

# Epithelium-derived cystatin SN enhances eosinophil activation and infiltration through IL-5 in patients with chronic rhinosinusitis with nasal polyps



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**Background:** The interaction between epithelial cells and immune cells plays an important role in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP); however, the mechanism or mechanisms underlying T<sub>H</sub>-biased inflammation in this process are largely unknown. Profiling protein expression in patients with CRSwNP by using shotgun proteomics suggested that cystatin SN (CST1), a type 2 cysteine protease inhibitor, might play a role because this was expressed with the greatest difference in patients with eosinophilic chronic rhinosinusitis with nasal polyps (ECRSwNP) and those with noneosinophilic chronic rhinosinusitis with nasal polyps (nonECRSwNP).

**Objectives:** We sought to investigate the expression and role of CST1 in modulating eosinophilic inflammation in patients with CRSwNP.

**Methods:** Sinonasal tissues were collected from 192 patients with ECRSwNP, 52 patients with nonECRSwNP, and 40 control

subjects. CST1 mRNA expression, localization, and concentration in the tissues were measured by using real-time PCR, *in situ* hybridization, immunohistochemistry, and an ELISA. Recombinant CST1 was used to further explore the function of the molecule in dispersed nasal polyp cells and eosinophils extracted from polyp tissues and peripheral blood. **Results:** CST1 was mainly expressed by epithelial cells and significantly increased in patients with ECRSwNP but decreased in patients with nonECRSwNP compared with that in control subjects. CST1 expression was further increased in patients with ECRSwNP and comorbid asthma and correlated with eosinophil percentages in tissue samples. CST1 was induced by IL-4 and IL-13 in tissue from both patients with ECRSwNP and those with nonECRSwNP and repressed by IL-17A in patients with nonECRSwNP in the presence of neutrophils. CST1 enhanced eosinophil activation and recruitment through induction of IL-5. **Conclusion:** Epithelium-derived CST1 modulates eosinophil activation and recruitment, expression of which could be regulated by T<sub>H</sub>2 and T<sub>H</sub>17 cytokines. (*J Allergy Clin Immunol* 2019;144:455-69.)

**Key words:** Chronic rhinosinusitis with nasal polyps, cystatin SN, eosinophil activation, eosinophil recruitment, epithelial cells, IL-5

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Chronic rhinosinusitis (CRS) is an inflammatory disease with high prevalence worldwide.<sup>1</sup> CRS can be divided into CRS without nasal polyps and chronic rhinosinusitis with nasal polyps (CRSwNP), with the latter type accounting for approximately 20% to 25% of patients with CRS, and is associated with greater recurrence after surgical or pharmaceutical treatment.<sup>2</sup> CRSwNP can be further classified into 2 distinct immunohistologic subtypes, eosinophilic chronic rhinosinusitis with nasal polyps (ECRSwNP) and noneosinophilic chronic rhinosinusitis with nasal polyps (nonECRSwNP), based on observed eosinophil infiltration.<sup>3</sup> The 2 subtypes are also characterized by differences in T<sub>H</sub> type inflammation.<sup>1,4-7</sup> ECRSwNP is pronounced as T<sub>H</sub>2 skewed and with relatively high recurrence and asthma comorbidity rate, whereas nonECRSwNP is characterized by a T<sub>H</sub>1 or T<sub>H</sub>17 milieu and a lower recurrence and asthma comorbidity rate.<sup>4,5,8,9</sup> The 2 subtypes also exhibit significant differences in terms of geographic and racial distribution. In particular, studies have demonstrated that although ECRSwNP is clinically more predominant in patients with CRSwNP in Europe and the United States, nonECRSwNP is more common in Asian patients.<sup>1</sup>

**Abbreviations used**

|             |                                                          |
|-------------|----------------------------------------------------------|
| CRS:        | Chronic rhinosinusitis                                   |
| CRSwNP:     | Chronic rhinosinusitis with nasal polyps                 |
| CST1:       | Cystatin SN                                              |
| CST2:       | Cystatin SA                                              |
| CST3:       | Cystatin C                                               |
| CSTA:       | Cystatin A                                               |
| CSTB:       | Cystatin B                                               |
| CSTF:       | Cystatin F                                               |
| DNPC:       | Dispersed nasal polyp cell                               |
| ECP:        | Eosinophil cationic protein                              |
| ECRSwNP:    | Eosinophilic chronic rhinosinusitis with nasal polyps    |
| EPX:        | Eosinophil peroxidase                                    |
| FENO:       | Fraction of exhaled nitric oxide                         |
| GAPDH:      | Glyceraldehyde-3-phosphate dehydrogenase                 |
| HNEC:       | Human nasal epithelial cell                              |
| nonECRSwNP: | Noneosinophilic chronic rhinosinusitis with nasal polyps |
| RNA-seq:    | RNA sequencing                                           |

Some studies have suggested that epithelial cells can also play a role in the pathogenesis of CRSwNP.<sup>10,11</sup> Although different T<sub>H</sub>-biased inflammatory pathways have been well established in patients with CRSwNP, relatively little is known about the influence of different T<sub>H</sub> status on expression of distinct epithelial pathways or mediator or mediators responsible for the crosstalk between the epithelium and immune cells in patients with the ECRSwNP and nonECRSwNP inflammatory subtypes. In this regard preliminary studies involving assessment of samples from patients receiving a diagnosis of ECRSwNP or non-ECRSwNP and healthy control subjects subjected to shotgun proteomics, followed by selection of specific proteins using the COMPARTMENTS and UniProt databases<sup>12,13</sup> and analysis of mRNA levels of each protein, indicated that the difference in expression of cystatin SN (CST1) was the most significant between patients with ECRSwNP and those with nonECRSwNP.

CST1 is a member of the type 2 cystatin proteins superfamily. Recent studies have proposed that the members of the cystatin superfamily can be involved in a number of immunologic processes.<sup>14–17</sup> However, the function of CST1 in the immunologic process is not fully understood. CST1 could inhibit the catalytic activity of clan C1 members, such as cathepsins B, H, L, and S.<sup>17</sup> It is reported that CST1 levels are increased in patients with childhood respiratory allergy and seasonal allergic rhinitis.<sup>18,19</sup> In patients with the latter disease, CST1 shows inhibition of histamine release. However, to our knowledge, very few studies have explored the function of CST1 in patients with CRSwNP. Therefore the aim of the present study was to investigate CST1 expression and its role in patients with CRSwNP.

**METHODS****Subjects and specimens**

A total of 284 subjects, including 192 patients with ECRSwNP, 52 patients with nonECRSwNP, and 40 healthy control subjects, were enrolled in the study. The diagnosis of CRSwNP was based on standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines.<sup>20</sup> The definition of ECRSwNP and nonECRSwNP was in accordance with the previously reported criteria.<sup>4</sup> Patients with immunodeficiency, coagulation disorder, fungal sinusitis, or cystic fibrosis and pregnant women did not participate in the study. None of the enrolled subjects had a history of aspirin

sensitivity. Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects.

Polyp tissues from patients with CRSwNP and inferior turbinate or uncinate mucosal tissues from control subjects were collected as biopsy specimens or during surgery. Detailed information on the subjects' characteristics is provided in the Methods section, Fig E1, and Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

This study was approved by the Ethics Committee of Beijing TongRen Hospital, Capital Medical University, and written informed consent was obtained from each patient before enrollment.

**Clinical assessment**

The diagnosis of asthma, allergic rhinitis, or atopy; measurements of routine hematologic parameters; and measurements of lower airway fraction of exhaled nitric oxide (FENO), a biomarker of eosinophilic inflammation, were made, as previously described.<sup>9</sup> Detailed information on these procedures is provided in the Methods section in this article's Online Repository.

**Hematoxylin and eosin staining and immunohistochemistry**

Hematoxylin and eosin and immunohistochemistry staining were performed, as previously described,<sup>7,21</sup> to determine the inflammation type and the location of CST1 in the tissue. Detailed information is provided in the Methods section and Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

**Assessment of protein profiles of tissues from patients with ECRSwNP, patients with nonECRSwNP, and control subjects by using shotgun proteomics and secreted protein selection**

Total proteins from 10 pooled samples each from patients with ECRSwNP, patients with nonECRSwNP, and control subjects were isolated and processed further for quality control. A minimum of 2 unique and nonredundant peptides per protein was used as the criterion for positive protein identification. Detailed information of these procedures is provided in the Methods section in this article's Online Repository.

Differential proteins were subjected to the COMPARTMENTS (<https://compartments.jensenlab.org>) and UniProt ([www.uniprot.org](http://www.uniprot.org)) databases for subcellular localization and secreted protein acquirement.<sup>12,13</sup> Detailed information on these procedures is provided in the Methods section in this article's Online Repository.

**RNA isolation, reverse transcription, and real-time PCR**

Total RNA from tissue samples and cultured cells were extracted with an RNA extraction kit (TaKaRa Biotechnology, Dalian, China). The quality of total RNA was assessed with the Nanodrop-2000 (Thermo Fisher Scientific, Waltham, Mass), and single-stranded cDNA was synthesized with the Prime-Script RT Master Mix (TaKaRa Biotechnology). Real-time PCR was then performed by using a SYBR Green method (TaKaRa Biotechnology) to assess mRNA levels of CST1 or mRNA coding eosinophilic granule proteins. Detailed information of the protocols and primer sequence are provided in the Methods section and Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

**Assessment of CST1 and eosinophilic proteins by using ELISA**

The concentration of CST1 in tissue samples or epithelial cells was measured by using a commercially available ELISA kit (Sigma-Aldrich, St Louis, Mo). Similarly, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) levels released from eosinophils in supernatant were determined by using specific ELISA kits obtained from Cusabio Life Sciences (Wuhan, China). Detailed information on these procedures is provided in the Methods section and Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Human nasal epithelial cell culture

Human nasal epithelial cells (HNECs) were prepared from nasal polyps or healthy mucosa after enzymatic digestion of tissue, and purified HNECs were cultured by using an air-liquid interface method.<sup>22</sup> Cell cultures were treated with several mediators, including cathepsins B and C, IL-3, IL-4, IL-5, CXCL8, IL-13, IL-10, IL-17A, GM-CSF, and IFN- $\gamma$ , for 24 hours and then harvested for assessment of CST1 mRNA by using quantitative RT-PCR, as described above. Detailed information on these procedures is provided in the Methods section in this article's Online Repository.

