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SHORT COMMUNICATION

Kinetics of asthma- and allergy-associated immune response gene expression in peripheral blood mononuclear cells from vaccinated infants after *in vitro* re-stimulation with vaccine antigen

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Summary The global expression of immune response genes in infants after vaccination and their role in asthma and allergy is not clearly understood. Pharmacogenomics is ideally suited to study the involved cellular responses, since the expression of thousands of genes can be assessed simultaneously. Here, array technology was used to assess the expression kinetics of immune response genes with association to asthma and allergy in peripheral blood mononuclear cells (PBMC) of five healthy infants after vaccination with Infanrix-Polio + Hib. At 12 h after *in vitro* re-stimulation of the PBMC with pertussis toxin (PT) antigen, 14 immune response pathways, 33 allergy-related and 66 asthma-related genes were found activated.

Introduction

Little is known about the gene expression changes involved in immune responses in adults and infants after vaccination, even less is known about asthma- and allergy-associated genes expressed during or after immune responses. An often-cited theory is that an imbalance between the Th1 and Th2 response is responsible for the development of asthma [1]. A pronounced T-reg and Th1 immune response has been found after specific immunotherapy of allergic rhinitis patients [2]. Immunisation with pertussis vaccines has been reported to induce both a Th1 and Th2 immune response [3].

Recent studies using microarray technology have lead to the discovery of several important markers for asthma and allergy [4–10], but it is unknown if they are expressed during or after immune responses. Pharmacogenomics is ide-

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ally suited to study cellular responses related to immune response and it was shown earlier that cytokine markers measured on the gene expression level by array technology are in good correlation with the corresponding protein levels measured by conventional technology [11]. Furthermore, additional gene markers for the adaptive as well as the innate immune responses can be evaluated [12,13], as well as genes involved in toxicity, inflammation, apoptosis, stress and oncogenesis [11—15]. Array technology holds the promise to find hitherto unknown immune response genes with associations to asthma and allergy-related inflammatory processes [16].

Earlier it was shown, that the ideal time-points for the evaluation of immune response gene expression in vaccinated mice are 4 and 24 h after *in vitro* re-stimulation of lymphocytes with the vaccine antigen [11]. But, the ideal time-point for the expression of immune response genes with an association to asthma and allergy in peripheral blood mononuclear cells (PBMC) of humans has not yet been determined.

Here, array technology was used in a small-scale study to determine the ideal time-point for the expression of immune response genes with association to asthma and allergy in PBMCs, which were isolated from five infants vaccinated with Infanrix-Polio + Hib and *in vitro* re-stimulated with the vaccine antigen, pertussis toxin (PT), for various time-points.

Materials and methods

Clinical subjects

Venous blood samples were collected from five infants at the age of 6 months (born November—December 2000). The infants were vaccinated with Infanrix-Polio + Hib vaccine (GlaxoSmithKline Beecham, Rixensart, Belgium) at the age of 3 and 5 months, respectively. The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping. The identities of the clinical subjects were blinded.

Isolation and *in vitro* re-stimulation of PBMC

The experiments were performed as described previously in detail [15]. Briefly, venous blood samples were drawn into heparin-treated tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria). PBMCs were separated on Ficoll Paque Density gradient (Amersham, Piscataway, NJ) according to standardised methodology and cryopreserved in freezing medium (50% fetal calf serum, 40% RPMI 1640 and 10% DMSO) (Sigma—Aldrich, St. Louis MO) until analysis. After thawing, the cell viability was checked with Trypan blue (Sigma—Aldrich) exclusion test. The cells were resuspended in AIM-V serum free medium (Life Technologies, Täby, Sweden) with 20 μM β-mercaptoethanol (Sigma—Aldrich, St. Louis MO) to a concentration of 1×10^6 cells/ml. Cell aliquots were *in vitro* cultivated for 0, 4, 12, 24 and 48 h at 37 °C in a humidified atmosphere with 5% CO_2, with serum free AIM-V medium alone (negative control, at 0 h) or with 1 μg/ml pertussis toxin at 4—48 h (GlaxoSmithKline Biologicals, Rixensart, Belgium). The PT was heat-inactivated for 20 min in an 80 °C water bath to avoid the antigen's high mitogenic potential. Because the blood volume that can be taken from infants is limited, the number of cells per infant was too low to allow for the isolation of a RNA quantity, which was sufficient for array experiments of four time-points and one control per infant. The cell aliquots per infant were therefore pooled after *in vitro* re-stimulation at each time-point, respectively. The cells were collected, washed in cold PBS, snap frozen in liquid N_2 and stored at −70 °C until RNA isolation.

