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Original Article

Inactivation of *CFTR* by CRISPR/Cas9 alters transcriptional regulation of inflammatory pathways and other networks

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ABSTRACT

Background: Individuals with cystic fibrosis (CF) experience elevated inflammation in multiple organs, but whether this reflects an inherent feature of CF cells or is a consequence of a pro-inflammatory environment is not clear.
Method: Using CRISPR/Cas9-mediated mutagenesis of *CFTR*, 17 subclonal cell lines were generated from Caco-2 cells. Clonal lines with functional CFTR ($CFTR^+$) were compared to those without ($CFTR^-$) to directly address the role of CFTR in inflammatory gene regulation.
Results: All lines maintained *CFTR* mRNA production and formation of tight junctions. $CFTR^+$ lines displayed short circuit currents in response to forskolin, while the $CFTR^-$ lines did not. Baseline expression of cytokines *IL6* and *CXCL8* (IL8) was not different between the lines regardless of CFTR genotype. All lines responded to TNFα and IL1β by increasing *IL6* and *CXCL8* mRNA levels, but the $CFTR^-$ lines produced more *CXCL8* mRNA than the $CFTR^+$ lines. Transcriptomes of 6 $CFTR^-$ and 6 $CFTR^+$ lines, before and after stimulation by TNFα, were compared for differential expression as a function of CFTR genotype. While some genes appeared to be differentially expressed simply because of CFTR's absence, others required stimulation for differences to be apparent.
Conclusion: Together, these data suggest cells respond to CFTR's absence by modulating transcriptional networks, some of which are only apparent when cells are exposed to different environmental contexts, such as inflammation. With regards to inflammation, these data suggest a model in which CFTR's absence leads to a poised, pro-inflammatory state of cells that is only revealed by stimulation.
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1. Introduction

Inflammation is a property of numerous tissues in CF, including the airways, the gut, pancreas, liver, and probably others. Elevated cytokines and other inflammatory molecules are characteristic of cystic fibrosis (CF) airways [21]. While altered airway surface environment and chronic pulmonary infections certainly contribute to the inflammatory response in the airway, there are studies that suggest this is not specific to airways, such that CF epithelial cells and cell models are inherently prone to hyper-inflammation [7]. Studies of the lungs of mice inoculated with bacteria showed that inflammatory markers rose higher in CF mice relative to wild type animals and failed to resolve inflammation as rapidly as non-CF animals [14], indicating inherent differences in inflammatory responses. Studies in transformed cell lines suggested this might reflect inherent properties of epithelial cells lacking CFTR [4]. We previously found reduced histone deacetylase 2 (HDAC2) in nasal epithelium freshly excised from CF patients [3] suggesting that epigenetic differences between CF and non-CF cells are involved. We also found that chromatin of the promoter *of CXCL8*, encoding IL8, was hyperacetylated in cell lines with reduced or absent CFTR expression [3,4]. These cell lines, however, were developed using heterologous CFTR expression [12] or other sequences to alter CFTR function [17] and thus may circumvent endogenous regulatory processes controlling CFTR gene expression.

Like the airway, the gastrointestinal tract of individuals with CF is also inflamed, coinciding with goblet cell hyperplasia and

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overproduction of mucus. The role of inflammation in CF gastrointestinal manifestations, such as malabsorption and poor nutritional status are being increasingly recognized [10,11,22]. In addition, individuals with CF are reported to be at much greater risk for inflammatory bowel disease [2,5] and subsequent gastrointestinal cancer (reviewed in [16] and [10]), both of which are associated with GI inflammation. We have shown previously in mouse models by conditional expression of CFTR that inflammation and goblet cell hyperplasia in the small intestines is due to CFTR's absence from epithelial cells [15]. However, we could not determine from these studies whether the inflammation is due to dehydrated mucosa, responses to obstruction, altered intracellular signaling caused by CFTR's absence, or some other mechanism.

Recently available genome editing technologies, particularly the CRISPR/Cas9 system, allow one to generate matched cell populations that differ at a single genomic locus. Unlike heterologous expression, cells engineered this way do not circumvent endogenous regulation of cellular processes. To study the effects of CFTR's absence on other genes and cellular processes such as inflammation, we used the CRISPR/Cas9 genome editing technology to generate subclonal cell lines from single cells of Caco-2 [13], a colonic adenocarcinoma-derived line that expresses high levels of CFTR [8]. Caco-2 cells have been reported to differ in cytokine induction upon siRNA-mediated knockdown of CFTR [9] and thus were used here to investigate cytokine regulation in models with the *CFTR* locus rendered inactive, akin to CF patients' cells.

