



Nasal interleukin 25 as a novel biomarker for patients with chronic rhinosinusitis with nasal polyps and airway hypersensitiveness A pilot study

Fenghong Chen, MD^{*}; Haiyu Hong, MD^{*†}; Yueqi Sun, MD[‡]; Xianting Hu, MD[‡]; Jia Zhang, MD[§]; Geng Xu, MD^{*}; Weidong Zhao, MD[‡]; Huabin Li, MD, PhD[‡]; Jianbo Shi, MD, PhD^{*}

^{*} Otorhinolaryngology Hospital, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

[†] Department of Otolaryngology, The Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai, China

[‡] Department of Otolaryngology, Head and Neck Surgery, Affiliated Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai, China

[§] Department of Otolaryngology, Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

ARTICLE INFO

Article history:

Received for publication March 29, 2017.

Received in revised form June 21, 2017.

Accepted for publication July 11, 2017.

ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease of the upper airway and is tightly linked with airway hyperresponsiveness (AHR) and asthma. However, the surrogate biomarkers for indicating AHR and asthma in patients with CRSwNP remain elusive.

Objective: To investigate the surrogate biomarkers for indicating AHR and asthma in patients with CRSwNP. **Methods:** In this study, sinonasal tissues were collected from 42 patients with CRSwNP (asthma, $n = 17$; asymptomatic AHR, $n = 11$; non-AHR, $n = 14$), 11 patients with chronic rhinosinusitis without nasal polyps (CRSsNP), and 13 controls. The protein and messenger RNA levels of interleukin (IL) 25 and other cytokines in nasal polyp (NP) and control sinonasal tissues were determined by quantitative real-time polymerase chain reaction and multiplex immunoassay, respectively. Multivariate logistic regression and receiver operating characteristic curve analysis were performed to assess the clinical relevance of IL-25.

Results: We found that the protein and messenger RNA levels of IL-25 were significantly increased in NP tissues compared with the control sinonasal tissues from patients with CRSwNP, patients with CRSsNP, and controls. Multivariate logistic regression revealed that the nasal IL-25 protein level and nasal and blood eosinophil counts were independent risk factors for AHR in patients with CRSwNP. According to receiver operating characteristic curve analysis, nasal tissue IL-25 had a sensitivity of 91.4% and a specificity of 62.8% (area under the curve, 0.845) at the cutoff level of 5 pg/ μ L for indicating AHR in this CRSwNP cohort.

Conclusion: Our findings indicated that IL-25 was significantly increased in NP tissues and may be considered as the molecular indicator for AHR in patients with CRSwNP.

Trial Registration: ClinicalTrials.gov Identifier: NCT02110654.

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Introduction

Chronic rhinosinusitis (CRS) is a common inflammatory condition that involves the nasal passages and cavities, potentially affecting 10% of the population worldwide¹ and 8% of the

population in China.² On the basis of the existence of visible nasal polyps (NPs) in the middle meatus, CRS can be generally divided into 2 subtypes: CRS without NPs (CRSsNP) and CRS with NPs (CRSwNP). CRSsNP and CRSwNP have been suggested to have distinct phenotypes and pathogenic mechanisms.^{3,4} CRSwNP was characterized by T_H2 -skewed eosinophilic inflammation in Western patients,⁵ whereas more neutrophilic inflammation and T_H1/T_H17 bias was found in Chinese patients with CRSwNP.⁶

CRSwNP is more likely than CRSsNP to be associated with asthma comorbidity. Asthma is also a chronic inflammatory disease of the airways, characterized by eosinophil accumulation, enhanced T_H2 response, and airway hyperresponsiveness (AHR).⁷ AHR could represent an ongoing airway inflammatory and remodeling process. Moreover, epidemiologic data have revealed that patients with asymptomatic AHR are at a high risk of

Reprints: Jianbo Shi, MD, PhD, The First Affiliated Hospital of Sun Yat-sen University, No. 74, Zhongshan 2nd Road, Guangzhou 510080, China; E-mail addresses: tsjbent@163.com or shijb@mail.sysu.edu.cn.

Drs Chen and Hong contributed equally to this study.

Disclosures: Authors have nothing to disclose.

Funding Sources: This study was supported by grants 81470673, 81460095, and 81670908 from the National Natural Science Fund of China, grant 16410723600 from the Shanghai Committee of Science and Technology, and grants 2016A020215030 and 2016A020215049 from Guangdong Province Science and Technology Planning Project.

<http://dx.doi.org/10.1016/j.anai.2017.07.012>

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developing asthma.⁸ Emerging evidence supports an active role for nasal T_H2-skewed, eosinophilic inflammation in patients with CRSwNP in the pathogenesis of concomitant asthma.^{9–11} Although local IgE and interleukin (IL) 5 production in NP tissues is related to the presence of comorbid asthma,^{12,13} the key factors initializing concomitant asthma in patients with CRS are not completely understood.

The aims of the current study were to characterize the inflammatory pattern of CRS subtypes among patients with CRSwNP, patients with CRSsNP, and healthy controls by synchronously measuring various cytokines within the same specimen. In addition, we evaluated the inflammatory profiles in NP subgroups with asthma or AAHR or normal low airway status to further explore the potential mechanisms that could drive the concomitant of AHR and NPs. Then we selected the most distinct cytokines as biomarkers to indicate concomitant CRSwNP with asthma.

Methods

This study was approved by the local ethical committee, and written informed consent was obtained from all the participants.

