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Airway and lung pathology due to mucosal surface dehydration in β-Epithelial Na+ Channel-overexpressing mice: role of TNFα and IL-4Rα signaling, influence of neonatal development, and limited efficacy of glucocorticoid treatment

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Abstract

Overexpression of the epithelial Na^+ channel β subunit (*Scnn1b* gene, βENaC protein) in transgenic (Tg) mouse airways dehydrates mucosal surfaces, producing mucus obstruction, inflammation, and neonatal mortality. Airway inflammation includes macrophage activation, neutrophil and eosinophil recruitment, and elevated KC, TNFα and chitinase levels. These changes recapitulate aspects of complex human obstructive airway diseases, but their molecular mechanisms are poorly understood. We used genetic and pharmacologic approaches to identify pathways relevant to the development of *Scnn1b*-Tg mouse lung pathology. Genetic deletion of tumor necrosis factor alpha (TNFα) or its receptor, TNFR1, had no measurable effect on the phenotype. Deletion of the interleukin-4 receptor alpha subunit (IL-4Rα) abolished transient mucous secretory cell (MuSC) abundance and eosinophilia normally observed in neonatal wild-type (WT) mice. Similarly, IL-4Rα deficiency decreased MuSC and eosinophils in neonatal *Scnn1b*-Tg mice, which correlated with improved neonatal survival. However, chronic lung pathology in adult *Scnn1b*-Tg mice was not affected by IL-4Rα status. Prednisolone treatment ablated eosinophilia and MuSC in adult *Scnn1b*-Tg mice, but did not decrease mucus plugging or neutrophilia. These studies demonstrate that: 1) normal neonatal mouse airway development entails an IL-4Rα-dependent, transient abundance of MuSC and eosinophils; 2) absence of IL-4Rα improved neonatal survival of *Scnn1b*-Tg mice, likely reflecting decreased formation of asphyxiating mucus plugs; and 3) in *Scnn1b*-Tg mice, neutrophilia, mucus obstruction, and airspace enlargement are IL-4Rα- and TNFα-independent, and only MuSC and eosinophilia are sensitive to glucocorticoids. Thus, manipulation of multiple pathways will likely be required to treat the complex pathogenesis caused by airway surface dehydration.

Keywords

Transgenic mice; Lung; Mucus obstruction; Airway inflammation; Neonatal airway development

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INTRODUCTION

Airway epithelial overexpression of the epithelial Na^+ channel β subunit (βENaC protein, *Scnn1b* gene), driven by the Clara cell secretory protein (CCSP) promoter in transgenic (Tg) mice, results in epithelial Na^+ hyperabsorption, airway surface liquid (ASL) dehydration, impaired mucus clearance, airway inflammation and early post-natal mortality (1). The *Scnn1b*-Tg mouse model recapitulates many features of cystic fibrosis (CF) and other human airway diseases associated with relative dehydration of airway surfaces (2), including chronic bronchitis (CB) and chronic obstructive pulmonary disease (COPD). At birth, the lungs of *Scnn1b*-Tg mice are morphologically normal, but rapidly develop time-dependent abnormalities (3). Tracheal mucus obstruction is associated with neonatal mortality and, in surviving mice, mucus plugging and mucous secretory cell (MuSC) metaplasia progressively extends into the intra-pulmonary bronchi. The inflammatory infiltrate is characterized by enlarged/highly vacuolated macrophages, persistent neutrophilia associated with elevated KC, MIP-2 and TNFα, and transient eosinophilia with increased levels of IL-13 and eotaxin-1 (from 2 to 6 weeks). YM1, YM2 and acidic mammalian chitinase, all associated with Th2 type inflammation in asthma and helmintic infection (4-7), are also elevated in *Scnn1b*-Tg mice. Moreover, *Scnn1b*-Tg mice exhibit transient and spotty necrotic degeneration of Clara cells in the intrapulmonary airways, peaking at day 3 and completely resolved by day 10, and early neonatal air-trapping that later results in emphysematous changes (3). As surviving *Scnn1b*-Tg mice age, lymphocytic aggregates similar to those described in the lungs of COPD patients (8) become more frequent, suggesting progressive development of adaptive immune responses (9). Collectively, the presence of MuSC metaplasia, airspace enlargement, and inflammatory markers of both Th1 and Th2 type responses suggests that the mucosal immune response in *Scnn1b*-Tg mice is multifactorial and shares features of both aerotoxin- and allergen- mediated lung pathologies, such as COPD and asthma.

One strategy to identify key signaling pathways in the development of lung pathology is to cross breed *Scnn1b*-Tg mice to mice deficient in putatively relevant inflammatory mediators. Based on the *Scnn1b*-Tg mouse phenotype, we focused our studies on two pathways, namely tumor necrosis factor α (TNFα) and IL-4 receptor alpha subunit (IL-4Rα). Exogenous administration or transgenic overexpression of TNFα in murine airways promotes mucus secretion, lymphoid hyperplasia and emphysema (10-13). Conversely, genetic ablation of TNFα-mediated signaling prevents cigarette smoke-induced matrix breakdown, macrophage and neutrophil influx, and late onset emphysema (14). IL-4Rα is a shared component of the receptors for IL-4 and IL-13, two cytokines that trigger Th2-type airway inflammation and remodeling (15,16) and have been highly implicated in the pathogenesis of allergy and asthma (17,18). Ablation of IL-4Rα signaling has been shown to suppress IL-4 and IL-13-induced airway MuSC metaplasia and eosinophilic inflammation (19) and inhibit accumulation of chitinases YM1/2 in bronchoalveolar lavage (BAL) of allergic mice (20). In particular, Clara cell-targeted deletion of IL-4Rα was sufficient to prevent allergen-induced MuSC metaplasia (21). Finally, *in vitro* studies showed that both TNFα (22-24) and IL-4/IL-13 (25) inhibited ENaC-mediated Na^+ absorption and could thus modify the *Scnn1b*-Tg mouse phenotype.

To study the contribution of TNFα and IL-4Rα signaling to the development of *Scnn1b*-Tg mouse lung pathology, we crossbred *Scnn1b*-Tg mice with mice deficient in TNFα, TNFα receptor 1 (TNFαR1) or IL-4Rα and studied survival, lung pathology, BAL cell, cytokine/chemokine and mucin content, and airway ion transport properties. As a complementary approach to our genetic studies, we tested whether established *Scnn1b*-Tg mouse lung disease was modulated by pharmacologic treatment with prednisolone, a broad spectrum anti-inflammatory glucocorticoid (26), shown to reduce eosinophilia and MuSC metaplasia in murine models of atopic asthma (27-31).

MATERIALS AND METHODS

Mice

All mice were housed in individually ventilated micro-isolator cages, in a specific pathogen free facility maintained at the University of North Carolina at Chapel Hill, on a 12-hour day/night cycle. They were fed a regular chow diet and given water *ad libitum*. Hemizygous *Scnn1b*-Tg mice (*Scnn1b*-Tg +/−, or *Scnn1b*-Tg) and littermate controls (*Scnn1b*-Tg negative, or WT) were obtained by breeding *Scnn1b*-Tg mice with C3H/HeN:C57Bl6/N (C3:B6) F1 mice (Taconic, Hudson, NY) and genotyped for *Scnn1b*-Tg expression by PCR of genomic DNA, as originally described (1). To generate *Scnn1b*-Tg/IL-4Rα deficient mice and appropriate littermate controls, we first bred *Scnn1b*-Tg mice with IL-4Rα KO mice [IL-4Rα −/−, strain BALB/c-*Il4ra*^{tm1Sz}/J (32), kindly provided to us by Dr. Beverly Koller, University of North Carolina at Chapel Hill. IL-4Rα deficient mice exhibit loss of IL-4 and IL-13-mediated responses upon challenge, but they do not exhibit phenotypic abnormalities at baseline (32)] and generated IL-4Rα heterozygous (IL-4Rα +/−), *Scnn1b*-Tg mice. IL-4Rα +/−, *Scnn1b*-Tg mice were then bred with IL-4Rα KO mice to obtain experimental animals of four predicted genotypes: IL-4Rα +/−, WT; IL-4Rα −/−, WT; IL-4Rα +/−, *Scnn1b*-Tg; and IL-4Rα −/−, *Scnn1b*-Tg. We note that this breeding strategy generated control mice heterozygous for the deleted gene of interest, which were not expected to be different from homozygous WT mice. However, we gained the advantage that experimental animals of all four genotypes, with the expected Mendelian distribution of 25% each, shared the identical environment (i.e. littermate controls). We used multiple breeders and multiple litters per breeder to minimize founder and litter order effects. Although non-random strain effects can never be ruled out, the use of littermate controls is the best possible approach for reducing the chance of misinterpreting transgene or knockout effects (33). The same breeding strategy was used to generate experimental animals for the TNFα KO × *Scnn1b*-Tg and TNFαR1 KO × *Scnn1b*-Tg crosses, with the exception that we used inbred C57Bl6/N *Scnn1b*-Tg mice, recently generated by backcrossing the original C3:B6 *Scnn1b*-Tg mouse (line 6608) with C57Bl/6N inbred mice for 12 generations (9). Both TNFα KO [B6;129-*Tnf*^{tm1Gkl}/J , (34), mixed 129S/SvEv:C57Bl/6J background, stock # 003008] and TNFαR1 KO [B6.129-*Tnfrsf1a*^{tm1Mak}/J (35), C57Bl/6J background, stock # 002818] mice were from The Jackson Laboratory (Bar Harbor, MN). Wild-type inbred C57Bl/6N, C3H/HeN and BALBc/J mice for studies of normal neonatal development were obtained from Jackson Laboratories and Taconic. For prednisolone treatment experiments, we used inbred C57Bl6/N *Scnn1b*-Tg mice and their WT littermates. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and performed according to the principles outlined by the Animal Welfare and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Prednisolone treatment

