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CFTR in a lipid raft-TNFR1 complex modulates gap junctional intercellular communication and IL-8 secretion

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Abstract

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause a chronic inflammatory response in the lung of patients with Cystic Fibrosis (CF). We have showed that TNF-α signaling through the Src family tyrosine kinases (SFKs) was defective as determined by an inability of TNF-α to regulate gap junctional communication (GJIC) in CF cells. Here, we sought to elucidate the mechanisms linking TNF-α signaling to the functions of CFTR at the molecular level. In a MDCKI epithelial cell model expressing wild-type (WtCFTR) or mutant CFTR lacking its PDZ-interacting motif (CFTR-ΔTRL), TNF-α increased the amount of WtCFTR but not CFTR-ΔTRL in detergent-resistant membrane microdomains (DRMs). This recruitment was modulated by SFK activity and associated with DRM localization of TNFR1 and c-Src. Activation of TNFR1 signaling also decreased GJIC and markedly stimulated IL-8 production in WtCFTR cells. In contrast, the absence of CFTR in DRMs was associated with abnormal TNFR1 signaling as revealed by no recruitment of TNFR1 and c-Src to lipid rafts in CFTR-ΔTRL cells and loss of regulation of GJIC and IL-8 secretion. These results suggest that localization of CFTR in lipid rafts in association with c-Src and TNFR1 provides a responsive signaling complex to regulate GJIC and cytokine signaling.

Keywords: Cystic fibrosis; CFTR; Lipid rafts; Tumor necrosis factor; Src tyrosine kinase; Inflammation; IL-8; Gap junctions

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease resulting from mutations of the CF transmembrane conductance regulator (*CFTR*) gene. CFTR, a member of the adenine nucleotide-binding cassette family, is an ATP- and cAMP-dependent protein kinase A (PKA)-regulated chloride channel present at the apical surface of epithelial cells. The ΔF508 mutation, which accounts for 70% of the cases in Caucasians, is retained at the endoplasmic reticulum and consequently is unable to function at the plasma membrane [1,2]. In CF lungs, the release of a variety of cytokines and chemokines, such as tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8), along with an excessive neutrophil influx and recurrent infection by *Pseudomonas aeruginosa* (*Pa*), lead to a progressively destructive inflammatory reaction and loss of pulmonary function [3]. CFTR is also a regulator of other membrane transport proteins, including ENaC sodium channels, outwardly rectifying chloride channels, bicarbonate exchangers/co-transporters, ROMK potassium channels, aquaporin3 water channels as well as gap junction channels [1,4,5]. Defective regulation of channel activity in CF cells is thus thought to contribute to the phenotypes of the disease.

Gap junctions contain hydrophilic membrane channels that allow direct communication between neighboring cells (GJIC) through the diffusion of ions, metabolites, and small cell signaling molecules. They are composed of a hexameric array of

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polypeptides encoded by the connexin multi-gene family. GJIC is crucial to tissue homeostasis, the alteration of which contributing to disease initiation and/or progression. In addition to their coupling function, recent studies suggest that connexin proteins may also mediate signaling. The integration of these cell functions is likely to be important in the role of gap junctions in development and disease [6]. We have reported that CFTR dysfunction is associated with altered TNF-α signaling in CF airway epithelial cells, resulting in the persistence of gap junction connectivity. While TNF-α induced the disruption of GJIC through a c-Src family kinase (SFK)-dependent pathway within minutes, the cytokine failed to modulate GJIC in CF cells [7,8]. Although these results suggest that c-Src is a central element in the signaling pathway connecting CFTR with Cx43 in airway epithelial cells, the mechanism by which CFTR intersects with SFKs during TNF-α stimulation is unknown.

Emerging evidence indicates that lipid microenviroments on the cell surface, known as lipid rafts, are critically involved in distal signaling events [9]. Lipid rafts are specialized membrane microdomains enriched in glycosphingolipids and cholesterol that resist detergent solubilization and can be found in low-density fractions on density gradients. These low-density detergent-resistant membranes (DRMs) provide a spatial compartmentalization of receptors and kinases that facilitates their interactions and the activation of downstream responses [10]. This is particularly relevant to the TNF-α signaling pathway, which triggers a variety of biological responses depending on the localization of the TNF-α receptor 1 (TNFR1) in lipid rafts [11,12]. Interestingly, two recent studies reported that CFTR associates with lipid rafts in epithelial cells [13,14]. Although these observations are consistent with the idea that localization of CFTR in lipid raft may trigger distinct intracellular cell responses, the role of CFTR in DRMs has only been evaluated so far by extraction of cell surface cholesterol [14], an approach that does not specifically targets CFTR in lipid rafts.

In an attempt to gain more insights into the role of CFTR in lipid rafts, we studied the dynamic partition of CFTR in DRMs in response to TNF-α and evaluated Cx43-mediated GJIC as well as IL-8 production in MDCKI cells expressing wild-type (WtCFTR) or a mutant CFTR lacking its last three amino acids (CFTR-ΔTRL) [15,16]. We report here that expression of CFTR, in association with c-Src and TNFR1 presented within the context of lipid rafts, provides a responsive signaling complex at the surface of MDCKI cells to regulate GJIC and cytokine signaling.

2. Materials and methods

2.1. Cell lines

Madin–Darby canine kidney cells type I (MDCKI) transfected with a plasmid expressing either a green fluorescent protein (GFP)-tagged to wild-type CFTR (WtCFTR), GFP-tagged to CFTR lacking its C-terminal PDZ (PSD-95, Dlg, and ZO-1)-binding domain (CFTR-ΔTRL) or GFP-tagged to the ΔF508 mutant (ΔF508CFTR) were described previously [15,16]. GFP, which is fused to the N-terminus of CFTR, has been shown to have no effect on CFTR localization, processing or function [15,16]. GFP-CFTR-expressing MDCKI and parental cells were treated overnight with 5 mM sodium butyrate (Sigma Chemical Co., St. Louis, MO) before experiments to increase CFTR expression. Most experiments were performed on sub-confluent cultures to prevent the down-regulation of Cx43 expression previously observed in confluent monolayers of MDCK cells [17]. In some experiments, MDCKI cells were cultured at high density on Transwell inserts to enhance cell polarization. Additional experiments were also performed on BHK cells stably expressing wild-type (untagged) CFTR and MDCKII cells expressing a mutant CFTR with GFP fused at its C-terminus [18]. Calu-3 cells, a human glandular cell line that endogenously expresses normal CFTR, were from the American Type Culture Collection (ATCC, Manassas, VA). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (SeraTech, Griesbach, Switzerland), 30 units/ml penicillin, and 30 μg/ml streptomycin (Invitrogen AG, Basel, Switzerland).

