

Genetic Approaches to Assessing Evidence for a T Helper Type 1 Cytokine Defect in Adult Asthma

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Recent evidence suggests that deficiency in the Th1 cytokine pathway may underlie the susceptibility to allergic asthma. This study examined whether (1) single-nucleotide polymorphisms exist in the promoter region of the two interleukin (IL)-12 subunit genes in patients with asthma; (2) messenger RNA and protein expressions of signal transducers and activators of transcription, IL-12, IFN- γ , and their receptors are altered in asthma; and (3) linkage to genes in the Th1 pathway is present in families with asthma in Iceland. The promoter regions of the IL-12 subunit genes were sequenced in 94 patients with asthma and 94 control subjects without asthma. Linkage was examined in 169 families that included over 570 patients with asthma and 950 of their unaffected relatives. The results demonstrate no evidence of linkage to microsatellite markers in close association with genes within the Th1 pathway, and no polymorphism was detected in the promoter regions of the two IL-12 subunit genes in the cohort with asthma patients. Moreover, we found no differences in the messenger RNA or protein expression signals of genes in the IL-12 pathway between the patients and control subjects. We conclude that decrease in Th1 type cytokine response is unlikely to present a primary event in asthma.

Keywords: asthma; genetics; polymorphism; linkage; expression

Bronchial asthma, the most common chronic disease affecting children and young adults, is a complex disorder with variable phenotype and aberrant Th2 cytokine profile (1, 2). The principal signs and symptoms of asthma including cough, wheezing, and shortness of breath are largely attributed to inflamed hyperresponsive airways (3, 4). Although the Th2 cytokines, interleukin (IL)-4, IL-13, and IL-5, have been strongly implicated in the pathogenesis of asthma, the potential role of a defective Th1 cytokine profile has been less well characterized. In this regard, IL-12 is secreted by antigen-presenting cells in response to variety of stimuli, including virus infections and allergen exposure, the two most common triggers of asthma. IL-12 plays a major role in the development of Th1 cells and, together with IL-18, stimulates activated T cells, natural killer cells, and fully differentiated Th1 cells to produce IFN- γ through activation of tyrosine kinase and signal transducers and activators of transcription (STAT4) protein signaling (5, 6). IFN- γ activates macrophages and amplifies the Th1-cytokine pathway by promoting the ability of naive T cells to respond to IL-12 (7, 8).

The overall balance between the Th1/Th2 cytokine phenotypes has been shown to be influenced by numerous parameters, including T cell activation, the specificity of the antigen-presenting

cells, genetic background, pathogen-derived material, and cytokines present in the priming milieu. In addition, the balance of Th1 and Th2 cytokines during antigen presentation and initiation of the T cell response has been shown to be critically important in determining the downstream effects of the antigen presentation process (9, 10). Because the initial cytokine response in this process is of the Th1 phenotype, genetic variations in this pathway could affect function and lead to polarization of the cytokine response toward the Th2 phenotype, which has been shown to dominate the response profile in patients with atopic asthma (11). Accordingly, a primary defect in the Th1 pathway could underlie the development of the atopic asthmatic phenotype. The latter concept was addressed in studies directed at identifying genetic variations in Th1 cytokine genes. A linkage study was also performed to examine if any microsatellite markers are closely associated with these genes. In addition we examined the capacity of peripheral blood mononuclear cells (PBMCs), isolated from patients with atopic asthma and control subjects without atopy and asthma to express and release Th1 cytokines, including both IL-12 and IFN- γ .

METHODS

Patients and Control Subjects

Patients were selected from the private and outpatient clinics of asthma specialists practicing at the Allergy and Pulmonary Divisions of the Vífilsstaðir University Hospital of Iceland in the years 1977 to 2001. Patients with physician-diagnosed asthma qualified for the study. Of 1982 medical charts reviewed, 1,185 patients, who belonged to 409 family clusters, were identified. For the single-nucleotide polymorphisms studies, 94 patients with atopy and asthma were randomly selected, 1 from each of 94 of the 409 available family clusters; for the linkage studies, 481 additional patients were selected from these same family clusters to ensure that each of the 575 patients was related to at least one other patient with asthma within four meioses. Ages ranged from 12 to 59 years (mean 38 years) and 59.9% were females. Information regarding the age at diagnosis, medications, hospital admissions, and family history of atopy and asthma were recorded. The atopy and asthma phenotypes were characterized on the basis of the medical history, physical examination, skin tests to 12 aeroallergens (including birch, grass, rumexcrispus, cat, dog, horse, *Cladosporium*, *Mucor*, *Alternaria*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Lepidoglyphus destructor*), total IgE levels, pulmonary function tests and, unless baseline FEV₁ was less than or equal to 70% predicted, a methacholine challenge (MCh) test. The phenotype assessments, pulmonary function tests, and methacholine tests were performed according to American Thoracic Society guidelines (12, 13). Patients were diagnosed as being atopic if their skin prick test reaction was more than or equal to 3 mm or 50% or more of the histamine positive control response. All patients signed an informed consent, donated blood samples, and completed a questionnaire and all tests necessary for proper phenotyping. Ninety-four unrelated subjects without history of asthma and atopy, who matched in age and sex with the 94 patients with atopy and asthma, were randomly selected from over 900 healthy subjects; they filled out a disease-oriented questionnaire to ensure they did not have asthma or atopy, donated blood samples, and served as control subjects for the association studies. The control subjects did not undergo a physical exam and no tests were performed either. All blood samples from the patients with asthma and control subjects were processed

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within 2 hours, from sampling, and the messenger RNA (mRNA) and proteins were isolated and examined for STAT4, IL-12, and IFN- γ expression. The study was approved by the Icelandic Data Protection Commission and the National Bioethics Committee. Personal information about the patients and their family members were subsequently encrypted by the Data Protection Commission of Iceland (14). All blood and DNA samples were also coded in the same way.

