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Abnormal Trafficking and Degradation of TLR4 Underlie the Elevated Inflammatory Response in Cystic Fibrosis

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Morbidity and mortality in cystic fibrosis (CF) are due not only to abnormal epithelial cell function, but also to an abnormal immune response. We have shown previously that macrophages lacking CF transmembrane conductance regulator (CFTR), the gene mutated in CF, contribute significantly to the hyperinflammatory response observed in CF. In this study, we show that lack of functional CFTR in murine macrophages causes abnormal TLR4 subcellular localization. Upon LPS stimulation, CFTR macrophages have prolonged TLR4 retention in the early endosome and reduced translocation into the lysosomal compartment. This abnormal TLR4 trafficking leads to increased LPS-induced activation of the NF- κ B, MAPK, and IFN regulatory factor-3 pathways and decreased TLR4 degradation, which affects downregulation of the proinflammatory state. In addition to primary murine cells, mononuclear cells isolated from CF patients demonstrate similar defects in response to LPS. Moreover, specific inhibition of CFTR function induces abnormal TLR4 trafficking and enhances the inflammatory response of wild-type murine cells to LPS. Thus, functional CFTR in macrophages influences TLR4 spatial and temporal localization and perturbs LPS-mediated signaling in both murine CF models and patients with CF. *The Journal of Immunology*, 2011, 186: 6990–6998.

Airway obstruction, chronic bacterial infection, and excessive inflammatory responses are major causes of morbidity and mortality in patients with cystic fibrosis (CF). CF is caused by homozygous mutation of the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel that is expressed in airway epithelial cells and, at lower levels, in other cell types (1). Although the development of CF lung disease is not fully understood, it is clear that abnormal

chloride transport on the apical membrane of airway epithelial cells leads to changes in the airway environment, such as water content, pH, and ion concentrations, resulting in airway obstruction by thick mucus and depletion of antimicrobial molecules (2). Together, these conditions may favor bacteria adaptation and chronic infection in the lungs, as observed with *Pseudomonas aeruginosa*. Less clear is the etiology of the robust inflammatory response that characterizes CF lung disease. The excessive inflammatory response was thought to be a consequence of chronic infection, but evidence suggests that the etiology of this exaggerated response may be more complex. Macrophages and mast cells are present at higher levels in CF airways even during fetal development (3), and airway inflammation is already present in CF infants prior to establishment of chronic infection (4–6). In addition, young children with CF have more alveolar macrophages and CC chemokines even in the absence of pulmonary infection (7). These observations support the hypothesis that intrinsic abnormalities in the innate immune system may contribute to the disease process and that CF lung pathology is due to intricate cross-talk between dysfunctional epithelial and immune cells.

Recent data show that CFTR has a direct role in the normal function of immune cells including macrophages (8–12), neutrophils (13, 14), dendritic cells (15), and lymphocytes (16–18). By creating bone marrow (BM) chimeras in which wild-type (WT) and CF mice were irradiated and transplanted with either WT BM or CF BM, we demonstrated that the increased levels of proinflammatory cytokines depend on lack of functional CFTR in immune rather than epithelial cells (11). We also found that, compared with WT, CFTR^{-/-} BM-derived macrophages (BMDM) and alveolar macrophages have elevated LPS-induced transcription and secretion of many proinflammatory cytokines (11). The lack of functional CFTR in macrophages has been associated with abnormal acidification of cell organelles (9), abnormal lipid metabolism (19), and alteration of transcription factors (10) that can contribute to the hyperinflammatory phenotype. In this study, we demonstrate that functional CFTR directly or indirectly affects the spatiotemporal compartmentalization of TLR4, which is necessary for well-controlled TLR4 signaling and degradation. In addition,

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E.M.B. designed the study, developed assays, performed experiments, analyzed data, and wrote the manuscript; P.-X.Z. developed the culture of primary human macrophages, helped in developing assays and in data analysis, and performed experiments; A. Satoh contributed to study design, data analysis, and performed the confocal experiments; C.C. provided technical help; A. Shenoy assayed the chloride efflux on primary macrophages; and R.M., M.E.E., and D.S.K. contributed to design of the study, interpretation of the data, and writing the manuscript.

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Abbreviations used in this article: BM, bone marrow; BMDM, bone marrow-derived macrophages; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CFTR_{inh} 172, CFTR inhibitor 172; EEA-1, early endosomal Ag-1; HD, healthy donor; IF, immunofluorescence; IRF-3, IFN regulatory factor-3; KC, keratinocyte chemoattractant; LAMP-1, lysosomal-associated membrane protein 1; MFI, mean fluorescence intensity; MQAE, *N*-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide; TRAM, Toll/IL-1R domain-containing adaptor inducing IFN- β -related adaptor molecule; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN- β ; WT, wild-type; WT_{inh}, wild-type cells treated with inhibitor.

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we show that macrophages from CF patients, as in mice, are hyperresponsive to acute LPS exposure. Thus, functional CFTR is necessary for controlling the innate immune response in macrophages, and intrinsic defects of such early players in the innate immunity may directly influence the cascade of events leading to CF lung disease.

Materials and Methods

Mouse breeding

Transgenic CFTR^{-/-} (B6.129P2-Cftr^{tm1Unc}) mice from The Jackson Laboratory were bred in the Yale University Animal Facility and are completely backcrossed to C57BL/6 mice. WT mice used in the experiments were littermate controls derived from breeding of CFTR^{+/-} pairs. Mice were fed with a liquid diet (Peptamen; Nestle, Deerfield, IL) as previously described (11). All procedures were performed in compliance with relevant laws and institutional guidelines and approved by the Yale University Institutional Animal Care and Use Committee.

Isolation and culture of murine BMDM and human peripheral blood progenitor cell-derived macrophages

Murine macrophages. After overnight culture of fresh BM cells (11), nonadherent cells were differentiated for 7 d in 20 ng/ml rM-CSF (Pepro-Tech, Rocky Hill, NJ). Approximately 1–3 × 10⁷ macrophages were obtained per mouse. Cells were plated at a concentration of 1 × 10⁶ cells/well in six-well plates overnight and challenged with *P. aeruginosa* LPS (25 ng/ml; Sigma-Aldrich) or Pam₃CSK₄ (50 ng/ml; Imagenex) for the times indicated. Supernatant, RNA or protein lysates were harvested for analysis at different time points. For time 0 h, untreated cells were used.