2.2. Cell treatment

Prior to an experiment, the culture medium was removed and cells were rinsed 2×15 min with an external solution (XP) containing (in mM): 136 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 2.5 glucose (pH 7.4). Cells were then incubated at 37 °C in XP supplemented with 100 U/ml TNF-α or isoproterenol for 5–30 min, 25 mM methyl-β-cyclodextrin (CD), 50 μM PP1, 0.4 mM sodium orthovanadate (VO4) and/or 10–40 μM of the CFTR inhibitor I-172 for 45 min. All agents were from Sigma except TNF-α (Bachem AG, Bubendorf, Germany).

2.3. Lipid raft preparation

Cells were washed twice in Dulbecco's phosphate buffer saline (PBS) containing $CaCl_2$ and $MgCl_2$ at 4 °C. Cells were then scraped off in the cell culture dish in cold PBS and spun for 2 min at 500×g. The supernatant was discarded and the cells lysed for 30 min in 1% Triton X-100 prepared in 25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 5 mM EDTA (TNE) in the presence of Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Rotkreuz, Switzerland). Detergent resistant membranes (DRMs) were prepared by sucrose density gradient isolation using the Optiprep method (Axon Lab AG, Baden, Switzerland). Briefly, cells were lysed in 200 μl of TNE, well mixed with 400 μl Optiprep 60% and transferred to an ultracentrifuge tube. Before centrifugation, 2× 600 μl Optiprep 30% followed by 600 μl TNE was added to the tube. Samples were spun 2 h at 148,000 ×*g* using a S55S rotor (*Sorvall Discovery* M150 GX). At the end of the centrifugation period, 8 fractions of 300 μl each were removed starting from the top of the Optiprep gradient. DRMs reach their isopycnic point at the interphase 0/30% OptiPrep (fractions 2 and 3). Fractions were stored at −20 °C before further processing. For these experiments, WtCFTR and CFTR-ΔTRL cells were grown to 70–80% confluence before cell lysis. No difference was observed in terms of protein content between WtCFTR (1.5±1 μg/μl, n=25 extractions) and CFTR-DTRL (1.3±0.2 μg/μl, n=10 extractions) cells. No correlation was found between protein load and CFTR recovered in lipid rafts in WtCFTR cells within the range 0.3–4.4 μg/μl of protein. Within this very same range of protein concentration, CFTR-ΔTRL was not detected in DRMs.

2.4. Western blotting

For each fraction, a sample was mixed with 4× NuPAGE lithium dodecyl sulfate (LDS) Sample buffer (Invitrogen) containing 100 mM 1,4-Dithio-DK-threitol (Fluka, Buchs, Switzerland) to reach 1X final concentration of the buffer and incubated for 30 min at 37 °C. From each of the 8 fractions, 15 μl of protein sample were electrophoresed on a 7.5% SDS-PAGE and electrotransferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore AG, Volketswill, Switzerland). Membranes were then blocked for 1 h in a 5% defatted milk saturation buffer containing 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1% Tween20 and 0.2% sodium azide. Blots were next incubated overnight at 4 °C with antibodies against flottilin-1 or caveolin-1 (kindly provided by Dr. G. Van Der Goot, Medical Faculty, Geneva, Switzerland), GFP (Molecular Probes, Eugene, OR), TNFR1 (Stressgen Bioreagents, Victoria, BC), v-Src (Calbiochem, EMD Biosciences Inc., San Diego, CA), EBP50, Fyn, c-Yes or Cx43 (Transduction Laboratories, Lexington, KY). This step was followed by 1-h incubation with goat anti-mouse or anti-rabbit IgG secondary antibodies conjugated to peroxidase (The Jackson Laboratories, West Grove, PA). Immunoreactivity was

detected through the Super Signal West Pico kit (Pierce Biotechnology, Rockford, IL). The GeneTools software (SynGene, Cambridge, UK) was used to determine relative band intensities. The M24.1 (directed against the C-terminal) and the M3A7 (directed against the second nucleotide-binding domain NBD2) antibodies were used to detect WtCFTR whereas only the M3A7 detected CFTR-ΔTRL. To ensure for equivalent expression of CFTR in these experiments, we took advantage that GFP is attached to the N-terminus of both WtCFTR and CFTR-ΔTRL by performing Western blots using antibodies against the fluorescent tag. Although there was some variability between experiments, the expression level of GFP-CFTR and GFP-CFTR-ΔTRL was within the same range (data not shown).

2.5. Biotinylation assay

For biotinylation studies, cells were incubated with 0.5 mg/ml of NHS-LC-biotin (Pierce Biotechnology) prepared in PBS for 30 min on ice. Cells were then washed in PBS and scraped to obtain a cell pellet. Cell pellet was then incubated in the lysis buffer and subjected to Optiprep gradients, as described above. In most of the experiments, fractions 1, 2 and 3 (DRMs) and 5, 6, 7, and 8 (non-raft fractions) were pooled. Monomeric avidin-coated beads were added to each pooled fractions (previously diluted to 50% in PBS), and incubated for 30 min at 4 °C under agitation. Samples were then thoroughly washed in lysis buffer with protease inhibitor cocktail. The final bead pellet was resuspended in Laemmli buffer, subjected to SDS-page, and detected by Western blot as described above.

2.6. Dye coupling

Intercellular coupling via gap junctions was evaluated by intracellular microinjection of Lucifer Yellow, as described in detail previously [17]. Lucifer Yellow (LY) was prepared in 150 mM LiCl and 10 mM HEPES (pH 7.2). Tracers were injected into MDCKI cells for 3 min using a thin-tip glass microelectrode. At the end of the microinjection, the electrode was removed and the number of LY labeled cells was immediately counted [8]. Experiments were performed on an inverted TMD-300 microscope (Nikon AG, Küsnacht, Switzerland) equipped for fluorescence with appropriate filters. Fluorescent cells were viewed with a 40× phase 3 DM objective with a numerical aperture of 0.7 (Nikon). Images were captured with a Visicam digital camera (Visitron systems, GmbH, Puchleim, Germany) connected to a personal computer running Metafluor 4.01 software (Universal Imaging Corp., Downington, PA).

