

A network-based analysis of the late-phase reaction of the skin

Mikael Benson, MD, PhD,^a Michael A. Langston, PhD,^b Mikael Adner, PhD,^c Bengt Andersson, MD, PhD,^d Åsa Torinsson-Nalwai, PhD,^e and Lars Olaf Cardell, MD, PhD^c

Gothenburg and Malmö, Sweden, and Knoxville, Tenn

Background: The late-phase reaction (LPR) of the skin is an *in vivo* model of allergic inflammation.

Objective: We sought to identify disease-associated pathways in the LPR using a network-based analysis.

Methods: The LPR was examined by means of DNA microarray analysis of skin biopsy specimens from 10 patients with allergic rhinitis and 10 healthy control subjects. The results were further analyzed in 2 different materials consisting of nasal fluids and allergen-challenged CD4⁺ T cells from patients with allergic rhinitis.

Results: The DNA microarray analysis revealed several genes of known relevance to allergy. The eosinophil marker Charcot-Leyden crystal protein (*CLC*) that encodes Charcot-Leyden crystal protein differed most in expression. A network-based analysis showed upregulation of IL-4- and CCL4-dependent pathways and downregulation of a TGF- β -induced pathway. CCL4 is expressed by CD4⁺ T cells and chemotactic for eosinophils. We hypothesized that allergen induces release of CCL4 from T_H2 cells and that this contributes to influx of eosinophils. Further analysis showed increase of CCL4 protein in nasal fluids from allergic patients during the season. Allergen challenge of PBMCs resulted in proliferation of T_H2 cells and increased production of CCL4 in CD4⁺ T cells from allergic patients. An analysis of the DNA microarray data revealed a significant correlation between CCL4 and the eosinophil marker *CLC*.

Conclusion: A network-based analysis of the LPR showed increased activity of IL-4- and CCL4- dependent pathways and downregulation of the TGF- β -induced pathway. Allergen-induced release of CCL4 from T_H2 cells might contribute to influx of eosinophils during the LPR.

Clinical implications: Involvement of multiple interacting pathways indicates that it might be difficult to identify one single mediator as a biomarker or drug target in allergic inflammation. (J Allergy Clin Immunol 2006;118:220-5.)

Key words: DNA microarrays, gene expression, late-phase reaction

The late-phase reaction (LPR) of the skin is an *in vivo* model of chronic allergic inflammation. The LPR is characterized by an IgE-dependent local influx of inflammatory cells, such as eosinophils and basophils.¹⁻⁴ These findings are in agreement with the T_H2 hypothesis, according to which allergic inflammation is orchestrated by increased release of cytokines from T_H2 cells. However, an increasing number of mediators other than those included in the T_H2 hypothesis have been implicated in the pathogenesis.⁵ This complexity is supported by DNA microarray studies, which have described hundreds of differentially expressed genes in allergic rhinitis, asthma, and eczema.⁶⁻¹¹ Although this complexity might be reduced in allergen challenge studies, a wide variety of mediators have still been reported in such studies of the nose, the lung, and the skin.^{5,10,12,13} These findings suggest that many different pathways are involved in the LPR. It seems plausible that the identification of these pathways could contribute to our understanding of the LPR.

Recently, Calvano et al¹⁴ described a network-based approach that was used to identify pathways in a DNA microarray study of the human inflammatory response to bacterial endotoxin. A bioinformatics program was used to organize differentially expressed genes into gene interaction networks. The program was based on manual review of more than 200,000 scientific publications. The resulting network of direct physical, transcriptional, and enzymatic interactions between mammalian orthologues was used as a template to organize the differentially expressed genes into networks and to find specific pathways in those networks. Such networks and pathways could be further examined to find individual genes with putative key functions.

In this study the same approach was used to identify significant pathways and genes in a DNA microarray study of cutaneous LPR in 10 patients with allergic rhinitis and 10 healthy control subjects. The results were further analyzed in 2 different materials, specifically nasal fluids and allergen-challenged CD4⁺ T cells from patients with

From ^athe Department of Pediatrics, Queen Silvia Children's Hospital, Gothenburg; ^bthe Department of Computer Science, University of Tennessee, Knoxville; ^cthe Laboratory for Clinical and Experimental Allergy Research, Department of Oto-Rhino-Laryngology, Malmö University Hospital, Lund University, Malmö; ^dthe Department of Clinical Immunology, Sahlgrenska Academy, Gothenburg; and ^ethe Department of Clinical Genetics and Göteborg Genomics, Sahlgrenska Academy, Gothenburg.

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Reprint requests: Mikael Benson, MD, PhD, Queen Silvia Children's Hospital, SE-41685 Gothenburg, Sweden. E-mail: mikael.benson@vgregion.se.

