

T Cell Phenotype in Allergic Asthma and Atopic Dermatitis

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Key Words

DNA array · Angiogenesis · Tissue remodeling ·
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Abstract

Background: T cells are key regulators of immunologic disease parameters. However, their contribution to the process of tissue remodeling is ill defined. In the present study, we investigated gene expression of allergy-characteristic, IL-4-rich T cell cDNAs to monitor expression of genes that might participate in the pathogenesis of allergic diseases. **Methods:** cDNAs of freshly isolated and restimulated CD4⁺ T cells from patients with allergic asthma (AA) or atopic dermatitis (AD) and healthy subjects were analyzed on Nylon membrane-based DNA arrays. Three patients were selected for an allergy-characteristic T cell phenotype with high IL-4 expression (AA) or IL-13 expression (AD). **Results:** Several gene families such as the TGF- β family, chemokines and chemokine receptors were found to be upregulated. Matrix metalloproteinases and their inhibitors were also found to be expressed in an enhanced manner. Furthermore, factors regulating tissue turnover such as fibroblast growth factors and neurotrophic as well as vasoactive factors were found to be expressed at a higher level in allergic patient

compared to healthy donors. **Conclusion:** The present study reveals and confirms genes relevant for allergy and highlights an approach to applying a DNA array technique for diagnostic discrimination of allergic diseases.

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Introduction

Allergy, some types of asthma and atopic dermatitis (AD) are T cell-dependent diseases induced by allergens. Allergic immune responses are characterized by the production of a distinct cytokine profile upon stimulation of allergen-specific T cells. In particular, IL-4, IL-5 and IL-13 are responsible for the initiation of allergic inflammation. Normal immune responses to antigen generate an equilibrated cytokine pattern containing increased IFN- γ and IL-2. Accordingly, specific T cells, mostly of the CD4 phenotype, represent the key regulatory immune cells in both allergic and normal conditions. Therefore, the current study focuses on the activity of peripheral blood CD4⁺ T cells.

AD and allergic asthma (AA) are characterized by an organ-specific inflammation in response to allergen. The T cell phenotype is known to correlate with the clinical

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profile as demonstrated repeatedly in asthmatic inflammation *in vivo* [1], *ex vivo* [2, 3] and *in vitro* [4]. Also, AD and the development of eczema depend on the activity of a subset of activated T cells bearing the skin homing receptor, cutaneous lymphocyte antigen [5–8]. However, other effector functions contribute to the pathogenesis of allergic disease locally and systemically.

Chemokines and their receptors have been shown to play an important role in asthma [9, 10] by attracting inflammatory cells such as neutrophils and eosinophils to the tissue [9–12]. Various local cells such as monocytes, basophils, eosinophils, neutrophils, NK cells, dendritic cells, endothelial cells and keratinocytes can also produce chemokines. In this respect, T cells play a dual role by inducing and producing chemokines [10]. The migration of lymphocytes from the bloodstream into sites of inflammation requires not only adhesion and chemotactic factors, but also the ability to penetrate the basement membrane. Lymphocytes require matrix metalloproteinase (MMP) expression to penetrate the endothelial lining and basement membrane [13]. Infiltrated T cells can directly cause tissue damage [14] and support neurogenic inflammation [15], fibrosis and angiogenesis as well as endothelial alterations.

In asthma, airway wall remodeling involves a number of changes including increased vascularity, vasodilation and microvascular leakage [16]. The pathogenetic role of neovascularization in AD is ill defined. However, increased expression of vascular endothelial cell growth factor has been described in acute contact dermatitis models [17].

Together, these considerations underline that many allergic disease parameters are influenced by T cells and their state of activation and are important to understand the pathogenesis of allergic diseases. DNA array technology provides an important tool to define these parameters. Several approaches were previously described [18]. DNA array analysis of nasal biopsies revealed increased IgA expression [19]. Studies analyzing the peripheral blood mononuclear cells of AD patients indicate pronounced Th1 cell activity [20]. Both AA and AD patients show increased IL-1R and IL-6 expression [21] and other genes which are essential for T-B cell interactions [21]. Lung tissue analysis of an experimental asthma model in monkeys by DNA array revealed increased expression of chemokines (e.g. MCP-1 and MCP-3) and tissue-modulating factors following allergen or IL-4 exposure [22]. Here, we demonstrate that gene expression of activated, peripheral blood CD4⁺ T cells of allergic patients is different from that of healthy donors.

Patients and Methods

Patients

Patients were selected on the basis of disease phenotype, showing either AD or AA. All asthmatic patients met the American Thoracic Society definitions of asthma [23]. At the time of the study, AA patients had stable bronchial asthma, were not in acute respiratory distress, had no evidence of chest infection and had never suffered from AD. AD patients fulfilling the criteria of Hanifin and Rajka [24] were selected for the study and did not show any symptoms of asthma or allergic rhinitis. The AD patients were polyallergic and reacted positively on a cutaneous test to at least three allergens. AD patients showed specific IgE antibodies on a radioallergosorbent test (class >2) and $5,150 \pm 1,114$ ng/ml (mean $\pm$ SEM) serum IgE. None of the patients had had systemic immune-suppressive treatment at least 3 weeks before peripheral blood was taken. Healthy subjects had never suffered from AA or AD and did not show any signs of other allergic or infectious disease. For DNA array analysis, 80 ml of blood was taken from the patients using a vacuum container containing heparin (VacutanerTM, Becton Dickinson, Plymouth, UK). Three AA and three AD patients were selected on the basis of their high IL-4 mRNA expression in CD4⁺ T cells, which was tested using RT-PCR.

