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IFRD1 polymorphisms in cystic fibrosis with potential link to altered neutrophil function

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Abstract

Lung disease is the major cause of morbidity and mortality in cystic fibrosis (CF), an autosomal recessive disease caused by mutations in *CFTR*. In CF, chronic infection and dysregulated neutrophilic inflammation lead to progressive airway destruction. The severity of CF lung disease has significant heritability, independent of *CFTR* genotype1. To identify genetic modifiers, we performed a genome-wide single nucleotide polymorphism (SNP) scan in one cohort of CF patients, replicating top candidates in an independent cohort. This approach identified *IFRD1* as a modifier of CF lung disease severity. IFRD1 is a histone deacetylase (HDAC)-dependent transcriptional co-regulator expressed during terminal neutrophil differentiation. Neutrophils, but not macrophages, from *Ifrd1*-deficient mice exhibited blunted effector function, associated with decreased NF-κB p65 transactivation. *In vivo*, IFRD1 deficiency caused delayed bacterial clearance from the airway, but also less inflammation and disease—a phenotype primarily dependent on hematopoietic cell expression, or lack of expression, of IFRD1. In humans, *IFRD1* polymorphisms were significantly associated with variation in neutrophil effector function. These data suggest that IFRD1 modulates the pathogenesis of CF lung disease through regulation of neutrophil effector function.

Attention to the role of *CFTR* in regulating epithelial ion transport has failed to illuminate the path from gene to pathogenesis in CF lung disease. In CF, colonization and infection (paradigmatically with *P. aeruginosa*) is associated with neutrophilic inflammation, the end result being progressive airway destruction2. This inflammatory response is out of proportion to inciting infectious stimuli3, which may well be due to compromise of lipid mediator pathways driving resolution of neutrophilic inflammation4.

To identify genetic modifiers of CF lung disease severity, we performed a genome-wide SNP scan in the Genetic Modifier Study Group (GMSG) cohort, followed by validation of top candidates in the US CF Twin and Sib Study (CFTSS) cohort. The former enrolled ΔF508-*CFTR* homozygotes with extremes of lung function for age, for case-control association approaches to modifier gene identification5. The latter enrolled CF-affected twins and siblings with any *CFTR* genotype, and their parents, for transmission-based approaches1. Severity status was quantified using lung function measures highly correlated with survival in CF6.

Genome-wide SNP analysis was performed using Affymetrix 100K microarrays in 320 patients from the GMSG cohort5: 160 with severe and 160 with mild lung disease, with DNA from 20 subjects pooled per microarray. To assess the robustness of the pooling approach, we first compared gene chip estimates of allele frequencies in pooled samples with individually genotyped frequencies in a subset of 93 SNPs. A high degree of correlation ($r^2 = 0.88$) was found. Second, comparison of genome-wide allele frequencies in these CF patients with those from a similar pooled genome-wide scan in asthma patients and controls (from an Isle of Wight birth cohort study7) unambiguously identified *CFTR* as the disease-causing locus in CF. Of the top-ranked polymorphisms distinguishing the two

cohorts, in terms of statistically significant differences in allele frequency, 34 out of 38 were clustered on chromosome 7, centered around *CFTR*, with a median uncorrected *P* value of 3×10^{-8} (Suppl. Fig. 1). Several of these SNPs would not pass Bonferroni correction for multiple testing, given the >100K SNPs on the microarrays—despite the known biological significance of *CFTR*. Thus, in addition to generating false positive results, correction for multiple testing in genome-wide SNP scans can generate false negative results as well.

To differentiate true from false association (or false lack of association), we prioritized follow-up efforts according to hierarchical criteria, focusing, firstly, on regional clusters of SNPs exhibiting different allele frequencies (Suppl. Fig. 2) and, secondly, on regions containing genes with biological coherence: regulators of transcription, genes with known function in the immune system or in lung biology, and genes implicated in biological functions abnormal in the CF airway (e.g., ion channels). This criterion was unlikely to facilitate identification of genes with unknown function or of unexpected pathogenetic pathways. However, we aimed to identify true modifiers amongst false positives, not to identify all modifiers. This heuristic approach was used to select 6 regions/genes for follow-up study: *IFRD1*, *CEBPA/CEBPG*, *CHI3L2*, *C6*, *SLC4A3*, and *ABCA1*.

The top-ranked locus in terms of clustering was *IFRD1*, 5 Mb away from *CFTR*, the 3′ region of which contained a cluster of 9 SNPs (Suppl. Table 1; Suppl. Fig. 2b) with significantly different allele frequencies between patient groups. Biology is addressed below. To confirm the genotyping in the pooled scan, as well as define which SNPs to pick for replication purposes, SNPs in *IFRD1* reaching significance in the pooling experiment, along with tagging SNPs throughout and flanking the region of the effect, were individually genotyped in the wider GMSG cohort. While no association signal was observed for *IFRD1*-flanking markers, retention of association signal for a cluster of SNPs on the haplotype block containing the 3′ *IFRD1* exons (Suppl. Tables 2 and 3, Suppl. Fig. 3) led us to pursue replication in a separate population.

Three *IFRD1* SNPs (rs7817, rs3807213, rs6968084) that linkage disequilibrium analysis suggested captured the bulk of the variation observed at this locus were genotyped in patients in the CFTSS cohort (Suppl. Table 4). Notably, the family-based association test demonstrated significant association between the rs7817 polymorphism and both cross-sectional and longitudinal measures of lung function (Table 1a). The other two SNPs showed trends toward significance (rs3807213, $P = 0.080$; rs6968084, $P = 0.082$; for longitudinal and cross-sectional measures, respectively, of lung function). A second, complementary method (quantitative transmission disequilibrium test) verified the result derived for rs7817 (Table 1b). Intriguingly, both methods revealed that the heterozygote genotype (“CT”) was associated with lower lung function than either homozygote (“CC” or “TT”; data not shown). However, other genotype models (additive, recessive and dominant) could not be conclusively excluded. It was important to exclude linkage with *CFTR* alleles as the cause of the observed association between *IFRD1* SNPs and CF lung function. No correlation was detected between genotypes composed of *IFRD1* SNPs and the presence of 0, 1 or 2 copies of the common mutation ΔF508, or when *CFTR* mutations were grouped according to their association with exocrine pancreatic status. Furthermore, there was no evidence of linkage between the SNPs and the pulmonary phenotypes8, important for

validating the association model used (a test of association in the absence, as opposed to the presence, of linkage). These data indicate that *IFRD1* polymorphisms contribute to lung function variation in CF independent of *CFTR*.

