ORIGINAL ARTICLE

X-Box–Binding Protein 1 and Innate Immune Responses of Human Cystic Fibrosis Alveolar Macrophages

Bob A. Lubamba[1], Lisa C. Jones[1], Wanda K. O'Neal[1,2], Richard C. Boucher[1,2], and Carla M. P. Ribeiro[1,2,3]

[1]Marsico Lung Institute/Cystic Fibrosis Research Center, [2]Department of Medicine, and [3]Department of Cell Biology and Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Abstract

Rationale: Alveolar macrophages (AMs) play a key role in host defense to inhaled bacterial pathogens, in part by secreting inflammatory mediators. Cystic fibrosis (CF) airways exhibit a persistent, robust inflammatory response that may contribute to the pathophysiology of CF. Recent findings have linked endoplasmic reticulum stress responses mediated by inositol-requiring enzyme 1α–dependent messenger RNA splicing (activation) of X-box–binding protein-1 (XBP-1s) to inflammation in peripheral macrophages. However, the role of XBP-1s in CF AM function is not known.

Objectives: To evaluate inflammatory responses of AMs from chronically infected/inflamed human CF lungs and test whether XBP-1s is required for AM-mediated inflammation.

Methods: Basal and LPS-induced inflammatory responses were evaluated in primary cultures of non-CF versus CF AMs. XBP-1s was measured and its function was evaluated in AMs using 8-formyl-7-hydroxy-4-methylcoumarin (4μ8C), an inhibitor of inositol-requiring enzyme 1α–dependent XBP-1s, and in THP-1 cells stably expressing XBP-1 shRNA, XBP-1s, or a dominant-negative XBP-1.

Measurements and Main Results: CF AMs exhibited exaggerated basal and LPS-induced production of tumor necrosis factor-α and IL-6, and these responses were coupled to increased levels of XBP-1s. In non-CF and CF AMs, LPS-induced cytokine production was blunted by 4μ8C. A role for XBP-1s in AM inflammatory responses was further established by data from dTHP-1 cells indicating that expression of XBP-1 shRNA reduced XBP-1s levels and LPS-induced inflammatory responses; and LPS-induced inflammation was up-regulated by expression of XBP-1s and inhibited by dominant-negative XBP-1.

Conclusions: These findings suggest that AMs contribute to the robust inflammation of CF airways via an up-regulation of XBP-1s-mediated cytokine production.

Keywords: cystic fibrosis; airway inflammation; alveolar macrophage; UPR; IRE1α/XBP-1

Cystic fibrosis (CF) airway disease is characterized by a chronic and robust inflammatory state often termed hyperinflammatory. In CF airways, the functional absence of the CF transmembrane conductance regulator (CFTR) results in an abnormal airway surface liquid hydration, adherence of thickened mucus to airway surfaces, and persistent airway infection leading to chronic inflammation (1, 2). As evidence of the robust inflammation in CF airways, cytokines and neutrophil elastase are elevated in sputa from patients with CF versus without CF (3). In particular, levels of proinflammatory cytokines and inflammatory cells triggered by bacterial infection are higher in patients with CF versus without CF with acute lung infection (4–6), including the cytokines tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 (7–10).

Alveolar macrophages (AMs) represent a first line of defense against inhaled

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Correspondence and requests for reprints should be addressed to Bob A. Lubamba, Ph.D., Marsico Lung Institute/Cystic Fibrosis Research Center, The University of North Carolina at Chapel Hill, 125 Mason Farm Road, CB #7248, Chapel Hill, NC 27599-7248. E-mail: lubamba@med.unc.edu; or Carla M. P. Ribeiro, Ph.D., Marsico Lung Institute/Cystic Fibrosis Research Center, Department of Medicine, Department of Cell Biology and Physiology, The University of North Carolina at Chapel Hill, 125 Mason Farm Road, CB #7248, Chapel Hill, NC 27599-7248. E-mail: carla_ribeiro@med.unc.edu

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At a Glance Commentary

Scientific Knowledge on the Subject: Alveolar macrophages (AMs) play a crucial role in pulmonary innate defense in part by secreting inflammatory mediators. The specific contribution of AMs to inflammatory responses observed in cystic fibrosis (CF) lung disease and the mechanisms underlying these responses are not well-established. The unfolded protein response mediated by activation of the inositol-requiring enzyme 1 (IRE1)-α–dependent X-box–binding protein-1 (XBP-1) pathway has been implicated in CF airway epithelial inflammation. However, the functional role of IRE1α/XBP-1 in inflammatory responses of AMs from CF lungs is not known.

What This Study Adds to the Field: This study indicates that primary cultures of human CF AMs exhibit a robust production of inflammatory mediators, and this response reflects an adaptation to the infectious/inflammatory environment of CF airways. The exaggerated response of CF AMs to LPS is mediated by activation of the IRE1α/XBP-1 arm of the unfolded protein response. Our findings offer the proof-of-concept that manipulation of the IRE1α/XBP-1 pathway in AMs may provide new therapeutic opportunities for CF airways disease.

pathogens, including *Pseudomonas aeruginosa* (11), via phagocytosis of pathogens and secretion of inflammatory mediators. Like neutrophils, persistent activation of AMs without resolution of infection could lead to lung damage. In one scenario, chronic exposure of AMs to the products of persistent intraluminal infection could produce AM-mediated lung damage. A second scenario has suggested that CFTR is expressed in AMs and loss of functional CFTR results in specific defects in AM function (12–17), including impaired bacterial killing linked to CFTR endolysosomal dysfunction (16, 18, 19) and alterations in inflammatory responses of AMs. Evidence for this scenario is provided by studies demonstrating that AMs from $CFTR^{-/-}$ mice exhibit exaggerated inflammatory responses to bacterial LPS (14), and inhibition or mutation of CFTR enhances production of cytokines in murine AMs (20). Moreover, $CFTR^{+/-}$ mice release larger amounts of IL-6 in response to transforming growth factor-β, as compared with wild-type mice (21). In agreement with the murine data, silencing of CFTR in human AMs results in increased secretion of IL-8 (22). These data suggest that human CF AMs might have abnormal function and contribute to an increased inflammatory response.

Inflammation of CF airway epithelia induces endoplasmic reticulum (ER) stress, which triggers the unfolded protein response (UPR) (23–26). Eukaryotic cells exhibit three UPR pathways: (*1*) inositol-requiring enzyme 1 (IRE1), which exists in two isoforms, α (ubiquitous) and β (present in mucous cells of the gut and respiratory tracts); (*2*) activating transcription factor 6; and (*3*) PKR-like ER kinase/pancreatic eIF2α kinase (24, 27, 28). In normal airways, the UPR constitutes an adaptive response to ER stress that provides cellular protection and survival. For instance, activation of IRE1α induces splicing (activation) of the mRNA of X-box–binding protein-1 (XBP-1s) (29, 30). XBP-1s is a transcription factor that up-regulates the ER protein folding capacity and expands the secretory pathway, facilitating increased production of inflammatory mediators involved in innate defense (24–26). In contrast, in obstructed CF airways, persistent high levels of ER stress can have a detrimental effect for airway homeostasis, including high levels of cytokine production that may mediate inflammation-induced airway wall damage.

Activation of the IRE1α-dependent XBP-1s pathway has been linked to cytokine production in macrophages from murine bone marrow or peripheral human monocytes (31). We hypothesized that IRE1α-mediated XBP-1s is required for AM cytokine production and activation of IRE1α-dependent XBP-1s is increased in CF AMs. Hence, this study addressed whether inflammatory responses are larger in primary cultures of AMs from CF versus noninfected/inflamed human lungs, and the role of IRE1α-dependent XBP-1s in inflammatory responses of non-CF and CF AMs. Some results from this study have been reported in the form of an abstract at the 2014 North American Cystic Fibrosis Conference (32).

Methods

For further details on the applied methods, *see* the online supplement.

Isolation and Culture of Primary Human AMs

Lungs were lavaged with phosphate-buffered saline (PBS) and the retrieved fluid centrifuged ($250 \times g$ for 10 min; 4°C). The cell pellet was resuspended in macrophage medium. AM isolation was performed as previously described (33). Non-CF and CF AMs were seeded onto 12-well plates (1×10^5 AMs per well) and cultured in macrophage medium.

Macrophage-like Differentiated THP-1 Cells

THP-1 human monocytic cells were exposed to 50 ng/ml of phorbol 12-myristate 13-acetate in the culture medium for 72 hours.

