Research Article

Differential allelic expression of *IL13* and *CSF2* genes associated with asthma

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Abstract

An important area of genetic research is the identification of functional mechanisms in polymorphisms associated with diseases. A highly relevant functional mechanism is the influence of polymorphisms on gene expression levels (differential allelic expression, DAE). The coding single nucleotide polymorphisms (SNPs) *CSF2*[rs25882] and *IL13*[rs20541] have been associated with asthma. In this work, we investigated whether the mRNA expression levels of *CSF2* or *IL13* were correlated with these SNPs. Samples were analyzed by mass spectrometry-based quantification of gene expression. Both SNPs influenced gene expression levels (*CSF2*[rs25882]: $p_{overall}$ = 0.008 and $p_{DAE\ samples}$ = 0.00006; *IL13*[rs20541]: $p_{overall}$ = 0.059 and $p_{DAE\ samples}$ = 0.036). For *CSF2*, the expression level was increased by 27.4% (95% CI: 18.5%-35.4%) in samples with significant DAE in the presence of one copy of risk variant *CSF2*[rs25882-T]. The average expression level of *IL13* was increased by 29.8% (95% CI: 3.1%-63.4%) in samples with significant DAE in the presence of one copy of risk variant *IL13*[rs20541-A]. Enhanced expression of CSF2 could stimulate macrophages and neutrophils during inflammation and may be related to the etiology of asthma. For IL-13, higher expression could enhance the functional activity of the asthma-associated isoform. Overall, the analysis of DAE provides an efficient approach for identifying possible functional mechanisms that link disease-associated variants with altered gene expression levels.

Key words: candidate gene, gene expression, gene polymorphism, QTL.

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Introduction

Genetic variation influences the susceptibility to disease. An important mechanism contributing to this effect is the influence of genetic polymorphisms on the expression levels of disease-related genes (differential allelic expression, DAE). DAE affects 20%-50% of genes (Yan *et al.*, 2002; Bray *et al.*, 2003; Lo *et al.*, 2003; Serre *et al.*, 2008) and is involved in gene-dosage compensation of sex chromosomes and imprinting on autosomes (Knight, 2004). Monoallelic expression with random choice between paternal and maternal alleles affects hundreds of autosomal genes and contributes to variability among individual cells (Gimelbrant *et al.*, 2007). To increase our understanding of the role of disease-related polymorphisms in complex disease traits it will be necessary to investigate the effect of these polymorphisms on the transcription levels of disease-related genes (Maniatis *et al.*, 2002; Orphanides and Reinberg, 2002).

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In humans, allelic heterogeneity and the polygenic nature of gene expression greatly limit the identification of *cis*-acting mechanisms by simple comparison of expression levels between individuals carrying different genotypes of the disease-related polymorphism. Genetic heterogeneity can be problematic when polymorphisms interfere with the assay or affect the expression of *trans*-acting factors such as transcription factors, or when copy number varies among individuals. The comparison of gene expression levels among different genotypes of a polymorphism is further complicated by the occurrence of various regulatory factors and pathways, tissue quality and cDNA preparation, as well as environmental influences (Stamatoyannopoulos, 2004).

These limitations in the identification of *cis*-directed DAE can be overcome by measuring the influence of disease associated genetic variants on gene expression levels directly in heterozygous individuals (Serre *et al.*, 2008). Since this method relies on comparison of the allelic ratio in DNA (which is expected to be 1:1) and RNA (which may differ from 1:1 if there is DAE) of each heterozygous individual the control for genetic heterogeneity can be obtained with a single sample. This approach markedly reduces con-

founding by environmental factors, tissue quality and cDNA preparation (Bray *et al.*, 2003; Stamatoyanno-poulos, 2004). In the absence of *cis*-regulatory polymorphisms (or imprinting), both copies of an autosomal gene will contribute equally to mRNA expression levels. However, expression levels will differ if there is DAE. These differences may reflect differences in transcriptional regulation, mRNA stability, processing and/or translational efficiency (Bray *et al.*, 2003).

Allele-specific gene expression levels can be quantified in individual heterozygous cDNA samples by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry. This technique allows accurate measurements and can detect differences as low as 5% in allele expression levels (Knight, 2004).

