Transcriptional regulation of the human interleukin-12 receptor beta2 gene
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A novel polymorphic GATA site in the human IL-12Rβ2 promoter region affects transcriptional activity

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ABSTRACT

Interleukin-12 (IL-12) is a potent inducer of IFNγ production by T cells and is a major factor for the development of Th1 cells. It exerts its biological effects through binding to the IL-12 receptor (IL-12R), a heterodimer composed of a β1 and a β2 subunit. The signaling β2 chain is expressed on Th1 cells, and to a lesser extent on Th0 cells, but not on Th2 cells, rendering these cells unresponsive to IL-12. Recently, polymorphisms in the coding region of the IL-12Rβ2 gene have been described that were associated with atopic disease. Here, we analyzed the 5’ regulatory region of the human IL-12Rβ2 gene by DHPLC (Transgenomic WAVE system). We found five novel single nucleotide polymorphisms (SNP) in the proximal 1.2 kb IL-12Rβ2 promoter region, i.e., -237C/T, -465A/G, -1023A/G, -1033T/C, and -1035A/G. SNP -465A/G is of particular interest as it determines the integrity of a GATA consensus site. By functional comparison of both -465 alleles in transient transfection assays, we show that promoter activity is increased in case of the -465G allele, disrupting the intact GATA site. In addition, only the -465A allele showed specific binding to (a) nuclear protein(s) extracted from Th cells. DNA-binding activity to an oligonucleotide containing the -465 GATA site was stronger in Th2 extracts as compared to Th1 extracts, in line with higher GATA3 expression levels in Th2 cells. Comparison of the prevalence of the -465A/G SNP alleles in small cohorts (n = 40) of allergic asthmatic and healthy control individuals provided no evidence for an altered distribution in the asthmatic population. In conclusion, we have identified a novel polymorphic GATA site that may affect transcriptional activity of the human IL-12Rβ2 gene under Th2 polarizing conditions.

INTRODUCTION

Interleukin-12 (IL-12) is produced by antigen-presenting cells and is a potent inducer of IFNγ production by T and NK cells and is a major factor for the induction of Th1 responses. The biological functions of IL-12 are mediated through the IL-12 receptor (IL-12R) consisting of two subunits, the IL-12Rβ1 and the IL-12Rβ2 chains, both in the human and the mouse. In the human system, both receptor subunits are able to bind IL-12 with low affinity, when expressed individually in COS-7 cells, but both chains are required to bind IL-12 with high affinity. Only the IL-12Rβ2 subunit contains intracellular tyrosine phosphorylation sites, necessary for IL-12 signaling. The non-signaling IL-12Rβ1 subunit is expressed after TCR stimulation of naive T cells and the protein is abundantly expressed by all differentiated T cells. In contrast, the expression pattern of the IL-12Rβ2 chain is more restricted and is not expressed by Th2 cells.
rendering these cells unresponsive to IL-12. So far, little is known about the transcriptional regulation of the IL-12Rβ2 gene. Transcription factors like NFATc2, SP1 and SP3 play a role in the regulation of the proximal promoter, but are probably not involved in the differential expression of the gene in Th1 and Th2 cells. Mouse experiments have implicated the polarizing masterswitch transcription factors GATA3 and T-bet as likely candidates to play a role in differential transcription of the IL-12Rβ2 gene.

It was shown by Rogge et al. that broncho-alveolar lavage (BAL) T cells from allergic asthma patients showed hardly any expression of the IL-12Rβ2 chain as compared to BAL T cells from sarcoidosis patients. The in situ IL-12Rβ2 mRNA expression in the asthmatic BAL T cells was even further down regulated upon allergen challenge. Furthermore, PBMC from atopic children expressed less IL-12Rβ2 mRNA after stimulation as compared to PBMC from non-atopic children. This data suggests a reduced capacity to respond to Th1-inducing stimuli in atopic patients, due to reduced IL-12 responsiveness of Th cells.

