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RNA INTERFERENCE AGAINST CFTR AFFECTS HL60-DERIVED NEUTROPHIL MICROBICIDAL FUNCTION

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Abstract

Biosynthesis of hypochlorous acid (HOCl), a potent anti-microbial oxidant, in phagosomes is one of the chief mechanisms employed by polymorphonuclear neutrophils (PMNs) to combat infections. This reaction, catalyzed by myeloperoxidase, requires chloride anion (Cl⁻) as a substrate. Thus, Cl⁻ availability is a rate-limiting factor that affects neutrophil microbicidal function. Our previous research demonstrated that defective CFTR, a cAMP-activated chloride channel, present in cystic fibrosis (CF) patients leads to deficient chloride transport to neutrophil phagosomes and impaired bacterial killing (Painter *et al.*, 2008 & 2010). To confirm this finding, here we used RNA interference against this chloride channel to abate CFTR expression in the neutrophil-like cells derived from HL60 cells, a promyelocytic leukemia cell line, with DMSO. The resultant CFTR deficiency in the phagocytes compromised their bactericidal capability, thereby recapitulating the phenotype seen in CF patient cells. The results provide further evidence suggesting that CFTR plays an important role in phagocytic host defense.

Keywords

HL60 cells; neutrophils; hypochlorous acid; siRNA; CFTR; bacterial killing

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are the first line of defense mobilized by the host in response to infections. To eliminate the invading microbes, PMNs initially contain the organisms in phagosomes via phagocytosis and then mount a coordinated and synergistic chemical attack with both pre-fabricated and *de novo* produced anti-microbial agents [1-2]. The *de novo* produced agents include various reactive oxygen species, among which

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hypochlorous acid (HOCl), the active component in common laundry bleach, is of the most potency. According to the chemical reaction ($\text{H}_2\text{O}_2 + \text{H}^+ + \text{Cl}^- \xrightarrow{\text{MPO}} \text{HOCl} + \text{H}_2\text{O}$), chloride is an indispensable substrate for myeloperoxidase (MPO)-catalyzed production of HOCl. Activated neutrophils generate copious amounts of HOCl, accounting for ~50-90% of the total oxygen consumption by neutrophils during the respiratory burst [3-5]. The rate of HOCl production in phagosomes is estimated to be ~134 mM/min [4]. Thus, we hypothesized that chloride availability to neutrophil phagosomes is essential for optimal oxygen-dependent neutrophil microbicidal function. Our previous research has shown that human peripheral blood neutrophils express cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel [6], and that the neutrophils from cystic fibrosis patients, which lack a functional CFTR, are defective in chloride transport to the phagosomes [7]. Such a defect leads to an impaired intraphagosomal chlorination and killing of *Pseudomonas aeruginosa* [6,8].

In the current study, a lentiviral vector system, expressing a short hairpin RNA (shRNA) against CFTR, was developed and CFTR-knockdown was achieved in HL60 cells, a human promyelocytic leukemia cell line. Neutrophil-like cells derived from the DMSO-induced differentiation of the gene-manipulated myeloid precursors demonstrated deficient CFTR expression and impaired bactericidal capacity. The results extend our previous findings and further demonstrate the significance of the CFTR chloride channel in normal phagocytic host defense.

MATERIALS AND METHODS

Chemicals

Human male AB serum, saponin, zymosan, and other common chemicals were obtained from Sigma (St. Louis, MO) and normal goat serum from Invitrogen (Carlsbad, CA). Mouse IgG_{2a} isotype antibody, CFTR 24-1 and CFTR 13-1 monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN). The AlexaFluor 488 F(ab')₂ fragment of goat anti-mouse IgG(H+L) antibody was from Molecular Probes (Carlsbad, CA). The custom oligonucleotides were ordered from IDT (Coralville, IA). The siRNA expression vector pSilencer 1.0-U6 was obtained from Ambion (Austin, TX).

Culture of HL60 cells and differentiation into neutrophils

The parental HL60 cells, CFTR-knockdown cells (HL60-shCFTR), and mock-control cells (HL60-shControl) were cultured at an initial density of 1.5×10^5 cells/ml in RPMI 1640 medium containing 2 mM GlutaMax (Gibco), 10% heat-inactivated bovine growth serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin and amphotericin B (0.25 µg/ml). For differentiation into PMN-like cells, the medium was supplemented with 1.25% DMSO. On day 3, the culture medium was replenished. Experiments were performed with the cells after differentiation for 5 days.

Reverse transcription PCR to confirm CFTR expression in HL60 cells

Total RNAs from HL60 cells and peripheral blood neutrophils (PMNs) purified by Percoll density gradient fractionation were isolated using Trizol RNA reagent (Invitrogen, Carlsbad, CA), followed by reverse transcription using the High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA). A reverse transcriptase-free reaction was included as a negative control. The reverse-transcribed cDNA was subjected to PCR amplification for CFTR using the ThermalAce DNA polymerase (Invitrogen) under the following conditions: 95°C for 3 min, 42 cycles of 95°C for 30 sec, 48°C for 45 sec, 74°C for 1.5 min and a final cycle of 74°C for 10 min. The CFTR-specific primers were the forward primer (5'-

GGATTTGGGGAATTATTTGAGAAG-3') specific for the 3' end of Exon 9, and the reverse primer (5'-CATCATAGGAAACACCAAA-3') specific for the 5' end of Exon 10. A primer-free reaction was also included to serve as a negative control to distinguish primer-specific amplification. The size of the correct amplicon was predicted to be 331 bp. To verify CFTR-specific amplification, the PCR products from both PMNs and HL60 cells were gel-purified and ligated into the pCRII-Blunt-TOPO vector (Invitrogen). Clones were confirmed by DNA sequencing.