Weaned 5-6 week-old C57Bl/6N *Scnn1b*-Tg mice and WT littermates were administered prednisolone (20 mg/kg/day) by intraperitoneal injection, for 2 weeks. Prednisolone (provided by Pfizer Inc., Chesterfield, MO) was administered twice a day, i.e. two injections of 10 mg/kg in 100 μl sterile vehicle (0.1% Pluronic P105 in sterile, pyrogen free 150 mM NaCl + 10% DMSO), approximately 10 hours apart. Control mice (not receiving prednisolone) were injected with 100 μl of vehicle on the same schedule. Mice were weighed every day before each injection. At the end of the 2 week treatment, bronchoalveolar lavage (BAL) was performed and lung tissue harvested for histological evaluation of lung pathology, as described below.

Bronchoalveolar lavage (BAL)

For 10 day-old or older mice, we used a standardized procedure to obtain both BAL cell counts and lung histology from each animal. Mice were euthanized by exsanguination under deep avertin (2-2-2 tribromoethanol) anesthesia and the chest cavity was opened to ligate the left main bronchus. A blunt needle (20 gauge for adults, 22 gauge for 5-10 day-old pups) was inserted through a small incision in the upper trachea and tied in place with 3.0 silk. After ligation of the left main stem bronchus, BAL was performed on the right lobes by instilling a volume of room temperature, sterile PBS determined by the formula [mouse weight (g) × 0.0175 ml = ml PBS instilled] (36). Due to their small size, 5 day-old pups were subject to either whole lung lavage [mouse weight (g) × 0.035 ml = ml PBS instilled] or fixation for histology, but not both. BAL was performed by gently injecting and retrieving the PBS volume three times. This procedure was carried out a second time with an equal volume of PBS and fractions were pooled. Return volume was consistently > 80% of the instilled volume. BAL cells were pelleted by centrifugation at 1,000 × g for 5 min at 4°C and the cell-free supernatant (BAL fluid or BALF) was collected and stored at −80°C for further analysis. BAL cells were resuspended in 100 μl PBS and total cells counted with a hemocytometer. Cytospin slides of 30,000 - 60,000 cells/slide were obtained (StatSpin Cytofuge 2, Norwood, MA), air dried, and stained with modified Giemsa for differential cell counts (NewComer Middleton, WI) of at least 200 cells per slide. After BAL, the left bronchial ligature was removed and the left lung was immersion-fixed in 10% neutral-buffered formalin to prevent dislodging of airway luminal contents.

Lung histology

Fixed lungs were embedded in paraffin oriented to maximize longitudinal sectioning of primary bronchi, sectioned to a thickness of 4-6 μm, and stained with hematoxylin and eosin (H&E) for assessment of lung morphology and Alcian Blue-Periodic Acid Schiff staining (AB-PAS) for mucopolysaccharides. The severity of lung pathology was graded semi-quantitatively on a scale ranging from 0 to 3 for the following features: 1) airway obstruction, i.e., airways obstructed by AB-PAS positive mucus: 0, no obstruction; 1, one airway partially or totally obstructed; 2, two airways partially or totally obstructed; 3, three or more airways partially or totally obstructed; 2) mucous secretory cell (MuSC) abundance, i.e., estimated percentage of AB-PAS positive cells in airway epithelium: 0, none; 1, 0-5% MuSC; 2, 5-20% MuSC; 3, ≥ 20% MuSC; 3) airspace enlargement, i.e., enlargement of the alveoli in the parenchymal space: 0, none; 1, spotty; 2, 50% of parenchyma; 3 > 50% of parenchyma; 4) lymphoid hyperplasia, i.e., peri-vascular, peri-bronchial or parenchymal lymphoid aggregates: 0, none; 1, one nodule/lung section; 2, two nodules/lung section; 3, three or more nodules/lung section; 5) airway inflammation, i.e., interstitial thickening and inflammatory cell infiltrate: 1, one airway; 2, two airways; 3, three or more airways. To confirm the results of the semi-quantitative score for MuSC abundance, we used MetaMorph image analysis software (MDS Analytical Technologies, Toronto, Canada) and determined the percentage of airway epithelial area positive for AB-PAS staining. Briefly, 3 random fields within the left lung proximal main stem bronchus were photographed with an Upright Nikon Microphot SA microscope interfaced with DXM 1200 color camera (Nikon Instruments, Melville, NY) at 20x magnification. The AB-PAS positive area was measured by thresholding, and was divided by the total epithelial area, to give the volume density of stored mucosubstances. Tissue blocks received a numerical code at time of embedding and scoring of the slides was performed by an investigator blinded to specimen genotype.

Agarose gel mucin western blot

This method was used to measure secreted mucins, as described in detail (37). BAL samples were centrifuged at low speed (1,000 × g for 5 min). Total protein concentration of BALF was

determined using the Microplate BCA Protein Assay, according to manufacturer instructions (Thermo Scientific, Rockford, IL), and was used to control for equivalent loading, since the large molecular sieve of agarose gels does not allow retention of globular proteins conventionally used for normalization. An equal volume of 8 M guanidine hydrochloride (GuHCl) was added to the BALF. GuHCl-dispersed samples were dialyzed against 6M urea, reduced with 10 mM DTT, and alkylated with 25 mM iodoacetamide. Alternatively, BALF was directly diluted 1:5 in 6M urea + 0.1%SDS, reduced, and alkylated. Equal volumes of reduced samples (20-25 microliters) were run on 1% agarose gel using a submerged gel electrophoresis apparatus with Tris Acetate EDTA/SDS buffer, at 80 V for 90-120 min. Gels were vacuum-blotted onto nitrocellulose membranes, blocked with Odyssey blocking buffer (OBB, Li-COR Biosciences, Lincoln, NE), and probed with a rabbit polyclonal antibody raised against purified cervical mucins ["reduced subunit antibody", described in (38,39)]. This antibody recognizes the Cys-rich domain of almost all mucins and is thus a "pan-mucin" detection reagent. Alternatively, blots were probed with a rabbit polyclonal antibody against murine Muc-5b, described in (40,41). Pan mucin and Muc5b antibodies were diluted 1:2,000 and 1:1,000 in OBB + 0.1% Tween 20 (OBBT), respectively. The secondary antibody was Alexa Fluor 680 goat anti-rabbit IgG, diluted 1:15,000 in OBBT. Detection and analysis of specific signals were performed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Measurement of cytokines and chemokines in BAL fluid

Aliquots of cell-free BALF, stored at −80°C, were used to measure mouse TNFα, KC, IL-4, IL-5, IL-13, IL-17, MCP-1, IL-6, MIP-2, and INFγ using a bead-based assay (Upstate-Millipore Beadlyte multiplex assays/Luminex, Billerica, MA), according to the manufacturer instructions.

Ussing chamber measurements of airway bioelectric properties

Freshly excised tracheas were mounted in Ussing chambers and equilibrated as described (42). After recording the basal short circuit current (I_{sc}), the following drugs were added sequentially to the chambers: amiloride (10^{-4} M, apical), forskolin (10^{-5} M, apical), UTP (10^{-4} M, apical), and bumetanide (10^{-4} M, basolateral), and changes in I_{sc} were recorded.

Statistical analyses

Statistical analyses were performed using SigmaStat 3.1 or GraphPad Prism 4.0. Survival curves were compared using Kaplan-Meier log rank analysis and Holm-Sidak multiple comparison. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons were used to determine significant differences among groups. $p<0.05$ was considered statistically significant and "n" represents the number of mice in each experimental group. All data are expressed as mean ± SEM.

RESULTS

TNFα signaling is not essential for airway inflammation and remodeling in *Scnn1b*-Tg mice

To investigate the role of TNFα in airway inflammation and remodeling in *Scnn1b*-Tg mice, we crossbred *Scnn1b*-Tg mice (inbred line C57Bl/6N) with mice lacking either TNFα ligand (TNFα −/− mouse, C57:129Sv mixed background) or TNFα receptor 1 (TNFαR1 −/− mouse, C57Bl/6J background), and produced four possible genotypes as described in the Methods. Both crosses gave similar results, and data for the TNFα KO cross are shown in Fig. 1 and Supplemental Fig. 1.D-E, while data for the TNFαR1 KO cross are provided in Supplemental Fig. 1.A-C.

Lack of TNFα or TNFαR1 did not alter survival of *Scnn1b*-Tg mice and all mice had comparable, high survival ranging between 80-95% (Fig. 1.A and Supplemental Fig. 1.A). Survival of *Scnn1b*-Tg mice differed from previously published studies (1), likely due to strain differences. In fact, ongoing backcross studies in our laboratory, aimed at obtaining inbred strains of *Scnn1b*-Tg mice, have shown that survival is significantly increased in the C57Bl/6N and 129S1/SvImJ backgrounds (9) compared to the original mixed C3:B6 background (1).