IFRD1 acts in an HDAC-dependent manner to mediate transcriptional co-repression and co-activation9. Expression and genetic deletion studies have implicated IFRD1 in cell differentiation and stress responses10. Available databases suggested highest expression in human blood cells11. Flow cytometric analysis of such cells revealed greatest expression in neutrophils (Suppl. Fig. 4a). Similarly, quantitative RT-PCR analysis of cells relevant to the CF airway indicated particular enrichment of expression in neutrophils (Suppl. Fig. 4b). Terminal differentiation of human and mouse neutrophils was associated with robust upregulation of IFRD1 expression (Suppl. Fig. 5), something mirrored in expression databases12. Neutrophilic differentiation of HL-60 cells also led to upregulation of IFRD1 expression (Suppl. Fig. 6a), and siRNA-mediated knockdown of IFRD1 in such cells blunted oxidative burst capacity (Suppl. Fig. 6b and 6c) without altering visual morphology or surface expression of CD11b (data not shown).

No alteration in peripheral blood neutrophil count, morphology, or CD11b and Gr-1 expression were observed in *Ifrd1*$^{-/-}$ mice13 (data not shown). However, neutrophils from Ifrd1$^{-/-}$ mice exhibited significant impairment of specific effector functions, including oxidative burst, bacterial killing, TNF-α, KC and LTB_4 production—but not chemotaxis—compared with wild type and/or heterozygote littermate controls (Fig. 1). *In vivo* stimulation led to similar results: after intratracheal LPS stimulation, *Ifrd1*$^{-/-}$ mice exhibited significantly less TNF-α production on a per cell basis in neutrophils, but not macrophages, compared with wild type controls (data not shown). No differences in airway neutrophil numbers or apoptosis were seen in these studies (data not shown). These functional effects exhibited specificity among myeloid cells; peritoneal macrophages from *Ifrd1*$^{-/-}$ mice exhibited normal oxidative burst capacity and LPS-driven TNF-α production (Suppl. Fig. 7). Thus, IFRD1 plays an important role in regulating neutrophil effector function.

We subsequently analyzed the role of IFRD1 in modulating airway infection with *P. aeruginosa*. Genetic deficiency of IFRD1 was associated with significantly slower bacterial clearance (Fig. 2a). Notably, however, *Ifrd1*$^{-/-}$ mice also had significantly ameliorated disease, with less weight loss, and less airway and systemic inflammation (Fig. 2b-f). To define whether this was due to hematopoietic cell IFRD1 expression, C57BL/6 (CD45.1) mice were lethally irradiated and reconstituted with bone marrow cells from CD45.2-expressing wild type or *Ifrd1*$^{-/-}$ mice. No differences in bone marrow reconstitution efficiency were observed (Supplementary Figure 8a). Indeed, competitive reconstitution assays formally demonstrated the lack of a role for IFRD1 in early neutrophil development (Suppl. Fig 8b). Wild type mice reconstituted with IFRD1-deficient bone marrow cells mirrored the phenotype of *Ifrd1*$^{-/-}$ mice during *P. aeruginosa* infection—with less efficient bacterial clearance, but less inflammation and disease (Suppl. Fig. 9). When reciprocal bone marrow transfers were performed (reconstituting lethally-irradiated CD45.2-expressing wild type and *Ifrd1*$^{-/-}$ mice with bone marrow cells from wild type C57BL/6 (CD45.1) mice), no such differences in bacterial burden, inflammation or disease course were seen (Suppl Fig.

10). Thus, IFRD1 modulation of the airway response to *P. aeruginosa* infection is largely dependent on hematopoietic cell expression of IFRD1.

Airway challenge with LPS was similarly associated with increased TNF-α and KC in BAL fluid from wild type, compared with *Ifrd1*$^{-/-}$, mice (Suppl. Fig. 11a-b). *In vivo* HDAC inhibition blunted LPS-driven airway TNF-α and KC production, specifically in wild type, not in *Ifrd1*$^{-/-}$, mice (Suppl. Fig. 11a-b). Furthermore, bone marrow transfer experiments revealed that the effects of HDAC inhibition on LPS-driven airway TNF-α production—in BAL and by airway neutrophils (Suppl. Fig. 11c-d); but not by airway macrophages (data not shown)—was dependent on hematopoietic cell IFRD1 expression.

The effector functions blunted in the absence of IFRD1 are dependent on NF-κBp6514,15. *Ifrd1*$^{-/-}$ mice exhibited significantly decreased LPS-stimulated neutrophil NF-κBp65 transactivation, compared with littermate controls (Suppl. Fig. 12a). As IFRD1, NF-κBp65 and HDAC1 were co-immunoprecipitable in neutrophil nuclear extracts (Suppl. Fig. 12b), it appears likely that IFRD1 mediates its effects on neutrophils, at least in part, by direct interactions with NF-κB. While our data are compatible with IFRD1 being a co-activator of transcriptional activity or a co-repressor of an inhibitor of transcriptional activity, the HDAC inhibition experiments suggest the latter. Co-immunoprecipitation analysis suggests the possibility of HDAC-mediated co-repression of an NF-κB-driven transcriptional inhibitor of NF-κB transactivation.

While we may have not identified the causal variant(s)16, cogent hypotheses exist for how the identified SNPs may alter *IFRD1* expression and/or function (Suppl. Discussion). In order to directly test the association of *IFRD1* polymorphisms with neutrophil effector function, we studied neutrophils from healthy subjects. Notably, analysis of human peripheral blood neutrophils revealed significant association of *IFRD1* polymorphisms with quantitative measures of neutrophil effector function (Fig. 3). Taken together, these data suggest that IFRD1 modulates the course of CF airway disease through regulation of neutrophil effector function.