Patient Selection

Forty-two patients with CRSwNP (asthma, *n* = 17; AAHR, *n* = 11; non-AHR [NAHR], *n* = 14) and 11 patients with CRSsNP (asthma, *n* = 1; AAHR, *n* = 1; NAHR, *n* = 9) were recruited from the First Affiliated Hospital of Sun Yat-sen University in Guangzhou. The diagnosis of CRSwNP and CRSsNP was established based on medical history, nasal endoscopy, and sinus computed tomography according to the current European Position Paper on rhinosinusitis and nasal polyps.³ All patients were subjected to lung expiratory volume tests and bronchial provocation or dilation tests and then divided into AHR and NAHR groups. AHR were defined as a 20% or greater reduction in the forced expiratory volume in 1 second after serial administration of histamine up to a concentration of 8 mg/mL or less. Asthma was diagnosed by a physician based on a history of recurrent dyspnea, wheezing or coughing episodes, and positive airway reversibility test results (forced expiratory volume in 1 second increasing $\geq 12\%$ and reaching 200 mL after the inhalation of 400 μ g of salbutamol) or positive provocation test results. Patients with AHR without asthma history and symptoms were considered as having AAHR. The atopic status was evaluated by a skin prick test or the detection of specific IgE (Phadia, Uppsala, Sweden) to common inhalant allergens (eg, pollens, house dust mites, pets, molds, and cockroaches). Patients with aspirin-exacerbated respiratory disease were excluded based on the history. During endoscopic surgery, the sinonasal tissues were sampled as follows: NPs (*n* = 42) and uncinate process (UP) (*n* = 12) in the CRSwNP group and UP (*n* = 11) in the CRSsNP group. As healthy controls, 13 patients with

traumatic optic neuropathy were enrolled, and UP tissues were sampled during endoscopic optic nerve decompression.

Each specimen was cut into 3 portions. The first portion was fixed overnight in freshly prepared fixative that contained 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and was embedded in paraffin wax for histologic staining. The second portion was stored in RNA stabilizing solution (RNAlater, Tiangen, Beijing, China) for quantitative reverse-transcription polymerase chain reaction. The third portion was stored at -80°C for Luminax-based multiplex assay.

Histologic Examination

Sinonasal tissues embedded in paraffin were cut into 5- μ m sections and placed onto glass slides. Hematoxylin-eosin staining was routinely performed for eosinophil. Immunohistochemical staining was performed with the avidin-biotin peroxidase method. Briefly, after antigen retrieval, sections were stained with the murine monoclonal antibodies antimyeloperoxidase (1:200; Abcam, Cambridge, Massachusetts). Then, streptavidin-biotin-peroxidase complex formation was used for detection. Immunohistochemical staining results were considered positive when the cells were stained brown after the addition of the 3',3'-diaminobenzidine reagent. Sections stained by isotype-matched IgG (Ab37355, Abcam) instead of primary antibodies were used as negative controls. The sections were examined and scored by 2 independent observers (F.C. and Y.S.) who were masked to the diagnosis and clinical data. The numbers of positive cells were counted in 10 randomly selected high-power fields ($\times 400$ magnification) and averaged. The ratio of eosinophils to total inflammatory cells higher than 10% was defined as eosinophilic CRS.¹⁴

Quantitative reverse-transcription polymerase chain reaction was performed according to the manufacturer's instructions, as we described elsewhere.¹⁵ Briefly, total RNA was extracted from sinonasal tissue with the TRIzol reagent (Invitrogen, Carlsbad, California) following the manufacturer's instructions. RT was performed, and the complementary DNA was synthesized from 2 μ g of total RNA using an oligo (dT)₁₈ primer and Moloney Murine Leukemia Virus RT (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. The messenger RNA (mRNA) expression levels were determined using an ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, California) and SYBR Premix Taq (Takara, Clinton, Mississippi). The primer sequences for target genes are available as requested (eTable 1). The PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L of 5 μ M primers, and 25 ng synthesized complementary DNA in a 25- μ L volume. The reactions were heated to 95 $^{\circ}\text{C}$ for 10 minutes followed by 40 cycles of denaturation at 95 $^{\circ}\text{C}$ for 10 seconds and annealing and extension at 60 $^{\circ}\text{C}$ for 60 seconds. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. The expression of the

Table 1
Demographic Characteristics of Patients With CRS and Controls^a

Characteristic	Control UP (n = 13)	CRSsNP UP (n = 11)	CRSwNP UP (n = 12)	CRSwNP NP (n = 42)	P value
Male sex	7 (53.9)	7 (63.6)	7 (58.3)	25 (59.5)	.97
Age, mean (SEM), y	33 (11)	50 (11)	34 (12)	39 (11)	.26
Atopic	0	3 (27.3)	4 (33.3)	15 (35.7)	.02
Asthmatic	0	1 (9.1)	4 (33.3)	17 (40.5)	<.001
AAHR	0	1 (9.1)	3 (25.0)	12 (28.6)	<.001
Oral steroid use	0	2 (18.2)	3 (25.0)	16 (38.0)	.048
Intranasal steroid use	0	5 (45.4)	6 (50.0)	26 (61.9)	<.001
Numbers of operations, mean (SEM)	0	0.27 (0.14)	0.83 (0.14)	0.86 (0.17)	.02
Blood eosinophil count, mean (SD) ^b	0.13 (0.03)	0.23 (0.05)	0.20 (0.05)	0.25 (0.04)	.54

Abbreviations: AAHR, asymptomatic airway hyperresponsiveness; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic sinusitis with nasal polyps; NP, nasal polyp; UP, uncinate process.

^aData are presented as number (percentage) of participants unless otherwise indicated.

^bBlood eosinophil count is expressed as number per high-power fields ($\times 400$ magnification).

Table 2Demographic Characteristics of Patients With NPs in the NAHR, AAHR, and Asthma Subgroups^a

Characteristic	NAHR-NP (n = 14)	AAHR-NP (n = 11)	AS-NP (n = 17)	P value
Male sex	9 (64.3)	7 (63.6)	9 (52.9)	.77
Age, mean (SEM), y	37 (8)	42 (11)	39 (14)	.46
Atopic	3 (21.4)	5 (45.4)	7 (41.2)	.37
Oral steroid use	3 (21.4)	5 (45.4)	8 (47.1)	.27
Intranasal steroid use	6 (42.9)	8 (72.7)	12 (70.6)	.20
No. of operations, mean (SEM)	0.57 (0.17)	0.82 (0.29)	1.12 (0.17)	.42
Blood eosinophil count, mean (SD) ^b	0.15 (0.07)	0.29 (0.22)	0.30 (0.07)	.27

Abbreviations: AAHR-NP, nasal polyps subjects with asymptomatic AHR; AHR, airway hyperresponsiveness; AS-NP, nasal polyps with asthma; CRSwNP, chronic sinusitis with nasal polyps; NAHR-NP, nasal polyps without AHR.

