Thymic stromal lymphopoietin (TSLP) secretion from human nasal epithelium is a function of *TSLP* genotype

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Recent candidate gene and genome-wide association studies have identified "protective" associations between the single-nucleotide polymorphism (SNP) rs1837253 in the *TSLP* gene and risk for allergy, asthma, and airway hyperresponsiveness. The absence of linkage disequilibrium of rs1837253 with other SNPs in the region suggests it is likely a causal polymorphism for these associations, having functional consequences. We hypothesized that rs1837253 genotype would influence TSLP secretion from mucosal surfaces. We therefore evaluated the secretion of TSLP protein from primary nasal epithelial cells (NECs) of atopic and nonatopic individuals and its association with rs1837253 genotype. We found that although atopic sensitization does not affect the secretion of TSLP from NECs, there was decreased TSLP secretion in NECs obtained from heterozygous (CT; 1.8-fold) and homozygous minor allele (TT; 2.5-fold) individuals, as compared with NECs from homozygous major allele individuals (CC; $P < 0.05$), after double-stranded RNA (dsRNA) stimulation (50 μg ml^{-1}). Our novel results show that rs1837253 polymorphism may be directly involved in the regulation of TSLP secretion. This may help explain the protective association of this genetic variant with asthma and related traits. Identifying functional consequences of SNPs in genes with previously reported clinical associations is critical in understanding and targeting allergic inflammation.

INTRODUCTION

Allergic diseases are often triggered by environmental stimuli that induce T helper type 2 cell (Th2) immune responses. For many years, the airway epithelium was recognized purely for its function as a physical barrier; however, it is now seen as an important initiator of the allergic response by secreting cytokines/chemokines that regulate innate immune cells.[1,2] An epithelial cell-derived cytokine, thymic stromal lymphopoietin (TSLP), is interleukin-7-like,[3] appears to be a key initiator of allergic inflammation, and also plays a role in the pathogenesis of allergic diseases—with effects on dendritic cells,[4] T lymphocytes,[5] and other effector cells such as mature eosinophils[6,7] and basophils[8] as well as their progenitors.[9,10] In asthmatics, TSLP is expressed at elevated levels in the lungs and is linked to disease severity.[11] Studies in murine models have demonstrated reduced airway disease in TSLP receptor-deficient mice, whereas experimental lung-specific expression of a TSLP transgene induced experimental asthma (Th2 cytokine-associated inflammation of the airways).[12] In a recent clinical trial, treatment with a human anti-TSLP antibody (AMG 157) reduced allergen-induced bronchoconstriction and airway inflammation in mild atopic asthmatics.[13]

Genome-wide association studies have shown associations between genetic variants in *TSLP* and allergic disease and phenotypes, including asthma[14–16] and eosinophilia.[17] Furthermore, a single-nucleotide polymorphism (SNP) rs1837253 in the *TSLP* gene has been shown to be associated with asthma, atopic asthma, and airway hyperresponsiveness.[14–16,18–19] It has further been reported that the minor T allele of rs1837253 in *TSLP* is associated with reduced risk of allergic rhinitis and asthma in males.[20,21] The absence of linkage disequilibrium between rs1837253 and other SNPs, 2 Mb up- or downstream in the chromosomal region, suggests that it is likely causal, having functional consequences.[18]

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Recent evidence suggests that SNPs in the TSLP pathway may play a critical role in the pathogenesis of allergic disease, but the functional role of rs1837253 SNP in *TSLP* has not been previously described. Given that the T allele of the rs1837253 SNP in *TSLP* is associated with reduced risk of allergic disease, and as there are no other SNPs in linkage disequilibrium with rs1837253, we hypothesized that there would be associations between rs1837253 genotype and *ex vivo* production of TSLP, a crucial cytokine for the induction of Th2 inflammatory responses. Specifically, we hypothesized that individuals with the minor allele would have a decreased propensity to induce a Th2 inflammatory response related to an altered mediator profile, leading to "protection" from asthma and related traits. We therefore evaluated the double-stranded RNA (dsRNA)-induced secretion of TSLP from primary nasal epithelial cells (NECs) from nonatopic and atopic individuals, examining associations among rs1837253 genotype, atopy, and TSLP secretion.