Polymerase Chain Reaction Amplification and Sequencing of IL-12 p35 and p40 Promoters

Five polymerase chain reaction (PCR) primer pairs were used to amplify a 1579-bp fragment from the promoter region of the IL-12 p35 subunit and seven PCR primer pairs were used to amplify a 1883-bp fragment from the promoter region of the IL-12 p40 subunit. Genomic DNA was extracted from whole blood using the phenol-chloroform method. The DNA was amplified for 30 cycles as follows: 95°C for 30 seconds, 55 to 60°C for 30 seconds, and 72°C for 1 minute. Final extension was performed for 12 minutes at 72°C. The PCR reactions were run on PTC-225 Peltier Thermal Cyclers (MJ-Research, Waltham, MA). Reactions were performed in a volume of 25 μ l, containing 20 ng of DNA from each patient or control subject, 0.5 pmol of each primer (TAG Copenhagen A/S, Copenhagen, Denmark and MWG-Biotech AG, Munich, Germany), 1.5 mM magnesium chloride, 0.2 pmol deoxynucleoside triphosphate, 2.5 μ l 10 $\times$ PCR buffer, 1 U/ μ l AmpliTaq Gold, and 13.9 μ l water. Sequencing was performed on PTC-225 Peltier Thermal Cyclers (MJ-Research), and the sequencing reactions were performed in a volume of 6.6 μ l, containing 4.8 μ l Big Dye Ready Reaction mix (Applied Biosystems, Foster City, CA), including buffer, enzymes, nucleotides, 6 pmol of each primer, and 15 μ M magnesium chloride. The PCR reactions were run for 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Excess dye terminators were removed from the sequencing reactions using Multiscreen 384Seq filter plates (Millipore, Billerica, MA). The sequences were read with ABI prism automated 3700 sequencer (Applied Biosystems). The products were analyzed using Gene Miner2 software (deCODE Genetics, Inc., Reykjavik, Iceland). Primers were designed using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All primers were verified for product specificity, by blasting to the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1>). All primers were also routinely tested in Gradient PCRs, to determine the optimal annealing temperatures, before running PCR assays on the study samples. Table 1 lists the primer pairs used for the sequencing of the promoter regions of p35 and p40 units, respectively.

RNA Expression Study

Real-time PCR was used to examine mRNA expression of the IL-12 receptor (IL-12 R) β 2 subunit, IFN- γ receptor 1, and STAT4 gene, in naive PBMCs and in cells after stimulation with cytokines. Total RNA was extracted from Ficoll-isolated PBMCs using TRIzol Reagent (Life Technologies A/S, Taastrup, DK). The PBMCs were isolated from the

blood of patients with asthma and control subjects without asthma. The RNA was extracted at baseline and after 4 hours stimulation *in vitro* with phytohemagglutinin (PHA) (5 μ g/ml). The RNA samples were cleaned using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and a stock concentration, (1.25 ng/ μ l) of RNA was made using RiboGreen RNA Quantitation Reagents and Kits (Molecular Probes, Eugene, OR). The real-time PCR reactions were performed using LightCycler (Roche Molecular Systems, Palo Alto, CA). Each reaction was performed in a total volume of 20 μ l, containing 4 μ l nuclease-free water, 4 μ l LightCycler real-time PCR Reaction Mix, SYBR Green I, 5 $\times$ buffer, 1.6 μ l magnesium chloride, 25 mM, 1 μ l forward and reverse primer, respectively (MWG-Biotech), and 0.4 μ l of LightCycler real-time PCR Enzyme Mix (Roche Molecular Systems). For each reaction, 10 ng of RNA was heated at 55°C for 10 minutes, denatured at 95°C for 30 seconds, amplified at 95°C and 55°C for 10 seconds each and 72°C for 5 seconds, followed by melting at 95°C and 65°C for 15 seconds and 95°C and cooling at 40°C for 30 seconds. The primers used were designed using the Oligo6 Primer Design software (LifeScience Software Resource, Long Lake, MN). The following primers were used for the mRNA quantification of the (1) IL-12 R β 2 subunit, sense 5'-AGTGGGAGACCCCGGATAA-3' and antisense 5'-CGTCGTCGGAGCTGGAA-3'; (2) IFN- γ receptor 1, sense 5'-TAAATGGAGACGAGCAGGAA-3' and antisense 5'-AATCGCTAACTGGCACTGAA-3'; and (3) STAT4, sense 5'-CAGAGGAATGTGGAG-CACAA-3' and antisense 5'-TAAGCATTTCTGCAGTGTCAA-3'.