Cell Culture

Peripheral blood mononuclear cells were isolated from the blood of healthy donors and AD or AA patients. The interphase cells of density gradients were washed and CD4⁺ T cells were purified using CD4-Dynal magnetic beads and Detach-a-Bead antibodies (Dynal, Hamburg, Germany) as previously described [25]. The cell purity was >95%, as initially tested by flow cytometry.

All cultures were carried out in RPMI-1640 supplemented with 1 mM *L*-glutamine, sodium pyruvate, nonessential amino acids, 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (all from Life Technologies, Basel, Switzerland). For RNA isolation, 5×10^6 Dynal-bead (Dynal)-purified CD4⁺ T cells were lysed with RNeasy lysis buffer (Qiagen, Hamburg, Germany) following overnight stimulation using plate-bound anti-CD3 (OKT3) and anti-CD28 (15E8). The OKT3 hybridoma was purchased from ATCC (Manassas, Va., USA) and supernatants were purified using affinity chromatography. The 15E8 monoclonal antibody was purchased from CLB (Amsterdam, The Netherlands). Of note, unstimulated cells were not suitable for gene expression profiling, resulting in very few and very low-intensity hybridizations [18].

cDNA Synthesis

RNA was isolated using the RNeasy Mini Kit (Qiagen) and eluted in 100 μ l of H₂O. The RNA was precipitated with sodium acetate and ethanol, washed with 70% ethanol and resuspended in 11 μ l of H₂O. The RNA was mixed with 4 μ l of human array-specific primers (R&D Systems, Abingdon, UK) and incubated in a thermal cycler at 90°C for 2 min. The temperature was ramped down to 42°C in 20 min. For reverse transcription, the following components were added: 6 μ l of 5 $\times$ reverse first-strand buffer (Gibco BRL, Basel, Switzerland), 2 μ l of 0.1 M DTT (Life Technologies), 20 U of RNase inhibitor (Roche, Mannheim, Germany), 1 μ l of dCTP, dGTP and dTTP (each 10 mM; Perkin Elmer), 5 μ l of [α -³²P]-dATP (0.4 MBq/ μ l; Hartmann, Braunschweig, Germany) and 1 μ l of Superscript II RNase H reverse transcriptase (Life Technologies). The mixture was incubated at 42°C for 3 h and filled up to 100 μ l with H₂O. The

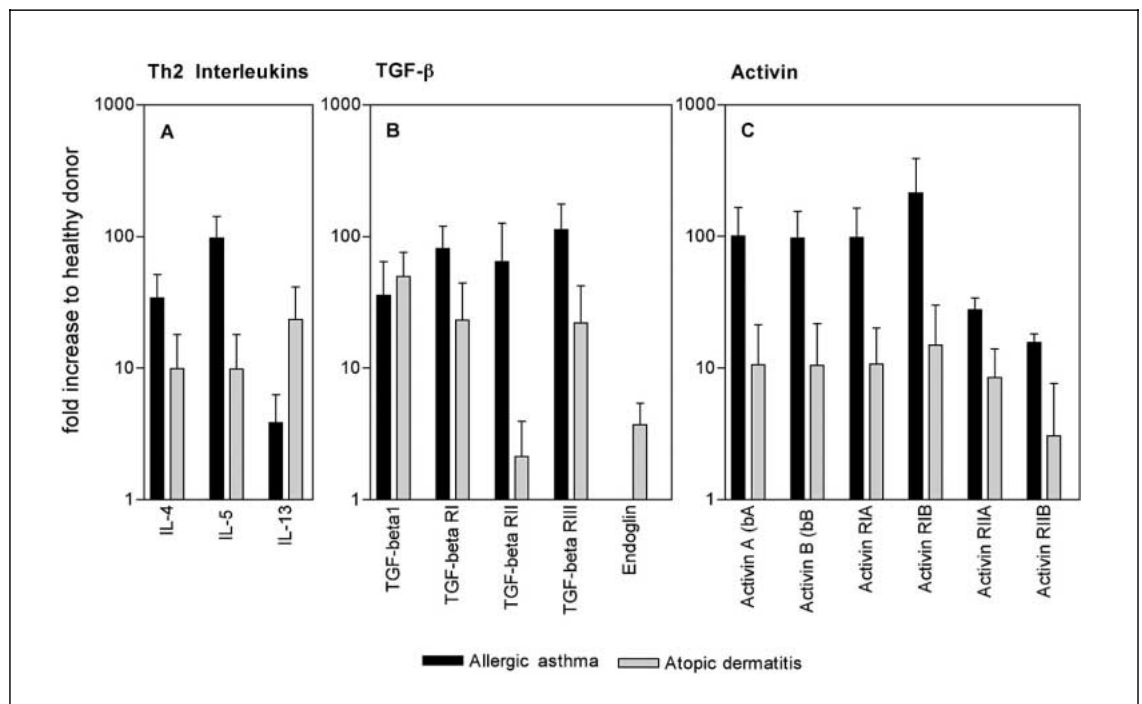


Fig. 1. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. TGF- β and the activin group are functionally assembled and also contain TGF and activin receptors. Error bars indicate the standard error of the mean.

cDNA was purified with the QIAquick Nucleotide Removal Kit (Qiagen) and eluted in 100 μ l of H₂O. An aliquot of 1 μ l was measured in a scintillation counter to estimate the percentage incorporation of labeled nucleotides in the cDNA.

