Modulation of cystic fibrosis lung disease by variants in *interleukin-8*

A.D. Hillian[1], **D. Londono**[2,*], **J.M. Dunn**[4], **K.A.B. Goddard**[2,**], **R.G. Pace**[3], **M.R. Knowles**[3], and **M.L. Drumm**[4,1,5]

[1]Department of Genetics, Case Western Reserve University, Cleveland, Ohio

[2]Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio

[3]Cystic Fibrosis Pulmonary Research Treatment Center, University of North Carolina, Chapel Hill, North Carolina

[4]Department of Pediatrics, Rainbow Babies and Children's Hospital, Cleveland, Ohio

[5]Reporting for the CF Gene Modifier Study Group

Abstract

Cystic fibrosis pulmonary disease is characterized by excessive and prolonged inflammation. CF Pulmonary disease severity exhibits considerable variation that, to some extent, appears to be due to the presence of modifier genes. Several components of the inflammatory response are known to have altered regulation in the CF lung. Genetic variants in 52 inflammatory genes were tested for associations with lung disease indices in a CF patient population (n=737) homozygous for the F508 *cystic fibrosis transmembrane conductance regulator* mutation. Variants in 3 inflammatory genes showed significant genotypic associations with CF lung disease severity, including *IL8* and previously reported *TGFβ1*[1] (p 0.05). When analyzed by gender, it was apparent that *IL8* variant associations were predominantly due to males. The *IL8* variants were tested in an additional CF population (n=385) and the association in males verified (p 0.01). The *IL8* variants were in strong linkage disequilibrium with each other (R^2 0.82), while variants in neighboring genes CXCL6, RASSF6 and PF4V1 did not associate (p 0.26) and were in weaker LD with each other and with the *IL8* variants (0.01 R^2 0.49). Studies revealed differential expression between the *IL8* promoter variant alleles (p<0.001). These results suggest that *IL8* variants modify CF lung disease severity and have functional consequences.

Keywords

cystic fibrosis; genetic modifier; interleukin-8; pulmonary

Corresponding Author: Mitchell Drumm, Ph.D., Associate Professor, Departments of Pediatrics and Genetics, Case Western Reserve University, 830 BRB, 10900 Euclid Avenue, Cleveland, OH 44106-4948, Phone: 216-368-6893, Fax: 216-368-2442, mitchell.drumm@case.edu.
*Current affiliation: Rockefeller University, New York, NY
**Current affiliation: The Center for Health Research, Kaiser Permanente Northwest, Portland, OR

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease that is caused by mutations in *cystic fibrosis transmembrane conductance regulator* (*CFTR*)[2]. Although CF is considered to be a classic monogenic disease, there is considerable phenotypic variation in CF among patients that share the same *CFTR* mutations; this is especially true with regards to pulmonary disease progression.[3] It has been suggested that the majority of the variation observed in pulmonary disease is due to genetic heterogeneity, independent of *CFTR* genotype[4]. Our previous work has revealed that *transforming growth factor β_1* (*TGF-β_1*), which is involved in immune responses and contributes to the progression of lung disease in animals, is a genetic modifier of CF lung disease[1]; however, *TGFβ_1* is predicted to only account for a small amount of the disease variation.

CF pulmonary disease is characterized by persistent inflammation and recurrent infection.[5] Several components of the inflammatory response (chemokines, cytokines, transcription factors, etc.) are known to have altered expression in the CF lung.[6–8] In general, dysregulation of the inflammatory responses is considered to be the primary source of much of the pathophysiology in CF.[9–13] The gender of a patient may also contribute to this phenomenon, as females are reported to display worse lung disease than males, which is demonstrated by earlier colonization with *P. aeruginosa*[14], reduced lung function[14], and shorter lifespan[14–16].

Here we report a survey of genetic variation in inflammatory pathway components, along with the effect of gender, and their effect on the severity of CF pulmonary disease. We selected 181 variants from a panel of 52 inflammatory genes and tested them for association with CF disease severity (see Supplementary Data). Common single nucleotide polymorphisms (SNPs) in these inflammatory genes were genotyped first in a large CF patient population (n=737) in which each subject is homozygous for the ΔF508 *CFTR* mutation, and associating variants then tested in a second cohort of 385 patients. Variants in the IL8 gene showed significant association with disease severity in males in both cohorts of CF patients. *In vitro* functional studies revealed differences in transcription due to the rs4073 *IL8* promoter variant.