Interleukin 13 is a cytokine located in the cytokine cluster on 5q31 and plays a crucial role in the etiology of asthma. IL-13 shares a common signaling pathway with IL-4, a key mediator of pro-inflammatory functions in asthma. Like IL-4, IL-13 induces the expression of CD23 and CD72 on B cells, as well as the expression of surface IgM, class II MHC antigens and IgE heavy-chain transcription (Zurawski and de Vries, 1994). The non-synonymous coding SNP *IL13*[rs20541] (R144Q) is associated with increased serum IgE levels and eosinophil counts in asthma (Hunninghake *et al.*, 2007) as well as asthma itself (Heinzmann *et al.*, 2000). *IL13*[rs20541] is also associated with psoriasis (Chang *et al.*, 2008). Detailed molecular modeling has shown that amino acid residue 144 of IL-13 might be important in the internal constitution of the ligand and crucial in ligand-receptor interaction. Vladich *et al.* (2005) examined the impact of *IL13*[rs20541] (R144Q) on the functional properties of IL-13 by comparing the activity of the glutamine (Q) variant to that of the common R (arginine) variant on primary effector cells of human allergic inflammation. The Q-variant was significantly more active than wild-type IL-13 in multiple effector assays. Based on their findings, these authors suggested that natural variation in the coding region of *IL13* might be an important genetic determinant of susceptibility to allergic inflammation. However, there has been no report of DAE in this variant. One of the aims of the present work was therefore to investigate whether *IL13*[rs20541] can influence *IL13* mRNA expression levels. Such an interaction could shed light on the contribution of *IL13*[rs20541] to the pathology and development of asthma.

Colony-stimulating factors are proteins necessary for the survival, proliferation and differentiation of hematopoietic progenitor cells. The colony-stimulating factor 2 (*CSF2*) gene encodes granulocyte/macrophage colony-stimulating factor (GM-CSF). CSF2 regulates macrophage and neutrophil function during inflammatory reactions (Fleetwood *et al.*, 2005) and the blockade of CSF2 signaling reduces airway inflammation and hyper-responsiveness in mouse models of environmentally-induced lung damage

and asthma (Ohta *et al.*, 1999; Yamashita *et al.*, 2002). The *CSF2* variant I117 (coding SNP *CSF2*[rs25882-T]) has been linked to a risk of atopic asthma (Rohrbach *et al.*, 1999) and is associated with increased lung cancer risk (Engels *et al.*, 2007). However, the functional mechanisms by which *CSF2* alleles influence the risk of disease are unknown. The aim of this study was therefore to investigate whether the non-synonymous coding variant *CSF2*[rs25882] (I117T) might be related to DAE of the *CSF2* gene.

Materials and Methods

For direct measurement of DAE, cell lines derived from Epstein-Barr virus (EBV)-transfected, immortalized B cells of 52 individuals were used. Genomic DNA was extracted (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany) and screened for heterozygosity of *IL13*[rs20541] and *CSF2*[rs25882] by mass spectrometry as described elsewhere (Burkhardt *et al.*, 2009). Individual genotyping primers (single base extension or SBE) containing a biotin tag and a UV-cleavable base (L) were used for purification purposes. Total RNA was extracted from heterozygous samples ($N_{rs20541} = 11$; $N_{rs25882} = 8$) and reverse transcribed using standard protocols (RevertAid H Minus first strand cDNA synthesis kit, Fermentas, Germany). PCR primers (Table 1) were designed to span exons 3 to 4 for IL13 and exons 1 to 4 for CSF2, thereby making amplification of genomic DNA (gDNA) improbable. PCR consisted of 40 rounds with an annealing temperature of 58 °C annealing temperature and a 15 s elongation per round. The PCR product size was monitored by agarose gel electrophoresis.

To quantitatively compare the expression of both alleles of an SNP in a heterozygous cDNA sample we compared the signal ratios of both alleles in cDNA with their corresponding signal ratios in simultaneously processed gDNA. Ratios of SNP allele expression within the range of gDNA allelic fluctuation (mean ± 1.96 SD), were indicative of no regulation.

To quantify allelic expression the MALDI-TOF-based genotyping system genoSNIP marketed by Bruker Daltonik GmbH (Bremen, Germany) was used, as described elsewhere (Burkhardt *et al.*, 2009), with minor modifications. Measurements were done at least in triplicate. The natural logarithm of the signal-to-noise ratios (intensity of allele-specific mass peaks divided by background noise) reported by Flexcontrol 3.0 (Bruker Daltonik GmbH, Bremen, Germany) was used for statistical analysis. Quality control ensured appropriate spectrum quality by excluding low-intensity spectra (signal to noise ration < 5) and spectra with abnormal ion-peaks.

