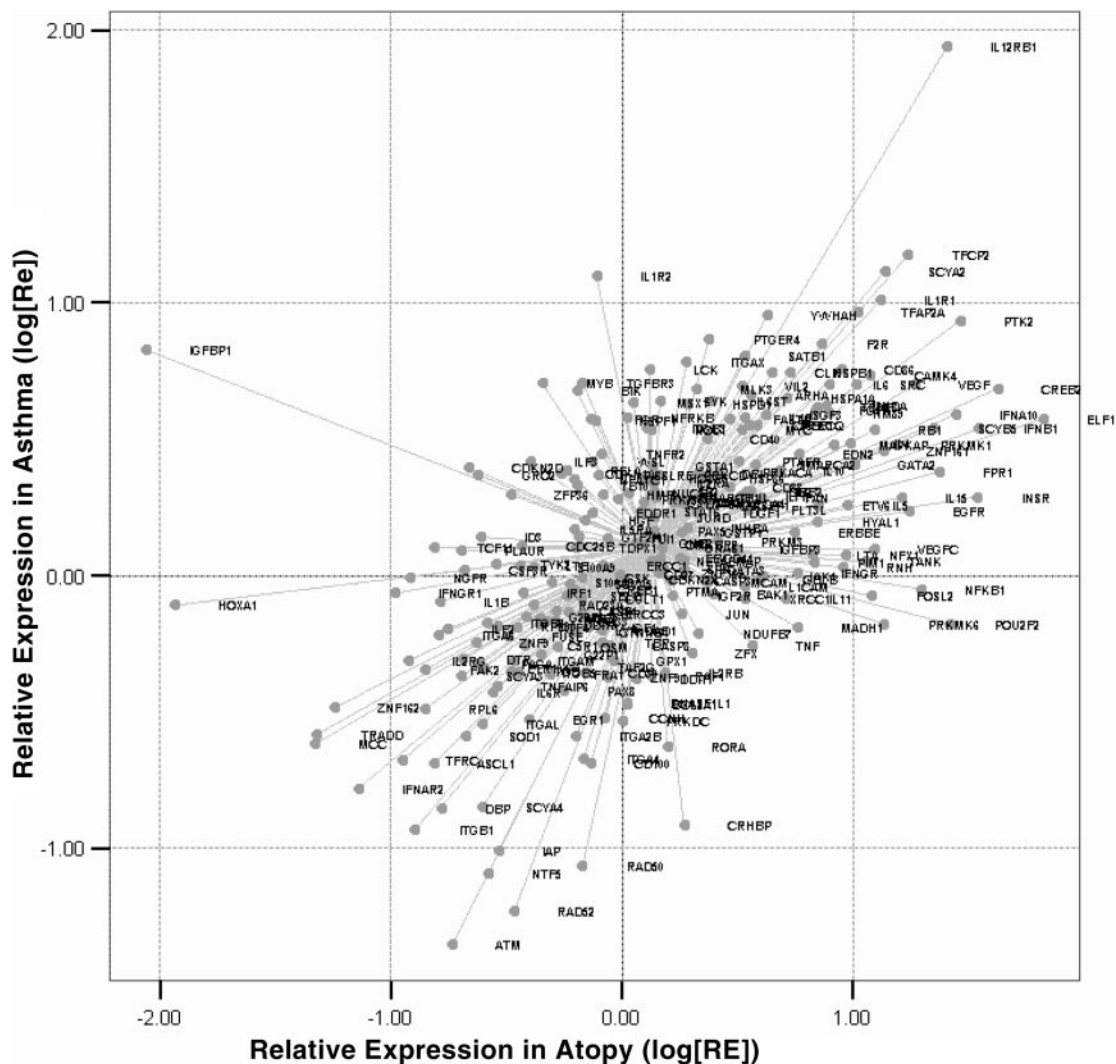
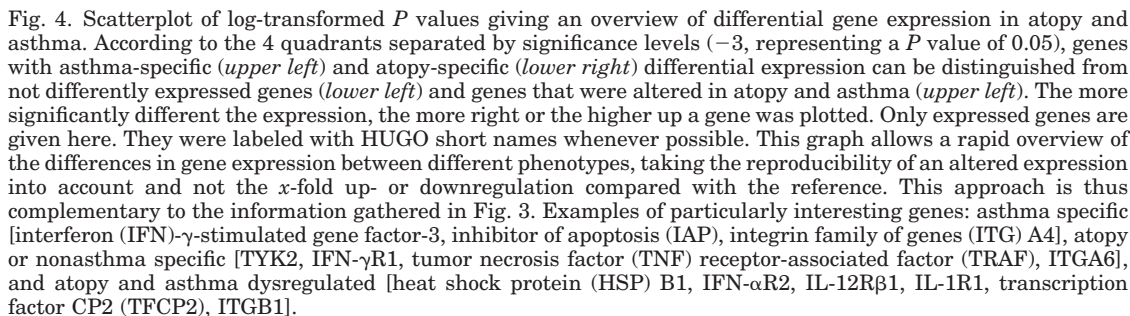


