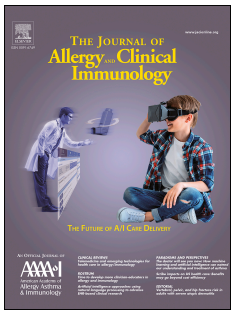
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Defective STING expression potentiates IL-13 signaling in epithelial cells in eosinophilic chronic rhinosinusitis with nasal polyps

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The regulation and function of STING in chronic rhinosinusitis with nasal polyps

CCL26: chemokine (C-C motif) ligand 26

cGAS: cyclic GMP-AMP synthase

2'3'-cGAMP: 2'3'-cyclic GMP-AMP

CRSwNP: chronic rhinosinusitis with nasal polyps

IFN: interferon

IL: interleukin

IRF3: interferon regulatory factor 3

P: Phosphorylation

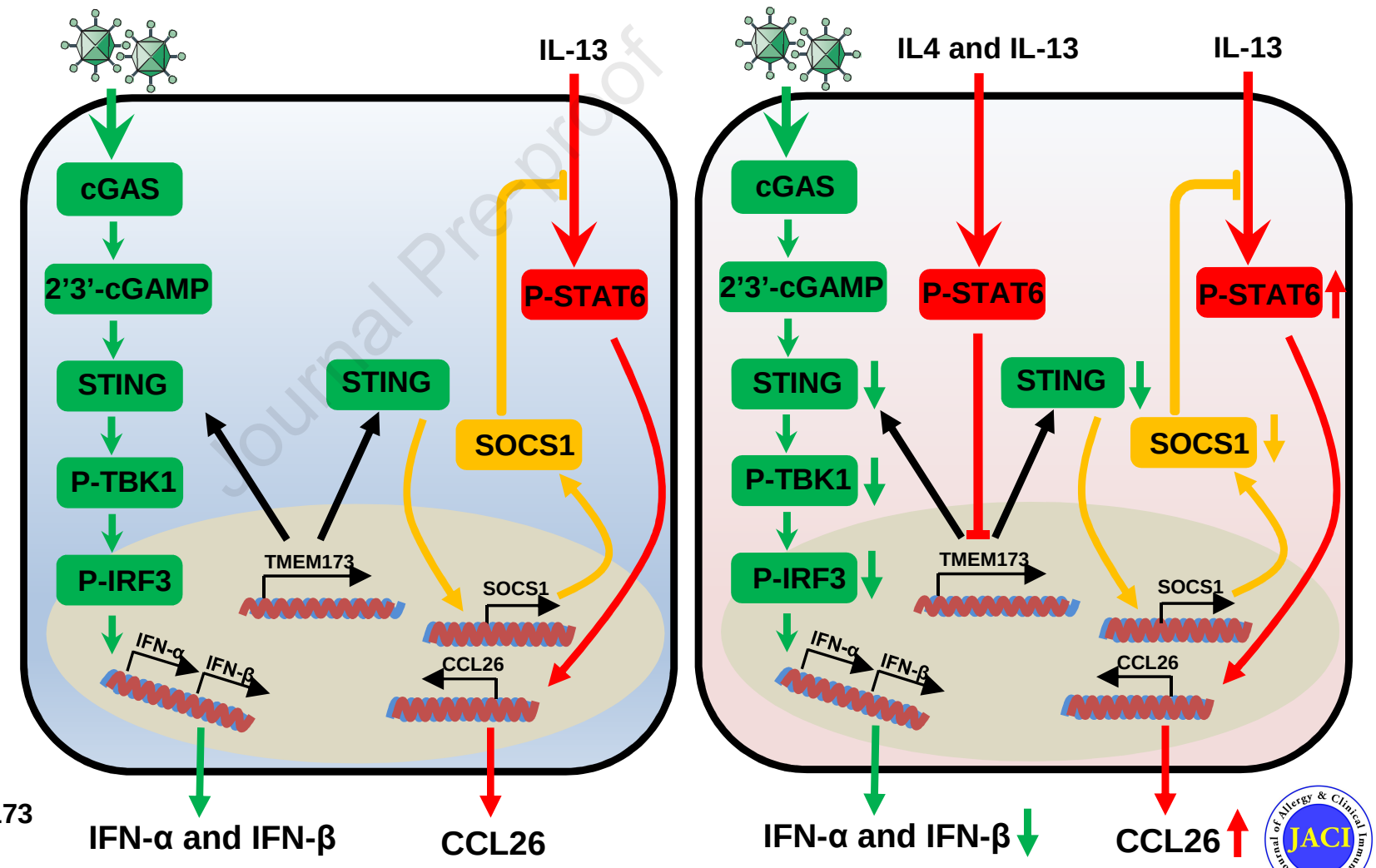
SOCS1: suppressor of cytokine signaling 1

STAT6: signal transducer and activator of transcription 6

STING: stimulator of interferon genes

TBK1: TANK binding kinase 1

TMEM173:transmembrane protein 173



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AUTHOR CONTRIBUTIONS

Hai Wang and Dan-Qing Hu performed flow cytometry, cell culture, immunohistochemistry and PCR assays, analyzed data, and prepared the manuscript; Qiao Xiao, Yi-Bo Liu and Jia Song performed cell culture; Yuxia Liang and Jian-Wen Ruan performed PCR assays; Zhe-Zheng Wang, Jing-Xian Li and Li Pan performed immunohistochemistry; Meng-Chen Wang, Ming Zeng, Li-Li Shi and Kai Xu participated in tissue sample collection and data discussion; Qin Ning, Guohua Zhen, Di Yu, De-Yun Wang and Sally E. Wenzel participated in data interpretation, experimental design, and manuscript preparation; and Zheng Liu designed the study and prepared the manuscript.

