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Conditional reprogramming of pediatric airway epithelial cells: a new human model to investigate early life respiratory disorders

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Abstract

Background—Airway epithelial cells (AEC) are quite difficult to access in newborns and infants. It is critically important to develop robust life-extended models to conduct translational studies in this age group. We propose the use of a recently described cell-culture technology (conditionally reprogrammed cells - CRC) to generate continuous primary cell cultures from nasal and bronchial AEC of young children.

Methods—We collected nasal and/or bronchial AEC from a total of 23 subjects of different ages including newborns/infants/toddlers (0–2 years; N=9), school age children (4–11 years; N=6), and a group of adolescent/adult donors (N=8). For CRC generation, we used conditioned medium from mitotically inactivated 3T3 fibroblasts and Rho-associated kinase (ROCK) inhibitor (Y-27632). Antiviral immune responses were studied using 25 key antiviral genes and protein production of type III epithelial interferon (IFN λ1) after double stranded (ds) RNA exposure.

Results—CRC derived from primary AEC of neonates/infants and young children exhibited: 1) augmented proliferative capacity and life-extension, 2) preserved airway epithelial phenotype after multiple passages, 3) robust immune responses characterized by expression of innate antiviral genes and parallel nasal/bronchial production of IFN λ1 after exposure to dsRNA, and 4) induction of airway epithelial inflammatory and remodeling responses to dsRNA (e.g. CXCL8 and MMP9).

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Conclusion—Conditional reprogramming of AEC from young children is a feasible and powerful translational approach to investigate early-life airway epithelial immune responses in humans.

Keywords

Airway; bronchial; CRC; children; epithelium; infants; nasal; newborns

INTRODUCTION

The airway epithelium is a crucial cellular interface that shapes airway immune responses in humans.(1–3) Airway epithelial cells (AEC) have evolutionary conserved innate receptors that control airway immune responses to viruses, allergens, and other enviromental challenges.(1–3) Thus, the epithelium is in a unique position to translate gene-environment interactions linked to the pathogenesis of respiratory conditions. In susceptible individuals, altered AEC function can make the airways vulnerable to viral infections and facilitate the establishment of the astmatic phenotype.(1–3) Studies using AEC derived from adults with asthma have led to the discovery of novel therapies and companion biomarkers based on epithelial-derived cytokines.(4, 5) In contrast, the airway epithelium of infants has been remarkably understudied, despite compelling evidence showing that asthma and other chronic respiratory disorders often begin in early life. (6)

One of the main limitations of studying the human infant airway epithelium is the lack of robust AEC culture systems. Prior studies have demonstrated that the use of human infant AEC cultures is a powerful strategy to investigate early life respiratory disorders,(7–10) however, airway sampling in this age group is a major challenge. Here we present a strategy to optimize nasal and bronchial infant AEC cultures using conditionally reprogrammed cells (CRC). (11, 12) The purpose of generating CRC is to enhance proliferative and survival capacity since primary AEC cannot survive multiple passages.(11–13) The CRC method combines exposure to a Rho kinase (ROCK) inhibitor Y-27632 and a fibroblast-derived feeder layer or conditioned medium (medium from irradiated fibroblasts) to reprogram primary AEC into progenitor cells.(11, 12, 14) The induction of CRC results from reprogramming of the entire cell population rather than the selection of a minor subpopulation, (11, 12) thus CRC are life-extended preserving the AEC phenotype. Although CRC generation has been described in human adult bronchial and nasal epithelium,(12, 15–17) our study is the first to adapt CRC technology to develop a human-based model of the newborn and infant airway epithelium. The impact of this work is that it presents a clinically and scientifically relevant human-based approach to investigate the developmental immunobiology of the airway epithelium and the pathogenesis of respiratory disorders that begin in early life.

METHODS

Study population and sampling

We collected nasal and/or bronchial AEC from 23 subjects of different ages including newborns/infants/toddlers (0–2 years; N=9), school age children (4–11 years; N=6), and

adolescent/adult donors (N=8) (Table 1). Samples were obtained during admissions, scheduled bronchoscopy, or surgical procedures in Children's National Health System (CNHS) in Washington D.C. We used two 2.7 mm cytology brushes rotated in each nostril for nasal AEC collection (inferior turbinate) or in the main carina for bronchial AEC collection. AEC were detached from the brushes by scrapping and agitation within the transport medium and subsequent centrifugation at 300g for 5 min at 4 °C. This study was approved by the Institutional Review Board of CNHS.

Conventional human airway epithelial cell cultures

Nasal and bronchial AEC were amplified on collagen, type IV from human placenta, (Sigma-Aldrich) coated T-75 flasks as described.(18) Cells were grown submerged using bronchial epithelial cell growth media (BEGM, Lonza) at 37°C and 5% CO2 with three changes of medium per week until 70–90% confluent in monolayer cultures.

Generation of CRC from human airway epithelial cells

NIH-3T3 fibroblasts were mitotically inactivated using 30 Gy irradiation. Irradiated NIH-3T3 cells were plated in 148-cm^2 tissue culture dishes (Corning) in 35 mL of BEGM. The medium was collected after 72 hours and was centrifuged at 1000 ×g for 5 minutes at 4°C. The resulting supernatant was passed through a 0.22-mm pore-size vacuum filter unit (VWR). The medium obtained is now referred to as conditioned BEGM. The conditioned BEGM was frozen and stored at −80°C in aliquots. Three volumes of conditioned BEGM were mixed with one volume of fresh BEGM; this mixture was supplemented with 10 μM Y-27632. Once combined the medium was immediately used to culture AEC (now referred to as CRC) at 37°C and 5% CO2. CRC were differentiated at air-liquid interface (ALI) as described,(18) applying conditioned media + Y-27632 to the basolateral compartment only.