CD4⁺ T cell cDNA probes prepared from patient's blood were hybridized in parallel with cDNA probes from healthy donors on a human cytokine expression array membrane (R&D) following the instructions of the manufacturer. Following hybridization, membranes were washed for 20 min with 30 ml of a 0.1 $\times$ SSPE, 1% (w/v) SDS solution. Subsequently, moistened arrays were sealed in plastic bags and exposed for 24 h on phosphorimager screens (Fuji Film, Tokyo, Japan). The screens were analyzed using the FLA 3000 phosphorimager system (Fuji) and evaluated with the AIDA program (Raytest, Urdorf, Switzerland). Confirmation of gene expression results was performed by real-time PCR as previously described [26].

Data Management

To eliminate artifacts, low-intensity spots (cutoff: 1% of housekeeping gene) were excluded from further consideration. To compare the intensity of spots containing different membranes, housekeeping genes are commonly used. From a selection of housekeeping genes, correction values were determined and used to correct the hybridization intensity of patients. As a cutoff value for unchanged expression, a factor of 2 was chosen, in line with previously published studies [27]; however, conclusions are generally based on at least 10-fold changes. Of note, lower cutoffs have been published (e.g. 0.7–1.3 [28]

and 1.2 [29]). Positive values indicate an increase in gene expression in the patient. Values below a ratio of 1 were negatively inverted to demonstrate a decrease of gene expression in the patient.

Results

The DNA array technology applied in the current study represents a semiquantitative method to monitor gene expression of CD4⁺ T cells. The validity of the results is supported by expression changes not only in one gene, but also in entire functional groups or gene families.

Gene Expression of IL-4 and IL-13 in AA and AD

The interleukins are well-characterized key factors in the regulation of allergic diseases and were therefore selected as markers for Th2-biased T cell populations. IL-4 and IL-13 mRNA expression was used as the patient selection criterion and array IL-4 expression confirmed successful selection (fig. 1A). This selection was chosen in order to focus on typical allergic diseases, which are associated with high IgE production, which in turn is associat-