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Abbreviations used

CLC: Charcot-Leyden crystal protein gene
C_T: Cycle threshold
IPA: Ingenuity Pathways Analysis application
LPR: Late-phase reaction

allergic rhinitis. The DNA microarray analysis of the skin resulted in identification of 3 main pathways, namely increased IL-4 and CCL4 signaling, as well as decreased TGF- β signaling. CCL4 is known to induce migration of eosinophils but had not been previously described in allergic disease. Further analysis showed that CCL4 increased in nasal fluids during the pollen season, as well as in allergen-challenged CD4⁺ T cells and correlated with the eosinophil marker Charcot-Leyden crystal protein (CLC). This supports the argument that allergen-induced release of CCL4 from T_H cells might contribute to the influx of eosinophils in allergic rhinitis.

METHODS

Subjects

Three different materials were used for analysis of inflammatory responses in the skin, PBMCs, and nasal fluids:

1. Allergen provocation study of skin: Ten patients with grass pollen-induced allergic rhinitis and 10 healthy control subjects were studied. Both patients and control subjects were seen outside the birch/grass pollen season. The median age of the patients and the control subjects was 34.5 years (range, 21-49 years) and 42.5 years (range, 21-51 years), respectively. Five patients and 4 control subjects were women.
2. Allergen provocation study of PBMCs: Ten patients with grass pollen-induced allergic rhinitis and 5 healthy control subjects were studied. Both patients and control subjects were seen outside the birch/grass pollen season. The median age of the patients and the control subjects was 36 years (range, 20-42 years) and 40 years (range, 21-46 years), respectively. Four patients and 3 control subjects were women.
3. Natural allergen exposure study of nasal fluids: Nasal lavage fluid was collected from 19 patients (11 women) outside and 16 patients (9 women) inside the birch/grass pollen season, as well as from 19 healthy control subjects (8 women). The median age was 37 years (range, 18-53 years) and 37.5 years (range, 18-61 years) for patients outside and inside the pollen season, respectively. The median age of the control subjects was 27 years (range, 18-51 years).

A detailed description of inclusion criteria for patients and control subjects is given in the Online Repository at www.jacionline.org.

Skin allergen challenge

Fifty standardized quality units (SQ-U) of grass pollen extract (AquaGen *Phleum pratense*; ALK-Abello, Horsholm, Denmark) was injected subcutaneously in the forearm, as previously described.¹⁵ A detailed description is given in the Online Repository at www.jacionline.org.

DNA microarray analysis and data analysis

Skin biopsy specimens from 10 patients with allergic rhinitis and 10 control subjects were analyzed as previously described⁸ by using

DNA microarrays measuring the expression of 22,283 genes and variants (HuGe U133A GeneChip; Affymetrix, Santa Clara, Calif). The hybridization and analysis were performed according to the manufacturer's instructions and the Minimum Information About a Microarray Experiment guideline. The Ingenuity Pathways Analysis application (IPA) was used to organize differentially expressed genes into networks of interacting genes and to find modules of functionally related genes that correspond to pathways.¹⁴ A detailed description is given in the Online Repository at www.jacionline.org.

Allergen challenge of PBMCs

A modification of the method by Picker et al¹⁶ was used as briefly described below. Heparinized blood was collected by means of venipuncture, and PBMCs were prepared by means of centrifugation on Ficoll-Hypaque, washed, and stimulated with grass pollen extract (ALK-Abelló A/S; 100 μ g/mL) at a density of 10⁶ cells/mL and in a total volume per well of 2 mL of cell culture medium. After 7 days of incubation at 37°C and 5% CO₂, the supernatant was removed and analyzed for the content of CCL4 by using a commercial ELISA (R&D Systems Ltd, Abingdon, United Kingdom). CD4⁺ cells were enriched from the allergen-challenged PBMCs by using anti-CD4-coated paramagnetic microbeads and a MACS system to examine whether CCL4 was expressed by T_H2 cells, according to the instructions of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). cRNA was extracted according to the procedure described in the Online Repository at www.jacionline.org.

Nasal lavage fluid

After clearing excess mucus by means of forceful exsufflation, room temperature sterile saline solution (0.9% NaCl) was alternately aerosolized into both nostrils.¹⁷ The nasal fluid was passively collected in a graded test tube until 7 mL was recovered. The fluid was centrifuged for 10 minutes at 1334g at 4°C. The supernatant was stored at -70°C until it was analyzed after 6 hours.