Biology is rarely simple, however. The predictive value of mouse knockout models for the more subtle biological differences likely to result from human allelic polymorphisms, as well as the power of the functional data from human neutrophils to account for the effects of these polymorphisms on lung function over time, should not be over-stated. It remains possible that neutrophils are not the only cells influenced by *IFRD1* polymorphisms in a fashion relevant to cystic fibrosis. There may, for example, be ways by which respiratory epithelia and neutrophils interact through *IFRD1* polymorphisms to modulate cystic fibrosis lung disease. On the whole, *Cftr* knockout and mutant mouse models have been somewhat disappointing; despite recapitulation of gut pathology, pulmonary phenotypes have been subtle. Whether this relates to different lung architecture in mice and humans, the influence of other genes in the mouse strains used, or stronger baseline immune counter-regulation in the mouse lung remains unclear. There is thus an essential problem with using such models to define whether the absence of *Ifrd1* (or the presence of mutant *Ifrd1* alleles) ameliorates cystic fibrosis lung disease, in the absence of a robust phenotype to ameliorate. In this light,

the recent report of pigs with targeted disruption of *CFTR*17 may point the way to informative models.

Of the other top-ranked genes/regions from the initial scan, *C6*, *SLC4A3* and *ABCA1* did not survive genotyping in the wider GMSG cohort; *CHI3L2* failed replication in the CFTSS cohort (data not shown). However, after refinement of the association signal from the pooled scan in the *CEBPA/CEBPG* locus (Suppl. Table 5) via individual genotyping in the wider GMSG cohort (Suppl. Tables 6 and 7), replication was pursued in the CFTSS cohort. As shown in Supplementary Table 8, polymorphisms in the 40 kb intergenic region between *CEBPA* and *CEBPG* (genes oriented 5′ to each other) were significantly associated with variation in CF lung function. While there are clearly other possible ways in which these transcription factors may affect CF lung disease severity18,19, CEBP-α is essential for neutrophil development20, a pathway that CEBP-γ has been implicated in as well21.

Despite considerable progress in CF therapy over recent decades, the norm is still an inexorable decline in pulmonary function. Identification of genes modifying CF lung disease, and delineation of the pathogenetic pathways that they influence, holds promise for the development of novel therapeutic strategies. The current data suggest likely utility for therapeutic targeting of neutrophils in this devastating disease. These data also suggest that the IFRD1/HDAC axis may provide a tractable therapeutic target in CF, and other diseases in which neutrophils play an important pathogenetic role.

Methods Summary

Genetic analysis

CF Patients were from the GMSG5 and CFTSS1 cohorts. Healthy controls were recruited at CCHMC. Studies were approved by the relevant Institutional Review Boards. Genome-wide analysis was performed using Affymetrix GeneChip 100K Human Mapping microarrays. Other genotyping was performed using Taqman PCR, AcycloPrime-FP SNP PCR, Illumina 610 Quad chips and llumina SNP beadarray genotyping. Follow-up association analysis (GMSG cohort) was performed using SNPGWA22. Association and transmission analysis (CFTSS cohort) was done using PEDSTATS v.0.6.6 (http://www.sph.umich.edu/csg/abecasis/Pedstats)23, Intercooled Stata 8, QTDT v.2.5.0 (http://www.sph.umich.edu/csg/abecasis/QTDT)24, Golden Helix software (http://www.goldenhelix.com). Quantitative Trait Analysis (Healthy Donor cohort) was performed using PLINK version 1.03 (http://pngu.mgh.harvard.edu/~purcell/plink/).

Cellular assays

Surface and intracellular FACS staining was performed as described25. Quantification of mRNA was performed by quantitative RT-PCR, as described25. Oxidative burst capacity was quantified by flow cytometry using the dihydrorhodamine 123 assay26. TNF-α production was quantified by ELISA (BDPharmingen) or by intracellular staining, as described27. LTB_4 production was quantified by ELISA (Neogen). Bacterial killing was quantified as described28. Neutrophil chemotaxis was quantified as described29. Nuclear NF-κB p65 DNA-binding activity was quantified using the EZ-Detect Transcription Factor

ELISA (Pierce). Co-Immunoprecipitation of nuclear proteins was performed using the Nuclear Complex Co-IP kit from Active Motif (Carlsbad, CA).

Mouse models

Mice were non-traumatically challenged intratracheally with *P. aeruginosa* (FRD1 strain). 48 h later, mice were sacrificed. BAL cell analysis, lung bacterial burden and myeloperoxidase activity were quantified as described4. BAL and serum cytokines were quantified by ELISA (BDPharmingen, R&D) or by the CCA ELISA30. Standard bone marrow cell transfer techniques were employed. SAHA, from Cayman, was administered intraperitoneally. Mice were non-traumatically challenged intratracheally with *P. aeruginosa* LPS (Sigma). Animal care was provided in accordance with National Institutes of Health guidelines. Studies were approved by the CCHMC Institutional Animal Care and Use Committee.

Methods

Cohorts

The GMSG cohort consists of CF patients homozygous for ΔF508 *CFTR* whose longitudinal FEV_1 measurements were in the highest or lowest quartile for age among ΔF508 homozygotes. Enrolment criteria, data collection and genotyping have been described5. The study was approved by the institutional review boards (IRB) of all participating institutions. Patients and parents of minors provided written informed consent.

CF twins and siblings (*N* =1,118) and their parents from 619 families were recruited by the CFTSS as previously described1. Twenty-one dizygous and 49 monozygous (MZ) twin pairs were included. Raw pulmonary function test data, *CFTR* genotypes, and height and weight measurements were obtained from medical records. In some cases in which genotypes were unavailable, *CFTR* exons were sequenced to identify mutations. Written informed consent or assent was obtained from all subjects. FEV_1 was used to derive cross-sectional ($MaxFEV_1CF\%$) and longitudinal ($AvgFEV_1CF\%$ and $EstFEV_1\%Pred$) measures, as previously described1. To include as many subjects as possible and to avoid randomly excluding one member of each pair, lung function measures were averaged for MZ twin pairs and included in analyses only if the twins' values were within ten percentiles of each other (or ten percent-predicted), as not to double-count genetically identical individuals. For MZ twin pairs in which only one of the twins had pulmonary data, that twin's data was included.