We screened for the presence of DAE in general (averaged across all samples) as well as for DAE in individual samples. For general screening, we determined whether the natural logarithms of allelic signal ratios for both transcripts differed between cDNA and gDNA by using a stan-

Table 1 - Primer sequences for *IL13*[rs20541] and *CSF2*[rs25882] assays.

	IL13[rs20541]	*CSF2*[rs25882]
cDNA	GAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGC TGGG*CAGTTTTCCAGCTTGCATGTCCGAGACACCAAAAT CGAGGTGGCCCAGTTTGTAAAGGACCTGCTCTTACATTTA *AAGAAACTTTTT(C)GCGAGGGAC*[A/G]GTTCAACTGAAACTT CGAAAGCATCATTATTTGCAGAGACAGGACCTGACTATTG AAGTTGCAG	TCCTGAACCTGAGTAGAGACACTGCTGCTGAGATG*AATG AAACAGTAGAAGTCATCTCAGAAATGTTTGACCTCCAG*G AGCCGACCTGCCTACAGACCCGCCTGGAGCTGTACAAGC AGGGCCTGCGGGGCAGCCTCACCAAGCTCAAGGGCCCCT TGACCATGATGGCCAGCCACTACAAGCAGCACTGCCCTCC AACCCCG**GAAACTTCCTGTG(C)AACCCAGA*[C/T]TATCACC TTTGAAAGTTTCAAAGAGAACCTGAAGGACTTTCTGCTTG TCATC
gDNA	TCTACTCATGTGCTGACCTCTTTGTCCTGCAGCAGTTTTCC AGCTTGCATGTCCGAGACACCAAAATCGAGGTGGCCCAG TTTGTAAAGGACCTGCTCTTACATTTA*AAGAAACTTTTT(C)G CGAGGGAC*[A/G]GTTCAACTGAAACTTCGAAAGCATCATT ATTTGCAGAGACAGGACCTGACTATTGAAGTTGCAGATTC ATTTTTCTTTCTGATGTCAAAAATGTCTTGGGTAGGCGGG AAGGAGG GTTAGGGAGGGGTAAAATTC	TGAGTTCTAAGAGGCAGTAGAGAAACATGCTGGTGCTTCC TTCCCCCACGTTACCCACTTGCCTGGACTCAAGTGTTTTTT ATTTTTCTTTTTTTAAAG*GAAACTTCCTGTG(C)AACCCAGA*[C /T]TATCACCTTTGAAAGTTTCAAAGAGAACCTGAAGGACT TTCTGCTTGTCATCCCCTTTGACTGCTGGGAGCCAGTCCA GGAGTGAGACCGGCCAGATGAGGCTGGCCAAGCCGGGGA GCTGCTCTCTC ATGAAACAAGAGCTAGAAACTCA

PCR-primer binding sequences are underlined, SNPs are indicated by [], SBE primer (single base extension; genotyping primer) binding sequences are underlined and italicized, SBE bases replaced by a photocleavable linker are indicated by () and exon borders are indicated by *.

dard linear model (R Development Core Team, 2010). In this case, allelic ratios that were averaged across 3-4 measurements were used. For individual samples, we assessed whether an observed allelic ratio in cDNA was more extreme than expected by chance given the variance of allelic ratios observed within gDNA samples. For this we initially estimated the distribution of allelic ratios within gDNA samples by calculating the mean and standard deviation. Subsequently, p-values indicating the presence of DAE in cDNA samples were determined by calculating the quantile of the distribution that corresponded to the measured allelic ratios of DAE in the samples. To avoid batch effects, the cDNA and gDNA analyses were always done simultaneously.

To facilitate the comparison of DAE between different studies we applied a method based on a one sample *t*-test, as described previously for DAE analysis (Antoun *et al.*, 1991). This method differs from the analysis described above as it does not take into account fluctuation of gDNA measurements. Briefly, in this method, significant deviation (based on a one sample Student's *t*-test) from one of the allelic ratios between cDNA and gDNA samples indicated DAE. A similar direction of altered allelic ratios across all heterozygous samples of a SNP indicated *cis*-regulation.

To verify the occurrence of *cis*-directed DAE purified PCR products (PCR purifying kit, Seqlab, Göttingen, Germany) from cDNA and genomic DNA of selected samples were analyzed by eurofins-MWG/operon (Ebersberg, Germany) using fluorescence-based sequencing (cycle sequencing technology with dideoxy chain termination in an Applied Biosystems ABI 3730XL-sequencer).

