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Preventive but Not Late Amiloride Therapy Reduces Morbidity and Mortality of Lung Disease in β ENaC-overexpressing Mice

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Rationale: Increased airway Na⁺ absorption mediated by epithelial Na⁺ channels (ENaC) is a characteristic abnormality in the pathogenesis of cystic fibrosis (CF) lung disease. However, inhalation therapy with the ENaC blocker amiloride did not have therapeutic benefits in patients with CF with established lung disease.

Objectives: We hypothesized that preventive inhibition of increased Na⁺ absorption in a structurally normal lung may be required for effective therapy of CF lung disease *in vivo*, and that therapeutic effects of late amiloride intervention may be impeded by the chronic disease process.

Methods: To test this hypothesis *in vivo*, we used the β ENaC-overexpression mouse as a model of CF lung disease and determined therapeutic effects of preventive versus late amiloride therapy on survival, airway mucus plugging, chronic bronchitis, and airway remodeling.

Measurements and Main Results: We show that early intervention, i.e., from the first day of life, with the intranasal administration of amiloride significantly reduced pulmonary mortality, airway mucus obstruction, epithelial necrosis, goblet cell metaplasia, and airway inflammation in β ENaC-overexpressing mice. In contrast, consistent with previous human trials in patients with CF, amiloride administration did not have benefits if treatment was started after the development of CF-like lung disease in β ENaC-overexpressing mice.

Conclusions: We conclude that preventive inhibition of increased airway Na⁺ absorption provides an effective therapy for CF-like lung disease *in vivo*. These results suggest that amiloride therapy may be an effective preventive therapy for patients with CF if initiated early in life before the onset of lung disease.

Keywords: epithelial Na⁺ channels; airway surface liquid; cystic fibrosis; pharmacotherapy; mucus

Cystic fibrosis (CF) lung disease remains one of the most frequent lethal hereditary diseases, and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (1, 2). *CFTR* encodes a cAMP-dependent Cl⁻ channel and regulates the epithelial Na⁺ channel (ENaC) (3–7). In CF airway epithelia, *CFTR*-mediated Cl⁻ secretion is defective and ENaC-mediated

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Increased airway Na⁺ absorption produces airway surface dehydration and deficient mucociliary clearance, and plays an important role in the pathogenesis of cystic fibrosis (CF) lung disease.

What This Study Adds to the Field

This study demonstrates that early, but not late, inhibition of increased airway Na⁺ absorption provides an effective therapy for CF-like lung disease in a murine disease model. This preventive paradigm may potentially be of benefit for the treatment of patients with CF.

Na⁺ absorption is increased (8–11). Evidence from *in vitro* studies demonstrated that these ion transport defects in CF airways cause airway surface liquid (ASL) volume depletion, defective mucus clearance, and mucus adhesion, suggesting that ASL volume depletion is a key mechanism in the pathogenesis of CF lung disease (12–14). Further, a selective increase of airway Na⁺ absorption by airway-specific overexpression of the β -subunit of ENaC (encoded by the *Scnn1b* gene) in mice demonstrated that accelerated Na⁺ transport alone is sufficient to produce ASL volume depletion and the subsequent spectrum of CF-like lung disease, including airway mucus obstruction, goblet cell metaplasia, chronic neutrophilic airway inflammation, impaired clearance of bacterial pathogens, and ultimately mortality (15–18). Further, recent studies identified additional abnormalities in the lungs of β ENaC-overexpressing mice that have been reported less frequently in patients with CF, including airway epithelial necrosis, transient airway eosinophilia, and emphysema (17). Taken together, these results indicated that increased airway Na⁺ absorption plays a critical role in the pathogenesis of CF and suggest ENaC as a pharmacologic target to improve airway surface hydration for treatment of a disease-initiating step in CF lung disease.

However, in previous clinical trials, inhalation therapy with the ENaC inhibitor amiloride (19) did not demonstrate significant therapeutic benefits in patients with CF with established lung disease (20, 21). Recent studies of antimicrobial agents in CF suggest that early intervention may be more effective in reducing bacterial infection than treatment of established infection in CF lung disease (22, 23). Accordingly, we hypothesized that therapeutic effects of aerosolized amiloride were also impeded by factors related to the chronic disease process and that preventive inhibition of increased Na⁺ absorption, started in a structurally normal neonatal CF lung, may be required to effectively treat CF lung disease. To test this hypothesis *in vivo*, we used the β ENaC-overexpressing mouse as a model of CF lung disease (15, 16) and

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compared the effects of preventive amiloride treatment versus amiloride intervention after the onset of lung disease on survival, airway mucus obstruction, epithelial necrosis, airway remodeling, and airway inflammation. Some of the results of this study have been reported previously in the form of abstracts (24, 25).

METHODS

Experimental Animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany. The generation of β ENaC-overexpressing mice (line 6608) has been described previously (15). Further details on experimental animals are provided in the online supplement.

Amiloride Treatment Studies

Amiloride hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in sterile distilled water (ddH₂O). Newborn, 5-day-old, and 4-week-old β ENaC-overexpressing mice and wild-type littermates were treated by intranasal instillation of amiloride (10 mmol/L; 1 μ l/g body weight; three times per day) or equal volumes of vehicle (ddH₂O) alone for a period of 13 to 14 days. Pulmonary deposition studies in newborn mice indicated that approximately 4% of the amiloride dose delivered by intranasal instillation was deposited into the lungs (*see* Figure E1 in the online supplement). During amiloride treatment, growth and survival were monitored, and deficits in body mass observed in amiloride-treated mice were replaced by subcutaneous injections of isotonic saline (NaCl 0.9%) as described in the online supplement.

Twelve hours after the last treatment, bronchoalveolar lavage (BAL) was performed; lungs were removed for histology, morphometry, and transcript expression studies; and serum and urine were sampled to determine renal effects of absorbed amiloride on Na⁺ and K⁺ concentrations as described in the online supplement. To determine the effect of amiloride on airway epithelial necrosis, β ENaC-overexpressing mice and wild-type littermates were treated from the first day of life for a period of 3 days and the lungs were removed for histology 12 hours after the last treatment. To examine if effects of preventive amiloride therapy during the neonatal period were sustained in the absence of continuous ENaC blockade, amiloride withdrawal studies were performed as described in the online supplement. Finally, to determine if systemic application of amiloride or NaCl 0.9% had effects on lung disease, β ENaC-overexpressing mice were treated with subcutaneous injections of amiloride or NaCl 0.9% alone as described in the online supplement (Figure E3). Endpoint studies were performed by investigators blinded to the genotype and the treatment of the mice.

BAL Cell Counts and Cytokine Measurements

BAL was obtained and cell counts were determined as previously described (15). Macrophage size was determined as described in the online supplement. IL-13 concentrations were measured in BAL using ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's instructions as previously described (17).

Histology and Airway Morphometry

Lungs were removed through a median sternotomy, fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) or Alcian blue periodic acid-Schiff (AB-PAS) as previously described (17). Lungs were sectioned transversally at the level of the proximal intrapulmonary main axial airway near the hilus, and at the distal intrapulmonary axial airway as described in the online supplement. Airway mucus obstruction was assessed stereologically by determining mucus volume density as previously described (17, 26–28). The volume density of the airway epithelium was determined as a measure of epithelial height. Goblet cells were identified by the presence of intracellular AB-PAS-positive material, degenerative airway epithelial cells were identified by morphologic criteria, and numeric cell densities were quantitated by counting epithelial cells per mm of the basement membrane. Further details on morphometry are provided in the online supplement.

Real-Time RT-PCR

Quantitative RT-PCR for Muc5ac, Gob5, β ENaC, and Gapdh was performed on an Applied Biosystems 7500 Real Time PCR System

using TaqMan universal PCR master mix and inventoried TaqMan gene expression assays according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). Relative fold changes in target gene expression were calculated from the efficiency of the PCR reaction and the crossing point deviation between samples from the four treatment groups, and determined by normalization to expression of the reference gene Gapdh, as previously described (17).

Statistics

Data were analyzed with SigmaStat version 3.1 (Systat Software, Erkrath, Germany) and are reported as mean \pm SEM. We performed statistical analyses using Student's *t* test, Mann-Whitney Rank Sum test, one-way ANOVA, Kruskal-Wallis ANOVA on Ranks, and Kaplan-Meier survival analysis as appropriate, and *P* < 0.05 was accepted to indicate statistical significance.