TNFα was significantly elevated in BAL from TNFα +/−, *Scnn1b*-Tg mice in comparison to TNFα +/−, WT littermates (Fig. 1.B, left panel), consistent with previous reports (3). As expected due to TNFα gene deletion, TNFα was undetectable in BAL samples from TNFα −/−, *Scnn1b*-Tg mice. KC was significantly elevated in both TNFα −/−, *Scnn1b*-Tg mice and TNFα +/−, *Scnn1b*-Tg mice (Fig. 1.B, right panel), indicating that absence of TNFα did not impact the production of this neutrophil chemo-attractant.

Histological lesions typically observed in *Scnn1b*-Tg mice are illustrated in Fig. 1.C. Semi-quantitative scoring of these lesions in 5 week-old mice from the TNFα KO × *Scnn1b*-Tg cross revealed significant mucus plugging, airspace enlargement, lymphoid hyperplasia and airway inflammation in *Scnn1b*-Tg mice compared to WT littermates, irrespective of TNFα or TNFαR1 status (Fig. 1.D and Supplemental Fig. 1.B). TNFα or TNFαR1 deficiency did not alter lung histology in WT mice. Absence of TNFα or TNFαR1 did not prevent neutrophil and eosinophil infiltration in *Scnn1b*-Tg mouse lung, as assessed by BAL differential cell counts (Fig. 1.E and Supplemental Fig. 1.C). Large, foamy alveolar macrophages (see Fig. 1.C10) were present in both TNFα sufficient and deficient *Scnn1b*-Tg mice. Furthermore, TNFα ablation did not affect lung histopathology or BAL differential cell counts in 10 day-old *Scnn1b*-Tg or WT mice (Supplemental Fig.1.D-E).

TNFα has been shown to downregulate ENaC activity in airway epithelial cells *in vitro* (22-24). To test whether lack of TNFα altered Na^+ transport, we studied freshly excised tracheas from 5 week-old mice in Ussing chambers (Fig. 1.F). Amiloride-sensitive short circuit current (I_{sc}) was significantly greater in *Scnn1b*-Tg mice than WT mice, consistent with increased ENaC activity and as previously reported (1), but was not affected by TNFα status. Collectively, these data indicate that TNFα or TNFR1 are not essential for development of airway and lung pathology in *Scnn1b*-Tg mice.

Absence of IL-4Rα enhances neonatal survival in *Scnn1b*-Tg mice

To determine the role of IL-4Rα signaling in the development of *Scnn1b*-Tg mouse lung pathology, we crossed *Scnn1b*-Tg (mixed C3:B6 background) and IL-4Rα knock-out mice (IL-4Rα KO, BALBc/J background), as described in the Methods. As expected, the survival of WT mice was high (100%) and was unaffected by the absence of IL-4Rα. In contrast, *Scnn1b*-Tg mice exhibited characteristic early postnatal mortality (Fig. 2.A). In agreement with our backcross studies, introduction of the BALBc/J background reduced *Scnn1b*-Tg mouse survival in comparison to the mixed C3:B6 background [32% vs. 50% for C3:B6:BALB and C3:B6 (1), respectively]. However, genetic ablation of IL-4Rα significantly increased survival of *Scnn1b*-Tg mice (54% vs. 32%, for IL-4Rα −/−, *Scnn1b*-Tg vs. IL-4Rα +/−, *Scnn1b*-Tg mice, Fig. 2.A). By genotyping pups at day 1-3, we verified that all four genotypes were present in the expected Mendelian proportions at birth (Supplemental Fig. 2.A). The improvement in survival occurred between 5 and 12 days, the window of peak mortality for *Scnn1b*-Tg mice, and stabilized thereafter (Fig. 2.A).

Absence of IL-4Rα decreases neonatal mucous secretory cell (MuSC) abundance and eosinophilia in wild-type and *Scnn1b*-Tg mice

To elucidate the reason(s) for improved survival in IL-4Rα deficient *Scnn1b*-Tg mice, we examined lung histology and BAL cell counts in 10 day-old pups. Although MuSC are rare in adult WT unchallenged mice, we detected luminal mucus and rather abundant MuSC in the bronchi of 10-day-old IL-4Rα +/−, WT mice (Fig. 2.B3, 2.C). However, both mucus and MuSC were virtually absent in IL-4Rα −/−, WT mice (Fig. 2.B4, 2.C). Although bronchial mucus plugging, as detected histologically, was similar in both IL-4Rα-sufficient and deficient *Scnn1b*-Tg mice (Fig. 2.C), MuSC were significantly reduced in IL-4Rα-deficient *Scnn1b*-Tg mice (Fig. 2.B7-B8, and 2.C), which was confirmed by computer image analysis of AB-PAS-positive stored mucosubstances (Supplemental Fig. 2.B). However, absence of IL-4Rα did not ameliorate the parenchymal air space enlargement and airway inflammatory lesions already evident in young *Scnn1b*-Tg mice (Fig. 2.C). Lymphoid hyperplasia was never observed in 10 day-old animals.

Absence of IL-4Rα decreased BAL eosinophils in 10 day-old *Scnn1b*-Tg mice in comparison to IL-4Rα +/−, *Scnn1b*-Tg mice (Fig. 2.D), but failed to reduce the pronounced macrophage and neutrophil infiltrate. Low, but readily detectable number of eosinophils was also present in BAL from 10 day-old IL-4Rα +/−, WT mice (4,890 ± 100 cells/ml), which was reduced in IL-4Rα −/−, WT mice (900 ± 260 cells/ml).

Similar to the results we obtained for genetic deletion of TNFα, we did not detect changes in airway ion transport properties of either WT or *Scnn1b*-Tg mice due to the absence of IL-4Rα (Supplemental Fig. 2.C).

Absence of IL-4Rα does not mitigate chronic lung pathology in surviving adult *Scnn1b*-Tg mice

We next tested whether absence of IL-4Rα modified chronic lung pathology or inflammation in surviving 5 week-old *Scnn1b*-Tg mice. As expected, no differences were detected in adult WT mice due to the presence or absence of IL-4Rα (Fig. 3.A1-A2). Absence of IL-4Rα minimally altered lung histology scores and BAL parameters in adult *Scnn1b*-Tg mice. Specifically, airway mucus plugging, MuSC abundance, air space enlargement, lymphoid hyperplasia and airway inflammation were equivalent in IL-4Rα-deficient and -sufficient *Scnn1b*-Tg mice (Fig. 3.A3-A4, B). As seen in 10 day-old animals, BAL eosinophils were reduced in IL-4Rα−/−, *Scnn1b*-Tg mice in comparison to IL-4Rα sufficient mice, but marked neutrophilia persisted in *Scnn1b*-Tg mice regardless of IL-4Rα status (Fig. 3.C).

Absence of IL-4Rα does not modify the BAL inflammatory mediator profile of neonatal or adult Scnn1b-Tg mice

Genetic deletion of IL-4Rα reduced neonatal MuSC and improved survival in *Scnn1b*-Tg mice, but did not eliminate chronic neutrophilic inflammation. To elucidate the IL-4Rα-independent mechanisms driving the development of chronic lung pathology, we analyzed BALF cytokines in 10 day- and 5 week-old *Scnn1b*-Tg mice. INFγ, MCP-1, and IL-17 were below the detection limit in all samples. KC and TNFα levels were elevated in *Scnn1b*-Tg mice compared to WT littermates at both early and late time points and were not altered by IL-4Rα status (Fig. 4.A, B). A trend towards higher IL-4, IL-5 and IL-13 levels was observed in 10 day-old *Scnn1b*-Tg mice in comparison to WT littermates, which normalized by 5 weeks of age (Fig. 4.C, D, E; IL-4, 2.0 ± 0.7 vs. 0.02 ± 0.02; IL-5, 127 ± 37 vs. 15.2 ± 7; IL-13, 17.6 ± 6 vs. 4.3 ± 1 pg/ml for 10 day-old *Scnn1b*-Tg and WT mice, respectively. n=10). Absence of IL-4Rα caused a small but significant decrease in IL-5 in 5 week-old *Scnn1b*-Tg mice. In agreement with a previous study (43), transient eosinophilia in 10 day-old WT mice was not associated with increased BALF levels of IL-4, IL-5 and IL-13 in comparison to adult mice.