Healthy controls (inclusion and exclusion criteria: standard for routine blood donation, plus exclusion for use of immunosuppressive medications or non-steroidal anti-inflammatory drugs in the 2 wk prior to blood donation for functional assays) were recruited at CCHMC. Blood for neutrophil function studies was obtained from 46 participants, 37 of whom self-reported European ancestry. Due to the small numbers of non-Europeans and the possibility of confounding due to stratification, analysis was restricted to these 37. Blood samples were blinded to haplotype and genotype status prior to functional analysis. All participants gave written informed consent; the study was approved by the CCHMC IRB.

Genotyping

Genome-wide analysis, using Affymetrix GeneChip 100K Human Mapping microarrays, was performed in 320 CF patients from the GMSG Study5: 160 with severe lung disease (lowest quartile of FEV_1 for age; 160 with mild lung disease (highest quartile of FEV_1 for age); 308 of whom self-reported European ancestry. Each group of 160 was comprised of 80 males and 80 females. Each such group of 80 was divided into groups of 20 (from across the relevant quartile) for pooling purposes. Equimolar amounts of DNA, with an A_{260}/A_{280} ratio between 1.65-2 and an A_{260}/A_{230} ratio between 1.0-2.2, as quantified by NanoDrop spectrophotometry (Wilmington, DE, USA), were combined into pools containing 250 ng DNA. DNA pools were digested with XbaI or HindIII, adapter-ligated, and PCR-amplified. Samples were separated on 4% agarose gels to ensure DNA fragmentation in the 100-300 bp range. PCR yields were compared between microarray chips to ensure uniformity (>1200 ng/ul accepted), and PCR products were separated on 2% agarose gels to ensure the proper range of amplified product. GeneChip Genotyping software (v.4.0, Affymetrix, Inc.) was used for relative quality control assessment, detection rates, and allele distributions. Hybridization intensity comparisons of the case and control pools were used to identify significant allele frequency differences for each SNP. A set of 100,198 (out of 111,664) SNPs provided data of sufficient quality on all microarrays.

Taqman PCR genotyping, using assays from ABI, was performed: (a) for assessment of the robustness of pooled estimates of allele frequency, by individual sample genotyping of the initial 320 patients in the GMSG cohort; (b) for individual genotyping of samples from 2194 subjects in the CFTSS cohort (see below); (c) for genotyping 91 normal healthy controls. AcycloPrime-FP SNP PCR assays (Perkin Elmer) were used (d) to genotype 100 healthy controls. Autoclustering algorithms were used ([a] SDS Version 1, [b, and c] SDS Version 2.3, both from ABI; and [d] FP Caller, from Johns Hopkins) to call SNPs. The call rates were (a) 99.9%, (b) 97.1%, (c) 98.6% and (d) 99.6% respectively.

Individual sample genotyping of an expanded sample set of patients in the GMSG cohort, comprised of the 320 samples from the pooling experiment, plus an additional 485 samples, for a total of 805 samples (261 severe, 541 mild), was performed. To minimize possible issues of population stratification, genetic analysis at this stage was confined to individuals self-reporting European ancestry (779 out of 805, including 241 and 538 patients with severe and mild lung disease, respectively). SNPs that reached significance in the pooling experiment, along with tagging SNPs31 throughout the region of the effect, were selected for follow-up. Tag SNPs were chosen based on HapMap data using Tagger (http://www.broad.mit.edu/mpg/tagger/) (minor allele frequency threshold = 0.05; pairwise R^2 threshold = 0.8). Additionally, a reported non-synonymous polymorphism in *IFRD1*, rs11542463, was assayed; it was monomorphic in the GMSG cohort. A final list of tag SNPs was chosen based on predicted assay design scores for the SNP beadarray. Genotyping was done by custom Illumina GoldenGate assays. An autoclustering algorithm was used on all SNPs. Clusters of SNPs were manually inspected when they had a low call rate (<98.5) or a low clustering score (<0.6). 3 individuals (out of an initial 808) with DNA quality or gender reporting problems were excluded. The genotype success rate for each SNP was >99.6%; the

overall call rate was 99.95%. 8 samples assayed in duplicate as technical replicates had >99.93% concordance.

CFTSS subjects were genotyped by two methods, TaqMan (Applied Biosystems, Foster City, CA) and the Illumina 610 Quad chips. In total, approximately 2200 individuals were typed for three *IFRD1* SNPs (rs6968084, rs3807213, and rs7817). Of these, 76% were typed by both methods, 20% were typed by TaqMan only, and 4% were typed by Illumina only. The discrepancy rates between the two methods were 0.42%, 0.06%, and 0.49% for rs6968084, rs3807213, and rs7817, respectively. No Mendelian errors were detected in families typed using the Illumina platform, while five Mendelian errors were detected in families typed by TaqMan. Because the former method appeared to be more reliable, Illumina genotypes were used in cases where calls made by the two methods were different.

Genetic association data analysis

Allele frequencies for Affymetrix data were determined using adjustment factors for pooled samples32. Z^2 P values were used to rank all SNPs. A cluster analysis of Z^2 statistics was performed. While a previous report has found evidence for minimal stratification in the GMSG cohort33, the possibility of confounding due to population substructure in the pooling step was investigated by applying the genomic control (GC) method to the Z^2 statistics for pooled DNA34. Direct application of GC to pooled data assumes the variance due to pooling has properties delineated by Devlin, et al.34 While the pooling experiment did not contain the technical replicates necessary to definitively satisfy these assumptions, GC was directly applied to the Z^2 statistics. Using this approach, the inflation factor $\lambda = 1.04$ when estimated using the mean—again suggesting that stratification is minimal in this population35.

Follow-up association analysis in the GMSG cohort was performed using SNPGWA22. Each SNP was tested for departures from Hardy-Weinberg equilibrium expectations. The additive genetic model test of association was the primary inference. Imputation analysis was performed using the gwas software, impute v0.4.2 and snptest v.1.1.5 (http://www.stats.ox.ac.uk/~marchini/software/gwas/gwas.html)35.

