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Abstract

Microbial lung infections are the major cause of morbidity and mortality in the hereditary metabolic disorder cystic fibrosis, yet the molecular mechanisms leading from the mutation of cystic fibrosis transmembrane conductance regulator (CFTR) to lung infection are still unclear. Here, we show that ceramide age-dependently accumulates in the respiratory tract of uninfected Cftr-deficient mice owing to an alkalinization of intracellular vesicles in Cftr-deficient cells. This change in pH results in an imbalance between acid sphingomyelinase (Asm) cleavage of sphingomyelin to ceramide and acid ceramidase consumption of ceramide, resulting in the higher levels of ceramide. The accumulation of ceramide causes Cftr-deficient mice to suffer from constitutive age-dependent pulmonary inflammation, death of respiratory epithelial cells, deposits of DNA in bronchi and high susceptibility to severe Pseudomonas aeruginosa infections. Partial genetic deficiency of Asm in Cftr(-/-)Smpd1(+/-) mice or pharmacological treatment of Cftr-deficient mice with the Asm blocker amitriptyline normalizes pulmonary ceramide and prevents all pathological findings, including susceptibility to infection. These data suggest inhibition of Asm as a new treatment strategy for cystic fibrosis.

Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis

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Abstract

Microbial lung infections are the major cause of morbidity and mortality in the hereditary metabolic disorder cystic fibrosis (CF), yet the molecular mechanisms leading from mutated CF Transmembrane Conductance Regulator (CFTR) to lung infection are still unclear. Here, we show that ceramide age–dependently accumulates in the respiratory tract of uninfected CF–mice due to an alkalinization of intracellular vesicles in *Cftr*–deficient cells that results in an imbalance of the activities of acid sphingomyelinase (Asm) cleaving sphingomyelin to ceramide, and acid ceramidase consuming ceramide. Accumulation of ceramide causes CF–mice to suffer from constitutive age–dependant pulmonary inflammation, death of respiratory epithelial cells, deposits of DNA in bronchi and high susceptibility to develop severe *Pseudomonas aeruginosa* infections. Partial inhibition of Asm in $Cftr^{-/-}/Smpd1^{+/-}$ mice or by pharmacological treatment of CF–mice with the Asm blocker amitriptyline normalizes pulmonary ceramide and prevents all pathological findings including susceptibility to infection. These data suggest inhibition of Asm as a novel treatment strategy in CF.

Introduction

The genetic disorder cystic fibrosis (CF), which affects approximately 80,000 individuals in Europe and North America, is caused by mutations in the CF Transmembrane Conductance Regulator (*CFTR*) gene[1–3]. Chronic microbial lung infections, most commonly caused by the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, reduce the life expectancy of CF subjects due to excessive lung tissue remodelling and destruction[4]. How mutation or absence of CFTR, which is primarily expressed in ciliated and submucosal gland epithelial cells of the respiratory tract[5,6], promotes pulmonary infections, is still incompletely understood. Several studies support the notion that CF cells and respiratory tissues exhibit a pro–inflammatory status, which may facilitate bacterial lung colonization and infection[7–10].

Here we investigated the role of sphingolipids in the pathogenesis of CF. CFTR belongs to the ATP binding cassette transporter family, which has been previously demonstrated to be involved in lipid transport[11,12]. Furthermore, studies indicate that defective CFTR leads to higher pH levels in intracellular organelles[13,14], which might be important for the regulation of the cellular sphingolipid metabolism by enzymes with an activity peak at acidic pH values. Since sphingolipids are critically involved in the regulation of cell survival[15,16], NFκB activation[17] and expression of pro–inflammatory cytokines[18], we hypothesized that an alteration of the sphingolipid metabolism may play an important role in the pathogenesis of CF lung disease.

We demonstrate that deficiency of *Cftr* results in an accumulation of cellular ceramide in the respiratory tract of uninfected CF–mice. The accumulation was caused by

the alkalinization of Cftr–deficient vesicles in respiratory cells leading to an imbalance of the activities of the acid sphingomyelinase (Asm) and acid ceramidase. A similar accumulation of ceramide was detected in respiratory epithelial cells and airways of CF subjects. In CF–mice, the increased sphingolipid concentrations triggered chronic pulmonary inflammation, death of respiratory epithelial cells and deposition of DNA in bronchi, and resulted in high susceptibility to severe pulmonary *P. aeruginosa* infections. Normalization of pulmonary ceramide levels that was achieved by pharmacological and genetic blockade of Asm, prevented all pathological findings and, most importantly, protected CF–mice from severe pulmonary *P. aeruginosa* infections.

Results

Cftr–deficiency and ceramide accumulation

To test the hypothesis that deficiency of functional Cftr results in alterations of the sphingolipid metabolism in the respiratory tract, we determined the concentration of ceramide in the lung of two wild type mouse strains and in the mouse strains $Cftr^{tm1Unc}$–$Tg^{(FABPCFTR)}$ (abbreviated $Cftr^{KO}$) and B6.129P2(CF/3)–C$ftr^{TgH(neoim)Hgu}$ (abbreviated $Cftr^{MHH}$, syngenic to C57BL/6). These mice lack functional *Cftr* in the respiratory tract (collectively named CF–mice). Our results demonstrated a significant increase of ceramide in lungs of *Cftr*-deficient mice compared to control animals (**Fig. 1a**). Notably, this increase was age–dependent, whereas ceramide levels did not differ with age in wild type mice.

To identify cells accumulating ceramide in $Cftr^{KO}$ and $Cftr^{MHH}$ mice, we stained isolated bronchial epithelial cells with two different monoclonal antibodies to ceramide. Excessive ceramide accumulation was observed in ciliated respiratory cells of CF–mice, but not wild–type mice (**Fig. 1b**). To further locate ceramide over–expressing cells, we stained paraffin sections of lungs from wild–type, $Cftr^{KO}$ and $Cftr^{MHH}$ mice for ceramide. Ceramide accumulation was observed in the respiratory tract epithelium and the submucosa of CF–mice (**Fig. 1c**). Control stainings with irrelevant Cy3–coupled antibodies were negative (not shown). Alveolar epithelial cells that constitute the majority of cells in the lung, did not contain substantial amounts of ceramide, neither in wild–type nor in $Cftr^{KO}$ and $Cftr^{MHH}$ mice (**Fig. 1c**). Concurrent with the presence of Cftr

in macrophages[14], we detected a 6–fold increase of ceramide in mouse lung macrophages obtained by pulmonary lavage (data not shown).