Immunostaining of CFTR in HL60-derived neutrophils during phagocytosis

Zymosan particles (10 mg/ml) in PBS were opsonized by incubation with pooled human male AB sera for 30 minutes at 37°C. DMSO-differentiated neutrophil-like cells were fed the opsonized zymosan particles at a ratio of 5:1 (zymosan/cells). The mixture was held on ice for 5 minutes to allow binding of the opsonized particles to the cells. Synchronized phagocytosis was initiated by incubating the mixture at 37°C for 15 minutes. After phagocytosis, the cells were placed on ice for 5 minutes to stop further phagocytosis and subsequently fixed with 2% paraformaldehyde for 30 minutes at room temperature. The fixed cells were permeabilized with 0.1% saponin in PBS, blocked with 5% normal goat serum, and stained with either mouse IgG_{2a} isotype or mouse anti-CFTR 24-1 primary antibody. The secondary antibody in both groups was AlexaFluor 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L). The stained cells were resuspended with Prolong Gold Antifade mounting reagent containing DAPI and mounted on glass microscope slides for visualization by standard fluorescence microscopy.

Generation of CFTR-knockdown and mock-control HL60 cell lines

Oligonucleotides for short hairpin RNA against CFTR or for sequence-scrambled control were synthesized according to the previous publication [9]. The short hairpin RNA expression cassettes consisting of the mouse U6 ribosomal RNA promoter driving transcription of the CFTR short-hairpin RNA (U6-shCFTR) or the sequence-scrambled control RNA (U6-shControl) were separately cloned into an HIV-1-based lentiviral vector system [10]. In the lentiviral vectors, there was another independent expression cassette containing an eGFP reporter gene driven by the cytomegalovirus (CMV) promoter. Vector production and concentration were performed according to the published protocol [11]. HL60 cells (ATCC, Manassas, VA) were, respectively, transduced with the vectors at an MOI of ~10:1. Two weeks after transduction, the cells from each vector transduction were sorted for eGFP expression by fluorescence-activated cell sorting (FACS) to a pure population. The resultant cell lines were referred to as HL60-shCFTR and HL60-shControl.

Quantitative real-time PCR to validate CFTR knockdown

Total RNA was isolated from DMSO-differentiated HL60-shControl and HL60-shCFTR cells using the RNeasy kit from Qiagen (Valencia, CA). We employed the two-step method for real-time PCR according to the Applied Biosystems protocol. First, 1 µg of total RNA from each cell line was reverse transcribed in 20 µl reactions using the iScript Select cDNA synthesis kit from BioRad (Hercules, CA) with either CFTR-specific primers from the commercially available TaqMan Gene Expression Assay kit for CFTR (HS00357011, Applied Biosystems, Foster City, CA) or random primers, respectively, according to the manufacturer's protocol. Next, to quantify CFTR mRNA levels, we used the aforementioned TaqMan Assay kit for CFTR (FAM-labeled). To quantify the levels of reference GAPDH mRNA, we used the target-specific primer-probe set from the TaqMan GAPDH control reagents kit (Applied Biosystems, #402869). Real-time PCR reactions contained 1 µl of reverse transcription product and were performed using the Mx3000P Real-Time PCR system (Stratagene).

Immunoprecipitation of CFTR and *in vitro* phosphorylation with [$\gamma^{32}\text{P}$]-ATP

DMSO-differentiated HL60-shControl and HL60-shCFTR cells were harvested by centrifugation at $350\times g$ for 10 minutes and resuspended in PBS at 1×10^7 cells per ml. 1×10^7 cells were pipetted into microfuge tubes and centrifuged at $350\times g$ for 5 minutes, and the pellets were resuspended in the lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 135 mM NaCl, one Roche complete mini multi-protease inhibitor tablet, 30 mM NaF and 3 mM β -glycerophosphate to inhibit phosphatases. After 10 minutes on ice, 25 μl of Pansorbin (Calbiochem) was added to each of the cell extracts and the samples were then incubated for 15 minutes on ice. Next, the samples were centrifuged at $20,000\times g$ for 20 minutes at 4°C . The supernatants were harvested and 1/5 volume of 5x radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) was added, and the samples were mixed well. One μg of a mouse IgG₁ monoclonal antibody M3A7 against CFTR (Millipore) was added to each supernatant, and after mixing, the samples were incubated on ice for 90 minutes. Paramagnetic beads coated with Protein G (20 μl ; Invitrogen) were added as supplied and incubated with rotation at 4°C for 2 hours. Using a magnet, the beads were washed 4 times with ice-cold 1x RIPA buffer and once with a transition buffer (50 mM Tris-HCl, pH 7.5, 135 mM NaCl). The final bead pellets were resuspended in 30 μl of PKA buffer (50 mM Tris-HCl, 135 mM NaCl, 0.01% BSA). To each sample, 20 μl of a cocktail containing 10 μCi [$\gamma^{32}\text{P}$]-ATP (3000 Ci/mmol, 10 mCi/ml; Perkin-Elmer) and 5 units of the catalytic subunit of PKA (Promega) in PKA buffer was added. After mixing, the samples were incubated at 30°C for 1 hour. The samples were magnetically washed with ice-cold 1x RIPA buffer twice and once with Tris-buffered saline (TBS). The final pellets were resuspended in 30 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing dithiothreitol (DTT), and after incubation at 37°C for 20 minutes, the samples were subjected to SDS-PAGE using the Invitrogen NuPAGE Bis-Tris gels (4-12% gradients SDS-PAGE gels) according to manufacturer's instructions using the provided MOPS running tank buffer. The gel was fixed by washing for 1 hour in 20% methanol, 5% acetic acid in water and then dried on a BioRad vacuum gel dryer at 80°C . The radioactive protein bands were revealed by autoradiography using an enhancing screen. A separate gel was run and stained with Ponceau S to reveal an equal protein input for immunoprecipitation. The control gel was imaged using an Alpha Innotech (San Leandro, CA) imager with a visible (white) light transilluminator.

