

Differential Expression of Genes Involved in Skin Homing, Proliferation, and Apoptosis in CD4 + T Cells of Patients with Atopic Dermatitis

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CD4 + T cells play a critical role in allergic diseases, both in the affected tissue as well as systemically. Our objective was to investigate the *in vivo* activation state of peripheral blood CD4 + T cells of atopic dermatitis (AD) patients by analyzing gene expression profiles of unstimulated CD4 + T cells. mRNA samples from blood CD4 + T cells, isolated from five AD patients and seven healthy controls (HC), were analyzed using oligonucleotide arrays. Differentially regulated genes were validated by quantitative PCR (Q-PCR) in a larger group of patients with AD, in a group of patients with allergic asthma (AA), and HC subjects. In addition, “typical” T helper type 1 (Th1)- and Th2-related genes were analyzed by Q-PCR. Microarray analysis revealed differential expression of 52 genes in AD patients. Q-PCR confirmed several differentially regulated genes in AD, including CCR10, CRTH2, C-JUN, and NR4A2. Two groups of genes with highly correlating gene expression levels involved in tissue homing and proliferation or apoptosis, respectively, were identified. No marked differences were found in gene expression levels of typical Th1 or Th2 genes in AD or in AA patients. This study demonstrates that peripheral blood, unstimulated CD4 + T cells in AD patients show differentially expressed genes involved in tissue homing, proliferation, and apoptosis. No marked expression differences of “typical” atopy genes were found.

Key words: allergic asthma/atopic dermatitis/atopy/CCR10/CCR4/CRTH2/microarray/TARC
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Atopic dermatitis (AD) and allergic asthma (AA) are common, chronic, allergic inflammatory diseases. AD is characterized by chronically persistent, as well as recurrent, pruritic, erythematous skin lesions. AD skin lesions typically show infiltration of immune cells, particularly CD4 + T cells, in the dermis and epidermis, suggested to induce apoptosis of keratinocytes, resulting in spongiosis, which is the histological hallmark of eczema (Trautmann *et al*, 2000). CD4 + T cells have also been shown to play a pivotal role in the initiation and maintenance of inflammation in the asthmatic airways (Larche *et al*, 2003).

CD4 + T cells can be divided into T helper type 1 (Th1) and Th2 subsets, according to their cytokine secretion profile. Atopic diseases have been associated with a systemic Th2-cytokine predominance, contributing to high IgE levels and blood eosinophilia. However, investigating T cell populations in lesional AD skin has revealed a more complex picture than suggested by the classic Th1/Th2 paradigm,

characterized by a Th2 profile in acute eczema and a Th1 profile in chronic eczema lesions (Grewe *et al*, 1998).

The restricted expression of cytokines by polarized T cell subsets arises from the differential expression of transcription factors. GATA-3 is expressed during Th2 differentiation via pathways that probably involve the interleukin (IL)-4-dependent activation of signal transducers and activators of transcription (STAT-6). In addition, GATA-3 transactivates the expression of genes for IL-5 and IL-13 directly in Th2 cells. T-BET is expressed exclusively in Th1 cells and its expression is mediated mainly by a STAT-4-dependent pathway inducing production of IFNG (Rengarajan *et al*, 2000).

Different subpopulations of CD4 + T cells have been shown to manifest preferential migration into tissues, such as skin *versus* lung. A preferential skin-homing memory T cell subset was characterized by expression of cutaneous lymphocyte antigen (CLA) (Picker *et al*, 1990). Compared with peripheral blood, CLA + memory T cells were shown to be highly enriched (expressed by 80%–90%) in skin, but not in other organs, like the lungs. Chemokines, a protein superfamily of structurally related small secreted proteins, have been shown to regulate the trafficking of distinct leukocyte subsets into peripheral tissues (Homey and Zlotnik, 1999). CC chemokine receptors (CCR4 and CCR10) have recently been suggested to play a sequential role in the recruitment pathway of skin-homing T cells in AD

Abbreviations: AA, allergic asthma; AD, atopic dermatitis; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CLA, cutaneous lymphocyte antigen; FDR, false discovery rate; HC, healthy control; IL, interleukin; LSS, Leicester sign score; Q-PCR, quantitative PCR (“real-time” RT-PCR); TARC, thymus and activation regulated chemokine; Th1, T helper type 1

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patients (Reiss *et al*, 2001). Furthermore, chemokines TARC (CCL17) and CTACK (CCL27) have been found to be increased in the serum of patients with AD, but not with AA (Hijnen *et al*, 2004).