RESULTS

Inflammatory Gene Screen

One hundred eighty-one single nucleotide polymorphisms (SNPs) in 52 genes involved in inflammation were examined for skewing of genotype frequency between severely and mildly affected ΔF508-homozygous CF patients ascertained by lung disease status[17]. These 52 genes were a subset of a larger project that included 810 variants in 300 gene regions, all selected as candidate modifiers of CF disease severity based on specific pathways thought to contribute to CF pathophysiology. Using a χ^2 test of independence, genotype frequencies of 49 of the 810 polymorphisms (6%) were significantly different between mild and severe patients ($P < 0.05$) while only 4 of the 181 inflammatory gene variants (2.2%) were significant. These 4 SNPs represented 4 different genes, including previously reported TGFB1[1], and therefore only the remaining 3 are included here, namely interleukin 8 (IL8),

interleukin 10 receptor B (IL10RB), and transforming growth factor beta-induced protein (TGFBI).

In addition to the test of independence, the cohort was assessed for possible population stratification using a genome control method (see Methods). The values of λ ranged from 1.0 to 1.29, suggesting stratification is minimal in this population and the adjusted p values are given for genotypes in Table 1.

Defining the associating interval

Alleles of 4 of the 52 inflammation-related genes tested showed significant associations with lung disease severity (p<0.05). Of the newly reported associations, *IL8* was selected for further characterization because its variants have been reported to associate with susceptibility to other airway disorders[18, 19]. Additionally, IL-8 is an integral component of CF lung disease; it is elevated, with substantial variation, in the lungs of CF patients[6, 20]. Because of the reported genetic association with CF lung function and the biological plausibility of *IL8* as a genetic modifier of CF airway pathophysiology, the *IL8* genomic region was investigated in more detail.

The strategy employed was to first define the genetic interval with the most significant association and evaluate the candidate interval in a second group of patients. To this end, we first examined markers in genes neighboring *IL8* to determine which interval contained the most significant association. As *IL8* is in proximity to several other chemokine genes in the chromosome 4q12–q13 region, and work by others suggests that some of these genes may be in linkage disequilibrium (LD) with *IL8*[21], one could not rule out flanking genes as the source of association in our initial screen.

There are 13 known CXC-family chemokine genes in the human genome, apparently the result of gene duplication[22]. Eight of these genes are in the *IL8* genomic region (4q13–21), therefore functional redundancy is of concern. Guided by the findings of Hull *et. al.* showing three distinct LD blocks (r^2>0.3) encompassing *IL8* and the genes immediately flanking it.[23], we selected the following variants for further evaluation: rs13131954 from *RASSF6* and rs941758 from *PF4V1* to represent the LD blocks adjacent to *IL8*[23] and rs4694639 from *CXCL6*, which is immediately 3′ of *IL8*, but for which the LD relationship has not been reported. The arrangement of these genes and the location of markers is shown in Figure 1. The pairwise linkage disequilibrium pattern was determined for all eight markers. The four *IL8* markers were all in strong LD (R^2>0.82, Table 2). The *CXCL6* marker is in moderate LD with the *IL8* markers (0.39 R^2 0.47), but the *RASSF6* and *PF4V1* markers are essentially in equilibrium with *IL8* and *CXCL6* (R^2 0.16). Our data are concordant with those of Hull[23] and show that not only are the *IL8* markers in very strong LD with each other, but also that the *CXCL6* marker is in modest LD with the *IL8* alleles (Table 2).

The markers across this region (240 kb) were tested for association with the lung disease phenotypes, severe and mild (see Methods), and the most significant associations were restricted to those markers in the IL8 gene (Table 3). There was no significant association between the *RASSF6* (p=0.25), *CXCL6* (p=0.26), or *PF4V1* (p=0.29) markers and lung function. These analyses indicate that the most significant markers are those in high linkage

disequilibrium with each other, and corresponding to the IL8 gene, strongly implicating only the *IL8* interval.

Because of the differences in pulmonary disease observed between males and females, we assessed males and females separately. Table 4 shows that the most significant findings are found in the male sub-population and do not extend outside of the *IL8* markers (Table 3).