Immunostaining of phagosomes and flow cytometry

Neutrophil-like cells differentiated from HL60-shControl and HL60-shCFTR cells were washed twice and resuspended in a chloride-free Ringer's buffer (122 mM sodium gluconate, 1.2 mM magnesium gluconate, 4 mM calcium gluconate, 2.4 mM K_2HPO_4 , 0.6 mM KH_2PO_4 , 20 mM HEPES, pH 7.4 and 10 mM dextrose). Next, the cells were fed serum-opsonized polystyrene beads (1 μm in diameter) which were pre-coated with BSA and coupled with a rabbit anti-BSA antibody for 15 minutes at 37°C prior to further opsonization with pooled human AB sera. The adherent cells containing phagocytosed polystyrene beads were washed 4 times with the Ringer's buffer to remove free beads. The cells were then treated with the serine protease inhibitor diisopropylfluorophosphate (DFP, 5mM) and scraped from the plates into 1 ml of relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2 , 10 mM PIPES, 1 mM ATP) supplemented with the Sigma eukaryotic protease inhibitor cocktail (1:20 cocktail to buffer) and were homogenized by nitrogen cavitation at 400 psi for 10 minutes. The homogenates were collected in tubes containing final concentrations of the following: EGTA (1.25 mM), phenylmethanesulfonylfluoride (PMSF; 1 mM), benzamidine (1 mM), and orthovanadate (1 mM). Whole-cell lysates were then stained with Cy3-rabbit anti-human LAMP-1 (lysosome-associated membrane protein-1) in combination with either mouse anti-human CFTR antibody 13-1 (R&D,

Minneapolis, MN) or its IgG₁ isotype control antibody. The secondary antibody was AlexaFluor 488 F(ab')₂ fragment of goat-anti-mouse IgG (H+L). The stained samples were analyzed by flow cytometry with dual gating on the polystyrene bead population with positive LAMP-1 staining to ensure that only phagolysosomes were analyzed.

Measurement of oxidant production in control and CFTR knockdown HL-60 cell lines

Hypochlorous acid production was quantified by a modified method based on previously described protocols [12-13]. DMSO-differentiated HL60-shCFTR and HL60-shControl cells were washed and resuspended in 1 ml Dulbecco's phosphate buffered saline (DPBS) containing excess taurine (15 mM). The cells were stimulated to release granule and lysosome contents extracellularly by stimulation with 30 ng/ml PMA, which results in extracellular HOCl production and chlorination of the taurine in the buffer to form a taurine chloramine intermediate (N-chlorotaurine). Because 1 mole of taurine chloramine retains 2 mole oxidizing equivalents, the chloramine concentration can be sensitively determined by the ability to oxidize 2 mole of I⁻ to I₂. So in order to measure HOCl production, 20 mM potassium iodide (KI) was added to the reaction, and oxidation of I⁻ to I₂ was measured immediately by reading the light absorbance at 350 nm. The concentration of taurine chloramine was calculated based on I₂ generation, and HOCl production was expressed as nmol of N-chlorotaurine produced in 30 minutes per 1×10⁶ cells. Diphenyleneiodonium sulfate (DPI) and 4-aminobenzoic acid hydrazide (ABAH) were added to the chlorination reactions to specifically inhibit the NADPH oxidase and myeloperoxidase, respectively. A no-PMA group served as the stimulation-free control. In one group, reactions were carried out in chloride-free NaGluconate Ringer's buffer to demonstrate the chloride dependence of HOCl production.