RNA isolation and array analysis

Total RNA was isolated from the lymphocytes, quality checked and used in cDNA array experiments as described previously [11—15]. Briefly, total RNA was isolated from pooled samples of PBMCs using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). DNase treatment was performed with DNase I (Promega, Madison, WI) and RNA quantification was performed with RiboGreen®RNA Quantitation kit (Molecular Probes, Eugene, OR). RNA (4 μg) was converted to cDNA (Clontech, Mountain View CA), radioactively labeled with α-^{32}P-dATP (PB10204) (Amersham Biosciences, Piscataway, NJ) and hybridized to Atlas Human cDNA Arrays (Clontech, Mountain View, CA) with 1176 genes, according to the manufacturers guidelines. The arrays were exposed to phosphor imaging screens for 2, 3 or 5 days (at room temperature). Gene expression levels were quantified by the CYCLONE®Storage Phosphor System (PerkinElmer, Waltham MA) and the image data were processed using the AtlasImage software version 2.0 (Clontech, Mountain View, CA).

Bioinformatics analysis

The data was treated statistically as described previously [11—15]. To identify genes with significant gene expression changes of at least two times above control and to group them according to their expression levels across all samples, the data was clustered using the software GeneCluster [17]. Data-mining tools from the World Wide Web, the software PubGene (version 2.6, Oslo, Norway) [18] and Ingenuity Pathways Analysis (version 5.0, Redwood City, CA) were used to assess the biological functions of the genes and their associations to asthma and allergy.

Results and discussion

Expression kinetics of genes reported in the literature

As a first step of evaluation the literature was searched for immune response genes that were reported to be up- or down-regulated in patients with asthma and allergy (see Table 1). The time-points of their gene expression levels (high, low or not detected) according to our array results were noted.

As shown in Table 1 the expression of the majority of these genes (34 genes out of a total of 52 genes) can be assessed at 12 h after *in vitro* re-stimulation with the PT antigen. This is not surprising, since these genes are involved

Table 1 Kinetics of immune response gene expression in human PBMC after *in vitro* re-stimulation with PT antigen for 4, 12, 24 and 48 h compared with negative control

