Original Paper

Regulation of the Inflammasome by Ceramide in Cystic Fibrosis Lungs

Heike Grassmé^a Alexander Carpinteiro^a Michael J. Edwards^b Erich Gulbins^{a,b}
Katrin Anne Becker^a

^aDept. of Molecular Biology, University of Duisburg-Essen, Essen, Germany, ^bDepartment of Surgery, University of Cincinnati, College of Medicine, Cincinnati, Ohio, USA

Key Words

Ceramide • Inflammasome • Cystic fibrosis • Tight junctions

Abstracts

Background: Cystic fibrosis (CF), the most common autosomal recessive disorder in Western countries, is characterized by chronic pulmonary inflammation, reduced mucociliary clearance, and increased susceptibility to infection. Our studies using *Cftr*-deficient mice and human CF specimens showed that ceramide accumulates in CF lungs and mediates increased cell death, susceptibility to infections, and inflammation. **Methods:** We used *Cftr*-deficient and syngenic wildtype mice as well as *Cftr*-deficient mice heterozygous for the acid sphingomyelinase. We determined activation and topology of inflammasome components as well as expression of tight junction proteins by confocal microscopy, western blotting and ELISA. **Results:** We demonstrate an upregulation and membrane recruitment of the adapter protein apoptosis-associated speck-like protein (Asc), a major component of the inflammasome, and caspase 1, an activation of Jun N-terminal kinase as well as an altered distribution and a degradation of the tight junction proteins ZO-1, ZO-2 and Occludin in lungs of CF mice. All of these events are abrogated in CF mice that are heterozygous for the acid sphingomyelinase and, therefore, show normal levels of ceramide in their lungs. These alterations indicate an activation of the inflammasome by ceramide in the lungs of CF mice. Consistent with this notion, we observe a normalization of the increased levels of the cytokines IL-1 β and KC/IL-8 in lungs of CF mice upon treatment with caspase 1 inhibitors. **Conclusion:** Our data suggest a signaling cascade from ceramide via the inflammasome to caspase 1, the release of cytokines and an alteration of tight junction proteins in CF epithelia.

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Introduction

Cystic fibrosis (CF) is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) and constitutes, with 1 case per 2500 births, the most common autosomal recessive disorder in the EU and the USA [1, 2]. In the EU, approximately 40 000 children and young adults are affected. The genetic defect of the CFTR molecule results in several clinical symptoms, in particular pulmonary and gastrointestinal problems. Gastrointestinal symptoms include the defective secretion of pancreatic enzymes and alterations in the liver; pulmonary problems include in particular chronic inflammation and recurrent and chronic infections with *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Burkholderia cepacia*, but also with *Staphylococcus aureus* and *Haemophilus influenzae*. The cause of the chronic inflammation experienced by CF patients and the reasons for their high sensitivity to pulmonary infections are unknown.

Because CFTR exhibits chloride channel activity, it was speculated that water absorption by the mucus present on the epithelial cells of the respiratory tract may be altered, and that these alterations may result in reductions in mucociliary clearance and in the ability to eliminate *P. aeruginosa* [3]. The increased viscosity of the mucus may also affect the ability of neutrophils to migrate to and kill bacteria in the respiratory tract. However, although *in vitro* experiments suggested the concept of reduced mucociliary clearance in cystic fibrosis, *in vivo* studies involving CF patients failed to demonstrate a significant and uniform reduction in mucociliary clearance [4]. Furthermore, CF patients do not seem to exhibit a general defect of neutrophil functions.

Recent studies have suggested that one of the leading causes of bacterial infections in CF patients is an imbalance between pro- and anti-inflammatory cytokines in the airways. It has been demonstrated that, compared with control cells, cells lacking functional CFTR secrete higher amounts of interleukin (IL)-1, IL-8/keratinocyte chemoattractant (KC), tumor necrosis factor (TNF)- α , and macrophage inflammatory protein (Mip)-2 even prior, but in particular upon infection with *P. aeruginosa* [5-10]. Levels of IL-8 in the trachea are already higher in naïve human tissue than in that from healthy controls [7], whereas the synthesis of anti-inflammatory cytokines, in particular IL-10 [11], has been shown to be lower in *Cftr*-deficient mice under basal conditions and after infection with *P. aeruginosa*. The increased release of proinflammatory cytokines from *Cftr*-deficient epithelial cells correlates with a constitutive activation of nuclear factor (NF)- κ B in these cells [12]. At present, the mechanisms by which a defect in *Cftr* increases the activity of NF- κ B, enhances the release of proinflammatory cytokines, and decreases the formation of anti-inflammatory cytokines are unknown.