Bacterial killing assays

Pseudomonas aeruginosa (PAO1) were cultured overnight in LB broth at 37°C with vigorous agitation. After centrifugation (5000× g) for 10 minutes, the cell pellet was suspended in PBS containing 10% fresh human male AB serum at a density of 1-2 ×10⁸ (A_{600nm}=0.2 OD) bacteria per ml and incubated for 30 minutes at 37°C with agitation for opsonization. The bacteria were then washed twice with the chloride-free Ringer's buffer, resuspended to 1-2 × 10⁹ per ml, and held on ice until use. Bacterial killing assays were performed using the published protocol [8,14]. Briefly, the opsonized PAO1 bacteria were dissociated by passing through a 25-gauge needle 3 times and mixed with DMSO-induced differentiated neutrophils (1 × 10⁷ per ml) at a ratio of 1:1 in the chloride-free Ringer's buffer containing 10% dialyzed, chloride-free human AB serum. After a 15-minute phagocytosis, the non-ingested bacteria were removed by low speed centrifugation (81× g) for 5 min at 4°C. The pellets were resuspended in buffers with either the chloride-free Ringer's buffer or a chloride-rich Ringer's buffer (122 mM NaCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 2.4 mM K₂HPO₄, 0.6 mM KH₂PO₄, 20 mM HEPES, pH 7.4 and 10 mM dextrose), followed by incubation at 37°C for 40 minutes for killing. The initial and remaining viable bacteria at different time points were determined. Data from multiple time points were normalized to 0 minutes. The percent relative viability of bacteria over time was compared between shControl and shCFTR groups (N=3 each) by two-way ANOVA with replication analysis. P-values of < or = 0.05 were considered statistically significant.

RESULTS

CFTR expression in HL60 cells and HL60-derived neutrophil-like cells

HL60 cells are a human promyelocytic cell line widely used to study neutrophil functions [15-16]. When induced with DMSO, retinoic acid or other organic compounds, the cells undergo myeloid differentiation. The terminally differentiated granulocytes after induction

share many functional features with peripheral blood mature neutrophils, including phagocytosis and microbicidal properties [17-18]. To test the hypothesis that CFTR is involved in neutrophil-mediated phagocytic defense, we first investigated if CFTR is expressed in HL60 cells. Total RNAs from non-differentiated HL60 cells and peripheral blood PMNs were extracted. Reverse-transcription PCR was performed to amplify the CFTR cDNA fragment by using the forward primer specific to exon 9 of human CFTR and the reverse primer to exon 10. Due to the large size of intron 9 which exceeds 10 Kb, this design only allows for amplification of CFTR cDNA under the used PCR condition. Gel electrophoresis revealed an amplified product with a size of ~331 bp, as expected if CFTR mRNA was present (Fig. 1A). The reverse transcriptase-free and the primer-free controls were negative in amplification of the same size product. DNA sequencing validated that the PCR amplicons from both PMNs and HL60 cells were identical and matched the predicted portion of human CFTR cDNA only (data not shown).

To identify the subcellular localization of CFTR in the HL60-derived neutrophil-like cells, we performed immunofluorescence staining of the differentiated cells during phagocytosis. As displayed, the serum-opsonized zymosan particles were ingested efficiently (Fig. 1 B & D). Immunostaining with either mouse IgG_{2a} isotype (Fig. 1C) or mouse anti-CFTR 24.1 primary antibody (Fig. 1E), followed by AlexaFluor-488-conjugated goat anti-mouse IgG secondary antibody, revealed the CFTR-specific intracellular immunofluorescence. Noticeably, the intracellular staining (Fig. 1F) was largely punctate, suggesting the granule-association of CFTR. The CFTR-positive granules were condensed around the phagocytosed particles (Fig. 1E & F, arrows), suggesting an ongoing granule recruitment and fusion.

Small interfering RNA to suppress CFTR expression

To genetically alter CFTR expression in HL60-derived neutrophils, we constructed two lentiviral vectors expressing either a short hairpin RNA (shRNA) against CFTR or a sequence-scrambled control. The structures of the transgene vectors are displayed (Fig. 2A). Each vector contained two independent expression cassettes: 1) an shRNA gene driven by the mouse U6 promoter and, 2) an eGFP reporter gene driven by the CMV promoter. High-titer VSVG-pseudotyped vectors were produced and concentrated as previously published [11]. HL60 cells were transduced with each vector at an MOI of ~10, individually. The cells were sorted by FACS for pure eGFP-expressing populations. These transduced cells, permanently expressing the transgenes, were confirmed by both fluorescent microscopy (Fig. 2E-F & H-J) and flow cytometry (Fig. 2D, G & J). The two established HL60 sublines were referred to as HL60-shCFTR and HL60-shControl.

We further examined whether the shRNA expression affects CFTR expression at both transcriptional and translational levels in the differentiated HL60-shCFTR and HL60-shControl cells. Similarly, the two sublines were cultured in the DMSO medium for 5 days, followed by RNA extraction and quantitative real-time RT-PCR. The differentiated HL60-shControl cells had 47.4 copies of CFTR-cDNA as normalized to the control GAPDH cDNA. In contrast, differentiated HL60-shCFTR cells had 7.5 copies, which represents ~84% knockdown (Fig. 3A). To compare the CFTR protein expression in the HL60-derived neutrophils, we used the approach of immunoprecipitation followed by *in vitro* phosphorylation with [γ ³²P]-ATP. Polyacrylamide gel electrophoresis (PAGE) and autoradiography allowed detection of a phospho-CFTR-specific band at ~180 KDa in the control neutrophils while detection of phospho-CFTR was dramatically diminished in the CFTR-knockdown cells (Fig. 3B). Even though we started the immunoprecipitation procedure from an equal number of cells for both knockdown and control, a Ponceau S staining of cell lysate proteins with an equal load of both cell lysates was performed to assess an equal amount of proteins used for the immunoprecipitation. The β -actin band at ~42 KDa is comparable, indicating that an even amount of proteins used for the