The goals of the work presented here were to determine if cells with and without functional CFTR were inherently pro-inflammatory in a non-stimulated context and whether they would respond differently to inflammatory stimuli, either of which might explain CF-associated inflammation. Caco-2 cells have been used to study inflammatory responses in gastrointestinal barrier function and those studies showed Caco-2 cells respond to stimuli such as TNFα by increasing CXCL8 (IL8) production [1,23,26], a key chemokine contributing to neutrophilia in CF lungs. We tested Caco-2 lines for temporal responses of pro-inflammatory genes to varying doses of inflammation-stimulating molecules such as *E. coli* lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and interleukin-1β (IL1β). Clonal lines that retained functional, intact *CFTR* were compared to those in which all *CFTR* alleles carried inactivating mutations. We found no difference in *IL6* or *CXCL8* mRNA expression prior to stimulation, but after stimulation the cells lacking CFTR appeared more sensitive to stimuli and achieved higher levels of *CXCL8* mRNA, but not *IL6*, than cells with functional CFTR. Transcriptome profiling indicated the expression differences were not restricted to *CXCL8* but rather a number of other networks were also affected by CFTR's absence.

2. Methods

2.1. Cell culture

Caco-2 cells obtained from the American Type Culture Collection (ATCC) were maintained in Eagle's minimum essential medium with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin. Cells were cultured at 37 °C in an atmosphere of 95% O_2–5% CO_2.

2.2. Cell mutagenesis

Plasmids were obtained from Addgene (pCas9 GFP, #44719; https://www.addgene.org/) or constructed using Invitrogen's GeneArt® Gene synthesis system.

pCas9 GFP nuclease was used with a guide RNA (gRNA) targeting exon 11 of *CFTR* and a gRNA targeting the G551 codon in exon 12.

A template plasmid carrying the G551D mutation was constructed and contained the G551D mutation along with 5 silent substitutions to prevent gRNA cleavage. The sequence for the template plasmid and gRNAs can be found in Table S1.

Mutagenesis was carried out using the CRISPR/Cas9 system [19]. To create loss of function mutations, 6 µg of exon 11 gRNA plasmid, 6 µg of exon 12 gRNA plasmid, and 12 µg of Cas9 GFP plasmid were added to the cells with 40 µl of Lipofectamine 2000. To create the G551D mutation, 6 µg of exon 12 gRNA plasmid, 6 µg of Cas9 GFP plasmid, and 12 µg of template plasmid were added with 40 µl of Lipofectamine.

2.3. Clonal isolation and sequencing

Forty-eight hours after transfection, transfected cells were isolated by florescence-activated cell sorting of GFP-expressing cells. The sorted cells were cultured using limiting dilution techniques to create subclonal populations. Each clone was grown and expanded. Once expanded, genomic DNA from each clone was isolated and amplified by PCR with primers recognizing sequences in exon 11 and/or exon 12 designed using Integrated DNA Technologies software. The sequences for the PCR primers can be found in Table S2. The most likely potential off-target cleavage sites for each gRNA, predicted by in silico analyses, were also sequenced in the lines and no sequence alterations were found. The use of two independent gRNAs and no detectable off-target changes strongly support the conclusion that cellular changes are the result of CFTR's loss of function.

PCR products were sequenced and if Sanger sequencing identified mutations of interest, next-generation sequencing was carried out to define alleles. Sequences for next-generation PCR primers can be found in Table S2. Next-Generation sequencing was performed using paired end, 125 base pair sequencing via Illumina MiSeq. >50,000 reads were obtained from each clone and clones were sequenced multiple times after passaging to determine stability. RNA sequencing data are available under GEO accession GSE130226 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130226

2.4. Functional characterization

Short circuit currents (I_{sc}) were measured with a modified Ussing chamber. Once confluent on transwell filters, cells were placed in asymmetric chloride solutions and 100 µM amiloride was added to block sodium absorption. Changes in transepithelial I_{sc} and conductance in response to 10 µM forskolin were measured. Clones were further characterized by examining the effects of CFTR inhibition with 10 µM Inh_{172} on I_{sc} induced by forskolin.

