It was reported recently that the cystic fibrosis conductance regulator protein (CFTR) is required for acidification of phagosomes in alveolar macrophages (Di et al. Nature Cell Biol. 8:933–944, 2006). Here we determined whether the CFTR chloride channel is a generalized pathway for chloride entry into phagosomes of macrophages and whether mutations in CFTR could contribute to alveolar macrophage dysfunction. The pH of mature phagolysosomes in macrophages was measured by fluorescence ratio imaging using a zymosan conjugate containing Oregon Green® 488 and tetramethylrhodamine (TMR). Acidification of phagolysosomes in J774A.1 macrophages (pH ≈ 5.1 at 45 min), murine alveolar macrophages (pH ≈ 5.3) and human alveolar macrophages (pH ≈ 5.3) was insensitive to CFTR inhibition by the thiazolidinone CFTR inh-172. Acidification of phagolysosomes in alveolar macrophages isolated from mice homozygous for ΔF508-CFTR, the most common mutation in cystic fibrosis, was not different when compared to those isolated from wild-type mice. We also measured the kinetics of phagosomal acidification in J774A.1 and murine alveolar macrophages using a zymosan conjugate containing fluorescein and TMR. Phagosomal acidification began within 3 minutes of zymosan binding and was complete within ~15 minutes of internalization. The rate of phagosomal acidification in J774A.1 cells was not inhibited by CFTR inh-172, and was not different in alveolar macrophages from wild-type vs. ΔF508 mice. Our data indicate that phagolysosomal acidification in macrophages is not dependent on CFTR channel activity, and do not support a proposed mechanism for cystic fibrosis lung disease involving defective phagolysosomal acidification and bacterial killing in alveolar macrophages.

Chronic lung infection and deterioration of lung function are the major causes of morbidity and death in Cystic Fibrosis (CF) (1,2). Although the genetic defect in CF was identified in 1989 - mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR) - the mechanisms by which CFTR mutations cause lung disease remain uncertain. Various mechanisms have been proposed to link defective CFTR function to CF lung disease, such as defective airway submucosal gland secretion, abnormal airway surface liquid (ASL) composition or oxygenation, Na⁺ hyperabsorption producing ASL dehydration (reduced ASL volume), loss of CFTR regulation of other transport proteins, and intrinsic hyperinflammation (reviewed in refs. 3-6). Determination of the mechanisms linking defective CFTR function to CF lung disease is of great importance in developing rational therapies to treat CF.

A recent report of defective phagolysosomal acidification in alveolar macrophages in CFTR null mice suggested a new mechanism to link defective CFTR function to CF lung disease (7). Macrophages are key protagonists of the innate immune system and patrol the body, including the alveolar surface, to engulf and destroy pathogens in their phagolysosomes (8-10). Di et al. (7) reported that phagolysosomes in alveolar macrophages acidify in a CFTR-dependent manner and that defective acidification of phagolysosomes of alveolar macrophages from CFTR null mice impaired their bactericidal activity. Interestingly, no defect in bactericidal activity was observed in peritoneal macrophages from CFTR null mice, suggesting that CFTR dependent acidification of phagolysosomes was specific to alveolar macrophages. Although the authors made no direct connection between CFTR mutations, acidification of phagosomes in alveolar macrophages, and CF disease progression, the prevailing view has emerged that defective alveolar macrophage function is important in the...
pathophysiology of CF lung disease (11-13).

Here we use ratiometric, fluorescent zymosan and dextran conjugates to determine the role of CFTR in the acidification of phagolysosomes in macrophages. In J774A.1 macrophages, phagolysosomal and lysosomal acidification were not dependent upon CFTR chloride conductance. We also found that phagolysosomal acidification was not dependent on CFTR channel activity in murine and human alveolar macrophages, and was not different in ΔF508-CFTR murine alveolar macrophages. As such, CFTR channel activity is not required for phagosomal acidification in macrophages.

**EXPERIMENTAL PROCEDURES**

*Cells*- J774A.1 murine macrophages (ATCC No. TIB-67), which are of peritoneal origin, were cultured in DMEM-H21 using standard procedures. Alveolar macrophages were freshly isolated from wild-type and CF (ΔF508-CFTR homozygous) mice in a CD1 genetic background. Briefly, mice were sacrificed by intraperitoneal injection of ketamine, and alveolar macrophages were obtained immediately by bronchoalveolar lavage (7,14). Mice were provided by the CF Animal Core facility at UCSF and experiments were done with institutional approval. Human alveolar macrophages were obtained by lavage of lungs that were rejected for lung transplants as part of the California Transplant Program. Alveolar macrophages were identified by standard morphological criteria and confirmed by the uptake of non-opsonized zymosan.