CRC passaging

Passage number was defined as the number of CRC sub-cultures. After reaching monolayer confluence, CRC were detached using 0.05% trypsin/EDTA solution (Sigma-Aldrich) and split in a 1:4 ratio. Cell counts were used to construct growth curves based on population doublings calculated as the log base 2 of the final cell number divided by the seeding cell number.

Whole mount immunofluorescence

Fixed AEC cells were permeabilized, blocked, incubated in primary antibodies against Cytokeratin 5 (CK5; ThermoFisher) and DAPI, (4',6-Diamidino-2-Phenylindole, Dihydrochloride; ThermoFisher) and visualized with a confocal microscope (Zeiss LSM 510).

Gene expression

Total RNA was extracted using an RNeasy kit (Qiagen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCRs were performed using TaqMan Assays and primers for detection of NOTCH-1

and DDL-1. (Applied Biosystems). 20 additional genes (Table S2) were quantified using nCounter digital analyzer (NanoString Technologies).

Cytokine measurements

IFNλ protein levels were quantified using electrochemiluminescence assays (MSD) after exposure to double-stranded (ds) RNA (poly I:C 10ug/ml) at different time points.

RESULTS

CRC induction enhances proliferation and prevents sencence in pediatric AEC

Following elution from cytology brushes the number of cells harvested ranged from $45 \times 10^3 – 200 \times 10^3$ cells. AEC were placed either in standard growth media (BEGM, Lonza) or conditioned media generated from irradiated 3T3 fibroblasts with the addition of a ROCK inhibitor (Y-27632), a process that we refer to here as *CRC induction* (Figure 1A). We observed that after CRC induction infant AEC had dramatic increased proliferative capacity relative to standard BEGM-based cultures (Fig 1B). This rapid CRC proliferation allowed sub-culturing of infant AEC in ≤7 days. As previously described in adult AEC, (11, 12) we found that pediatric CRC lost their increased proliferative capacity and stopped dividing when the medium was changed to simple BEGM after passage 2 (Fig 1B). We next examined if this enhanced proliferative capacity was sustained over multiple passages. For this we compared population doublings in CRC generated from adult (N=8) and pediatric (N=15) nasal AEC donors. In the absence of CRC induction, infant nasal AEC ceased to divide after only two sub-cultures (passage 2). In contrast, infant nasal AEC grown in conditioned media plus ROCK inhibitior had continuous proliferation over multiple passages to the same extend of what was achieved in adult nasal AEC (Figure 2A and 2B). Using this CRC approach we had a substantial high-yield of cells for bio-banking from each infant donor, totaling 5–6 passages over 30 days of continuous primary nasal AEC cultures.

ROCK inhibition enhaces epithelial cell proliferation via downregulation of NOTCH1 signaling,(19) which occurs during CRC induction.(12, 14, 17) In agreement with this notion, we identified that infant nasal AEC treated with conditioned media plus ROCK inhibitior had reduced gene expression of NOTCH1 and its receptor DDL-1. (Figure 2C) We also found that following 30 days of continuous sub-culturing (passage 5), CRC derived from infant nasal AEC still exhibited abundance of cytokeratin 5 (CK5), a cytoskeleton protein of basal epithelial cells used as a molecular marker of the basal AEC phenotype (Figure 2D).(20) In addition, as reported by other groups,(12, 15–17) we found that pediatric CRC derived from primary AEC were able to differentiate at ALI after multiple passages (Figures 2E and 2F).

CRC induction enables AEC cultures from infants born prematurely and bronchial specimens of young children

We next examined if this approach could be used to culture AEC in two challenging but clinically relevant conditions: airway samples from infants born prematurely and bronchial speciments from young children. AEC sampling in small premature infants has not been reported, likely due to airway size limitations and underlying comorbidities. Similarly,

obtaining bronchial AEC in infants is limited by the need for pediatric bronchoscopy and sedation. Figure 3A shows that we were able to generate CRC from nasal AEC of a neonate born prematurely at 29 weeks gestation (sampled at 1 month of age; weight 2.5 kg) and that these CRC mantained typical AEC morphology after multiple sub-cultures (passage 5). We also were able to generate CRC cultures from bronchial AEC in two infants in which we had cultured nasal AEC. Samples were obtained at the same time (paired nasal/bronchial brushing; Figure 3B). CRC induction in bronchial AEC resulted in continuous proliferation over 30 days after multiple passages with a cell division rate (population doublings) similar to nasal AEC (Figure 3C).

CRC is a feasible method to study infant AEC innate immune responses

We used a sub-group of infant donors (N=4) in whom we generated CRC and sub-cultures (passage 5) from nasal AEC to examine if CRC maintain typical epithelial innate immune responses after multiple passages. We exposed these cells to dsRNA (poly I:C, 10 ng/ml), a viral mimic that stimulates the production of antiviral cytokine/chemokine in the airway epithelium.(18) In these CRC experiments we found that dsRNA induced the expression of *MYD88, MDA-5, RIG-I*, and *TLR-3*, (Figure 4A) which are classical innate immune signaling responses observed in AEC.(1, 2, 21, 22) The induction of these genes was coupled with downstream expression of *STAT1* and the induction of AEC antiviral chemokines such as *CXCL11, CXCL10*, and *CCL5* (Figure 4B), (21) as well as interferon (IFN) stimulated genes such as *IFIT1, IFI44, OAS2*, and *MX1* (Figure 5A). (21, 23) Notably IFN regulatory transcription factor 7 (IRF-7), which regulates transcriptional activation of virus-inducible IFN genes in AEC, (22, 24) was also overexpressed. IRF-7 induction was coupled with the expression of *IFNL*, (Figure 5A) the gene that encodes the IFN λ1 protein (also known as IL-29), an epithelial type III IFN secreted by AEC during viral respiratory infections.(25, 26) CRC also showed a robust production of IFN λ1 after exposure to dsRNA with basically identical IFN λ1 production in nasal and bronchial infant CRC paired cultures from the same donor (Figure 5B).

