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The βENaC-overexpressing mouse as a model of cystic fibrosis lung disease

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Abstract

Chronic lung disease remains the major cause of morbidity and mortality of cystic fibrosis (CF) patients. Cftr mutant mice developed severe intestinal obstruction, but did not exhibit the characteristic CF ion transport defects (i.e. deficient cAMP-dependent Cl^- secretion and increased Na^+ absorption) in the lower airways, and failed to develop CF-like lung disease. These observations led to the generation of transgenic mice with airway-specific overexpression of the epithelial Na^+ channel (ENaC) as an alternative approach to mimic CF ion transport pathophysiology in the lung. Studies of the phenotype of βENaC-transgenic mice demonstrated that increased airway Na^+ absorption causes airway surface liquid (ASL) depletion, reduced mucus transport and a spontaneous CF-like lung disease with airway mucus obstruction and chronic airway inflammation. Here, we summarize approaches that can be applied for studies of the complex *in vivo* pathogenesis and preclinical evaluation of novel therapeutic strategies in this model of CF lung disease.

Keywords: Cystic fibrosis; Mouse model; ENaC; Airway surface liquid; Mucus obstruction; Airway inflammation

1. Introduction

Cystic fibrosis (CF) is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR)* gene and remains one of the most common lethal hereditary diseases in Caucasian populations. Although CF is a complex multi-organ disease, chronic lung disease still determines more than 90% of morbidity and mortality in CF patients and remains a primary focus of CF research [1]. Previous studies demonstrated that CF lungs are structurally normal at birth, with an early onset of lung disease in infancy evolving from small airway mucus obstruction to chronic airway inflammation, bacterial infection, progressive lung damage and ultimately death due to respiratory failure [1–3]. Despite the detailed pathological and clinical description, important questions regarding the initiation and spontaneous progression of CF lung disease remain unsolved, and effective therapies targeting its root cause are still pending.

Genetically engineered mice offer unique opportunities to unravel the *in vivo* pathogenesis of inherited diseases and test novel therapies in a living organism in the preclinical area. The first CF mouse models, in which Cftr function was either disabled by homologous recombination or impaired by introduction of specific Cftr mutations were generated shortly after cloning of the *CFTR* gene [4–6]. As reviewed by Wilke et al. in this issue [7], Cftr mutant mice exhibited characteristic defects in intestinal cAMP-dependent Cl^- secretion producing a severe CF-like gastrointestinal phenotype. However, even when it became possible to prevent early intestinal mortality by treatment with osmotic laxatives,

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Cftr mutant mice did not develop CF-like lung disease [6]. Careful histopathological examination revealed that Cftr mutant mice on some genetic backgrounds and at older age developed signs of alveolitis with thickening of alveolar walls [8,9], but none of the Cftr mutant mouse models develops airway mucus plugging, goblet cell metaplasia and spontaneous airway inflammation characteristic of CF lung disease in humans [1,6]. Subsequently, a series of functional studies demonstrated that the lower airways of CF mice do not recapitulate the basic ion transport and mucus clearance defects characteristic of CF patients [10,11]. In human CF airways, CFTR malfunction causes deficient cAMP-dependent Cl^- secretion and increased ENaC-mediated Na^+ absorption in the superficial airway epithelium [12] and abnormal fluid secretion by submucosal glands [13–15], and these alterations in fluid transport are associated with reduced mucociliary clearance [16,17]. In Cftr mutant mice, these basic CF ion transport defects are detected in the nasal epithelium [18] but not in the superficial epithelium of lower airways where lack of Cftr-mediated Cl^- secretion is likely compensated by alternative Ca^{2+}-activated Cl^- channels (CaCC), and airway Na^+ transport and mucus clearance remain normal [6,10,11,19]. In addition, in contrast to humans, intrapulmonary conducting airways of mice do not possess submucosal glands [6]. These studies indicated that the differences in airway ion transport (patho)physiology and anatomy between mice and man protects Cftr mutant mice from the development of CF-like lung disease. Further, these observations formed the rationale for the generation of transgenic mice with airway-specific overexpression of ENaC to mimic the increased Na^+ absorption across the superficial epithelium observed in human CF airways to determine the relative role of this abnormality in CF pathogenesis and as an alternative strategy to produce CF-like lung disease in mice [20–24].

2. Development of a mouse model with airway-specific overexpression of ENaC

2.1. Generation and functional characterization of transgenic mice with airway-specific overexpression of ENaC

To mimic increased ENaC activity observed in CF airways and prevent unrelated pathologies in other organs that may result from systemic overexpression of ENaC such as arterial hypertension due to increased absorption of Na^+ and fluid in the kidney [25], we used the Clara cell secretory protein (CCSP) promoter element to target expression of ENaC to mouse airways [26]. Clara cells are the most abundant cell type in mouse airways constituting ~50% of epithelial cells in the trachea and up to ~80% in the small airways [27]. Therefore, the CCSP promoter enables tissue-specific transgene expression throughout the conducting airways of mice. Because ENaC is a heteromultimeric protein composed of three subunits (α, β, γ), and heterologous expression studies indicate that co-expression of all three subunits is required for maximal Na^+ channel activity [28], we generated transgenic mice with airway-specific overexpression of the three individual subunits (α, β and γ) of mouse ENaC [29] by pronuclear injection (Fig. 1A, B). *In situ* hybridization of lung sections confirmed that CCSP-driven overexpression of α, β and γENaC transgenes was localized to airway epithelia (Fig. 1C). Surprisingly, measurements of bioelectric properties in freshly excised tracheal tissues from neonatal and adult mice demonstrated that overexpression of βENaC, but not αENaC or γENaC alone, was sufficient to produce a ~2 to 3-fold increase in amiloride-sensitive Na^+ transport (Fig. 1D–F, Table 1), whereas cAMP-induced (forskolin) and Ca^{2+}-activated (UTP) Cl^- secretion remained unchanged in airways from βENaC-overexpressing (βENaC-Tg) mice [30].