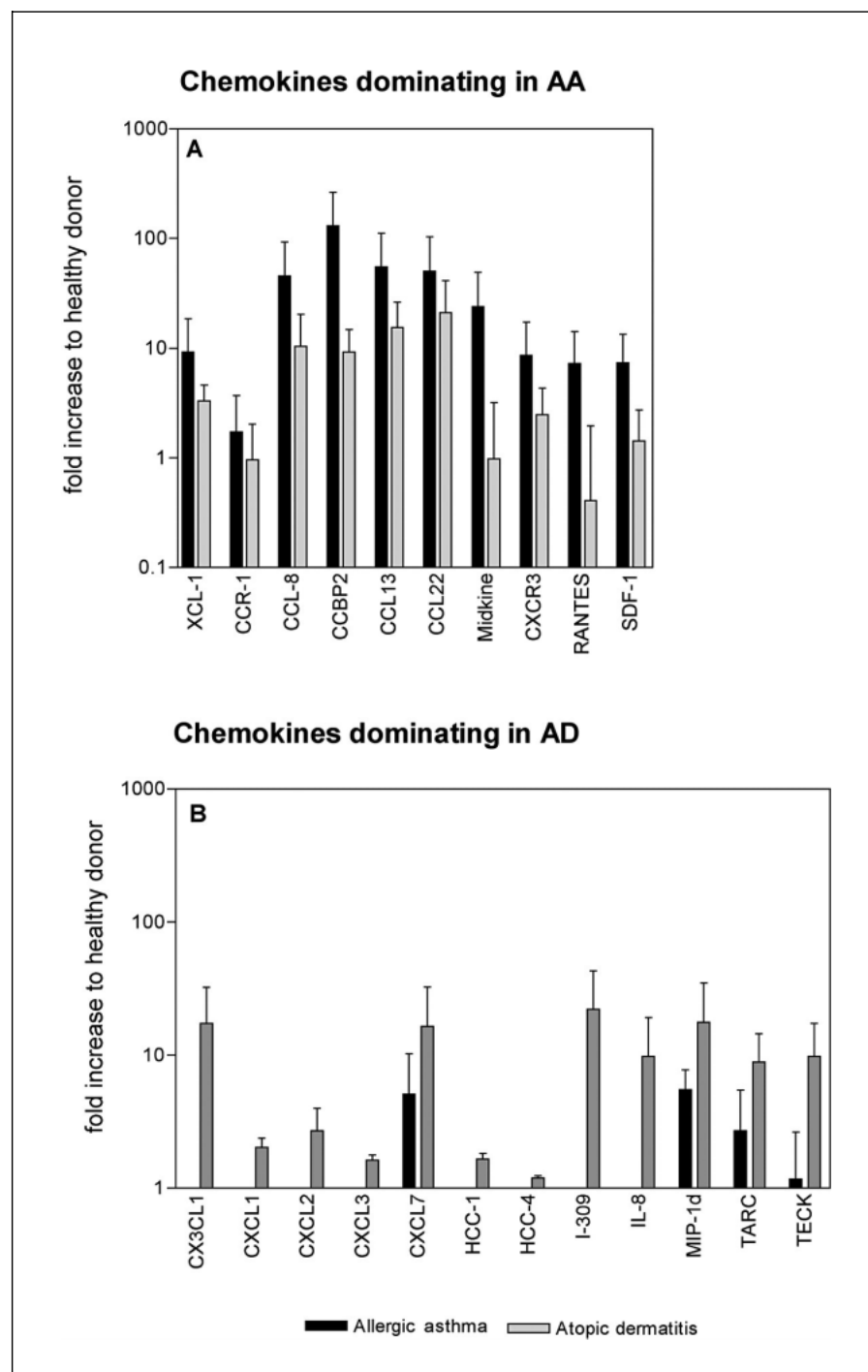


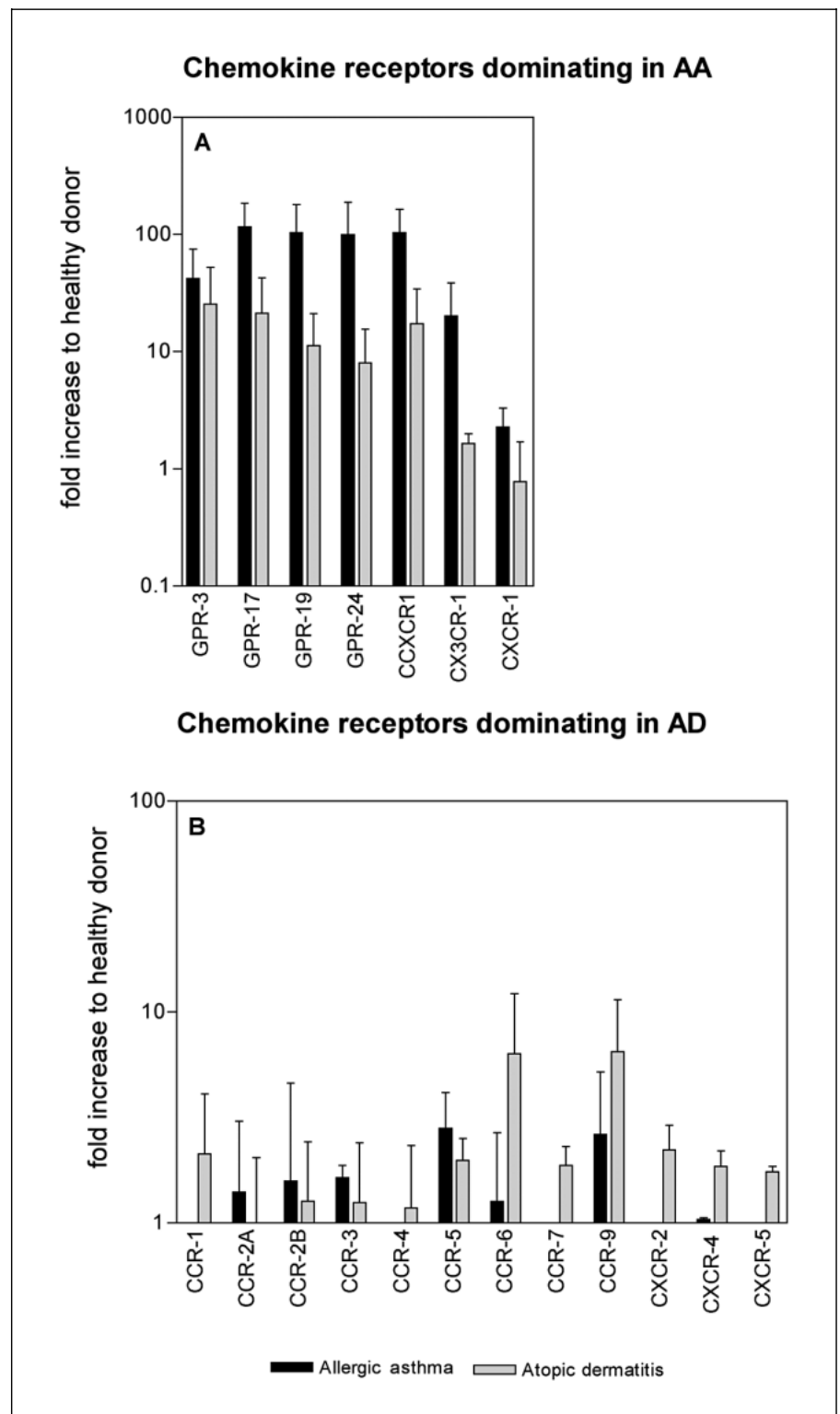
Fig. 2. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. The chemokines were grouped into those which were differentially expressed in AA (**A**) and AD patients (**B**) in comparison to healthy control subjects. Error bars indicate the standard error of the mean.

ed with high IL-4 expression by T cells. For AD, IL-13 expression was used as the entry criterion, as it is meant to play an important role in AD [30, 31]. Of note, IFN- γ was also detectable, but not different compared to healthy donors.

