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. $Cftr^{+/+}$ 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 effective 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.
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 Gunther, 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-L, Sigma, St. Louis, MO), fumonisin-B1 (FB1; Cayman Chemicals, Ann Arbor, MI), amitriptyline (ATM; Sigma), methyl-β-cyclodextrin (CD; Sigma), TNF-α (Invitrogen), and cigarette smoke extract (CSE; Marry 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 ATM, 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 ATM, 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), B6-129S6-Cftr+/+ mice purchased from the Tobacco Research Institute (University of Kentucky, Lexington, KY) for 5 h/d for 5 d. An average total particulate matter of 150 g/ml CSE as described above, and levels of mature (C form) and immature (B form) CFTR were quantified by Western blotting. The total protein extract was collected using the M-PER protein lysis buffer and total protein was determined using the Bio-Rad Protein Assay Kit. The Western blots were performed with or without 50 ng/ml TNF-α stimulation. For LPS binding experiments, total protein was collected using the M-PER protein lysis buffer and the protein concentration was determined using the Bio-Rad Protein Assay Kit. The Western blots were performed with or without 50 ng/ml TNF-α stimulation. For LPS binding experiments, the cells were treated with 10 ng/ml LPS (Molecular Probes, Eugene, OR) for 12 h. The total protein content was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The CFBE41o−→ΔTRL-CFTR cells were treated with PBS or 5 mM CD for 24 h on a 24-well plate, and IL-8 secretion in the cell supernatants was quantified by sandwich ELISA (R&D Biosystems, Minneapolis, MN). The HEK-293 cells were transiently transfected with WT-CFTR and incubated with increasing doses (0, 40, 80, 120, and 160 μg/ml) CSE for 12 h. The total protein content was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The CFBE41o−→ΔTRL-CFTR cells were treated with PBS or 5 mM CD for 24 h on a 24-well plate, and IL-8 secretion in the cell supernatants was quantified by sandwich ELISA (R&D Biosystems, Minneapolis, MN).

In vitro and ex vivo experiments

The macrophages and neutrophils from Cftr+/+ and Cftr+/− mice were isolated by i.p. injection of 1 ml 4% thiglycollate broth (Fluka, St. Louis, MO). The peritoneal cavity was flushed as indicated after 6 h (32) (neutrophils) or 4 d (33) (macrophages) with 10 ml RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 10% FBS (Life Technologies) and 1% PSA (Life Technologies) (complete RPMI medium). The lavage was centrifuged at 1200 rpm for 8–10 min at 4°C followed by RBC lysis in LCK lysis buffer (Quality Biologicals, Gaithersburg, MD). The 3 × 106 cells per well was plated in a 6-well plate and cultured overnight in complete RPMI medium. The culture supernatants were collected for cytokine ELISAs as well as myeloperoxidase (MPO) measurements. The spleens were dissected from Cftr+/+ and Cftr+/− mice and macerated using the plunger of a 5 ml BD (San Diego, CA) syringe. The suspension was subjected to RBC lysis as described above, and 2 × 106 spleenocytes per well were cultured in a 96-well plate. The cells were treated with 5 or 10 μg/ml Con A for 72 h. For spleocyte proliferation assay, 20 μl of the Cell Titer 96 AQueous One Solution reagent (Promega, Madison, WI) was added at the same time point, and the plate was incubated at 37°C. 5% CO2 for another 2 h. The OD at 490 nm was recorded by a 96 well microplate reader (Molecular Devices, Sunnyvale, CA) using SOFT-MAX-Pro software (Molecular Devices) as a measure of cell proliferation. For immunoblotting, spleenocytes (2 × 106 cells/well) were treated with 5 μg/ml Con A for 12 h, and the total protein extract was collected using the M-PER protein lysis buffer and 1× protease inhibitor mixture (Pierce, Rockford, IL). The human CF bronchial epithelial cells, CFBE41o−→CFBE41o−→WT-CFTR were cultured in MEM medium supplemented with 10% FBS (Life Technologies) and 1% PSA (Life Technologies). The CFBE41o−→WT-CFTR cells were cultured in the presence of 500 μg/ml Hygromycin B (Invitrogen) to maintain the stable expression of WT-CFTR. For fluorescence or confocal microscopy, equal numbers of cells were cultured in glass-bottom, collagen-coated, 35-mm Petri dishes (MatTek, Ashland, MA) at 10 ng/ml Pa-LPS, 5 mM CD, and/or 10 ng/ml TNF-α. The CFBE41o−→WT-CFTR cells were treated with PBS or 5 mM CD for 24 h on a 24-well plate, and IL-8 secretion in the cell supernatants was quantified by sandwich ELISA (R&D Biosystems, Minneapolis, MN). The HEK-293 cells were transiently transfected with WT-CFTR and incubated with increasing doses (0, 40, 80, 120, and 160 μg/ml) CSE for 12 h. The total protein content was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The CFBE41o−→ΔTRL-CFTR cells were treated with PBS or 5 mM CD for 24 h on a 24-well plate, and IL-8 secretion in the cell supernatants was quantified by sandwich ELISA (R&D Biosystems, Minneapolis, MN).

