DISEASE CAUSING MUTATIONS IN THE CYSTIC FIBROSIS TRANSMEMBRANE
CONDUCTANCE REGULATOR DETERMINE THE FUNCTIONAL RESPONSES OF
ALVEOLAR MACROPHAGES

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Alveolar macrophages (AMs) play a major role in host defense against microbial infections in the lung. In order to perform this function, these cells must ingest and destroy pathogens, generally in phagosomes, as well as secrete a number of products that signal other immune cells to respond. Recently, we demonstrated that murine alveolar macrophages employ the CFTR (Cystic Fibrosis Transmembrane conductance Regulator) Cl⁻ channel as a determinant in lysosomal acidification (1). Lysosomes and phagosomes in murine cftr-/− AMs failed to acidify and the cells were deficient in bacterial killing compared to wild-type controls. Cystic fibrosis is caused by mutations in CFTR and is characterized by chronic lung infections. The information about relationships between CFTR genotype and disease phenotype is scarce both on the organismal and cellular level. The most common disease-causing mutation, ΔF508, is found in 70% of CF patients. The mutant protein fails to fold properly and is targeted for proteosomal degradation. G551D, the second most common mutation causes loss of function of the protein at the plasma membrane. In this study, we have investigated the impact of CFTR ΔF508 and G551D on a set of core intracellular functions including organellar acidification, granule secretion, and microbicidal activity in the AM. Utilizing primary AMs from wild-type, cftr+/−, as well as mutant mice, we show a tight correlation between CFTR genotype and levels of lysosomal acidification, bacterial killing and agonist-induced secretory responses all of which would be expected to contribute to a significant impact on microbial clearance in the lung.

Macrophages and neutrophils are key cells of the innate immune system. Blood monocytes infiltrate different tissues and then differentiate into tissue-specific macrophages that perform vital host defense functions while neutrophils (PMNs) are recruited from the blood to sites of infection. Mature macrophages from distinct sources exhibit significant variation in molecular and cellular properties as well as gene expression profiles specific for their host tissue, while maintaining a common set of core functions (2). One such cell type is the alveolar macrophage (AM) that resides in the terminal airway alveoli of the lung from where they recruit PMNs that respond chemotactically to microbial insult. PMNs are generally considered the dominant component of innate immunity because of their sheer numerical superiority. While less numerous than the mobile PMNs, AMs live longer and are a more potent source of the cytokines that orchestrate the immune response to bacterial pathogens. Ultimately, macrophages are also responsible for clearing apoptotic PMNs from infection sites, also by phagocytosis (3,4). Delayed removal of the dying cells results in chronic inflammation with resultant tissue damage as seen in several chronic inflammatory lung diseases including cystic fibrosis (CF) (for review see Henson et al. (5)).

Professional phagocytes have specialized pathways that ensure efficient killing of pathogens in phagosomes (6). A common element in these pathways is organellar acidification that facilitates the optimal functioning of various degradative enzymes, particularly in phagosomes (7). Indeed, low pH is required in several organelles for diverse functions in many cell types: e.g. maturation of secretory products and their final secretion by the exocytotic pathway and dissociation and recycling
in the endosomal pathway. Generation of low organellar pH is primarily driven by the V-ATPases, proton pumps that use cytoplasmic ATP to load H⁺ into the organelle (8,9). Alongside the pumps are various channels that shunt the transmembrane potential generated by movement of protons; in different organelles these comprise H⁺ channels, K⁺ channels and Cl⁻ channels. Without these shunt pathways acidification is limited and organelle function is compromised (10).

Cl⁻ channels are central to the function of several intracellular organelles (11) and recently we have shown definitively that the specialized Cl⁻ channel CFTR is important in lysosomal and phagosomal acidification in murine and human AMs (1). In parallel, others have shown that CFTR expressed on secretory granules may be involved in human neutrophil phagocytic function, being expressed on secretory granules (12). Painter et al. showed that CFTR is also expressed on phagolysosomes in neutrophils where it may contribute Cl⁻ crucial to the chlorination reactions involved in bacterial killing (12). They demonstrated that neutrophils from CF patients exhibited a defect in chlorination of *Pseudomonas aeruginosa* proteins, presumably due to poor production of hypochlorous acid in phagolysosomes. Interestingly, clinical studies also suggest a phagocytic defect in pulmonary neutrophils from CF patients, although the mechanism is not known (13). Combined with our own observations these results suggested that CFTR function may operate at several levels in regulating normal innate immunity.

In the present study, we have used AMs from both *cftr<sup>+/+</sup>* and *cftr<sup>-/</sup>-* mice as well as AMs from mutant mice expressing the two most common disease-causing CFTR mutations, ΔF508 and G551D. The ΔF508 is an in frame deletion of phenylalanine at the position 508 of exon 10 in the *cftr* gene. In most tissues, this mutated protein is rapidly degraded in the endoplasmic reticulum due to misfolding; however, if the channel reaches the plasma membrane (or perhaps the lysosomal/endosomal compartment) it is functional with a 7-fold decrement in PKA-dependent activation over that observed for wild-type channels (14). CFTR G551D is a missense mutation in exon 11 which is located in the signature sequence of the first nucleotide binding domain. The G551D mutation results in a channel that is appropriately localized to the plasma membrane but also has an extremely low open-state probability in response to cAMP stimulation as compared to cells expressing the wild type protein (15,16). Genetically engineered G551D mice display a defect in the innate immunologic response to inflammatory stimuli, with higher than normal levels of neutrophils in the lung as well as a hypersensitive macrophage phenotype following intravenous lipopolysaccharide (LPS) challenge (17). Recently, McMorran and colleagues have reported that these mice have an impaired clearance of *Pseudomonas* suggesting that this mouse might closely model CF lung disease (18). We compared the functional response of primary AMs for the four CFTR genotypes with respect to phagosomal and lysosomal acidification, bacterial killing, phagocytic index and GTP-γ-S induced secretion.

**Experimental Procedures**

*Materials-* The murine macrophage-like cell line, J774, was propagated as previously described (19). *P. aeruginosa/EGFP* (27853-EGFP) (20) was a kind gift from Dr. John C. Alverdy and Dr. Olga Zaborina at The University of Chicago. Unlabeled zymosan, fluorescein (494/518)-labeled zymosan, Alexa-fluor-488-labeled zymosan, fluorescein-tetramethylrhodamine (TMR)-tagged dextran (average MW 70,000), nigericin and valinomycin were obtained from Molecular Probes (Eugene, OR). pHrodo™ *E.coli*, a pH-sensitive Rhodamine-based pHrodo™ dye conjugated to *E.coli* BioParticles®, as well as 10 kD dextran doubly conjugated to pHrodo™ and Rhodamine green were received as a gift from Molecular Probes. The thiazolidinone CFTR inhibitor (CFTR<sub>inh-172</sub>) and GTP-γ-S were obtained from Sigma (St. Louis, MO).