^aData are presented as number (percentage) of participants unless otherwise indicated.

^bBlood eosinophil count is expressed as number per high-power fields ($\times 400$ magnification).

housekeeping gene β -actin was used to normalize the expression of the transcripts of target genes. Melt curves are provided in eFigure 1.

Multiplex Immunoassay

Freshly obtained sinonasal tissue specimens were weighed, supplemented with phosphate-buffered saline and a protease inhibitor cocktail (Keygentec Com, Nanjing, Jiangsu, China) (1 mL of phosphate-buffered saline per 100 mg of tissue), and homogenized for 1 minute on ice. The suspension was then centrifuged at $1,574 \times g$ for 20 minutes at 4°C . The supernatants were stored at -80°C for the multiplex immunoassay. Briefly, analysis was performed on a MAGPIX clinical diagnostics instrument (Luminex, Austin, Texas) by using a commercial MILLIPLEX MAP kit according to the manufacturer's instructions (EMD Millipore, Billerica, Massachusetts). Data were analyzed with xPONENT software (Luminex). The following inflammatory mediators were examined:

IL-4; IL-5; IL-9; IL-25; IL-33; interferon γ (IFN- γ); eotaxin 1; eotaxin 2; eotaxin 3; IL-8; IL-10; thymic stromal lymphopoietin; monocyte chemotactic protein 4; regulated on activation, normal T-cell expressed, and secreted; thymus and activation regulated chemokine; tumor necrosis factor α ; and epithelial-derived neutrophil-activating peptide 78 (ENA-78).

Statistical Analysis

Data are expressed as mean (SEM). Because values of items were not normally distributed, statistical significance was analyzed by the Kruskal-Wallis test. Binary logistic regression analysis was performed for multivariate analysis for indicators of AHR. A receiver operating characteristic (ROC) curve was generated to find the best cutoff of an indicator for the diagnosis of AHR. The area under the curve (AUC) for each potential indicator was calculated; an AUC with a value close to 1 indicates an excellent ability to discriminate. $P < .05$ was considered statistically significant.

Results

The demographic data from all the study participants are listed in Table 1 and Table 2. As shown in Figure 1A–D and Table 3, 10 of 19 cytokines were differentially expressed among NPs and UP from CRSwNP, CRSsNP, and control subjects. These mediators included the classic type 2 mediators IL-4 ($P = .02$), T_H2 regulators thymic stromal lymphopoietin ($P = .02$) and IL-25 ($P = .04$), and the eosinophil chemokines eotaxin 1 ($P = .02$), monocyte chemotactic protein 4 ($P < .001$), and thymus and activation regulated chemokine ($P < .001$). Other growth factors, including the classic type 1 mediators IL-12p40 ($P < .001$), neutrophils chemokine IL-8 ($P < .001$) and ENA-78 ($P < .001$), and IL-9 ($P = .03$), were also found to differ significantly among the sinonasal tissues. However, no significant differences in IL-5, IL-13, eotaxin 2, eotaxin 3, and IFN- γ protein levels were observed between these 4 sinonasal tissue subsets.

Next, we evaluated whether cytokine expression in one particular sinonasal tissue group significantly differed from the other groups. The protein level of IL-25 was significantly increased in NP

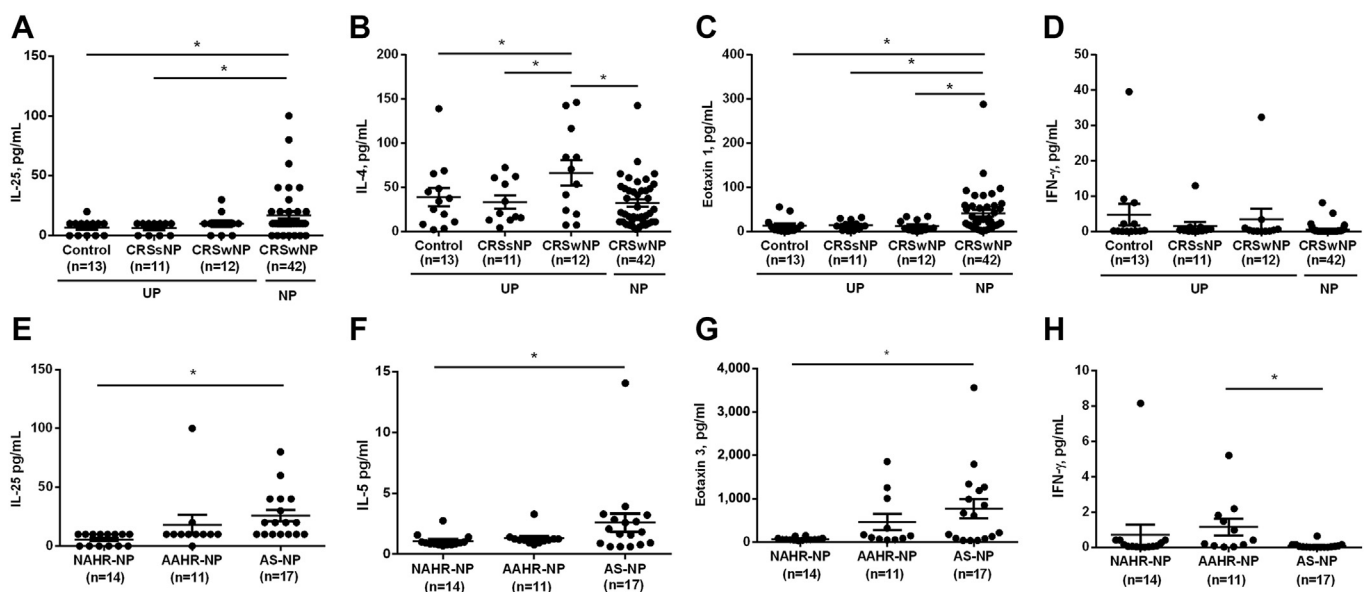


Figure 1. The protein levels of interleukin (IL) 25 and other cytokines in sinonasal tissues by Luminex-based multiplex assay. (A–D) Protein expression was measured in the tissue homogenates from the uncinate process (UP) in controls and patients with chronic rhinosinusitis without nasal polyps (CRSsNP) and the UP and nasal polyps (NPs) in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). (E–H) Protein expression in patients with NP homogenates from non-airway hyperresponsiveness (NAHR-NP), asymptomatic airway hyperresponsiveness (AAHR-NP), and asthma (AS-NP). Asterisk indicates ($P < .05$) as determined by Kruskal-Wallis test with post hoc analysis using the Dunn test for multiple comparison. Dot plots represent individual data points, and solid lines represent the mean (SEM). IFN- γ , interferon γ .