Immunofluorescence microscopy and flow cytometry

The longitudinal tissue sections from murine or human lungs or CFBE41o−→CFBE41o−→WT-CFTR cells were immunostained with the primary Abs (1:50 to 1:200 dilution) for CFTR (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), ceramide (mouse monoclonal; Alexis Biochemicals, Plymouth Meeting, PA), Fosp3 (rabbit polyclonal; Santa Cruz Biotechnology), NF-κB (rabbit polyclonal; Santa Cruz Biotechnology), ZO-1 (rabbit polyclonal; Santa Cruz Biotechnology), and neutrophil marker MIMP-R14 (rat monoclonal; Abcam, Cambridge, MA) followed by the secondary Abs (1:200 dilution), using our previously described protocol (34). The secondary Abs used were goat anti-rabbit IgG FITC (Santa Cruz Biotechnology), goat anti-rat IgG (H+L) PE, goat anti-mouse IgG/IgM (H+L), Alexa Fluor 488, donkey anti-goat Alexa Fluor 488 (Invitrogen), donkey anti-mouse Dylight 594, donkey anti-rabbit Dylight 594, and donkey anti-mouse Dylight 488 (Jackson ImmunoResearch, West Grove, PA). Nuclei were detected by Hoechst (Invitrogen) staining, and H&E was used to evaluate lung morphology and inflammatory state. Images were captured with an Axiovert 200 Carl Zeiss (Thornwood, NY) fluorescence microscope using the Zeiss Axiovid HRC camera and Axiovision software. The membrane localization of ZO-1 and ceramide in CFBE41o−→WT-CFTR cells was detected by confocal microscopy. The same protocol for confocal microscopy was similar to the immunofluorescence staining protocol. The images were captured using a Zeiss LSM 510 Meta confocal microscope and analyzed by Zeiss LSM Image Browser software. All fluorescent and confocal images were captured at room temperature.
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-Apochromat (×200/0.40 Korr Phz, fluorescence), LD Plan-Neofluar (×40/×0.6 Phz Korr, fluorescence), and LD Plan-Achromat (×63/1.4 oil), respectively, with 1.6× optic. Splenocytes were isolated from Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup> mice for flow cytometry, and non-specific Ab binding was blocked by incubating them with either donkey or goat serum. Cells were washed in FACS buffer (PBS in PBS) and double stained with CD4-PE (rat monoclonal; Santa Cruz Biotechnology), and CFT or intracellular Fop3 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-2 (rat monoclonal; Abcam) or 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 Permabilization kit (Intronigene) was used for IFN-γ, Fop3, and ceramide intracellular staining following the manufacturer’s protocol. The cells were acquired using the BD FACScalibur instrument, and analysis was done with the BD Cell Quest Pro software.

**ELISA, MPO activity, and reporter assay**

The BALF and cell culture supernatants (n = 3–5) were quantified in triplicate for mouse IL-6, IL-1β, or human IL-8 using ELISA kits (R&D Systems, or Ebsioscience, San Diego, CA) following the manufacturer’s instructions. MPO levels in neutrophil culture supernatant or mouse BALF were similarly quantified using the MPO ELISA kit (Hycult Biotechnology, Uden, The Netherlands). For reporter assays, CFBE410–WT, CFT or CFBE410– cells were transfected with NF-κB firefly luciferase promoter (pGL2) 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 transfected with 10 ng of TFP and/or 50 μM FB1 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 CFT over-expression were calculated.