To address the topology of ceramide in respiratory epithelial cells, we performed confocal microscopy. These studies indicated a marked accumulation of ceramide–containing vesicles in respiratory epithelial cells of *Cftr*–deficient mice, and also the accumulation of ceramide in other cell membranes (**Fig. 1d**). Fluorescence activated cell sorting (FACS) data confirmed the accumulation of ceramide–containing vesicles in lung extracts from *Cftr*–deficient mice (**Fig. 1e**) as indicated by the increased percentage of ceramide–positive vesicles. In addition, we detected an increase of the absolute ceramide amount in these vesicles as indicated by the increased Cy3–signal that measures binding of the Cy3–labelled antibodies to ceramide in the FACS studies. The FACS data demonstrated that the accumulation of ceramide–containing lysosomes already occured in young, 12 week old *Cftr*–deficient mice, but to a lower degree than in older mice. Analysis of samples from $Cftr^{KO}$ revealed the same results.

To further support the accumulation of ceramide in *Cftr*–deficient cells and to exactly localize ceramide, we performed immunogold electron microscopy on lung sections from wild–type and *Cftr*–deficient mice. Ceramide predominantly located to intracellular vesicles in *Cftr*–deficient respiratory epithelial cells versus normal control cells (**Fig. 1f**). The accumulation of ceramide was not restricted to cathepsin D positive lysosomes, but also observed in other vesicles.

To test whether these findings in CF mouse strains are also relevant for CF subjects, we isolated nasal respiratory epithelial cells from adult CF subjects and healthy individuals and stained the cells for ceramide. Similarly, lung transplant material from

three CF subjects and lung material from three normal donors was stained. The results showed an accumulation of ceramide in the membrane of nasal epithelial cells (**Fig. 1g**), respiratory epithelial cells and submucosal glands (**Fig. 1h**) compared to the respective cells or tissues from healthy individuals. Quantification of the fluorescence in epithelial cells from 17 healthy and 18 CF individuals revealed a 4 ± 0.4–fold increase of the ceramide signal in cells from CF subjects ($P < 0.01$, t–test).

Defective acidification and ceramide accumulation

Recent studies showed that CFTR–deficiency in alveolar macrophages results in a lysosomal pH shift from pH 4.5 to at least pH 5.9[14]. Other studies demonstrate that an increase of the pH to 5.9 reduces the Asm activity by only ~35%, while the activity of the acid ceramidase is reduced by more than 90% at this pH, in some respects mimicking Farber disease, which is caused by a deficiency of the acid ceramidase and results in an accumulation of ceramide[19,20]. Furthermore, at a pH of 5.9, the acid ceramidase exhibits a reverse activity producing ceramide, instead of consuming it[19]. The imbalance of the Asm and the Ac and the reverse activity of the acid ceramidase at a vesicular pH of 5.9 should result in a net accumulation of ceramide in an *in vivo* situation.

To investigate this hypothesis, we determined the pH in cellular vesicles of respiratory epithelial cells from wild–type and 24–week old CF–mice. Measurements of the vesicular pH in *Cftr*–deficient respiratory epithelial cells employing lysosensor–green revealed an increase of the pH in these vesicles to a value of 5.9 (**Fig. 2a**). This increase was mimicked by adding the Cftr–inhibitor 172 to wild–type cells (**Fig. 2a**).

Next, we confirmed the pH dependency of Asm and acid ceramidase activity in mouse lung preparations (**Fig. 2b**) as well as the reverse activity of the acid ceramidase at ph 4.5, 5.0 and pH 5.9 (**Fig. 2b**). The data showed an almost complete inhibition of the Ac at pH 5.9. In fact, at this pH, the enzyme showed a reverse activity producing ceramide. In contrast, the activity of the Asm was reduced by only 35% at pH 5.9.

To show that an inhibition of the acid ceramidase results in cellular ceramide accumulation, we inhibited the acid ceramidase in respiratory epithelial cells of wild type mice *in vivo* by inhalation of n–oleoylethanolamine. Alternatively, intracellular vesicles were alkalinized by inhalation of bafilomycin, nigericin, chloroquin or NH_4Cl. The results confirmed that direct inhibition of the acid ceramidase or alkalinization of lysosomes increase concentrations of ceramide in wild–type respiratory cells (**Fig. 2c**).

To directly demonstrate a critical role of the lysosomal pH for the accumulation of ceramide in *Cftr*–deficient cells, we acidified isolated vesicles from *Cftr*–deficient lung cells *in vitro.* In the FACS studies on isolated vesicles from *Cftr*–deficient lung cells that were either left at a pH of 5.9 or acidified to pH 4.5 (lower panels in Fig. 2d) the intensity of the Cy3–coupled antibodies to ceramide staining served to measure the amount of ceramide in a single vesicle. The data demonstrated that acidification of vesicles isolated from *Cftr*–deficient cells reduced the vesicular ceramide amount (**Fig. 2d**). Vice versa, alkalinisation of vesicles isolated from wild–type cells from pH 4.5 to pH 5.9 resulted in ceramide–accumulation.

Previous studies demonstrated that Cftr also functions as a direct transmembrane transporter or regulator of the transmembrane transport of sphingosine–1–phosphate[12]. However, we did not detect a significant increase of sphingosine–1–phosphate levels in

CF–mice. Sphingosine was increased in lungs of CF–mice, but the total sphingosine amounts were 0.5 ‰ of those of ceramide suggesting that a defect in uptake of sphingosine–1–phosphate via Cftr might not be the major cause for ceramide accumulation in *Cftr*–deficient cells (for further details see **supplementary note 1** online).