Allergic asthma is a Th2 associated disease, of which the etiology is complex and involves the interaction of multiple genetic loci and a variety of environmental factors. Quite a number of chromosomal regions and genes have been identified that suggest linkage which allergy and asthma. Candidate asthma genes that may be linked or associated with the asthmatic phenotype, include cytokine genes, receptor genes, transcription factors and many others. A well documented example is chromosome 5 where linkage is found in a region (5q31-33) representing a gene cluster with IL-4, IL-5 and IL-13 and the β2-adrenergic receptor. The IL-12Rβ2 gene is located on chromosome 1 (1p31.2). So far, there have been no reports suggesting linkages between allergy or asthma and chromosome 1. This, of course does not rule out the possibility that possible functional polymorphisms in the IL-12Rβ2 gene, will be linked or associated with an asthmatic phenotype.

Indeed, several polymorphisms in the coding region of the IL-12Rβ2 gene have been described, resulting in truncated IL-12Rβ2 protein leading to decreased STAT4 phosphorylation and IL-12 signaling. The presence of these mutations, that only occurred heterozygously in atopic individuals, were associated with decreased IFNγ production following IL-12 stimulation. Polymorphisms within the 5' and 3' regulatory sequences or introns of the IL-12Rβ2 gene may have a significant effect on transcription since they may alter the structure of regulatory elements and affect the affinity of transcription factor binding. So far, no polymorphisms in the IL-12Rβ2 regulatory regions have been reported.
Here, we set out to identify DNA sequence variation in the 1.2 kb 5' flanking region of the human IL-12Rβ2 gene in a small cohort of allergic asthma patients, to identify possible genetic determinants that may characterize allergic asthmatic subpopulations. We used denaturing HPLC (DHPLC). This technique depends on detecting heteroduplexes in PCR products by HPLC and therefore is very useful to identify unknown polymorphisms. We identified novel single nucleotide polymorphisms (SNPs) in the promoter region of the human IL-12Rβ2 gene (update of promoter sequence is available from GenBank AF349574). Strikingly, three of these SNPs are located in GATA consensus sequences. It is known, at least in the mouse, that GATA3 inhibits IL-12RB2 expression. We show that the -465A/G SNP in the GATA consensus site affects transcription and transcription factor binding. Preliminary comparison of the prevalence of these polymorphic alleles in small cohorts of forty healthy control individuals and forty patients suffering from allergic asthma, provided no evidence for an altered distribution of any of these SNPs in the asthmatic population.

**Materials & Methods**

**Subjects**

Forty allergic patients with mild to moderate asthma (according to the criteria of the American Thoracic Society) were recruited either by advertisement or from the outpatient respiratory clinic of the Academic Medical Center, Amsterdam. All patients gave written informed consent to participate in the study, which was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam. Forty healthy subjects, without specific IgE for common inhalant allergens (RAST negative), with serum IgE levels below 100 IU/ml were selected as controls.

**Genomic DNA isolation**

Genomic DNA was isolated from heparinized total blood with the Qiagen blood isolation kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions with a minor modification: instead of Qiagen protease we used 0.5 mg Proteinase K (Merck, Darmstadt, Germany) / 200 μl total blood.

**Denaturing high-performance liquid chromatography (DHPLC)**

Primers (BioSource, Nivelles, Belgium) used for the amplification of the -1210 through +107 IL-12Rβ2 promoter fragment of each subject are listed in Table 1. PCR reactions were performed in
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a final volume of 50 µl, using 125 ng of genomic DNA as template, 250 µM of each dNTP, 3 pmol of the forward and reverse primers, 1.25 U of Taq DNA polymerase (Roche Diagnostics, Almere, The Netherlands) and Taq buffer. Reactions were carried out in the Peltier Terminal Cycler-200 (BIOzym, Landgraaf, The Netherlands), as follows: denaturation for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C or 64°C (primer dependent) for 30 s and elongation at 72°C for 1 min, and finally, elongation at 72°C for 10 min. PCR products were analyzed for size by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. Amplified fragments were denatured by incubating at 94°C for 10 min, reannealed at 65°C for 10 min and slowly cooled down to 4°C to allow formation of heteroduplexes. DHPLC analysis was performed on an automated DHPLC instrument: the Transgenic WAVE System (Transgenic Inc., San Jose, CA). The stationary phase consisted of a DNA Sep® column, which binds DNA during analysis. The mobile phase consisted of two eluents (pH 7.0). Buffer A contained triethylammonium acetate (TEAA), which interacts with the negatively charged phosphate groups on the DNA as well as with the surface of the column. Buffer B contained TEAA with 25% of the denaturing agent acetonitrile. Fragments were eluted with a linear acetonitrile gradient of 2% per min at a flow rate of 0.9 ml/min. Increasing the concentration of acetonitrile at a fixed temperature will denature the fragments. Temperatures for successful resolution of heteroduplexes were calculated by the DHPLC Melt program (http://insertion.stanford.edu/cgi-bin/melt.pl) and are listed in Table 1. Fragments of the -1210/+107 IL-12Rβ2 promoter region that showed variation based on changed peak formation due to heteroduplex formation were further analyzed.