Analysis of IL-5, IL-13, and CCL4 in nasal fluids and supernatants from allergen-challenged PBMCs

Analyses of IL-5, IL-13, and CCL4 were performed by using commercial ELISA kits, according to the manufacturer's instructions (R&D Systems Ltd).

Statistical analysis of cytokines and chemokines in nasal fluids and allergen-challenged PBMCs

The levels of IL-5, IL-13, and CCL4 in nasal fluids or supernatants from allergen-challenged PBMCs from patients and control subjects were compared by using the nonparametric Mann-Whitney *U* test.

Quantitative real-time PCR analysis of CCL4 in CD4⁺ T cells

For a detailed description, see the Online Repository at www.jacionline.org.

RESULTS

Overview of gene expression data

This DNA microarray study of allergen-challenged skin was performed on skin biopsy specimens from 10 patients with allergic rhinitis and 10 healthy control subjects. The analysis revealed several genes of known relevance to

allergy (see Table E1 in the Online Repository at www.jacionline.org). In fact, the gene that had the highest expression in patients was the eosinophil marker Charcot-Leyden crystal protein (*CLC*), which had a ratio of 17.6 compared with that seen in control subjects. The early T-cell activation antigen *CD69* had a ratio of 8.3, and the neutrophil chemotaxin *IL8* had a ratio of 3.4. The list also included several cytokines and their receptors, such as *IL4R*, *IL13A1*, and *IL13RA2*. These findings are in agreement with a T_H2 -dependent influx of inflammatory cells during the LPR, as previously described.^{1-4,13,18} However, the T_H2 -associated genes only represented a small fraction of the 1840 differentially expressed genes. A network-based analysis was performed to obtain a functional overview of all these genes.

A network-based analysis of the LPR

The IPA was used to organize the differentially expressed genes into networks of interacting genes and to find modules of functionally related genes that correspond to known pathways.¹⁴ The IPA contains a network model of gene interactions that is based on manual review of scientific articles. This model serves as a template to organize the differentially expressed genes into functionally annotated networks and pathways. Statistical algorithms were then used to rank the resulting networks according to how well they corresponded to the template. Each network contained a maximum of 35 genes. After building one network, the remaining genes were used to form new networks. This process was repeated, with each network statistically scored. In this material 50 significant networks were formed. Network 1 and its subcellular location are shown in Fig 1, A. The main functions associated with this network were cellular growth and tissue development. The network included several downregulated growth factors and their receptors or binding proteins, including the insulin-like growth factor genes *IGF1*, *IGF1R*, *IGFBP5*, and *IGFBP6* and the bone morphogenic protein genes *BMP2*, *BMP4*, *BMP7*, *BMPRI1A*, and *BMPRI2*. By contrast, several proinflammatory cytokines and cytokine receptors were upregulated in network 1, for example, small inducible cytokine subfamily E (*SCYE1*) and IL-3 receptor α (*IL3A*). This might be consistent with inflammatory remodeling events, as previously reported on the LPR and asthma.^{18,19} The main function of network 2 was cellular signaling. In this network a distinct interaction pattern could be seen (Fig 1, B). The plasma membrane contained several upregulated proinflammatory genes that were linked to signal transducer and activator of transcription 3 (*STAT3*). These proinflammatory genes included a subnetwork of known relevance to T_H2 responses, *IL4R*, *IL13A1*, and *IL13RA2*, as well as selectin P (*SELP*) and selectin P ligand (*SELPLG*). *SELP* and *SELPLG* increase the expression of *IL4R*.²⁰ Other genes of known relevance to allergy that were linked to *STAT3* were the chemokine receptor *CCR2* and the eosinophil granule protein *CLC*. These findings are consistent with recent data indicating T_H2 cytokine-induced upregulation of *STAT3* in eosinophils.²¹ The next 5 networks were

associated with functions such as cell cycle, cellular growth, and inflammatory response. These 7 networks were merged into one network (see Fig E1 in the Online Repository at www.jacionline.org). The complexity of these interactions was not readily accessible for empiric analysis. We therefore proceeded to identify canonical pathways. This was done by correlating the differentially expressed genes with canonical pathways within the IPA. The resulting inflammatory pathways with the highest scores were IL-4 signaling, TGF- β signaling, and chemokine signaling. The first pathway included *IL4R*, *IL13A1*, and *IL13RA2*, all of which were upregulated. By contrast, all but one of 10 genes in the TGF- β pathway were downregulated. The chemokine signaling pathway included *CCL4* and its receptor *CCR5*, both of which were upregulated. *CCL4* is chemotactic for eosinophils²²⁻²⁴ and has been reported to increase in both T_H1 and T_H2 cells on T-cell receptor stimulation.²⁴ Although it is not known whether *CCL4* is involved in allergic inflammation, these findings suggested the hypothesis that allergen stimulation induces *CCL4* expression in T_H2 cells, which in turn contributes to influx of eosinophils during the LPR.

