

ORIGINAL ARTICLE

A network-based analysis of allergen-challenged CD4⁺ T cells from patients with allergic rhinitis

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We performed a network-based analysis of DNA microarray data from allergen-challenged CD4⁺ T cells from patients with seasonal allergic rhinitis. Differentially expressed genes were organized into a functionally annotated network using the Ingenuity Knowledge Database, which is based on manual review of more than 200 000 publications. The main function of this network is the regulation of lymphocyte apoptosis, a role associated with several genes of the tuber necrosis factor superfamily. The expression of TNFRSF4, one of the genes in this family, was found to be 48 times higher in allergen-challenged cells than in diluent-challenged cells. TNFRSF4 is known to inhibit apoptosis and to enhance Th2 proliferation. Examination of a different material of allergen-stimulated peripheral blood mononuclear cells showed a higher number of interleukin-4⁺ type 2 CD4⁺ T (Th2) cells in patients than in controls ($P < 0.01$), as well as a higher number of non-apoptotic Th2 cells in patients ($P < 0.01$). The number of Th2 cells expressing TNFRSF4, TNFSF7 and TNFRSF1B was also significantly higher in patients. Treatment with anti-TNFSF4 resulted in a significantly decreased number of Th2 cells ($P < 0.05$). A logical inference from all this is that the proliferation of allergen-challenged Th2 cells is associated with a decreased apoptosis of Th2 cells and an increase in TNFRSF4 signalling.

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Introduction

Type 2 CD4⁺ T helper (Th2) cells play a central role in orchestrating the allergic response. The differentiation of Th cells is determined by a complex interplay between multiple factors such as antigen type and dose, dendritic cells, the T-cell receptor, co-stimulatory signals and the cytokine microenvironment as well as apoptotic regulators.^{1–7}

DNA microarray studies have described hundreds of differentially expressed genes in CD4⁺ T cells stimulated by cytokines,^{8,9} taken from transgenic mice,¹⁰ or from peripheral blood of patients with atopic dermatitis.¹¹ These findings indicate that the proliferation of Th2 cells depends on multiple, interacting genes. Thus, it is difficult to obtain a functional overview of these genes and their relative importance to Th2 cell proliferation. There has been considerable recent interest in representing complex systems, such as gene interactions in a cell, as networks.¹² Such a network can be functionally

annotated and dissected to identify genes with key regulatory functions. This principle has been validated for protein networks in *Saccharomyces cerevisiae*,¹³ but has proved elusive to demonstrate in the more complex networks in human cells. Nevertheless, network-based analyses have recently been described in DNA microarray studies of human disease.^{11,14} The latter report, published in *Nature*, describes a bioinformatic program that was used to organize all differentially expressed genes into a gene interaction network.¹⁴ This program was based on the manual review of over 200 000 scientific publications. The networks were functionally annotated and further examined to find individual genes with putative key functions.

In this study, a similar approach is used to identify significant networks and genes in DNA microarray data for allergen-stimulated Th cells taken from patients with allergic rhinitis. The analysis indicates that altered regulation of apoptosis plays an important role in proliferation of Th2 cells, and that this is associated with several genes of the tuber necrosis factor (TNF) superfamily. Examination of a different material of allergen-stimulated peripheral blood mononuclear cells (PBMC) shows a higher number of non-apoptotic Th2 cells in patients than in control. Blocking TNFRSF4, one of the TNF receptors, significantly inhibited proliferation of Th2 cells. Although significant, this inhibition was not

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complete. Further analysis indicates that this can be explained by interactions from other members of the TNF superfamily. Thus, this work supports an hypothesis that the allergen-induced proliferation of Th2 cells is associated with a decreased apoptosis of Th2 cells, and an altered activity of TNFRSF4 and other members of the TNF superfamily.