Table 3
Mean (SEM) Protein Levels of Inflammatory Mediators in Chronic Rhinosinusitis and Controls

Protein	Control UP (n = 13)	CRSSNP UP (n = 11)	CRSwNP UP (n = 12)	CRSwNP NP (n = 42)	P value
IL-5	1.7 (0.6)	2.1 (0.5)	2.6 (1.1)	1.7 (0.3)	.15
IL-13	5.9 (0.7)	11.1 (2.6)	9.1 (1.4)	6.5 (0.6)	.07
IL-9 ^a	4.26 (0.62)	4.09 (0.31)	4.33 (0.59)	3.16 (0.21)	.04
IL-33	439.9 (70.5)	428.9 (66.3)	483.4 (113.4)	449.9 (45.2)	>.99
TSLP ^a	3.6 (0.4)	3.9 (0.3)	4.7 (0.3)	4.3 (0.9)	.02
Eotaxin 2	244.7 (146.6)	269.0 (79.1)	170.9 (44.6)	203.6 (35.0)	.14
Eotaxin 3	744.4 (387.1)	930.2 (312.2)	777.4 (180.4)	452.0 (107.8)	.10
MCP-4 ^a	16.3 (2.7)	17.8 (1.9)	24.5 (1.8) ^b	16.0 (1.2) ^d	.001
RANTES	5,030.4 (788.9)	6,314.7 (992.6)	4,750.7 (831.7)	4,186.7 (452.4)	.17
TARC ^a	7.2 (3.2)	5.5 (1.2)	2.6 (0.3) ^b	2.2 (0.2) ^{b,c}	<.001
IL-12p40 ^a	5.8 (1.3)	5.7 (0.9)	5.1 (1.1)	2.3 (0.2) ^{b,c,d}	<.001
TNF- α	1.6 (0.2)	1.9 (0.3)	1.6 (0.2)	1.6 (0.1)	.87
IL-8 ^a	87.7 (9.7)	222.6 (73.1)	278.1 (83.2)	310.6 (40.3) ^b	<.001
ENA-78 ^a	523.0 (218.5)	836.5 (335.3)	2,062.3 (702.1) ^{b,c}	432.5 (175.2) ^d	<.001
IL-10	4.7 (1.2)	3.1 (1.2)	1.7 (1.0)	1.3 (3.9)	.07

Abbreviations: CRSSNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic sinusitis with nasal polyps; ENA-78, epithelial-derived neutrophil-activating peptide 78; IL, interleukin; MCP-4, monocyte chemotactic protein-4; RANTES, regulated on activation, normal T-cell expressed, and secreted; TARC, thymus and activation regulated chemokine; TNF- α , tumor necrosis factor α ; TSLP, thymic stromal lymphopoietin; UP, uncinate process.

^aP < .001, Kruskal-Wallis test for statistical significance.

^bSignificantly different (P < .05) compared with control UP according to post hoc analysis using the Dunn multiple-comparisons test.

^cSignificantly different (P < .05) compared with CRSSNP UP according to post hoc analysis using the Dunn multiple-comparisons test.

^dSignificantly different (P < .05) compared with control UP according to post hoc analysis using the Dunn multiple-comparisons test.

tissues compared with the UP tissues from patients with CRSSNP and controls (P < .05). However, no difference was noted between NP tissues and UP tissues in patients with CRSwNP (P > .05) (Fig 1A). The T_H2 cytokine protein level of IL-4 was significantly increased in UP tissues of patients with CRSwNP compared with the other 3 sinonasal tissue groups (P < .05) (Fig 1B). In addition, the eosinophil chemokine eotaxin 1 in NP tissue was higher than in the other 3 sinonasal tissue groups (P < .05) (Fig 1C). Besides, the neutrophil chemokine ENA-78 was significantly increased in UP tissue of patients with CRSwNP compared with the other 3 tissue subsets (P < .05) (Table 3).

When NP tissues were divided into 3 subgroups (asthma, AAHR, and NAHR), we found that only 5 of 19 cytokines were significantly different in these 3 NP subgroups. IL-25, IL-5, and eotaxin 3 levels were consistently elevated in the asthmatic NP subgroup compared with the NAHR subgroup (P < .05) but did not differ from the AAHR subgroup (P > .05) (Fig 1E–G). Conversely, IFN- γ was significantly decreased in the asthmatic subgroup compared with the AAHR subgroup (P < .05) but did not differ from the NAHR subgroup (P > .05) (Fig 1H). However, the T_H2 cytokines IL-4 and IL-13 did not reach statistically significant differences in the 3 subgroups (P > .05) (Table 4).

For gene expression levels of the transcription factors of T_H1 and T_H2 cells, we found the mRNA level of GATA-3, but not T-bet, was significantly increased in NP tissues compared with the control UP tissues from patients with CRSwNP, patients with CRSSNP, and controls (P < .05) (Fig 2A and B). Consistent with the protein levels, the mRNA level of IL-25 was elevated in NP tissue of patients with CRSwNP compared with UP tissues in controls, patients with CRSSNP, and patients with CRSwNP (P < .05) (Fig 2C). Besides, the mRNA level of IL-4 was increased in UP tissue of patients with CRSwNP compared with the UP tissue in controls and patients with CRSSNP and NP tissue (P < .05) (Fig 2D).

When we divided the NP tissues into 3 subgroups (asthma, AAHR, and NAHR), we found that GATA-3 was significantly increased in the asthmatic NP subgroup compared with the AAHR and NAHR subgroups (P < .05) (Fig 2E and F). Furthermore, in the NP subgroup comparisons, the mRNA level of IL-25 was increased in the asthmatic NP subgroup compared with the NAHR NP subgroup (P < .05) (Fig 2G).