immunoprecipitation and *in vitro* phosphorylation assay (Fig. 3B). We then asked if the knockdown of CFTR mRNA results in a diminished CFTR protein presentation to phagosomes. The neutrophil-like cells derived from the control and CFTR-knockdown cells were fed with opsonized 1 μ m-diameter latex beads for 15 minutes and were then homogenized by nitrogen cavitation. The cell lysates were double-immunostained with Cy3-labeled rabbit anti-human LAMP-1 in combination with either a CFTR or an isotype control antibody. Both Cy3-LAMP-1 and CFTR 24-1 antibodies recognize the epitopes on the cytoplasmic C-termini of the LAMP-1 and CFTR proteins, respectively. Because the phagosomes are an “inside-out” organelle, the epitopes are accessible by adding the antibodies directly to the homogenate. When gated on both the 1- μ m bead population (Fig. 3C & F) and the LAMP-1 and CFTR dual-positive fraction (Fig. 3D & G), the CFTR and isotype control antibody staining of phagosomes can be assessed in a quantitative manner. The results show that the CFTR knockdown cells had a weaker CFTR staining on phagosomes with a mean channel fluorescence (MCF) of 65.1 (Fig. 3H) and the control cells had a MCF of 141.7 (Fig. 3E), representing ~54% reduction in CFTR protein presentation to the phagosomes.

CFTR knockdown in HL60 cells does not affect their differentiation and phagocytic capability

We next investigated whether CFTR knockdown affects HL60 myeloid differentiation. Wright-Giemsa staining was performed to appreciate the nuclear morphologic changes, according to which the cells were classified into the following 4 categories: promyelocytes, metamyelocytes, banded neutrophils and segmented neutrophils. The HL60-shControl and HL60-shCFTR cells after differentiation consisted of approximately 55.0 $\pm$ 12.7% and 57.0 $\pm$ 14.1% of metamyelocytes, respectively, 25.5 $\pm$ 19.1% and 16.5 $\pm$ 17.7% of banded neutrophils, respectively, and 6.0 $\pm$ 1.4% and 5.5 $\pm$ 6.4% of segmented neutrophils, respectively (Fig. 4A). Even with 5 days of DMSO-induced differentiation, 13.5 $\pm$ 7.8% of HL60-shControl and 20.5 $\pm$ 9.2% of HL60-shCFTR cells remained undifferentiated (promyelocytes). No significant difference was detected by Student's t-test between the control and knockdown cells in each differentiation category (Fig. 4A). These data are in accord with previously published results [19], of which 38% metamyelocytes, 26% of banded neutrophils and 2% segmented neutrophils were reported. Thus, our lentiviral vector transduction and transgene expression did not significantly alter DMSO-induced HL60 differentiation. Furthermore, the DMSO-differentiated neutrophils were fed serum-opsonized zymosan particles at a ratio of 5-10:1 (particles to cells). The number of ingested particles in the differentiated HL60-shCFTR and HL60shControl cells were counted microscopically and analyzed statistically. The mean number of particles ingested per cell was 4.0 $\pm$ 0.6 for HL60-shControl and 3.2 $\pm$ 0.5 for HL60-shCFTR (Fig. 4B). The results demonstrated no significant difference between the groups ($P = 0.15$, $n=3$).

CFTR knockdown does not affect PMA-stimulated oxidant production by HL60-derived neutrophils

Phorbol-12-myristate-13-acetate (PMA) can stimulate phagocytes to produce superoxide and H₂O₂. PMA also stimulates the release of MPO to the extracellular media [20-21]. Thus, in the presence of chloride, hypochlorous acid (HOCl) is produced extracellularly. The produced HOCl can be trapped by extracellular taurine, resulting in N-chlorotaurine which can be measured colorimetrically after the addition of potassium iodide. To compare oxidant production by the neutrophil-like cells derived from HL60-shCFTR and HL60-shControl cells, we similarly treated the cells with 1.25% DMSO for 5 days. As shown in Figure 5, when 127 mM chloride was present in the medium, the neutrophil-like cells derived from the HL60-shCFTR and HL60-shControl cells had abundant and comparable oxidant release, suggesting that neither the lentiviral vectors nor CFTR knockdown had any

effect on the oxidant production machinery. To validate that the NADPH oxidase-MPO-Cl⁻ system components are responsible for the extracellular oxidant production, DPI and ABAH, which are specific inhibitors for NADPH oxidase and MPO, respectively, were applied, and the HOCl production was abrogated. In the absence of PMA stimulation, the cells produced little to no HOCl. Moreover, when no chloride was provided in the system, the extracellular HOCl production was diminished, further indicating the requirement of this anion in HOCl production.