RESULTS

Subject characteristics

From 30 October 2013 to 10 February 2014, 61 subjects were recruited. On visit 1, all 61 subjects received skin prick tests and had mouthwash samples collected and genotyped for the SNP rs1837253: 29 were homozygous for the major allele (CC), 9 were homozygous for the minor allele (TT), and 23 were heterozygous (CT). On visit 2, only 43 of these subjects returned and received nasal scrapes. Of these latter 43 subjects, 36 long-term expansions of NECs *in vitro* were successful. Of these 36 subjects, the breakdown by genotype was as follows: 14 CC, 8 TT, and 14 CT. Of the 18 subjects who dropped out before visit 2, 14 were because of studying/exam and holiday schedule conflicts and 4 were because of a common cold. Further subject characteristics are shown in **Table 1**.

PolyI:C induces TSLP expression in NEC

Polyinosinic:polycytidylic acid (polyI:C) has been previously reported to induce TSLP production (messenger RNA (mRNA) or protein) in human airway epithelial cells.[22–24] To investigate whether NECs behave similarly, the epithelial nature of the cultured cells was confirmed using immunofluorescence staining for cytokeratin-5 (**Figure 1**), and cultured cells were stimulated with polyI:C for 0, 3, 6, and 24 h. PolyI:C (25 μg ml^{-1}) induced a time-dependent expression of TSLP mRNA ($P<0.001$ at 6 h; **Figure 2a**). Furthermore, a dose-dependent release of TSLP protein was observed following 24 h stimulation with polyI:C ($P<0.01$; **Figure 2b**).

Effects of atopy and *TSLP* rs1837253 genotype on TSLP expression

No statistically significant association was found between allergic sensitization and production of TSLP by NECs at baseline. Furthermore, polyI:C did not induce differential TSLP secretion from NECs derived from nonatopic, as compared with atopic, individuals (**Figure 3**). However, polyI:C did induce differential TSLP secretion according to genotype (**Figure 4a**). When subjects were stratified by genotype, decreased TSLP secretion was observed in NECs obtained from heterozygous (CT; 1.8-fold; $P<0.05$) and homozygous minor allele (TT; 2.5-fold; $P<0.05$) individuals, as compared with NECs from homozygous major allele (CC) individuals, after stimulation with polyI:C (25 and 50 μg ml^{-1}; **Figure 4b,c**).

DISCUSSION

There is increasing evidence implicating *TSLP* polymorphisms in the development of allergy and asthma.[25–27] Previous work has documented inverse associations between the T allele of rs1837253 and asthma;[18,20] however, the reasons for this association have remained unclear. Genetic polymorphisms are proposed to exert effects that can be measured through a variety of "intermediate" steps and outcomes ("phenotypes" that could be biological or clinical); however, information on these "intermediate phenotypes" is currently lacking.

In this study, our aim was to examine a key intermediate phenotype—specifically, TSLP secretion from NECs in response to stimulation *ex vivo*—that could shed insight into the underlying involvement of rs1837253 in the pathogenesis of

Table 1 Subject characteristics

Characteristics	Homozygous major allele (CC)	Heterozygous (CT)	Homozygous minor allele (TT)
Sample size, *n*	14	14	8
Sex, male:female	5:9	11:3	3:5
Age,[a] years	28.4 (15.0)	30.4 (15.4)	27.0 (11.0)
Atopy, *n*	6	4	4
Ethnicity			
Asian	3 (21.4)	4 (28.6)	6 (75.0)
Caucasian	10 (71.4)	8 (57.1)	2 (25.0)
Other	1 (7.2)	2 (14.3)	0 (0.0)

Values in parentheses indicate percentages.
[a]Mean (s.d.).