Cells were assessed for CFTR protein by immunoblot. Cells were lysed with RIPA buffer containing protease inhibitors and incubated on ice for 30 min. Protein was quantified by BCA assay (BioRad). Samples were prepared in 2× Leammli sample buffer (BioRad) and incubated 30 min at room temperature. Ten micrograms of protein were loaded in a 7.5% precast polyacrylamide gel (BioRad). Protein was transferred to polyvinylidene fluoride microporous membranes (Millipore) using Trans-Blot Turbo Blotting System (BioRad). Membranes were blocked in 5% nonfat milk prepared in PBS with 0.01% Tween-20 for 2 h at room temperature. Membranes were incubated overnight with anti-CFTR (Antibody 596, 1:2000, CFTR Antibody Distribution Program). Goat anti-mouse IgG antibody, HRP conjugate (Millipore, AP181P) was used as the secondary antibody. Immunoblot signal was detected using a ChemDoc imaging system (BioRad).

2.5. Cytokine stimulation

Cells were exposed to either *E. coli* LPS, IL1β, or TNFα. Two sub-maximal stimulation doses were chosen to stimulate the cells

based on dose response curves. The cells did not increase *CXCL8* expression in response to LPS. The sub-maximal stimulating doses for IL1β were 0.1 ng/ml and 10 ng/ml and the sub-maximal stimulating doses for TNFα were 10 ng/ml and 100 ng/ml. Time courses were performed to determine time to peak response. Cells were stimulated for 2 h prior to harvesting RNA for RT PCR and RNAseq.

2.6. RT qPCR and RNAseq

CFTR, *CXCL8* and *IL6* mRNA were quantified by Real-Time Polymerase Chain Reaction (RT-PCR). RNA was harvested using RNAeasy kit per manufacturer instructions. One microgram of total RNA was reverse-transcribed into cDNA using qScript cDNA Synthesis Kit per manufacturer instructions. Two microliters of synthesized cDNA was amplified by quantitative PCR. Quantitative PCR was carried out using Taqman gene expression assays (Applied Biosystems). Relative expression was analyzed with the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). For *CFTR* expression, values were expressed as fold change compared to the average of $CFTR^{+}$ group. For *IL6* and *CXCL8* expression, ΔCT was normalized to β-actin for each individual sample and then compared to the individual sample's untreated control.

2.7. RNA expression analyses

RNA seq data were analyzed for individual genes (*CXCL8, IL6, ACTB*) to compare with RT-PCR values and gene set enrichment was assessed to evaluate responses to TNFα, as well as to compare CF and non-CF cells under basal and stimulated conditions. The Molecular Signatures Database Hallmark Gene Set [18] was analyzed for enrichment of coordinately regulated genes. Those gene sets with nominal *p*-values below 0.05 and a conservative false discovery rate q-value of <0.1 were considered significant.

3. Results

This study was designed to examine the effects of CFTR's absence on inflammatory processes in epithelial cell models. Caco-2 cells have been used extensively for studies of CFTR mRNA expression and protein function and we found these cells to be particularly amenable to subcloning. Using gRNAs targeting exon 11 and/or exon 12, we generated a panel of clonal lines with all, some, or no *CFTR* alleles mutated. Caco-2 cells, derived from a colonic carcinoma, are aneuploid as are many such cancer-derived lines [20]. *CFTR* genotyping by next generation sequencing showed that *CFTR* copy number ranged from 2 to 4, indicative of the genomic heterogeneity of this line. Next-generation sequencing was performed over multiple passages to evaluate stability and consistency of the genotype and allele ratios. The genotypes of these clonal lines are given in Table S3 and S4, supplementary data.

The clonal lines were assessed for several features, including CFTR mRNA, CFTR protein, transepithelial resistance and forskolin-induced short-circuit currents. As Fig. 1 shows, all lines maintained CFTR mRNA expression (Fig. 1A), measured after multiple passages and found to be reproducible. Lines without wild type *CFTR* alleles ($CFTR^{-}$) had no detectable CFTR protein (Fig. 1B) or forskolin-stimulated function (Fig. 1C), while both were readily detected in lines with wild type CFTR alleles ($CFTR^{+}$).

