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## Effects of common atopy-associated amino acid substitutions in the IL-4 receptor alpha chain on IL-4 induced phenotypes

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**Abstract** The human IL-4 receptor alpha chain gene (*IL4R*) is highly polymorphic and controversial reports have been published with respect to the association of different single nucleotide polymorphisms (SNPs) with atopy markers. Here we analyzed the functional and associational relevance of common *IL4R* coding SNPs. Transfection of B cell lines expressing the IL-4R variant V75+R576 did not result in enhanced IL-4 induced CD23 expression compared to cell lines expressing the wild type IL-4R alpha chain. Transfection of the IL-4R variant P503 into a murine T cell line did not influence IL-4 induced T-cell proliferation compared to wild type constructs. Analysis of six *IL4R* coding SNPs (*I75V*, *E400A*, *C431R*, *S436L*, *S503P*, *Q576R*) and common haplotypes (frequency  $\geq 0.05\%$ ) in blood donors ( $n=300$ ) did not indicate a significant association with elevated serum IgE level. Moreover, the most informative *IL4R* coding SNPs (*I75V*, *C431R*, *Q576R*) and related two- and three-point haplotypes (frequency  $\geq 0.05\%$ ) were analyzed in a second, extended group of blood donors ( $n=689$ ). Again, no significant association with elevated serum IgE was detectable. We conclude that common coding SNPs in

the *IL4R* gene are unlikely to contribute significantly to increased IgE levels and variations outside the coding region may influence atopy susceptibility.

**Keywords** Human · Cytokine receptors · Signal transduction · Allergy

### Introduction

Atopy is a common complex disorder characterized by the presence of specific IgE antibodies to common allergens and elevated total serum IgE levels. Genome-wide studies have detected linkage of a region on chromosome 16p12 with the atopy-related phenotype total serum IgE level (Daniels et al. 1996; Ober et al. 2000). However, this linkage has not been replicated in numerous genome screens (Cookson et al. 1989; Noguchi et al. 1999a; Wjst et al. 2002), for a review see (Wills-Karp and Ewart 2004).

One biological candidate gene within this region is the interleukin-4 receptor gene (*IL4R*), which codes for the alpha chain of the IL-4R heterodimer. Targeted disruption of the *IL4R* gene in mice abrogates the IgE immune response, which is consistent with its essential role in the pathogenesis of the allergic immune response (Noben-Trauth et al. 1997). The IL-4R is activated through binding of the Th2 cytokine IL-4, which promotes proliferation and survival of lymphocytes, immunoglobulin isotype switching to IgE, Th2 lineage commitment of CD4<sup>+</sup> T cells and expression of class II MHC and Fc epsilon RII (CD23) (Chomarat and Banchereau 1997; Coffman et al. 1993).

The binding of IL-4 to its receptor induces the phosphorylation of Janus kinases (Jak) and activated Jaks in turn mediate phosphorylation of conserved tyrosine residues of the cytoplasmic tail of the IL-4R alpha chain. The proximal Y1 residue (Y497) is surrounded by a motif that is referred to as the insulin/IL-4R (I4R) motif and interacts upon its phosphorylation with insulin receptor substrate (IRS)-1/2 and several phosphotyrosine binding domain signaling proteins (Keegan et al. 1994).

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The I4R-IRS-1/2 pathway has been implicated in growth-promoting signals in response to IL-4 (Keegan et al. 1994).

The three conserved tyrosines Y2, Y3, and Y4 (Y575, Y603, Y631) serve as docking sites for signal transducer and activator of transcription 6 (STAT6), a Src homology domain 2 (SH2) transcription factor that is indispensable for induction of gene transcription by IL-4 (Blaeser et al. 2003; Pan and Rothman 1999).

Several single nucleotide polymorphisms (SNPs) have been identified in the *IL4R* gene, of which eight result in amino acid exchanges in the IL-4R alpha chain (Deichmann et al. 1997; Hershey et al. 1997; Ober et al. 2000). Genetic association studies have led to controversial findings. Some showed association of different *IL4R* SNPs with total serum IgE levels (Beghe et al. 2003; Cui et al. 2003; Hackstein et al. 1999; Hershey et al. 1997; Howard et al. 2002; Izuhara et al. 2000; Kauppi et al. 2001; Kruse et al. 1999; Liu et al. 2004; Mitsuyasu et al. 1998; Nakamura et al. 2003; Rogala et al. 2001; Takabayashi et al. 2000; Tan et al. 1999), whereas other studies found no association between these variants and serum IgE levels (Bottini et al. 2002; Dupre et al. 2000; Grimbacher et al. 1998; Haagerup et al. 2001; Malerba et al. 1999; Migliaccio et al. 2003; Noguchi et al. 1999b; Patuzzo et al. 2000; Wjst et al. 2002).

Two common coding SNPs of the *IL4R* gene alter the amino acid sequences surrounding the effector tyrosine residues Y1 and Y2. The common serine to proline polymorphism *S503P* is located six amino acids downstream of the I4R docking site Y497. The second common polymorphism, *Q576R*, is situated one amino acid downstream of the Y2 docking site implicated in STAT6 phosphorylation.

Targeted mutation of the Y1 residue of the *IL4R* gene in mice was associated with increased allergen-induced IgE, airway responsiveness, tissue eosinophilia, and mucus production (Blaeser et al. 2003). In humans, the *S503P* polymorphism may also affect the function of the I4R motif, and this may provide a physiological mechanism resulting in susceptibility to allergic disorders in carriers of the *P503* allele.