## Assessment of cytokines using the Luminex system

Concentrations of cytokines, including IL-5, IL-17A, IFN- $\gamma$ , GM-CSF, CXCL8, and TNF- $\alpha$  in tissue samples and IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1 $\beta$ , IFN- $\gamma$ , CCL5, CCL11, CCL24, CCL26, and GM-CSF in cultured cells, were determined by using the Luminex assay (Luminex, Austin, Tex). Specific details of the cytokines analyzed are provided in the Methods section and Table E4 in this article's Online Repository.

## Assessment of the CST1 mRNA transcriptional site by using *in situ* hybridization

The CST1 mRNA transcriptional site in tissue sections of samples from patients with ECRSwNP was analyzed by using *in situ* hybridization. Detailed information on the procedure is provided in the Methods section in this article's Online Repository.

## Eosinophil isolation and culture

Peripheral blood eosinophils were purified by using an eosinophil isolation kit (Miltenyi Biotec, San Diego, Calif). Eosinophil purity was assayed by using flow cytometry. Isolated eosinophils were cultured in RPMI 1640 medium (Thermo Fisher Scientific), supplemented with 10% FBS (Thermo Fisher Scientific), and stimulated with recombinant CST1, IL-5, GM-CSF (R&D Systems, Minneapolis, Minn), or E64d (Cayman Chemical, Ann Arbor, Mich) for 24 hours. At the end of this incubation, eosinophils were harvested and investigated further by using quantitative RT-PCR for expression of ECP, EPX, and PU.1 mRNAs; by using ELISA for the concentration of ECP and EPX in the supernatants; by using a Luminex assay for synthesis of IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1 $\beta$ , IFN- $\gamma$ , CCL5, CCL11, CCL24, CCL26, and GM-CSF; and by using flow cytometry for CD69 expression. Proliferation was determined up to 48 hours. Detailed information on this protocol and procedures is provided in the Methods section in this article's Online Repository.

## Flow cytometry

WBCs were stained for allophycocyanin-conjugated CST1 antibody under baseline and IL-4 stimulation. Isolated eosinophils were stained for Alexa Fluor 700-conjugated Siglec-8 for purity detection or fluorescein isothiocyanate-conjugated CD69 antibody for activation after treatment with recombinant CST1 (R&D Systems) or E64d (Cayman Chemical). Then the cells were subjected to flow cytometry. Detailed information on the procedures used is provided in the Methods section in this article's Online Repository, and the antibodies used for flow cytometry are shown in Table E2.

## Dispersed nasal polyp cell culture

Dispersed nasal polyp cells (DNPCs) were cultured and treated with recombinant CST1 (R&D Systems) and E64d (Cayman Chemical) for 24 hours. At the end of this incubation, cells and cell-free supernatants were harvested for further determination of mRNA levels of ECP, EPX, and PU.1 and for cytokine detection. Detailed information on these procedures is provided in the Methods section in this article's Online Repository.

## Eosinophil migration assay

Eosinophils isolated from peripheral blood of subjects were added into the upper compartments of transwells at a  $5 \times 10^5$  eosinophils in 200  $\mu$ L of RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific). The lower chambers were added with the same medium containing 100 ng/mL recombinant CST1 or 10  $\mu$ mol/L E64d. The cells were incubated at 37°C and 5% CO<sub>2</sub> for 1 to 4 hours. At the end of incubation, the medium in the lower chambers was collected and assessed for the number of eosinophils present. Detailed information on the assay is provided in the Methods section in this article's Online Repository.

## Eosinophil recruitment

Polyp tissues were rinsed in PBS containing 1% penicillin, 1% streptomycin, and 1% amphotericin and then weighed and cut into smaller sections of about 0.1 g in weight. Ten microliters of 10  $\mu$ g/mL recombinant CST1, 10  $\mu$ g/mL recombinant IL-5, 10  $\mu$ g/mL recombinant GM-CSF, 10  $\mu$ g/mL recombinant CCL24, 1 mmol/L E64d, or PBS were injected separately or together with 10  $\mu$ L of 10  $\mu$ g/mL IL-5 blocking antibody into each section and cocultured with WBCs ( $10^6$  cells in each well) for 96 hours for assessment of eosinophils infiltrating the tissue. Detailed information on the assay is provided in the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Statistical analysis

All statistical analyses were performed with SPSS software (version 23.0; IBM, Armonk, NY). All data are presented as means  $\pm$  SEMs, unless otherwise noted. Differences between groups were analyzed by using Kruskal-Wallis ANOVA. The Pearson  $\chi^2$  or Fisher exact test was applied to sets of categorical data. Correlations were assessed by using Spearman correlation and defined as strong when the *R* value was greater than 0.7, moderate when the *R* value was in the range of 0.5 to 0.69, or weak when the *R* value was in the range of 0.3 to 0.49. A paired *t* test was used to test the significance of cell-culture data. Samples before and after glucocorticoid treatment were compared by using a paired *t* test. Cells treated with recombinant CST1, E64d, and/or IL-5 neutralizing antibody were analyzed by using 2-way ANOVA. A *P* value of less than .05 was considered statistically significant. All significance levels presented are 2-tailed.

## RESULTS

### Protein profiles and screening of secreted proteins in nasal polyp tissue

Polyp tissues from patients with ECRSwNP and patients with nonECRSwNP and mucosa from healthy control subjects subjected to shotgun proteomics demonstrated a total of 1022, 872, and 944 proteins, respectively. To characterize inflammatory types present among these groups, IL-5, IL-17A, IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , and CXCL8 (IL-8) were assessed in appreciable quantities. Levels of IL-5, GM-CSF, and IFN- $\gamma$  were significantly greater in tissue from patients with ECRSwNP compared with control tissue, whereas levels of CXCL8, IL-17A, and IFN- $\gamma$  were significantly greater in tissue from patients with nonECRSwNP compared with tissue from control subjects (see Fig E1).

Assessment of secreted proteins in nasal polyp tissues by using the COMPARTMENTS and UniProt databases followed by RT-PCR to assess the genes encoding secreted proteins present exclusively in one group or with over 5-folds difference in between-group comparison demonstrated that CST1 was expressed, with the greatest difference between patients with ECRSwNP and those with nonECRSwNP.

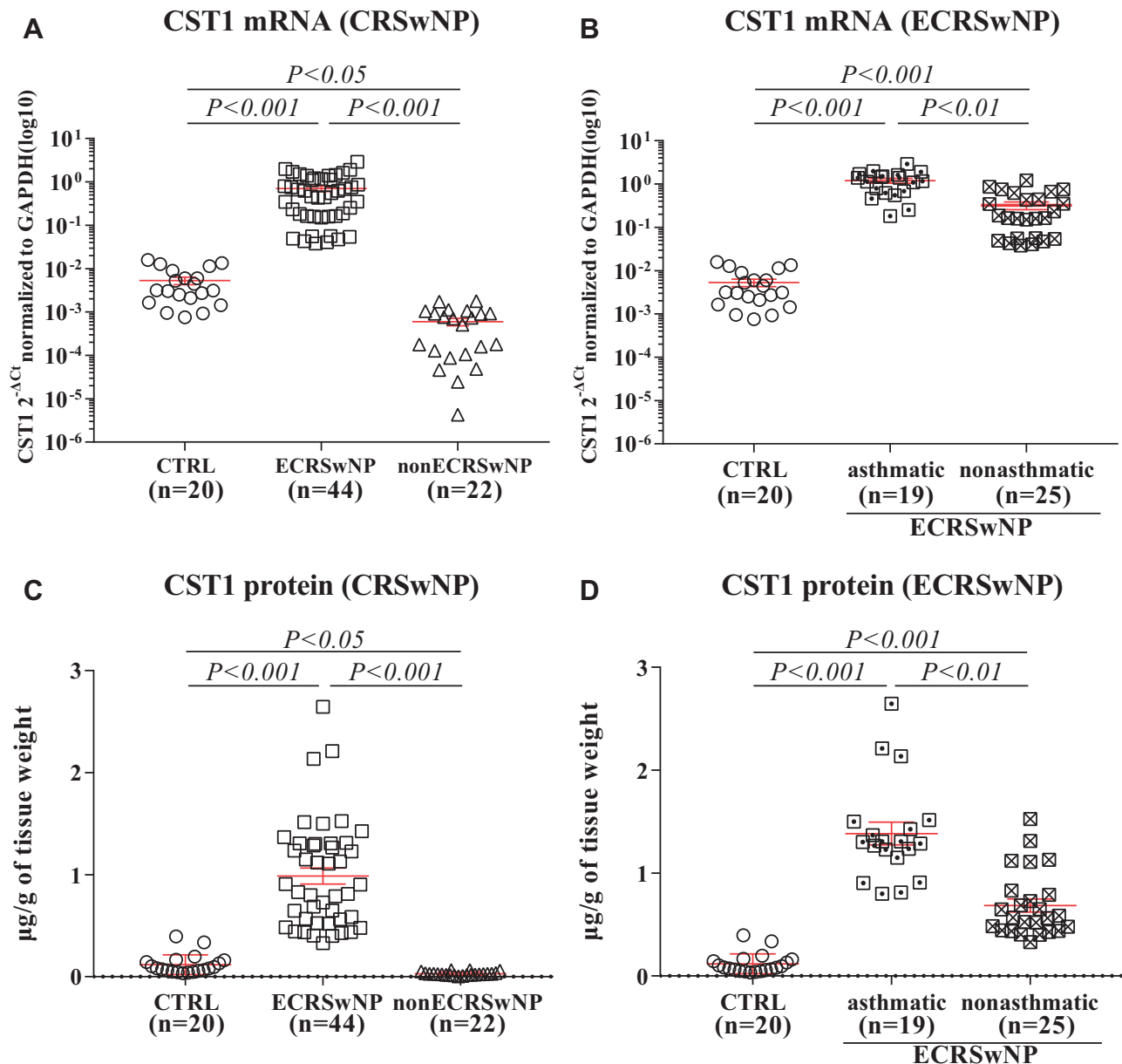


**FIG 1.** CST1 expression in nasal tissue from patients with CRSwNP. **A**, CST1 mRNA levels in tissues from control subjects, patients with ECRSwNP, and patients with nonECRSwNP. **B**, CST1 mRNA levels in tissues from control subjects and patients with ECRSwNP with or without asthma. **C**, CST1 protein levels in tissues from control subjects, patients with ECRSwNP, and patients with nonECRSwNP. **D**, CST1 protein levels in tissues from control subjects and patients with ECRSwNP with or without asthma.