Protein Expression—Cytokine Assays

PBMCs were isolated from whole blood of patients and control subjects using the Ficoll-Paque isolation method (Pharmacia, Uppsala, Sweden). The cells were cultured in RPMI 1640 medium (Life Technologies), containing 0.1% fetal bovine serum, penicillin, gentamycin, and fungizone, to a concentration of 1×10^6 cells/ml media. For the IL-12 measurements, cells from patients and control subjects were stimulated for 40 hours with 0.1% wt/vol *Staphylococcus aureus* Cowan 1 (SAC) (Calbiochem, San Diego, CA) and 10 ng/ml of rhIFN- γ (R&D Systems, Minneapolis, MN). For the IFN- γ measurements, cells from patients with asthma and control subjects were stimulated with 10 μ g/ml PHA (Sigma Chemicals Co., St. Louis, MO) and 2.5 ng/ml rhIL-12 (R&D Systems) for up to 40 hours. The supernatant was collected and assayed for these two cytokines, using ELISA IL-12 and IFN- γ Quantikine kits (R&D Systems). The plates were read at 450 nm using a spectrometer and correction was made at 570 nm. The minimum detectable level was 5 and 8 pg/ml for IL-12 and IFN- γ , respectively.

Flow Cytometry

Intracellular IL-12 and IFN- γ and IL-12 cell surface receptor expressions were examined in both resting and IFN- γ -activated PBMCs using flow cytometry. A total of 10^5 cells were used per sample.

TABLE 1. LISTS OF PRIMER PAIRS USED TO SEQUENCE THE PROMOTER REGIONS OF THE p35 AND p40 SUBUNITS

No.	Forward Primer	Reverse Primer	Product Size (bp)
Interleukin-12 p35 subunit			
1	ATTAGAAGGGGCTTAGAGATCAA	AGTAGAGCAGAGGGAAGAGTTGAC	503
2	TCCAAATGGAACCTCTCAC	GAGTCTTCTGGGTTTAGG	300
3	CCAGGAAGACTCCCACTACT	GGATGAGAGCTGGCTGACTC	406
4	GGAGCCTTTGCTGACTCCT	AGAGGAGTGCCTCCCACTTT	395
5	TAGGCTGGAGGTCGTGGTTA	CAGGGGGCCACATTTTAT	436
Interleukin-12 p40 subunit			
1	AAATTTCAAGGTGCTTTACGAAC	GTTATCCGACCTTGCTCTG	502
2	GGTGCACTTACACATACG	AGATTTCCGGGCTAACTTT	386
3	AAAGTTAGCCCGGAAATCT	ATGTACCTGCACGTATACCA	278
4	AGGGATGGAGAAGTGACCT	CCTCAGGGAGGCATACAGAC	417
5	TGCATACCTCTTAGCAACTTGAAA	TAAAAACAAAATCCTGGTTCTTC	501
6	TGGTACACGCAGTTTATCATCTC	GTGTATCAATAACGAGGAGGGAAC	500
7	TATTCCCCACCCAAAAGTCA	CTGCTGTTGCTGGTACTGGA	232

Intracellular Staining of IL-12 in Asthmatic PBMCs

To study intracellular expression of IL-12, isolated PBMCs from patients with asthma and control subjects were resuspended in RPMI 1640 medium (Life Technologies), containing 2% fetal bovine serum, and stimulated with 10 ng/ml of rhIFN- γ for 24 hours. After incubation, the samples were treated with 1 μ l Golgi Stop (Pharmingen, Becton Dickinson, San Diego, CA) for every 1.5 ml of media. The cells were then washed and resuspended in 100 μ l of staining buffer (Dulbecco's phosphate-buffered saline without magnesium ion or calcium ion, 1% heat-inactivated fetal calf serum, 0.09% [wt/vol] sodium azide and pH adjusted to 7.4–7.6) and stained for CD14 with phycoerythrin (PE)-labeled mouse antihuman CD14 monoclonal antibody (Serotec, Oxford, UK). The cells were stained for 30 minutes at 4°C, centrifuged for 10 minutes at 1,400 rpm, at which time they were fixed and permeabilized with Cytotfix/Cytoperm solution (Pharmingen). To keep the cells permeable they were washed two times in Perm/Wash solution (Pharmingen) and centrifuged at 1,400 rpm for 7 minutes between washes. The cells were then stained for IL-12 using fluorescein isothiocyanate-labeled anti-human IL-12 (p40/p70) mouse monoclonal antibody (Pharmingen). Incubation at 4°C for 30 minutes was then repeated and the cells were washed two times with Perm/Wash solution (Pharmingen) and centrifuged for 8 minutes at 1,400 rpm between washes. The cells were then fixed with 0.5% formalin solution and examined using a flow cytometer (fluorescence-activated cell sorter) (Becton Dickinson). A fluorescein isothiocyanate-labeled mouse anti-human IgG1 and PE-labeled IgG2 isotype control antibodies were used to measure background staining.