CFTR knockdown impairs neutrophil-mediated bacterial killing

We then analyzed the ability of the neutrophils derived from HL60-shCFTR and HL60-shControl cells to kill phagocytosed *Pseudomonas aeruginosa* (PAO1). Opsonized PAO1 bacteria were fed to the derived neutrophils at a ratio of ~1:1 in the chloride-free Ringer's buffer. After phagocytosis, the bacteria-containing neutrophils were placed in either the chloride-free or the chloride-rich Ringer's buffer for different periods of time. Viable bacteria at each time point were quantified by saponin lysis and plating for overnight culture. This design allowed us to assess the contribution of the extracellular chloride to bacterial killing in the system. Percent viability relative to time 0 was obtained for each time point and compared by two-way ANOVA analysis. The results, shown in Figure 6, demonstrate that the bactericidal capability of the control cells (shControl) in the 127 mM chloride solution was significantly greater than that in the 0 mM chloride solution, indicating that there existed a component of chloride-dependent killing. However, when CFTR was abated, such chloride-dependent killing diminished, suggesting that CFTR is a major channel transporting chloride to the phagosomal lumen and the chloride-dependent component is also CFTR-dependent. This conclusion was also validated by no difference in bacterial killing between the CFTR Knockdown cells in 127 mM chloride buffer and the wild-type control cells in 0 mM chloride buffer.

Proposed model to explain CFTR involvement in neutrophil microbial killing

Because of the need for chloride by MPO to produce HOCl in neutrophils [4], chloride anion has to be supplied to the phagosomes. We explain our results by the following proposed model (Fig. 7). CFTR and perhaps other intracellular chloride channels allowed chloride to flow from the cytosol to the phagosomal lumen. There were potentially two forces to drive the anion movement: 1) gradient chemical force due to the high cytosolic chloride [22] and the low phagosomal chloride after MPO consumption, and 2) electric force due to the relative positive charge inside the phagosomal lumen [23]. Therefore, under our designed experimental condition, if the intracellular and extracellular chloride was depleted (**Panels A and C**), neither the control (ShControl) nor the CFTR-Knockdown (ShCFTR) HL60-neutrophils obtained any significant chloride for HOCl synthesis, thus demonstrating no difference in bacterial killing. However, when the intracellular chloride was depleted and the extracellular chloride was supplied after bacterial phagocytosis (**Panels B and D**), the wild-type control provided more chloride to the phagosomes for HOCl production, thus showing a significantly higher bacterial killing rate than the CFTR-Knockdown cells.

Discussion

This report provides direct evidence to suggest that CFTR channel deficiency in neutrophil-like cells affects their microbicidal function, which recapitulates our previous finding that CFTR defect in the neutrophils of CF patients impairs phagocytic innate immunity. To date, multiple chloride transporters have been identified in neutrophils, such as Ca²⁺-activated chloride channels [24], CIC-3 (Chloride channel-3) [25], the Na⁺-K⁺-2Cl⁻ exchanger [22,26], the Cl⁻-HCO₃⁻ exchanger [26], the swelling-induced chloride channels (ICl_{swell}) [27-28] and the PKA-activated CFTR chloride channel [6]. CIC-3 and CFTR have been

found on neutrophil phagosomes. Loss of function of CIC-3 impairs neutrophil chemotaxis ability, and inhibition of $\text{ICl}_{\text{swell}}$ affects cell volume regulation [28]. However, the CFTR channel has been predominantly associated with phagosomal chloride acquisition [7]. The limited distribution of CFTR in secretory vesicles of resting PMNs and its later redistribution to phagosomes during phagocytosis explains the very selective role of this molecule in chloride transport to phagosomes. Therefore, individual chloride transporters may play different roles in neutrophil functions. Further studies are needed to fully understand the complexity of chloride regulation in neutrophils.

Chloride is the most abundant anion in the human body. The common function of this anion includes adjustment of cell volume, modulation of intracellular pH and stabilization of resting membrane potential [29]. In addition to the general function of chloride, neutrophils utilize this anion in a unique way to produce the HOCl oxidant for microbial killing [30]. The importance of chloride in neutrophil function is reflected by the significant intracellular Cl^- concentration (~80 mEq) in neutrophils, which is much higher than in other cells [22]. It is well-defined that the biosynthesis of HOCl and microbicidal activity largely occurs in phagosomes where chemical assaults are mounted against the phagocytosed bacteria. We measured the steady state level of chloride in neutrophil phagosomes to be ~73 mEq per liter [31]. Apparently, the high cytoplasmic chloride level favors phagosome chloride acquisition and thus oxidant production. However, it is not completely understood how chloride anion is delivered to the phagosomes of neutrophils. There are three possible pathways for the organelle to attain chloride: 1) inclusion of extracellular medium during phagocytosis, 2) release of granule-stored chloride during granule fusion with the nascent phagosomes and, 3) transport either passively or actively of chloride from the cytosol into the phagosome lumen. The first two sources are limited and believed to be consumed within the first several minutes after phagocytosis [4]. Only the third pathway can provide a continuous supply of this anion. Our previous research demonstrated that CFTR is a major channel transporting chloride to neutrophil phagosomes [7] and defects of this channel in CF patients cause bactericidal deficiency [8]. In this report, we genetically altered the expression of CFTR in HL60 cells, a promyelocytic cell line, and their derived neutrophils by small RNA interference. This gene manipulation and *in-vitro* differentiation procedure resulted in CFTR-deficient neutrophil-like cells with an identical genetic background. We found that CFTR deficiency in the HL60-derived neutrophils, mimicking the CFTR defect in CF patient neutrophils, impedes neutrophil microbicidal function.

