Critical Modifier Role of Membrane-Cystic Fibrosis Transmembrane Conductance Regulator-Dependent Ceramide Signaling in Lung Injury and Emphysema

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Critical Modifier Role of Membrane-Cystic Fibrosis Transmembrane Conductance Regulator-Dependent Ceramide Signaling in Lung Injury and Emphysema

Manish Bodas,* Taehong Min,* Steven Mazur,* and Neeraj Vij*†

Ceramide accumulation mediates the pathogenesis of chronic obstructive lung diseases. Although an association between lack of cystic fibrosis transmembrane conductance regulator (CFTR) and ceramide accumulation has been described, it is unclear how membrane-CFTR may modulate ceramide signaling in lung injury and emphysema. Cftr/fl and Cftr−/− mice and cells were used to evaluate the CFTR-dependent ceramide signaling in lung injury. Lung tissue from control and chronic obstructive pulmonary disease patients was used to verify the role of CFTR-dependent ceramide signaling in pathogenesis of chronic emphysema. Our data reveal that CFTR expression inversely correlates with severity of emphysema and ceramide accumulation in chronic obstructive pulmonary disease subjects compared with control subjects. We found that chemical inhibition of de novo ceramide synthesis controls *Pseudomonas aeruginosa*-LPS–induced lung injury in Cftr−/− mice, whereas its efficacy was significantly lower in Cftr−/− mice, indicating that membrane-CFTR is required for controlling lipid-raft ceramide levels. Inhibition of membrane-ceramide release showed enhanced protective effect in controlling *P. aeruginosa*-LPS–induced lung injury in Cftr−/− mice compared with that in Cftr+/+ mice, confirming our observation that CFTR regulates lipid-raft ceramide levels and signaling. Our results indicate that inhibition of de novo ceramide synthesis may be effective in disease states with low CFTR expression like emphysema and chronic lung injury but not in complete absence of lipid-raft CFTR as in ΔF508-cystic fibrosis. In contrast, inhibiting membrane-ceramide release has the potential of a more effective drug candidate for ΔF508-cystic fibrosis but may not be effectual in treating lung injury and emphysema. Our data demonstrate the critical role of membrane-localized CFTR in regulating ceramide accumulation and inflammatory signaling in lung injury and emphysema. The Journal of Immunology, 2011, 186: 602–613.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: Asm, acid sphingomyelinase; AMT, amitriptyline; BALF, bronchoalveolar lavage fluid; CD, methyl-β-cyclodextrin; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, cigarette smoke extract; ΔF508-CF, ΔF508-cystic fibrosis; FB1, fumonisin-B1; FEV1, forced expiratory volume; FVC, forced vital capacity; i.t., intratracheal; MPO, myeloperoxidase; Pa-LPS, *Pseudomonas aeruginosa*-LPS; PBA, penicillin, streptomycin, and amphotericin B; SPE, signal protein extraction; Treg, regulatory T cell; ZO, zona occludens.

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that expression of the mutant form of CFTR lacking the PDZ-interacting domain (ΔTRL), modulates its role as a pattern recognition molecule (26) and results in ceramide accumulation.

Our current work supports and expands these important findings and correlates the expression of membrane and lipid-raft (27, 28) localized CFTR with ceramide signaling and severity of lung disease. Our data show that CFTR regulates tight junction formation (29), ceramide accumulation, and inflammatory signaling in lung injury and emphysema.

**Materials and Methods**

**Reagents and treatments**

The cells were cultured at 37°C with 5% CO2 in MEM (CFBE41o−, CFBE41o−→WT-CFTR [from Dr. Dieter Gruener, University of California, under material transfer agreement]), DMEM/F12 (HEK-293), or RPMI 1640 (sphingolipids, neutrophils, and macrophages) media, supplemented with 10% FBS and 1% penicillin, streptomycin, and amphotericin B (PSA) from Invitrogen (Carlsbad, CA). The *P. aeruginosa*-LPS (PS-LPS; Sigma, St. Louis, MO), fumonisin-B1 (FB1; Cayman Chemicals, Ann Arbor, MI), amitriptyline (AMT; Sigma), methyl-β-cyclodextrin (CD; Sigma), Con A (Sigma), TNF-α (Invitrogen), and cigarette smoke extract (CSE; Marly Pharmaceuticals, Lexington, KY) treatments were used for the indicated time points. For in vitro experiments, cells were treated with 10 ng/ml Pa-LPS, 50 μM FB1, 50 μM AMT, 5 mM CD, 5 or 10 μg/ml Con A, 10 ng/ml TNF-α, and/or 0–160 μg/ml CSE as described. Mice were treated by intratracheal (i.t.) instillation with 20 μg Pa-LPS, 50 μg FB1, 50 μg AMT, and/or 50 μg CD as indicated in 100 μl total volume of PBS, and control mice received PBS alone.

**Murine experiments and human subjects**

All animal experiments were carried out in accordance with Johns Hopkins University (Baltimore, MD) Animal Care and Use Committee-approved protocols. We used age-, weight-, and sex-matched (24 wk old), B6-Cftr+/+ male mice and correlates the expression of membrane and lipid-raft (27, 28) localized CFTR with ceramide signaling and severity of lung disease. Our data show that CFTR regulates tight junction formation (29), ceramide accumulation, and inflammatory signaling in lung injury and emphysema.