Gene name	Genbank accession no.	4 h	12 h	24 h	48 h	Asthma association
CCR1	NM001925	—	++	—	—	Yes
CCR2	NM000647	++	+	—	—	Yes
CD4	M35160	—	+	—	+	Yes
CD8	NM001768	—	—	—	++	Yes
CD27 (T-cell antigen)	M63928	—	+	—	—	No
CD30	M83554	++	—	—	++	Yes
CD33	M23197	—	+	—	—	Yes
CD40L	X67878	++	+	+	—	Yes
CD70	L08096	—	+	—	—	No
CD72	M54992	—	+	—	—	Yes
CD102	NM000873	—	—	++	+	No
CD103	L25851	—	—	+	—	Yes
CD104	NM001005619	—	—	++	+	No
CD106	M60335	—	+	—	—	Yes
ICAM-1 (CD54)	NM000201	—	+	—	—	Yes
IL-1Rbeta	X59770	++	—	—	++	No
IL-2	A14844	—	++	+	—	Yes
IL-2Rbeta (CD122)	M26062	+	+	—	—	No
IL-2R gamma	D11086	—	++	+	+	Yes
IL-3	M14743	—	++	—	—	Yes
IL-4	M13982	—	++	—	—	Yes
IL-4Ralpha (CD124)	X52425	—	+	—	—	Yes
IL-5	X04688	—	—	++	+	Yes
IL-5 R alpha	M75914	—	+	—	—	Yes
IL-6R	M20566	+	—	—	—	Yes
IL-6	X04602	+	++	+	—	Yes
IL-7	J04156	—	++	+	—	Yes
IL-8	Y00787	—	++	—	—	Yes
IL-10	M57627	++	++	—	—	Yes
IL-11	M57765	—	—	—	++	Yes
IL-10R	U00672	—	—	++	—	No
IL-12 R	U03187	++	—	—	++	Yes
IL-13	L06801	—	++	+	—	Yes
IL-15	U14407	—	—	++	+	Yes
IL-16	M90391	—	++	+	—	Yes
IFN beta	M28622	—	++	—	—	No
IFN gamma	X01992	++	++	—	—	No
IFN gamma R	J03143	++	—	—	—	No
IRF-1	X14454	—	++	—	—	Yes
ITGB8	M73780	+	+	—	—	No
Jak3	U31631	—	—	++	—	Yes
CCL13	NM005408	+	+	—	—	Yes
PAX-5	NM016734	—	++	+	—	No
STAT1	NM007315	—	+	++	—	Yes
STAT2	NM005419	—	—	++	—	No
TGF beta R1	X11695	—	++	+	+	No
TGF beta II	P61862	—	+	++	—	Yes
TGF beta III	NM003239	++	—	—	++	No
TGF beta R III	L07594	++	+	—	—	No
TNF beta	P01374	—	—	—	++	No
TNF R	M67454	—	—	++	+	No
YES	M15990	—	++	++	—	Yes

Expression levels of the genes are shown as high (++), low (+) or no expression (—). The genes were chosen for their association with asthma and allergies according to Refs. [4—9,20—23]. Only genes with sequences represented on the Human 1.2 array are shown. Association of the genes with asthma according to the literature was assessed using the data-mining tool PubGene (see also Fig. 2).