Testing the association in additional populations

As Table 4 indicates, the evidence for association is more significant in the males than females, but is modest even in males. Therefore, an additional population was examined to determine if the association, particularly in males, could be replicated. Three of the *IL8* markers were typed in a second cohort. Patients in Cohort 2 (n=385, 219 males) were ascertained by the same phenotypic criteria as Cohort 1, but were not all homozygous for the F508 mutation. They did, however, carry at least one copy of the F508 *CFTR* mutation and another pancreatic insufficiency-associated mutation[24] on the second allele. As Table 5 shows, the males in these two cohorts are not significantly different across indices of pulmonary function.

Males and females in Cohort 2 were assessed separately using a χ^2 test of association. In accordance with the first population, only male genotypes showed significant associations (0.01>p>0.08) in this second cohort (Table 4). The reproducibility of the association only in males indicates that gender is a major factor in *IL8*'s role as a genetic modifier of CF. We also examined the IL8 SNPs for departures from Hardy-Weinberg proportions and found no evidence of such departures in either of the cohorts, nor in males alone (P > 0.6).

The *IL8* markers display high LD with each other and are therefore largely redundant in their information. An example of the genotype distribution in the studied subjects is provided for the promoter polymorphism A-251T (rs4073) in Table 6. The frequency of males homozygous for the A allele in the mild group is greater than in the severe group (25.6% vs. 18.9%), while the frequency of T homozygotes is greater for the male subjects in the severe group (38.9% vs. 25.1%). Heterozygosity is found more often in the mildly affected males (49.3% vs. 42.1%), suggesting a dominant or semi-dominant effect of the A allele on the mild phenotype.

Effect of other loci and phenotypes on IL8 association

We previously reported an association between variants in the transforming growth factor-β1 gene, TGFB1, and pulmonary function. Logistic regression was used to determine if there were evidence of interaction between these two genes, Using the TGFB1 promoter and codon 10 polymorphisms, no significant interactions were identified with any of the 4 IL8 variants (0.96 > P > 0.46).

To determine if the association found in males might be reflective of other pulmonary conditions, the incidence of physician-reported asthma was assessed by genotype as well and was not significant (0.05 < P <0.4).

Effect of the IL8 promoter variant on expression

A significant statistical association between *IL8* gene polymorphisms and CF pulmonary disease has been demonstrated in two populations; however these associations do not address the functional mechanism by which these variants modify phenotype.

A luciferase-reporter assay was used to assess the *IL8* promoter variant for effects at the level of transcription. *IL8* promoter-luciferase constructs with either the A or T allele at variant rs4073 (rs4073A or rs4073T) were transiently transfected into 9HTEo⁻ cells (pCEP-2), treated with a pro-inflammatory cytokine mix, and luciferase output measured at 6, 12, and 24 hours post-treatment.

Treatment with the cytokine mix induced a significant increase in luciferase expression for both constructs (p<0.001) (Figure 2A), but under all conditions the T allele expressed 2–3 times more than the A allele (p<0.001) (Figure 2B). The fold induction of both constructs by the cytokine mix was similar and non-significant, however (p>0.56, data not shown).

DISCUSSION

Pulmonary disease in CF, which is characterized by recurrent infections and excessive inflammation[5, 12], exhibits marked phenotypic variability, due in part to genetic variation, or so-called modifier genes.[25–27] In general, a dysregulation of inflammatory responses (with an overall increase in pro-inflammatory and decrease in anti-inflammatory response) is thought to be a key contributor to the lung pathophysiology in CF[10, 11]. Due to the central role of inflammation in the lung, we attempted to determine whether variants in inflammatory genes could act as modifiers of CF lung disease.

A panel of polymorphisms in candidate inflammatory genes (n=52) thought to be relevant to cystic fibrosis (CF), as determined through a literature review, were screened for associations with level of lung function in a cohort of CF patients (n=737). Polymorphisms in three inflammatory pathway genes showed nominal associations in the initial survey: *IL10 receptor* (*IL10R*), *TGFβ-induced protein* (*TGFβI*), and *interleukin-8* (*IL8*). *IL8* was selected for further characterization because its variants have been reported to associate with susceptibility to other airway disorders[18, 19]. Additionally, IL-8 is an integral component of CF lung disease; it is elevated, with substantial variation, in the lungs of CF patients[6, 20]. The primary function of IL-8 is to mediate neutrophil chemotaxis, and CF lung inflammation is largely characterized by neutrophilic infiltrates, leading to subsequent tissue damage[28–31]. Thus, the role of *IL8* polymorphisms in CF pathophysiology should be more readily understood than that of the other candidates.