The *Q576R* polymorphism also has been associated with elevated IgE levels and allergic diseases. An enhanced IL-4 induced expression of CD23 on B lymphocytes from individuals bearing the *R576* allele compared to individuals bearing the *Q576* allele has been reported (Hershey et al. 1997). In contrast, analysis of responsiveness to IL-4 of transfectants with gene constructs carrying either *Q576* or *R576* did not reveal any difference in IL-4 induced gene transcription (Mitsuyasu et al. 1999; Wang et al. 1999). Recently, it was shown that the *Q576R* variant alone did not affect IL-4 induced CD23 expression. However, the combination of the *Q576R* amino acid exchange together with the extracellular substitution I75V resulted in enhanced sensitivity of transfectants to IL-4 (Risma et al. 2002). The I75V variant has previously been implicated in enhanced CD23 expression and IgE synthesis (Mitsuyasu et al. 1999). In contrast, in the study by

Risma et al. (2002), the I75V substitution alone did not affect IL-4 induced CD23 expression.

In this study, we analyzed whether the *S503P* polymorphism alters the I4R docking site of the IL-4R alpha chain and has an effect on IL-4 induced cell proliferation by transduction of murine cell lines with human *IL4R* gene constructs. Second, we asked whether the combined mutation I75V+Q576R alters the Y2 STAT6 docking site and has an effect on IL-4 induced CD23 gene transcription.

The functional studies were complemented by genetic association studies. We investigated the association of all known *IL4R* coding SNPs and extended haplotypes with the phenotype total serum IgE level. Our results suggest that common amino acid polymorphisms of the IL-4R alpha chain do not measurably affect signalling functions of the IL-4R.

## Materials and methods

### Antibodies

The phycoerythrin (PE)-coupled rat anti-mouse CD23 monoclonal antibody (mAb) and the fluorescein isothiocyanate (FITC)-coupled rat anti-mouse CD45R (B220) mAb were purchased from Caltag Laboratories (Hamburg, Germany). The PE-coupled mouse anti-human CD124 (IL-4R) mAb was purchased from Immunotech (Beckman Coulter, Krefeld, Germany). All isotype-matched control Abs were purchased from BD-PharMingen (Heidelberg, Germany).

### Cell lines

The A20 murine B cell line, originally derived from a BALB/c mouse, was maintained in complete RPMI, consisting of RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 2 mM L-glutamine (Life Technologies) and 10% fetal calf serum (FCS) (PAA Laboratories, Linz, Austria). The murine CTLL-2 cell line, derived from cytotoxic T-cells of a C57BL/6 mouse was cultured in RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine, 10–20 IU/ml murine recombinant IL-2 (Boehringer Mannheim, Mannheim, Germany) and 10% fetal bovine serum (FBS) (PAN Systems, Aidenbach, Germany). The ecotropic packaging cell line Phoenix for the generation of ecotropic retroviruses is based on the 293T cell line, a human embryonic kidney line transformed with adenovirus E1a and carrying a temperature-sensitive T antigen co-selected with neomycin. The Phoenix cell line was obtained from Dr. Slany (Erlangen, Germany). The maintenance of Phoenix cells is outlined elsewhere (<http://www.stanford.edu/group/nolan>).

## Cloning of the human *IL4R* cDNA

A total of  $1 \times 10^6$  peripheral blood mononuclear cells (PBMC) derived from a healthy, adult voluntary blood donor were incubated with recombinant human IL-4 (Boehringer Mannheim) for 24 h at 37°C and 5% CO<sub>2</sub> in RPMI 1640 containing 2 mM-glutamine, 10 ml/l penicillin/streptomycin (PAA Laboratories) and 10% FCS. Total RNA was extracted (RNeasy Mini Kit, Qiagen, Hilden, Germany) and cDNA was synthesized using the First Strand cDNA purification kit (Amersham Pharmacia Biotech, Freiburg, Germany).

The polymerase chain reaction (PCR) was used to amplify the *IL4R* cDNA. The amplification was carried out in a total volume of 50 µl, containing  $3.3 \times$  XL buffer II (Gene Amp, Perkin Elmer, Langen, Germany), 0.2 mmol/l each of the dNTPs (Gene Amp dNTP Blend, Perkin Elmer), 0.4 µmol/l of each primer (sense primer 5'-AGGGGCGCGCAGATAATTAAA-3', antisense primer 5'-AGTGGGGCCAATCACCTTCATA-3'; MWG Biotech, Ebersberg, Germany), 1.2 µmol/l Mg(OAc)<sub>2</sub> (Gene Amp, Perkin Elmer), 2 U *rTth* DNA polymerase (Gene Amp, Perkin Elmer) and 50 ng cDNA prepared as described. After initial denaturation (94°C, 1 min) the PCR reaction proceeded, consisting of 33 cycles of 20 s at 94°C, 40 s at 60°C, 3 min at 72°C.

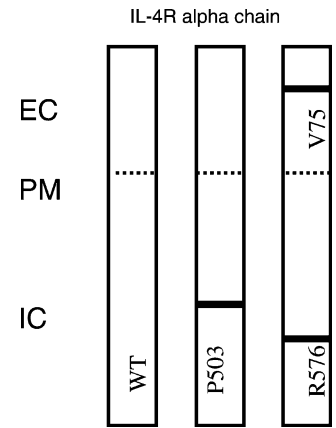
A nested PCR was used to add two *Bam*HI restriction sites to the *IL4R* fragment (sense primer 5'-GGATCCGCGCAGATAATTAAAGA-3', antisense primer 5'-GGATCCAAATCACCTTCATACCAT-3'). The amplified cDNA was diluted and subjected to an initial denaturation of 1 min at 94°C followed by 31 cycles of 20 s at 94°C, 45 s at 59°C, 3 min at 72°C. The *IL4R* cDNA fragment was ligated into the cloning vector pGEM-T (Promega, Mannheim, Germany) and transfected into JM109 competent cells (Promega). Plasmid DNA was prepared by using the Plasmid Mini Kit (Qiagen).