Human macrophages. Blood was obtained from healthy donors (HD) or from patients (age range 3–18 y, all pancreatic insufficient) with CF carrying at least one deltaF508 allele during their annual checkup with informed consent in accordance with the Yale University Medical School Human Investigation Committee. Human mononuclear cells were isolated by Histopaque-1077 (Sigma-Aldrich) from 5–10 ml blood and seeded at 5 × 10⁶ cells/well in 24-well plates in RPMI 1640 supplemented with 10% FBS and 40 ng/ml recombinant human M-CSF (PeproTech). Cells were split 1:1 every 3 to 4 d. After 2 to 3 wk, cells were characterized by flow cytometry (CD14⁺/CD45⁺) and morphology analyzed on cytospin (Supplemental Fig. 4). We obtained ~1 × 10⁶ macrophages from 5 ml blood. Before LPS treatment, cells were washed extensively with PBS, detached with Accutase (Innovative Cell Technology), and seeded at 0.25 × 10⁶ cells per well of 24-well plates. The day after, cells were challenged with LPS (50 ng/ml) for the times indicated in the text, and the supernatant and cells were harvested for analysis. For time 0 h, fresh media without LPS was harvested after 12 h of incubation.

Flow cytometry

For plasma membrane flow cytometry, WT and CF macrophages were detached with Accutase and washed in wash buffer (PBS/2% serum). After 10 min of incubation with Fc-block, cells were stained on ice for 30 min with either rat monoclonal allophycocyanin-F4-80 (eBioscience) and rat monoclonal FITC-TLR4 (Imagenex) or allophycocyanin-F4-80 and FITC-TLR2 (Imagenex). Dead cells were excluded with either propidium iodide or dead/live staining (L10119; Invitrogen). Appropriate isotype controls were used. For experiments with CFTR inhibitor 172 (CFTR_{inh}172) (kind gift from Dr. Alan Verkman), cells were pretreated with 20 μM inhibitor or vehicle alone (DMSO) overnight before the experiment.

Human cells were stained with the mouse monoclonal FITC-TLR4 (Imagenex) and run on an FACSCalibur (BD Biosciences) instrument followed by analysis using FlowJo software (Tree Star).

Immunofluorescence and pulse-chase experiments for TLR4 colocalization with endosomal vesicles by immunofluorescence analysis and Imagestream

Immunofluorescence. WT and CF macrophages were grown on poly-L-lysine-coated 12-mm round coverslips at 80% confluence. Untreated and LPS (25 ng/ml) treated cells were fixed for 15 min in 4% PFA, permeabilized for 5 min in PBS/0.1% Triton, and stained with mouse monoclonal anti-TLR4 Ab (Imagenex; 1:200) and rat monoclonal anti-lysosomal-associated membrane protein 1 (LAMP-1) Ab (Southern Biotechnology Associates; 1:100) for 30 min at room temperature, following

by staining with secondary Alexa 488 anti-mouse and Alexa 555 anti-rat (Invitrogen; 1:400) for 30 min at room temperature. Pictures were taken with a confocal microscope (Leica TCS SP5 Spectral Confocal Microscope; Leica Microsystems).

Pulse-chase experiments. WT and CF macrophages were grown in six-well plates for Imagestream or on poly-L-lysine-coated 12-mm round coverslips at 80% confluence. Keeping the cells at 4–6°C, cells were washed in PBS, incubated with Fc block for 5 min, and labeled with rat anti-TLR4 Ab (Imagenex) while also binding to LPS. After washing in cold PBS, 37°C media was added, and cells were incubated at 37°C still in the presence of LPS to allow internalization of TLR4 bound to Abs for the time indicated in the text. For confocal immunofluorescence (IF) analysis, after incubation at 37°C, cells were fixed, permeabilized, and stained with anti-early endosomal Ag-1 (EEA-1) Ab (PA1063A; Affinity BioReagents; dilution 1:250), which recognizes endosomal compartments. TLR4 was detected with a biotin-anti-rat Ab (Invitrogen; 1:200) followed by tyramide signal amplification according to the manufacturer's instructions (Invitrogen). Pictures were taken with a confocal microscope (TCS SP5; Leica Microsystems). For ImageStream, after incubation at 37°C, cells were detached using PBS/2 mM EDTA on ice for 15 min, fixed, permeabilized, and stained with anti-EEA-1 Ab and FITC anti-rat Ab. We gated on single cells and focused on cells that were positive for both FITC (TLR4) and PE (EEA-1). Within this population, we assessed the location of the TLR4 Ab and its colocalization with the EEA-1 compartment. Using the Imagestream software, we also assessed the median fluorescence intensity for FITC (TLR4) in the EEA-1 compartment (5000 cells/experiment) during LPS stimulation and determined the number and the size of EEA-1-positive vesicles over time.

Protein analysis

For TLR4 Western blots, 1 × 10⁶ cells were lysed, and an equal amount of protein was separated by electrophoresis on 12% Bis-Tris Gels, transferred to nitrocellulose membrane (Bio-Rad), and incubated with a mouse anti-TLR4 Ab (1:100; Imagenex). HRP-conjugated goat anti-mouse IgG secondary Ab (1:5000; Santa Cruz Biotechnology) and Amersham ECL Plus Western blotting System (GE Healthcare Biosciences) were used for detection. For the degradation experiments, cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich) for 30 min before harvesting controls (no LPS) and LPS-treated (1 h, 3 h) cell lysates.

Two different assays were performed to detect the phosphorylated proteins: Western blot and a bead-based phosphoprotein assay (8-Plex Multi-Pathway Signaling Kit; Millipore) using a Luminex instrument (Luminex). In all phosphoprotein experiments, cells were serum starved for 1 h before LPS addition. Phosphorylated protein levels were normalized to total target and/or β-actin. Quantification of the phosphorylated form was assessed using ImageJ software (National Institutes of Health) for Western blot and calculating the median fluorescence intensity for the Multi-Pathway Signaling (Millipore) assay. All Abs used for phosphoprotein Western blot were purchased from Cell Signaling Technology.

Cytokine quantification

Cytokine concentrations in the media of cultured murine or human macrophages were assessed either by ELISA (R&D Systems) or by Milliplex following the manufacturer's instructions (MPXHCYTO-60K-16; Millipore).

Expression analysis

Real-time PCR analysis was performed with a Bio-Rad iCycler (Bio-Rad) using TaqMan technology. Copy numbers were normalized to 18S expression, and the fold increase over time was calculated by the ΔΔ cycle threshold method. Primers were purchased from Applied Biosystems (data shown in Supplemental Fig. 1).