Airway inflammatory and remodeling responses of infant CRC

We also examined inflammatory and remodeling responses of CRC derived from nasal infant AEC. For these experiments we used a sub-group of infant donors (N=4) with CRC bio-banked cells after multiple sub-cultures (passage 5). After exposing these cells to dsRNA (poly I:C), we found significant upregulation of *MUC5AC, CXCL8 (IL-8), IL-13*, and *TNFα*, (Figure 6A) which are signature genes of airway epithelial inflammatory responses (2, 21). We also identified that dsRNA induced the expression of *MMP9* and *TGFβ1*, (Figure 6B) markers of remodeling responses in asthma and other respiratory conditions.(27, 28)

DISCUSSION

The main findings of this study are that conditionally reprogrammed cells (CRC) derived from the nasal or bronchial epithelium of infants and young children exhibit: 1) augmented proliferative capacity and absence of senecense after multiple passages with preserved airway epithelial phenotype, 2) robust immune responses characterized by expression of

innate antiviral genes and parallel nasal/bronchial production of type III epithelial interferon (IFN λ1) after exposure to a dsRNA, and 3) induction of typical airway epithelial inflammatory and remodeling responses to a viral stimuli (e.g. CXCL8 and MMP9). Collectively, our findings provide evidence that the use of CRC is a potentially powerful translational approach to elucidate the molecular mechanisms that control airway epithelial immune responses in infants and young children.

Prior studies have demonstrated that modelling the human airway epithelium using AECs from infants is a feasible approach to study early life antiviral responses.(7–10) Unfortunately, a major limitation to develop human-based epithelial models is that primary AEC typically do not survive in the lab after 2–3 passages due to epithelial cell senescence.(13) Importantly, epithelial cell senescence has recently been overcome using a novel system that generates CRC from primary AEC of adult donors.(12, 14, 15, 17) In our current study we have adapted this new approach to generate CRC from primary AEC of neonates and infants, including those born prematurely. We found that pediatric AEC-derived CRC have enhanced initial proliferative capacity and are life-extended (without viral vectors) in a reversible process that preserves AEC phenotype after multiple passages. The original CRC induction method combines exposure to a Rho kinase (ROCK) inhibitor and a fibroblast-derived feeder layer or conditioned medium (medium from irradiated fibroblasts) to reprogram primary AEC.(12, 14) In our study we used the conditioned medium approach to bypass the need to have a constant stock of growing fibroblasts, continuous irradiation of cells, and the risk of mixing cell types. Notably, life-extended CRC preserved their original epithelial phenotype allowing multiple experiments in AEC from the same donors. Accordingly, the application of the CRC system to primary AEC derived from infants and children provides an attractive new approach to study the genetics and phenotype of the early life airway epithelium in translational studies.

To date, few studies have examined the phenotype of primary AEC after CRC induction. Reynolds et al. recently identified that adult CRC have differential expression of gene pathways implicated in the formation of cytoskeleton, cell-cell junctions, and extracellular matrix interactions.(15) In our current work, we expanded the phenotypical characterization of pediatric CRC examining their immune responses *in vitro*. As expected, pediatric nasal CRC exposed to dsRNA showed upregulation of cannonical antiviral AEC signaling pathways activated by innate receptors including TLR-3, RIG-I, and MDA-5, and downstream transcription of IFN genes and other NFκβ-related pro-inflammatory genes. In addition, dsRNA induced CRC production of IFN λ1, a signature molecule for epithelial IFN responses during viral respiratory infections.(25, 26) Importantly, the fact that antiviral and inflammatory genes are upregulated under CRC conditions is promising but it does not prove that these responses are the same as the *in vivo* AEC responses. Although transcriptomic profiles have not identified major effects of CRC induction in antiviral IFN stimulated genes,(15, 17) changes in some inflammatory genes (e.g. IL-8) have been reported.(15) Accordingly, further studies are still needed to define the precise differences and similarities in the immune responses of CRC and non-CRC derived from primary AEC.

Our current study has important limitations. Given that the main focus was to describe the methodology, feasibility, and potential use of CRC technology to culture AEC in young