## Site-directed mutagenesis

Site-directed mutagenesis was performed at nucleotide 1682 [according to the IL-4R alpha chain nucleotide sequence of Idzerda et al. (1990)] with a substitution of thymidine for cytidine (S503P) using the QuikChange site-directed mutagenesis kit (Stratagene Europe, The Netherlands) according to the manufacturer's instructions (Fig. 1). In the same way, site-directed mutagenesis was conducted at nucleotide 398 with a substitution of adenine for guanine (I75V) and in addition at nucleotide 1902 with an adenine to guanine substitution (Q576R).

To generate the construct 1682T→C, the primers were 5'-CCGCAGCTTCAGCAACCCCTGAGCCAGTCACC-3' (sense) and 5'-GGCGTCGAAGTCGTTGGGGGACTCGGTCAAGTGG-3' (antisense). The primers for the construct 398A→G were 5'-GCCACACGTGTGTCCCTGAGAACACGG-3' (sense) and 5'-CCGTTGTTCTCAGGGACACACGTGTGGGC-3' (antisense). For 1902A→G, the primers used were 5'-CCCACCAGTGGCTATCGGGAGTTTGTA

**Fig. 1** Schematic representation of the IL-4R alpha chain and the different IL4R constructs used for transfection. The horizontal lines denote amino acid substitutions compared to the wild type (WT) shown for the extracellular domain (EC) and the intracellular domain (IC). The dotted line between the extracellular and the intracellular domain denotes the plasma membrane (PM).



CATGCG-3' (sense) and 5'-CGCATGTACAAACTCCCCG ATAGCCACTGGTGGG-3' (antisense).

## Sequencing of constructs

The integrity of the cDNA constructs (wild type, S503P, I75V+Q576R) was confirmed by DNA sequencing on an ABI Prism 310 Genetic Analyzer by using the ABI Prism Big Dye Terminator Sequencing Kit (Applied Biosystems, Darmstadt) according to the manufacturer's protocol.

## Retrovirus production

Supernatants containing retrovirus were produced by transient transfection of the packaging cell line Phoenix-E. In short,  $1 \times 10^6$  Phoenix cells per 10 cm plate were seeded into 10 ml medium containing DMEM (Life Technologies), 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM-glutamine. At 90% confluence, the cells were transfected with DNA of the *IL4R* constructs carrying either I75+Q576 (wild type) or V75+R576 (variant), using a standard calcium phosphate transfection protocol (Schreiner et al. 2001). After 2–3 days, the supernatants containing retrovirus were harvested, cleared from cell debris by centrifugation at 200 g for 5 min, and frozen at -80°C.

## Generation of transfected B cell lines

For retroviral infection, 1.8 ml of the virus stock,  $0.5 \times 10^6$  A20 cells and 2 µl Polybrene (stock 4 mg/ml) (Sigma-Aldrich, Germany) were combined and centrifuged at 2500 g for 3.5 h at 37°C. Cell pellets were resuspended in medium containing RPMI 1640 and 10% FCS. After 24 h cells were selected for resistance to neomycin at 1 mg/ml for 12–21 days. Stable transfectants were selected and characterized for their expression of human IL-4R by flow cytometry using a PE-conjugated mouse anti-human CD124 mAb. Untransfected cells and cells stained with the appropriate isotype-matched IgG were used as negative controls.

### Human IL-4 induced expression of CD23 in huIL-4R transfected murine B cell lines

A total of  $1 \times 10^6$  A20 transfected cells were cultured in complete RPMI medium alone or treated with increasing doses of recombinant human IL-4 (Boehringer Mannheim) for 48 h. Thereafter the transfected cells were stained with the PE-conjugated rat anti-mouse CD23 mAb in the presence of FITC-coupled CD45R mAb for 30 min on ice. Cells stained with the appropriate isotype-matched IgG were used as a negative control. After the final wash, CD45R positive cells were gated and then analyzed for surface expression of murine CD23 by flow cytometry (FACSCalibur, Heidelberg, Germany).

### Generation of transfected T cell lines

Full length wild type huIL-4R cDNA (*S503*) and mutated huIL-4R cDNA (*P503*) were cloned into the expression vector pM5neo (Laker et al. 1987). For transfection, DNA of the two *IL4R* constructs (*S503*, *P503*) were electroporated into CTLL-2 cells with a Genepulser II electroporation device at a field intensity of 2 kV/cm. After electroporation cells were maintained in RPMI 1640 containing 10% FCS and 50 U/ml IL-2. After 24 h cells were selected for resistance to neomycin (PAA Laboratories) at 1 mg/ml for 12–21 days. Stable transfectants were selected and screened by flow cytometry for CD124 expression using FITC-coupled mouse anti-human CD124 mAb. Controls included untransfected CTLL-2 cells and isotype-matched control antibodies.

### Human IL-4 induced proliferation of huIL-4R transfected murine T cell lines

A total of  $1 \times 10^4$  CTLL-2 cells/well, which were transfected with the DNA of the two *IL4R* constructs (*S503*, *P503*), were incubated in 96-well plates for 48 h with different concentrations of recombinant human IL-4 (40 ng/ml–0.15 ng/ml) in a final volume of 200  $\mu$ l. Afterwards, [ $^3$ H]-thymidine was added (0.5  $\mu$ Ci/well in 50  $\mu$ l medium) followed by a further incubation at 37°C for 16 h. After washing, [ $^3$ H]-thymidine incorporation was measured. CTLL-2 cells incubated with recombinant mIL2 (5 ng/ml) served as positive control, and cells maintained in medium without growth factors served as a negative control.