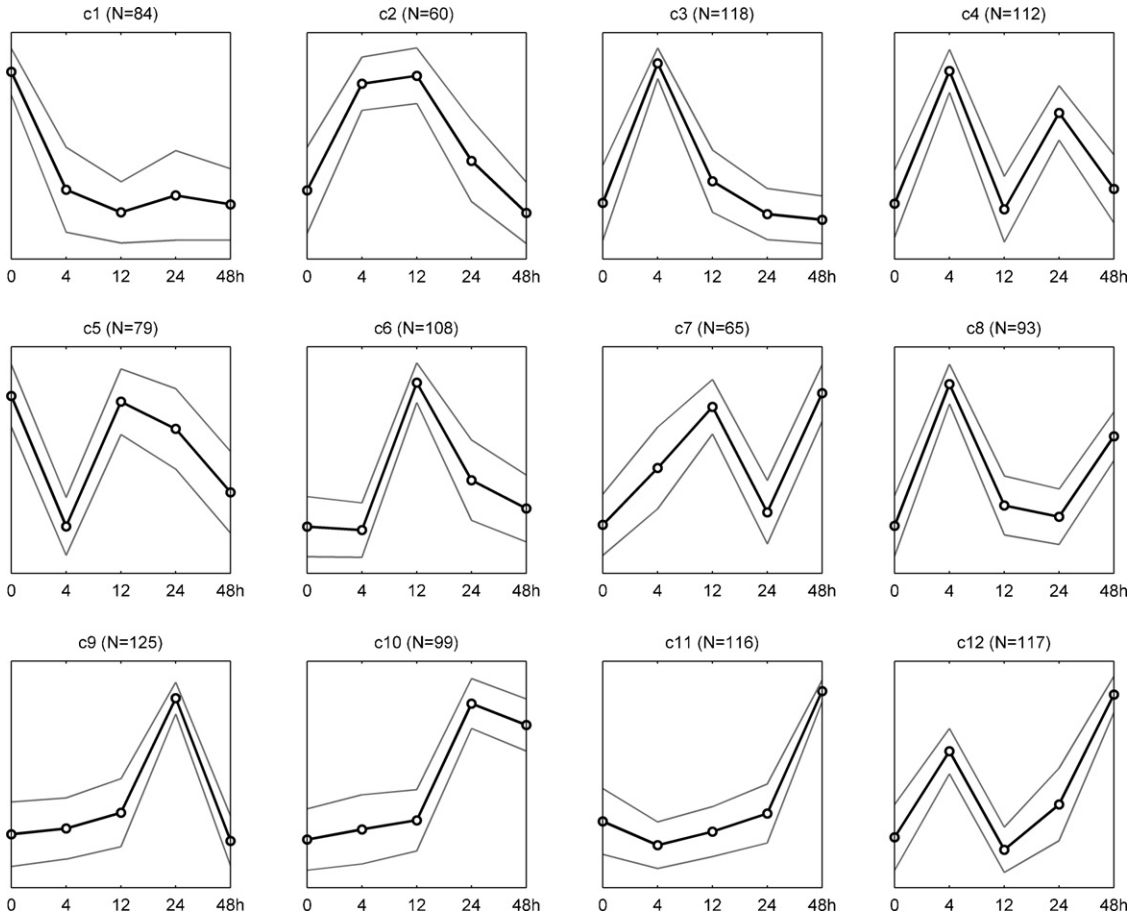

Figure 1 Gene expression differences in lymphocytes from infants at 4, 12, 24 and 48 h after *in vitro* re-stimulation with PT antigen and non-treated lymphocytes. Self-organizing map clusters according to Tamayo's algorithm [17] using the criteria for significant gene expression described in ''Materials and methods''. Bold black lines indicate the mean expression profiles; grey lines indicate the SD. *N* represents the number of genes in each cluster.

in immune response and it is known from earlier array experiments in mice that immune response genes are highly activated at 4 h and 24 h after *in vitro* re-stimulation with tetanus toxoid or diphtheria toxoid, respectively [11,12]. In addition, it has been shown earlier in mice, that the expression level of genes involved in Th1 responses is high between 2 and 18 h after stimulation with ovalbumin peptide [19]. There were only 18 genes without measurable gene expression at 12 h after *in vitro* re-stimulation of PBMCs with PT antigen, of which 9 genes had no association with asthma in the literature (see Table 1).

Other non-immune response genes with association to allergies, which were reported in the literature (see references of Table 1), were also detected at 12 h after *in vitro* re-stimulation with PT antigen. These genes comprised thymosin-β4, HMG-1 (high mobility group box 1), DPP4 (dipeptyl peptidase 4), MMP7 (matrix metalloproteinase 7) and SOD1 (superoxide dismutase 1) (data not shown). MMP7 and SOD1 have also an additional association with asthma according to the literature (see Table 1).

In the next step, the analysis was focused on finding additional genes with significant expression at 12 h after *in vitro* re-stimulation with PT-antigen and possible involvement in asthma and allergy. Clustering of the gene expression data using the self-organizing map software [17], which groups genes according to their expression behavior, resulted in 12 clusters (c1—c12) containing between 60 and 125 genes (Fig. 1). No clusters were found with a significant down-regulation in the 12 h-sample versus the control (0 h) and all the other time-point samples. The clusters containing genes with up-regulation at 12 h after *in vitro* re-stimulation with PT antigen were c2, c6 and c7 with a total number of genes of 233. These three clusters contained the 33 genes listed in Table 1, resulting in 200 additional genes with up-regulation at 12 h.