THP-1 Cells Expressing shRNA Targeting XBP-1

THP-1 cells were infected with a control shRNA or an anti–XBP-1 shRNA lentiviral vector. Positive cells were selected with 1 μg/ml puromycin.

Stable Expression of Control, XBP-1s, and Dominant Negative XBP-1 Vectors

THP-1 cells were infected with a control retroviral vector or retroviral vectors containing XBP-1s or dominant-negative XBP-1 (DN-XBP-1). Positive cells were selected with 200 μg/ml neomycin G418.

Immunohistochemistry

Cytocentrifuge slides were fixed with 4% paraformaldehyde, blocked with 1% bovine serum albumin in PBS with 0.1% Tween-20, incubated overnight at 4°C with a HAM-56 antibody, followed by incubation with a secondary antibody for 1 hour at room temperature.

Flow Cytometry

One hundred thousand 3-day-old cells were incubated for 30 minutes at 4°C with anti–CD11c-APC and/or anti–CD163-PerCP/Cy5.5 conjugated antibody. Data were analyzed using FlowJo X.0.7 (Ashland, OR).

Treatment with Pharmacologic Agents

AMs were washed with PBS and treated with 100 ng/ml LPS from *P. aeruginosa*. $CFTR_{inh}$-172 (3-[(3-trifluoromethyl) phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone) was used at the concentration of 10 μM. 8-Formyl-7-hydroxy-4-methylcoumarin (4μ8C), an IRE1α inhibitor, was used at the concentration of 50 μM.

Treatment with Supernatant of Mucopurulent Material

Mucopurulent material was harvested from the airways of human CF lungs and processed as previously described (23–25, 34). Filtered supernatant from mucopurulent material (SMM) was pooled from five CF lungs. Non-CF AMs were exposed to 30 μl of PBS or SMM (1:3 dilution in culture medium) for 3 hours.

Quantitative Real-Time Polymerase Chain Reaction

RNA isolation, cDNA preparation, and real-time polymerase chain reaction were performed as previously described (27). mRNA values were normalized to 18S mRNA values.

ELISA Assays

TNF-α and IL-6 secretion was evaluated by ELISA.

Statistical Analysis

Data are reported as mean ± SD with a *P* value less than 0.05 considered statistically significant.

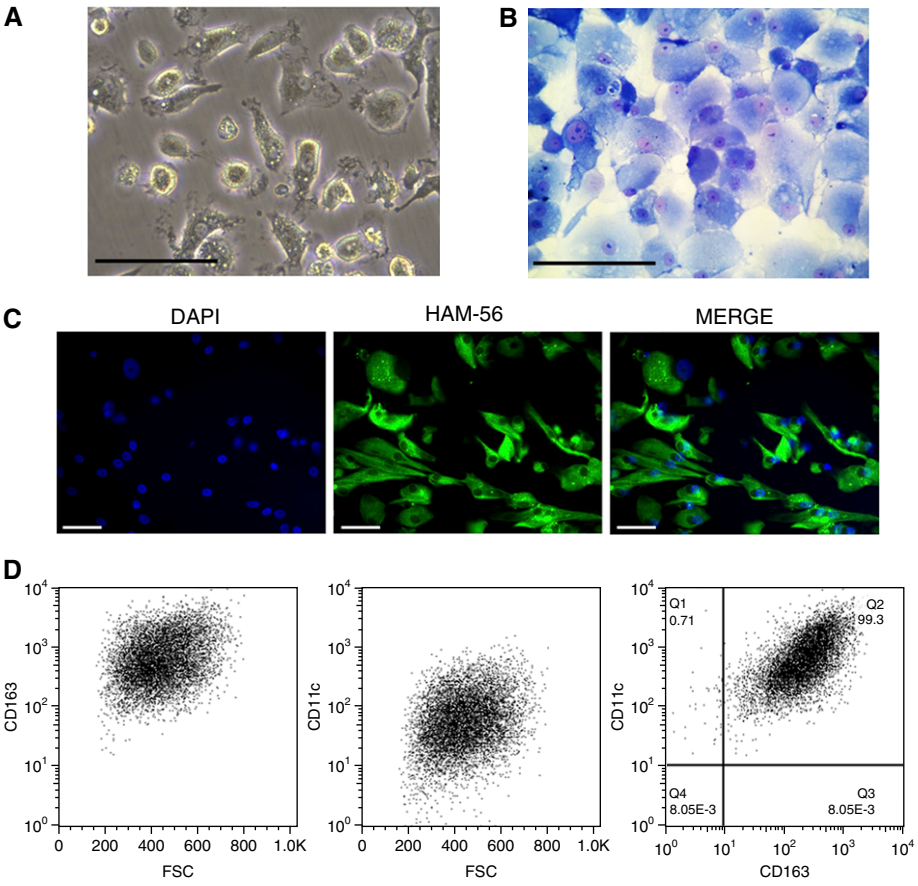

Figure 1. Characterization of alveolar macrophages (AMs) isolated from resected human lungs. (*A*) Adherent cells were isolated as described in the METHODS. Examination of cell morphology by phase-contrast inverted light microscopy. (*B*) Morphology of sedimented AMs. Cells were sedimented onto slides (cytospin), stained (Diff-Quik), and examined by light microscopy. (*C*) Immunofluorescence microscopy of AMs. *Green* = macrophage migration inhibitor factor HAM-56 (human macrophage marker). *Blue* = nuclei stain with 4'6-diamidino-2-phenylindole (DAPI). (*D*) Flow cytometric analysis of AMs. The preparations were analyzed according to their forward scatter characteristics (FSC), CD163 (macrophage marker), and CD11c (macrophage marker) expression in human AMs. Number in *right panel* indicates the percentage of cells positive for the macrophage markers. *Scale bars* = 100 μm.

Results

Isolation and Characterization of AMs

AM isolation and their attachment to plastic dishes was performed as previously described (33). Each lung tissue yielded 2–20 × 10^6 AMs, which consisted mostly of large, round cells heterogeneous in size, as observed by light microscopy (Figure 1A). AM evaluation using Diff-Quik (Polysciences, Inc., Warrington, PA) indicated that the preparation consisted of more than 99% AMs (Figure 1B). The purity of the AM samples was further indicated by the expression of the macrophage marker HAM-56 (35) in all isolated cells (Figure 1C).

Flow cytometry was next performed to establish the purity of the AMs. The preparations were analyzed according to their forward scatter characteristics, and for their expression of $CD163^+$ and $CD11c^+$ (Figure 1D, *left* and *middle*). CD163 is expressed exclusively on monocytes and macrophages (36), and CD11c is expressed on many monocytic-derived cells, including macrophages (37). The combination of $CD11c^+$ and $CD163^+$ expression, which is necessary and sufficient to accurately distinguish macrophages from other lung cells, indicates that our isolation procedure yielded a highly purified preparation of AMs (Figure 1D, *right*).

Primary Cultures of CF AMs Express a Robust Inflammatory Phenotype

We next evaluated the basal and LPS-induced cytokine production in non-CF versus CF AMs in primary cultures for 3 days. Treatment with vehicle (0.1% dimethyl sulfoxide) did not affect the expression of inflammatory markers, as compared with untreated AMs (data not shown). Therefore, cytokine values obtained under vehicle treatment were considered baseline values.

We first compared the baseline mRNA levels of TNF-α and IL-6 in primary cultures of CF versus non-CF AMs. These inflammatory mediators were evaluated because of their relevance to CF airways disease (7, 8). Under baseline conditions, CF AMs exhibited 25- and 24-fold higher mRNA levels of TNF-α and IL-6, as compared with non-CF AMs (Figures 2A and 2B). We then determined the effect of LPS from *P. aeruginosa* on the mRNA