Results

Proliferation of Th2 cells following allergen stimulation

To determine whether allergen stimulation results in proliferation of Th2 cells, the protein levels of the Th2 cytokines interleukin (IL)-5 and IL-13 were analyzed in supernatants from PBMC that had been challenged with allergen or diluent.¹⁻⁷ Supernatants from 11 patients and five controls were studied. In supernatants from patients, IL-5 was significantly higher in supernatants from allergen-challenged PBMC, 100 (0–500) vs 0 (0–0), $P < 0.01$, as was IL-13, 13 (0–270) vs 0 (0–0), $P < 0.05$. In supernatants from controls, no significant difference in IL-5 levels was found, 12 (0–66) vs 0 (0–0) pg/ml. To search for signs of antigenic stimulation other than allergen, macrophage inhibitory factor (MIF) was measured in supernatants from both patients and controls.¹⁵ In supernatants from patients, no significant difference in MIF levels was found between allergen-challenged and diluent-treated PBMC, 850 (680–1200) vs 870 (650–1700) pg/ml. The corresponding values for controls were 710 (760–940) vs 660 (710–950) pg/ml, NS.

DNA microarray analysis of allergen-challenged CD4⁺ T cells from patients with allergic rhinitis

CD4⁺ cells were enriched from PBMC in three patients. Gene expression in the CD4⁺ T cells was analyzed with DNA microarrays and compared in cells that had been challenged with allergen or diluent. Thus, a total of six DNA microarray analyses were performed. Of the thousand most differentially expressed genes, 768 were upregulated (see online supplement for a complete list). Several of the up-regulated genes had known relevance for proliferation of Th2 cells, for example, the IL-4 receptor and the Vitamin D3 receptor.¹⁶ Interestingly, not only Th2- but also Th1-related genes, such as 2'5'-oligoadenylate synthetase 1 40/46 kDa and interferon (IFN)- γ -inducible protein 16, were upregulated. These genes, however, represented only a small fraction of all those that were differentially expressed. A network-based analysis was therefore performed in order to obtain a functional overview of how these genes relate to the proliferation of Th2 cells.

A network-based analysis of the late-phase reaction

The Ingenuity Knowledge Database (KB) was used to organize the differentially expressed genes into networks of interacting genes.¹⁴ Statistical algorithms were then used to rank the resulting networks according to how well they corresponded to gene interactions described in the literature. Owing to KB idiosyncrasies, each network contained a maximum of 35 genes. Fifty significant networks were formed. The seven top-scoring networks were integrated into one, showing the sub-cellular location and interactions of each gene (Figure 1). In this network, a distinct interaction pattern can be seen. The gene with the highest number of interactions was TNF,

which interacts with two receptors known to affect proliferation of Th2 cells, namely, the receptors for IL-2 and IL-4, respectively. TNF is known to increase expression of both receptors^{17,18} TNF also increases the expression of the CCR2 receptor on Th2 lymphocytes,¹⁹ as well as of CC-chemokine ligand4 that is chemotactic for eosinophils.²⁰

The above-mentioned genes and interactions represent only a small part of those included in the seven top-scoring networks. Moreover, these networks were chosen from a total of 50 networks that were identified as significant. An obvious question arises: how would it be possible to acquire from this a functional understanding of how these networks relate to proliferation of Th2 cells? KB assigned biological functions to all the significant networks, based on information extracted from the scientific literature. Therefore, a statistical algorithm was used to score these functions. The top-scoring function was 'death of lymphocytes', which contained several genes of the TNF superfamily, specifically, TNF, FAS, TNFRSF4 (= OX40; CD134), TNFRSF25, TNFRSF1B (= CD120b) and TNFSF7 (= CD70). These genes are involved in regulating T-cell apoptosis or proliferation. All were upregulated in allergen-stimulated Th2 cells. TNFRSF4, however, differed most in expression. In fact, it was ranked as the second most regulated gene of all those that were differentially expressed. The median gene expression level of TNFRSF4 was 48 times higher in allergen-stimulated than diluent-treated cells, that is, 411 (317–525) compared to 7 (5–37). TNFRSF4 is known to inhibit apoptosis in T cells and to enhance Th2 proliferation.²¹ Recent data have shown that, depending on the antigenic stimulus, TNFRSF4 can also induce Th1 proliferation.²² It is not known if allergen-induced proliferation of Th2 cells in allergic patients involves TNFRSF4 signalling.

