# Inflammatory response of asthmatic epithelial air-liquid-interface cultures to mechanical wounding, respiratory syncytial virus and particulate matter


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## Abstract


**Rational**: There is limited information on how the airway epithelium responds to common environmental insults, especially in individuals with asthma. The objective of this study was to characterize air-liquid interface (ALI) epithelial cultures derived from asthmatic and non-asthmatic donors and their response to mechanical wounding, respiratory syncytial virus (RSV) and particulate matter air pollutants (EHC-93).

**Methods**: ALI cultures were generated from asthmatic (n=6) and non-asthmatic (n=6) airway epithelial cells. Tracheal tissues and patient matched ALI cultures were analyzed by immunohistochemistry for Cytokeratin-5, E-cadherin, Ki67, Muc5AC, and for apoptosis using Apotag™. ALI cultures were either mechanically wounded or incubated with RSV (Multiplicity of Infection=3) or EHC-93 (100µg/ml) for 24, 48, and 96 hours and supernatants were analyzed for a panel of pro-inflammatory cytokines using Luminex technology.

**Results**: Tracheal tissue and ALI cultures demonstrated increased staining for Cytokeratin-5 and decreased staining for E-cadherin. Asthmatic ALI cultures exhibited a delayed wound closure compared to non-asthmatic ALIs ($p<0.05$). Asthmatic and non-asthmatic ALI cultures released similar concentrations of IL-6, IL-8 and GM-CSF in response to mechanical wounding and EHC-93 exposure. Following RSV infection asthmatic ALI cultures exhibited enhanced expression of GM-CSF, Eotaxin and IL-6 compared to non-asthmatics ALI cultures.

**Conclusions**: This parallel *ex vivo* and *in vitro* study of ALI cultures demonstrates that the asthmatic epithelium displays an altered phenotype and asthmatic derived ALI cultures respond differentially to RSV infection but not to other environmental insults such as wounding and air pollutants. in vitro.


## Introduction

Asthma remains a heavy burden on health-care systems, despite significant advances in our understanding of the disease pathogenesis and the availability of more effective therapies. The cost related to hospital admissions during asthma exacerbations are a substantial proportion of the cost associated with the disease[1].

Epidemiological studies suggest that exposure to urban particulate matter air pollution promotes the development of asthma[2] and is associated with asthma exacerbations (3-5). Many studies have demonstrated that acute increases in air pollution result in a greater use of asthma medication, more consultations with general practitioners and increased hospital admissions for asthma[3,4].

Similarly, viral respiratory tract infections particularly with respiratory syncytial virus (RSV) or rhinovirus (RV) are most commonly associated with bronchiolitis, childhood wheeze and induction of asthma exacerbations (80% in children, 50-76% in adults)[5,6].

Within the airway, the epithelium is a major target for RSV infections and these cells are also critically important in processing inhaled airborne particles. Accordingly, it is important to understand how environmental exposures such as virus infection and ambient particulate matter exacerbate the pathophysiology of asthma.

## Specific Aim

The objectives of this study were to characterize the responses of asthmatic and non-asthmatic derived ALI epithelial cultures to the following environmental insults: mechanical wounding to mimic epithelial denudation with wound repair, and exposure to ambient particulate matter and RSV infection.

Table 1. Patient characteristics

| Patient ID | Gender | Age (yrs) | Disease | Medication | Cell Source |
|---|---|---|---|---|---|
| N1 | Female | 5 | None | None | Donor Lung |
| N2 | Male | 23 | None | None | Donor Lung |
| N3 | Male | 22 | None | None | Donor Lung |
| N4 | Male | 18 | None | None | Donor Lung |
| N5 | Male | 24 | None | None | Donor Lung |
| N6 | Female | 4 | None | None | Donor Lung |
| A1 | Female | 8 | Asthma | Albuterol, Monteleukast | Donor Lung |
| A2 | Male | 11 | Asthma | Albuterol | Donor Lung |
| A3 | Female | 21 | Asthma | Albuterol, Salmeterol/Fluticazone | Donor Lung |
| A4 | Female | 15 | Asthma | Albuterol, Salmeterol/Fluticazone | Donor Lung |
| A5 | Male | 26 | Asthma | Albuterol | Donor Lung |
| A6 | Male | 6 | Asthma | None | Donor Lung |

Table 1. Patients are identified as non-disease (N) or asthmatic (A).