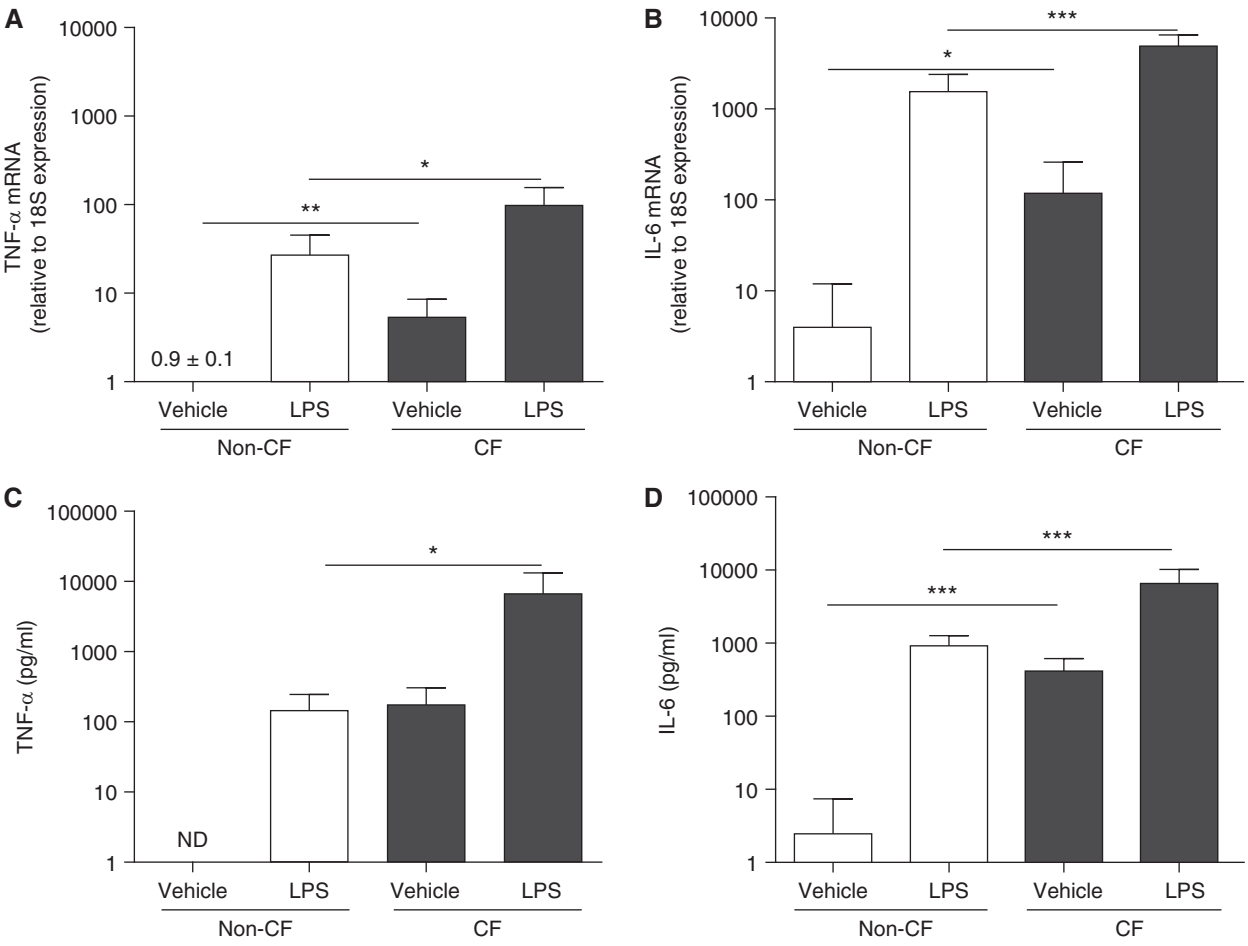

Figure 2. Primary cultures of cystic fibrosis (CF) alveolar macrophages (AMs) exhibit robust basal and LPS-induced tumor necrosis factor (TNF)-α and IL-6 production. Non-CF and CF AMs were stimulated for 6 hours with 0.1% dimethyl sulfoxide (vehicle) or 100 ng/ml LPS from *Pseudomonas aeruginosa*. Levels of TNF-α (*A*) and IL-6 (*B*) mRNA were determined by quantitative reverse transcriptase polymerase chain reaction and expressed as fold change relative to 18S mRNA. TNF-α (*C*) and IL-6 (*D*) protein secretion into the culture media were determined by ELISA. *Open bars* correspond to primary cultures of non-CF AMs. *Solid bars* correspond to primary cultures of CF AMs. The *y-axis* uses a logarithmic scale. Data are from six independent experiments and represent mean ± SD. Unpaired *t* test was used to compare non-CF with CF AMs and paired *t* test was used to compare vehicle with LPS treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ND = not detected. The fold change of TNF-α mRNA from cells exposed to the vehicle is 0.9 ± 0.1.

levels of TNF-α and IL-6 in CF and non-CF AMs. Pilot studies indicated that 100 ng/ml LPS was a maximal dose for inducing cytokine mRNA expression and secretion (*see* Figure E1 in the online supplement). Moreover, 6-hour LPS led to maximal cytokine mRNA up-regulation associated with increased cytokine secretion (*see* Figure E2). The 100 ng/ml (6-h treatment) LPS-up-regulated TNF-α and IL-6 mRNA levels were higher in CF versus non-CF AMs (Figures 2A and 2B).

To address whether the higher mRNA expression of TNF-α and IL-6 corresponded to higher TNF-α and IL-6 protein levels, the secretion of TNF-α and IL-6 was evaluated. Under basal conditions, CF AMs secreted 175- and 260-fold higher levels of TNF-α and IL-6 than non-CF AMs (Figures 2C and 2D). In addition, LPS-up-regulated TNF-α and IL-6 secretion was 49-fold and sevenfold higher in CF versus non-CF AMs (Figures 2C and 2D). These data indicate that CF AMs exhibit a greater inflammatory phenotype than non-CF AMs under basal condition and following LPS exposure.

CFTR Expression Levels in Human AMs

Because it has been suggested that macrophages express CFTR at a low, but functional level (12), we addressed whether the absence of CFTR function is coupled to the robust inflammatory response of CF AMs. We first evaluated CFTR expression in primary non-CF AMs from human lungs versus primary human bronchial epithelial (HBE) cells known to express CFTR. CFTR mRNA levels were very low in AMs as compared with HBE cells (Figure 3). Notably, the low CFTR expression levels in AMs did not differ from the hypothetical value zero, based on one-sample *t* test (Figure 3). These data suggest that primary cultures of human AMs either do not express CFTR or their CFTR expression is extremely low.

The putative very low level of CFTR expression in non-CF AMs could be, nevertheless, important for AM function. Hence, we considered that the loss of functional CFTR in CF AMs could be implicated in the exaggerated inflammatory

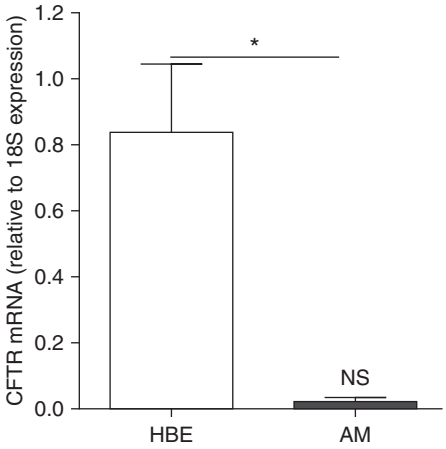

Figure 3. Cystic fibrosis transmembrane conductance regulator (CFTR) expression levels in human alveolar macrophages (AMs). *Bars* represent mRNA levels, determined by quantitative reverse transcriptase polymerase chain reaction, of CFTR in primary cultures of human bronchial epithelial (HBE) cells and primary cultures of human AMs. Data are expressed as fold change relative to 18S mRNA and represent mean ± SD from three independent experiments. Unpaired *t* test was used for the statistical analysis. $^*P < 0.05$, HBE versus AMs. NS = not significant, CFTR levels in AMs versus the hypothetical value 0, based on one-sample *t* test.

response to LPS (Figure 2). To address this issue, we used $CFTR_{inh}$-172 to pharmacologically inhibit putative CFTR function (12, 38) in non-CF AMs. Treatment of non-CF AMs for 72 hours with a maximal dose of $CFTR_{inh}$-172 (10 μM) (39) had no effect on basal IL-6 mRNA levels and protein secretion (Figures 4A and 4B). We also determined the effect of 100 ng/ml LPS on AMs pretreated with $CFTR_{inh}$-172 for 72 hours. Although LPS significantly increased IL-6 mRNA levels (Figure 4A) and IL-6 secretion (Figure 4B), pretreatment with $CFTR_{inh}$-172 did not potentiate the LPS-induced inflammatory response (Figures 4A and 4B). These data suggest that the robust inflammatory phenotype with respect to these cytokines of CF AMs is not linked to a defective CFTR function.

