Coexpression of type 2 immune targets in sputum-derived epithelial and dendritic cells from asthmatic subjects

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Background: Noninvasive sputum sampling has enabled the identification of biomarkers in asthmatic patients. Studies of discrete cell populations in sputum can enhance measurements compared with whole sputum in which changes in rare cells and cell-cell interactions can be masked.

Objective: We sought to enrich for sputum-derived human bronchial epithelial cells (sHBECs) and sputum-derived myeloid type 1 dendritic cells (sDCs) to describe transcriptional coexpression of targets associated with a type 2 immune response.

Methods: A case-control study was conducted with patients with mild asthma (asthmatic cases) and healthy control subjects. Induced sputum was obtained for simultaneous enrichment of sHBECs and sDCs by using flow cytometry. Quantitative PCR was used to measure mRNA for sHBEC thymic stromal lymphopoietin *(TSLP)*, *IL33*, *POSTN*, and *IL25* and downstream targets in sDCs (OX40 ligand *[OX40L]*, *CCL17*, *PPP1R14A*, *CD1E*, *CD1b*, *CD80*, and *CD86*).

Results: Final analyses for the study sample were based on 11 control subjects and 13 asthmatic cases. Expression of *TSLP*, *IL33*, and *POSTN* mRNA was increased in sHBECs in asthmatic cases ($P = .001$, $P = .05$, and $P = .04$, respectively). Expression of sDC *OX40L* and *CCL17* mRNA was increased in asthmatic cases ($P = .003$ and $P = .0001$, respectively). sHBEC *TSLP* mRNA expression was strongly associated with sDC *OX40L* mRNA expression ($R = 0.65$, $P = .001$) and less strongly with sDC *CCL17* mRNA expression. sHBEC *IL33* mRNA expression was associated with sDC *OX40L* mRNA expression ($R = 0.42$, $P = .04$) but not sDC *CCL17* mRNA expression.

Conclusions: Noninvasive sampling and enrichment of select cell populations from sputum can further our understanding of cell-cell interactions in asthmatic patients with the potential to enhance endotyping of asthmatic patients. (J Allergy Clin Immunol 2015;136:619-27.)

Key words: *Asthma, sputum, bronchial epithelial cells, dendritic cells, thymic stromal lymphopoietin, IL-33, OX40 ligand, CCL17*

Asthma is a disease of type 2 immunity variably characterized by eosinophilic inflammation, mucus production, and airway hyperresponsiveness.[1,2] Although great strides in understanding type 2 immunity have been made in animal models, human studies are limited by the difficulty in obtaining samples, which often involves invasive sampling of the airway by means of bronchoscopy. Induced sputum samples provide substantial information on airway inflammation in asthmatic patients, but mRNA measurements from total cells in whole sputum might mask changes in rare cells and obscure cell-cell interactions. There is an unmet need for noninvasive sampling of human airway epithelial cells and interacting cells to further our understanding of cell-cell interactions in innate immune pathways in human subjects, with the potential to lead to the development of biomarkers and targeted therapy for asthma.

Airway epithelial cells link innate and adaptive immune responses that initiate a type 2 immune response in part through cytokines, which include thymic stromal lymphopoietin (TSLP), IL-33, and IL-25.[1,2] TSLP participates in sensitization and priming of allergic airway disease.[3-5] Predominantly expressed by epithelial cells, TSLP expression is regulated by infectious and environmental stimuli.[6-9] *TSLP* mRNA and protein expression in human lung biopsy specimens correlate with asthma severity.[10,11] *IL33* expression is upregulated in both lung epithelial cells and other cells of asthmatic patients and polarizes naive $CD4^+$ T cells to produce IL-5 but not IL-4.[1,12-14] The importance of TSLP and IL-33 in human asthma is further supported by genetic studies of *TSLP*, *IL33*, and the IL-33 receptor *ST2*.[15-17] In human subjects and in murine models IL-25 is released by epithelial cells in response to allergen challenge.[18-21] Increased *IL25* mRNA expression has been reported in analysis of total sputum samples of patients with uncontrolled asthma.[22]

Epithelial cell–derived cytokines target multiple innate immune cell types, including group 2 innate lymphoid cells and dendritic cells (DCs).[2,23] In murine models DCs participate in both the initiation and maintenance of allergic airway inflammation and asthma.[24,25] Multiple DC subtypes have been described in mice and from surgically resected human lungs or bronchoalveolar lavage fluid[26-28]; less is known about the role of mucosal DCs in human asthma. Both myeloid and plasmacytoid DC

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Abbreviations used
CK-7: Cytokeratin-7
Ct: Cycle threshold
DC: Dendritic cell
EGFR: Epidermal growth factor receptor
FACS: Fluorescence-activated cell sorting
FSC: Forward scatter
GAPDH: Gyceraldehyde-3-phosphate dehydrogenase
IQR: Interquartile range
OX40L: OX40 ligand
qPCR: Quantitative PCR
sDC: Sputum-derived myeloid type 1 dendritic cell
sHBEC: Sputum-derived human bronchial epithelial cell
SSC: Side scatter
TSLP: Thymic stromal lymphopoietin

numbers are increased in induced sputum after allergen challenge.[29] Myeloid DC numbers in BAL fluid are reduced in asthmatic patients after treatment with humanized anti-IgE.[30]