**Immunoblotting and lipid-raft isolation**

Splenocytes from Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup> 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 Fop3 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 Pico Chemiluminescent Substrate kit (Pierce). Similarly, the total cell lysates from HEK-293 cells transiently transfected with the WT-CFT or those in the Cfr<sup>−/−</sup> mice were used to detect constitutive neutrophil-MPO levels in BALF of Cfr<sup>−/−</sup>− mice compared with those in the Cfr<sup>−/−</sup> mice. To test the outcome of CFT deficiency on the adaptive immune response, we quantified differences in cell proliferation and IL-6 secretion in splenocytes from Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup> mice. We did not find a significant difference in the nonactivated splenocytes, but Con A induced a significantly higher (***p < 0.01) in constitutive neutrophil-MPO levels (Fig. 1B) in Cfr<sup>−/−</sup>− compared with those in the Cfr<sup>−/−</sup> mice. We did not confirm that CFT is expressed on murine splenocytes (Fig. 1F). The CFT-deficient splenocytes demonstrate higher numbers of CD4<sup>+</sup>IFN-γ<sup>+</sup>T cells (Fig. 1Fii) supporting the notion that the absence of CFT 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 Fop3 in Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup>− mice and found constitutively higher numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> splenocytes in the Cfr<sup>−/−</sup>− (0.55%) compared with that in the Cfr<sup>−/−</sup> mice (0.32%) (Fig. 1Fii). We also confirmed this by Foxp3 immunostaining and Western blotting in lung sections and splenocytes, respectively (Fig. 1G, 1H). The data substantiate the previous observations (28, 36–40) and strongly suggest that CFT is a critical regulator of both innate and adaptive immune responses.

**CFT expression in inflammatory cells inversely correlates with the levels of ceramide and lipid-raft marker (Z0-1)**

Ceramide is a critical regulator of inflammatory and apoptotic signaling (20) and mediates these processes in lung injury (41), asthma (21), emphysema, COPD (20), and CF (7). Moreover, CFT is present in the lipid rafts (27, 42), and its role in regulating TNF-R1 and lipid-raft signaling has been examined previously (27). We tested the hypothesis that CFT may be regulating inflammatory signaling via ceramide by inhibiting the formation of membrane and lipid-raft platforms, which would hamper proper clustering of signaling receptor complexes on the plasma membrane. Evidence from previous studies (7) and our data show that macrophages from Cfr<sup>−/−</sup> mice have significantly higher ceramide levels compared with those of the Cfr<sup>−/−</sup> mice (Fig. 2A, left panel), immunoblotted with CFT 570 Ab (mouse polyclonal Ab; procured from University of North Carolina, Chapel Hill and Cystic Fibrosis Foundation Therapeutics under a material transfer agreement).

**Results**

CFT regulates innate and adaptive immune response

To confirm and expand the hypothesis that functional CFT is a critical regulator of inflammatory signaling (28), we compared the immune profile of the gut-correction Cfr<sup>−/−</sup> mice with that of the Cfr<sup>−/−</sup> mice. We quantified the constitutive levels of proinflammatory cytokine IL-6 ex vivo in peritoneal macrophages and neutrophils isolated from Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup> mice (n = 3) and found significantly (p < 0.001) higher basal IL-6 levels in Cfr<sup>−/−</sup>− compared with that in the Cfr<sup>−/−</sup> mice (Fig. 1A). We also found a significant increase (p < 0.01) in constitutive neutrophil-MPO levels (Fig. 1B) in Cfr<sup>−/−</sup>− compared with those in the Cfr<sup>−/−</sup> mice. We did not confirm that CFT may be regulating the innate immune response. We quantified differences in cell proliferation and IL-6 secretion in splenocytes from Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup> mice. The CFTR-deficient splenocytes demonstrate significantly higher numbers of CD4<sup>+</sup>IFN-γ<sup>+</sup>T cells (Fig. 1Fii) supporting the notion that the absence of CFT results in a constitutive hyperinflammatory state by inducing proinflammatory response. In addition, prevalence of T regulatory cells is reported in the hyperinflammatory COPD lungs (35). We compared the expression of Fop3 in Cfr<sup>−/−</sup> and Cfr<sup>−/−</sup>− mice and found constitutively higher numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> splenocytes in the Cfr<sup>−/−</sup>− (0.55%) compared with that in the Cfr<sup>−/−</sup> mice (0.32%) (Fig. 1Fii). We also confirmed this by Foxp3 immunostaining and Western blotting in lung sections and splenocytes, respectively (Fig. 1G, 1H). The data substantiate the previous observations (28, 36–40) and strongly suggest that CFT is a critical regulator of both innate and adaptive immune responses.