*Fluorescent probe synthesis*- Ratioable, pH-sensitive fluorescent probes for measurement of phagolysosomal and lysosomal pH were synthesized using standard succinimidyl ester chemistry. Oregon Green® 488-X or 5-(and-6)-carboxyfluorescein (FAM) were covalently coupled to zymosan (Sigma) together with 5-(and-6)-tetramethylrhodamine (TMR). For lysosomal pH determination, Oregon Green® 488-X and TMR were conjugated to 40 kDa amino dextran (Invitrogen). For each probe, TMR is a pH-insensitive marker of conjugate uptake to allow ratio imaging.

*Cell labeling, in situ calibration and quantitative fluorescence imaging*- For measurement of the pH of mature phagolysosomes, cells were incubated with 0.5 mg/ml Oregon Green® 488/TMR-zymosan for 30–45 min at 37 °C in PBS supplemented with 6 mM glucose and 1 mM pyruvate (PBSglu/pyr), washed 3–5 times in PBS, and imaged. To label lysosomes, cells were incubated with 4–10 mg/ml Oregon Green® 488/TMR-dextran for 30 min at 37 °C in PBSgluc/pyr, washed, and incubated in PBSgluc/pyr for 2 h at 37 °C to chase dextran to lysosomes. The kinetic of phagosomal acidification was measured using a method based on published protocols to synchronize the uptake of phagocytic substrates (12,15,16). Cells were washed with 4 °C PBSgluc/pyr and incubated with 0.5 mg/ml FAM/TMR-zymosan at 4 °C for 20–30 min. Five volumes of 37 °C PBSgluc/pyr were then added to cells and internalization of FAM/TMR-zymosan was allowed to proceed for 2 min at 37 °C before washing cells 4 times in 4 °C PBSgluc/pyr to remove zymosan that had not internalized. Cells were kept at 4 °C for up to 2 hours prior to experiments. Temperature was raised to 37 °C just prior to microscopy and initial images were acquired 3 min after internalization began. All imaging experiments were performed in PBSgluc/pyr. In some experiments the thiazolidinone CFTR inhibitor CFTRinh-172 was used at 10 µM, or the vacuolar ATPase inhibitor bafilomycin A1 was used at 100 nM. Inhibitors were present during cell loading, chase and experiments.

*In situ* calibration experiments were done to relate fluorescence ratios to pH. After loading (as described above) cells with labeled phagolysosomes or lysosomes were incubated for 30–40 min at 37 °C in calibration buffers (5 mM Hepes, 5 mM K citrate, 120 mM KCl, 20 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 100 mM bafilomycin A1, 10 µM nigericin, 10 µM valinomycin and 10 µM CCCP) at specified pH in the range 2.5–8.6. Background-corrected fluorescence ratios at different pH were fitted to the equation: \( F_{\text{green}}/F_{\text{red}} = A + B \left(1 + 10^{(pK_a - \text{pH})/n}\right)^{-1} \).

*Microscopy*- Fluorescence images were acquired on a Nikon TE2000U microscope equipped with a 100×/NA 1.45 Plan Apo TIRF objective, Exfo Xcite light source, Hamamatsu EM-CCD camera,
Uniblitz shutter and micro-incubator to maintain cells at 37 °C during data acquisition. Sequential, wide-field images of cells were captured using 31001 and 31002 filter sets (Chroma). Neutral density filters were placed in the excitation light path to attenuate illumination intensity and the absence of photobleaching was confirmed in control experiments by stable fluorescence of labeled cells during continuous illumination. For kinetic experiments, illumination light was shuttered in between data acquisition. Images of representative cells containing phagocytosed Oregon Green® 488/TMR-zymosan were obtained using a Nikon TE2000S microscope equipped for phase contrast and fluorescence imaging and a Hamamatsu EM-CCD camera. Cells were incubated with fluorescent zymosan for 45 minutes and fixed in 4 % paraformaldehyde in PBS prior to imaging. As we reported in prior studies of endosomal pH and chloride (17-19), standard image analysis was performed by computing area-integrated, background-subtracted fluorescence intensities for specified regions-of-interest using NIH ImageJ software.