Elements of *Scnn1b*-Tg airway and lung pathology are glucocorticoid resistant

In view of the minimal impact of TNFα, TNFR1 or IL-4Rα genetic removal on adult *Scnn1b*-Tg mouse lung pathology/inflammation, we investigated the effects of glucocorticoid treatment. Daily systemic administration of 20 mg/kg prednisolone for two weeks, starting at 5-6 weeks of age, inhibited the body weight gain (2.5 g/week) typical of both *Scnn1b*-Tg and WT littermates (Supplemental Fig. 3.A-B), likely reflecting catabolic effects of high dose systemic glucocorticoids (48) and indicating effective drug delivery. Prednisolone administration markedly diminished MuSC abundance and eosinophilia and significantly reduced the incidence of lymphoid aggregates in *Scnn1b*-Tg mice, but failed to ameliorate mucus plugging, airway inflammation and neutrophilia (Fig. 5.A, B, C). BAL lymphocyte counts were decreased by prednisolone, but not significantly (2,620 ± 867 and 993 ± 580 lymphocytes/ml BAL, for vehicle and prednisolone, respectively). In *Scnn1b*-Tg mice, prednisolone administration increased the BALF total protein content (Fig. 5.D), but did not affect the levels of KC, MIP-2, TNFα and IL-6, which were significantly elevated in comparison to WT littermates (data not shown). Interestingly, prednisolone also reduced the normal abundance of bronchial MuSC in WT mice (Fig. 5.A, 5.B2). We assessed whether the prednisolone-induced reduction in MuSC correlated with decreased BAL mucin protein content, as assessed by agarose gel western blots. As shown in Fig. 5.E, BAL mucin content was significantly higher in *Scnn1b*-Tg mice compared to WT mice, and was not reduced as a function of prednisolone administration.

Normal postnatal development of mouse airways entails a spatially and temporally confined abundance of MuSC and transient eosinophilia

The presence of AB-PAS positive MuSC and eosinophils in the airways of 10 day-old IL-4Rα sufficient WT mice and their absence in IL-4Rα deficient mice prompted us to investigate whether the appearance of MuSC and eosinophils was a normal developmental feature of the mouse respiratory system. We analyzed tracheal and lung histology in 5 day-, 10 day-, and 5 week-old mice from C57BL/6N, C3H/HeN and BALB/cJ inbred strains, and found a distinct developmental pattern. At day 5, when submucosal glands are rudimentary (44,45), AB-PAS positive cells were abundant in the trachea (illustrated for the C57BL/6N strain in Fig. 6.A1) and absent in the bronchi (Fig. 6.A4), whereas by day 10 their frequency diminished in the trachea (Fig. 6.A2), and increased in the proximal portion of the main stem bronchi (Fig. 6.A5). At 5 weeks, MuSC were virtually absent in the trachea (Fig. 6.A3) and the number of bronchial MuSC stabilized at the low levels found in adult mice (Figure 6.A6). This pattern was conserved amongst inbred strains (Fig. 6.B for C57BL/6N and Supplemental Fig. 4.A-B for C3H/HeN and BALB/cJ).

BAL differential cell counts from 5 day-, 10 day-, and 5 week-old C57BL/6N, C3H/HeN and BALB/cJ mice revealed mainly macrophages, although we detected rare neutrophils in 5-10 day old mice, which were absent in adult animals (Fig. 6.C for C57BL/6N and Supplemental Figure 4.C-D for C3H/HeN and BALB/cJ). Notably, we also found a subtle but consistent degree of eosinophilia in 10 day-old C57BL/6N and C3H/HeN mice. Eosinophil counts were significantly lower in 10 day-old BALB/cJ mice (Supplemental Fig. 4.D) compared to C57BL/6N and C3H/HeN mice, which is consistent with previous studies comparing allergic BALB/cJ and C57BL/6N mice and the reported lower affinity of the BALB/c IL-4Rα variant for IL-4 (46,47).

BAL secreted mucin content is increased in *Scnn1b*-Tg mice

To test for a biochemical correlate of the time-dependent changes in MuSC observed in WT mice, and to assess how BALF mucin content may be affected by airway surface dehydration, we performed a time-course analysis in WT mice and their *Scnn1b*-Tg littermates (C57Bl/6N line). At all ages, the BALF mucin content, assessed with antibodies that detect either all mucins

(Fig. 7.A) or murine Muc5b (Supplemental Fig. 5.A), were greater in *Scnn1b*-Tg mice than in WT mice, whereas the BALF total protein content for *Scnn1b*-Tg mice was only slightly increased in comparison to WT mice at 5 days and 8 weeks of age (Fig. 7.B). In both WT and *Scnn1b*-Tg littermates, BALF mucins were greatest in 5 and 10 day-old mice and declined in older animals. This temporal pattern of mucin glycoprotein expression is consistent with prior Muc5ac, Muc5b, Muc4 and Gob5 mRNA expression studies (3) and suggests that relative mucin abundance is a consistent feature of neonatal airway development which is further augmented by impaired mucus clearance in *Scnn1b*-Tg mice. Analysis of BALF mucin content in 5 day-, 10 day-, and 5 week-old WT mice of diverse genetic backgrounds (inbred C57BL/6N, C3H/HeN and BALB/cJ) revealed modest, age- and strain-dependent changes in BALF total and Muc5b mucin content (Supplemental Fig. 5.B-D).

DISCUSSION

Adequate airway mucosal surface hydration is essential for effective mucus clearance and lung health. The dynamic progression of lung disease following disruption of mucus clearance in *Scnn1b*-Tg mice suggests a complex host response to airway surface dehydration (3). We investigated the role of TNFα and IL-4Rα signaling during postnatal lung development and identified aspects of *Scnn1b*-Tg lung pathology that are uniquely susceptible to modification of these pathways and to corticosteroid treatment.

Our studies indicated that TNFα signaling is not required for triggering or sustaining inflammation, airway remodeling and distal lung pathology (air space enlargement) in *Scnn1b*-Tg mice. The early appearance of air trapping/airspace enlargement in *Scnn1b*-Tg mice suggests that neonatal airway inflammation might shift the protease/antiprotease balance, impairing alveolarization and generating pro-inflammatory signals via extracellular matrix degradation, e.g., Pro-Gly-Pro peptide (49) and hyaluronan (50). Development of pulmonary lymphoid aggregates in *Scnn1b*-Tg mice was also TNFα-independent. Since similar nodules are found in lungs of mice repetitively challenged with aerosolized allergen (51,52) or *H. influenzae* lysate (53), we speculate that lymphoid hyperplasia is caused by greater and/or more sustained exposure to environmental antigens due to mucus stasis, which in turn enhances adaptive immune responses. Finally, TNFα inhibits ENaC expression and activity *in vitro* (22-24), and can down-regulate the rat CCSP promoter (54), which in *Scnn1b*-Tg mice drives βENaC overexpression. However, we found that absence of TNFα did not alter airway epithelial ion transport properties in excised tracheal tissue from WT or *Scnn1b*-Tg mice, suggesting that TNFα does not affect either ENaC activity or the CCSP promoter *in vivo*.

Breeding *Scnn1b*-Tg mice with IL-4Rα gene deleted mice revealed the existence of IL-4Rα-dependent and -independent elements of *Scnn1b*-Tg mouse lung pathology and provided new insights regarding the role of IL-4Rα signaling during normal neonatal airway development.

In murine models of allergic asthma, eosinophils are involved in collagen deposition and airway smooth muscle hyperplasia, but they are not required for airway hyperreactivity or MuSC metaplasia (55,56). Our data suggest that eosinophils are not a major determinant of lung pathology in *Scnn1b*-Tg mice. Decreased eosinophils correlated with fewer MuSC in neonatal but not adult IL-4Rα −/−, *Scnn1b*-Tg mice, and lack of eosinophils did not ameliorate other aspects of lung pathology in *Scnn1b*-Tg mice. Moreover, although a time-dependent role for eosinophils in promoting MuSC abundance is conceivable based on correlations observed between neonatal MuSC and eosinophilia (see Fig. 2.C, D; Fig. 6.B-C; and Supplemental Fig. 4.A, C), the abundant MuSC found in normal 10 day-old BALBc/J mice (Supplemental Fig. 4.B), despite the virtual absence of eosinophils (Supplemental Fig. 4.D), suggests that a causal association is unlikely.

The transient and spatially restricted appearance of MuSC in the airways of neonatal WT mice was completely ablated in the absence of IL-4Rα (Fig. 2.C). We speculate that neonatal MuSC expansion is driven by local, basal levels of Th2 cytokines (IL-4, IL-13 and IL-5, Fig. 4.C-E), which are difficult to detect once diluted in BAL. In comparison to WT littermates, neonatal *Scnn1b*-Tg mice had higher levels of Th2 cytokines (Fig. 4.C-E), which likely increased MuSC (Fig. 2.C). However, absence of IL-4Rα mitigates this response. The residual MuSC in neonatal IL-4Rα −/−, *Scnn1b*-Tg mice as compared to IL-4Rα −/−, WT mice (Fig. 2.C) indicates the existence of an IL-4Rα-independent pathway that triggers MuSC in neonatal *Scnn1b*-Tg mice. Indeed, this pathway could also be active in adult *Scnn1b*-Tg mice, in which MuSC are abundant (Fig. 3.B) but Th2 cytokine levels return towards baseline (Fig. 4.C-E).

The similarity in the MuSC distribution pattern among neonatal WT mice (Fig. 2.B3 and 6.A5), *Scnn1b*-Tg mice (Fig. 2.B7), and OVA challenged mice (57), namely abundance in the proximal main stem bronchi and a gradual decrease distally, suggests that specific populations of airway epithelial cells are primed to differentiate into MuSC in response to external stimuli. Reports of temporal and spatial expression of transcription factors involved in promoting or suppressing MuSC, such as SPDEF (58) and FOXA2 (59), support this hypothesis.

