Heterozygosity for the F508del Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator Anion Channel Attenuates Influenza Severity

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Background. Seasonal and pandemic influenza are significant public health concerns. Influenza stimulates respiratory epithelial Cl[−] secretion via the cystic fibrosis transmembrane conductance regulator (CFTR). The purpose of this study was to determine the contribution of this effect to influenza pathogenesis in mice with reduced CFTR activity.

Methods. C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (HET) and wild-type (WT) controls were infected intranasally with 10 000 focus-forming units of influenza A/WSN/33 (H1N1) per mouse. Body weight, arterial O₂ saturation, and heart rate were monitored daily. Pulmonary edema and lung function parameters were derived from ratios of wet weight to dry weight and the forced-oscillation technique, respectively. Levels of cytokines and chemokines in bronchoalveolar lavage fluid were measured by enzyme-linked immunosorbent assay.

Results. Relative to WT mice, influenza virus-infected HET mice showed significantly delayed mortality, which was accompanied by attenuated hypoxemia, cardiopulmonary dysfunction, and pulmonary edema. However, viral replication and weight loss did not differ. The protective HET phenotype was correlated with exaggerated alveolar macrophage and interleukin 6 responses to infection and was abrogated by alveolar macrophage depletion, using clodronate liposomes.

Conclusions. Reduced CFTR expression modulates the innate immune response to influenza and alters disease pathogenesis. CFTR-mediated Cl[−] secretion is therefore an important host determinant of disease, and CFTR inhibition may be of therapeutic benefit in influenza.

Keywords. Influenza; cystic fibrosis; CFTR; alveolar macrophage; pulmonary edema; hypoxemia; lung function.

Seasonal influenza is the eighth leading cause of attributable annual mortality in the United States, accounting for approximately 200 000 hospitalizations and

>36 000 excess deaths per year [1]. Moreover, in the recent H1N1 swine influenza pandemic, approximately 61 million people were infected in the United States between April 2009 and April 2010, resulting in approximately 12 500 excess deaths [2]. Emergence of novel and more highly virulent influenza strains may result in pandemics with a devastating loss of life. For example, the 1918 Spanish influenza pandemic was estimated to have caused approximately 50 million deaths worldwide [3]. Influenza is therefore a significant public health concern.

Respiratory epithelial cells are the main infection target and site of replication for influenza viruses [4, 5].

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A primary function of these cells is to regulate the depth of the thin layer of fluid lining the airspaces, thereby facilitating normal gas exchange and effective mucociliary clearance. Apical epithelial sodium (Na^+) channels (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) anion channels play a central role in this process [6]. ENaC-mediated active transport of Na^+ ions from the alveolar lining fluid to the interstitial space creates a transepithelial gradient for Cl^- absorption via CFTR. CFTR also regulates ENaC activity—reduced CFTR function induces ENaC hyperactivity [7]. Trans-epithelial NaCl transport creates an osmotic gradient, driving passive movement of water from the airspace to the interstitium.

ENaC and CFTR activity are primary determinants of normal lung function. ENaC inhibition and/or stimulation of CFTR-mediated Cl^- secretion leads to accumulation of fluid in the alveolar space, impaired gas exchange, and hypoxemia [8, 9]. Patients with acute lung injury who have intact transepithelial ion transport have lower morbidity and mortality [10]. In contrast, the absence of functional CFTR and/or ENaC-mediated Na^+ hyperabsorption result in cystic fibrosis, which is characterized by decreased airspace lining fluid depth, mucus dehydration, and impaired mucociliary clearance [11, 12]. Cystic fibrosis afflicts an estimated 30 000 people in the United States and its most common cause is homozygosity for the F508del CFTR mutation (a phenylalanine deletion at position 508) [13]. Respiratory complications account for nearly 85% of cystic fibrosis-associated deaths [13].