Cell Surface Staining of IL-12 and IFN- γ Receptors

To examine IL-12 and IFN- γ receptor expression, isolated PBMCs from patients with asthma and control subjects were resuspended in RPMI 1640 medium (Life Technologies) containing 2% fetal bovine serum and stimulated for 48 hours with 5 μ g/ml of PHA (Sigma Chemicals Co). The cells were washed and resuspended in 100 μ l phosphate-buffered saline before staining with specific antibodies. The antibodies used included a PE-labeled mouse anti-hIL-12 receptor β 1 and PE-labeled mouse anti-hIFN- γ receptor monoclonal antibodies (Pharmingen). The cells were stained for 30 minutes at 4°C and then washed with phosphate-buffered saline, fixed in 0.5% formalin and examined by a flow cytometer (fluorescence-activated cell sorter) (Becton Dickinson). A PE-labeled mouse anti-human IgG2 isotype control antibody was used to assess background staining.

Genotyping

Apart from the 94 patients who were analyzed for single-nucleotide polymorphisms, DNA was also extracted from the peripheral blood of additional 481 patients with asthma and atopy and 958 of their unaffected relatives in 169 families. The latter included both parents in about 70% of the cases, one parent together with one or two siblings, or two to three siblings if neither parent was available for the rest of the cases. All DNA samples were genotyped using specific fluorescently labeled primers with an average spacing of 3 to 4 cM in the chromosomal regions of the 10 genes studied in the Th1 pathway (15).

PCRs were set up, run, and pooled on Perkin Elmer/Applied Biosystems 877 Integrated Catalyst Thermocyclers as described previously (16). The PCR conditions used were 95°C for 10 minutes to activate the Amplitaq Gold, then 34 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute. The pooled products were supplemented with the internal size standards and detected on an Applied Biosystems 3700 model capillary Sequencer using Genescan v3.0 peak calling software. The genotypes were defined and edited in the Applied Biosystems Genotyper v2.0 program. The marker orders and genetic distances used were obtained from both publicly available genetic maps and genetic maps constructed at deCODE genetics Inc (15–19). Two separate reference samples of Northern European descent from the CEPH (<http://www.cephb.fr/>) collection (1331–01 and 1331–02; Coriell Cell Repositories) were used.

Statistical Analysis

Statistical analysis of RNA and protein expressions were performed in GraphPad InStat version 3.05 using the unpaired *t* test and the

Mann–Whitney test. A *p* value less than 0.05 was considered significant. All linkage results reported were produced by the ALLEGRO program, which includes enhanced statistical analytical features of the GENEHUNTER-PLUS program. The ALLEGRO program implements a nonparametric method of linkage analysis and produces nonparametric lod scores, which indicate the amount of excess identity by descent sharing among related affecteds as measured by a chosen scoring function (20–22). The scoring function used for this manuscript was S_{pairs} , which is found to be quite powerful for a wide range of inheritance models with or without locus heterogeneity (22, 23).

RESULTS

Sequencing

A 1579 nucleotide segment corresponding to the –1592 to –13 site relative to the transcription start of the IL-12 p35 gene was amplified in 94 patients with asthma and 94 control subjects, using five primer pairs. A fragment of 1883 nucleotides, corresponding to the –1883 to +1 site relative to the transcription start of the IL-12 p40 gene was screened in the same cohort, using seven primer pairs. A total of 3.5 kb of genomic sequence was analyzed. No polymorphisms were detected in the promoter regions of p35 and p40 subunits.

Real-Time PCR

To examine for differences in mRNA expression of the IL-12 R β 2 unit, IFN- γ receptor 1, and STAT4 genes between the patients with asthma and control subjects without asthma, kinetic PCR reactions were run on total RNA samples isolated from PBMCs at baseline and after stimulation with cytokines (*see METHODS*). The mRNA expressions were examined in 10 patients with asthma and 10 control subjects without atopy and asthma. The results from the IL-12 R β 2 unit expression demonstrated no difference between patient and control groups, wherein the baseline values amounted to 363.51 ± 77.44 in the patients versus 263.33 ± 56.50 in the control subjects (*p* = 0.29). No significant difference was detected after cytokine stimulation, and the expression values amounted to 4210.80 ± 1187.30 versus 2507.90 ± 464.29 in the patients and control subjects, respectively (*p* = 0.18) (Figure 1A).

The expression values of the IFN- γ receptor 1 studies were similar in both groups, amounting to 499.02 ± 204.73 in the patients versus 381.75 ± 96.36 in the control subjects, (*p* = 0.59), at baseline and 727.58 ± 337.87 in the patients versus 643.73 ± 158.21 in the control subjects, (*p* = 0.77), after cytokine stimulation, respectively (Figure 1B). As shown in Figure 1C, STAT4 mRNA expression was similar in both groups, wherein the baseline value amounted to 0.08 ± 0.03 in the patients versus 0.05 ± 0.02 in the control subjects, (*p* = 0.54). Moreover, no significant difference was detected after cytokine stimulation, wherein the expression values amounted to 5.45 ± 1.71 in the patients versus 3.22 ± 1.59 in the control subjects (*p* = 0.35).