Next, we investigated the eosinophilic and neutrophilic inflammation in sinonasal tissue. Figure 3 reveals that eosinophil count

was significantly increased in NP tissue compared with UP tissue in the control, CRSSNP, and CRSwNP subgroups. Among the 42 patients with CRSwNP, 36 (85.7%) were eosinophilic and 6 (14.3%) were noneosinophilic. However, in subgroups analysis, although there was a trend for level of eosinophils count to increase in the asthmatic NP subgroup, this finding did not reach statistical significance. Previous publication have reported that neutrophilic inflammation was elevated in Chinese patients with NPs.⁶ In the further investigation, we analyzed the level of neutrophilia in sinonasal tissue used myeloperoxidase-positive cells in immunohistochemical staining as a marker. We found that the neutrophil count was increased in UP tissue of the CRSSNP group and NP tissue of the CRSwNP group when compared with UP tissue in controls and patients with CRSwNP. Notably, in subgroup analysis, we found that the NAHR and NP subgroups had the highest neutrophils count (P < .05).

Table 4
Mean (SEM) Protein Levels of Inflammatory Mediators in NPs of NAHR, AAHR, and Asthma by Multiplex Immunoassay

Protein	NAHR-NP (n = 14)	AAHR-NP (n = 11)	Asthma-NP (n = 17)	P value
IL-4	37.1 (9.5)	37.9 (5.2)	23.0 (5.7)	.25
IL-13	7.7 (1.3)	7.2 (1.1)	5.1 (0.6)	.16
IL-9 ^a	2.5 (0.3)	3.8 (0.3) ^b	3.4 (0.4)	.03
IL-33	370.9 (65.5)	573.0 (97.4)	398.5 (65.9)	.18
TSLP	3.6 (0.3)	3.6 (0.2)	5.3 (2.8)	.66
Eotaxin 1	44.9 (8.9)	46.9 (25.0)	35.8 (8.2)	.83
Eotaxin 2	109.3 (242.2)	245.0 (92.3)	264.8 (58.3)	.14
MCP-4	14.4 (1.6)	20.8 (3.7)	14.6 (1.2)	.10
IL-12p40	2.6 (0.4)	2.0 (0.1)	1.9 (0.2)	.30
RANTES	4,387.5 (862.1)	2,925.0 (729.2)	4,583.7 (708.1)	.32
TARC	1.6 (0.6)	2.4 (1.7)	2.5 (1.6)	.18
TNF- α	1.4 (0.1)	1.6 (0.2)	1.9 (0.2)	.08
IL-8	376.3 (84.3)	230.0 (56.2)	314.5 (65.1)	.41
ENA-78	261.3 (83.9)	186.5 (60.0)	117.3 (50.2)	.47
IL-10	1.5 (63.7)	81.0 (61.7)	1.6 (79.3)	.71

Abbreviations: CRSSNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic sinusitis with nasal polyps; ENA-78, epithelial-derived neutrophil-activating peptide 78; IL, interleukin; MCP-4, monocyte chemotactic protein-4; RANTES, regulated on activation, normal T-cell expressed, and secreted; TARC, thymus and activation regulated chemokine; TNF- α , tumor necrosis factor α ; TSLP, thymic stromal lymphopoietin; UP, uncinate process.

^aP < .001, Kruskal-Wallis test for statistical significance.

^bSignificantly different (P < .05) compared with control UP according to post hoc analysis using the Dunn multiple-comparisons test.