Our studies found that three polymorphisms within *IL8* associate with CF pulmonary disease severity, rs4073, rs2227306 and rs2227307, and the effect was most obvious in males. With respect to pulmonary phenotype, associations between the rs4073 variant and infant susceptibility to bronchiolitis subsequent to infection with respiratory syncytial virus (RSV)[18, 32] and bronchial asthma[19] have been identified. The studies by Hull and Hacking found that homozygosity for the rs4073A allele is associated with increased occurrence of bronchiolitis following acute RSV infection, while the rs4073T allele is protective against

this condition[18, 32]. In our CF groups, we find that the rs4073A allele is protective and the rs4073T allele is associated with the more severe phenotype. A similar finding was reported in a study in which the rs4073T allele was associated with bronchial asthma, which is a chronic pulmonary condition[19]. That the same variant seems to have contradicting associations suggests that the functional impact of this variant is context dependent. We propose that the context of inflammation (i.e., acute as in innate defense versus chronic stimulation) may dictate the direction of the association.

The reason for the observed gender effect is not clear but a phenomenon that is not novel in CF. Females with CF have an overall worse prognosis as demonstrated by earlier colonization with *P. aeruginosa*[14], reduced lung function[14], and more rapid decline than males, suggesting that the inflammatory burden is greater in the female lung than the male[14, 15, 34–36] and culminating in shorter lifespan[14–16] for females. Sex hormones are able to regulate cytokine expression[33], but there is no evidence that *IL8* expression is directly regulated by sex hormones or that there are no known sex hormone-responsive elements in the *IL8* promoter. Thus, the sex-specific association is likely more complicated than hormone-responsive transcription. Whether our results reflect an ascertainment bias due to our study design (selecting males and females using the same criteria for both may impose a bias) or a true sex-specific effect could be addressed by replication in populations without exclusion of intermediate phenotypes. In the populations studied here, the relative risk of having severe lung disease for patients homozygous for the "severe" allele of rs4073 is 1.7 (p=0.04) but increases to 2.25 when considering only males (P=0.001).

As there are no known common variants in the coding sequence one of IL8, variation is most likely due to quantitative effects. Two of the variants are located in introns (rs2227307 and rs2227543) while the remaining variant (rs4073) is found in the promoter region of *IL8* and has been shown to associate with several pulmonary phenotypes[18, 19, 32] and to have demonstrated functional effects[18, 32]. We therefore felt this variant to be the most likely candidate to impart the functional effect and rs4073 was therefore the target for functional testing. We found that the rs4073T variant induces a 2–3 fold increase in transcriptional activity relative to the rs4073A allele. Although such a modest fold change may not be usually considered biologically significant, context must be considered when investigating genetic modifiers of disease. As stated, CF patients are known to have abnormally high levels of IL-8 in their lungs[6, 20]. Given a scenario in which elevated IL-8 is associated with severe pulmonary disease, it is entirely plausible that a moderate, yet significant, and chronic increase in IL-8 could account for more severe lung disease. Based on our findings, we propose that regardless of colonization or exacerbation status, CF patients with the rs4073T allele have higher levels of *IL8* expression and are thus more likely to have severe pulmonary disease.

Interpretation of previous evidence regarding *IL8* transcript regulation is somewhat unclear. Hacking *et. al.*[32] found that when assessed in basal and TNF-α stimulated reporter constructs, the T allele at −251 (rs4073T), which was predominant in our severe phenotypic groups, has more than twice the promoter activity of the A allele, which is similar to our findings[32]. However, when endogenous transcript levels are compared in primary epithelial

cells, the TNF-α stimulation results in marginally higher levels from the haplotype containing the A allele[32].