Inhibition of the Asm normalizes ceramide

Ceramide is generated in cellular membranes from sphingomyelin by the constitutive activity of Asm or by de–novo synthesis[21–23]. The data described above indicate that the accumulation of ceramide within the lung of *Cftr*–deficient mice is caused by an imbalance between Asm and acid ceramidase activities. Thus, the Asm might be a potential pharmacological target to manipulate the sphingolipid metabolism in CF–mice. We, therefore, tested whether inhibition of Asm normalizes the increased ceramide concentration in the respiratory tract of $Cftr^{KO}$ and $Cftr^{MHH}$ mice. Partial inhibition of Asm was achieved by intraperitoneal injection of amitriptyline (10 mg/kg twice daily for 2.5 d), which induces a proteolytic degradation of Asm[24] or by crossing $Cftr^{KO}$ mice with Asm knock–out mice ($Smpd1^{-/-}$) to obtain mice deficient for Cftr and heterozygous for Asm ($Cftr^{KO}/Smpd1^{+/-}$) (**Fig. 2e**). Biochemical assays and immunofluorescence studies on respiratory epithelial cells from $Cftr^{KO}$, $Cftr^{MHH}$ and $Cftr^{KO}/Smpd1^{+/-}$ mice revealed that amitriptyline–mediated degradation or heterozygosity of Asm almost normalized pulmonary ceramide levels in these mice (**Fig. 2f,g**).

Amitriptyline has been recently shown to result in degradation of the acid ceramidase in addition to the Asm[25]. Our data indicated that amitriptyline in fact also reduced the

already very low acid ceramidase activity at pH 5.9, but also the ceramide–producing reverse activity of the acid ceramidase at this pH (**Fig. 2e**). Thus, inhibition of ceramide generation via Asm and acid ceramidase was the predominant effect of amitriptyline at pH 5.9 resulting in net–reduction of cellular ceramide. Since the specific activity of the Asm is approximately 40–fold higher than the reverse activity of the Ac and 250–fold higher than the ceramide–consuming activity of the Ac at a pH of 5.9, we assumed the Asm as the primary target of amitriptyline. This is also consistent with the observation that heterozygosity of the Asm, which does not affect acid ceramidase expression, also reduced ceramide levels in CF mouse lungs.

Normal ceramide prevents *P. aeruginosa* infection

To test the significance of these findings for bacterial lung infections in CF, we determined the susceptibility of wild–type, $Cftr^{KO}$ and $Cftr^{MHH}$ mice to develop pulmonary *P. aeruginosa* infections. Increased susceptibility of CF mice for *P. aeruginosa* lung infection has been reported previously[26,27]. While wild–type mouse lungs contained only ~10^3 CFU of *P. aeruginosa* per 100 mg of homogenized lung tissue 2 h after inoculation, $Cftr^{KO}$ and $Cftr^{MHH}$ mice were significantly more susceptible to infection, revealing ~10^6 CFU of *P. aeruginosa* strain 762/100 mg lung tissue (**Fig. 3a**). Pharmacological or genetic inhibition of Asm protected $Cftr^{KO}$ and $Cftr^{MHH}$ mice from pulmonary *P. aeruginosa* infections. Amitriptyline–treated $Cftr^{KO}$ or $Cftr^{MHH}$ mice or $Cftr^{KO}/Smpd1^{+/-}$ mice, respectively, displayed substantially less *P. aeruginosa* CFU in the lung than the respective CF–mouse strains 2 h after intranasal inoculation with 10^8 CFU of *P. aeruginosa* 762 (**Fig. 3a**). Similar data were obtained after infection of $Cftr^{KO}$ or

Cftr^{MHH} mice with *P. aeruginosa* strains 769 and ATCC 14115 (**Fig. 3b**). The susceptibility of *Cftr*^{KO} and *Cftr*^{MHH} mice to develop pulmonary *P. aeruginosa* infections increased with the age of the mice (**Fig. 3a**), corresponding to the age–dependent increase of pulmonary ceramide concentrations. A slightly increased susceptibility to bacterial infection was already detectable in CF animals at an age of 8 and 12 weeks (**Fig. 3a**) although the susceptibility of younger mice was much lower than in mice with 16 and 24 weeks of age consistent with the lower accumulation of ceramide in young mice lungs.

Control experiments revealed that when mice were intranasally challenged with 10^8 CFU of *P. aeruginosa*, almost all bacteria could be recovered from the noses of the mice 2 min after application, while the lungs were still free of pathogens (**Fig. 3c**). There was no difference in recovered CFUs between wild–type and CF–mice at this early time point. In the early phase, i.e. 15, 30 and 60 min after infection, *P. aeruginosa* migrated to the lower airways and bacterial numbers increased in the lungs of all mice to a similar degree (**Fig. 3c**). After approximately 60 min wild–type mice started to eliminate the bacteria, whereas in CF–mice bacteria started to grow exponentially. Infection of the mice with only 10^7 CFU resulted in approximately lower absolute numbers in the lung 2 h after the infection, revealing again a marked difference in CFUs between wild–type and CF–mice as observed for the higher infections dose (**Fig. 3c**).

We also challenged the lungs of *Cftr*–deficient and wild–type mice with *Streptococcus pneumoniae*, a pathogen, which rarely infects CF subjects. The data revealed no difference between the pulmonary infection of wild–type and CF–mice with *S. pneumoniae* (not shown).

Next, we explored the relation of *P. aeruginosa* lung colonization rates to mouse mortality. The analysis of the 7 d survival rates demonstrated that initially high *P. aeruginosa* CFU correlated with mortality, which was significantly reduced ($P < 0.05$) by pharmacological or genetic heterozygosity of Asm (**Fig. 3d**).

Accumulated ceramide causes inflammation

Next, we aimed to identify mechanisms how accumulated ceramide mediates the hypersusceptibility of CF–mice to develop *P. aeruginosa* infections. To this end we tested typical immunological changes in the respiratory tract that are known to increase the susceptibility to *P. aeruginosa* and/or have been demonstrated to be altered in CF cells or lung tissues[7–10]. $Cftr^{KO}$ or $Cftr^{MHH}$ mice exhibited a constitutive increase of interleukin (IL)–1 and keratinocyte–derived chemokine (KC), the mouse homologue of human IL–8, in their lungs (**Fig. 4a**). Genetic or pharmacological inhibition of Asm normalized levels of pulmonary IL–1 and KC/IL–8 in CF mice (**Fig. 4a**), suggesting that cytokine up–regulation is linked to ceramide accumulation in CF–mice. Of note, the mice, held in a pathogen–free environment, were free of any infection as defined by repeated microbial cultures of lung homogenates and serological testing for multiple microbial species.