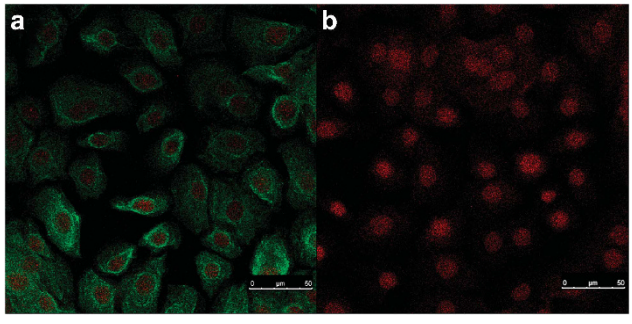

Figure 1 Immunofluorescence staining of cultured primary nasal epithelial cells (NECs). (**a**) Representative image of primary NECs stained for cytokeratin (green staining with AlexaFluor 488) to assess for the epithelial nature of the cells. (**b**) Representative image of negative control of primary NECs stained without primary antibody. Nuclei in all images stained red with DRAQ5 (original magnification × 63).

allergic disease. We selected the *TSLP* SNP rs1837253, based on the fact that it was the most significant signal in a candidate gene association study on asthma and related phenotypes,[18] which has been confirmed in recent global genome-wide association study analyses.[14–16] Although the role of TSLP in allergic rhinitis is definitely not as strong as the one it has been shown to play in asthma, we elected to use NECs in this study because of the ease in which NECs can be obtained compared with bronchial epithelial cells (BECs). Furthermore, although Zhang *et al.*[28] failed to find an association between specific SNPs in the *TSLP* gene and allergic rhinitis susceptibility in the Chinese population, Bunyavanich *et al.*[21] reported the *TSLP* SNP rs1837253 to have reduced associations with allergic rhinitis in three independent cohorts for asthma. There is also accumulating evidence that suggests elevated levels of TSLP in nasal lavage,[29] higher levels of TSLP expression,[30,31] and greater number of TSLP + cells[32] in the nasal mucosa of subjects with allergic rhinitis compared with healthy controls. Admittedly, a study with asthmatic subjects would more clearly elucidate the functional significance of the *TSLP* SNP rs1837253; however, this was beyond the scope of this study.

Consistent with previous studies on small airway epithelial cells,[22] NECs,[33] and BECs from healthy and asthmatic individuals,[23,34,35] we demonstrated that exposure to dsRNA *in vitro* induced a time-dependent expression of TSLP mRNA and a dose-dependent release of TSLP protein in NECs. We[35] and others[34,36,37] have previously reported differences in immune response between the BECs of healthy and asthmatic individuals, with enhanced TSLP secretion in BECs from asthmatics compared with their healthy counterparts following "viral" exposure. The higher capacity of asthmatic BECs to sense and respond to viral infections and thus to release elevated levels of TSLP was reported to be due, in part, to increased expression of the retinoic acid-inducible gene 1 (RIG-1) in asthmatic airway epithelial cells following viral infections.[36] In this study, dsRNA did not induce differential TSLP secretion in NECs obtained from nonatopic compared with atopic individuals, suggesting that there is no discrepancy in RIG-1 expression in NECs between these two groups. Further study examining differential RIG-1 expression in relation to clinical phenotypes will provide additional mechanistic insights into the function of SNP rs1837253.

Previous data demonstrate that the long form of TSLP is highly inducible by polyI:C in BECs.[26] A functional SNP, rs3806933, has been identified in the regulatory region of the *TSLP* gene that enhances the binding of activator protein-1, a prominent transcription factor in airway diseases that regulates the expression of multiple inflammatory proteins.[38] Furthermore, the rs3806933 SNP influences activator protein-1-driven promoter activity of the long form of TSLP in BECs.[26] Activator protein-1 expression is enhanced in the asthmatic airway,[38] consistent with reports that SNP rs3806933 is associated with adult asthma and childhood atopic asthma.[27]

In this study, we demonstrate that clinical atopic status does *not* affect NEC-derived TSLP. We however did not examine whether clinical atopic status was associated with rs1837253, as we would need a very large sample size in order to reach such a conclusion. Although it would be interesting to know other clinical details of the participants so that we could examine for