Protein Expression—ELISA

To search for difference in protein expression, IL-12 and IFN- γ proteins were examined in the cells media of activated PBMCs isolated from both patients with asthma and control subjects. As shown in Figure 2A, we found no difference in the IL-12 levels of the cell media between patients and control subjects, after exposure to SAC/IFN- γ . The latter amounted to 10.8 ± 5.7 pg/ml in the patients with asthma versus 9.5 ± 4.8 pg/ml in the control group (*p* = not significant). Furthermore, as shown in Figure 2B, no differences were detected in IFN- γ production, wherein the levels amounted to 1803 ± 157 pg/ml and 1788 ± 108 pg/ml in the asthma and control groups, respectively (*p* = not significant). Thus, the trend in these results suggests that it

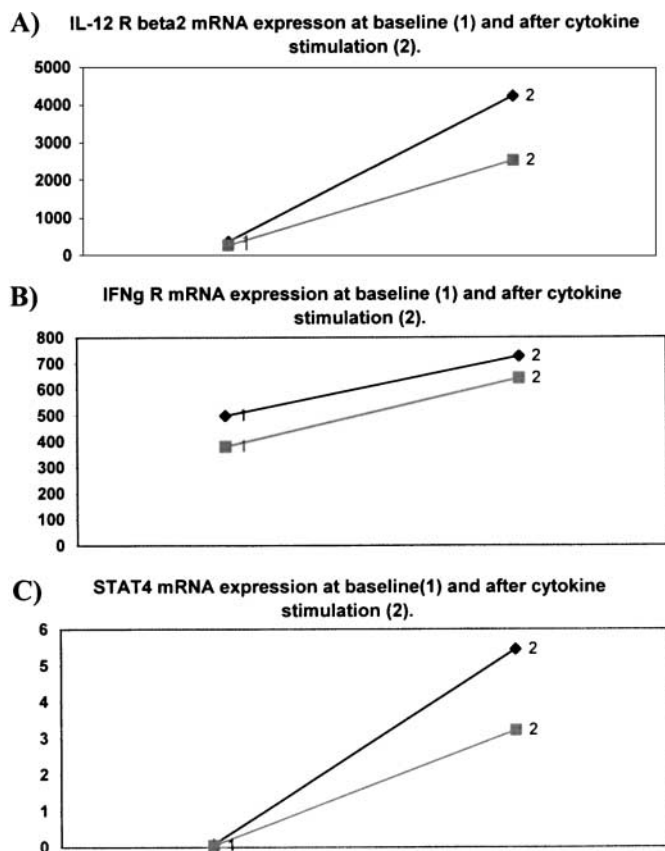


Figure 1. (A) The messenger RNA (mRNA) expression of interleukin (IL)-12 receptor $\beta 2$ unit was similar in patients with asthma ($n = 10$) and control subjects ($n = 10$), wherein the mean baseline expression values amounted to 363.51 ± 77.44 versus 263.33 ± 56.50 , respectively ($p = 0.29$). In addition, no significant difference was detected after cytokine stimulation, wherein the mean expression values amounted to 4210.80 ± 1187.30 versus 2507.90 ± 464.29 , in the patients and control subjects, respectively ($p = 0.18$). (B) The mRNA expression of the IFN- γ receptor 1 unit was similar in the patients with asthma ($n = 10$) and control subjects ($n = 10$), wherein the mean baseline expression values amounted to 499.02 ± 204.73 versus 381.75 ± 96.36 , respectively ($p = 0.59$). In addition, no significant difference was detected after cytokine stimulation, wherein the mean expression values amounted to 727.58 ± 337.87 versus 643.73 ± 158.21 in the patients and control subjects, respectively ($p = 0.77$). (C) The mRNA expression of STAT4 was similar in the patient with asthma ($n = 10$) and control ($n = 10$) groups, wherein the mean baseline expression values amounted to 0.08 ± 0.03 versus 0.05 ± 0.02 , respectively ($p = 0.54$). In addition, no significant difference was detected after cytokine stimulation, wherein the expression values amounted to 5.45 ± 1.71 versus 3.22 ± 1.59 in the patient and control groups, respectively ($p = 0.35$). Data are expressed as mean $\pm$ SE. *Diamonds* = patients with asthma; *squares* = control subjects.

would be unlikely that we would find lower levels in the asthma cohort by expanding the number of study participants in Iceland.

Flow Cytometry

Intracellular staining of IL-12 and the IL-12 and IFN- γ cell surface receptors was examined in the PBMCs using a flow cytometer. As shown in Figure 3A, there were no differences in expression intensities of the IL-12 cytokine between the patient and control cells, wherein the mean fluorescent intensity values amounted to 33.1 ± 1.9 for the patients with asthma