In addition to the age–dependent increases in ceramide, IL–1 and IL–8 expression, lung tissues of CF–mice exhibited an age–dependent increase of macrophage and neutrophil cell numbers that was corrected by genetic or pharmacological inhibition of Asm (**Fig. 4b,c**). Compared to wild–type, amitriptyline-treated or $Cftr^{KO}/Smpd1^{+/-}$ mice, $Cftr^{KO}$ and $Cftr^{MHH}$ mice exhibited significantly increased macrophage (**Fig. 4b**) and

neutrophil (**Fig. 4c**) cell counts in the lung at an age of 30 and 52 weeks (*$P < 0.05$, ** $P < 0.01$). The high macrophage and neutrophil cell numbers were normalized in $Cftr^{KO}/Smpd1^{+/-}$ mice and upon treatment of $Cftr^{KO}$ mice with amitriptyline (**Fig. 4b,c**). In $Cftr^{KO}$ mice, macrophages clustered around mouse submucosal glands (**Fig. 4d**), the principal site of CFTR expression in humans[6]. Macrophages (**Fig. 4e**) and neutrophils (**Fig. 4f**) were also present in high numbers in the bronchial associated lymphatic tissue, while only marginal numbers of macrophages and neutrophils were present in the lungs of wild–type mice.

Accumulated ceramide, cell death and deposition of DNA

Besides its impact on cytokine expression and inflammatory cell recruitment, ceramide has previously been shown to be critically involved in the induction of cell death[15,16]. Therefore, we tested whether CF–mice display an increased death rate of respiratory epithelial cells and whether this contributes to the hypersusceptibility of CF–mice to develop *P. aeruginosa* infections.

Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick–end labeling (TUNEL) staining of lung sections of $Cftr^{KO}$ or $Cftr^{MHH}$ mice revealed a higher number of dead cells, dispersed in the respiratory mucosa of large and medium–sized bronchi when compared to wild type mice, in which cell death was rarely observed (**Fig. 5a,b**). The rate of respiratory epithelial cell death was increased in CF–mice in an age–dependent manner starting with a few dead cells in the respiratory tract in 12 week old mice (**Fig. 5b**). The increased death rate of respiratory epithelial cells in $Cftr^{KO}$ or $Cftr^{MHH}$ mice was normalized by pharmacological or genetic inhibition of Asm (**Fig.**

5a,b) or application of the broad–spectrum caspase inhibitor benzyloxycarbonyl-valin-alanin-aspartat-fluoromethylketon (zVAD) that has been previously shown to block apoptosis *in vivo*[28] (**Fig. 5a,b**).

Next, we investigated whether increased cell death, caused by the accumulation of ceramide in nasal and bronchial epithelial cells, resulted in DNA deposits on the respiratory epithelium. DNA deposits were detectable on the mucosal lung surface in 12%–17% of bronchi in $Cftr^{KO}$ and $Cftr^{MHH}$ mice (**Fig. 5c,d**), compared to maximal 2% DNA deposits in wild type mice. Young, 12–week old mice displayed a small, but distinct increase of DNA deposits in bronchi (**Fig. 5d**). To test whether the increased cell death rate in CF–mice was linked to deposition of DNA in the airways of these mice, we treated $Cftr^{KO}$ and $Cftr^{MHH}$ mice with zVAD. ZVAD inhibited the formation of DNA deposits within airway mucus in CF–mice (**Fig. 5c,d**). Notably, pharmacological inhibition of Asm by amitriptyline, genetic heterozygosity of *Smpd1* or inhalation of recombinant DNase also abrogated DNA deposition on the mucosal lung surface (**Fig. 5c,d**).

DNA deposits, *P. aeruginosa* adhesion and infection

To investigate whether DNA deposits on the respiratory epithelium of CF–mice mediate adherence of *P. aeruginosa*, we distributed DNA on a respiratory cell line, which led to an increased adherence of *P. aeruginosa* along DNA fibers (**Fig. 6a**) indicating the importance of DNA for bacterial adhesion. Therefore, we treated both $Cftr^{KO}$ and $Cftr^{MHH}$ mice with either recombinant human DNase (rhDNase) or with zVAD prior to bacterial challenge. Both, rhDNase or zVAD treatment effectively reduced *P. aeruginosa* cell

numbers in lungs of *Cftr*^{KO} or *Cftr*^{MHH} mice (**Fig. 6b**), suggesting that DNA deposits derived from dead epithelial cells facilitate *P. aeruginosa* adhesion and infection. Neither rhDNase nor zVAD changed pulmonary ceramide levels (not shown).

Accumulation of ceramide in CF–mice is reduced by a diet

A recent study[29] shows a decrease of ceramide in the lungs of *Cftr* knock out mice compared to their littermate controls, which seems to be inconsistent with our data. However, these studies employed mice that are completely deficient for *Cftr.* Mice completely lacking *Cftr* in the intestinum usually die after birth due to intestinal obstruction and therefore require a special liquid diet, for instance with Peptamen^{R} [29]. We demonstrate that this diet reduced ceramide levels and inhibited the Asm by a ~300% increase of cholesterol in the lungs of CF mice (**Supplementary Fig. 1a-d** online), effects that are consistent with the previously published interference of cholesterol with Asm-activity[30] and were prevented by treatment with simvastatin[31].

Discussion

In the present study we demonstrate an age–dependent hypersusceptibility of CF–mice to *P. aeruginosa* infection that is caused by increased cellular ceramide levels in the lungs of CF mice. Inhibition of Asm and/or acid ceramidase by treatment of $Cftr^{KO}$ or $Cftr^{MHH}$ mice with amitriptyline or by generation of $Cftr^{KO}/Smpd1^{+/-}$ mice normalized pulmonary ceramide levels, prevented pulmonary *P. aeruginosa* infections and increased survival of infected animals.