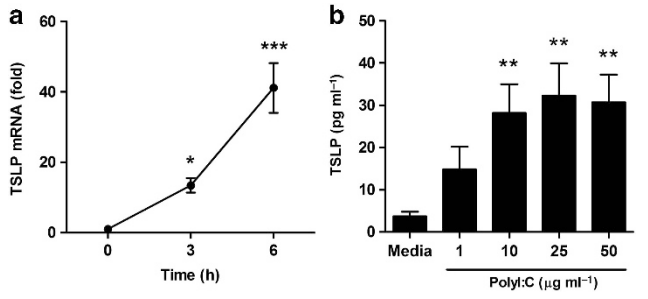

Figure 2 Polyinosinic:polycytidylic acid (polyI:C) induces thymic stromal lymphopoietin (TSLP) expression and secretion in human primary nasal epithelial cells (NECs). (**a**) Kinetics of TSLP expression in polyI:C (25 μg ml^{-1})-stimulated NECs ($n=8$). Results shown are expressed as fold change in the TSLP mRNA level in polyI:C-stimulated NECs at 3 and 6 h relative to the level in unstimulated NECs (0 h). (**b**) Primary NECs were incubated for 24 h with increasing concentrations of polyI:C (0–50 μg ml^{-1}). Concentrations of TSLP protein in the culture supernatant were measured using enzyme-linked immunosorbent assay (ELISA). Results shown are mean ± s.e.m. of duplicates ($n=8$). One independent experiment performed per subject. Significant difference from unstimulated cells (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

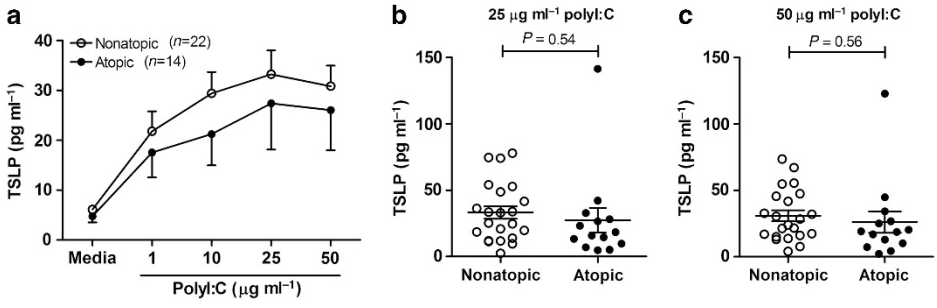

Figure 3 Relationship between atopy and polyinosinic:polycytidylic acid (polyI:C)-induced thymic stromal lymphopoietin (TSLP) secretion in nasal epithelial cells (NECs). Concentrations of TSLP protein in NEC culture supernatant from nonatopic ($n=22$) and atopic ($n=14$) individuals were measured using enzyme-linked immunosorbent assay (ELISA) following overnight stimulation with (**a**) increasing concentrations of polyI:C (0–50 μg ml^{-1}); (**b**) 25 μg ml^{-1} of polyI:C; and (**c**) 50 μg ml^{-1} of polyI:C. Results shown are mean ± s.e.m. of duplicates. One independent experiment performed per subject.

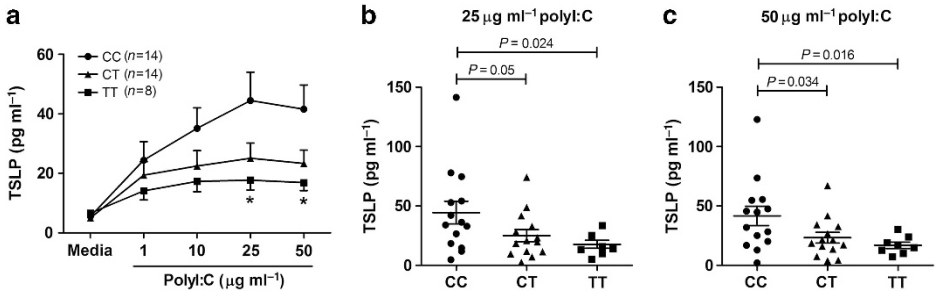

Figure 4 Relationship between *TSLP* rs1837253 genotype and polyinosinic:polycytidylic acid (polyI:C)-induced thymic stromal lymphopoietin (TSLP) secretion in nasal epithelial cells. Concentrations of TSLP protein in nasal epithelial cell culture supernatant from rs1837253 homozygous major allele (CC; $n=14$), heterozygous (CT; $n=14$), and homozygous minor allele (TT; $n=8$) individuals were measured using enzyme-linked immunosorbent assay (ELISA) following overnight stimulation with (**a**) increasing concentrations of polyI:C (0–50 μg ml^{-1}); (**b**) 25 μg ml^{-1} of polyI:C; and (**c**) 50 μg ml^{-1} of polyI:C. Results shown are mean ± s.e.m. of duplicates. One independent experiment performed per subject. Significant difference from CC genotype (*$P<0.05$).