children, we used a small sample size that did not allow us to correlate CRC data with *in vivo* responses and/or clinical sub-groups. We did not have enough donors to make conclusions about the relevance of atopy or history of prematurity in AEC immune responses or to examine the effect of CRC methods in the success/failure rates of AEC cultures. However, our experiments demonstrate that CRC derived from AEC of neonates and infants have significantly increased proliferative capacity, life-extension, and are capable of mounting an innate immune response that resembles that seen in primary human AEC *in vitro*, (25) and *in vivo* during viral respiratory infections in children.(26) Accordingly, pediatric nasal CRC might potentially represent a valuable research approach to investigate the molecular mechanisms that control airway epithelial immune responses in early life. Given that most chronic respiratory conditions may have their origins during early childhood,(6) the use of CRC derived from infants and children may establish a new paradigm to investigate respiratory disease initiation and chronic progression beyond childhood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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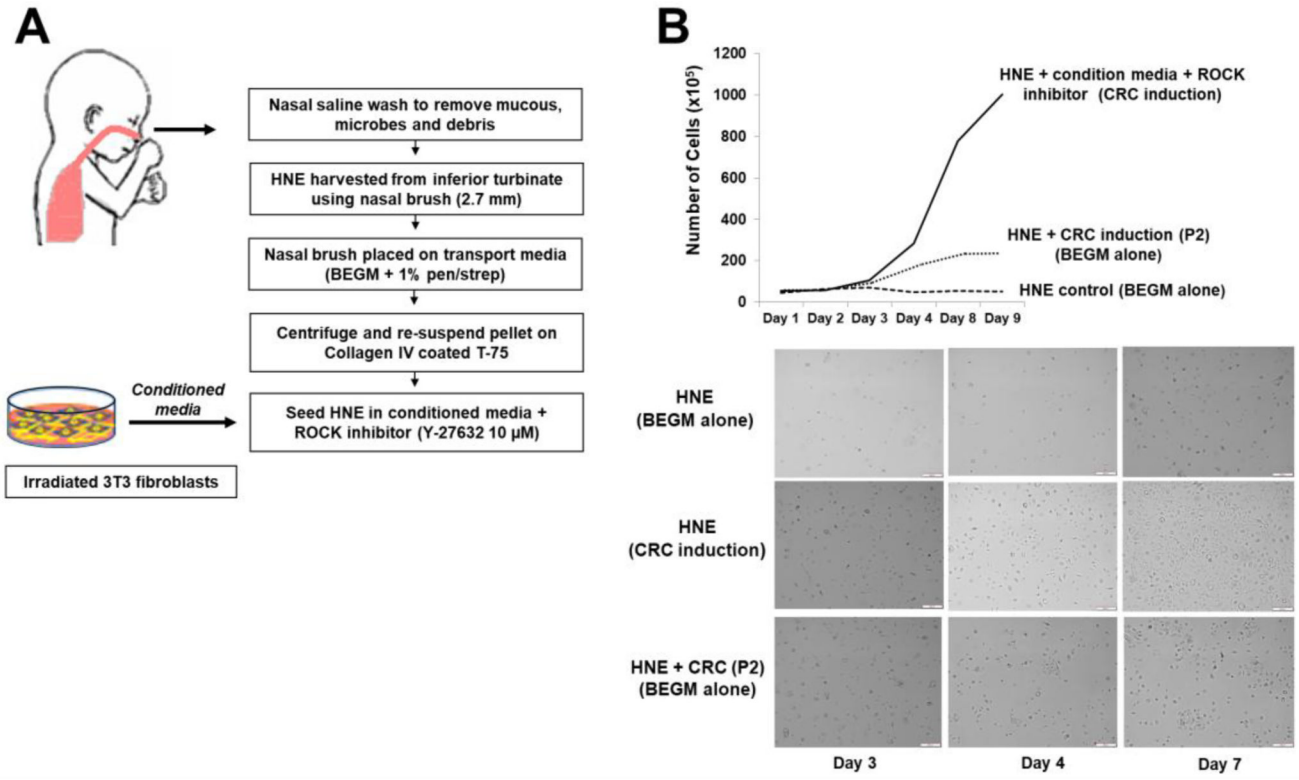

Figure 1. Conditional reprogrammed cells (CRC) exhibit augmented proliferative capaticity

(A) Schematic protocol for the development of CRC using primary human nasal epithelial cells (HNE) from infants. (B) Representative cell counts and light microscopy images from an infant donor showing that relative to fresh HNE grown in BEGM alone (top row), CRC induction accelerate initial HNE proliferation (middle row). The effect of CRC induction is reversed if media is changed to BEGM alone in HNE passage 2 (P2) (bottom row).

