Supplemental Information

METHODS

Patients and tissue samples

Sinonasal and polyp tissues were obtained from patients with CRS during routine functional endoscopic sinus surgery. All subjects provided written informed consent for study participation, and the study was approved by the internal review board of the Seoul National University Hospital, Boramae Medical Center (No. 06-2012-109). The diagnosis of CRS was based on personal history, physical examination, nasal endoscopy, and CT findings of the sinuses according to the 2012 European position paper on rhinosinusitis and nasal polyps (EPOS) guidelines. Exclusion criteria were as follows: (1) younger than 18 years of age, (2) prior treatment with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs for 4 weeks before surgery, and (3) unilateral rhinosinusitis, antrochoanal polyp, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease. Control tissues were obtained during other rhinologic surgeries, such as skull base, lacrimal duct, or orbital decompression surgery, from patients without any sinonasal diseases. Uncinate process (UP) tissue was obtained from control subjects and those with CRSsNP or CRSwNP. NP tissue in patients with CRSwNP also was evaluated. Each sample was divided into three parts: one third was fixed in 10% formaldehyde and embedded in paraffin for histological analysis, another third was immediately frozen and stored at -80ºC for subsequent isolation of mRNA, and the final third was submersed in 1 mL phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (Sigma-Aldrich, St Louis, MO) and 1% PIC (Sigma-Aldrich) per 0.1 g of tissue. The latter tissue was homogenized with a mechanical homogenizer at 1,000 rpm for 5 min on ice. After homogenization, the suspensions were centrifuged at 3,000 rpm for 10 min at 4ºC and the supernatants were separated and stored at -80ºC for further analysis of cytokines and other inflammatory mediators. The atopic status of study subjects was evaluated using the ImmunoCAP® assay (Phadia, Uppsala, Sweden) to detect IgE antibodies against six mixtures of common aeroallergens (house dust mites; molds; trees; weeds and grass pollen; and animal danders). Subjects were considered atopic if the allergen-specific IgE level was greater than 3.51 KU/L to any one or more of the allergens. Also, total IgE levels in nasal tissue homogenates were measured using the ImmunoCAP® assay. A diagnosis of asthma was based on the medical history and lung function analysis, including challenge tests by an allergist. In this study, CRSwNP were classified as eosinophilic NP (E-NP) if eosinophils comprised more than 10% of the inflammatory cell population and as non-eosinophilic NP

(NE-NP) if eosinophils comprised less than 10% of the inflammatory cells.

Immunohistochemistry (IHC)

Single immunohistochemical staining was performed using the polink-2 plus polymerized horseradish peroxidase (HRP) broad DAB-Detection System (Golden Bridge International Labs.,WA).Briefly,after deparaffinization, sections were incubated in 3% hydrogen peroxidase to inhibit endogenous peroxidases. Heat-induced epitope retrieval was then performed by microwaving samples in 10 mmol/L citrate buffer (pH 6.0). The sections were incubated for 60 minutes at room temperature in a primary antibody. The primary antibodies were rabbit anti-periostin (1:2000; Abcam, Cambridge, UK), rabbit anti-CD51 as marker for integrin αV (1:150; Abcam) and TSLP (1:1000; Abcam). The sections were incubated in broad antibody enhancer and polymer-HRP and then stained with the DAB Detection System. Finally, slides were counterstained with hematoxylin. The numbers of positive cells were counted in the five densest visual fields (400×) by two independent observers, and the average values were determined. Sequential double IHC was employed using polymer-HRP and alkaline phosphatase (AP) kits to detect mouse anti-mast-cell tryptase (1:500; Abcam) and rabbit anti-periostin primary antibodies for human tissue with permanent-Red and Emerald (Polink DS-MR-Hu C2 Kit; Golden Bridge International Labs).