Our reporter construct assays are in accord with similar studies, but we have not yet assessed endogenous transcript levels. Sequence analysis suggests the rs4073 variant alters binding for the Oct-1 transcription factor, which has been shown to regulate the expression of several cytokines[37, 38] including *IL8*[32, 39]. The *IL8* variants do appear to alter expression levels, but mechanism of the regulation may be context dependent and will be explored in the future.

Other studies have identified potential genetic modifiers of CF; Hull *et. al.* showed variants in *tumor necrosis factor-α* (*TNF-α*) associate with FEV$_1$ in CF patients,[40–43] although we have not been able to replicate these findings in a larger group of subjects[1]. We have, however, found that variants within the *transforming growth factor-β$_1$* (*TGF-β$_1$*) gene correlate with CF patient disease severity[1]. The findings presented here show that variants in *IL8* associate reproducibly with lung disease severity in males with cystic fibrosis and that there is functional variation associated with one of the polymorphism. Taken in context with the biological role of IL-8, this gene likely modifies lung function in CF through different levels of neutrophil chemotaxis and subsequent differences in destruction of lung tissue.

METHODS

Patient Recruitment

The initial study cohort was made up of 737 CF patients enrolled in the Gene Modifier Study (GMS), ascertained by methods described previously[1, 17]. Briefly: All study subjects in cohort 1 are CF patients homozygous for the F508 *CFTR* mutation. Based on data obtained from the National CF Patient Registry, upper and lower quartiles of lung function for age were determined. Using these values as a guide, subjects were designated as "mild" (n=491) or "severe" (n=246), respectively, to reflect their lung function profile. A case-control study design was used to compare these groups, with patients with severe lung disease arbitrarily set as the case population and the mild group as the control population.

A second cohort ascertained for the same clinical criteria as the first, but including F508 compound heterozygous patients, were used for the replication study (n=385). Cohort 2 patients not homozygous for F508 carry one copy of the F508 *CFTR* mutation and a mutation recognized as severe on the second allele, with severe defined as being associated with pancreatic exocrine insufficiency[1]. The same criteria for "mild" and "severe" used for cohort 1 were applied to the second cohort.

Both cohorts were assessed for indices of pulmonary function, including average forced expiratory volume in 1 second (FEV$_1$), rate of decline in FEV$_1$ and predicted FEV$_1$ at age 20 (EBint20[17]), as well as bacterial colonization and body mass index.

Genotyping

In the initial screen, genotyping was performed by SNP BeadArray™ (Illumina, Incorporated, San Diego, CA) technology and included the following polymorphic loci

within *IL8* (position relative to the transcriptional start site, number of patients genotyped): rs4073 (A-251T, n=733), rs2227307 (G396T, n=732), and rs2227543 (T1632C, n=737). Patients not genotyped by Illumina, Inc. were genotyped at the following *IL8* loci using restriction fragment length polymorphism (RFLP) assays. Primers and restriction enzymes used for the RFLP genotyping were (SNP, forward, reverse, product size, restriction endonuclease): rs4073, 5′-TGAAAAGTTGTAGTATGCCCC-3′, 5′-CATTTAAAATACTGAAGCTCCACAATTTGGTGAATTATCgA-3′, 310 bp, *ClaI*; and rs2227543, 5′-TGGTTAAAAAAAAAGGAATAGCATCAATAGTGAGTTTGTTGTcCT-3′, 5′-ACCCTACAACAGACCCACACAATA-3′, 420 bp, *MnlI*. Lower case letters indicate a sequence change in order to introduce a restriction endonuclease recognition site. Digestion products were analyzed on a 2% agarose gel. Reactions were performed using Invitrogen Platinum *Taq* polymerase enzyme, 10× buffer, deoxyribonucleotides (2.5mM), and $MgCl_2$ (25mM). Annealing temperature, $MgCl_2$ concentration, and amplification cycles were optimized for each primer set.

Variants in the *RASSF6* (rs13131954, n=717), *IL8* (rs2227306, n=727), *CXCL6* (rs4694639, n=709), and *PF4VI* (rs941758, n=716) genes were genotyped using an Assay-on-Demand™ assay (Applied Biosystems, Foster City, CA).