Expression of Genes of the TGF- β Family

TGF- β -related genes have been recognized as crucial mediators for peripheral tolerance [32], but they also play an important role in the regulation of tissue growth or fibrosis. Expression of the members of the TGF- β family

Fig. 3. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. The chemokine receptor group contains genes (GPRs) which are only assumed but not proven to be chemokine receptors. The chemokine receptors were grouped into those which were differentially expressed in AA (**A**) and AD patients (**B**). Error bars indicate the standard error of the mean.



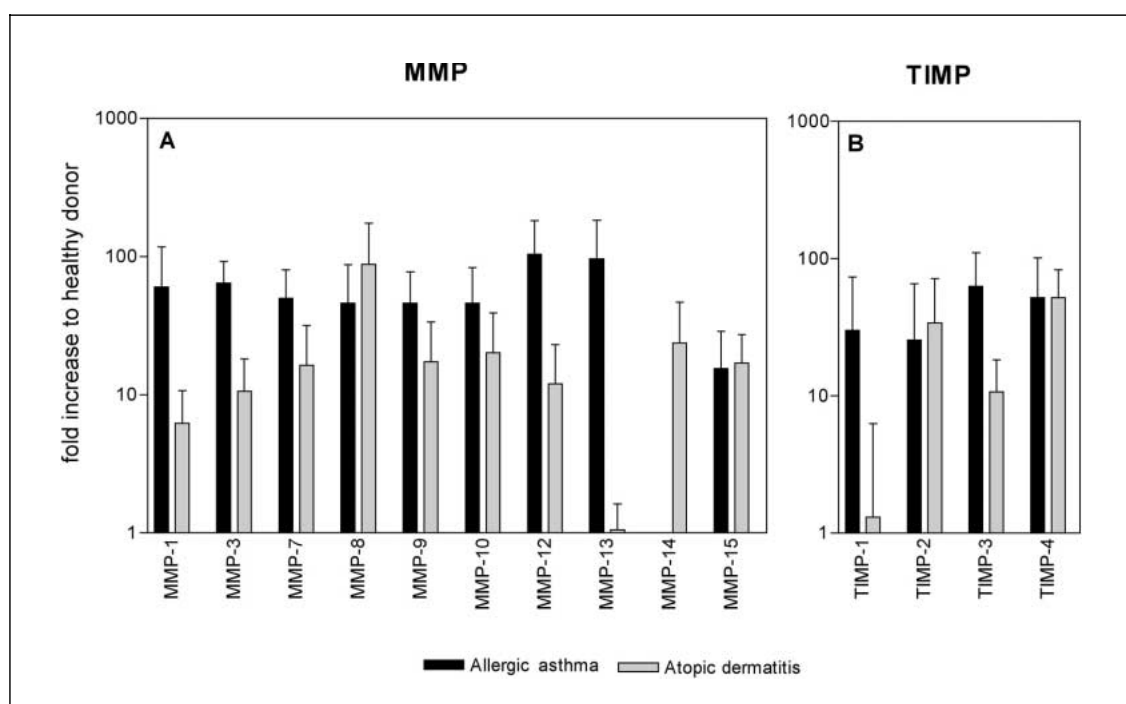


Fig. 4. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. MMPs and TIMPs are shown in **A** and **B**, respectively. Error bars indicate the standard error of the mean.

was at least 10 times higher in AA and AD patients. Remarkable exceptions were TGF- β RII and endoglin (CD105), which were only moderately upregulated or only increased in AD patients, respectively (fig. 1B). This result was confirmed by flow cytometry (data not shown). Interestingly, activin and the activin RI/RII were more dramatically (100-fold) upregulated in AA compared to AD patients (fig. 1C).

Expression of Genes for Chemokines and Chemokine Receptors

The chemokine family represents a complex group of genes, and their expression creates an expression profile which is likely to differ from disease to disease, depending on the target organ to which cells are supposed to migrate. The infiltration pattern and the composition of infiltrates is characteristic for different diseases and also for disease activity.

Interestingly, chemokine expression showed some clear-cut differences between AA and AD patients: CX3CL1, I-309, IL-8, TECK, midkine and RANTES were upregulated by at least 10-fold in AD patients in comparison to healthy controls, whereas the AA group did

not show any increased expression of these genes. In contrast, midkine and RANTES were only found to be increased in the AA group (fig. 2A). Surprisingly, many chemokine receptor genes were only marginally upregulated, except for a group of G protein-coupled receptors (GPRs), which are not yet characterized functionally (GPR3, 17, 19, 24, CCXCR1). GPR5, 17, 19 and 24 showed a more dramatic upregulation in AA patients (fig. 3A) in relation to normal donors than in AD patients (fig. 3B).

Genes for MMPs and Tissue Inhibitors of Metalloproteinase

MMPs are assumed to be essential for transmigration through extracellular matrix components. The MMP genes are not well known as T cell products, but may – as with chemokines – reflect disease-specific expression profiles, depending on the target tissue which needs to be transmigrated.