The software Genevar 3.5.0 was used to locate expression quantitative trait loci (eQTL) related to *IL13* or *CSF2* within published genome-wide datasets (Dimas *et al.*, 2009; Nica *et al.*, 2011). The Gene Expression Atlas maintained by the European Bioinformatics Institute v.

2.0.5 05/11 was searched for published mRNA expression of *IL13* and *CSF2* in asthma relevant tissues or animal models.

Results

We found *cis*-directed DAE for *CSF2*[rs25882] and *IL13*[rs20541] (Figures 1 and 2). For *CSF2*[rs25882] a significant effect of DAE (p = 0.008) was found across all samples with allele *CSF2*[rs25882-T] (corresponding to isoleucine) and was overexpressed by an average of 17.7% (95% CI: 5.7%-28.2%). The one sample *t*-test method showed similar significant results (p = 0.008). Of the eight heterozygous samples analyzed, four showed individual DAE, all in the same direction (*cis*-effect). When only those samples with a significant DAE were analyzed the p value was 0.00006, with *CSF2*[rs25882-T] being overexpressed by an average of 27.4% (95% CI: 18.5%-35.4%) compared to allele A. The results obtained by sequencing agreed with those of mass spectrometry (Table 2).

We also found evidence for *cis*-directed DAE of *IL13*[rs20541]. We found DAE across all samples to be marginally significant (p = 0.059) with higher expression of allele A; the one sample *t*-test method showed p = 0.001. Two out of 11 samples were individually significant for DAE, with both showing similar direction (*cis*-effect; p = 0.036). Within these samples, allele A was overexpressed by an average of 29.8% (95% CI: 3.1%-63.4%). The results obtained by sequencing selected samples agreed with these findings (Table 3).