Figure 4 demonstrates that the extracellular HOCl production was comparable between the knockdown and control cells, when MPO was released to the extracellular media with chloride available. These data indicate that the CFTR knockdown did not affect NADPH oxidase activity and the differentiated neutrophils are capable of generating oxidants. We previously reported that CFTR is a major channel in transporting chloride to the phagosome but not the cytosol [7], suggesting that CFTR is largely an intracellular chloride channel in neutrophils. The CFTR knockdown cells had significantly less CFTR on the phagosomes which presumably limits chloride availability in the organelle. Such a deficiency would lead to less intraphagosomal HOCl production. *Pseudomonas aeruginosa* bacteria possess tough polysaccharide capsules which are resistant to non-oxidant killing mechanisms, such as protease and hydrolase digestion [32]. Therefore, HOCl is a key neutrophil-produced agent for the eradication of this pathogen by the phagocytes [32-34].

Mature neutrophils are terminally differentiated with a very short life span. Furthermore, these end-stage cells are intrinsically refractory to gene manipulation because most of the gene expression is completed by the end of terminal differentiation. These properties make it difficult to study gene functions in these cells. HL60 is a promyelocytic cell line, which can be expanded in culture and induced to differentiate. These cells have been widely used for a

variety of studies of neutrophil maturation and function. In spite of lacking expression of the secondary granule proteins presented in the differentiated mature cells, these cells are able to generate reactive oxygen species including H_2O_2 and $HOCl$. Therefore, the cell model can be used to study phagocytic oxidant production and oxidant-mediated microbicidal activity. In the current report, we permanently transduced HL60 cells with a lentiviral vector expressing siRNA against CFTR, and the expression of this chloride channel was abated in their derived neutrophil-like cells after DMSO-induced differentiation. The results validate a method for genetically engineering myelogenic progenitor cells to achieve gene manipulation in terminally differentiated cells to study their gene functions.

In summary, CFTR is expressed in HL60 cells and their derived neutrophil-like cells. This cAMP-activated chloride channel pronouncedly localizes to phagosomes after phagocytosis. Lentiviral vectors expressing the small interfering RNA against CFTR in the HL60-derived phagocytes significantly impairs their bactericidal function. The observation is consistent with the previous finding that neutrophils from CF patients exhibit deficient chloride-mediated microbicidal function. Therefore, the CFTR chloride channel plays an important role in phagocytic innate immunity.

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ABBREVIATIONS

ABAH	4-aminobenzoic acid hydrazide
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
DFP	Diisopropylfluorophosphate
DMSO	Dimethyl sulfoxide
DPI	Diphenylene iodonium
DTT	Dithiothreitol
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
HIV-1	Human immunodeficiency virus type 1
HOCl	Hypochlorous acid
LAMP-1	Lysosome-associated protein-1
MPO	Myeloperoxidase
NaF	Sodium fluoride
NaGluconate	Sodium gluconate
PAO1	<i>Pseudomonas aeruginosa</i>
PMNs	Polymorphonuclear neutrophils
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

shCFTR	Short hairpin RNA against CFTR
shControl	Short hairpin RNA scrambled, non-binding control
shRNA	Short hairpin ribonucleic acid
MOI	Multiplicity of infection

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Figure 1. CFTR expression in HL60 cells and their derived neutrophil-like cells

A) Transcription of CFTR – Reverse transcription PCR using exon-specific primers produced cDNA-specific amplification of CFTR fragment, confirming that PMNs and HL60 cells express CFTR at the mRNA level. The reverse transcriptase-free [RT(–)] and primer-free [Primer(–)] controls showed no specific amplification. **B–F)** Subcellular localization of CFTR – DMSO-differentiated HL60 cells were fed serum-opsonized zymosan at a ratio of 5:1 (zymosan to neutrophil) and were subsequently stained with either mouse IgG_{2a} isotype or mouse anti-CFTR 24-1 primary antibody. **B & D)** Relief contrast images show engulfment of zymosan particles by the differentiated neutrophils. **C & E)** Fluorescence microscopy shows that CFTR-positive granules distribute around the phagocytosed particles (**arrows**). **F)** An enlarged image of CFTR staining shows the intracellular punctate staining pattern and potential granule fusion (**arrows**).

Figure 2. Small interfering RNA to suppress CFTR expression

A) Structures of the transgene constructs – The short hairpin CFTR sequence (shCFTR) and the scrambled control sequence (shControl) were under the transcriptional control of the mouse U6 promoter. The eGFP reporter gene is an independent expression cassette driven by the cytomegalovirus (CMV) promoter. Micrographs by relief contrast and fluorescent microscopy of the parental HL60 (**B & C**), HL60-shControl (**E & F**) and HL60-shCFTR (**H & I**) cells showed expression of eGFP in the transduced cells which was confirmed by flow cytometry analysis of each cell subtype (**D, G, & J**, respectively).