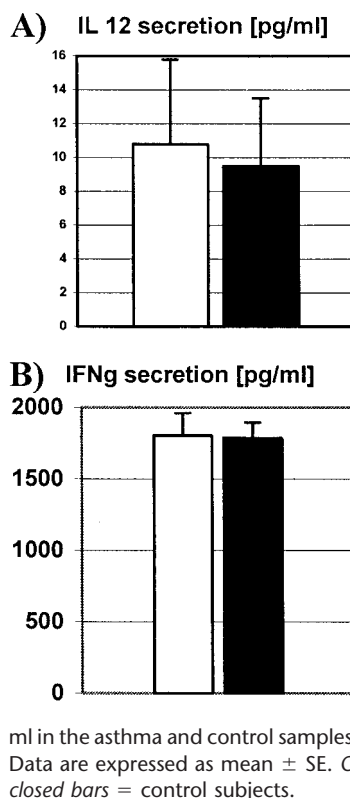


Figure 2. (A) Measurements of IL-12 production by peripheral blood mononuclear cells (PBMCs) isolated from patients with asthma ($n = 6$) versus control subjects ($n = 5$) using ELISA. No difference was detected in IL-12 production in SAC/IFN- γ -stimulated PBMCs between the two groups, wherein the pg/ml concentration amounted to 10.8 ± 5.7 versus 9.5 ± 4.8 in the asthma and control samples, respectively ($p =$ not significant). (B) Measurement of IFN- γ production by PBMCs from patients with asthma ($n = 11$) and control subjects ($n = 6$) using ELISA. No difference was detected in IFN- γ production from the PHA/IL-12-stimulated PBMCs between the two groups, wherein the pg/ml concentration amounted to 1803 ± 157 pg/ml versus 1788 ± 108 pg/ml in the asthma and control samples, respectively ($p =$ not significant). Data are expressed as mean $\pm$ SE. *Open bars* = patients with asthma; *closed bars* = control subjects.

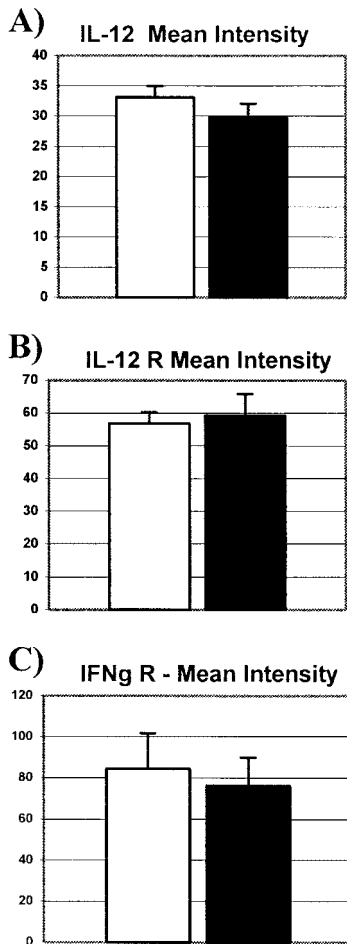
versus 29.8 ± 2.3 in the control group ($p = 0.28$). Moreover, as shown in Figure 3B, no differences were detected in the IL-12 R expression, wherein the mean levels amounted to 56.9 ± 3.3 for the asthma group versus 59.3 ± 6.6 for the control group, respectively ($p = 0.37$). As shown in Figure 3C, no differences were detected in the IFN- γ receptor expression between the groups, wherein the mean levels amounted to 84.5 ± 17.2 versus 76.3 ± 13.6 , in the patient versus the control groups, respectively ($p = 0.76$).

Linkage Analysis

As shown in Figure 4, we uncovered no evidence of linkage to the regions of the chromosomes studied for these Th1 cytokines and their receptors including: IL-12 p35 and IL-12 p40, IL-12 R β units 1 and 2, IFN- γ and IFN- γ receptor units 1 and 2, Toll-like receptor 4, IL-18, and the transcription factor STAT4. The lod scores obtained for these 10 loci were all under one. Although the power of the linkage approach is relatively poor in mapping genes that have low relative risk, the 169 families, including 575 patients with asthma used in this study provide excellent power to detect linkage, if one exists, if the relative risk of any of these Th1-pathway genes is 1.8 or higher.

DISCUSSION

Several studies suggest that dysregulation of the IL-12 cytokine pathway resulting in loss of responsiveness to IL-12 may underlie allergic airway responses in asthma. Indeed, numerous studies have reported decreased production of IL-12 and IFN- γ in PBMCs from patients with asthma compared with control subjects without asthma (24–29). To address this issue, we examined for genetic variations and differences in the expression and action of the cytokines IL-12, IFN- γ , and their receptors in PBMCs isolated from patients with asthma versus control subjects without asthma. Accordingly, we sequenced the two promoter



between the patients and control subjects, wherein the mean expression intensities amounted to 84.5 ± 17.2 versus 76.3 ± 13.6 in the asthma and control samples, respectively ($p = 0.76$). Data are expressed as mean $\pm$ SE. Open bars = patients with asthma; closed bars = control subjects.

regions of the IL-12 subunit genes, measured mRNA and protein expression, and examined linkage to 10 Th1-type genes in the Th1 pathway in 575 patients with asthma.