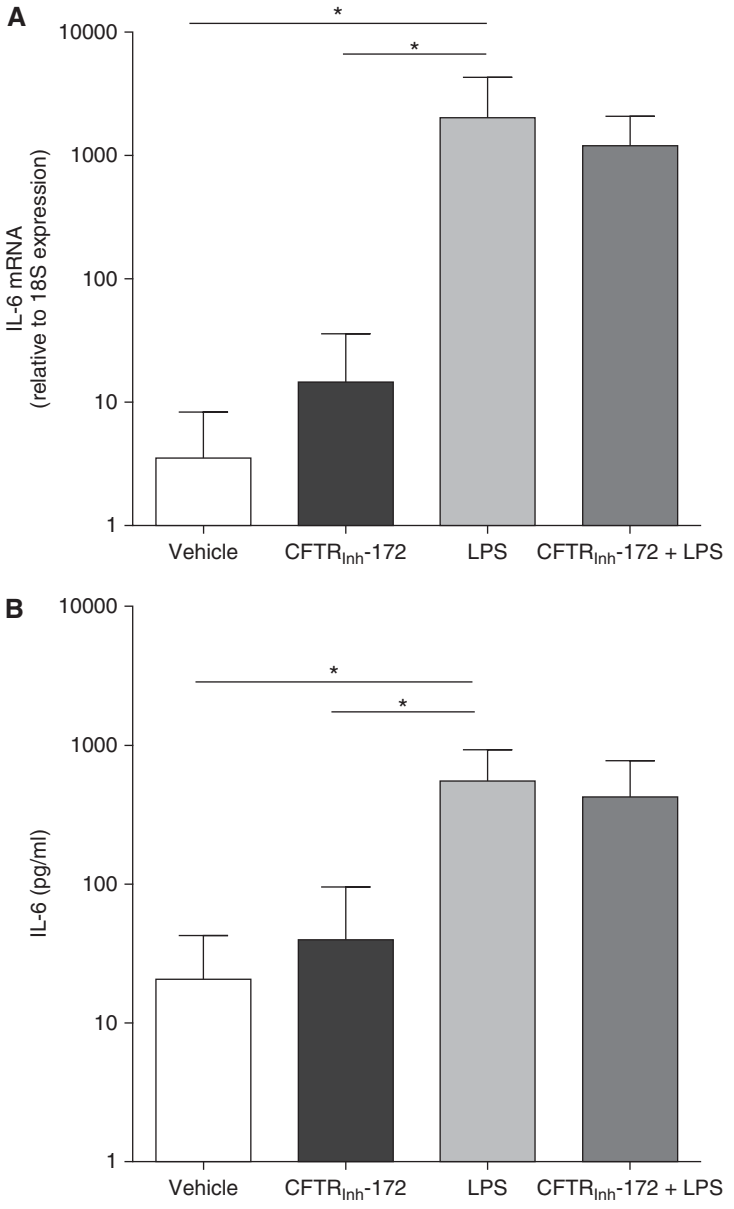

Figure 4. Cystic fibrosis transmembrane conductance regulator (CFTR) inhibition does not induce an inflammatory response in non-CF alveolar macrophages. Non-CF alveolar macrophages were pretreated with $CFTR_{inh}$-172 (10 μM) for 72 hours. Where applicable, 100 ng/ml LPS from *Pseudomonas aeruginosa* was added during the last 6 hours. (*A*) Quantitative reverse transcriptase polymerase chain reaction was used to determine the levels of IL-6 mRNA, which are expressed as fold change relative to 18S mRNA. (*B*) IL-6 secretion into the culture media was evaluated by ELISA. The *y-axis* uses a logarithmic scale. Data are from six independent experiments and represent mean ± SD. Paired *t* test was used for the statistical analysis. $^*P < 0.05$.

AM Inflammatory Responses Are Coupled to UPR Activation

To investigate whether the inflammatory response of AMs is linked to activation of IRE1α-dependent XBP-1 mRNA splicing (XBP-1s), a UPR pathway implicated in inflammatory responses of peripheral macrophages (31), we compared the levels of XBP-1s in non-CF versus CF AMs. Under basal conditions, CF AMs exhibited fourfold higher mRNA levels of XBP-1s versus non-CF AMs. LPS up-regulated the levels of XBP-1s in non-CF and CF AMs. Notably, the absolute magnitude of response of CF AMs to LPS was higher than non-CF AMs (Figure 5).

We also evaluated the effect of $CFTR_{inh}$-172 on the mRNA levels of XBP-1s. Under basal and LPS-stimulated conditions, $CFTR_{inh}$-172 did not affect XBP-1s levels (Figure 6), suggesting that loss of CFTR function *per se* was not associated with the higher levels of XBP-1s in CF AMs (Figure 5).

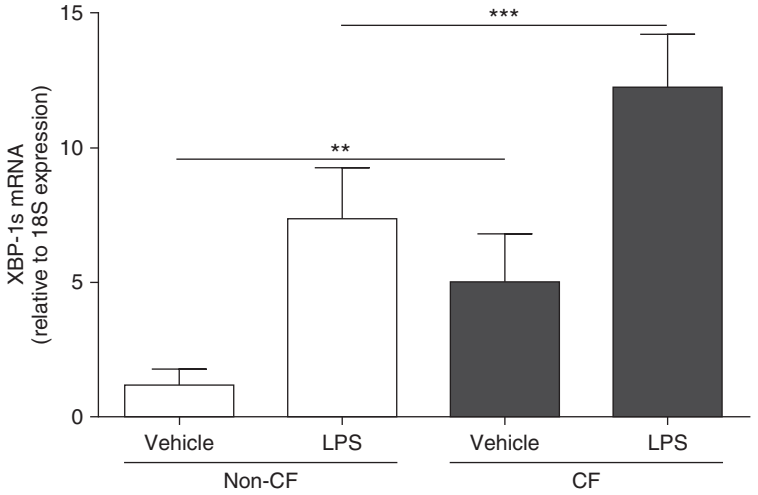

Figure 5. Primary cultures of cystic fibrosis (CF) alveolar macrophages (AMs) express high levels of X-box–binding protein-1 mRNA splicing (XBP-1s). Non-CF and CF AMs were stimulated for 6 hours with 100 ng/ml LPS from *Pseudomonas aeruginosa*. Quantitative reverse transcriptase polymerase chain reaction was used to determine the mRNA levels of XBP-1s. Data are expressed as fold change relative to 18S mRNA and represent mean ± SD from six independent experiments. *Open bars* correspond to primary cultures of non-CF AMs. *Solid bars* correspond to primary cultures of CF AMs. Unpaired *t* test was used to compare non-CF with CF AMs, and paired *t* test was used to compare vehicle with LPS treatment. $^{**}P < 0.01$, $^{***}P < 0.001$.

To address whether the robust inflammatory response coupled to higher XBP-1s levels in CF AMs resulted from an acquired response to the CF airway infectious/inflammatory milieu, non-CF AMs were exposed to SMM from human CF airways (23–25). SMM up-regulated XBP-1s, TNF-α, and IL-6 (Figures 7A–7C) mRNA levels. Moreover, SMM up-regulated TNF-α and IL-6 protein secretion (Figures 7D and 7E), reproducing the robust inflammatory phenotype of CF AMs (Figure 2) associated with higher XBP-1s levels (Figure 5).

Together, these data suggest that (*1*) activation of IRE1α-dependent XBP-1s is coupled with inflammatory responses in both non-CF and CF AMs; (*2*) the higher levels of XBP-1s in CF AMs are proportionate to their robust inflammatory phenotype; and (*3*) the greater inflammatory response of CF AMs linked to increased levels of XBP-1s is not associated with the absence of CFTR function but, rather, reflects a response to persistent inflammatory stimulation.

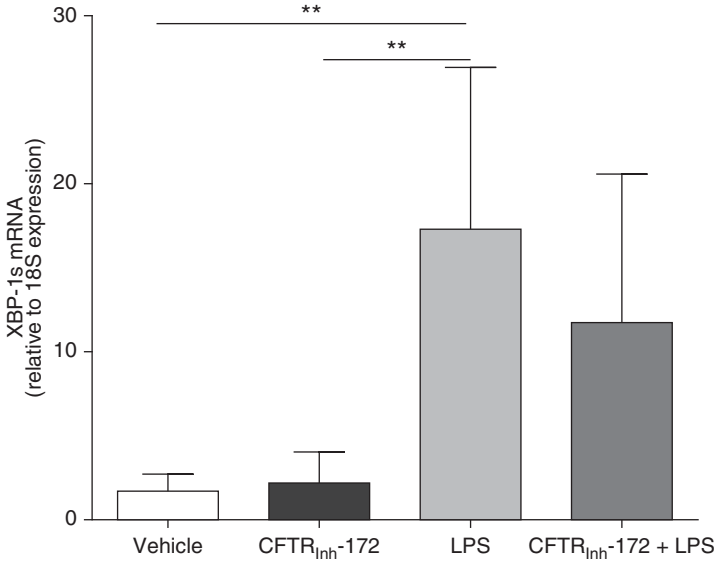

Figure 6. Cystic fibrosis transmembrane conductance regulator (CFTR) inhibition does not induce X-box–binding protein-1 mRNA splicing (XBP-1s) in primary cultures of non-CF alveolar macrophages. Non-CF alveolar macrophages were pretreated with vehicle or $CFTR_{inh}$-172 (10 μM) for 72 hours and, when applicable, stimulated during the last 6 hours with 100 ng/ml LPS from *Pseudomonas aeruginosa*. Quantitative reverse transcriptase polymerase chain reaction was used to determine the mRNA levels of XBP-1s. Data are expressed as fold change relative to 18S mRNA and represent mean ± SD from six independent experiments. Paired *t* test was used for the statistical analysis. $^{**}P < 0.01$.