In an attempt to elucidate the selective functional effect of overexpression of βENaC, transcript levels of endogenous α, β and γENaC subunits were compared by quantitative real-time PCR. These studies demonstrated that βENaC is expressed at relatively low levels in mouse lower airways suggesting that the βENaC subunit is rate limiting for airway Na^+ absorption *in vivo* [30]. Recent studies indicate that the increased airway Na^+ and fluid absorption produced by selective overexpression of βENaC (in airway cells expressing α, β, and γENaC endogenously) was not only caused by an increase in the number of αβγENaC channels inserted into the luminal membrane of airway epithelial cells, but also by a shift in subunit stoichiometry resulting in a subpopulation of αβENaC channels. These αβENaC channels are known to have an increased open probability ($P_o \sim 1.0$) [31] and were found to be constitutively active in native airway tissues from βENaC-Tg mice [32].

Of note, hemizygosity for the βENaC transgene is sufficient to produce increased Na^+ transport, which facilitates breeding and genotyping of βENaC-Tg mice. Another important consideration for studies using transgenic mouse models is the genetic background and the use of an appropriate control group. Since βENaC-Tg were generated on a mixed genetic background (C3H x C57BL/6), it seems prudent to breed βENaC-Tg with C3B6 F1 hybrid wild-type mice to prevent genetic drifts in the colony, and use wild-type littermate controls for all experiments to control for possible effects of strain background on the phenotype.

2.2. Increased airway Na^+ absorption causes ASL volume depletion and reduced mucociliary clearance

Evidence from *in vitro* studies in cultured primary normal and CF airway epithelia suggested that increased ENaC-mediated Na^+ absorption and defective CFTR-mediated Cl^- secretion cause volume depletion of the thin film of liquid (~7 μm) covering airway surfaces, and that this abnormality results in mucus adhesion and impaired mucociliary function [33]. These observations led to the hypothesis that airway surface liquid (ASL) volume depletion may play an important role in the pathogenesis of CF lung disease. However, the results of these *in vitro* studies were discussed controversially [34] and it was not feasible to study the thin (~7 μm) ASL layer in humans. Therefore, the βENaC-Tg mouse provided a