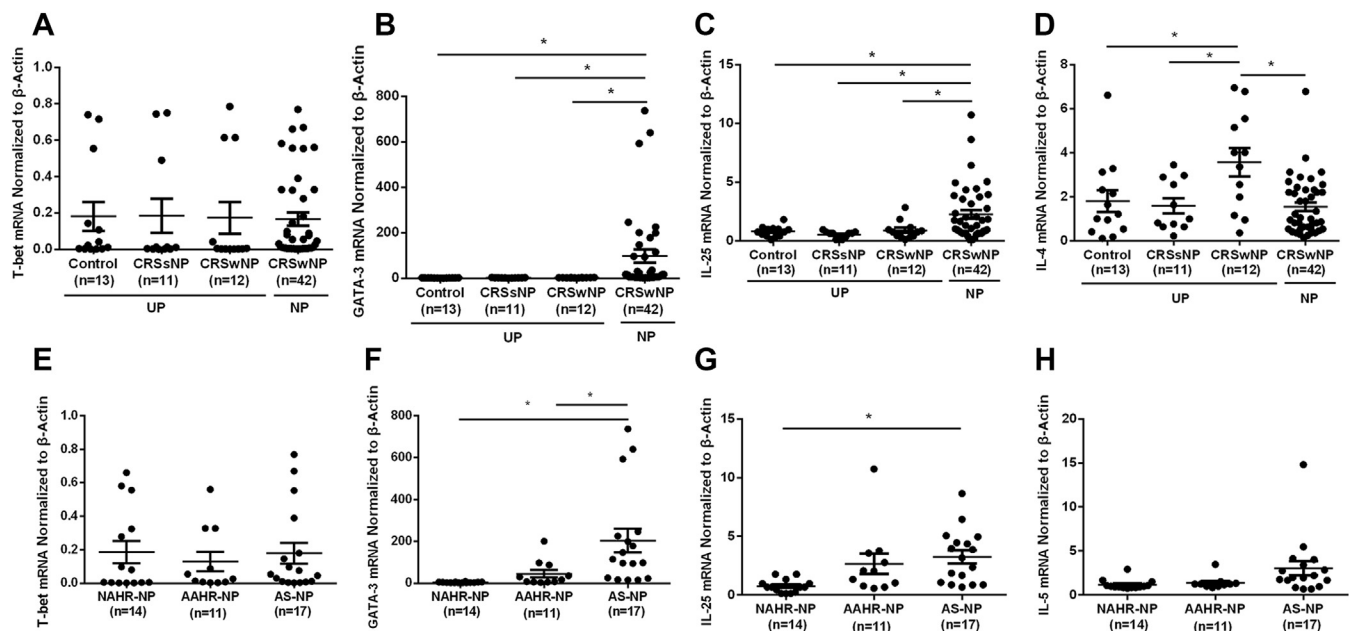


Figure 2. The messenger RNA (mRNA) levels of T-bet, GATA-3, interleukin (IL) 25, and other cytokines in sinonasal tissues determined by real-time polymerase chain reaction. (A–D) Total RNA was extracted from the uncinate process (UP) from controls and patients with chronic rhinosinusitis without nasal polyps (CRSsNP) and UP and nasal polyps (NPs) of patients with chronic rhinosinusitis with nasal polyps (CRSwNP). (E–H) Total RNA was extracted from NPs from patients with non–airway hyperresponsiveness (NAHR-NP), patients with asymptomatic airway hyperresponsiveness (AAHR-NP), and asthma (AS-NP). Asterisk indicates ($P < .05$) was determined by the Kruskal-Wallis test with post hoc analysis using the Dunn test for multiple comparison. Dot plots represent individual data points, and solid lines represent the mean (SEM).

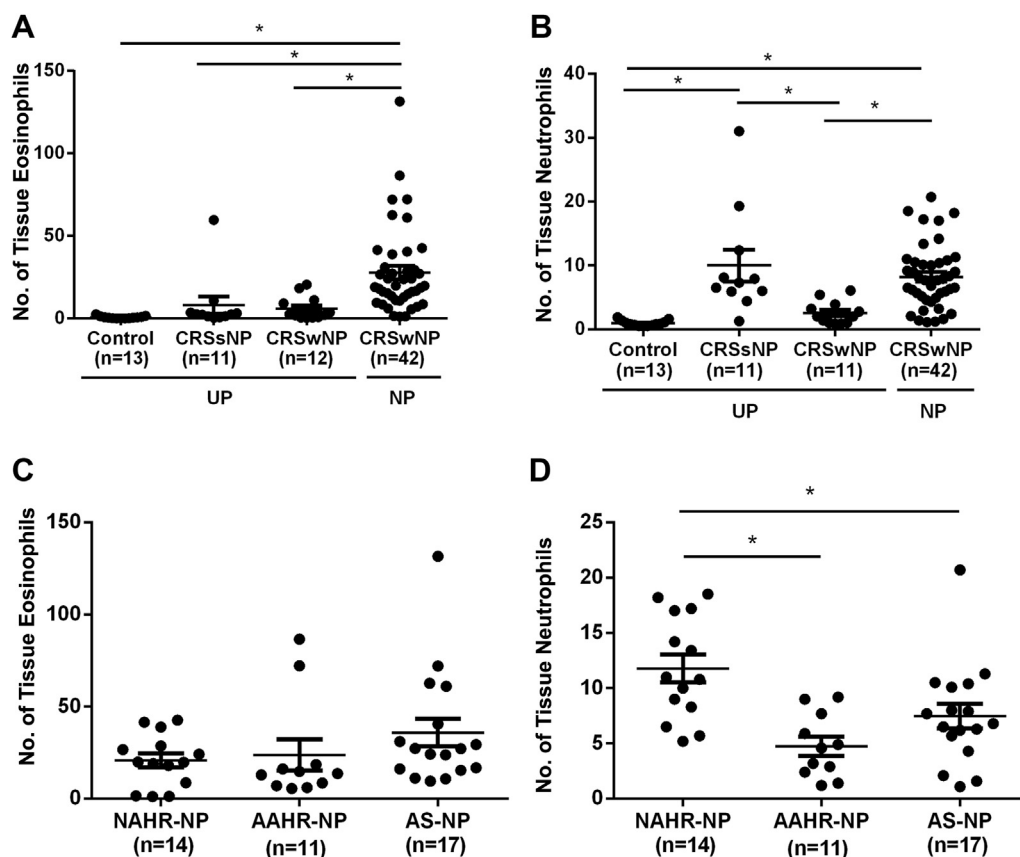


Figure 3. Tissue eosinophil and neutrophil counts in sinonasal tissues and different phenotypes of nasal polyps (NPs) in 10 randomly selected high-power fields ($\times 400$ magnification). Tissue eosinophil count (A) and neutrophils count (B) in the uncinate process (UP) from controls and patients with chronic rhinosinusitis without nasal polyps (CRSsNP) and UP and NPs in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). Tissue eosinophil count (C) and neutrophils count (D) in NP tissues from patients with non–airway hyperresponsiveness (NAHR-NP), asymptomatic airway hyperresponsiveness (AAHR-NP), and asthma (AS-NP). Asterisk indicates ($P < .05$) as determined by the Kruskal-Wallis test with post hoc analysis using the Dunn test for multiple comparison. Dot plots represent individual data points, and solid lines represent the mean (SEM).

Moreover, by applying multivariate logistic regression analysis, we found that nasal IL-25 protein level and tissue and blood eosinophil counts were independent risk factors for AHR in patients with CRSwNP ($P < .05$). In the following ROC curve analysis, we examined the sensitivity and specificity of nasal tissue IL-25 protein level and tissue and blood eosinophil counts in the diagnosis of AHR in this CRSwNP cohort. Consequently, at the cutoff tissue IL-25 protein level of 5.0 pg/ μ L, the sensitivity was 93.5%, the specificity was 60.0%, and the AUC of IL-25 was 0.803 (95% confidence interval [CI], 0.700–0.907). On the other hand, when the cutoff tissue eosinophil count was set as 14 cells per high-power field, the sensitivity was 87.1% and the specificity was 62.5%. As well, when the cutoff blood eosinophil count was set as $0.13 \times 10^9/L$, the sensitivity was 67.7% and the specificity was 62.5%. The AUC of tissue and blood eosinophils counts were 0.743 (95% CI, 0.629–0.857) and 0.699 (95% CI, 0.575–0.824), respectively (Fig 4).