As quantitated histologically, neonatal IL-4Rα-deficient *Scnn1b*-Tg mice exhibited mucus plugging equivalent to IL-4Rα-sufficient *Scnn1b*-Tg mice, despite decreased MuSC (Fig. 2.C). Similar results obtained upon prednisolone treatment suggest that airway surface dehydration and defective mucus clearance are major determinants of mucus accumulation over a wide range of MuSC abundance. Moreover, even in WT mice, the high degree of variation in MuSC abundance detected histologically at 5 days, 10 days and 5 weeks of age (Fig. 6.A, B and Supplemental Fig. 4.A, B) in comparison to the modest changes observed in BALF total and Muc5b mucins (Supplemental Fig. 5.B-C) suggests that BAL mucin content depends on factors other than the intracellular content of stored mucosubstances alone. We hypothesize that constitutively secreted mucins (40), including Muc5b as detected in BAL (Supplemental Fig. 5.A, C) and shed cell surface mucins (Muc1, 4 and 16), are an integral part of secreted mucus in WT mice and contribute to mucus obstruction in *Scnn1b*-Tg mouse airways. However, the survival advantage of IL-4Rα-deficient *Scnn1b*-Tg mice suggests that, especially during the neonatal period, modifications of the mucus secretory system can be beneficial to prevent fatal airway obstruction when mucus clearance is impaired. The absence of significant differences in tracheal epithelial ion transport properties due to the absence of IL-4Rα in *Scnn1b*-Tg mice (Supplemental Fig. 2.C) supports our conclusion that the increased survival of IL-4Rα deficient, *Scnn1b*-Tg mice reflects modifications in MuSC and mucus secretion, not Na^+ transport.

Although IL-4Rα removal had an impact on the neonatal phenotype, lung lesions in adult IL-4Rα-sufficient and -deficient *Scnn1b*-Tg mice were indistinguishable. In contrast to allergen challenge (21) or the late response to viral infection (60), which require the IL-13/IL-4Rα signaling axis to elicit lung pathology, our findings strongly suggest that airway surface dehydration/mucus stasis is a unique stimulus that activates multiple, IL-4Rα-independent effector pathways leading to inflammation and remodeling in *Scnn1b*-Tg mice.

Given the complexity of the inflammatory responses in *Scnn1b*-Tg mice, we tested whether a broad-spectrum anti-inflammatory agent could ameliorate or reverse adult lung lesions. Glucocorticoids are widely used both clinically and experimentally to blunt inflammation and mucus hypersecretion (27-31,61,62), and they can effectively reduce eosinophils, mast cells, CD4+ T lymphocytes, dendritic cells, and pro-inflammatory cytokines in asthma (63,64). However, in obstructive lung diseases characterized by neutrophilic inflammation, e.g., chronic bronchitis and COPD, inflammation appears to be corticosteroid-resistant (65,66). In *Scnn1b*-Tg mice, prednisolone treatment blunted eosinophilia and MuSC (Fig. 5.A-C), similar to other

mouse models (62,67,68), but did not reduce neutrophil influx, the appearance of large foamy macrophages, or mucus accumulation in the airway lumen. In COPD, unresponsiveness to glucocorticoids has been attributed to inhibition of histone deacetylase 2 activity by oxidative and nitrosative stress (26), a condition that may also occur in *Scnn1b*-Tg mice. Alternatively, glucocorticoids have been shown to spare or enhance innate immunity while repressing adaptive responses (69,70). We suggest that chronic neutrophil recruitment and macrophage activation are due to a glucocorticoid-insensitive, innate immune response triggered by accumulation of environmental and endogenous stimuli in stagnant mucus, which likely involves signaling through pattern recognition receptors, such as Toll-like receptors.

Our studies with neonatal *Scnn1b*-Tg mice and WT littermates led us to discover a temporal and spatial pattern of MuSC and inflammatory cell abundance in the airways of unchallenged WT mice suggestive of an active phase of maturation and adaptation of innate immune responses (Fig. 5). Perinatal changes in MuSC/mucus composition and BAL leucocytes have been described in several species (71-76), including humans (77-81). Surprisingly, we found little information regarding these normal developmental changes in mouse airways. In agreement with our studies, one study focused on mouse submucosal gland development incidentally shows abundant MuSC in the superficial epithelium at postnatal day 4 (44), and there is one report of eosinophilia in 10 day-old C3HeB/FeJ mice as compared to adult animals (43). Our histological sectioning protocol and the multiple time points studied enabled us to visualize the dynamic changes in MuSC and BAL cells. We hypothesize that immediately after birth, when the acquired immune system is still immature (82,83), airway mucosal immunity relies on secreted mucus as a primary barrier to protect the host from inhaled, potentially pathogenic particles. A growing body of evidence indicates that neonatal events, e.g., bacterial colonization (84,85), viral infection (86,87), or allergen exposure (88), have a profound impact on later immune responses. In this context, effective mucus clearance is critical because it determines the concentration and dwell time of particles/pathogens/chemical mediators, the extent of inflammatory and parenchymal cell activation, and the propensity for airway obstruction. While mucus abundance may offer broad airway protection (89), it may become life-threatening when coupled with defective clearance, as in *Scnn1b*-Tg mice. Indeed, different survival rates observed in *Scnn1b*-Tg mice of different genetic backgrounds (Dr. Wanda O'Neal, personal communication and (9)) likely reflect diversity in the amount/physical properties of secreted mucus or in the architecture of the proximal airways.

In summary, we propose that in neonatal *Scnn1b*-Tg mice, impaired mucus clearance strongly interacts with the normal, IL-4Rα-dependent abundance of MuSC and eosinophils. This interaction produces both potentially lethal mucus obstruction and delays the normal regression of MuSC and eosinophils. In addition to exogenous aerotoxins and endogenous mediators trapped in static mucus, epithelial cell necrosis, transiently present in the lower airways of newborn *Scnn1b*-Tg mice (3), contributes to promote local inflammation (90) and disease onset. Although unresolved/lingering MuSC abundance and eosinophilia can be blunted by corticosteroid treatment, neutrophilia and mucus obstruction persist and are likely responsible for maintaining airway and lung disease in *Scnn1b*-Tg mice. Future studies elucidating the interaction of mucus clearance with other components of airway mucosal immune system during postnatal development, and identifying the molecular mechanisms that sustain airway inflammation and remodeling in adults, will suggest therapeutic approaches for common human obstructive airway diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ASL, airway surface liquid
Tg, transgenic
CF, cystic fibrosis
CB, chronic bronchitis
COPD, chronic obstructive pulmonary disease
MuSC, mucous secretory cells
TNFα, tumor necrosis factor alpha
IL-4Rα, interleukin-4 receptor alpha subunit
CCSP, Clara cell secretory protein
BAL, bronchoalveolar lavage
BALF, bronchoalveolar lavage fluid
Scnn1b, Sodium Channel Non-Voltage Gated 1, beta subunit

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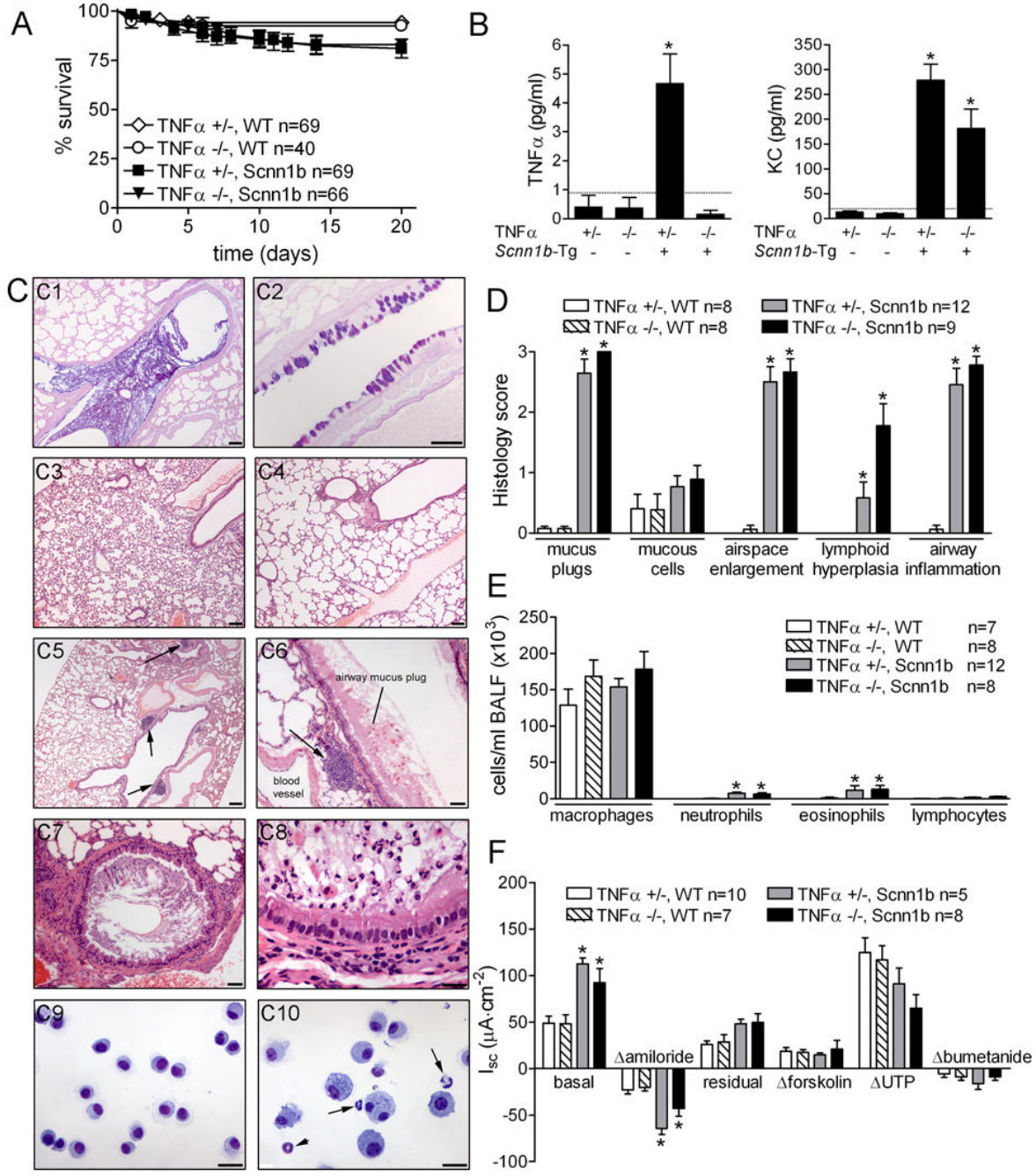