To achieve a better understanding of the underlying processes in atopic diseases, several molecular approaches are currently being utilized. Genetic studies have identified several genes related to allergic diseases. In AD linkage was demonstrated to the high-affinity IgE receptor (FCER1B) and to genes on chromosomes 1q21, 17q25, and 20p, previously found to be related to psoriasis (Folster-Holst *et al*, 1998; Cookson *et al*, 2001). ADAM33 was recently found to show linkage to AA, although its role in AA largely remains unknown (Van Eerdewegh *et al*, 2002). Despite the identification of several atopic disease-related susceptibility loci, specific genes that play a causative role in disease progression still have to be identified. Genomics represents a promising approach that is being increasingly used to unravel the pathogenesis of complex diseases by the observation of gene expression. Much effort has been put in the investigation of gene expression in differentiated T cells of various types. Most data have been obtained by using established T cell lines, or at least T cells that have been cultured *in vitro* for some time (Nagata *et al*, 2003; Wohlfahrt *et al*, 2003). Relatively few experiments have addressed the question of gene expression of unstimulated peripheral blood T cells in atopic diseases (Matsumoto *et al*, 2002).

The aim of this study was to investigate the *in vivo* activation state of peripheral blood CD4⁺ T cells in AD, by comparing differential gene expression profiles of unstimulated peripheral blood CD4⁺ T cells of AD and AA patients with gene expression patterns of HC.

Results

In order to investigate differential gene expression in CD4⁺ T cells from AD patients, we compared gene expression profiles of CD4⁺ T cells from AD patients with healthy control (HC). Using SAM software, 52 genes with a false discovery rate (FDR) of less than 50% were found to show at least 1.5-fold increased or decreased expression in AD compared with HC (Table I). Expressed sequence tags, hypothetical genes, and genes with an unknown function were excluded from this list. From the list of genes found to be differentially expressed, 20 demonstrated a higher expression in AD compared with HC. Furthermore, 32 genes were found to have a decreased expression in AD compared with HC. To confirm gene expression changes observed by microarray analysis, quantitative PCR (Q-PCR) analysis was performed on a selection of genes in larger groups of AD patients and HC. Furthermore, Q-PCR analysis was extended to a group of patients with AA (without AD).

For Q-PCR validation we chose 10 genes that showed a relatively low Q-value and a high fold change (TYMS, FUCT-VII, NR4A2, TNFAIP3, ZNF331, GADD45A, JUNB, DUSP2, ZFP36, CXCR4, C-JUN), two genes (CRTH2 and CCR10) that have been suggested to be involved in the pathogenesis of AD (Iwasaki *et al*, 2002; Vestergaard *et al*, 2003), and one gene (FCER1A) that was unexpectedly showing

differential regulation in the microarray analysis. Q-PCR analysis showed statistically significant increased expression of CCR10 (3.0-fold) and CRTH2 (1.8-fold), and decreased expression of NR4A2 (4.9-fold), GADD45A (2.4-fold), TNFAIP3 (3.4-fold), JUNB (2.5-fold), and C-JUN (5.7-fold) in AD patients. But, Q-PCR analysis could not confirm statistically significant differential expression for FUCT-VII, FCER1A, TYMS, DUSP2, ZFP36, CXCR4, and ZFP331.

None of the 13 genes studied by Q-PCR analysis were found to be differentially expressed in the AA patient group. By Q-PCR no differential expression of FCER1A was found.

Because AD and AA are classically defined as Th2-mediated diseases, and the microarray analysis did not reveal differential expression of genes known to be associated with a Th2 phenotype, a selection of Th1- and Th2-related genes was analyzed by Q-PCR. We studied expression of transcription factors controlling Th1 and Th2 development (STAT-4, T-BET, STAT-6, and GATA-3), Th1 and Th2 cytokines (IFN- γ , IL-4, IL-5, IL-10, and IL-13), and CCR4 (which has been described to be preferentially expressed on Th2 cells (Bonecchi *et al*, 1998) (Table II). By Q-PCR analysis no statistically significant differential expression of typical Th1 or Th2 transcription factors and cytokines was found. CCR4 showed a statistically significant 2.3-fold increased expression in AD patients compared with HC. Th2-cytokines IL-4, IL-5, and IL-13 were expressed below the detection level.

Correlation coefficients between gene expression levels, determined by Q-PCR, of a selection of genes (showing at least 1.5-fold increased or decreased expression) are listed in supplementary Table S3. Two groups of genes with highly correlating gene expressions were found (Fig 1). The first group includes genes, with increased expression in AD compared with HC, that are suggested to be important for homing of T cells to the skin: FUCT-VII, CRTH2, and CCR10. Gene expression levels of a second group of genes, including NR4A2, CJUN, JUNB, GADD45A, TNFAIP3, and DUSP2, showing decreased expression in AD compared with HC, were also found to correlate highly (Fig 1B).