qRT-PCR analysis

Total RNA was extracted from tissue samples using the TRI reagent (Invitrogen, Carlsbad, CA). One microgram total RNA was reverse transcribed to cDNA using the cDNA Synthesis Kit (amfiRivert Platinum cDNA Synthesis Master Mix, GenDEPOT). Quantitative real-time PCR (qRT-PCR) was performed. For analysis of POSTN (Hs01566734_m1), CD51 (integrin αV, Hs00233808_m1), IL-4 (Hs00174122_m1), IL-5 (Hs01548712_g1), IL-13 (Hs00174379_m1), IL-17A (Hs00174383_m1), IFN-γ (Hs00989291_m1), TSLP (Hs00263636_m1), IL-25 (Hs03044841_m1), IL-33 (Hs00369211_m1) and GAPDH (Hs02758991_g1), pre-developed assay reagent (PDAR) kits of primers and probes were purchased from TaqMan assays (Life Technologies Korea, Seoul, Korea). Pre-developed assay reagent kits containing primers and probes were purchased from Applied Biosystems (Foster City, CA). Expression of GAPDH was used as an internal control for normalization. Cycling conditions were as follows: 95°C for 5 min followed by 60 cycles at 95°C for 15 sec, 60°C for 20 sec, and 72°C for 20 sec. To analyze the data, Sequence Detection Software version 1.9.1 (Applied Biosystems) was utilized. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

Measurement of cytokines and total IgE in tissue homogenates

The protein concentrations for tissue extracts were determined using the Pierce 660nm Protein Assay Kit (Thermo Scientific Inc., NY). Samples were thawed at room temperature and vortexed to ensure well-mixed sample. Tissue homogenates were then assayed for periostin proteins by using commercially available ELISA kits (R&D systems, MN). Multiple cytokine analysis kits (IL-4, IL-5, IL-13, IL-17A, IL-25, IL-33, CXCL-8, CCL-11, and IFN-γ) were obtained from R&D systems (Cat. No. LMSAHM) and data collected using Luminex 100 (Luminex, Austin, TX). Data analysis was performed using the MasterPlex QT version 2.0 (MiraiBio, Alameda, CA). Total IgE levels in nasal tissue homogenates were measured by using the ImmunoCAP® assay. All kinds of assays were run in duplicate according to the manufacturers' protocol. All the protein levels in the tissue homogenate were normalized to the concentration of total protein.

Human mast cell culture and measurement of cytokine production

LAD2 mast cells (MCs) were cultured in serum-free media (StemPro-34 SFM, Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin and 100 ng/ml stem cell factor (SCF). The cell suspensions were cultured at a density of 10^5 cells/ml and maintained at 37^{o}C and 5% CO2. LAD2 cells were suspended at 0.2 x 10^6 cells per well in fresh media containing 100 ng/mL of SCF, and sensitized with 0.5 µg/mL of biotinylated IgE (bIgE; Abbiotec, San Diego, CA) overnight at 37^{o}C. Sensitized cells were resuspended at 0.5 x 10^6 cells/mL in fresh media and stimulated with 0.05-0.5 µg/mL streptavidin (Life Technologies), recombinant human IL-4 (10 ng/ml), recombinant human IL-13 (10 ng/ml) or recombinant human TNF (10 ng/mL) for 3 hr at 37^{o}C. RNA was isolated using Trizol. Reverse transcription of total RNA and amplification of target mRNA were performed using the iScript Select cDNA synthesis kit from (BioRad). The gene target, periostin was amplified using gene specific primers, designed for end point evaluation using Primer Express software (Perkin Elmer). The primer sequences for periostin were as follows: 5'-GATGCAGTGCCTGTGGAAATA-3' Forward, 5'- CTGAGAACGACCTTCCCTTAATC-3' Reverse, Housekeeping gene, GAPDH primer set: 5'-GAAGGTGAAGGTCGGAGTC-3' Forward, 5'-GAAGATGGTGATGGGATTTC-3' Reverse. qRT-PCR analysis was performed using Perfecta Sybr supermix from Quanta Bioscience (VWR). The reaction conditions were as follows: 95^{o}C for 3 min (Hold) 1 cycle and 95^{o}C for 10 sec, 55^{o}C for 30 sec, 72^{o}C for 60 sec (cycling) 40 cycles. Resulting PCR products were measured by the sequence detector Qiagen Rotor-Gene Q (Qiagen). Quantitation of gene expression by real-time RT-PCR

was evaluated using the copy number method. Secreted periostin was measured in cell supernatants using a sensitive and specific ELISA according to the manufacturer's instructions (BioVision, Inc). The detection limit for the assay was 15 pg/ml.