## Methods

**Airway epithelial cell isolation and culture:** Airway epithelial cells (AEC) obtained from de-identified human lungs from asthmatic and non-asthmatic donors not suitable for transplantation and donated for medical research were obtained though the International Institute for the Advancement of Medicine (Edison, NJ). AEC were isolated by protease digestion as previously described (28). The study was approved (#H0-50110) by the relevant ethics committees within each institution and the clinically relevant information for each of the subjects is listed in Table 1. AECs were maintained in Bronchial Epithelial Growth Medium (BEGM, Cambrex Bio Science, Charles city, IA, USA) containing 100U/mL penicillin and 100ug/mL streptomycin, at 37°C in a humidified 5% CO2 atmosphere. AECs were differentiated into ALI Cultures on a fee-for-service basis by MatTek Corporation, Ashland, MA, using serum-free AIR-100-MM medium (MatTek Corporation).

**Viral screening:** To detect the presence of latent or previous infection with RSV, RNA was extracted from epithelial cells and screened with the Luminex's xTAG® Respiratory Viral Panel (RVP) assay according to the manufacturer's instructions. For a positive control AEC were infected with RSV stain A2 multiplicity of infection (MOI) =3 for 48 h in culture prior to RNA extraction.

**Epithelial denudation experiments:** ALI cultures were mechanically wounded in a cross-hatch manner using a small rubber GUM® Stimulator. After wounding, cells were washed with PBS to remove cell debris and then re-incubated with AIR-100-MM ALI media (MatTek Corporation). Wounds were imaged at t = 0, 6, 12, 24, 48 and 96 h using a Nikon Eclipse TE200 inverted microscope equipped with a Nikon Coolpix E995 (Mississauga, Ontario, Canada). Wound area was calculated by manual tracing and area calculation software ImagePro Plus™ (Media Cybernetics, Silver Spring, MD).

**RSV challenge experiments:** Before challenge of ALI-AEC cultures, AIR-100-MM ALI media was replaced in the basal compartment. ALI-AECs were then stimulated with or without RSV stain A2 (MOI=3) in 100 mL of AIR-100-MM ALI media at the apical surface of the ALI-AEC culture for 90 min and then removed. The ALI culture and supernatant were harvested at 24, 48 and 96 h time points. ALI cultures were embedded for immunohistochemical analysis (see below) and supernatant was stored at -80°C.

**EHC-93 challenge experiments:** Ambient particulate matter (EHC-93) was provided by the Environmental Health Directorate, Health Canada (Ottawa, ON, Canada). The particles were collected over Ottawa in 1993, are biochemically well characterized (29) particles which have a mass median diameter of 2.5 to 10 um. The endotoxin content of the EHC-93 suspension is <3.0 ng/mL [10, 13]. This dose of LPS has been shown not to activate either alveolar macrophages or lung epithelial cells to produce cytokines (30). EHC-93 was suspended at a concentration of 100 µg/mL in AIR-100-MM ALI media and sonicated 3 times for 1 min each at maximal power to disperse all aggregates prior to adding to the cells. ALI-AECs were either stimulated with 100 µL of 100 µg/mL EHC-93 or ALI media at the apical surface of the ALI culture for 24, 48 and 96 h. Supernatants and ALI cultures were harvested at each time point and stored at -80°C or embedded for immunohistochemical analysis.

**Periodic acid Schiff (PAS) staining**
Paraffin embedded sections of donor trachea biopsy specimens and donor matched ALI cultures were oxidized in 0.5% periodic acid solution for 5 min, and rinsed before staining with Schiff reagent for 15 min then counterstained with Mayer's Hematoxylin.

**Apoptosis Detection:** Apoptosis was detected in isolated airways and ALI sections by the *in situ* oligo ligation (ISOL) assay, using an ApopTag ISOL assay kit (Millipore, CA), as per the manufacturer's instructions.