Detection of IL-12Rβ2 promoter polymorphisms

Fragments of the 5' region that showed heteroduplex formation were further analyzed. To this aim the particular 5' flanking region fragment of the appropriate patient was sequenced, by sequencing. To this aim, PCR fragments were purified with the Concert™ gel extraction system according to the manufacturer's instructions (Life Technologies, Paisley, UK), sequenced using BigDye Terminator Reaction Kit (PE Applied Biosystems, Warrington, UK) and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). In this way, five novel SNPs were found in the 1.2 kb proximal promoter region. For all subjects, further analysis of the -237C/T SNP and the -1023A/G SNP was performed by restriction fragment length polymorphism (RFLP) assays. This was performed by 40 cycles of PCR with primer 1S and primer 7AS (Table 1). PCR fragments were electrophoresed on a 2% agarose gel following Ital (-237) or
### Table 1

<table>
<thead>
<tr>
<th>Fr.</th>
<th>Location</th>
<th>Length (bp)</th>
<th>Primer Sequence</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1210/-766</td>
<td>434</td>
<td>S 5' -TCATGCTCTTCTTATACAAAGCGTGATTGG-3' AS 5' -CCTTTAAAACGCTCTCATTTGTGAGAT-3'</td>
<td>53/54/55</td>
</tr>
<tr>
<td>2</td>
<td>-853/-682</td>
<td>171</td>
<td>S 5' -CCCTTAAGAGACAAATCTGTACATGTTGGA-3' AS 5' -AGTTTTCCTTATGTCACGAGCCCC-3'</td>
<td>56/57/60/61</td>
</tr>
<tr>
<td>3</td>
<td>-707/-409</td>
<td>298</td>
<td>S 5' -CCCTGACTCGGCACTGACTAGAGCAAG-3' AS 5' -CCCTCCAAGCACCGTAGGATT3'</td>
<td>53/54/55/59</td>
</tr>
<tr>
<td>4</td>
<td>-525/-291</td>
<td>234</td>
<td>S 5' -CCTGACGGTTTTGAATGCTGACGTCG-3' AS 5' -CTGTTTTTAACCCCTGAAAGACAGCTC-3'</td>
<td>55/56/60/61</td>
</tr>
<tr>
<td>5</td>
<td>-402/-136</td>
<td>266</td>
<td>S 5' -CCTGATTACCGCTACACTTG-3' AS 5' -CTGGACAGAGCGTGGTGACGTC-3'</td>
<td>57/58/60/61</td>
</tr>
<tr>
<td>6</td>
<td>-173/+7</td>
<td>180</td>
<td>S 5' -GACCTATACGGGATGTTGACAGCC-3' AS 5' -CTCCTGCCAGCCAGAGCTCCTTA-3'</td>
<td>58/59/62/63</td>
</tr>
<tr>
<td>7</td>
<td>-56/+107</td>
<td>163</td>
<td>S 5' -CGTCTGACAGCAGCTTGGGC-3' AS 5' -GGCGCGATAAAAATCTG GTGCT-3'</td>
<td>62/63/64</td>
</tr>
</tbody>
</table>

a Fr., designated identification number of the amplified fragment. b Location of the oligonucleotide sequence in the IL-12Rβ2 gene. c S, sense oligonucleotide; AS, anti-sense oligonucleotide. d Temp (°C), Temperatures for successful resolution of heteroduplexes by DHPLC analysis (Transgenomic WAVE System) were calculated by the DHPLC Melt program as described in Materials and Methods.