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ABSTRACT

Background: Stimulator of interferon genes (STING) activation favors effective innate immune responses against viral infections. Its role in chronic rhinosinusitis with nasal polyps (CRSwNP) remains unknown.

Objective: To explore the expression, regulation, and function of STING in CRSwNP.

Methods: STING expression in sinonasal mucosal samples was analyzed by means of quantitative RT-PCR, immunohistochemistry, flow cytometry and western blotting. STING expression regulation and function were explored by using cultured primary human nasal epithelial cells (HNECs) and BEAS-2B cell lines *in vitro*.

Results: STING expression was reduced in eosinophilic nasal polyps compared with that in noneosinophilic nasal polyps and control tissues. STING was predominantly expressed by epithelial cells in nasal tissue and was downregulated by IL-4 and IL-13 in a STAT6-dependent manner. HNECs derived from eosinophilic polyps displayed compromised STING-dependent type I interferon production, whereas heightened IL-13-induced STAT6 activation and CCL26 production as compared to those from noneosinophilic polyps and control tissues, which were rescued by exogenous STING overexpression. Knocking down or overexpressing STING decreased or enhanced SOCS1 expression in BEAS-2B cells, respectively, independent of canonical STING pathway elements, TBK1 and IRF3. Knocking down SOCS1 abolished the inhibitory

effect of STING on IL-13 signaling in BEAS-2B cells. STING expression positively correlated with SOCS1 expression, but negatively correlated with CCL26 expression in nasal epithelial cells in CRSwNP patients.

Conclusions: Reduced STING expression caused by the type 2 milieu not only impairs STING-dependent type I interferon production, but also amplifies IL-13 signaling by decreasing SOCS1 expression in nasal epithelial cells in eosinophilic CRSwNP.

Key words: Chemokine (C-C motif) ligand 26, interleukin 13, nasal polyp, stimulator of interferon genes, suppressor of cytokine signaling 1, signal transducer and activator of transcription 6.

Key messages:

- STING expression is reduced in nasal epithelial cells in eosinophilic nasal polyps compared with that in noneosinophilic nasal polyps and control tissues, which may be induced by IL-4 and IL-13 in a STAT6-dependent manner.
- Nasal epithelial cells of eosinophilic nasal polyps display compromised STING-dependent type I interferon production, but heightened IL-13-induced STAT6 activation and subsequent CCL26 production compared to those from noneosinophilic polyps and control tissues.
- STING restricts IL-13 signaling by inducing SOCS1 expression in airway epithelial cells.

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Capsule summary: Downregulated STING expression caused by type 2 cytokines impairs type I interferon production but exaggerates eosinophilic inflammation in CRSwNP. STING functions as a suppressor of IL-13 signaling in airway epithelial cells.

Abbreviations:

CCL: chemokine (C-C motif) ligand

CDN: cyclic dinucleotides

2'3'-cGAMP: 2'3'-cyclic GMP-AMP

CRSwNP: chronic rhinosinusitis with nasal polyps

dsDNA: double-stranded DNA

GUSB: beta-glucuronidase

HNECs: human nasal epithelial cells

IL: interleukin

IRF3: interferon regulatory factor 3

LPS: lipopolysaccharide

Poly (I:C): polyinosinic: polycytidylic acid

RT-PCR: reverse transcription-polymerase chain reaction

siRNA: small interfering RNA

SOCS: suppressor of cytokine signaling

STAT6: signal transducer and activator of transcription 6

STING: stimulator of interferon genes

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TBK1: TANK binding kinase 1

TLR: Toll-like receptor

INTRODUCTION

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a multifactorial disease characterized by exaggerated and persistent inflammation in sinonasal mucosa and polyp formation.[1, 2] Type 2 response-dominated eosinophilic inflammation has been considered a major pathologic hallmark of CRSwNP in Caucasians; however, over half of CRSwNP patients in East Asia, including China, demonstrate noneosinophilic inflammation.[3-7] Despite this heterogeneity, tissue eosinophilia is consistently linked with poor clinical outcome in both populations.[8, 9] Therefore, elucidating the factors leading to eosinophilic inflammation in CRSwNP remains a research focus.

Viral infection is thought to be a potential contributor to the initiation and development of CRSwNP.[10-12] A high rate of common respiratory viruses, including rhinovirus, influenza A virus and respiratory syncytial virus, was detected in nasal epithelial cells or lavage fluids of CRSwNP patients.[11, 13] The impaired innate response to these RNA viruses has been reported in patients with CRSwNP.[14] In addition to eliciting innate immunity, these RNA viruses may promote eosinophilic inflammation by inducing thymic stromal lymphopoietin production via specific pattern recognition receptors, such as Toll-like receptor 3 (TLR3), in airway epithelial

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cells.[15] Other than RNA viruses, several DNA viruses, such as herpes virus, Epstein-Barr virus and adenoviruses, have been locally detected in patients with CRSwNP as well.[11, 16] Wang et al. reported that herpes simplex virus 1 infection may disrupt the epithelial barrier and lead to subsequent bacterial infection in *ex vivo* cultured polyp tissues, suggesting a pathogenic role of DNA viruses in CRSwNP.[17] However, the innate responses against DNA viruses are very poorly understood in patients with CRSwNP.