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with oil (×40 confocal and ×63 fluorescence) and air (×20 and ×40 fluorescence) as the imaging medium. The magnifications for the confocal and fluorescence microscopes were EC Plan-Neofluar (×40/1.3 oil, confocal), LD Plan-Achromat (×200/0.40 Korr Phz, fluorescence), LD Plan-Neofluor (×40/×0.6 Phz Korr, fluorescence), and LD Plan-Achromat (×63/1.4 oil), respectively, with ×1.6 opticvar. Splenocytes were isolated from Cftr+/+ and Cftr−/− mice for flow cytometry, and non-specific Ab binding was blocked by incubating them with either donkey or goat IgG (1:100; Cell Signaling Technology, Danvers, MA) or FACS buffer (PBS in PBS) and double stained with CD4-PE (rat monoclonal; Santa Cruz Biotechnology), and CFTR or intracellular FcR III primary Abs followed by anti-rabbit FITC secondary Ab or stained with CD4-PE followed by intracellular IFN-γ–FITC (rat polyclonal; Invitrogen). The macrophages and neutrophils were double stained with the respective cell surface markers, Mac-3 (rat monoclonal; Abcam) and NIMP-R14 (rat monoclonal; Abcam) and ceramide or ZO-1 primary Abs followed by anti-rat R-PE, anti-mouse Alexa Fluor 488, or anti-rabbit FITC secondary Abs. The cells were stained and washed twice in FACS buffer and resuspended in 0.1% paraformaldehyde (USB, Cleveland, OH). Appropriate secondary Ab controls were used in all the flow cytometry experiments. The Fix & PermCell Permeabilization kit (Intronigene, Uden, The Netherlands). For reporter assays, CFBE41o−/− WT, CFTR or CFBE41o− cells were transfected with NF-κB firefly luciferase promoter (pGRL2) and renilla luciferase (pRLTK) control using Lipofectamine 2000 (Invitrogen). Renilla luciferase was used as an internal control for normalization of DNA and transfection efficiency of reporter constructs. Cells were treated with 10 ng/ml TNF-α and/or 50 μM FBL for 12 h, and luciferase activities were measured after overnight treatment using the Dual-Luciferase Reporter Assay System (Promega) as described previously (28). Data were normalized with internal renilla luciferase control for each sample, and the changes in reporter activities with CFTR over-expression were calculated.

Immunoblotting and lipid-raft isolation

Splenocytes from Cftr+/+ and Cftr−/− mice were isolated and stimulated with 5 μg/ml Con A for 12 h. Cells were washed in PBS, and total protein was isolated using the 1× M-PER Mammalian protein extraction reagent (Pierce) supplemented with protease inhibitor mixture (Sigma). The protein lysate was immunoblotted for FcR3 primary (Santa Cruz Biotechnology) or β-actin (Sigma) loading control and anti-rabbit IgG HRP secondary Abs (Amersham, Piscataway, NJ) and developed using the Super Signal West Pico Chemiluminescent Substrate kit (Pierce). Similarly, the total cell protein was quantified in each sample, and equal amount of protein (cells, 300 μg; and lung tissue, 500 μg) was used to purify the raft fraction. The SPE buffer-II was added followed by incubation on ice for 15 min with intermittent vortexing. The lysate was centrifuged at 20,000 × g for 15 min and the supernatant discarded. The pellet containing signal proteins was solubilized in a small amount of fucoidin solubilization buffer and used for immunoblotting of ZO-2 (Santa Cruz Biotechnology), goat primary and anti-goat IgG HRP and β-actin (Sigma, rabbit primary and anti-rabbit IgG HRP). The raft protein from mouse lungs or HEK-293 cells was immunoblotted with CFTR 570 Ab (mouse polyclonal Ab; procured from University of North Carolina, Chapel Hill and Cystic Fibrosis Foundation Therapeutics under a material transfer agreement).

Statistical analysis

Data are presented as the mean ± SEM of at least three experiments, and Student t test and ANOVA were used to determine the statistical significance. The murine and human microscopy data were analyzed by densitometry (MATLAB R2009b; Mathworks, Natick, MA) followed by Spearman’s correlation coefficient analysis to calculate the significance among the indicated groups.