Figure 1. Minimal effect of TNFα deletion on the *Scnn1b*-Tg mouse phenotype

A) Survival curves. Introduction of the 129S/SvEv:C57Bl/6J background (TNFα KO) decreases mortality of *Scnn1b*-Tg mice compared to the original C3H/HeN:C57Bl/6N strain, irrespective of TNFα status. See text for details. B) BAL cytokine/chemokine profile in 5 week-old mice. * $p<0.05$ vs. TNFα +/−, WT mice. The dotted line represents the assay lower detection limit. C.1-10) Representative photomicrographs illustrating characteristic lesions in 5 week-old TNFα −/−, *Scnn1b*-Tg mice. H&E stain unless indicated. C1) Mucus plugs, AB-PAS stain, scale bar = 100 μm; C2) Mucous secretory cells, AB-PAS stain, scale bar = 50 μm; C3-C4) Distal airspace enlargement, comparing WT (C3) and *Scnn1b*-Tg mice (C4), scale bar = 200 μm; C5-C6) Lymphoid hyperplasia, at low (C5, scale bar = 200 μm) and high (C6, scale bar

= 50 μm) magnification; C7-C8) Airway inflammation, at low (C7, scale bar = 50 μm) and high (C8, scale bar = 20 μm) magnification; cytospins from WT (C9) and *Scnn1b*-Tg mice (C10), illustrating large/foamy macrophages, neutrophils (arrows) and eosinophils (arrowhead), Giemsa stain, scale bar = 20 μm. D) Semi-quantitative histopathology scores indicating similar lesions in TNFα +/− and TNFα −/−, *Scnn1b*-Tg mice. * $p<0.05$ vs. TNFα +/−, WT mice. E) Differential BAL cell counts. * $p<0.05$ vs. TNFα +/−, WT mice. F) Ion transport properties of freshly excised tracheal tissues. “Basal” indicates the I_{sc} before drug application. The change in I_{sc} (Δ) after sequential drug addition is shown. “Residual” I_{sc} is the I_{sc} remaining after amiloride application. * $p<0.05$ vs. TNFα +/−, WT mice.

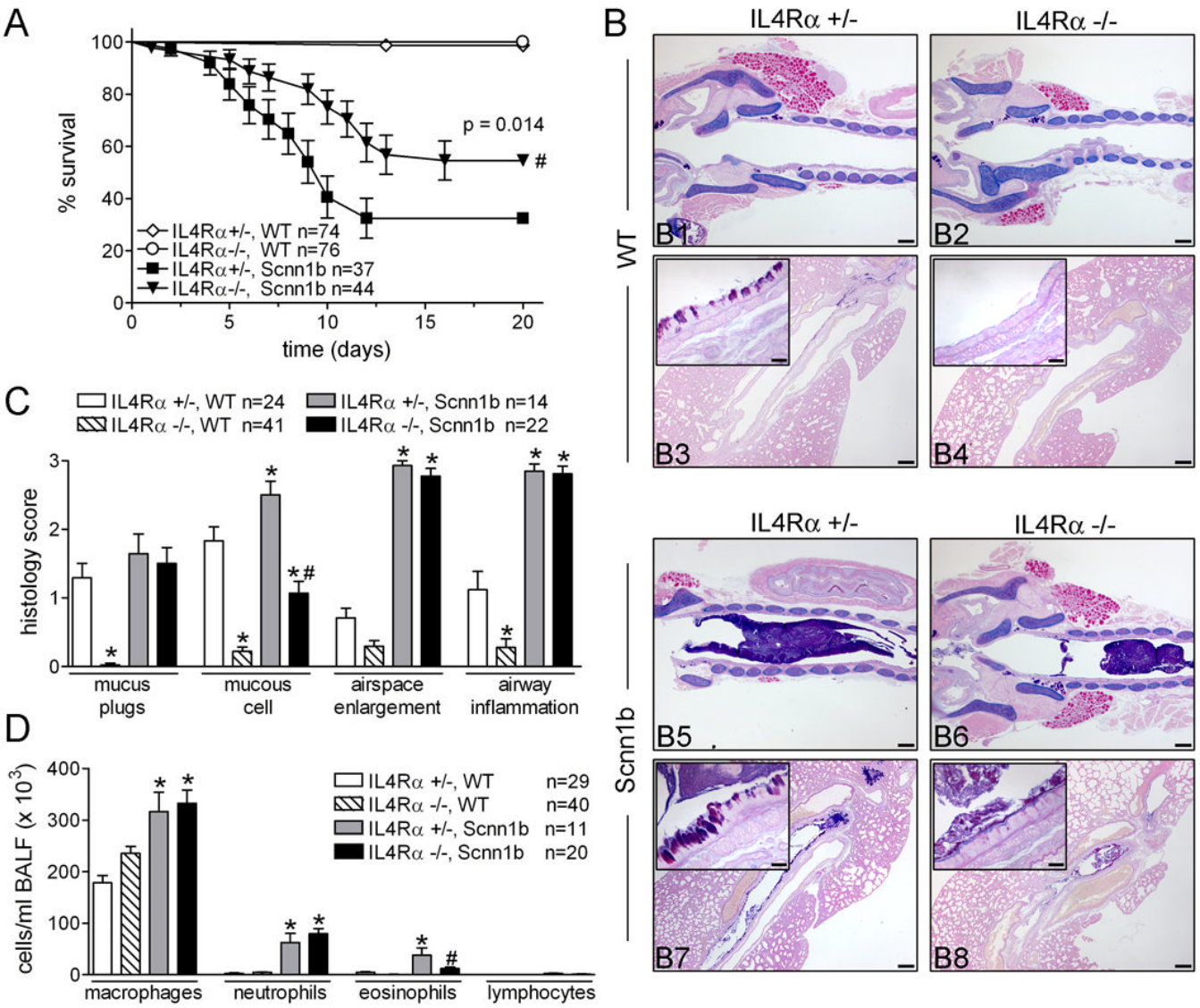

Figure 2. Genetic deletion of IL-4Rα improves neonatal survival and decreases mucous secretory cells and eosinophils in 10 day-old *Scnn1b*-Tg mice

A) Survival curves. Introduction of the BALBc/J background (IL-4Rα KO) increases mortality of *Scnn1b*-Tg mice compared to the original C3H/HeN:C57Bl/6N strain and absence of IL-4Rα improved survival. See text for details. # p=0.014 vs. IL-4Rα +/−, *Scnn1b*-Tg mice. B) Representative photomicrographs of trachea (B1-B2 for WT and B5-B6 for *Scnn1b*-Tg mice) and lung (B3-B4 for WT and B7-B8 for *Scnn1b*-Tg mice) of IL-4Rα +/− and IL-4Rα −/− mice, AB-PAS stain. Scale bars: low magnification = 200 μm, high magnification insets = 20 μm. C) Semi-quantitative histopathology scores. * p<0.05 vs. IL-4Rα +/−, WT mice. # p<0.05 vs. IL-4Rα +/−, *Scnn1b*-Tg mice. D) Differential BAL cell counts. * p<0.05 vs. IL-4Rα +/−, WT mice. # p<0.05 vs. IL-4Rα +/−, *Scnn1b*-Tg mice.