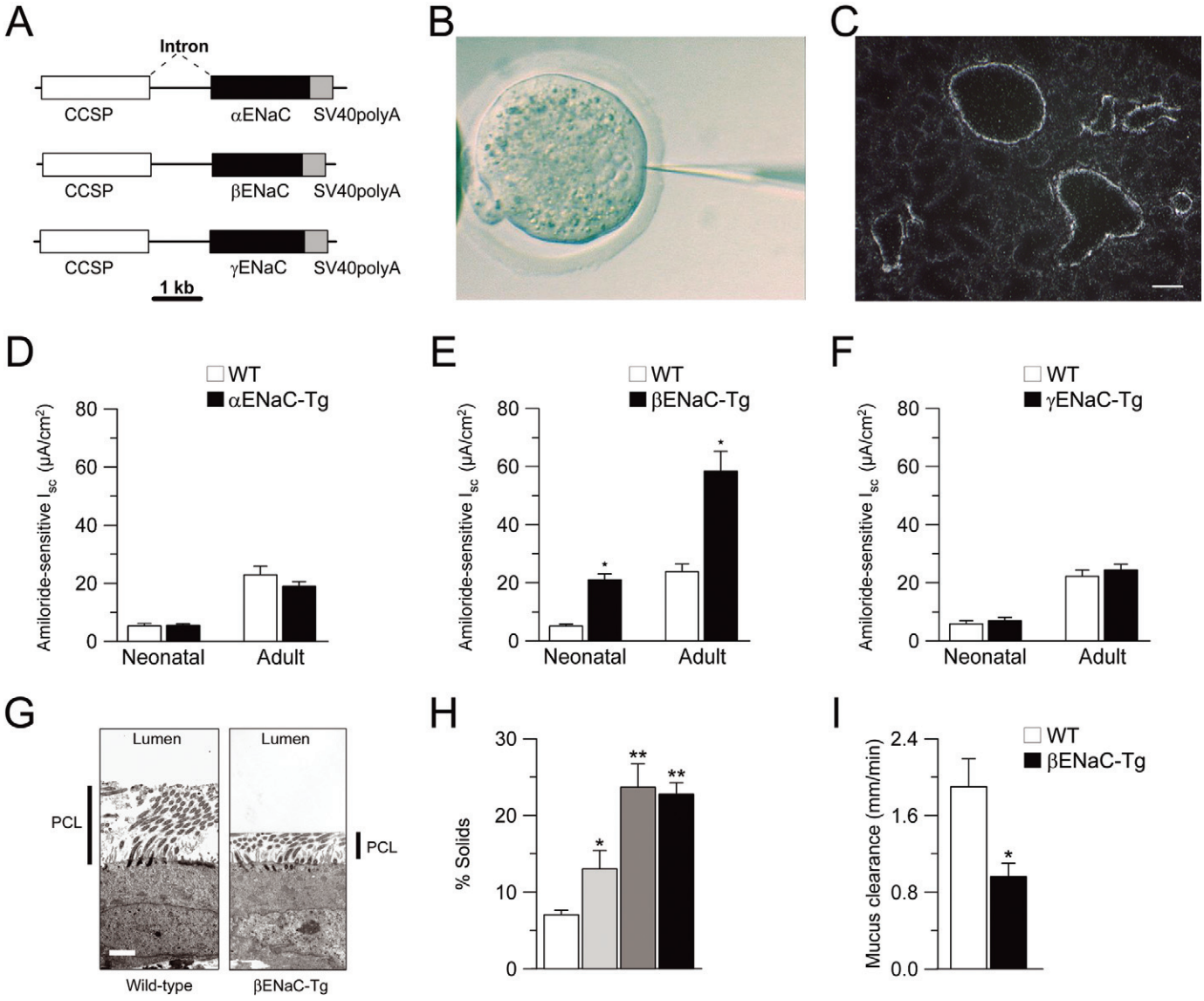

Fig. 1. Generation and functional characterization of transgenic mice (Tg) with airway-specific overexpression of the subunits (α, β and γ) of the epithelial Na^+ channel (ENaC). (A) Transgenic (Tg) constructs for airway-specific overexpression of individual ENaC subunit genes, containing the rat CCSP promoter (white), αENaC, βENaC or γENaC cDNAs (black), followed by the SV40 polyadenylation signal (gray). (B) Generation of Tg mice by pronuclear injection of constructs into fertilized oocytes. (C) *In situ* hybridization of a lung section from a neonatal βENaC-Tg mouse, using a Tg-specific anti-sense probe. Scale bar, 100 μm. (D–F) Amiloride-sensitive short-circuit current (I_{sc}) in neonatal and adult αENaC-, βENaC- and γENaC-Tg mice and wild-type (WT) littermates $^*P < 0.0001$ compared with WT. (G) PCL height in bronchi from WT and βENaC-Tg mice, as visualized by transmission electron microscopy after fixation with OsO4/PFC. Scale bar, 2.5 μm. (H) Percent solids of mucus sampled from airways of adult WT (white bar) and βENaC-Tg mice (light gray bar), and of mucus plugs removed from the tracheobronchial tree of βENaC-Tg adult (dark gray bar) and neonatal (black bar) mice. $^*P < 0.05$ compared with wild-type; $^{**}P < 0.05$ compared to mucus samples from WT and βENaC-Tg mice. (I) Mucus clearance in WT and βENaC-Tg mice. $^*P < 0.01$ compared with WT Adapted, with permission, from Mall et el. [30].

model to study the effect of raised ENaC-mediated absorption of Na^+ and fluid on ASL *in vivo*. To preserve the ASL *in situ*, lungs from βENaC-Tg mice were inflation fixed with 1 % osmium tetroxide in perfluorocarbon (OsO4/PFC) [35]. Morphometric studies by transmission electron microscopy of airway surfaces demonstrated that the height of the periciliary liquid (PCL) layer is significantly reduced on lower airway surfaces of βENaC-Tg mice (Fig. 1G) [30]. Independent evidence that increased Na^+ absorption causes ASL depletion came from measurements of airway mucus concentration in βENaC-Tg mice. Similar to CF patients [36], the water content of mucus specimens sampled from the airways *in vivo* as determined by measuring the ratio of wet to dry mucus weights, was significantly reduced in βENaC-Tg mice compared to wild-type littermate controls (Fig. 1H, Table 1). These results demonstrated that both compartments of the ASL, i.e. the PCL layer and the mucus layer were dehydrated by increased airway Na^+ absorption [30]. The pathophysiological significance of ASL dehydration was demonstrated by *in vivo* measurements of mucociliary clearance (MCC). Using a new technique that allows the determination of MCC in living mice from the clearance

Table 1
Pulmonary phenotypes of CF patients, Cftr-deficient mice and βENaC-Tg mice

	CF patients	Cftr mutant mice	βENaC-Tg mice
Genetic defect	CFTR mutations	Cftr knockout Specific Cftr mutations	βENaC overexpression
Ion transport in lower airways	Increased ENaC-mediated Na^+ absorption Deficient cAMP-dependent Cl^- secretion	Normal ENaC-mediated Na^+ absorption Normal cAMP-dependent Cl^- secretion	Increased ENaC-mediated Na^+ absorption Normal cAMP-dependent Cl^- secretion
ASL homeostasis in lower airways	Increased mucus concentration Reduced PCL height (*in vitro*)	Not determined	Increased mucus concentration Reduced PCL height (*in vivo*)
Mucociliary clearance	Reduced	Normal	Reduced
Airway mucus obstruction	Mucus plugging Goblet cell metaplasia	Absent	Mucus plugging Goblet cell metaplasia
Airway inflammation	Chronic neutrophilia	Absent	Chronic neutrophilia Transient eosinophilia
Airway infection	Chronic bacterial infection (*Pseudomonas aeruginosa* and other pathogens)	No spontaneous infection	No spontaneous infection
Disease mortality	>90% pulmonary mortality	No pulmonary mortality Variable gastrointestinal mortality	~50% pulmonary mortality

rate of a fluorescent dye deposited into the lower airways and measurements of the appearance of the dye in the trachea with microdialysis [11], it was demonstrated that ASL volume depletion reduced MCC in βENaC-Tg mice by ~50% (Fig. 1I, Table 1) [30]. Taken together, these studies showed that increased airway Na^+ transport in βENaC-Tg mice causes ASL depletion similar to CF airway cultures and CF sputum and that this mechanism impaired the mucociliary clearance component of lung defense similar to CF patients [16,33,36] (Table 1).

3. Modeling CF lung disease in βENaC-Tg mice

3.1. βENaC-Tg mice develop airway mucus plugging and pulmonary mortality

A first clue about the disease-causing effects of increased airway Na^+ absorption and impaired mucociliary clearance came from the observation that ~50 % of βENaC-Tg mice die within the first weeks of life (Fig. 2A). Observations of clinical signs of respiratory distress, such as intercostal and subdiaphragmatic retractions, indicated that the mortality was caused by airflow obstruction and asphyxia. This was confirmed by post mortem histopathological examination that revealed severe airway mucus plugging in the lung, but no pathology in other organ systems of βENaC-Tg mice (Fig. 2B, Table 1) [30]. Subsequently, longitudinal studies of lung morphology were performed to elucidate the onset and spontaneous progression of lung disease in βENaC-Tg mice. These studies demonstrated that lungs are structurally normal at birth and identified mucus obstruction of the trachea as an early and invariable lesion in the airways of βENaC-Tg mice [37]. Of note, mucus plugs in βENaC-Tg neonates form in the absence of goblet cell metaplasia and elevated mucin gene expression [37]. These results indicate that initial mucus obstruction results from mucus that is constitutively secreted onto airway surfaces where it accumulates due to lack of proper clearance. Thus ASL depletion alone, i.e. in the absence of mucus hypersecretion, is sufficient to cause mucus obstruction leading to severe airflow limitation and spontaneous pulmonary mortality in βENaC-Tg mice [37]. Of note, airway mucus plugging in the absence of goblet cell metaplasia was also the earliest change detected in the lungs of CF infants [2] (Table 1). The similarity in the early mucus plugging phenotype between βENaC-Tg mice and CF infants supports the concept that ASL depletion may also be sufficient to produce severe airway mucus obstruction in the human lung.

Juvenile and adult βENaC-Tg mice exhibit chronic lung disease with mucus obstruction throughout the conducting airways, substantial goblet cell metaplasia and elevated expression of airway mucins, including the secreted mucins Muc5ac and Muc5b and the membrane-tethered mucin Muc4, which likely contribute to airway mucus obstruction in βENaC-Tg mice with chronic CF-like lung disease (Fig. 2C, D) [37–39]. In contrast, Cftr mutant mice do not develop mucus obstruction, mucus hypersecretion or other CF-like abnormalities in the conducting airways (Fig. 2C, D) [4–6]. Thus the βENaC-Tg mouse provides an experimental model to study both the development of initial airway mucus plugging, as well as secondary goblet cell metaplasia and mucus hypersecretion observed in CF patients (Table 1).

3.2. Chronic airway inflammation in βENaC-Tg mice

In parallel to airway mucus obstruction and mucus hypersecretion, βENaC-Tg mice develop chronic airway inflammation. Longitudinal bronchoalveolar lavage (BAL) studies

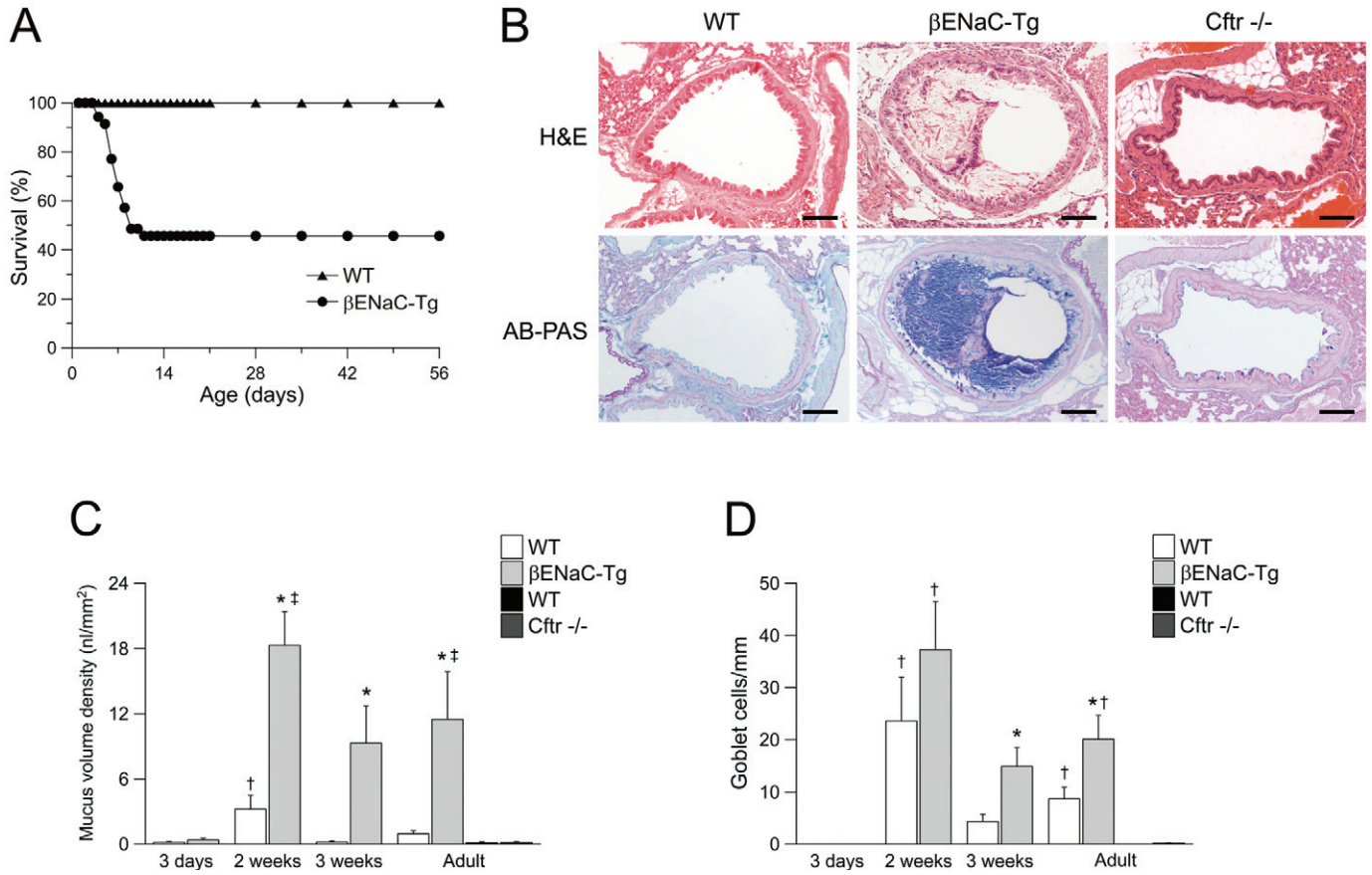

Fig. 2. Pulmonary mortality and development of airway mucus obstruction and goblet cell metaplasia in βENaC-Tg mice. (A) Survival curves for βENaC-Tg mice and wild-type (WT) littermates. (B) Representative airway histology from WT, βENaC-Tg and Cftr$^{-/-}$ mice. Sections were cut at the level of the proximal main axial airway and stained with Alcian blue-periodic acid Schiff (AB-PAS) to visualize mucus. Scale bars, 100 μm. (C, D) Summary of longitudinal development of airway mucus obstruction and goblet cell metaplasia in bronchi from neonatal (3-day-old), juvenile (2- and 3-week-old) and adult (6-week-old) βENaC-Tg mice, adult Cftr$^{-/-}$ mice and their respective WT littermates. n = 5–10 mice per group. (C) Airway mucus content, as determined by mucus volume density. $^{*}P < 0.01$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus 3-day-old and 3-week-old WT mice; $^{\ddagger}P < 0.05$ versus 3-day-old βENaC-Tg mice. (D) Goblet cell counts in proximal main axial airways. $^{*}P < 0.05$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus 3-day-old mice of same genotype. Note that Cftr$^{-/-}$ mice did not show airway mucus obstruction or goblet cell metaplasia. Adapted from Mall et al. [37] and reprinted with permission of the American Thoracic Society.

demonstrated that airway inflammation is characterized by an early influx of macrophages associated with transient elevation of TNFα and chronic neutrophilic inflammation with elevated levels of the neutrophil attractant chemokines KC and Mip-2 (constituting the murine homologues of human IL-8), findings consistent with the inflammatory response observed in human CF airways (Fig. 3A–H, Table 1). Surprisingly, these longitudinal BAL studies also identified transient eosinophilic airway inflammation with increased expression of the Th2 signaling molecule IL-13 (Fig. 3F, I) as well as the eosinophil attractant eotaxin-1 and other Th2 signature genes [37]. Likely, this inflammatory response, together with secondary goblet cell metaplasia and mucus hypersecretion was triggered by inhaled stimuli, such as particulate matter or bacterial products, and effector molecules released from inflammatory cells (e.g. neutrophil elastase) and/or airway epithelial cells (e.g. agonists of the epidermal growth factor receptor) [40–43] that were concentrated in mucostatic airways. The surprising finding that juvenile βENaC-Tg mice with a Th2 polarized immune system [44] develop transient allergic airway inflammation suggests that ASL depletion results in reduced clearance of inhaled allergens and is consistent with the increased prevalence of concomitant allergic airway disease such as allergic bronchopulmonary aspergillosis (ABPA) in CF patients.

3.3. Impaired clearance of bacterial pathogens, but no spontaneous respiratory tract infection in βENaC-Tg mice

Impaired mucociliary clearance [17,33,45], together with several other factors that compromise innate airways defense against bacterial pathogens, including anaerobic conditions in mucus obstructed airways [46], or neutrophil elastase mediated cleavage of the chemokine receptor CXCR1 on neutrophils disabling their bacterial-killing capacity [47] likely contribute to the chronic bacterial colonization and infection of the CF lung. Initial attempts to mimic bacterial infection in βENaC-Tg mice by intrapulmonary challenge with *Pseudomonas aeruginosa* or *Haemophilus influenzae*, isolated from airway secretions of CF patients indeed demonstrated that pulmonary clearance of these pathogens was substantially slowed in βENaC-Tg mice (Fig. 4A, B) [30]. Whereas

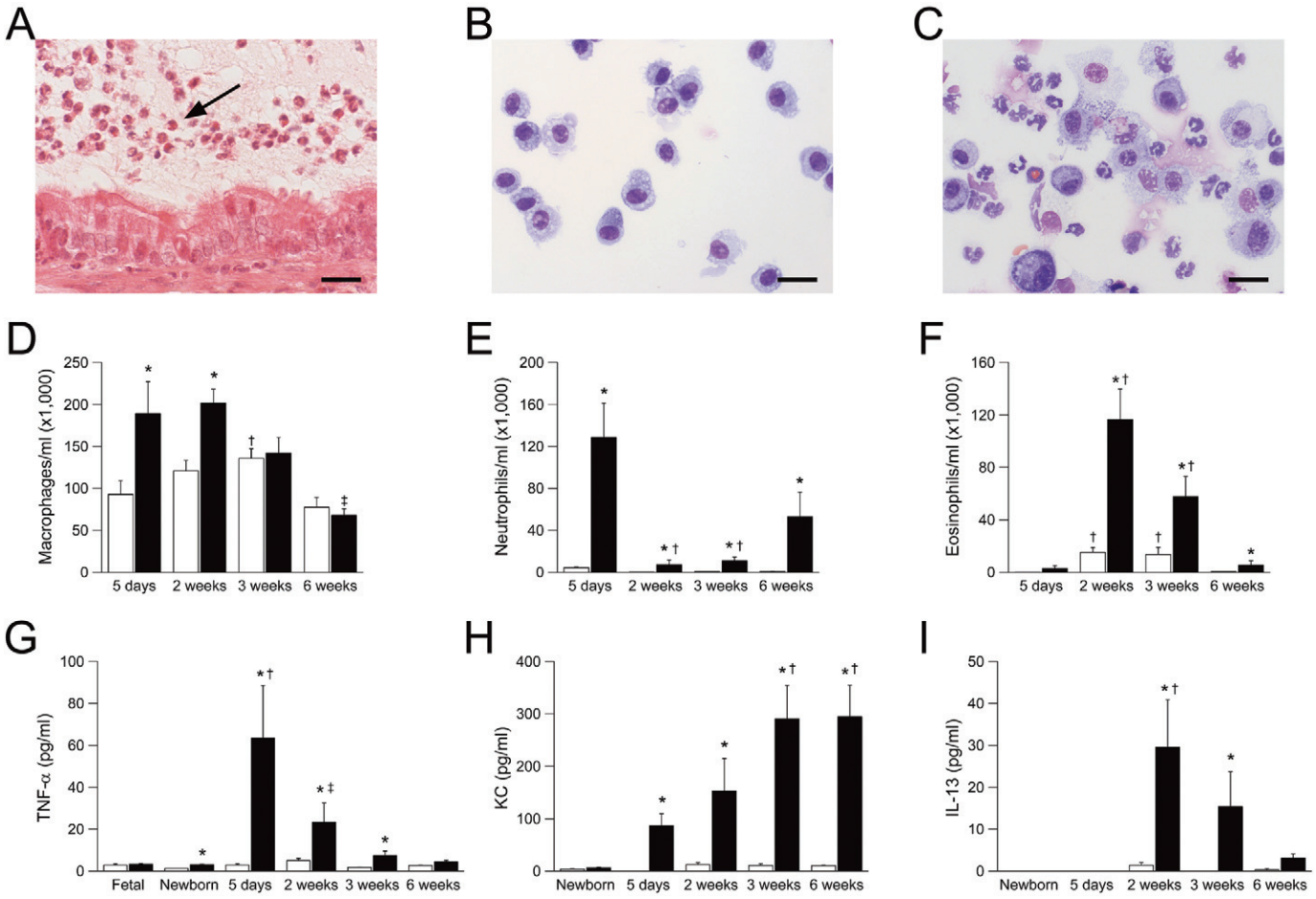

Fig. 3. Development of airway inflammation in βENaC-Tg mice. (A) Neutrophils (arrow) in airway mucus plaque in an adult βENaC-Tg mouse (stained with hematoxylin and eosin). Scale bar, 100 μm. (B, C) Cytospin preparations of bronchioalveolar lavage (BAL) cells (stained with May Grünwald Giemsa) from adult wild-type (WT) (B) and βENaC-Tg mice (C) showing activated macrophages and neutrophilia in βENaC-Tg mice. Scale bars, 100 μm. (D–F) Summary of longitudinal studies of BAL inflammatory cell counts in neonatal (5-day-old), juvenile (2- and 3-week-old) and adult (6-week-old) WT (open bars) and βENaC-Tg (solid bars) mice. (D) Macrophages. $*P < 0.01$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus 5-day-old and 6-week-old WT mice; $^{\ddagger}P < 0.05$ versus 5-day-old, 2-week-old, and 3-week-old βENaC-Tg mice. (E) Neutrophils. $*P < 0.001$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus 5-day-old βENaC-Tg mice. (F) Eosinophils. $*P < 0.001$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus 5-day-old and 6-week-old mice of same genotype. (G–I) Time course of expression of proinflammatory cytokines TNF-α, KC and IL-13 in lungs from fetal, neonatal, juvenile and adult WT (open bars) and βENaC-Tg (solid bars) mice. (G) TNF-α. $*P < 0.01$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus newborn βENaC-Tg mice; $^{\ddagger}P < 0.05$ versus fetal and newborn βENaC-Tg mice. (H) KC. $*P < 0.01$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus newborn βENaC-Tg mice. (I) IL-13. $*P < 0.05$ versus WT mice of same age; $^{\dagger}P < 0.05$ versus newborn and 5-day-old βENaC-Tg mice. Adapted from Mall et al. [37] and reprinted with permission of the American Thoracic Society.

wild-type mice cleared bacteria almost completely within 24 hours following a single intratracheal challenge, significant numbers of bacteria were detected in the lungs of βENaC-Tg mice for periods of 3 days up to 2 weeks. However, bacteria were cleared thereafter, and single challenges did not result in chronic bacterial infection in βENaC-Tg mice. Further, in contrast to CF patients, βENaC-Tg mice do not develop spontaneous bacterial infections, as evidenced by culturing lung homogenates on various growth media used in the diagnostic setting including blood agar, MacConckey agar and thioglycolate broth [30]. In the majority of lungs, no bacterial growth was detected by routine microbiological culture. Low grade growth ($\leq$ 30 cfu/lung) of lactobacilli, α-hemolytic streptococci, coagulase-negative staphylococci or micrococcus was detected in the lungs of some animals; however the bacterial counts were not different in βENaC-Tg mice compared to wild-type mice. More recently, in order to address the possibility that low numbers of bacteria escaped detection by these culture techniques, we performed quantitative analyses of bacterial DNA load by quantitative real time PCR that amplifies 16S ribosomal DNA in lungs from βENaC-Tg mice and wild-type littermates by using established protocols [48]. With this highly sensitive technique, we detected bacterial DNA in the lungs from most (∼70%) mice. However, bacterial DNA load was low and did not differ between βENaC-Tg and wild-type mice (Fig. 4C, Table 1).

Several possibilities may explain why βENaC-Tg mice, in contrast to CF patients, do not appear to develop spontaneous bacterial airway infections. First, it is possible that the conditions under which these murine experiments were performed (i.e. specific pathogen free environment and single challenge with human pathogens) did not adequately mimic the conditions under which chronic bacterial infections are acquired by CF patients (i.e. repetitive challenge and/or coin-

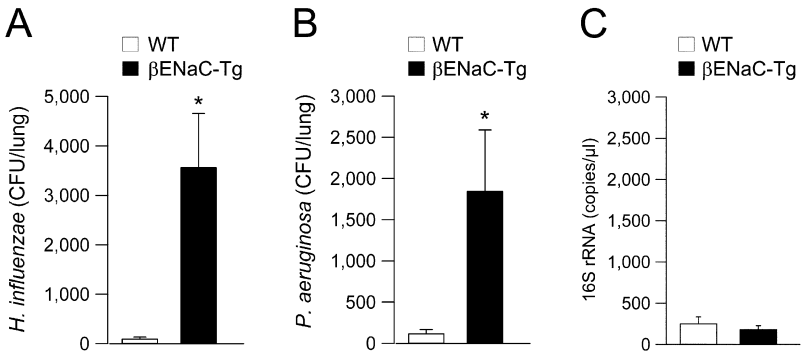

Fig. 4. Slowed clearance of bacterial pathogens, but no spontaneous bacterial infection in lungs of βENaC-Tg mice. (A, B) Increased bacterial burden 3 days after intratracheal instillation with clinical isolates of *Haemophilus influenzae* (A) or *Pseudomonas aeruginosa* (B) in lungs from βENaC-Tg mice compared with wild-type (WT) littermates. $*P < 0.01$. Adapted, with permission, from Mall et al. [30]. (C) Quantitative analysis of bacterial DNA load in lungs from WT and βENaC-Tg mice in the absence of bacterial challenge. DNA was extracted from lungs of adult WT and βENaC-Tg mice (n = 16 each) and total bacterial load was determined by quantitative real-time PCR that amplifies 16S ribosomal DNA, and quantified by applying a recombinant plasmid standard, as previously described [48]. Using this sensitive method, low levels of bacterial DNA were detected in both groups, however, bacterial load did not differ in lungs from WT versus βENaC-Tg mice.

fection with different pathogens). Second, mice may have host defense mechanisms that enable efficient bacterial killing in the lung even in the presence of impaired mucociliary clearance and chronic inflammation. Third, besides dysregulation of cAMP-mediated Cl^- secretion and ENaC-mediated Na^+ absorption, CFTR malfunction has been implicated in a number of other cellular dysfunctions including abnormal clearance of *Pseudomonas aeruginosa*, phagosome acidification in macrophages and ceramide metabolism [49–51]. These abnormalities may contribute to the high susceptibility for bacterial infection in lungs from CF patients, but not βENaC-Tg mice, in which Cftr function is normal. However, it should be stressed that Cftr mutant mice, similar to βENaC-Tg mice, also fail to develop spontaneous pulmonary bacterial infections [6]. The βENaC-Tg mouse should be a useful model to test these possibilities in future studies, which may provide novel insights into host–pathogen interactions in mucostatic airways *in vivo* and contribute to the development of novel anti-bacterial therapies for CF patients.

4. Application of the βENaC-Tg mouse in basic and translational research of CF lung disease

4.1. βENaC-Tg mice as a model to elucidate modifiers of CF lung disease

Clinical studies demonstrated that the severity of lung disease can vary substantially in CF patients carrying the same *CFTR* genotypes [52]. These observations suggest that the disease severity can be modulated by modifier genes that may alter airway ion transport, mucus secretion, inflammation or other important abnormalities in the CF lung. The βENaC-Tg mouse can be used as a tool to elucidate potential modifiers of CF lung disease by two different general strategies. First, cross-breeding of the βENaC-Tg mouse with knockout mouse models can be used to elucidate the role of specific candidate genes in the *in vivo* pathogenesis. So far, this approach has been used to determine the selective roles of TNF-α and IL-4Rα signaling in chronic airway inflammation in βENaC-Tg mice [38]. Although TNF-α and IL-13 were markedly elevated at early time points [37] genetic deletion of their respective receptors, i.e. TNFR1 and IL-4Rα, did not affect airway neutrophilia and mucus obstruction in adult βENaC-Tg mice [38]. These results suggest that other signaling pathways are responsible for the development of chronic inflammation in mucostatic airways. Second, phenotypic and genetic analyses of βENaC-Tg mice with different genetic backgrounds provide additional opportunities to study the role of modifier genes. Recent experiments in which the βENaC-Tg mouse was backcrossed from its original mixed genetic background (C3H x C57BL/6 hybrids) onto the parental strains and other commonly used inbred strains such as BALB/c demonstrated that genetic background has profound effects on the severity of pulmonary mortality [53]. Therefore, we predict that further functional and genetic studies of βENaC-Tg mice with different genetic backgrounds and divergent pulmonary phenotypes have a high potential to identify genetic modifiers of CF lung disease [54].

4.2. In vivo evaluation of therapies for CF lung disease in βENaC-Tg mice

The βENaC-Tg mouse also provides a powerful tool for preclinical evaluation of treatment strategies targeting ASL depletion, airway mucus obstruction and chronic inflammation in CF lung disease. In a recent study, we were able to demonstrate that pharmacological inhibition of increased Na^+ absorption and ASL depletion by preventive intrapulmonary treatment with the ENaC blocker amiloride had significant therapeutic benefits in this model of CF lung disease [24,55]. Preventive administration of amiloride, from the first day of life for a period of 2 weeks, had significant mucolytic effects as evidenced by a substantial reduction of airway mucus obstruction, goblet cell metaplasia and mucus hypersecretion, and reduced the spontaneous pulmonary mortality of βENaC-Tg mice by ∼70% (Fig. 5A–C). Interestingly, preventive amiloride treatment also had potent anti-inflammatory effects in βENaC-Tg mice, likely due to improved clearance of

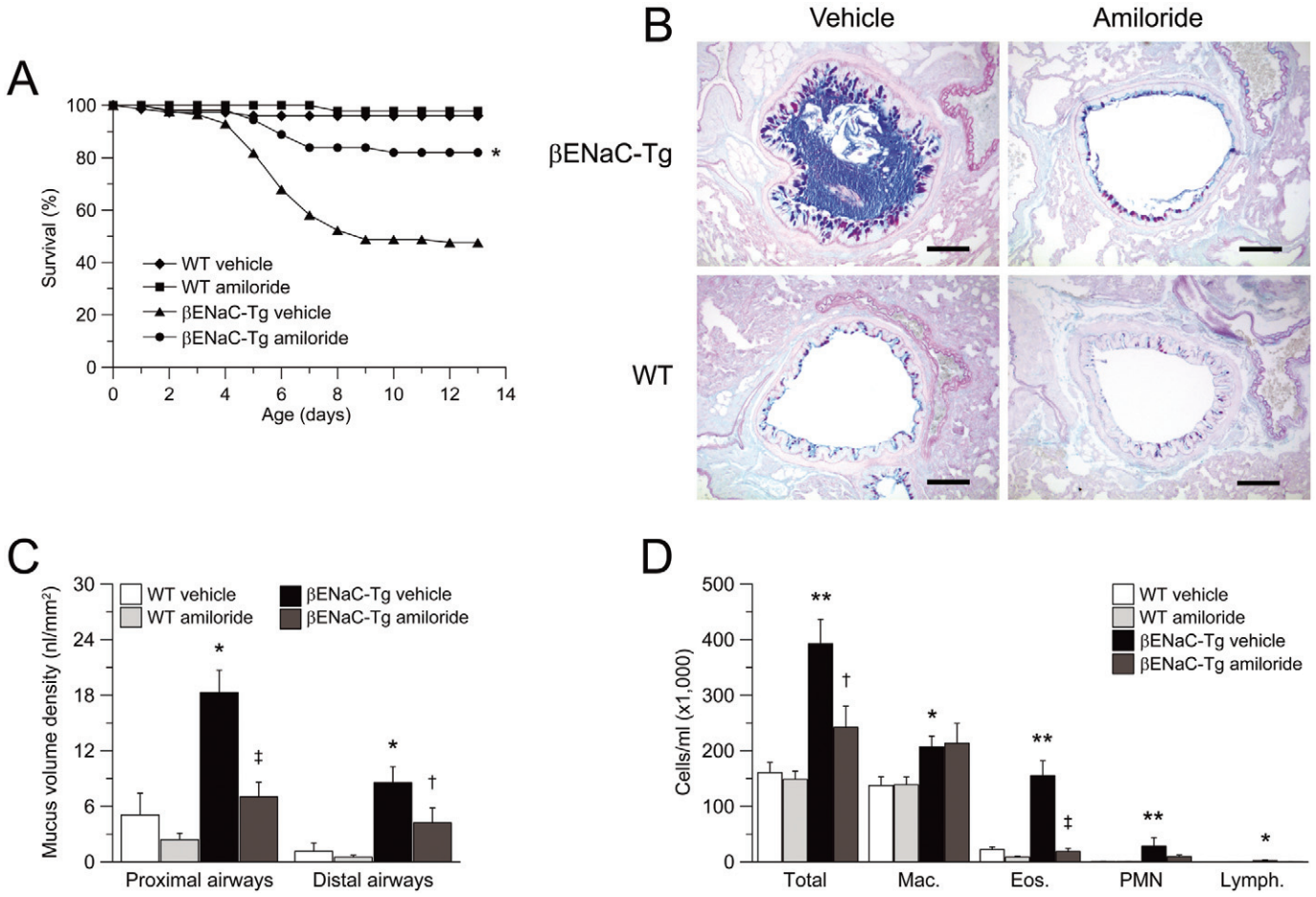

Fig. 5. Preventive amiloride therapy reduces mortality, airway mucus obstruction and airway inflammation in βENaC-Tg mice. Effects of preventive amiloride treatment, administered from the first day of life for a period of 2 weeks on (A) survival, (B and C) airway mucus obstruction and (D) airway inflammation in βENaC-Tg mice and wild-type (WT) littermates. (A) Survival curves for βENaC-Tg and WT mice treated with amiloride or vehicle alone. $^{*}P < 0.001$ compared with vehicle-treated βENaC-Tg mice. (B) Airway histology of βENaC-Tg and WT mice after preventive treatment with amiloride or vehicle alone. Sections were stained with Alcian blue-periodic acid Schiff (AB-PAS) to visualize intraluminal mucus and goblet cells. Scale bars, 100 μm. (C) Airway mucus content was determined by measuring the volume density of AB-PAS-positive material in proximal and distal main axial airways. $^{*}P < 0.001$ compared with vehicle-treated WT; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated βENaC-Tg mice. (D) BAL inflammatory cell counts. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with vehicle-treated WT mice; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated βENaC-Tg mice. Adapted from Mall et al. [37] and reprinted with permission of the American Thoracic Society.

inhaled irritants and allergens that otherwise accumulated and triggered chronic inflammation in mucostatic airways [55]. Taken together, these results provide a proof-of-concept that preventive treatment with amiloride is an effective mucolytic and anti-inflammatory therapy in this *in vivo* model of CF lung disease, and suggest that this preventive strategy may be beneficial for CF patients who are diagnosed in early infancy by CF newborn screening [3,56].

However, similar to observations in previous clinical trials [57,58], amiloride had no effect on airway mucus obstruction or inflammation when treatment was started in βENaC-Tg mice with chronic lung disease [55]. We predict that the βENaC-Tg mouse will be useful to determine if the lack of therapeutic effects of amiloride in chronic lung disease is caused by its low potency and rapid absorption from airway surfaces [59,60] or, alternatively, by the persistence of anatomical and functional abnormalities in chronically inflamed airways [61], even if ASL is restored. With the recent development of novel highly potent and long-acting ENaC blockers, such as 552-02 [62–64] it will be possible to test if ENaC-directed pharmacotherapy can be beneficial in established CF lung disease. Besides the preclinical evaluation of *in vivo* effects of specific ENaC blockers, the βENaC-Tg mouse may also be used to test the efficacy of other mucolytic and anti-inflammatory agents. For example, DNAse and hypertonic saline were shown to improve lung function and quality of life as indirect outcome measures and are already used for the treatment of CF patients [65,66]. However, no information is available as to what extent these therapeutic agents reduce airway mucus obstruction and airway inflammation in the CF lung. Further, a direct comparison of the efficacy of DNAse and hypertonic saline is still pending. With an increasing number of new mucolytic and anti-inflammatory compounds becoming available for clinical testing, systematic and comparative preclinical evaluation in the βENaC-Tg mouse could help to pre-select the most promising compounds and stratify future clinical trials and new therapeutic strategies in CF patients. Such a strategy of systematic pre-selection of new

mucolytic and/or anti-inflammatory therapies should help to reduce the number of compounds that have to be tested in CF patients, and thus reduce the overall development costs, as well as the burden for patients associated with participation in multiple clinical trials. Of note, CFTR expression and function are not altered in the airways of βENaC-Tg mice. Therefore, this model does not allow testing of therapeutic effects of drugs designed to correct and/or potentiate the function of mutated CFTR.

5. Summary and conclusions

In summary, mimicking increased epithelial Na^+ absorption, i.e. a basic defect in CF airways, by airway-specific overexpression of βENaC in mice resulted in the first animal model with spontaneous CF-like lung disease characterized by airway mucus obstruction, goblet cell metaplasia, mucus hypersecretion, chronic airway inflammation, reduced clearance of bacterial pathogens and high pulmonary mortality [24,30,37,67]. Studies of the phenotype of the βENaC-Tg mouse provided a proof-of-concept that ASL depletion, due to an imbalance of ENaC-mediated Na^+ absorption and CFTR/CaCC-mediated Cl^- secretion, causes mucociliary dysfunction and that this mechanism plays a critical role in initiating CF-like lung disease *in vivo*. Thus, the βENaC-Tg mouse provides a useful tool for further elucidation of the complex *in vivo* pathogenesis, identification of modifier genes, and the *in vivo* evaluation of novel therapies targeting ASL depletion, mucus obstruction and chronic inflammation in CF lung disease [24,53,55]. Because overexpression of ENaC is restricted to the lung, and CFTR function is normal in βENaC-Tg mice, this model does not allow to study CF pathogenesis in other organ systems, nor to study the role of cellular dysfunctions caused by mutant CFTR that are independent of deficient Cl^- secretion and ASL homeostasis, e.g. phagosome acidification in macrophages, cellular lipid trafficking or ceramide metabolism [50,51,68]. However, this limitation could be overcome by crossbreeding of the βENaC-Tg mouse with Cftr mutant mice to produce double-mutant mice exhibiting both increased airway Na^+ absorption and CFTR malfunction in the lung.

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Conflict of interest

Z.Z., J.D., B.J., S.C.S., D.T., M.H., S.G., A.D., and C.S. have no financial or personal relationship with people or organizations that could inappropriately influence their work. M.A.M. is co-inventor on a patent filed on the βENaC-Tg mouse. Of note, the βENaC-Tg mouse has been deposited at the Jackson Laboratory for general disposition.

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