Human bronchial epithelial cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersvile, MD) and cultured in serum-free bronchial epithelial growth medium (BEGM, Lonza) at 37^{o}C in a humidified environment containing 5% CO2. NHBE cells were plated in 12-well culture plates coated with collagen (Corning, NY) and grown 40-50% confluence. Before treatment, NHBE cells were maintained in BEGM without hydrocortisone for at least 2 days. NHBE cells were treated with recombinant human IL-4 or IL-13 (100ng/ml, each; R&D systems, Minneapolis, MN) with or without periostin (10 or 100 ng/ml; R&D systems) for 72 hours. Additionally, in each experiment, NHBE cells were stimulated with poly(I:C)(5 μg/ml; InvivoGen, San Diego, CA) at 1 hour after treatment with IL-4, IL-13, and/or periostin. Anti-integrin αVβ3 and anti-integrin αVβ5 (2.5μg/ml; R&D systems) were added to confirm reversibility of periostin-induced TSLP production. Cell culture supernatants were collected and used for TSLP protein ELISA (R&D systems, Minneapolis, MN).

Statistical analysis

Statistical analyses were performed using IBM SPSS 21 (SPSS, Inc., Chicago, IL) and GraphPad Prism software 6.0 (GraphPad software Inc, La Jolla, CA). The Kruskal-Wallis test and the Mann-Whitney *U*-test with a 2-tailed test for unpaired comparisons were used. For comparisons between groups, the Kruskal-Wallis test was used to establish significant intergroup variability. The Mann-Whitney *U*-test was then used for between-group comparisons. The Pearson correlation test was also used to determine variable relationships. If the data were not normally distributed, the Spearman correlation coefficient was utilized. The significance level was set at an α value of 0.05.

Figure Legends

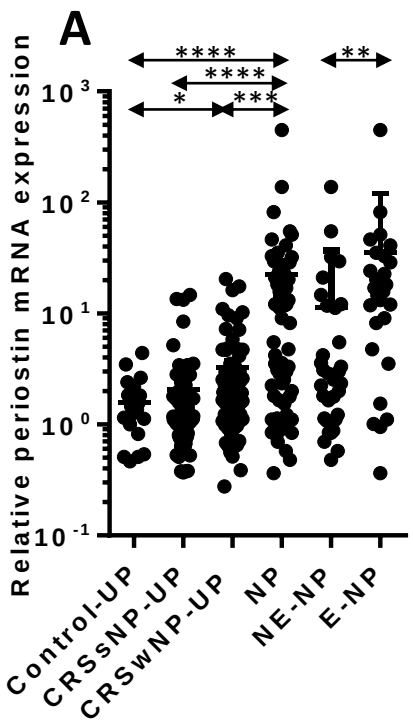
Supplementary Fig 1. Expression of periostin in eosinophilic nasal polyps