HindIII (-1023)/EcoRI double digestion. For analysis of the -465A/G SNP, 40 cycles of PCR were performed with two separate PCR reactions using allele specific sense primer G 5’-ACTAGC GCA TAA ATA ATG GTA TG-3’ or A 5’-ACTAGC GCA TAA ATA ATG GTA TA-3’, both with anti-sense primer 6AS (Table 1). The annealing temperatures used in the reactions with allele specific primers A and G were 59°C and 60°C, respectively.

**Site-directed mutagenesis in reporter gene constructs**

The 5’ flanking region of the human IL-12Rβ2 gene (GenBank AF349574) was amplified by PCR using genomic clone PAC104 (obtained as described 4) as a template. For cloning purposes, the 5’ sense primer -1191 (5’-GCC CGA GCT CCG GTA TCA CTT GTG GTG AAA ATT-3’) or -591 (5’-GCC CGA GCT CGA TAT CTA AAT AAA ATC TCT-3’) were designed with an additional SacI restriction site (underlined) and the 3’ anti-sense primer (5’-GCC CGT TGA
CAG CCA TCA GGG AAC T-3') with a natural HinII site (underlined), resulting in PCR products spanning -1191 through +54 or -591 through +54, relative to the start of the reported cDNA sequence 1. Site-directed mutagenesis in the IL-12Rβ2 promoter constructs was carried out as described before 6. The internal forward primers used, containing the targeted sites (core-binding elements underlined, with substitutions indicated in italics and bold) were: -1035 non-GATA 5'-TTG GGG TTT TTT TGT AAA CTT AAA GAA AAT GAC TCA CT-3' and -465 GATA 5'-ATA ATG GTA TAT CTT ATA ATT A-3'. Products from this procedure were cloned into pGL3e (Promega, Leiden, The Netherlands) and sequenced to confirm the introduction of the desired mutations. Plasmid DNA was prepared from bacterial cultures using Qiagen Plasmid Midi Kits (Hilden, Germany).

**Cell culture and transient transfection**

Jurkat cells were grown in complete medium (Iscove's modified Dulbecco's medium, Bio-Whittaker, Walkersville, MD) supplemented with 5% pooled, C-inactivated fetal calf serum (Bio-Whittaker) and gentamycin (80 μg/ml; Duchefa, Haarlem, The Netherlands). Transfection and promoter-driven luciferase activity experiments were performed as described before 6.

**Electrophoretic Mobility Shift Assays (EMSA)**

Nuclear protein extraction and EMSA were performed as described before 6. The ds oligonucleotides used in this study were (core-binding elements underlined): -1035 GATA 5'-TTG GGG TTT TTT TGT AAA GAT AAA GAA AAT GAC TCA CT-3', -1035 non-GATA as described above, -465 non-GATA 5'-ATA ATG GTA TGT CTT ATA ATT A-3' and -465 GATA as described above.

**Detection of GATA3 protein expression by immunocytochemistry**

Full length GATA3 cDNA, kindly provided by Dr. L. Rogge (Roche Ricerche, Milan, Italy), was subcloned into the pcDNA3.1/Myc-His(+) expression vector (Invitrogen, Leek, The Netherlands), further referred to in the text as GATA3 x pcDNA3 myc. Thus obtained GATA3 protein contains a myc-His tag for detection purposes. For cloning purposes, the 5' sense primer (5'-GCG CGA ATT CCC ACC ATG GAG GTG ACG GCG GAC-3') was designed with an additional EcoRI restriction site (underlined) and a Kozak sequence and the 3' anti-sense primer (5'-GCG CTC TAG AAC CCA TGG CGG TGA CCC TGC T- 3') with an XbaI restriction site.
The cloned product was checked by sequencing and used for co-transfection with reporter constructs to increase GATA3 protein level in transfected cells.