We have shown that infection of mice with respiratory syncytial virus resulted in ENaC inhibition and moderate pulmonary edema [14, 15]. Although infection with H1N1 influenza A/WSN/33 led to a comparable reduction in ENaC-mediated Na^+ transport, influenza also stimulated CFTR-mediated Cl^- secretion by the respiratory epithelium and induced severe pulmonary edema and hypoxemia [16–18]. We therefore hypothesized that increased disease severity in influenza virus-infected mice is a consequence of CFTR stimulation. Furthermore, we speculated that presence of the F508del mutation would reduce CFTR-mediated Cl^- secretion, attenuating pulmonary edema and disease severity. We found that F508del CFTR-heterozygous (HET) C57BL/6-congenic mice infected with influenza virus had improved survival and less severe lung dysfunction than wild-type (WT) littermate controls, despite comparable viral replication and weight loss. Bronchoalveolar lavage fluid (BALF) alveolar macrophage counts and interleukin 6 (IL-6) levels were significantly increased in HET mice. Alveolar macrophage depletion with clodronate liposomes attenuated the IL-6 response and increased influenza severity to WT levels. Our findings suggest that CFTR-mediated Cl^- secretion is an important determinant of disease severity and that CFTR inhibition may be of therapeutic benefit in influenza.

METHODS

Breeding and Genotyping of F508del Mice

C57BL/6-congenic F508del CFTR-heterozygous and -homozygous mutants and WT controls were generated by breeding B6.129S7-Cftr^{tm1Kth} mice [19]. All procedures were approved by the institutional animal care and use committees at both The Ohio State University and the University of Alabama at Birmingham.

Infection of Mice

Mice aged 8–12 weeks of each genotype were infected intranasally with 10 000 focus-forming units (FFU) per mouse of egg-grown H1N1 influenza A/WSN/33 in 50 μL of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin [17, 18]. Mice were individually marked and weighed daily. Data for each experimental group were derived from a minimum of 2 independent infections.

Depletion of Alveolar Macrophages

Mice were treated intranasally with 100 μL of clodronate liposomes under light isoflurane anesthesia 48 hours prior to infection and every 72 hours thereafter [20]. Clodronate was encapsulated in phosphatidylcholine/cholesterol liposomes at approximately 5 mg/mL of liposome suspension [21]. Clodronate liposomes were a gift from Dr. Nico van Rooijen, Vrije Universiteit of Amsterdam, Amsterdam, the Netherlands. Pilot studies indicated that undiluted clodronate liposomes and those diluted 1:1 in PBS caused comparable macrophage depletion, but undiluted clodronate liposomes induced more respiratory compromise and occasional mortality. As in prior studies [20], clodronate liposomes were therefore administered in diluted form, which caused no deaths. The degree of alveolar macrophage depletion was determined by measuring total and differential cell counts in BALF.

Measurement of Lung Mechanical Properties

Mechanical properties of the mouse lung were assessed in valium/ketamine-anesthetized, tracheotomized mice, using the forced-oscillation technique [22] as in our previous studies [23]. Mice were mechanically ventilated on a flexiVent computer-controlled piston ventilator (SciReq, Montreal, Canada), with a tidal volume of 8 mL/kg and a frequency of 150 breaths/minute, against a positive end-expiratory pressure of 2–3 cm H_2O . Total lung resistance, static lung compliance, and dynamic lung compliance were calculated using the single-compartment model [22]. Maximal airway responsiveness to bronchoconstrictors was measured following exposure to increasing doses of nebulized methacholine (0.1–50 mg/mL).

Measurement of BALF Inflammatory Mediators

The total level of protein in BALF was determined by a bicinchoninic acid assay. Murine interferon γ (IFN- γ), interleukin 6

(IL-6), interleukin 10 (IL-10), and CXCL-1/KC levels were quantified by an ultrasensitive mouse proinflammatory multiplex electrochemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD). Murine interferon α (IFN- α), CXCL-10/IP-10, CCL-2/MCP-1, and CCL-5/RANTES levels were measured using Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). All assays were performed in accordance with the manufacturers' instructions.