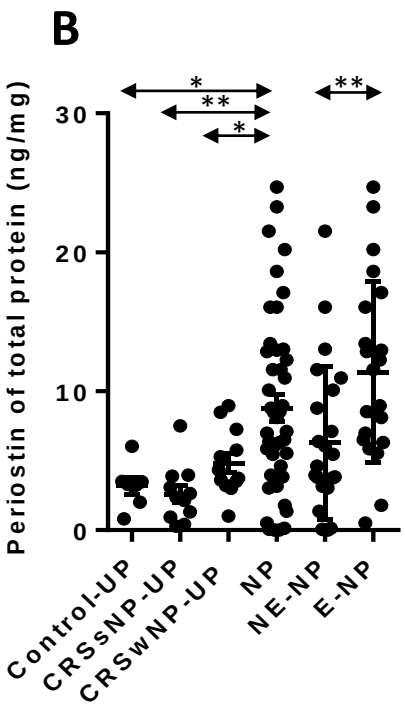
(A) Relative periostin mRNA expression was measured by qRT-PCT in tissues from each group (n=22 for Control-UP, n=62 for CRSsNP-UP, n=68 for CRSwNP-UP, and n=67 for CRSwNP-NP). **(B)** Protein levels of periostin were measured by ELISA (n=7 for Control-UP, n=11 for CRSsNP-UP, n=12 for CRSwNP-UP, and n=43 for CRSwNP-NP). **(C)** Correlation between Lund-MacKay CT score and protein levels of periostin in different subtypes of CRSwNP

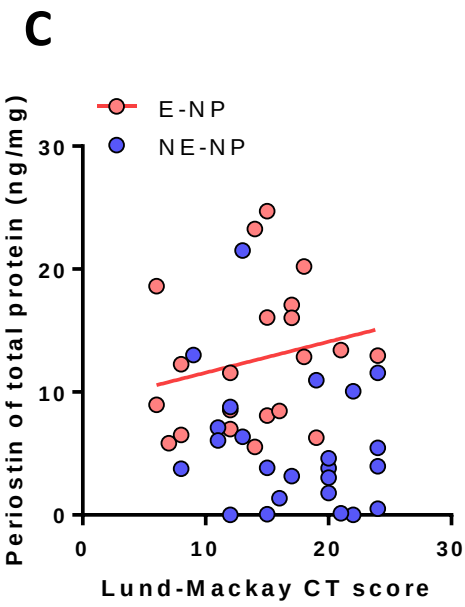
Supplementary Fig 2. Correlation between periostin expression and major cytokines in NP.