To assess if two cytokines commonly elevated in lungs of individuals with CF were inherently elevated as a consequence of CFTR's absence, *IL6* and *CXCL8* mRNA, encoding IL6 and IL8, respectively, were measured in the clonal lines. *CFTR* genotype had no detectable effect on baseline expression of these cytokine genes (Fig. 2). It has also been proposed that CF cells are more sensitive to pro-inflammatory stimuli than non-CF cells [24]. To explore this concept, the clonal lines were exposed to either *E. coli* lipopolysaccharide (LPS), IL1β, or TNFα. LPS had no detectable effect on *IL6* or *CXCL8* mRNA expression and was not pursued further. Both IL1β and TNFα induced expression markedly. When exposed to TNFα the $CFTR^{-}$ lines appeared more sensitive than $CFTR^{+}$ lines,

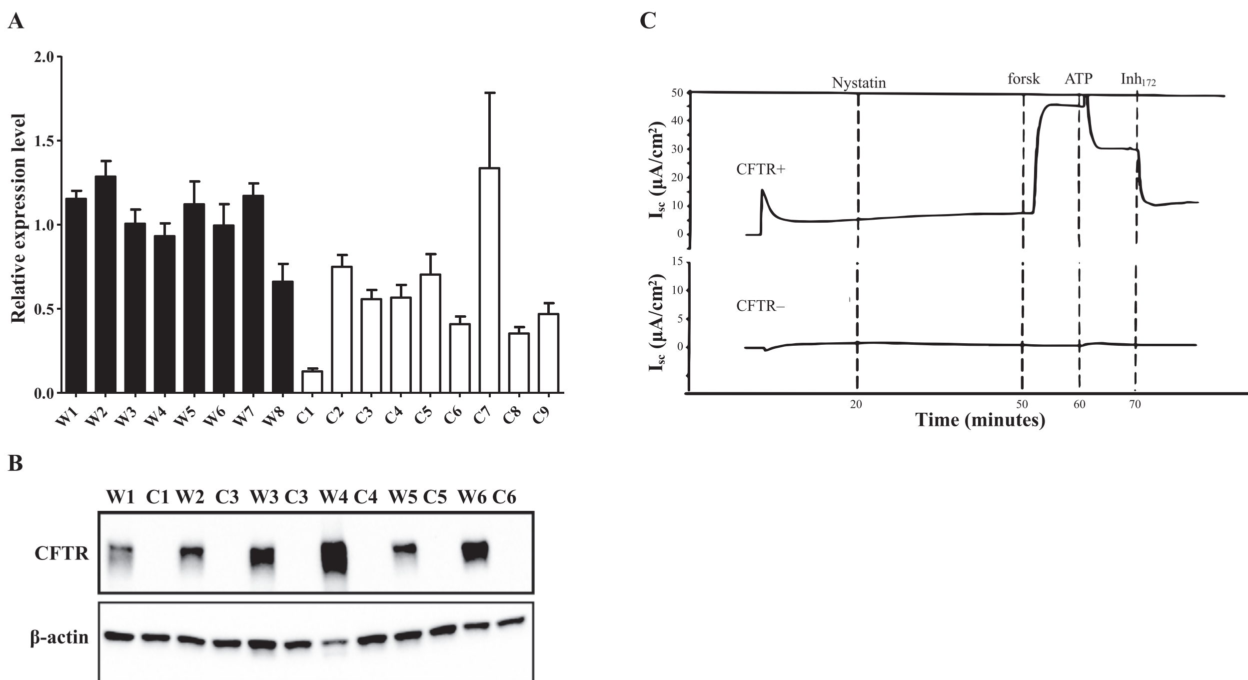

Fig. 1. Characterization of clonal lines. Clonal lines were tested after multiple passages, to evaluate stability and consistency of phenotypes. "W" denotes $CFTR^{+}$ clonal lines, "C" denotes $CFTR^{-}$ clonal lines. (A) CFTR mRNA expression detected by RT-PCR was present and stable over multiple passages in both $CFTR^{+}$ and $CFTR^{-}$ lines. (B) CFTR protein was detected by Western Blot analysis in all $CFTR^{+}$ lines and was not detected in any of the $CFTR^{-}$ lines. Only clones that were used for RNA profiling are shown here. (C) $CFTR^{+}$ lines showed an increase in the transepithelial short circuit current after stimulation with forskolin (forsk). The current was due to CFTR, as it was inhibited by Inh_{172}, a selective CFTR inhibitor. $CFTR^{-}$ lines showed no response to forskolin.