However, the first clear evidence of chronic inflammation in the lung of CF patients before infection was provided by studies of aborted fetuses [13]. These studies showed that the levels of some markers of inflammation were significantly higher than normal in the lungs of these fetuses. In addition, inflammatory markers have been detected in bronchoalveolar lavage fluid from CF patients as young as four weeks of age, even though cultures tested negative for CF-related bacteria, viruses, and fungi [14].

Here, we investigated the role of ceramide in inflammation in the lungs of CF mice. At present the role of ceramide in aseptical inflammation has been only poorly characterized. Previous findings by R. Kolesnick et al. have demonstrated that stimulation of cells via the IL-1 receptor activates acid sphingomyelinase [15]. Furthermore, stimulation of macrophages results in a release of secretory sphingomyelinase [16, 17]. Studies of microglial cells, fibroblasts and enterocytes demonstrated that acid sphingomyelinase is required for the synthesis and secretion of IL-1 [18] and metalloproteases [19, 20]; these findings strongly suggest that the enzyme is involved in inflammatory processes. However, none of these findings defines the molecular mechanisms by which a constitutive accumulation of ceramide triggers inflammation. Many recent studies have determined that a multiprotein complex consisting of Nod-like receptor (NLR) proteins, the adapter protein apoptosis-associated speck-like protein (ASC), and caspase 1 are crucial for the induction of inflammatory

responses in the cell [for a recent review see 21]. The NLR proteins NLRC4, NLRP1, and NLRP3, which detect bacterial proteins or factors, stress stimuli, reactive oxygen species, etc., associate with ASC and caspase 1 to form a multiprotein complex and to activate caspase 1 [21]. NLRP1 and NLRP3 require ASC if they are to associate with and activate caspase 1, whereas NLRC4 seems to be able to activate caspase 1 independent of ASC [21, 22]. The multiprotein complex of NLR proteins, ASC, and caspase 1 is termed inflammasome. The stimulation of caspase 1 is the final event in the formation of inflammasomes. Caspase 1 is essential for the cleavage of IL-1 and IL-18 and the maturation of pro-proteins [2]. At present it is unknown whether the inflammasome is activated and whether ceramide is involved in the regulation of the inflammasome in CF lungs.

We and others have previously shown that *Cftr*-deficiency results in an accumulation of ceramide in the lung, trachea, intestine and in macrophages [23-31]. The accumulation of ceramide in *Cftr*-deficient cells is corrected by pharmacological inhibition of the acid sphingomyelinase or upon reduction of the gene dose of the acid sphingomyelinase in mice deficient for *Cftr* and only heterozygous for the acid sphingomyelinase. The beneficial effect of a pharmacological inhibition of the acid sphingomyelinase in CF lungs was recently also demonstrated in clinical studies treating CF patients with the pharmacological inhibitor of the acid sphingomyelinase amitriptyline [32].

Here, we investigated the role of ceramide in inflammasome activation in CF lungs and the molecular consequences of this process, in particular consequences on the cytoskeleton and tight junctions within the lung.

Materials and Methods

Mice

B6.129P2(CF/3)-*Cftr*^{TgH(neoim)Hgu} (abbreviated *Cftr*^{-/-}) congenic mice were previously described [23]. Syngenic B6 littermates were used as controls. *Cftr*^{-/-} mice were crossed with acid sphingomyelinase-deficient mice that are also on a C57BL/6 background to obtain mice that were deficient in *Cftr* and heterozygous for acid sphingomyelinase (called *Cftr*^{-/-}/*Smpd1*^{+/-}; *Smpd1* is the gene symbol for acid sphingomyelinase). As control mice we used syngenic wildtype littermates. All studies were performed with mice at an age of 16-20 weeks. Mice were housed in the Central Laboratory Animal Facility of the University Hospital Essen, University of Duisburg-Essen, Germany, in isolator cages that provided a pathogen-free environment. The hygienic status of the mice was repeatedly tested by a panel of common murine pathogens according to the 2002 recommendations of the Federation for Laboratory Animal Science Associations. Bacterial and parasite culturing and serology were always negative. All procedures performed on mice were approved by the Animal Care and Use Committee of the Bezirksregierung Duesseldorf, Duesseldorf, Germany.