Other Methods

Preparation of histopathologic images, BALF, and measurements of carotid arterial O₂ saturation, heart rate, lung homogenate viral titers, and ratios of lung wet weight to dry weight were performed as in our previous studies [15, 16].

Statistical Analysis

Survival data were analyzed by a log-rank (Mantel-Cox) test, using GraphPad Prism 5.04 (GraphPad Software, San Diego, CA). Descriptive statistics were calculated using InStat 3.05 (GraphPad Software). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Differences between group means were analyzed by 1-way analysis of variance, with Tukey-Kramer multiple comparison posttests. A *P* value of <.05 was considered statistically significant. All data are presented as mean $\pm$ standard error of the mean.

RESULTS

Heterozygosity for the F508del CFTR Mutation Delays Mortality Following Influenza Virus Infection

Compared with WT littermate control mice, HET mice showed significantly delayed mortality following infection with a lethal dose of H1N1 influenza virus (A/WSN/33). Median times to death increased from 7 days in WT mice to 8 days in HET mice (Figure 1A). All WT controls died by 8 days after infection, while 25% of HET mice survived. In contrast, median times to death did not differ between WT controls and congenic F508del CFTR-homozygous mice, and all homozygotes died (Figure 1B). Given the lack of impact of homozygosity on influenza-associated mortality, subsequent studies focused solely on differences between WT and HET animals.

Pulmonary Histopathology Is Attenuated in HET Mice

On day 2 after infection, moderate interstitial pneumonitis was present in both WT mice (Figure 2A) and HET mice (Figure 2D). On day 6 after infection, widespread, severe interstitial bronchoalveolar pneumonitis was present in WT animals, and alveoli contained large amounts of protein-rich edema fluid (Figure 2B and 2C). In contrast, only moderate interstitial alveolitis was evident in HET lungs 6 days after infection, and alveolar spaces contained large numbers of alveolar macrophages but no edema fluid (Figure 2E and 2F).

Figure 1. Heterozygosity for the F508del cystic fibrosis transmembrane conductance regulator (CFTR) mutation delays mortality following influenza virus infection. Effect of intranasal infection with H1N1 influenza A/WSN/33 (10 000 focus-forming units/mouse) on mortality in 16 F508del CFTR heterozygotes (HET; A) and 5 F508del CFTR homozygotes (HOM; B). [#]*P* < .0005, versus 18 wild-type C57BL/6 littermate controls (WT).

Infection With Influenza A Virus Induces an Exaggerated, Protective Alveolar Macrophage Response in the Lungs of HET Mice

Over 95% of cells in BALF from uninfected WT mice and HET mice were alveolar macrophages, and total cell counts did not differ between the 2 strains. However, BALF alveolar macrophage counts in HET mice were almost 3-fold higher than those in WT mice at 6 days after infection (Figure 3A). BALF lymphocyte and neutrophil counts also increased in both WT and HET mice 2 and 6 days after infection, but to a comparable degree in both strains at either time point (Figure 3B and 3C, respectively).

To determine whether an exaggerated alveolar macrophage response contributed to delayed mortality in influenza virus-infected HET mice, we depleted alveolar macrophages, using clodronate liposomes [21]. As in prior studies [20], intranasal clodronate liposome administration (250 μ g/mouse in 100 μ L) depleted approximately 70% of BALF alveolar macrophages in uninfected WT mice at 48 hours, without inducing any loss of body weight or other toxicity. BALF macrophage counts declined from a mean of $7.3 \pm 0.5 \times 10^4$ to $2.4 \pm 0.8 \times 10^4$ (*P* < .005; *n* = 4).

Figure 2. Pulmonary histopathology is attenuated in C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (HET). Representative parenchymal and airway histology in hematoxylin-eosin–stained lung tissues, showing pathologic effects of influenza virus infection after 2 days (original magnification $\times 10$; *A*) and 6 days (original magnification $\times 10$ [*B*] $\times 20$ [*C*]) in wild-type C57BL/6 littermate controls and after 2 days (original magnification $\times 10$; *D*) and 6 days (original magnification $\times 10$ [*E*] $\times 20$ [*F*]) in HET mice.