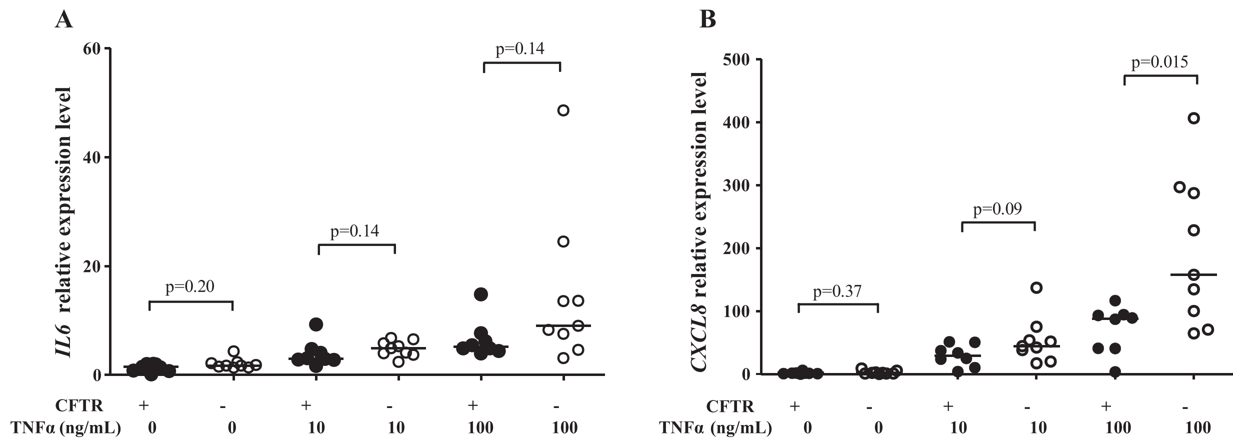

Fig. 2. Cytokine gene expression. Expression of *IL6* and *CXCL8* mRNA levels were assessed by RT-PCR at baseline and after stimulation with TNFα. Solid circles represent individual $CFTR^+$ clonal lines while open circles represent individual $CFTR^-$ clonal lines. Each circle represents the average of 3 stimulation assays performed on each clonal line over multiple passages. The median is denoted by the horizontal bar. (A) No statistically significant difference was seen in *IL6* mRNA expression between $CFTR^+$ and $CFTR^-$ lines at baseline or after stimulation with TNFα. (B) No statistically significant difference was seen in *CXCL8* mRNA expression between $CFTR^+$ and $CFTR^-$ clonal lines at baseline; however, $CFTR^-$ lines appeared more sensitive than $CFTR^+$ lines, producing statistically greater *CXCL8* mRNA amounts after stimulation with 100 ng/ml TNFα. Statistical analysis was performed by Mann-Whitney *U* test.

producing greater *CXCL8* mRNA amounts at each dose, reaching statistical significance at a dose of 100 ng/ml (Fig. 2). *IL6* mRNA expression between the $CFTR^+$ and $CFTR^-$ clonal lines was not different ($p > .05$).

The stimulation-dependent, differential expression of *CXCL8,* but not *IL6,* suggested epigenetic remodeling as a consequence of CFTR's absence. We previously reported differential acetylation of the *CXCL8* promoter in CF model cell lines [4] and reasoned that such a phenomenon is not likely restricted to a single gene. Transcriptome profiling (RNA-seq) was carried out to compare mRNA from a panel of clonal lines, 6 $CFTR^+$ and 6 $CFTR^-$, to address inter-line variation that might exist. RNA profiles pre- and post-TNFα stimulation were compared and relatedness of lines and genotypes were assessed by hierarchical clustering (Fig. 3). Each line was more closely related to itself pre- and post-stimulation than to any other line under the same conditions (Fig. 3A). This likely reflects the heterogeneity of cells within the parent cell line and each subclone's unique genomic complement, underscoring the importance of comparing multiple, independent lines. When grouped by *CFTR* genotype (Fig. 3B), the $CFTR^+$ lines pre- and post-stimulation were more similar to each other than the $CFTR^-$ lines, indicating CFTR's absence does influence gene expression of multiple genes.

To better understand the expression patterns contributing to the clustering, expression levels of individual genes showing significantly different expression between any two groups were plotted as a heat map. As (Fig. 3C) shows, there are groups of genes that differentiate $CFTR^+$ from $CFTR^-$, groups of genes that discriminate

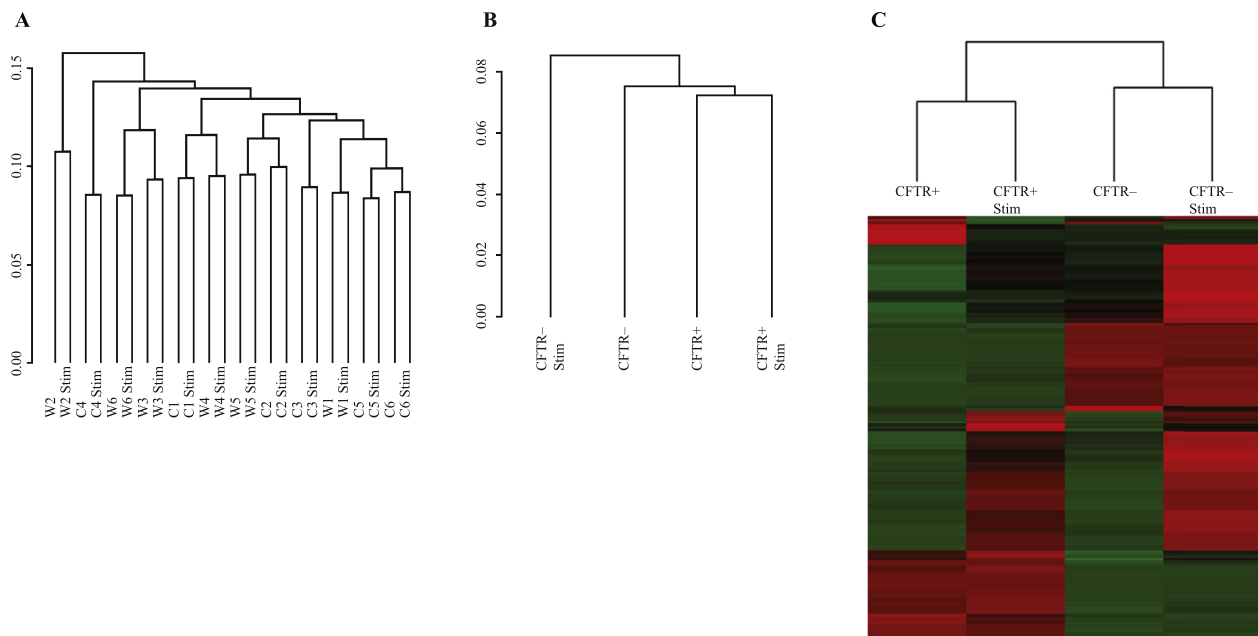

Fig. 3. Gene expression comparisons. RNA-seq was performed on 6 $CFTR^+$ and 6 $CFTR^-$ lines before and after stimulation with 100 ng/ml TNFα. (A) Hierarchical clustering showed each line was more closely related to itself before and after stimulation than to any other line under the same conditions. Samples did not segregate based on genotype or stimulation status. (B) Hierarchial clustering based on genotype and stimulation status showed $CFTR^+$ lines before and after stimulation were more similar to each other than $CFTR^-$ lines. $CFTR^-$ lines before stimulation were more similar to both the unstimulated and stimulated $CFTR^+$ lines than they were to the $CFTR^-$ stimulated lines. (C) Heat map displaying those genes showing significant, differential expression by genotype or by stimulation. Those in red indicate increased expression and those in green indicate decreased expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Gene sets responding to TNFα stimulation.

Enriched by TNFα in CFTR+ or CFTR−	Gene set	# genes in set	CF p-val	WT p-val	CF FDR q-val	WT FDR q-val
Both	TNFα SIGNALING VIA NFκB	195	<0.001	<0.001	<0.001	<0.001
Both	APOPTOSIS	155	<0.001	<0.001	<0.001	<0.001
Both	INFLAMMATORY RESPONSE	195	<0.001	<0.001	<0.001	<0.001
Both	UV RESPONSE UP	153	<0.001	<0.001	<0.001	<0.001
Both	INTERFERON GAMMA RESPONSE	195	<0.001	<0.001	<0.001	0.004
Both	IL2 STAT5 SIGNALING	194	<0.001	<0.001	<0.001	0.002
Both	KRAS SIGNALING UP	191	<0.001	<0.001	<0.001	<0.001
Both	EPITHELIAL MESENCHYMAL TRANSITION	195	<0.001	<0.001	<0.001	0.008
Both	P53 PATHWAY	195	<0.001	<0.001	<0.001	0.004
Both	HYPOXIA	193	<0.001	<0.001	<0.001	0.004
Both	ANDROGEN RESPONSE	99	<0.001	<0.001	<0.001	0.005
Both	INTERFERON ALPHA RESPONSE	93	<0.001	<0.001	<0.001	0.001
Both	IL6 JAK STAT3 SIGNALING	87	<0.001	<0.001	<0.001	<0.001
Both	MYC TARGETS V1	190	<0.001	<0.001	<0.001	<0.001
Both	CHOLESTEROL HOMEOSTASIS	71	<0.001	<0.001	<0.001	0.002
Both	MTORC1 SIGNALING	195	<0.001	<0.001	<0.001	0.007
Both	COMPLEMENT	190	<0.001	<0.001	0.001	0.008
Both	COAGULATION	134	<0.001	<0.001	0.007	0.012
Both	UNFOLDED PROTEIN RESPONSE	110	0.002	<0.001	0.013	0.008
Both	OXIDATIVE PHOSPHORYLATION	193	0.007	<0.001	0.044	0.008
CFTR−	ALLOGRAFT REJECTION	197	<0.001	NS	<0.002	NS
CFTR−	TGF BETA SIGNALING	52	<0.001	NS	<0.002	NS
CFTR−	MITOTIC SPINDLE	194	<0.001	NS	0.002	NS
CFTR−	APICAL JUNCTION	195	<0.001	NS	0.004	NS
CFTR−	PROTEIN SECRETION	93	0.002	NS	0.004	NS
CFTR−	ESTROGEN RESPONSE EARLY	197	<0.001	NS	0.01	NS
CFTR−	UV RESPONSE DN	140	0.002	NS	0.012	NS
CFTR−	G2M CHECKPOINT	194	0.003	NS	0.018	NS
CFTR−	ADIPOGENESIS	193	0.007	NS	0.018	NS
CFTR−	ESTROGEN RESPONSE LATE	197	0.007	NS	0.029	NS
CFTR+	MYC TARGETS V2	57	NS	0.009	NS	0.01
CFTR+	E2F TARGETS	190	NS	<0.001	NS	0.01

NS = not significant.
Cells with functional CFTR (CFTR+) and with CFTR inactivated (CFTR−) were examined for gene expression responses to TNFα exposure, utilizing gene sets as indicators of transcriptional coordination. The number of genes in each set are listed as well as the nominal p values and false discovery rate (FDR) adjusted for the number of genes examined and number of comparisons made.

between stimulated and non-stimulated and groups of genes that separate both genotype and stimulation status.

To better understand the mRNA expression patterns and develop hypotheses regarding transcriptional regulatory pathways that might explain the gene expression clustering, we carried out gene set enrichment analyses. These types of analyses provide clues regarding co-regulation and counter-regulation of genes by comparing with expression patterns from other contexts or scenarios [18,25].

We first compared each clonal line pre- and post-stimulation by TNFα. As Table 1 shows, *TNFα SIGNALING* VIA *NFκB* was the most significantly enriched gene set regardless of *CFTR* genotype, and serves as a control for the process. Overall, 20 gene sets were significantly enriched from stimulation in both CFTR+ and CFTR− cells, 6 of which are clearly involved in inflammation and therefore could be expected. Ten other sets were enriched in CFTR− cells, but not CFTR+, and two sets enriched in CFTR+ but not CFTR−.

To investigate effects of CFTR function, CFTR+ lines were compared to CFTR− lines pre- and post-stimulation (Table 2). Of the 6 gene sets enriched in CFTR− cells pre-stimulation, 5 were still enriched after stimulation, indicating that the genes involved are sensitive to CFTR genotype. Interestingly, *TNFα SIGNALING* VIA *NFκB* was enriched in stimulated CFTR− lines relative to stimulated CFTR+, even though this gene set was enriched in all cells and suggesting that it is enhanced by absence of CFTR. Three gene sets were enriched in unstimulated CFTR+ cells relative to CFTR− cells, but no sets were significantly enriched in stimulated CFTR+ cells relative to stimulated CFTR− cells.

4. Discussion

Cystic fibrosis is a multisystem disease caused by absence of CFTR function in many different cell types and organs. Recognizing that no single model is likely to be representative of all cell types, we compared the effects of removing CFTR function from an epithelioid cell line on inflammatory gene regulation and the broader cellular transcriptome. Multiple clonal lines were examined to evaluate inter-sample variation and establish whether or not CFTR function influences cytokine mRNA regulation.

Cell attributes were not detectably affected by the mutagenesis and cloning processes, as all subclonal lines maintained CFTR mRNA expression, electrophysiologic properties and response to inflammatory stimuli. Forskolin-stimulated, short-circuit current was the only measured phenotype that changed, and absent only from those lines lacking CFTR. No detectable difference was found between cells with and without CFTR in baseline expression of the cytokine genes tested, while stimulation by TNFα, elicited differential expression. CFTR− cells had, on average, higher *CXCL8* mRNA and much greater variation in response to TNFα stimulation than CFTR+ cells. These result bear similarity to a report showing in-

Table 2
Gene sets differentially enriched when stratified by *CFTR* genotype.

Genotype	Gene set	# genes in set	Unstim p-val	Unstim FDR q-val	Stim p-val	Stim FDR q-val
CFTR−	TNFα SIGNALING VIA NFκB	195	NS	NS	<0.001	<0.001
CFTR−	E2F TARGETS	190	<0.001	<0.001	<0.001	<0.001
CFTR−	MYC TARGETS V1	190	<0.001	<0.001	<0.001	<0.001
CFTR−	G2M CHECKPOINT	194	<0.001	<0.001	<0.001	<0.001
CFTR−	MYC TARGETS V2	57	<0.001	0.002	NS	NS
CFTR−	MTORC1 SIGNALING	195	0.005	0.03	<0.001	0.004
CFTR−	ESTROGEN RESPONSE LATE	197	0.002	0.064	0.005	0.056
CFTR+	IL6 JAK STAT3 SIGNALING	87	0.004	0.118	NS	NS
CFTR+	INTERFERON GAMMA RESPONSE	195	0.002	0.076	NS	NS
CFTR+	EPITHELIAL MESENCHYMAL TRANSITION	195	0.004	0.1	NS	NS

NS = not significant.
Gene set comparisons were carried out to assess the effect of CFTR's presence on baseline (unstimulated) mRNA expression of genes in a set and expression in response to TNFα (stimulated). Gene sets significantly different between CFTR+ and CFTR− cells are listed here. The number of genes in each set is listed as well as the nominal p values and false discovery rate (FDR) adjusted for the number of genes examined and number of comparisons made.

creased *CXCL8* (IL8) expression in response to siRNA interference of CFTR in Caco-2 cells. However, that report found differential expression even without exogenous stimulation [9], a significant difference from the work shown here. Whether the siRNA treatment itself acted as an inflammatory stimulus is unclear, as those were acute experiments, while our cultures were carried without exposure to any exogenous stimuli for baseline expression measurements.

The differential expression of *CXCL8* led us to examine the broader transcriptome of these cells. This analysis identified genes that appear to be differentially expressed simply from CFTR's absence and another set of genes that, like *CXCL8*, are only apparent as differentially expressed when stimulated. Heatmaps derived from the RNA-seq data show that the patterns of expression are quite different between groups when all expressed genes are examined (data not shown). Restricting the analysis to genes showing significant, differential expression in any of the comparisons, reveals more apparent patterns of genes that may be inherently different and those that that reveal themselves only after stimulation (Fig. 3C). We propose this differential expression may represent a mechanism in which the genome is remodeled in response to CFTR's absence, altering chromatin in a way that alters active expression or puts genes in a poised position to undergo transcription.

The data presented here provide a model to help explain CF-associated inflammation and show that the presence or loss of CFTR impacts cellular gene expression much more broadly than just inflammation. The data suggest that CF cells are not overtly different in cytokine expression until provoked, an example of a "gene by environment" interaction. This model also implies that there are epigenetic or other transcriptional regulatory processes affected by CFTR's absence. Caradonna et al. [6] showed that DNA methylation of *CXCL8* and *IL6* promoters in Caco-2 cells decreases and gene expression increases upon stimulation by IL1β, but *IL10* is unaffected. We reported previously that CF primary nasal epithelial cells and several CF cell models exhibit reduced promoter acetylation of the *CXCL8* gene. Clearly additional studies will be needed to more completely address epigenetic effects.

Based on data presented here, we propose a threshold model to help explain the chronic inflammation in CF in which absence of CFTR confers changes on the epigenome that influence transcriptional regulation of genes such as *CXCL8*. For these genes, such chromatin modifications make these genes more sensitive to stimulation. Ex vivo and without stimulation, CF cells do not behave very differently from non-CF cells. Once stimulation is initiated in vivo, however, pro-inflammatory immune cells would be recruited and contribute to the cytokine/inflammatory mediator environment. In a non-CF context, levels of these mediators would diminish as inflammation is resolved, along with neutralization of the inciting stimulus. However, because of the increased sensitivity of the CF cells, amounts of cytokines that would cease to stimulate WT cells and allow resolution of inflammation would still be able to stimulate CF cells and chronic inflammation would result. Future studies, including co-culture and/or in vivo experiments are needed to test this hypothesis.

Conflict of interest statement

MD is a consultant for the Cystic Fibrosis Foundation. There are no other potential conflicts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2019.05.003.

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