2.7. NF-κB localization

The localization of the nuclear factor (NF)-κB was investigated by immunofluorescence. To this end, cells grown on glass coverslips were exposed for 5–15 min to XP supplemented or not with TNF-α and then fixed in 4% paraformaldehyde for 15 min followed with cold methanol (−20 °C) for 10 min. The coverslips were rinsed and with 0.2% Triton X-100 for 1 h, 2% bovine serum albumin for another 60 min. Cells were the rinsed and incubated for 1 h with a polyclonal antibody raised against the p65 subunit of NF-κB (Santa Cruz Biotech. Inc., Santa Cruz, CA). After washing in PBS, the coverslips were incubated with a secondary Alexa Fluor® 568 goat anti-rabbit antibody (Molecular Probes Inc., Eugene, OR) for 1 h, and then examined using fluorescent microscopy. Images were acquired as described above. No staining was observed in the absence of primary antibody.

2.8. IL-8 secretion

IL-8 secretion was evaluated by dot blots. Cells were grown in 6-well culture plates. The culture medium was removed and cells were rinsed twice for 15 min in XP. Cells were then incubated with or without (control) 100 U/ml TNF-α for 15 min. At the end of the treatment, the cell culture medium was removed and replaced with 500 µl of fresh XP supplemented with 0.4% BSA for 2 h. At the end of the 2-h period the supernatant was collected and frozen at −20 °C before further processing. Plates were washed twice in PBS and exposed with 3 cycles of freeze-thaw in 500 µl distilled water for cell lysis. Protein content was then determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories AG, Reinach, Switzerland). To detect the amount of secreted IL-8, 100 µl of the collected supernatant was spotted on Immobilon-P polyvinylidene difluoride membranes using the Bio-Rad Bio-Dot™ Apparatus. Increasing concentrations (0.1–1 ng/100 µl) of recombinant canine IL-8 (R and D) were also spotted on the membrane to generate a standard curve. Membranes were blocked in PBS supplemented with 3% bovine serum albumin (PBS-BSA) for 1 h. Blots were next incubated overnight at 4 °C with a polyclonal antibody raised in goat against canine IL-8 (R and D). This step was followed by 1-h incubation with a biotin-SP-conjugated donkey anti-goat IgG secondary antibody and followed by additional 1-h incubation with extravididin conjugated to peroxydase (The Jackson Laboratories). Immunoreactivity was detected through the Super Signal West Pico kit (Pierce Biotechnology). The GeneTools software was used to determine relative spot intensities. IL-8 was expressed as pg/µg of protein.

2.9. Statistical analysis

Experiments were compared using Student's *t* test. Values are expressed as mean ± SEM. $P < 0.05$ was considered to be significant.

3. Results

3.1. GJIC in WtCFTR and CFTR-ΔTRL MDCKI cells

We have previously shown that TNF-α reduced GJIC in normal airway cells expressing Cx43. This mechanism, however, was defective in CF airway cells expressing the ΔF508 mutant of CFTR [7,8]. To evaluate GJIC in MDCKI cells, which endogenously express Cx43 [17], we performed dye

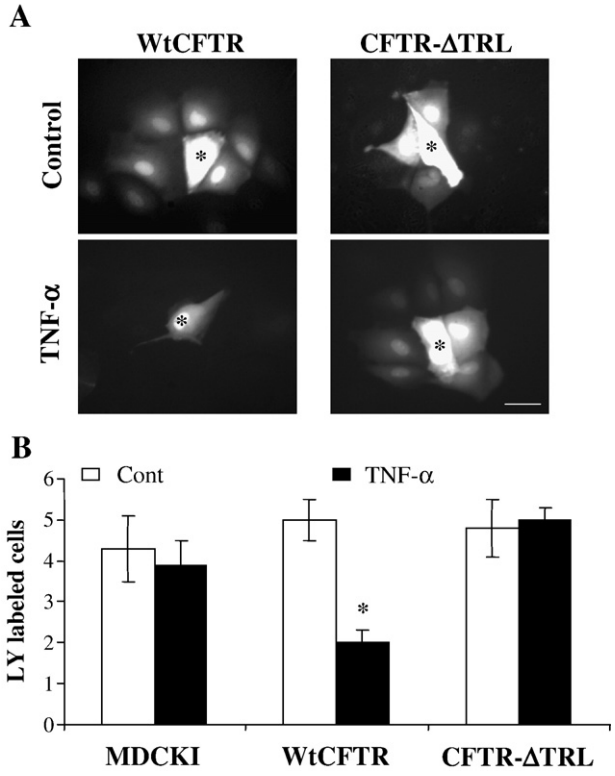

Fig. 1. GJIC was investigated by dye coupling. The number of Lucifer Yellow (LY) labeled cells was measured in parental and MDCKI cells expressing WtCFTR or CFTR-ΔTRL and exposed to TNF-α. A) TNF-α markedly decreased GJIC in WtCFTR-expressing cells but not in CFTR-ΔTRL-expressing cells. Asterisk indicates the injected cell from which Lucifer Yellow diffused to neighboring cells. Bar represents 20 µm. B) Quantitative analysis of dye coupling in CFTR-expressing MDCKI cells. Mean ± SEM of the number of cells labeled with Lucifer Yellow are indicated for parental ($n = 11$), WtCFTR ($n = 18$) and CFTR-ΔTRL ($n = 13$). Asterisk indicates $p < 0.001$.

coupling experiments in WtCFTR- and CFTR-ΔTRL-expressing cells and compared the results with those obtained in parental MDCKI cells. Under basal conditions, Lucifer Yellow rapidly spread from the injected cells to several neighbors in all three cell types (Fig. 1). TNF-α, however, markedly reduced ($p<0.001$) the extent of the dye diffusion in cells expressing WtCFTR (Fig. 1A and B). In contrast, TNF-α did not affect dye coupling in parental or CFTR-ΔTRL cells (Fig. 1). Similarly, GJIC was unaffected by TNF-α in a clone of MDCKI cells expressing ΔF508CFTR (4.1±0.2 cells as compared to 3.7±0.5 cells under control conditions, $n=11$). These results confirm in MDCKI cells our previous findings that WtCFTR confers sensitivity of Cx43 gap junction channels to TNF-α. In addition, these results demonstrate the importance of expression of an intact CFTR protein in mediating TNF-α-dependent regulation of GJIC.