The group of MMPs generally showed clearly increased expression in both diseases. However, MMP-13 was more strongly expressed in AA patients, while MMP-14 was more strongly expressed in AD patients (fig. 4A).

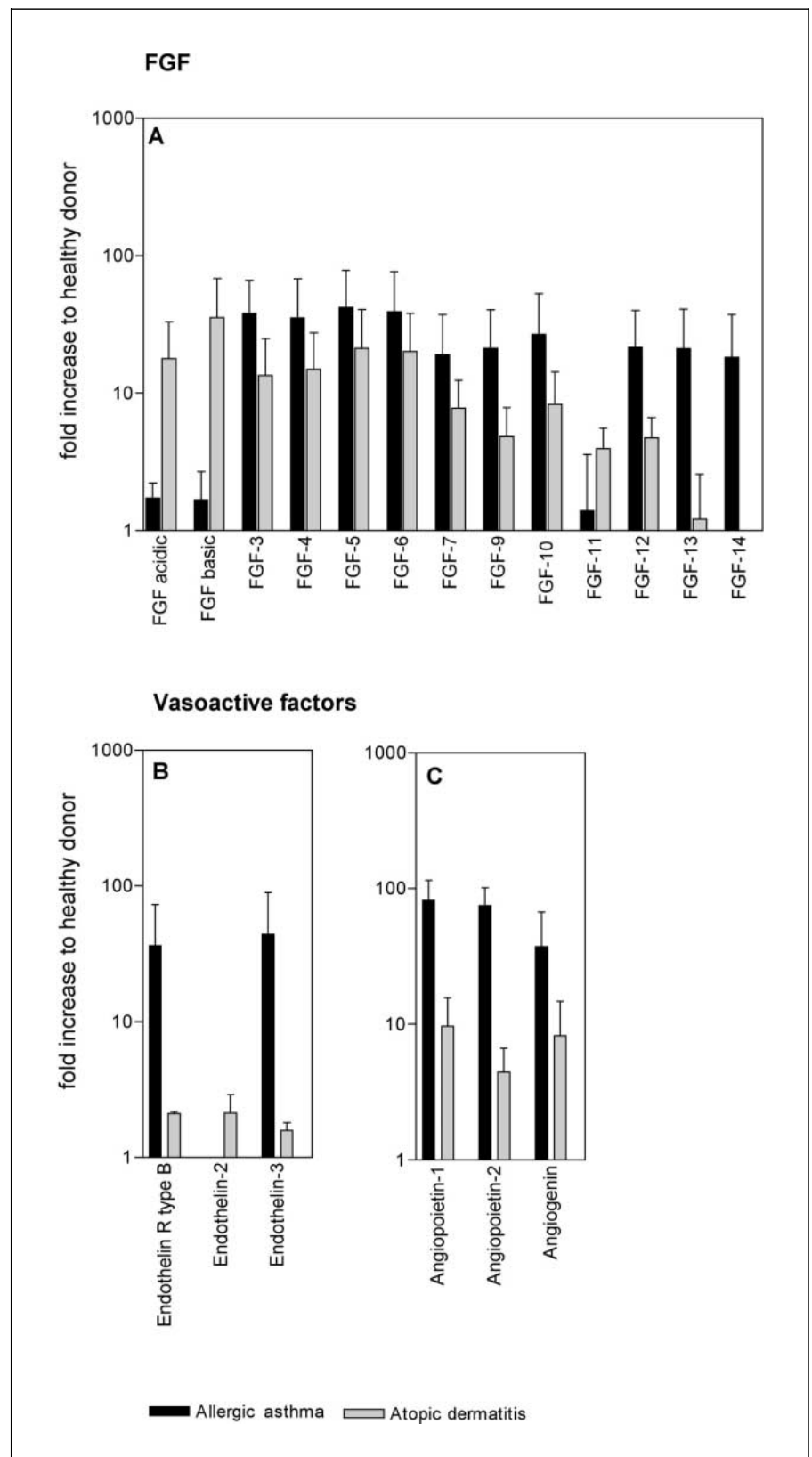
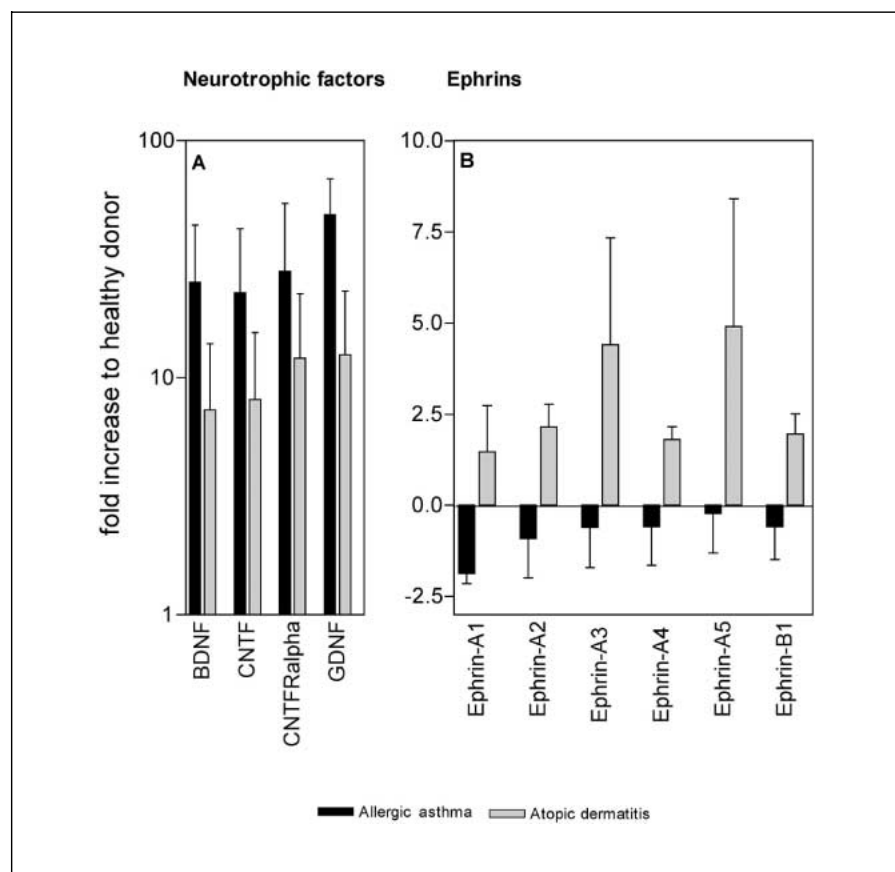