Examination of the role of apoptosis and TNFRSF4 signalling in allergen-induced proliferation of Th2 cells

Network-based analysis had indicated that altered regulation of apoptosis was the most important function of the networks formed by the differentially expressed genes. This was further examined in a different material consisting of allergen-stimulated PBMC from eight patients with allergic rhinitis and 10 healthy controls. Stimulation of PBMC with allergen, and post-stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin resulted in a higher number of Th2 cells/ μ l in patients than in controls, 35 vs 12 ($P < 0.01$). To examine if allergen challenge affected apoptosis in Th1 and Th2 cells, the numbers of non-apoptotic (terminal deoxynucleotidyl transferase uridine triphosphate nick end labeling (TUNEL) negative) IL-4 CD4⁺ cells (Th2) and IFN- γ CD4⁺ T cells (Th1) were analyzed. There were significantly higher numbers of non-apoptotic Th2 cells in patients than in controls after allergen challenge. The mean number of TUNEL-negative Th2 cells/ μ l was eight in patients and one in controls ($P < 0.001$). The corresponding figures for Th1 cells were 31 and 19 ($P < 0.05$).

The role of TNFRSF4 in proliferation of Th2 cells was further examined. The number of allergen-challenged Th2 cells/ μ l expressing TNFRSF4 was significantly higher in patients than in controls, five vs one, respectively ($P < 0.05$) (Figure 2). Treatment with anti-TNFRSF4 resulted in a significantly lower number of Th2