3.2. DRM-associated proteins in MDCKI cells

Proteins from MDCKI were extracted in cold Triton-X100 and fractioned by density gradient ultracentrifugation. The quality of the DRM preparation was first examined by Western blots for specific markers of lipid rafts. As shown in Fig. 2A, caveolin-1 and flotillin-1 were detected in fractions 2 and 3, which correspond to the DRMs. EBP50 (ezrin, radixin and merlin (ERM)-binding phosphoprotein 50; also referred to as NHERF1), the adapter molecule that bridges CFTR to the N-terminal domain of ezrin [16], was detected in all fractions (Fig. 2A). To determine whether CFTR localized to DRMs, fractions were prepared from WtCFTR-expressing cells and then immunobloted for CFTR using an antibody against the NBD2 domain (M3A7) or the C-terminus domain (M24.1) of the protein, respectively (Fig. 2B). Although a small amount of GFP-CFTR was found in lipid rafts, the large majority of the protein was excluded from DRMs. In all experiments, the identification of CFTR was confirmed by immunoblots using an antibody against GFP (data not shown). Quantitative analysis on 10 distinct DRM preparations revealed that the amount of CFTR detected in lipid rafts averaged 6–7% of the total CFTR protein (Fig. 5B). The amount of CFTR in DRMs did not increase further in WtCFTR cells cultured on permeable support (5.1±0.3 % of total CFTR). However, this proportion decreased markedly in the presence of methyl-β-cyclodextrin (CD), a membrane cholesterol scavenger that reduces DRMs (Figs. 2B and 5B). None of the antibodies used (M24.1, M3A7) detected endogenous CFTR in parental MDCKI cells (not shown).

3.3. Effect of TNF-α on CFTR and Cx43 in DRMs from WtCFTR and CFTR-ΔTRL MDCKI cells

We next evaluated the dynamic partition of CFTR and Cx43 in DRMs from WtCFTR and ΔTRL-CFTR cells. Treatment of WtCFTR cells with TNF-α markedly increased the amount of CFTR in DRMs (Fig. 3A). We observed a significant translocation as early as 2 min after TNF-α addition, which peaked at 5 min and progressively declined to control levels after 20–30 min (not shown). Quantitative analysis of the immunoblots revealed that the amount of CFTR detected in DRMs increased ($p<0.01$) from 6.6±1.8 % of total CFTR ($n=10$) to 21.2±4.2 % ($n=7$) after 5 min of TNF-α (Fig. 5B). The translocation of CFTR to DRMs, however, was not observed in MDCKI cells expressing CFTR-ΔTRL. Although the mutant CFTR was expressed in these cells, CFTR-ΔTRL was weakly detected in DRMs in the absence or presence of TNF-α (Figs. 3B and 5B). We performed additional control experiments on cells expressing CFTR alone (not fused to GFP) or CFTR with GFP fused to the C-terminus of the protein, a situation that abolishes PDZ-dependent interactions [18]. Although the amount of CFTR detected in DRMs was very variable in these two cell lines, we observed that TNF-α facilitated the movement of CFTR into lipid rafts only in cells expressing a CFTR with an intact C-terminus (not shown).

We also studied the DRM partition of Cx43 in WtCFTR and CFTR-ΔTRL cells. On Western blot, Cx43 exhibits several bands corresponding to the non-phosphorylated (lower band) and phosphorylated (higher bands) forms of the protein

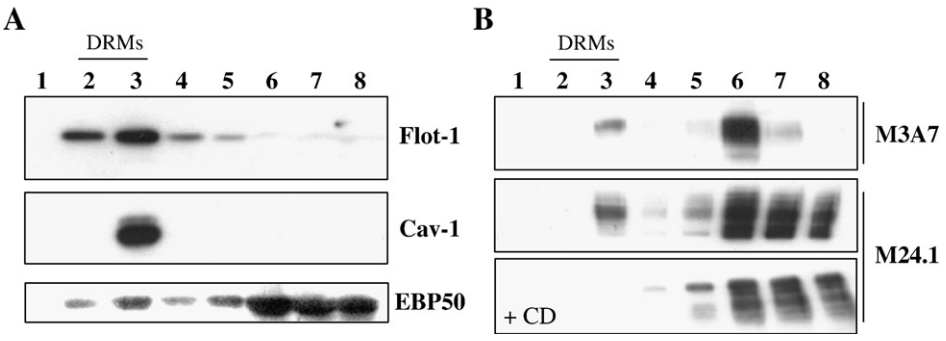

Fig. 2. Detergent resistant membranes (DRMs) were prepared by ultracentrifugation of Optiprep gradients as described in Materials and methods. Eight fractions [1–8] were collected and subjected to Western bloting. Fractions 2 and 3 contain the DRMs, whereas fractions 5–8 contain the Triton-soluble material. Fraction 4 represents the transition between Triton-insoluble and soluble materials. A) Western blots showing the presence of flottilin-1 (Flot-1) and caveolin-1 (Cav-1) in lipid rafts prepared from MDCKI cells. The EBP50, a transmembrane adaptor, is found in raft and non-raft fractions. B) Under control conditions, a small proportion of GFP-CFTR can be detected in DRMs by the M3A7 and the M24.1 antibodies. Inhibition of raft formation by extracting cholesterol from the cell membrane with methyl-β-cyclodextrin (+CD) reduced the basal amount of CFTR in DRMs. Blots are representative of at least 4 experiments.

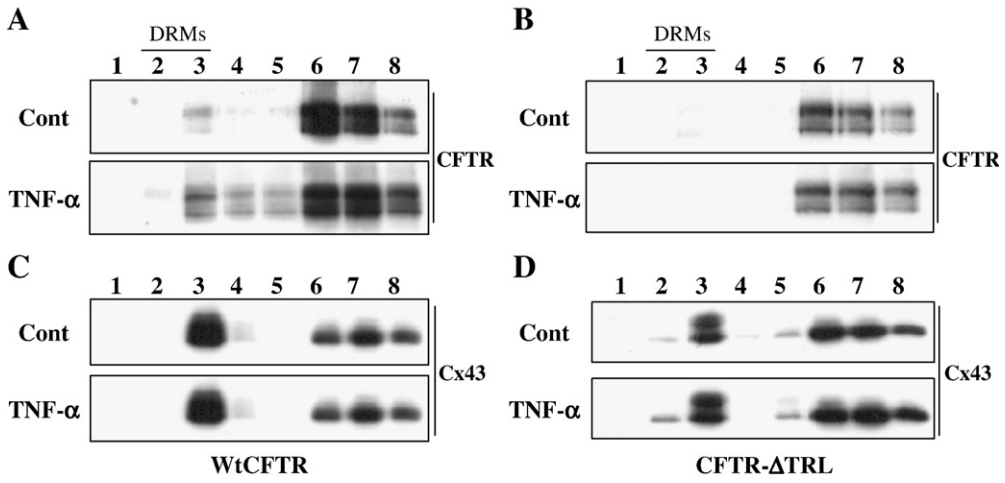

Fig. 3. Effects of TNF-α on CFTR and Cx43 distribution in lipid rafts. A) Within 5 min, TNF-α increased the amount of CFTR in DRMs isolated from WtCFTR-expressing cells. This phenomenon, however, was never observed in CFTR-ΔTRL-expressing cells. The M24.1 and the M3A7 antibody were used to detect WtCFTR and CFTR-ΔTRL, respectively (B). The Triton-insoluble/soluble distribution of Cx43, another membrane channel-forming protein of the gap junction family, was not altered by TNF-α and was similar in WtCFTR-expressing (C) and CFTR-ΔTRL-expressing (D) cells.