PCR

Cftr was analyzed by using the primers 5'-ccttccatgtaccctcctcactt-3' and 5'-cccggcataatccaagaaattg-3' to detect wildtype *Cftr* and 5'-ctcgtgctttacggtatcgcc-3' 5'-tgctgtagtggcaagctttga-3' to detect the knock-out genotype. *Asm* was detected by the primers 5'-cgagactgttgccagacatc-3', 5'-ggctaccgtgatattgctg-3' and 5'-agccgtgtcctcttcttac-3'.

Ceramide measurements

Ceramide concentrations in the lung were determined by a DAG-kinase. To this end, the lungs were removed, shock frozen, and homogenized under liquid nitrogen. The homogenates were transferred to 300 μ L CH₃OH, an aliquot was removed to normalize for protein by a Bradford assay. Homogenates were then brought to 600 μ L CHCl₃:CH₃OH:1N HCl (100:100:1, v/v/v), 200 μ L H₂O were added and the samples were vigorously vortexed. The samples were centrifuged for 5 min at 14,000 rpm to promote phase separation, the lower phase was collected and dried. The samples were resuspended in 20 μ L of a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid), bath-sonicated for 10 min, and the kinase reaction was initiated by the addition of 70 μ L of a reaction mixture consisting of 10 μ L DAG kinase (GE Healthcare Europe, Munich, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2

mM diethylenetriaminepentaacetic acid (pH 6.6), 70 mM NaCl, 17 mM MgCl₂ and 1.4 mM EGTA, 2 mM DTT, 1 μM ATP, and 10 μCi [³²P]γATP. The kinase reaction was performed for 60 min at room temperature. Lipids were then extracted by the addition of 1 mL CHCl₃:CH₃OH:1N HCl (100:100:1, v/v/v), 170 μL buffered saline solution (135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.2), and 30 μL of a 100 mM EDTA solution. The samples were vortexed, phases separated, the lower phase was collected, dried, dissolved in 20 μL CHCl₃:CH₃OH (1:1, v/v), and separated on Silica G60 Thin Layer Chromatography (TLC) plates by using chloroform, acetone, methanol, acetic acid, and H₂O (50:20:15:10:5, v/v/v/v/v). The TLC plates were analyzed by autoradiography. Ceramide spots were identified by co-migration with a ceramide standard and were removed from the plates. The incorporation of [³²P] into ceramide was quantified by liquid scintillation counting. Ceramide amounts were determined by comparison with a standard curve using C₁₆-ceramide as substrate.

Immunostainings

Lungs were removed and immediately fixed in PBS-buffered 4% paraformaldehyde (PFA; pH 7.4) for 36 hrs. Samples were embedded in paraffin, sectioned, dewaxed, and incubated for 30 min with pepsin (Digest All, Invitrogen) at 37°C. They were then washed, blocked for 10 min with PBS, 0.05% Tween 20, and 5% FCS, and immunostained for 45 min with polyclonal antibodies directed against caspase 1, p45 and the active form p20 (diluted 1:100, Santa Cruz Biotechnology Inc. #M-20), anti-ZO-1 (diluted 1:100, Santa Cruz Biotechnology Inc. #sc-10804), anti-ZO-2 (diluted 1:100, Santa Cruz Biotechnology Inc. #sc-11448), occludin (1:100, Invitrogen # 71-1500), Asc (diluted 1:100, Santa Cruz Biotechnology Inc. sc-22514-R) or phospho-JNK (1:100, Cell Signaling #9251). All antibodies were diluted in HEPES/Saline (H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄) supplemented with 1% FCS. The samples were washed 3-times in PBS + 0.05% Tween 20 and incubated for 45 min with a Cy3 coupled donkey anti-rabbit IgG (Jackson ImmunoResearch), washed again 3 times in PBS + 0.05% Tween 20 and once in PBS, embedded in Mowiol and analyzed by confocal or fluorescence microscopy on a Leica DMIRE2.

Western blots

For western blot studies, lungs were removed, shock-frozen in liquid nitrogen, homogenized under liquid nitrogen and the homogenates were lysed in 150 mM NaCl, 25 mM TrisHCl, pH 7.4, 10 mM EDTA, 10 mM sodium-pyrophosphate, 3% NP40, each 10 μg/ml aprotinin and leupeptin, 5 μL 5x SDS Laemmli buffer was added, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), incubated with anti-phospho-JNK antibodies, anti-Asc antibodies, anti-ZO-1 or anti-ZO-2 (all antibodies as above, diluted 1:1000), and developed with alkaline phosphatase-coupled secondary antibodies and the Tropix chemoluminescence system.