### Subjects

Six hundred and eighty-nine random volunteer blood donors from the region of Giessen were included after they gave informed consent. The procedure was approved by the local ethics committee.

**Table 1** Primer sequences for detecting *IL4R* SNPs

| SNP               | Orientation | Primer sequence (5'→3') |
|-------------------|-------------|-------------------------|
| <i>IL4R I75V</i>  | Sense       | CGCGCCTCCGTTGTTC        |
| <i>IL4R I75V</i>  | Antisense   | ACCCAGCCCTGTGTCT        |
| <i>IL4R Q576R</i> | Sense       | TGCTCCACCGCATGTACAA     |
| <i>IL4R Q576R</i> | Antisense   | GCCGAAATGTCTCCAGCAT     |

### Genotyping of *IL4R* sequence variants

Genomic DNA was extracted from blood samples using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, USA). Genotyping of *IL4R E400A*, *IL4R C431R*, *IL4R S436L* and *IL4R S503P* was performed according to a published PCR-SSP protocol with minor modifications (Hackstein et al. 1999).

Genotypes for *IL4R I75V* and *IL4R Q576R* were assigned using sequence specific probes (ABI PRISM 7000 Sequence Detection System, Taqman). Genotyping primers and probes are listed in Tables 1 and 2.

PCR was carried out following the universal protocol supplied by ABI. Fluorescence emission was measured on an ABI PRISM 7000 Sequence Detection System at 60°C. Known DNA samples of each genotype and water samples were used as positive and negative controls for each run.

### Measurement of serum IgE levels and definition of atopic phenotype

A subgroup of individuals was assessed by a physician who administered a questionnaire for a medical history of atopy ( $n=286$ ) (Hackstein et al. 1999; Hecker et al. 2003). We found a highly significant correlation between a positive clinical history of atopy and the prevalence of elevated serum IgE levels. Therefore, we used an elevated total serum IgE level of  $>100$  kU/l as an intermediate phenotype of atopy. Serum IgE levels were determined by nephelometry (Behring Nephelometer Analyzer, Marburg, Germany).

**Table 2** Probe sequences for detecting *IL4R* SNPs

| NCBI SNP cluster ID <sup>a</sup> | SNP                   | Probes for Taqman analysis |
|----------------------------------|-----------------------|----------------------------|
| rs1805010                        | <i>IL4R I75V</i> VIC  | ACACGTGTATCCCTG            |
| rs1805010                        | <i>IL4R I75V</i> FAM  | CACGTGTGTCCCTG             |
| rs1801275                        | <i>IL4R Q576R</i> VIC | CAGTGGCTATCAGGAGT          |
| rs1801275                        | <i>IL4R Q576R</i> FAM | CAGTGGCTATCGGGAGT          |

<sup>a</sup>The polymorphisms here have been assigned accession numbers as follow: rs1805010 (*I75V*), rs1801275 (*Q576R*)

## Statistical analysis

To evaluate deviation from the Hardy-Weinberg equilibrium, observed and expected genotype frequencies were compared by a Monte Carlo simulation-based goodness of fit test.

To investigate the possible associations of genotype and haplotype frequencies with serum IgE level, serum IgE level was dichotomized as being high ( $\text{IgE} > 100$ ) or low ( $\text{IgE} \leq 100$ ). Genotype frequencies were then compared using the Cochran-Armitage trend test as recommended by Sasieni (1997) assuming a linear increase in risk from one homozygous genotype via the heterozygous genotype to the other homozygous genotype. In addition, odds ratios (OR) and exact 95% confidence intervals (CI) are reported.

Frequencies of haplotypes were estimated with the expectation-maximization algorithm (Schaid et al. 2002) and compared between probands with high and low serum IgE levels using a score test with simulated  $P$  values from  $10^6$  replications. A linkage disequilibrium units (LDU) map was constructed adopting a Malecot model as implemented in LDMap (Maniatis et al. 2002) to select SNPs for further haplotype reconstruction.

## Results

Generation of stably transfected B cell lines expressing the wild type IL-4R alpha chain or the variant I75V+Q576R

To investigate the combined effect of IL-4R alpha chain amino acid substitutions at position 75 and 576 on IL-4 induced signaling, we transfected *IL4R* cDNA constructs (wild type, I75V+Q576R) into the murine B-cell line A20. The respective cell line expresses its own common gamma chain and is capable of forming a functional receptor with the human IL-4R alpha chain (Andrews et al. 2001; Harada et al. 1992; Idzerda et al. 1990; Lischke et al. 1995). The successful transfection was confirmed by flow cytometric detection of huIL-4R surface expression employing PE-conjugated mouse anti-human CD124 mAb (Fig. 2a).

The amino acid substitution I75V in combination with the amino acid substitution Q576R did not enhance IL-4 induced CD23 expression

Transfected B cells were incubated with increasing doses of huIL-4 for 48 h, and murine CD23 surface expression