Analysis of CCL4 in nasal fluids and CD4⁺ T cells from patients with allergic rhinitis

Nasal fluid levels of CCL4 protein were examined in a different material to investigate whether CCL4 is involved in allergic rhinitis. The CCL4 concentration was similar in healthy control subjects (3.4 pg/mL; range, 0.5-24.0 pg/mL) compared with that seen in allergic patients outside the pollen season (3.1 pg/mL; range, 1.5-7.3 pg/mL). A significantly higher protein level of CCL4 was measured in the nasal lavage fluid from symptomatic allergic patients during the pollen season (4.7 pg/mL; range, 0.1-45.5 pg/mL; $P < .05$).

The hypothesis that allergen stimulation resulted in increased production of CCL4 in CD4⁺ T lymphocytes was tested in another material consisting of allergen-stimulated PBMCs from 10 patients with allergic rhinitis. In supernatants from the patients, CCL4 levels were significantly higher in allergen-challenged PBMCs compared with in diluent-challenged PBMCs (12,000 pg/mL [range, 1500-38,000 pg/mL] compared with 5600 pg/mL [680-6500 pg/mL], $P < .01$). The T_H2 cytokines IL-5 and IL-13 were also analyzed to examine whether this was associated with a T_H2 response. IL-5 levels were significantly higher in supernatants from allergen-challenged PBMCs (100 pg/mL [range, 0-500 pg/mL] vs 0 pg/mL [range, 0-0 pg/mL], $P < .01$), as were IL-13 levels (13 pg/mL [range, 0-270 pg/mL] vs 0 pg/mL [range, 0-0 pg/mL], $P < .05$). In supernatants from 5 control subjects, no significant difference was found for CCL4 between allergen-challenged and diluent-challenged PBMCs (3300 pg/mL [range, 680-6200 pg/mL] vs 49 pg/mL [range, 46-76 pg/mL]). Similarly, no significant difference in IL-5 levels was found (12 pg/mL [range, 0-66 pg/mL] vs 0 pg/mL [range, 0-0 pg/mL]). Then expression of *CCL4* was analyzed in CD4⁺ T cells extracted from the



FIG 1. Network representation of interactions between differentially expressed genes from a DNA microarray analysis of allergen-challenged skin from allergic patients and healthy control subjects. Of 1840 differentially expressed genes, a total of 50 statistically significant networks were formed. The networks were scored according to significance. The figure shows the top-scoring networks 1 (**A**) and 2 (**B**). Genes that are colored *red* have higher expression in patients than in control subjects, and genes colored *green* have lower expression in patients.

allergen-challenged PBMCs by using real-time PCR and normalized to the reference gene *GUSB*. The median Δ cycle threshold value for allergen-challenged CD4⁺ T cells was 6.2 (range, 1.7-7.5) compared with 1.4 (range, 0.5-3.4) in diluent-challenged CD4⁺ T cells ($P < .0001$). This corresponds to a more than 10-fold increase of *CCL4* expression in allergen-challenged CD4⁺ T cells.

Finally, analysis of the DNA microarray data revealed a significant correlation between *CCL4* and the eosinophil granule protein *CLC* ($r = 0.8$, $P < .001$).

DISCUSSION

In this DNA microarray study of the LPR in skin biopsy specimens from 10 patients with allergic rhinitis and 10 healthy control subjects, the gene that differed most in expression was the eosinophil marker *CLC*. The differentially expressed genes also included the T-cell activation markers and *IL4R*, *IL13RA1*, and *IL-13RA2*. These receptors are essential for IgE production. To our knowledge, increased expression of *IL4R*, *IL13RA1*, and *IL-13RA2* has not been previously described in allergic disease. However, in an earlier report we found increased levels of the soluble form of IL-4 receptor protein in patients with allergic rhinitis.²⁵ Taken together, our findings are in agreement with an IgE-dependent influx of eosinophils during the LPR, as previously described.^{1-4,13,18} It is, however, of note that the role of eosinophils in allergic inflammation is complex.^{26,27} Moreover, these genes only represented a small fraction of the differentially expressed genes. It is difficult to obtain an overall functional understanding of such complexity on the basis of detailed analysis of individual genes. One approach to address this problem might be to start by organizing the differentially expressed genes into networks based on functional relationships. These networks can be used for a top-down analysis, where the networks are dissected to find individual disease-associated pathways. Finally, individual upstream genes in the pathways can be selected for functional studies. This approach was recently described by Calvano et al¹⁴ in a study of the human inflammatory response to endotoxin using the IPA, a Web-based analytic tool. The IPA correlated the differentially expressed genes to a manually curated network of gene interactions that had been described in the literature. By using statistical algorithms, networks comprising a maximum of 35 genes were identified and ranked according to statistical significance. The underlying assumption was that if more differentially expressed genes than expected by chance mapped to the same network, then this was likely to represent biologically relevant processes. In other words, the global molecular network within the IPA was used as a template to find matching, significant networks among the differentially expressed genes. In this study the LPR was analyzed with the same program. Fifty significant networks were identified. Empiric analysis of the top-ranking networks reflected potentially relevant processes. Network 1 contained several downregulated growth factors