correlations between the clinical characteristics and TSLP production *ex vivo*, and/or *TSLP* genotype, unfortunately, aside from clinical atopic status, we do not have these. However, we[35] and others[34,36,37] have previously demonstrated that primary BECs from patients with asthma release more TSLP than primary BECs from healthy controls after dsRNA stimulation. Furthermore, Harada *et al.*[26] showed that individuals with the rs3806933 SNP in the promoter region of long-form TSLP have increased TSLP expression and protein secretion. Nonetheless, in this study, we related the differential secretion of TSLP protein to the SNP rs1837253 genotype, and our data suggest that this SNP in the upstream region of *TSLP* has functional effects on TSLP protein production. SNP rs1837253 is located 5.7 kb upstream of the TSLP transcription start site and is predicted to disrupt a number of potential transcription factor binding sites.[39] It is possible the rs1837253 T allele downregulates microbe-induced production of TSLP in NECs through inhibiting the binding of transcription factors to regulatory elements. In order to better understand the mechanism of SNP rs1837253 associations with TSLP secretion, potential differential binding of possible regulatory proteins to this important SNP should be examined. As such, rs1837253 SNP-associated epigenetic alterations, such as DNA methylation in airway epithelial cells, merits investigation.

In a recent proof-of-concept study,[13] in addition to reducing allergen-induced bronchoconstriction in both early and late asthmatic responses, AMG 157, a human anti-TSLP monoclonal antibody, was demonstrated to reduce markers of systemic and airway inflammation measured as fraction of exhaled nitric oxide and sputum eosinophil levels, as well as levels of circulating eosinophils. The capacity for AMG 157 to decrease baseline blood eosinophil counts and exhaled nitric oxide[13] suggests a pivotal role for TSLP in the development or persistence of asthma in the absence of allergen exposure. *In vivo*, TSLP levels are elevated in asthmatics and are associated with disease severity and eosinophilic inflammation.[40] Relevantly, we recently demonstrated the ability of TSLP to mediate eosinophil–basophil differentiation from human peripheral blood hemopoietic progenitor cells (hemopoiesis) *ex vivo* in relation to clinical atopic status, a process that was dependent on tumor necrosis factor-α and TSLP–TSLP receptor interactions.[10] In this study, we demonstrate for the first time diminished TSLP secretion in NECs derived from individuals who are carriers of the rs1837253 T allele, a finding that provides key insight into allergic disease pathogenesis and explains, at least in part, the inverse association between rs1837253 and asthma and related phenotypes, and the recently reported therapeutic effects of TSLP antagonism. However, to what extent a reduction in TSLP levels is functionally and/or clinically significant *in vivo* in humans in the context of allergic disease is unknown. Nonetheless, several clinical outcomes and phenotypes of allergic diseases such as allergic rhinitis, total immunoglobulin E, and airway hyperresponsiveness have also been reported to be associated with SNPs in *TSLP*.[18,20,21,25–27,41] Collectively, these data suggest a critical role for TSLP in regulating multiple downstream effector pathways involved in allergic disease.

Finally, asthma exacerbations are a major cause of mortality, and repeated exacerbations can cause permanent impairment of lung function.[42] Rhinoviruses, single-stranded RNA viruses that synthesize dsRNA during replication at the respiratory epithelium,[43] are a major cause of asthma exacerbations in both children and adults.[44] PolyI:C is a highly accepted dsRNA surrogate commonly used to simulate viral infection, as it mimics many aspects of rhinovirus infection in primary BECs,[34] allowing for the yield of robust and reproducible data. However, other strains of viruses, rhinovirus[23] and respiratory syncytial virus,[36] have demonstrated similar responses by inducing TSLP from airway epithelial cells. As such, our findings may have implications in explaining virus-induced asthmatic exacerbations, especially those because of rhinovirus infections. Of note, *TSLP* has recently been demonstrated to be a methylation-sensitive gene.[45,46] Along these lines, our findings demonstrate differential secretion of TSLP between rs1837253 genotypes after polyI:C stimulation, suggesting that epithelial cells may be susceptible to epigenetic alterations induced by environmental factors such as infection. These epigenetic changes may, in turn, alter the threshold for epithelial activation in response to environmental stimuli such as viruses and allergens.[47] For example, Toll-like receptor