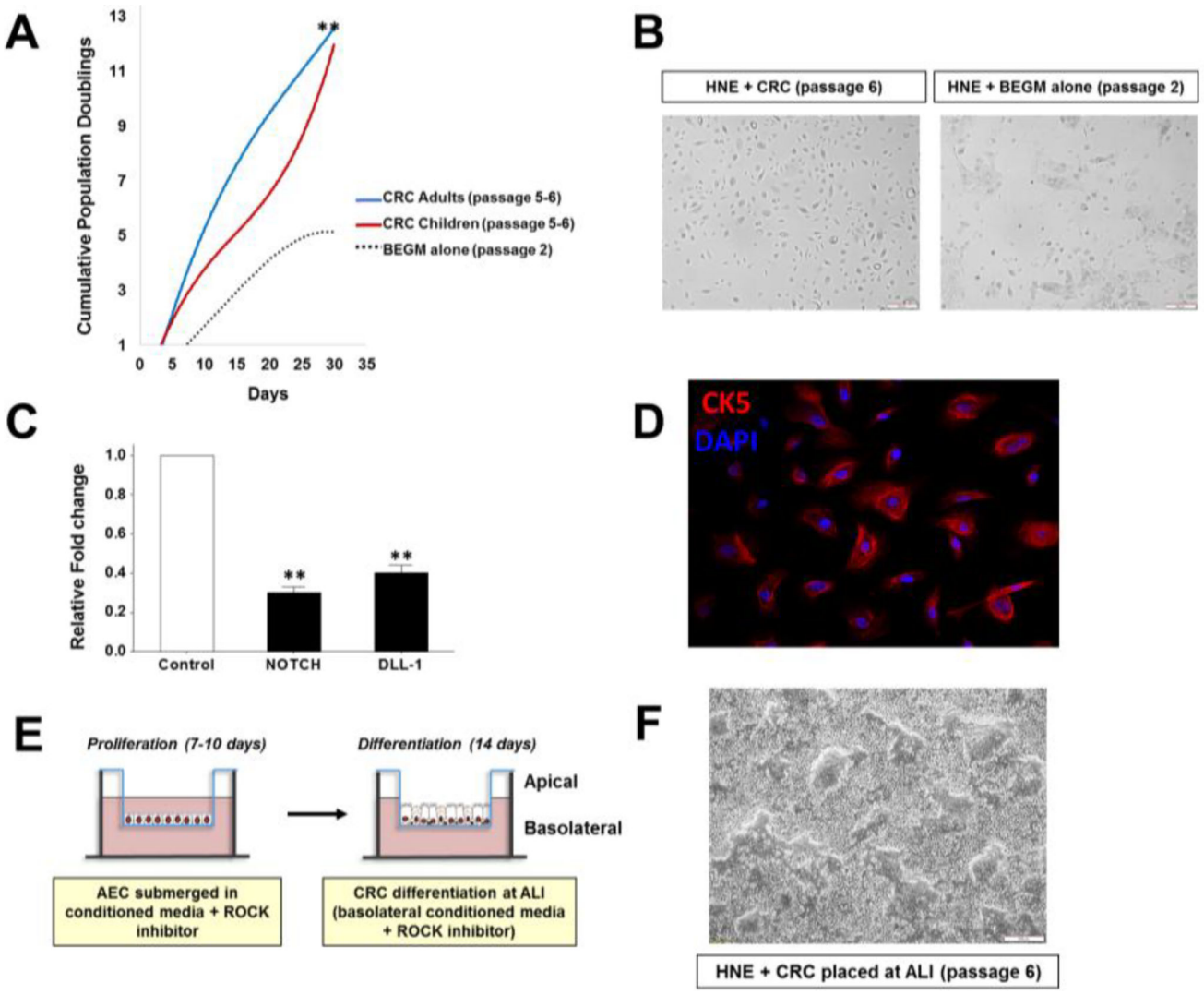

Figure 2. CRC-derived from primary HNE are life-extended

(A) CRC-derived from primary HNE of adults (N=8) or infants/children (N=15) continue proliferating after multiple passages. (B) Light microscopy images show that CRC induction prevents HNE senescence after passage 2. (C) NOTCH signaling is downregulated in CRC-derived from primary infant HNE; bars represent mean +/− SE (N=4 infants; ** $p<0.05$). (D) Confocal microscopy illustrates that infant CRC express basal epithelial cell markers after multiple passages (HNE passage 5; CK5= Cytokeratine 5). (E) Schematic protocol for air-liquid interface (ALI) differentiation of CRC-derived from primary infant HNE (passage 6) and (F) light microscopy image showing multilayered HNE culture at ALI.