### CST1 expression in patients with CRSwNP

Assessment of CST1 expression relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), indicated that CST1 mRNA levels were significantly increased in tissues from patients with ECRSwNP compared with those from patients with nonECRSwNP ( $P < .001$ ) or control subjects ( $P < .001$ ; Fig 1, A). Furthermore, CST1 mRNA levels were significantly decreased in tissue from patients with non-ECRSwNP compared with those in control subjects ( $P < .05$ ; Fig 1, A). We further subclassified patients with ECRSwNP into asthmatic and nonasthmatic groups and found that the asthmatic patients demonstrated even greater levels of CST1 mRNA compared with the nonasthmatic subjects ( $P < .01$ ; Fig 1, B). Measurement of the concentration of CST1 protein by using ELISA

demonstrated that CST1 levels were significantly increased in patients with ECRSwNP ( $P < .001$ ) and decreased in patients with nonECRSwNP ( $P < .05$ ) compared with control subjects (Fig 1, C). Similarly, CST1 levels were significantly increased in asthmatic patients with ECRSwNP compared with nonasthmatic patients with ECRSwNP ( $P < .01$ ; Fig 1, D), thus confirming the findings for increased expression of CST1 mRNA in patients with ECRSwNP.

### CST1 is mainly expressed in nasal epithelial cells

Immunohistochemical staining of tissues from the 3 groups of subjects indicated that CST1 was localized mainly to the epithelium (Fig 2, A). Furthermore, semiquantitative analysis of

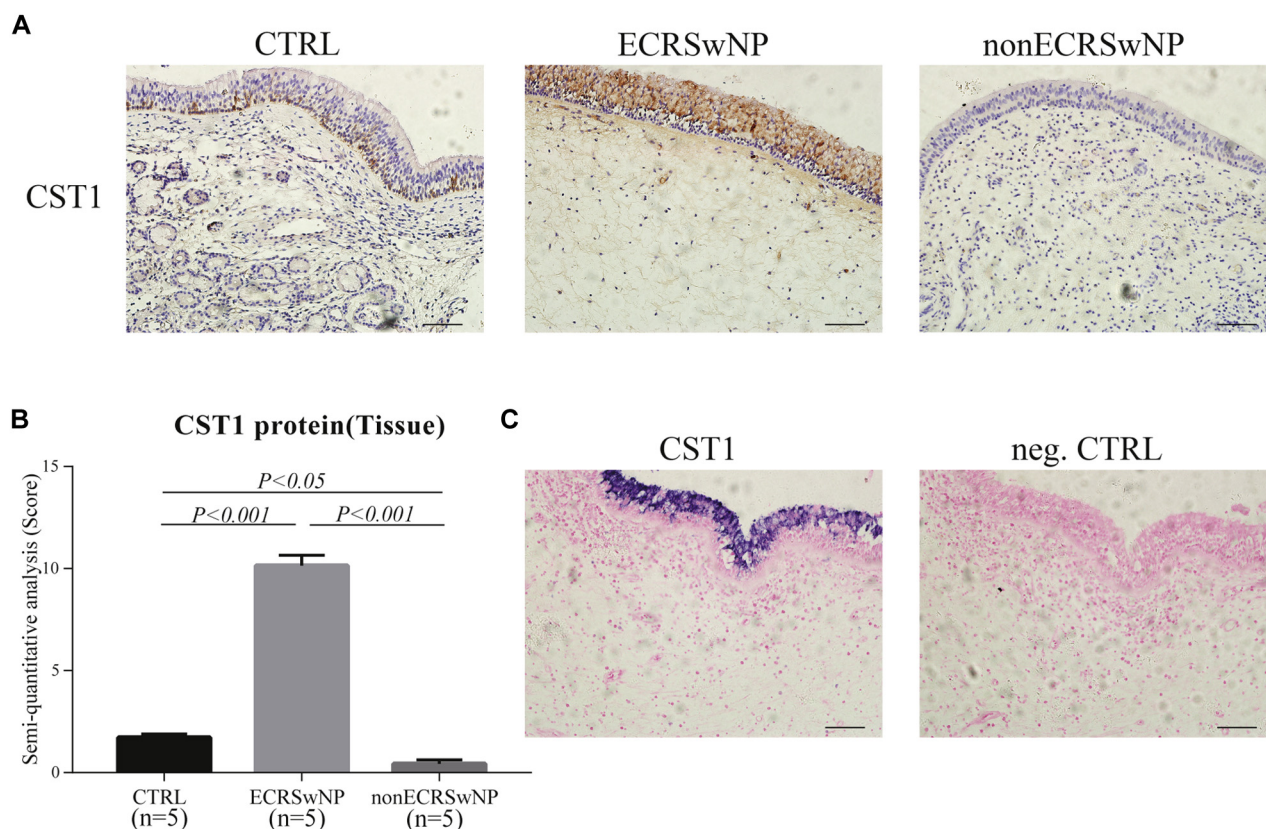


**FIG 2.** Localization of CST1 in nasal tissue. **A**, Representative figures showing CST1 staining of nasal mucosa or polyps tissue from control subjects, patients with ECRSwNP, and patients with nonECRSwNP. Bars = 20  $\mu$ m ( $\times 400$  magnification). **B**, Semi-quantitative analysis of stained epithelial cells in control subjects, patients with ECRSwNP, and patients with nonECRSwNP (for each group,  $n = 5$ ). **C**, *In situ* hybridization of CST1 mRNA in tissue from patients with ECRSwNP. Purple color represents positive expression of CST1. Bars = 20  $\mu$ m.

the staining indicated that consistent with the results obtained by using ELISA, CST1 levels were significantly increased in patients with ECRSwNP ( $P < .001$ ) and decreased in patients with non-ECRSwNP ( $P < .05$ ) compared with those in control subjects (Fig 2, B). *In situ* hybridization for determination of the CST1 mRNA transcriptional site further confirmed epithelial cells to be the major site in this regard (Fig 2, C).

Flow cytometric assessment to detect potential expression of CST1 in hematopoietic cells indicated that very low levels of CST1 were expressed in these cells, both at baseline and after stimulation with IL-4 (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Correlation of clinical parameters with CST1 mRNA and protein levels

For patients with ECRSwNP, both tissue CST1 mRNA and protein levels were significantly but weakly correlated positively with percentages of eosinophils infiltrating the tissues ( $P = .003$ ,  $R = 0.443$  for mRNA and  $P = .026$ ,  $R = 0.335$  for protein) and FENO values ( $P = .013$ ,  $R = 0.411$  for mRNA and  $P = .005$ ,  $R = .461$  for protein; Fig 3). In contrast, no significant correlation was observed between CST1 mRNA or protein levels and

percentages of peripheral blood eosinophils ( $P = .382$  for mRNA and  $P = .068$  for protein).

### Regulation of CST1 in patients with CRSwNP

We explored factors to regulate the production of CST1. Because cathepsin family members act as the substrate for CST1, we assessed expression of these proteins by using data mining of microarrays, RNA sequencing (RNA-seq), and our proteomic data and found that 2 members of the family, cathepsins B and C, were upregulated in patients with ECRSwNP (see Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Thus we further assessed the function of cathepsins B and C in CST1 expression. In this regard the recombinant cathepsins B and C were not able to induce CST1 expression in epithelial cells (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Next, typical  $T_H$  cytokines, which affect CST1 expression, were assessed, and in accordance with previous reports,<sup>18,19</sup> we found that IL-4 induced CST1 expression (Fig 4). IL-13 also induced CST1 production, although not to the same level as IL-4. In contrast, IL-17A significantly decreased CST1 expression in polyps infiltrated with neutrophils but not in polyps from patients with ECRSwNP (Fig 4). However, IL-3, IL-5, IL-8, IL-