Additional genes with significant expression levels at 12 h and their functions

To determine the function of these additional genes found in clusters c2, c6 and c7, the expression data of these genes were submitted to the bioinformatics tool ingenuity, which searches submitted gene lists against a pathway database. The software's output shows the number of submitted genes per pathway and the corresponding statistical error. According to this software, the main functions associated with the 233 genes obtained by GeneCluster (some genes were listed

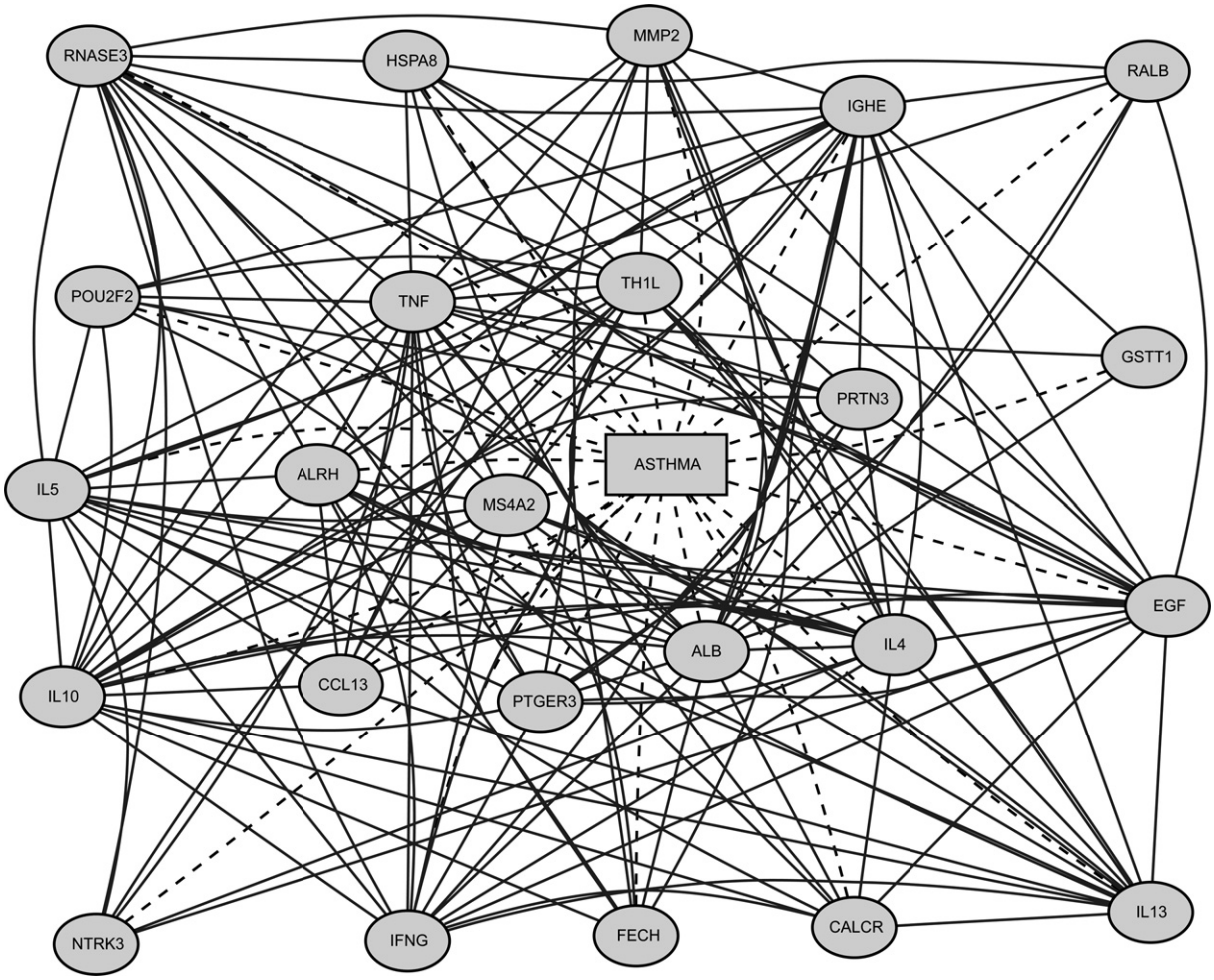

Figure 2 Literature network of cluster c2 genes. A list of 60 genes was submitted to PubGene to identify the association of co-expressed genes with asthma in the literature. A dark gray node represents one gene. Black lines indicate co-citation in the literature (the number of articles varied between 5 and 2320). The association with the keyword asthma is shown as a black-dotted line.

with more than one function) were immune response (33 genes; 9.42 E^{-17}), cellular growth and proliferation of lymphocytes (37 genes; 3.34 E^{-24}), T lymphocytes (29 genes; 1.12E^{-19}), B lymphocytes (17 genes; 1.15E^{-14}), leukocytes (37 genes; 1.75 E^{-24}), activation of lymphocytes (23 genes; 4.29 E^{-17}), T lymphocytes (21 genes; 1.62 E^{-17}); B lymphocytes (8 genes; 2.57E^{-8}), activation of phagocytes (11 genes; 5.88 E^{-9}), activation of mononuclear cells (5 genes; (2.54E^{-8})), inflammatory disease (32 genes; 2.84E^{-17}), cytotoxic reaction (8 genes; 4.65E^{-10}), antibody response (7 genes; 4.78E^{-9}), phagocytosis (11 genes, 5.29E^{-9}). Furthermore, cancer genes were up-regulated (67 genes; 7.07 E^{-18}), apoptosis genes involved in hematological disease (35 genes; 1.5 E^{-12}) and immunological disease (25 genes; 1.5 E^{-12}).

Taken together, this analysis elucidates the suitability of this time-point for the evaluation of immune response reactions as well as allergy-related processes.