[19]. As shown in Fig. 3 (C and D), Cx43 was separated in two distinct pools. One pool, which was primarily the non-phosphorylated form of Cx43, was detected in the soluble fraction. The second pool, which exhibited non-phosphorylated and phosphorylated forms of Cx43, was present in DRMs. This distribution, which was similar in WtCFTR and CFTR-ΔTRL cells, was not affected by TNF-α. These results demonstrate that, in transfected MDCK cells, TNF-α does not induce the movement of all membrane channel-forming proteins into DRMs. They also indicate that TNF-α-dependent regulation of Cx43 connectivity is independent of its recruitment to DRMs.

It is possible that detection of CFTR in lipid rafts, however, is a consequence of forced expression of the chimeric GFP-CFTR protein. To rule out this possibility, we performed control experiments on Calu-3 cells, which endogenously express a normal CFTR protein. As shown in Fig. 4A, the recruitment of CFTR to DRMs in response to TNF-α was also observed in these cells. In addition, lipid rafts were isolated from Calu-3 cells after biotinylation of their cell surface. Under these conditions, biotinylated CFTR was recovered in detergent-resistant membranes (Fig. 4B). These results demonstrate in an endogenous human glandular cell system the presence of surface CFTR in lipid rafts.

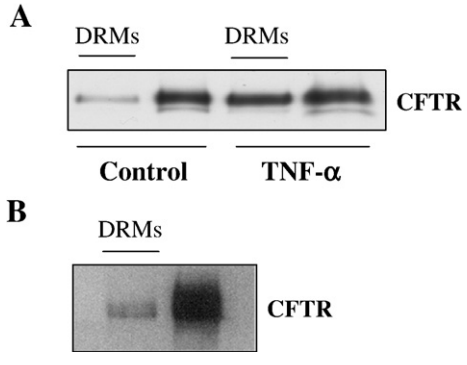

Fig. 4. Detergent resistant membranes (DRMs) were prepared by ultracentrifugation of Optiprep gradients from Calu-3 cells. A) Western blots showing the presence of CFTR in raft (DRMs) and non-raft fractions by the M24.1 antibody. Within 10 min, TNF-α increased the amount of CFTR in DRMs isolated from Calu-3 cells. B) Western blots showing the presence of CFTR (using the M24.1 antibody) in DRMs prepared from Calu-3 cells after biotinylation of their cell surface.

3.4. The amount of WtCFTR in DRMs is dependent of SFK activity

Members of the SFK family have been involved in TNF-α signaling [20–22]. We next examined whether pharmacological modulation of SFK activity could affect the localization of CFTR in DRMs. To this end, the effects of pervanadate (VO4), a tyrosine kinase activator, and of PP1, a broad inhibitor of SFKs, were studied on the TNF-α-dependent recruitment of CFTR to DRMs. Interestingly, we found that PP1 enhanced the proportion of CFTR in DRMs induced by TNF-α while VO4 reduced this proportion (Fig. 5A). In addition, PP1 alone did recruit CFTR to DRMs to an amount representing 12.9± 4.7% of total CFTR (Fig. 5B). In contrast, experiments with VO4 alone, cAMP-elevating agents or the CFTR inhibitor I-172 did not change the amount of CFTR in DRMs (not shown). These results indicate that, in MDCKI cells, SFK activity modulates the amount of CFTR in DRMs and suggest that modulation of CFTR channel activity is independent of its association with lipid rafts.

To search for possible SFK candidates involved in TNF-α signaling in MDCKI cells, fractions from WtCFTR cells exposed to TNF-α were immunobloted for Fyn, c-Yes or c-Src (Fig. 6A, B and C, respectively). The location of Fyn and c-Yes, which are present in DRMs as well as in soluble fractions, was not affected by TNF-α (Fig. 6A and B). In contrast, the amount of c-Src detected in DRMs was increased by TNF-α (Fig. 6C). Interestingly, the presence of c-Src and CFTR in DRMs showed

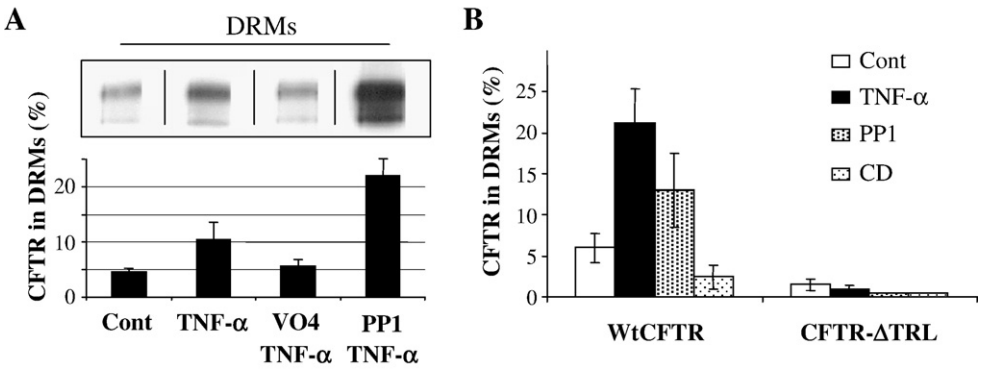

Fig. 5. Pharmacological modulators of SFKs alter the proportion of CFTR in lipid rafts. A) WtCFTR-expressing cells were pre-treated with pervanadate (VO4), a broad inhibitor of tyrosine phosphatase, or PP1, an inhibitor of tyrosine kinase, and stimulated with TNF-α. The DRM fractions shown in A are from the very same experiment. WtCFTR cells were processed in parallel in terms of culture, treatment with the different stimuli, DRM isolation and Western blotting. The relative amount of CFTR in DRMs (expressed in % of total GFP-CFTR) is illustrated the bottom panel from 3 independent measurements. The M24.1 antibody was used to detect WtCFTR. B) The amount of CFTR in DRMs (expressed in % of total CFTR) was measured in WtCFTR- and CFTR-ΔTRL-expressing cells. Under control conditions, CFTR in DRMs represented only 5% of total CFTR ($n=10$). Exposure of WtCFTR cells to TNF-α ($n=7$) or PP1 ($n=3$) increased the percentage of CFTR detected in DRMs. Methyl-β-cyclodextrin (CD) treatment reduced both the basal and stimulated amount of CFTR in DRMs ($n=5$). In contract to WtCFTR-expressing cells, CFTR-ΔTRL was not present in DRMs prepared from CFTR-ΔTRL-expressing cells treated with vehicle, TNF-α or PP1 ($n=6$). The M24.1 and the M3A7 antibody were used to detect WtCFTR and CFTR-ΔTRL, respectively. Asterisk indicates significance at $p<0.05$.