For association and transmission analysis in the CFTSS cohort, genotype distributions were tested for Hardy-Weinberg equilibrium using the '--unrelatedsOnly' option in PEDSTATS v.0.6.6 (http://www.sph.umich.edu/csg/abecasis/Pedstats)23, which performs an exact test in a subset of unrelated individuals, so as to avoid bias from correlated genotypes within families. Because correlation among sibling marker genotypes may invalidate the results of family-based tests of association in the presence of linkage, linkage between SNPs and pulmonary phenotypes was evaluated using Merlin software (MERLIN v. 1.1.2; http://www.sph.umich.edu/csg/abecasis/Merlin/).

Association between three *IFRD1* SNPs (rs6968084, rs3807213, and rs7817) and the three pulmonary phenotypes was analyzed using the PBAT module implemented within Golden Helix software (Golden Helix, Inc. Bozeman, MT, USA. Golden Helix PBAT Software http://www.goldenhelix.com). Four genetic models were tested: additive, dominant, recessive, and heterozygote distortion. The best associated SNP and phenotypes resulting

from PBAT analysis were tested for transmission disequilibrium by a second method, QTDT (http://www.sph.umich.edu/csg/abecasis/QTDT)24, using the orthogonal model of association and assuming dominance. Complete and incomplete trios were utilized in both analyses. General statistics were performed in Intercooled Stata 8 (StataCorp, College Station, TX).

Analysis of data on neutrophil oxidative index and TNF-α production in healthy donors was performed using PLINK version 1.03 (http://pngu.mgh.harvard.edu/~purcell/plink/) standard quantitative trait association options for genotypes (--assoc) and haplotypes (--hap-assoc) as indicated. Estimated Haplotypes were imputed using the Expectation-Maximization algorithm as implemented in PLINK using the (--hap-phase) option.

Cellular phenotypic and functional assays

Human neutrophils and mononuclear cells were isolated by Ficoll-Hypaque sedimentation, monocytes by leukapheresis and counter-current elutriation. CD34+ cells (CCHMC Normal Donor Repository) were differentiated *in vitro* with rG-CSF (50 ng/ml) plus rSCF (50 ng/ml, both from Peprotech) for 8 d, followed by rG-CSF alone for 8 d. Primary tracheobronchial cells were harvested from bronchial brushings from normal subjects (UCCOM Bronchoscopy Core). THP-1, BEAS-2B and HL-60 cells were from ATCC. HL-60 cells were differentiated with 1.5% DMSO or with retinoic acid (1 μg/ml). Mouse peripheral blood leukocytes were isolated from whole blood after lysis of erythrocytes with ACK buffer. Neutrophils were isolated immunomagnetically from mouse bone marrow using Gr-1 beads (Miltenyi), a purification strategy yielding a highly purified population of mature neutrophils, as demonstrated by stained cytospins (data not shown). Mouse peritoneal exudate macrophages were isolated after thioglycollate elicitation25. Mouse hematopoietic progenitor ($Lin^{-}c\text{-}kit^{+}sca\text{-}1^{+}$) cells were purified by flow-cytometric sorting29, and differentiated *in vitro* for 11 d with rSCF (100 ng/ml), rMGDF (100 ng/ml) and rG-CSF (100 ng/ml; all from Amgen).

Surface and intracellular FACS staining was performed as described25, using antibodies from Sigma (IFRD1), Molecular Probes (mouse IgG2a), eBioscience (CD11b, Gr-1, CD16, CD3, CD4, CD8, CD19), an LSRII flow cytometer and FACSDiVa Software (BDPharmingen). Fc-receptor blockade was performed with human AB serum Gemini Bio; human cells) or blocking Ab to CD16 and CD32 (Fc block, eBioscience; mouse cells). Quantification of mRNA was performed by qRT-PCR25, using a LightCycler (Roche) and the following primers: IFRD1 5′ TGCAGCGTTAGCATCTGTTC, IFRD1 3′ ACCAAAGCAAGTTGCACAAG; IFRD2 5′ TGTTTTCAGCCGGTTCTATGG, IFRD2 3′ TGCCTGTCAAGGATGTGGC; ubiquitin 5′ CACTTGGTCCTGCGCTTGA, ubiquitin 3′ CAATTGGGAATGCAACAACTTTAT. KC 5′ ACCCAAACCGAAGTCATAGC, KC 3′ TCTCCGTTACTTGGGGACAC. Oxidative burst capacity was quantified by flow cytometry in mouse cells treated with PMA (Sigma), using the dihydrorhodamine 123 assay26. HL-60 cells were mock transfected, or transfected by Nucleofection (Amaxa) with 90 pmol (45nM) synthetic siRNA against IFRD1, or negative control siRNA, and incubated for 48h, during differentiation to a neutrophil phenotype with DMSO. IFRD1 siRNA sense/antisense: r(GGU GAG UUC UGA UUA UUA A)dTdT/r(UUA AUA AUC AGA ACU

CAC C)dAdG; Control (non-silencing) siRNA sense/antisense: r(UUC UCC GAA CGU GUC ACG U) dTdT/r(ACG UGA CAC GUU CGG AGA A) dTdT. Oxidative burst was quantified by flow cytometry in human neutrophils by the dihydrorhodamine 123 assay26. Fluorescence was quantified in neutrophils ($CD11b^+CD15^+$ cells; antibodies from Biolegend; within the granulocyte gate set based on forward and side scatter characteristics) using an LSRII flow cytometer. TNF-α production by cells or in airways was quantified by ELISA (BDPharmingen) or by intracellular staining27 (anti-TNF-α from eBioscience). KC was quantified by ELISA (R&D) or qRT-PCR. LTB_4 was quantified by ELISA (Neogen) after stimulation of neutrophils with *P. aeruginosa* LPS or GM-CSF, followed by incubation with arachidonic acid36. Killing of *P. aeruginosa* (FRD1 strain) was quantified as described28. Neutrophil chemotaxis was quantified as described29. Nuclear NF-κB p65 DNA-binding activity was quantified using the EZ-Detect Transcription Factor ELISA (Pierce). Co-Immunoprecipitation of nuclear proteins was performed using the Nuclear Complex Co-IP kit from Active Motif (Carlsbad, CA). Immunoprecipitating and immunoblotting antibodies were from Santa Cruz. Immunoreactive proteins were visualized by ECL (Amersham).