RESULTS

To measure the pH of mature phagolysosomes we synthesized a fluorescent pH indicator consisting of Oregon Green® 488 (pKₐ ~4.7) and tetramethylrhodamine (TMR) covalently bound to zymosan (Oregon Green® 488/TMR-zymosan, Fig. 1A, inset). The pKₐ of Oregon Green® 488 renders it pH-sensitive at or near the acidic pH reported previously for mature macrophage phagolysosomes and allowed accurate measurement of phagolysosomal pH by two-color ratio imaging. Unlike Di et al., we chose a ratiometric approach to measure phagosomal pH such that our measurements were insensitive to probe concentration. To calibrate the pH probe we incubated J774A.1 macrophages with the zymosan conjugate for 45 minutes to allow internalization of the probe and subsequent maturation of phagolysosomes. Labeled cells were then washed and exposed to solutions of specified pH containing ionophores to equilibrate the extracellular and intraphagosomal pH. The pKₐ determined for Oregon Green® 488 by ratio imaging of cells at different pH agreed with that determined in vitro, indicating that the zymosan conjugate faithfully reports phagolysosomal pH (Fig. 1A).

We initially measured the pH of phagolysosomes in J774A.1 murine macrophages, which were reported previously to express CFTR by biochemical, electrophysiological and immunocytochemical criteria (7). Following incubation of J774A.1 macrophages with fluorescent zymosan, fluorescently labeled phagolysosomes were seen through the green and red channels as shown in Fig. 1B, which are shown along with a pH pseudocolored ratio image. The intensity of individual phagolysosomes varied greatly indicating that zymosan uptake and/or size varied in phagosomes. Averaged data for many cells indicated that phagolysosomes attained an acidic pH of 5.08 ± 0.08 (Fig. 1C, left), in agreement with prior studies (12,20,21). To assess the CFTR dependence of phagolysosomal acidification in J774A.1 macrophages we labeled cells with Oregon Green® 488/TMR-zymosan in the presence of 10 µM CFTRinh-172, a selective inhibitor active against CFTR from different species including human and mouse (22). Phagolysosomal acidification was insensitive to CFTR inhibitor in J774A.1 macrophages with a final pH of 5.10 ± 0.03 (Fig. 1C, middle). To confirm that our fluorescent indicator was sensitive to pH changes, phagolysosomal pH was measured following incubation with bafilomycin A1, an inhibitor of the vacuolar ATPase required for phagosomal acidification (15). As expected, phagolysosomes failed to acidify completely, reaching a pH of ~6.5 (Fig. 1C, right) in agreement with prior data (15,20).

To determine the role of CFTR channel activity in acidification of phagolysosomes in alveolar macrophages, similar measurements were done in alveolar macrophages lavaged from mouse lungs. In alveolar macrophages from wild-type mice, phagolysosomal acidification was not impaired by CFTR inhibitor (pH 5.34 ± 0.05 vs. 5.37 ± 0.07; Fig. 2, left). Further, phagolysosomal pH in alveolar macrophages from CF mice containing the most common CF mutation, ΔF508-CFTR, was not significantly different than that in alveolar macrophages from wild-type mice (pH 5.34 ± 0.05 vs. 5.40 ± 0.06, Fig. 2, middle). As expected, phagolysosomal acidification was sensitive to bafilomycin, confirming dependence on the vacuolar H⁺-ATPase (Fig. 2, left and
middle). We also found that the CFTR-selective inhibitor CFTRinh-172 did not impair acidification of phagolysosomes in (non-CF) human alveolar macrophages (pH 5.30 ± 0.08 vs. 5.35 ± 0.07; Fig. 2, right).

To account for their finding of defective phagolysosomal acidification in CF alveolar macrophages, Di et al. (7) reported that (i) lysosomal acidification was defective in CFTR knockout macrophages, and that (ii) lysosome fusion to phagosomes was responsible for phagolysosomal acidification. To assess whether lysosomal acidification was dependent on CFTR channel activity we measured lysosomal acidification in J774A.1 macrophages using a dextran-conjugate containing Oregon Green® 488 and TMR. This ratiometric, fluid-phase marker is sensitive at the acidic pH values found in lysosomes (Fig. 3A). Lysosomes were labeled by a standard pulse-chase protocol (Fig. 3B, inset). We found that lysosomal acidification in J774A.1 murine macrophages was not altered by CFTR inhibitor (10 μM CFTRinh-172, pH 4.48 ± 0.03 vs. 4.42 ± 0.03). In a control study, lysosomes failed to acidify completely in the presence of bafilomycin (Fig. 3B).