ELISA

Lungs of the indicated mouse strains were homogenized in liquid nitrogen and lysed in 25 mM Tris/HCl, pH 7.4, 2% Nonidet P40, 125 mM NaCl, 10 mM EDTA and 10 mM sodiumpyrophosphate. Cytokine concentrations were determined by commercial ELISA assays according to the manufacturer's instructions (R&D, Wiesbaden-Nordenstedt, Germany).

Statistics

Values are expressed as mean ± SD. Significant differences were determined by using t-test.

Results

In an attempt to identify molecular mechanisms by which ceramide accumulation mediates (aseptic) inflammation in cystic fibrosis lungs, we investigated whether ceramide accumulation results in the activation of caspase 1 and JNK. Our findings revealed an upregulation of caspase 1 in bronchial epithelial cells of cystic fibrosis mice and a translocation of caspase 1 to the apical membrane of these epithelial cells (Fig. 1A) consistent with previous findings [33]. The translocation of caspase 1 correlates with an activation of caspase 1 in CF lungs as previously shown by us [33]. Likewise, we observed membrane translocation

Fig. 1. Ceramide accumulation results in membrane recruitment of caspase 1 and phospho-JNK in *Cftr*-deficient bronchial epithelial cells. Paraffin-embedded sections from lungs of wild-type, *Cftr*^{-/-}, and *Cftr*^{-/-}/*Smpd1*^{+/-} mice were stained with Cy3-coupled anti-caspase 1 (A) or Cy3-coupled anti-phospho-JNK antibodies (B) and analyzed by confocal microscopy. Shown are representative studies from groups of 5 animals. (C) Western blot studies show a phosphorylation of JNK in cystic fibrosis lungs that is corrected by heterozygosity of the acid sphingomyelinase. Shown is a representative western blot from 5 animals. (D) Ceramide measurements in lung homogenates confirm the accumulation of ceramide in CF lungs and the normalization of ceramide levels in CF mice heterozygous for the acid sphingomyelinase. Shown is the mean ± SD of 5 mice/group. * indicates a significant difference between wildtype and CF mice, Δ between *Cftr*^{-/-} and *Cftr*^{-/-}/*Smpd1*^{+/-} mice. Significance was set at the level of $P \leq 0.05$, as determined by analysis of variance (ANOVA).

and phosphorylation of JNK reflecting activation of the protein (Fig. 1B, C). The cellular reorganisation of caspase 1 and JNK in cystic fibrosis epithelia as well as the phosphorylation/

Fig. 3. Intraperitoneal injection of the caspase 1 inhibitor Ac-YVAD-cmk reduces inflammation in cystic fibrosis mice. Mice were injected intraperitoneally with Ac-YVAD-cmk (10 mg/kg) twice daily, and the concentrations of IL-1 and IL-8 in the lung were determined by ELISA. Shown is the mean $\pm$ SD of 5 mice/group. * indicates a significant difference between wildtype and CF mice, Δ between treated and untreated CF mice. Significance was set at the level of $P \leq 0.05$, as determined by analysis of variance (ANOVA).

activation of JNK were abolished by heterozygosity of acid sphingomyelinase (*Smpd1*) in *Cfr*-deficient mice (Fig. 1A-C), a finding suggesting that these events are downstream of ceramide. Heterozygosity of the acid sphingomyelinase has been previously shown to normalize pulmonary ceramide levels, which was confirmed here (Fig. 1D).

To further confirm the relation between ceramide and the inflammation in CF cells, we investigated the cellular distribution of a key component of the inflammasome, i.e. adapter protein apoptosis-associated speck-like protein (Asc). Asc has been shown to associate with caspase 1 upon induction of the inflammasome [21, 22]. We, thus, investigated whether Asc is clustered on the cell membrane in *Cfr*-deficient cells. The results (Fig. 2A) show clustering

Fig. 4. The distribution pattern and expression of tight junction proteins is altered in the bronchi of *Cftr*-deficient mice and is corrected by normalization of ceramide levels. (A, B) Paraffin-embedded sections from the lungs of wild-type, *Cftr*^{-/-}, and *Cftr*^{-/-}/*Smpd1*^{+/-} mice were stained with Cy3-coupled anti-ZO-2 or Cy3-coupled anti-occludin antibodies and were analyzed by confocal microscopy. Shown are representative studies from groups of 5 animals. (C) The western blot studies are from lung homogenates that were lysed as above, separated on 8.5% SDS-polyacrylamide gel electrophoresis (PAGE), and developed with anti-ZO-1 or anti-ZO-2 antibodies. The results demonstrate a down-regulation of ZO-1 and ZO-2 in the lungs of *Cftr*-deficient mice, which is corrected by genetic inhibition of acid sphingomyelinase. Shown are representative studies from groups of 8 animals.

of Asc in bronchial epithelial cells of *Cftr*-deficient mice, which was absent in epithelial cells from wildtype mice and corrected by heterozygosity of the acid sphingomyelinase. In addition, Asc is up-regulated in lungs of *Cftr*-deficient mice, a phenotype that was also corrected by heterozygosity of the acid sphingomyelinase (Fig. 2B).