One day after co-transfection, cells were stimulated with PMA (10 ng/ml, Sigma Aldrich Co.) and ionomycin (1 μg/ml, Sigma Aldrich Co.) for 16 hours. Hereafter, cytospins were made (Cytospin 2, Shandon, Pittsburgh, PA) by spinning at 300 x g for 5 min. Cells were fixed with acetone (-20°C) for 10 min and, after blocking with 1% FCS in PBS, incubated with mouse anti-human GATA3 (1 μg/ml, HG3-31, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-myc (1 μg/ml; Invitrogen) or no antibody for 45 min at RT. Cytospins were washed in PBS containing 1% FCS and incubated with FITC-conjugated goat anti-mouse IgG FITC (1μg/ml, Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 45 min at RT. Nuclei were stained with Hoechst 33342 (Sigma-Aldrich Co). Slides were analyzed with a fluorescence microscope (Leica, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands) to determine the percentage of GATA3 expressing cells.

RESULTS

Identification of polymorphisms in the 1.2 kb 5' flanking region of the human IL-12Rβ2 gene

The IL-12Rβ2 -1210 through +107 promoter region of 40 allergic asthma patients was analyzed by DHPLC, in which heteroduplexes in PCR products are detected by HPLC. These analyses revealed heteroduplexes in PCR fragments 1, 3, 4 and 5 (Table 1), suggesting the presence of polymorphic sites. Subsequent sequence analyses of these fragments revealed five novel SNPs: -237C/T, -465A/G, -1023A/G, -1033T/C and -1035A/G, as shown in Fig. 1.

Genotyping for five novel SNPs in the human IL-12Rβ2 promoter

To determine the genotypes of subjects with respect to SNPs -237C/T and -1023A/G, RFLP assays were performed on the PCR products of the respective promoter fragments. For analysis of position -237 we used the polymorphic Ital site. The fragment from -853 through +107 was amplified with primers 2S and 7AS and digested with Ital. The -237-allele lacking the Ital site generated a single product of 960 bp, whereas the -273C-allele with the intact Ital site was digested into two fragments of 617 bp and 343 bp, respectively (Fig. 2A). Position -1023 is part of a polymorphic HinfI site. The PCR-amplified promoter fragment obtained with primer 1S
Fig. 1 Five novel SNPs in the 5' flanking region of the human IL-12RR2 gene. The region from -1210 through +107 in exon 1 was divided into seven overlapping fragments that were amplified by PCR and used to detect heteroduplexes by DHPLC. Each polymorphic nucleotide identified by subsequent sequence analyses is indicated by a grey triangle and is named after its position.

Table 2 shows the allele frequencies of all five SNPs within the cohort of 40 allergic asthma patients. All genotyping either by restriction analysis, allele-specific PCR or sequencing analysis confirmed the results obtained with the DHPLC assay. Striking is the very low frequency of the -237T allele, that occurred homozygously in only one single patient.

and 7AS was double digested with Hinfl and EcoRV to allow size separation on a 2% agarose gel. The -1023A-allele lacks the intact Hinfl site and produced 7 fragments of 7, 48, 110, 227, 230, 264 and 436 bp, respectively. The -1023G allele with the intact Hinfl site produced 8 fragments, with the 264 bp fragment cut into 83 and 181 bp (Fig. 2B). For analysis of the -465A/G genotype we designed an allele-specific PCR with sense primers containing either a G or an A nucleotide at their 3' ends, selectively amplifying the -465G or A allele (Fig. 2C). The SNPs -1033T/C and -1035A/G were genotyped by additional sequence analysis.
Functional polymorphic GATA site in IL-12Rβ2 promoter