Using live cell imaging, Di et al. (7) reported that phagosomal acidification occurred after fusion with lysosomes but that this fusion event occurred 20–30 min after an initial zymosan acidification event (~0.5 pH units lower than cytoplasmic or external pH, see DISCUSSION). In the case of CFTR null macrophages, although fusion of lysosomes to phagosomes was observed, acidification did not occur as lysosomes were not acidic. To reassess these unexpected findings, we measured the kinetics of phagosomal acidification in murine and human alveolar macrophages. Further, using alveolar macrophages isolated from ΔF508-CFTR mice, we found no evidence for defective phagolysosomal acidification with mutated CFTR. As such, our experimental approach does not formally rule out the possibility that residual channel activity is responsible for the observed acidification in phagosomes and lysosomes. However, for several reasons we believe that significant residual channel activity is unlikely. The targeting of ΔF508-CFTR to phagosomal membranes is very unlikely given that this mutant resides in the endoplasmic reticulum (2,23,24), and a complete absence of endoplasmic reticulum-derived components in phagosomes has been proven (25). Further, ΔF508-CFTR is strongly inhibited at acidic pH (26), subject to degradation in lysosomes (27), and requires a chemical potentiator (such as genistein) to produce contrary to the kinetic data of Di et al. (7). Further, the rate of acidification in J774A.1 macrophages was not altered by CFTR inhibition by CFTRinh-172, nor was rate of acidification different in wild-type vs. ΔF508-CFTR alveolar macrophages (Fig. 4B). The final pH values obtained using FAM/TMR-zymosan were in good agreement with those determined using Oregon Green® 488/TMR-zymosan. Taken together, these data indicate that phagolysosomal acidification in macrophages is not dependent on CFTR chloride channel activity.

**DISCUSSION**

The aim of this study was to investigate the role of CFTR channel activity in phagosomal acidification in macrophages. Our study was motivated by the critical role for CFTR in phagosomal acidification as recently reported (7), and the important implications of this finding to bacterial killing by alveolar macrophages and mechanisms of CF lung disease. A CFTR-dependent defect in the acidification of lysosomes and phagolysosomes (by 1–2 pH units) in alveolar, but not peritoneal, macrophages from CFTR null mice was reported to be responsible for reduced bacterial killing (7). Using the CFTR inhibitor CFTRinh-172, we found pharmacological evidence against CFTR involvement in phagosomal acidification in J774A.1 macrophages, and in murine and human alveolar macrophages. Further, using alveolar macrophages isolated from ΔF508-CFTR mice, we found no evidence for defective phagolysosomal acidification with mutated CFTR. As such, our experimental approach does not formally rule out the possibility that residual channel activity from ΔF508-CFTR or after CFTR inhibition is responsible for the observed acidification in phagosomes and lysosomes. However, for several reasons we believe that significant residual channel activity is unlikely. The targeting of ΔF508-CFTR to phagosomal membranes is very unlikely given that this mutant resides in the endoplasmic reticulum (2,23,24), and a complete absence of endoplasmic reticulum-derived components in phagosomes has been proven (25). Further, ΔF508-CFTR is strongly inhibited at acidic pH (26), subject to degradation in lysosomes (27), and requires a chemical potentiator (such as genistein) to produce...
significant chloride channel activity (28). For experiments with the inhibitor CFTRinh-172 (used at 10 µM), which has IC50 ~300 nM, complete CFTR inhibition has been found at ~5–10 µM in a variety of cellular and tissue preparations (22,29). Finally, we cannot formally rule out the unlikely possibility that CFTR performs an alternative function, other than ion conductance, that is not directly assessed by our studies. For example, CFTR has been reported to participate in the regulation of endocytosis, exocytosis and endosome fusion (30,31). However, taken together, our data indicate that CFTR does not constitute a universal pathway for chloride ion conductance in the phagosomal or lysosomal membranes of macrophages and so defective acidification of alveolar macrophage phagosomes does not contribute to CF disease progression in CF subjects having the common ΔF508-CFTR mutation.