Stimulator of interferon genes (STING), encoded by *TMEM173*, was originally identified as a cytosolic nucleic-acid sensor that controls DNA virus and retrovirus infection.[18, 19] This process requires cyclic GMP-AMP synthase, a DNA-binding protein, to synthesize the second messenger 2'3'-cyclic GMP-AMP (2'3'-cGAMP), which in turn activates STING.[20] Activated STING subsequently recruits and activates TANK-binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3) via a phosphorylation-dependent mechanism. STING also activates NF-κB, which functions together with IRF3 to initiate the transcription of type I interferon (IFN).[18, 19] Beyond the classic function in innate immunity, several novel functions of STING have recently been revealed. For example, STING has been implicated in the regulation of adaptive $CD4^+$ T cell proliferation and function.[21, 22]

The present study was undertaken to explore (1) STING expression in the sinonasal mucosa and whether its expression level is altered in CRSwNP; (2) whether the CRSwNP-related inflammatory milieu has a role in modifying STING expression; (3) whether STING-dependent antiviral function is disturbed in CRSwNP; and (4)

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whether STING has other novel functions in CRSwNP.

METHODS

Subjects

This study was approved by the Ethics Committee of Tongji Hospital and conducted with written informed consent from every patient. A total of 96 control subjects, 91 patients with eosinophilic CRSwNP and 85 patients with noneosinophilic CRSwNP, were included in this study. The diagnosis of CRSwNP was made according to the current European and American guidelines.[1, 2] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of the total infiltrating cells, as previously reported.[3, 6, 23, 24] This cutoff was calculated as twice the standard deviation of the mean eosinophil percentage in controls.[3] Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects. During surgery, nasal polyp tissues from CRSwNP patients and inferior turbinate mucosal tissues from control subjects were collected. Human nasal epithelial cells (HNECs) were scraped from the inferior turbinate of controls or nasal polyps of CRSwNP patients as previously described.[6, 13, 25, 26] Not all samples were included in every experimental protocol because of limited quantity. More information is provided in the Online Repository, including Table E1.

Histology and immunohistochemistry

Hematoxylin and eosin staining, immunohistochemical and immunofluorescence

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staining, and cell quantification were performed as previously described.[6, 13, 25] More information is provided in the Online Repository, including Table E2 and Table E3.

Quantitative RT-PCR

Quantitative RT-PCR was performed as stated elsewhere.[13, 26] More information is provided in the Online Repository, including Table E4.

Dispersed nasal tissue cell preparation and flow cytometry

Sinonasal mucosal samples were dissociated mechanically.[6, 24] Flow cytometric analysis of STING expression in dispersed nasal tissue cells was performed as previously described.[6, 27] More information is provided in the Online Repository.

Western blot analysis

Western blotting analysis was performed as previously reported.[6, 26] More information is provided in the Online Repository, including Table E5.

Cell culture

Primary HNECs and the human bronchial epithelial BEAS-2B cell line (American Type Culture Collection, Manassas, VA) were cultured with an air-liquid interface (ALI) method or submerged as previously mentioned.[13] For expression regulation experiments, HNECs from control subjects were cultured with an ALI method and treated with various cytokines and TLR agonists. To retain the

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disease-specific expression pattern of STING, HNECs were cultured submerged when comparing the differences among the control, eosinophilic CRSwNP and noneosinophilic CRSwNP groups. BEAS-2B cells were cultured submerged and used for the transfection assay. More information is provided in the Online Repository.

Transfection and function assay

BEAS-2B cells and primary HNECs were transfected with small interfering RNA targeting STING (si-STING), STAT6 (si-STAT6), or STING expression plasmids as stated elsewhere.[28, 29] In some experiments, cells were further stimulated with 2'3'-cGAMP (Thermo Fisher Scientific, Waltham, MA, USA) and/or IL-13 (R&D systems, Minneapolis, MN, USA) after transfection. More information is provided in the Online Repository.

Generation of TBK1, IRF3, and SOCS1 knockout BEAS-2B cells

TBK1-knockout (KO), IRF3-KO and SOCS1-KO BEAS-2B cells were generated by the CRISPR/Cas9 system as previously described.[29, 30] More information is provided in the Online Repository.

Promoter-luciferase reporter construct and luciferase assay

Putative STAT6-binding elements on the STING promoter were predicted by the JASPAR database (http://jaspar.binf.ku.dk/). DNA fragments containing wild-type and mutated STAT6 binding sites of the STING promoter were generated by PCR and

cloned into a firefly luciferase reporter construct (pGL3 vector; Promega, Madison, WI). BEAS-2B cells were transfected with the indicated constructs together with Renilla luciferase plasmids as previously reported.[13] In some experiments, transfected cells were treated with a STAT6 inhibitor or si-STAT6 before IL-13 stimulation. Cell lysates were analyzed for luciferase activity. More information is provided in the Online Repository.