N-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide indicator studies

Macrophages were incubated at 37°C with N-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide ([MQAE] 30 mM; Invitrogen) for 30 min. Subsequently, cells were examined using an Olympus IX-71 inverted microscope (Olympus) with a digital imaging system collecting data every 5 s in arbitrary fluorescent intensity units. MQAE was excited at 340 ± 10 nm, and emitted fluorescent light was measured at 460 ± 10 nm. Data were analyzed using Metafluor 7.0R program in conjunction with Microsoft Excel (Microsoft). Cl⁻ is secreted from the cell as [Cl⁻]_{EC} exceeds [Cl⁻]_{IC}, resulting in an increase in the intensity of fluorescence of MQAE. Initially, macrophages were perfused with chloride-containing buffer

(Krebs Bicarbonate Ringers Solution, 125 mM NaCl; 2.4 mM K_2HPO_4 , KH_2PO_4 , 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 25 mM $NaHCO_3$ [pH 7.4], 295–305 mOsm) at a perfusion rate of 3 to 4 ml/min. Following plateau of background fluorescence, the perfusion buffer is changed to a chloride-free solution: 150 mM Na cyclamate, 2 mM $MgSO_4$, 0.5 mM Ca cyclamate, 1 mM EGTA, and 5 mM HEPES [pH 7.4], 298 mOsm). The CFTR contribution to the observed Cl^- efflux was assessed by treating cells with forskolin (20 μ M) to activate CFTR with the subsequent addition of CFTR_{inh}172 (50 μ M) to inhibit CFTR-dependent Cl^- efflux. The subsequent decrease in intensity of fluorescence in the presence of CFTR inhibitor is representative of Cl^- secretion likely due to CFTR. Lastly, CFTR inhibitor is removed from the perfusate, and the increase in efflux is assessed to determine if there is restoration of CFTR activity (data shown in Supplemental Fig. 3B).

Statistical analysis

Statistical analysis of the results was analyzed using a one-sided two-sample *t* test. Data are expressed as means \pm SE. A *p* value <0.05 was considered significant.

Results

Abnormal TLR4 localization in untreated CFTR^{-/-} macrophages

We have previously demonstrated that CFTR^{-/-} BMDM have increased transcription and secretion of proinflammatory cytokines in response to LPS (11).

To investigate the nature of this hyperinflammatory response, we examined extracellular TLR4 expression by flow cytometry, total TLR4 protein expression by Western blot, and TLR4 cell localization by IF in WT and CF untreated macrophages.

CF macrophages displayed significantly higher levels of plasma membrane-associated TLR4 (Fig. 1A). Because forward and side scatter analysis did not reveal differences in macrophage size between genotypes (data not shown), the mean fluorescence intensity (MFI) for TLR4 assessed on 10,000 live F4/80⁺ cells can be used to compare the relative TLR4 per cell. Although these differences may appear to be modest, this degree of increase in TLR4 expression is known to cause a profound increase in the LPS response by immune cells (20, 21). There was no difference in TLR4 mRNA expression assessed by quantitative PCR between WT and CF macrophages (Supplemental Fig. 1A) nor was there a difference in total TLR4 protein (Fig. 1B), suggesting that TLR4 may have abnormal posttranslational regulation in CF macrophages. IF revealed that while in WT macrophages TLR4 is widely distributed in vesicular compartments throughout the cells, CF macrophages have a remarkably higher distribution of TLR4 in the periplasma membrane area (Fig. 1C). In contrast, no differences were observed in expression of plasma membrane TLR2 between WT and CF BMDM in the absence (Supplemental Fig. 1C) or presence of its specific ligand Pam₃CSK (data not shown).

CF macrophages have more rapid and prolonged plasma membrane TLR4 signal transduction

As a consequence of abnormal TLR4 distribution, we expected to see differences in TLR4 signal transduction via the TIRAP–MyD88 pathway in CF cells (22, 23). BMDM were exposed to LPS, and phosphoproteins were analyzed by Western blot (two to four mice per genotype) and/or Luminex (four mice per genotype) technology (Luminex). Untreated WT and CF cells served as controls. Consistent with findings in CF airway epithelial cells (24) and CF alveolar macrophages (12), untreated CF macrophages had higher steady-state levels of p-I κ B α than WT macrophages. After LPS activation, I κ B α phosphorylation was more robust and prolonged (20 min) in CF than WT macrophages (Fig. 2A). At the transcriptional level, LPS treatment led to a >10-fold increase in I κ B α mRNA expression in both CF and WT cells. However, from

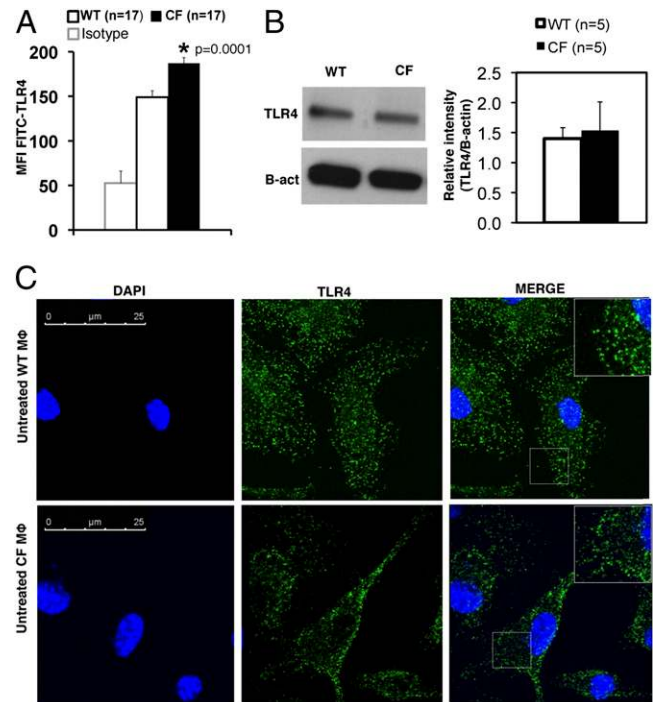


FIGURE 1. CF macrophages have abnormal TLR4 localization compared with WT mouse macrophages. *A*, Flow cytometry of plasma membrane TLR4 on untreated WT (white bars) and CF (black bars) macrophages. The isotype control is also shown. Data are representative of 17 independent experiments. *B*, Western blot of total TLR4 protein from untreated WT or CF macrophages; gel is representative of five independent experiments. Mean \pm SEM relative quantification normalized to β -actin is shown in the *right panel*. Statistical analysis was performed by two-sample *t* tests. *C*, Representative IF for TLR4 on untreated WT (*upper panels*) and CF (*lower panels*) macrophages; nuclei were stained with DAPI. In the merged microphotographs, the inserts represent a higher magnification of specific cell regions (gray boxes). Confocal images were taken using a 63 \times oil immersion lens.