Figure 3. CFTR expression in the control and CFTR-knockdown HL60-derived neutrophils

A) Quantitative RT-PCR of CFTR transcripts as normalized to GAPDH – HL60-shCFTR derived cells had 84.2% less CFTR expression than that of the HL60-shControl cells, confirming CFTR knockdown by the siRNA. **B)** Immunoprecipitation and *in vitro* phosphorylation to assess CFTR protein expression – an equal number of DMSO-differentiated HL60-shControl and HL60-shCFTR neutrophil-like cells were lysed and the cell lysates were immunoprecipitated for CFTR protein. *In vitro* phosphorylation with radioactive [γ^{32} P]-ATP was performed to label CFTR followed by SDS-PAGE. Autoradiography displays CFTR-specific band at ~180 KDa (top panel). Ponceau S staining of a membrane transferred with the total cell lysate proteins after gel electrophoresis was performed. The β -actin band at ~42 KDa is specifically displayed to shown an equal protein application to the immunoprecipitation (bottom panel). **C-H)** Flow cytometry analysis of CFTR expression on phagosomes – Both HL60-shControl- and HL60-shCFTR-derived neutrophils were allowed to phagocytose serum-opsonized 1- μ m latex beads. After phagocytosis, the cells were homogenized by nitrogen cavitation. Antibody staining and flow cytometry were performed on the cell lysates by adding a Cy3-conjugated antibody against human lysosome-associated membrane protein-1 (LAMP-1) and a mouse anti-human CFTR antibody. **C & F)** The forward scatter (FSC) and side scatter (SSC) parameters of the stained cell lysates were adjusted to gate on the 1- μ m bead population. **D & G)** The 1- μ m beads were analyzed for LAMP-1 and CFTR expression. The dual-positive population represents phagolysosome membranes containing both LAMP-1 and CFTR. **E & H)** The dual-positive beads were further analyzed for CFTR expression levels. The shaded lines represent the CFTR antibody staining. The black lines represent the control isotype antibody staining.

Figure 4. Differentiation and phagocytosis of the DMSO-differentiated HL60-shControl and HL60-shCFTR cells

A) Differentiation of the differentiated cells – Both control and CFTR-knockdown cell lines were differentiated into neutrophil-like cells by exposure to 1.25% DMSO for 5 days and stained with Wright-Giemsa stain. Differentiation was classified based on the degree of nuclear polymorphism as follows: round nuclei – promyelocytes (undifferentiated), nuclei with apparent invaginations – metamyelocytes, elongated nuclei with distinct banding – banded neutrophils, polymorphic nuclei – segmented neutrophils. Percentages of each cell type in the total cell populations were calculated and compared between cell lines by Student's t-test. There was no statistical difference in the proportion of the differentiated cells in each culture between the control and CFTR-knockdown cells. **B)** Phagocytosis by the differentiated cells – Differentiated cultures of HL60-shControl and HL60-shCFTR cells were fed serum-opsonized zymosan particles at a ratio of ~5-10:1 (particles to cells) for 15 minutes at 37°C. After phagocytosis, the cells were fixed and mounted onto glass slides by cytospin. The number of particles per phagocytic cell was determined microscopically and compared between control and CFTR-knockdown groups by Student's t-test ($P = 0.15$, $n = 3$).

Figure 5. Hypochlorous acid (HOCl) production by control and CFTR-knockdown HL60-derived neutrophils

Both DMSO-differentiated HL60-shControl and HL60-shCFTR cells produced abundant HOCl when stimulated with PMA and supplied with physiological chloride in the extracellular media. The MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) and the NADPH oxidase inhibitor diphenylene iodonium (DPI) diminish the extracellular production of HOCl in this system. There is little HOCl produced when the cells are incubated in a chloride-free medium (NaGluconate Ringer's buffer, 0 mM Cl⁻). Throughout, there was no statistical difference in extracellular HOCl production between the control and CFTR-knockdown cell lines suggesting that the oxidant production machinery is unaffected by the CFTR knockdown genetic manipulation.

Two-way ANOVA Analysis of Different Group Combination

	shControl 0 mM	shControl 127 mM	shCFTR 0 mM	shCFTR 127 mM
shControl 0 mM	N/A	< 0.0001	0.2295	0.7333
shControl 127 mM	< 0.0001	N/A	< 0.0001	< 0.0001
shCFTR 0 mM	0.2295	< 0.0001	N/A	0.3596
shCFTR 127 mM	0.7333	< 0.0001	0.3596	N/A

Figure 6. Killing of *Pseudomonas aeruginosa* by neutrophil-like cells derived from HL60-shControl and HL60-shCFTR cells

Percent viability (relative to time 0) of the phagocytosed bacteria by the control (shControl) or the knockdown (shCFTR) cells was plotted against time (Minutes). The graph represents 3 independent experiments. Two-way ANOVA analyses of the different group combinations were performed and the results shown below. In the absence of chloride, neutrophils differentiated from the control and knockdown cells had a low bacterial killing efficiency and showed no statistical difference. However, in the presence of 127 mM chloride, the control cells, but not the knockdown cells, showed an increased bactericidal activity with a statistically significant difference ($p < 0.0001$, $n = 3$). Therefore, the loss of CFTR in the knockdown cells impaired the chloride-dependent microbicidal capacity of the cells.

Figure 7. Proposed model to explain CFTR involvement in phagocytic microbicidal function

When intracellular and extracellular chloride was depleted (**Panels A & C**), the phagosomes of both control HL60-neutrophils (ShControl) and CFTR Knockdown (ShCFTR) HL60-neutrophils acquired little chloride. Thus, HOCl production was affected for both cells, showing no bacterial killing difference. However, when extracellular chloride was supplied after phagocytosis, only the control HL60-neutrophils (ShControl) (**Panel B**), but not the CFTR Knockdown (ShCFTR) HL60-neutrophils (**Panel D**) provided significant chloride to the phagosomes to generate HOCl, thus showing greater bacterial killing than the knockdown cells.