Enzyme-linked immunosorbent assay

IFN-α, IFN-β, and CCL26 levels in cell culture supernatants were measured by using commercial kits (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Data distribution was evaluated by using a Kolmogorov-Smirnov or Shapiro-Wilk test to assess the normality and homogeneity of variance. For normally distributed data, ANOVA with Tukey's *post hoc* test was applied to adjust for multiple comparisons, and unpaired Student's *t* test was used for binary comparisons. For data not normally distributed, a Kruskal-Wallis test with the Dunn *post hoc* test was used to adjust for multiple comparisons, and binary comparisons were carried out with a Mann-Whitney *U* 2-tailed test. Data derived from tissue studies are presented in dot plots with horizontal bars representing medians and error bars showing interquartile ranges. Data from cell culture experiments are expressed as the mean and SEM. For categorical variables, a chi-square test or Fisher's exact test was performed.

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Correlations were performed using Spearman's correlation. $P < 0.05$ was considered significant.

RESULTS

Decreased expression of STING in eosinophilic nasal polyp tissues

Consistent with previous studies,[3, 6] we found that the mRNA expression levels of IL-17A and IFN-γ were elevated in both eosinophilic and noneosinophilic nasal polyp tissues (Fig E1, A), whereas the mRNA expression of IL-5 and IL-13 was upregulated only in eosinophilic nasal polyp tissues (Fig 1, A), as compared with those in control tissues. Moreover, we found that in comparison with those in control tissues, the mRNA expression level of STING was significantly decreased in eosinophilic nasal polyps, but not noneosinophilic nasal polyps (Fig 1, A). The reduction of STING in eosinophilic nasal polyps was confirmed at the protein level by western blot analysis (Fig 1, B). In addition, we discovered negative correlations of STING mRNA expression with IL-5 and IL-13 mRNA expression (Fig 1, C) in nasal polyp tissues in patients with CRSwNP. Nevertheless, there was no correlation of STING mRNA levels with IL-17A or IFN-γ mRNA levels in patients with CRSwNP (Fig E1, B).

Decreased expression of STING in epithelial cells in eosinophilic nasal polyps

To examine the tissue-specific cellular expression of STING, we first performed immunofluorescence staining of nasal polyp tissue sections and found that STING

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289 was expressed by nasal epithelial cells (Fig 2, A) as well as infiltrating immune cells
290 in the lamina propria, including $CD3^+$ T cells, $CD20^+$ B cells, $CD11c^+$ dendritic cells,
291 $CD68^+$ macrophages, $CD138^+$ plasma cells, MBP^+ eosinophils, and MPO^+ and
292 $elastase^+$ neutrophils (Fig E2). Compared to those in control tissues, the numbers of
293 $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells, $CD11c^+$ dendritic cells,
294 $CD68^+$ macrophages, MPO^+ neutrophils, and $elastase^+$ neutrophils were elevated in
295 both eosinophilic and noneosinophilic nasal polyps (Fig E3). The numbers of MBP^+
296 eosinophils were higher in eosinophilic nasal polyps than in noneosinophilic nasal
297 polyps and control tissues (Fig E3). The numbers of MPO^+ and $elastase^+$ neutrophils
298 were further increased in noneosinophilic nasal polyps compared with eosinophilic
299 nasal polyps (Fig E3). We also studied the cell type with STING expression in nasal
300 epithelium, and we found that $KRT5^+$ basal cells, $KRT8^+$ apical cells, and $Foxj1^+$
301 ciliated cells all had significant STING expression (Fig E4).

302 Then, we compared the expression intensity of STING in epithelial cells and
303 immune cells by flow cytometric analysis of dispersed nasal tissue cells. We found
304 that $CD45^+CD326^-$ hematopoietic cells had lower STING expression than
305 $CD45^-CD326^+$ epithelial cells in nasal polyp tissues (Fig 2, B). Therefore, we focused
306 on STING expression in nasal epithelial cells; compared to those in noneosinophilic
307 nasal polyps and control tissues, we found a lower proportion of STING-positive cells
308 by flow cytometry (Fig 2, C) and decreased immunoreactivity of STING by
309 immunohistochemical staining (Fig E5) in nasal epithelial cells in eosinophilic nasal
310 polyps.

311

312 **Type 2 cytokines downregulate STING expression in a STAT6-dependent**
313 **manner in airway epithelial cells**