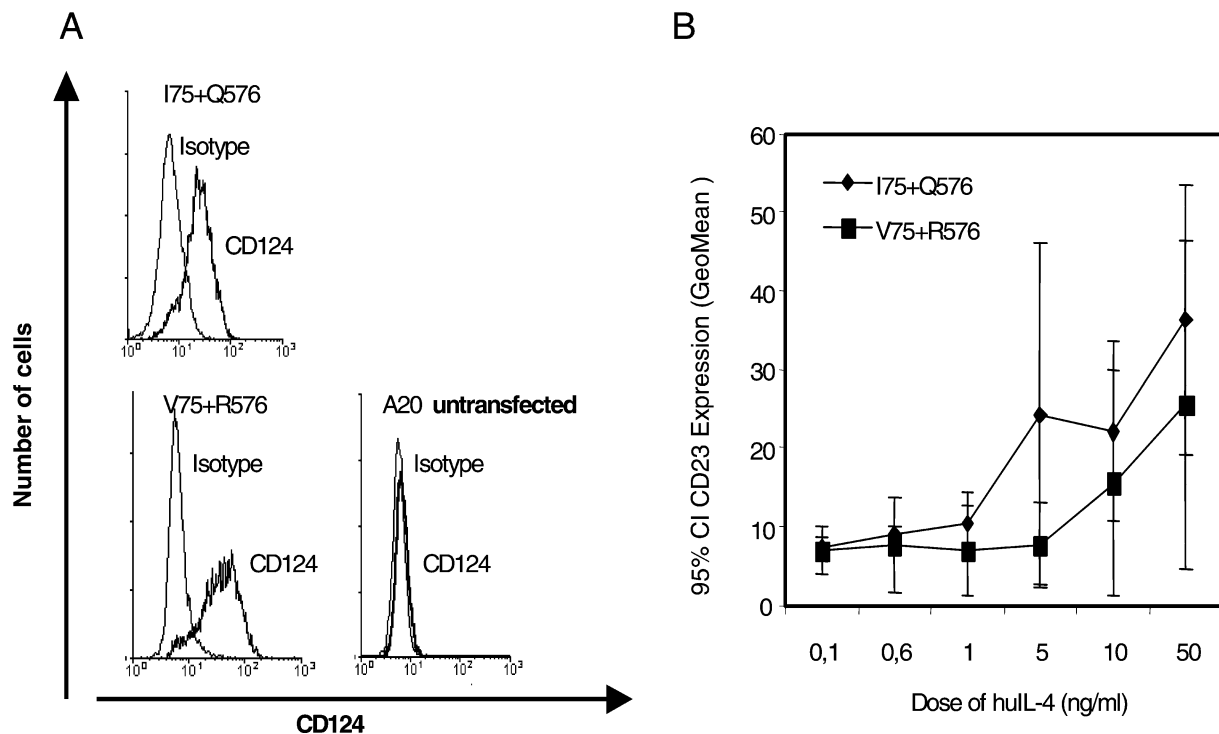


**Fig. 2** Human IL-4R surface expression in transfected B-cell lines and their responsiveness to huIL-4. Murine A20 cells were transfected with the *IL4R* wild type construct (I75+Q576) or the IL4R variant construct coding for the amino acid substitutions V75 combined with R576 (V75+R576). Stable clones were selected and then assayed in triplicate for huIL-4R alpha chain surface expression by flow cytometry using a PE-conjugated mouse anti-human CD124 mAb. Controls included untransfected A20 cells and isotype-matched

control antibodies (a). The A20 transfectants were then incubated with increasing doses of huIL-4 for 48 h and examined for expression of murine CD23 by flow cytometry (b). The results of three experiments displayed no enhanced responsiveness of transfected B-cell lines to huIL-4 (mean values of geometric mean fluorescence intensity  $\pm$  95% confidence interval;  $P=0.26$ ; two-tailed Mann-Whitney  $U$  test)

was determined by flow cytometry. Untransfected murine A20 cells did not respond to huIL-4. Cells expressing either the wild type huIL-4R alpha chain (I75+Q576) or the variant huIL-4R alpha chain (V75+R576) showed dose dependent CD23 expression upon stimulation with huIL-4. However, B cells expressing the double mutant huIL-4R alpha chain (V75+R576) did not reveal enhanced huIL-4 induced CD23 expression compared to B cells expressing the wild type huIL-4R alpha chain (I75+Q576) (Fig. 2b).

Generation of stably transfected T cell lines expressing the wild type huIL-4R alpha chain or the variant S503P

We transfected the huIL 4R cDNA constructs coding either for the wild type (S503) or the variant (P503) IL-4R alpha chain into the murine T-cell line CTLL-2 and screened for stable expression. The respective cell line expresses its own gamma chain and can therefore interact with the huIL-4R alpha chain (Andrews et al. 2001; Harada et al. 1992; Idzerda et al. 1990; Lischke et al. 1995). Figure 3a shows

the two T-cell lines expressing the different huIL-4R alpha chain variants, which were determined by flow cytometry using PE-coupled mouse anti-human CD124.

The amino acid substitution S503P did not enhance IL-4 induced cell proliferation upon stimulation with huIL-4

Stable murine CTLL-2 cell lines expressing huIL-4R alpha chains were treated with increasing doses of recombinant human IL-4 for 48 h and their proliferation was measured by [<sup>3</sup>H]-thymidine incorporation. Untransfected murine CTLL-2 cells did not respond to huIL-4 (Fig. 3b). After analyzing cells expressing either the wild type IL-4R alpha chain (S503) or the IL-4R alpha chain variant (P503), we detected dose-dependent cell proliferation in response to huIL-4. However, the cell line expressing the IL-4R alpha chain variant bearing the amino acid substitution S503P did not reveal higher huIL-4 induced cell proliferation compared to that of the cell line expressing the wild type IL-4R alpha chain (Fig. 3b).