RESULTS

Preventive Amiloride Therapy Reduces Mortality of CF-like Lung Disease in β ENaC-overexpressing Mice

The lungs of β ENaC-overexpressing mice are structurally normal at birth, but develop central airway mucus obstruction in the first days of life (15). To evaluate effects of preventive amiloride therapy on CF-like lung disease, amiloride administration to β ENaC-overexpressing mice was started on the first day of life (*i.e.*, before the onset of lung disease) using a protocol of intranasal administration of amiloride (10 mmol/L; 1 μ l/g body weight) or vehicle (ddH₂O) alone three times daily for a period of 2 weeks. Wild-type littermates were treated with the same protocol to assess for pulmonary toxicity of amiloride therapy. Renal effects of absorbed amiloride were determined by measuring Na⁺ and K⁺ concentrations in serum and urine, and weight loss due to diuresis. Volume losses were replaced by subcutaneous injections of isotonic saline (NaCl 0.9%) (Figure E1).

Using this treatment protocol, we first measured the effect of preventive amiloride therapy on survival. Similar to the spontaneous pulmonary mortality observed in previous studies (15, 17), vehicle-treated β ENaC-overexpressing mice exhibited a mortality rate of approximately 50% (Figure 1A). Preventive amiloride treatment resulted in a delayed onset, with an overall reduction of pulmonary mortality by approximately 70% in β ENaC-overexpressing mice. Amiloride had no adverse effects on survival in wild-type littermates (Figure 1A).

Early Amiloride Therapy Prevents Airway Mucus Obstruction, Goblet Cell Metaplasia, and Mucus Hypersecretion in β ENaC-overexpressing Mice

Our previous studies indicated that mortality in β ENaC-overexpressing mice is caused by asphyxia due to severe postnatal central airway mucus plugging, and demonstrated that surviving mice develop mucus obstruction, epithelial remodeling with goblet cell metaplasia and epithelial thickening, and mucus hypersecretion in intrapulmonary airways during the first weeks of life (15, 17). We, therefore, next measured the effects of preventive amiloride therapy on these characteristic pathologic features in surviving β ENaC-overexpressing mice. As predicted from previous studies, airway mucus content was significantly elevated in vehicle-treated β ENaC-overexpressing mice versus wild-type littermates (Figures 1B and 1C) (17). Preventive amiloride treatment significantly reduced airway mucus obstruction to near-normal values in proximal and distal airway regions of β ENaC-overexpressing mice (Figures 1B and 1C). Further, early amiloride therapy prevented goblet cell metaplasia and epithelial thickening observed in distal airways of vehicle-treated β ENaC-overexpressing mice (Figures 1D and 1E). Inhibition of goblet cell metaplasia and airway mucus obstruction was paral-

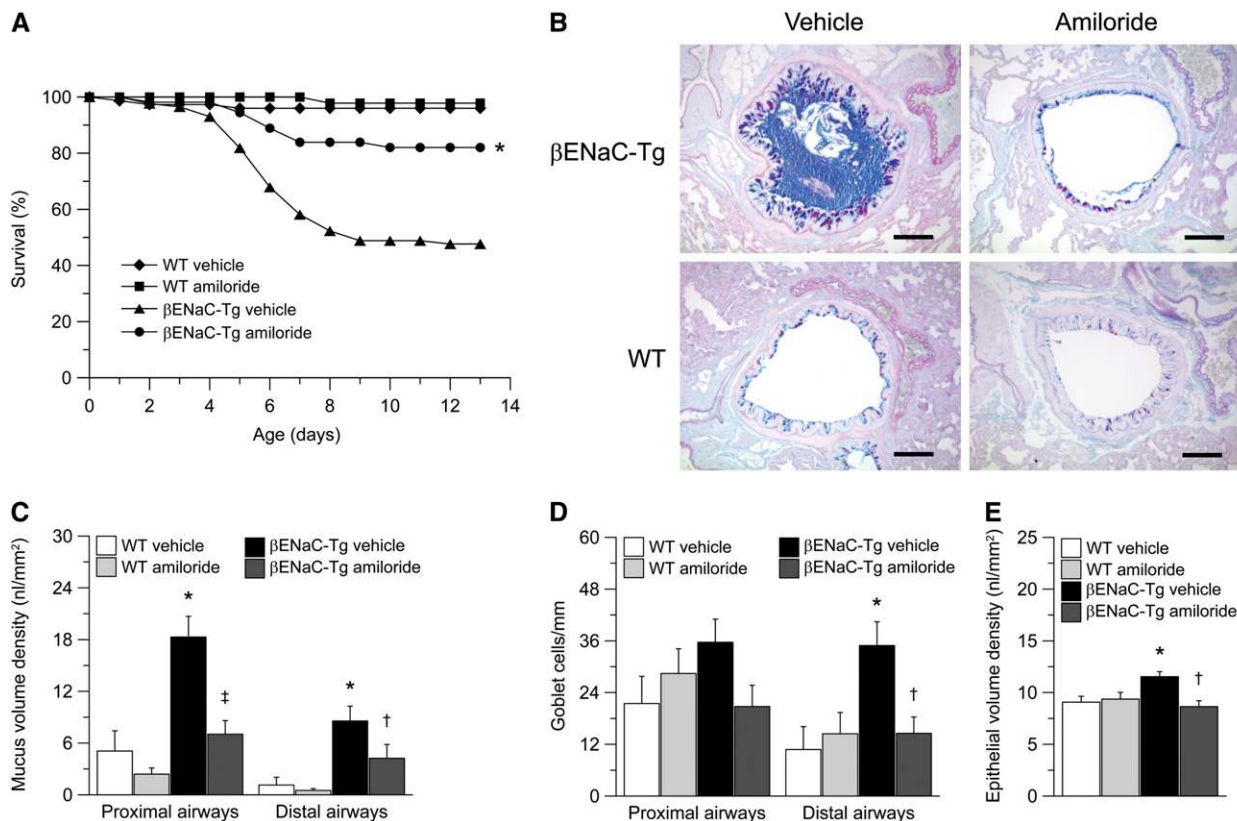


Figure 1. Preventive amiloride therapy reduces mortality, airway mucus obstruction, and epithelial remodeling in β -subunit of the epithelial Na^+ channel (β ENaC)-overexpressing mice. Effect of preventive amiloride treatment, administered from the first day of life for a period of 2 weeks on (A) survival; (B and C) airway mucus content, (D) goblet cell counts, and (E) epithelial height in β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates. (A) Survival curves for β ENaC-overexpressing and wild-type mice treated with amiloride or vehicle alone ($n = 46$ – 86 mice for each group; $*P < 0.001$ compared with vehicle-treated β ENaC-overexpressing mice). (B) Airway histology of β ENaC-overexpressing and wild-type mice after preventive treatment with amiloride or vehicle. Sections were stained with Alcian blue–periodic acid Schiff (AB-PAS) to determine the presence of intraluminal mucus and goblet cells. Representative of $n = 15$ – 27 mice for each group. Scale bars = $100 \mu\text{m}$. (C) Mucus content was determined by measuring the volume density of AB-PAS-positive material in proximal and distal main axial airways ($n = 15$ – 27 mice for each group; $*P < 0.001$ compared with vehicle-treated wild-type, $^\dagger P < 0.05$ compared with vehicle-treated β ENaC-overexpressing mice, $^\# P < 0.001$ compared with vehicle-treated β ENaC-overexpressing mice). (D) Goblet cell densities in proximal and distal main axial airways were determined from the number of AB-PAS-positive epithelial cells per mm of the basement membrane ($n = 15$ – 27 mice for each group; $*P < 0.01$ compared with vehicle-treated wild-type, $^\dagger P < 0.05$ compared with vehicle-treated β ENaC-overexpressing mice). (E) Epithelial height was determined by measuring the volume density of the epithelium in main axial airways ($n = 15$ – 27 mice for each group; $*P < 0.001$ compared with vehicle-treated wild-type, $^\dagger P < 0.01$ compared with vehicle-treated β ENaC-overexpressing mice).

led by a significant reduction of transcript levels of the goblet cell marker *Gob5* and the airway mucin *Muc5ac* in lungs from amiloride-treated compared with vehicle-treated β ENaC-overexpressing mice (Figures 2A and 2B). In contrast, preventive ENaC blocker therapy had no effect on expression of β ENaC mRNA in lungs from β ENaC-overexpressing mice (Figure 2C), indicating that therapeutic effects of amiloride were conferred by pharmacologic inhibition of ENaC-mediated Na^+ absorption rather than reduced β ENaC expression.

Evaluation of lungs from vehicle- and amiloride-treated wild-type mice did not reveal any signs of pulmonary toxicity caused by amiloride therapy. Specifically, preventive amiloride therapy did not alter airway mucus content, goblet cell numbers, epithelial height, or *Gob5* and *Muc5ac* expression in amiloride-treated compared with vehicle-treated wild-type mice (Figures 1 and 2).

Loss of body mass due to increased diuresis and excretion of Na^+ in the urine of amiloride-treated β ENaC-overexpressing and wild-type mice (Figure E1) indicated that intranasally administered amiloride was at least partially absorbed, resulting

in systemic effects including ENaC blockade in the kidney. To examine the possibility that therapeutic benefits of intranasal amiloride treatment (Figures 1 and 2) were caused by systemic rather than by topical inhibition of ENaC in the airways, we performed studies in which β ENaC-overexpressing mice and wild-type littermates were treated systemically with subcutaneous injections of amiloride (0.1 mmol/l , dissolved in NaCl 0.9% ; $100 \mu\text{l/g}$ body weight; 3 times per day), or equal volumes of NaCl 0.9% alone, for a period of 2 wk. Of note, the dose of subcutaneously administered amiloride was equivalent to the total dose used in intranasal treatment studies (Figures 1 and 2). Systemic amiloride treatment caused more severe growth retardation in mice of both genotypes than topical treatment, but did not have therapeutic effects on pulmonary mortality, airway mucus obstruction, or goblet cell metaplasia in β ENaC-overexpressing mice (Figures E3A–E3D). Treatment with subcutaneous NaCl 0.9% alone also had no effect on the pulmonary phenotype in β ENaC-overexpressing mice (Figure 1, and Figures E3A–E3D).

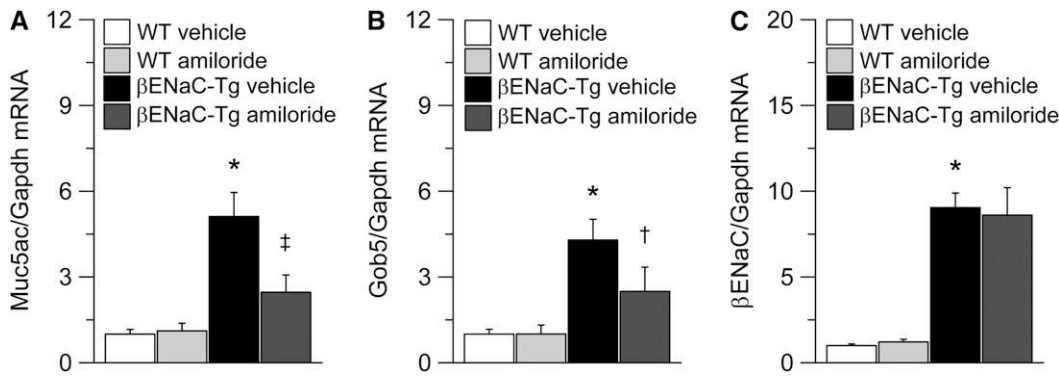


Figure 2. Preventive amiloride therapy reduces increased mucin expression in β ENaC-overexpressing mice. (A–C) Expression levels of Muc5ac, Gob5, and β ENaC transcripts in lungs from wild-type and β ENaC-overexpressing mice after 2 weeks of preventive amiloride treatment ($n = 13$ – 15 mice for each group; * $P \leq 0.001$ compared with vehicle-treated wild-type, † $P < 0.05$ compared with vehicle-treated β ENaC-overexpressing mice, * $P < 0.01$ compared with vehicle-treated β ENaC-overexpressing mice).

Amiloride Treatment Has No Effect on Mucus Obstruction, Goblet Cell Metaplasia and Mortality in β ENaC-overexpressing Mice with Established CF-like Lung Disease

Based on the therapeutic benefits of preventive intranasal amiloride therapy, we next mimicked the paradigm of previous clinical trials in patients with CF (20, 21, 29) and tested the effect of amiloride administration on mucus obstruction and mucus

hypersecretion in adult β ENaC-overexpressing mice with established CF-like lung disease. We started treatment at the age of 4 weeks, when β ENaC-overexpressing mice exhibit chronic airway mucus obstruction and remodeling with goblet cell metaplasia and epithelial hypertrophy (15, 17), and continued treatment of β ENaC-overexpressing mice and their wild-type littermates by intranasal instillation of amiloride or vehicle alone for a period of

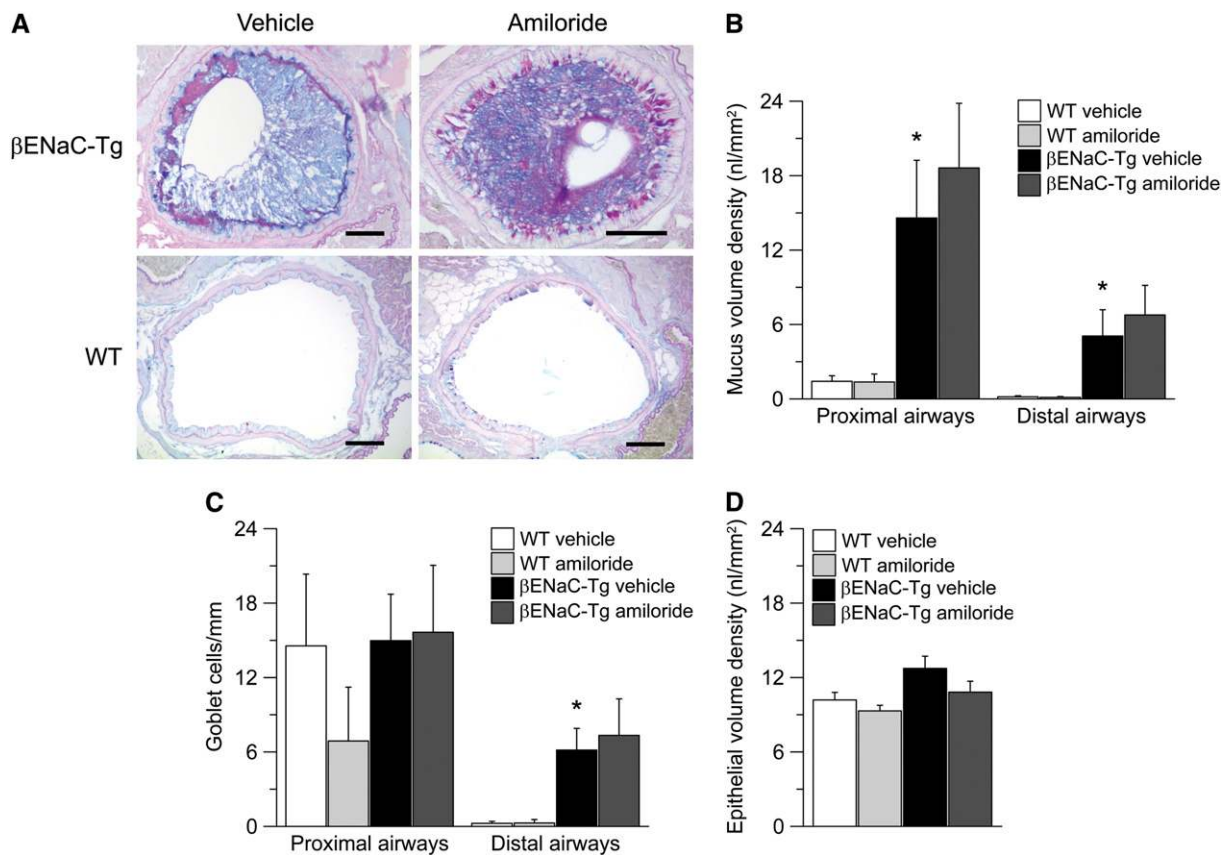


Figure 3. Late amiloride treatment in adult β ENaC-overexpressing mice with chronic CF-like lung disease has no therapeutic effects on airway mucus obstruction or goblet cell metaplasia. Effects of late amiloride treatment, administered from the age of 4 weeks for a period of 2 weeks on (A and B) airway mucus content, (C) goblet cell counts, and (D) epithelial height in adult β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates ($n = 9$ – 11 mice for each group). (A) Airway histology from adult β ENaC-overexpressing mice and wild-type littermates after administration of amiloride or vehicle for 2 weeks stained with AB-PAS to determine the presence of intraluminal mucus and goblet cells. Scale bars = 100 μ m. (B) Mucus content was determined by measuring the volume density of AB-PAS-positive material in proximal and distal main axial airways (* $P < 0.01$ compared with vehicle-treated wild-type). (C) Goblet cell densities in proximal and distal main axial airways were determined from the number of AB-PAS-positive epithelial cells per mm of the basement membrane (* $P < 0.001$ compared with vehicle-treated wild-type). (D) Epithelial height, as determined from measurement of epithelial volume density in main axial airways.

2 weeks as for the preventive amiloride study (Figure 1). In contrast to preventive therapy, initiating amiloride treatment in adult mice reduced neither proximal nor distal airways mucus obstruction (Figures 3A and 3B), nor goblet cell metaplasia (Figure 3C) and epithelial thickening (Figure 3D) in amiloride-treated versus vehicle-treated β ENaC-overexpressing mice.

Because recent studies demonstrated that mucus plugging in β ENaC-overexpressing mice originates in the trachea during the first week of life in the absence of goblet cell metaplasia and intrapulmonary mucus obstruction (17), we asked if amiloride therapy was still effective when treatment was started at the age of 5 days—that is, after the onset of proximal mucus plug formation, but before the establishment of chronic lung disease in β ENaC-overexpressing mice. In contrast to preventive therapy administered from the first day of life (Figure 1), initiating amiloride treatment in mice that were alive at the age of 5 days for a period of 2 weeks failed to reduce mortality in amiloride-treated versus vehicle-treated β ENaC-overexpressing mice (Figure 4A). Further, initiating amiloride treatment at the age of 5 days had no effect on airway mucus obstruction (Figure 4B), goblet cell metaplasia (Figure 4C), or epithelial thickening (Figure 4D) in β ENaC-overexpressing mice. Collectively, these data demon-

strate that preventive amiloride treatment is effective in reducing airway mucus obstruction, airway remodeling, mucin hypersecretion, and pulmonary mortality (Figures 1 and 2), but that these therapeutic effects were abrogated when treatment was started after the onset of CF-like lung disease (Figures 3 and 4) in β ENaC-overexpressing mice.

Preventive Amiloride Therapy Reduces Airway Inflammation in β ENaC-overexpressing Mice

In addition to airway mucus obstruction and mucus hypersecretion, chronic airway inflammation is an invariable feature of CF lung disease, leading to progressive damage and remodeling of the lung (30–32). We, therefore, next asked if preventive intranasal amiloride therapy had therapeutic effects on airway inflammation in β ENaC-overexpressing mice. Consistent with a Th2-biased immune system in the neonatal period (33, 34), we have previously shown that spontaneous airway inflammation in 2-week-old β ENaC-overexpressing mice is predominated by eosinophils associated with morphologically activated macrophages (i.e., foam cells), elevated numbers of neutrophils, and increased levels of the Th2-signaling molecule IL-13 (17). Evaluation of BAL fluid for inflammatory cells at the end of the

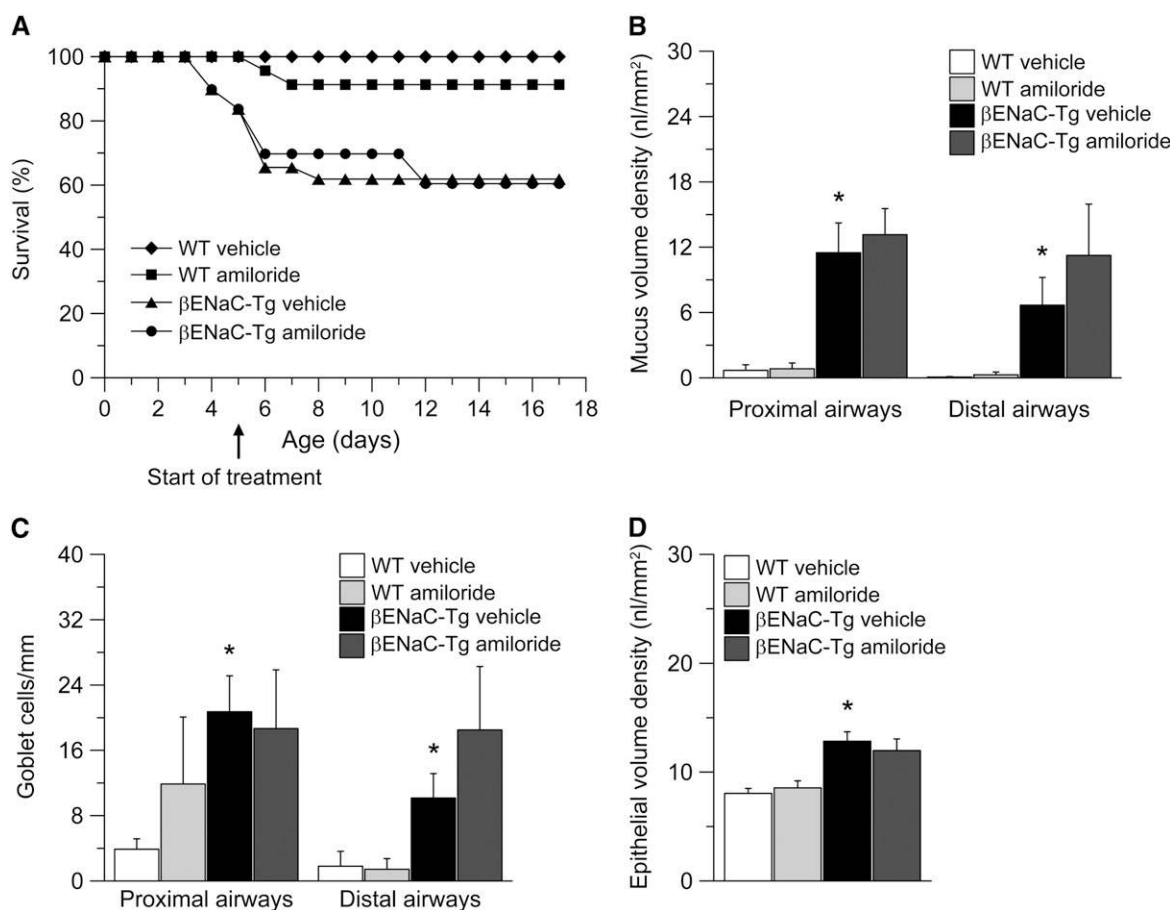


Figure 4. Initiating amiloride treatment in juvenile β ENaC-overexpressing mice with established mucus plugging has no therapeutic effects on airway mucus obstruction, goblet cell metaplasia, or pulmonary mortality. Effect of amiloride treatment, administered from the age of 5 days for a period of 2 weeks on (A) survival, (B) airway mucus content, (C) goblet cell counts, and (D) epithelial height in juvenile β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates. (A) Survival curves for β ENaC-overexpressing and wild-type mice treated with amiloride or vehicle alone from the age of 5 days ($n = 18$ –34 mice for each group). (B) Mucus content in proximal and distal main axial airways ($n = 7$ –11 mice for each group; $*P < 0.001$ compared with vehicle-treated wild-type). (C) Goblet cell counts in proximal and distal main axial airways ($n = 7$ –11 mice for each group; $*P < 0.01$ compared with vehicle-treated wild-type). (D) Epithelial volume density as a measure of epithelial height was determined in main axial airways ($n = 7$ –11 mice for each group; $*P < 0.001$ compared with vehicle-treated wild-type).

2-week treatment period revealed that total cells and eosinophil numbers were significantly reduced in amiloride-treated versus vehicle-treated β ENaC-overexpressing mice (Figures 5A and 5B). Notably, total macrophage numbers were not changed, but macrophage activation was significantly reduced by preventive amiloride therapy in β ENaC-overexpressing mice (Figures 5A–5C). The reduction of airway eosinophilia was paralleled by a significant reduction in IL-13 levels in BAL from amiloride-treated versus vehicle-treated β ENaC-overexpressing mice (Figure 5D). In wild-type littermates, preventive amiloride therapy did not have adverse effects on BAL cellularity, macrophage morphology, or IL-13 concentration (Figures 5A–5D).

In contrast to intranasal treatment, subcutaneous administration of amiloride had no effect on BAL inflammatory cell counts in β ENaC-overexpressing mice (Figure E3E). Taken together, our results show that preventive topical inhibition of airway Na^+ hyperabsorption was efficient in reducing the chronic airway inflammation characteristic of the CF-like lung disease in β ENaC-overexpressing mice.

Amiloride Treatment Has No Effect on Airway Inflammation in β ENaC-overexpressing Mice with Established CF-like Lung Disease

Next, we evaluated the effects of amiloride intervention on airway inflammation in 5-day-old (Figures 6A, 6C, and 6E) and 4-week-old (Figures 6B, 6D, and 6F) β ENaC-overexpressing mice with established CF-like lung disease (15, 17). In contrast to the antiinflammatory effects provided by preventive amiloride therapy, starting amiloride treatment after the onset of lung disease had no effect on elevated BAL inflammatory cell counts (Figures 6A and 6B), morphologic macrophage activation (Figures 6C and 6D), or IL-13 levels in BAL (Figures 6E and 6F) from amiloride-treated versus vehicle-treated β ENaC-overexpressing mice.

Therapeutic Effects of Preventive Amiloride Treatment Were Not Sustained if Treatment Was Discontinued in β ENaC-overexpressing Mice

Next, we performed amiloride withdrawal studies to determine if therapeutic effects of preventive amiloride treatment (Figures 1 and 5) were sustained, or if airway mucus obstruction and airway inflammation recurred, when amiloride treatment was stopped in β ENaC-overexpressing mice. In these studies, amiloride or vehicle alone were applied from the first day of life for a period of 2 weeks as described for the experiments shown in Figures 1 and 5. Then treatment was discontinued, mice were monitored for survival, and BAL was performed and lungs of mice from all treatment groups were evaluated for mucus obstruction at the age of 6 weeks. Withdrawal of amiloride treatment did not cause recurrence of mortality in β ENaC-overexpressing mice (data not shown). However, the severity of airway mucus obstruction, goblet cell metaplasia, epithelial hypertrophy, and BAL inflammatory cell counts were not different in β ENaC-overexpressing mice that were temporarily treated with either amiloride or vehicle alone (Figure 7). These results demonstrate that early but temporary amiloride therapy reduced neonatal mortality, but did not have sustained therapeutic effects on mucus obstruction and airway inflammation in β ENaC-overexpressing mice.

Preventive Amiloride Therapy Reduces Epithelial Necrosis in β ENaC-overexpressing Mice

The mechanistic links between increased epithelial Na^+ absorption, reduced ASL volume, and airway inflammation in the β ENaC-overexpressing mouse are likely multiple. One clue to such a link emanates from recent studies that demonstrated that neonatal (but not 4-wk-old) β ENaC-overexpressing mice develop airway epithelial hypoxia and epithelial cell necrosis, likely

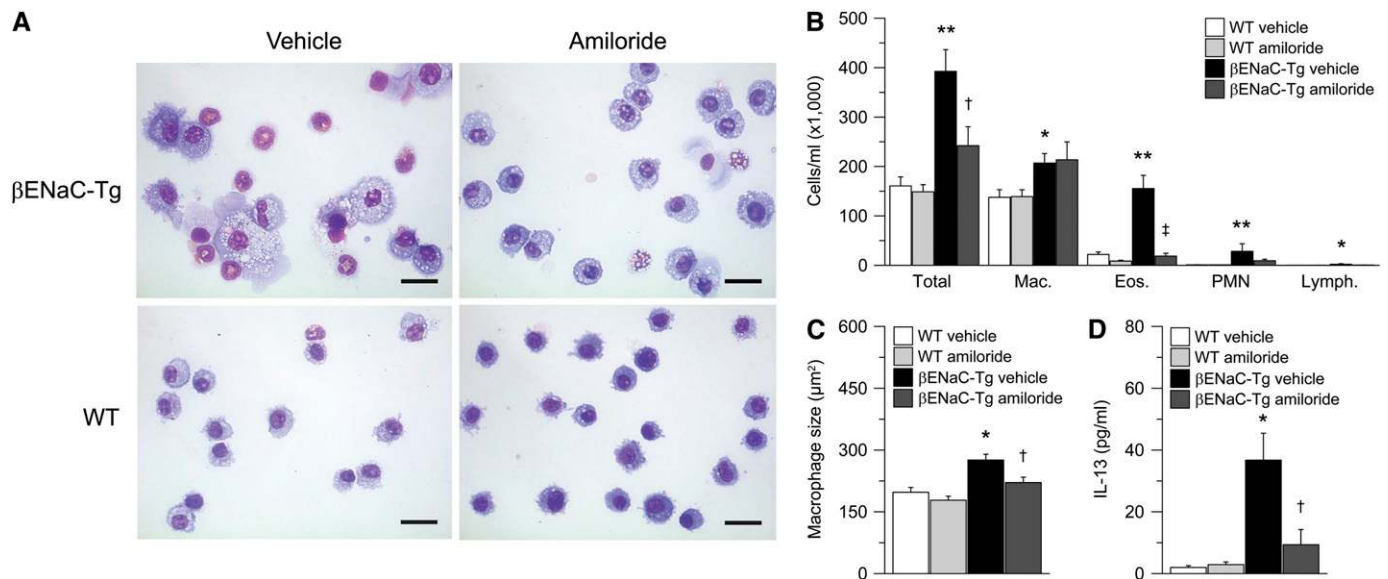


Figure 5. Preventive amiloride therapy reduces airway inflammation in β ENaC-overexpressing mice. Effect of preventive treatment with amiloride or vehicle alone, administered from the first day of life for a period of 2 weeks, on (A) macrophage morphology, (B) inflammatory cell counts, (C) macrophage size, and (D) concentration of the Th2 cytokine IL-13 in bronchoalveolar lavage (BAL) from β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates. (A) Morphology of BAL macrophages (stained with May Grünwald Giemsa). Representative of $n = 24$ –40 mice for each group. Scale bars = 20 μm . (B) BAL cell counts ($n = 24$ –40 mice for each group; * $P < 0.05$ compared with vehicle-treated wild-type, ** $P < 0.001$ compared with vehicle-treated wild-type, † $P < 0.05$ compared with vehicle-treated β ENaC-overexpressing mice, ‡ $P < 0.001$ compared with vehicle-treated β ENaC-overexpressing mice). (C) Size of BAL macrophages as a marker for macrophage activation ($n = 14$ –30 mice for each group; * $P < 0.001$ compared with vehicle-treated wild-type, † $P < 0.01$ compared with vehicle-treated β ENaC-overexpressing mice). (D) IL-13 concentration in BAL ($n = 7$ –28 mice for each group; * $P < 0.01$ compared with vehicle-treated wild-type, † $P < 0.01$ compared with vehicle-treated β ENaC-overexpressing mice).

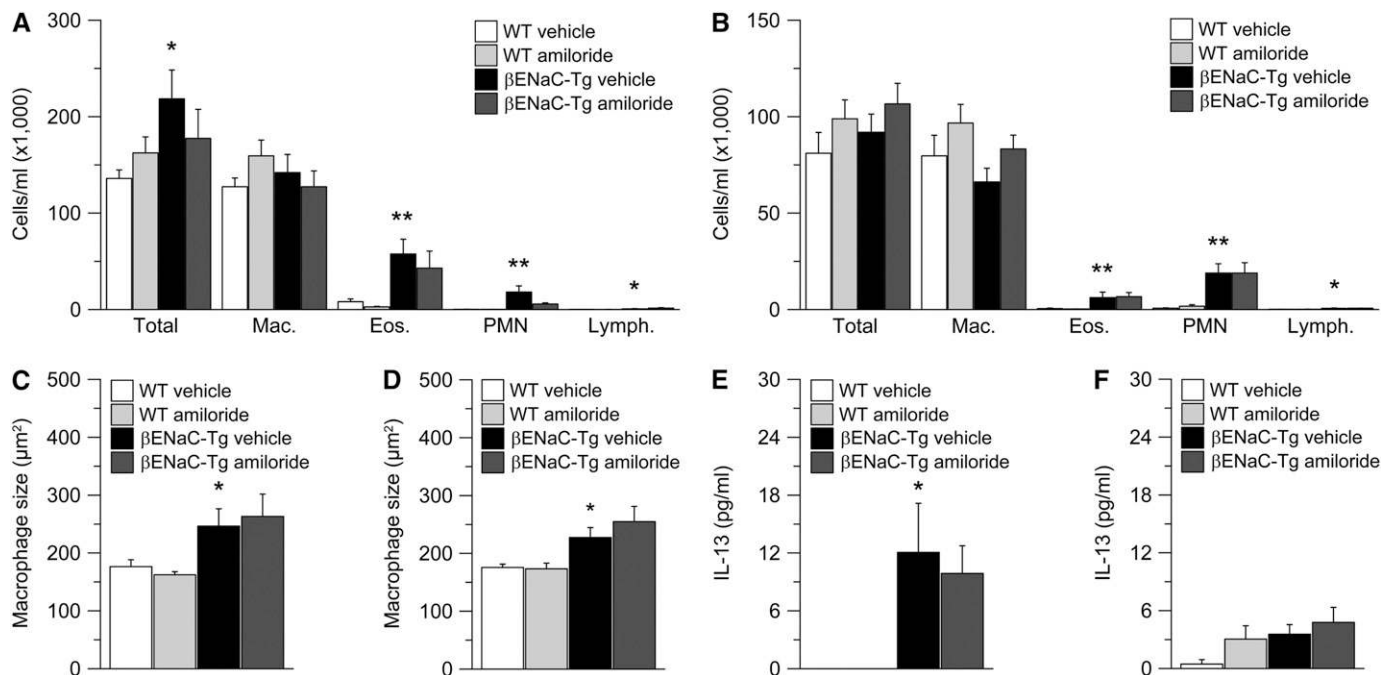


Figure 6. Late amiloride therapy has no effect on airway inflammation in β ENaC-overexpressing mice. (A–E) Effect of late amiloride treatment, administered from the age of (A, C, and E) 5 days or (B, D, and F) 4 weeks for a period of 2 weeks, on (A and B) cell counts, (C and D) macrophage size, and (E and F) IL-13 concentrations in BAL from β ENaC-overexpressing and wild-type mice. (A and B) BAL cell counts after treatment with amiloride or vehicle alone from the age of (A) 5 days or (B) 4 weeks ($n = 13$ –34 mice for each group; * $P < 0.01$ compared with vehicle-treated wild-type, ** $P < 0.001$ compared with vehicle-treated wild-type). (C and D) Size of BAL macrophages after treatment from (C) 5 days or (D) 4 weeks ($n = 7$ –11 mice for each group; * $P < 0.05$ compared with vehicle-treated wild-type). (E and F) IL-13 concentration in BAL after treatment from (E) 5 days or (F) 4 weeks ($n = 4$ –17 mice for each group; * $P < 0.01$ compared with vehicle-treated wild-type).

resulting from combined effects of increased epithelial O_2 consumption due to Na^+ hyperabsorption and decreased O_2 delivery due to airway mucus plugging (17). Because necrotic debris is a consistent finding in the small airways of patients with CF (35), and cellular necrosis is a potent trigger for inflammation (36), we evaluated the effects of preventive amiloride treatment starting on the first day of life on the occurrence of necrotic epithelial cells in airways of 3-day-old β ENaC-overexpressing neonates (Figure 8). Notably, compared with vehicle treatment, preventive administration of amiloride significantly reduced the frequency of necrotic airway epithelial cells in neonatal β ENaC-overexpressing mice (Figures 8A and 8B), demonstrating that preventive ENaC blocker therapy protected epithelial cells from necrosis and, thus, reduced a strong stimulus for airway inflammation.

DISCUSSION

In the present study, we used the β ENaC-overexpressing mouse as a model of CF lung disease (15, 16) to test the timing of strategies that inhibit increased ENaC-mediated airway Na^+ absorption to treat CF lung disease *in vivo*. Our results show for the first time that preventive inhibition of accelerated Na^+ absorption by the ENaC blocker amiloride is an effective therapy for CF-like lung disease in a murine disease model. Preventive amiloride administration exhibited significant therapeutic benefits by reducing airway mucus obstruction, goblet cell metaplasia, mucus hypersecretion, airway epithelial necrosis, and pulmonary inflammation in β ENaC-overexpressing mice (Figures 1, 2, 5, and 8). These therapeutic benefits resulted in an approximately 70% reduction in spontaneous pulmonary mortality, and provide a proof of concept for a novel therapeutic strategy for CF lung disease (Figures 1, 2, 5, and 8). In contrast to currently available CF therapies that target secondary pathogenetic events (i.e.,

antiinfective compounds for the treatment of bacterial infections [32, 37] and inhaled DNase to antagonize increases in sputum viscoelasticity caused by high levels of DNA released from inflammatory cells [38, 39]), preventive inhibition of increased Na^+ absorption constitutes a pharmacologic strategy that targets a proximal mechanism involved in the pathogenesis of CF lung disease.

We used the β ENaC-overexpressing mouse for these studies because previous work demonstrated that CF mice, either deficient of CFTR or carrying specific CFTR mutations, do not mimic increased Na^+ absorption in the lower airways and do not develop spontaneous airway mucus plugging, goblet cell metaplasia, and airway inflammation characteristic of CF lung disease in humans (8, 10, 40, 41). Therefore, CF mice were not directly useful for preclinical evaluation of ENaC directed therapies in CF lung disease. However, besides dysregulation of cAMP-dependent Cl^- secretion and ENaC-mediated Na^+ absorption, CFTR deficiency and/or malfunction has been implicated in various other cellular dysfunctions, including abnormal receptor-mediated clearance of bacteria, phagosome acidification in macrophages, cellular lipid trafficking, and ceramide metabolism (42–46). Because CFTR function is normal in β ENaC-overexpressing mice, efficient therapy of CF lung disease may be more complex in humans, and may require correction of other cellular dysfunctions in addition to increased airway Na^+ absorption. The development of compounds that correct and/or potentiate dysfunctional CFTR (47–49) will help elucidate the relative importance of increased airway Na^+ absorption versus defects in other CFTR-mediated functions as targets for CF pharmacotherapy.

Interestingly, in addition to mucolytic effects, preventive amiloride therapy had potent effects on airway inflammation in β ENaC-overexpressing mice (Figure 5). We speculate that the antiinflammatory effects of amiloride may be mediated by several mechanisms. First, we previously hypothesized that chronic

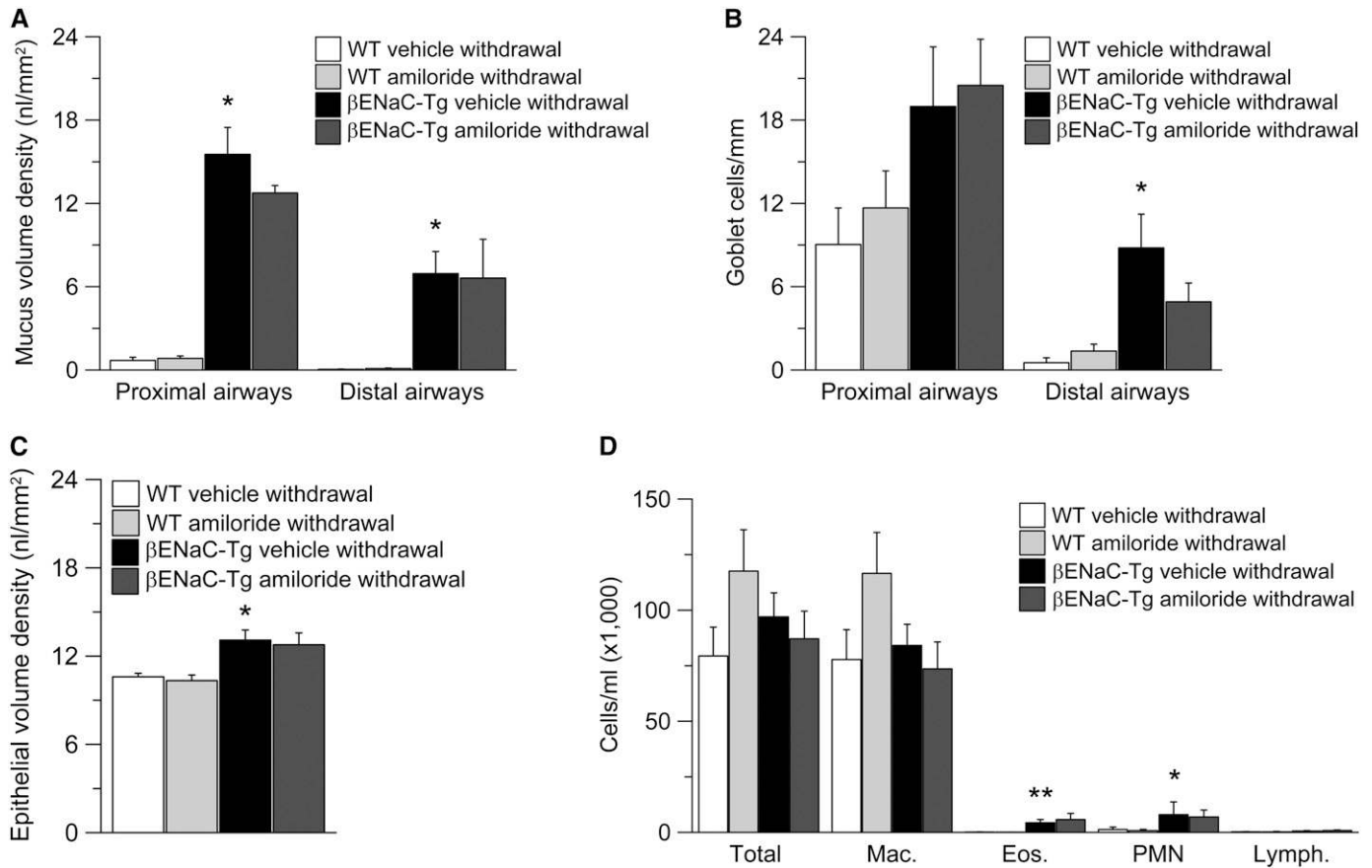


Figure 7. Therapeutic effects of preventive amiloride treatment are not sustained if treatment is withdrawn in β ENaC-overexpressing mice. Summary of amiloride withdrawal studies. β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates were treated with amiloride from birth for a period of 2 wk, then treatment was withdrawn and effects on (A) airway mucus content, (B) goblet cell counts, (C) epithelial height, and (D) inflammatory cell counts were evaluated in 6-week-old adult mice ($n = 6-10$ mice for each group). (A) Mucus content in proximal and distal main axial airways ($*P \leq 0.001$ compared with vehicle-treated wild-type). (B) Goblet cell counts in proximal and distal main axial airways ($*P < 0.05$ compared with vehicle-treated wild-type). (C) Epithelial height was determined by measuring epithelial volume density in main axial airways ($*P < 0.05$ compared with vehicle-treated wild-type). (D) Total and differential BAL cell counts ($*P < 0.05$ compared with vehicle-treated wild-type, $**P \leq 0.001$ compared with vehicle-treated wild-type).

airway inflammation may be triggered by a failure to clear inhaled particulates and irritants, which induce expression of proinflammatory cytokines like keratinocyte chemoattractant (KC) by macrophages and/or airway epithelia in β ENaC-overexpressing mice (15, 50). We therefore predict that antiinflammatory effects may be conferred by improved clearance of proinflammatory stimuli when airway Na^+ hyperabsorption and ASL depletion were prevented by amiloride. Second, we recently demonstrated that neonatal β ENaC-overexpressing mice develop airway epithelial necrosis, likely reflecting increased epithelial O_2 consumption due to ENaC-mediated Na^+ hyperabsorption and/or decreased O_2 delivery due to airway mucus plugging (17). Because necrosis is a strong trigger for inflammation (36), we predict that inhibition of this phenotype by preventive amiloride shown in this study (Figure 8) also reduced a potent stimulus for airway inflammation in β ENaC-overexpressing mice. Third, amiloride has been shown to inhibit secretion of proinflammatory cytokines including IL-8 and TNF- α in alveolar macrophages, and that this antiinflammatory action is mediated by inhibition of the Na^+/H^+ exchanger (NHE) (51), rather than by ENaC blockade. Future studies with more selective blockers (51, 52) are required to determine the relative roles of ENaC versus NHE as targets for antiinflammatory therapy of CF-like lung disease in β ENaC-overexpressing mice.

Our observation that amiloride therapy became ineffective when treatment was started after the onset of CF-like lung disease in β ENaC-overexpressing mice (Figures 3, 4, and 6) is consistent with previous clinical trials in patients with established CF lung disease (20, 21). The failure of amiloride inhalation therapy in patients with CF was mainly attributed to: (1) insufficient pulmonary delivery of amiloride due to limited solubility restricting the amount that could be delivered by a nebulizer; (2) limited potency; and (3) limited half-life of amiloride on airway surfaces (53–55). Our findings showing that amiloride can be delivered to the lung in therapeutically active quantities before the onset of lung disease suggest that airway mucus obstruction and/or airway remodeling were contributing factors to the absence of therapeutic benefits in older β ENaC-overexpressing mice and patients with CF with established lung disease.

In this context, previous studies demonstrated that single viral infections of the respiratory tract not only cause acute bronchiolitis, but can also trigger chronic responses with goblet cell metaplasia and mucus hypersecretion that persist for many years after viral clearance (56). Emerging evidence suggests that persisting airway disease may be caused by viral reprogramming of the epithelium and/or components of the innate and adaptive immune system in a susceptible host, and that this mechanism may be implicated in the pathogenesis of chronic airway diseases

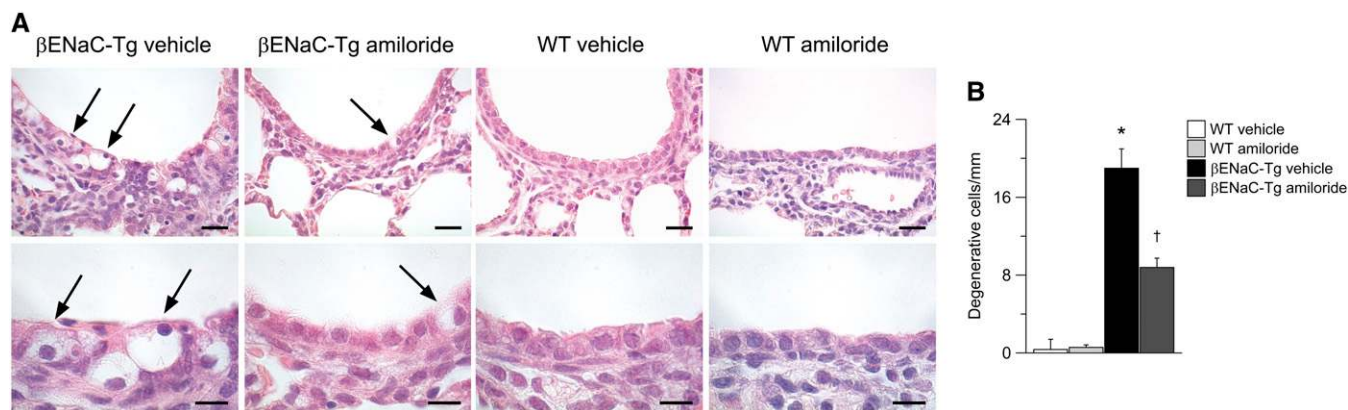


Figure 8. Preventive amiloride therapy reduces airway epithelial necrosis in β ENaC-overexpressing mice. Effect of preventive amiloride treatment, administered from the first day of life for a period of 3 days, on (A) airway histology and (B) numbers of degenerative airway epithelial cells in β ENaC-overexpressing (β ENaC-Tg) mice and wild-type (WT) littermates. (A) Airway histology of 3-day-old β ENaC-overexpressing and wild-type mice after preventive treatment with amiloride or vehicle alone. Sections were stained with hematoxylin and eosin to determine the numbers of degenerative airway epithelial cells (arrows). Representative of $n = 7$ – 12 mice for each group. Scale bars = $20\ \mu\text{m}$ (upper panels) or $10\ \mu\text{m}$ (lower panels). (B) Severity of airway epithelial necrosis was determined from the number of degenerative epithelial cells per mm of the basement membrane ($n = 7$ – 12 mice for each group; * $P \leq 0.001$ compared with vehicle-treated wild-type, † $P < 0.001$ compared with vehicle-treated β ENaC-overexpressing mice).

such as asthma and chronic bronchitis (56, 57). We speculate that early mucus obstruction and inflammation caused by ASL depletion in CF lung disease may provide an alternative stimulus that reprograms host cell behavior to produce chronic airway disease. While future studies are required to elucidate the mechanisms that impede therapeutic effects of amiloride in β ENaC-overexpressing mice and patients with CF with established airway disease, our results point to the possibility that a preventive ENaC blocker treatment paradigm may be required for effective therapy of CF lung disease.

The concept that a single viral infection in a vulnerable period could trigger prolonged airways disease (56) led us to speculate that the lung disease in adult β ENaC-overexpressing mice may reflect a sustained change in airway cell populations and/or function that resulted from early (3–10 d) pathologic events (17). We, therefore, tested the hypothesis that early inhibition of airway epithelial dysfunction by preventive ENaC blocker therapy may have durable effects by performing amiloride withdrawal protocols, in which preventive amiloride therapy was discontinued in juvenile mice and lungs were evaluated after a 4-week off treatment period. These studies demonstrated that stopping amiloride therapy resulted in recurrence of airway mucus obstruction, epithelial remodeling, and airway inflammation in adult β ENaC-overexpressing mice (Figure 7) (17). However, withdrawal of amiloride treatment in surviving juvenile β ENaC-overexpressing mice did not cause recurrence of mortality. Our previous studies demonstrated that death in neonatal β ENaC-overexpressing mice is caused by severe mucus plugging of the trachea (17). We speculate that the growth-related increase in cross-sectional surface area of the trachea protected juvenile β ENaC-overexpressing mice from life-threatening airflow limitation after amiloride therapy was discontinued and mucus accumulation recurred. Taken together, the observation that temporary inhibition of increased airway Na^+ absorption prevented early mortality, but did not have lasting therapeutic effects on airway mucus obstruction and inflammation in β ENaC-overexpressing mice, suggests that continuous ENaC blocker therapy may be required to optimize long-term treatment benefits in CF lung disease.

Regarding the feasibility of preventive amiloride therapy in patients with CF, only limited information is available on the timing of the onset and early progression of CF lung disease in

humans. Pathologic studies demonstrated that lungs were structurally normal in CF neonates who died of intestinal obstruction, but that airway mucus plugging associated with air trapping and atelectasis developed in the first months of life (58, 59). These findings suggest that inhaled amiloride therapy is unlikely to be beneficial for patients with CF who are diagnosed in infancy or early childhood on the basis of clinical symptoms. On the other hand, these pathologic studies indicate that in the first weeks and months of life, there is a window for preventive therapy in the human CF lung. Importantly, recent developments in CF neonatal screening allow establishing a CF diagnosis in this critical life span (60, 61). We, therefore, predict that near-birth initiation of preventive amiloride therapy may become feasible in a larger number of patients with CF in the near future.

Previous pharmacokinetic studies in healthy subjects demonstrated that targeting the lung with aerosols produced therapeutic amiloride concentrations on airway surfaces in a structurally normal lung (53, 62). Importantly, previous clinical studies documented that long-term inhalation of aerosolized amiloride at concentrations of 1 to 10 mmol/L (equivalent to doses of 0.1 to 1 mg/kg body weight per day), was well tolerated, safe, and did not cause diuresis or electrolyte imbalance in humans (20, 21, 29). In mice, intranasal instillation is commonly used for pulmonary delivery of drugs or allergens (63). Pulmonary delivery is likely mediated by generation of an aerosol within the nose, which is deposited onto the lower airways. Our deposition studies indicated that intranasal instillation in mice produced a pulmonary deposition fraction ($\sim 4\%$) that was substantially smaller than that achieved by aerosol inhalation in humans (Figure E1) (20, 21, 29, 53, 64, 65). Further, initial dose–response studies indicated that high doses of intranasal amiloride ($\sim 8\ \text{mg/kg}$ body weight per day) were required to reduce pulmonary mortality in neonatal β ENaC-overexpressing mice (Figure E2). These results indicate that the high intranasal amiloride dose administered in our study was required to compensate for inefficient intrapulmonary deposition. The diuretic effects caused by intranasal amiloride instillation in mice (Figure E1) indicated that much of the intranasally deposited amiloride was swallowed and absorbed from the gastrointestinal tract, causing systemic effects by inhibition of ENaC in the kidney.

Gastrointestinal absorption, therefore, can account for the diuretic effects, but also raised the possibility that therapeutic effects of intranasal amiloride therapy in β ENaC-overexpressing mice (Figures 1 and 2) were mediated by systemic, rather than topical, ENaC inhibition in airway epithelia. To address this possibility, we treated mice systemically by subcutaneous amiloride injections with doses equivalent to the doses used for intranasal therapy (Figure E3). In these studies, preventive systemic amiloride treatment caused more severe weight loss, likely due to profound diuresis, but did not have therapeutic effects on the lung disease in β ENaC-overexpressing mice. Taken together, these findings support the concept that therapeutic benefits of preventive intranasal amiloride in β ENaC-overexpressing mice were conferred by topical blockade of ENaC in the airways, and predict that substantially lower doses will be required to deliver amiloride in pharmacologically active concentrations to the lungs of CF infants with efficient aerosol delivery systems (64, 65).

The low potency of amiloride and its short half-life on airway surfaces limited the ability of our studies, as well as previous clinical trials (20, 21), to address whether ENaC-directed therapies can be beneficial in established CF lung disease (53–55). These limitations have led to the development of a series of novel more potent and durable amiloride analogs designed specifically for inhalation therapy of CF lung disease (52, 66). Recent *in vitro* studies demonstrated that the selected compound 552-02 was 60- to 100-fold more potent, up to fivefold less reversible, and twofold more slowly absorbed from surfaces of airway epithelia than amiloride (52). Compared with amiloride, compound 552-02 produced greater ASL expansion in airway cultures *in vitro* and was more effective at increasing mucus clearance in sheep. These results indicate that highly potent and long-acting ENaC blockers may be more effective than amiloride, both in the prevention and therapy of CF lung disease. We predict that future studies in β ENaC-overexpressing mice will help to evaluate the efficacy and safety of these compounds, including their therapeutic effects in established lung disease, at the preclinical level.

As novel chemical entities, compounds such as 552-02 will first have to undergo preclinical evaluation and toxicity testing and are, therefore, years away from routine clinical use, especially for preventive therapy in very young patients with CF. In contrast, amiloride is inexpensive, readily available, and has been in clinical use as a diuretic for many years. We predict that these properties, together with a widespread implementation of CF newborn screening programs (60, 61), and recent improvements in nebulizer technology allowing enhanced aerosol delivery to the neonatal and infant human lung (64, 65), may facilitate the translation of preventive amiloride therapy for CF lung disease from mice to the clinic.

We hypothesized that the predominant eosinophilic airway inflammation observed in juvenile (2- to 3-wk-old) β ENaC-overexpressing mice was triggered by impaired clearance of inhaled airborne allergens in a Th2-biased host (17, 33, 34). Here, we demonstrate that characteristic features of this allergic airway inflammation, including airway eosinophilia and elevated IL-13, were effectively reduced by preventive amiloride therapy. Furthermore, recent evidence indicated that exposure to cigarette smoke decreases CFTR expression and cAMP-dependent Cl^- secretion in airway epithelia *in vivo*, suggesting that ASL depletion may also be implicated in the pathogenesis of reduced airway mucus clearance observed in smokers and patients with chronic bronchitis (67–69). In summary, our results suggest that improving ASL hydration by preventive inhalation therapy with the ENaC blocker amiloride may be of benefit for the treatment of CF, and potentially other chronic lung diseases associated with mucociliary dysfunction, including chronic bronchitis and asthma.

Conflict of Interest Statement: Z.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.C.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.C.B. is named as a co-inventor on a patent filed on the β ENaC-overexpressing mouse in 2004; M.A.M. is listed on a patent application filed by the University of North Carolina at Chapel Hill, describing the β ENaC-overexpressing mouse. Of note, the β ENaC-overexpressing mouse has been deposited at JAX for general disposition.

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