The findings described above were confirmed by Genevar, which revealed a nominally significant *cis*-directed DAE for *IL13* in the MuTHER pilot project (Figure 3). The expression of *IL13* mRNA was increased in carriers of *IL13*[rs20541-A] (p = 0.04). All other SNPs showing evidence for *cis*-directed eQTL in *IL13* (< 5 kbp distance) were in close linkage disequilibrium based on HapMap (release
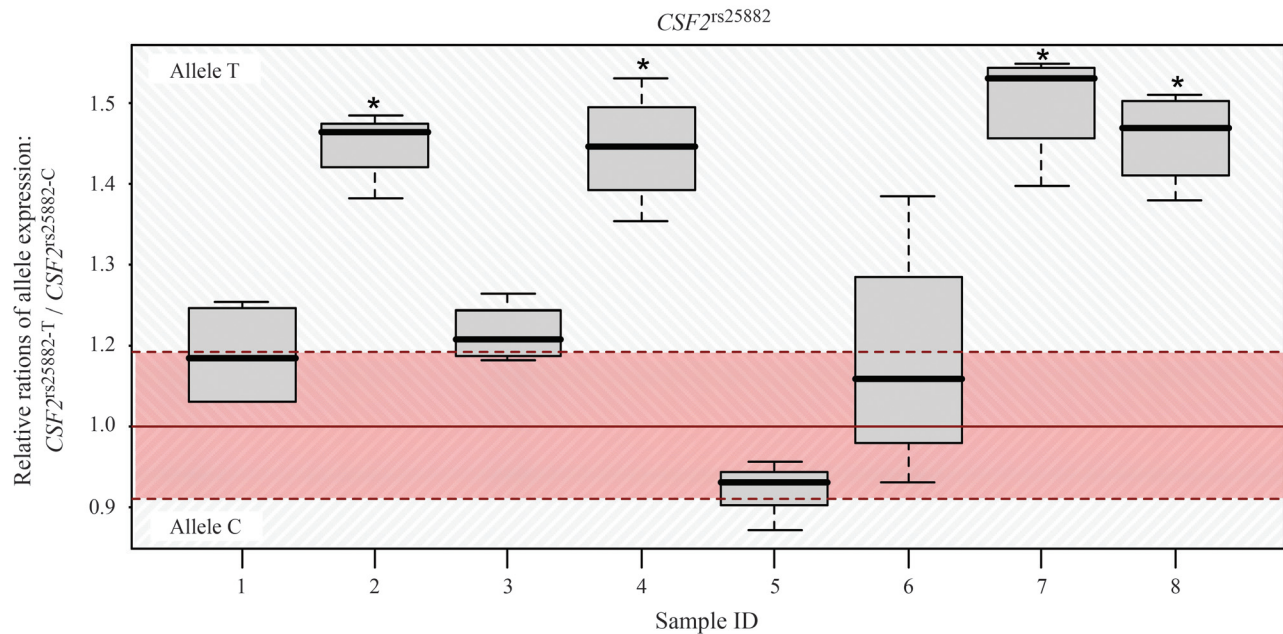
Figure 1 - Differential *cis*-directed expression in samples heterozygous for *CSF2*^rs25882. Boxplots show the allelic expression in individual samples; horizontal lines indicate the median and whiskers indicate the 1.5 x interquartile range. The minor allele is C (T, threonine) and the major allele is T (I, isoleucine). The gDNA fluctuation range (mean ± 1.96 SD) is indicated in red and represents the range (defined in the Methods) in which there is no differential allelic expression. Allelic ratios were calibrated relative to the mean for gDNA samples, which corresponded to an allelic ratio of 1. *p < 0.05. SNR – signal-to-noise-ratio.
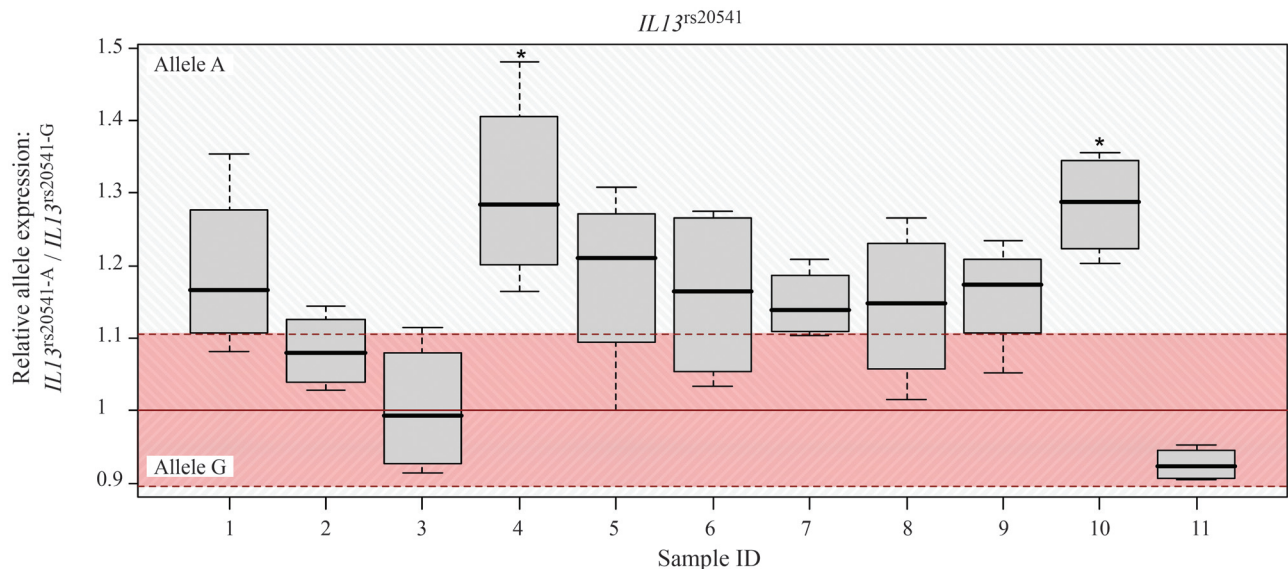
Figure 2 - Differential *cis*-directed expression in samples heterozygous for *IL13*^rs20541. Boxplots show the allelic expression in individual samples; horizontal lines indicate the median and whiskers indicate the 1.5 x interquartile range. The minor allele is A (Q, glutamine) and the major allele is G (R, arginine). The gDNA fluctuation range (mean ± 1.96 SD) is indicated in red and represents the range (defined in the Methods) in which there is no differential allelic expression. Allelic ratios were calibrated relative to the mean for gDNA samples, which corresponded to an allelic ratio of 1. *p < 0.05. SNR – signal-to-noise-ratio.

#27) data (D': 1.0 and r2 > 0.88 with rs847, rs848, rs1295685, rs1295686 and rs1881457).