**Immunohistochemical Staining:** Donor trachea biopsy specimens and donor matched ALI culture sections were deparaffinized, rehydrated and endogenous peroxidase was quenched with 3% H2O2. Non-specific interactions were blocked with 10% goat serum. Antibodies directed against human cytokeratin-5 4µg/mL (D5/16 B4, Dako, ON, Canada), E-cadherin 4µg/mL (SC8426, Santa Cruz Biotechnologies, USA), Muc5AC 2µg/mL (ab24070), 2µg/mL Ki67 (ab15580, both from AbCAM, USA), monoclonal RSV (NCL-RSV3, NovoCastra Labs, Newcastle upon Tyne, UK), Mouse IgG control (MCA928, Serotec, Oxford, UK) were added overnight at 4°C in 5% goat serum. Sections were then incubated with either biotinylated goat anti-mouse or goat anti-rabbit antibody (1:100, Vector Labs Burlingame, CA) for 60 min followed by a 10 min treatment with Streptavidin-HP (Dako, Mississauga, ON. Canada) or MACH4 universal AP polymer kit (BIOcare medical, ON. Canada). The antigen of interest was visualized by using 3,3-diaminobenzidine, brown colour generated (Dako, Mississauga, ON. Canada) or Vulcan fast red chromogen kit 2, red colour generated (BIOcare medical) and counterstained with Harris Hematoxylin Solution (Sigma, Oakville, ON, Canada). For quantification of staining, colour segmentation was used with ImagePro.

**Luminex Multiplex Cytokine Panel**
A multiplex cytokine array analysis was performed using the Human CYTOKINE LINCOplex kit (Millipore, Ontario) for Eotaxin, GM-CSF, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α, mediators which have previously been shown to be released following wounding, RSV infection or EHC-93 exposure(31-35). The assay was run according to the manufacturer's instructions.

**Transmission electron microscopy (TEM):** ALI cultures were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, then post-fixed in 1% osmium tetroxide in 0.1 M phoshate buffer for 1 h. Following dehydration with ethanol, samples were infiltrated with 1:1 solution of EMBed 812 and Propylene Oxide for1-2 hour and then a 2:1 solution of EMBed 812:Propylene Oxide overnight and then baked in 60 °C oven for 48 h. Ultrathin sections (60 nm) were then mounted on copper grids and stained with 5% uranyl acetate/2.6% lead citrate and then observed under a Tecnai 12 transmission electron microscope (FEI Inc, Hillsboro, Oregon, USA).

**Statistics:** Data are presented as the mean ± SEM of 6 or more independent experiments. Students unpaired t-test was used for pair-wise comparisons and ANOVA with Dunnett's post test for comparison of group data (Prism Version 4.0, Software Inc., USA). P values <0.05 were considered as statistically significant.

## Results

### Figure 1. Phenotype of ALI cultures from asthmatic and non-asthmatic airways

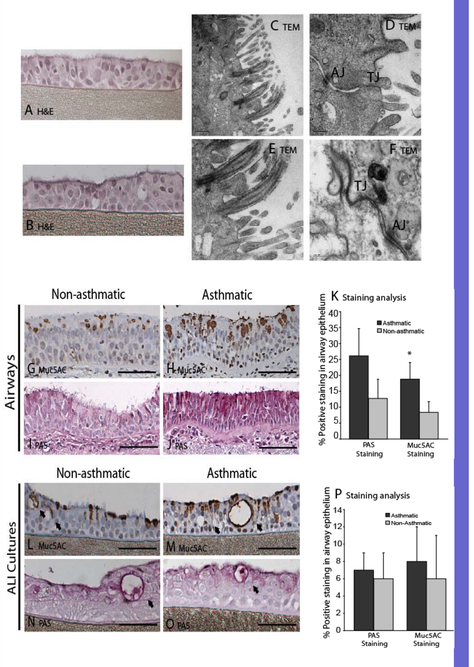


**Figure 1. Generation of ALI cultures from asthmatic and non-asthmatic airways.** ALI-AEC cultures derived from non-asthmatic (A, C, D) and asthmatic donors (B, E, F) were analyzed for morphology by H&E sections or transmission electron microscopy for formation of cilia (C, E) and formation of adherens junctions (AJ) and tight junctions (TJ) (D, F). Patient matched airway sections and ALI cultures from non-asthmatic and asthmatic donors were immuno stained for Muc5AC in brown (G,H, L, M) and periodic acid-Schiff (PAS) stained in purple (I, J, N, O). The expression of Muc5AC and PAS staining within the epithelium was quantified using Image pro plus software (K and P). Scale bar is equal to 100 µM, arrows indicate glandular-like structures in ALI cultures. Data are expressed as % of positive staining in airway epithelium ±SEM. * indicates p<0.05 compared to % positive staining in non-asthmatic airways (n=6).