According to current models, sphingomyelin is constitutively metabolized to ceramide by Asm and further degraded to sphingosine by acid ceramidase [32]. This process is thought to be located predominantly in lysosomes but also in other membranes such as the plasma membrane. Our data indicate that acidification of intracellular vesicles mediated by Cftr presumably through the provision of counter ions to permit higher lumenal H^+ concentrations[14,33,34] is critical for the concerted regulation of ceramide by the activities of Asm and acid ceramidase. The alkalinization of Cftr–deficient vesicles in respiratory cells of CF mice to a pH of 5.9, results in an imbalance of the Asm and Ac activities with a reduced consumption of ceramide and, finally, a net accumulation of ceramide. Our FACS, confocal and electron microscopy studies indicate an increase in the number of ceramide–containing vesicles and increased ceramide concentrations within those vesicles and in the cell membranes of *Cftr*–deficient cells, predominantly in cells from older mice.

Two previous reports support our notion of defective acidification as a result of diminished Cl^- conductance in other CF cell types. These studies demonstrated defective

acidification in the Golgi/trans–Golgi network, prelysosomes and endosomes of CF–cell lines leading to abnormal glycosylation of membrane proteins[13], and showed the same phenomenon in phagolysosomes of alveolar macrophages from *Cftr*–null mice leading to impaired bactericidal activity[14]. These results have been questioned.[35,36] Methodological differences, particularly concerning the thiazolidinone CFTR inhibitor $CFTR_{inh}$–172, which is very sensitive to storage in stock DMSO solutions at low temperature, may account for some of the discrepant results[14,36]. Independent of this controversy, it is difficult to compare data employing zymosan conjugates containing fluorescein and TMR in macrophages or cultured cells, [36] with our data employing lysosensor green on ex vivo epithelial cells.

A decrease of ceramide levels rather than an accumulation of ceramide was observed by other investigators in CF–mice, fed with the liquid diet Peptamen^{R} [29]. Here we demonstrate that Peptamen^{R} treatment results in a very high cellular concentration of cholesterol that in turn results in reduction of Asm activity and pulmonary ceramide concentrations. CF–mice might be in particular sensitive to changes in the cholesterol metabolism[37], since cultured *Cftr*–deficient cells already display 1.5–fold higher cholesterol levels than normal cells. These levels are too low to alter the activity of the Asm as demonstrated in the present study, however when CF mice are fed the Peptamen^{R} diet, cholesterol concentrations increase to such levels that Asm is severely affected. The increase in cholesterol concentrations is due to an impressive consumption of the diet by the mice (~75% of body weight in a 24–hour cycle) leading to liver steatosis[38,39]. It is obvious that such a consumption is hard to achieve in humans, with the result that cholesterol levels will not increase in humans and, hence, Asm levels will not be

changed. Further, any diet that requires a 300-400% increase of cellular cholesterol concentrations to be effective can not be used in humans due to the severe adverse effects of high cholesterol.

According to our data, showing a marked shift in ceramide and cholesterol levels, Peptamen^{R} may have also been responsible for the observed essential fatty acid imbalance in CF–mice[40]. This notion is supported by studies showing that rats, fed diets containing cholesterol, significantly increase the cellular level of arachidonic acid, and the high arachidonic acid levels in hypercholesterolemic subjects, revealing high cellular cholesterol relative to the protein content[41,42].

We identified two mutually non–exclusive pathways, which may facilitate bacterial lung infection in CF as a consequence of abnormal ceramide accumulation: (i) the increased release of dead cells into respiratory airways, resulting in the formation of DNA deposits, which may serve as bacterial adherence factors, and, (ii) an increased inflammatory response. The latter may negatively affect lung function, innate immune responses and change the airway architecture to allow enhanced bacterial adhesion, but also lung fibrosis.

While the link between ceramide and the induction of cell death has been extensively documented[15,16,43], conflicting results with regard to the rate of apoptosis in *Cftr*–deficient cells and mice prior[44] and after[45] *P. aeruginosa* infection have been previously described. Here, we show that an increased rate of cell death occurs only in the lungs of aged CF–mice, which was never investigated before. Increase of cell death is most probably caused in cells lacking functional Cftr by accumulated ceramide, since

Asm inhibition by amitriptyline or Asm heterozygosity in uninfected CF–mice reduce cell death to rates observed in wild type mice.

The increased rate of cell death causes DNA deposition on the airway epithelium of CF–mice. DNA/mucus deposits may facilitate adhesion of *P. aeruginosa* to abiotic surfaces[46], an event critical for the high susceptibility of CF–mice to develop *P. aeruginosa* infections. Accumulated ceramide per se does not seem to promote adhesion, since our experiments demonstrate that the adhesion of *P. aeruginosa* strains to washed nasal epithelial cells cultures from CF subjects and control individuals do not differ (unpublished data).

Furthermore, it is conceivable that the accumulation of ceramide might severely impact the function of membrane rafts that were shown to be required for cell activation upon *P. aeruginosa* lung infections[47]. Curiously, feeding CF–mice with Peptamen^{R} may also induce hypersusceptibility to infection. While ceramide selectively displaces cholesterol from ordered lipid rafts,[48] high cholesterol levels may turn this reaction in the other direction, resulting in ceramide–poor cholesterol–rich rafts. Indeed, a significant improvement of *Cftr*–deficient mice to combat *P. aeruginosa* infection was reported when ceramide levels were normalized by treatment with Fenretinide[29].

Besides cell death, ceramide is also critically involved in the regulation of transcription factors[17], which could subsequently lead to the release of cytokines and induce inflammation with age–dependent recruitment of effector cells in uninfected CF–mice. How the accumulation of macrophages and neutrophils around submucosal glands contributes to the hypersusceptibility of CF–mice to *P. aeruginosa* lung infection remains to be defined. Both cell types have been implied in tissue remodeling mediated by the

production of reactive oxygen species, serine and metallo–proteases[49], and their continuous exposure may affect submucosal gland structure and antimicrobial, anti–inflammatory and anti–oxidant gland function and finally provoke fibrosis and emphysema[49].

As direct or indirect consequences of the increased ceramide concentrations in cells of the respiratory tract, our results unify several previously published observations in CF mouse strains with regard to inflammation[9], apoptosis[44,45] and hypersusceptibility to *P. aeruginosa* infection[26,27]. Furthermore, our findings emphasize that older animals are hypersusceptible to severe pulmonary disease, an observation in accordance with previous studies[26].