Statistical Analysis

The distribution of the marker alleles and genotypes in the severe group was compared to that in the mild group using a chi-square test of independence. A goodness of fit test for Hardy-Weinberg Equilibrium (HWE) was performed by comparing the genotype frequencies with those expected on the basis of the observed allele frequencies[44]. SNPs with a $p<0.05$ for the test of HWE were noted as possibly requiring careful consideration of the possible causes of departure from HWE, but were not removed from the analysis. The *IL8* and flanking polymorphisms were found to exist in HWE, $0.27<p<0.96$. All analyses were conducted in SPlus version X.0 (Insightful Corporation, Seattle, WA).

Population stratification

The current study was part of a larger project in which 737 subjects were typed for 810 markers. The genomic control method[45] was performed using these markers to examine the potential impact of population structure in this study. The observed values of the inflation factor λ ranged from 1 to 1.29, and were used to adjust the p-values of the IL-8 regional markers.

Pairwise Linkage Disequilibrium (LD)

The pattern of pairwise LD between SNPs was measured by the correlation coefficient (R^2)[46]. R^2 varies from 0 to 1 and measures the correlation between alleles on the same chromosome. R^2 will take the value of 1 when the allele at one locus can always be predicted by the allele at the second locus (that is, if only two haplotypes are present).

IL8 Promoter Luciferase Assay

The *IL8* promoter (1.5kb) was cloned into a pGL3 vector (Promega Corporation, Madison, Wisconsin) containing the firefly luciferase gene. Using site-directed mutagenesis, two versions of the construct were generated to reflect the polymorphism at the −251 position (relative to the start of transcription), corresponding to SNP rs4073. The constructs are designated −251A/rs4073A and −251T/rs4073T.

These constructs were transiently co-transfected with the Renilla luciferase reporter construct (Promega) into control (pCEP-2) and CF-like (pCEP-RF) 9HTEo- cell lines[47] using FuGene 6 Transfection Reagent (Roche Applied Science, Indianapolis, Indiana). As results from the pCEP-2 and pCEP-RF cells lines were similar, only data from pCEP-2 cells are shown.

To induce *IL8*-promoter driven luciferase expression[48], transfected cells were treated with physiologically relevant[49] quantities of a pro-inflammatory cytokine mixture (interleukin-1β [0.167 ng/μl] and tumor necrosis factor-α [0.33 ng/μl]) in serum-free culture media 24 hours after transfection. Treatment with the cytokine mix was intended to mimic the pro-inflammatory state in CF airway in which there are increased levels of IL-1β and TNF-α, both of which are considered key pro-inflammatory cytokines. Cells were also treated with hydrogen peroxide (H_2O_2), as oxidative stress has been shown promote inflammation[50], which yielded a similar expression pattern to cytokine mix treatment (data not shown). Control cells were not exposed to stimulus, but rather to the phosphate-buffered saline diluent.

Cell lysates were harvested using the Dual-Luciferase Kit (Promega) at 6, 12, 24, 36 and 48, 60, and 72 hours after treatment and luciferase activity measured in a luminometer (Turner BioSystems, Sunnyvale, California). All samples returned to baseline by 24 hours and thus data are shown for 6, 12, and 24 hour time points only. The average relative luminescence was obtained for each condition (n=30 wells). Comparisons were made between treated and untreated cells, as well as between alleles. In all cases comparisons were assessed by a two-tailed, student's t-test.

The cytokine mix was found to significantly repress Renilla expression ($p<0.04 – p<0.005$), thereby falsely inflating the magnitude of the observed response to the cytokine mix. Therefore, Renilla expression was only used to determine if transfection efficiencies were comparable for each time point. While expression estimates from the control samples were normalized to the co-transfected Renilla construct, the level of expression from the treated samples was not. Rather, multiple replicates were performed to ensure accuracy (30 individually transfected wells for each condition).