Discussion

IL-25 was suggested as a novel therapeutic target in patients with CRSwNP because it plays a crucial role in polypogenesis.¹⁶ In the current study, we provide evidence that IL-25 expression is significantly enhanced in polyp tissues of patients with CRSwNP, and, notably, in NP subgroups analysis, IL-25 expression were significantly increased in the asthmatic subgroup compared with the NAHR subgroup. Furthermore, the NP IL-25 level was associated with AHR (concomitant asthma). These findings, therefore, expand our understanding of the concept of united airway diseases.

Emerging evidence supports an active role for nasal T_H2 -skewed, eosinophilic inflammation in patients with CRSwNP in the pathogenesis of concomitant asthma.^{9–11} However, the key factors initializing concomitant asthma in patients with CRS are not completely understood. IL-25 (also known as IL-17E) is a member of the IL-17 cytokine family and has been reported to play various

roles in different inflammatory murine models, such as asthma, atopic dermatitis, and pulmonary fibrosis.¹⁷ Intraperitoneal or intranasal administration of IL-25 protein resulted in the production of eosinophils or T_H2 cytokines, such as IL-4, IL-5, and IL-13, in bronchoalveolar lavage fluid and lung tissue.^{18,19} Conversely, blocking IL-25 decreased T_H2 cytokine production in an animal model of asthma.²⁰ Besides, Shin et al²¹ reported that IL-25 was significantly increased in human NP tissues and the murine CRSwNP model, and anti-IL-25 treatment reduced the number of polyps, mucosal edema thickness, collagen deposition, and infiltration of inflammatory cells, such as eosinophils and neutrophils. Taking together, the previous studies and our present study revealed IL-25 may play a crucial role in initializing concomitant asthma in CRS. In addition, IL-25 has the potential to be a therapeutic target in concomitant asthma and CRS.

To further validate the clinical potential of nasal IL-25 in indicating AHR in patients with CRSwNP, we next compared the sensitivity and specificity of nasal IL-25 protein level and tissue and blood eosinophil counts in the diagnosis of AHR in this CRSwNP cohort by applying ROC curve analysis. Consequently, when the cutoff tissue IL-25 protein level was 5.0 pg/ μ L, the sensitivity was 93.5%, the specificity was 60.0%, and the AUC of IL-25 was 0.803 (95% CI, 0.700–0.907). Several studies have reported the potential role of blood eosinophil counts and nasal symptoms in indicating AHR in CRSwNP, reporting moderate sensitivity and specificity.^{22–24} In our previous study,²⁵ we found that the olfactory cleft score had a higher indicative value for AHR than blood eosinophil count. The olfactory cleft score was evaluated on the coronal images of sinus computed tomography at the posterior ethmoid sinus and superior turbinate level and was awarded 0 to 2 points in each side. Our study found that the IL-25 protein level had better predict value than eosinophil count.

IL-5 production in NP tissues is related to the presence of comorbid asthma.^{12,13} Consistently, we also found that IL-5 was increased in asthmatic NP. However, it was IL-4 protein level, but not IL-5, significantly increased in the NP group compared with UP tissues from patients with CRSwNP, patients with CRSsNP, and controls. IL-5 expression level was diverse in different races and regions. Wang et al⁵ compared IL-5 levels in NP tissues from 6 cities in China, Japan, Europe, and Australia. They reported that Asiatic regions had lower level of IL-5 than European regions and Chinese patients with NPs had lower level of IL-5 than Japanese patients. Moreover, in Chengdu, China, the level of IL-5 from NP tissue was not higher than sinonasal tissue from patients with CRSsNP and controls. The discrepancy among different regions was possibly caused by a combination of genetic and environmental factors.

In the current study, we found that eosinophil count was significantly increased in NP tissue compared with UP tissue in controls, patients with CRSsNP, and patients with CRSwNP. Among the patients with CRSwNP, 85.7% were eosinophilic and 14.3% were noneosinophilic. However, in subgroups analysis, although there was a trend for level of eosinophils count to increase in the asthmatic NP subgroup, this finding did not reach statistical significance. On the other hand, we found that neutrophil count was increased in UP tissue of the CRSsNP group and NP tissue. Meanwhile, the levels of chemokines of neutrophils, including IL-8 and ENA-78, were elevated in NP tissue and UP tissue of patients with CRSwNP. Notably, in subgroup analysis, we found that the NAHR-NP subgroups had the highest neutrophil counts ($P < .05$). Several studies reported that Chinese NPs had more prominent neutrophil inflammation.^{5,6} It suggested that environmental factors, such as microbiota and air pollution, can result in IL-8 and ENA-78 synthesis and neutrophil accumulation.^{26,27} The results reminded us that eosinophils play a pivotal role in concomitant CRS with asthma.

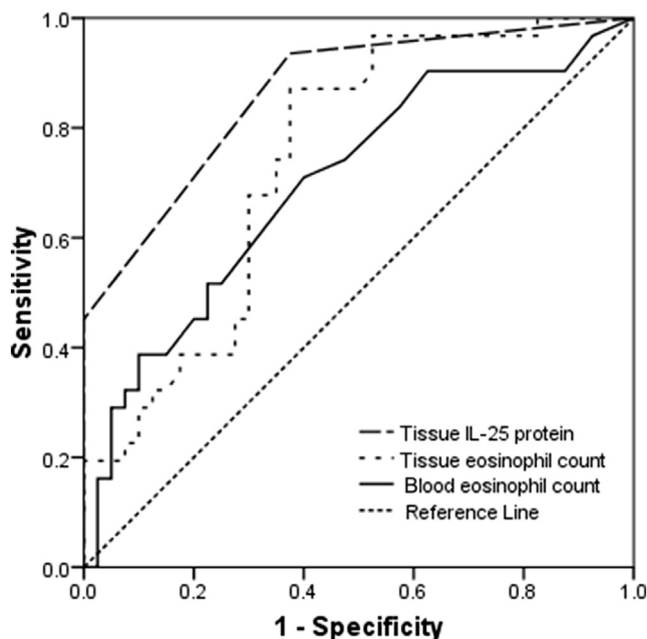


Figure 4. The receiver operating characteristic (ROC) curve for diagnosis of airway hyperresponsiveness in the cohort with chronic rhinosinusitis with nasal polyps using tissue interleukin (IL) 25 and eosinophil counts. When the tissue IL-25 protein level was 5.0 pg/ μ L or greater, the sensitivity was 93.5%, and the specificity was 60.0%. At the cutoff tissue eosinophil count of 14 cells per high-power field or greater, the sensitivity was 87.1%, and the specificity was 62.5%. At the cutoff blood eosinophil count of $0.13 \times 10^9/L$ or higher, the sensitivity was 67.7%, and the specificity was 62.5%.