Results

CFTR regulates innate and adaptive immune response

To confirm and expand the hypothesis that functional CFTR is a critical regulator of inflammatory signaling (28), we compared the immune profile of the gut-corrected Cftr−/− mice with that of the Cftr+/+ mice. We quantified the constitutive levels of proinflammatory cytokine IL-6 ex vivo in peritoneal macrophages and neutrophils isolated from Cftr+/+ and Cftr−/− mice (n = 3) and found significantly (p < 0.001) higher basal IL-6 levels in Cftr−/− compared with that in the Cftr+/+ mice. We also found a significant increase (p < 0.01) in constitutive neutrophil-MPO levels (Fig. 1B) in Cftr−/− compared with those in the Cftr+/+ mice, which is indicative of the activated state of neutrophils in the absence of CFTR. We confirmed this in vivo using the murine model and observed a significant increase (p < 0.05) in basal and PaLPS-induced MPO levels in BALF of Cftr−/− mice compared with those in the Cftr+/+ mice (Fig. 1C). To test the outcome of CFTR deficiency on the adaptive immune response, we quantified differences in cell proliferation and IL-6 secretion in splenocytes from Cftr+/+ and Cftr−/− mice. We did not find a significant difference in the nonactivated splenocytes, but Con A induced a significantly higher (**p < 0.01, ***p < 0.001) splenocyte proliferation and IL-6 secretion in Cftr−/− compared with that in Cftr+/+ (Fig. 1D, 1E). We confirmed that CFTR is expressed on murine splenocytes (Fig. 1F). The CFTR-deficient splenocytes demonstrate higher numbers of CD4+IFN-γ T cells (Fig. 1Fii) supporting the notion that the absence of CFTR results in a constitutive hyperinflammatory state by inducing the proinflammatory response. In addition, prevalence of regulatory T cells is reported in the hyperinflammatory COPD lungs (35). We compared the expression of FcR3 in Cftr+/+ and Cftr−/− mice and found constitutively higher numbers of CD4+FceRI+ T cells (Fig. 1Fii) by guest on April 25, 2022 http://www.jimmunol.org/ Downloaded from
which concurs with increased expression of lipid-raft marker ZO-1 (Fig. 2A, right panel). Although the Cftr<sup>−/−</sup> neutrophils show a similar increase in ZO-1 expression, ceramide levels remain unchanged (Fig. 2B). We speculate that other mechanisms may be involved in constitutive increase of neutrophil (MPO) activity in the absence of CFTR (14, 17). Our data indicate a mechanism by which CFTR regulates lipid-raft signaling and inflammatory cell function(s). The constitutive defect in the absence of CFTR compromises the ability of these inflammatory cells to respond to infection or injury resulting in pathogenesis of chronic lung disease.

**CFTR regulates membrane-ceramide signaling and pathogenesis of chronic emphysema**

Ceramide upregulation was recently correlated with emphysema (20), and it is known that CFTR deficiency leads to increased ceramide accumulation and lung injury (7). We verified this obser-

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**FIGURE 1.** CFTR regulates innate and adaptive immune responses. A and B, The macrophages and neutrophils isolated from Cftr<sup>−/−</sup> mice show significant increase in constitutive (A) IL-6 and (B) MPO (myeloperoxidase levels, only in neutrophils) secretion in the culture supernatants compared with that of the Cftr<sup>+/+</sup>. ***p < 0.001. C, The BALF from Cftr<sup>−/−</sup> mice show significant increase in the basal and Pa-LPS (20 μg i.t., 24 h) induced MPO levels compared with those of the Cftr<sup>+/+</sup>. *p < 0.05. D, The splenocytes from Cftr<sup>−/−</sup> mice show significantly higher Con A (5 or 10 μg/ml) induced cell proliferation compared with that of the Cftr<sup>+/+</sup>. **p < 0.01; ***p < 0.001. E, The culture supernatants from the splenocytes of D have significantly higher IL-6 levels in the Cftr<sup>−/−</sup> compared with those of the Cftr<sup>+/+</sup>. ***p < 0.001. F, The flow cytometry analysis shows Cftr expression in CD4<sup>+</sup> Cftr<sup>+/+</sup> mice splenocytes (i), and Cftr<sup>−/−</sup> splenocytes were used as a negative control. The significant increase in percentage of CD4<sup>+</sup>IFN-γ (ii) and CD4<sup>+</sup>Foxp3<sup>+</sup> (iii) cells in the Cftr<sup>−/−</sup> splenocytes compared with that of the Cftr<sup>+/+</sup> is indicative of the constitutive T cell activation in the absence of CFTR. G, Immunofluorescence staining verifies the increase in constitutive and Pa-LPS–induced Foxp3 expression (primary-rabbit polyclonal, secondary-goat anti-rabbit IgG-FITC) and nuclear localization in Cftr<sup>−/−</sup> mice lungs compared with that of the Cftr<sup>+/+</sup>. Original magnification ×20; scale bar, 50 μm. H, Differences in basal and Con A (5 μg/ml) induced Foxp3 expression in Cftr<sup>+/+</sup> and Cftr<sup>−/−</sup> splenocytes is confirmed by Western blotting. β-Actin blot shows the equal loading. I, Densitometry analysis of Foxp3 expression (in H) normalized to β-actin. Data represent n = 3 in each group, and error bars depict mean ± SEM.
significantly downregulated. We anticipate this as an outcome of is not completely absent in severe COPD lungs, its expression is expression of lipid-raft marker ZO-1 shows a significant increase in both the primary Abs indicated. Data from significant increase in ceramide-positive cells in cells. The ceramide, NF-κB immunostaining of lung sections from these mice for ceramide, NF-

FIGURE 2. Ceramide and ZO-1 expression is elevated in immune cells of Cfr⁻/⁻ mice. Flow cytometry analysis showing ZO-1 and ceramide expression in macrophages (A) and neutrophils (B) from Cfr⁺/+ and Cfr⁻/⁻ mice. Thioglycolate-elicited peritoneal macrophages and neutrophils were immunostained for Mac-3 (macrophage) and NIMP-R14 (neutrophil) markers, and co-staining with ceramide (left panels) or ZO-1 (right panels) Abs was used to quantify the percentage changes in the number of positive cells. The upper right quadrants show the percentage gated cells positive for both the primary Abs as indicated. Data from n = 3 mice show a very significant increase in ceramide-positive cells in Cfr⁻/⁻ mice (97.85%) derived macrophages compared with that of the Cfr⁺/+ mice (0.99%) (A, left panel), whereas neutrophils (B, left panel) have no change. In contrast, expression of lipid-raft marker ZO-1 shows a significant increase in both the cell types (A and B, right panels) in the absence of CFTR (Cfr⁻/⁻) indicating the role of CFTR in tight junction formation.