Fig. 3. Scatterplot comparing relative expression (RE) levels of atopic nonasthmatic (AN) and atopic asthmatic (AA) subjects. Not differentially expressed genes are plotted around the center of this graph ($x, 0; y, 0$). They do not seem to contribute to the tested phenotypes. Genes with the same trend in AN and AA subjects follow a 45° diagonal line (*bottom left to top right*). Such genes point to similarly dysregulated pathways in both phenotypes. However, genes that fall out of this pattern, like HOXA1, insulin growth factor binding protein 1 (IGFBP1), interleukin (IL)-1 receptor type 2 (IL-2R2), and nuclear factor (NF)- κ B1, may be of particular interest. They could indicate pathways with some phenotype specificity. However, this way of looking at the data potentially overemphasizes genes with a large expression variability. An alternate way to screen the large amount of information is given in Fig. 4.

present on the cDNA arrays used in this study, which were related to the human B-cell isotype control and the production of IgE, had significantly altered expression in atopy and asthma compared with that in the healthy subjects (Table 1, Figs. 2–4). Most of those genes were similarly altered in both atopy and asthma compared with those in control subjects. We did not identify any individual gene products, which were expressed in all normal control subjects and not at all in atopy or asthma or vice versa. Figure 5 shows an attempt to integrate the results obtained in AN and AA subjects in a model of a human B-cell isotype control.

In accordance with the two-signal model, where a minimum of two distinct signals is needed to induce

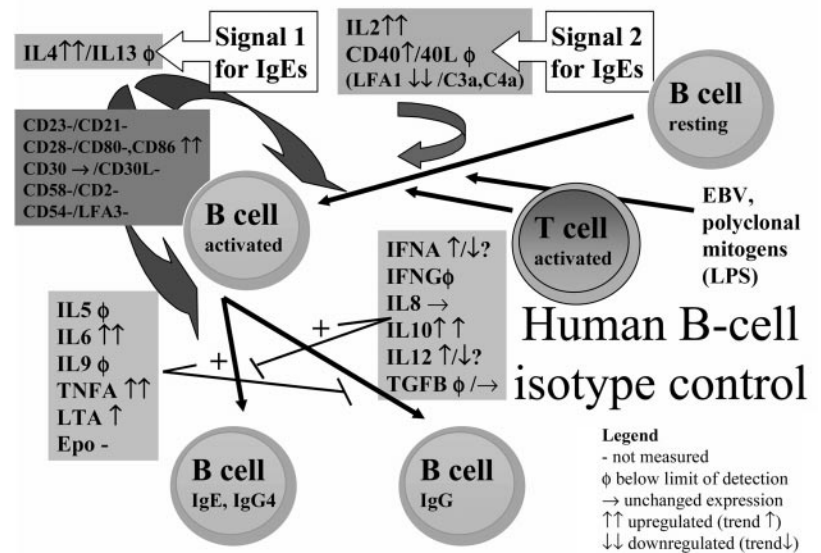
IgE synthesis, we found an upregulation of the IL-4 receptor (IL-4R; AA and AN subjects). IL-4 itself had a twofold increased expression in AN and AA subjects. This increase, however, was not significant due to expression variability. IL-13 was not expressed in all groups. The intracellular effector of IL-4, IL-13, or signal transducer and activator of transduction (STAT) 6 had higher levels of expression in both atopic groups, which, however, did not reach significance. The expression of the gene for CD40 together with the CD40 receptor-associated factor-1 [also called the tumor necrosis factor (TNF) receptor-associated factor (TRAF-3)] was higher in both AN and AA subjects compared with that in the control group. CD40L was not expressed in all groups. Formation of a high-affinity