Inhibition of IRE1α-Dependent XBP-1s Decreases LPS-induced Inflammation

We next evaluated whether inhibition of IRE1α-dependent XBP-1s decreased AM cytokine secretion. The effect of the IRE1α inhibitor 4μ8C (40, 41) was tested on LPS-induced IRE1α activation–dependent XBP-1s and cytokine production in non-CF and CF AMs. AMs were pretreated for 1 hour with 50 μM 4 μ8C (40, 41) before induction of inflammation with 100 ng/ml LPS. Our pilot studies indicated that this dose promotes the highest inhibition of LPS-induced IL-6 mRNA associated with IL-6 secretion (*see* Figure E3). 4μ8C significantly decreased LPS-induced XBP-1s mRNA levels in non-CF and CF AM cultures, as compared with LPS-treated cultures that were not administered 4μ8C (Figure 8A). Pretreatment with 4μ8C significantly decreased LPS-increased TNF-α mRNA levels by 57% and 83%, and IL-6 mRNA levels by 52% and 87%, respectively, in non-CF and CF AMs (Figures 8B and 8C). In addition, 4μ8C significantly decreased LPS-up-regulated TNF-α secretion by 45% and 89%, and IL-6 secretion by 41% and 76%, respectively, in non-CF and CF AM cultures (Figures 8D and 8E). Notably, the decrease of TNF-α and IL-6 protein secretion resulting from 4μ8C pretreatment was more marked in CF versus non-CF AMs. These findings suggest that 4μ8C has antiinflammatory properties resulting from its inhibitory effect on IRE1α-mediated XBP-1s in AMs.

XBP-1 Is Required for LPS-induced AM Cytokine Production

The previously mentioned data indicate that activation of IRE1α-dependent XBP-1s is

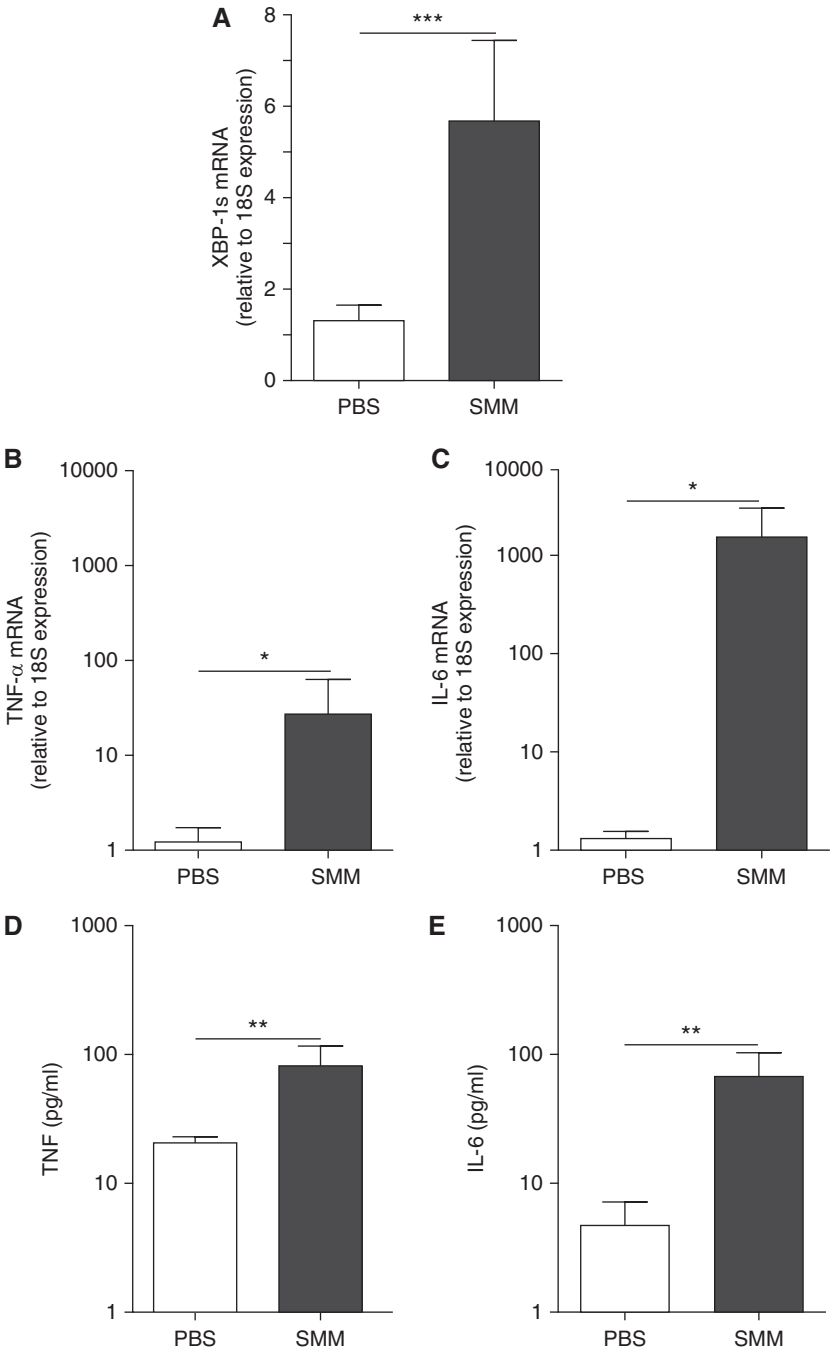

Figure 7. The increased X-box–binding protein-1 mRNA splicing (XBP-1s) and the robust inflammation of cystic fibrosis (CF) alveolar macrophages reflect an acquired response to the luminal infectious and inflammatory milieu of CF airways. Primary cultures of non-CF alveolar macrophages were exposed to phosphate-buffered saline (PBS) or supernatant from mucopurulent material (SMM; 1:3 dilution; pooled from the airways of five human CF lungs) for 3 hours. Quantitative reverse transcriptase polymerase chain reaction was used to evaluate the levels of XBP-1s (*A*), tumor necrosis factor (TNF)-α (*B*), and IL-6 (*C*) mRNA. Data are expressed as fold change relative to 18S mRNA. TNF-α (*D*) and IL-6 (*E*) protein secretion into the culture media was determined by ELISA. *Open bars* correspond to PBS exposure. *Solid bars* correspond to SMM exposure. The *y-axis* uses a logarithmic scale for TNF-α and IL-6 mRNA and protein secretion. Data are from six independent experiments and represent mean ± SD. Paired *t* test was used for the statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

required for AM inflammatory responses, and that XBP-1s mediates, at least in part, the robust inflammatory phenotype of CF AMs. To further evaluate the functional role of XBP-1 in AM inflammatory responses, a XBP-1-specific short hairpin RNA (shRNA) was used to knockdown XBP-1 in cultures of dTHP-1 cells. In the absence of LPS stimulation, the XBP-1 shRNA knocked down the baseline mRNA levels of XBP-1s by approximately 60% as compared with control shRNA (Figure 9A). LPS-up-regulated XBP-1s was significantly blunted in XBP-1 shRNA expressing cells as compared with control cells (Figure 9A). The XBP-1 shRNA also blunted LPS-increased TNF-α and IL-6 mRNA levels (Figures 9B and 9C) and LPS-up-regulated TNF-α and IL-6 protein secretion (Figures 9D and 9E), as compared with cells expressing the control shRNA. Notably, the inhibitory effect of the XBP-1 shRNA was stronger for LPS-stimulated TNF-α versus IL-6 secretion.