*Animals-* The studies detailed herein conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research and were approved by The University of Chicago Institutional Animal Care and Use Committee. CFTR null mice (STOCK Cfr<sub>tm1Unc</sub>/TgN(FABPCFTR)#Jaw/Cwr and ΔF508 mutant mice (C57BL/6 Cfr<sub>tm1Kth</sub>-TgN(FABPCFTR)#Jaw/Cwr homozygous for ΔF508 (21) were purchased from Case Western Reserve University’s Cystic Fibrosis Animal Core.
These mice express the hCFTR protein in the gut under the influence of the rat FABP promoter and are referred to as “gut corrected”. Mice carrying the CFTR mutation G551D (22) were a kind gift from Dr. Gerald B. Pier (Harvard Medical School, Boston, MA). Animals were housed in a specific pathogen-free biohazard level 2 facility maintained by The University of Chicago Animal Resources Center (Chicago, IL). Genotyping of G551D mice was performed by Embark Scientific (Austin, TX).

Bronchoalveolar lavage (BAL) and peritoneal lavage fluids were collected from mice using standard methods. A total number of AMs recovered from BAL depended on the genotype and ranged from approximately 0.6 x 10^5 per mouse in WT mice and 1.1 x 10^5 per mouse in ΔF508 mutant mice.

Before analysis, cell suspensions were washed 3 times with PBS. After fixation with 4% paraformaldehyde, cells were permeabilized using 0.1% Triton X-100.

**Analysis of zymosan uptake (phagocytic index)-**

Macrophages were incubated with fluorescein-conjugated zymosan at 0.5 mg/ml at 37°C for 30 min; cells were then washed 5 times with PBS. Cells with phagocyted particles were imaged with a Leica SP2 AOB inverted confocal microscope using a 488 nm argon laser, a 63X oil objective lens (NA = 1.4), and an emission bandwidth of 500-535 nm. The ingested zymosan particles were counted and their intracellular localization confirmed by scanning z-series sections through the whole cell (one section about 1 μm). Phagocytic index is defined as the number of ingested particles per cell.

**Analysis of time course of phagosomal acidification-**

Alveolar and peritoneal macrophages and blood monocytes were isolated from WT and cftr^-/- mice as well as ΔF508 and G551D CFTR mutant mice and incubated with fluorescein-TMR-tagged dextran (average MW 70,000; 5 mg ml^-1) for 1h at 37°C. Labeled cells were washed several times with serum-free medium and chased in fresh medium for 2 h. Live cell imaging was carried out in medium buffered with 20 mM HEPES, pH 7.4. Cells were visualized with a Leica SP2 AOB inverted confocal microscope using a 488 nm laser and a 594 nm laser, a 63X oil objective lens (NA = 1.4), and emission bandwidths of 500-535 nm and 607-686 nm, respectively. Precautions were taken to minimize the 488 nm laser intensity to prevent photobleaching of fluorescein (the laser was used at 9% power or less and the images were taken within 10 sec). No photobleaching was registered during this time interval in our pilot experiments (data not shown).

**Determination of lysosomal pH calibration-**

Calibration curve for determining lysosomal pH was created by incubating cells for 15-20 min at room temperature in triple component calibration buffer at different temperatures.
H+/ATPase pump inhibitor, bafilomycin (0.1 μM), monensin (1 μM), valinomycin (10 μM) and the H^+/ATPase pump inhibitor, bafilomycin (0.1 μM). The pH was adjusted from 4.5 to 7.5 in 0.5 pH unit increments. Cells were imaged using a Leica SP2 AOBS inverted confocal microscope (see above). TMR/FITC ratios were plotted against pH values fitted to an exponential decay function. The ratio of pHrodo™ (pH sensitive) to Rhodamine Green (pH-insensitive) emission was calibrated as described above, and the experimental values obtained from samples were determined by interpolation.

**Determination of phagosomal acidification** - We used two experimental techniques to determine acidification of phagosomes. In the first one, J774A.1 murine macrophages were seeded at 70,000 cells per well in a 96 well assay plate and cultured overnight in DMEM +10% FCS. The cells were pretreated in complete medium at 37°C in the absence or presence of CFTRinh-172 at 10 or 20 μM concentrations for 30 minutes before removing the medium and switching to 100 microliters of uptake buffer (HBSS buffered with 20 mM HEPES, pH 7.4) containing 1 mg/mL pHrodo® E. coli bioparticles (Molecular Probes, Invitrogen Detection Technologies) in the absence or presence of CFTRinh-172 at the indicated concentrations. The assay plate was incubated for 30 minutes at 37°C in a humidified chamber, and then read on a Flex Station® plate reader (Molecular Devices, Sunnyvale CA). Net phagocytosis was calculated by subtracting the baseline fluorescence from wells containing 100 microliters of 1 mg/mL pHrodo® E. coli bioparticles, but no cells.

In the second approach, AM isolated from mice with different CFTR genotypes were incubated with the fluorescein-conjugated zymosan (Molecular Probes, OR) 0.5 mg/ml at 37°C for 30 min, washed five times and incubated for another hour in complete DMEM at 37°C. During data acquisition, the medium was buffered at pH 7.4 with 20 mM HEPES. In all experiments with CFTRinh-172, the inhibitor at indicated concentrations was present at all times during the zymosan loading, further incubation, and image acquisition. Images were collected on a Leica SP2 AOBS laser confocal microscope employing a DMIRE2 platform and a 63X (NA 1.4) oil objective. During microscopy, cells were maintained at 37°C using a stage heater in the medium buffered at pH 7.4 with 20 mM HEPES. Samples were located with minimum light exposure. Image capture used excitation via the 488 nm line of the argon laser (5% AOTF power) and was recorded in the 500-535 nm emission range. Single zymosan particles found in a single focal plane were taken as ROIs and represented data points.

**Phagosomal pH calibration** - Phagosomal pH was determined using an in situ calibration. Cells which had ingested fluorophore-conjugated zymosan particles were equilibrated in the three-component buffer with ionophores as used in the lysosomal calibration (see above). Cells were incubated for 15-20 min at room temperature in calibration buffers (pH 4.5 – 7.5) containing ionophores as above. Data were analyzed using Image J software. ROIs were drawn around single zymosan particles found in a focal plane and represent data points. The calibration curve was fit with a dose-response function using Origin (Microcal Software, Inc., Northampton, MA). In further analysis, the ratio of fluorescent particle intensity, inside versus outside the cells, was used to compare fluorescent intensity in the absence and presence of 10 μM CFTRinh-172.