Mouse models

Six to 8 wk-old *Ifrd*$^{-/-}$ mice13 on a C57BL/6 background (> 10 generations) and wild type controls, were challenged intratracheally (non-traumatically, as described4) with 6×10^6 CFU of *P. aeruginosa* (FRD1 strain). 48 h after challenge, mice were sacrificed, bronchoalveolar lavage (BAL) was performed, and serum and lungs were harvested. Lung bacterial burden and myeloperoxidase activity were quantified by standard techniques4. BAL and serum cytokines were quantified by ELISAs (BDPharmingen, TNF-α; R&D, KC). $CD45.1^+$ congenic C57BL/6 (B6.SJL-PtprcaPep3b/BoyJ) mice were lethally irradiated, and rescued with 2×10^6 bone marrow cells from wild type or IFRD1-deficient C57BL/6 ($CD45.2^+$) mice. In these experiments, TNF-α was measured by the CCA ELISA30. Similarly, wild type and *Ifrd1*$^{-/-}$ C57BL/6 ($CD45.2^+$) mice were lethally irradiated, and rescued with 2×10^6 bone marrow cells from wild type ($CD45.1^+$) mice. Reconstitution was monitored by flow cytometric analysis of peripheral blood cell populations, using mAb to CD45.2, CD11b, Gr-1, TCR, B220, and NK1.1 (eBioscience). Reconstituted mice were challenged with *P. aeruginosa* ≥ 2 months after transplantation. To formally test the ability the relative reconstitution ability of bone marrow cells from *Ifrd1*$^{-/-}$ and wild type mice, bone marrow cells from *Ifrd1*$^{-/-}$ or wild type mice (both $CD45.2^+$) were transplanted into lethally irradiated wild type ($CD45.1^+$) recipient mice, along with an equal number (1×10^6) of competitor bone marrow cells ($CD45.1^+$). Mice were treated with SAHA (10 mg/kg; Cayman) i.p., followed 1 h later by intratracheal challenge with *P. aeruginosa* LPS (2 mg/kg; Sigma). Animal care was provided in accordance with National Institutes of Health guidelines. Studies were approved by the CCHMC Institutional Animal Care and Use Committee.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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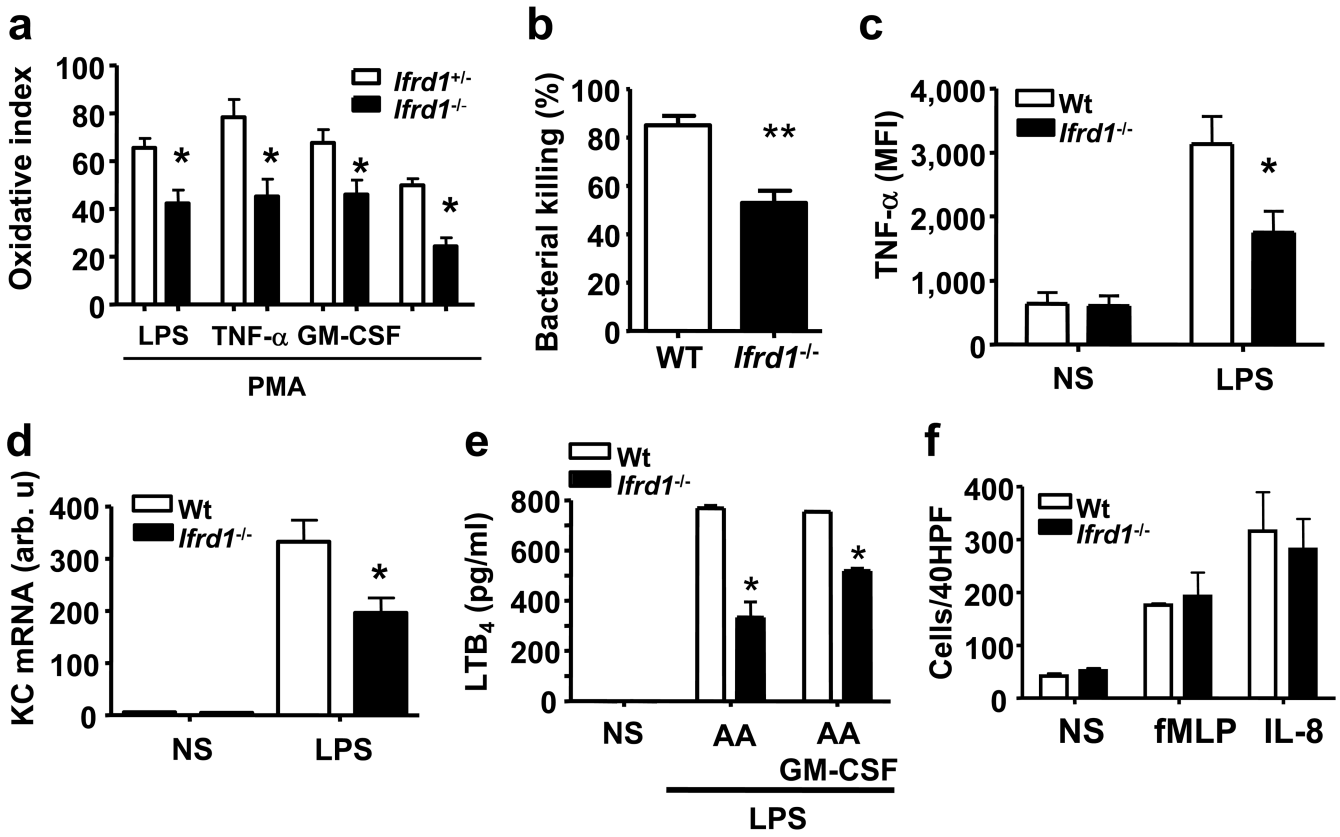