These findings suggested that in dTHP-1 cells the temporal and quantitative regulation of TNF-α and IL-6 by XBP-1 is different. To investigate this disparity, we evaluated the mRNA levels and protein secretion of TNF-α and IL-6 in control and XBP-1 shRNA expressing dTHP-1 cells at an earlier time point, after 3 hours LPS stimulation. LPS up-regulated the mRNA and protein secretion levels of TNF-α (*see* Figure E4). The mRNA levels of TNF-α were greatly decreased and TNF-α protein secretion was not detectable after 3 hours LPS in cells expressing the XBP-1 shRNA (*see* Figure E4). At 3 hours postexposure to LPS the IL-6 mRNA levels and IL-6 secretion were decreased in cells expressing the XBP-1 shRNA, but these inhibitory responses were not as robust as those for LPS-induced TNF-α (*see* Figure E4). We speculate that, unlike the rapid inhibition of TNF-α transcription, there was a delay in inhibition of IL-6 mRNA levels after 3 hours LPS in XBP-1 shRNA expressing cells that produced a pool of IL-6 mRNAs to be translated into IL-6 protein at 3 hours and, then, at 6 hours LPS exposure.

To directly test the role of XBP-1s in LPS-induced inflammatory responses relevant to CF AMs, dTHP-1 cells stably overexpressing a retroviral pQCXIN vector (control), a pQCXIN vector expressing a DN-XBP-1 construct, or a pQCXIN vector expressing a XBP-1s construct (24, 27, 42) were used. LPS up-regulated XBP-1s levels

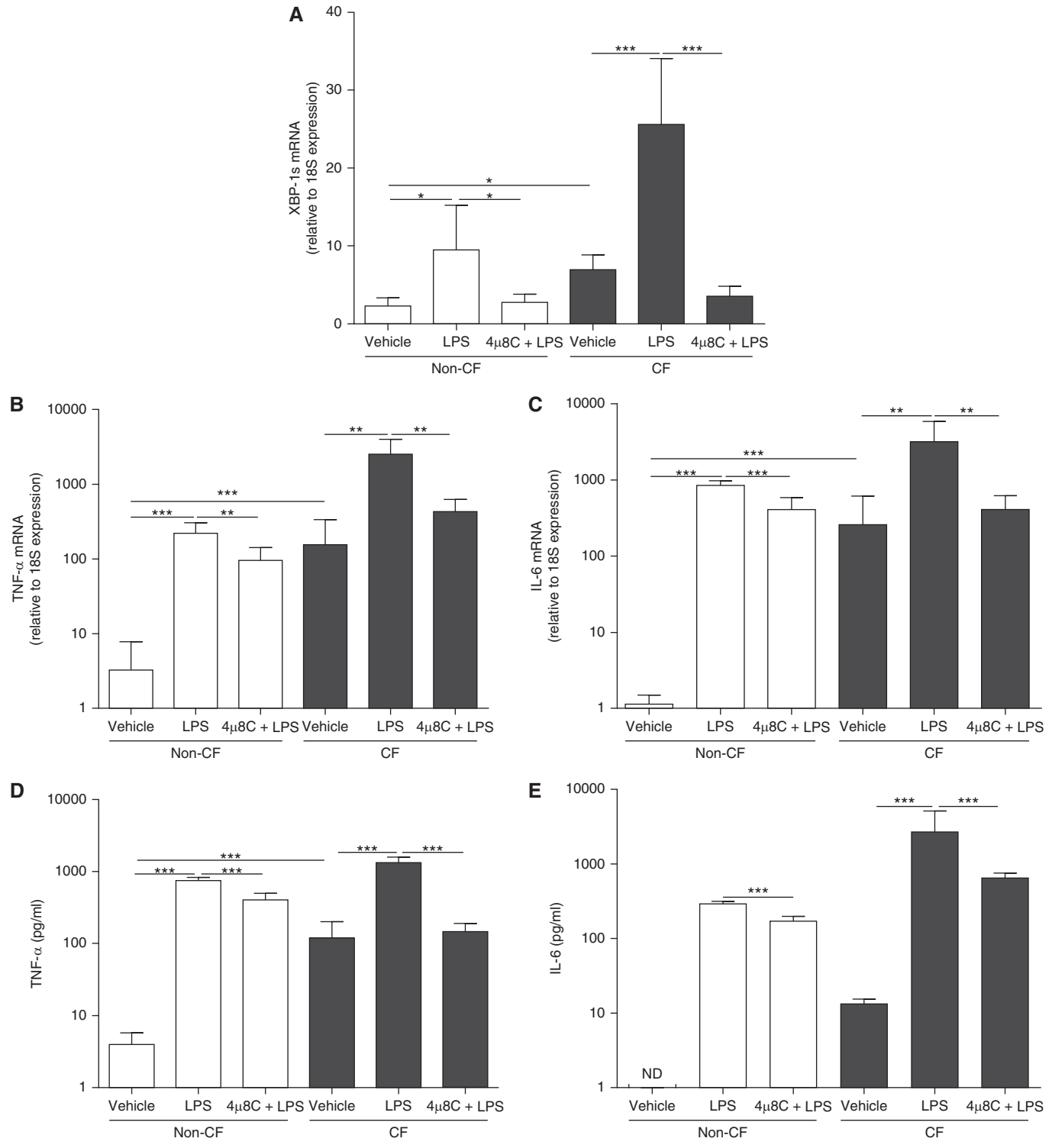

Figure 8. Inhibition of inositol-requiring enzyme 1 α–dependent X-box–binding protein-1 mRNA splicing (XBP-1s) suppresses LPS-induced cytokine secretion in primary cultures of alveolar macrophages (AMs). Non–cystic fibrosis (CF) and CF AMs were stimulated for 6 hours with 100 ng/ml LPS from *Pseudomonas aeruginosa* in the absence or presence of the Inhibition of inositol-requiring enzyme 1 α inhibitor 8-formyl-7-hydroxy-4-methylcoumarin (4μ8C) (50 μM; 1 h pretreatment). Quantitative reverse transcriptase polymerase chain reaction was used to evaluate the levels of XBP-1s (*A*), tumor necrosis factor (TNF)-α (*B*), and IL-6 (*C*) mRNA. Data are expressed as fold change relative to 18S mRNA. TNF-α (*D*) and IL-6 (*E*) secretion into the culture media was determined by ELISA. *Open bars* correspond to primary cultures of non-CF AMs. *Solid bars* correspond to primary cultures of CF AMs. The *y-axis* uses a logarithmic scale for TNF-α and IL-6 mRNA and protein secretion. Data are from five independent experiments and represent mean ± SD. Unpaired *t* test was used to compare non-CF with CF AMs and paired *t* test was used to compare the different treatments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. ND = not detected.