viation in lung sections from control (Gold 0, at risk) and COPD (Gold I, mild; II, moderate; and III–IV, severe and very severe emphysema) human subjects (Table I) and found that CFTR expression significantly decreases with disease severity while ceramide levels increase (Fig. 3A, 3R, p < 0.001). Although CFTR is not completely absent in severe COPD lungs, its expression is significantly downregulated. We anticipate this as an outcome of lung injury. These data imply that lipid-raft localization of CFTR (Fig. 3A, inset) controls ceramide accumulation and possibly severity of emphysema. We confirmed our findings in HEK-293 cells transfected with WT-CFTR and show that CS exposure treatment decreased cell surface expression of CFTR (mature, band C) in a dose-dependent manner (Fig. 3C, left panel). The non-transfected HEK-293 cells do not show the CFTR at this Ab concentration (Fig. 3C, right panel). Extending our findings in the murine model (C57BL/6 mice), we found that acute CS exposure (5 h/d for 5 d) diminished CFTR expression both in the mouse lung lysate (Fig. 3D, upper panel, and Fig. 3E, left panel, p < 0.01) and in the purified lipid-raft fraction (Fig. 3D, lower panel, and Fig. 3E, right panel, p < 0.001). Moreover, we also demonstrate that lungs of CS-exposed mice have significantly (p = 0.004, p = 0.9316) increased ceramide accumulation that is colocalized with ZO-1 (Fig. 3F), which implies that CS-mediated decrease in CFTR expression results in lipid-raft ceramide accumulation. Therefore, in accord with our previous observation (28), the current data verify that decreased cell surface and lipid-raft expression of CFTR correlates with the increased inflammation and emphysema (Fig. 3A, H&E staining, bottom panel).

CFTR expression regulates ceramide signaling in lung injury

To verify whether CFTR regulates ceramide signaling and outcome of lung injury, we used the Pa-LPS–induced mouse model of lung injury. We treated Cfr⁺/+ and Cfr⁻/⁻ mice with 20 μg/mouse Pa-LPS i.t. for 12 h, followed by either FB1 or AMT (50 μg/mouse) for another 24 h. We inhibited either the de novo ceramide synthesis (FB1) or membrane-ceramide release (AMT), as they have been shown to mediate the pathogenesis of emphysema and CF lung disease, respectively (7, 20). We measured BALF cytokines IL-6 and IL-1β in all the groups as a marker of Pa-LPS–induced proinflammatory insult and the efficacy of the drugs. We found that inhibition of de novo ceramide synthesis by FB1 in Cfr⁺/+ mice shows a 2-fold reduction (p < 0.05) in the Pa-LPS–induced IL-6 levels (Fig. 4Ai) and a very significant decrease (p < 0.001) in IL-1β secretion (Fig. 4Aii). In the absence of Cfr (Cfr⁻/⁻ mice), FB1 treatment decreased Pa-LPS–induced IL-6 (Fig. 4Aiii), but the magnitude of rescue was not as efficient as that in Cfr⁺/+ mice. In addition, IL-1β levels were unaltered by FB1 treatment in the Cfr⁻/⁻ mice (Fig 4Aiv). This was also verified by immunostaining of lung sections from these mice for ceramide, NF-κB, and neutrophil marker NIMP-R14 (Supplemental Fig. 2A, 2B).

In contrast, inhibition of membrane-ceramide release by AMT was unable to rescue Pa-LPS–induced IL-6 or IL-1β secretion in Cfr⁻/⁻ mice (Fig. 4Bii, 4Biii), whereas inhibition of membrane-ceramide in the Cfr⁻/⁻ mice showed a significant decrease (p < 0.05) in Pa-LPS–induced IL-6 and IL-1β levels (Fig. 4Biii, 4Biv). The ceramide, NF-κB, and NIMP-R14 immunostaining of murine lungs verified these findings (Supplemental Fig. 2A, 2B). Our data concur with findings of Teichgräber et al. (7) who showed that normalization of acid sphingomyelinase (Asm) levels by AMT treatment or partial genetic deficiency reduced pulmonary ceramide levels that protected Cfr-deficient mice from

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Table I. Patient characteristics
*P. aeruginosa* infection. Our results indicate that inhibition of de novo ceramide synthesis (not the release) by FB1 may be effective in disease states with low CFTR expression like emphysema and lung injury but not in total absence of apical or lipid-raft CFTR, for instance in ΔF508-cystic fibrosis (ΔF508-CF), where phenylalanine mutation impairs the folding and trafficking of CFTR to the plasma membrane.