**Determination of bactericidal activity of alveolar macrophages** - AMs from WT, cftr^-/-, ΔF508 or G551D mutant mice were incubated with P. aeruginosa-EGFP (27853/pUCP24-EGFP) (20) (MOI<10) for 40 min at 37°C with 5% CO₂. Non-ingested bacteria were carefully washed away with culture medium lacking FBS. Cells were incubated with a ceftazidime/amikacin mixture at 1 mg/ml each for 20 min at room temperature in order to eliminate remaining non-ingested bacteria adherent to the plastic. In our pilot experiments, 95% of P.a. was killed under these conditions (data not shown). After two washings, complete DMEM buffered with 20 mM HEPES, pH 7.4 was added to the cells; 1 ml of mineral oil was carefully layered on the surface of the medium in the dish to prevent evaporation. The dish was kept in a microincubator (Warner) at 37°C during image acquisition. Time lapse series were recorded on Olympus IX81 microscope using minimal light exposure (0.25% lamp output). Z-stacks were taken every 2 hrs (0, 2, 4, and 6 hrs) with approximately 1 μm step. During analysis, ROIs were drawn around the entire cell and the fluorescence intensities in all slices determined and subsequently summed. Increase in fluorescence
intensity over time was indicative of bacteria proliferation inside the cell.

*Live cell image Data Analysis* - Images were analyzed using Fluoview software, Image J (NIH), and MetaMorph (Universal Imaging Corp, Downingtown PA). Data are expressed as means ± standard error of the mean. Significance between groups was determined using the Student’s t-test.

*Electrophysiological recording* - Electrophysiological data were acquired using an Optiplex 755, Core 2 Quad PC. Capacitance recordings were made in the whole-cell configuration using an EPC-9 amplifier with a built-in data acquisition interface (ITC-16, Instrutech, Port Washington, NY) in combination with PULSE acquisition software (HEKA Electronik, Lambrecht, Germany) and Igor Pro (WaveMetrics, Lake Oswego, OR) for graphics and data analysis. Recordings were made in the “sine + dc” stimulus mode under the LOCK-IN module of PULSE. The temporal resolution of the capacitance data was 10 ms per point using a 1 kHz 30 mV sine wave. The holding potential was -10 mV. All experiments were conducted at room temperature (22-24°C). Pipettes with resistances of 6-8 MΩ were obtained using quartz glass (Sutter Instrument Co., Novato, CA) and a Sutter model P-2000 puller. Experiments were carried out using a standard pipette solution that contained (in mM): 80 K-MES, 40 KCl, 2 MgCl₂, 1.1 EGTA, 0.2 CaCl₂ and 10 HEPES, pH 7.2. The bath solution contained (in mM): 100 NaCl, 50 KCl, 2 MgCl₂, 2 CaCl₂ and 10 HEPES, pH 7.4. Solution osmolarities were monitored with a vapor pressure osmometer (Model 5500; Wescor, Logan, UT), and measured to be 290 mOsm.

*Electrophysiological data analysis* - Electrophysiological data were acquired and analyzed off-line using PC-computer based IGOR Pro (WaveMetrics, Lake Oswego, OR). The number and size of the step changes in the capacitance recordings were obtained using a automated step detection detection routine in Igor written in the laboratory (23). Amplitude histograms were constructed and fitted using Origin (Microcal Software, Inc., Northampton, MA) with a multiple Gaussian algorithm. Step changes in capacitance were not distinguishable from noise at levels below 10 fF, therefore histograms were not fit below 10 fF. In addition, step changes with a rise time of greater than 0.3 fF/s were ignored. The histogram bin size was 0.20 fF.

*Capacitance change (ΔCm) and delay (Δt) measurement* - In analyzing capacitance (Cm) changes with time, we constructed averaged Cm-time curves by zeroing the initial Cm value and averaging all Cm-time recordings for each CFTR genotype. We measured ΔCm at t = 0 and t = 450-500 s where the Cm of WT CFTR reached a constant Cm value. A threshold value of ΔCm = 100 fF was used to determine the lag time before the initiation of an increase in Cm.

All average results are presented as mean ± S.E.M. with the number of experiments in parenthesis followed by the number of mice used in each experimental data set. Student’s t test as well as the Kolmogorov-Smirnov test was used to analyze data. A probability level of < 0.05 was considered significant. All experiments were conducted at room temperature with the exception of the phagocytosis experiments which were conducted at 37°C.

**RESULTS**

The central tenet of our previously published studies, namely that the Cl⁻ conductance introduced into the AM lysosome and phagosomal membrane by CFTR is used for charge neutralization of the primary H⁺ pump, thus enabling these organelles to develop an internal pH of ~5.6 (1) is the platform for the current investigation. A recent report from Haggie and Verkman presented data that appeared to conflict with our previously published data on the involvement of CFTR in lysosomal acidification (24). In their studies, they used the inhibitor CFTRinh-172 in a variety of cell types to re-examine whether or not CFTR was involved in phagolysosomal acidification. In their studies, the inhibitor was ineffective in producing a decrease in phagolysosomal acidification at the concentration of 10 μM. Their findings are in contrast to our published findings in which CFTR inh-172 inhibited lysosomal acidification in *cftr*<sup>+/−</sup> mice (1), a conclusion further supported by data obtained from *cftr*<sup>−/−</sup> AMs. To address the disparate results obtained by Haggie and Verkman, we carried out fluorescence ratio imaging experiments on phagolysosomal acidification in wild-type murine alveolar macrophages in the presence of CFTR inh-172. In agreement with our previous data, we observed a significant decrement in phagosomal...
acidification in the inhibitor-treated cells following engulfment of FITC-conjugated zymosan particles (Fig. 1). The concentration dependence of the CFTRinh-172-induced defect in acidification was further verified in studies using cells from the murine macrophage cell line J774A.1 (Fig. 2A). In these studies, cells were fed pHrodo® E. coli bioparticles where a decrease in phagosomal pH is associated with an increase in fluorescence. Averaged fluorescence following bacterial uptake was read in a plate reader and compared in the presence of both 10 and 20 µM CFTRinh-172. As can be seen in the summary data in Fig. 2A, we observed a significant dose-dependent decrease in fluorescence (corresponding to an alkalinized phagosomal compartment) in the presence of the inhibitor. The data in Fig. 2A suggested that perhaps handling or cold storage of the highly hydrophobic CFTRinh-172 might shift the dose-response curve of the drug. We, therefore, investigated whether cold storage of the solubilized CFTRinh-172 resulted in a shift in the dose required to inhibit CFTR in cftr+/− AMs as has been observed for respiratory epithelial cells (25). In these studies, we used dextran labeled with pHrodo (as the pH sensor) and Rhodamine green (pH insensitive) as the compartment marker. The doubly conjugated dextran has the advantage that the pH marker pHrodo, in contrast to fluorescein, increases in fluorescence with increasing acidification of the compartment in which it is concentrated. A calibration curve and a summary of our data obtained with the pHrodo-Rhodamine green dextran double conjugate in murine AMs is given in Fig. 2B and C. We show that there was a significant shift in the concentration of the inhibitor required to produce maximal CFTR inhibition and, thus, an alkalinizing shift in lysosomal pH, between the freshly solubilized inhibitor and that reconstituted from frozen DMSO stock solution. In the freshly solubilized solution, inhibition/alkalinization is seen at 10 µM; whereas, 30 µM was needed to achieve a similar level of inhibition/alkalinization when the inhibitor was reconstituted from frozen stock regardless of storage time.