Among other relevant IgE-promoting cytokines, IL-6 (AN and AA subjects), lymphotoxin A (formerly TNF- β ; AN subjects only), and TNF- α (AN subjects only) were found to be upregulated. IL-5, IL-9, and their receptors were not expressed in all groups. Among the cytokines, which normally preferentially promote IgM and IgG

synthesis, IL-10 was upregulated in AN subjects only. Apart from IFN- γ receptor 1 (IFN- γ R1), which is downregulated in AN subjects, IFN- γ itself and related products were not differentially expressed across all groups. TGF- β 1, - β 2, and - β 3 and TGF- β receptor 3 (TGF- β R3) were unchanged in all groups, and IL-8 was downregulated in a nonsignificant fashion. We found the type I IFNs IFN- α 10 and IFN- β 1 upregulated in AN subjects and IFN- α receptor 2 (IFN- α R2) downregulated in AN and AA subjects. IFN- α R2 (AN and AA subjects), a potent inhibitory receptor for IFN- α and IFN- β , and the tyrosine protein kinase TYK2 (AN

Fig. 5. Model of human B-cell isotype control with respect to IgE production in atopy and asthma with integrated gene expression results. Arrows, gene expression in AN and AA individuals relative to the expression in healthy control subjects (see Figs. 3 and 4, Table 1). The main results found in atopy and asthma were summarized and pooled together and thus represent a simplification of the real observations. EBV, Epstein-Barr virus; LPS lipopolysaccharide; Epo, erythropoietin; LFA, lymphocyte function-associated antigen; LTA, lymphotoxin A; TGF, transforming growth factor; CD30L and CD40L, CD30 and CD40 ligands, respectively. Genes were named by HUGO short names, and GenBank accession numbers are given in Table 1.



subjects only), involved in type I IFN signaling, were significantly downregulated compared with those in the control subjects. Additionally, we found higher levels of the IFN type I consensus sequence binding protein IFN-stimulated gene factor 3 in AA subjects. Thus there seems to be a differential expression for type I IFNs with, however, an unclear net effect but at least a partial upregulation of type I IFN signaling. IL-12 was below the limit of detection but had an upregulated receptor in AN and AA subjects (Figs. 3 and 4).

Higher levels for CD86 were seen in atopic and asthmatic individuals. ITG-AL, also known as CD11A or lymphocyte function-associated antigen-1α chain, was significantly downregulated in both AN and AA subjects. CD30 showed unchanged expression in all groups.

The receptor for prostaglandin E₂ (PTGER-2) was significantly upregulated in AA subjects.

Comparison of gene expression between asthmatic individuals and atopic subjects without asthma. In general, expression for genes related to IgE production was very similar in atopic and asthmatic individuals, with mostly gradual differences between those groups. For several genes with significant differences in expression in AN compared with control subjects, AA subjects showed similar but attenuated trends as AN subjects.

Differences between AA and AN individuals were identified in relation to TNF pathways. Subjects with atopy without asthma had higher TNF-α levels, with unchanged TNF receptor (TNFR) 1 or TNFR2 levels, whereas asthmatic individuals had unchanged TNF-α and TNFR1 expression levels but higher TNFR2, the high-affinity TNFR, expression levels. Furthermore, asthma-specific upregulated genes include IL-2Rα, a marker of T-cell activation, PTGER-4, a receptor for PGE involved in tissue remodeling, and the protein

tyrosine kinases LCK and SYK, involved in T- and B-cell receptor signaling. For these gene products, AN subjects had similar but attenuated trends. Furthermore, AA subjects had normal nuclear factor (NF)-κB1 expression and increased NF-κB receptor (NFR-κB; ER 1.79), whereas AN subjects had upregulated NF-κB1 (ER 3.66) and normal NFR-κB expression.

Genes of the heat shock protein (HSP) group were more dysregulated in AA than in AN subjects compared with control subjects. This was true for HSPB1, HSPD1, and HSPA1A.

Altered gene expression according to asthma severity. Comparing subjects with Aas (1) severity scores of ≤3 vs. >3, we identified higher CD86 levels in the more severe group. Within the TNF pathway, AA subjects in the more severe group had higher TRAF-3 and lower TNF-α inositol hexaphosphate levels. More severe AA subjects had greater expression of extracellular signal-regulated kinase 1 (PRKM3; also called ERK1), and mitogen-activated protein kinase-activated protein kinase. The same difference was observed for HSPF1 and the lymphoid-specific transcription factor POU2F2.