**FIGURE 3.** Severity of inflammatory lung disease inversely correlates with the membrane-CFTR levels. A, Human lung tissue sections from each group at Gold stage 0 (at risk), I (mild), II (moderate), and III–IV (severe and very severe) COPD (n = 4 to 10) were stained with H&E (bottom row) showing a significant increase in inflammatory cells and emphysema in moderate and severe COPD compared with that in mild COPD. The lung tissue sections immunostained with CFTR (green, top row) or ceramide (green, third row) show significant decrease in membrane CFTR expression at advanced stage of COPD lung disease while ceramide levels increase. Nuclear (Hoechst) staining is shown in blue (second and fourth rows). Original magnification ×20 and ×63; scale bars: white, 50 μm; red, 10 μm; black, 100 μm. B, Densitometric analysis confirms the statistical significance (p < 0.001) and illustrates the correlation of CFTR and ceramide expression with severity of lung emphysema. C, The HEK-293 cells transfected with WT-CFTR and treated with increasing doses of CSE for 12 h (n = 3) show an inverse relationship between increasing CSE dose and expression of membrane CFTR (mature C band, left panel). The total cell lysates from HEK-293 cells, either control (a) or transfected with WT-CFTR (b), show the absence of CFTR (B and C bands) in the control cells (right panel). D, The lung lysates from air and CS exposed mice (n = 3) were used for either *Cftr* immunoprecipitation (CFTR-169, upper panel) or lipid-raft isolation, and CFTR protein levels were detected by Western blotting. The data show a significant decrease in membrane and lipid-raft CFTR protein expression in the lungs of CS-exposed mice. E, Densitometry analysis of membrane and raft CFTR expression from control and CS groups (in D) is shown as mean ± SEM of triplicate samples. **p < 0.01; ***p < 0.001. F, The longitudinal lung sections from air or CS exposed mice (same experiment as D) show an increased ceramide and ZO-1 co-staining (red arrow) in the CS-exposed lungs verifying that CS modulates lipid-raft and ceramide signaling in murine lungs.
the cell surface. In contrast, inhibition of Asm activity or membrane-ceramide release by AMT has potential application as a more effective drug treatment for ΔF508-CF but may not be effective in treating lung injury and emphysema.

**CFTR expression negatively regulates membrane-ceramide and lipid-raft signaling**

To elucidate the mechanism by which CFTR regulates lipid-raft signaling, we quantified the expression of tight junction protein ZO-2 in purified raft-protein extract from CFBE41o−WT-CFTR and CFBE41o− cells with or without Pa-LPS or FB1. We found that ZO-2 expression was downregulated by Pa-LPS or FB1, only in the presence of WT-CFTR (Fig. 5A). It is possible that Pa-LPS may induce more recruitment of WT-CFTR to the raft (41, 43), which in turn inhibits raft formation (low ZO-2). FB1 is also able to modulate ZO-2 expression by an unknown mechanism that needs further investigation. Moreover, in the absence of functional CFTR in CFBE41o− cells, we observed higher basal expression of ZO-2 compared with that in CFBE41o−WT-CFTR cells. We also observed that neither Pa-LPS nor FB1 is able to modulate ZO-2 expression in these cells (Fig. 5A). To confirm these data, we stained the lung sections from Cfr+/+ and Cfr−/− mice with ZO-2 and found a constitutively higher ZO-2 expression in the Cfr−/− deficient mouse lungs (Fig. 5B). We also tested another marker of tight junctions, ZO-1, and analyzed it by co-immunostaining with ceramide using the lung sections from Cfr+/+ and Cfr−/− mice that were treated with Pa-LPS or PBS. We found a constitutive increase in ceramide levels in the Cfr−/− mice lungs compared with that in the Cfr+/+ mice, which was significantly enhanced by Pa-LPS treatment. Moreover, ceramide was colocalized with ZO-1 indicating its presence in the membrane lipid-rafts. (Fig. 5C, 5D).

**Lack of PDZ binding domain modulates CFTR-dependent ceramide accumulation**

Our data demonstrate the importance of cell surface and lipid-raft CFTR in regulating ceramide-mediated inflammatory signaling. The C-terminal PDZ-interacting domain of CFTR protein is crucial for its apical membrane polarization and functional robustness (24, 25). To investigate the role of this domain in CFTR-dependent inflammatory responses, we overexpressed WT- or ΔTRL-CFTR (CFTR lacking the PDZ binding domain) in HEK-293 cells and quantified ceramide levels by flow cytometry. We found that expression of ΔTRL-CFTR triggers higher ceramide accumulation (Fig. 6A, upper panel), which is more prominent upon CSE treatment (Fig. 6A, lower panel). Expression of ΔTRL-CFTR also decreases the binding of E. coli LPS-Alexa Fluor 488 to the plasma membrane (Fig. 6B). Because CFTR has been described as a pattern recognition molecule for LPS binding (26), our data demonstrate that the PDZ binding domain of CFTR may be crucial for its function as a pattern recognition molecule. We also demonstrate that expression of ΔTRL-CFTR leads to less CFTR protein reaching the lipid-raft fraction (Fig. 6C, 6D). Treatment with TNF-α induces the localization of CFTR to the lipid rafts, but ΔTRL-CFTR mutation compromises its translocation to lipid-raft. Our data suggest that PDZ binding domain is required for CFTR membrane stability, and lipid-raft-localization and signaling (Fig. 7). We anticipate binding to PDZ domain-containing proteins (ZO-1/2) may be critical for this process.