ligands have been demonstrated to alter methylation processes.[48] Given that reduction of DNA methylation facilitates transcription by permitting transcription factors or coactivators to bind to regulatory elements (promoter or enhancer regions),[49,50] we hypothesized that there would be enhanced methylation of the TSLP promoter in NEC obtained from heterozygous (CT) and homozygous minor allele (TT) individuals, following stimulation with polyI:C.[45,46] Therefore, the effects of dsRNA and/or Toll-like receptor-mediated production of TSLP in NECs from patients with or without asthma in relation to rs1837253 genotype merits investigation. Activation of the innate immune system in the epithelium involving rs1837253 may ultimately influence the development of allergic diseases by modulating TSLP downstream effector pathways. Further investigation into SNP rs1837253-associated epigenetic alterations in airway epithelial cells will also be critical in understanding the mechanisms underlying the involvement of rs1837253 in regulating TSLP secretion.

METHODS

Study subjects. All studies were approved by Hamilton Integrated Research Ethics Board and subjects provided written informed consent to participate. Eligible subjects were healthy, nonsmoking individuals, 18 to 63 years of age, with no history of use of any nasal, oral, or inhaled corticosteroids within the previous 3 months. All recruited study participants had mouthwash samples collected and skin prick tests performed at visit 1. Atopy was defined as a positive skin prick test response (>2-mm wheal) to at least one of 14 common aeroallergens. In addition, all subjects were confirmed to have no prior history of upper respiratory tract infection within 6 weeks of sample collection. Further subject characteristics are shown in **Table 1**.

DNA extraction and genotyping. The SNP rs1837253 in the *TSLP* gene was selected based on previous associations with asthma and related traits as well as the absence of significant linkage disequilibrium with any other SNP.[18,20,21] DNA was extracted from mouthwash samples using QIAamp DNA Blood Mini Kits (Qiagen, Toronto, ON, Canada) according to the manufacturer's supplementary protocol for mouthwash samples. Genotyping was performed using a commercially available TaqMan genotyping assay for rs1837253 (Assay C__11910823_20, Life Technologies, Burlington, ON, Canada). The genotyping data were verified for Hardy–Weinberg equilibrium by the χ^2 test.

Nasal epithelial cell isolation and culture. Primary NECs were derived from the inferior nasal turbinate using Rhino-Probe nasal curettes (Arlington Scientific, Springville, UT). NECs were expanded and cultured as previously described with modification.[35] Briefly, NECs were maintained in bronchial epithelial growth medium (BEGM; Clonetics, Allendale, NJ) supplemented with SingleQuots (Clonetics). The SingleQuots comprised 2 ml bovine pituitary extract (52 $\mu g\,ml^{-1}$) and 0.5 ml each of insulin (5 $\mu g\,ml^{-1}$), human epidermal growth factor (0.5 $ng\,ml^{-1}$), hydrocortisone (0.5 $\mu g\,ml^{-1}$), epinephrine (0.5 $\mu g\,ml^{-1}$), transferrin (10 $\mu g\,ml^{-1}$), retinoic acid (0.1 $ng\,ml^{-1}$), triiodo-L-thyronine (6.5 $ng\,ml^{-1}$), and Gentamicin/Amphotericin-B (GA1000; 50 $\mu g\,ml^{-1}$). Collected cells were centrifuged and resuspended in BEGM with 10% 100 × DNase 1 (Sigma Aldrich, St Louis, MO; at room temperature for 20 min). Cells were washed twice and plated on 35 mm culture dishes (Corning Costar, Corning, NY), coated with Purecol (Inamed, Fremont, CA), and incubated at 37 °C, 5% CO_2. Media were changed 24 h after plating; thereafter, media were replaced every second day until 80–90% confluence. Subsequently, cells were passaged (P1) using trypsin (Gibco, Burlington, ON, Canada) and further expanded in 75 cm^2 flasks (Corning) in BEGM. In this study, second and third passaged cells were used. Viability was assessed by exclusion of Trypan blue dye (Gibco) and the epithelial nature of cells assessed by immunocytochemistry as previously described with modification.[51] Briefly, selected cultures were fixed in 4% paraformaldehyde and permeabilized using Triton-X 100 (Sigma). Subsequently, cells were incubated with rabbit anti-cytokeratin 5 antibody (Abcam, Cambridge, UK) overnight at 4 °C. Cells were then exposed to AlexaFluor 488 goat anti-rabbit IgG antibody (Life Technologies) at room temperature for 1 h in the dark. Nuclei were stained with DRAQ5 (Abcam) at room temperature for 15 min and slides were subsequently mounted using Fluoroshield Mounting Medium (Abcam). Negative control slides were prepared by omitting the primary antibody (**Figure 1**). Fluorescent images were viewed using a Leica SP5 confocal inverted microscope (Leica Microsystems, Concord, ON, Canada) using a 63 × oil immersion objective. Images were captured and viewed using LAS AF (Leica) acquisition software.