To further demonstrate the role of caspases in the induction of inflammation in the lungs of *Cftr*-deficient mice, we treated these mice with the caspase 1 inhibitor Ac-YVAD-cmk, which was intraperitoneally injected twice daily over a period of 7 days. The results show that the application of this inhibitor markedly reduced the concentration of proinflammatory mediators in the lungs of *Cftr*-deficient mice (Fig. 3).

Because activation of caspase 1 and stimulation of the inflammasome is able to influence tight junctions, we investigated whether the integrity of tight junctions is altered in *Cftr*-deficient mice. A previous report has demonstrated a downregulation of ZO-1 and JAM in biopsy materials from *CFTR*-deficient human patients [34]; however, no further defects are known.

Our fluorescence and confocal microscopy data show that the subcellular distribution of the tight junction proteins ZO-2 and occludin is altered and appears fragmented in the bronchi of *Cftr*-deficient mice (Fig. 4A,B), whereas the staining of claudin 1 and cadherin did not differ between wild-type and *Cftr*-deficient mice (not shown). Genetic heterozygosity of acid sphingomyelinase in *Cftr*^{-/-}/*Smpd1*^{+/-} mice normalized the staining for ZO-2 and occludin (Fig. 4A,B).

In accordance, western blot studies revealed a decrease in the expression of ZO-1 and ZO-2 in the lungs of *Cftr*-deficient mice, which was corrected by pharmacological inhibition of acid sphingomyelinase (Fig. 4C). However, the expression of cadherin and occludin was not altered (not shown).

These findings indicate a distinct defect of some tight junction proteins in *Cftr*-deficient cells, which is mediated by the accumulation of ceramide.

Discussion

Here we demonstrate that the accumulation of ceramide in lungs of CF mice results in membrane recruitment of inflammasome proteins and activation of caspase 1. Caspase 1 seems to trigger a release of typical inflammatory mediators in the lung, such as IL-1 β and KC/IL-8, which have been previously shown to be elevated in the lungs of CF mice and patients. This data links the accumulation of ceramide in CF lungs with an activation of the inflammasome, a general and fundamental mechanism of inflammation.

CF lungs are characterized by chronic inflammation that may begin in individuals with CF as young as 4 weeks of age [14]. Bronchiolar lavage samples from CF individuals revealed increased levels of inflammatory cytokines and neutrophils in airways prior to measurable colonization of bacteria [14]. Studies on aborted fetuses [13] show an increase of inflammatory molecules, i.e. ICAM-1 and cyclooxygenase 2 (COX-2) in bronchial epithelial cells of CF fetuses, NF κ B activation as indicated by a nuclear translocation of the p65 subunit and increased metalloprotease-1 activity in the cartilage of these airways. These findings suggest pulmonary inflammation prior to infection. The development of pulmonary inflammation seems to be slightly different in CF-pigs that develop infection first followed by pulmonary inflammation within a few months after birth [35]. However, finally, it seems to be irrelevant whether inflammation or infection is first, since they will form a vicious cycle in CF.

Here we provide a novel pathway to explain inflammation and in particular the release of IL-1 β and KC/IL-8 in CF airways, i.e. the activation of the inflammasome by the increased ceramide levels in CF airways. The notion that ceramide activates the inflammasome is consistent with the previous findings that silencing the NLRP3-inflammasome attenuates ceramide-induced transepithelial permeability [36].

At present it is unknown how ceramide activates the inflammasome. Ceramide may directly recruit and cluster Asc and thereby initiating activation of the inflammasome. However, we have previously shown that ceramide accumulation in CF airways also results in clustering and activation of CD95 [33]. Since clustering of CD95 in ceramide-enriched membrane domains is pre-requisite for signaling via this receptor [37, 38], ceramide may also activate the inflammasome by clustering and stimulation of CD95. In addition, ceramide may trigger a slow, but constant release of reactive oxygen species in epithelial cells from cystic fibrosis mice by clustering NADPH-oxidase in ceramide-enriched membrane domains as previously shown for instance in podocytes, endothelial cells, monocytes and macrophages [39-43].