IL-12 is a heterodimeric cytokine, composed of 35- and 40-kD peptides, located on chromosomes 3p12-q13.2 and 5q31-q33, respectively. Both units are required for cytokine signaling (8). The IL-12 R is composed of two subunits, $\beta 1$ and $\beta 2$, and coexpression of both units is necessary to bind IL-12 with high affinity. The $\beta 2$ subunit is the signaling component and is only expressed in Th1 cells. Its expression is also upregulated by IFN- γ and downregulated by IL-4 (10). IL-12 stimulates IFN- γ production through tyrosine kinase activation and STAT4 protein signaling (STAT4) in synergy with IL-18 (IFN- γ -inducing factor) (5, 6, 30). Impairment in IL-12 production has been reported in both whole blood and PBMCs from patients with atopy and asthma, whereas steroid-mediated improvement in lung function is associated with increased numbers of IL-12+ cells in patients with asthma and reduced number of cells expressing IL-13 in bronchial biopsy specimens (1, 6, 8, 30, 31). Taken together, these studies suggest that deficiencies in the Th1 cytokine axis may underlie susceptibility to allergic airway disease. However, it is unclear whether the altered IL-12 deficiency is of primary or secondary origin. The results from this study suggest that the alterations in the Th1 cytokine pathway are not of primary origin.

Figure 3. (A) Comparison of intracellular staining of IL-12 in PBMCs isolated from patients with asthma ($n = 20$) versus control subjects ($n = 12$), using flow cytometry. No difference was detected in the intensity of IL-12 expression between the groups, wherein the mean expression intensities amounted to 33.1 ± 1.9 versus 29.8 ± 2.3 in the asthma and control samples, respectively ($p = 0.28$). (B) Comparison of IL-12 receptor cell surface protein expression in PBMCs isolated from patients with asthma ($n = 24$) and control subjects ($n = 13$) using a flow cytometry. No difference was detected in the intensity of IL-12 receptor expression between the groups, wherein the mean expression intensities amounted to 56.9 ± 3.3 versus 59.3 ± 6.6 in the asthma and control samples, respectively ($p = 0.28$). (C) Comparison of IFN- γ receptor 1 cell surface protein expression in PBMCs isolated from patients with asthma ($n = 17$) and control subjects ($n = 10$) using a flow cytometry. No difference was detected in the intensity of IFN- γ receptor 1 expression

Indeed, the promoter sequence of the IL-12 gene, its expressivity, cytokine levels, and responsiveness to IL-12 were no different in PBMCs that were isolated from patients with asthma compared with control subjects. Although these results would be in favor of the Th2 hypothesis in asthma, it should be noted, that our experiments were performed on cells from patients with mild to moderate asthma, therefore a primary decrease in IL-12 or IFN- γ secretion in patients with more severe phenotype cannot be ruled out. Likewise, we examined a population with well established, physician-diagnosed asthma, in the age range of 12 to 59 years, and we did not observe any differences in the expression levels of these Th1-type cytokines or their receptors on the basis of age. However, a decrease in Th1 cytokine production cannot be ruled out in younger children because we examined a population with a mean age of 38 years. In addition, because the cells tested were PBMCs isolated from peripheral blood, we cannot exclude potential differences in expression levels of these genes in cells infiltrating the lungs.

Pravica and coworkers reported a relatively rare polymorphism within the promoter region of the IL-12 p35 gene (-916 , C $\rightarrow$ T) together with a transition within the promoter part of p40 (-1287 , C $\rightarrow$ T), which was unrelated to the capacity of the cells to produce IL-12 (32). Although potentially important in certain subgroups of individuals with asthma, we were unable to confirm this polymorphism in the Icelandic population. A recent study reported a polymorphism in the IL-12 p40 promoter in children with asthma together with reduced production of the IL-12 p70 protein (26). A rare polymorphism in the IL-12 p40 gene was also reported but with no evidence of altered function (33). We could neither detect polymorphism in the promoter regions of IL-12 p35 and IL-12 p40 nor could we detect expression differences in IL-12 mRNA or protein levels in PBMCs between patients with asthma and control subjects, suggesting that it is unlikely that the sequence alterations in these genes that are of functional relevance are common.

In further support of a lack of a primary role of Th1-type cytokine genes in asthma, the results from our genome-wide scan demonstrated no evidence of linkage to microsatellite markers that are closely associated with the genes that code for the IL-12 p35 and p40 subunits, IL-12 R β units 1 and 2, IFN- γ and IFN- γ receptor units 1 and 2, Toll-like receptor 4, IL-18, and STAT4, in the Th1 pathway, suggesting that it is unlikely that variants within these genes confer a significant susceptibility to asthma. Although environmental factors cannot be excluded, it should be noted that the promoter sequences of the IL-12 gene, its expressivity, cytokine levels, and responsiveness to IL-12 were no different in PBMCs that were isolated from patients with asthma compared with control subjects, suggesting that the function of IL-12 is unaltered. Whereas these results would be in favor of the Th2 hypothesis in asthma, it should be noted that our experiments were performed on cells from patients with mild and moderate asthma, and a primary decrease in IL-12 or IFN- γ secretion in patients with more severe asthma cannot be ruled out. Likewise, we examined a population with well established, physician-diagnosed asthma, in the age range of 12 to 59 years, and we did not observe any differences in the expression levels of these Th1-type cytokines or their receptors on the basis of age. However, a decrease in Th1 cytokine production cannot be ruled out in younger children because we examined a population with a mean age of 38 years. In addition, because the cells tested were PBMCs isolated from peripheral blood, we cannot exclude potential differences in the functional activities of these genes in cells infiltrating the lungs. We conclude that a perturbation in the Th1 type cytokine pathway is unlikely to be a primary event in adult asthma.