a linear correlation in WtCFTR cells exposed to TNF-α (Fig. 6D). These results suggest that c-Src is involved in TNF-α signaling in WtCFTR MDCKI cells.

3.5. CFTR contributes to the formation of a TNFR1 signaling complex

We found that SFKs activity modulates the proportion of CFTR and c-Src present in DRMs in WtCFTR MDCKI cells. Because PP1, like TNF-α, recruited CFTR to DRMs, we tested the hypothesis that inhibition of SFKs could partially mimic the effect of TNF-α on TNFR1 activation. TNFR1 can be transiently mobilized to lipid rafts where it oligomerizes and recruits protein partners to form a receptor signaling complex in response to TNF-α [12]. This was indeed the case in WtCFTR cells exposed to TNF-α. As shown in Fig. 7A, TNF-α recruited TNFR1 to DRMs within 5 min. As previously described [12], the recruitment of TNFR1 to DRMs was transient, such that after 20–30 min following TNF-α, TNFR1 was not longer present in DRMs (data not shown). Interestingly, PP1 treatment was sufficient to recruit TNFR1 to DRMs (Fig. 7A). These data indicate that inhibition of SFKs represents a key step in the mechanism regulating the translocation of TNFR1 and c-Src into DRMs in WtCFTR cells. Of note, PP1 did also recruit c-Src

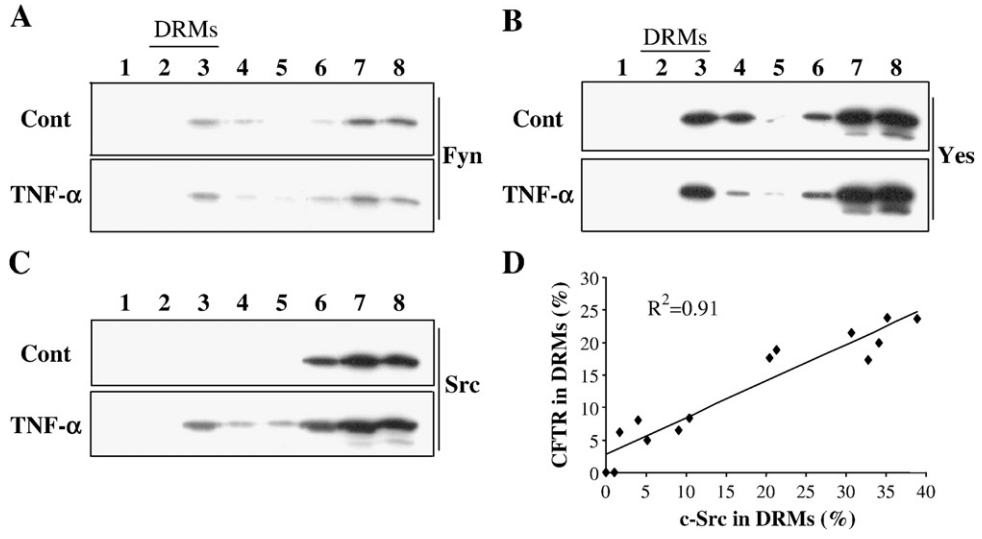

Fig. 6. The Triton-insoluble/soluble distribution of SFKs Fyn (A), Yes (B), and Src (C) was examined in GFP-CFTR-expressing cells. All three SFKs were detected but recruitment to DRMs in response to TNF-α was only observed for c-Src. The blots are representative of 6 experiments. D) The amount of CFTR and c-Src detected in DRMs was measured in 14 individual experiments. The distribution of these values were plotted and a linear relationship ($R^2=0.91$) was established between the amount of CFTR and c-Src in DRMs.

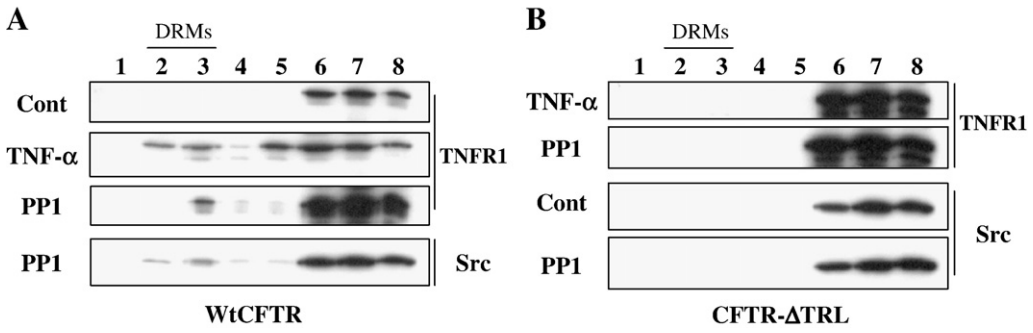

Fig. 7. TNFR1 and c-Src in DRMs is stimulated by inhibitors of SFKs. A) Under control conditions, TNFR1 is present in non-raft fractions. However, TNFR1 is recruited to DRMs within 5 min in the presence of TNF-α. Translocation of TNFR1 to the Triton-insoluble fractions was also observed with PP1. Similarly, c-Src was recruited to DRMs in response to PP1 treatment. B) Translocation of TNFR1 or c-Src to DRMs, however, was not observed in CFTR-ΔTRL-expressing cells. TNFR1 and c-Src remained in the non-raft fractions like under control conditions. Blots are representative of at least 4 experiments.

to DRMs (Fig. 7A). In contrast, PP1 did not stimulate the recruitment of TNFR1 or c-Src to DRMs in CFTR-ΔTRL cells (Fig. 7B). Finally, the observation that neither TNF-α nor PP1 recruited TNFR1 or c-Src to DRMs isolated from parental MDCKI cells (not shown) indicates that expression of an intact CFTR protein contributes to the formation of a TNFR1 signaling complex in DRMs from MDCKI cells.