Figure 3. Infection with influenza A virus induces an exaggerated, protective alveolar macrophage response in the lungs of C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (HET). Effects of influenza virus infection after 2–6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on bronchoalveolar lavage fluid (BALF) alveolar macrophages (AMs; $n = 10$ –20 per group; *A*), BALF lymphocytes ($n = 10$ –20 per group; *B*), BALF neutrophils ($n = 10$ –20 per group; *C*), and mortality in untreated HET mice ($n = 16$; *D*) and CL-LIP–treated HET mice ($n = 5$; *D*). Data are presented as mean $\pm$ standard error of the mean. * $P < .05$, ** $P < .005$, # $P < .0005$, versus uninfected wild-type C57BL/6 mice (WT) mice; $^{\parallel}P < .0005$, versus WT mice at the same time point; $^{\S}P < .0005$, versus non-CL-LIP–treated HET mice 6 days after infection.

Intranasal clodronate liposome administration reduced BALF alveolar macrophage counts (and, thus, total cell numbers) 6 days after infection by 50% in WT mice and 75% in HET mice (Figure 3A). Final BALF alveolar macrophage numbers did not differ between clodronate liposome-treated WT and HET animals. Likewise, following clodronate liposome treatment, BALF neutrophil and lymphocyte numbers 6 days after infection did not differ between strains (Figure 3B and 3C, respectively).

Treatment of WT mice with clodronate liposomes did not alter the median time to death after infection ($n = 5$; data not shown). In contrast, alveolar macrophage depletion reduced the median time to death to 5.5 days in influenza virus-infected HET mice (Figure 3D). Because clodronate liposome treatment did not alter BALF neutrophil counts in HET mice but normalized macrophage counts in a fashion that also resulted in increased lethality, these data suggest that the heterozygous advantage in influenza virus-infected mice results from an exaggerated influx of alveolar macrophages into the lungs. Moreover, the lack of effect of clodronate liposomes on mortality in

WT mice indicates that the detrimental effects of this drug on survival in HET mice were not due to nonspecific toxic effects of clodronate.

Influenza Virus–Infected HET Mice Exhibit Macrophage-Dependent, Replication-Independent Amelioration of Cardiopulmonary Dysfunction

Despite the protective effect of heterozygosity for the F508del CFTR mutation on influenza-associated mortality and the reversal of this effect by alveolar macrophage depletion, the rate of postinfection weight loss did not differ between WT mice, untreated HET mice, and macrophage-depleted HET mice (Figure 4A). Carotid arterial oxygen saturation and heart rate did not differ between WT mice, untreated HET mice, and clodronate liposome-treated HET mice prior to influenza virus infection. However, untreated HET mice experienced less severe hypoxemia than WT mice and macrophage-depleted F508del mice 6 days after infection (Figure 4B). Likewise, moderate tachycardia was observed in all groups 2 days after infection, but only WT controls and macrophage-depleted HET

Figure 4. Influenza virus–infected C57BL/6-congenic mice heterozygous for the F508del cystic fibrosis transmembrane conductance regulator (CFTR) mutation (HET) exhibit macrophage-dependent, replication-independent amelioration of cardiopulmonary dysfunction. Effects of influenza virus infection after 2–6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on body weight (BWT; % change from day 0; $n = 20$ per group; A), carotid arterial oxygen saturation (S_{aO_2} ; $n = 20$ per group; B), heart rate (beats/minute [bpm]; $n = 20$ per group; C), and log viral titers in lung homogenates (log focus-forming units/g; $n = 8$ –10 per group; D). Data are presented as mean $\pm$ standard error of the mean. $**P < .005$, $^{\#}P < .0005$, versus wild-type C57BL/6 mice at the same time point; $^{\#}P < .0005$, versus non-CL-LIP-treated mice of the same genotype 6 days after infection.

mice exhibited physiologically significant bradycardia 6 days after infection (Figure 4C).