Figure 1. IFRD1-deficient neutrophils exhibit blunted effector functions

(a) Oxidative burst, in peripheral blood neutrophils from *Ifrd1*$^{-/-}$ mice and heterozygote littermate controls. **(b) Bacterial Killing.** Killing of *P. aeruginosa* by bone marrow neutrophils, calculated as [(control cfu–experimental cfu)/control cfu] × 100. Means ± S.E.M. are shown, of 6 different mice/genotype, tested in duplicate. Similar results were seen with whole blood neutrophil killing assays in 3 separate experiments. **(c) TNF-α production.** Intracellular TNF-α expression by neutrophils, quantified by flow cytometry in LPS-stimulated whole blood isolated from *Ifrd1*$^{-/-}$ mice and wild type controls. **(d) KC production**. KC mRNA expression, quantified by qRT-PCR in bone marrow neutrophils from wild type or *Ifrd1*$^{-/-}$ mice. Means ± S.E.M. are shown, of 6 different mice/genotype, tested in duplicate. **(e) LTB_4 production.** LTB_4, quantified by ELISA in supernatants from bone marrow neutrophils from wild type or *Ifrd1*$^{-/-}$ mice incubated with arachidonic acid (AA), and stimulated with LPS in the presence or absence of GM-CSF. **(f) Chemotaxis.** Quantification of chemotaxis by bone marrow neutrophils from wild type or *Ifrd1*$^{-/-}$ mice in response to fMLP (1 μM) or IL-8 (1 μg/ml). Means ± SE are shown, representative of 3 separate experiments for a, c and e. **P* < 0.05, ***P* < 0.01. NS, mock stimulation; PMA, phorbol-12-myristate-13-acetate.

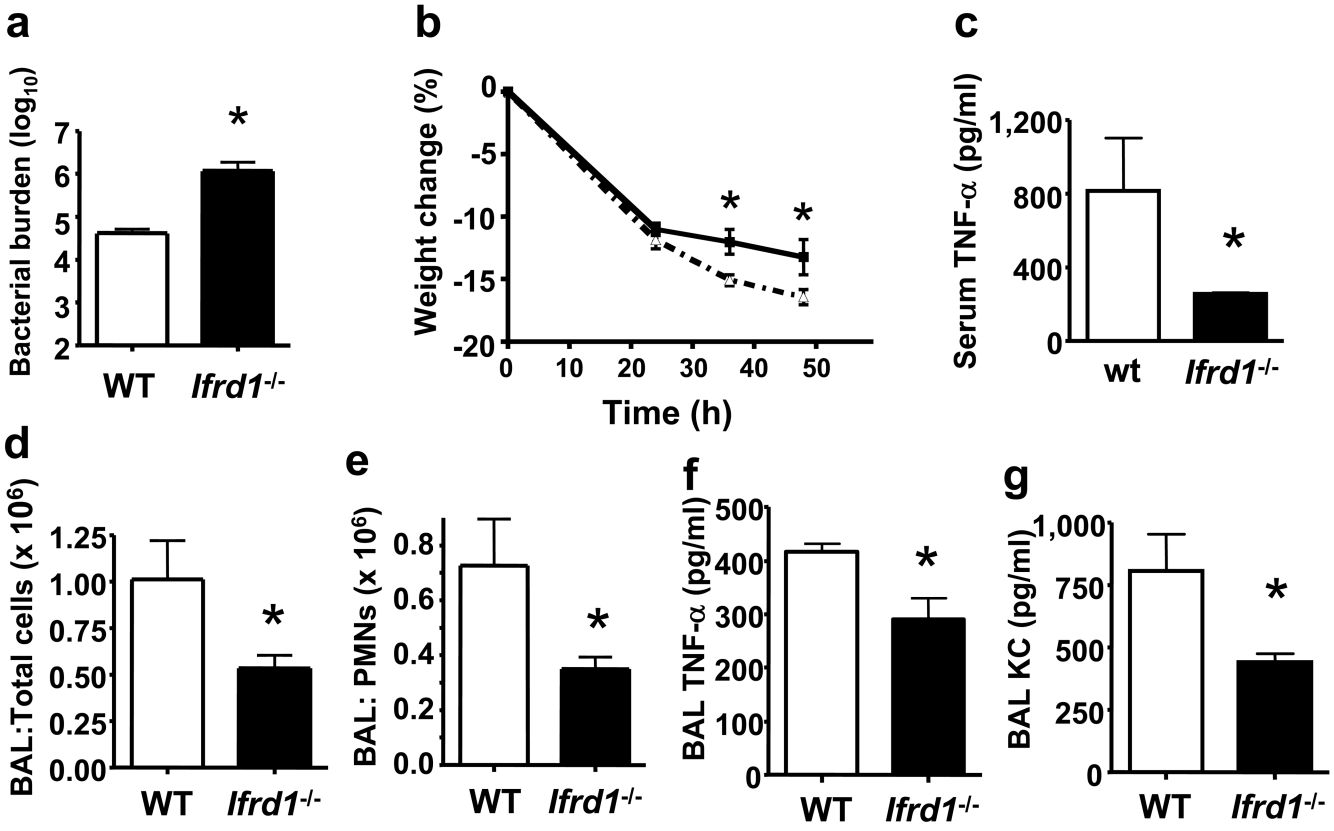

Figure 2. Genetic deficiency of IFRD1 is associated with delayed bacterial clearance, but decreased neutrophilic inflammation and ameliorated disease, after airway challenge with mucoid *P. aeruginosa*

Wild type and *Ifrd1*$^{-/-}$ mice were challenged intratracheally with *P. aeruginosa* (FRD1 strain), and analyzed 48 h later. **(a) Lung bacterial burden**. **(b) Weight loss**. Solid line, *Ifrd1*$^{-/-}$; dashed line, WT. **(c) Serum TNF-α. (d) BAL: total cells**. **(e) BAL: neutrophils**. **(f) BAL TNF-α. (g) BAL KC**. No differences in parenchymal accumulation of neutrophils (quantified by analysis of myeloperoxidase activity in blanched lungs) were observed. Means ± S.E.M. of 6 mice/group are shown; data are representative of 3 separate experiments. *$P < 0.05$.