Fig. 2 Genotyping subjects for novel SNPs in the IL-12Rβ2 gene. A) The polymorphic *Ita* site was used to identify the genotype at -237. PCR products from -853 through +107 were cleaved with *Ita* and analyzed on an agarose gel. The -237T allele lacks an intact *Ita* site and generates a single product of 960 bp (lane 1), whereas the -237C allele generates two fragments of 617 and 343 bp (lane 2). Only the -237 CC or TT genotypes were observed in our cohort. B) The polymorphic *Hinfl* site was used in a double digest with *EcoRV* on the complete PCR product to identify the genotype at -1023. Allele -1023A has a disrupted *Hinfl* site and therefore the double digest results in fragments of 7, 48, 110, 227, 230, 264 and 436 bp (lane 1). The -1023G allele contains a *Hinfl* site resulting in digestion of the 264 bp fragment in 181 and 83 bp (lane 2). Only the -1023 AA or AG genotypes were identified. Fragments smaller than 80 bp are too small to detect. C) SNP -465 was genotyped with an allele specific PCR as described in Materials and Methods.

Three polymorphisms are located within two GATA consensus sites

We next screened the sequences of the polymorphic sites by TRANSFAC MathInspector V2.2 data base analysis for possible involvement of transcription factor binding sites. Interestingly, three of the five SNPs either create or disrupt a GATA consensus site (WGATAR\(^{16}\)). -465A→G disrupts a reverse GATA consensus site, and -1035G in combination with -1033T create a GATA site (these two GATA sites will be referred to as -465 and -1035 from here on). Of the other two SNPs, -237C/T disrupts a potential binding site for AP4, whereas neither of the alleles of the -1023 SNP have affinity for any known transcription factor. The transcription factor GATA3 plays a key role in directing Th2 cell development and, in the mouse, has been associated with down regulation of IL-12Rβ2 expression \(^{6,16}\). Therefore, polymorphisms disrupting or creating GATA binding sites might affect IL-12Rβ2 expression.
### Table 2

Allele frequencies of IL-12Rβ2 promoter polymorphisms in a cohort of 40 allergic asthmatics

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele frequencies n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-237C/T</td>
<td>C-allele 78 (97.5%) T-allele 2 (2.5%)</td>
</tr>
<tr>
<td>-465A/G</td>
<td>A-allele 64 (80%) G-allele 16 (20%)</td>
</tr>
<tr>
<td>-1023A/G</td>
<td>A-allele 68 (85%) G-allele 12 (15%)</td>
</tr>
<tr>
<td>-1033T/C</td>
<td>T-allele 69 (86.3%) C-allele 11 (13.7%)</td>
</tr>
<tr>
<td>-1035A/G</td>
<td>A-allele 67 (83.8%) G-allele 13 (16.2%)</td>
</tr>
</tbody>
</table>

* SNPs were traced by DHPLC analysis and identified by sequencing. Patients were genotyped for SNPs as follows: -237C/T by HinfI digestion, -465A/G by allele-specific PCR, -1023A/G by HinfI/EcoRV digestion and -1033T/C and -1035A/G by additional sequencing. ** n (%), represents number of both alleles, respectively, calculated as percentages. Forty patients represent 80 alleles.

**Effects of the -465A/G and -1035A/G SNPs on IL-12Rβ2 promoter activity**

The effects of the polymorphic -465 and -1035 GATA sites were analyzed by functional comparison in transient transfection assays, using the luciferase reporter system to test promoter activity. Transfections were performed in the human Jurkat cell line as described before 4.