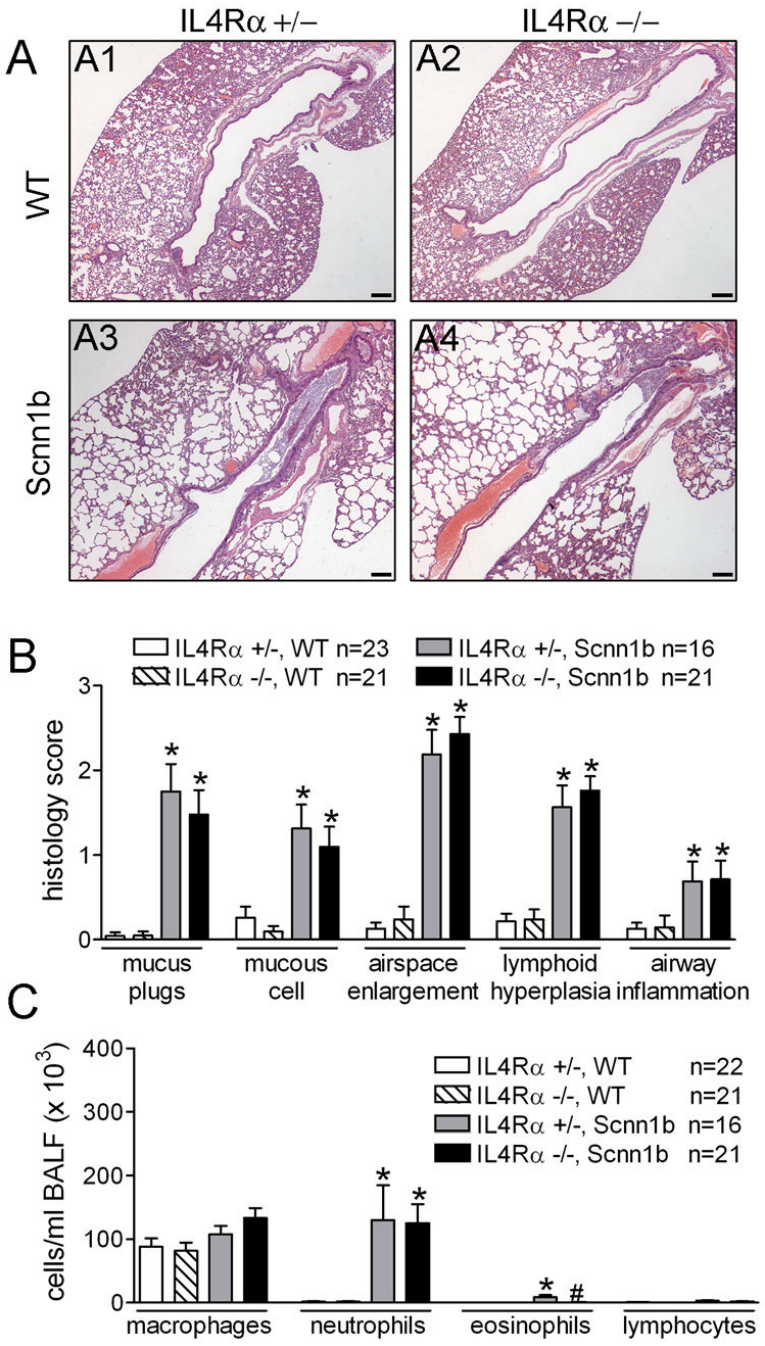

Figure 3. Minimal effect of IL-4Rα deletion on the adult *Scnn1b*-Tg mouse phenotype

A) Representative photomicrographs of lung histology in WT (A1-A2) and *Scnn1b*-Tg (A3-A4) mice, H&E stain, scale bar = 200 μm. B) Semi-quantitative histopathology scores. * $p<0.05$ vs. IL-4Rα +/−, WT mice. C) Differential BAL cell counts. * $p<0.05$ vs. IL-4Rα +/−, WT mice. # $p<0.05$ vs. IL-4Rα +/−, *Scnn1b*-Tg mice.

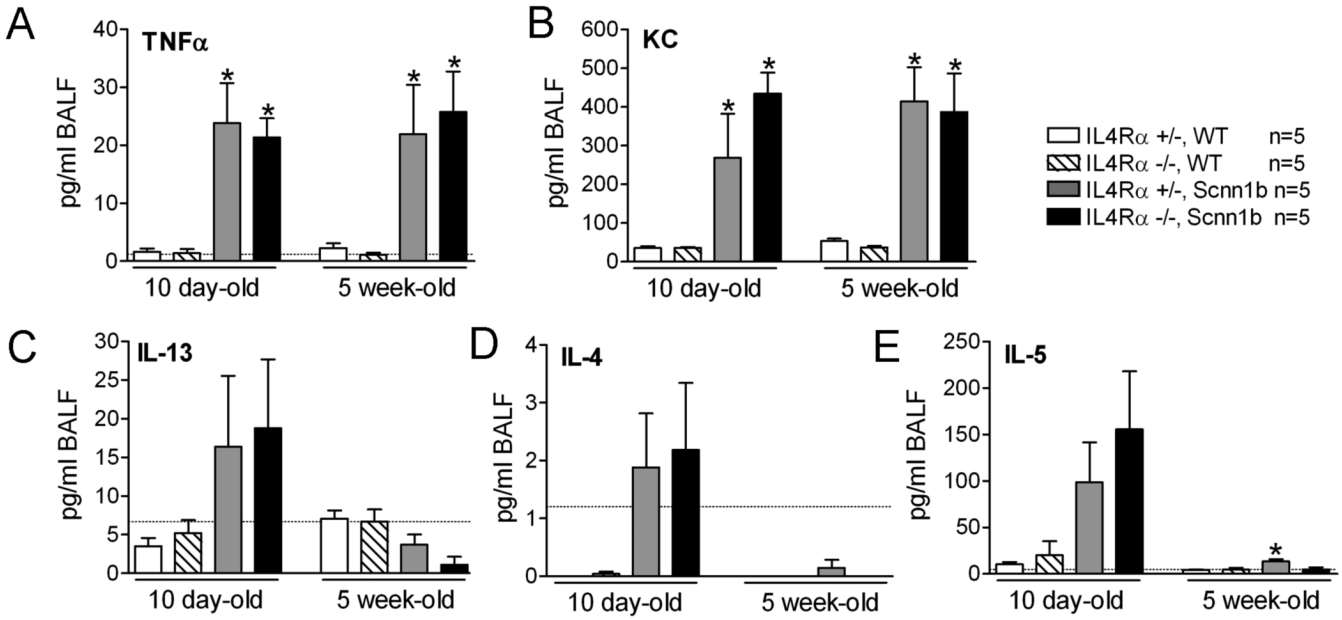

Figure 4. IL-4Rα deletion in *Scnn1b*-Tg mice has minimal effect on sustained BAL TNFα and KC and transient Th-2 cytokines

A-E) BAL cytokines in 10 day-old and 5 week-old mice. The dotted line represents the assay lower detection limit. * $p<0.05$ vs. age-matched IL-4Rα +/−, WT mice.

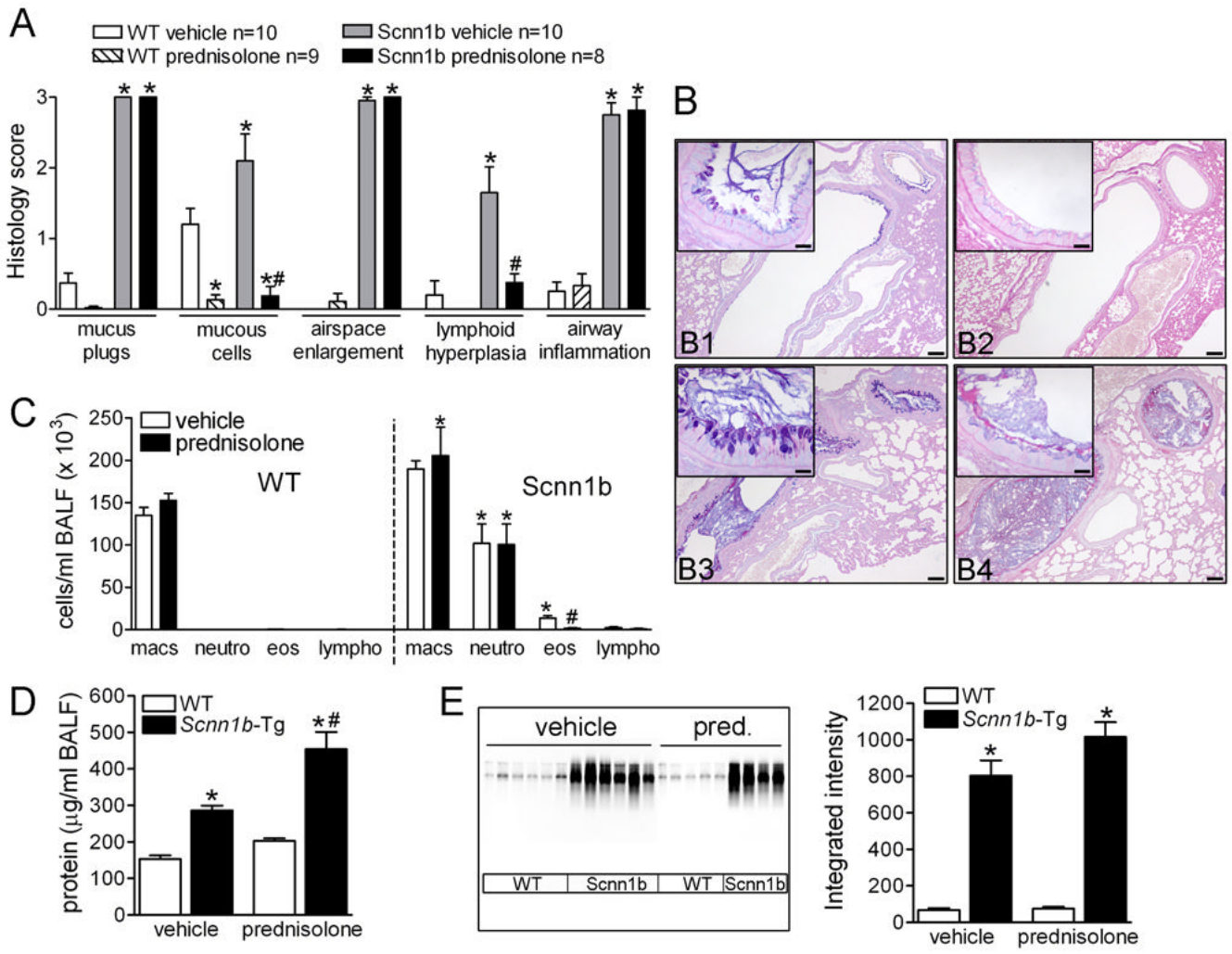

Figure 5. Prednisolone treatment of *Scnn1b*-Tg mice reduces MuSC and eosinophils but not neutrophils or BAL mucin content

