Atypical Activation of the Unfolded Protein Response in Cystic Fibrosis Airway Cells Contributes to p38 MAPK-Mediated Innate Immune Responses

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Inflammatory lung disease is the major cause of morbidity and mortality in cystic fibrosis (CF); understanding what produces dysregulated innate immune responses in CF cells will be pivotal in guiding the development of novel anti-inflammatory therapies. To elucidate the molecular mechanisms that mediate exaggerated inflammation in CF following TLR signaling, we profiled global gene expression in immortalized human CF and non-CF airway cells at baseline and after microbial stimulation. Using complementary analysis methods, we observed a signature of increased stress levels in CF cells, specifically characterized by endoplasmic reticulum (ER) stress, the unfolded protein response (UPR), and MAPK signaling. Analysis of ER stress responses revealed an atypical induction of the UPR, characterized by the lack of induction of the PERK–eIF2α pathway in three complementary model systems: immortalized CF airway cells, fresh CF blood cells, and CF lung tissue. This atypical pattern of UPR activation was associated with the hyperinflammatory phenotype in CF cells, as deliberate induction of the PERK–eIF2α pathway with salubrinal attenuated the inflammatory response to both flagellin and *Pseudomonas aeruginosa*. IL-6 production triggered by ER stress and microbial stimulation were both dependent on p38 MAPK activity, suggesting a molecular link between both signaling events. These data indicate that atypical UPR activation fails to resolve the ER stress in CF and sensitizes the innate immune system to respond more vigorously to microbial challenge. Strategies to restore ER homeostasis and normalize the UPR activation profile may represent a novel therapeutic approach to minimize lung-damaging inflammation in CF.   *The Journal of Immunology*, 2012, 189: 000–000.

I n cystic fibrosis (CF) lung disease, chronic airway infection and inflammation are the primary cause of morbidity and mortality (1). Clinical interventions targeting inflammation have proved to be beneficial, supporting the concept that lung inflammation is an attractive therapeutic target for improving CF outcomes (2–4). The only currently available approaches to reducing inflammation are global anti-inflammatories such as corticosteroids or ibuprofen, but the use of these medications is limited by unwanted side effects (5). To develop effective anti-inflammatory therapies, it is crucial to understand the specific biochemical mechanisms underlying the propagation of the inflammatory response in CF triggered by clinically relevant pathogens, such as *Pseudomonas aeruginosa.*

The lung epithelium relies on innate immune receptors such as TLRs to recognize pathogens. We and others have shown that TLR5 (ligand: flagellin) plays a critical role in mediating much of the immune response to *P. aeruginosa* in vitro (6–8) and that a single nucleotide polymorphism rendering TLR5 less responsive is associated with improved clinical status in adult CF patients (7). TLR5 activation triggers signaling cascades such as the ERK (9) and p38 MAPK pathways (10, 11), which are potentially involved in mediating inflammatory responses to CF pathogens, yet fundamental questions remain concerning the links between *CFTR* mutations and dysregulated inflammatory signaling.

The endoplasmic reticulum (ER) is a sensitive organelle, and perturbation of protein homeostasis (proteostasis) due to deficiencies in folding, trafficking, and degradation potentially contributes to the severity of a broad range of inflammatory conditions, such as chronic obstructive pulmonary disease (12) and autoimmune and neurodegenerative diseases (13, 14). In CF cells, many factors are likely to have an impact on proteostasis to trigger ER stress, including misfolded CFTR protein (15), chronic infection, and oxidant–antioxidant imbalance (16, 17). Nevertheless, the literature is not unanimous and data are conflicting on whether ER stress is induced in CF (18, 19).

In the case of ER stress, the unfolded protein response (UPR) is activated to prevent overloading of the compartment. The classical UPR consists of three pathways initiated by the sensor proteins

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Abbreviations used in this article: ATF6, activating transcription factor 6; CF, cystic fibrosis; DE, differentially expressed; ER, endoplasmic reticulum; GO, gene ontology; IRE-1, inositol-requiring enzyme 1; ORA, overrepresentation analysis; PERK, eukaryotic translation initiation factor 2-α kinase 3; TF, transcription factor; UPR, unfolded protein response.

IRE-1α (inositol-requiring enzyme 1α), PERK (eukaryotic translation initiation factor 2-α kinase 3), and the transcription factor (TF) ATF6 (activating TF 6) (20). Together these three arms of the UPR mitigate ER stress by attenuating protein synthesis, facilitating protein folding, and increasing protein degradation.

A number of studies have identified cross-talk mechanisms between the UPR and proinflammatory pathways, including NF-κB signaling (20–22). Consequently, the goal of this study was to investigate how the UPR and ER stress might contribute to the damaging hyper-inflammatory phenotype in CF. By combining computational with biochemical approaches, we identify dysregulation of the homeostatic UPR system in CF, which appears to potentiate TLR5 signaling. Thus strategies to correct dysregulated stress response pathways may represent a novel therapeutic approach to alleviate lung-damaging inflammation in CF.

Materials and Methods
Human biological samples

The well-characterized, immortalized airway epithelial cell lines IB3-1 (compound heterozygote for CF mutations ΔF508 and W1282X) and C38 (IB3-1 transfected with CFTR by adeno-associated viral vector) were cultured as described (23). PBMCs were isolated from whole blood obtained from healthy control and CF donors (sweat test and CF mutation positive) using published techniques (24). All CF patients were clinically stable at the time of blood donation, and subjects receiving systemic corticosteroid medications were excluded.