Verification experiments with FACS analysis for IL-2Rα (CD25). IL-2Rα, also called CD25, the marker for T-cell activation, which was found upregulated in AA subjects by cDNA hybridization, was also significantly upregulated on lymphocytes of asthmatic individuals ($P < 0.001$) as determined with FACS technology. This proved to be the case due to increased expression on CD4⁺ lymphocytes. The percentage of CD4⁺ lymphocytes positive for CD25 was increased 3.2-fold in AA subjects. In addition, the analysis of the staining intensity for CD25 showed higher staining intensities on CD4⁺ lymphocytes in AA subjects ($P = 0.002$) and also in the AN subjects ($P = 0.03$), although there was a true upregulation of CD25 on CD4⁺ lymphocytes in AA subjects in terms of percent positivity and the degree of staining for CD25 on those cells. In CD8⁺ and CD19⁺

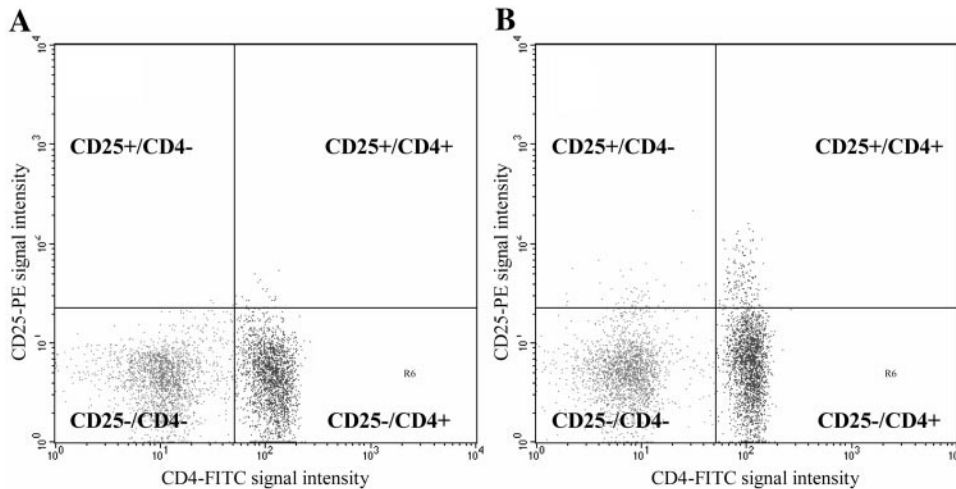


Fig. 6. Box plots of typical fluorescence-activated cell sorter double-staining experiments for CD4 and IL-2RA (CD25). A: healthy subject. B: AA individual. PE, phycoerythrin. As seen in B, a small proportion of CD4⁺ lymphocytes are positive for CD25, which is not the case in the healthy individual. This phenomenon was also detected by cDNA array technology.

cells, no significant difference for CD25 could be found. Figure 6 shows the staining results from a representative normal and AA individual. Thus these findings represent a validation of cDNA technology.

DISCUSSION

Until now, studies have focused on a very limited number of selected gene products based on availability and current understanding. With cDNA or oligonucleotide arrays, several hundred to thousands of gene products can be assayed in a single experiment, opening a new, eventually genomewide dimension to gene expression studies (4, 7, 9, 13, 21). Using this technique, we were able to identify substantial numbers of differentially expressed genes in the peripheral blood of AN and AA subjects compared with those in healthy control subjects. With this approach, many reported observations could be verified simultaneously for their relative *in vivo* importance. Also, several new hypotheses could be generated. For the sake of clarity, we focused on gene products related to B-cell isotype control.

Many of the differences in gene expression were similar in AN and AA individuals and were thus related to atopy rather than to asthma. Along these lines, all atopic individuals had a marked activation of proinflammatory cascades in peripheral blood. Therefore, our findings confirm the hypothesis that atopy is characterized by a significant proinflammatory drive, which is in accordance with the fact that atopy is a major risk factor for the subsequent development of different atopic diseases in children (23) and adults (8). With respect to the different aspects of B-cell isotype control, we were able to document the presence of all minimal requirements for the induction of IgE production (Fig. 5). Also, there seems to be a balance between "pro-IgE" and "anti-IgE" cytokines, of which members of both types were found upregulated in peripheral blood of AN and AA subjects. However, in an attempt to weigh the expression of pro-IgE against anti-IgE cytokines, clearly pro-IgE cytokines dominate in AN and AA subjects.