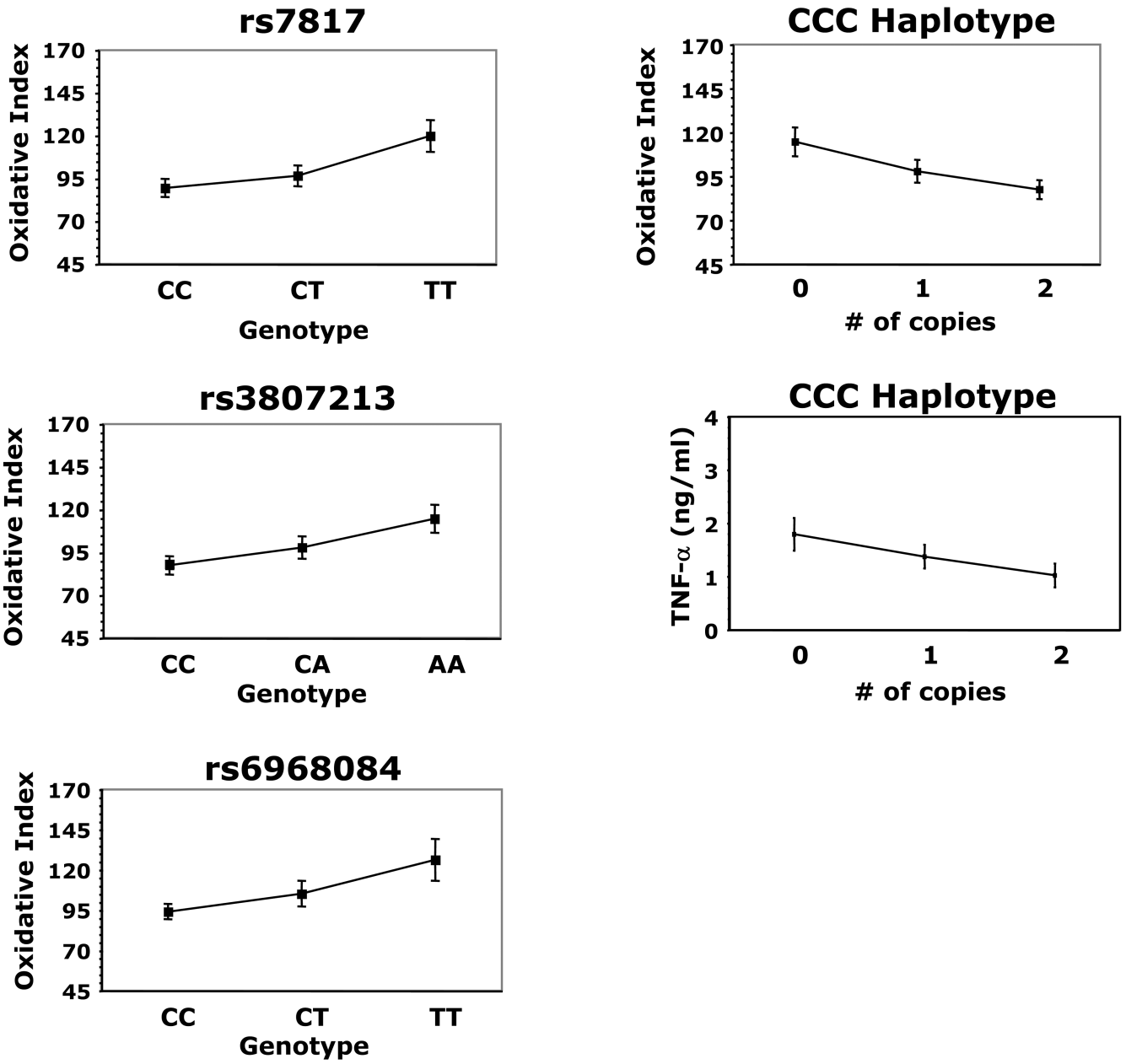

Figure 3. *IFRD1* polymorphisms are associated with variation in human neutrophil effector function

Oxidative burst capacity following PMA stimulation, and LPS-driven TNF-α secretion, was quantified in neutrophils from healthy donors of self-reported European descent (n = 36). **(a) Oxidative index, SNP rs7817**. C/C (*N* = 13); T/C (*N* = 13); T/T (*N* = 10). *P* =0.005 (Wald test). **(b) Oxidative index, SNP rs3807213**. C/C (*N* = 12); A/C (*N* = 11); A/A (*N* = 13). *P* = 0.007. **(c) Oxidative index, SNP rs6968084.** C/C (*N* = 19); T/C (*N* = 15); T/T (*N* = 2). *P* = 0.06. **(d) Oxidative index, 3-Marker Haplotype (rs7817, rs3807213, rs6968084).** 0 (*N* = 13), 1 (*N* = 11) or 2 (*N* = 12) copies of CCC haplotype. *P* = 0.007. **(e) TNF-α, 3-Marker**

Haplotype (rs7817, rs3807213, rs6968084). CCC haplotype copy *N* as in (d). $P = 0.03$. Data represent means ± S.E.M.

Table 1

Table 1a. Transmission analysis of *IFRD1* rs7817 in CF patients using family based association testing (PBAT module, Golden Helix®, Bozeman, MT)						
SNP	**Allele**	**Genetic Model**	**n**	***P* value**	**Clinical outcome measure**	**Effect**
rs7817	C/T	Het. Distortion	248	0.004	Cross-sectional lung function[a]	-
	C/T	Het. Distortion	186	0.016	Longitudinal lung function[b]	-
Table 1b. Transmission analysis of *IFRD1* rs7817 in CF patients using Quantitative Transmission Disequilibrium Testing (QTDT24)						
SNP		F	N	*P* value	Clinical outcome measure	
rs7817		4.10	467	0.0168*	Cross-sectional lung function[a]	
		4.00	314	0.0187*	Longitudinal lung function[b]	

Het., heterozygous; n, number of informative families; N, number of informative individuals; F, QTDT test statistic24; Effect, phenotypic effect associated with the over-transmitted allele (+, better function).

[a] BayesFEV$_1$%Pred@20yrs, estimated FEV$_1$%-predicted at age 20 years as described in1.

[b] MaxFEV$_1$CF%, maximum CF-specific percentile for FEV1 in patient's most recent year of available data, as described in1.

* $P < 0.05$ after Bonferroni correction for two tests.

Minor allele frequencies: rs7817, 0.48 C; rs3807213, 0.40 C; rs6968084, 0.14 T