Discussion

The results of this study demonstrate an association between two previously identified asthma-related SNPs (*CSF2*^rs25882 and *IL13*^rs20541) and the expression levels of the genes *CSF2* and *IL13*; both of these genes are important candidates for involvement in the etiology of asthma and other diseases. Overall, asthma-related *CSF2*^rs25882-T and *IL13*^rs20541-A were associated with higher gene expression levels of *CSF2* and *IL13*, respectively. One copy of the

Table 2 - Differential expression of the allelic ratio $CSF2^{rs25882-T}/CSF2^{rs25882-C}$ in individual heterozygous B cell line samples. Two techniques were used to determine the allelic expression ratios, with sequencing being applied to selected samples. Allelic ratios that differed significantly from a cDNA/gDNA ratio of 1 are indicated in bold.

Sample	p value	cDNA/gDNA ratio	
		Genotyping	Sequencing
1	0.34	1.10	-
2	**0.002**	1.37	-
3	0.23	0.89	
4	**0.002**	1.12	1.41
5	1	0.93	-
6	0.19	1.14	1.05
7	**0.0005**	1.41	1.35
8	**0.002**	1.37	-

Table 3 - Differential expression of the allelic ratio $IL13^{rs20541-A}/IL13^{rs20541-G}$ in individual heterozygous B cell line samples. Two techniques were used to determine the allelic expression ratios, with sequencing being applied to selected samples. Allelic ratios that differed significantly from a cDNA/gDNA ratio of 1 are indicated in bold.

Sample	p value	cDNA/gDNA ratio	
		Genotyping	Sequencing
1	0.07	1.20	-
2	0.38	1.09	-
3	0.95	1.01	-
4	**0.006**	1.31	1.11
5	0.08	1.18	-
6	0.12	1.16	-
7	0.14	1.15	-
8	0.15	1.15	-
9	0.12	1.16	-
10	**0.009**	1.29	1.52
11	1	0.93	-

$CSF2^{rs25882-T}$ allele increased expression by an average of 17.7% in all samples and by 27.4% in samples that showed individually significant DAE of the two alleles. However, not all of the samples displayed DAE. This finding may reflect (1) the presence of a different, possibly unknown causative variant in linkage disequilibrium with $CSF2^{rs25882-T}$, (2) complex modulation by various transcription factors or other means of gene-gene interaction or (3) interaction with extrinsic environmental factors.

Data from the Gene Expression Atlas (experiment E-MEXP-980; Verhaeghe *et al.*, 2007) showed increased expression of *CSF2* in a human cystic fibrosis cell line (CFT-2) compared to a control cell line (NT-1) (p = 0.024). The CFT-2 cell line shares similar characteristics with inflamed asthmatic bronchoepithelial tissue. *CSF2* was also up-regulated after LPS-induced lung injury in mice (E-
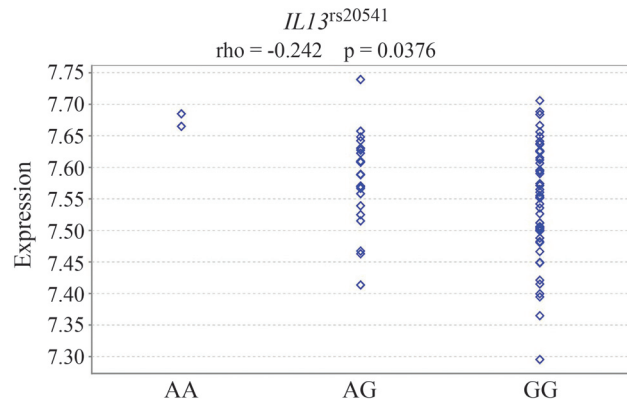

Figure 3 - Genevar eQTL analysis for $IL13^{rs20541}$ in which the rare allele A was significantly associated with elevated *IL13* mRNA expression (Spearman's rank correlation coefficient, rho = -0.242 and p = 0.0376). Analysis of expression quantitative trait loci (eQTL) was based on the genotyping data and mRNA expression data available from the MuTHER (multiple tissue human expression resource) project, lymphocyte twin set 1 (N = 156; Nica *et al.*, 2011).

GEOD-1871; Jacobson *et al.,* 2005), a model for airway inflammation similar to that in asthmatic diseases (p = 1.25 x 10^{-9}) (Figure 4). Therefore, our finding that known risk variant $CSF2^{rs25882-T}$ and not $CSF2^{rs25882-C}$ increased the expression of *CSF2* agreed with published results.