314 We next investigated the regulation of STING expression in nasal epithelial cells.
315 HNECs from control subjects were cultured with an ALI method and stimulated with
316 allergens, TLR agonists, and cytokines related to the pathogenesis of CRSwNP.[9, 25]
317 We found that STING mRNA levels were upregulated by CpG and TNF-α but were
318 downregulated by IL-4 and IL-13 in HNECs (Fig E6, A). We further found that IL-4
319 and IL-13 downregulated the mRNA (Fig E6, B) and protein (Fig 3, A and B)
320 expression of STING in a dose-dependent manner in HNECs. Similar results were
321 found in BEAS-2B cells cultured submerged (data not shown). Considering the high
322 transfection efficiency and cell viability of cell lines, we selected BEAS-2B cells for
323 siRNA and plasmid transfection assays unless specifically stated. STAT6 is a master
324 regulator of IL-4 and IL-13 signaling.[31, 32] We confirmed the enhanced
325 phosphorylation of STAT6 in response to IL-13 treatment in BEAS-2B cells, which
326 was inhibited by pretreatment with AS1517499, a STAT6 inhibitor (Fig 3, C). We
327 further found that IL-13-induced STING downregulation was abolished by
328 AS1517499 pretreatment in BEAS-2B cells, indicating a STAT6-dependent effect
329 (Fig 3, D). This notion was further proven by the luciferase reporter assay. We found
330 that IL-13-induced suppression of STING promoter activity was abolished by
331 pretreatment with the STAT6 inhibitor and si-STAT6 in BEAS-2B cells (Fig 3, E). The
332 knockdown efficiency of si-STAT6 is shown in Fig E7. Inspection of the STING

promoter sequence revealed four putative STAT6 binding sites (-1808 to -1794, -1573 to -1559, -474 to -460, and -93 to -79 relative to the transcriptional start site) (Fig 3, F). We found that mutation in the first STAT6 binding site (-1808 to -1794) abolished the inhibitory effect of IL-13 on STING promoter activity in BEAS-2B cells, suggesting that the STING promoter indeed harbors a functional STAT6 binding site (Fig 3, F).

Compromised STING-dependent type I interferon production in epithelial cells in eosinophilic nasal polyps

Classically, upon activation, STING rapidly undergoes translocation to perinuclear-associated endosomal regions, leading to phosphorylation of TBK1, IRF3 and NF-κB and subsequent type I IFN production.[18, 33] After stimulation with 2'3'-cGAMP, a well-known STING activator, we found punctate aggregations of STING and phosphorylated TBK1 in the perinuclear area, nuclear translocation of phosphorylated IRF3 and p65 (Fig E8, A), and upregulation of IFN-α and IFN-β mRNA expression (Fig E8, B) in BEAS-2B cells. These results underline a functional canonical STING machinery in airway epithelial cells. Since HNECs may lose the *in vivo* disease-specific expression pattern of STING under long-term ALI culture, we cultured HNECs submerged when comparing the differences among the different study groups. In line with the defective expression of STING, we found that HNECs from patients with eosinophilic CRSwNP showed compromised phosphorylation of TBK1, IRF3 and p65 after 2'3'-cGAMP stimulation as compared to HNECs from

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control subjects and patients with noneosinophilic CRSwNP (Fig 4, A). Eventually, mRNA and protein production of IFN-α (Fig 4, B) and IFN-β (Fig 4, C) were significantly impaired in HNECs from patients with eosinophilic CRSwNP as compared with those from noneosinophilic CRSwNP and control subjects in response to 2'3'-cGAMP stimulation.

Loss of STING expression augments IL-13 signaling in airway epithelial cells

In addition to type 2 cytokines, we found that STING expression negatively correlated with tissue eosinophilia in patients with CRSwNP (Fig 5, A), which drove us to explore whether STING in turn regulates type 2 inflammation. Epithelial cell-derived CCL11, CCL24 and CCL26 are critical for local eosinophil recruitment in CRSwNP,[34, 35] and can be induced by IL-13 via STAT6.[21, 36] Among them, CCL26 had the highest upregulation in expression level in eosinophilic nasal polyps and the tightest correlation with polyp eosinophilia (Fig E9, A and B). We therefore selected CCL26 as our target mediator for further investigations. We discovered that STING mRNA expression negatively correlated with CCL26 mRNA expression in nasal epithelial cells in patients with CRSwNP (Fig 5, A), indicating that STING may be involved in the regulation of CCL26 expression in nasal epithelial cells. To substantiate this possibility, we transfected airway epithelial cells with si-STING or STING expression plasmid, and the transfection efficiencies in BEAS-2B cells are shown in Fig E10. We found that si-STING treatment promoted IL-13-induced CCL26 production in BEAS-2B cells at both the mRNA and protein levels (Fig 5, B),

which was not affected by 2'3'-cGAMP treatment (Fig 5, B). Consistently, we found that submerged cultured HNECs from eosinophilic CRSwNP patients had enhanced CCL26 production under IL-13 stimulation in comparison to HNECs from control subjects and noneosinophilic CRSwNP patients (Fig 5, C). STING expression plasmid transfection significantly inhibited IL-13-induced CCL26 production in HNECs from patients with eosinophilic CRSwNP (Fig 5, D).

To explore the mechanisms by which STING regulates IL-13-induced CCL26 production, we studied the influence of STING on STAT6 activation. We found that STING overexpression markedly attenuated, whereas STING knockdown significantly promoted STAT6 phosphorylation induced by IL-13 in BEAS-2B cells (Fig 5, E-F). Moreover, we found that HNECs from eosinophilic CRSwNP patients showed enhanced STAT6 phosphorylation under IL-13 stimulation in comparison to HNECs from control subjects and noneosinophilic CRSwNP patients (Fig 5, G).