3.6. IL-8 secretion in WtCFTR and CFTR-ΔTRL MDCKI cells

To examine whether formation of a CFTR-dependent TNFR1 signaling complex in DRMS affect the biological response of MDCKI cells, we first studied the activation of NF-κB, a transcriptional activator of immunomodulatory genes, including IL-8. Under control conditions, NF-κB was detected by

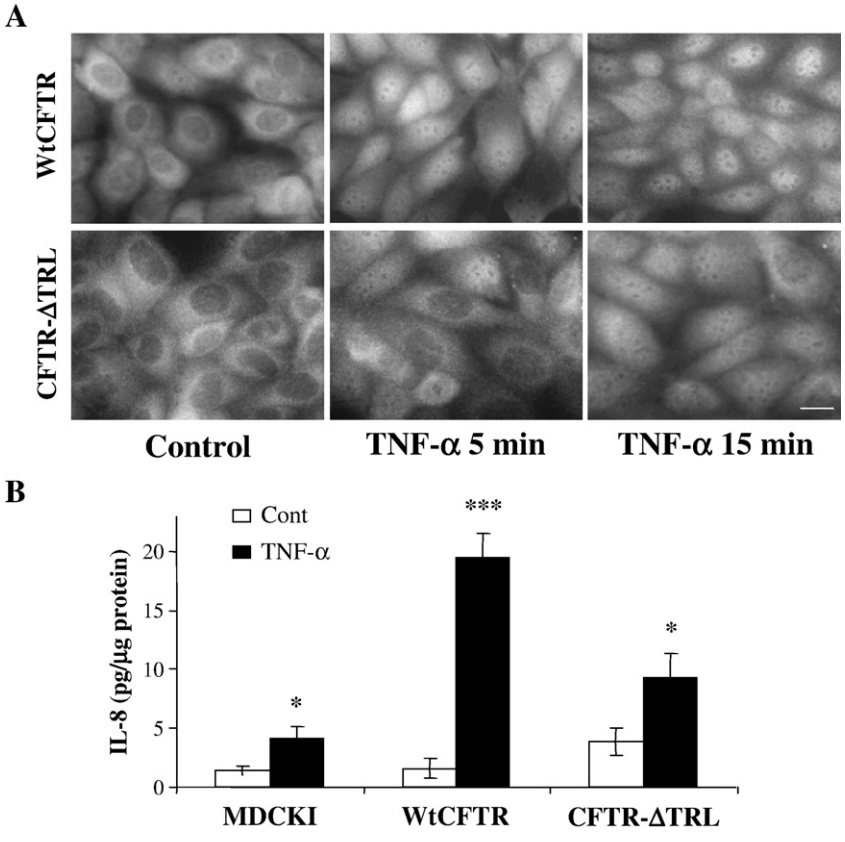

Fig. 8. Inflammatory response evoked by TNF-α was investigated by NF-κB immunodetection and IL-8 secretion. A) In WtCFTR and CFTR-ΔTRL-expressing cells, NF-κB was mostly localized in the cytoplasm under control conditions. Translocation of NF-κB to the nucleus was detected after 5 and 15 min of TNF-α exposure in all WtCFTR cells. In contrast, many nuclei of CFTR-ΔTRL cells were not immunostained for NF-κB after TNF-α treatment, suggesting for a less efficient activation of the nuclear transcription factor. B) IL-8 secretion was investigated by immunoblots in parental and transfected MDCKI cells exposed to TNF-α. Whereas TNF-α markedly enhanced IL-8 secretion by WtCFTR-expressing cells, the pro-inflammatory mediator modestly stimulated the production of IL-8 by parental (MDCKI) and CFTR-ΔTRL-expressing cells. Values were obtained from at least 6 experiments. Asterisk indicates significance at $p<0.05$.

immunofluoresence in the cytoplasm of WtCFTR and CFTR-ΔTRL cells. In the presence of TNF-α, NF-κB was rapidly and massively translocated to the nucleus of all WtCFTR cells, a phenomenon that was still observed after 15 min of treatment (Fig. 8A). This, however, was not the case in CFTR-ΔTRL MDCKI cells whereby nuclear detection of NF-κB was heterogeneous with many nuclei that were not immunostained even after 15 min of TNF-α treatment (Fig. 8A).

We next studied the secretion of IL-8 by immunoblot in WtCFTR and CFTR-ΔTRL cells exposed to TNF-α. As shown in Fig. 8B, TNF-α markedly stimulated IL-8 secretion (12.4 fold) in WtCFTR cells. In contrast, TNF-α-induced a 2.4 fold increase of IL-8 secretion in CFTR-ΔTRL cells. IL-8 production was also examined in MDCKI cells expressing ΔF508CFTR. In this cell clone, TNF-α also only modestly stimulated (1.6 fold) IL-8 secretion. These results suggest that localization of the CFTR/TNFR1/c-Src complex to lipid rafts participate in signal transduction, affecting the inflammatory response of MDCKI cells to TNF-α.

4. Discussion

In this study, we have demonstrated in an epithelial cell model that TNF-α recruits WtCFTR but not CFTR-ΔTRL to DRMs in association with TNFR1 and c-Src. The recruitment of these proteins to lipid rafts was dependent on SFK activity and required an intact C-terminus of CFTR. Moreover, the translocation of CFTR, TNFR1 and c-Src into lipid rafts triggered by TNF-α stimulated an intracellular signaling pathway that regulated GJIC and IL-8 secretion in WtCFTR- but not in CFTR-ΔTRL-expressing cells.

Several observations indicate that CFTR is present in DRMs prepared from WtCFTR MDCKI cells. First, CFTR was detected in DRMs using three different antibodies (GFP, M24.1 and M3A7). Second, CFTR was located in fractions containing proteins that are known to be permanently associated with lipid rafts, such as caveolin-1 and flottilin-1. Third, disruption of lipid rafts by cholesterol depletion abolished the detection of CFTR in the insoluble fractions. These observations, which are in agreement with a previous study [14], suggest that at least two pools of CFTR exist with one small population being associated with DRMs. Consistent with this interpretation is the detection in lipid rafts of biotinylated cell surface CFTR in Calu-3 cells, a human cell line endogenously expressing CFTR. The amount of CFTR that associates with lipid rafts was temporarily increased by TNF-α in WtCFTR-expressing MDCKI cells as well as in Calu-3 cells. This phenomenon, however, was not observed in MDCKI cells expressing a mutant CFTR lacking its C-terminal PDZ-binding domain. In contrast, TNF-α did not affect the raft and non-raft partition of Cx43 in either WtCFTR or CFTR-DTRL cells. Several members of the connexin family have been detected in lipid rafts but it is believed that only the gap junction precursor hemichannels are localized in DRMs [23,24]. Our data are thus consistent with the idea that TNF-α-dependent regulation of Cx43 channel activity does not involve association of gap junctions with lipid rafts in MDCKI cells.