Time course of phagosomal acidification. There appears to be a controversy in the literature as to the precise timing of the phagosomal acidification process (24). We developed a dynamic assay investigating the kinetics of individual phagosomes undergoing the acidification process. Phagosomal acidification in macrophages is intimately tied to the acquisition of the vesicular proton-ATPase (v-ATPase) conferred by endo-lysosomal fusion (1, 26, 27). The point at which the phagosome reaches the pH of the mature lysosome, approximately pH 5.0, is one of the markers that can be used to determine full phagosomal maturation (28). Once the lysosome fuses with the nascent phagosome, the time course over which phago-lysosomal acidification occurs is reported to be approximately 12-15 min (1, 28-30) and consistent with the time course of phagosomal acidification in experiments where particle uptake is synchronized (24, 31). Synchronized particle uptake uses a cooling cycle to allow particles to bind followed by a heating cycle in which it is assumed that once warmed, all particles are taken up into cells as a “synchronous wave” (32) and the resultant acidification kinetics measured on a population of particles is assumed to reflect that of individual phagosomes.

In order to determine whether, in fact, all particles taken up into AMs acidify at identical rates, we examined AMs exposed to pHrodo-Rhodamine Green-conjugated zymosan in real time using video imaging. Cells were maintained at 37°C while particles were dropped onto the cells. Particles reached the cells at different rates and were taken up into the cells with a variable time course. Not all zymosan particles that attached to the cell surface were taken up immediately, some particles resided bound to the cell surface for periods of minutes before entering the cell. The kinetics of acidification were determined offline using particle tracking software. Time zero was determined visually by entry of the particle into the cell. Phagosomal acidification was initiated with a variable lag time once the particle entered the cell presumably by the acquisition of the lysosomal v-ATPase (see Fig. 3A) We compared rates of acidification in 15 phagosomes in a total of 2 cells. Surprisingly, once acidification started, the rate was fast and relatively constant from phagosome to phagosome as determined by exponential fits to individual acidification curves as in Fig. 3C. The average lag time between particle uptake and onset of acidification was 72 ± 13 s (range was 30 s to 210 s). The average τ derived from single exponential fits to the acidification time course was 50.9 ± 10 s (summary statistics are given in Fig. 3C).

Macrophage tissue source defines dependence of lysosomal acidification on CFTR expression. In
order to determine whether all macrophages, regardless of tissue source, showed lysosomal acidification to be sensitive to CFTR expression, we carried out a comparative analysis of lysosomal acidification using macrophages from three sources isolated from both WT and cftr–/– mice. We examined AMs, peritoneal macrophages and circulating monocytes which all express CFTR (1). Interestingly, only AMs showed a dependence of lysosomal acidification upon CFTR expression (Fig. 4). In the WT AMs, fluorescein emission is quenched due to the acidic nature of the compartment (pH 5.16 ± 0.06, n = 58 cells (3 mice)) while in CFTR KO (pH 6.91 ± 0.05, n = 161 (8)) very little quenching is observed (left panel Fig. 4). Lysosomal compartments in peritoneal macrophages (WT pH 5.16 ± 0.06, n = 58 (4); CFTR KO pH 5.27 ± 0.07, n = 58 (3)) and blood monocytes (WT pH 5.61 ± 0.15, n = 12 (1); CFTR KO pH 5.65 ± 0.14, n = 14 (1)) did not depend on CFTR expression. We were unable to observe a significant difference in lysosomal pH in AMs isolated from WT and clc-3–/– animals (see Supplementary Fig. 1) demonstrating that a Cl– channel important in intracellular acidification in primary hepatocytes (33) and neutrophils (34) did not play a significant role in AM acidification.

Mutational analysis of CFTR in the functional response of the murine alveolar macrophage. Phenotype-genotype relationships as a function of CFTR expression and mutations were examined in murine AMs. We compared lysosomal acidification, bacterial killing, phagocytic index, and stimulus-evoked secretion in AMs derived from cftr+/+, cftr–/–, ΔF508, ΔF508 ΔF508, and G551D mice.

Lysosomal acidification. We examined and compared lysosomal acidification in AMs as a function of CFTR genotype using live cell fluorescence ratio imaging. These data are summarized in Fig. 5. Our data show a clear phenotypic relationship between lysosomal acidification and CFTR genotype, with the most severe lysosomal alkalinization in absence of CFTR expression in the AMs isolated from the cftr–/– mice (pH 6.91 ± 0.05, n = 161 (8)). The ΔF508 CFTR mutation also resulted in significantly impaired lysosomal AM acidification (pH 6.11 ± 0.06, n = 77 (3)). Acidification was the least affected in the G551D AMs (pH 5.91 ± 0.04, n = 73 (4)) where the protein is expressed at the plasma as well as intracellular membranes but is only marginally functional as a Cl– conductance.