**Discussion**

We and others have recently shown that alipid-raft–localized functional WT-CFTR is critical for controlling the innate immune response (7, 28, 36, 39, 44). Although the link between CFTR dysfunction and inflammatory pathophysiology of CF lung disease has been a subject of debate (45), recent work clarifies and discusses these findings that we have recently reviewed in detail (11, 28). In this study, we verify that CFTR is not only critical for regulating the innate immune response in epithelial cells but also regulates the adaptive immune response as lack of functional CFTR confers a hyperinflammatory phenotype to the splenocytes. It has been reported that CD4+ T cells from CF patients have lower IFN-γ response (46). We report in this study that mouse CD4+ T cells lacking CFTR (Cfr−/−) secrete higher amounts of IFN-γ compared with that of the Cfr+/+. A recent study by Carrigan et al. (47) showed that although natural regulatory T cells (Tregs) were increased in P. aeruginosa-infected Cfr−/− mice, depletion of Tregs did not alter the disease outcome. Our original finding shows that lack of functional CFTR was able to modulate Foxp3 expression in the lungs and the peripheral tissues indicative of increased number of Tregs. The lungs of COPD patients similarly harbor higher number of Tregs that are proposed to be
The proinflammatory response in the Cftr-deficient mice is known to be mediated by neutrophils and macrophages, the primary cells of the innate immune response (40, 51–54). We evaluated whether the defect in lipid metabolism in the absence of CFTR (7) extends to these immune effector cells. For these studies, we used the common Pa-LPS–induced acute lung injury model (55) that is also a component of air pollutants that cause lung inflammation (56). Notably, we observed increased ceramide staining in macrophages (Fig. 2A, left panel) but not neutrophils (Fig. 2B, left panel) from uninfected Cftr−/− mice, which correlates with the higher constitutive and Pa-LPS–induced proinflammatory cytokine levels. We also observed an increase in ZO-1 staining in both macrophages and neutrophils in the absence of CFTR. Some recent studies support our finding and have shown the expression of tight junction proteins like ZO-2 in human macrophages (57, 58). Our data support the recent findings that CFTR inhibition by CFTR small interfering RNA in human alveolar macrophages renders them a proinflammatory phenotype along with an increase in caveolin-1 expression, as it is related to inflammation and apoptosis of macrophages (59). Although constitutive activation of neutrophils in CF is well documented (44, 60), CFTR expression in neutrophils is a subject of debate. Based on current literature, CFTR expression in neutrophils is either very low or absent. It may be possible that lack of CFTR regulates neutrophil function in a ceramide-independent manner. The lower expression of functional CFTR protein on murine and human neutrophils compared to that of epithelial or other inflammatory cells (52) may account for lack of ceramide accumulation in the Cftr−/− over Cftr+/+. Moreover, a recent study inversely correlates CFTR-mediated SCN(−) transport with the MPO activity (14). We anticipate this as a potential mechanism of neutrophil activation in the Cftr−/− mice that mediates the pathogenesis of chronic lung disease in the presence of Pseudomonas aeruginosa infection or lung injury.

It is proposed that changes in sphingosine and sphingosine-1-phosphate uptake in the absence of CFTR may result in membrane-ceramide accumulation (13) that triggers a proinflammatory and proapoptotic response in the respiratory tract. Ceramide forms membrane platforms and alters small lipid rafts that consist of sphingomyelin and cholesterol. We anticipate that involved in controlling pulmonary inflammation or autoimmunity (48). We anticipate that a similar mechanism may be triggered in the absence of functional CFTR, and strategies directed to modulate functional Tregs to revert acute or chronic lung disease warrant further investigation (49, 50).