Acknowledgments

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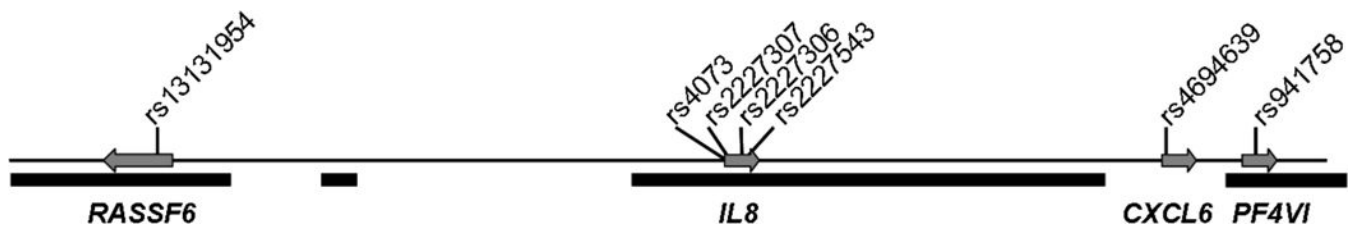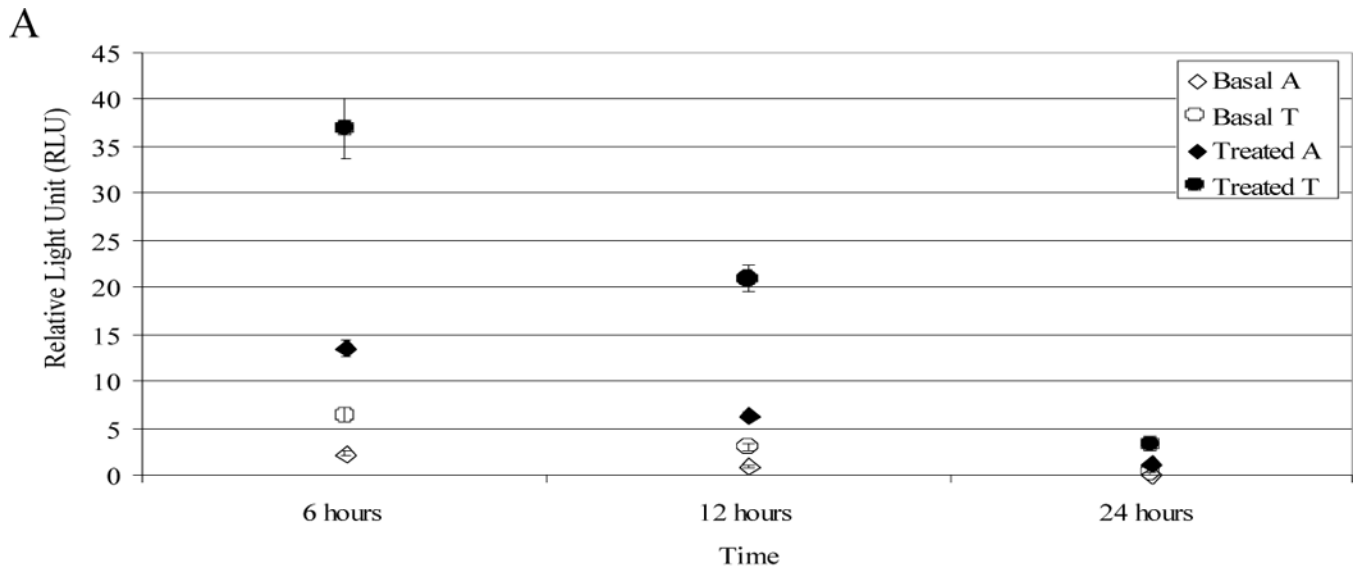
Figure 1.
The surveyed *IL8* genomic region (240 kb). Markers in the three genes most proximal to *IL8* were tested for an association with pulmonary disease severity. Arrows indicate direction of gene transcription. Solid black bars denote haplotype blocks as defined by the Haplotype Map Project[34].

A

B

Time Post-Treatment (hours)	T:A No Treatment	T:A Cytokine mix
6	2.73	3.10
12	2.72	3.26
24	1.8*	2.5**

Figure 2.
pCEP-2 cells transfected with *IL8* promoter-luciferase constructs (rs4073A or rs4073T) were treated with a pro-inflammatory cytokine mix. (a) Luciferase expression at 6, 12, and 24 hours post-treatment. Treatment with the cytokine mix induced a significant increase in luciferase expression for both constructs, (b) Values represent relative luciferase activity of the rs4073T allele to the rs4073A allele (*p=0.08 p=, **p=0.01, all others p<0.0001).