To test whether the integrity of the -1035 GATA site affected promoter activity, we mutated the GATA site AGATAA into ACTTAA (core binding site underlined, mutation in italics and bold) in the context of the -1191/+54 promoter construct. Although it should be noted that within this promoter fragment three additional GATA consensus sites are present (unpublished observations), disruption of this GATA site did not result in a significant change of transcriptional activity (Fig. 3A). The reversed GATA site ATATCT (-465A; core binding site underlined, mutation in italics and bold) was mutated into the disrupted-GATA consensus site.
Fig. 3 Functional analysis of polymorphic GATA sites -1035 and -465 in the presence of overexpressed GATA3. Jurkat cells were transiently co-transfected with reporter gene constructs, containing either the GATA or disrupted GATA sites, and a plasmid that constitutively expressed the full length GATA3 cDNA. These cultures were stimulated one day after transfection with PMA and ionomycin for 16 hours. Luciferase activity values were corrected for transfection efficiency (Renilla luciferase), calculated as relative luciferase activity units and expressed as the percentage of promoter activity of -1035 GATA (100%) and -465 GATA (100%), respectively. Luciferase activity is shown as the mean luciferase activity ± S.D. of at least three independent duplicate experiments. A) Here, the -1035 GATA (AGATAA) and the -1035 non-GATA (ACTTAA) were tested in the context of the -1191/+54 promoter construct. B) The -465 reversed GATA (AT4TCT) and -465 non-GATA (ATGTCT) alleles were tested for promoter activity in the context of the -591/+54 promoter fragment. C) Overexpression of GATA3 in Jurkat cells. As a control to confirm GATA3 expression, cytospins were made of Jurkat cells co-transfected with empty vector (left) as a control or GATA3 x pcDNA3 myc (right). The cytospins were stained to show GATA3 expression with anti-myc.
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ATGTC (-465G) in the context of a -591/+54 IL-12Rβ2 promoter construct. Interestingly, disrupting the -465 GATA site resulted in an increase of promoter activity of almost 200%, as compared to -465A (Fig. 3B).

Because Jurkat cells express GATA3 protein only at very low levels, a full-length GATA3 cDNA under the control of a strong constitutive promoter was co-transfected, to ensure high levels of GATA3 protein. GATA3 was produced as a GATA3/myc fusion protein to confirm expression by immunocytochemistry with anti-myc antibody (Fig. 3C). These results suggest that the activity of the promoter of the human IL-12Rβ2 gene is upregulated when the -465 GATA consensus site is disrupted (-465G).

Effects of the -465A/G SNP on binding of nuclear proteins

Next, we tested whether the -465A/G polymorphism affects binding of nuclear factors. To this aim, we performed EMSA analysis using the sequence -475 through -454 as ds oligonucleotide containing either a A or an G (disrupting a GATA site) at position -465. Incubation with nuclear

![Fig. 4 Transcription factor binding at the -465 GATA site. The radiolabeled -465G and -465A probes were incubated with nuclear Th cell extracts as described in the text. A) Incubation of the -465A oligo with Th2 cell nuclear extracts showed the formation of a specific complex (C1), indicated by the arrowhead. B) Comparison of nuclear extracts of Th1 and Th2 cells showed more intense formation of C1 to probe -465A (GATA) with Th2 cell extracts.](image-url)
protein extracts of TCR-stimulated CD4+ Th2 cells produced a complex (C1) with the -465A oligonucleotide (Fig. 4A, lane 2) and not with the GATA-disrupting -465G oligonucleotide (lane 1). The addition of anti-GATA3 antibody did not result in a clear supershift (data not shown). As Th2 cells express higher levels of GATA3, we tested for differential formation of C1, comparing Th1 and Th2 cell extracts. Indeed, nuclear extracts of both unstimulated and stimulated Th2 cells formed a more intense complex C1 with the -465A sequence (Fig. 4B, lane 3 and lane 4), as compared to Th1 extracts. This indicated that the A to G substitution at position -465, which disrupts a GATA consensus site, diminished the binding of a transcription factor, that is preferably expressed in Th2 cells.

Prevalence of -465A/G in allergic asthma patients versus healthy controls
Because the -465A to G substitution resulted in a disruption of a consensus GATA site with functional consequences for expression of the IL-12Rβ2 gene, we compared the prevalence of this allele in the asthmatic cohort to that in a cohort of 40 healthy control individuals. The frequencies of the -465A and G alleles in the healthy cohort (78.8 % and 19.2 %, respectively) were not different. Also the frequency of the heterozygous and homozygous genotypes was quite similar (Table 3). In conclusion, the comparison of the prevalence of these polymorphic alleles provided no evidence for an altered distribution in the asthmatic population.