Our results strongly suggest that increased ceramide concentrations are deleterious for CF subjects and that reduction of such levels by amitriptyline might be beneficial. However, it is important to note that ceramide concentrations should not be reduced under a critical cellular level by a future drug to treat CF, which would impair the biological functions of ceramide. For example, we have previously shown that mice completely lacking Asm (*Smpd1*$^{-/-}$) fail to adequately respond to acute infection with *P. aeruginosa*[18]. These mice are unable to clear bacteria from airways by epithelial cell internalization via ceramide–dependent rafts and subsequent degradation and also fail to control the infection[18]. Previous studies demonstrated that 5–15% residual activity of the Asm are sufficient to permit a normal response of epithelial cells to *P. aeruginosa* infection. Therefore, we employed in the present study mice heterozygous for the Asm. Thus, Asm–heterozygosity and treatment with amitriptyline in doses used here do not affect the ability of epithelial cells to adequately react to acute *P. aeruginosa* infections,

but they are sufficient to normalize ceramide levels in CF–mice. Consequently, a future drug, targeting ceramide to treat CF, should be carefully titrated to normalize ceramide levels in CF–lungs. A complete suppression of ceramide would paralyse early defense mechanisms of the host. This situation is reminiscent for many highly effective drugs, for instance anti–coagulants: given at doses that moderately suppress coagulation, these drugs may prevent heart and brain infarcts, while doses that result in a stronger blockade of coagulation may result in life threatening bleeding.

In CF subjects expressing normal levels of Asm, the described pathophysiological events may provoke a vicious cycle, since *P. aeruginosa* triggers Asm activation, and, hence, ceramide release leading to apoptosis[18]. Thus, the accumulation of ceramide on the respiratory epithelium, caused by the basic defect in CF, may still increase after the onset of chronic *P. aeruginosa* infection. In such a scenario, the increasing deposition of DNA (as a consequence of increased apoptosis) would further facilitate bacterial adhesion, impair mucociliary clearance by increasing the viscoelasticity of the airway surface liquid, and augment tissue destruction and remodeling by increasing inflammation.

In summary, our data identify ceramide as one of the key regulators of inflammation and subsequent infection in CF airways. Normalization of ceramide levels by amitriptyline represents a novel strategy to prevent bacterial infections in CF subjects.

Methods

Human subjects. We isolated ciliated epithelial cells from 18 CF subjects and 17 healthy control individuals (mean age 27 ± 7.8 years and 33 ± 6.5, respectively), attending the Eberhard–Karls–Universität, Universitätsklinikum, Tübingen, Germany. The study was approved by the ethic committee of the University of Tübingen, Tübingen, Germany. We obtained samples of normal lungs from three succumbed donors (mean age 26 years) in which the contralateral lung was not utilized for transplantation, and samples from the explanted lungs from three CF subjects receiving a double lung transplant, from the Deptartment of Pathology, University of Southern California, Los Angeles, USA, in compliance with the University of Southern California Institutional Review Board, Los Angeles, USA. We fixed bronchial tissue blocks (~ 1 cm^3) in 10% paraformaldehyde, and embedded them in paraffin.

Mice and treatments. For various treatment regimens, infection protocols and lung tissue investigations, different *Cftr* mutant mouse strains and their respective littermates were used (detailed methods in **supplementary note 2** online). Amitriptyline was dissolved in distilled H_2O and injected i.p. into mice at a dose of 10 mg/kg twice daily for 2.5 d. ZVAD was injected i.p. at a dose of 8 mg/kg twice daily for 2.5 d. We fed mice if indicated with Peptamen^R ad libitum. A single mouse consumed approximately 17–20 ml of the liquid diet daily, which is in accordance with previous reports[38]. In other experiments, mice inhaled 1 ml of the drugs Bafilomycin (1 μM), Nigericin (2.5 μM), NH_4Cl (25 mM), chloroquine (10 μM), n–oleoylethanolamine (100 μM) and DNase (2.5

μg/ml, 25 μg/ml, 100 μg/ml and 1 mg/ml). Drugs were dissolved in 0.9% NaCl and inhaled for approximately 20 min. Furthermore, mice were intranasally infected with 10^7 or 10^8 CFU of *P. aeruginosa* strains 762, 769 and ATCC 14115 or *S. pneumoniae* strain R6, employing a plastic–covered 30–gauge needle, which was inserted 2 mm into the nose with very short and light ether anesthesia[50]. *P. aeruginosa* cell numbers were determined in mouse lungs 15, 30, 60 or 120 min after challenge. Additionally, the survival of infected mice was determined for seven days after challenge. To reduce the variation associated with *in vivo* infectious experiments, we employed a standardized protocol (**supplementary note 2** online). All procedures performed on mice were approved by the Animal Care and Use Committee of the Bezirksregierung Duesseldorf, Duesseldorf, Germany.

Measurements of enzymes, ceramide, cell death, neutrophils, macrophages and DNA. We measured enzyme activities of Asm, acid cermidase and concentrations of ceramide and cholesterol in homogenized lungs from different mouse strains. Mouse lungs were homogenized in PBS in a dounce homogenisator for 30 sec. Additionally, we stained human or mouse lung sections for cell death using TUNEL, for ceramide using indirect immunofluorescence and electron microscopy, for neutrophils and macrophages using indirect immunofluorescence and for DNA. Finally, we determined concentrations of the cytokines IL–1 and KC in aliquots of homogenized mouse lungs using commercial ELISA assays, following the instructions of the manufacturer (R&D) and determined the adhesions of *P. aeruginosa* to DNA (**supplementary note 2** online).

Isolation of cellular vesicles. We mechanically homogenized mouse lung tissue and incubated it for 30 min at 4 ^{o}C in 0.3 M sucrose, 10 mM TES, pH 7.4, and 0.5 mM EGTA to swell the cells. We disintegrated cells by 40x Dounce–homogenization, pelleted nuclei and unbroken cells by centrifugation for 5 min at 600 x g and 4 ^{o}C, and permeabilized the vesicles by 5 min incubation with 0.05% Triton X–100. Then we washed and stained vesicles with antibodies to ceramide (1:100) for 45 min at 4 ^{o}C, followed by staining with Cy3–coupled anti–mouse IgM antibodies and analysis by FACS.