Downstream TSLP targets in DCs include OX40 ligand *(OX40L)*, thymus and activation-regulated chemokine/*CCL17*, *PPP1R14A*, *CD1E*, and *CD1b* and DC maturation genes.[31] IL-33 signals through engagement of the T1/ST2 receptor and the IL-1 receptor accessory protein expressed on DCs,[32,33] and in murine models engagement of the IL-33 receptor increases survival, activation, or both of DCs with increased type 2 immunity.[1,34] Downstream IL-33 targets in DCs also include *OX40L* and *CCL17*.[32,35] Studies in human subjects have been limited, with inconsistent reports of increased expression of *OX40L* detected in the airways in patients with mild but not severe asthma.[11,36]

The limited information about the human airway epithelial cell–DC network provided by invasive studies suggests the need for noninvasive techniques to study these rare cells. We now describe the enrichment of sputum-derived human bronchial epithelial cells (sHBECs) and sputum-derived myeloid type 1 dendritic cells (sDCs) and describe transcriptional coexpression of cytokines associated with a type 2 immune response. These studies suggest that flow cytometric isolation of sputum cells can be used to improve our understanding of human mucosal epithelial cell–DC interactions with the potential to improve our ability to identify and target new therapeutics.

METHODS

Study design and participant selection

A case-control study was conducted with patients with mild asthma and healthy control subjects. Our primary outcome was *TSLP* mRNA expression in sHBECs and *OX40L* mRNA expression in sDCs. The study was approved by the New York University School of Medicine Institutional Review Board (10-01191), and all participants signed consent forms. Volunteers (18-55 years) were recruited from the Bellevue Hospital Asthma Clinic or by advertisement. Inclusion criteria for asthmatic cases is based on a standard research definition for asthma that included a physician's diagnosis of asthma and either spirometric testing with a 12% and 200-mL improvement in FEV_1 after bronchodilator (n = 6) or a 20% or greater decrease in FEV_1 after inhalation of methacholine (≤8 mg/mL, n = 8). Inclusion criteria for control subjects included the absence of an asthma diagnosis, as well as absence of a bronchodilator response on spirometric testing and a negative methacholine bronchoprovocation test result. The presence or absence of atopy was not used for inclusion/exclusion criteria. Sputum induction was delayed for 2 weeks after the end of an upper respiratory tract infection. Exclusion criteria for all participants included use of inhaled or oral corticosteroids and current or more than 5 pack years of tobacco use. Two populations of asthmatic cases and control subjects were studied: a pilot group (n = 18) of asthmatic cases and control subjects and a study group of asthmatic cases and control subjects (n = 24). The pilot group was used to develop the initial technique. For the pilot group, cells obtained from sputum were first fixed in 1% (wt/vol) formalin, and fluorescence-activated cell sorting (FACS) was performed within 1 day. Samples from the study group were not formalin fixed and were processed within 3 hours to improve RNA yield. All results in the print article are from the study group, and data from the pilot group are provided in the Methods section in this article's Online Repository at www.jacionline.org. Three asthmatic cases and 4 control subjects participated in both the pilot and study groups.

Procedures

Prebronchodilator and postbronchodilator (360 μg of albuterol sulfate) spirometry was performed with a Vitalograph spirometer (Vitalograph, Lenexa, Kan), according to American Thoracic Society standards.[37,38] Assessment of asthma control was performed with the Asthma Control Test (ACT; Optum, Eden Prairie, Minn).[39]

Complete blood counts with cell differentials were performed by using an automated laboratory procedure. Measurements of total and allergen-specific serum IgE for allergens common to the Northeastern United States were performed in a commercial laboratory (ImmunoCAP, Pharmacia, Uppsala, Sweden; Quest Diagnostics, Madison, NJ). Atopy was defined as the presence of at least 1 increased allergen-specific IgE level (≥0.35 kU/L).

Sputum induction

Sputum induction was performed according to standard methods.[40] Briefly, asthmatic cases and control volunteers inhaled albuterol sulfate (360 μg), and sputum was induced by inhalation of nebulized hypertonic saline solution (3%, NOUVAG 2000; Nouvag AG, Goldach, Switzerland) for 2-minute intervals (20 minutes). Volunteers were encouraged to cough and expectorate throughout the procedure. Participants were excluded from the study group if samples had a total cell count of less than 800,000 cells before flow sorting or a sorted cell count of less than 800 sHBECs or if mucus could not be broken down after standard dithiothreitol treatment.

Sputum processing

Sputum samples from asthmatic cases and control subjects were treated in the same manner. For the pilot group, sputum plugs were treated (at 37°C for 15 minutes) with 1% dithiothreitol.[40] For the study group, whole sputum samples were used because squamous epithelial cells were subsequently removed during the sorting procedure. The sample was filtered (70-μm nylon cell strainer) to remove mucus and debris and centrifuged (at 300*g* for 10 minutes), and the pellet was resuspended in cell dissociation buffer with 10% FCS. A total cell count was performed (hematocytometer), and cell viability was determined (trypan blue). Specimens with greater than 90% cell viability were used, and differential cell counts were performed on cytospin slides from 400 nonsquamous cells.

Flow cytometry

Washed cells were labeled with pretitered mAbs (4°C for 30 minutes). Cells were simultaneously reacted with LIVE/DEAD stain (L23105; Life Technologies, Carlsbad, Calif). Compensation for spillover and spectral overlap was performed with CompBeads (BD Biosciences, San Jose, Calif), as well as the ArC Amine Reactive Compensation Bead Kit (Life Technologies).

For the pilot samples, cells were fixed (1% formalin) in Dulbecco PBS (overnight at 4°C) and flow sorted within 1 day. For the study sample, cells were sorted immediately after labeling on a FACSAria II (BD Biosciences; injection and collection temperatures set at 4°C). Forward scatter (FSC)

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