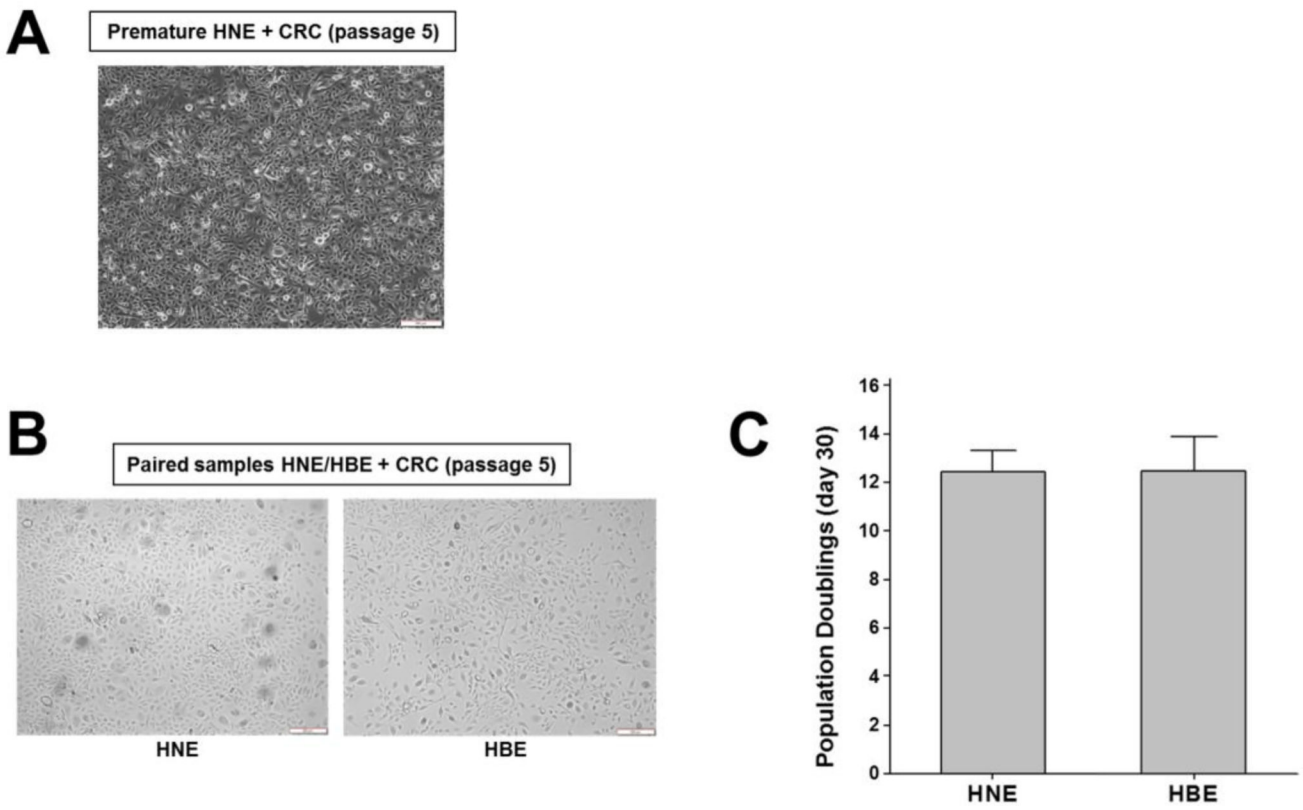

Figure 3. CRC-derived from premature nasal epithelial cells and nasal/bronchial epithelial pairs

Light microscopy images showing (A) CRC derived from HNE of a 1 month old infant born 29 weeks gestation and (B) paired infant HNE/HBE samples mantaining typical airway epithelial morphology after CRC induction and multiple passages. (C) CRC derived from nasal and bronchial epithelial cells from the same donor (HNE/HBE pairs; N=2 donors) show identical life-extension response; bars represent mean +/− SE.

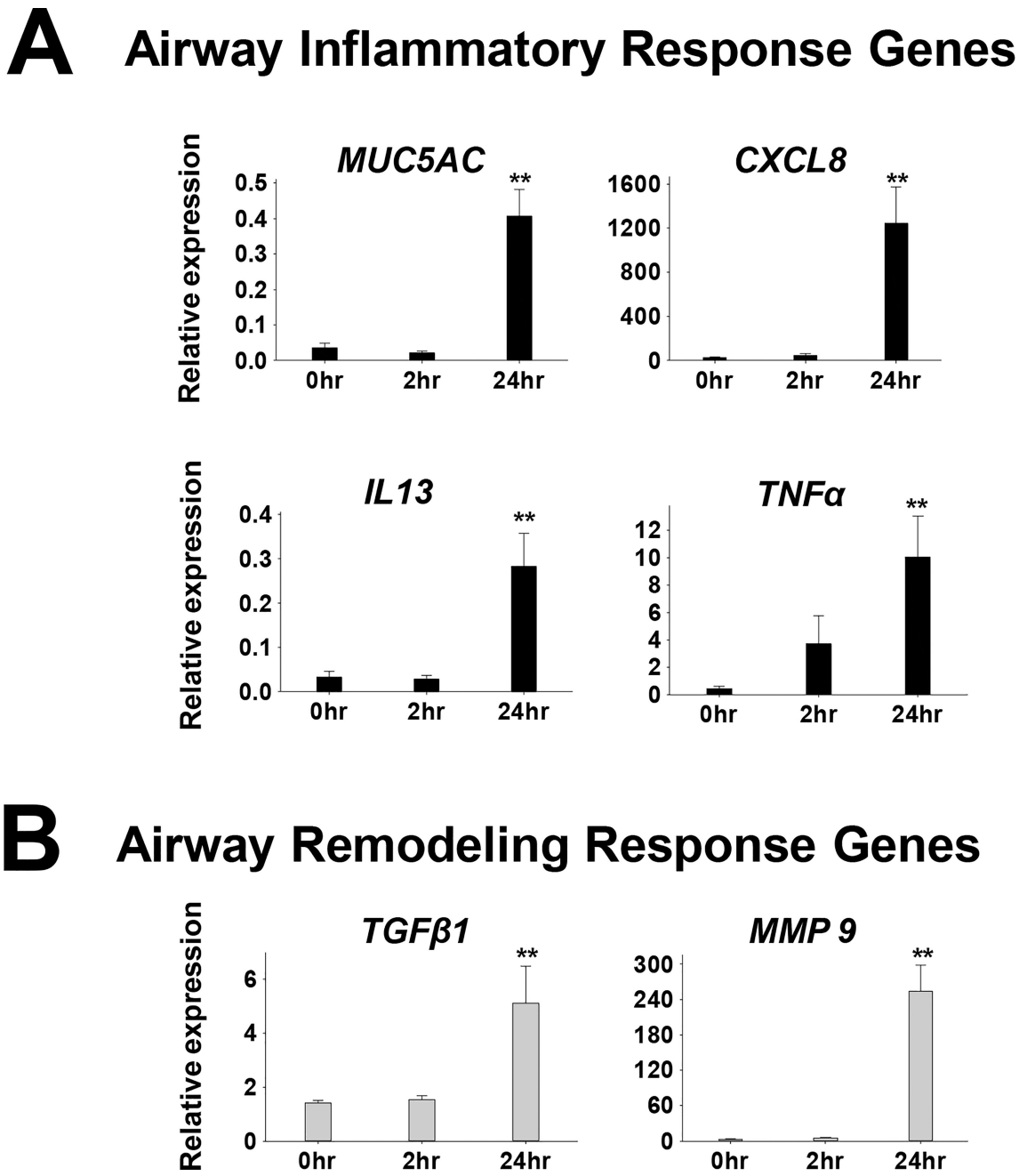

Figure 4. CRC innate antiviral immune responses

CRC-derived from primary HNE (passage 5) exposed to dsRNA (poly I:C; 10 ug/ml) exhibit robust induction of genes associated with airway epithelial (A) innate immunity and (B) antiviral response. Bars represent mean +/− SE of normalized gene/β-actin mRNA expression from 4 infants; ** $p<0.05$.