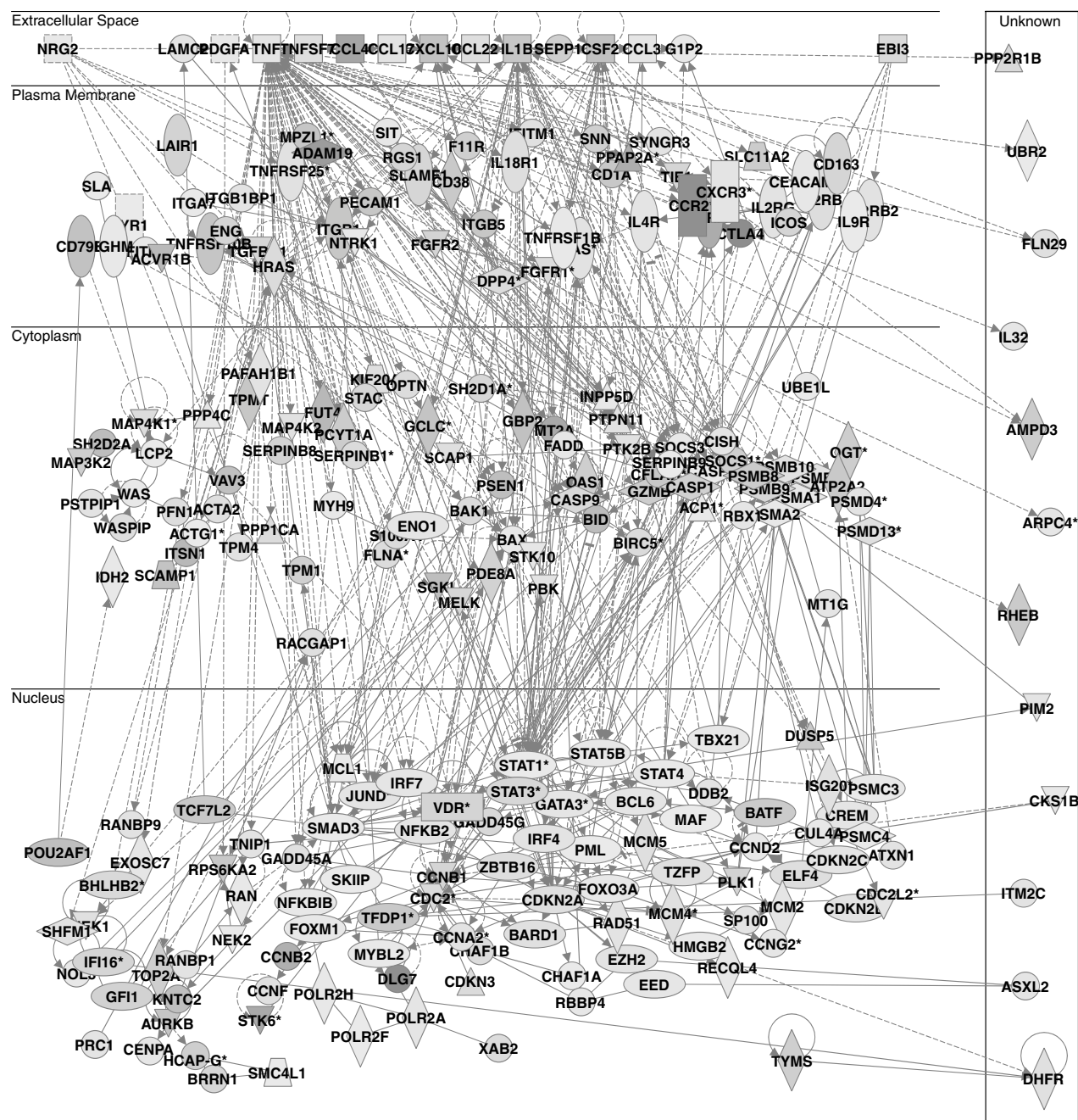


Figure 1 Network representation of interactions between differentially expressed genes from a DNA microarray analysis of allergen-challenged CD4⁺ T cells from patients with allergic rhinitis. Out of 1000 differentially expressed genes, a total of 50 statistically significant networks were formed. The networks were scored according to significance. The seven top-scoring networks were merged into a single network. Genes colored red (green) have higher (lower) expression in allergen-challenged cells than in diluent-treated cells.

cells/ μ l, 14 vs 35 ($P < 0.05$). Although significant, this decrease was not complete. This was not owing to a compensatory increase of TNFRSF4 expression; treatment with anti-TNFSF4 resulted in lower number of Th2 cells expressing TNFRSF4, six vs 18 in untreated cells ($P < 0.01$). Another explanation could be that other genes of the TNF ligand and receptor family contributed to T-cell proliferation. The DNA microarray analysis had shown increased expression of two such genes, TNFSF7 and TNFRSF1B. Both of these augment T-cell proliferation. Further analysis showed that the number of

allergen-challenged Th2 cells expressing TNFSF7 was 18 in patients and five in controls ($P < 0.01$). The corresponding figures for TNFRSF1B were six and one, respectively ($P < 0.01$). Thus, these genes could contribute to allergen-induced proliferation of Th2 cells from allergic patients. Interestingly, expression of these genes also decreased following treatment with anti-TNFSF4, although the decrease was less pronounced than for TNFRSF4 (Figure 3).

As previously described by Akdis *et al.*,²³ Th cells from both allergic patients and controls respond to allergen.

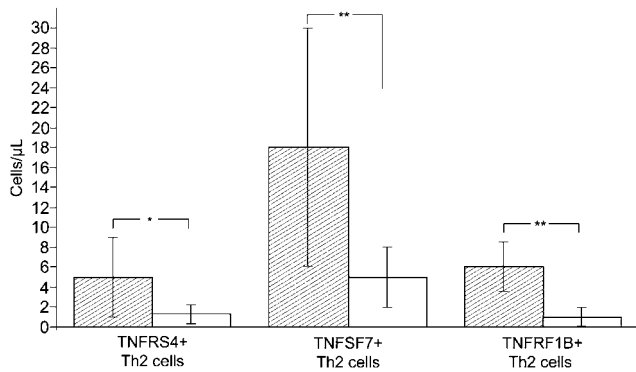


Figure 2 The numbers of allergen-challenged Th2 cells/ μ l expressing TNFRSF4, TNFSF7 and TNFRSF1B in patients (shaded bars) and controls (empty bars). * $P < 0.05$, ** $P < 0.01$, Mann-Whitney *U*-test.

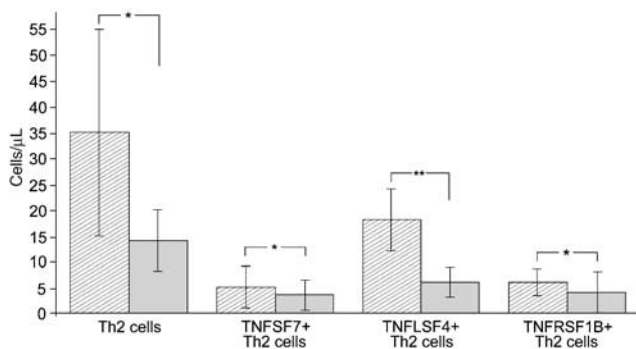


Figure 3 The numbers of allergen-challenged Th2 cells/ μ l with anti-TNFSF4 treatment (shaded bars) and without treatment (hatched bars). The first pair of bars shows total numbers of Th2 cells. The following bars show Th2 cells expressing TNFRSF4, TNFSF7 and TNFRSF1B, respectively. * $P < 0.05$, ** $P < 0.01$, Wilcoxon's signed-rank test.