A) Semi-quantitative histopathology scores. * $p<0.05$ vs. vehicle-treated WT mice. # $p<0.05$ vs. vehicle-treated *Scnn1b*-Tg mice. B) Representative photomicrographs of lung sections from WT (B1, B2) and *Scnn1b*-Tg mice (B3, B4) treated with vehicle (B1, B3) or prednisolone (B2, B4), AB-PAS stain, scale bars: low magnification = 100 μm, high magnification insets = 20 μm. C) BAL differential cell counts. * $p<0.05$ vs. vehicle-treated WT mice. # $p<0.05$ vs. vehicle-treated *Scnn1b*-Tg mice. D) BALF total protein content. * $p<0.05$ vs. vehicle-treated WT mice, # $p<0.05$ vs. vehicle-treated *Scnn1b*-Tg mice. E) Agarose gel western blots and corresponding densitometry of BAL samples from vehicle- and prednisolone-treated mice. Blots were probed with Muc5b antibody. * $p<0.05$ vs. vehicle-treated WT mice.

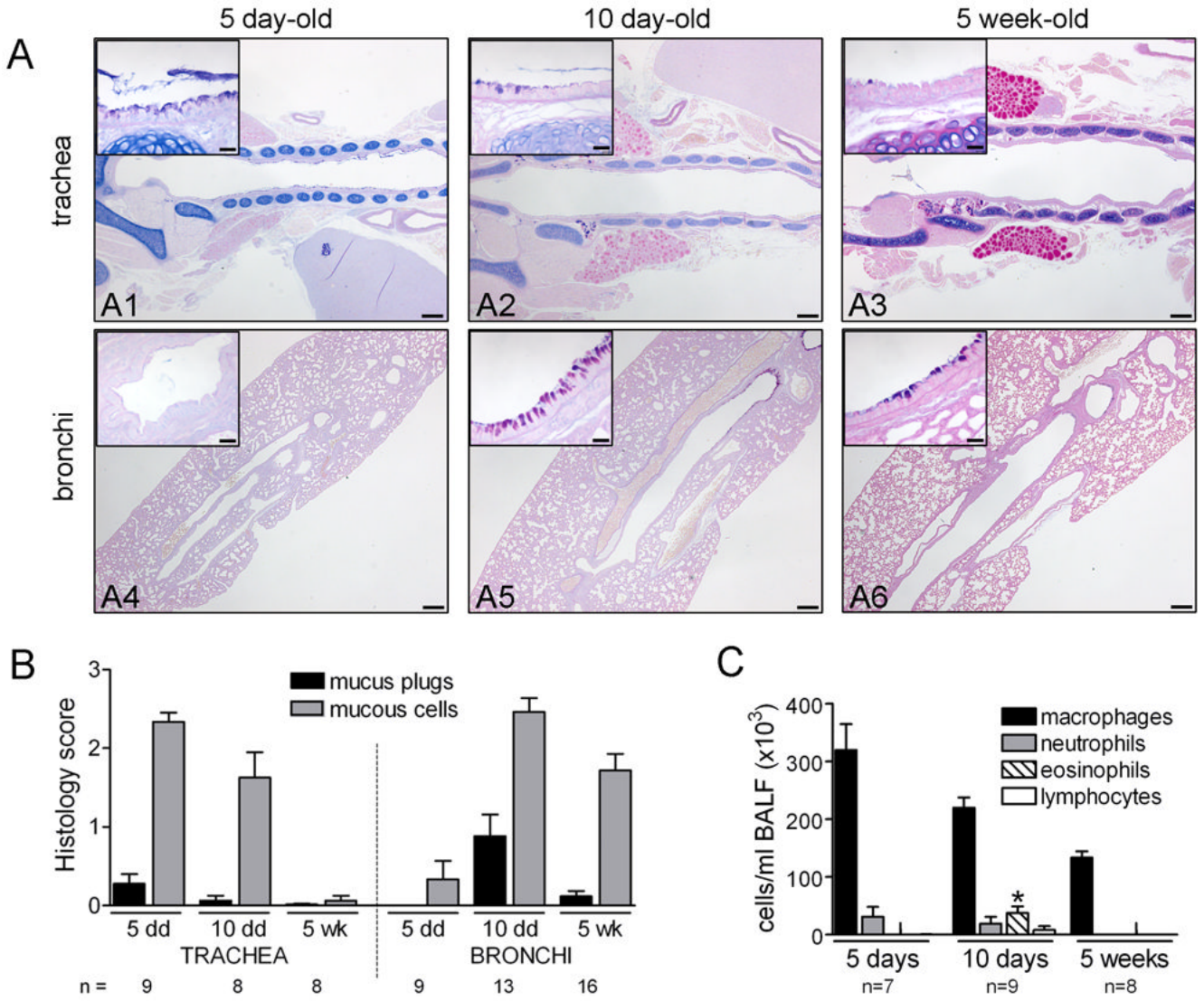

Figure 6. MuSC and eosinophils are transiently abundant during normal mouse airway development

A) Representative photomicrographs of trachea and bronchi from C57Bl/6N mice at age 5 days (A1, A4), 10 days (A2, A5) and 5 weeks (A3, A6), AB-PAS stain, scale bars: low magnification = 200 μm, high magnification insets = 20 μm. B) Semi-quantitative histology scoring of mucus and MuSC in tracheas and bronchi of C57Bl/6N mice at age 5 days (5 dd), 10 days (10 dd), and 5 weeks (5 wk). C) Differential BAL cell counts in C57Bl/6N mice at 5 days, 10 days, and 5 weeks of age. * $p<0.05$ vs. 5 day- and 5 week-old.

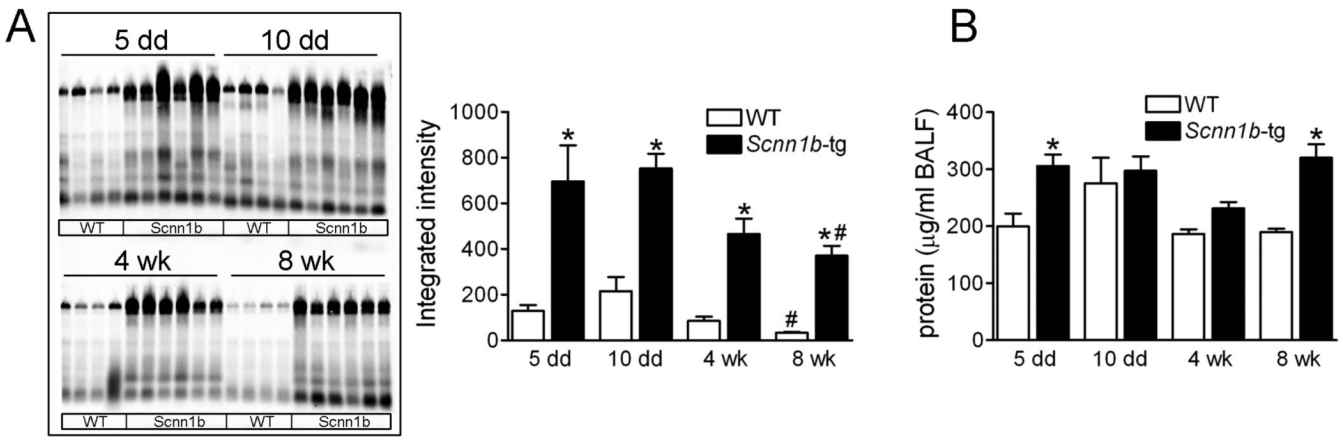

Figure 7. BALF total mucin content varies as a function of age and is elevated in neonatal and adult *Scnn1b*-Tg mice

A) Agarose gel western blots and corresponding densitometry of BAL samples from C57Bl/6N *Scnn1b*-Tg mice and WT littermates at age 5 days (5 dd), 10 days (10 dd), 4 weeks (4 wk), and 8 weeks (8 wk). Membranes were probed with pan-mucin antibody. * $p<0.05$ vs. WT mice of corresponding age; # $p<0.05$ vs. 10 day-old mice of corresponding genotype. B) BALF total protein content. * $p<0.05$ vs. WT mice of corresponding age.