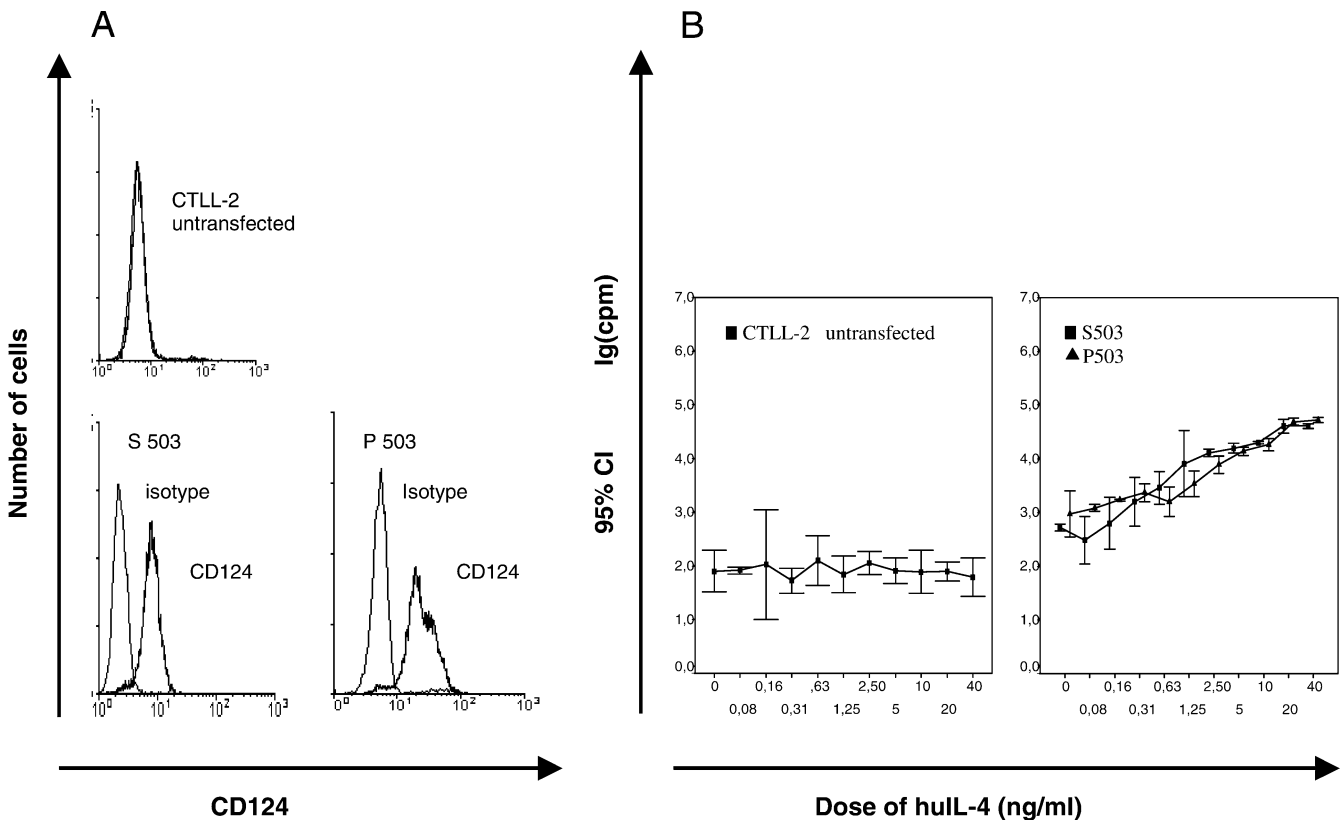


**Fig. 3** Human IL-4R surface expression in transfected T-cell lines and their measurement of cell proliferation in response to huIL-4. Murine CTLL-2 cells were transfected with the IL-4R wild type construct *S503* or the IL-4R variant construct *P503*. Stable clones were selected and then in triplicates assayed for huIL-4R alpha chain expression by flow cytometry using PE-conjugated mouse anti-human CD124 mAb. Controls included untransfected CTLL-2 cells and isotype-matched control antibodies (a). Transfected and control

untransfected CTLL-2 cells were then incubated with increasing doses of huIL-4 for 48 h, and their levels of [<sup>3</sup>H]-thymidine incorporation were measured. The results of three experiments showed that the amino acid substitution at position 503 (*P503*) of the huIL-4R alpha chain did not enhance cell proliferation in response to huIL-4 (mean values of logarithmized counts per minute  $\pm$  95% confidence interval;  $P=0.28$ ; two tailed Mann-Whitney *U* test) (b)

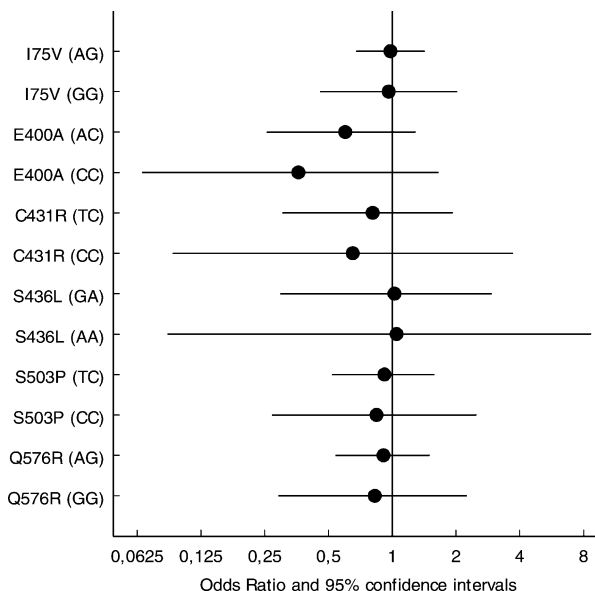


**Fig. 4** No association between the odds ratios of the different *IL4R* SNP genotypes and total serum IgE levels. Six SNPs (*I75V*, *E400A*, *C431R*, *S436L*, *S503P*, *Q576R*) were genotyped in a group of 300 blood donors. The odds ratios and exact 95% confidence intervals were calculated to compare genotype frequencies. Genotype frequencies were investigated using the Cochran-Armitage trend test

Genetic variations in the coding region of the human *IL4R* gene are not associated with total serum IgE levels

Six SNPs (*I75V*, *E400A*, *C431R*, *S436L*, *S503P*, and *Q576R*), which are located in the coding region of the human *IL4R* gene, were genotyped in 300 German blood donors. The rate of homozygosity and heterozygosity for all SNPs did not deviate from Hardy-Weinberg equilibrium, indicating random assortment.