Discussion

AD and AA are systemic diseases in which CD4⁺ T cells play a pivotal role (Kay, 2001; Leung *et al*, 2004). Much effort has been put in the investigation of established T cell lines, or primary T cells that have been cultured *in vitro*. The limited amount of RNA that can be retrieved from primary T cells has been a pitfall in the investigation of gene expression in primary cells (Wohlfahrt *et al*, 2003). But, the introduction of mRNA amplification has enabled gene profiling of primary cell populations, with the possibility to understand the physiologic status of these cells *in vivo*. Using mRNA amplification, Matsumoto *et al* (2002) recently showed differential gene expression (using fluorescent differential display) in CD3⁺ T cells from children with AD or AA. In their study, 15 genes being differentially expressed in both AD and AA children were described. However, no genes exclusively regulated in AD patients were found. By additional RT-PCR analysis, on genes known to be involved in atopy, increased expression of CCR4, decreased expression of

Table I. Differentially expressed genes in atopic dermatitis compared to healthy controls

Abbreviation	Genbank	Fold change	q-value (%)
<i>Increased</i>			
TYMS	NM_001071	2.7	12.9
FUT7	NM_004479	1.9	12.9
S100A11	NM_005620	1.6	12.9
ANX2P2	—	1.9	23.0
FCER1A	NM_002001	2.0	27.9
CST3	NM_000099	1.7	27.9
IFI30	NM_006332	1.6	27.9
HLA-DRB1	NM_002124	1.5	27.9
SPI1	NM_003120	1.5	27.9
FCER1G	NM_004106	2.0	30.4
LGALS1	NM_002305	1.9	30.4
CRTH2	NM_004778	1.8	30.4
CD74	NM_004355	1.5	30.4
HN1	NM_016185	1.6	33.2
SPAG1	NM_003114	1.6	33.2
CPVL	NM_031311	1.6	36.3
HLA-DRB5	NM_002125	1.5	36.3
AHNAK	—	1.6	39.7
HLA-DRA	NM_019111	1.5	39.7
CCR10	NM_016602	1.8	47.5
<i>Decreased</i>			
NR4A2	NM_006186	−9.1	6.7
ZNF331	NM_018555	−3.4	6.7
GADD45A	NM_001924	−3.2	6.7
TNFAIP3	NM_006290	−3.2	6.7
JUNB	NM_002229	−3.1	6.7
DUSP2	NM_004418	−2.4	6.7
ZFP36	NM_003407	−2.2	6.7
BRF2	NM_006887	−2.2	6.7
SBDS	NM_016038	−1.9	6.7
CXCR4	NM_003467	−1.8	6.7
CJUN	NM_002228	−2.9	12.9
RNF139	NM_007218	−1.9	12.9
NY-REN-7	NM_173663	−1.6	12.9
FC(GAMMA)BP	NM_003890	−2.2	16.7
HLA-DQA1	NM_002122	−1.8	16.7
TAF7L	NM_024885	−1.7	16.7
RRN3	NM_018427	−1.6	16.7
PER1	NM_002616	−2.3	23.0
LAP70	NM_004901	−1.9	23.0
PHF1	NM_002636	−1.7	23.0
H3F3A	NM_002107	−1.6	23.0

Table I. Continued

Abbreviation	Genbank	Fold change	q-value (%)
SUI1	NM_005801	−1.6	23.0
RNF138	NM_016271	−1.6	27.9
IDI1	NM_004508	−1.6	33.2
PDE4B	NM_002600	−1.6	33.2
HSPC297	NM_021237	−1.6	36.3
HSF2	NM_004506	−1.9	39.7
ZNF395	NM_018660	−1.5	39.7
DEFA1	NM_004084	−2.5	43.9
DUSP1	NM_004417	−1.8	43.9
PCDHGB4	NM_003736	−1.5	43.9
IGKV1D-8	—	−1.7	47.5

In bold genes validated by quantitative PCR (Q-PCR).

GATA3, and no differences for TBET and IFNG in CD3+ T cells from AD children were found.

This study is the first to investigate gene expression patterns that characterize freshly isolated, unstimulated CD4+ T cells from adult AD patients. Microarray analysis demonstrated 52 differentially expressed genes in AD patients. Subsequent Q-PCR analysis confirmed differential expression of several genes in AD. As these genes were found differentially expressed in AD and not in AA patients, they seem to be related specifically to AD, rather than associated with atopy.

Microarray analysis revealed differential gene expression of FCER1A in CD4+ T cells from AD patients. This might suggest contamination by FCER1A expressing plasmacytoid dendritic cells (pDC). But, FACS analysis excluded

Table II. Fold changes in differential gene expression of Th1- and Th2-related genes in CD4+ T cells in AD (n = 8) and AA (n = 8) patients compared with HC (n = 11)

	AD versus HC		AA versus HC	
	Fold change	p-value ^b	Fold change	p-value ^b
CCR4	2.3	0.005	1.2	NS
GATA-3	1.2	NS	1.1	NS
IFNG	−1.3	NS	1.2	NS
IL-10	−1.4	NS	1.3	NS
IL-13	^a	^a	^a	^a
IL-4	^a	^a	^a	^a
IL-5	^a	^a	^a	^a
STAT-4	1.5	NS	1.4	NS
STAT-6	1.0	NS	−1.1	NS
T-BET	−1.0	NS	1.2	NS

^aNot detectable.