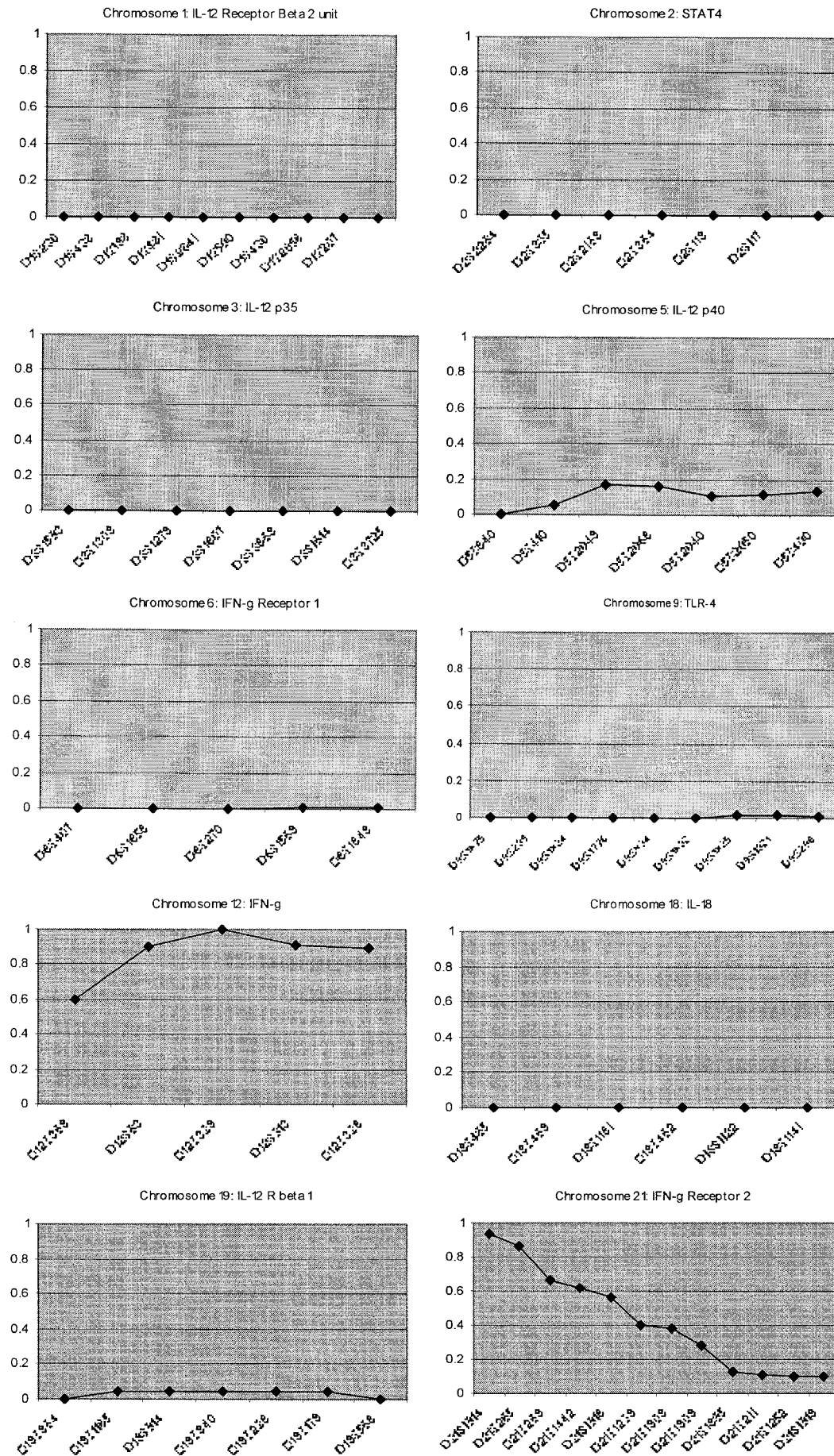


Figure 4. Linkage analysis to 10 candidate Th1 cytokine pathway genes. No evidence of linkage was uncovered to the regions of the chromosomes studied, which contained the following genes: IL-12 p35 and IL-12 p40, IL-12 receptor β units 1 and 2, IFN- γ and IFN- γ receptor units 1 and 2, Toll-like receptor 4, IL-18, and the transcription factor STAT4. The lod score obtained for each of these 10 genes ranged from 0 to 1.0. The Y axis represents the lod score. The microsatellite markers are on the X axis.

Conflict of Interest Statement: I.F.B. has no declared conflict of interest; E.H. has no declared conflict of interest; U.S.B. has no declared conflict of interest; D.L.S. has no declared conflict of interest; E.A. has no declared conflict of interest; T.A. was a DeCode Genetics employee from 1999 to 2002; D.G. has no declared conflict of interest; T.G. has no declared conflict of interest; J.G. has no declared conflict of interest; K.S. has no declared conflict of interest; H.H. has no declared conflict of interest.

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