Comparable influenza virus titers were detected in lung homogenates from WT and HET mice 2–6 days after infection (Figure 4D). As in previous studies [20, 24], day 6 influenza virus titers increased by ≥ 1.5 logs in both WT mice and HET mice following clodronate liposome treatment. However, titers did not differ between clodronate liposome-treated WT controls and HET mice 6 days after infection.

Heterozygosity for the F508del CFTR Mutation Prevents Influenza Virus–Induced Pulmonary Edema in a Macrophage-Dependent Fashion

Influenza virus infection of WT mice for 2 or 6 days resulted in a progressive increase in lung water content (as measured by the ratio of wet weight to dry weight), indicating development of severe pulmonary edema (Figure 5A). By 6 days after infection, mean lung water content in both untreated and clodronate liposome-treated WT controls had increased by 160% from baseline values. In contrast, no pulmonary edema developed in influenza virus–infected HET mice, even at 6 days after infection. However, day 6 lung ratios of wet weight to dry weight increased to WT levels in alveolar macrophage-depleted HET mice.

Development of pulmonary edema in both WT mice and HET mice was accompanied by an increase in the total protein level in BALF 6 days after infection, indicative of increasing damage to the bronchoalveolar epithelial barrier (Figure 5B). However, BALF protein levels remained lower in HET mice than in WT controls at day 6. Finally, BALF protein levels 6 days after infection were not significantly affected by alveolar macrophage depletion in either strain.

Detrimental Effects of Influenza Virus Infection on Airway Resistance and Lung Compliance Are Attenuated in HET Mice

Static and dynamic lung compliance are indices of lung tissue stiffness and resistance to inflation on inspiration, which are measured at a fixed lung volume and during normal ventilation, respectively. As in previous studies [25], static and dynamic lung compliance were higher in uninfected HET mice than in WT controls, but this effect was not statistically significant (Figure 6A and 6B, respectively). Following infection, both static and dynamic compliance progressively declined in WT mice over the course of infection but remained normal in untreated, influenza virus–infected HET mice. Clodronate liposome treatment of HET mice reduced both static and dynamic compliance to WT levels 6 days after infection.

Baseline total lung resistance to airflow did not differ between uninfected WT mice and HET mice. Infection induced a progressive and significant increase in total lung resistance 2–6 days after infection in both groups, but this effect was attenuated in HET mice (Figure 6C). Alveolar macrophage

Figure 5. Heterozygosity for the F508del cystic fibrosis transmembrane conductance regulator (CFTR) mutation prevents influenza-induced pulmonary edema in a macrophage-dependent fashion. Effects of influenza virus infection after 2–6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on lung water content (ratio of wet weight to dry weight; $n=6-9$ per group; A) and bronchoalveolar lavage fluid (BALF) protein content ($\mu\text{g/mL}$; $n=6-10$ per group; B). Data are presented as mean $\pm$ standard error of the mean. ** $P < .005$, # $P < .0005$, versus uninfected wild-type C57BL/6 mice (WT) mice; $^{\dagger}P < .005$, $^{\parallel}P < .0005$, versus WT mice at the same time point; $^{\P}P < .0005$, versus non-CL-LIP-treated C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (HET) 6 days after infection.

depletion had no effect on baseline total lung resistance in WT mice 6 days after infection but induced a significant increase in HET mice. Finally, airway hyperresponsiveness to methacholine was present 2 days after infection in WT controls but not in HET mice (Figure 6D), indicating that influenza does not induce exaggerated bronchoconstrictive responses in HET mice.