**FIGURE 5.** CFTR regulates lipid-raft expression and signaling via ceramide. A, CBFBE41o− WT-CFTR (WT-CFBE) and CBFBE41o− cells were stimulated with Pa-LPS (10 ng/ml) or FB1 (50 μM) for 24 h. The lipid-raft protein extracts were isolated from these cells, and expression of lipid-raft marker ZO-2 was quantified by Western blotting. Data show significant downregulation (>2-fold) of lipid-raft ZO-2 expression with Pa-LPS or FB1 treatment only in the presence of WT-CFTR indicating that CFTR is a critical regulator of Pa-LPS or ceramide mediated lipid-raft expression and signaling. The same membrane was blotted with α-actin as a loading control. B, Immunostaining for ZO-2 shows its increased expression in lung tissue sections from Cftr−/− mice (n = 3) compared with that of the Cftr+/+ (n = 3) (top panels) verifying that CFTR regulates the expression of lipid-raft protein, ZO-2. Nuclear staining is shown in blue (bottom panels). C, The lung sections from Cftr+/+ and Cftr−/− mice (n = 4–5), treated with PBS or Pa-LPS (20 μg/mouse; 24 h), immunostained for ZO-1 (green, goat anti-rabbit IgG FITC) and ceramide (red, donkey anti-mouse Dylight 594), show significant increase in constitutive and Pa-LPS–induced ZO-1 and ceramide levels (top row) in Cftr−/− compared with those in Cftr+/+. The colocalization of ceramide with ZO-1 verifies the lipid-raft localization of ceramide in the absence of CFTR. The CFTR immunostaining (green, third row, goat anti-rabbit IgG FITC) shows the CFTR expression levels in the Cftr−/− mice lungs, and Cftr−/− are shown as a negative control. Nuclear (Hoechst) staining is shown in blue (second and fourth rows) and H&E staining shows increase in constitutive and Pa-LPS induced inflammation (bottom row). Original magnification ×20; scale bars: white, 50 μm; red, 10 μm; black, 100 μm. D, The densitometry and Spearman’s correlation coefficient analysis of ZO-1 and ceramide staining (C) shows the statistical significance of immunostaining data.
ceramide accumulation in the absence of CFTR might change the function of proteins in the membrane by altering the composition of sphingomyelin–cholesterol-rich lipid rafts. In favor of this hypothesis, WT-CFTR expression in Cfr−/− cells controls ceramide accumulation and inflammatory signaling (13). The data also support the critical role of membrane or lipid-raft CFTR in ceramide biogenesis and pathogenesis of lung disease (21, 41). This raises the important question whether modulation of CFTR expression in airway diseases can contribute to pathogenesis of chronic lung disease. Notably, CSE has previously been shown to inhibit chloride secretion in human bronchial epithelial cells (61). A more direct correlation between CSE and CFTR expression was established by Cantin et al. (62), showing that CSE decreased CFTR expression in airway diseases can contribute to pathogenesis of chronic lung disease. Notably, CSE has previously been shown to inhibit chloride secretion in human bronchial epithelial cells (61). A more direct correlation between CSE and CFTR expression was established by Cantin et al. (62), showing that CSE decreased CFTR expression in airway diseases can contribute to pathogenesis of chronic lung disease. Notably, CSE has previously been shown to inhibit chloride secretion in human bronchial epithelial cells (61). A more direct correlation between CSE and CFTR expression was established by Cantin et al. (62), showing that CSE decreased CFTR expression in airway diseases can contribute to pathogenesis of chronic lung disease. Notably, CSE has previously been shown to inhibit chloride secretion in human bronchial epithelial cells (61). A more direct correlation between CSE and CFTR expression was established by Cantin et al. (62), showing that CSE decreased CFTR expression.

The previous clinical studies showing the association of CFTR mutations with asthma and COPD (18, 19, 65, 66) were not conclusive due to lack of sufficient controls. Moreover, only few reports have verified emphysema development in CF subjects (67). Our data suggest the critical modifier role of membrane CFTR and ceramide levels in pathogenesis of severe emphysema. An interesting question here is why CF subjects with severe emphysema develop or is recognized. Nonetheless, our data suggest that pathogenetic changes in membrane and lipid-raft CFTR may have a modifier function in pathogenesis of COPD and emphysema. Based on our data, we propose that the association of apical and lipid-raft CFTR expression with COPD disease severity and ceramide accumulation and signaling has a clinical application as both prognostic marker and therapeutic. Further clinical studies are warranted to confirm the role of CFTR as a modifier or pathogenetic susceptibility factor for COPD, emphysema, and asthma.

Because ceramide is an important component of lipid rafts (43), we hypothesized that disruption of raft CFTR by CD (28) may trigger ceramide accumulation and NF-κB activation. We selected CD treatment as a method to deplete selectively CFTR from the lipid-rafts over CFTR small interfering RNA or inhibitor as it would result in an overall decrease of CFTR expression and/or lipid-raft translocation. The transient expression of CFTR lacking the PDZ binding domain results in reduced binding of LPS to the plasma membrane. We anticipate that less LPS binding to ∆TRL expressing cells is a direct consequence of its reduced cell surface expression and/or lipid-raft translocation. C. The lipid-raft proteins from HEK-293 cells expressing WT-CFTR or ∆TRL-CFTR were analyzed for CFTR expression by Western blotting (Fig. 3A). We verified that acute CS exposure of Cfr−/− (C57BL/6) mice decreases cell surface and lipid-raft expression of CFTR (Fig. 3D). We also found an increase in colocalization of ceramide and ZO-1 (Fig. 3F) in the murine lungs after CS exposure. In support of our findings, a recent study (63) demonstrates that CS induces ceramide accumulation in human bronchial epithelial cells. We anticipate based on these data that ceramide accumulation and chronic P. aeruginosa infections in severe COPD patients (5) and CF (5, 64) may be an outcome of decreased CFTR expression.