Fig. 5. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. The FGF family is shown (**A**), and vasoactive factors such as endothelin (**B**) and angiopoietin or angiogenin (**C**) are also depicted. Error bars indicate the standard error of the mean.

Fig. 6. Bar charts of AA (black bars) and AD (gray bars) patients, showing the difference in hybridization intensity of patients in terms of the -fold difference relative to healthy controls, normalized for housekeeping genes. Factors involved in neuronal regulation (**A**) and the ephrin family members (**B**) are shown. Error bars indicate the standard error of the mean. CNTF = Ciliary neurotrophic factor; GDNF = glial cell line-derived neurotrophic factor.



The tissue inhibitors of metalloproteinases (TIMPs) were also more strongly expressed in AA patients (fig. 4B).

Genes for Fibroblast Growth Factors

Fibrosis, keratinocyte growth and smooth muscle cell expansion are regulated by fibroblast growth factor (FGF) gene family members, but are rarely described as T cell-released factors.

Profibrogenic factors were upregulated in AD and AA patients in comparison to healthy donors (fig. 5A). However, FGF acidic and FGF basic were increased only in AD patients, while FGF-13 and -14 were upregulated only in AA patients.

Genes for Vasoactive Factors

The allergic reaction is characterized by pronounced vascularization of the tissue and increased extravasation of inflammatory cells following allergen contact. Endothelin-3 was increased in AA but not in AD patients (fig. 5B). Angiogenic factors were found to be increased in AA and to a lesser extent in AD patients (fig. 5C).

Genes for Neurotrophic Factors and Ephrins

The hyperreactibility of the asthmatic lung is caused by a process called neurogenic inflammation, which is characterized by increased innervation of the lung, which in turn depends on neuronal growth and survival factors.

Four neurotrophic factors were found to be more highly expressed (10-fold) in AD and AA patients, of which brain-derived neurotrophic factor (BDNF) was previously suggested to be an important T cell-released factor increasing neurogenic inflammation in AA (fig. 6A). Some genes of the group of ephrins and their receptors showed a large abundance of mRNA and a clear decrease in all three AA patients, although the decrease was only about 2-fold (fig. 6B).

Discussion

Allergic diseases are systemic diseases affecting large populations of immune-regulatory, antigen-presenting and effector cells. T cells differentiate upon antigen con-

tact into mature cells, expressing a defined subset of cytokines and other mediators [27]. Allergic diseases are characterized by T cell populations secreting the Th2 cytokines IL-4, IL-5 and IL-13 following activation. The T cell phenotype is known to correlate with clinical profile as demonstrated repeatedly in asthmatic inflammation *in vivo* [1], *ex vivo* [2, 3] and *in vitro* [4]. The IL-4 secretion of T cells is crucial for the switch of IgM to IgE of allergen-specific B cells [33–35]. Therefore, CD4+ T cells are generally accepted to be key regulators of allergic diseases. The present study describes the expression profiles of activated CD4+ T cells of AA and AD patients, who were selected on the basis of high IL-4 and IL-13 expression, respectively.

The gene expression of TGF- β was found to be increased in AA and AD patients, which confirms previous findings describing TGF- β as a cytokine which is regulated by IL-4 [36, 37]. The group of activin-related genes was also more dramatically upregulated in AA patients. Both TGF- β and activin are well known for their lymphocyte-suppressive capacity; however TGF- β is also an important inducer of extracellular matrix production of fibroblasts [38, 39]. Thus, increased TGF- β expression could indicate an increased suppressive potential of the lymphocytic compartment, but could also support tissue fibrinogenesis. Interestingly, TGF- β RII was only increased in AA and not in AD patients. An interesting exception is represented by endoglin, which was confirmed by alternative techniques and which appears to play an important role in TGF- β -dependent lymphocyte regulation [Schmidt-Weber, C.B., unpubl. results]. The importance of SMAD2/3-inducing ligands TGF- β and activin lies particularly in their potency as inducers of fibrosis, which is one of the major complications of AA. T cells may contribute to or even trigger the TGF- β -dependent induction of extracellular matrix deposition. However, TGF- β is also known as an important suppressor in T-T cell [40, 41] and T cell-monocyte interactions [32]. Current investigations in our laboratory are focusing on activin, which may help to find target molecules for interception in the inflammatory and fibrotic process in lung tissues.