BALF Cytokine and Chemokine Responses to Influenza Virus Infection Differ Significantly Between WT Mice and HET Mice

Minimal levels of inflammatory cytokines and chemokines were detectable in BALF from uninfected WT or HET mice (not shown). BALF IFN- α , IL-6, CCL-2/MCP-1, CXCL-1/KC, and CXCL-10/IP-10 levels increased significantly in WT mice 2

Figure 6. Detrimental effects of influenza virus infection on airway resistance and lung compliance are attenuated in C57BL/6-congenic mice heterozygous for the F508del CFTR mutation. Effects of influenza virus infection after 2–6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on static lung compliance (C_{ST} ; mL/cm H₂O, × 10; A), dynamic lung compliance (C_{DYN} ; mL/cm H₂O, × 10; B), baseline total lung resistance (R_{BASAL} ; cm H₂O.s/mL; C), and maximal lung resistance following nebulization of 50 mg/mL methacholine (R_{MAX} ; cm H₂O.s/mL; D). There were 7–10 values per time point for both mouse strains. Data are presented as mean ± standard error of the mean. ** P < .005, # P < .0005, versus uninfected wild-type C57BL/6 mice (WT) mice; † P < .005, ‡ P < .0005, versus WT mice at the same time point; ‡ P < .0005, versus non-CL-LIP-treated HET mice 6 days after infection.

days after infection (Table 1). However, with the exception of CCL-2, these responses were either absent or attenuated in HET mice. In both strains, BALF contained minimal amounts of IFN- γ , IL-10, and CCL-5/RANTES at this time point.

Six days after infection, IFN- α , IL-6, and CXCL-1/KC levels declined in WT mice, but all increased significantly in HET mice. The difference in BALF IL-6 levels between WT and HET mice 6 days after infection was particularly dramatic. IFN- γ , IL-10, CCL-2/MCP-1, CCL-5/RANTES, and CXCL-10/IP-10 levels increased to a comparable degree in both strains 6 days after infection. Finally, alveolar macrophage depletion attenuated cytokine responses on day 6 in both WT and HET mice but further increased the CXCL-10/IP-10 level in BALF.

DISCUSSION

Despite vaccination and use of antiviral drugs, seasonal influenza is a significant threat to public health [26]. Although the 2009 H1N1 swine influenza pandemic did not cause significant

excess mortality, the 1918 pandemic may have killed >50 million people worldwide, and there is no reason to believe that future outbreaks may be any less severe [27–29]. However, our understanding of basic mechanisms underlying the pathogenesis of severe cardiopulmonary dysfunction and lung injury during influenza remains limited [30]. In the current study, we found that influenza virus-infected HET mice had improved survival and less severe lung dysfunction than wild-type WT controls, despite comparable viral replication and weight loss. This unprecedented protective phenotype in HET mice was associated with exaggerated alveolar macrophage and IL-6 responses to infection and was lost following alveolar macrophage depletion with clodronate liposomes. Our findings show for the first time that altered anion channel activity can modulate the pathogenesis of any pulmonary viral infection of the lung. They also suggest that CFTR-mediated Cl[−] secretion is an important host determinant of influenza severity. Finally, our results suggest that CFTR inhibitors such as glibenclamide and CF_{inh}-172 may be of therapeutic benefit in influenza. However,

Table 1. Heterozygosity for the F508del Cystic Fibrosis Transmembrane Conductance Regulator Mutation Modulates Cytokine and Chemokine Responses to Influenza

Time After Infection, d	IFN- α	IFN- γ	IL-6	IL-10	CCL-2	CCL-5	CXCL-1	CXCL-10
2								
WT	460 $\pm$ 70	20 $\pm$ 3	760 $\pm$ 70	50 $\pm$ 4	760 $\pm$ 100	140 $\pm$ 20	330 $\pm$ 30	1940 $\pm$ 60
HET	50 $\pm$ 20 ^a	6 $\pm$ 3	20 $\pm$ 8 ^b	20 $\pm$ 3	630 $\pm$ 142	60 $\pm$ 20 ^c	9 $\pm$ 2 ^a	1100 $\pm$ 230 ^c
6								
WT	20 $\pm$ 2	3690 $\pm$ 840	500 $\pm$ 50	540 $\pm$ 80	2320 $\pm$ 140	320 $\pm$ 30	160 $\pm$ 20	2920 $\pm$ 240
HET	480 $\pm$ 70 ^a	3980 $\pm$ 650	5580 $\pm$ 470 ^a	480 $\pm$ 60	2350 $\pm$ 340	400 $\pm$ 30	320 $\pm$ 20 ^a	2860 $\pm$ 180
WT + CL-LIP	0	300 $\pm$ 70 ^a	240 $\pm$ 50 ^c	320 $\pm$ 80	2570 $\pm$ 380	250 $\pm$ 40	70 $\pm$ 7	5130 $\pm$ 730 ^c
HET + CL-LIP	80 $\pm$ 40	380 $\pm$ 110 ^a	1190 $\pm$ 280 ^b	240 $\pm$ 40 ^c	2220 $\pm$ 310	440 $\pm$ 70	170 $\pm$ 40	4620 $\pm$ 340 ^c