6–16 h of LPS stimulation, CF macrophages had lower I κ B α expression with a more rapid decline over time, suggesting slower NF- κ B inactivation (Supplemental Fig. 1B). ERK1/2 phosphorylation, similar to I κ B α phosphorylation, was robust and prolonged in CF compared with WT cells (Fig. 2B). After 20 min of LPS, p-ERK1/2 decreased in WT cells, whereas it remained elevated in CF cells. These data were confirmed by Western blot analysis for p-ERK1/2 (Fig. 2B, *inset*). Other MAPKs analyzed, JNK and p38, did not show differences in the kinetics of LPS-induced activation in CF and WT macrophages (Supplemental Fig. 1E–H). To confirm that these phosphorylated proteins were functionally associated with an increase in signal transduction, we measured the expression and secretion of cytokines that are predominantly activated by the two specific pathways assessed. At the transcriptional level, CF macrophages had more LPS-induced IL-6 and CCL-2 mRNA than WT cells (data not shown). Consistent with the expression profile, LPS-treated CF macrophages also secreted more proinflammatory cytokines, including IL-6 and GM-CSF, than WT cells (Fig. 2C). In conclusion, these data suggest that untreated CF macrophages have higher TLR4 expression on the plasma membrane and in the periplasma membrane area compared with WT cells and that this abnormal distribution may contribute to the proinflammatory state with more robust LPS-mediated TIRAP–MyD88 signal transduction. In contrast and consistent with the unchanged TLR2 plasma membrane expression in the CF cell, there was no difference in Pam₃CSK₄-induced

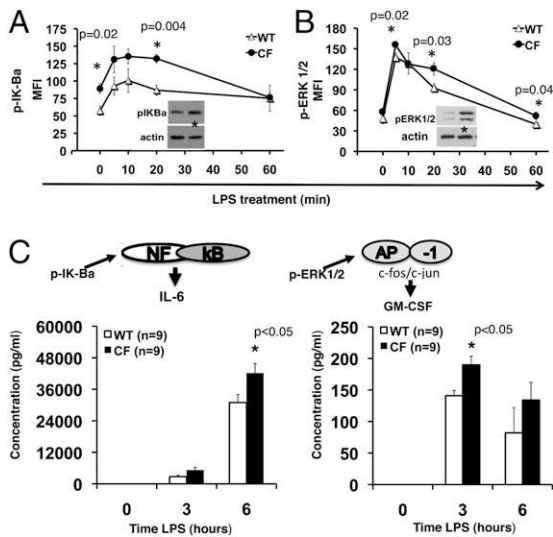


FIGURE 2. CF macrophages have more robust and prolonged TLR4 signal transduction. p-IκBα (A) and p-ERK1/2 (B) assessed in untreated (0) and LPS treated (5, 10, 20, and 60 min) WT (open triangles) and CF (closed circles) macrophages by Luminex-based (Luminex) phosphoprotein assay (y-axis: MFI); representative Western blots are also shown (20 min). C, Mean \pm SEM of IL-6 and GM-CSF concentration (pg/ml) in the supernatant of LPS-treated WT (white bars) and CF (black bars) macrophages untreated (0) or treated with LPS for 3 and 6 h.

TIRAP/MyD88-dependent signal transduction between WT and CF cells, as assessed by IL-6 expression over time (Supplemental Fig. 1D).

Increased and prolonged localization of active TLR4 in the endosomal compartment of CFTR^{-/-} macrophages

Upon activation on the plasma membrane, TLR4 is internalized into endosomal compartments, where it activates the Toll/IL-1R domain-containing adaptor inducing IFN- β -related adaptor molecule (TRAM)-Toll/IL-1R domain-containing adaptor inducing IFN- β (TRIF) pathway (25). We hypothesized that the trafficking and endosomal signaling may be aberrant in CF cells as well. We performed pulse-chase studies using both confocal microscopy and Imagestream to follow the trafficking of LPS-activated plasma membrane TLR4 into early endosomes (EEA-1 positive). The experimental design is described in detail in *Materials and Methods*. After labeling the cell membrane with fluorescent TLR4 Ab and inducing internalization in the presence of LPS, cells were stained with anti-EEA-1 Ab and anti-rat Ab. After 30 min of LPS stimulation, activated TLR4 was localized within EEA-1-positive endosomes predominantly in CF cells, and there was significantly less TLR4 colocalized with the EEA-1-positive compartment in WT macrophages (Fig. 3), suggesting that internalized TLR4 receptors are retained in CF endosomal compartments during LPS stimulation.

To accurately quantify the subcellular localization of TLR4, we used Imagestream technology in which flow cytometry technology is combined with imaging of single cells (26). Gating on single cells that were positive for both FITC (TLR4) and Alexa 568 (EEA-1), we correlated the location of the TLR4 Ab (extra- versus intracellular; y-axis, Fig. 4A) with the colocalization of TLR4 with the EEA-1 compartment (x-axis, Fig. 4A). There were three populations: 1) extracellular TLR4 shown in Fig. 4A in pink; 2) internalized TLR4 not colocalized with EEA-1 in red (Fig. 4A); and 3) internalized TLR4 colocalized with EEA-1 in orange (Fig. 4A). We assessed the median fluorescence intensity for FITC (TLR4) in the EEA-1 compartment (5000 cells/experiment) dur-

ing LPS stimulation. The time course from a representative experiment is shown in Fig. 4B. The mean difference (Δ = CF - WT) in median TLR4 IF between WT and CF during LPS stimulation of three independent experiments (Fig. 4C) revealed that although accumulation of TLR4 in the endosomes was the same for WT and CF cells at 5 min, CF macrophages had statistically significantly greater accumulation of internalized TLR4 in the endosomal compartment than WT macrophages after 15 (p = 0.03) and 30 min (p = 0.03) of LPS treatment. Consistent with the finding that CF macrophages have retention of internalized TLR4 in the endosomal compartment, TRAM-TRIF signal transduction from the CF endosomal compartment, assessed by Western blot for phosphorylated IFN regulatory factor-3 (IRF-3) (25), was more robust in CF than WT cells (p = 0.004; Supplemental Fig. 1I). Secretion of RANTES, which is predominantly activated by this specific pathway, was also higher in LPS-treated CF macrophages than WT cells (p = 0.005; Supplemental Fig. 1J). Together, these data suggest that in CF cells, TLR4 in endosomal compartments engages the TRIF adaptor and is actively signaling for a prolonged period compared with WT cells.

Decreased TLR4 degradation in CFTR^{-/-} macrophages

Once in the endosomal compartment, TLR4 is targeted to the lysosomal compartment for degradation, which is fundamental for resolution of signaling (21, 27). One potential explanation for the prolonged retention of active TLR4 in endosomal compartments and the subsequent increase in signal transduction in CF cells is that TLR4 degradation is slower in CF cells. To test this hypothesis, WT and CF macrophages, treated with the protein synthesis inhibitor cycloheximide, were exposed to LPS (1 h and 3 h), and total TLR4 protein was assessed by Western blot (Fig. 5A). Indeed, during LPS stimulation, at 3 h post-LPS treatment, TLR4 was decreased by >50% in WT cells. In contrast, in CF macrophages, the rate of degradation was slower than in WT cells; after 3 h of LPS exposure, only 20% of the protein was degraded (Fig. 5A, 5B).