Lung tissue from end-stage CF patients was obtained following transplantation. Control lung tissue was obtained from normal portions of the lung from patients undergoing surgery for lung cancer. All tissues were cryopreserved in tissue blocks. Studies were approved by the University of British Columbia Clinical Research Ethics Board (H09-01192), and informed consent was obtained from participants.

Cell stimulation assays

Cells were seeded into 96-well plates 16 h prior to simulation and stimulated with optimized concentrations of flagellin (100 ng/ml; Invivogen), tunicamycin (20 μg/ml; Calbiochem), and/or P. aeruginosa PAO1 [multiplicity of infection (MOI) = 50:1]. For inhibitor studies, cells were incubated with a p38 MAPK inhibitor (SB203580, 1 μM; Cell Signaling) 1 h prior to exposure to tunicamycin (20 μg/ml) or flagellin (100 ng/ml). A specific inhibitor of the eIF2α phosphatase GADD34, salubrinal (100 μM; EMD), was applied 48 h prior to stimulation with flagellin or P. aeruginosa PAO1. IL-6 concentration was determined by ELISA (eBioscience). Cells were challenged with tunicamycin (20 μg/ml) for 2, 4, 6, 8, and 24 h or with salubrinal (100 μM) for 18, 24, and 30 h before mRNA was extracted for gene expression studies. Cell viability based on mitochondrial metabolic rate was measured by MTS assay (Promega).

Gene and protein expression quantification

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) from IB3-1, C38, fresh PBMCs, or lung tissue cores and transcribed to cDNA with oligo-dT primers (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Quantitative PCR primers were designed for SYBR Green chemistry to amplify cyclophilin A (PPIA), IL-6, XBP-1u, XBP-1s, GRP78, DDIT3, ATF4, NRF2, and GADD34 (Table I) by standard cycling protocols, using a 7300 Real-Time PCR System (Applied Biosystems). Relative

gene expression in the CF compared with non-CF cells was analyzed by the $2^{-\Delta\Delta Ct}$ Livak method (25), using PPIA as a housekeeping gene. To accurately depict up- and downregulated gene expression patterns, the expression ratios were displayed as $\log_2$ transformed values. Cell lysates were analyzed by standard Western blotting protocols and were probed for p38, phospho-p38, phospho-eIF2α, and β-actin (#9212, 9211, 3597, 4967; Cell Signaling). Band densitometry was calculated with an Odyssey Infrared Imaging System (LI-COR).

Global gene expression profiling

Cells were stimulated in six-well plates (BD Biosciences) with 1 μg/ml flagellin for 2 h. Cells were lysed, and total RNA was harvested. RNA quality was determined using Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized using an RNA ampULSe kit (Kreatech), hybridized to human HT-12 expression bead chips and raw data processed with BeadStudio v3.4.0 (Illumina). Microarray data were deposited to the publically viewable National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE35391, www.ncbi.nlm.nih.gov/geo/).

Differential gene expression and network analysis

Further processing was performed using Bioconductor (www.bioconductor.org/) for quantile normalization. Differential expression of gene probes was calculated using the limma package with empirical Bayes (26). Adjusted p values were calculated using the Benjamini-Hochberg method (27). Gene ontology (GO; http://geneontology.org) overrepresentation analysis (ORA) was calculated using GOstats (28), whereas ORA of TFs based on differentially expressed (DE) targets was calculated using CORNA (29), with data from TRANSFAC PRO (29), processed using the InnateDB Project (30). Network analysis was carried out as previously described (30, 31). Briefly, a list of protein-level interactions between DE genes (FC > ±1.4; p < 0.05) was obtained using InnateDB (32). Network analysis was carried out by visualizing these interactions into a single integrated network using Cytoscape (2.8.0 for Windows) and the software plugin Cerebral to appreciate cellular directionality, as previously described (33). Hub degree (or the number of protein-level interactions with other members of the network) was determined for each gene "node" in the network using cytoHubba (34). Further enrichment of the network was performed through the manual addition of interactions based on primary literature (35), focusing on genes curated in the KEGG pathway Protein processing in endoplasmic reticulum [KEGG:HSA04141] (36).

Results
Immortalized CF airway cells display evidence of ER stress, UPR activation, and enhanced inflammatory responsiveness

It remains unclear which molecular events in CF lead to excessive immune responses to flagellated pathogens such as P. aeruginosa. To generate testable hypotheses concerning the mechanisms underlying this increased immune response, gene expression profiling was performed on IB3-1 and C38 cells at baseline and 2 h after flagellin stimulation. The data were subjected to computational analysis using complementary approaches of GO and TF ORA methods (Tables II–IV). In addition, a comprehensive network of protein-level interactions between DE genes in IB3-1 compared to C38 cells was constructed (Fig. 1). The goal of this analysis was to facilitate unbiased identification of the transcriptional processes contributing to the increased responsiveness of immortalized CF airway cells to flagellin.