### Figure 2. The asthmatic epithelium is composed of an altered epithelial phenotype

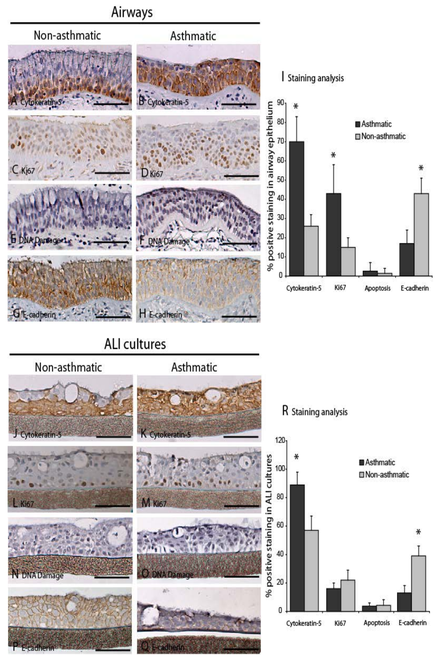


**Figure 2. The asthmatic epithelium is composed of an altered epithelial phenotype.** Patient matched airway sections and ALI cultures derived from asthmatic and non-asthmatic airway epithelial cells were differentiated in culture for 28 days and then fixed, embedded in paraffin and then sectioned for immunohistochemical analysis. Sections were stained with cytokeratin-5 (A, B, J, K), Ki67 (C, D, L, M), *in situ* oligo ligation analysis of apoptosis (E, F, N, O), or E-cadherin (G, H, P, Q). Scale bar is equal to 100 µm. The expression of cytokeratin-5, Ki67, apoptosis and E-cadherin within the epithelium (I) and ALIs (R) was quantified using Image pro plus software. Data are expressed as % of positive staining in airway epithelium SEM. * indicates p<0.05 compared to % positive staining in non-asthmatic epithelium (n=6).

### Figure 3. Wound closure is delayed in asthmatic derived ALI cultures

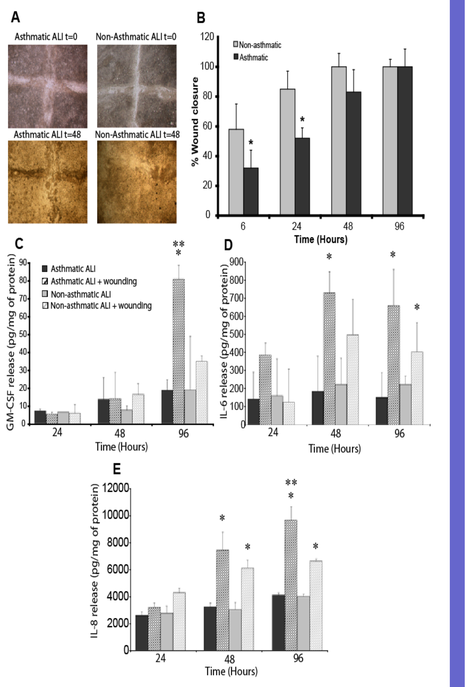


**Figure 3. Wound closure is delayed in asthmatic derived ALI cultures.** ALI cultures derived from asthmatic and non-asthmatic airway epithelial cells were wounded (t=0) and epithelial repair imaged over 96 h using DCI images (A). The percentage of wound closure was analyzed by Image Pro plus for asthmatic (dark grey bars) and non-asthmatic (light grey bars) ALI cultures (B). Data are expressed as % wound closure ±SEM. * indicates p<0.05 compared wounded non-asthmatic ALI cultures (n=12). Supernatants from asthmatic ALI cultures (dark grey bars), wounded asthmatic ALI cultures (dark striped bars), non-asthmatic ALI cultures (light grey bars) and wounded non asthmatic cultures (light striped bars) were collected at 24, 48 and 96 hours and analyzed for release of (C) GM-CSF, (D) IL-6 and (E) IL-8. Data are expressed for each cytokine as pg/mg of protein ±SEM. * indicates p<0.05 compared to un-wounded control.
** indicates p<0.05 compared to wounded non-asthmatic ALI culture (n=6).

### Figure 4. Viral screening in epithelial cultures

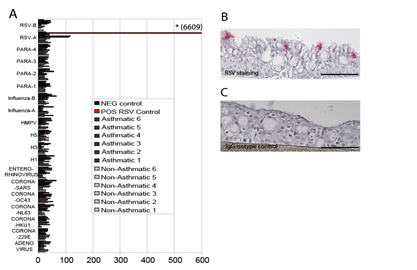


**Figure 4. Viral screening of epithelial samples.** Airway epithelial RNA from asthmatic (dark grey bars) and non-asthmatic (light grey bars) donors was screened for prior viral infection using the Luminex's xTAG® Respiratory Viral Panel. Samples were compared to a negative control (black bars) and positive control (red bars) which consisted of epithelial cells infected with RSV-A for 48 hours. Infection of ALI cultures with RSV-A was confirmed by staining for RSV in airway epithelial cells (B) compared to IgG isotype control (C).