and upregulated proinflammatory genes. This might be consistent with remodeling events, as previously described in both the LPR and asthma.^{18,19} Network 2 contained upregulated proinflammatory genes, such as *IL4R*, *IL13A1*, and *IL-13RA2*, that interacted with *STAT3* and the eosinophil granule protein *CLC*. These findings were consistent with a T_H2-induced eosinophilic response, as discussed above.

It should be noted that although each gene interaction in the IPA was linked to at least one publication, functional annotation of gene interactions is far less developed than that of individual genes. Moreover, there are no established principles on how to analyze networks. These are important research directions, and the IPA is one example of databases attempting to annotate interactions and networks systematically.^{28,29}

To our knowledge, this is the first comprehensive network-based analysis of allergic inflammation. The large number of gene interactions in the networks indicates that many pathways might contribute to the LPR. A clinical implication of this is that it might be difficult to identify one single mediator as a biomarker or drug target in allergic disease. In the present project we proceeded by searching for known pathways among the differentially expressed genes. This was done by correlating the differentially expressed with canonical pathways in the IPA. The resulting inflammatory pathways with the highest scores were IL-4 signaling, TGF- β signaling, and chemokine signaling. The first pathway included *IL4R*, *IL13A1*, and *IL-13R*, again underscoring the central role of these genes in allergic inflammation. By contrast, all but one of 10 genes in the TGF- β pathway were downregulated. TGF- β has anti-inflammatory effects. Decreased activity of the TGF- β pathway has previously been described in a DNA microarray study of allergic rhinitis.⁶ Moreover, TGF- β is released from T_R1 cells, which are important regulators of peripheral tolerance. Decreased activity of T_R1 cells was recently shown in allergic patients.³⁰ Thus decreased activity of the anti-inflammatory TGF- β pathway could contribute to the LPR. It is of note that because this study was performed in skin biopsy specimens, we do not know the cellular origin of the differentially expressed genes. Moreover, it is not possible to validate all pathways with new experiments. Because increased IL-4 signaling and decreased TGF- β signaling were consistent with previous induced data, we decided to focus on chemokine signaling, which included *CCL4* and its receptor *CCR5*, both of which increased in expression. *CCL4* is chemotactic for eosinophils²²⁻²⁴ and has been reported to increase in T_H1 and T_H2 cells after anti-CD3 stimulation.²⁴ Moreover, stimulation of the high-affinity Fc ϵ RI in mast cells resulted in increased expression of *CCL4*.³¹ Although little is known about *CCL4* in allergy, these data suggested the hypothesis that release of *CCL4* from allergen-stimulated T_H2 cells could contribute to influx of eosinophils during the LPR. This hypothesis was examined in 2 different materials consisting of nasal fluids and allergen-stimulated PBMCs from patients with allergic rhinitis and healthy control subjects. First, *CCL4* levels were examined in

nasal fluids and found to be significantly higher in symptomatic patients during the pollen season than in asymptomatic patients after the season. To our knowledge, this is the first demonstration of involvement of CCL4 in human allergic inflammation. Second, we examined whether allergen stimulation caused increased production of CCL4 in T_H2 cells. PBMCs were challenged with allergen to mimic natural allergen challenge of CD4⁺ T cells, which is mediated by antigen-presenting cells. In supernatants from patients, this resulted in a significant increase of CCL4. A parallel increase of the T_H2 cytokines IL-5 and IL-13 in the patients showed that this was associated with proliferation of T_H2 cells. CCL4 was analyzed in CD4⁺ T cells extracted from the allergen-challenged PBMCs to examine whether CCL4 production was derived from CD4⁺ T cells. Examination with real-time PCR showed a more than 10-fold increase of CCL4 in CD4⁺ T cells from the patients. Finally, analysis of the DNA microarray data showed a strong correlation between CCL4 and the eosinophil protein CLC. Taken together, these data support the hypothesis that allergen challenge results in increased CCL4 production in T_H2 cells from patients with allergic rhinitis and that this might contribute to influx of eosinophils.