Increased chemokine expression could be expected, because T cells migrate intensively into inflamed lung or skin. The expression is anticipated to be different between AA and AD, since target tissues and the type of inflammation are different. However, receptor expression showed only minor differences, such as upregulated CX3CR-1 for AA patients. CX3CR-1 is the receptor for fractalkine and is known to act as a coreceptor for HIV envelope protein for CD4+ T cells [42]. Greater increases were observed for

several GPRs with unknown functions (GPR3, 17, 19, 24, CCXCR1), which showed the largest increases in AA patients. This result shows that although no chemokine profile for lung- or skin-infiltrating T cells has been described yet, it might nonetheless exist and consist of receptors/chemokines which currently belong to uncharacterized groups of genes.

Chemokine expression by T cells once they have arrived at the site of inflammation will determine the composition of newly recruited inflammatory cells. CX3CL1 (fractalkine), I-309 (SCYA-1), IL-8 and Teck (SCYA-25) were found to be increased in AD. IL-8 is already known to be increased in the skin of AD patients [43], and serum levels are correlated with disease severity [44]. Interestingly, CX3CL1, I-309 and Teck are assumed to act on monocytes or dendritic cells [45].

Midkine and RANTES were selectively upregulated in AA patients. RANTES is well known to be increased in serum in acute asthma attacks [46], and anti-RANTES treatment reduces peribronchial inflammation in experimental asthma models [47]. Although midkine is known to be expressed by T cells [48], its contribution to lung disease is unclear. Interestingly, the activity of midkine is not restricted to chemotaxis, but may also positively regulate neuronal growth [49], which might contribute to airway hyperresponsiveness. The differences in the ability of T cells to produce chemokines indicates that allergic diseases, and in particular AD, are characterized by attraction of other cells. In fact, AD is known to be rich in T cell infiltrates. Further experiments are now required to analyze which cells are attracted by the given chemokine pattern.

MMPs and TIMPs are well-known products of macrophages, but T cells are also known to express these genes, which allows them to transmigrate through the extracellular matrix [50]. Current data show clear expression results for multiple members of the MMP family. It is tempting to speculate that T cells targeting different tissues express different MMPs depending on the composition of the extracellular matrix. Of note, inhibitors of MMP can prevent the onset of asthma in experimental asthma models [51].

Expression profiles showed a clear upregulation of most FGF family members, except FGF acidic and FGF basic, which were not upregulated in AA, and FGF-13 and FGF-14, which were not upregulated in AD. Fibrosis is a complication of AA and AD and is caused by the inflammation-induced deposits of extracellular matrix components at the basal membranes. FGF-2 and -4 can directly induce collagen-1 synthesis [52, 53]. FGF-7 is

also known as keratinocyte growth factor [54] and is supposed to be important for the growth of epithelial cells in general. FGF-13, which was only upregulated in AA, is known to induce cell growth of lung fibroblasts and aortic smooth muscle cells but has no effect on dermal vascular endothelial cells [55]. As suggested for TGF- β and activin, FGF production may be an important factor for the induction or perpetuation of the fibrotic process.

Angiogenic and vasoactive regulators were more prominently upregulated in AA patients. The important role of neovascularization and the role of angiopoietin-1 as well as angiogenin were previously described for AA [56]. Endothelins are a family of peptide mediators that have a number of biological properties, including the ability to act as bronchoconstrictors and vasoconstrictors of isolated airways and vessels, and are therefore important mediators of asthma pathophysiology [57]. Neurotrophic factors were observed to be upregulated in AD and AA patients, of which BDNF was previously described to be a potential T cell mediator of neurogenic inflammation in asthma [58–60]. These neurogenic factors are likely to be involved in lung hyperreactivity. Along with vasoactive and fibrinogenic factors, T cells appear to have a broader role in tissue remodeling than has been discussed so far.

The group of ephrins showed a significant reduction in their gene expression only in AA patients and are currently not known to be expressed by peripheral blood CD4+

cells. To confirm this finding, we performed real-time PCR with ephrin A1 and confirmed the expression of this gene. Although little is known about this gene family, especially in the field of immunology, it is remarkable that receptors for ephrin A1 are expressed in lung tissue. Therefore, ephrins represent a group of genes allowing interaction of the immune system with lung tissues. Currently, work at the Swiss Institute of Allergy and Asthma Research (SIAF) is being performed to characterize the nature of this interaction.

The current study clearly shows that T helper cells express several gene families, which may contribute to tissue remodeling in allergic inflammation. These factors were more dramatically upregulated in AA patients, where tissue remodeling is more pronounced than in AD. The current study describes several gene groups which have so far not been investigated in the context of T cell biology and give new insights into the pathophysiology of allergic diseases.

Acknowledgments

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