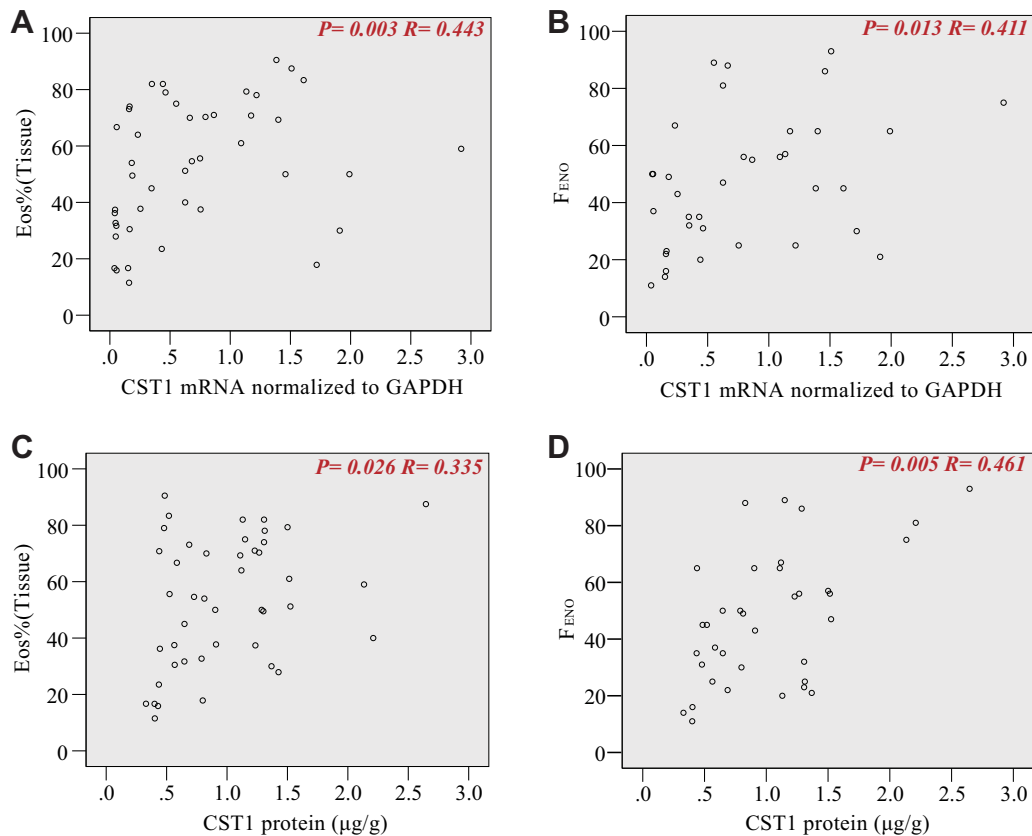


**FIG 3.** Correlation of CST1 expression with the clinical index in patients with ECRSwNP. **A** and **B**, Correlation of CST1 mRNA levels with tissue eosinophil percentages and FENO levels, respectively. **C** and **D**, Correlation of CST1 protein levels with tissue eosinophil percentages and FENO levels, respectively.

10, GM-CSF, and IFN- $\gamma$  had little effect on CST1 expression (see Fig E3).

### CST1 activates eosinophils

The positive correlation noted in polyp tissue for CST1 expression and eosinophil percentages suggested that CST1 can affect eosinophils. In this regard incubation of DNPCs for 24 hours with recombinant CST1 or E64d, a chemical cysteine protease inhibitor that can mimic the activity of cystatins,<sup>23</sup> demonstrated that both these treatments significantly increased mRNA levels of EPX and ECP (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although EPX and ECP proteins were also detected in medium, levels of these proteins were lower than the limit of detection in several samples, and thus these data were not available. However, incubation of eosinophils isolated from peripheral blood of patients with ECRSwNP with recombinant CST1 or E64d demonstrated that, consistent with the results from studies using DNPCs, levels of mRNAs for EPX and ECP were significantly increased after recombinant CST1 or E64d treatment for 24 hours in comparison with control medium (mock; Fig 5, A). Furthermore, levels of EPX and ECP proteins were also significantly increased after recombinant CST1 treatment for 24 hours (Fig 5, B). Similarly, assessment of expression of CD69, a protein with increased levels during eosinophil activation that is necessary for degranulation, on eosinophils demonstrated that both recombinant CST1 and E64d significantly enhanced the number of CD69<sup>+</sup> eosinophils (Fig 5, C). The purity

of the blood eosinophil preparation was assessed by using flow cytometry and found to be 93.4% (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Eosinophils isolated from several patients were also treated with recombinant IL-5 or GM-CSF as a positive control (see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Because activated eosinophils secrete granule contents through a mechanism termed piecemeal degranulation, which ensures that the cells do not die after degranulation and can produce more granules,<sup>24</sup> we assessed the mechanism by which CST1 can promote the synthesis of eosinophils granule (ie, by enhancing the proliferation of eosinophils or by stimulating synthesis). Assessment of numbers of cultured eosinophils incubated with recombinant CST1 and E64d demonstrated that eosinophil numbers were not significantly greater after 48 hours compared with those in control subjects (Fig 5, D), suggesting that CST1 can enhance granule synthesis of "resident eosinophils." Because PU.1, a transcriptional factor, has been reported to be crucial for the synthesis of eosinophil granule proteins, such as ECP and EPX,<sup>25</sup> we also assessed the effect of recombinant CST1 and E64d on PU.1 expression and demonstrated that this was also significantly increased by CST1 and E64d compared with control medium (Fig 5, A).

### CST1 promotes eosinophil recruitment

Assessment of the effect of CST1 on eosinophil recruitment using transwells with eosinophils treated with recombinant CST1 or E64d for up to 4 hours demonstrated no significant increase in

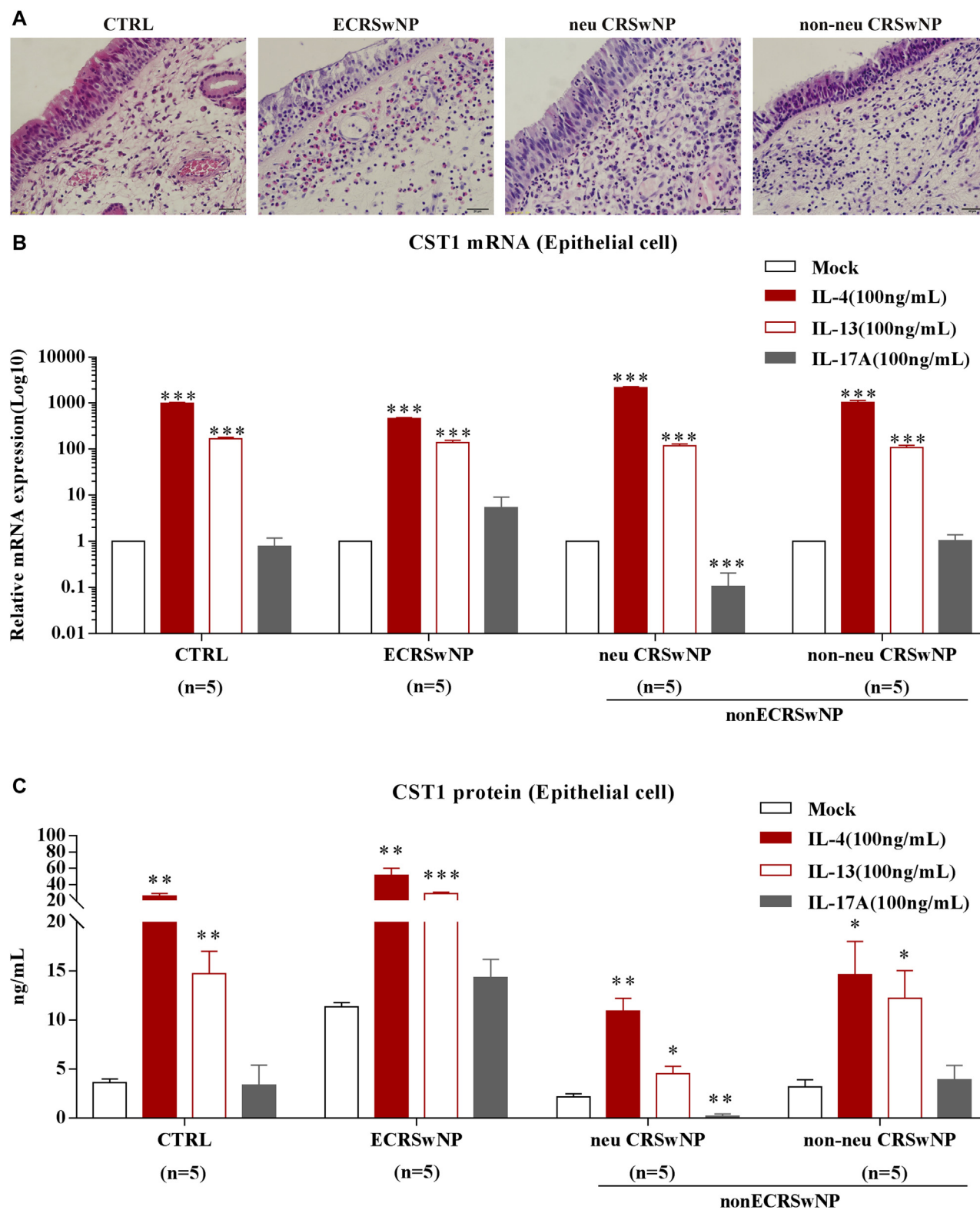


**FIG 4.** Regulation of CST1. **A**, Representative figures showing hematoxylin and eosin staining for each type of CRSwNP. Bars = 20  $\mu$ m ( $\times 400$  magnification). **B** and **C**, mRNA (Fig 4, B) and protein (Fig 4, C) levels of CST1 measured in epithelial cells cultured from nasal mucosa from control subjects or polyp tissues stimulated with IL-4, IL-13, and IL-17A. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