In this material, the number of control Th2 cells that responded to allergen was 12/ μ l (compared to 35 in patients and two in samples treated with medium only and post-stimulated with PMA and ionomycin). A small number of Th2 cells from controls also expressed TNFRSF4 (1 cell/ μ l), TNFSF7 (5 cells/ μ l) and TNFR2 (1 cell/ μ l). However, treatment with anti-TNFSF4 resulted in a significant decrease of Th2 cells/ μ l, 12 vs four ($P < 0.01$).

Discussion

Multiple interacting genes are involved in proliferation of Th2 cells. IL-4 signalling plays an important role, as do several other cytokines and co-stimulatory molecules.^{1,21} Functional interpretation of previous studies is complicated not only by the large number of genes involved but also by variable gene expression profiles. A possible explanation for this may be that these studies have been performed with different stimulation methods and on different species. As the present study aimed to examine proliferation of Th2 cells in human allergic disease, it was based on an analysis of allergen-stimulated PBMC from patients with allergic rhinitis vs healthy controls. DNA microarray experiments were then performed on

Th cells enriched from the PBMC. In this way, Th2 proliferation was induced by allergen and by antigen-presenting cells. This may be more relevant for allergic disease than direct stimulation with, for example, Th2 cytokines as performed in previous DNA microarray studies.⁸

Determination of Th2 cytokines in supernatants and by flow-cytometric analysis of CD4⁺ T cells showed that an allergen challenge resulted in significant proliferation of Th2 cells in patients compared to controls. The analysis also revealed several genes of known relevance to proliferation of Th2 cells, for example, the IL-4 and Vitamin D3 receptors.¹⁶ Network-based approaches to analyze functionally gene interactions have recently been described in DNA microarray studies of allergic and infectious disease.^{11,14} This was carried out using KB, a web-based analytical tool that mapped the differentially expressed genes to a network model of all mammalian gene interactions that had been described in the literature. Significant networks and functions were identified by statistical algorithms.^{24,25}

Before proceeding, it is important to recognize the various limitations of network analysis based on KB. First, the networks are generated via a manual review of the scientific literature. Thus, their design may be heavily influenced by the particular choice of journals and articles employed. Also, the analysis points to association only; it does not attempt to describe causality. Another limitation is that relative mRNA expression levels may not correspond to relative protein presence levels. Finally, a variety of other factors (e.g. post-translational modification) cannot be measured with microarray technology. Therefore, this analysis can be viewed primarily as a tool to generate hypotheses for experimental validation.

In this study, 50 significant networks were identified. Biological functions were assigned to each of these networks using the findings that had been extracted from the scientific literature and stored in the KB. The functions were then scored using a statistical algorithm. The top-scoring function of the networks was 'death of lymphocytes.' This is in agreement with previous data analyses that appear to associate Th2 proliferation with altered regulation of apoptosis.²⁻⁶ Lymphocyte death was associated with several genes of the TNF ligand and receptor superfamilies, most notably TNF, FAS, TNFRSF4, TNFRSF25, TNFRSF1B and TNFS7. These genes are involved in regulating T-cell apoptosis and proliferation. TNF, FAS and TNFRSF25 are proapoptotic, whereas TNFRSF1B, TNFSF7 and TNFRSF4 promote survival.²⁶ All were upregulated in allergen-stimulated Th2 cells from allergic patients. TNFRSF4 was ranked as the second most highly regulated of all the differentially expressed genes. In fact, its expression levels were 48 times higher in allergen-challenged than in diluent-challenged cells. TNFRSF4 and its ligand, TNFS4, have been reported to inhibit apoptosis in T cells and to enhance Th2 proliferation.^{2,27} However, some infectious agents may also induce Th1 proliferation through TNFRSF4 signals.^{22,28} In the context of allergy, however, recall responses were shown to be dependent on TNFRSF4 signals in an animal model of asthma.²⁹ TNFRSF4 has therefore been suggested as a therapeutic candidate in allergic disease.³⁰ Despite this, there has been only one previous report on TNFRSF4 in human