We also used MatInspector and Genomatix to assess whether transcription factor binding sites were created or lost in the presence of $CSF2^{rs25882-T}$. Interestingly, a probable binding site for Smad3 was lost when the T allele was present (core similarity = 1; matrix similarity = 0.997). Smad3 is a negative regulator of the normal TGF-β response (Zhang *et al.*, 1996) and a direct mediator of transcriptional activation by the TGF-β receptor. This effect is mediated either by induction of cyclin-dependent kinase inhibitors or by repression of other growth controlling genes such as MYC, a key oncogene (Chen *et al.*, 2002). In asthma, airway remodeling following immune driven inflammation is thought to contribute to airway dysfunction and there is evidence that TGF-β, expressed mainly by eosinophils in asthmatic lung tissue, is a major mediator in airway remodeling (Fattouh and Jordana, 2008). In summary, the loss of a Smad3 binding site through the presence of $CSF2^{rs25882-T}$ may be a pathologically relevant event for the development of asthma.

$IL13^{rs20541-A}$ was associated with higher expression in some of the samples investigated. This association may have arisen for reasons similar to those stated for $CSF2^{rs25882}$, *i.e.*, the influence of different variants and transcription factors, imprinting, gene-gene interactions or extrinsic factors. In samples with evidence of DAE allele A was overexpressed by an average of 29.8%.

Based on *in silico* analyses using Genomatix and MatInspector (Tasheva *et al.*, 2004) the presence of the asthma risk allele $IL13^{rs20541-A}$ was predicted to create two transcription factor binding sites: EN1 and MYT1. How-
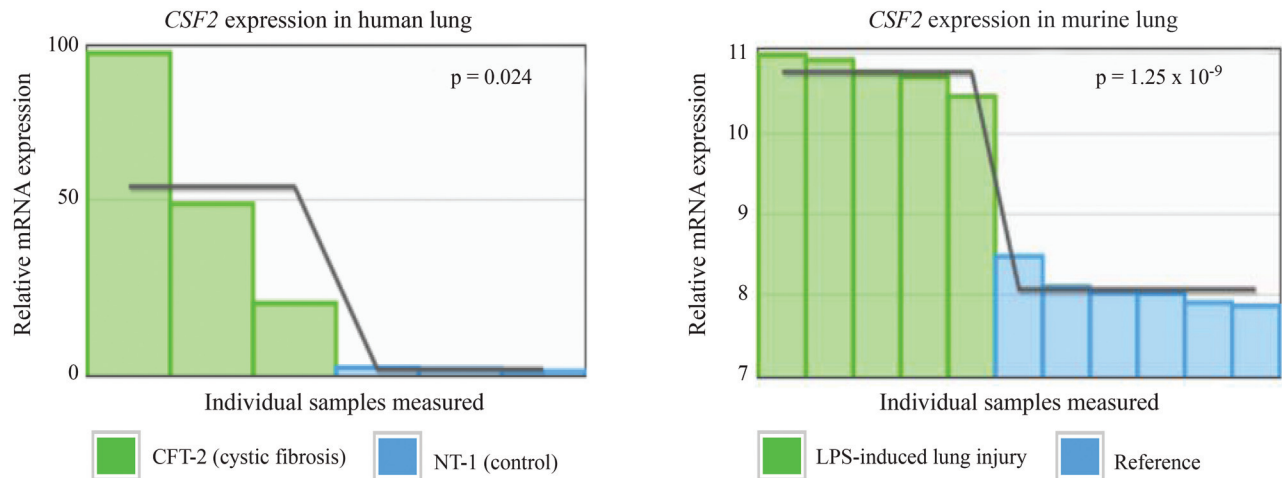
Figure 4 - *CSF2* expression in human and murine lung. Left panel – *CSF2* expression in the human cystic fibrosis cell line CFT-2 and human NT-1 cells (control); right panel – *CSF2* expression in murine lung before and after LPS-induced lung injury. The relative *CSF2* mRNA expression is shown based on data derived from the Gene Expression Atlas, experiments E-MEXP-980 (Verhaeghe *et al.*, 2007) and E-GEOD-1871 (Jacobson *et al.*, 2005). Each bar represents an individual sample, the moving average is shown as solid line.

ever, a role for these two transcription factors in asthma is not immediately obvious as they are both commonly expressed in neuronal, but not peripheral, tissues (Kim *et al.*, 1997; Logan *et al.*, 1992). Since SNPs in linkage disequilibrium with *IL13*[rs20541] could be responsible for the observed effect we repeated this analysis with all SNPs in linkage disequilibrium with *IL13*[rs20541], according to the HapMap CEU population ($r^2 > 0.8$), and additionally displaying *cis*-directed eQTL in the Genevar database. Our findings revealed several new or lost transcription factor binding sites in the presence of these linked SNPs (Table 4).