To test whether decreased TLR4 degradation in CF macrophages was due to reduced translocation to the lysosomal compartment, WT and CF macrophages treated with LPS for 45 min were analyzed for colocalization of TLR4 with the LAMP-1 by IF. Whereas WT macrophages had a robust TLR4 colocalization in the lysosomal compartment, in CF cells, there was minimal TLR4 associated with LAMP-1-positive vesicles (Fig. 5C, Supplemental Fig. 2). LAMP-1-positive vesicles (red signal) were clustered in the nuclear area in WT cells, whereas the signal was present diffusely throughout the cytoplasm in CF cells (Fig. 5C, additional samples and a representative z-stack in Supplemental Fig. 2). Finally, the small GTPase Rab7, which is implicated in the transport from early to late endosomes (28) and a known key regulator for proper aggregation and fusion of late endocytic structures in the perinuclear region and consequently for the biogenesis and maintenance of functional lysosomal compartment (29), failed to increase during LPS stimulation in CF compared with WT macrophages (Fig. 5D). Together, these data suggest that lack of functional CFTR in macrophages leads to aberrant maturation of vesicles in the endosomal-lysosomal axis, which may explain why TLR4 is retained in the endosomal compartment in its active state.

The abnormal trafficking and signaling in CFTR^{-/-} macrophages is CFTR dependent

In WT mice, although CFTR expression is \sim 100-fold lower in immune cells than lung (9, 15) (Supplemental Fig. 3A), it is still functional. Function of CFTR in WT macrophages is shown by forskolin-stimulated Cl⁻ secretion that can be inhibited with the

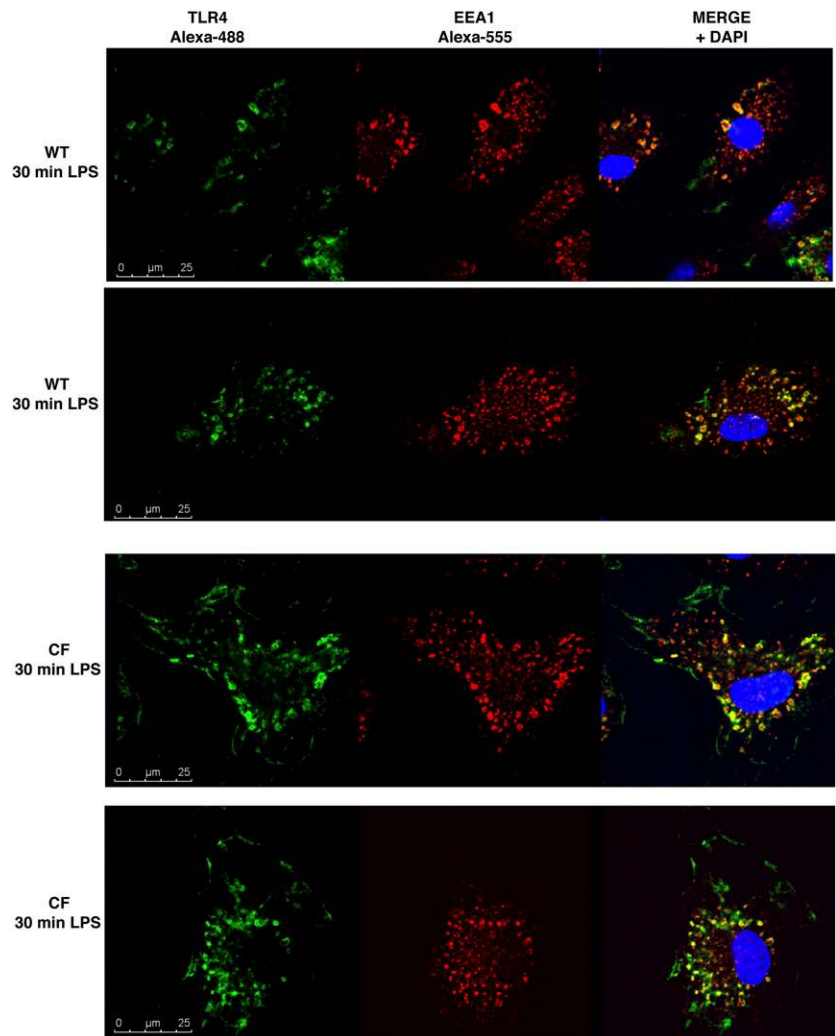


FIGURE 3. Accumulation of activated TLR4 in the endosomal compartment of CF macrophages. After labeling plasma membrane TLR4, cells were incubated at 37°C in the presence of LPS for 30 min. Internalized TLR4 was detected by IF for TLR4 (green), EEA-1 (red), and DAPI (blue) in two representative WT (*upper panels*) and CF (*bottom panels*) cells. Yellow staining represents TLR4 and EEA-1 co-localization.

specific thiazolidinone CFTR inhibitor [CFTR_{inh}172 (30)] and is not detected in CF cells (Supplemental Fig. 3B). To determine whether lack of CFTR function contributes directly to abnormal TLR4 signaling and trafficking, WT and CF macrophages were pretreated overnight with CFTR_{inh}172, as described previously for airway epithelial cells (31). Plasma membrane TLR4 expression was significantly increased ($p = 0.03$) on WT cells treated with inhibitor (WT_{inh}) compared with untreated WT macrophages, but remained less than CF macrophages. The expression of plasma

membrane TLR4 in CF macrophages treated with the inhibitor was not different compared with CF cells treated with vehicle (DMSO; Fig. 6A). Secretion of keratinocyte chemoattractant (KC), IL-6, and G-CSF, which we previously demonstrated are hypersecreted by CF macrophages (11), was assessed by ELISA ($n = 3$ mice/genotype). For all cytokines analyzed, WT_{inh} had increased secretion levels, which were greater than those of WT but less than those of CF macrophages. The secretion of KC and G-CSF was significantly increased in WT_{inh} macrophages