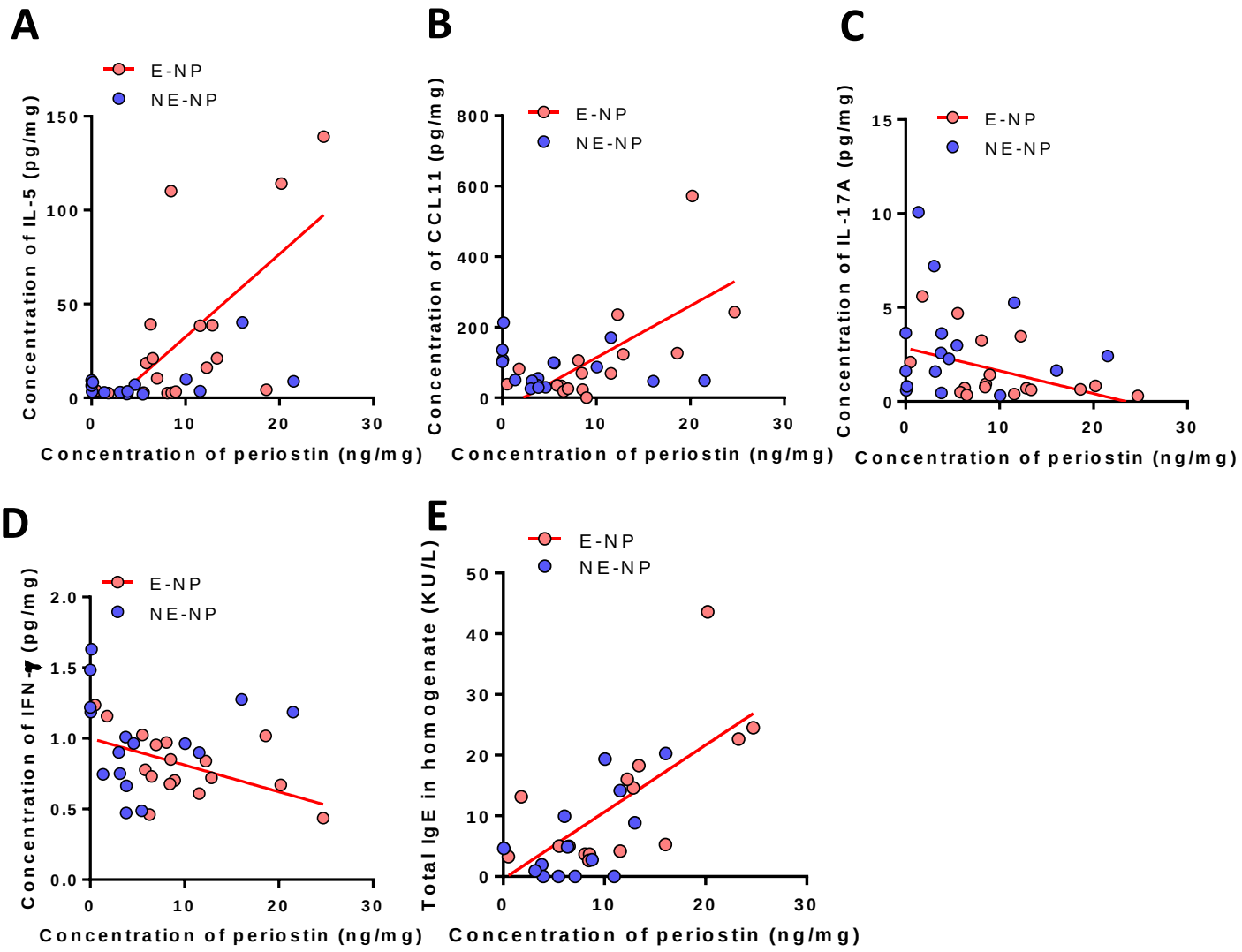
(A) IL-5 (n=18 for E-NP and n=16 for NE-NP). **(B)** CCL-11 (n=18 for E-NP and n=16 for NE-NP). **(C)** IL-17A (n=18 for E-NP and n=16 for NE-NP). **(D)** IFN-γ (n=18 for E-NP and n=26 for NE-NP). **(E)** Periostin levels in comparison with total IgE in NP homogenates (n=16 for E-NP and n=15 for NE-NP).