JNK has been shown to be involved in many stress and inflammatory signaling pathways [44]. However, the role in cystic fibrosis is poorly characterized. Activation of JNK might be involved in regulation of IL-8 formation [45] and thereby contribute to the release of cytokines elicited by activation of the inflammasome.

Many inflammatory diseases that affect epithelial cell layers result in changes in the tight junctions. These changes in the function of tight junctions may be crucially involved in

the initiation and perpetuation of inflammatory diseases.

Experiments using lung biopsy tissues from CF patients showed that ZO-1 and JAM are downregulated in the bronchial epithelial cell layers [34], consistent with our findings. We, furthermore demonstrate a reduced expression of ZO-2 and a change of the cellular distribution of occludin in CF cells. Claudin 1, 3 and 5 expression levels have been reported to be unchanged in cystic fibrosis, and the transepithelial resistance in the epithelium of human CF airways is also not significantly different from that of non-CF samples, at least in *in vitro* cultures [46]. These findings suggest that tight junctions are distinctly altered in cystic fibrosis, whereas the overall barrier function of tight junctions does not seem to be affected.

Treatment of cystic fibrosis patients with amitriptyline, a functional inhibitor of the acid sphingomyelinase, has been shown to improve lung functions, in particular the forced expiratory volume [32, 47]. The present studies suggest that normalization of ceramide levels should also reduce or even prevent the chronic inflammation present in the airways of cystic fibrosis patients.

In summary, we provide evidence for a novel pathway triggered *in vivo* in CF airways upon accumulation of ceramide, i.e. the stimulation of the inflammasome, caspase 1 and finally the release of pro-inflammatory cytokines as well as a distinct alteration of the cytoskeleton.

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Disclosure Statement

The authors have no competing interests.

References

- 1 Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: Identification of the cystic fibrosis gene: genetic analysis. *Science* 1989;245:1073-1080.
- 2 Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N, Zsiga M, Buchwald M, Riordan JR, Tsui L-C, Collins FS: Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 1989;245:1059-1065.
- 3 Matsui H, Grubb BR, Tarran R, Randell SH, Gatzky JT, Davis CW, Boucher RC: Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 1998;95:1005-1015.
- 4 Boucher RC: Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* 2007;58:157-170.
- 5 Griesse M, Essl R, Schmidt R, Rietschel E, Ratjen F, Ballmann M, Paul K: BEAT Study Group. Pulmonary surfactant, lung function, and endobronchial inflammation in cystic fibrosis. *Am J Respir Crit Care Med* 2004;170:1000-1005.
- 6 Schultz MJ, Rijneveld AW, Florquin S, Edwards CK, Dinarello CA, van der Poll T: Role of interleukin-1 in the pulmonary immune response during *Pseudomonas aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L285-290.
- 7 Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J: Relationship between Ikappa-Balpha deficiency, NFkappaB activity and interleukin-8 production in CF human airway epithelial cells. *Pflügers Arch* 2001;443:S40-44.
- 8 Tirouvanziam R, de Bentzmann S, Hubeau C, Hinnrasky J, Jacquot J, Peault B, Puchelle E: Inflammation and infection in naive human cystic fibrosis airway grafts. *Am J Respir Cell Mol Biol* 2000;23:121-127.