To investigate the role of the pH for ceramide accumulation, we isolated vesicles as above and incubated intact vesicles in 50 mM PIPES–KOH, 40 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 μg/ml each aprotinin and leupeptin, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, 50 μg/ml creatine kinase and 1% fetal calf serum at a pH of 4.5 or 5.9. Then we incubated vesicles at 37 ^{o}C for 8 h, and analysed vesicles for ceramide by the DAG kinase assay (**supplementary note 2** online). Alternatively, we fixed vesicles for 15 min with 2% paraformaldehyde, permeabilized them with 0.05% Triton X–100, stained them with Cy3–coupled antibodies to ceramide and analysed them by FACS. The intensity of the staining with Cy3–coupled ceramide-specific antibodies served to measure the amount of ceramide in a single vesicle.

pH measurements. We incubated isolated respiratory epithelial cells from CF– and wild–type mice with 70 nM lysosensor–green D189 (Molecular Probes) for 15 min and immediately analyzed dye emission by fluorescence microscopy. To isolate intact epithelial cells, large bronchi and the trachea were removed, and cells were gently removed from the tissue surface. Typically, a total of 500 cells (100 epithelial cells from

five different mice) were analysed. To determine the pH, the fluorescence intensity in vesicles was determined by measuring the fluorescence intensity in areas of 1.5 x 1.5 mm. Samples were located with minimum light exposure. Images were captured with excitation at 488 nm employing an argon laser and were recorded at 505 nm emission. The measured fluorescence intensity was compared with a standard curve. To obtain the standard curve, we permeabilized cells and resuspended them in H/S with a given pH of 4.5, 4.7, 4.9, 5.1, 5.3, 5.5, 5.7, 5.9, 6.1, 6.3 and 6.5. We incubated cells for 15 min with 70 nM lysosensor–green and determined the fluorescence intensity in an area of 1.5 x 1.5 mm. The standard curve (random fluorescence units) then served to determine fluorescence intensity and, therefore, the vesicular pH in intact cells. If indicated, we incubated intact cells with 10 μM of the CFTR inhibitor $CFTR_{inh}$–172 (Sigma) for 15 min prior to the addition of lysosensor–green. The inhibitor was present throughout the whole experiment. Since $CFTR_{inh}$–172 is very sensitive to storage in stock dimethyl sulfoxid (DMSO) solutions at low temperature for prolonged periods of time, we used it immediately after solubilisation.

Statistics. We expressed data as arithmetic means ± SD and performed statistical analysis as indicated. In the case that values were not normally distributed, exact Mann–Whitney tests were performed. For normal distributed values, one–way ANOVA was applied. Significances are indicated. Kaplan–Meyer curves were analyzed by the log rank test ($P < 0.05$). All data are obtained from independent measurements.

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Author Contributions

V.T. determined ceramide, sphingosine and sphingosine-1-phosphate, M.U. and C.C.D.O–M. stained ceramide, neutrophils and macrophages and quantified neutrophils and macrophages in mouse or human lung tissues and conducted *P. aeruginosa* adherence studies on A549 cells, N.E conducted electron microscopy studies, J.R. contributed and stained human epithelial cells, A.M. v.H. and B. T. raised CF mice of different backgrounds, M. L. B provided human CF and normal lung tissues, G.v.K., K.W.S. and M.W. performed tissue sections and stainings, H.G. performed confocal and fluorescence microscopy, E.G. conducted infection experiments in mice, determined ceramide in mouse lungs and performed stainings, F.L., G.D. and E.G. wrote the manuscript and supervised parts of the project.

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Legends

Figure 1 Cftr–deficiency results in pulmonary ceramide accumulation. (**a**) Ceramide concentrations in homogenized lung tissues of *Cftr*^{KO} and *Cftr*^{MHH} mice of different ages. Wild type (wt) mice were either C3H (WT) or C57BL/6 mice (B6). Asterisks indicate significant differences compared to the respective wt mice (mean ± SD; *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (**b–c**) Accumulated ceramide, detected by fluorescence microscopy, in isolated epithelial cells (**b**) and epithelial cells and submucosa of lung tissues (**c**), from *Cftr*^{KO} and *Cftr*^{MHH} mice compared to WT and B6 mice. Alveolar epithelial cells do not show ceramide accumulation. Shown are representative results from 6 independent experiments. (L = lumen of the bronchus, E = epithelial cells, S = submucosa). (**d–f**) Accumulated ceramide in vesicles of *Cftr*–deficient epithelial cells versus wild type cells, detected by fluorescence confocal microscopy (**d**), FACS analysis (**e**) and immunogold electron microscopy (**f**). Confocal and FACS results represent 10, electron microscopy results three independent experiments. (**g**,**h**) Ceramide accumulation in nasal epithelial cells (**g**, stained with Cy3-coupled antibodies to ceramide) or in epithelial cells (**h**, upper panel) and submucosal glands (**h**, lower panel; arrow indicates submucosal glands, stained with Cy2-coupled antibodies to ceramide) of lung tissues from CF subjects versus healthy individuals. Shown are representative results from 18 CF and 17 healthy individuals (nasal respiratory cells) or three lung samples, respectively.

Figure 2 Influence of vesicular pH on ceramide levels. (**a**) Increased vesicular pH in *Cftr*–deficient mouse respiratory epithelial cells (~ pH 5.9), and in respective cells of WT mice treated with the CFTR–inhibitor $CFTR_{Inh}$–172 (CFTR-INH), detected by lysosensor–green staining. Shown are typical examples (upper panel) and the mean ± SD (lower left panel) of 5 independent experiments. Vesicular pH was calculated from a standard curve (lower right panel). (**b**) Influence of pH levels on activities of acid sphingomyelinase (Asm) and acid ceramidase (Ac). A pH shift from pH 4.5 to pH 6.0 reduces Ac activity to cleave ceramide by ~90%, while its activity to generate ceramide from sphingosine (reverse activity) increases and Asm activity is reduced by 35%, resulting in an Asm/Ac imbalance. (**c**) Ceramide accumulation in lung cells of wild type mice, treated with bafilomycin, nigericin, chloroquin or NH_4Cl by inhalation. **b** and **c** show the mean ± SD of 5 independent experiments. (**d**) Acidification of vesicles from *Cftr*–deficient mice normalizes ceramide, whereas alkalinization of vesicles from wild type mice increases ceramide levels. Left panel: Data represent mean ± SD of 5 independent studies with one mouse each. Right plots: Representative FACS plots from 3 independent experiments display ceramide levels in untreated and acidified vesicles from 16–week old $Cftr^{MHH}$ mice. Three vesicle populations containing different ceramide levels are visible. Arrows indicate vesicles with high ceramide levels at pH 5.9, which are reduced by acidification to pH 4.5. (**e-g**) Treatment of *Cftr*–deficient mice with amitriptyline (Ami, indicated by +) or heterozygosity of Asm ($Cftr^{-/-}/Smpd1^{+/-}$) reduces Asm and acid ceramidase activities at pH 5.9 (**e**) and almost normalizes pulmonary ceramide levels in 24 week old mice (**f**,**g**). Ceramide levels were determined by DAG–kinase assay (**f**) or fluorescence microscopy (**g**). Displayed are the mean ± SD or