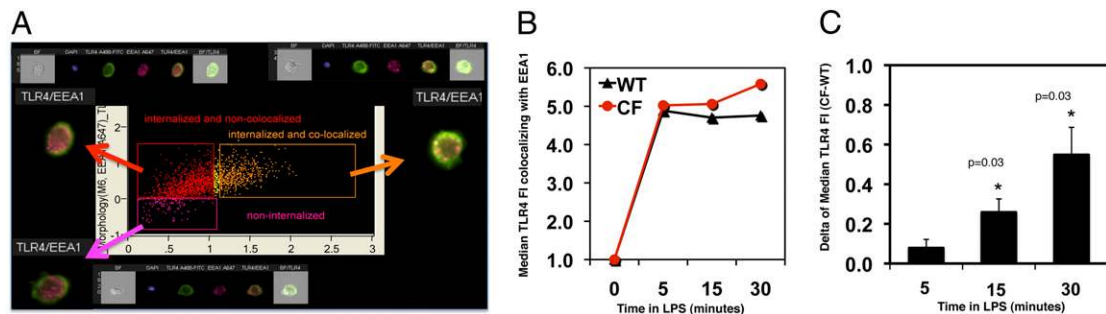


FIGURE 4. TLR4 quantification in the endosomal compartment of CF macrophages. *A*, Representative Imagestream analysis as described in the text. *B*, Graph from a representative experiment showing the median TLR4 fluorescence intensity in EEA-1–positive vesicles normalized to time 0 for WT (black) and CF (red) macrophages. *C*, Mean \pm SEM difference ($\Delta = \text{CF} - \text{WT}$) in TLR4 MFI between WT and CF during LPS stimulation from three independent experiments. In each experiment, 5000 cells were analyzed. Statistical analysis was performed by two-sample *t* tests.

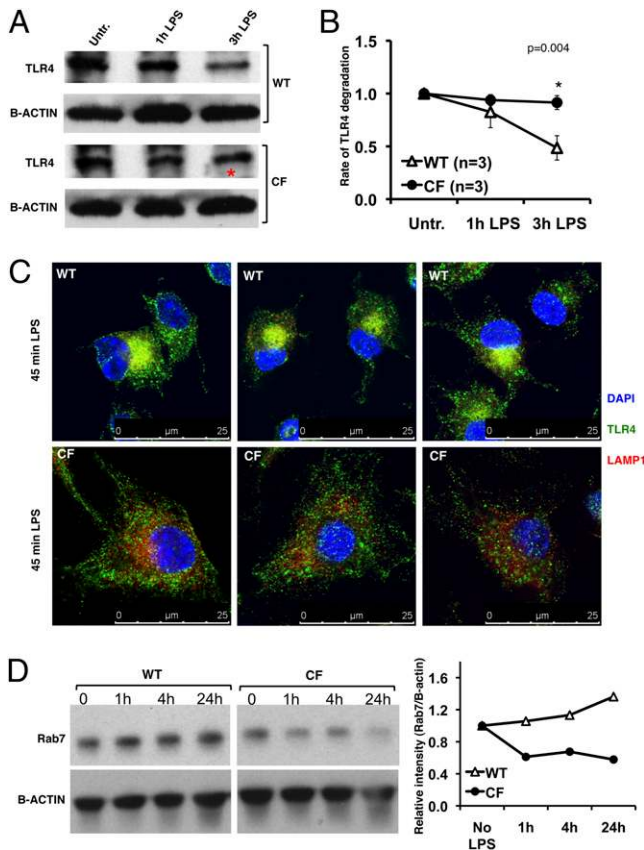


FIGURE 5. CF macrophages have reduced TLR4 protein degradation and translocation to the lysosomal compartment. *A*, Representative Western blot of TLR4 from WT and CF macrophages treated with protein synthesis inhibitor and challenged with LPS. Note higher TLR4 expression in CF cells (red asterisk). *B*, Mean \pm SEM relative quantification normalized to β -actin with time zero set at 1 for three independent experiments. Statistical analysis was performed by two-sample *t* tests. *C*, IF for TLR4 (green) and LAMP-1 (red) in WT (*top panels*) and CF (*bottom panels*) macrophages treated with LPS for 45 min; nuclei were stained with DAPI. Yellow staining represents TLR4 and LAMP-1 co-localization (see also Supplemental Fig. 2). *D*, Representative Western blot (*left panel*) and relative quantification normalized to β -actin (*right panel*) for Rab7 in WT and CF macrophages untreated or challenged with LPS as indicated.

compared with WT cells, whereas IL-6 showed an increasing trend (Fig. 6C). Lastly, using Imagestream technology, WT_{inh} macrophages displayed an increased accumulation of TLR4 in the EEA-1–positive compartment after 30 min of LPS stimulation ($p = 0.02$; Fig. 6B). These data confirm that the lack of functional CFTR affects TLR4 localization and trafficking and has repercussions on the inflammatory response.

Human macrophages from CF patients are hyperresponsive to LPS

We next assessed whether primary CF human macrophages are also hyperresponsive to LPS. Human mononuclear cells from the peripheral blood of three HD and five CF patients carrying at least one deltaF508 allele were differentiated with M-CSF *in vitro* into adherent cells with macrophage-like morphology and immunophenotype (CD45; CD14 positive) (Supplemental Fig. 4A, 4B). CFTR was detectable in cells from HD (Supplemental Fig. 4C). Despite the fact that the human cells were derived from the peripheral blood and the murine cells that we have studied were matured *in vitro* from BM precursors, the human cells still showed higher levels of TLR4 in CF compared with HD macro-

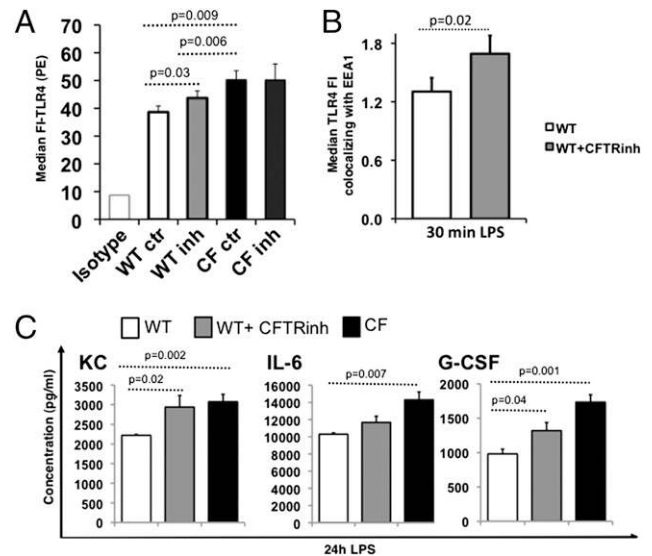


FIGURE 6. The abnormal trafficking and signaling in CFTR^{-/-} macrophages is CFTR dependent. *A*, TLR4 plasma membrane expression as indicated for WT, WT treated with CFTR_{inh}172, CF, and CF treated with CFTR_{inh}172 cells: average results for three independent experiments (isotype control, leftmost bar). *B*, KC, IL-6, and G-CSF concentration (pg/ml) in the supernatant of LPS-treated WT (white bars), WT treated with CFTR_{inh}172 (gray bars), and CF (black bars) macrophages. *C*, Average \pm SEM MFI of TLR4 localized to the endosomal compartment after 30 min of LPS stimulation as assessed by Imagestream for three independent experiments (WT is white, WT treated with CFTR_{inh}172 is light gray). Statistical analysis was performed by two-sample *t* tests.