STING induces the expression of the negative regulator SOCS1 in airway epithelial cells

The SOCS family has a critical role in inhibiting cytokine signaling by regulating the JAK-STAT pathway, and SOCS1, SOCS2, and SOCS3 are the best-characterized members in suppressing type 2 cytokine signaling.[36-38] We found that the mRNA (Fig 6, A) and protein (Fig 6, B) levels of SOCS1, but not those of SOCS2 or SOCS3, were downregulated by si-STING and upregulated by STING plasmid transfection in BEAS-2B cells, which was not affected by 2'3'-cGAMP treatment.

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We further interrogated whether STING exerts its suppressive effect on IL-13 signaling through SOCS1. Indeed, we found that STING overexpression could not inhibit STAT6 phosphorylation (Fig 6, C) and CCL26 production (Fig 6, D and Fig E11) in BEAS-2B cells when SOCS1 was knocked out. The representative results of SOCS1 knockout in BEAS-2B cells are shown in Fig E12.

The effect of STING on SOCS1 (Fig 6, A and B) and CCL26 (Fig 5, B) production was not influenced by 2'3'-cGAMP treatment, suggesting that this effect of STING is unlikely to be related to its classic DNA sensing function. We then manipulated the expression of key elements, TBK1 and IRF3, in the classic pathway by which STING activates host defense. Representative results of TBK1 and IRF3 knockout in BEAS-2B cells are shown in Fig E13. Not surprisingly, we found that TBK1 and IRF3 knockout impaired the upregulation of type I IFN in response to 2'3-cGAMP (Fig E14). However, no significant effect on STING-induced SOCS1 upregulation was observed in BEAS-2B cells (Fig E15, A and B). Consistent with our previous report,[13] we found that IFN-β induced BAFF, CXCL10 and CXCL11 mRNA expression in BEAS-2B cells (Fig E15, C); nevertheless, IFN-β had no effect on SOCS1 expression (Fig E15, C).

SOCS1 positively correlates with STING expression in nasal epithelial cells in nasal polyps

The expression of SOCS1 in nasal epithelial cells in sinonasal mucosa was confirmed by immunofluorescence staining (Fig E16). We discovered that compared

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to those in control subjects and patients with noneosinophilic CRSwNP, the mRNA (Fig 7, A) and protein expression of SOCS1 (Fig 7, B), but not SOCS2 or SOCS3 (Fig E17, A), in nasal epithelial cells were significantly decreased in patients with eosinophilic CRSwNP. We found a positive correlation between the mRNA expression of STING and SOCS1 (Fig 7, A), but not SOCS2 or SOCS3 (Fig E17, B), in nasal epithelial cells in patients with CRSwNP. In addition, a negative correlation between SOCS1 and CCL26 mRNA expression in nasal epithelial cells in patients with CRSwNP was observed (Fig 7, A).

DISCUSSION

Previous studies have concentrated on the function of STING in myeloid populations given its instrumental role in antiviral innate immunity. The expression and function of STING in tissue structural cells including epithelial cells are limited studied. Airway epithelial cells form part of the first line of defense and play a key role in the initiation of immune responses against airborne allergens and pathogens.[39, 40] In this study, we provide the first evidence for a dual role of STING in innate immune activation and type 2 inflammation in airway epithelial cells in CRSwNP.

Protein posttranslational modifications, including phosphorylation and ubiquitination, have been reported to regulate STING levels during the antiviral response.[41] However, the regulation of STING expression at the transcriptional level has rarely been studied. In this study, we revealed that STING mRNA expression was significantly downregulated by type 2 cytokines in a STAT6-dependent manner in

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airway epithelial cells. This finding was reinforced by the discovery of a STAT6 binding site on the STING promoter. Thus, defective STING expression in epithelial cells in eosinophilic CRSwNP likely results from the local type 2 milieu. However, whether there are other mechanisms regulating STING expression in airway epithelial cells requires further investigation. For example, it is interesting to explore whether there is any association between genetic variants of type-2 cytokine genes and STING encoding the *TMEM173* gene in the future.

A high detection rate of viruses, including DNA viruses, in nasal epithelial cells has been reported in patients with CRSwNP,[16] suggesting a potential accumulation of cytoplasmic DNA in epithelial cells. In this study, we found that defective STING expression in nasal epithelial cells led to blunt type I IFN production in response to 2'3'-cGAMP in patients with eosinophilic CRSwNP, suggesting impaired defense against DNA viruses in patients with eosinophilic CRSwNP. Our finding is consistent with a previous observation,[42] in which impaired type I IFN production in response to herpes simplex virus 1, a DNA virus, was observed in *ex vivo* cultured nasal polyp tissues characterized by a type 2 response, although the underlying mechanisms had not been investigated in that study. The impaired defense against DNA viruses may lead to sustained inflammation, virus-related exacerbation, and subsequent bacterial infection in eosinophilic CRSwNP.[17] Nevertheless, more epidemiological and mechanistic studies are needed to clarify the role of viral infection in the pathogenesis of CRSwNP with a focus on the difference between eosinophilic and noneosinophilic types.