Table I. Quantitative PCR primer sequences

Gene	NCBI Accession	Forward (5′ → 3′)	Reverse (5′ → 3′)	CDS Region	Size (bp)	Tm (°C)
ATF4	NM_001675.2	AACAACAGCAAGGAGGATGCC	TTGGGTTCACCGTCTGGGGG	c. 214–421	208	55, 58
DDIT3	NM_001195053.1	GAAATGAAGAGGAAGAATCA	TTCTCCTTCATGCGCT	c. 197–437	241	46, 47
GADD34	NM_014330.3	CCTCCTAGGCTGCCCCTCCG	TCCCGAGCAAGCTGCTCCCA	c. 1570–1763	194	60, 60
GRP78	NM_005347.3	TGCCGTTCAAGGTGGTTGAAA	ACTGCATGGGTAACCTTCTTTCCC	c. 344–506	163	55, 57
IL-6	NM_000600	TGTGAAAGCAGCAAAGAGGCACTG	ACCAGGCAAGTCTCCTCATTGAAT	c. 232–338	107	58, 56
NRF2	NM_006164.3	GGACATTGAGCAAGTTTGGGAGGAGC	TGTGGAGAGGATGCTGCTGAAGGA	c. 477–711	235	60. 59
PPIA	NM_021130	TAAAGCATACGGGTCCTGGCATCT	ATCCAACCACTCAGTCTTGGCAGT	c. 269–369	101	58, 58
XBP-1s	NM_001079539.1	ATGGATGCCCTGGTTGCTGAAGA	TGCACCTGCTGCGGACTCA	c. 415–504	90	58, 59
XBP-1u	NM_005080.3	AGCACTCAGACTACGTGCACCTCT	CCAGAATGCCCAACAGGATATCAG	c. 495–624	130	59, 55

CDS Region, Coding sequence region; NCBI, National Center for Biotechnology Information; Tm, temperature.
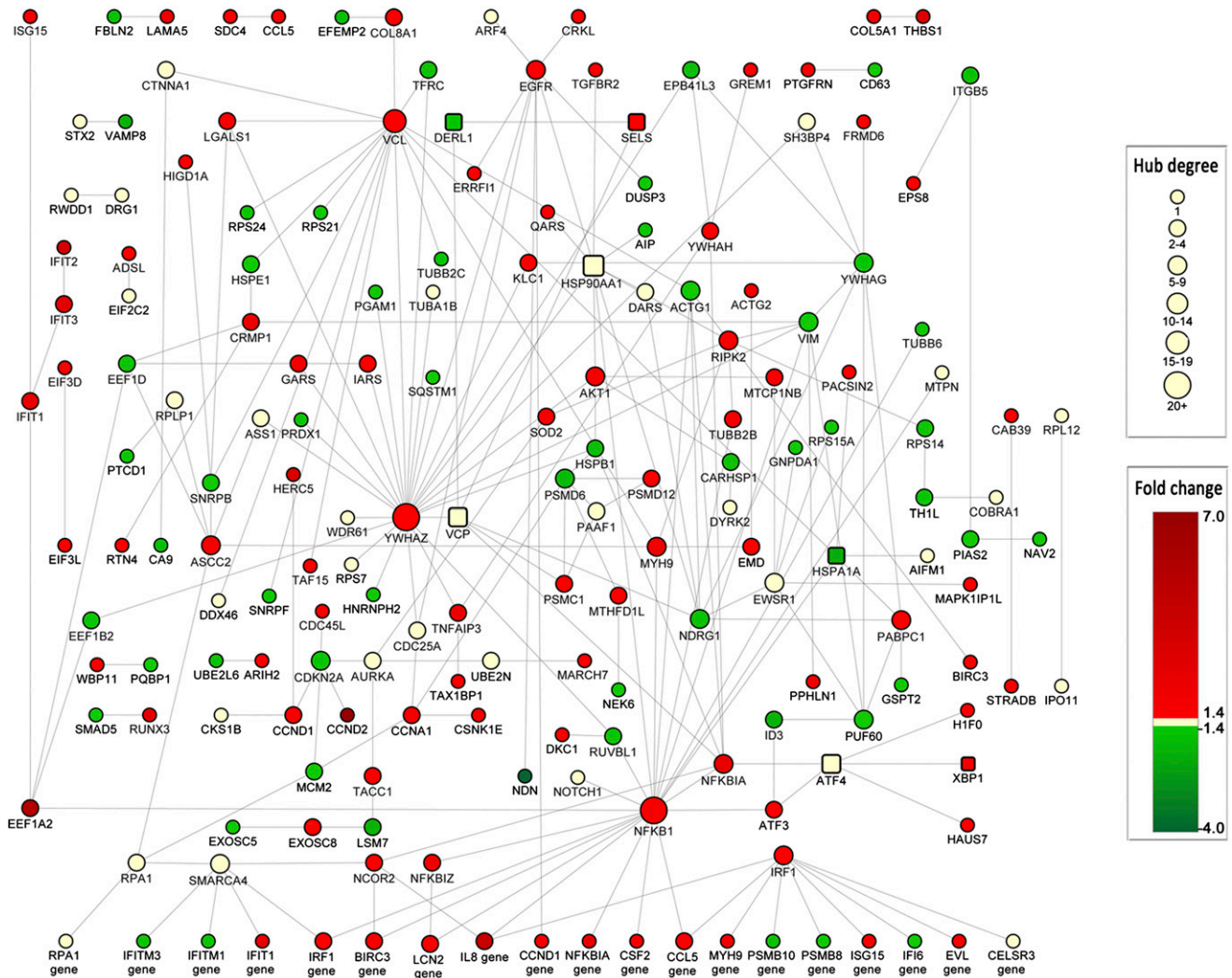
FIGURE 1. Protein-interaction network after flagellin stimulation. Nodes curated as components of ER stress responses are shown as squares. Node size is proportional to its hub degree (interconnectivity within the network), whereas node color is proportional to the fold change of expression in IB3-1 cells compared with C38 cells (red, upregulated; green, downregulated).

We investigated GO terms to identify global molecular themes dysregulated in IB3-1 compared with C38 cells. Multiple GO terms (categorized as *Biological Process*) related to both ER stress and the innate immune response were significantly different between the cell lines (Tables II, III). These findings were further reinforced by the TF ORA, which returned a signature of TFs associated with the UPR (XBP-1, ATF6, DDIT3) as well as TFs activated downstream of the p38 MAPK signaling pathway related to inflammatory cytokine production (NF-κB, CREB1, AP-1) (Table IV).

When DE genes between cell lines were visualized graphically, DE genes related to ER stress and UPR were present in the network

(Fig. 1). NF-κB (*NFKB1*) was not significantly different between IB3-1 and C38 under unstimulated conditions but, after flagellin treatment, was upregulated in IB3-1 and centered as one of the largest and most prominent hubs in the network, orchestrating the downstream upregulation of immune genes. Most strikingly, multiple gene nodes related to ER stress and UPR were found to be interacting with NF-κB, either directly through *NFKB1* (such as *HSP90AA1* and *HSPA1A*) or indirectly through *NFKBIA* (such as *VCP* and *XBP1-ATF4*). Taken together, this analysis revealed a pattern of enhanced inflammatory signaling in conjunction with ER stress, the UPR, and general stress response pathways in

Table II.   GO terms categorized as "biological process" associated with ER stress and the UPR, overrepresented by DE genes in CF (IB3-1) compared with non-CF (C38) cells at baseline

GO Term ID	*p* Value	Term
GO:0032874	0.022	Positive regulation of stress-activated MAPK cascade
GO:0017148	0.035	Negative regulation of translation
GO:0050727	0.035	Regulation of inflammatory response
GO:0080134	0.016	Regulation of response to stress
GO:0070374	0.016	Positive regulation of ERK1 and ERK2 cascade
GO:0000165	0.033	MAPKKK cascade

ID, identification.

Table III. GO terms categorized as "biological process" associated with ER stress and the UPR, overrepresented by DE genes in CF (IB3-1) compared with non-CF (C38) cells following flagellin stimulation

GO Term ID	p Value	Term
GO:0030970	3.92E-04	Retrograde protein transport, ER to cytosol
GO:0034612	0.003	Response to TNF
GO:0006983	0.027	ER overload response
GO:0009306	0.031	Protein secretion
GO:0032469	0.030	Endoplasmic reticulum calcium ion homeostasis
GO:0050850	0.006	Positive regulation of calcium-mediated signaling
GO:0002764	0.025	Immune response–regulating signal transduction
GO:0045089	0.006	Positive regulation of innate immune response
GO:0002221	0.044	Pattern recognition receptor signaling pathway
GO:0000187	0.044	Activation of MAPK activity
GO:0043123	0.039	Positive regulation of I-κB kinase/NF-κB cascade

flagellin-stimulated immortalized CF cell lines. On this basis, we hypothesized that the integration of these pathways in CF cells was contributing to their heightened proinflammatory responses.

Atypical activation of the UPR is a feature of CF cells

To validate the computational predictions, we established the reliability of a panel of commonly accepted ER stress/UPR markers by treating IB3-1 and C38 cells with the ER stress inducer tunicamycin and measuring the expression of genes influenced by the three arms of the UPR: IRE-1 (*XBP-1u*, *XBP-1s*), ATF6 (*GRP78*), and PERK (*GADD34, ATF4, NRF2, DDIT3*) (37, 38). Tunicamycin induces ER stress by inhibiting *N*-linked glycosylation. Consistent with current understanding of the UPR, all gene expression increased in a time-dependent fashion except *XBP-1u*, which decreased as expected because under conditions of ER stress, *XBP-1u* is processed by IRE-1 to generate *XBP-1s*. *NRF2* changes were modest, and this marker was not included in subsequent UPR analysis (Fig. 2A–D).

To ensure the validity and generalizability of our findings, we next profiled UPR activation in three complementary human model systems: immortalized CF and control airway epithelial cells, primary PBMCs from CF patients and healthy controls, and CF and control primary lung tissue (Fig. 3A, 3C, 3D, respectively). The active form of *XBP-1*, *XBP-1s*, was consistently overexpressed in the CF cells, validating our computational predictions and indi-

cating that the IRE-1 arm of the UPR was active in these CF samples. In contrast, *ATF4, GADD34, DDIT3,* and *GRP78* expression was not induced in parallel with *XBP-1s,* suggesting the PERK–eIF2α arm of the UPR is not activated in CF cells in all three model systems. A significant lack of activation of the PERK–eIF2α arm of the UPR in immortalized CF airway epithelial cells was confirmed at the protein level, as phosphorylation of eIF2α was lower in CF than in control cells (Fig. 3B). This unanticipated finding provides evidence of both ER stress in CF cells and an atypical activation of the UPR in immortalized CF airway cells.

Pharmacological activation of the PERK–eIF2α pathway attenuates proinflammatory immune responses in CF airway cells

Having identified an atypical pattern of UPR activation in CF cells in which the IRE-1 pathway was activated, in contrast to the absence of PERK–eIF2α pathway induction, we examined whether deliberate activation of the PERK–eIF2α pathway would beneficially modulate the inflammatory response pattern of CF airway cells. Upon activation of PERK, eIF2α is phosphorylated, which leads to ATF4-dependent transcription of *DDIT3*. This pathway is negatively regulated by the phosphatase GADD34, which dephosphorylates eIF2α. Salubrinal, a specific pharmacological inhibitor of GADD34, upregulates this pathway, as demonstrated by increased phosphorylation of eIF2α and induction of *DDIT3*

Table IV. TFs associated with the UPR and proinflammatory signaling pathways, overrepresented by DE genes in CF (IB3-1) compared with non-CF (C38) cells

TF	p Value	Description
Baseline		
CREB1	0.05	Involved in inflammation
CEBPD	0.004	Dimerization partner with CHOP
CEBPE	0.009	Dimerization partner with CHOP
DDIT3	0.007	CHOP
NFYA	0.01	Dimerization partner with ATF6, XBP-1
RELA	0.01	NF-κB
RELB	0.05	NF-κB
Flagellin stimulation		
FOSL1	0.03	AP-1
FOSL2	0.03	AP-1
JUN	0.004	AP-1
TFAP2A	0.03	AP-2
RELA	<0.001	NF-κB
REL	0.04	C-Rel
CEBPD	<0.001	Dimerization partner with CHOP
CEBPE	0.01	Dimerization partner with CHOP
DDIT3	0.007	CHOP
NFYA	0.007	Dimerization partner with ATF6, XBP-1
ATF6	0.007	ER stress sensor
XBP1	0.02	UPR TF

FIGURE 2. Tunicamycin induces expression of UPR markers. IB3-1 (**A**, **C**) and C38 (**B**, **D**) cells were treated with tunicamycin (20 μg/ml) for 24 h ($n = 3$), and *XBP-1u, XBP-1s, GRP78, GADD34, ATF4, NRF2,* and *DDIT3* expression was measured. Expression values were normalized to the housekeeping gene *PPIA* and are relative to baseline. Data are expressed as a log$_2$ ratio and represent mean ± SEM.
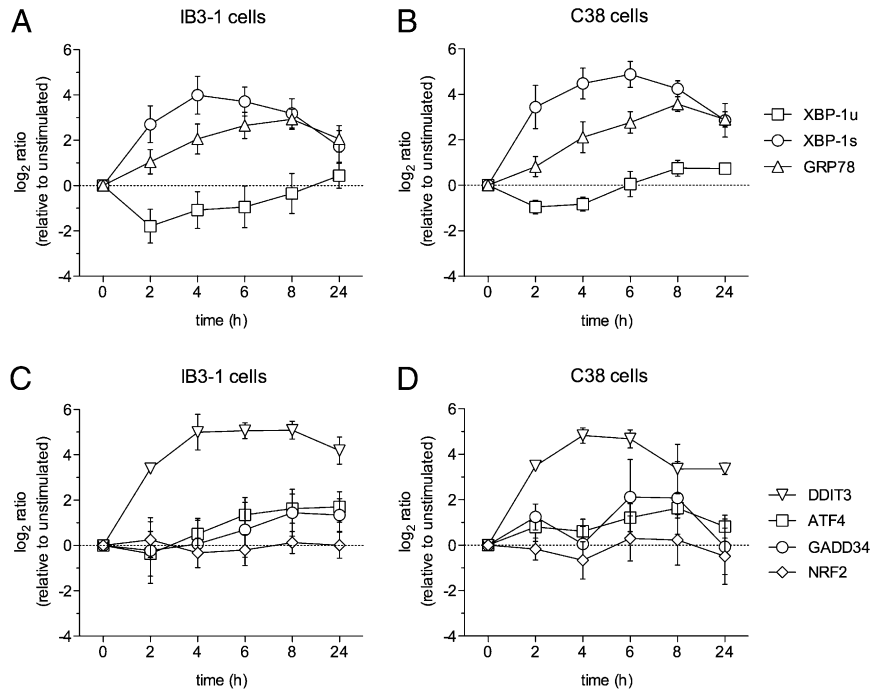
expression (Fig. 4A, 4B). Pharmacological activation of the PERK–eIF2α pathway through preincubation of IB3-1 cells with salubrinal (30 h) attenuated the inflammatory response to flagellin ($p < 0.001$) and *P. aeruginosa* (PAO1, MOI = 50:1; $p < 0.001$) (Fig. 4C, 4D). Importantly, salubrinal treatment did not significantly affect cell viability, as measured by MTS assay (data not shown).

ER stress, the UPR, and proinflammatory innate immune responses are mechanistically linked through p38 MAPK signaling

With the goal of increasing our mechanistic understanding of inflammation in CF and ascertaining potential anti-inflammatory targets, we sought to identify the molecular pathways integrat-

ing ER stress, the UPR, and innate immune signaling. Informed by the results of our gene expression profiling (Tables II–IV) and literature reports implicating p38 MAPK signaling in CF inflammation (11, 39), the activity of p38 MAPK was evaluated in response to flagellin stimulation. Flagellin triggered an increased and prolonged phosphorylation of p38 MAPK in IB3-1 cells compared with control cells (Fig. 5). This differential signaling was biologically relevant, as treatment of CF cells with the specific p38 MAPK inhibitor SB203580 reduced the exaggerated proinflammatory immune response to baseline (Fig. 6A). These data validate the results from the gene expression array analysis, suggesting that p38 MAPK activity plays a significant role in the proinflammatory immune response of IB3-1 cells to flagellin.
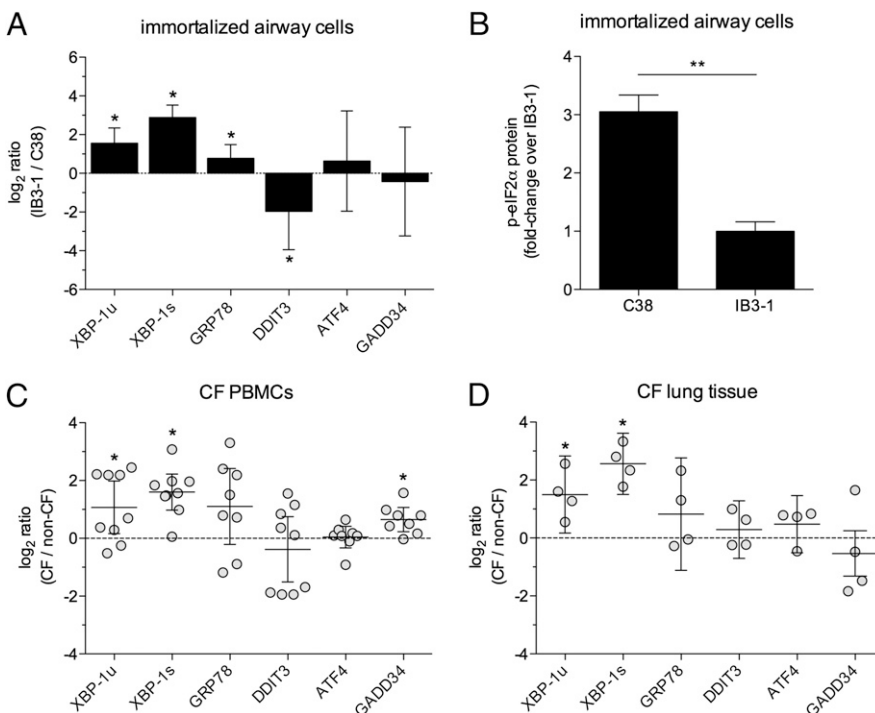
FIGURE 3. Pattern of UPR activation in immortalized CF airway cells, blood cells, and lung tissue. Expression of UPR markers *XBP-1u, XBP-1s, GRP78, GADD34, ATF4,* and *DDIT3* at unstimulated conditions in (**A**) IB3-1/C38 cells ($n = 3$), (**C**) CF PBMCs ($n = 8$), and (**D**) tissue from lung explants ($n = 4$) compared with their non-CF controls. Expression values were normalized to the housekeeping gene *PPIA* and are relative to baseline. Data are expressed as a log$_2$ ratio and represent mean ± 95% confidence interval. *$p < 0.05$. (**B**) Phospho(p)-eIF2α protein levels in CF (IB3-1) and non-CF (C38) airway cells were determined by Western blot and quantified by densitometry ($n = 3$). Statistical comparison was performed by t test. **$p < 0.01$.

FIGURE 4. Salubrinal attenuates pro-inflammatory cytokine production in immortalized CF cells. Activation of the PERK–eIF2α pathway attenuates the inflammatory immune response to flagellin and *P. aeruginosa* (PAO1). (**A**) The PERK–eIF2α pathway showing phospho-eIF2α protein levels in IB3-1 cells by Western blot following salubrinal treatment (6 h, 100 μM). (**B**) Treatment of IB3-1 cells with salubrinal (30 h, 100 μM) induces *DDIT3* expression, with the change in *DDIT3* expression from unstimulated and salubrinal treated (inset, *n* = 3). Expression values were normalized to the housekeeping gene *PPIA* and are relative to baseline. Data are expressed as a $\log_2$ ratio and represent mean ± 95% confidence interval. *$p < 0.05$. (**C** and **D**) IB3-1 cells were treated with salubrinal (48 h, 100 μM) prior to flagellin (14 h, 100 ng/ml) or live PAO1 (4 h, MOI = 50:1) stimulation (*n* = 3). Data are expressed as mean ± SEM and analyzed by ANOVA using the Bonferroni posttest. ***$p < 0.001$.
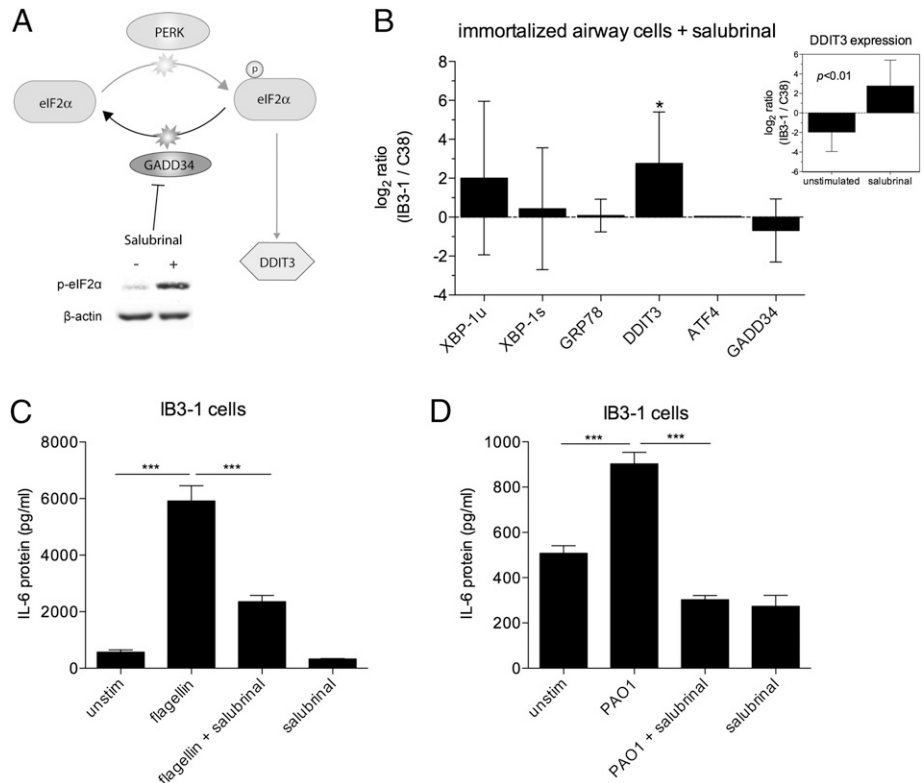
To investigate a potential link between ER stress and inflammation, we next sought to determine if ER stress–augmented inflammatory responses were also mediated by p38 MAPK. The level of ER stress was artificially enhanced by preincubation with tunicamycin. Subsequent stimulation with flagellin resulted in an augmented IL-6 response in both cell lines (Fig. 6A, 6B), suggesting that existing and increasing perturbation of the ER can potentiate the inflammatory immune response of immortalized airway epithelial cells. Similar to flagellin responses, the differences in tunicamycin-induced IL-6 were greater in IB3-1 than in C38 ($p < 0.05$). This ER stress–induced IL-6 production was dependent upon p38 MAPK activity, as pretreatment with SB203580 abrogated the IL-6 response to tunicamycin (Fig. 6A,

6B). Finally, to confirm that ER stress contributes to flagellin-induced p38 MAPK-dependent inflammatory gene expression, we demonstrated that SB203580 abolished IL-6 production from cells treated with both tunicamycin and flagellin (Fig. 6A, 6B). Together these data suggest that in IB3-1 cells, signaling and transcriptional pathways activated by ER stress and flagellin are mechanistically linked through p38 MAPK signaling, and that this pathway convergence in turn contributes to augmented inflammatory responses of immortalized CF airway cells.

Discussion

Inflammatory lung disease is the major cause of morbidity and mortality in CF. Although nonspecific anti-inflammatory medi-
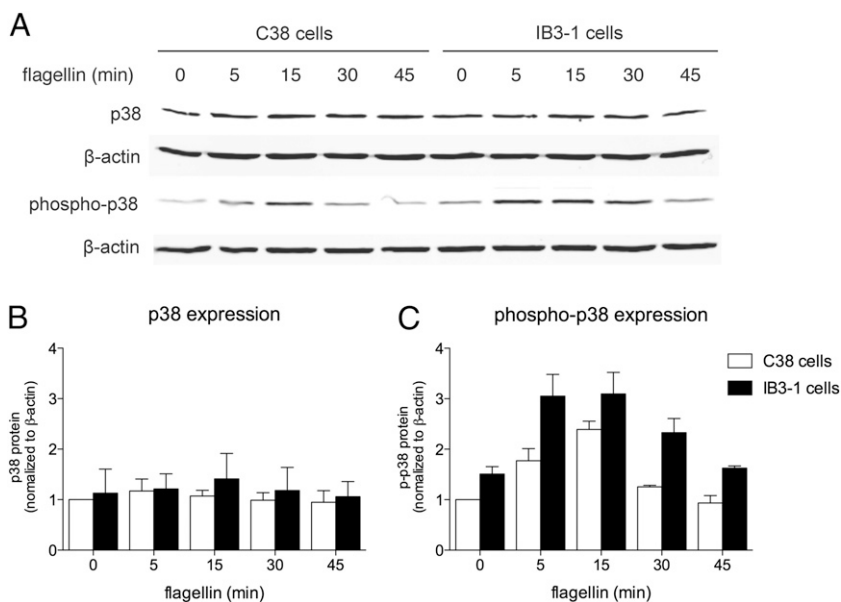
FIGURE 5. Increased activation of p38 MAPK signaling in immortalized CF cells. Phosphorylation of p38 MAPK is increased and prolonged upon flagellin stimulation in IB3-1 compared with C38 cells. (**A**) Cells were stimulated with flagellin for 0, 5, 15, 30, and 45 min. Total and phospho-p38 MAPK were evaluated by Western blot. Densitometry for both cell lines was calculated and normalized to β-actin and unstimulated C38 and is shown for (**B**) p38 and (**C**) phospho-p38 (*n* = 3). Statistical comparison between C38 and IB3-1 of phospho-p38 was performed by *t* test after calculating the area under the curve ($p < 0.05$).

FIGURE 6. The flagellin- and tunicamycin-induced IL-6 responses are dependent on p38 MAPK activity. (**A**) Induction of ER stress with tunicamycin (6 h, 20 μg/ml) prior to flagellin stimulation significantly potentiates the proinflammatory immune response in immortalized CF cells. Pretreatment of IB3-1 cells with SB203580 (1 h, 1 μM) prior to flagellin (14 h, 100 ng/ml) or tunicamycin stimulation significantly decreases IL-6 production in (A) CF and (**B**) non-CF cell lines. Statistical analysis was performed by ANOVA using the Bonferroni posttest. Data are derived from at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
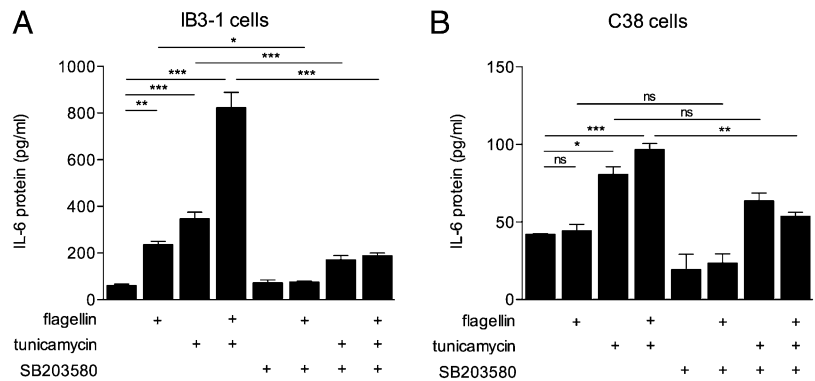
cations (i.e., corticosteroids, ibuprofen) have been beneficial in clinical trials in slowing the decline in lung function in CF, clinical uptake is low owing to unacceptable side effects, such as growth impairment, weight gain, and risk of gastrointestinal bleeding. However, this "proof-of-concept" evidence has been the rationale in many studies for understanding the specific pathways mediating the increased inflammatory response to bacterial infection in CF, all with the goal of guiding the development of safe and effective anti-inflammatory therapies. Our group and others have generated evidence highlighting the importance of innate immune receptors, particularly TLR5, in mediating inflammatory responses to *P. aeruginosa* in CF (7, 9, 40). The mechanism or mechanisms underlying the increased immune response in CF, however, remain unknown.

This study aimed to identify pathways involved in the increased proinflammatory cytokine production occurring in CF in response to microbial stimuli. Our global gene expression profiling reflected a general theme of activated stress response mechanisms in immortalized CF airway cells that was already present at baseline, suggesting disturbance of cellular homeostasis. In particular, ER stress response mechanisms and the UPR were indicated in the GO and TF ORA (Tables II–IV), consistent with previous studies identifying an increased level of ER stress in CF cells, potentially due to misfolded CFTR (41, 42). In addition to these results, network analysis revealed direct and indirect interactions between ER stress pathway mediators and NF-κB–dependent transcription of inflammatory mediators (Fig. 1).

In the case of ER stress, the UPR is activated by a variety of insults that disturb proteostasis. Three general mechanisms have evolved to restore ER homeostasis—increase in the protein folding capacity, attenuation of translation, and upregulation of the degradation machinery (ER-associated degradation, autophagy) (43). It is important to appreciate that in addition to misfolding of mutated CFTR protein, many factors are likely to have an impact on proteostasis to trigger ER stress in CF, including oxidative stress (11, 16, 17, 44–46), chronic infection (47), and decreased functionality of ER stress relief mechanisms, such as autophagy (21). Nevertheless, the literature contains conflicting results concerning the presence of ER stress in CF and the potential contribution of ER stress to CF inflammatory disease. Several studies report that ΔF508, the mutation found in 70% of all CF patients, is retained within the ER, leading to upregulation of protein degradation and ER stress markers indicative of unbalanced ER homeostasis (15, 42, 48–51). More specifically, Martino et al. (52, 53) showed that XBP-1s is overexpressed in CF cells and the inflammatory responses in CF cells were dependent upon the action of this TF. In contrast, Hybiske et al. (19) reported that no difference can be noted in ER stress, NF-κB signaling, and IL-8 production in CF cells compared with control cells; whereas

Nanua et al. (18) found that neither *XBP-1* splicing nor eIF2α phosphorylation was increased in CF cells compared with controls, suggesting that classical UPR activation is absent in CF cells.

Our study expands the literature concerning ER stress and the UPR in CF. Although we confirmed an increased activity of the IRE-1 pathway in CF (evident by overexpression of *XBP-1s*), we also observed a lack of induction of the PERK–eIF2α pathway (evident by the lack of *DDIT3* induction and low levels of phospho-eIF2α at baseline conditions) (Fig. 3). However, we were able to induce the PERK–eIF2α pathway in the immortalized CF airway cells with ER stress inducer tunicamycin, indicating that in this model system this pathway is not defective but, rather, inactivated. A potential weakness of our study design was the use of immortalized airway cells. Indeed, C38 cells, although "corrected," express not only wild-type CFTR but also mutant CFTR that still has the potential to induce ER stress. To specifically address this weakness, we validated this atypical pattern of UPR activation in both fresh blood cells and lung tissue from patients with CF (Fig. 3C, 3D). We also acknowledge that inflammation is a potential cause for ER stress (52–54), and with respect to the CF lung tissues (Fig. 3D), a clear interpretation of whether the increased UPR evident in these samples is due to inflammation or protein misfolding cannot be made. It has been suggested that this atypical pattern of UPR activation may be a fundamental cellular adaptation to chronic ER stress (55–57). PERK–eIF2α signaling is rapidly downregulated as a consequence of negative feedback loops within the UPR, which might indicate that long-term activation of this signaling axis is deleterious perhaps because of upregulation of CHOP and its targets (58, 59).

We hypothesized that this incomplete activation of the UPR was associated with the increased proinflammatory phenotype in CF by dysregulating inflammatory signaling events. We used two complementary approaches to test this hypothesis. First, we artificially increased ER stress in IB3-1 cells and subsequently stimulated with flagellin. Increased levels of ER stress exacerbated the IL-6 response to TLR5 signaling (Fig. 6A), supporting the hypothesis that imbalanced ER homeostasis augments proinflammatory TLR5 signaling. Second, on the basis of the evolutionary assumption that activation of the UPR network is beneficial in restoring cellular homeostasis, we elected to activate the PERK–eIF2α arm of the UPR rather than inhibiting the overactivated IRE-1–XBP-1 arm [as was performed by Martino et al. (52)]. Salubrinal inhibits GADD34 (60) and thus constitutively activates this pathway, as evidenced by the phosphorylation of eIF2α and the induction of *DDIT3* expression (Fig. 4). Intriguingly, preconditioning of immortalized CF airway cells with salubrinal significantly reduced the proinflammatory IL-6 response following exposure to both flagellin and live *P. aeruginosa* (Fig. 4C, 4D). Although reports

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