^bPairwise fixed reallocation randomization test.

NS, not statistically significant; Th1, T helper type 1; AD, atopic dermatitis; AA, allergic asthma; CCR4, CC chemokine receptor 4; IL, interleukin; STAT, signal transducers and activators of transcription.

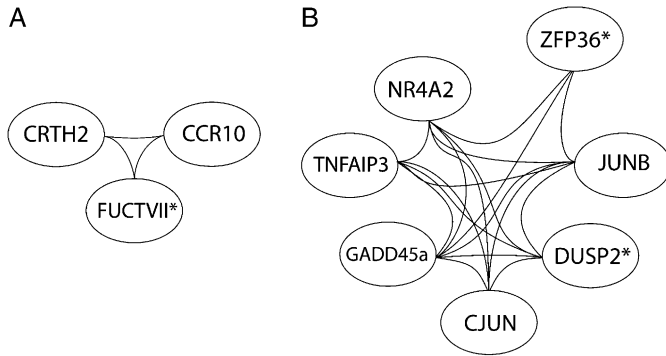


Figure 1
Identification of two groups of genes with highly correlating gene expressions. Lines represent statistically significant correlations (Pearson's correlation coefficient $r > 0.70$, $p < 0.01$, Methods) between gene expression levels. (A) Genes involved in skin homing of T cells. (B) Genes involved in apoptosis or proliferation. *No statistically significant differential expression levels found with Q-PCR analysis.

contamination by pDC (data not shown). Q-PCR confirmed expression of FCER1A in CD4⁺ T cells (which had also been described by Nakajima *et al*, 2004); however, no differential expression of FCER1A between AD patients and HC could be detected.

In addition, Q-PCR analysis revealed two groups of genes with highly correlating gene expression levels. One group includes genes known to be involved in skin homing of T cells, whereas the other group includes genes suggested to be involved in apoptosis or proliferation. The first group includes FUCTVII, CRTH2, and CCR10, showing increased expression in AD (Fig 1A). Expression of FUCT-VII has been suggested to regulate CLA expression (Nakayama *et al*, 2000), important for homing of CD4⁺ T cells to the skin in AD. Chemokine receptor CCR10 has also been proposed as a critical mediator of skin-specific T cell homing (Homey *et al*, 2002). Furthermore, CTACK (CCL27), the ligand for CCR10, was found to be increased in the serum of AD patients (Kakinuma *et al*, 2001, 2002; Hijnen *et al*, 2004). The receptor for prostaglandin D2, CRTH2, has also been suggested to be involved in skin homing of T cells. Furthermore, a preferential increase of CRTH2 within the CLA⁺ T cell population has been described in AD patients (Iwasaki *et al*, 2002). Taken together, increased expression of these genes in peripheral blood CD4⁺ T cells from AD patients suggests a "pre-activated" status with increased potential to migrate to the skin. This idea was further supported by the detection of increased gene expression of CCR4. As CCR4 expression on CD4⁺ T cells has been shown to correlate with disease severity in AD (Vestergaard *et al*, 2000) and Matsumoto *et al* (2002) also showed increased expression in CD3⁺ T cells, RT-PCR analysis for CCR4 was performed. Furthermore, CLA expression has been shown to correlate with CCR4 expression (Campbell *et al*, 1999), and the ligands for CCR4 (TARC and MDC) were found to be increased in the serum of patients with AD (Galli *et al*, 2000; Kakinuma *et al*, 2001; Hijnen *et al*, 2004).

Gene expression levels of a second subgroup of genes, including NR4A2, DUSP2, TNFAIP3, C-JUN, JUNB, and GADD45A, were also found to show significant correlating gene expression levels. None of these genes have been

associated with AD before, and are thought to be involved in regulating either proliferation or apoptosis (Fig 1B). A role for NR4A2 in a signal transduction pathway mediating multiple inflammatory signals was suggested, after it was found to show increased expression in the synovial tissue in rheumatoid arthritis patients (McEvoy *et al*, 2002). TNFAIP3 has been related to activation-induced cell death in T cells via NF- κ B (Malewicz *et al*, 2003). DUSP2 has been reported to be involved in p53-mediated apoptosis (Yin *et al*, 2003). Jun family members are involved in T cell activation, IL-2 expression and have been implicated in Th2 polarization (Petrak *et al*, 1994; Hartenstein *et al*, 2002). T cells in GADD45A knockout mice, which develop an autoimmune disease similar to human systemic lupus erythematosus, were found to have a lower threshold for activation and proliferate to a greater extent after primary T cell receptor stimulation (Salvador *et al*, 2002). In a recent study, Akdis *et al* (2003) suggested that the Th2 predominance in AD is because of increased apoptosis of peripheral blood Th1-type cells. Taken together, differentially regulated genes involved in apoptosis or proliferation described in this study might be related to this phenomenon.