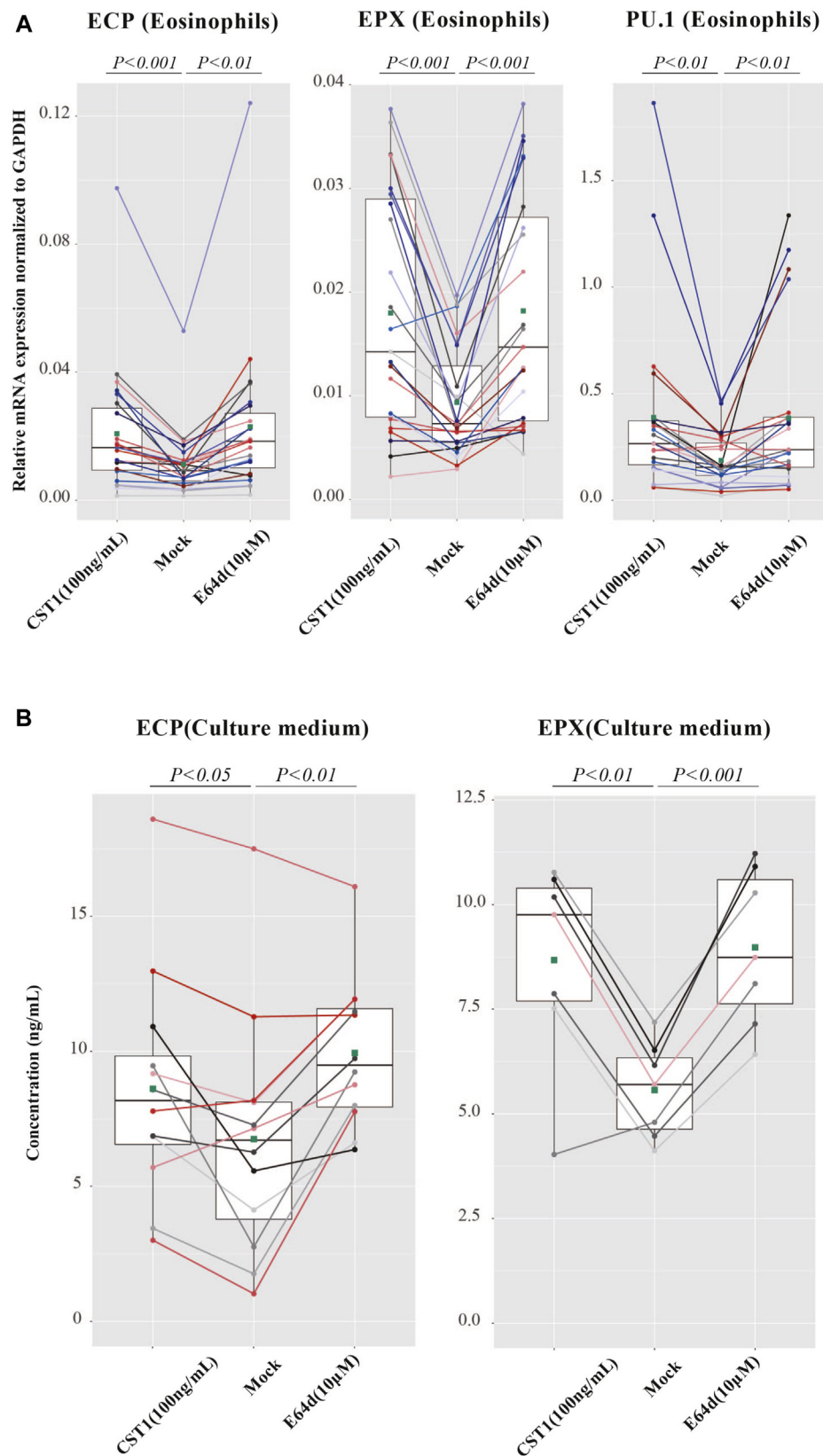


**FIG 5.** Effect of CST1 on activation of eosinophils isolated from peripheral blood of patients with ECRSwnP. Isolated eosinophil cultures were treated with 100 ng/mL recombinant CST1 or 10  $\mu$ M E64d for 24 (**A-C**) or 48 (**D**) hours and then assessed for relative expression of ECP, EPX, and PU.1 mRNA in eosinophils (Fig 5, **A**), concentrations of ECP and EPX released in culture medium (Fig 5, **B**), CD69 expression in eosinophils (Fig 5, **C**), and eosinophil proliferation (Fig 5, **D**). For Fig 5, **A** and **B**, samples from the same subject are shown in the same color, and mean values for each group are shown as green squares. FITC, Fluorescein isothiocyanate.

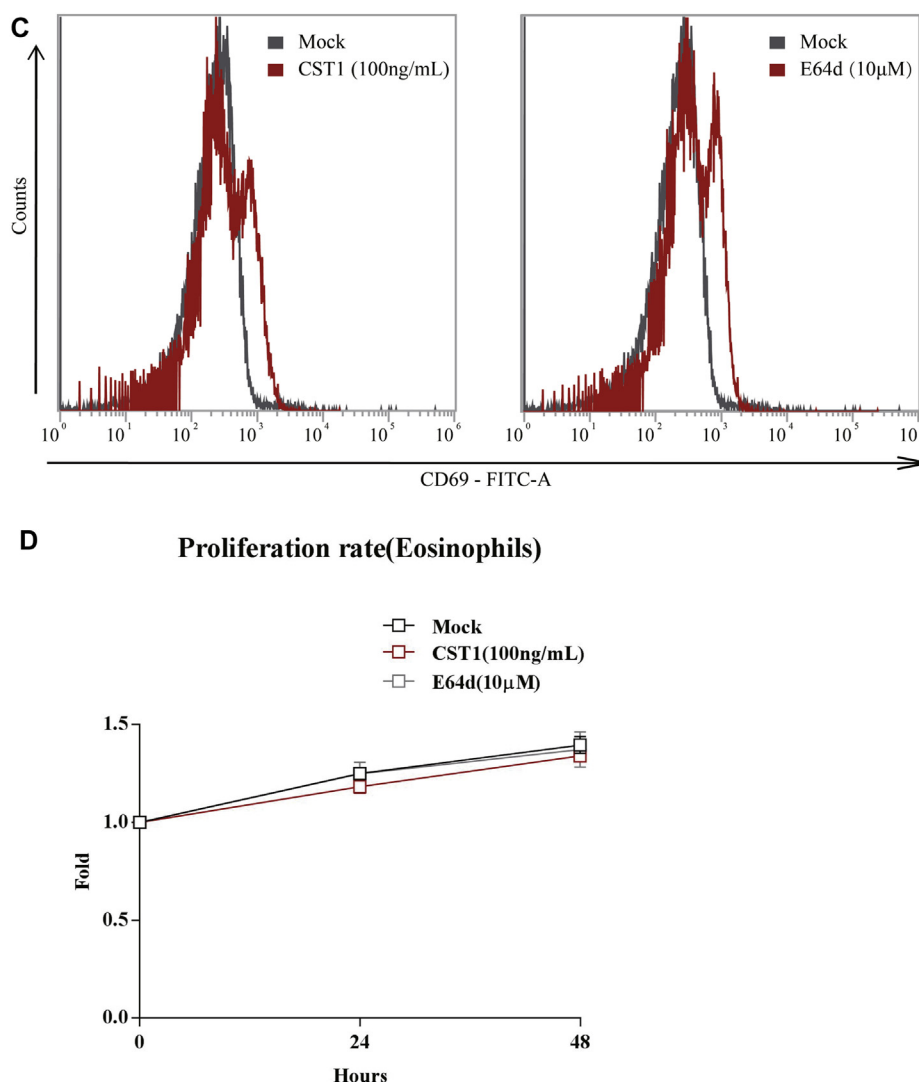


FIG 5. (Continued).

numbers of migrating eosinophils compared with control values (see Fig E7 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). However, polyps injected with recombinant CST1 or E64d and cultured with WBCs of the same subject from whom the polyps were obtained demonstrated that after 96 hours, large numbers of eosinophils were present in tissue sections treated with recombinant CST1 and E64d compared with PBS (Fig 6 and see Figs E8 and E9 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Injection of recombinant IL-5, GM-CSF, and CCL24 (eotaxin-2) as positive controls also demonstrated significantly increased numbers of eosinophils compared with PBS and thus confirmed the effects of CST1, as well as the validity of the model in assessing the efficacy of specific mediators in the recruitment of eosinophils (see Fig E8). This finding suggests that recruitment of eosinophils by CST1 is unlikely to be due to ligand or receptor binding on the eosinophils but is probably mediated through cytokines or molecules.

### CST1 enhances IL-5 expression

Because previous studies have indicated that CST1 mainly plays a role in eosinophilic inflammation through some mediators, to illustrate the whole vision of the inflammation bias, we assessed the effect of CST1 on induction or inhibition of a variety of  $T_H$  cytokines, including IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1 $\beta$ , and IFN- $\gamma$ . Incubation of DNPCs from tissues and eosinophils of patients with ECRSwNP isolated from patients with ECRSwNP with both recombinant CST1 and E64d for 24 hours significantly upregulated expression of IL-5 in both DNPCs and eosinophils (Fig 7). IL-17A and IFN- $\gamma$  were not detectable in the eosinophils by using the Luminex system in the present study.

We further explored the effect of CST1 on concentrations of CCL5, CCL11, CCL24, CCL26, and GM-CSF, which are also known to be effective mediators in recruitment and activation of eosinophils. Our study demonstrated that incubation of eosinophils isolated from patients with ECRSwNP with recombinant

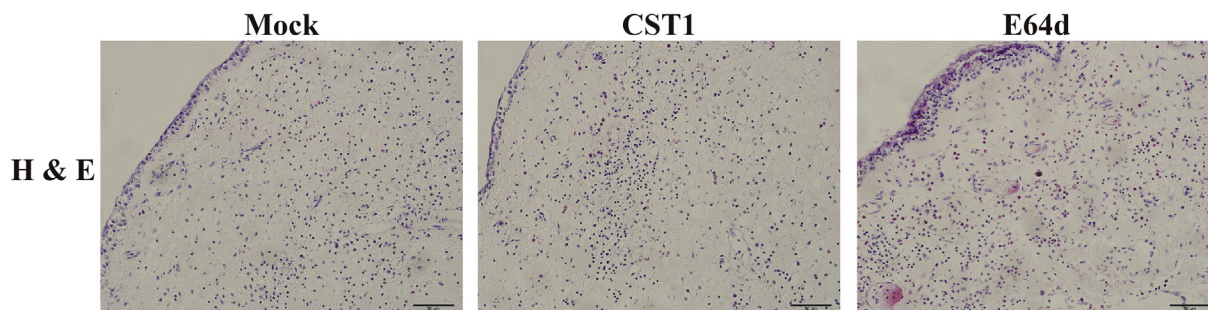


**FIG 6.** CST1 promotes eosinophil recruitment. Nasal polyp sections were injected with 10  $\mu$ g/mL recombinant CST1 or 1 mmol/L E64d in the volume of 10  $\mu$ L per 0.1 g of tissue and then incubated with WBCs isolated from the subject from whom the polyp tissue was obtained. After incubation for 96 hours, polyp sections were collected assessed for the number of eosinophils present after hematoxylin and eosin (H&E) staining. Bars = 50  $\mu$ m ( $\times 200$  magnification).