representative results of 6 independent experiments. Asterisks indicate significant differences compared to wt (**a,e**) or untreated mice (**c,e**) or as indicated (**d**,**f**) (ANOVA, $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$).

Figure 3 Ceramide accumulation mediates hypersusceptibility of CF–mice to *P. aeruginosa* infection. **(a, b**) *Cftr*–deficient ($Cftr^{KO}$, $Cftr^{MHH}$) mouse lungs contain high colony forming units (CFU) of *P. aeruginosa* strain 762 (**a**), 769 or 14115 (**b)** 2 h after intranasal application, compared to wild–type mice (WT, B6), *Cftr*–deficient mice treated with amitriptyline (+), or Asm–heterozygous mice ($Cftr^{-/-}/Smpd1^{+/-}$). (**c**) Upper left panel: recovery of *P. aeruginosa*, strain 762, 2 min after intranasal application of 10^8 CFU in different mouse strains. Lower panel: *P. aeruginosa* CFU in lungs of different mouse strains 15, 30, 60 and 120 min after challenge with 10^8 CFU of *P. aeruginosa*. Upper right panel: *P. aeruginosa* CFU in lungs of different mouse strains 120 min after challenge with 10^7 CFU of *P. aeruginosa.* (**d**) Increased mortality of *Cftr*–deficient mice, infected intranasally with 10^8 CFU of *P. aeruginosa* strain 762, compared to wild type mice, is prevented by pharmacological inhibition or heterozygosity of Asm. Data (**a-c**) represent means ± SD of 6 independent experiments ($*P < 0.05$, $** P < 0.01$, $*** P < 0.002$; exact Mann–Whitney test) and Kaplan–Meyer curves (**d**) ($*P < 0.05$; log–rank test).

Figure 4 CF–mice suffer from constitutive pulmonary inflammation corrected by normalization of pulmonary ceramide levels. Amitriptyline-treatment (+) or *Smpd1*-heterozygosity normalizes (**a**) cytokine levels (24 week old mice) and (**b**) macrophage

and (**c**) neutrophil cell numbers in lungs of aged CF–mice that are constitutively increased in uninfected aged *Cftr*^{KO} and *Cftr*^{MHH} mice versus wild type mice. Data represent means ± SD of six independent experiments (ANOVA, **P* < 0.05, ** P < 0.01 between age-matched wild–type, amitriptyline-treated CF–mice or *Cftr*^{KO}*/Smpd1*$^{+/-}$ and *Cftr*^{KO} or *Cftr*^{MHH} mice). Numbers of fluorescent cells were determined microscopically. (**d**) Fluorescent-labelled macrophages (light blue, left panel, arrow) accumulate around submucosal glands, stained with periodic acid Schiff reagent (red, right panel, arrow) (b = bronchus). (**e**) Fluorescent-labelled macrophages (green, upper left panel) accumulate in the submucosal lung tissue of uninfected CF–mice but not wild–type mice (lower left panel). Staining with 4,6–diamidino–2–phenylindole–dihydrochloride (DAPI) is shown for CF–mice in the upper right panel and for wild type mice in the lower right panel. (**f**) Fluorescent-labelled neutrophils (green, upper left panel) accumulate in the submucosal lung tissue of uninfected CF–mice but not wild–type mice (lower left panel). DAPI staining is shown for CF–mice in the upper right panel and for wild type mice in the lower right panel.

Figure 5 Pulmonary ceramide accumulation results in constitutive increase of respiratory cell death and deposition of DNA on the respiratory epithelium. (**a**) Increased death of epithelial cells (red stained nuclei), dispersed in the mucosa of large and medium–sized bronchi in *Cftr*^{KO} and *Cftr*^{MHH} mice, stained by TUNEL. Amitriptyline-treatment, *Smpd1*-heterozygosity or application of the caspase inhibitor zVAD normalizes the death rate. Representative stainings from 6 independent studies are shown. (**b)** Quantitative analysis of dead respiratory epithelial cells in different mouse strains. Data represent means ± SD,

counted in at least 10 bronchi/mouse of 6 mice per group. (**c**) CF–mice display increased DNA–deposits (arrows) on the mucosal surface of bronchi, which are prevented by pharmacological or genetic inhibition of the Asm, treatment with zVAD or inhalation of recombinant human DNase. DNA was stained in paraffin sections either by TUNEL (upper panel) or with ethidium bromide (lower panels) resulting in bright DNA staining. Arrows: DNA–deposits, E: epithelial cell layer; L: bronchial lumen. Representative stainings are shown of at least 10 bronchi/mouse from 6 mice/group. (**d)** Percentage of bronchi displaying one or more DNA–deposits (mean ± SD). (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA).

Figure 6 DNA deposits are critically involved in the high susceptibility of CF–mice to *P. aeruginosa* infection. (**a**) DNA distributed on A549 respiratory cells significantly increases adherence and growth of *P. aeruginosa*. Shown are representative photographs from 3 independent experiments. *P. aeruginosa* was stained by indirect immunofluorescence and A549 cells were counterstained with 4,6–diamidino–2–phenylindole–dihydrochloride. (**b**) Treatment of $Cftr^{KO}$ and $Cftr^{MHH}$ mice with the caspase inhibitor zVAD or recombinant human DNase prior to bacterial challenge prevents pulmonary infection with *P. aeruginosa*. Data represent means ± SD from 6 mice/group, studied in 6 independent experiments. Significant differences are indicated (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA).