Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung

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Cystic fibrosis (CF) is a life-shortening disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene1. Although bacterial lung infection and the resulting inflammation cause most of the morbidity and mortality, how the loss of CFTR function first disrupts airway host defence has remained uncertain2–4. To investigate the abnormalities that impair elimination when a bacterium lands on the pristine surface of a newborn CF airway, we interrogated the viability of individual bacteria immobilized on solid grids and placed onto the airway surface. As a model, we studied CF pigs, which spontaneously develop hallmark features of CF lung disease2,7,8. At birth, their lungs lack infection and inflammation, but have a reduced ability to eradicate bacteria4. Here we show that in newborn wild-type pigs, the thin layer of airway surface liquid (ASL) rapidly kills bacteria in vivo, when removed from the lung and in primary epithelial cultures. Lack of CFTR reduces bacterial killing. We found that the ASL pH was more acidic in CF pigs, and reducing pH inhibited the antimicrobial activity of ASL. Reducing ASL pH diminished bacterial killing in wild-type pigs, and, conversely, increasing ASL pH rescued killing in CF pigs. These results directly link the initial host defence defect to the loss of CFTR, an anion channel that facilitates HCO3− transport5–13. Without CFTR, airway epithelial HCO3− secretion is defective, the ASL pH falls and inhibits antimicrobial function, and thereby impairs the killing of bacteria that enter the newborn lung. These findings suggest that increasing ASL pH might prevent the initial infection in patients with CF, and that assaying bacterial killing could report on the benefit of therapeutic interventions.

Proposed origins of CF lung disease include reduced mucociliary clearance due to defects such as decreased ASL volume or altered mucus, reduced bacterial killing by ASL antimicrobials, defective bacterial elimination by phagocytes, abnormal inflammatory responses, and reduced or increased bacterial binding by airway epithelia2–6. One or more of these defects could be responsible. Two factors have made it difficult to distinguish among hypotheses and identify the initiating insults. First, with many diseases, the clinical manifestations may not reflect the original defects, but it is problematic to study CF at its insults. First, as with many diseases, the clinical manifestations may not reflect the original defects, but it is problematic to study CF at its original insults. Second, mice with disrupted Cfr genes do not develop typical CF4,14.

To circumvent these obstacles, we generated CFTR−/− pigs (CF pigs)7. Within months of birth, CF pigs spontaneously develop hallmark features of CF lungs such as airway inflammation, infection, tissue remodelling, mucus accumulation and airway obstruction8–10. Although at birth they exhibit none of these features, they already manifest a host defence defect against bacteria. Thus, newborn CF pigs provide an unprecedented opportunity to investigate mechanisms impairing host defence and initiating disease, because they allow CF/non-CF comparisons without secondary confounders.

In previous work, we instilled Staphylococcus aureus into airways and four hours later found more bacteria in CF than in non-CF pigs8. However, that study revealed little about the responsible mechanisms; we do not know whether bacteria were removed or killed within the lung, whether bacteria grew after instillation, whether phagocytic cells eliminated bacteria, whether bacteria bound to surfaces, or whether deposition and sampling were identical in all animals.

To investigate initial host defence defects, we developed a simple assay that tested viability of individual bacteria. We chemically linked biotin to S. aureus, bound streptavidin to gold grids, and combined them to attach S. aureus to the grids (Fig. 1a, b). We chose S. aureus because we frequently isolate it from porcine CF lungs, and it is the most common organism isolated from young children with CF8,15. A fluorescent live/dead stain showed the state of bacteria. Exposing grids to ethanol killed most S. aureus (Fig. 1c). Importantly, placing grids on the porcine tracheal surface in vivo also killed bacteria.

In 6–15-h-old pigs, we made a small tracheal incision and placed bacteria-coated grids on the airway surface. Even 30-s applications on non-CF airways killed S. aureus (Fig. 1d). Applying grids to littermate CF pigs killed approximately half as many bacteria. We administered methacholine to stimulate the secretion of submucosal glands, which produce substantial amounts of antimicrobials16,17, and to allow us to collect ASL for other studies. After methacholine application, the CF/non-CF differences persisted (Fig. 1e). We predicted that antimicrobial activity would also be detected if we removed methacholine-stimulated ASL and studied it with conventional colony-forming unit (CFU) assays. Indeed, bacterial killing was reduced in CF secretions (Fig. 1f).

We also applied S. aureus-coated grids to primary cultures of porcine airway epithelia and found reduced killing in CF (Fig. 1g). Previous data suggest that the host defence defect involves many different bacteria8,15. Therefore, we tested Pseudomonas aeruginosa-coated grids and found defective killing by CF epithelia (Fig. 1h). We also added S. aureus directly to cultured epithelia. Most non-CF epithelia eliminated low inocula of bacteria, but bacteria grew on most CF epithelia (Fig. 1i). At the highest inocula, S. aureus infected both CF and non-CF epithelia.

These data indicate that ASL rapidly kills bacteria, and that CF impairs killing. The defect was partial, as CF ASL retained some activity. The assays allow several conclusions. Defective bacterial killing was not due to dysfunctional mucociliary clearance or abnormal killing by phagocytes; neither would explain the results with grids in vivo or studies of cultured epithelia. Also, we cannot attribute CF/non-CF differences to altered bacterial–epithelial binding, because we saw the difference with bacteria attached to grids and with ASL studied.
Figure 1 | Bacterial killing is impaired in CF ASL. 

**a** Schematic showing biotin/streptavidin linking *S. aureus* to gold grids that were placed on the airway surface. After removal, bacteria were exposed to fluorescent live/dead stain (SYTO 9/propidium iodide), imaged and counted. 

**b** Scanning electron photomicrographs of bacteria-coated grid (top), grid bar (middle) and individual bacteria (bottom). 

**c** Bacteria-coated grid (green = live, red = dead) after placement for 5 min on the tracheal surface of 1-month-old, wild-type pig. Bottom shows percentage of bacteria that were dead after immersion in saline, after placement for 5 min on the tracheal surface of 1-month-old, wild-type pig.

**d** Dead (%)

| Time (min) | Non-CF | CF |
|-----------|-------|----|
| 0         | 100   | 100|
| 1         | 75    | 50 |
| 2         | 50    | 25 |
| 3         | 25    | 12.5|
| 4         | 12.5  | 6.25|
| 5         | 6.25  | 3.125|

**e** Dead (%)

| Time (min) | Non-CF | CF |
|-----------|-------|----|
| 0         | 100   | 100|
| 1         | 75    | 50 |
| 2         | 50    | 25 |
| 3         | 25    | 12.5|
| 4         | 12.5  | 6.25|
| 5         | 6.25  | 3.125|

**f** Dead (%)

| Time (min) | Non-CF | CF |
|-----------|-------|----|
| 0         | 100   | 100|
| 1         | 75    | 50 |
| 2         | 50    | 25 |
| 3         | 25    | 12.5|
| 4         | 12.5  | 6.25|
| 5         | 6.25  | 3.125|

**g** Dead (%)

| Time (min) | Non-CF | CF |
|-----------|-------|----|
| 0         | 100   | 100|
| 1         | 75    | 50 |
| 2         | 50    | 25 |
| 3         | 25    | 12.5|
| 4         | 12.5  | 6.25|
| 5         | 6.25  | 3.125|

**h** Dead (%)

| Time (min) | Non-CF | CF |
|-----------|-------|----|
| 0         | 100   | 100|
| 1         | 75    | 50 |
| 2         | 50    | 25 |
| 3         | 25    | 12.5|
| 4         | 12.5  | 6.25|
| 5         | 6.25  | 3.125|

**i** Dead (%)

| Log(inoculum) | Non-CF | CF |
|---------------|-------|----|
| 1.8           | 100   | 100|
| 2.8           | 75    | 50 |
| 3.8           | 50    | 25 |
| 4.8           | 25    | 12.5|

**e**, **f**, **g**, **h** and **i** show the percentage of dead bacteria after treatment with saline, H_2O, EtOH or ASL. The data are from different pigs per genotype.

We investigated antimicrobials by measuring messenger RNA, protein and aggregate activity under optimal conditions. The abundance of transcripts for secreted antimicrobial proteins (Supplementary Table 1 and Supplementary Fig. 1) and proteins with known host defence functions showed no consistent differences between genotypes (Supplementary Table 2). In methacholine-stimulated ASL, concentrations of the two most abundant antimicrobials, lysozyme and ex vivo. Our earlier finding that newborn CF airways lack inflammation and the killing defect in cultured epithelia indicate that abnormal inflammation was not responsible. Furthermore, our bacteria-coated grid method also excludes differences in bacterial delivery, sampling or growth. Therefore, we reasoned that defective killing arose from either reduced amounts of ASL antimicrobial factors or inhibition of their function.
lactoferrin, as well as palate, lung and nasal epithelial clone (PLUNC) and surfactant protein SP-A, did not differ by genotype (Fig. 2a). To assay aggregate ASL antimicrobial function, we performed four experiments in which we maximized activity by reducing the ionic strength close to zero. First, we added isotonic, salt-free buffer to the apical surfaces of cultured airway epithelia. Under these control conditions, both genotypes showed equivalent killing of bacteria on grids (Fig. 2b). Second, ASL removed with water from cultured CF and non-CF epithelia killed bacteria to the same extent (Fig. 2c and Supplementary Fig. 2). Third, ASL removed from pigs and diluted 1:100 with water showed genotype-independent killing (Fig. 2d). Fourth, radial diffusion assays with 10 mM sodium phosphate in 1% agarose revealed areas of clearance for S. aureus and Escherichia coli that were similar for both genotypes (Fig. 2e). These data indicate that non-CF and CF ASL had similar amounts of antimicrobials. Thus, they suggested that CF:non-CF bacterial killing disparities derived from other differences in ASL composition.

An increased ionic strength inhibits activity of many antimicrobials. Studies of human CF airway epithelia in culture or xenografts reported either higher or the same ASL NaCl concentrations as non-CF epithelia, but an in vivo study reported similar concentrations. Therefore, we measured Na+ and K+ concentrations in ASL collected from newborn pigs and found that they did not differ by genotype (Fig. 3a). In addition, ASL collected after methacholine stimulation showed similar ion concentrations to those measured under basal conditions, and only minor differences in K+ concentration between CF and non-CF ASL (Fig. 3b). Thus, different ASL Na+ and K+ concentrations do not explain the defective bacterial killing in CF.

Earlier studies indicated that pH can affect antimicrobial activity. Human and porcine airway epithelia exhibit CFTR-dependent HCO3− secretion and CF reduces ASL pH. To assess ASL pH in vivo, we placed a planar pH-sensitive probe on the tracheal surface. pH was lower in CF than in non-CF ASL (Fig. 3c). Methacholine-stimulated ASL removed from CF pigs and measured with an optical pH probe was more acidic than ASL from non-CF pigs (Fig. 3d); pH was measured 10 min after removal in ambient CO2, probably contributing to the higher absolute pH values. We also measured ASL pH in primary airway epithelial cultures using a fluorescent pH indicator and found reduced pH in CF (Fig. 3e). Although absolute pH values varied in different preparations, in all three, CF ASL was more acidic.

We tested whether pH affects ASL antimicrobial activity by removing ASL from newborn non-CF pigs, adjusting the pH and applying S. aureus-coated grids. Killing was pH-dependent, increasing as pH increased (Fig. 4a and Supplementary Fig. 3). We also tested lysozyme and lactoferrin; increasing pH increased S. aureus and E. coli killing (Fig. 4b and Supplementary Figs 4 and 5).

If pH is responsible for the differences in bacterial killing, we predicted that reducing ASL pH would inhibit bacterial killing in wild-type pigs, and raising pH would enhance killing in CF pigs. In non-CF pigs, increasing airway CO2 reduced ASL pH and inhibited bacterial killing (Fig. 4c). In CF pigs, we aerosolized NaHCO3 into the trachea. Compared with NaCl, NaHCO3 increased ASL pH and enhanced killing (Fig. 4d).

Our results directly link CFTR mutations to defective bacterial eradication. CFTR is an anion channel that conducts HCO3− and works together with Cl−/HCO3− exchangers and H+ secretion to regulate ASL pH. Its loss prevents airway epithelia from secreting HCO3−, thereby impairing killing of bacteria that enter the lung. Our findings with bacteria-coated grids in vivo, ASL removed from pigs, and primary epithelial cultures all point to this defect.

What about other defects that might commence CF lung disease? Progression from the pristine lung of a newborn to the chronically
**Methods**

**Bacteria**—coated grids were prepared by chemically modifying gold electron microscopy grids (200 mesh) and coating them with streptavidin. Bacteria (*S. aureus* isolate 43SA or *P. aeruginosa* PA01) were cultured to log-phase growth and mixed with *N*-hydroxysulfosuccinimide-biotin. Streptavidin-coated grids were then incubated with biotinylated bacteria and rinsed in PBS. Bacteria–coated grids were placed on airway surfaces, removed, immediately rinsed with PBS, and then immersed in PBS containing the fluorescent indicators SYTO 9 and propidium iodide (Live/Dead Bacterial Viability Assay, Invitrogen) to assess viability. After 15 min with grids were rinsed with PBS and imaged with a laser-scanning confocal microscope (Olympus). The numbers of live and dead bacteria were counted in multiple fields by operators blinded to genotype. pH measurements were done in vivo using non-invasive dual lifetime referencing and a planar pH-sensitive optode. A needle-type fibre optic pH meter was used for pH measurements ex vivo. A ratiometric pH indicator was used in primary cultures of airway epithelia.

**Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.
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Author Information Microarray data have been deposited in the Gene Expression Omnibus under accession numbers GSE36906 and GSE21071. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.J.H. (michael-welsh@uiowa.edu) or J.Z. (joseph-zabner@uiowa.edu).
Bacteria-coated grids were also prepared for scanning electron microscopy using standard procedures. In brief, the grids were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, followed by post-fixation in 1% osmium tetroxide. The grids were then dehydrated in a graded series of ethanol, transitioned to hexamethyldisilazane, air dried overnight and mounted on aluminum stubs. After sputter coating with gold/palladium, the samples were imaged in a Hitachi S-8000 scanning electron microscope.

**Micro-CFU and radial diffusion assays.** We used *S. aureus* strain 43SA and *E. coli* strain pCGS1-Micro-CFU assays were performed as previously described. When ASL was collected in vivo, each micro-CFU assay contained an initial inoculum of approximately $1 \times 10^5$ CFU ml$^{-1}$. Radial diffusion assays with 10 mM sodium phosphate, pH 7.4, in 1% agarose were performed with a final attendance of $D_{500\text{nm}}$ of bacteria of 0.02 as previously described.

**Collection of ASL for protein and antimicrobial studies.** ASL was collected from pigs anaesthetized with ketamine (20 mg kg$^{-1}$, intramuscularly (i.m.)) and xylazine (2 mg kg$^{-1}$, i.m.), and maintained with propofol (2 mg kg$^{-1}$, i.v.). The neck was dissected to expose the trachea. Tracheal secretion was stimulated by administering methacholine (2.5 mg kg$^{-1}$, i.v.). After approximately 5 min, tracheal secretions were collected by making a small incision in the anterior tracheal wall and inserting a sterile polyethylene catheter (Puritan Medical Products) to collect ASL. The probe was then inserted into a microcentrifuge tube and secretions were recovered by centrifugation. This procedure produced approximately 10–20 μl of ASL fluid from each animal. For assays of ASL proteins, samples were immediately placed on ice and frozen at −80 °C until use. For assays of antimicrobial activity, samples were used immediately.

ASL was also collected from primary cultures by rinsing the apical surface with 100 μl H$_2$O. On the basis of our earlier work, we estimate that collection produced an approximate 1:80 to 1:125 dilution of ASL.

**Measurement of amounts of ASL antimicrobial proteins.** To immunoblot for lactoferrin, PLUNC and SP-A, 10 μl of a 1:10 dilution of the ASL was separated on 4–15% Tris-HCl gels and transferred to polyvinylidene difluoride membranes, followed by blocking in TBS-Tween containing 2% BSA. Membranes were incubated with a primary antibody (rabbit anti-human lactoferrin, Immunology Consultants; monoclonal anti-human PLUNC, R&D Systems; or polyclonal antiserum against porcine SP-A$^-^3$). Membranes were washed four times using TBS-Tween, then incubated with secondary antibody conjugated to horseradish peroxidase (Thermo Fisher Scientific) at a 1:20,000 dilution for 1 h. After five more washes in TBS-Tween, protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were then exposed to film and densitometry performed. The same ASL samples were used for each western blot and stripped between westerns with Restore Western Blot Stripping Buffer (Thermo Scientific). Lysozyme was measured using lysoplates as previously described. Undiluted methacholine-stimulated secretions (5 μl) were used and compared with a standard of human lysozyme (Sigma).

**Collection and determination of ASL Na$^+$ and K$^+$ concentrations.** Pigs were anaesthetized with ketamine (20 mg kg$^{-1}$, i.m.) and xylazine (2 mg kg$^{-1}$, i.m.), followed by propofol (2 mg kg$^{-1}$, i.v.). The trachea was surgically exposed and accessed anteriorly using heat cautery. We then made a small anterior incision through the tracheal rings using heat cautery to prevent bleeding. To ensure that air was completely humidified, the animal was studied in a humidified chamber (100% relative humidity, 25–30 °C).

We collected ASL using a procedure designed to minimize the generation of excessive capillary forces during sampling. We fused thin lens paper (VWR Scientific Products) with Parafilm M (Pechiney Plastic Packaging) in an oven (205 °C) for 70–90 s. This procedure further reduced the volume of liquid that the paper would absorb and minimized evaporation from the surface not touching ASL. We prepared 0.5 × 2 cm strips, washed them three times in double distilled water, and dried them overnight at 40 °C. The Parafilm-fused paper strips were weighed and then gently placed in contact with the luminal surface of the posterior trachea for 15s. Immediately after removal from the trachea, Parafilm-fused paper strips were placed (paper side up) on a precision balance (Mettler-Toledo XP26DR). Mass measurements were recorded by a synchronized computer ten times per second for 200 s (BalanceLink, Mettler-Toledo) while evaporation occurred. The amount of ASL collected was determined by plotting mass versus time, fitting a one-phase exponential decay to the data (GraphPad Prism 5; GraphPad Software), and extrapolating to mass at time 0, that is, the time at which the strip was removed from the airway surface. We then dried the strips overnight at 40 °C and measured the dry mass; 3.0 ± 0.5 μl of ASL (n = 14) was collected per cm$^2$.
To dissolve the dried ASL contents, the strips were placed in 1 ml flame photometer internal standard solution (Instrumentation Laboratory) overnight. We then measured Na\(^+\) and K\(^+\) content with an IL943 Flame Photometer and compared output with calibration curves of solutions containing known mole contents (Instrumentation Laboratory). ASL Na\(^+\) and K\(^+\) concentrations were calculated by dividing the mole content of cation by the mass of solvent. The mass of ASL solvent was determined as the difference between the initial ASL sample mass and the dry sample mass.

We also measured Na\(^+\) and K\(^+\) concentrations after methacholine administration (2.5 mg kg\(^{-1}\), i.v.) to stimulate submucosal gland and goblet cell secretion. To collect the readily visible secretions, we lightly applied a sterile polystyrene applicator (Puritan Medical Products) to the tracheal surface. The applicator tip was then suspended and sealed in a microcentrifuge tube, and liquid was isolated by centrifugation through a layer of water-saturated oil. We diluted secretions 1:10 in double distilled H\(_2\)O, added 10 l to 1 ml of flame photometer internal standard, measured Na\(^+\) and K\(^+\) content by flame photometry, and calculated the ion concentrations.

**Measurement of ASL pH.** To assess pH in vivo, we used non-invasive dual lifetime referencing to interrogate a 3 x 3 mm planar optode (pH sensitive foil, PreSens GmbH), applied directly to the tracheal surface. The device used to transmit and receive the excitation and emission light was a single channel pH meter (pH-1 mini; PreSens GmbH). The tip of the fibre optic pH meter was kept at the same distance from the tracheal surface in all samples and confirmed by recording the amplitude registered by the device. Calibration before each set of measurements was done by placing the planar optode on the surface of a flat filter soaked in standard pH buffers. To minimize alterations in CO\(_2\) during placement of the probe, the experiments were done in an environment of 5% CO\(_2\); hence the CO\(_2\) concentration in ASL was probably >5% owing to CO\(_2\) production by the pigs. Thus, the pH values are probably lower than occur normally.

To assess pH ex vivo, secretion was stimulated with methacholine, and 5 min later, ASL was removed using the same methods as described for measurement of ion concentrations. Ten minutes after removal from the pig, ASL pH was measured using a needle-type fibre optic pH meter (World Precisions Instruments). The pH meter was calibrated before each set of measurements. After removal and during measurement, the CO\(_2\) over the sample was ambient, that is, approximately zero, which probably accounts for the higher pH values in the ex vivo ASL.

To assess pH in primary cultures of airway epithelia, we used the fluorescent ratiometric pH indicator SNARF conjugated to dextran (Molecular Probes). pH in non-CF pigs, CO\(_2\) concentration was controlled in a humidified chamber that reductions in luminescence have an excellent correlation with a decrease in pH. Thus, the pH values are probably lower than occur normally. The pH values are probably lower than occur normally.

**Microarray analysis.** Trachea and bronchus tissue samples were dissected from newborn piglets within 12 h of birth. Samples were cut into ~5-mm\(^3\) pieces and stored in RNAlater RNA stabilization reagent (Ambion) using the manufacturer’s recommended protocols. Primary cultures of differentiated CF and non-CF tracheal epithelia were prepared as described above. Total RNA was isolated with TRIzol reagent (Invitrogen). Only RNA samples obtaining a minimum of 7.0 RNA integrity number on the Agilent 2100 Bioanalyzer (Agilent Technologies) were processed. Five micrograms of total RNA was used to generate biotinylated complementary RNA using the Affymetrix GeneChip one-cycle target labelling kit (Affymetrix) according to the manufacturer’s recommended protocols, and then hybridized to the Affymetrix Porcine GeneChip (23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 Sus scrofa genes). Each hybridization sample (trachea, bronchus and cultured tracheal epithelia) was performed on a separate day, with all genotypes represented in each run. The arrays were washed, stained and scanned using the Affymetrix Model 450 Fluidics Station and Affymetrix Model 3000 scanner, and data were collected using the GeneChip Operating Software (GCOS), v.1.4, using the manufacturer’s recommended protocols. Partek Genomics Suite Software (Partek) (one-way analysis of variance analysis) was used to analyse the data.

**Quantitative RT–PCR.** Primers to amplify porcine GAPDH, lactoferrin, lysozyme, S100A9 and PBD-2 were designed and validated using standard procedures. Total RNA was isolated using TRIzol (manufacturer’s recommended protocol) from the trachea and bronchus of 6 CF\(^+\) and 6 CF\(^−\) pigs and from primary cultures of tracheal epithelia from 8 CF\(^+\) and 8 CF\(^−\) pigs. Reverse transcription with 1 μg total RNA was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Ten nanograms of cDNA and 50 pm of both forward and reverse primers were used per reaction for quantitative PCR. Power SYBR green PCR master mix (Applied Biosystems) was used for quantification. GAPDH cDNA levels were used to normalize expression of lactoferrin, lysozyme, S100A9 and PBD-2. Fold change was generated using average ΔΔC\(_v\) values for each genotype. Error bars were generated using standard error of the mean \(±\)SD values of each genotype. The primers used were: GAPDH, forward: 5′-GACCTTCAGGAGGAGAGGTCG-3′; reverse: 5′-GCCAGGTTTGGTGTTGGGTCAGG-3′; lactoferrin, forward: 5′-AGCCATCTGCTACGAAATCG-3′; reverse: 5′-ATCATGAAGCACAGGCTTCAG-3′; lysozyme, forward: 5′-TGCAAGAAGGTTGTTCAGGACG-3′; reverse: 5′-AAGAGAACAGTGTTGCAAGG-3′; S100A9, forward: 5′-TCAAGGGAGGAGCCCTATAAATGCTG-3′; reverse: 5′-TCTTCTGCAGTCTGTTCAACCG-3′; PBD-2, forward: 5′-GAGATTGAGGAAGAGCCCTTTAGACG-3′; reverse: 5′-GGAAATACTCTCACCTTGGCCTG-3′.

**Statistical analysis.** Data are presented as mean \(±\) s.e.m. Unless otherwise indicated, statistical analysis used an unpaired Student’s t-test. Differences were considered statistically significant at P < 0.05.