Abbreviations: HET, C57BL/6-congenic mice heterozygous for the F508del CFTR mutation; HET + CL-LIP, C57BL/6-congenic mice heterozygous for the F508del CFTR mutation following alveolar macrophage depletion with clodronate liposomes; IFN- α , interferon α ; IFN- γ , interferon γ ; IL-6, interleukin 6; IL-10, interleukin 10; WT, wild-type C57BL/6 mice; WT + CL-LIP, wild-type mice following alveolar macrophage depletion with clodronate liposomes.

^a $P < .0005$, by analysis of variance, versus WT mice at the same time point.

^b $P < .005$, by analysis of variance, versus WT mice at the same time point.

^c $P < .05$, by analysis of variance, versus WT mice at the same time point.

this possibility remains untested and may necessitate infection with an influenza virus dose that induces lower mortality in untreated WT mice and thereby maximizes the sensitivity for detecting improved outcomes in treated animals.

CFTR expressed on the apical surface of respiratory epithelial cells plays a central role in regulation of alveolar lining fluid depth. Consequently, altered CFTR activity can significantly impact normal lung function. We have shown previously that influenza virus stimulates CFTR-mediated Cl⁻ secretion, which contributes to reduced clearance of excess intra-alveolar fluid [16]. However, CFTR is also expressed on mononuclear leukocytes [31]. Multiple studies have shown that, as well as its detrimental effects on mucociliary clearance, cystic fibrosis results in altered respiratory epithelial cell and alveolar macrophage innate immune responses to bacterial pathogens [32]. These include attenuation of type I IFN production by respiratory epithelial cells and increased production of IL-6 and CXCL-1/KC by alveolar macrophages [33, 34]. We and others have shown that lung function is not adversely affected by F508del CFTR heterozygosity in either mice or humans [25]. However, there is currently very little information regarding the impact of heterozygosity for F508del CFTR on immune responses to either bacterial or viral lung pathogens. We found that baseline cell, cytokine, and chemokine levels in BALF did not differ between uninfected WT mice and HET mice, which suggests that the immune function of these cells is not intrinsically abnormal. However, both respiratory epithelial (type I IFN) and alveolar macrophage (IL-6) cytokine responses to influenza virus are altered in HET mice. Cellular mechanisms underlying these alterations have not yet been defined, and it is unclear whether they are specific to influenza virus or extend to other pulmonary pathogens: since epithelial and alveolar macrophage expression of Toll-like receptors 2 and 4 is altered in cystic

fibrosis, it is possible that HET mice might also respond differently to bacterial pathogens [35, 36]. Finally, the impact of homozygosity for F508del CFTR on influenza pathogenesis is unknown—although mortality among homozygotes did not differ from that among WT mice, this does not necessarily imply that both strains responded similarly to infection. However, given the lack of precedent in the literature regarding effects of F508del CFTR homozygosity on responses to viral infection, further studies will be necessary to address this point.