allergic disease in which increased expression of TNFSF4 and TNFRSF4 were described on airway smooth muscle cells in asthma.³¹ It is not known if allergen-induced proliferation of Th2 cells involves TNFRSF4 signalling. The present analysis proceeded by examining a different material of allergen-stimulated Th cells from patients with allergic rhinitis and healthy controls. The resultant experiments showed a significantly higher number of Th2 cells in patients than in controls, and a higher number of non-apoptotic Th2 cells in patients. This was associated with higher expression of TNFRSF4. Moreover, treatment with anti-TNFSF4 resulted in significant decrease of allergen-induced proliferation of Th2. Although significant, this decrease was by no means complete. A possible explanation may be a compensatory increase of TNFRSF4. Yet, anti-TNFSF4 resulted in decreased expression of TNFRSF4. Another possible explanation may be increased expression of other genes that induce T-cell proliferation. DNA microarray analysis had identified two such genes, TNFSF7 and TNFRSF1B. To our knowledge, these genes have not been described previously in allergic disease. Further analysis showed that the corresponding genes both increased in protein expression in allergen-challenged Th2 cells and decreased following treatment with anti-TNFSF4. It should also be noted that DNA microarray analysis had previously shown increased expression of proapoptotic genes like TNF and FAS, both of which have been described in allergic disease.^{2,3,5} These findings support the hypothesis that Th2 proliferation can be seen as a net effect of multiple interacting genes, with TNFRSF4 and other genes of the TNF superfamily playing important roles.

A similar level of network complexity was found in the study of endotoxin-challenged leukocytes, where the inflammatory response was associated with changes in gene expression levels in many different networks. It is hard not to conclude that a possible clinical implication of all this is that, it may be difficult to identify single genes that can be used as diagnostic markers or drug targets. It is possible that a network-based analysis may contribute to our understanding of the complex web of enhancing and inhibiting signals that underlie inflammatory responses. This would be significantly enhanced if the relevant networks could be annotated to show whether an interaction is positive or negative. If so, this could be implemented graphically or by an interactive model. For example, by clicking on such a model, facts about how interactions relate to an effect such as apoptosis could be revealed. More advanced models could display temporal and other types of information.

Interestingly, allergen-stimulation resulted in an increase of Th2 cells in controls, although the number of Th2 cells was lower than in patients. Moreover, allergen also caused an increase in the number of Th1 cells in patients compared to controls. These observations are consistent with DNA microarray data that had shown an increased expression of IFN-induced genes. These findings are also in agreement with recent data reported by Akdis *et al.*,²³ in which it was shown that Th2 cells from controls respond to allergen in a manner that is qualitatively similar to patients, although quantitatively the response is smaller. The authors also reported that decreased inhibitory activity of T-regulatory cells seems to play an important role in the response to allergens

in allergic patients. Further work delineating gene expression signatures in this T-cell subset may contribute to a better and more complete understanding of the regulation of allergic inflammation.

In summary, the network-based analysis reported here indicates that proliferation of allergen-stimulated Th2 cells in allergic patients is associated with decreased apoptosis and increased activity of TNFRSF4 and other members of the TNF superfamily.

Materials and methods

Subjects

This study included two different materials:

1. Eleven patients with asymptomatic birch pollen-induced allergic rhinitis and five healthy controls. Both patients and controls were seen outside of the pollen season. The median (range) age of the patients and the controls were 19 (17–34) years and 44 (20–48) years, respectively. Six patients and three controls were women. This material was used to validate that allergen stimulation resulted in proliferation of Th2 cells in patients and for DNA microarray analysis.
2. Eight patients with asymptomatic birch pollen-induced allergic rhinitis and 10 healthy controls. This material was used to validate results from the DNA microarray analysis.

The diagnosis of birch pollen-induced allergic rhinitis was based on a positive history of birch pollen-induced allergic rhinitis and positive skin prick tests against birch pollen. All patients were asymptomatic at the time of inclusion in the study. The controls were all symptom-free, had no history of allergic rhinitis or any other atopic disease, and had a negative skin prick tests against birch pollen. None of the patients and none of the controls had received any local or systemic treatment with glucocorticoids or antihistamines within 2 weeks immediately before samples were taken. The study was approved by the Ethics Committee of the Medical Faculty, Göteborg University.