### Figure 5. RSV stimulates an enhanced inflammatory response in asthmatic ALI cultures

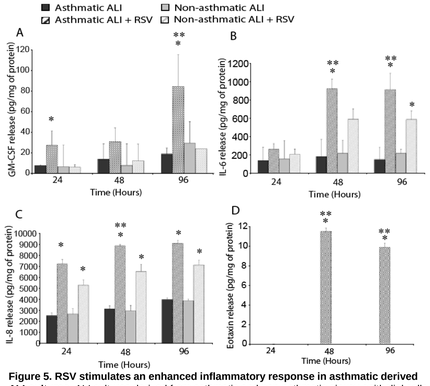


**Figure 5. RSV stimulates an enhanced inflammatory response in asthmatic derived ALI-cultures.** ALI cultures derived from asthmatic and non-asthmatic airway epithelial cells wereinfected with or without RSV-A MOI=3 for 24, 48 and 96 h. Following which supernatants from asthmatic ALI cultures (dark grey bars), RSV treated asthmatic ALI cultures (dark striped bars),non-asthmatic ALI cultures (light grey bars) and RSV treated non asthmatic cultures (light striped bars) were collected and analyzed for release of GM-CSF (A), IL-6 (B), IL-8 (C), and Eotaxin (D)m using Luminex. Data are expressed for each cytokine as pg/mg of protein ±SEM. * indicates p<0.05 compared with non-infected control. ** indicates p<0.05 compared with infected non-asthmatic ALI cultures (n=6).

### Figure 6. Inflammatory profiles following exposure to EHC-93

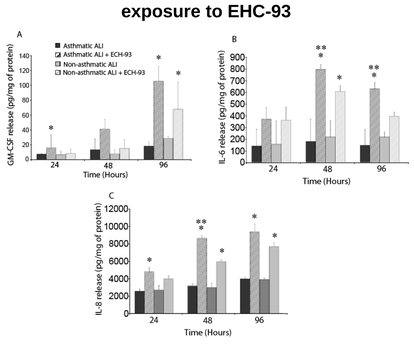


**Figure 6. Asthmatic derived ALI cultures demonstrate enhanced innate inflammatory profiles following exposure to EHC-93.** ALI cultures derived from asthmatic and non-asthmatic airway epithelial cells were treated with or without EHC-93 for 24, 48 and 96 h. Following which supernatants from asthmatic ALI cultures (dark grey bars), EHC-93 treated asthmatic ALI cultures (dark striped bars), non-asthmatic ALI cultures (light grey bars) and EHC-93 treated non asthmatic cultures (light striped bars) were collected and analyzed for release of GM-CSF (A), IL-6 (B), IL-8 (C) using the Luminex multiplex cytokine panel. Data are expressed for each cytokine as pg/mg of protein ±SEM. * indicates p<0.05 compared to non-infected control. ** indicates p<0.05 compared to infected non-asthmatic ALI cultures (n=6).

## Summary

- Our study characterizes the use of air-liquid interface (ALI) epithelial cultures derived from asthmatic and non-asthmatic donors and their response to common environmental insults such as mechanical wounding, respiratory syncytial virus (RSV) and particulate matter air pollutants (EHC-93).
- We demonstrate for the first time that that tracheal tissue and ALI cultures derived from the same donor display an altered epithelial phenotype. In particular asthmatic epithelial cells have increased expression of basal cell marker cytokeratin-5, MUC5AC and decreased expression of adherens junction protein E-cadherin.
- Importantly we demonstrate that asthmatic ALI cultures exhibit enhanced expression of GM-CSF, Eotaxin and IL-6 compared to non-asthmatics ALI cultures in response to RSV infection. In contrast this exuberant response was not observed for all environmental stimuli as both asthmatic and non-asthmatic ALI cultures released similar concentrations of IL-6, IL-8 and GM-CSF in response to mechanical wounding and to EHC-93 exposure.
- This study highlights the use of air-liquid interface culture systems to evaluate the response of differentiated epithelial cells to airborne environmental stimuli.

### References


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