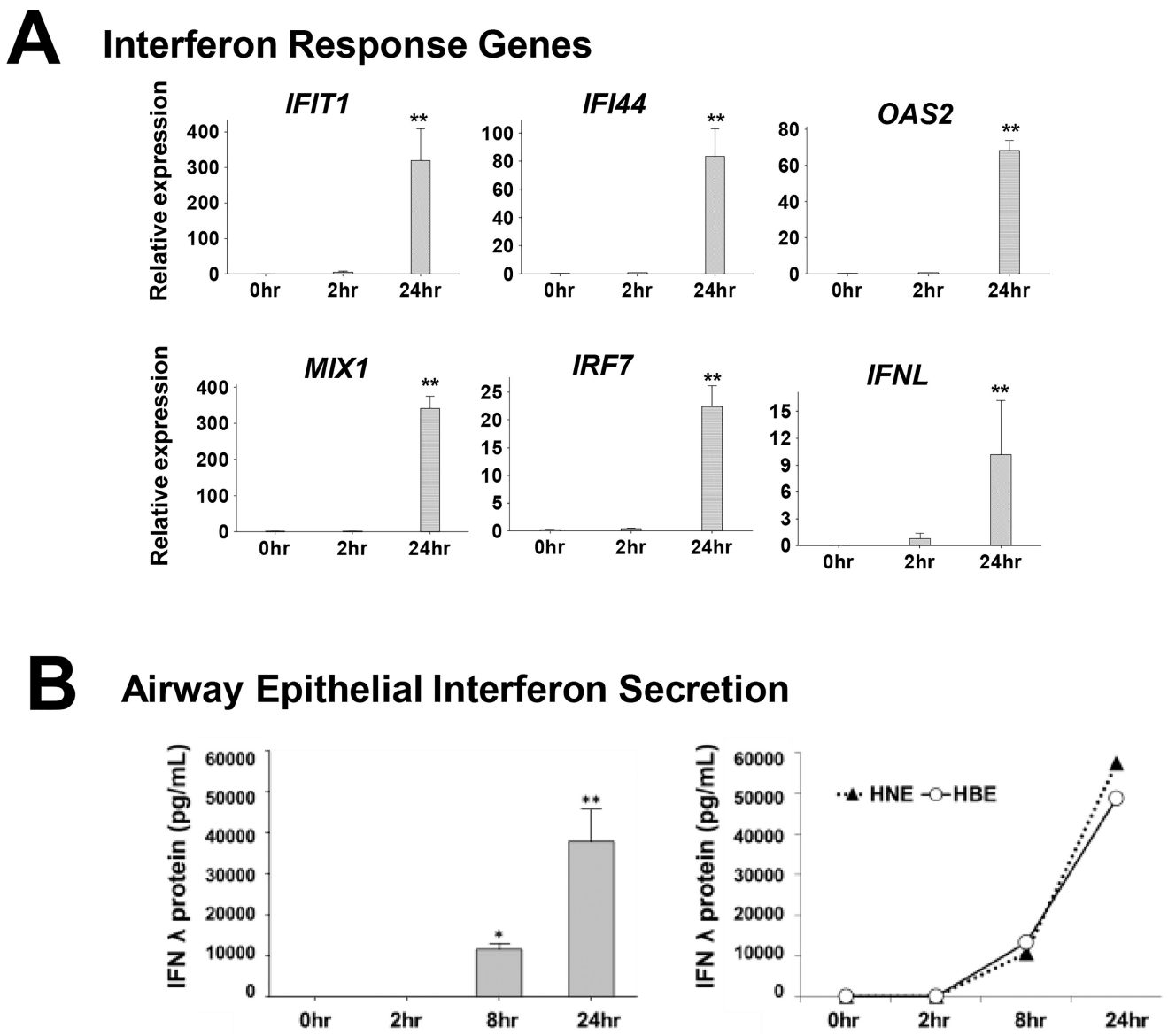

Figure 5. CRC activation of interferon signaling responses

CRC-derived from primary HNE (passage 5) exposed to dsRNA (poly I:C; 10 ug/ml) have induction of genes associated with (A) interferon (IFN) signaling and secretion of (B) IFN lambda (IFNλ), which occurs in parallel in nasal and bronchial epithelial cells (representative HNE/HBE paired response from the same donor). Bars represent mean +/− SE of normalized gene/β-actin mRNA expression (A) or protein levels (B) from 4 infants; ** $p<0.05$.