In vitro stimulation of PMBC with grass extract

A modification of the method by Picker *et al.*³² was used as briefly described below. PBMCs were prepared by centrifugation on Ficoll-Hypaque, washed and stimulated with grass pollen extract (ALK-Abelló A/S, Hørsholm, Denmark; final concentration: 100 µg/ml) at a density of 10⁶ cells/ml, in polystyrene tubes and a total volume of 2 ml of cell culture medium (RPMI1640 with human AB serum (5% (v/v)), gentamicin (100 µg/ml), L-glutamine (1% (v/v)) and mercaptoethanol (1% (v/v))). After 7 days of incubation at 37°C and 5% CO₂, the supernatants were removed and analyzed for the content of IL-5 and IL-13 (see below).

In experiments using DNA microarrays, CD4⁺ cells were enriched using anti-CD4-coated paramagnetic microbeads and an MACS (magnetic cell sorter) system according to the instructions of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). cRNA was extracted according to the procedure described below.

In experiments where the numbers of different subpopulations of CD4⁺ cells expressing intracellular IL-4

(Th2) or IFN- γ (Th1) were determined by flow cytometry, post-stimulation of allergen-stimulated cells with PMA (final concentration: 25 ng/ml) and ionomycin (final concentration: 1 μ g/ml) was performed on day 7. In some experiments, polyclonal goat antibodies to TNFS4 (R&D Systems Ltd, Abingdon, UK; final concentration: 10 μ g/ml) were added to stimulated and unstimulated cells.

Flow cytometric assay

The method of Picker *et al.*³² was modified as described. Briefly, cells were harvested by centrifugation on day 7, and the cell number adjusted to 2×10^6 cells/ml. Post-stimulation with PMA and ionomycin was performed with brefeldin A (final concentration: 10 μ g/ml) present during the last 4 h. After washing, the cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences, San Jose, CA, USA) during incubation for 20 min at 4°C. Washing with buffer containing detergent (Permashield, BD Biosciences) was followed by the addition of the following monoclonal antibodies: anti-IFN- γ ^{FITC}, anti-CD134^{FITC}, anti-CD70^{PE}, anti-CD120b^{Biotin}, anti-CD4^{PerCP} and anti-IL-4^{APC} (all from BD Biosciences).

A fraction of the cells was analyzed for apoptosis as described below. After incubation for 20 min at 4°C, some samples were incubated with streptavidin^{PE} for 20 min at 4°C. After washing, the cells were analyzed in a flow cytometer (FACSCalibur, BD Biosciences) as described below.

Analysis for apoptosis was performed using a commercial kit for a TUNEL-based apoptosis detection assay using flow cytometry (R&D Systems). The method followed the instructions from the manufacturer and is briefly described. After fixation and permeabilization, cytonin (100 μ l), anti-IFN- γ ^{FITC} and anti-IL-4^{APC} were added. The cells were then incubated for 30 min in room temperature and in the dark. After washing in labelling buffer, a mix for the detection of terminal deoxynucleotidyl transferase enzyme activity was added. Positive (containing Trevigen apoptotic cell system (TACS) nuclease) and negative apoptosis controls were made. The cells were incubated for 60 min at 37°C. A stop solution was added. The cells were then centrifuged and resuspended. Streptavidin^{PE} and anti-CD4^{PerCP} were added, after which the cells were incubated for 15 min in room temperature and in the dark. After washing, the cells were analyzed in a flow cytometer as described below.

Flow cytometer analysis was performed by collecting a defined volume and setting a gate around CD4⁺ cells as has been described previously³³ (not by collecting the same number of cells from the different samples). The sample volume was calculated from the flow rate determined using TruCount beads (BD) and collection time. Different sub-populations of CD4⁺ cells were defined by antibody staining. Results were expressed as the number of stained cells per μ l. The purity of the CD4⁺ T cells was assessed by examining the percentage of CD4⁺ non-T cells, which was less than 5%.

Analysis of IL-5, IL-13 and MIF in supernatants from allergen-challenged PBMC

Analyses of IL-5, IL-13 and MIF were performed using commercial enzyme-linked immunosorbent assay kits

according to the manufacturer's instructions (R&D Systems Ltd, Abingdon, UK).