rophages (Fig. 7A). As observed in murine cells, phosphorylation of ERK1/2 in response to LPS was more robust in CF cells compared with HD controls. Three independent experiments and a representative Western blot are shown in Fig. 7B. Though there was donor variability in the kinetics of ERK1/2 activation, in all three experiments, CF patients had more robust ERK1/2 phosphorylation than HD after 20 and 60 min of LPS stimulation. In some of the experiments, we observed higher p-ERK1/2 in untreated CF cells compared with WT cells. The detection of other phosphoproteins was not possible due to the low number of human cells available for the assay. The supernatants of LPS-treated and untreated human macrophages were analyzed for cytokine content by Luminex. Similar to our observations in murine cells (11), human CF macrophages had significantly ($p < 0.05$) higher secretion of TNF- α (6 h), IL-6 (3 h), GM-CSF (3 and 6 h), MCP-1 (6 h), and IP-10 (3 and 6 h) (Fig. 7C). G-CSF (6 and 24 h), RANTES (6 h), and IL-1b (6 and 24 h) were also elevated (data not shown). Consistent with the findings of Zaman et al. and Xu et al. (8, 12), the basal level of IL-8 in the supernatant of WT and CF macrophages was abundant and significantly higher for CF than HD cells. However, during activation, the secretion of IL-8 increased similarly in both CF and HD cells. No differences were observed in secretion of IL-1a and growth-related oncogene (data not shown). These data suggest that, similar to murine cells, human CF macrophages are hyperresponsive to LPS and further support the hypothesis that immune cells may contribute directly to the development of CF lung disease in humans.

Discussion

We previously reported that CF macrophages are hyperresponsive to LPS (11). In this study, we demonstrate that abnormal trafficking of TLR4 contributes to this exaggerated response. Tight

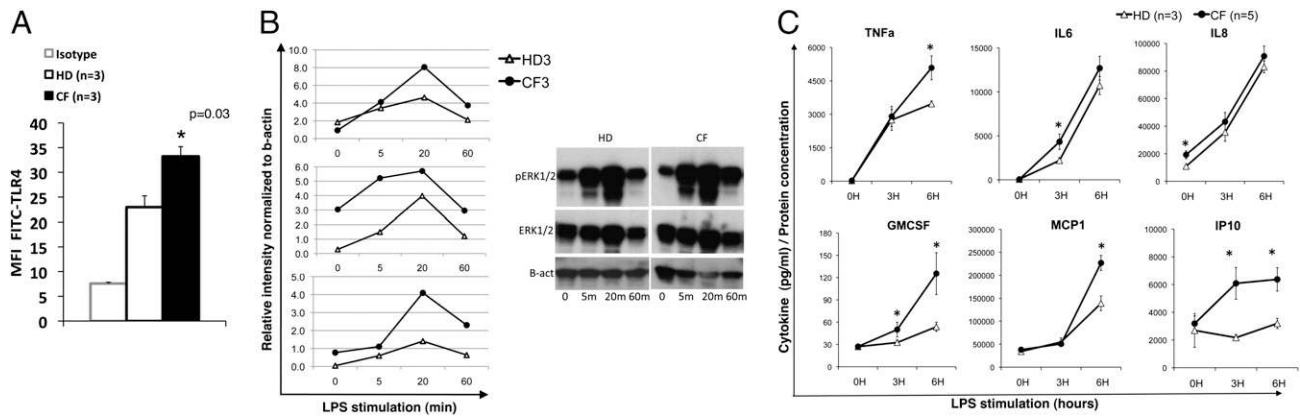


FIGURE 7. Human macrophages from CF patients are hyperresponsive to LPS. **A**, Mean \pm SD of TLR4 expression in macrophages from HD and patients with CF; isotype control is also shown. **B**, p-ERK1/2 assessed in untreated (0) and LPS treated (5, 20, and 60 min) HD and CF macrophages by Western blot normalized to β -actin; data are from three separate experiments with different CF and HD samples. A representative blot is shown (*right panel*). **C**, Mean \pm SEM cytokine concentration in the supernatant of HD (open triangle) and CF patient (closed circles) macrophages untreated (0) and treated with LPS as indicated. Statistical analysis was performed by two-sample *t* tests. Asterisks indicate statistically significant differences between WT and CF cells ($p < 0.05$).

control of active TLR4 signaling is critical for an adequate inflammatory response to LPS and is essential for preventing injury to the host (32). In resting cells, TLR4 is located both in the Golgi and at the plasma membrane, and TLR4–MD–2–CD14 constantly cycles between the plasma membrane and the Golgi (27, 33). In response to LPS binding, the TLR4 complex composed of CD14, TLR4, and MD-2 assembles on lipid rafts and signals through the TIRAP–MyD88 adaptors (34, 35), activating the NF- κ B and MAPK pathways (22, 23). After LPS engagement, TLR4 is ubiquitinated and associates with the ubiquitin-binding endosomal sorting protein hepatocyte growth factor-regulated tyrosine kinase substrate, which sorts TLR4 into EEA-1–positive early endosomes (27, 36). In this location, TLR4–TRAM engages TRIF and activates the IRF-3 pathway, leading to transcription of targets including RANTES, IP-10, and IFN genes (25). Translocation to endosomes is also associated with TLR4 dedimerization, detachment from LPS (37), and engagement of the TAG protein, which is required for trafficking of TLR4 to Rab7-positive late endosomes (38). These events drive receptors to the degradation pathway (21, 27, 38) required for resolution of the inflammatory response. Minimal perturbation of any of these steps causes an abnormal inflammatory response (20, 21).

In this study, we have demonstrated that naive CF macrophages have abnormal TLR4 subcellular localization and increased TLR4 plasma membrane expression compared with WT cells. Because CF cells do not have more TLR4 mRNA or total TLR4 protein than WT cells, it is likely that trafficking regulation is responsible for the increased plasma membrane TLR4 protein levels. A possible explanation for the increased plasma membrane TLR4 of untreated CF macrophages could be abnormal vesicular sorting and trafficking between the Golgi and the plasma membrane. Although this hypothesis remains to be fully verified, it may explain why untreated WT macrophages pretreated with CFTR inhibitor display increased plasma membrane TLR4, whereas this effect does not occur in CF cells pretreated with the CFTR inhibitor.