It is well established that the PDZ domain containing adaptor molecules, such as NHERF1/EBP50 and NHERF2, associates with the C-terminal PDZ-binding motif of CFTR and tether the channel to the actin cytoskeleton via the ERM binding domain [4]. Fluorescence recovery after photobleaching measurements as well as fluorescence correlation spectroscopy suggests that about 50% of CFTR is immobile at the plasma membrane [25]. Single-particle tracking using quantum-dot labeled CFTR showed that the channel diffusion is confined to <200 nm in both non-polarized cells and epithelia [25,26]. It was shown in these studies that the immobile fraction and the diffusional mobility of CFTR were significantly increased when CFTR binding to EBP50 was eliminated. Thus, our results are consistent with the idea that transient PDZ interactions contributes to the dynamic recruitment or clustering of CFTR to lipid rafts in response to TNF-α. Elimination of these interactions, which may alter specific localizations of CFTR to the membrane or prevent the formation of multiprotein complexes [18], may explain the defective recruitment of CFTR-ΔTRL to DRMs in MDCKI cells.

The mechanism underlying TNF-α-dependent mobilization of CFTR to lipid raft has not been established in this study. Engagement of TNF-α with its cognate receptor results in the release of the inhibitory protein silencer of death domains (SODD), oligomerization of TNFR1 receptors and recruitment of additional protein partners to form a receptor signaling complex [27]. TNF-α signaling triggers a variety of biological responses as diverse as proliferation, differentiation, inflammation and apoptosis. Recent findings have suggested that association of TNFR1 with lipid rafts determines the outcome of TNF-α-triggered signal pathways [12]. In activated T lymphocytes, translocation of TNFR1 into lipid raft results in anti-apoptosis and a NF-κB-dependent pro-inflammatory response. We found that TNF-α not only recruited CFTR to DRMs within minutes in WtCFTR cells but also TNFR1 and c-Src, a phenomenon that was also reproduced by inhibitors of SFKs activity. It is therefore possible that an early step in the recruitment of TNFR1 and protein partners into lipid rafts involves SFK inhibition by TNF-α. These observations suggest that WtCFTR confers to TNFR1 and c-Src the ability to mobilize into lipid rafts upon TNF-α stimulation. c-Src has been shown to interact with the cytoplasmic domain of TNFR1 [28]. Although the molecular mechanisms remain to be elucidated, our results suggest that CFTR contributes the formation of a TNFR1/c-Src signaling complex in DRMs. This possibility is strengthened by the recent report of Chen and collaborators who showed that transient anchorage of CFTR to microdomains depends on its PDZ-binding domain and SFK inhibition [29]. In contrast, the absence of CFTR in DRMs was associated with abnormal TNFR1 signaling as revealed by no recruitment of TNFR1 and c-Src to lipid rafts in cells MDCKI expressing CFTR-ΔTRL.

There is only one study so far reporting biochemical and functional evidence for a role of CFTR in DRMs [14]. In this study, the non specific CD molecule was used to extract cholesterol and assess lipid raft function. The differential effect of TNF-α on CFTR recruitment to DRMs in WtCFTR and CFTR-ΔTRL MDCKI cells that we report here therefore provides a genetic mean to evaluate the contribution of DRM-associated

CFTR on the biological responses elicited by TNFR1 signalling in an epithelial cell line. In this context, we evaluated TNF-α responses on GJIC and IL-8 production. In keeping with previous reports [7,8], we observed that TNF-α decreased Cx43-mediated GJIC in MDCK1 expressing WtCFTR. Interestingly, this regulation was absent in CFTR-ΔTRL cells, suggesting that recruitment of CFTR to DRMs is an important step for the TNFR1-dependent regulation of Cx43 channel activity. It is well established that c-Src tyrosine kinase activity can abolish GJIC in Cx43-expressing cells, including normal airway epithelial cells [8,30]. It is interesting to note that, in MDCKI cells, activation of TNFR1 signaling is associated with the parallel recruitment of CFTR and c-Src in DRMs. It is therefore tempting to hypothesize that complexes containing CFTR and c-Src in DRMs may transduce signals from membrane receptors to Cx43 channels. Whether DRM-associated CFTR modulates the activity of c-Src in response to TNFR1 activation remains, however, to be investigated. As a second biological parameter in MDCKI cells, we have studied the secretion of IL-8 in response to TNF-α. We observed that only WtCFTR cells strongly responded to TNF-α in terms of IL-8 secretion whereas MDCKI cells expressing the CFTR-ΔTRL showed modest increase in their IL-8 production. This observation is consistent with one previous study reporting that a cytokine signaling cascade is regulated by the PDZ-binding domain of CFTR and its interaction with EBP50 [31]. These results suggest, therefore, that WtCFTR in lipid rafts intersects with the TNFR1 signaling cascade to regulate cytokine expression. Consistent with this interpretation is the finding that nuclear translocation of NF-κB was less efficient in MDCKI cells expressing the CFTR-ΔTRL protein.

TNFR1 signaling is central in the transmission of the innate immune response and subsequent activation of the adaptive immune system. The functioning of both systems is required for optimal clearance of pathogens from the airways. Previous results have shown that cultured human airway epithelial cells as well as airway epithelial cells of mice expressing wild-type CFTR better internalize *Pa* than cells or mice unable to express CFTR [32]. In addition, CFTR-expressing cells showed rapid nuclear translocation of NF-κB and enhanced rate of apoptosis in response to infection [33,34]. Our observations support therefore a scenario in which CFTR, in association with TNFR1 and c-Src presented within the context of lipid rafts, provides a determinant step to coordinate inflammatory response and maintain tissue homeostasis. Alteration in the formation of this complex may provide an explanation to some dysfunction of CF epithelial cells. In this regards, we have observed that MDCKI cells expressing the ΔF508CFTR, which does not reach the plasma membrane and is not detected in lipid rafts [14], showed defective TNF-α-dependent regulation of GJIC and IL-8 secretion. Thus, disruption of CFTR interactions with component of the TNFR1 complex may alter c-Src and NF-κB activities, leading to abnormalities that may be relevant for the CF pathogenesis.

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