The increased expression of *IL13* in *IL13*[rs20541-A] carriers agreed with a previous report showing that variant *IL13*[rs20541-A] was significantly more active in effector assays (Vladich *et al.*, 2005). The latter authors suggested that the amino acid switch from R to Q increased the activity of the IL-13 Q144 protein variant. Our data provide evidence for an additional effect, namely that the enhanced activity of IL-13 in carriers of Q144 may be amplified by increased expression via DAE.

Increased IL-13 levels may contribute to the development of asthma by inducing the expression of CD23 and CD72 in B cells, both of which are key molecules in B cell activation and differentiation. IL-13 also enhances the expression of surface IgM and class-II MHC antigen and induces germline IgE heavy-chain gene transcription (Zurawski and de Vries, 1994), indicating a possible role for IL-13 in the pathogenesis of immune disorders such as asthma. The finding that risk variant *IL13*[rs20541-A] was related to increased IL-13 levels therefore agrees with our current understanding of the role of IL-13 in asthma.

A limitation of this study is that we used immortalized B-cell lines that did not originate from affected individuals. Further studies are therefore necessary to validate our findings in other tissues, *e.g.*, diseased primary tissue.

Serre *et al.* (2008) examined the possible influence of lymphoblastoid culture conditions and passages on DAE but found very little evidence for a bias attributable to technical aspects in a genome-wide study. In addition, data ob-

Table 4 - Loss or gain of transcription factor (TF) binding sites as predicted by Genomatix for *IL13*[rs20541] or SNPs in linkage disequilibrium. The loss or gain was predicted to occur in carriers of the rare allele compared to the common allele (common allele → rare allele). The similarity scores for the core and matrix sequences represent the similarity between expected TF binding sites (the core TF binding sequence and the TF family binding sequence) and the corresponding sequences altered by the indicated SNPs.

SNP rs #	Allele	Loss/gain	TF family/ matrix	Core similarity	Matrix similarity
rs1881457	A → C	gain	MIF1	0.8	0.772
rs847	A → G	loss	EN1	1	0.775
rs1881457	A → C	loss	CTCF	0.787	0.781
rs848	G → T	loss	ZNF76_143	1	0.789
rs1881457	A → C	loss	DICE	0.783	0.806
rs20541	C → T	gain	EN1	0.782	0.816
rs20541	C → T	loss	MYBL1	1	0.837
rs1295685	C → T	loss	AML3	1	0.84
rs1295685	C → T	loss	MYOD	1	0.881
rs1295686	A → G	gain	XBP1	1	0.887
rs1295686	A → G	loss	PARAXIS	0.882	0.914
rs1295685	C → T	loss	USF	0.851	0.927
rs20541	C → T	gain	MYT1L	1	0.93
rs1295686	A → G	gain	BHLHB2	1	0.933
rs1295686	A → G	loss	MIT	1	0.956
rs1295686	A → G	gain	NMYC	1	0.976
rs1295686	A → G	gain	HRE	1	0.984
rs1295686	A → G	gain	HIF1	1	0.991

tained from the MuTHER pilot project (Nica *et al.*, 2011) indicated DAE for *IL13*^{rs20541}, as also observed here; unfortunately, *CSF2*^{rs25882} was not genotyped in that dataset. The *IL13* mRNA expression reported in the MuTHER project was based on fresh blood lymphocytes, thereby eliminating any influence of immortalization. Together, these findings indicate that changes in gene expression seen with DAE-based techniques tend to reflect true biological responses rather than technical artifacts.

The effects of both SNPs on gene expression can be considered moderate. However, common diseases are expected to be influenced by many risk factors and it is the sum of the effects of these factors that accounts for a substantial part of phenotypic variation (Morley *et al.*, 2004). In general, observed effect sizes are similar to known sizes of effects of genetic variants on gene expression. Thus, in a genome-wide study of DAE, only 18.1% of 349 SNPs with DAE (excluding X-linked SNPs) showed fold changes in DAE ≥ 1.5 and only 5.4% showed fold changes ≥ 3 (Serre *et al.*, 2008).

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