The mechanism of phagosomal maturation in macrophages is incompletely understood; however, a variety of biophysical, proteomic and genetic approaches have begun to elucidate this process (10,32,33). To the best of our knowledge, in all studies that rigorously investigated the kinetics of phagosomal acidification, irrespective of macrophage origin or the precise assay protocol, acidification of phagosomes to ~pH 5 is observed to begin within 2–3 minutes of probe binding and is complete within 5–15 minutes of particle ingestion (12,15,16,20,21,34). Phagosomal acidification in macrophages is mediated by the bafilomycin-sensitive V-ATPase (15,16); however, there is no consensus on the cellular source for delivery of this proton pump to phagosomes. Indeed, V-ATPases are found in many cellular membranes including the plasma membrane, early endosomes and the Golgi (35). Several studies do, however, indicate that lysosomes are probably not the source of V-ATPase for phagosomal acidification. In murine peritoneal macrophages, phagosomal acidification was complete prior to fusion with lysosomes, as assessed by fluorescence imaging and electron microscopy (15). Electron microscopy of J774A.1 macrophages (in which phagosomes acidify rapidly after particle ingestion (20) and this study) indicates that lysosomal fusion to phagosomes is not observed until >10 minutes after phagocytic uptake (Fig. 2 in ref. 36). Live cell fluorescence microscopy of RAW 264.7 macrophages (in which phagosomes acidify rapidly after particle ingestion, ref. 12) also demonstrated that the lysosomal marker LAMP-1 was not enriched at phagosomes until ~20 minutes after particle ingestion (37). Finally, in bone marrow derived macrophages, fluorescence resonance energy transfer-based assays of lysosome-phagosome fusion indicated that fusion lagged acidification by 5–10 minutes (21,34). Thus, a large body of data indicates that macrophage phagosomes acidify rapidly after particle ingestion and that lysosomal contents are delivered to the maturing phagosome at some time (~10 min) after acidification has initiated (10). As such, our measurements of rates of phagosomal acidification are in accord with previously published data.

In contrast to this consensus mechanism, Di et al. (7) report that mouse alveolar macrophages maintain zymosan particles at pH ~6.8 (~0.5 pH units lower than extracellular or cytoplasmic pH) for 20–30 minutes prior to complete phagosomal acidification, which occurs only after lysosomal fusion and delivery of acidic contents. The data of Di et al. does not clearly indicate the time of zymosan uptake (Fig. 7 and associated movies in Di et al. (7)). As such, it is not clear why zymosan initially reports pH < 7. Further, as no statistics were provided for these experiments, it is not possible to assess the generality of this observation.

Major difference between this report and the study of Di et al. (7) exist in the methods used to measure phagolysosomal and lysosomal pH. Whereas the present study applied a ratiometric approach with pH-sensitive (Oregon Green® 488 or fluorescein) and pH-insensitive (TMR) dyes conjugated to zymosan, Di et al. relied upon a single pH-sensitive fluorophore (fluorescein) and no marker of probe uptake. Single wavelength determination of pH has been reported (38,39); however, such applications relied upon post calibration of fluorescence intensity with high K+/ionophore-containing solutions, which does not appear to have been done by Di et al. (7). Ratiometric methods are clearly preferable to single wavelength methods in the determination of physiological parameters such as pH and Ca2+ (40,41). In terms of the fluorophores used in the respective studies, maximum sensitivity is afforded when the pKₐ of a fluorophore is similar
to the pH of the compartment being investigated. In the case of phagosomes and lysosomes (pH 4.5–5.3), Oregon Green® 488 (pKa ~4.7) is a much superior fluorophore than fluorescein (pKa ~6.5) for steady-state pH measurements. For measurements of phagosomal acidification rate (Fig. 4), fluorescein was chosen as a pH probe because initial pH in early phagosomes is >7, where the Oregon Green® 488 fluorescence signal is saturated. Despite the relatively lower sensitivity of fluorescein at ~pH 5, our measurements of final pH in phagosomes using fluorescein-TMR zymosan (Fig. 4) were in excellent agreement with the steady-state measurements (Figs. 1 and 2). For lysosomes, pH of ~4.5 was reported in this study and by Di et al. (7); such acidic pH quenches fluorescein fluorescence by ~95%, rendering this dye quite insensitive. Further, Di et al. (7) used confocal microscopy to image samples, whereas the present study relied upon wide-field detection. Because of the substantial height of macrophages (~10–15 µm), the intrinsic optical sectioning of confocal microscopy (42) and the mobility of phagosomes (36,43), movement and chromatic artifacts can confound quantitative imaging applications done at a single wavelength.