In summary, this network-based analysis of the LPR in allergic patients showed that allergen challenge results in increased activity of IL-4- and CCL4-signaling pathways but decreased TGF- β signaling activity. Increased production of CCL4 from allergen-stimulated T_H2 cells might contribute to the influx of eosinophils during the LPR.

REFERENCES

- Dolovich J, Hargreave FE, Chalmers R, Shier KJ, Gaudie J, Bienenstock J. Late cutaneous allergic responses in isolated IgE-dependent reactions. *J Allergy Clin Immunol* 1973;52:38-46.
- Solley GO, Gleich GJ, Jordan RE, Schroeter AL. The late phase of the immediate wheal and flare skin reaction. Its dependence upon IgE antibodies. *J Clin Invest* 1976;58:408-20.
- Frew AJ, Kay AB. Eosinophils and T-lymphocytes in late-phase allergic reactions. *J Allergy Clin Immunol* 1990;85:533-9.
- Ong YE, Menzies-Gow A, Barkans J, Benyahia F, Ou TT, Ying S, et al. Anti-IgE (omalizumab) inhibits late-phase reactions and inflammatory cells after repeat skin allergen challenge. *J Allergy Clin Immunol* 2005;116:558-64.
- Bochner BS, Busse WW. Allergy and asthma. *J Allergy Clin Immunol* 2005;115:953-9.
- Benson M, Carlsson B, Carlsson LM, Mostad P, Svensson PA, Cardell LO. DNA microarray analysis of transforming growth factor-beta and related transcripts in nasal biopsies from patients with allergic rhinitis. *Cytokine* 2002;18:20-5.
- Benson M, Carlsson B, Carlsson LM, Wennergren G, Cardell LO. Increased expression of vascular endothelial growth factor-A in seasonal allergic rhinitis. *Cytokine* 2002;20:268-73.
- Benson M, Carlsson L, Adner M, Jernas M, Rudemo M, Sjogren A, et al. Gene profiling reveals increased expression of uteroglobin and other anti-inflammatory genes in glucocorticoid-treated nasal polyps. *J Allergy Clin Immunol* 2004;113:1137-43.
- Groneberg DA, Welker P, Fischer TC, Dinh QT, Grutzkau A, Peiser C, et al. Down-regulation of vasoactive intestinal polypeptide receptor expression in atopic dermatitis. *J Allergy Clin Immunol* 2003;111:1099-105.
- Nomura I, Gao B, Boguniewicz M, Darst MA, Travers JB, Leung DY. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. *J Allergy Clin Immunol* 2003;112:1195-202.
- Zimmermann N, King NE, Laporte J, Yang M, Mishra A, Pope SM, et al. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J Clin Invest* 2003;111:1863-74.
- Hansen I, Klimek L, Mosges R, Hormann K. Mediators of inflammation in the early and the late phase of allergic rhinitis. *Curr Opin Allergy Clin Immunol* 2004;4:159-63.
- Menzies-Gow A, Ying S, Sabroe I, Stubbs VL, Soler D, Williams TJ, et al. Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers. *J Immunol* 2002;169:2712-8.
- Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, et al. A network-based analysis of systemic inflammation in humans. *Nature* 2005;437:1032-7.
- Plewako H, Arvidsson M, Petruson K, Oancea I, Holmberg K, Adelroth E, et al. The effect of omalizumab on nasal allergic inflammation. *J Allergy Clin Immunol* 2002;110:68-71.
- Pickler LJ, Singh MK, Zdravski Z, Treer JR, Waldrop SL, Bergstresser PR, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 1995;86:1408-19.
- Benson M, Strannegard IL, Strannegard O, Wennergren G. Topical steroid treatment of allergic rhinitis decreases nasal fluid TH2 cytokines, eosinophils, eosinophil cationic protein, and IgE but has no significant effect on IFN-gamma, IL-1beta, TNF-alpha, or neutrophils. *J Allergy Clin Immunol* 2000;106:307-12.
- Phipps S, Ying S, Wangoo A, Ong YE, Levi-Schaffer F, Kay AB. The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. *J Immunol* 2002;169:4604-12.
- Hoshino M, Nakamura Y, Sim JJ. Expression of growth factors and remodelling of the airway wall in bronchial asthma. *Thorax* 1998;53:21-7.
- Mahoney TS, Weyrich AS, Dixon DA, McIntyre T, Prescott SM, Zimmerman GA. Cell adhesion regulates gene expression at translational checkpoints in human myeloid leukocytes. *Proc Natl Acad Sci U S A* 2001;98:10284-9.
- Stout BA, Bates ME, Liu LY, Farrington NN, Bertics PJ. IL-5 and granulocyte-macrophage colony-stimulating factor activate STAT3 and STAT5 and promote Pim-1 and cyclin D3 protein expression in human eosinophils. *J Immunol* 2004;173:6409-17.
- Adams DH, Harvath L, Bottaro DP, Interrante R, Catalano G, Tanaka Y, et al. Hepatocyte growth factor and macrophage inflammatory protein 1 beta: structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. *Proc Natl Acad Sci U S A* 1994;91:7144-8.
- Borchers MT, Ansay T, DeSalle R, Daugherty BL, Shen H, Metzger M, et al. In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. *J Leukoc Biol* 2002;71:1033-41.
- Sallusto F, Kremmer E, Palermo B, Hoy A, Ponath P, Qin S, et al. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol* 1999;29:2037-45.
- Benson M, Strannegard IL, Wennergren G, Strannegard O. Increase of the soluble IL-4 receptor (IL-4sR) and positive correlation between IL-4sR and IgE in nasal fluids from school children with allergic rhinitis. *Allergy Asthma Proc* 2000;21:89-95.
- Wills-Karp M, Karp CL. Biomedicine. Eosinophils in asthma: remodeling a tangled tale. *Science* 2004;305:1726-9.
- Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. *Trends Immunol* 2004;25:477-82.
- Jenssen TK, Laegreid A, Komorowski J, Hovig E. A literature network of human genes for high-throughput analysis of gene expression. *Nat Genet* 2001;28:21-8.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13:2498-504.
- Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.
- Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, et al. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor 1 cross-linking: an interspecies comparison. *Blood* 2002;100:3861-8.