- 9 Inoue H, Massion PP, Ueki IF, Grattan KM, Hara M, Dohrman AF, Chan B, Lausier JA, Golden JA, Nadel JA: Pseudomonas stimulates interleukin-8 mRNA expression selectively in airway epithelium, in gland ducts, and in recruited neutrophils. *Am J Respir Cell Mol Biol* 1994;11:651-663.
- 10 Oceandy D, McMorran BJ, Smith SN, Schreiber R, Kunzelmann K, Alton EW, Hume DA, Wainwright BJ: Gene complementation of airway epithelium in the cystic fibrosis mouse is necessary and sufficient to correct the pathogen clearance and inflammatory abnormalities. *Hum Mol Genet* 2002;11:1059-1067.
- 11 Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M: Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol* 1995;13:257-261.
- 12 Venkatakrishnan A, Stecenko AA, King G, Blackwell TR, Brigham KL, Christman JW, Blackwell TS: Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2000;23:396-403.
- 13 Verhaeghe C, Delbecq K, de Leval L, Oury C, Bours V: Early inflammation in the airways of a cystic fibrosis foetus. *J Cyst Fibros* 2007;6:304-308.
- 14 Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW: Early pulmonary inflammation in infants with cystic fibrosis. *Am J Respir Crit Care Med* 1995;151:1075-1082.
- 15 Mathias S, Younes A, Kan CC, Orlov I, Joseph C, Kolesnick RN: Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. *Science* 1993;259:519-522.
- 16 Marathe S, Schissel SL, Yellin MJ, Beatini N, Mintzer R, Williams KJ, Tabas I: Human vascular endothelial cells are a rich and regulatable source of secretory sphingomyelinase. Implications for early atherogenesis and ceramide-mediated cell signaling. *J Biol Chem* 1998;273:4081-4088.
- 17 Wong ML, Xie B, Beatini N, Phu P, Marathe S, Johns A, Gold PW, Hirsch E, Williams KJ, Licinio J, Tabas I: Acute systemic inflammation up-regulates secretory sphingomyelinase *in vivo*: a possible link between inflammatory cytokines and atherogenesis. *Proc Natl Acad Sci U S A* 2000;97:8681-8686.
- 18 Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E, Saggiotti L, Schuchman EH, Furlan R, Clementi E, Matteoli M, Verderio C: Acid sphingomyelinase activity triggers microparticle release from glial cells. *Embo J* 2009;28:1043-1054.
- 19 Bauer J, Huy C, Brenmoehl J, Obermeier F, Bock J: Matrix metalloproteinase-1 expression induced by IL-1beta requires acid sphingomyelinase. *FEBS Lett* 2009;583:915-920.
- 20 Bauer J, Liebisch G, Hofmann C, Huy C, Schmitz G, Obermeier F, Bock J: Lipid alterations in experimental murine colitis: role of ceramide and imipramine for matrix metalloproteinase-1 expression. *PLoS One* 2009;4:e7197.
- 21 Brodsky IE, Monack D: NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens. *Semin Immunol* 2009;21:199-207.
- 22 Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G: The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 2009;10:241-247.
- 23 Teichgraber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC, van Heeckeren AM, Barr ML, von Kürthy G, Schmid KW, Weller M, Tümmler B, Lang F, Grassme H, Döring G, Gulbins E: Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat Med* 2008;14:382-391.
- 24 Becker KA, Riethmüller J, Lüth A, Döring G, Kleuser B, Gulbins E: Acid sphingomyelinase inhibitors normalize pulmonary ceramide and inflammation in cystic fibrosis. *Am J Respir Cell Mol Biol* 2010;42:716-724.
- 25 Becker KA, Tümmler B, Gulbins E, Grassmé H: Accumulation of ceramide in the trachea and intestine of cystic fibrosis mice causes inflammation and cell death. *Biochem Biophys Res Commun* 2010;403:368-374.
- 26 Bodas M, Min T, Mazur S, Vij N: Critical modifier role of membrane-cystic fibrosis transmembrane conductance regulator-dependent ceramide signaling in lung injury and emphysema. *J Immunol* 2011;186:602-613.
- 27 Bodas M, Min T, Vij N: Critical role of CFTR-dependent lipid rafts in cigarette smoke-induced lung epithelial injury. *Am J Physiol Lung Cell Mol Physiol* 2001;300:L811-820.
- 28 Brodlie M, McKean MC, Johnson GE, Gray J, Fisher AJ, Corris PA, Lordan JL, Ward C: Ceramide is increased in the lower airway epithelium of people with advanced cystic fibrosis lung disease. *Am J Respir Crit Care Med* 2010;182:369-375.