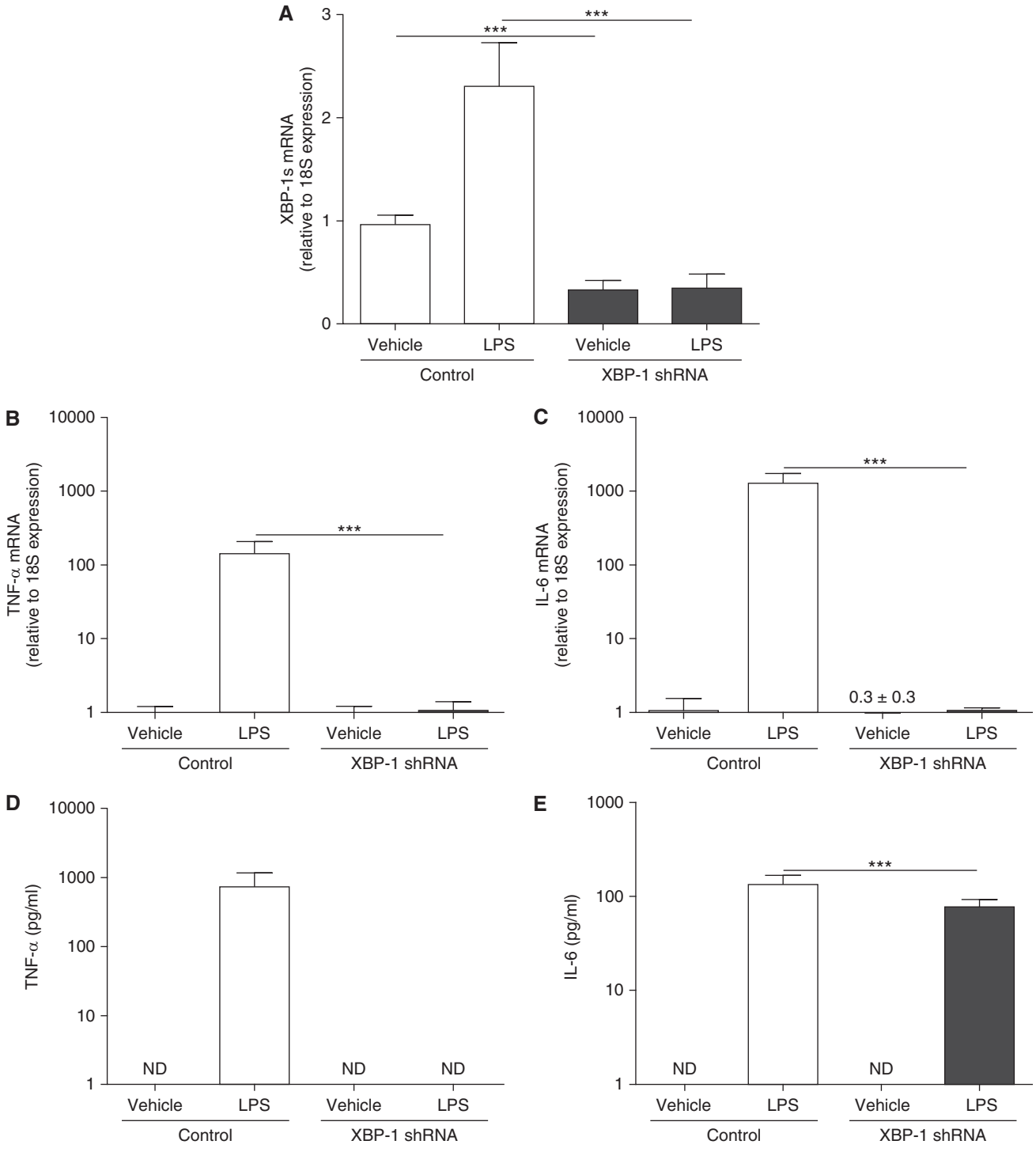

Figure 9. Knockdown of X-box–binding protein-1 (XBP-1) suppresses LPS-induced XBP-1 mRNA splicing (XBP-1s) and cytokine production. Macrophage-like differentiated THP-1 cultures stably expressing a control vector or a XBP-1-specific short hairpin RNA (shRNA) were stimulated during 6 hours with 100 ng/ml LPS from *Pseudomonas aeruginosa*. Quantitative reverse transcriptase polymerase chain reaction was used to evaluate the levels of XBP-1s (*A*), tumor necrosis factor (TNF)-α (*B*), and IL-6 (*C*) mRNA. Data are expressed as fold change relative to 18S mRNA. TNF-α (*D*) and IL-6 (*E*) protein secretion into the culture media was determined by ELISA. The *y-axis* uses a logarithmic scale for TNF-α and IL-6 mRNA and protein secretion. Data are from six independent experiments and represent mean ± SD. One-way analysis of variance was used for the statistical analysis. ***$P < 0.001$. ND = not detected. The fold change of IL-6 mRNA from dTHP-1 cells expressing XBP-1 shRNA and exposed to vehicle is 0.3 ± 0.3.

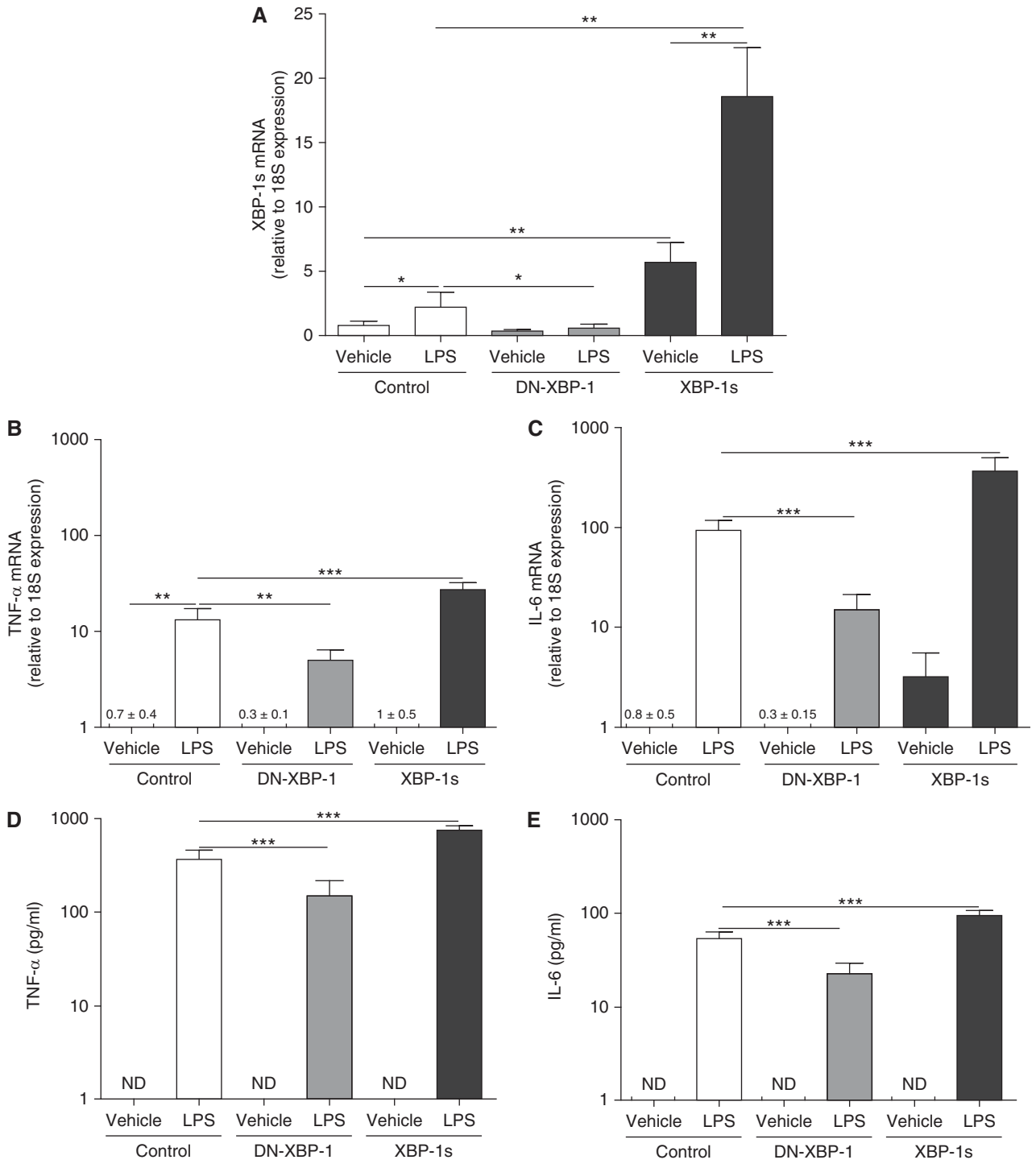

Figure 10. LPS-dependent cytokine production is mediated by X-box–binding protein-1 mRNA splicing (XBP-1s). Macrophage-like differentiated THP-1 cultures stably expressing a control pQCXIN vector, a pQCXIN vector containing a dominant negative XBP-1 (DN-XBP-1), or a pQCXIN vector containing XBP-1s were stimulated for 6 hours with vehicle or 100 ng/ml LPS from *Pseudomonas aeruginosa*. The levels of XBP-1s (*A*), tumor necrosis factor (TNF)-α (*B*), and IL-6 (*C*) mRNA were analyzed by quantitative reverse transcriptase polymerase chain reaction and expressed as fold change relative to 18S mRNA. TNF-α (*D*) and IL-6 (*E*) protein secretion into the culture media was determined by ELISA. The *y-axis* uses a logarithmic scale for TNF-α and IL-6 mRNA and protein secretion. Data are from six independent experiments and represent mean ± SD. One-way analysis of variance was used for the statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ spliced XBP-1 or DN-XBP-1 expressing cells versus control cells. ND = not detected. The fold changes of TNF-α mRNA in dTHP-1 cells expressing control, DN-XBP, and XBP-1s and exposed to vehicle are 0.7 ± 0.5, 0.3 ± 0.1, and 1 ± 0.5, respectively. The fold changes of IL-6 mRNA in dTHP-1 cells expressing control and DN-XBP-1 and exposed to vehicle are 0.8 ± 0.5 and 0.3 ± 0.15, respectively.

in control cultures, and this response was attenuated in cells expressing the DN-XBP-1 (Figure 10A). The inhibitory effect of the DN-XBP-1 was also observed for LPS-up-regulated TNF-α and IL-6 mRNA levels (Figures 10B and 10C) and protein secretion (Figures 10D and 10E). Assessment of XBP-1s mRNA confirmed that XBP-1s mRNA levels were increased in dTHP-1 cell overexpressing XBP-1s (Figure 10A). Importantly, LPS-increased XBP-1s, TNF-α, and IL-6 mRNA levels (Figures 10A–10C) and TNF-α and IL-6 protein secretion (Figures 10D and 10E) were potentiated in dTHP-1 cells overexpressing XBP-1s. These findings further establish the functional role of XBP-1s in AM inflammatory responses.