Inclusion and exclusion criteria for patients and control subjects

The diagnosis of grass pollen-induced allergic rhinitis was based on a positive history of seasonal allergic rhinitis and positive skin prick test responses against grass pollen. Except for the symptomatic patients in material 3, all patients were outside the pollen season and were asymptomatic at the time of inclusion in the study. Before inclusion, all subjects, both patients and control subjects, were evaluated by an experienced ear, nose, and throat consultant, performing nasoscopy. Individuals with signs or symptoms of chronic rhinitis, nasal polyposis, or recurrent sinusitis were excluded, along with subjects with a history of concurrent purulent nasal infection within 6 weeks before the study or nasal surgery during the last year.

Other exclusion criteria included a history of perennial symptoms, positive skin prick test responses against house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) and molds (*Cladosporium* and *Alternaria* species), and treatment with local or systemic corticosteroids or antihistamines for the last 2 months. The control subjects were all symptom free, had no history of allergic rhinitis or any other atopic disease, and had negative skin prick test responses against a panel of allergens, including birch, timothy, mugwort, house dust mite (*D. pteronyssinus* and *D. farinae*), horse, dog, cat, and molds (*Cladosporium* and *Alternaria* species).

Informed consent was obtained from all subjects involved in the study. The study was approved by the Ethics Committee of the Medical Faculties of the Universities of Lund and Göteborg.

Skin allergen challenge

Fifty standardized quality units (SQ-U) of grass pollen extract (Aquagen *Phleum pratense*; ALK-Abello, Hørsholm, Denmark) was injected subcutaneously in the forearm. Albumin diluent (Aquagen, ALK SQ) was injected in the opposite forearm and served as a negative control. The size of the skin prick test response was measured ($36 \pm 8 \text{ mm}^2$ for the allergic patients and no reactions for control subjects), and skin biopsy specimens were obtained after 6 hours. One punch biopsy specimen (4 mm diameter) was taken after achievement of local anesthesia from each patient and control subject.

The biopsy specimens were snap-frozen in liquid nitrogen and stored at -70°C until RNA preparation.

Preparation of cRNA and hybridization to microarrays

The biopsy specimens were homogenized for 60 seconds in Heidolph Diox 900 (Heidolph Instrument GmbH & Co, Schwabach, Germany). RNA was isolated by using the acid guanidinium thiocyanate, phenol-chloroform protocol^{E1} and purified with an RNeasy kit (Qiagen, Hilden, Germany). The quality of RNA samples was verified by means of agarose gel electrophoresis and by checking the A_{260}/A_{280} ratio.

RNA samples from duplicate biopsy specimens were pooled for each individual. cRNA was prepared from synthesized cDNA according to the Affymetrix manual (Affymetrix Inc, Santa Clara, Calif). Briefly, double-stranded cDNA was prepared by using the Life Technologies Superscript Choice system (Life Technologies, Paisley, United Kingdom) and an oligo(dT)24-anchored T7 primer by using 1.8 to 2 μg of total RNA. This was followed by *in vitro* transcription of biotin-labeled cRNA. Hybridization and visualization with the streptavidin-phycoerythrin assay was done according to the manufacturer's instructions, with a fluidic station and scanner.