Increased mortality rates following infection with highly pathogenic influenza strains have been correlated with several factors, including a higher rate and extent of viral replication [37], a greater neutrophil response to infection [24, 38, 39], development of a cytokine storm [40–42], and an attenuated alveolar macrophage response [20, 24, 37]. Our findings suggest that the protective effect of heterozygosity for F508del CFTR during influenza is associated with the last of these factors. Attenuation of postinfection cardiopulmonary dysfunction in HET mice was not a consequence of differences in viral replication kinetics or neutrophil counts in BALF between WT mice and HET mice. Likewise, the IFN- γ response to infection did not differ between WT controls and HET mice 6 days after infection, suggesting that reduced disease severity in HET mice was not associated with a reduced cytokine storm per se. In contrast, exaggerated alveolar macrophage and IL-6 responses to infection were only present in HET mice. Moreover, alveolar macrophage depletion with clodronate liposomes both reduced IL-6 production and increased disease severity 6 days after infection in HET mice but had no such effects in WT controls. Furthermore, as in previous studies, clodronate liposome treatment increased viral titers and lung edema and reduced BALF neutrophils in both WT mice and HET mice but only altered disease progression in the latter group [20, 24].

IL-6 is a common component of the cytokine storm in influenza, but its role in pathogenesis is not fully understood: some investigators have reported an association between a high IL-6 level in BALF and increased mortality [43], while others have found that disease severity is not reduced in IL-6-knockout mice [44, 45] or even that IL-6 is protective [46]. We found a profound increase in BALF levels of IL-6 6 days after infection in HET but not WT mice, which was ablated by alveolar macrophage depletion. This enhanced IL-6 response to influenza virus in HET mice may reflect altered MAP kinase signaling in alveolar macrophages [47]. Importantly, IL-6 can stimulate ENaC activity [48]. Hence, we hypothesize that attenuation of cardiopulmonary dysfunction in influenza virus-infected HET mice results from a combination of the effects of this mutation on both bronchoalveolar epithelial and alveolar macrophage function: following infection, mutant CFTR expressed in the epithelium has a reduced capacity for Cl^- secretion and ENaC inhibition, while expression of this mutation on alveolar macrophages results in increased IL-6 production and a subsequent increase in epithelial ENaC activity. Together, these improve transepithelial ion transport, reduce the depth of the alveolar lining fluid, attenuate hypoxemia, and improve outcome. By extension, we propose that increased CFTR-mediated Cl^- secretion and inadequate alveolar macrophage and/or IL-6 responses may contribute to the greater disease severity associated with infection by highly pathogenic influenza virus strains.

Homozygosity for the recessive F508del CFTR mutation is the predominant cause of cystic fibrosis in the Western hemisphere [13]. Interestingly, the F508del CFTR mutant allele is estimated to have a carrier frequency of 2%–4% in populations of European descent [49]. The carrier incidence is significantly lower in the Middle East and India, while this allele is almost completely absent from Southeast Asia and sub-Saharan Africa. Maintenance of this allele at such high frequency among northern Europeans suggests that heterozygosity imparts a survival advantage [49]. However, the specific selection pressure underlying the continued persistence of this mutation has not been identified to date [50]. Our data suggest the intriguing possibility that a survival advantage against influenza imparted by heterozygosity for the F508del CFTR mutation may account for persistence of this allele at high levels in persons of European descent. Clearly, however, this possibility cannot be confirmed without studies in humans.

In conclusion, our results indicate that heterozygosity for the F508del mutation in CFTR attenuates influenza-induced cardiopulmonary dysfunction in mice. Moreover, they demonstrate that this effect is not associated with altered viral replication, an attenuated proinflammatory cytokine response, or a reduction in pulmonary neutrophil infiltrates but is correlated with an approximately 3-fold increase in BALF alveolar macrophages and a 10-fold greater IL-6 response to infection. Taken together, these entirely unprecedented observations show for the first

time that, as in pulmonary bacterial infections, altered CFTR function can modulate the innate immune response to, and alter the pathogenesis of, viral infections of the lung. Furthermore, they support our hypothesis that CFTR-mediated Cl^- secretion is an important determinant of influenza severity and that CFTR inhibition may be of therapeutic benefit in this disease. Finally, our findings suggest that the high carrier frequency of the F508del CFTR mutation might result from a heterozygous survival advantage against influenza.

Notes

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