**Statistical analysis**

Data are presented as the mean ± SEM of at least three experiments, and Student’s 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.
which concurs with increased expression of lipid-raft marker ZO-1 (Fig. 2A, right panel). Although the Cfr⁻/⁻ 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-

**FIGURE 1.** CFTR regulates innate and adaptive immune responses. A and B, The macrophages and neutrophils isolated from Cfr⁻/⁻ 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 Cfr⁺/⁺. ***p < 0.001. C, The BALF from Cfr⁻/⁻ mice show significant increase in the basal and Pa-LPS (20 μg i.t., 24 h) induced MPO levels compared with those of the Cfr⁺/⁺. ***p < 0.001. D, The splenocytes from Cfr⁻/⁻ mice show significantly higher Con A (5 or 10 μg/ml) induced cell proliferation compared with that of the Cfr⁺/⁺ mice. ***p < 0.001; **p < 0.01. E, The culture supernatants from the splenocytes of D have significantly higher IL-6 levels in the Cfr⁻/⁻ compared with those of the Cfr⁺/⁺. ***p < 0.001. F, The flow cytometry analysis shows Cfr expression in CD4⁺ Cfr⁺/⁺ mice splenocytes (i), and Cfr⁻/⁻ splenocytes were used as a negative control. The significant increase in percentage of CD4⁺IFN-γ⁺ (ii) and CD4⁺Foxp3⁺ (iii) cells in the Cfr⁻/⁻ splenocytes compared with that of the Cfr⁺/⁺ 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 Cfr⁻/⁻ mice lungs compared with that of the Cfr⁺/⁺. Original magnification ×20; scale bar, 50 μm. H, Differences in basal and Con A (5 μg/ml) induced Foxp3 expression in Cfr⁺/⁺ and Cfr⁻/⁻ 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.
Lung injury. These data imply that lipid-raft localization of CFTR significantly downregulated. We anticipate this as an outcome of is not completely absent in severe COPD lungs, its expression is significant increase in ceramide-positive cells in derived macrophages compared with that of the lipid-raft marker ZO-1 shows a significant increase in both the upper right quadrants (Fig. 3A, left panel) or 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).

**FIGURE 2.** Ceramide and ZO-1 expression is elevated in immune cells of Cfr<sup>−/−</sup> mice. Flow cytometry analysis showing ZO-1 and ceramide expression in macrophages (A) and neutrophils (B) from Cfr<sup>+/+</sup> and Cfr<sup>−/−</sup> 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<sup>−/−</sup> mice (97.85%) derived macrophages compared with that of the Cfr<sup>+/+</sup> 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<sup>−/−</sup>) indicating the role of CFTR in tight junction formation.

**Table I.** Patient characteristics

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<th>Parameters</th>
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<th>Gold II (Moderate)</th>
<th>Gold III-IV (Severe)</th>
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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<sup>+/+</sup> and Cfr<sup>−/−</sup> 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<sup>+/+</sup> 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<sup>−/−</sup> mice), FB1 treatment decreased Pa-LPS–induced IL-6 (Fig. 4Aiii), but the magnitude of rescue was not as efficient as that in Cfr<sup>+/+</sup> mice. In addition, IL-1β levels were unaltered by FB1 treatment in the Cfr<sup>−/−</sup> mice (Fig 4Aiv). This was also verified by immunostaining of lung sections from these mice for ceramide, NF-kB, and neutrophil marker NIMP-R14 (Supplemental Fig. 1A, 1B).

In contrast, inhibition of membrane-ceramide release by AMT was unable to rescue Pa-LPS–induced IL-6 or IL-1β secretion in Cfr<sup>+/+</sup> mice (Fig. 4Bi, 4Bii), whereas inhibition of membrane-ceramide in the Cfr<sup>−/−</sup> mice showed a significant decrease (p < 0.05) in Pa-LPS–induced IL-6 and IL-1β levels (Fig. 4Biii, 4Biv). The ceramide, NF-kB, 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 Cftr-deficient mice from...
*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

![Image](https://example.com/image.png)

**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.
CFTR regulates de novo and membrane ceramide signaling.

BALF from three to five C57BL/6 Cftr+/+ or Cftr−/− mice, treated intratracheally with PBS (control), Pa-LPS (20 μg/mouse; 12 h), FB1 (50 μg/mouse; 24 h) and/or AMT (50 μg/mouse; 24 h) was used to quantify the IL-6 and IL-1β levels. A. Inhibition of de novo ceramide synthesis by FB1 treatment significantly decreases Pa-LPS–induced IL-6 and IL-1β in Cftr+/+ mice (i, ii), but FB1 has a modest effect on Pa-LPS–induced IL-6 levels in Cftr−/− mice (iii, iv). B. Inhibition of membrane-ceramide release by AMT treatment is relatively less protective against Pa-LPS–induced lung injury in Cftr+/+ mice (i, ii) but effectively controls the inflammatory cytokines in Cftr−/− mice (iii, iv). The data show that inhibition of de novo and membrane-ceramide release can control Pa-LPS–induced lung injury in the presence or absence of CFTR, respectively. This also indicates that CFTR can regulate de novo and membrane-ceramide signaling. Data represent the averages of triplicate ELISAs from n = 3 to 5 samples and are shown as mean ± SEM. *p < 0.05; **p < 0.1; ***p < 0.001.

Discussion

We and others have recently shown that apical lipid-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 (Cftr−/−) secrete higher amounts of IFN-γ compared with that of the Cftr+/+. A recent study by Carrigan et al. (47) showed that although natural regulatory T cells (Tregs) were increased in P. aeruginosa-infected Cftr+/+ 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
FIGURE 5. CFTR regulates lipid-raft expression and signaling via ceramide. A, CFBE41o−/−WT-CFTR (WT-CFBE) and CFBE41o−/− 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.

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).

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
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 expression of CFTR- gene, protein, and function in Calu-3 cells. Our in vitro studies in HEK-293 cells transfected with WT-CFTR confirm their observation. We document here the first report to our knowledge showing that decrease in CFTR expression correlates with severity (Gold 0 [at risk] versus Gold I [mild]; II [moderate], and Gold III–IV [severe and very severe]) of lung emphysema and ceramide accumulation (Fig. 3A). We verified that acute CS exposure of Cfr+/+ (C57BL/6) mice decreases cell surface and lipid-raft expression of CFTR in murine lungs (Fig. 3D, 3E). 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.

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 ΔF508-mutation, resulting in very low membrane-CFTR levels, do not develop severe emphysema? The paucity of emphysema in ΔF508-CF patients may be due to the absence of other contributors like CS or lack of detection as the patients die before severe emphysema develops 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. C. The lipid-raft proteins from HEK-293 cells expressing WT-CFTR or ΔTRL-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 ΔTRL-CFTR groups in C.

**FIGURE 6.** The PDZ-interacting domain of CFTR regulates ceramide accumulation. A, The HEK-293 cells were transiently transfected with pEGFP WT-CFTR or ΔTRL-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 binding domain results in reduced binding of LPS to the plasma membrane. We anticipate that less LPS binding to cell surface may be an outcome of decreased CFTR expression. B, The HEK-293 cells transiently overexpressing WT-CFTR or ΔTRL-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 ΔTRL 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 ΔTRL-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 ΔTRL-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 Cfr2−/− 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 Cfr2−/− 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 prognosticator of the aforementioned therapeutic strategy. It remains an open question whether the development of a potent CFTR corrector (CF-ΔF508) and putator (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

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.

Fig. 3A, 3B. In vivo depletion of lipid-raft CFTR also showed an increase in ceramide, NF-κB, and neutrophil (Supplemental Fig. 3C) levels and activity, confirming our hypothesis that lipid-raft–localized CFTR controls ceramide and NF-κB mediated proinflammatory signaling. We further verified these results by depleting (CD treatment) (28, 42) or inducing (TNF-α) (41, 43) lipid-raft CFTR in CFBE41o−WT-CFTR cells and observed that lipid-raft CFTR expression controls membrane-ceramide accumulation (Supplemental Fig. 4), although we understand that CD and TNF-α may modulate NF-κB signaling by CFTR-independent mechanisms, which warrants further investigation and identification of small molecules that can selectively modulate lipid-raft CFTR expression. Nonetheless, our preliminary studies demonstrate that membrane-localized WT-CFTR inhibits lipid-raft formation as the expression of lipid-raft marker ZO-1/2 was elevated in the absence of CFTR, which is known to induce immune receptor clustering and signaling (68). We anticipate this as a potential mechanism by which CFTR regulates ceramide-mediated NF-κB signaling.

We and others observed that ceramide-mediated lung injury and NF-κB signaling is prevented by inhibiting de novo ceramide synthesis (FB1) (20); therefore, we tested the efficacy of FB1 to suppress TNF-α–induced NF-κB reporter activity in the presence or absence of CFTR. FB1 was able to suppress NF-κB reporter activity only in the CFBE41o−WT-CFTR cells but not in the

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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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