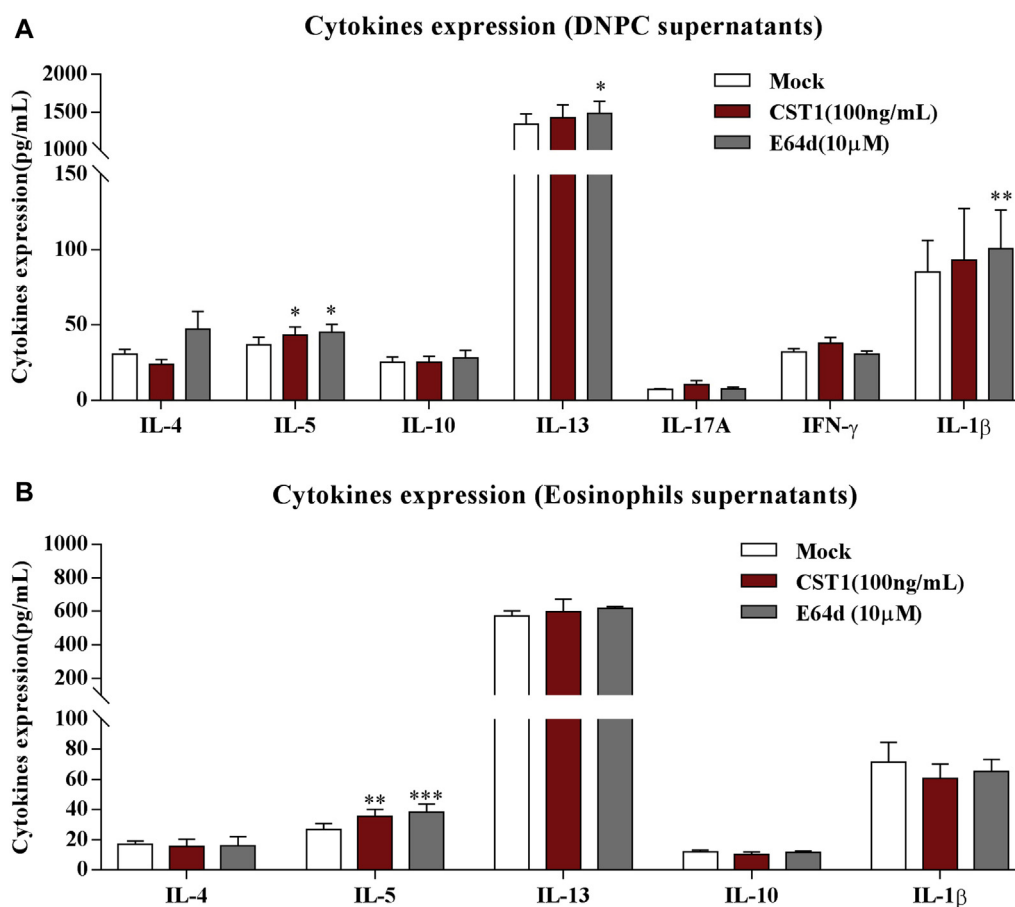


**FIG 7.** Effect of recombinant CST1 and E64d on cytokine expression in nasal polyps and eosinophils. DNPCs (A) and cultured eosinophils (B) were treated with 100 ng/mL recombinant CST1 or 10  $\mu$ mol/L E64d for 24 hours, and cell-free supernatants were collected for assessment of cytokines by using Luminex. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

CST1 did not significantly alter the concentrations of CCL5, CCL11, CCL24, and CCL26 released from eosinophils, whereas the concentration of GM-CSF was less than the detection limit for the assay (see Fig E10 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### IL-5 is involved in patients with CST1-induced eosinophilic inflammation

Eosinophils were pretreated with medium containing 10  $\mu$ g/mL IL-5 blocking antibody (R&D Systems) or medium alone for 1 hour and then incubated for a further 24 hours with recombinant

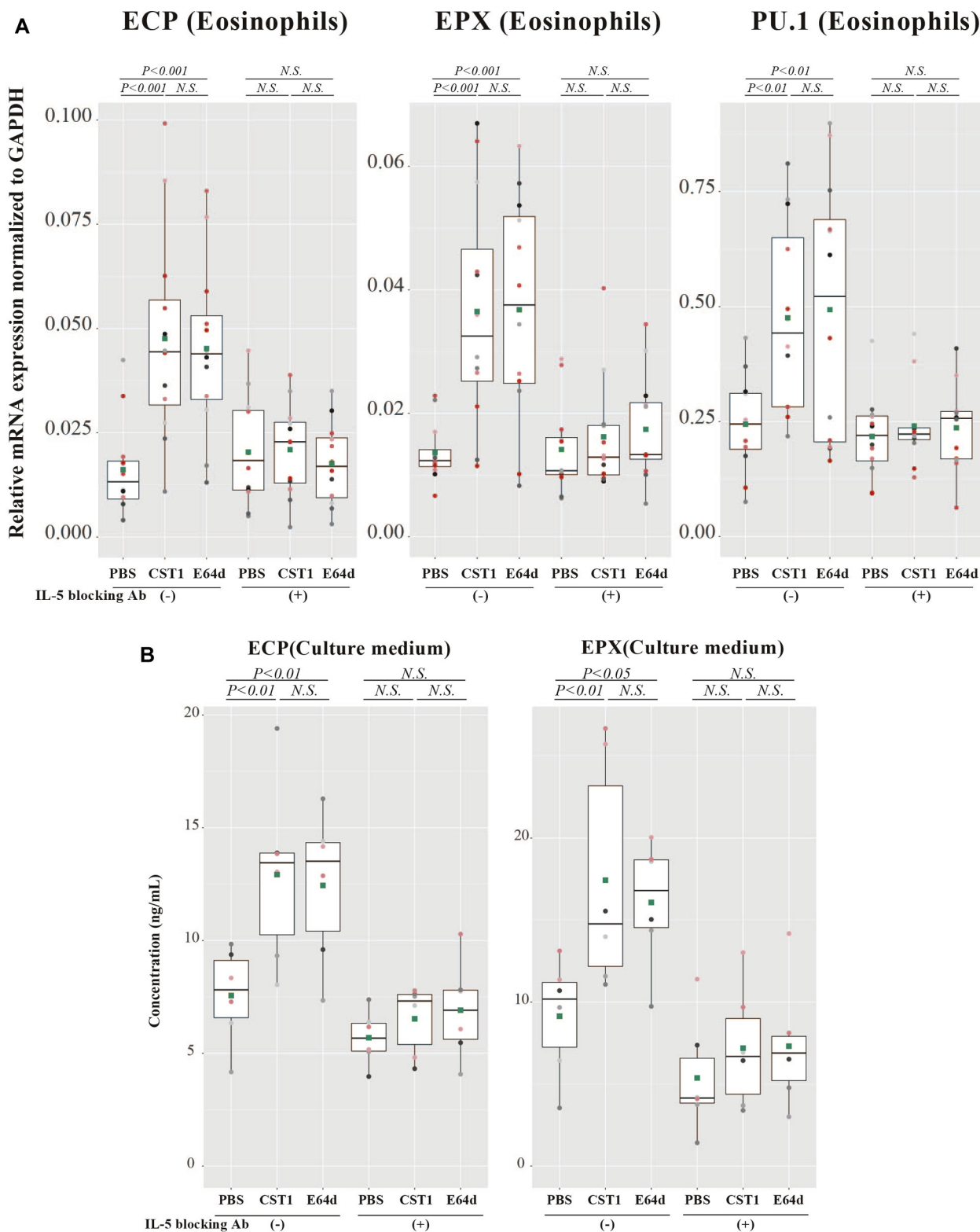


**FIG 8.** Effect of IL-5 blocking antibody (10  $\mu$ g/mL) on CST1- or E64d-induced relative mRNA expression of ECP, EPX, and PU.1 (**A**); ECP and EPX protein expression (**B**); and eosinophil recruitment (**C** and **D**). For Fig 8, **A** and **B**, the mean value for each group is shown as the green square. Fig 8, **C**, shows representative images for recruitment of eosinophils induced by recombinant CST1 or E64d. Ab, Antibody. Bars = 50  $\mu$ m ( $\times 200$  magnification). Fig 8, **D**, shows average numbers of eosinophils recruited in each group of 5 high-power fields (HPF) at  $\times 400$  magnification. N.S., Not significant.

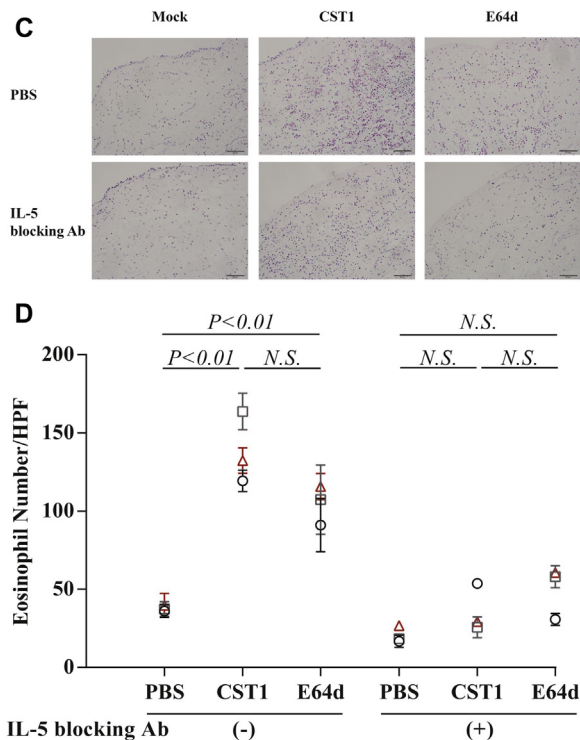


FIG 8. (Continued).

CST1 or E64d to determine the role of IL-5 in the function of CST1. Both CST1 and E64d significantly increased EPX, ECP, and PU.1 mRNA expression (Fig 8, A) and release of EPX and ECP protein in cell-culture media (Fig 8, B) in the absence of IL-5 blocking antibody. However, these CST1- and E64d-induced increases were abolished by IL-5 blocking antibody (Fig 8, A and B, respectively).