Using several assays, Di et al. reported that alveolar, but not peritoneal, macrophages from CFTR null mice were less efficient at bacterial killing than those from wild-type mice (Fig. 3 of Di et al.). In addition to assessing phagolysosomal pH, several additional processes necessary for bacterial killing by macrophages were assessed to determine the mechanism by which CFTR-dependent defects impair killing (7). The generation of oxidative species by macrophages was assessed by fluorescence imaging of CFTR null and wild-type cells loaded with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (7). This probe is readily taken up by cells and is ultimately oxygenated to generate the fluorescent species 2',7'-dichlorofluorescein, the intensity of which is generally taken to be a readout of reactive oxygen species activity. Di et al. reported that the intensity of 2',7'-dichlorofluorescein in phagolysosomes from CFTR null mice was identical to that from wild-type mice (Fig. S2 from (7). However, 2',7'-dichlorofluorescein is pH-sensitive (pK_a ~5), such that their measured pH of phagolysosomes in CFTR null (pH ~6.5) vs. wild-type mice (pH ~4.7) would confound interpretation of this data. Indeed, the quenching of 2',7'-dichlorofluorescein by the acidic environment of phagosomes in wild-type mice relative to that in CFTR null mice would account for a significant difference in fluorescence signal, suggesting that wild-type mice produce much more reactive oxygen species than CFTR null mice. A role for CFTR in transport of chloride ions into neutrophil phagosomes for the generation of oxidative species was recently proposed (44). As such, the mechanism of CFTR-dependent bacterial killing in CFTR null macrophages warrants further, definitive investigation.

There is a long-standing interest about the possibility of defective organellar acidification in CF cells. Defective acidification (by ~0.25 pH units) of the trans-Golgi, endosomes and pre-lysosomes in CF was initially reported by Al-Awqati and colleagues (45), suggesting a role for CFTR in organellar counter ion conductance. This work was subsequently refuted by multiple laboratories (46-50). Of direct relevance to this study, CFTR was found to play no role in the acidification of lysosomes in pancreatic epithelial cells (50). More recently, Deretic and colleagues have reported that endosomes and the Golgi hyperacidify in CF due to defective regulation of organellar sodium transport (51-53). Direct evidence to indicate that chloride is the major counter ion in the acidification of endosomes and the Golgi has been obtained using chloride-sensitive fluorophores (17,18). Evidence from knockout mice and direct measurements of chloride ion concentration have also indicated a role for the CIC-3 and CIC-5 chloride channels in the acidification of endosomes (54,55). Synaptic vesicles in CIC-3 knockout mice also show defective acidification (56). A general role of CIC proteins in organellar acidification has, however, been challenged as the acidification of lysosomes in neurons is normal in mice with CIC-6 deletion (57) and CIC-7 deletion (58). With regard to the immune system, contradictory evidence for CFTR function in neutrophil phagosomes has been reported (7,44) and compelling evidence from knockout mice suggests that CIC-3 activity is necessary for neutrophil function (59). A complete understanding of the molecular determinants of organelle acidification in different
tissues, including cells of the immune system, will likely require the combined application of knockout mice/knockdown technology, highly specific small-molecule inhibitors, and selective reporters of ionic content (i.e. H⁺, Cl⁻, K⁺, etc. selective probes).

In summary, our findings do not support the contention that phagolysosomal acidification in alveolar macrophages is dependent on CFTR channel activity, nor that it is impaired in CF. The mechanism of phagosomal acidification proposed by Di et al. (7) is also not in accord with our data or precedents in the literature. Because phagolysosomal acidification is central to a proposed mechanism linking defective CFTR chloride channel function with CF lung disease, our results do not support a direct role for CFTR in defective macrophage function in the pathogenesis of CF lung disease. Our findings thus underscore the need for further evaluation of mechanisms such as airway surface liquid dehydration and defective submucosal gland fluid secretion in the pathophysiology of CF.