Our study suggests a "primed" state of peripheral blood CD4⁺ T cells in AD patients. Previously, an *in vivo* activated state of peripheral blood skin homing CD4⁺ T cells in AD patients was described by Akdis *et al* (1997) characterized by a spontaneous release of Th2 cytokines. Remarkably, the microarray experiments revealed no increased expression of typical Th2-related genes, which was confirmed by Q-PCR analysis (Table II). These results suggest that peripheral blood CD4⁺ T cells from AD patients, although being "primed" for migration to the skin and showing differential expression of genes involved in activation or proliferation, do not show polarization towards a Th1- or Th2-cytokine profile *in vivo*. Several studies that investigated AD or AA patients support this observation. Differential gene expression analysis of unstimulated PBMC or CD3⁺ T cells from AD patients did not show a marked Th2-type gene expression pattern (Heishi *et al*, 2002; Matsumoto *et al*, 2002). Furthermore, in a genomic linkage analysis of a large population of AA patients, no Th2 gene involvement was found (Van Eerdewegh *et al*, 2002). These results support our results that, although being primed for skin homing, peripheral blood CD4⁺ T cells from AD patients are not expressing typical Th2-related genes.

Gene expression differences described in this study are the result of differential expression of genes in the total population of CD4⁺ T cells. Future research will elucidate if these genes are preferentially expressed in subpopulation(s) of CD4⁺ T cells, and how they contribute to the pathogenesis of AD.

In conclusion, our results show that peripheral blood, unstimulated CD4⁺ T cells in AD patients show differentially expressed genes involved in tissue homing, proliferation, and apoptosis, but no marked expression differences of "typical" atopy genes. Importantly, in peripheral blood CD4⁺ T cells from AD patients, we describe two groups of differentially expressed genes involved in T cell migration to the skin and in proliferation or apoptosis, that might be important in the pathogenesis of AD.

Table III. Clinical parameters of individuals in the (A) microarray experiments and (B) real-time PCR analysis

	Age (years)	Total IgE (kU per liter)	LSS	PC ₂₀	TARC (pg per mL)
(A) Microarray					
Healthy controls (n = 7)	33 ± 9	37 ± 42	—	—	136 ± 46
Atopic dermatitis (n = 5)	43 ± 10	8850 ± 11281	34 ± 6	—	6485 ± 5449
(B) RT-PCR					
Healthy controls (n = 11)	34 ± 8	34 ± 30	—	—	128 ± 35
Atopic dermatitis (n = 8)	36 ± 11	4189 ± 3176	26 ± 17	—	1842 ± 2019
Allergic asthma (n = 8)	37 ± 9	363 ± 485	—	3.7 ± 3.1	171 ± 184

LSS, Leicester sign score.

PC₂₀, provocative concentration of histamine causing a 20% fall in FEV₁.

All values are shown as mean ± SD.

Materials and Methods

Subjects for microarray analysis For the microarray analysis, five patients, three females and two males, with chronic (extrinsic) AD experiencing an exacerbation of disease (mean age, 43 y), who fulfilled the criteria of Hanifin and Rajka (1980), were selected (Table IIIA). Two patients also had allergic rhinitis, but none of the AD patients had AA. All were polyallergic and had positive skin prick tests to at least three aeroallergens. The patients were allowed to use topical corticosteroids. None of the AD patients had systemic immunosuppressive treatment at least 3 mo before peripheral venous blood was taken. Seven healthy non-allergic individuals, four females and three males (mean age, 33 y), with no history of AD or AA were included in the microarray study as a normal control group. In AD patients, severity of the eczema was evaluated by the Leicester sign score (LSS; range, 0–108), in which severity is scored by six clinical features (erythema, purulence, excoriation or crusting, dryness or scaling, cracking or fissuring, and lichenification) graded at six defined body sites on a scale of 0 (none) to 3 (severe) (Sowden *et al*, 1991). Furthermore, serum TARC (CCL17) levels were determined as an objective parameter for the severity of eczema in AD patients, as previously described (Table IIIA) (Hijnen *et al*, 2004).

Subjects for RT-PCR analysis Real-time RT-PCR analysis was performed with AD patients and HC in addition to those included for microarray analysis. Because RNA samples from two HC subjects and one AD patient were no longer available for RT-PCR analysis, six samples from different HC and four samples from different AD patients were added to make up the RT-PCR analysis set (Table IIIB). Furthermore, eight patients with a medical history of AA (mean age, 37 y), as defined by the American Thoracic Society (1987), were included, all patients had allergic rhinitis, but none of the AA patients had AD. All AA subjects were polyallergic and had positive skin prick tests to at least three aeroallergens. The AA patients were allowed to use inhaled corticosteroids. The PC₂₀, histamine challenge test, was determined as a parameter for bronchial hyperresponsiveness in AA patients. Eight patients, five females and three males, with chronic (extrinsic) AD (mean age, 36 y), who fulfilled the criteria described above, were included. Three patients also had allergic rhinitis, but none of the AD patients had AA. None of the AD or AA patients had systemic immunosuppressive treatment at least 3 mo before peripheral venous blood was taken. Eleven healthy individuals, seven females and four males (mean age, 34 y), with no history of AD or AA, were included in the study as a healthy non-allergic control group (HC). Disease severity parameters for AD (LSS and serum TARC levels) were determined as described above. The medical ethical committee of the University Medical Center Utrecht approved all described studies and the

participants gave their written informed consent. The study was conducted according to the Declaration of Helsinki Principles.