Similarly, to determine whether IL-5 blocking antibody could decrease CST1- and E64d-induced infiltration of eosinophils, polyp tissues were injected with PBS, recombinant CST1, or E64d in the absence or presence of IL-5 blocking antibody. Similar to the findings for EPX, ECP, and PU.1, recombinant CST1 or E64d significantly increased eosinophil numbers infiltrating the polyp tissues compared with mock PBS, and this increase in eosinophil counts was significantly attenuated in the presence of IL-5 blocking antibody (Fig 8, C and D).

### Glucocorticoids partially suppress expression of epithelial CST1

We investigated the effect of glucocorticoids on epithelial CST1 mRNA and protein expression. Samples were acquired before and after treatment by means of biopsy and after surgery. Patients underwent a 2-week oral corticosteroid therapy (24 mg of methylprednisolone once daily for 7 days, followed by a 4-mg reduction every other day during the other 7 days).<sup>26,27</sup> This study demonstrated that glucocorticoids significantly suppressed both CST1 mRNA and protein expression compared with baseline values. However, regular doses of glucocorticoids could not attenuate CST1 expression in patients with ECRSwNP to the level of healthy control subjects, and even after treatment, CST1 expression on epithelial cells of patients with ECRSwNP was

significantly higher compared with that in healthy control subjects (Fig 9).

### DISCUSSION

Research into CRSwNP over the last few decades has shown that this is a complex and heterogeneous disease.<sup>1</sup> A recent study has indicated that several clusters of CRSwNP can be identified based on distinct clinical pathobiological features and differences in response to treatment.<sup>7,28-31</sup> Immunohistologically, CRSwNP can be divided into ECRSwNP and nonECRSwNP,<sup>3,7</sup> with ECRSwNP predominating in white patients, demonstrating T<sub>H</sub>2-biased cytokine profiles, and nonECRSwNP predominating in mostly Asian patients, demonstrating T<sub>H</sub>1/T<sub>H</sub>17-biased patterns.<sup>4-6</sup> Furthermore, ECRSwNP is associated with a greater likelihood of comorbid asthma and thus can be further divided into the subtypes according to asthma comorbidity or not. As a clustering axis extending from nonECRSwNP to ECRSwNP without asthma to ECRSwNP with comorbid asthma, peripheral blood eosinophil counts and eosinophil percentages are generally increased, suggesting that the probability of the disease to be a systemic disorder is increased.

For the purpose of treatment, confirming the disease subtype is possibly the first and most important thing because this helps in deciding the strategy for the route of administration, as either local or systemic treatment, in minimizing the systemic risks and reducing the drug load as well as the costs. Hence determining an effective biomarker for the clustering is likely to aid in the diagnosis and appropriate treatment for CRSwNP.

The present study has indicated that CST1 might be a novel useful biomarker for ECRSwNP because its expression was significantly increased in tissue from patients with ECRSwNP and decreased in tissue from patients with nonECRSwNP compared with inferior turbinate or uncinate tissue from control subjects. Furthermore, CST1 expression was significantly increased in patients with ECRSwNP and comorbid asthma compared with that seen in patients with ECRSwNP without asthma. Indeed, levels of both CST1 mRNA and protein were also found to be positively correlated with percentages of tissue eosinophils and with FENO levels in patients with ECRSwNP. Similar to our findings, Kato et al<sup>32</sup> have recently demonstrated that the expression of CST1 was significantly increased in patients with ECRSwNP compared with that in patients with non-ECRSwNP. Collectively, these findings suggest that CST1 might serve as a reliable biomarker for ECRSwNP.

Activation and recruitment of eosinophils are major features of eosinophilic inflammation, and in this regard it is likely that CST1 plays a role in both processes. Activated eosinophils undergo degranulation, releasing proteins, such as EPX, ECP, and eosinophil-derived neurotoxin, that can be toxic to invading cells and also be responsible for tissue damage and remodeling. It has been reported that apoptosis of eosinophils is delayed in patients with CRSwNP and that infiltrating eosinophils release the granules without undergoing self-destruction.<sup>33</sup> Although different mechanisms of eosinophil degranulation have been postulated,<sup>33</sup> mechanisms underlying the regeneration/sustainability of granules/granule proteins in activated eosinophils are presently not fully understood. Findings from the present study suggest that epithelial cell-derived CST1 promotes eosinophil granule protein synthesis by inducing IL-5. Although several cells, including T<sub>H</sub>2 and group 2 innate lymphoid cells, could

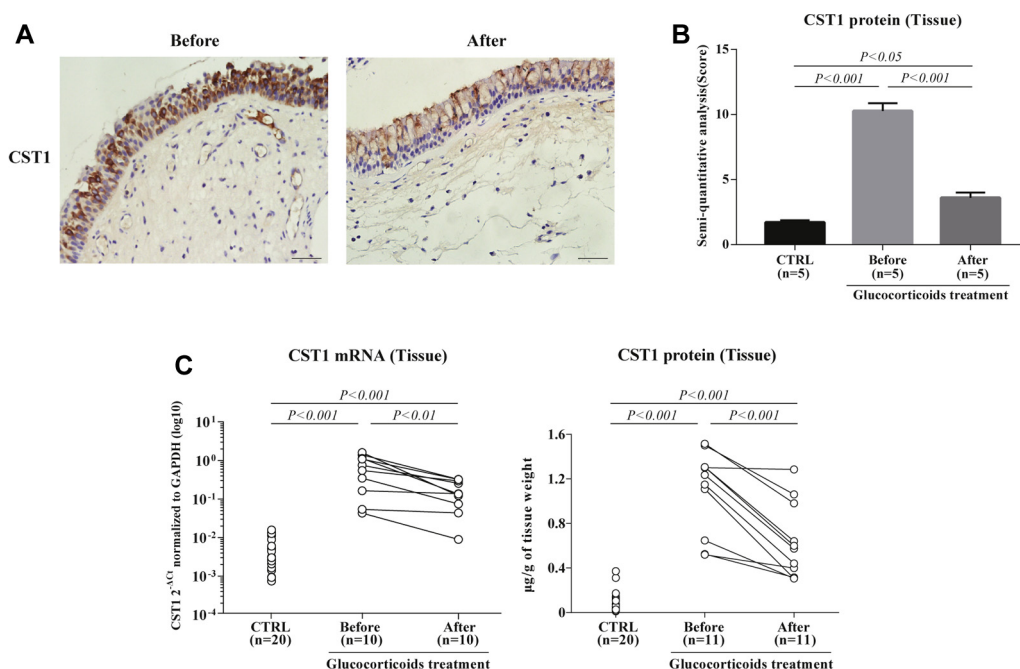


**FIG 9.** Effect of glucocorticoids on epithelial CST1 expression. **A** and **B**, Representative CST1 staining in nasal tissue and quantitative analysis before and after glucocorticoid treatment ( $n = 5$ ). Bars = 20  $\mu\text{m}$  ( $\times 400$  magnification). **C**, relative mRNA level and protein expression of CST1 before and after glucocorticoid treatment ( $n = 10$  and 11, respectively). N.S., Not significant.

be the source of IL-5, our study has further demonstrated that CST1 also enhances production of IL-5 in eosinophils. Thus it is possible that CST1-induced IL-5 synthesis in eosinophils can be involved in autocrine regulation of granule protein synthesis. Furthermore, the present study has demonstrated that CST1-mediated activation and recruitment of eosinophils through IL-5 can be reciprocated by IL-4 or IL-13 released from eosinophils.

Our study has further suggested that CST1 expressed in tissue from patients with CRSwNP also has the potential to enhance the recruitment and infiltration of eosinophils directly from the blood through involvement of IL-5 and activation of eosinophils involving degranulation. Eosinophil infiltration was significantly enhanced by treatment of recombinant CST1 or E64d for 96 hours, which reflects the promotion of eosinophilic inflammation of CST1 *in vivo*. Overall, these data indicate that CST1 is involved in activation of existing eosinophils in the tissue and is responsible for eosinophil recruitment under  $T_H2$  microenvironments.

Furthermore, the finding from the present study that recombinant CST1 did not recruit eosinophils effectively over a relatively short period of 4 hours but significantly increased eosinophil recruitment over a longer period of 96 hours suggests that CST1 is unlikely to affect eosinophils directly by binding to specific receptor or receptors on eosinophil surfaces. Indeed, although eosinophils are known to express numerous cell-surface receptors, including chemokine receptors, Fc receptors, pattern recognition receptors, lipid mediator receptors, cytokine receptors, complement receptors, and adhesion receptors,<sup>34</sup> to our knowledge, there are no reports of cystatin receptors on eosinophils.

Studies in cystatin F (CSTF) knockout mice have reported that eosinophils from these mice had reduced lifespans, reduced granularity, and disturbed granule morphology.<sup>35</sup> Furthermore,

cysteine protease inhibitors restored granularity, demonstrating that control of cysteine protease activity by CSTF was critical for normal eosinophil development. However, microarray, RNA-seq, and proteomics assessments have not detected the expression of either CSTF mRNA or protein in patients with CRSwNP, whereas CST1, cystatin SA (CST2), cystatin A (CSTA), cystatin B (CSTB), and cystatin C (CST3) have been shown to be present.<sup>36-38</sup> RNA-seq assays indicated that CST1 was upregulated in patients with ECRSwNP compared with that in healthy control subjects, and the fold change of CST1 for patients with ECRSwNP versus control subjects is 1732.2, which also indicated that CSTA and CSTB were upregulated in both patients with ECRSwNP and patients with non-ECRSwNP compared with healthy control subjects, with no significant difference between the 2 subtypes: the fold change of CSTA for patients with ECRSwNP versus control subjects is 4.32, and that for patients with non-ECRSwNP versus control subjects is 3.95; the fold change of CSTB for patients with ECRSwNP versus control subjects is 1.34, and that for patients with non-ECRSwNP versus control subjects is 2.28.<sup>36</sup> Proteomics demonstrated CSTB and CST3 to be present in the non-ECRSwNP group, but they were not detectable in control subjects and patients with ECRSwNP (unpublished data). Similarly, microarray analysis demonstrated CST1, CSTA, and CST2 expression to be increased in patients with CRSwNP; however, expression of CSTA and CST2 is relatively low compared with that of CST1. The fold change of CST1, CSTA, and CST2 for patients with CRSwNP versus control subjects is 31.53, 2.1, and 8.34, respectively.<sup>37,38</sup> Thus, unlike the internal signaling of CSTF for mice, the CST1-mediated external signaling might be the way to regulate eosinophils by cystatins in human subjects and reflects interaction between the epithelium and eosinophil counts.