Bacterial killing. The consequence of a decrease in steady-state lysosomal acidification in AMs is a decrease in bacterial killing (1). Thus, the next set of experiments was carried out to examine whether the lysosomal alkalinization seen in cells expressing mutant CFTR results in a similar decrement in their microbicidal capacity. Bacterial killing among the CFTR genotypes was examined using confocal video microscopy. Macrophages were allowed to ingest P. aeruginosa expressing EGFP over a 40 min period, washed with mixture of 2 antibiotics at 1 mg/ml each in culture medium without FBS to kill remaining undigested bacteria adherent to the dish, then observed microscopically for 6 hours to assess intracellular bacterial proliferation as we have published previously (1). Whereas, wild type cells exhibited a steady restriction of bacterial proliferation, cftr–/– cells showed continued bacterial growth as assayed by increasing levels of intracellular fluorescence over time. These data are summarized in Fig. 6A and B. Note the strong genotypic dependence on the extent of bacterial killing over time with the G551D cells showing the greatest microbicidal efficacy over time among the mutant cells. Experiments done to examine the effect of the CFTRinh-172 on bacterial killing demonstrated that the inhibitor was itself an antibiotic in bacterial cultures at the micromolar concentrations which are used to inhibit channel function (data not shown).

Phagocytic index. A defect in bacterial killing could be due to a decrease in the phagocytic index, or due to a defect in the degradative process once bacterial uptake is complete. Therefore, we examined the phagocytic index as a function of CFTR genotype. As in our original findings (1) comparing wild type and cftr–/– cells, the phagocytic response to zymosan uptake was identical in cells isolated from the two animals. Interestingly, the AMs isolated from the ΔF508 animals exhibited a significant decrease in their phagocytic index as summarized in Fig. 6C indicating that the capacity for bacterial proliferation in the killing studies would be underestimated in the mutant ΔF508 expressing AMs due to a significant decrement in bacterial uptake.

Secretion. Filling, priming and subsequent exocytosis of synaptic vesicles and large dense core granules is dependent upon chloride channel expression as well as granule-vesicle acidification (35-37). Macrophages are secretory cells containing a
panoply of vesicles with respect to both size and cargo (38). Although there is no data as to the acidification level of these granules, their release in macrophages is dependent upon a signaling process that generates both changes in small GTPases and intracellular Ca\(^{2+}\) levels (19,23,39) as is the case, for all hematopoietic cells (19,40-43). In order to determine whether the release of membrane bound vesicles from AMs was a function of CFTR expression perhaps linked to acidification, we conducted high resolution membrane capacitance recordings of the stimulus-induced secretory response in AMs derived from both WT and mutant AMs. Capacitance (Cm) measurements track surface membrane area (in pF) in real time and can detect both exocytosis and endocytosis. For these studies, we used GTP-\(\gamma\)-S as a secretory stimulus added to the electrophysiological pipette solution. The average GTP-\(\gamma\)-S secretory response from both mutant and WT cells and cells exposed the vesicular H-ATPase inhibitor bafilomycin (BAF) is given in Fig. 7A. When WT cells were exposed to BAF prior to capacitance recording in order to induce vesicular alkalinization, GTP-\(\gamma\)-S driven exocytosis/secretion was completely abolished indicating that vesicle acidification is critical for release. Cells expressing mutant CFTR showed a smaller increase in cell size (as assayed as an increase in surface area or pF) or secretory response following stimulation with GTP-\(\gamma\)-S as compared to the control cells (Fig. 7A and B). The time to the initial increase in cell size thresholded at 100 fF also increased significantly in the mutants \(\Delta F508\) and G551D over that observed in WT cells (Fig. 7C). These data are consistent with a model in which vesicle acidification is necessary for release in AMs and CFTR appears to play a role in the acidification process.

The size of the step changes in capacitance is proportional to the size of the vesicle fusion events at the plasma membrane. As detectable increases in capacitance were only observed in WT and \(\Delta F508\) cells, we performed a step size analysis between records obtained from the two genotypes (Fig. 8). Steps were detected using an automated program which accepted events based upon the slope and minimal size (100 fF) of the capacitance increase. Events were summed over cells from each genotype. Records in Fig. 8A demonstrate the variability in step size during stimulus-induced secretion. Summation of the detected events in all cells for a given genotype is displayed in histogram format in Fig. 8B, and fit with a single Gaussian distribution in the case of the WT AMs and a double Gaussian distribution in the case of the mutant \(\Delta F508\) expressing cells. The mean amplitude of the small vesicle population was between 105 and 108 fF in both cell types. The mutant cells secreted a second larger vesicle population of approximately 382 fF which was not seen in the WT cells.

**DISCUSSION**

Intracellular acidification plays a key role in a broad spectrum of cellular functions including maintaining proteolytic enzymes in the lysosomal compartment at optimal pH (44), processing prohormones in large dense core granules to the active state (45), determining neurotransmitter loading of synaptic vesicles (35), and contributing to pathogen killing in the phagolysosomal compartment (1,28,29). The vesicular ATPase drives the influx of protons into the various membrane bound organelles; however, the magnitude of the proton gradient inside each organelle is determined by the co-expression and activation of chloride channels that act as charge shunt pathways in the same organelle. The number and identity of chloride channels subserving this role is the subject of active investigation and appears to include members of the CIC (46,47) family of anion channels as well as CFTR (1,25,48-50). Perhaps one of the most interesting of the unsolved questions is how the diversity of chloride channels involved in these processes are targeted to a given organelle and whether their activity is regulated by the development of an intragranular proton gradient.

We have determined in this study that CFTR is involved in a number of processes integral to AM function that are driven by vesicular acidification and that the most common disease-causing mutations of CFTR, \(\Delta F508\) and G551D result in: 1) a secretion defect; 2) a decrease in the killing of internalized bacteria, 3) a decrease in the phagocytic response in the cells isolated from the \(\Delta F508\) mutant mice; and 4) down regulation in acidification of an endosome/lysosome-like compartment in resting cells and in the phagosomal compartment in phagocytosing cells.

Regulated secretory activity in macrophages is **influenced by CFTR**. The AM contribution to innate immunity is first and foremost due to their...
phagocytic and microbicidal activity. Accompanying this critical function, they also recruit other inflammatory cells by secreting a variety of products including enzymes, cytokines and reactive oxygen intermediates. Hematopoietic cells, in general, and macrophages in particular, contain several distinct secretory organelles that are selectively mobilized by different stimuli (38). Macrophages contain different types of dense core granules, in addition to phagosomes and phagolysosomes (38). It has been known for some time that macrophages possess a class of lysosomes that is specialized for secretion (the so-called “secretory lysosome”; for review see (51)). Using electrophysiological techniques, we have examined the intracellular requirements for secretion of these organelles and others in a series of studies (19,23,39). In our current study, we determined that CFTR plays an integral role in determining the secretory response in AMs. The response scales with the expression of CFTR as well as CFTR mutations common in human disease. Although we have not measured the steady-state pH of the secretory granules themselves in the AMs expressing the mutant forms of CFTR, the response to the absence of CFTR expression is equivalent to that evoked by a complete inhibition of the v-ATPase and, therefore, strongly linked to secretory granule alkalinization. A comparison of capacitance step size during the exocytic response revealed that both WT and ΔF508 expressing macrophages secrete a population of vesicles which have a mean amplitude of approximately 105 fF. Given a membrane capacitance of 10 fF/µm², transformation of vesicle capacitance predicts a spherical structure of approximately 0.9 µm in diameter. Thus, most steps in Fig. 9A result from the regulated release of small vesicles. The ΔF508 expressing cells showed an additional capacitance peak at 382 fF corresponding to a larger vesicle population with an average diameter of 1.7 µm. Normalization of peak size to number of cells studied, revealed that the number of small vesicles secreted was equivalent in both WT and ΔF508 cells (data not shown). Thus, the mutant cells appeared to secrete a larger vesicle population absent in the WT cells. Although it is tempting to hypothesize that this unique vesicle population might be responsible for the heightened level of inflammatory cytokines released by CF alveolar macrophages (52) it is difficult to establish a direct correlation between vesicle size and associated cargo.

**CFTR is critical to normal phagosomal function in AMs.** Recently, we established that AMs express functional CFTR and that cells from CFTR null-mice exhibit defective bactericidal activity (1). The cause of this deficiency is apparently a failure of lysosomes and phagosomes to acidify properly in the knockout. The phagocytic index in the cfr<sup>−/−</sup> cells per se is not affected and it does not appear that CFTR affects phago-lysosomal fusion or reactive oxygen species (ROS) production. In the current study, we have extended those observations to show that mutations in CFTR also modulated in a graded manner both lysosomal acidification as well as bactericidal events in AMs. The defect in bacterial killing appears to scale with mutant channel trafficking to plasma membrane sites. The ΔF508 mutation which is rapidly degraded due to misfolding produces a defect in killing equivalent to that seen in the cfr<sup>−/−</sup> cells. The defect in bacterial killing in the ΔF508 cells is undoubtedly exacerbated by the fact that the phagocytic index in these cells is reduced over that seen in the cfr<sup>−/−</sup> and G551D expressing cells.

Two recent reports (24,31) present data comparing the kinetics of acidification in WT, cfr<sup>−/−</sup> and ΔF508 expressing cells. Both groups studied the acidification time course of multiple phagosomes taken up into macrophages using differing dyes and particles e.g. zymosan or labeled bacteria. The time course of phagosomal acidification was highly variable between the two studies but appeared to show little difference as a function of CFTR expression. Two important points should be noted when comparing our results with theirs. Barriere and colleagues (31) did show in pH dissolution assays that in AMs a PKA-activated counteranion permeability associated with acidification of what they termed immature phagosomes formed within 5 min after ingestion of fluorescein-conjugated *P. aeruginosa*. This counteranion conductance was undetectable in the cfr<sup>−/−</sup> cells. In addition, they were also able to show that recycling endosomes in AMs as well as in peritoneal macrophages demonstrated a proton efflux which was assumed to represent alkalinization dependent upon CFTR expression and consistent with our earlier observation of a considerable CFTR conductance at the plasma membrane of AMs (1). This observation is not consistent with our study showing that peritoneal
endo-lysosomal acidification is not dependent upon CFTR expression. Quantification of acidification levels in intracellular organelles is highly dependent upon a number of factors which are variably agreed upon among investigators in the field. Of prime importance is the cellular loading with fluid phase markers, dyes which are conjugated to dextrans and which traffic along the endocytic pathway with a predicted time constant. In some investigations, the loading time can be as long as overnight (53) or as little as 30 min (24) with an equally variable chase time. The time chosen to load cells with the fluid phase marker can have a significant impact on the steady state pH measured in a given compartment. As an example, loading overnight can shift the observed steady-state lysosomal pH to a more alkalinized level by over a half of a pH unit than that observed after 1 hour of incubation with equivalent chase times (see Supplementary Fig. 2). The alkalinized compartment seen with the longer loading time could be due to an overload with lysosomotropic agents which are all weak bases. The fluid phase markers can be a doubly conjugated dextran (24) or two single conjugated dextrans which are assumed to label the targeted organellar population with a single kinetic time constant (31) which may or may not be the case with resultant inaccuracies in normalizing pH changes to compartment size. Some investigations have used a single dye which has a pH sensitive excitation range as well as a relatively pH insensitive range (but not zero) which can be used as a ratioable indicator to normalize for compartment loading. This technique has a disadvantage that small changes in the relatively pH-insensitive range can cause profound changes in the ratiometric data. Once cells are loaded, an in situ calibration curve is constructed for conversion of fluorescence ratios or intensity to pH. Analysis schemes can vary as well. One method is to utilize averaged whole cell fluorescence so as to not to introduce a detection bias to regions within the cell that are brighter than others. Regions of interest within a cell can vary widely throughout the pH range and thereby introduce a bias which may affect the collective observed pH. And finally, not all cellular populations may acidify in the same way even within a given classification. As we have seen in the current study, tissue macrophages differ in the Cl channel type which is expressed in a given vesicle compartment acting as a charge shunt pathway. Cell lines made from primary cell populations as in the case of the bone marrow macrophages in the studies of Lamothe et al (53) may also express a different population of intraorganellar ion channels. Thus, in order to compare acidification data observed between laboratories, one must compare across similar loading criteria, dye usage and analysis procedures in similar cell types. The mice used in such studies could also be a source of inconsistencies as a result of breeding strategies and genetic drift/ectopic expression of region-specific promoter-driven human CFTR used in gut-corrected mice. We examined the possibility of significant transgene ectopic expression in the population of cftr<sup>−/−</sup> mice that have been gut corrected by expression of the human CFTR protein driven by the rat L-type FABP promoter. The animals available from Case Western Reserve University as well as animals bred in small colonies outside of Case Western have been bred as homozygotes. In colonies which are inbred for a significant period of time, the possibility exists that AMs may acquire aberrant transgene expression which is then passed on to future generations quite rapidly if the colony is not re-derived regularly and bred as heterozygotes. In order to test for this, we carried out a genotype-phenotype experiment with a cftr<sup>−/−</sup> (STOCK Cfr<tm1Unc> TgN FABPCFTR) mouse which was purchased from Case Western’s facility within last year (unlike mice used in our earlier experiments that were acquired about two years ago and were phenotypically dissimilar). In this study, we isolated AMs from a single animal, isolated RNA from a fraction of the cells and cultured the remaining cells for fluorescence imaging. These data are shown in Supplementary Data (Fig.3S). In Fig. 3SA, PCR analysis of macrophage cDNA using primers specific to human CFTR revealed that indeed the AMs from the “inbred” cftr<sup>−/−</sup> mouse expressed the human transgene CFTR transcript not present in the cftr<sup>−/+</sup> mouse used as a control. We probed only for the human gene as routine genotyping for the murine homolog revealed that it was absent in the mouse. Fluorescence imaging experiments of cells loaded with doubly conjugated FITC-TMR dextran revealed that, in fact, AMs isolated from the WT mouse and from the “inbred” cftr<sup>−/−</sup> mouse showed identical levels of acidification. As a positive control, we compared the “inbred” cftr<sup>−/−</sup> and cftr<sup>−/+</sup>
lysosomal acidification data to data obtained in ΔF508 AMs which are bred as heterozygotes and show a significant shift in lysosomal acidification levels (Fig. 3C).