A variety of cytokines can modulate IL-4- and IL-13-mediated IgE synthesis. IgE synthesis can be enhanced by the cytokines IL-5 (17) and IL-6 (26) *in vitro*. Although IL-5 and its receptor were not expressed in our samples, IL-6 was consistently upregulated in both AN and AA subjects. An involvement of TNF- α and its receptors TNFR1 and TNFR2 for the induction of IgE synthesis was suggested in a study of Aversa et al. (3). They demonstrated that TNF- α expressed on T cells can promote IgE synthesis *in vitro*. Upregulation of TNF- α in AN subjects and the high-affinity TNFR2 in AA subjects was found in the present study. Thus TNF pathways are dysregulated in atopy and asthma and may contribute to increased IgE production. On the other hand, factors that have been shown to downregulate IgE synthesis *in vitro* include IFN- α (16), IFN- γ (16), IL-8 (11), IL-10 (18), IL-12 (12), and TGF- β (29). In our study, we found an upregulation of IL-10 in AN subjects, and there are arguments of a net increased type I IFN signaling in both AN and AA subjects. The upregulation of the receptor for IL-12 is more difficult to interpret. However, with an ER of 6.95 in AA subjects, this gene was among the most dysregulated genes. IL-12 levels could not be detected in peripheral blood in all groups. It is unclear whether a lack of pro-Th1 IL-12 signaling led to an upregulation of its receptor. IL-8, IFN- γ , TGF- β , and related genes were not differentially expressed or not expressed in our assay.

Asthma-specific alterations in gene expression could be identified, including members of the TNF family of genes and the NFR- κ B gene, which is known to induce the transcription of the high-affinity IL-2R2 (CD25) (2). And consistently, IL-2R2, a marker of T-cell activation, was upregulated in an asthma-specific way, compatible with an increased level of T- (and B-) cell activation in asthmatic individuals.

We were able to verify these results on a protein level with FACS double staining, underlying the validity of cDNA hybridization technology. CD25 has been found to be upregulated on CD4⁺ lymphocytes of AA subjects. This documents evidence of increased activation

of circulating CD4⁺ T cells in stable asthma without allergen challenge. Validation of cDNA array data, as shown here, is not only important to confirm a true up- or downregulation of the many "significant" genes but may also help to attribute the changes to specific cell types.

From an analysis of peripheral blood, limited conclusions can be made about the phenomena, which might occur at the site of inflammation. The picture in the circulation might represent a mirror image of the true situation where "disease-modulating" cells have migrated into the tissue. On the other hand, atopy is clearly a systemic phenomenon involving activation of many genes. Recently, Till et al. (24) found an equivalent cytokine production and proliferative response of T cells from bronchoalveolar lavage fluid and peripheral blood in AA subjects after segmental allergen challenge. Minshall et al. (15) reported an increased IL-5 expression by T cells in the bone marrow of ovalbumin-sensitized BALB/c mice 6 h after allergen challenge compared with that in nonsensitized control mice. We therefore hypothesized that a potential primary alteration in gene regulation, either genetically anchored or triggered by environmental factors, would also be present away from the actual site of inflammation, the lung. Also, an approach in peripheral blood is cheap, easy, and noninvasive and therefore widely applicable. Cell sequestration and the contribution of the different cell types to a specific gene expression pattern need to be further investigated in gene expression studies comparing lung tissue and peripheral blood samples.

Taken together, our results document a clear pro-IgE climate in atopic and asthmatic individuals compared with that in healthy control subjects. Most of the differences in gene expression were related to atopy, being similar in atopic and asthmatic individuals. Different therapeutic targets for an intervention in the regulation of IgE, such as surface markers involved in T- or B-cell interaction (e.g., CD86, cytotoxic T-lymphocyte antigen-4, CD28, CD40), cytokines (e.g., TNF- α , IL-8), intracellular signaling molecules (e.g., SYK, TYK, PYK), and transcription factors (e.g., STAT6, TFCP2, NF- κ B), could be identified. Finally, cDNA array technology proved to be useful and may be complementary to DNA-based studies to analyze interactive and multidimensional pathways as shown here for B-cell isotype control and the production of IgE.

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