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Recently, functions beyond the canonical innate antiviral effect have been reported for STING. Wu et al. uncovered an IFN-independent function of STING in calcium homeostasis, ER stress, and T cell survival in mice.[43] A study of patients with STING-associated vasculopathy identified an IRF3-independent effect of STING on $CD4^+$ T cell proliferation.[21] IL-13 signaling plays an important role in inducing the expression of eosinophil chemokines and subsequent tissue eosinophilia. In this study, we provided several converging lines of evidence for a novel and interesting function of STING in restricting IL-13 signaling in airway epithelial cells. First, we found that knockdown or overexpression of STING led to upregulation or suppression of IL-13-induced STAT6 phosphorylation and CCL26 production in BEAS-2B cells. Second, compared to nasal epithelial cells from control tissues and noneosinophilic nasal polyps, those from eosinophilic polyps with defective STING expression demonstrated enhanced phosphorylation of STAT6 and CCL26 production in response to IL-13, which could be suppressed by exogenous STING overexpression. Third, we found a negative association between STING and CCL26 expression in nasal epithelial cells in patients with CRSwNP.

Several negative regulators, particularly SOCS1, SOCS2, and SOCS3, are involved in the regulation of type 2 cytokine signaling. In this study, we found that STING induced the expression of SOCS1, but not SOCS2 or SOCS3, in airway epithelial cells. This is in line with the specific downregulation of SOCS1 in epithelial cells in eosinophilic CRSwNP, suggesting a SOCS1-mediated mechanism for the inhibitory effect of STING on IL-13 signaling in airway epithelial cells. Indeed, we

found that SOCS1 knockout abrogated the inhibitory effect of STING on IL-13 signaling. The upregulation of SOCS1 by STING has also been reported in macrophages and nasopharyngeal carcinoma cells with undefined mechanism[29, 44] How does STING regulate SOCS1 expression? Here, we found that SOCS1 expression, STAT6 phosphorylation, and CCL26 production in airway epithelial cells were not affected by 2'3'-cGAMP, an activator of the STING-TBK1-IRF3-type I IFN pathway. In addition, we discovered that STING retained the ability to induce SOCS1 expression despite the knockout of TBK1 or IRF3 in BEAS-2B cells. Finally, we discovered that type I IFN was unable to induce SOCS1 expression in airway epithelial cells. Collectively, these data suggest a novel mechanism for STING to induce SOCS1 expression in airway epithelial cells, which is unrelated to the classic pathway activated by STING during host defense. Recently, Cerboni et al. reported that the TBK1/IRF3 and IFN-independent effect of STING on T cell proliferation was possibly mediated through a novel miniCTT motif (aa343-354),[21] which is distinct from the known domains of STING required for type I IFN production.[45] Wu et al. identified an unfolded protein response motif (aa322-343) of STING that controls calcium homeostasis, endoplasmic reticulum stress and T cell survival.[43] These studies suggest that different C-terminal domains of STING may possess distinct functions. Obviously, the precise mechanisms underlying STING-induced SOCS1 expression in airway epithelial cells merit further investigation.

Our study has several limitations. Recently, STING has been found to directly recognize cyclic dinucleotides from invading bacteria, including *Staphylococcus*

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aureus.[46] It will be interesting to explore the role of STING in the response to *Staphylococcus aureus* in CRSwNP. In addition, we observed STING expression in infiltrating immune cells in nasal polyps. The role of STING in immune cells in the context of CRSwNP remains to be studied further. Moreover, the structural motif and signaling pathway responsible for SOCS1 induction by STING in airway epithelial cells await to be defined.

These comments notwithstanding, for the first time, we discovered that STING is profoundly expressed by airway epithelial cells, which are downregulated by type 2 cytokines via STAT6. The defective expression of STING in airway epithelial cells may not only impair innate immunity to viruses but also augment IL-13 signaling by modulating SOCS1 expression. The mutual regulation between type 2 inflammation and STING may exaggerate the development of eosinophilic inflammation in CRSwNP. STING is a potential therapeutic target for eosinophilic CRSwNP.

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REFERENCES

1. Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2020. Rhinology 2020; 58:1-464.
2. Orlandi RR, Kingdom TT, Hwang PH, Smith TL, Alt JA, Baroody FM, et al. International Consensus Statement on Allergy and Rhinology: Rhinosinusitis. Int Forum Allergy Rhinol 2016; 6 Suppl 1:S22-209.
3. 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. Journal Of Allergy And Clinical Immunology 2009; 124:478-U135.
4. Van Bruaene N, Perez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. J Allergy Clin Immunol 2008; 121:1435-41, 41 e1-3.
5. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy 2006; 61:1280-9.
6. Wang H, Li ZY, Jiang WX, Liao B, Zhai GT, Wang N, et al. The activation and function of IL-36gamma in neutrophilic inflammation in chronic rhinosinusitis. J Allergy Clin Immunol 2018; 141:1646-58.
7. 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.

8. Soler ZM, Sauer D, Mace J, Smith TL. Impact of mucosal eosinophilia and nasal polyposis on quality-of-life outcomes after sinus surgery. Otolaryngol Head Neck Surg 2010; 142:64-71.

9. 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.

10. Hoggard M, Wagner Mackenzie B, Jain R, Taylor MW, Biswas K, Douglas RG. Chronic Rhinosinusitis and the Evolving Understanding of Microbial Ecology in Chronic Inflammatory Mucosal Disease. Clin Microbiol Rev 2017; 30:321-48.