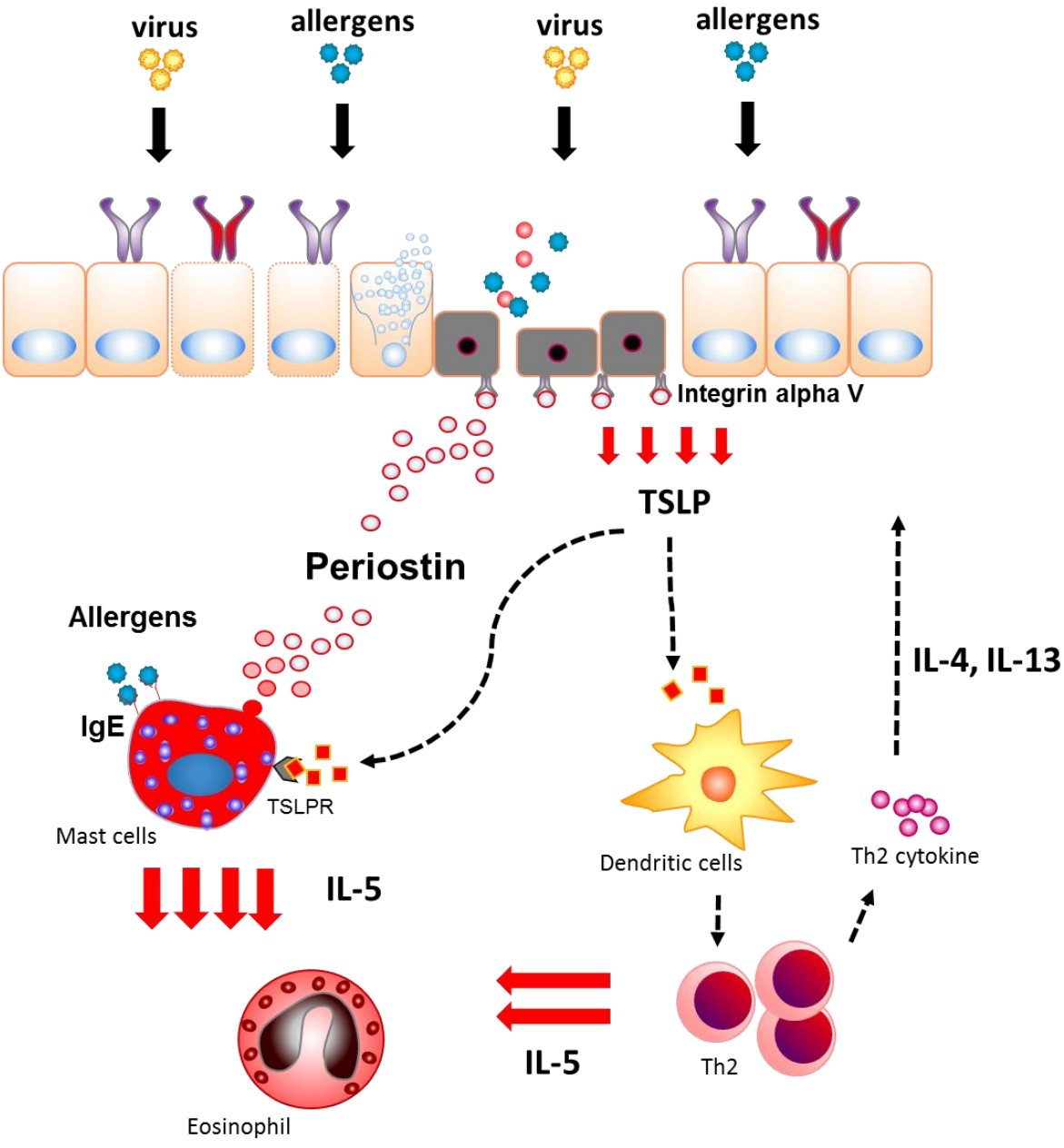
Supplementary Fig 3. Schematic depiction of periostin-mediated cross-talk between mast cells and epithelium.


A
Relative periostin mRNA expression
10^3
10^2
10^1
10^0
10^{-1}
****
****
*
***
**
Control-UP
CRSsNP-UP
CRSwNP-UP
NP
NE-NP
E-NP


B
Periostin of total protein (ng/mg)
30
20
10
0
*
**
*
**
Control-UP
CRSsNP-UP
CRSwNP-UP
NP
NE-NP
E-NP


C
Periostin of total protein (ng/mg)
E-NP
NE-NP
30
20
10
0
0
10
20
30
Lund-Mackay CT score


A
E-NP
NE-NP
Concentration of IL-5 (pg/mg)
150
100
50
0
0
10
20
30
Concentration of periostin (ng/mg)
B
E-NP
NE-NP
Concentration of CCL11 (pg/mg)
800
600
400
200
0
0
10
20
30
Concentration of periostin (ng/mg)
C
E-NP
NE-NP
Concentration of IL-17A (pg/mg)
15
10
5
0
0
10
20
30
Concentration of periostin (ng/mg)
D
E-NP
NE-NP
Concentration of IFN-γ (pg/mg)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Concentration of periostin (ng/mg)
E
E-NP
NE-NP
Total IgE in homogenate (KU/L)
50
40
30
20
10
0
0
10
20
30
Concentration of periostin (ng/mg)


virus
allergens
virus
allergens
Integrin alpha V
TSLP
Periostin
Allergens
IgE
TSLPR
Mast cells
IL-5
Eosinophil
IL-5
Th2
Dendritic cells
Th2 cytokine
IL-4, IL-13