These data differ from a recent study (39) in which TLR4 expression in CF epithelial cells was lower than WT airway epithelial cells. This is not surprising because epithelial cells and macrophages play unique roles during an inflammatory response. For instance, epithelial cells express TLR4 mainly on the basolateral membrane of the cell rather than on the apical membrane where TLR2 and TLR5 predominate (40). However, consistent

with data from human CF airway epithelial cells (24, 31), we found that prior to LPS challenge, CF macrophages have increased p-I κ B α , suggesting that absence of functional CFTR activity leads to a proinflammatory state. The increase of plasma membrane TLR4 observed in untreated CF macrophages is likely responsible for the robust plasma membrane TIRAP–MyD88–mediated signal transduction that we observed after LPS stimulation. Increased phosphorylation of I κ B α and ERK1/2 resulted in increased production of downstream inflammatory cytokines such as IL-6 and GM-CSF. This exuberant TLR4-dependent inflammatory response is CFTR dependent, based on data showing that WT cells pretreated with a CFTR inhibitor exhibit abnormalities analogous to those in CF cells. In contrast, there was no difference in plasma membrane TLR2 at rest or after stimulation with its ligand Pam₃CSK₄. TLR2 primarily signals from the plasma membrane and does not internalize in response to stimulation. These data provide support that CFTR affects TLR4 signaling/internalization in a specific manner, perhaps due to the fact that TLR4 regulation is highly dependent on its trafficking between the plasma membrane and intracellular compartments.

We also found that after activation and internalization, TLR4 was retained in early endosomes of CF macrophages. In the CF endosomes, TLR4 engages the TRIF adaptor, as shown by the robust phosphorylation of IRF-3 and RANTES production after LPS stimulation. TLR4 retention in the endosomes could also explain the prolonged phosphorylation of I κ B α and ERK1/2 in CF macrophages as a second wave of signaling. Translocation to endosomes is associated with TLR4 dedimerization and detachment from LPS (37), which is required for the engagement of degradation adaptors (e.g., TAG) as well as TLR4 shuffling to Rab7-positive late endosomes for degradation (38). Importantly, Wang et al. (21) have shown that blocking the endosomal–lysosomal TLR4 trafficking in macrophages causes decreased TLR4 degradation and increased plasma membrane TLR4 expression, which leads to a more robust inflammatory response to LPS, similar to what we have observed in CF cells. In our studies, Rab7 fails to increase during LPS stimulation in CF macrophages. Rab7 is a crucial GTPase implicated in the delivery of receptors from early to late endosomes (28). In addition, Rab7 is a key regulatory protein for proper aggregation and fusion of late endocytic structures in the perinuclear region and consequently for the biogenesis and maintenance of the lysosomal compartment (29).

Therefore, a decreased expression of Rab7 in CF cells may account for reduced trafficking of TLR4 to the degradation pathway. Consistent with this hypothesis, we found that CF macrophages after LPS stimulation (45 min) had a minimal TLR4 translocation in LAMP-1-positive vesicles, whereas WT macrophages had robust trafficking of TLR4 to the lysosomal compartment. In addition, LAMP-1-positive vesicles were clustered in the nuclear area of WT cells, whereas the signal was more diffusely distributed in the cytoplasm of CF cells (Fig. 5C, Supplemental Fig. 2). Not surprisingly, we found that during LPS stimulation, the rate of TLR4 degradation was slower in CF cells compared with WT controls. These data suggest that, in the CF environment, there is reduced TLR4 translocation to lysosomes due to aberrant maturation of vesicles in the endosomal-lysosomal axis during LPS stimulation. Taken together, these data show that lack of functional CFTR affects TLR4 signaling and degradation and that it is related to the accumulation of active receptor in the endosomal compartments.

A potential alternative explanation for TLR4 retention in early endosomes in CF cells could be altered endosomal acidification as suggested by Saitoh and Kobayashi et al. (37, 41), who found that impaired acidification was associated with retention of TLR4 in the endosomal compartment. However, the role of CFTR in vesicle acidification is controversial (42). Multiple studies from experienced investigators have resulted in conflicting conclusions, with some reports providing evidence for such a role in airway epithelial cells (43) and macrophages (9, 44), and others finding no role for CFTR in organelle acidification (45–47). The data presented in this study examine the fate of TLR4 in a broader context than endosomal acidification and provide additional investigative lines of study that may lead to a better understanding of the relationship among CFTR, lysosome formation, and TLR4 trafficking and degradation.

It has been reported that CF epithelial cells have abnormal trafficking and accumulation of unesterified cholesterol in endolysosomes (48). Importantly, perturbation of cholesterol trafficking is associated with accumulation of TLR4 in the endosomal compartment with subsequently increased signal transduction from this cell compartment (49). Therefore, cholesterol trafficking may be one of the causes of activated TLR4 retention in the endosomes of CF macrophages.

In the current study, we demonstrate that human CF macrophages are intrinsically more responsive to LPS stimulation than HD cells, similar to our findings in murine CF macrophages. In 2004, Zaman and collaborators (8) first reported that monocytes from CF patients (and from heterozygous subjects) were more sensitive to low-dose LPS stimulation. They demonstrated that CF monocytes had elevated secretion of IL-8 and more robust activation of the MAPK pathway. More recently, Xu et al. (12) obtained similar results by silencing CFTR expression in human alveolar macrophages. To determine if the findings we observe in the murine model are similar to what is observed in CF patients, we differentiated macrophages from peripheral blood cells, which allowed us to overcome the difficulties in previous studies with low monocyte numbers and the heterogeneous immunophenotype of freshly isolated cells. We found that macrophages from CF patients respond to LPS in a manner similar to that observed in murine CF macrophages, including increased TLR4 and an hyperresponsiveness to LPS with more robust signal transduction and elevated secretion of several proinflammatory cytokines compared with healthy donor cells. During the preparation of this article, Sturges et al. (50) published a study in which the expression of TLR4 and TLR2 was assessed on peripheral blood monocytes of a cohort of 66 young children with CF and com-

pared with both children and adults without CF. TLR4 expression analyzed using flow cytometry was significantly higher in patients with CF compared with healthy controls ($p = 0.017$) and non-CF disease controls ($p = 0.025$), whereas no differences were seen in TLR2 expression (50). The similarities in the LPS response of murine and human macrophages lead us to hypothesize that the abnormal molecular mechanisms observed in CF murine macrophages can be extended to CF human cells.

In this study, we report that CF macrophages have intrinsically abnormal TLR4 signaling and trafficking. Thus, our findings further support the hypothesis that there is a primary role of immune cells in development of CF lung disease. Because inflammatory cells such as macrophages are key to an appropriate innate immune response, abnormal activity of these cells due to the lack of CFTR may be one of the upstream causes of the chronic infection that characterizes CF lung disease. This study may help to identify new molecular targets for therapeutic intervention of the CF lung disease.

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Disclosures

The authors have no financial conflicts of interest.

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