Our study participants were not restricted from taking oral steroids. However, some of our participants were using corticosteroids at the time of the study. Moreover, even with some oral corticosteroid use, IL-25, IL-5 and eotaxin 3 continued to be increased in asthma-NP, suggesting that plethoric eosinophil recruitment was still happening. We acknowledged that the potential limitations of this study, such as the small sample size and result bias, cannot be ignored. Therefore, a subsequent study performed with a larger cohort is necessary to further validate our findings.

In summary, we found that increased IL-25 expression in NP tissue, and especially in asthmatic NP tissues compared with NAHR NP tissues, and IL-25 level may act as biomarkers for concomitant asthma in patients with CRSwNP.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2017.07.012>.

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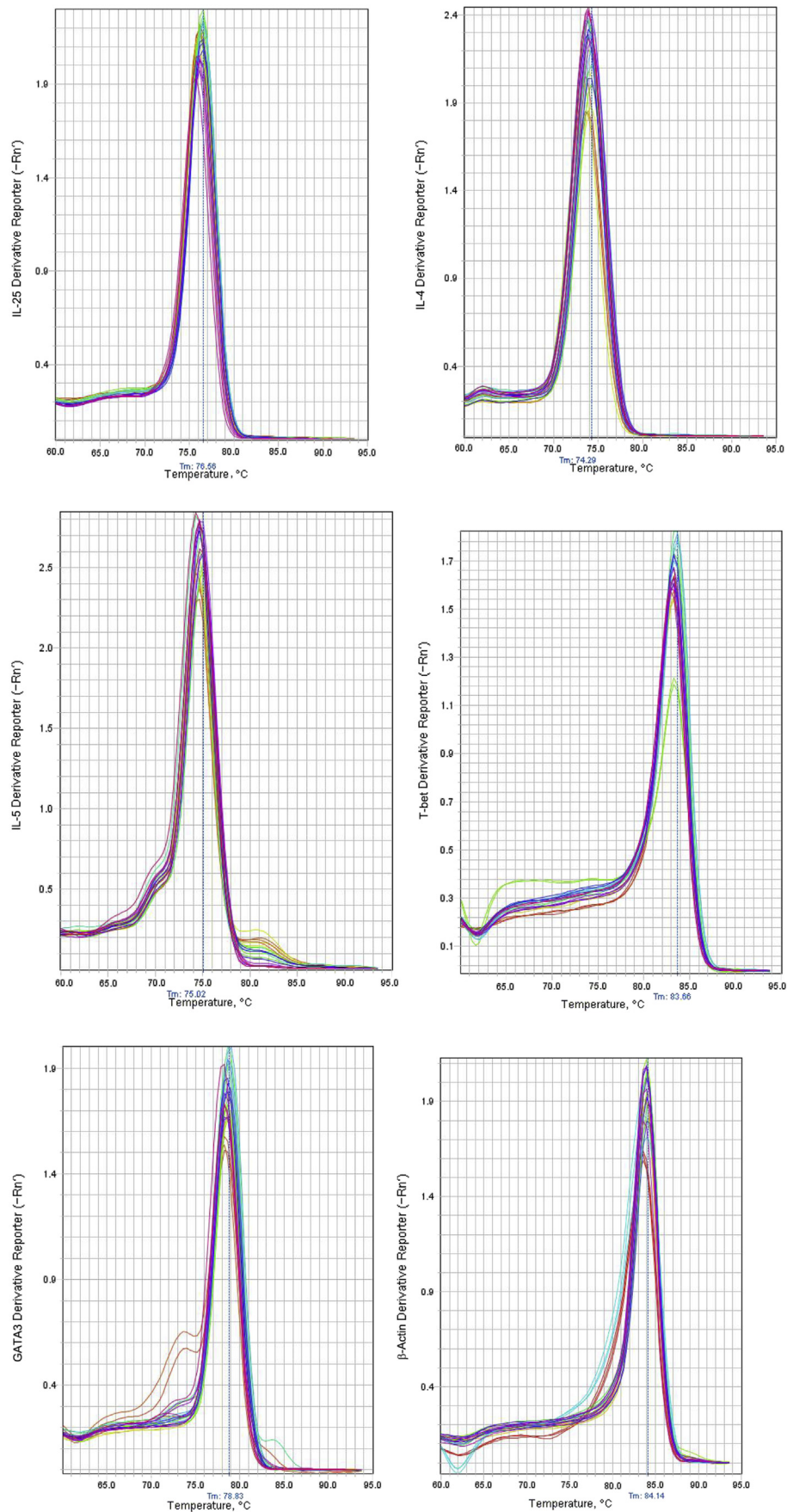
Supplementary Data

eTable 1

Sequences of Primers

Primer	Forward	Reverse
T-bet	GTCAATTCCTTGGGGGAG AT	TCATGCTGACTGCTCGAA AC
GATA-3	ACCACAACCACACTCTGGAGGA	TCGGTTTCTGGTCTGGATGCCT
IL-25	CCAGGTGGTTGCATTCTTGG	TGGCTGTAGGTGTGGGTTCC
IL-4	GCAGTTCTACAGCCACCATG	ATGATCGTCTTTAGCCTTTCC
IL-5	ATCTTTCAGGGAATAGGCACAC	CCGTCTTCTTCTCCACACTTT
IL-13	AGCATGGTATGGAGCATCAA	CAGCATCCTCTGGGTCTTCT
IFN- γ	TTTTGAAGAATTGGAAAGAGGA	CACTTGGATGAGTTCATGTATTG
β -actin	GTACCAACTGGGACGACA	CACAGCCTGGATAGCAACG

Abbreviations: IL, interleukin; IFN, interferon.



eFigure 1. Melt curves. IL, interleukin.