The data-mining tool PubGene [18] was used to search the clusters c2, c6 and c7 with up-regulation at 12 h for genes, which are associated with asthma in the literature (the keyword allergy was not recognized by the software). The gene names were translated into the primary gene symbols used by the software and submitted together with the keyword asthma. In clusters c2: 15 genes out of 60; c6: 31 genes out of 108 and c7: 20 genes out of 65 were found to be associated with asthma. Of the total of 233 submitted genes were 66 genes (28%) reported to be involved in asthma.

The results for cluster c2 are shown in Fig. 2. Beside the 15 on the array represented and up-regulated genes, the software found 9 additional genes, which are associated by co-citation in the literature with the keyword asthma and with the other genes of the literature network. These 9 genes were: ALB (serum albumin); IL-4 and IL-13, which were represented on the array and measurable at 12 h; IL-5 which was represented on the array, but was not detectable at 12 h; ALRH, IGHE (heavy immunoglobulin component of IgE), MS4A2 (Fc fragment of IgE, high affinity I, receptor for), RNASE3 (eosinophil cationic protein) and TNF, which were not represented on the array, but are well-known to be involved in asthma. In cluster c6 and c7, these 9 genes were listed again, respectively; but two additional genes for each cluster were found: AKT1 (v-akt murine thymoma viral oncogene homolog 1); FLT1 (vascular endothelial growth factor receptor); MAPK1 (mitogen-activated protein kinase 1)

and TBP (TATA box-binding protein) were found by the software (data not shown). Taken together, the software found 13 additional genes with involvement in asthma.

In summary, these results point towards the suitability of the 12 h time-point after *in vitro* re-stimulation of PBMCs with PT antigen for the detection of significantly expressed immune genes with an association to asthma and allergy. These comprise the 33 allergy- and asthma-related genes shown in Table 1, the 14 pathways (containing between 5 and 37 genes) involved in immune response, related cellular reactions and allergic reactions, as well the 66 genes associated with asthma in the literature.

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