FIGURE 6. The PDZ-interacting domain of CFTR regulates ceramide accumulation. A. The HEK-293 cells were transiently transfected with pEGFP WT-CFTR or ∆TRL-CFTR plasmid constructs, and one experimental group was treated with 100 μg/ml CSE for 12 h. The cells were stained and analyzed for ceramide (R-PE, FL-2) and GFP expression (FL-1) by flow cytometry. The data represent three independent experiments. Expression of CFTR lacking the PDZ-interacting domain shows an increase in basal (49.85–56.48%) and CSE-induced ceramide accumulation (69.22–80.56%), indicating the crucial role of PDZ binding domains in regulating CFTR-dependent ceramide signaling. B. The HEK-293 cells transiently overexpressing WT-CFTR or ∆TRL-CFTR plasmids (n = 3) were incubated with FITC-labeled E. coli LPS for 3 h and analyzed by flow cytometry (unpermeabilized cells). The transient expression of CFTR lacking the PDZ binding domain results in reduced binding of LPS to the plasma membrane. We anticipate that less LPS binding to ∆TRL expressing cells is a direct consequence of its reduced cell surface expression and/or lipid-raft translocation. C. The lipid-raft proteins from HEK-293 cells expressing WT-CFTR or ∆TRL-CFTR were analyzed for CFTR expression by Western blotting (a, 30-s exposure; b, 20-min exposure). The data show that lack of the PDZ-interacting domain of CFTR compromises its membrane expression (b, left panel) and translocation to the lipid-rafts (a and b, right panel). D. Densitometry analysis of membrane- and raft-CFTR expression from WT-CFTR and ∆TRL-CFTR groups in C.
lipid-raft CFTR in CFBE41o– cells (Supplemental Fig. 3D). We speculated that WT-CFTR might be regulating membrane-ceramide levels by its interaction with lipid-raft signaling complex (TNF-R1–sphingomyelin) while FB1 suppresses the de novo ceramide hydrolysis. We anticipated that in the CFTR-deficient scenario, membrane-ceramide accumulation is catalyzed by ASM; hence, inhibition of de novo ceramide synthesis is rendered ineffective. The importance of the ASM pathway in several disease models has been comprehensively reviewed (69). Recently, Teichgräber et al. (7) demonstrated that Cfr−/− mice induce lung ceramide accumulation via Asm, and its inhibition by AMT rescued the mice from P. aeruginosa infection. A clinical trial using AMT in CF patients also demonstrates its safety and efficacy as a potent drug candidate (70).

In the current study, we demonstrate that inhibition of de novo (FB1) or membrane-ceramide (AMT) synthesis/release has differential outcomes in controlling the Pa-LPS–induced lung injury in the presence and absence of CFTR. We found that in the presence of WT-CFTR, inhibition of de novo ceramide synthesis by FB1 inhibits Pa-LPS–induced NF-κB activity and recruitment of neutrophils in the lungs of Cfr−/− mice while its inhibitory effect was significantly lower in Cfr−/− mice indicating that WT-CFTR depletes NF-κB activity by controlling TNF-R1 or sphingomyelin (Fig. 7). Moreover, treatment with FB1 may not only prevent the ceramide synthesis but also deplete sphingomyelin levels. This may indirectly modulate the function of ASM that leads to lower ceramide generation and thereby decreased inflammation in Cfr−/− mice. In contrast, inhibition of ASM by AMT showed an enhanced protective effect in controlling the Pa-LPS–induced lung injury in Cfr−/− mice compared with that in the Cfr−/− indicating that inhibition of de novo ceramide synthesis by FB1 can be a more potent therapeutic strategy in lung injury, emphysema, and COPD where CFTR raft expression is depleted but not absent, whereas AMT may be more effective in absence of cell surface CFTR, as in the case of ΔF508-CF.

The previous observations that PDZ-interacting domain in CFTR is required for its apical polarization and Cl− channel function (24, 25) led us to investigate its role in CFTR-dependent ceramide and lipid-raft signaling. Our data demonstrate that the absence of CFTR PDZ binding domain (ΔTRL) leads to 1) reduction in membrane CFTR levels (Fig. 6C), 2) decrease in binding of E. coli LPS to the plasma membrane (Fig. 6B), and 3) increased ceramide accumulation in both constitutive and CSE-induced states (Fig. 6A). These findings elucidate a potential mechanism by which CFTR may be sequestered to the lipid-rafts, where it regulates ceramide-mediated inflammatory signaling. We anticipate binding to PDZ domain-containing proteins (ZO-1/2) is required for CFTR membrane stability and lipid-raft translocation. The current study not only describes the critical role of CFTR in pathogenesis of obstructive lung diseases but also demonstrates the scope of an intervention strategy targeting CFTR-dependent lipid-rafts and ceramide for treatment of lung injury and emphysema. In addition, we evaluate CFTR-dependent lipid-rafts as a biomarker for lung injury and emphysema and demonstrate its potential utility as a prognosticor of the aforementioned therapeutic strategy. It remains an open question whether the development of a potent CFTR corrector (CF-ΔF508) and potentiator (COPD and emphysema) drug (currently under Phase II–III clinical trial for CF by Vertex Pharmaceuticals, Cambridge, MA) may serve as an effective therapeutic strategy to overcome the ceramide-induced pathological conditions emerging from decreased membrane or lipid-raft CFTR expression. Because the Vertex drugs were identified based on their ability to correct CFTR chloride transport function only (71), we anticipate that the development of selective strategies to modulate CFTR-dependent

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**FIGURE 7.** Schematic of CFTR-mediated ceramide signaling. Schematic illustrates the critical role of lipid-raft CFTR in controlling ceramide (sphingomyelin) and inflammatory (TNF-α) or apoptotic (CD95) signaling. Our model predicts that the absence or decrease in lipid-raft CFTR expression culminates these regulatory functions, resulting in NF-κB-mediated hyperinflammatory response. Environmental factors such as P. aeruginosa infection or CS exposure further exaggerate the lipid-raft signaling and contribute to the pathogenesis of chronic inflammatory or apoptotic signaling by modulating CFTR lipid-raft expression that controls ceramide accumulation. We anticipate that in the absence of lipid-raft CFTR, membrane-ceramide accumulation induces lipid-raft fusion and large-scale clustering of the membrane receptors that result in lung injury and emphysema.
lipid-rafts and cystic fibrosis signaling as proposed in this study will have a more specific therapeutic outcome for treating the chronic stages of lung disease.

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Disclosures
The authors have no financial conflicts of interest.

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