Microarray data analysis

Signal values and arrays were background corrected and normalized by using the robust multiarray average. Differentially expressed genes were identified by analyzing genes that differed in expression by a factor of 1.5. A 2-tailed Student *t* test was used, and the results were corrected for multiple testing with the Benjamini and Hochberg false discovery rate. The analyses were performed with the Genesifter (VizX Labs, Seattle, Wash). A complete list of differentially expressed genes can be downloaded here (Table E1).

The IPA was used to organize differentially expressed genes into networks of interacting genes and to find modules of functionally related genes that correspond to pathways. The IPA consists of a global network that is based on reviewing and extracting mammalian gene interactions from review of more than 200,000 articles in 275 peer-reviewed journals. The findings for the IPA were manually curated and assigned to ontologic classes by content and modeling 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 done in a stepwise manner:

1. Differentially expressed genes are scored on the basis of the number of specific interactions found within the IPA global network. Focus genes were identified as those having direct or indirect interactions with other genes in the IPA database.
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 for the remaining genes to construct new networks. 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 those new networks.
3. A statistical algorithm based on the Fischer exact test was used to rank the networks. Identification of pathways was based on using canonical pathways in the IPA as templates for the differentially expressed

genes. Again using the Fischer exact test, the resulting pathways were ranked according to their correlation with the template in the IPA.

Nasal lavage fluid

After clearing excess mucus by means of forceful exsufflation, room temperature sterile saline solution (0.9% NaCl) was alternately aerosolized into both nostrils.^{E1} The nasal fluid was passively collected in a graded test tube until 7 mL was recovered. The fluid was centrifuged for 10 minutes at 1334g at 4°C. The supernatant was stored at -70°C until it was analyzed after 6 hours.

Quantitative real-time PCR analysis of *CCL4* in CD4⁺ T cells

Double-stranded cDNA was prepared by using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, Calif) and random hexamer primers supplied in the kit.

Quantitative PCR was performed with the ABI7900HT Sequence Detection System. Validated expression assays from Applied Biosystems were used: Hs00605740_g1 for *CCL4* and human *GUSB* (β -glucuronidase; FAM/MGB Probe, Non-Primer Limited) for the endogenous control, which also was predesigned by Applied Biosystems. The assays and TaqMan PCR mastermix (Applied Biosystems) were pipetted in a 384-well plate by using a liquid-handling robot (Biomek FX; Beckman Coulter Inc, San Diego, Calif). A total reaction volume of 10 μ L was used, and each sample was pipetted in triplicate for accuracy measurements.

Reactions are characterized by comparing threshold cycle (C_T) values. The C_T is a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold. Samples with high starting copy number show an increase in fluorescence early in the PCR process, resulting in a low C_T number, whereas a lower starting copy number results in higher C_T numbers. A C_T value for each sample was set at the exponential phase of the PCR.

Statistical analysis of real-time PCR data and *CCL4* expression

The relative standard curve method was used. Two standard curves with known amounts of total RNA were analyzed for the gene of interest (*CCL4*) and for the endogenous control gene (*GUSB*). For each unknown sample, the relative amount was calculated by using linear regression analysis from their respective standard curves, and relative and normalized *CCL4* expression values were obtained by subtracting the *CCL4* C_T value from the *GUSB* C_T value (ΔC_T). The significance of the different mean ΔC_T value between patients and control subjects was assessed by using a *t* test for equality of means, and a 2-tailed *P* value was obtained. The reference gene *GUSB* was equally expressed in the 2 groups.

REFERENCE

- E1. Benson M, Strannegard IL, Strannegard O, Wennergren G. Topical steroid treatment of allergic rhinitis decreases nasal fluid TH2 cytokines, eosinophils, eosinophil cationic protein, and IgE but has no significant effect on IFN-gamma, IL-1beta, TNF-alpha, or neutrophils. *J Allergy Clin Immunol* 2000;106:307-12.

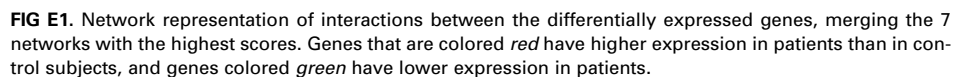


FIG E1. Network representation of interactions between the differentially expressed genes, merging the 7 networks with the highest scores. Genes that are colored *red* have higher expression in patients than in control subjects, and genes colored *green* have lower expression in patients.