Table 1

Significantly associating SNPs in the initial inflammatory gene screen

Gene	SNP	n	Genotypic p-value
IL8	rs2227307	732	0.04
IL10RB	rs1467845	733	0.04
TGFBI	rs248158	657	0.04

Table 2

Correlation coefficients (R^2) from pairwise disequilibrium analysis

	RASSF6 (rs13131954)	IL8 (rs4073)	IL8 (rs2227307)	IL8 (rs2227306)	IL8 (rs2227543)	CXCL6 (rs4694639)	PF4V1 (rs941758)
RASSF6 (rs13131954)	1.00						
IL8 (rs4073)	0.00	1.00					
IL8 (rs2227307)	0.00	0.95	1.00				
IL8 (rs2227306)	0.00	0.82	0.85	1.00			
IL8 (rs2227543)	0.00	0.86	0.88	0.95	1.00		
CXCL6 (rs4694639)	0.01	0.50	0.47	0.39	0.40	1.00	
PF4V1 (rs941758)	0.01	0.12	0.10	0.10	0.09	0.16	1.00

Table 3

Analysis of association testing for SNPs in *IL8*-flanking genes

Gene	SNP	n	Genotypic p-value
RASSF6	*rs13131954*	717	0.54
	rs4073	733	0.07
	rs2227307	732	0.04
IL8			
	rs2227306	727	0.19
	rs2227543	737	0.06
CXCL6	*rs4694639*	709	0.27
PF4V1	*rs941758*	716	0.40

Table 4

Comparison of *rs4073* genotypic p-values between cohort 1 and 2 reveals gender effect

	rs4073	*rs2227307*	*rs2227306*	*rs2227543*
Patient Cohort 1:				
Total (n=737)	0.07	0.04	0.19	0.06
Males (n=392)	0.07	0.06	0.15	0.12
Females (n=345)	0.25	0.12	0.36	0.19
Patient Cohort 2:				
Total (n=385)	0.25	0.15	0.18	0.52
Males (n=219)	0.01	0.08	0.01	0.06
Females (n=166)	0.82	0.59	0.84	0.70

Table 5

Clinical characteristics of Study Cohorts

Variable	Severe Males		Mild Males	
	Cohort 1 (n=130)	Cohort 2 (n=69)	Cohort 1 (n=303)	Cohort 2 (n=151)
Age – Mean (± SD) (yrs)	16.3 ± 4.5	17.1 ± 5.1	29.7 ± 9.9	31.5 ± 10.3
Age – Range (yrs)	8.1–32	8–25.9	15.1–54.1	15.1–58.2
FEV_1 (± SD) (% predicted)	47.0 ± 17.1	46.5 ± 17.8	69.8 ± 30.0	64.0 ± 29.9
FEV_1 (± SD) (decline %/year)	−3.59 ± 2.27	−3.63 ± 2.01	−1.41 ± 1.52	−1.30 ± 1.59
EBint20 (± SD)	34.5 ± 17.4	35.0 ± 17.3	84.8 ± 15.3	82.0 ± 17.0
Body mass Index (± SD) (percentile)	17.5 ± 2.05	17.8 ± 2.80	22.4 ± 2.76	22.4 ± 2.78
P. aeruginosa Positive (%)	85.4	85.7	85.8	90.4
% F508/ F508	100.0	63.0	100.0	66.7

Table 6

Comparison of *rs4073* genotypes between cohort 1 and 2 reveals gender effect

	%AA	%AT	%TT	Genotypic p-value
Patient Cohort 1:				
All (n=733):				
Mild (n=490)	25.6	48.8	25.6	0.07
Severe (n=243)	19.2	50.8	30.0	
Males (n=389):				
Mild (n=271)	26.5	50.0	23.5	0.07
Severe (n=118)	19.5	46.9	33.6	
Patient Cohort 2:				
All (n=379):	22.7	46.7	30.6	
Mild (n=242)	20.4	42.3	37.2	0.25
Severe (n=137)				
Males (n=219):				
Mild (n=149)	23.6	47.9	28.6	0.01
Severe (n=70)	17.5	33.3	49.2	
Patient Cohorts 1 and 2:				
All(n=l 112):				
Mild (n=732)	24.7	48.2	27.1	0.02
Severe (n=380)	19.6	47.9	32.5	
Males (n=608):				
Mild (n=420)	25.6	49.3	25.1	0.001
Severe (n= 18 8)	18.9	42.1	38.9	