In summary, we have detected CFTR protein in peritoneal macrophages (PMs) and observed no acidification defect in PMs from cftr/− animals. We surmise that other Cl− channels may play a similar role in phagosomal function in these and possibly other innate immune cells. We determined that mice null for CIC-3 express normal lysosomal acidification in AMs. However, mice null for CIC-3 are susceptible to sepsis and Moreland et al. suggest that CIC-3 is crucial for normal host defense by mechanisms that may involve phagolysosomal and secretory behavior in neutrophils (34). Our hypothesis is that Cl− channel function is intimately connected to phagosomal acidification in innate immune cells and that the molecular species of Cl− channel may vary between cell types.
# REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig. 1. CFTR- inh 172 induced inhibition of phagosomal acidification in AMs isolated from WT mice. A. Fluorescein-conjugated zymosan was exposed to WT AMs in the absence (control) of inhibitor or to cells that had been pre-treated with 10 µM CFTR inh-172 for 30 min. Images were collected on a Leica SP2 A0BS laser confocal microscope (see Supplementary Data Methods). Particles not ingested by the cells are marked with white arrows. B. Calibration curve was performed on the same cells using a three-component buffer with ionophores. Data were analyzed using Image J software. Single zymosan particles found in focal plane were taken as ROIs and represent data points on the curve. Data are expressed as a mean ± standard error. Calibration curve was fitted using Origin and the dose-response model. C. The ratio of green fluorescence between particles inside vs. outside cells (white arrows in A) in control cells (upper panel) and in the presence of 10 µM CFTR inh-172 (lower panel). For analysis, fluorescence intensity of each inside particle was divided by the mean intensity of all outside particles. Bars represent the mean of these ratios ± standard error.

Fig. 2. Concentration dependence of the CFTR inh172-induced inhibition of acidification in phagosomes and lysosomes. A. J774A.1 murine macrophages were fed with 1 mg/ml E.coli bioparticles conjugated to a pH-sensitive dye pHrodo® (Molecular Probes, Invitrogen Detection Technologies, Eugene, OR) in the absence or presence of CFTR inhibitor at indicated concentrations. The assay was performed in 96-well plate format. The fluorescence was read on a Flex Station plate reader (Molecular Devices, Sunnyvale, CA). Mean values are relative fluorescence units (RFU), calculated from 4 wells per sample. Error bars show standard deviation. B. AMs from WT or cftr /- (KO) were incubated with dextran (MW 10 kDa) doubly conjugated to a pH sensitive dye pHrodo® and rhodamine green as described in Supplementary Methods. The ratio of pHrodo (pH sensitive) to rhodamine green (pH-insensitive) emission was calibrated in situ using a triple buffer system, and the experimental values obtained from samples in C were determined by interpolation. C. Data summary is presented as the mean ± SEM where the number of the cells examined is indicated over each bar. Note a significant shift in the concentration of the inhibitor required to produce the maximum inhibition of acidification between freshly solubilized ("fresh") and reconstituted from stored frozen DMSO stock ("stored").

Fig. 3. Kinetics of phagosomal acidification. A. The kinetics of phagosomal acidification was determined in experiments in which zymosan particles doubly conjugated to Rhodamine green and pHrodo where pipetted onto cells and particle uptake followed with live cell video microscopy. Movies of particle uptake were analyzed and particles followed with particle tracking software. B. Fluorescence ratio imaging for the particle/phagosome outlined by boxes in A was calculated and plotted normalized to initial values obtained upon particle entry into the cell as a function of time. Fluorescence ratios (proportional to acidification) were determined every 30s following particle uptake. The acidification time course of the particle/phagosome isolated by the boxes in A is depicted in the curve with the open boxes. The data were fitted with a smooth line through the time points. The two curves represent the
acidification time course of two phagosomes which showed distinctly different time courses for acidification. The time course data for the phagosome depicted with open boxes is representative of a class that acidified rapidly upon entry. The data represented by the closed boxes is representative of a phagosome that acidified with a significant lag following particle uptake. C. The initiation of acidification was determined as the time at which the pH/fluorescence ratio changed to $\leq 90\%$ of its initial value. The rate of acidification ($\tau$) was determined from exponential fits to the data with the initial time point taken at a value that was $\leq 70\%$ of the total change in pH. These kinetic data are summarized in the box plots for a total of 15 phagosomes from cells isolated from 2 WT mice. The average lag time for the onset of acidification was $72.0 \pm 13$ s and the average time constant ($\tau$) was $50.9 \pm 10$ s.

Fig. 4. Lysosomal acidification in AM, peritoneal macrophages and blood monocytes as a function of CFTR expression. Lysosomal pH was compared between cftr$^{+/+}$ and cftr$^{-/-}$ alveolar and peritoneal macrophages and blood monocytes loaded with dextran doubly conjugated with FITC and TMR. Representative DIC and fluorescent images are given in A-C. Lower Panel: Data comparison between cell types using ratiometric pH determination. ROIs for quantification were drawn around the whole cell. Summary data are expressed as pH means $\pm$ standard error of the mean where the number of cells examined under each condition is shown over each data point (the number of animals from which cells were taken for each experimental point is given in parentheses). Insert: Ratiometric data for each cell was calibrated in vivo using a multi-buffer system and the pH values obtained from the samples were determined by interpolation.

Fig. 5. The relationship between CFTR genotype and phenotype in the regulation of lysosomal acidification. A. Murine AMs were obtained from homozygous $\Delta F508$ and G551D animals and compared to data obtained from AM isolated from WT and cftr$^{-/-}$ animals. Representative cell images from each of the cell types are shown. B. Determination of intra-organelle pH in lysosome-like compartments. Fluorescence was averaged over the whole cell at a single focal plane and then background subtracted. Summary data are expressed as pH means $\pm$ standard error of the mean where the number of cells examined under each condition is shown over each data point (the number of animals from which cells were taken for each experimental point is given in parentheses). C. The ratio of TMR to fluorescein emission was calibrated in situ using a multi-buffer system and the values obtained from the samples shown in A. were determined by interpolation.