Table 3
Genotypes of -465A/G in allergic asthmatics versus healthy control individuals

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymorphism</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
</tr>
<tr>
<td>Allergic asthmatics</td>
<td>-465A/G (40)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>-465A/G (40)</td>
</tr>
</tbody>
</table>

^ n, represents number of individuals.
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DISCUSSION

In the present study, we report the identification of five novel SNPs in the 1.2 kb proximal promoter region of the human IL-12Rβ2 gene, i.e., -237C/T, -465A/G, -1023A/G, -1033T/C and -1035A/G. These are the first polymorphisms described in 5' regulatory region of human IL-12Rβ2 gene.

Computer assisted TRANSFAC database analysis indicated that the -237 C>T substitution disrupts a putative AP4 binding site. However, this SNP was found only in one individual, albeit homozygously, and we performed no functional experiments on this site, so far. Neither of the -1023 allelic variants form binding sites for known transcription factors, whereas the almost adjacent SNPs at -1033 and -1035 are part of a putative AP1 site and an overlapping GATA site. The integrity of only the GATA site is influenced by these SNPs, but disruption of this GATA site did not result in altered transcriptional activity. The -465 A>G substitution may be of particular interest, as it disrupts a GATA consensus sequence, reducing transcription factor binding activity and resulting in increased IL-12Rβ2 transcription.

So far, we could not confirm the identity of the transcription factor(s) binding at the -465A GATA site. TRANSFAC database searches suggested that the -465A/G SNP does not affect binding of any known T cell transcription factor, other than GATA3. Although the polymorphic basepair is situated within a putative Oct1 binding element, it does not affect the actual Oct1 binding site 19, arguing against involvement of Oct1 in the different binding patterns of the -465A and -465G oligonucleotides. Still, we could not detect GATA3 protein in complex C1 on the -465A probe by supershift analysis, using a commercially available GATA3 antibody. To the best of our knowledge, a clear GATA3 supershift in experiments with human cells has never been reported before by others.

Previous studies have shown that GATA3 expression is increased in Th2 cells, both in the mouse 20 and in humans 21, and that ectopic expression of GATA3 in developing mouse Th1 cells suppresses IL-12Rβ2 gene expression 20,22. Our own unpublished results indicated four other GATA sites in the 1.2 kb 5' regulatory region (including the polymorphic one at -1033), none of which affected promoter activity as indicated by site-directed mutagenesis and reporter gene assays (Chapter 5). Based on the above findings, it is tempting to speculate that GATA3 binds at the A-allele of the -465 GATA site and that, in Th2 cells, this results in stronger suppression of the IL-12Rβ2 gene.

As allergic asthma is a Th2-mediated disease with a considerable genetic predisposition, we analyzed the prevalence of the -465A allele in a small cohort of 40 allergic asthmatic patients and
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found a high incidence of 80%. Although this allele may contribute to the downregulation of the IL-12Rβ2 chain and facilitate GATA3-mediated Th2 polarization of allergen-specific Th cells in allergic asthmatic patients, it is probably not an important risk factor for the development of asthma, as almost the same -465A allele-frequency was found in a cohort of 40 healthy control individuals. Furthermore, both cohorts showed largely the same distribution of homozygous and heterozygous genotypes but, notably, homozygous -465G individuals were only found in the asthmatic cohort.

However, firm conclusions along this line cannot be drawn yet, as the cohorts in this study were much too small. Polymorphisms that have small to moderate effects are often difficult to link to multifactorial diseases, like allergic asthma, due to their relatively minor contribution. This does not exclude the possibility that the -465A allele has a facilitating role in the suppression of IL-12Rβ2 expression under Th2 polarising conditions, as induced for example upon allergen exposure in allergic asthmatics. A prerequisite for this effect to occur is that GATA3 needs to be activated and, therefore, the -465 SNP is not expected to affect IL-12-responsiveness in Th1-mediated responses. To test the net impact of the -465A/G SNP in a controlled and more physiological setting, allele-specific IL-12Rβ2 expression should be investigated in naive Th cells from -465 heterozygous individuals upon stimulation under graded Th2-polarizing conditions, using allele-specific transcript quantification (ASTQ) based on the coding polymorphism reported by Matsui et al., as a read out. The genotypes for the other SNPs in the 5' regulatory region should preferably be identical. These experiments are underway.

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Chapter 6

REFERENCE LIST