REFERENCES

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Phagolysosomal acidification in J774A.1 macrophages is not dependent on CFTR chloride channel function. A, In situ calibration of zymosan-conjugate containing Oregon Green® 488 and tetramethylrhodamine (inset). J774A.1 macrophages were labeled with fluorescent zymosan and probe fluorescence ratio (F_{green}/F_{red}) measured using perfusates at specified pH containing high K+/ionophores (see EXPERIMENTAL PROCEDURES). Average data shown for three separate experiments at each calibration point, with 20–63 phagosomal regions analyzed per experiment. Phagolysosomal pH from one experiment is overlayed on the calibration curve (dashed lines on calibration curve). B, fluorescence micrographs of labeled J774A.1 macrophages showing Oregon Green® 488 (green) and TMR (red) fluorescence, and pseudocolored ratio image (bar, 5 µm). C, phagolysosomal pH in J774A.1 macrophages measured by ratio imaging. pH values for individual control experiments (black circles) and mean ± S.E.M. for the data sets (red) are shown. Where indicated, CFTRinh-172 (10 µM, unfilled circles) or bafilomycin (100 nM, grey circles) was included during incubations. * p < 0.001 by ANOVA with Bonferroni post-hoc test. 25–112 phagolysosomal regions were analyzed per experiment.

FIGURE 2. Phagolysosomal acidification in murine and human alveolar macrophages is not dependent on CFTR chloride channel function. Phagolysosomal pH was measured by ratio imaging and pH values for individual control experiments (black circles) and mean ± S.E.M. for data sets (red) are shown. Where indicated, CFTRinh-172 (10 µM, unfilled circles) or bafilomycin (100 nM, grey circles) was included during incubations or alveolar macrophages from ΔF508 CF mice were studied. * p < 0.001 by ANOVA with Bonferroni post-hoc test. 28–146 phagolysosomal regions were analyzed per experiment Insets show phase-contrast images of paraformaldehyde fixed murine and human macrophages with overlayed fluorescence from phagolysosomes (bar, 5 µm).

FIGURE 3. Lysosomal pH in J774A.1 macrophages is not dependent on CFTR chloride channel activity. A, In situ calibration of 40 kDa dextran-conjugate containing Oregon Green® 488 and tetramethylrhodamine. J774A.1 macrophages were labeled with the fluorescent dextran for 30 minutes followed by 2 hour chase at 37 °C to allow probe accumulation in lysosomes. Calibration of F_{green}/F_{red} vs. pH done using solutions at specified pH containing high K+/ionophores. Average data shown for 3 separate experiments at each calibration point with 21–103 lysosomal regions analyzed per experiment. B, lysosomal pH in J774A.1 macrophages determined by ratio imaging. pH values for individual control experiments (black circles) and mean ± S.E.M. for data sets (red) shown. Where indicated, CFTRinh-172 (10 µM, unfilled circles) or bafilomycin (100 nM, grey circles) were included during incubations. * p < 0.001 by ANOVA with Bonferroni post-hoc test. 35–89 lysosomal regions were analyzed per experiment Insets show fluorescence images of J774A.1 macrophages (bar, 5 µm).
FIGURE 4. **CFTR-independent phagosomal acidification kinetics in J774A.1 and alveolar macrophages.** *A*, *In situ* calibration of zymosan-conjugate containing fluorescein and tetramethylrhodamine (*inset*). Average data shown for 4–5 separate experiments at each calibration point with 22–64 phagosomal regions analyzed per experiment. *B*, acidification kinetics of phagosomes in J774A.1 macrophages (*left*) and murine alveolar macrophages (*right*). Cells were pulse-labeled with zymosan-conjugate and initial images of phagosomes were taken 3 min after initiation of phagocytosis. Where indicated, J774A.1 macrophages were incubated with CFTRinh-172 (10 µM, open circles, *left*) and murine macrophages from ΔF508 CF mice (open circles, *right*) were studied. *Insets* show averaged rate constants (mean ± S.E.M. for 4–5 separate experiments with 3–8 phagosomal regions analyzed per experiment). Differences not significant.
Figure 1
Figure 2
Figure 3
Figure 4

A

Carboxyfluorescein TMR
zymosan

B

Mouse alveolar macrophages

J774

control
+inhibitor

WT
CF

WT
CF

pH

2 min

F_{green}/F_{red}

pH

F_{green}/F_{red}

(min^{-1})

0

0.15

0.3

WT

control
+inhibitor

CF

CFTR inhibitor
CFTR-independent phagosomal acidification in macrophages
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