DNA microarray analysis of CD4⁺ T cells

CD4⁺ T cells were enriched from PBMC that had been challenged with either allergen or diluent as described above. DNA microarray analyses were performed on CD4⁺ T cells from three patients (a total of six microarray analyses) as described,⁷ using DNA microarrays measuring the expression of 22 283 genes and variants (HuGe U133A GeneChip, Affymetrix, Santa Clara, CA, USA). RNA isolation was performed using Qiagen Lipid Tissue kit (Qiagen, Hilden, Germany). The concentration was measured spectrophotometrically, with an A_{260}/A_{280} ratio of 1.8–2.0 and the quality was verified by agarose gel electrophoresis. Preparation of cRNA and hybridization to DNA microarrays was performed according to Affymetrix Gene Chip Expression Analysis manual. Briefly, RNA was reverse transcribed into cDNA, and biotin-labelled target cRNA was prepared by *in vitro* transcription (Enzo Diagnostics Inc., Farmingdale, NY, USA) followed by hybridization to DNA microarrays. The microarrays were scanned with Hewlett Packard confocal laser scanner (Hewlett Packard, GeneArray scanner G2500A) and visualized using Affymetrix Genechip 4.0 software (Affymetrix, Santa Clara, CA, USA). The analyses were performed according to manufacturer's instructions and the MIAME guidelines (<http://www.mged.org/Workgroups/MIAME/miame.html>). The raw array data can be found at the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>).

Identification of differentially expressed genes in DNA microarray data from CD4⁺ T cells

Differentially expressed genes are often identified using a fixed cutoff value, for example a two-fold difference, or straightforward *t*-tests, which may lead to the spurious selection of genes with highly variable gene expression levels. In this work, the selection was based instead on a statistic designed to control for such variability. Starting from the Affymetrix CEL files, data at the probe level were first corrected for background noise and normalized. Probe values were then summarized to get a single value for each probe set. These three steps were carried out using R (R Development Core Team, 2005) with the R package *affy*²⁴ available from Bioconductor (www.bioconductor.org). Then, we estimated the effect of stimulation on each gene using a linear model with a Bayesian smoothing described by Smyth and implemented in the R package *LIMMA*.²⁵ Finally, the probe sets were ranked according to their evidence of being differentially expressed.

Network-based analysis of differentially expressed genes

The Ingenuity Pathways KB was used to organize differentially expressed genes into networks of interacting genes. The KB currently consists of a global network, which is based on reviewing and extracting mammalian gene interactions from reviewing over 200 000 articles in 275 peer-reviewed journals. The findings for the KB were manually curated and assigned to ontological classes by content and modelling experts. Interactions were also inferred algorithmically by combining functional and structural information about proteins. Every interaction can be referenced back to a MedLine ID within the

application. Identification of networks formed by the differentially expressed genes was performed in the following stepwise manner:

- (1) Differentially expressed genes were scored based upon the number of specific interactions found within the interpretative phenomenological analysis global network. Focus genes were identified as those having direct or indirect interactions with other genes in the KB.
- (2) The specificity of connections for each focus gene was calculated by the percentage of its connections to other differentially expressed genes. A network was then constructed starting with the gene with the highest specificity of connections. Each network contained a maximum of 35 genes. This process was repeated to construct new networks for the remaining genes. As the number of focus genes is reduced as a result of their inclusion in previous networks, genes from the global network not in the original list, but with significant specific connectivity to the growing network, can be included into new networks.
- (3) A statistical algorithm based on the Fisher's exact test was used to score and rank the networks. The score was derived from a *P*-value, and indicates the likelihood that focus genes in a network might be found together owing to random chance. A score of 2 indicates that there is a 1 in 100 chance that focus genes are together owing to chance.
- (4) Biological functions and disease associations were assigned to all significant networks by using findings extracted from the scientific literature and stored in the KB. Each function was ranked according to its significance to the network. A Fisher's exact test was used to calculate a *P*-value estimating the probability that the biological function assigned to the network was explained by chance alone.

Statistical analysis of cytokines and chemokines in allergen-challenged PBMC or CD4⁺ T cells

The levels of cytokines in supernatants from allergen-challenged PBMC and in CD4⁺ T cells from patients and controls were compared using the non-parametric Mann-Whitney *U*-test for unpaired comparisons and Wilcoxon's signed-rank test for paired comparisons.

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