PolyI:C stimulation. NEC were seeded onto 24-well plates (Corning) and allowed to grow to 80–90% confluence. Thereafter, BEGM was replaced with bronchial epithelial basal media (Clonetics) and 1% fetal bovine serum (R&D Systems, Minneapolis, MN) before experimental stimulation. NECs were treated with polyI:C (EMD Chemicals, Gibbstown, NJ) as indicated or vehicle control for 24 h at 37 °C, 5% CO_2.

RNA isolation and reverse transcription. RNA was extracted from a number of different human nasal epithelial samples using the RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA in each sample was quantified using a NanoDrop Spectrophotometer (Thermo Fisher, Wilmington, DE). The RNA in each sample time point (0, 3, and 6 h) from each participant was diluted based on the lowest concentration sample using RNase-free water. The samples were reverse-transcribed using the Quantitect Reverse Transcription kit with genomic DNA wipeout buffer (Qiagen) and complementary DNA were aliquoted and stored at −80 °C.

Quantitative reverse transcription-PCR. The expression of TSLP was determined by quantitative reverse transcription-PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. The PrimePCR Human Reference Gene Panel (Bio-Rad) was used to determine *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,* (*YWHAZ*) to be the optimal reference genes. We used the following primer set for quantitative reverse transcription-PCR: (NM_033035.4 and NM_138551.4), 5′-CT AAGGCTGCCTTAGCTATC-3′ and 5′-AAGCGACGCCACAATC CTTG-3′ as previously described.[26] PrimePCR SYBR green assay for Human *GAPDH* and *YWHAZ* were used for reference gene primers (Bio-Rad). All TSLP primer sets (Integrated DNA Technologies, Coralville, IA) were designed and evaluated as per the minimum information for publication of quantitative reverse transcription-PCR experiments (MIQE) guidelines.[52] Pooled complementary DNA was used to optimize annealing temperature (found to be 60 °C for all primer sets). The 8-point standard curves were used to verify amplification efficiency for each TSLP isoform primer sets and the *GAPDH/YWHAZ* reference genes using Sso Advanced SYBR green Supermix (Bio-Rad). Melt curves were used to verify expected single peak amplicons. The threshold cycle (Ct) is inversely correlated with the target mRNA expression level and was defined as the cycle number at which the reporter fluorescence emission exceeded the midpoint along the amplification curve.[53] The standard $2^{(-\Delta\Delta Ct)}$ formula was used to calculate arbitrary TSLP mRNA concentrations.[53] The level of TSLP mRNA was normalized to the level of GAPDH/YWHAZ mRNA.

TSLP measurement. The release of TSLP in cell-free supernatant after 24 h stimulation with polyI:C was measured using DuoSet enzyme-linked immunosorbent assay (ELISA) Development kits (R&D) as per the manufacturer's instructions.

Statistical analysis. All data are expressed as mean ± s.e.m. Significance was assumed at $P<0.05$. All analyses were performed with Prism version 5 (GraphPad Software, La Jolla, CA) using nonparametric tests. Differences within groups were assessed by Friedman test with Dunn's *post hoc* test. Differences between groups (CC and CT vs. TT) were assessed by Mann–Whitney *U*-test.

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DISCLOSURE

The authors declared no conflict of interest.

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