- 29 Ulrich M, Worlitzsch D, Viglio S, Siegmann N, Iadarola P, Shute JK, Geiser M, Pier GB, Friedel G, Barr ML, Schuster A, Meyer KC, Ratjen F, Bjarnsholt T, Gulbins E, Döring G: Alveolar inflammation in cystic fibrosis. *J Cyst Fibros* 2010;9:217-227.
- 30 Caretti A, Bragonzi A, Facchini M, De Fino I, Riva C, Gasco P, Musicanti C, Casas J, Fabriàs G, Ghidoni R, Signorelli P: Anti-inflammatory action of lipid nanocarrier-delivered myriocin: therapeutic potential in cystic fibrosis. *Biochim Biophys Acta* 2014;1840:586-594.
- 31 Itokazu Y, Pagano RE, Schroeder AS, O'Grady SM, Limper AH, Marks DL: Reduced GM1 ganglioside in CFTR-deficient human airway cells results in decreased β 1-integrin signaling and delayed wound repair. *Am J Physiol Cell Physiol* 2014;PMID: 24500283.
- 32 Nährlich L, Mainz JG, Adams C, Engel C, Herrmann G, Icheva V, Lauer J, Deppisch C, Wirth A, Unger K, Graeppler-Mainka U, Hector A, Heyder S, Stern M, Döring G, Gulbins E, Riethmüller J: Therapy of CF-patients with amitriptyline and placebo – a randomised, double-blind, placebo-controlled phase IIb multicenter, cohort study. *Cell Physiol Biochem* 2013;31:505-512.
- 33 Becker KA, Henry B, Ziobro R, Tümmeler B, Gulbins E, Grassmé H: Role of CD95 in pulmonary inflammation and infection in cystic fibrosis. *J Mol Med (Berl)* 2012;90:1011-23.
- 34 Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG: Regulation of airway tight junctions by proinflammatory cytokines. *Mol Biol Cell* 2002;13:3218-3234.
- 35 Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, Hanfland RA, Wohlford-Lenane C, Dohrn CL, Bartlett JA, Nelson GA 4th, Chang EH, Taft PJ, Ludwig PS, Estin M, Hornick EE, Launsbach JL, Samuel M, Rokhlina T, Karp PH, Ostedgaard LS, Uc A, Starner TD, Horswill AR, Brogden KA, Prather RS, Richter SS, Shilyansky J, McCray PB Jr, Zabner J, Welsh MJ: Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* 2010;2:29ra31.
- 36 Kolliputi N, Galam L, Parthasarathy PT, Tipparaju SM, Lockey RF: NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability. *J Cell Physiol* 2012;227:3310-3316.
- 37 Grassmé H, Jekle A, Riehle A, Schwarz H, Berger J, Sandhoff K, Kolesnick R, Gulbins E: CD95 signaling via ceramide-rich membrane rafts. *J Biol Chem* 2001;276:20589-20596.
- 38 Grassmé H, Cremesti A, Kolesnick R, Gulbins E: Ceramide-mediated clustering is required for CD95-DISC formation. *Oncogene* 2003;22:5457-5470.
- 39 Zhang C, Boini KM, Xia M, Abais JM, Li X, Liu Q, Li PL: Activation of NOD-like receptor protein 3 inflammasomes turns on podocyte injury and glomerular sclerosis in hyperhomocysteinemia. *Hypertension* 2012;60:154-162.
- 40 Xu M, Zhang Y, Xia M, Li XX, Ritter JK, Zhang F, Li PL: NAD(P)H oxidase-dependent intracellular and extracellular O₂^{•-} production in coronary arterial myocytes from CD38 knockout mice. *Free Radic Biol Med* 2012;52:357-365.
- 41 Wei YM, Li X, Xiong J, Abais JM, Xia M, Boini KM, Zhang Y, Li PL: Attenuation by statins of membrane raft-redox signaling in coronary arterial endothelium. *J Pharmacol Exp Ther* 2013;345:170-179.
- 42 Abais JM, Zhang C, Xia M, Liu Q, Gehr TW, Boini KM, Li PL: NADPH oxidase-mediated triggering of inflammasome activation in mouse podocytes and glomeruli during hyperhomocysteinemia. *Antioxid Redox Signal* 2013;18:1537-1548.
- 43 Zhang Y, Li X, Grassmé H, Döring G, Gulbins E: Alterations in ceramide concentration and pH determine the release of reactive oxygen species by *Cfr*-deficient macrophages on infection. *J Immunol* 2010;184:5104-5111.
- 44 Sabio G, Davis RJ: TNF and MAP kinase signalling pathways. *Semin Immunol* pii: S1044-5323(14)00022-0. doi: 10.1016/j.smim.2014.02.009.
- 45 Roque T, Boncoeur E, Saint-Criq V, Bonvin E, Clement A, Tabary O, Jacquot J: Proinflammatory effect of sodium 4-phenylbutyrate in deltaF508-cystic fibrosis transmembrane conductance regulator lung epithelial cells: involvement of extracellular signal-regulated protein kinase 1/2 and c-Jun-NH2-terminal kinase signaling. *J Pharmacol Exp Ther* 2008;326:949-956.
- 46 Coyne CB, Gambling TM, Boucher RC, Carson JL, Johnson LG: Role of claudin interactions in airway tight junctional permeability. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L1166-1178.
- 47 Riethmüller J, Anthonysamy J, Serra E, Schwab M, Döring G, Gulbins E: Therapeutic efficacy and safety of amitriptyline in patients with cystic fibrosis. *Cell Physiol Biochem* 2009;24:65-72.