Figure 4 demonstrates the odds ratios for association between the *IL4R* SNP genotypes and the dichotomized total serum IgE level phenotype. No statistically significant associations were detected between the different genotypes and total serum IgE levels. The same was true for allele positivity (11) versus (12+22).

Haplotypes estimated from the SNPs in the coding region of the human *IL4R* gene are not associated with serum IgE level

To investigate the association of the *IL4R* haplotypes with total serum IgE levels the most frequent haplotypes ( $f \geq 0.01\%$ ) estimated from the six coding SNPs in the *IL4R* gene region were determined. Furthermore, the dichotomized total serum IgE levels were compared across the estimated haplotype frequencies using a score test with simulated  $P$  values from  $10^6$  replications. The data of the three estimated *IL4R* haplotypes (only haplotypes with a  $f \geq 0.05\%$  were depicted) are presented in Table 3. This analysis did not reveal an association between the estimated haplotypes and the phenotype total serum IgE level.

In addition, separate analyses for each gender did not reveal association of *IL4R* haplotypes with serum IgE levels (data not shown).

*IL4R* haplotypes, constructed from selected SNPs, are not associated with the total serum IgE levels in an extended population sample

Next, we constructed a LDU map and identified a set of the most informative coding SNPs in the human *IL4R* gene (results not shown). We genotyped an extended group of blood donors ( $n=689$ ) for the SNPs *I75V*, *C431R*, and *Q576R* and determined the most frequent haplotypes ( $f \geq 0.01$ ). Again, we did not find an association between the estimated haplotypes and the dichotomized total serum IgE level (data not shown). Separate analyses for each gender did not detect significant an association of the *IL4R* haplotypes with the phenotype total serum IgE level.

The *IL4R* haplotype *V75+R576* is not associated with total serum IgE levels

Since it has been reported that the *IL4R* haplotype *V75+R576* is associated with atopy phenotypes (Risma et al. 2002), we investigated separately whether the haplotype frequencies (*I75V+Q576R*) were associated with total serum IgE levels in the extended sample of 689 normal blood donors. The pairwise combinations of the *I75V* and *Q576R* alleles did not differ between individuals with serum IgE levels greater than 100 kU/l and individuals

**Table 3** Estimated six-locus *IL4R* haplotypes observed in German blood donors ( $n=300$ ). Allele 1 corresponds to the allele in the reference sequence derived from the human chromosome 16 BAC clone *CIT987SK-58J2* and allele 2 is defined as variation. The hap-

lotype frequencies were estimated with the expectation-maximization algorithm. Dichotomized serum IgE levels were compared across the estimated haplotype frequencies using a score test with simulated  $P$  values from  $10^6$  replications

| Haplotype      | <i>I75V</i> | <i>E400A</i> | <i>C431R</i> | <i>S436L</i> | <i>S503P</i> | <i>Q576R</i> | Haplotype frequency (%) | Haplotype score | Simulated $P$ value |
|----------------|-------------|--------------|--------------|--------------|--------------|--------------|-------------------------|-----------------|---------------------|
| <i>IL4R*01</i> | 1           | 1            | 1            | 1            | 1            | 1            | 38.22                   | 0.37            | 0.71                |
| <i>IL4R*02</i> | 2           | 1            | 1            | 1            | 1            | 1            | 26.79                   | 0.41            | 0.70                |
| <i>IL4R*03</i> | 2           | 2            | 2            | 1            | 2            | 2            | 5.56                    | -0.97           | 0.34                |

**Table 4** Estimated two locus haplotypes observed in German blood donors ( $n=689$ ). Allele 1 corresponds to the allele in the reference sequence derived from the human chromosome 16 BAC clone *CIT987SK-58J2* and allele 2 is defined as variation. The haplotype

| Haplotype      | <i>I75V</i> | <i>Q576R</i> | Haplotype frequency (%) | Haplotype score | Simulated <i>P</i> value |
|----------------|-------------|--------------|-------------------------|-----------------|--------------------------|
| <i>IL4R*01</i> | 1           | 1            | 44.11                   | -0.24           | 0.82                     |
| <i>IL4R*02</i> | 1           | 2            | 9.81                    | -0.38           | 0.82                     |
| <i>IL4R*03</i> | 2           | 1            | 36.30                   | -0.01           | 0.99                     |
| <i>IL4R*04</i> | 2           | 2            | 9.78                    | 0.88            | 0.38                     |

with IgE levels less than or equal to 100 kU/l (data not shown). Furthermore, dichotomized total serum IgE levels were compared across the estimated haplotype frequencies using a score test with simulated  $P$  values from  $10^6$  replications (Table 4). This analysis did not show any association between the estimated frequency of the four possible haplotypes and the IgE level phenotype.

## Discussion

Here we did not confirm a functional or associational relevance for common *IL4R* coding SNPs.

Our study demonstrated that transfection of the human IL-4R coding variant P503 into a murine T cell line did not influence IL-4 induced T cell proliferation compared to wild type constructs. This finding is consistent with the conservation of the I4R motif. The amino acid at position 503 of the human IL-4R alpha chain is located outside of the conserved I4R core sequence (Fig. 5), making a functional role for this common IL-4R variant questionable.

Furthermore, the results of our study revealed that transfected murine B cell lines expressing the common IL-4R variant R576 in combination with the common variant V75 did not enhance IL-4 induced CD23 expression compared to B cell lines expressing the wild type human IL-4R alpha chain.

Recently, it was reported that the Q576R variant alone did not affect IL-4 induced CD23 expression on transfected murine B cell lines. However, the combination of the Q576R amino acid exchange together with the extracel-

frequencies were estimated with the expectation-maximization algorithm. Dichotomized serum IgE levels were compared across the estimated haplotype frequencies using a score test with simulated  $P$  values from  $10^6$  replications

lular I75V substitution resulted in enhanced sensitivity of transfectants to IL-4 (Risma et al. 2002). Our results did not confirm this observation and this is in agreement with the current knowledge regarding IL-4 induced STAT6 induction. The three equidistantly spaced and conserved tyrosine residues Y2, Y3, and Y4 are components of the “gene regulation motif” of the IL-4R alpha chain (Ryan et al. 1996). However, mutation of any single tyrosine or any two tyrosine residues had no effect on IL-4 induced CD23 expression (Ryan et al. 1996). Only mutation of all three tyrosine residues resulted in receptors that no longer induced CD23 (Ryan et al. 1996). The redundant capacity of the tyrosine residues Y2, Y3, and Y4 to serve as docking sites for STAT6 may explain the lack of a functional role for the amino acid substitution Q576R.

In conclusion, we did not detect effects of IL-4R amino acid substitutions on the intermediate phenotypes IL-4 induced proliferation and CD23 expression. However, we cannot rule out an effect of the amino acid substitutions on IL-4 induced phosphorylation patterns or other IL-4 induced signaling pathways (e.g., the SOCS pathway).

Finally, we analyzed the effect of all known common amino acid variants (I75V, E400A, C431R, S436L, S503P, and Q576R) of the IL-4R alpha chain on the proximal phenotype *in vivo*, total serum IgE level. Neither any of the variants alone nor any of the estimated six point haplotypes ( $f > 0.05$ ) were significantly associated with serum IgE levels. Specifically, all possible two point haplotypes of the variants I75V and Q576R were not associated with IgE levels (Risma et al. 2002). The possibility for a false negative finding in our study is rather low. Based on the previous results from Hackstein et al. (1999), we calculated the power of our study to detect effects similar to those previously reported. To be specific, we determined the effects of carrying a mutation at the SNPs *E400A*, *C431R* and *S503P* on the mean IgE in non-atopic blood donors ( $n=90$ ). In our study sample, applying a conservative significance level of 0.001, the power to detect similar effects was greater than 90%. In conclusion, the genetic association analysis agreed very well with our experimental studies, which demonstrated no effect of the IL-4R variants S503P and Q576P (together with I75V on the same haplotype) on the physiological function of the IL-4 receptor.

In contrast to our results, a number of authors have reported association between the *IL4R* coding SNPs with the total serum IgE level phenotype (see above). Non-replication of statistical association between a genetic locus and the investigated phenotype of complex disease

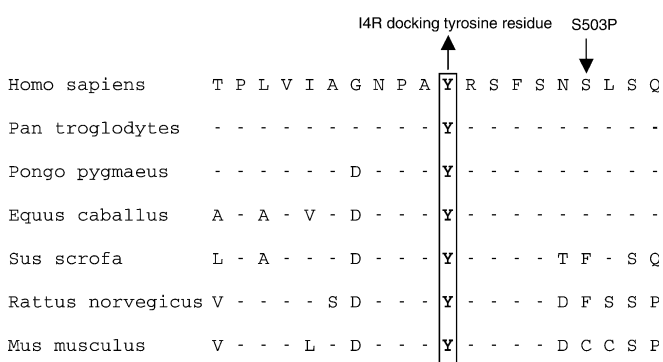


**Fig. 5** Amino acid sequence alignment of the IL-4R alpha chain I4R motif and the tyrosine docking site (Y1) in seven species. The amino acid 503 of the human IL-4R alpha chain is located outside of the conserved I4R core sequence

has been observed frequently in the past and it has only recently been acknowledged that genetic association studies need very stringent significance levels to assure reproducibility (Colhoun et al. 2003; Freimer and Sabatti 2004; Thomas and Clayton 2004; Wacholder et al. 2004). Many causes have been identified for the phenomenon of non-replication. The most likely reason for failure of replication is that false positive results have occurred by chance (type I error; Colhoun et al. 2003). It has been emphasized that controlling the type I error at conventional significance levels ( $P < 0.05$ ) does not control for a low posterior error rate or false positive report probability, i.e., the lack of true association when an apparent significant association has been observed (Wacholder et al. 2004). These quantities are also dependent on the power of the statistical test and the prior probability of a true association. For example, in the report by Risma et al. (2002), the lowest  $P$  value reported was 0.015, and this value was not Bonferroni-corrected, which would be about 0.15. This value is far greater than the  $P$  values needed to assure a low posterior error rate (Colhoun et al. 2003; Freimer and Sabatti 2004; Thomas and Clayton 2004; Wacholder et al. 2004).

However, variations in other parts of the *IL4R* gene, e.g., the 5' promoter region, may affect gene transcription and disease susceptibility (Hackstein et al. 2001; Hosomi et al. 2004). Thus, if the true susceptibility variants reside within the non-coding regions, they may be missed and association studies will result in false negative findings (Hakonarson and Wjst 2001).

In conclusion, we reject the common variant/common disease hypothesis regarding common coding variants of the *IL4R* gene. This hypothesis states that common alleles without deleterious effects in the healthy population may lead to a disease phenotype by genetic interactions and environmental factors (Becker 2004). An alternative hypothesis has been proposed, where numerous independent rare mutations contribute to disease susceptibility (Smirnova et al. 2003). If the findings of Smirnova and co-workers are to be generalized, resequencing of the entire *IL4R* gene of several hundred atopic individuals and controls will be necessary to reliably reject or prove a role for this gene in human disease pathogenesis.

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