Cell isolation and RNA extraction PBMC were isolated by Ficoll (Amersham, Uppsala, Sweden) density gradient centrifugation of peripheral venous blood. Cells were washed three times and resuspended in PBS containing 2 mM EDTA (Merck, Germany) and 0.5% BSA (Sigma, St Louis, Missouri). After CD14 depletion using the magnet activated cell separation system (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany), CD4⁺ cells were isolated as indicated by the manufacturer. In brief, anti-CD14-depleted cells were incubated with MACS microbead-conjugated anti-CD4.

Table IV. Fold changes of differentially expressed genes in AD patients (n = 8) and AA patients (n = 8), compared to healthy controls (n = 11)

	AD versus HC		AA versus HC	
	Fold change	p-value ^a	Fold change	p-value ^a
<i>Upregulated</i>				
CCR10	3.0	0.001	1.2	NS
CRTH2	1.8	0.045	−1.0	NS
FUCT-VII	1.5	NS	1.1	NS
FCERIA	1.1	NS	1.1	NS
TYMS	1.0	NS	1.1	NS
<i>Downregulated</i>				
C-JUN	−5.7	0.016	1.2	NS
NR4A2	−4.9	0.020	1.2	NS
TNFAIP3	−3.1	0.014	2.2	NS
JUNB	−2.5	0.046	1.1	NS
GADD45A	−2.4	0.050	−1.1	NS
DUSP2	−2.4	NS	1.0	NS
ZFP36	−1.5	NS	−1.1	NS
CXCR4	−1.3	NS	1.1	NS
ZFP331	−1.2	NS	1.1	NS

^aPairwise fixed reallocation randomization test.

NS, not statistically significant; AD, Atopic dermatitis; AA, allergic asthma; HC, healthy controls.

Microbead-conjugated CD4⁺ T cells were then recovered from a second MACS column. CD4⁺ cells were $\geq 95\%$ pure as assessed by FACS analysis. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, California), according to the manufacturers protocol.

RNA amplification, microarray analysis, and quantitative real-time PCR RNA amplification and labeling were performed as described previously (Roepman *et al*, 2005). The exact procedure is available as supplementary data (Methods). Results of the microarray analysis are shown as fold change. The q-value determined for each gene is the lowest FDR at which that gene is called significant. It is like the well-known p-value, but adapted to multiple-testing situations. q-values of the lowest possible FDR ($< 50\%$) were used to generate a list of most relevant up- and downregulated genes. Finally, genes with an intensity value of at least 50 (which is about three times above the background signal), and showing differential expression of at least 1.5-fold, were selected.

RT-PCR was performed for 25 genes (detailed information is available as supplementary data (Methods), primers are shown in Table S2). Results were normalized for PCR efficiency and for the reference genes β -actin and GAPDH using relative expression software tool (REST) (Pfaffl *et al*, 2002).

Statistical analysis Statistical significance of real-time PCR data (Table IV, Table S3) was determined by REST software, using a pairwise fixed reallocation randomization test (Pfaffl *et al*, 2002). An associated probability (p-value) $p < 0.05$ was considered significant. Correlations between mRNA expression levels were calculated using Pearson's correlation coefficient. Correlation coefficients $r > 0.7$ ($p < 0.01$) were considered relevant.

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Supplementary Material

The following material is available online for this article.

Supplemental text. Supplemental text includes supplemental Abbreviations, detailed Methods, additional References, Primers, and Pearson's correlation coefficients of mRNA expression levels (Q-PCR) between selected genes.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1. Supplemental Abbreviations

▪ ADAM33	A desintegrin and metalloprotease 33
▪ AHNAK	AHNAK nucleoprotein (desmoyokin)
▪ ANX2P2	human lipocortin (LIP) 2 pseudogene mRNA
▪ BRF2	butyrate response factor 2 (EGF-response factor 2)
▪ CCR10	CC chemokine receptor 10
▪ CCR4	chemokine receptor 4
▪ CD74	CD74 antigen
▪ C-JUN	v-jun avian sarcoma virus 17 oncogene homolog
▪ CPVL	carboxypeptidase, vitellogenic-like
▪ CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
▪ CST3	cystatin C (amyloid angiopathy and cerebral hemorrhage)
▪ CTACK	Cutaneous T-cell attracting chemokine
▪ CXCR4	chemokine (C-X-C motif), receptor 4 (fusin)
▪ DEFA1	defensin, alpha 1, myeloid-related sequence
▪ DUSP1	dual specificity phosphatase 1
▪ DUSP2	dual specificity phosphatase 2
▪ FC(GAMMA)BP	IgG Fc binding protein
▪ FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
▪ FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
▪ FUCT-VII	fucosyltransferase 7 (alpha (1,3) fucosyltransferase)
▪ GADD45A	growth arrest and DNA-damage-inducible, alpha
▪ GPR44/CRTH2	G protein-coupled receptor 44
▪ H3F3A	H3 histone, family 3A
▪ HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1
▪ HLA-DRA	major histocompatibility complex, class II, DR alpha
▪ HLA-DRB1	major histocompatibility complex, class II, DR beta 1
▪ HLA-DRB5	major histocompatibility complex, class II, DR beta 5
▪ HN1	hematological and neurological expressed 1
▪ HSF2	heat shock transcription factor 2
▪ HSPC297	protein HSPC030 (protein HSPC297)
▪ IDI1	isopentenyl-diphosphate delta isomerase
▪ IFI30	interferon, gamma-inducible protein 30
▪ IFNG	interferon gamma
▪ IGKV1D-8	immunoglobulin kappa variable 1D-8
▪ IL-10	interleukin 10
▪ IL-13	interleukin 13
▪ IL-4	interleukin 4
▪ IL-5	interleukin 5
▪ JUNB	jun B proto-oncogene
▪ LAP70	apyrase, lysosomal
▪ LGALS1	lectin galactoside-binding soluble 1 (galectin 1)
▪ NR4A2	nuclear receptor subfamily 4, group A, member 2
▪ NY-REN-7	NY-REN-7 antigen
▪ PCDHGB4	protocadherin gamma subfamily B, 4
▪ PDE4B	phosphodiesterase 4B, cAMP-specific
▪ PER1	period (Drosophila) homolog 1
▪ PHF1	PHD finger protein 1

▪ RNF138	strin protein
▪ RNF139	patched related protein translocated in renal cancer
▪ RRN3	RNA polymerase I transcription factor RRN3
▪ S100A11	S100 calcium binding protein A11 (calgizzarin)
▪ SBDS	CGI-97 protein
▪ SPAG1	sperm associated antigen 1
▪ SPI1	spleen focus forming virus (SFFV) proviral integration oncogene spi1
▪ STAT-4	signal transducer and activator of transcription 4
▪ STAT-6	signal transducer and activator of transcription 6
▪ SUI1	putative translation initiation factor
▪ TAF7L	TAF7-like RNA polymerase II
▪ T-BET	T-box expressed in T-cells
▪ TNFAIP3	tumor necrosis factor, alpha-induced protein 3
▪ TYMS	thymidylate synthetase
▪ ZFP36	zinc finger protein 36
▪ ZNF331	zinc finger protein 331
▪ ZNF395	zinc finger protein 395

Appendix S2

RNA amplification and Microarray analysis

Total RNA was converted to double-stranded cDNA through the use of a T7 oligo(dT)₂₄VN primer (Ambion, Austin, Texas USA), Superscript II RNase H-reverse transcriptase (Invitrogen), DNA polymerase1, RNase H, and DNA ligase (Promega, Madison, Wisconsin, USA). cDNA was amplified using a T7 Megascript Kit (Ambion) (modified Eberwine (Van Gelder et al., 1990) RNA amplification), supplemented with aminoallyl-UTP (Sigma). Amplified RNA was purified using RNeasy mini cleanup kit (Qiagen, Venlo, The Netherlands). RNA integrity was verified by spectrophotometry and by the Agilent 2100 Bioanalyser (Agilent, Geneva, Switzerland). Amplified RNA was then labeled with Cy3 or Cy5 dye (Amersham, Uppsala, Sweden), purified using Chroma Spin columns (Clontech, Franklin Lakes, NJ, USA) and hybridized to spotted oligonucleotide microarrays (Human Version 1, Operon, Department of Physiological Chemistry, UMC Utrecht, The Netherlands, <http://www.genomics.med.uu.nl>). The microarray contained 70-mer oligo-nucleotides representing 16,659 genes as well as 2541 additional features for control purposes. The slides were scanned on a microarray scanner (Agilent) followed by interpretation of the scanned image using Imagen software (Silicon Genetics, Redwood city, CA, USA). A lowess normalization per print tip was performed (Yang et al., 2002). Ratios (LOG₂) of QQCC data per dye swap were defined as one measurement and permuted using Significance Analysis of Microarrays (SAM) software (Tusher et al., 2001) using the following parameters: one class response, row average imputer, 500 permutations. The one class option tests whether the mean gene expression ratio differs from zero. The q-value determined for each gene is the lowest False Discovery Rate (FDR) at which that gene is called significant. It is like the well-known p-value, but adapted to multiple-testing situations. Q-values of lowest possible FDR (<50%) were used to generate a list of most relevant up- and downregulated genes. Finally genes were selected with an intensity value of at least 50 (which is about 3 times above background signal) and showing differential expression of at least 1.5-fold.

Quantitative real-time PCR

Real-time PCR was performed using a MyiQ real-time PCR detection system (Biorad, Hercules, CA, USA) using SYBR Green I (Biorad). Primers (Sigma-Genosys, The Woodlands, TX, USA) were designed using Primer 3 software of the whitehead institute/ MIT center for genome Research (supplementary data S3)(Rozen and Skaletsky, 2000). Amplification was performed using 0.2 µl of cDNA, 10 µl of 2× SYBR Green I mastermix and 300 nM of each primer, according to the following conditions: 3 minutes at 95°C to activate DNA polymerase, 45 cycles of 10 seconds at 95°C, 20 seconds at 62°C and 25 seconds at 72°C. PCR was checked for a-specific products by performing dissociation curves after each PCR run. The signal of SYBR Green I was measured against the passive signal of the reference dye, fluorescein, to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Each assay included a standard curve of four serial concentrations of a pool of all cDNA samples to determine linearity and PCR efficiency. Results were normalized for PCR efficiency and for the reference genes β-actin and GAPDH using relative expression software tool (REST[®])(Pfaffl et al., 2002), according to the following method:

$$\text{ratio} = (E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control} - \text{sample})} / (E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control} - \text{sample})}$$

Reference genes were checked for stable expression using Best-Keeper[®] software (Pfaffl et al., 2004).

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Appendix S3. Primers used in this study.

Name	Forward Primer	Reverse Primer
CCR4	GCACCAAAGAAGGCATCAAG	AGGACCAGAACCACCACAGAA
CCR10	GAGGCCACAGAGCAGGTTTC	GGAGACACTGGGTTGGAAGG
CJUN	GTCCCAGGAGCGGATCAA	TCTGAGCTTTCAAGGTTTTCACTT
CXCR4	GCCACCGCATCTGGAGAA	CATGGAGTCATAGTCCCCTGAG
DUSP2	CTGCGAGGAGGCTTCGAC	TGGTTTTGTCCCCTGTTGG
FCERIA	TCCCATTGTTGGTGGTGATT	TGGCTTAGGATGTGGGTTCA
FUTVII	CTCGGGTCTCTTGGCTGAC	CGTGCCACAGCATTATTCATC
GADD45A	CAGAAGACCGAAAGGATGGATAAG	TCGTACACCCCGACAGTGAT
GATA-3	GGCAGGGAGTGTGTGAACTG	CCTTCGCTTGGGCTTAATG
HIP55	CAACTGGACAGGCGAGGG	TGCGTTGATGGTCACATGG
IFNG	TTTGGGTTCTCTTGGCTGTTACT	GTTCCATTATCCGCTACATCTGAAT
IL-4	AGCAGTTCCACAGGCACAAG	ACTCTGGTTGGCTTCCTTCAC
IL-10	GAGAACCAAGACCCAGACATCA	GTGGAGCAGGTGAAGAATGC
IL-12	ATGCCGTTCAACAAGCTCAAGT	GGTGGGTCAGGTTTGATGATG
IL-13	CACGGTCATTGCTCTCACTTG	AGCGGAGCCTTCTGGTTCT
JUNB	CTAAATGGAACAGCCCTTCTACC	GGCTCGGTTTCAGGAGTTTG
NR4A2	CGAAGAGCCCACAGGAGC	TCGCCTGGAACCTGGAATAG
STAT-4	GCAACCGAAGATTGTACTTTGTG	GACAGCCCTCATTTCTTTACCT
STAT-6	GTGTACCCACCACACTCTCACTC	GGTCAAAGGGCAGGCTCAT
T-BET	GGGAACTAAAGCTCACAAACAAC	GCACAATCATCTGGGTCACATT
TNFAIP3	GGGCGTTCAGGACACAGAC	CATCATTCCAGTTCCGAGTATCA
TYMS	AATTCAGCTTCAGCGAGAACC	TGGATGCGGATTGTACCCCT
VEGFB	GAGGAAAGTGGTGTATGGATAGA	CTCCACAGTCAAGGGCACC
ZNF331	CGCCGACGTAGCCATAGAC	TGCTGACTCCAAATCCAGTGA
ZNF36	GATCTGACTGCCATCTACGAGAG	GGAGTCGGAGGGGCTCAG

Sequences are shown from 5' (left) to 3' (right)

Appendix S4. Pearson's correlation coefficients of mRNA expression levels (Q-PCR) between selected genes (all correlations $p < 0.01$). Correlation coefficients > 0.7 were considered relevant. * not relevant: < 0.7

[illegible]