Discussion

AMs are critical for the maintenance of airway homeostasis, but their role in CF lung disease has not been fully studied. AMs clear the air spaces of infectious, toxic, and allergic particles; regulate innate alveolar defense against infection by secreting cytokines (43); and initiate inflammatory responses by recruiting activated neutrophils into air spaces (44). However, AM dysregulation could impair resolution of inflammation via a failure to act as suppressor cells, leading to chronic pulmonary infection and inflammation (38). Furthermore, in chronically obstructed CF airways, the persistent exposure of AMs to the infectious/inflammatory milieu can lead to persistent and, possibly, inappropriate activation of their inflammatory responses, contributing to lung damage.

Activation of the UPR has been implicated in airway epithelial inflammatory responses characteristic of CF airways (25, 26). Freshly isolated airway epithelia from chronically infected/inflamed human CF lungs exhibit increased levels of XBP-1s, and XBP-1s is required for cytokine production by inflamed human airway epithelia (24). These findings suggested that the IRE1α–XBP-1 arm of the UPR plays a pivotal role in CF airway epithelial inflammation. Based on the role of XBP-1s in inflammatory responses of airway epithelia, we hypothesized that AMs from chronically infected/inflamed human CF lungs also exhibit robust inflammation (e.g., increased basal and LPS-stimulated cytokine production), and XBP-1s is required for these responses.

Our data demonstrate that primary cultures of human CF AMs indeed exhibit a robust inflammatory phenotype (Figure 2). The greater inflammatory response of CF AMs required IRE1α activation–dependent generation of XBP-1s based on these findings: (*1*) increased XBP-1s levels were associated with increased LPS-induced cytokine production (Figures 5, 8–10); (*2*) treatment with the IRE1α inhibitor 4μ8C reduced LPS-increased XBP-1s, TNF-α, and IL-6 mRNA levels and TNF-α and IL-6 protein secretion (Figure 8); (*3*) knockdown of XBP-1 inhibited LPS-increased XBP-1s, TNF-α, and IL-6 mRNA levels and TNF-α and IL-6 protein secretion (Figure 9); (*4*) overexpression of DN-XBP-1 decreased LPS-up-regulated XBP-1s and inhibited LPS-induced TNF-α and IL-6 production (Figure 10); and (*5*) macrophage cultures overexpressing XBP-1s exhibited increased LPS-induced TNF-α and IL-6 production (Figure 10).

The baseline levels of IL-6 and TNF-α mRNA and protein secretion in CF AMs were higher than non-CF AMs, and the absolute increase after LPS stimulation was greater in CF than non-CF AMs (Figure 2). The same effect was observed with the mRNA levels of XBP-1s (Figure 5). These data suggest that native CF AMs exhibit higher XBP-1s-mediated TNF-α and IL-6 production in response to acute and chronic exposure to bacterial infection (and LPS) in CF lungs. Note, the *in vitro* exposure to LPS resulted in a lower fold increase of these genes in CF versus non-CF AMs, perhaps reflecting the higher basal levels in CF AMs.

It is generally accepted that LPS promotes cytokine production via activation of the transcription factor nuclear factor (NF)-κB during innate immune responses to pathogens (45). Our study suggests that TLR activation by LPS triggers IRE1α activation–dependent XBP-1 mRNA splicing, which identifies a novel pathway required for LPS-induced cytokine production and secretion in human AMs.

These findings agree with a report describing the role of XBP-1 in innate immune responses of peripheral macrophages based on the observations that transgenic mice deficient for XBP-1 in peripheral macrophages exhibited decreased inflammatory responses (31). This study also suggested that LPS (via TLR-4) and IRE1α/XBP-1 are interconnected and cooperate to maximize innate immune responses to pathogens (31). Activation of TLRs can couple to activation of IRE1α via TRAF6- and NOX2-dependent pathways (46). The activated IRE1α interacts via TRAF2 with the IKK complex and with ASK and JNK protein kinases (47), thereby modulating activation of the transcription factors NF-κB and AP-1 (48). Deletion of the IRE1α gene reduces proinflammatory cytokine production because of impairment of JNK activation and the lack of functional XBP-1s in bone marrow–derived macrophages from IRE1α knockout mice (41). In parallel, the XBP-1s resulting from IRE1α activation can directly promote transcriptional up-regulation of cytokine genes, contributing to maximal cytokine production (46). These findings suggest that the two mechanisms (e.g., IRE1α-dependent XBP-1s and IRE1α-mediated NF-κB and/or JNK activation) can coexist with canonical LPS–NF-κB signaling in AMs. Additional studies are necessary to define the pathways for IRE1α-regulated NF-κB and JNK activation in AM inflammatory responses.

The IRE1α inhibitor 4μ8C, a synthetic coumarin derivative, blocks substrate access to the active site of IRE1α and selectively inactivates IRE1α-dependent mRNA splicing of XBP-1 (40). Several studies have shown that coumarin compounds have strong antiinflammatory effects (49, 50), including findings suggesting that coumarin compounds can also target NF-κB (51) and mitogen-activated protein kinase (52). Our data demonstrated that 4μ8C decreased the levels of XBP-1s and suppressed LPS-induced cytokine production (Figure 8). Additional studies are needed to expand the understanding of the mechanism underlying the antiinflammatory action of 4μ8C in CF airways disease.

Previous studies have reported that CFTR is expressed in murine and human AMs (12) and CFTR malfunction in macrophages is directly linked with the robust inflammation in CF (14–16). It has been suggested that altered properties of murine CF AMs may contribute to uncontrolled lung inflammation (14, 15). For instance, it has been reported that functional CFTR is critical for regulation of phagosomal pH in murine AMs (12), and CFTR-deficient macrophages fail to acidify lysosomes and phagolysosomal

compartments and display altered bactericidal activity (13, 16, 19, 53). Furthermore, malfunction of CFTR and excessive inflammation in human and murine macrophages have been associated with a higher proinflammatory cytokine secretion (14, 38).

Our findings suggest that the robust inflammatory response of human CF AMs reflects an adaptive response to the chronic infectious/inflammatory milieu of CF airways *in vivo* and is independent of CFTR function, based on the following observations. First, as compared with the levels of CFTR expression in HBE cultures, the levels of CFTR expression in non-CF AMs are close to zero (Figure 3). Second, pretreatment of non-CF AMs with $CFTR_{inh}$-172 neither increased basal PS-induced cytokine production (Figure 4). Third, exposure of non-CF AMs to SMM, the infectious/inflammatory milieu of native CF airways, reproduced the robust inflammatory phenotype of CF AMs coupled to larger XBP-1s levels (Figure 7). Hence, although very low levels of CFTR expression may be important for regulation of other AM functions (12, 13), the present studies indicate that the exaggerated inflammation of primary cultures of human CF AMs is not linked to defective CFTR function.

In summary, our study revealed that AMs harvested from chronically infected/inflamed human CF lungs exhibit increased cytokine production and secretion. These data indicate that this response reflects a cellular adaptation to the infectious/inflammatory milieu of CF airways and requires activation of IRE1α/XBP-1. The observation that CF AMs exhibit a larger response to LPS-induced inflammation in an XBP-1s–dependent manner supports the notion that XBP-1s is a positive regulator of genes resulting from TLR-4 activation in AMs. These findings offer the proof-of-principle that targeting the IRE1α/XBP-1 pathway may be a therapeutic strategy to decrease the robust inflammatory response of AMs in chronically infected/inflamed CF lungs. This view is supported by recent studies suggesting that targeting the IRE1α/XBP-1 pathway may improve clinical outcomes for patients with drug-resistant pre-B-cell acute lymphoblastic leukemia (54) and patients with multiple myeloma (55). ■

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