The present study shows that CST1 could be regulated by a variety of cytokines, including IL-4 and IL-13, which promoted the transcriptional level of CST1 in epithelial cells, whereas IL-17A decreased its expression. Interestingly, because this decrease could only be observed in neutrophil-infiltrated polyps, it might reflect that IL-17A does not directly regulate CST1 but acts through another molecule that is absent or undetectable in healthy subjects or patients with ECRSwNP. Nevertheless, these results further support the concept that CRSwNP is a heterogeneous condition, and each subtype presents with the distinct genetic background. However, because activated eosinophils could also release IL-4, IL-5, and IL-13, this suggests that there might be a CST1-mediated positive feedback loop that propagates eosinophil recruitment, activation, and synthesis of eosinophil granular proteins in patients with ECRSwNP (see Fig E11 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Thus CST1 might be an effective biomarker for rapid detection of CRSwNP subtypes for several reasons. First, the threshold window is wide enough that the mRNA relative expression ratio of typical ECRSwNP to nonECRSwNP is as high as hundreds-fold.

Second, the absolute expression of CST1 is high in patients with ECRSwNP, which is as high as or even greater than the expression of housekeeping genes, such as *GAPDH*. The wide threshold window and the large amount of CST1 in patients with ECRSwNP make it easy for clinical examination.

Third, our research provides positive correlation of CST1 expression and eosinophil percentages, which could be the basis for the biomarker development.

Taken together, these data indicate that CST1 participates in eosinophilia and that the cysteine protease inhibitor ability is required, particularly because tissue levels of CST1 were profoundly increased in tissue from patients with ECRSwNP and decreased in tissue from patients with nonECRSwNP. This difference in expression results from the different T<sub>H</sub> type of inflammation. Epithelial cells are the major CST1-producing cell in patients with ECRSwNP, and CST1 expression is positively correlated with tissue eosinophil percentages and FENO levels. Overproduction of CST1 might lead to eosinophil granule protein synthesis and recruitment of eosinophils by IL-5. Collectively, these findings suggest that CST1 is a valuable biomarker for systemic CRSwNP and that targeting CST1 might be of therapeutic value in treating patients with T<sub>H</sub>2-dominated CRSwNP.

**Clinical implications: CST1 might be a valuable biomarker and a potential therapeutic target for management of CRSwNP.**

## REFERENCES

- Bachert C, Zhang L, Gevaert P. Current and future treatment options for adult chronic rhinosinusitis: focus on nasal polyposis. *J Allergy Clin Immunol* 2015; 136:1431-40.
- Schleimer RP. Immunopathogenesis of chronic rhinosinusitis and nasal polyposis. *Annu Rev Pathol* 2017;12:331-57.
- Ishitoya J, Sakuma Y, Tsukuda M. Eosinophilic chronic rhinosinusitis in Japan. *Allergol Int* 2010;59:239-45.
- Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, e1-2.
- Zhang N, Van Zele T, Perez-Novo C, Van Bruene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;122:961-8.
- Wang X, Zhang N, Bo M, Holtappels G, Zheng M, Lou H, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: a multicenter study in Europe, Asia, and Oceania. *J Allergy Clin Immunol* 2016;138: 1344-53.
- Lou H, Meng Y, Piao Y, Zhang N, Bachert C, Wang C, et al. Cellular phenotyping of chronic rhinosinusitis with nasal polyps. *Rhinology* 2016;54:150-9.
- Kim JW, Hong SL, Kim YK, Lee CH, Min YG, Rhee CS. Histological and immunological features of non-eosinophilic nasal polyps. *Otolaryngol Head Neck Surg* 2007;137:925-30.
- Lou H, Meng Y, Piao Y, Wang C, Zhang L, Bachert C. Predictive significance of tissue eosinophilia for nasal polyp recurrence in the Chinese population. *Am J Rhinol Allergy* 2015;29:350-6.
- Rinia AB, Kostamo K, Ebbens FA, van Drunen CM, Fokkens WJ. Nasal polyposis: a cellular-based approach to answering questions. *Allergy* 2007;62: 348-58.
- Kato A. Immunopathology of chronic rhinosinusitis. *Allergol Int* 2015;64:121-30.
- The UniProt Consortium. UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 2018;46:2699.
- Binder JX, Pletscher-Frankild S, Tsafou K, Stolte C, O'Donoghue SI, Schneider R, et al. COMPARTMENTS: unification and visualization of protein subcellular localization evidence. *Database (Oxford)* 2014;2014:bau012.
- Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002;20: 621-67.
- Sun Y, Liu G, Li Z, Chen Y, Liu Y, Liu B, et al. Modulation of dendritic cell function and immune response by cysteine protease inhibitor from murine nematode parasite *Heligmosomoides polygyrus*. *Immunology* 2013;138:370-81.
- Zi M, Xu Y. Involvement of cystatin C in immunity and apoptosis. *Immunol Lett* 2018;196:80-90.
- Kopitar-Jerala N. The role of cystatins in cells of the immune system. *FEBS Lett* 2006;580:6295-301.
- Imoto Y, Tokunaga T, Matsumoto Y, Hamada Y, Ono M, Yamada T, et al. Cystatin SN upregulation in patients with seasonal allergic rhinitis. *PLoS One* 2013;8: e67057.
- Giovannini-Chami L, Marcet B, Moreillon C, Chevalier B, Illie MI, Lebrigand K, et al. Distinct epithelial gene expression phenotypes in childhood respiratory allergy. *Eur Respir J* 2012;39:1197-205.
- Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
- Wang M, Wang X, Zhang N, Wang H, Li Y, Fan E, et al. Association of periostin expression with eosinophilic inflammation in nasal polyps. *J Allergy Clin Immunol* 2015;136:1700-3.
- Soyka MB, Wawrzyniak P, Eiwegger T, Holzmann D, Treis A, Wanke K, et al. Defective epithelial barrier in chronic rhinosinusitis: the regulation of tight junctions by IFN-gamma and IL-4. *J Allergy Clin Immunol* 2012;130: 1087-96.
- Wilcox D, Mason RW. Inhibition of cysteine proteinases in lysosomes and whole cells. *Biochem J* 1992;285:495-502.
- Melo RC, Weller PF. Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. *Histol Histopathol* 2010;25:1341-54.
- Doyle AD, Jacobsen EA, Ochkur SI, McGarry MP, Shim KG, Nguyen DT, et al. Expression of the secondary granule proteins major basic protein 1 (MBP-1) and eosinophil peroxidase (EPX) is required for eosinophilopoiesis in mice. *Blood* 2013;122:781-90.
- Van Zele T, Gevaert P, Holtappels G, Beule A, Wormald PJ, Mayr S, et al. Oral steroids and doxycycline: two different approaches to treat nasal polyps. *J Allergy Clin Immunol* 2010;125:1069-76.
- Milara J, Morell A, Ballester B, Armengot M, Morcillo E, Cortijo J. MUC4 impairs the anti-inflammatory effects of corticosteroids in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2017;139:855-62.
- Liao B, Liu JX, Li ZY, Zhen Z, Cao PP, Yao Y, et al. Multidimensional endotypes of chronic rhinosinusitis and their association with treatment outcomes. *Allergy* 2018;73:1459-69.
- Turner JH, Chandra RK, Li P, Bonnet K, Schlundt DG. Identification of clinically relevant chronic rhinosinusitis endotypes using cluster analysis of mucus cytokines. *J Allergy Clin Immunol* 2018;141:1895-7.
- Tomassen P, Vandeplas G, Van Zele T, Cardell LO, Arebro J, Olze H, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. *J Allergy Clin Immunol* 2016;137:1449-56.
- Ikeda K, Shiozawa A, Ono N, Kusunoki T, Hirotsu M, Homma H, et al. Subclassification of chronic rhinosinusitis with nasal polyp based on eosinophil and neutrophil. *Laryngoscope* 2013;123:E1-9.

32. Kato Y, Takabayashi T, Sakashita M, Imoto Y, Tokunaga T, Ninomiya T, et al. The expression and functional analysis of CST1 in intractable nasal polyps. *Am J Respir Cell Mol Biol* 2018;59:448-57.
33. Acharya KR, Ackerman SJ. Eosinophil granule proteins: form and function. *J Biol Chem* 2014;289:17406-15.
34. Diny NL, Rose NR, Cihakova D. Eosinophils in autoimmune diseases. *Front Immunol* 2017;8:484.
35. Matthews SP, McMillan SJ, Colbert JD, Lawrence RA, Watts C. Cystatin F ensures eosinophil survival by regulating granule biogenesis. *Immunity* 2016;44:795-806.
36. Wang W, Gao Z, Wang H, Li T, He W, Lv W, et al. Transcriptome analysis reveals distinct gene expression profiles in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps. *Sci Rep* 2016;6:26604.
37. Plager DA, Kahl JC, Asmann YW, Nilson AE, Pallanch JF, Friedman O, et al. Gene transcription changes in asthmatic chronic rhinosinusitis with nasal polyps and comparison to those in atopic dermatitis. *PLoS One* 2010;5:e11450.
38. Liu Z, Kim J, Sypek JP, Wang IM, Horton H, Oppenheim FG, et al. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. *J Allergy Clin Immunol* 2004;114:783-90.