Fig. 6. Capacity of macrophages to eliminate internalized bacteria is a function of AM genotype. A comparison of intracellular bacterial growth in single murine AMs was carried out using live cell microscopy. Cells were allowed to ingest EGFP-expressing P. aeruginosa for 30 min (m.o.i. < 10). Adherent and non-ingested bacteria were then removed by washing and incubation with antibiotics and live AMs were observed microscopically for approximately 6 hours. Representative AMs from $\Delta F508$ CFTR mutant mouse with ingested bacteria at the initiation of the incubation period and after 6 hours are seen in A. B. Summary data from at least 3 separate experiments. C. Comparison of phagocytic index across CFTR genotypes. AMs isolated from mice with different CFTR genotype were incubated with fluorescein-conjugated zymosan A (Invitrogen, Molecular Probes, Eugene, OR)) at the concentration of 0.5 mg/ml for 30 min at 37°C with $5\%$ CO$_2$. Non-ingested particles were removed by excessive washings. The cells were visualized by confocal microscopy that allowed scanning each cell through its depth in order to count all ingested particles and discriminate them from those attached but not ingested by the cell. The data are represented as the mean $\pm$ standard error. The number of cells analyzed is given above each bar. The phagocytic index of the WT cells was significantly different from the $\Delta F508$ cells ($p<0.001$) using the student’s t-test.

Fig. 7. Mutations in CFTR alter the GTP-$\gamma$-S induced secretory response in AM. Secretion was stimulated by the intracellular introduction of 400 $\mu$M GTP-$\gamma$-S through the patch clamp pipette. A.
Whole-cell capacitance recordings were obtained with an EPC-9 computer-controlled patch clamp amplifier (HEKA Electronik, Lambrecht, Germany) running PULSE software (HEKA). The EPC-9 includes a built-in data acquisition interface (ITC-16, Instrutech, NY). The software package controlled the stimulus and data acquisition for the software lock-in amplifier in the ‘sine + dc’ mode. The temporal resolution of the capacitance data was 40 msec per point with a 1-kHz, 20-mV sine wave. The holding potential in the capacitance experiments was −10 mV. All electrophysiological experiments were performed at room temperature. A comparison of averaged secretory responses in voltage clamped AMs over time. The gray shading over the smooth line represents the standard error of the average membrane capacitance increase as a function of mutant CFTR genotype over time. Cell number (number of mice used in parenthesis) in the WT experiments was 6(4); ΔF508 was 12(6); G551D was 6(3); cftr−/− KO was 3(2) and in the BAF experiments 4(1). B. A summary of the average change in membrane capacitance as well as percent change in membrane capacitance increase as a function of cell genotype. C. Data summary of the time to initial secretory response thresholded at 100 fF.

Fig. 8. Analysis of step size during the GTP-γ-S induced secretory response from wild type and ΔF508 AMs. Time dependent changes in membrane capacitance were obtained from cultured AMs isolated from WT and ΔF508 mice following intracellular introduction of 400 μM GTP-γ-S as in Fig. 7. A. Representative capacitance traces showing step changes due to small vesicle fusion (less than 0.2 pF) and large vesicle fusion (greater than 0.2 pF). B. Histograms of the total number of step changes in capacitance recorded during experiments obtained from 6(4) WT and 12(6) ΔF508 cells. The number and amplitude of step changes in capacitance were obtained with an automated step analysis detection routine in IGOR (Wavemetrics, Lake Oswego, OR) written in the laboratory. Steps observed in the capacitance traces were summed for each genotype. Data were fit in the case of WT with a single Gaussian and in the events detected in the ΔF508 cells with a double Gaussian. The solid lines through the bars indicate the best Gaussian fit with the peak average above each peak. The X axis indicates both step size in pF and vesicle diameter in μm (in red). C. Cumulative amplitude histogram of step changes in capacitance pooled from WT (solid black circles) and ΔF508 cells (solid green circles. The amplitude distribution for the ΔF508 cells is shifted to the tight indicating larger vesicle release in the mutant cells (p<0.01, Kolmogorov-Smirnov).
Figure 2

### A

**CFTR<sup>ω1-172</sup>**

<table>
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<tr>
<th>Control</th>
<th>10μM</th>
<th>20μM</th>
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<td>Mean Subtracted RFU (x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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**E. coli + pHondo**

pH 4  pH 7

### B

**pHondo/RG Ratio**

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<th>6.0</th>
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<td>1.2±0.1</td>
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**pHondo**

**Dextran**

**RG**

### C

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<th>7.0</th>
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<td>22±1</td>
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</table>

**WT**  **KO**

10 μM fresh inhibitor

20 μM stored inhibitor

30 μM stored inhibitor
Figure 3
Figure 4
Figure 5
Figure 6

A

ΔF508 AMs

0 hrs

6 hrs

1

2

3

4

5

B

Increase in fluorescence intensity (%)

Time (hrs)

GFP-P. aeruginosa

ΔF508

n=48

KO

n=42

G551D

n=29

WT

n=17

C

p<0.001

n=21

n=27

n=22

Zymosan particles per cell

WT

ΔF508

KO

G551D
Figure 7

A

\[ \Delta C_m \text{(pF)} \]

\[ \text{Time (s)} \]

\[ \text{WT} \]

\[ \Delta F508 \]

\[ G551D \]

\[ BAF \]

\[ KO \]

B

Change in cell size

\[ \Delta C_m \text{(pF)} \]

\[ \% C_m \text{ increase} \]

\[ \text{WT} \]

\[ \Delta F508 \]

\[ G551D \]

\[ KO \]

\[ BAF \]

C

Time to initial response

\[ \text{Latency (s)} \]

\[ \text{WT} \]

\[ \Delta F508 \]

\[ G551D \]
Figure 8

A

B

C

WT

ΔF508

Cumulative Probability

0.0 0.5 1.0 1.5 2.0

Vesicle Size (pF)

WT

ΔF508

105.2 fF

108.6 fF

382 fF
Disease causing mutations in the cystic fibrosis transmembrane conductance regulator determine the functional responses of alveolar macrophages
Ludmila V. Deriy, Erwin A. Gomez, Guangping Zhang, Daniel W. Beacham, Jessika A. Hopson, Alexander J. Gallan, Pavel D. Shevchenko, Vytautas P. Bindokas and Deborah J. Nelson

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