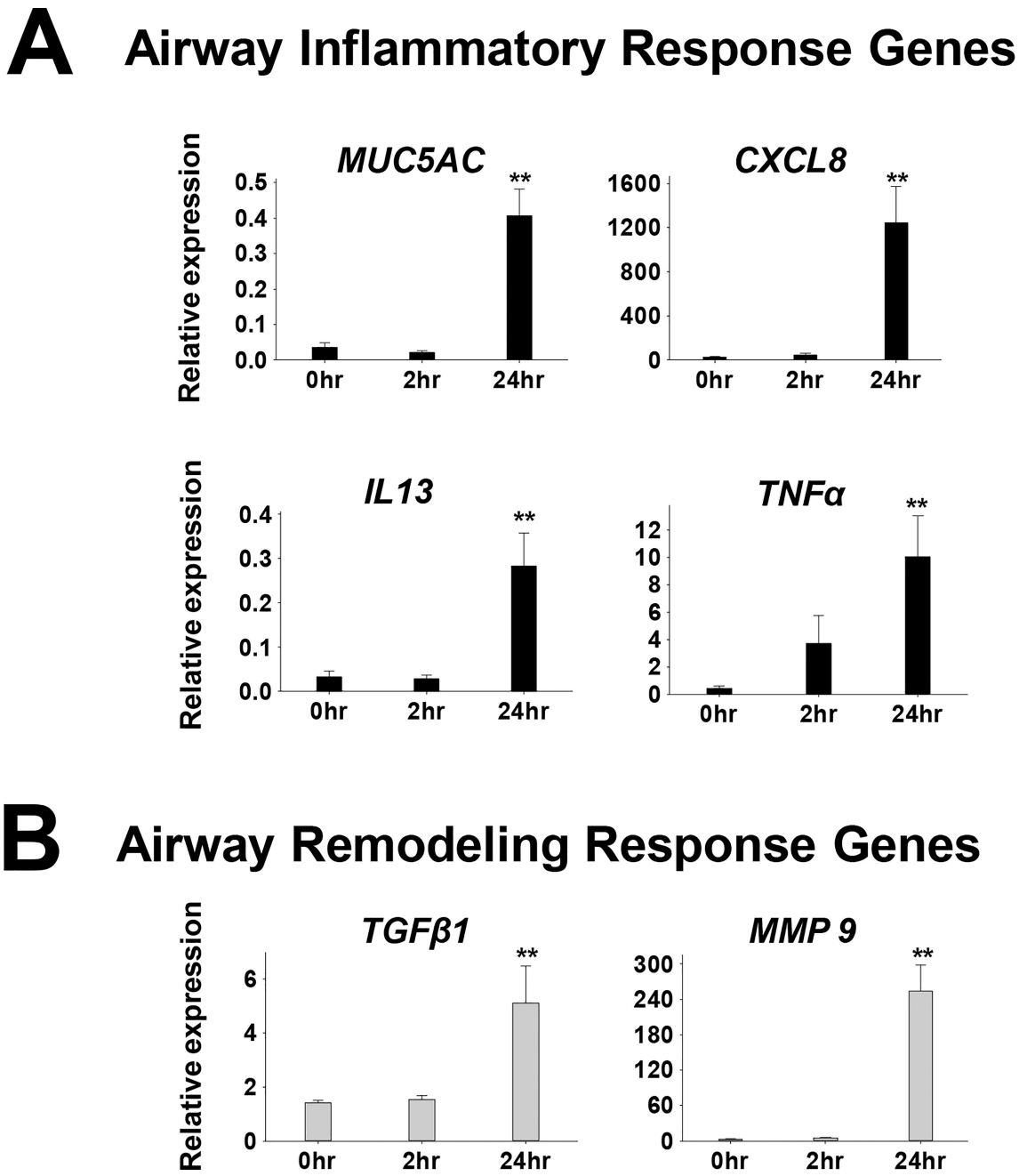

Figure 6. CRC show airway epithelial inflammatory and remodeling responses

CRC-derived from primary HNE (passage 5) exposed to dsRNA (poly I:C; 10 ug/ml) have induction of genes typically associated with airway epithelial (A) inflammatory and (B) remodeling responses. Bars represent mean +/− SE of normalized gene/β-actin mRNA expression from 4 infants; ** $p<0.05$.

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Table 1

Characteristics of study subjects

Subject	Age	Sex	Atopy	Medications	Procedure	Sample	Indication
Infants/children (n=15)							
FT	13 m	F	N	None	NB only, surgery (TT)	NEC	OM
FT	9 m	M	N	None	NB only, surgery (TT)	NEC	OM
FT	8 m	M	N	None	Bronchoscopy	NEC, BEC	Stridor
FT	2 yrs.	M	N	None	NB only, surgery (AT)	NEC	OSA
FT	1 m	F	N	None	NB only, inpatient	NEC	Fever
FT	19 m	M	N	IC, β2 agonist	NB only	NEC	Wheezing
FT	2 yrs.	M	N	β2 agonist	Bronchoscopy	NEC, BEC	Pneumonia
FT	2 yrs.	M	N	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
PM (29 wks. GA)	1 m	M	N	None	NB only, inpatient	NEC	Prematurity
PM (23 wks. GA)	3 yrs.	F	Y	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
PM (32 wks. GA)	4 yrs.	M	N	β2 agonist	Bronchoscopy	BEC	Pneumonia
HC	7 yrs.	M	N	None	NB only, surgery (AT)	NEC	OSA
Asthma	8 yrs.	M	Y	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
Asthma	10 yrs.	M	N	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
Asthma	11 yrs.	F	Y	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
Adults/adolescents (n=8)							
Asthma	16 yrs.	M	Y	IC, β2 agonist	Bronchoscopy	NEC, BEC	Wheezing
HC	26 yrs.	M	N	None	NB, outpatient	NEC	N/A
HC	38 yrs.	M	N	None	NB, outpatient	NEC	N/A
HC	37 yrs.	M	N	None	NB, outpatient	NEC	N/A
HC	27 yrs.	F	N	None	NB, outpatient	NEC	N/A
HC	24 yrs.	F	N	None	NB, outpatient	NEC	N/A
HC	37 yrs.	M	N	None	NB, outpatient	NEC	N/A
HC	35 yrs.	M	N	None	NB, outpatient	NEC	N/A

NEC= Nasal epithelial cells, BEC= Bronchial epithelial cells, FT= Full-term, PM= premature, HC= healthy control, IC= inhaled corticosteroids, NB= nasal brushing, TT= tympanostomy tubes, AT= adenotonsillectomy, OM= otitis media, OSA, obstructive sleep apnea.