11. Lan F, Zhang N, Gevaert E, Zhang L, Bachert C. Viruses and bacteria in Th2-biased allergic airway disease. Allergy 2016; 71:1381-92.

12. Hamilos DL. Host-microbial interactions in patients with chronic rhinosinusitis. J Allergy Clin Immunol 2014; 133:640-53 e4.

13. Zhang XH, Zhang YN, Li HB, Hu CY, Wang N, Cao PP, et al. Overexpression of miR-125b, a novel regulator of innate immunity, in eosinophilic chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med 2012; 185:140-51.

14. Hwang JW, Lee KJ, Choi IH, Han HM, Kim TH, Lee SH. Decreased expression of type I (IFN-beta) and type III (IFN-lambda) interferons and interferon-stimulated genes in patients with chronic rhinosinusitis with and without nasal polyps. J Allergy Clin Immunol 2019; 144:1551-65 e2.

15. Liu JX, Liao B, Yu QH, Wang H, Liu YB, Guo CL, et al. The IL-37-Mex3B-Toll-like receptor 3 axis in epithelial cells in patients with eosinophilic chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2020; 145:160-72.

16. Tan KS, Yan Y, Ong HH, Chow VTK, Shi L, Wang DY. Impact of Respiratory Virus Infections in Exacerbation of Acute and Chronic Rhinosinusitis. Curr Allergy Asthma Rep 2017; 17:24.

17. Wang X, Zhang N, Glorieux S, Holtappels G, Vaneechoutte M, Krysko O, et al. Herpes simplex virus type 1 infection facilitates invasion of Staphylococcus aureus into the nasal mucosa and nasal polyp tissue. PLoS One 2012; 7:e39875.

18. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 2008; 455:674-8.

19. Sun W, Li Y, Chen L, Chen H, You F, Zhou X, et al. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. Proc Natl Acad Sci U S A 2009; 106:8653-8.

20. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 2013; 339:786-91.

21. Cerboni S, Jeremiah N, Gentili M, Gehrmann U, Conrad C, Stolzenberg MC, et al. Intrinsic antiproliferative activity of the innate sensor STING in T lymphocytes. J Exp Med 2017; 214:1769-85.

22. Zhao Q, Manohar M, Wei Y, Pandol SJ, Habtezion A. STING signalling protects against chronic pancreatitis by modulating Th17 response. Gut 2019; 68:1827-37.

23. Song J, Wang H, Zhang YN, Cao PP, Liao B, Wang ZZ, et al. Ectopic lymphoid tissues support local immunoglobulin production in patients with chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2018; 141:927-37.

24. Zhai GT, Wang H, Li JX, Cao PP, Jiang WX, Song J, et al. Immunoglobulin D-Activated Mast Cells Induce IgE Synthesis in B Cells in Nasal Polyps. J Allergy Clin Immunol 2018.

606 25. Liao B, Cao PP, Zeng M, Zhen Z, Wang H, Zhang YN, et al. Interaction of thymic stromal
607 lymphopoietin, IL-33, and their receptors in epithelial cells in eosinophilic chronic
608 rhinosinusitis with nasal polyps. Allergy 2015; 70:1169-80.
609 26. Wang BF, Cao PP, Wang ZC, Li ZY, Wang ZZ, Ma J, et al. Interferon-gamma-induced insufficient
610 autophagy contributes to p62-dependent apoptosis of epithelial cells in chronic rhinosinusitis
611 with nasal polyps. Allergy 2017; 72:1384-97.
612 27. Dong G, You M, Ding L, Fan H, Liu F, Ren D, et al. STING Negatively Regulates Double-Stranded
613 DNA-Activated JAK1-STAT1 Signaling via SHP-1/2 in B Cells. Mol Cells 2015; 38:441-51.
614 28. Liu JX, Liao B, Yu QH, Wang H, Liu YB, Guo CL, et al. IL-37-Mex3B-TLR3 axis in epithelial cells in
615 eosinophilic chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2019.
616 29. Zhang CX, Ye SB, Ni JJ, Cai TT, Liu YN, Huang DJ, et al. STING signaling remodels the tumor
617 microenvironment by antagonizing myeloid-derived suppressor cell expansion. Cell Death
618 Differ 2019.
619 30. Luo P, Xu Z, Li G, Yan H, Zhu Y, Zhu H, et al. HMGB1 represses the anti-cancer activity of
620 sunitinib by governing TP53 autophagic degradation via its nucleus-to-cytoplasm transport.
621 Autophagy 2018; 14:2155-70.
622 31. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential role of
623 Stat6 in IL-4 signalling. Nature 1996; 380:627-30.
624 32. Pesu M, Takaluoma K, Aittomaki S, Lagerstedt A, Saksela K, Kovanen PE, et al.
625 Interleukin-4-induced transcriptional activation by stat6 involves multiple serine/threonine
626 kinase pathways and serine phosphorylation of stat6. Blood 2000; 95:494-502.
627 33. Konno H, Konno K, Barber GN. Cyclic dinucleotides trigger ULK1 (ATG1) phosphorylation of
628 STING to prevent sustained innate immune signaling. Cell 2013; 155:688-98.
629 34. Min JY, Ocampo CJ, Stevens WW, Price CPE, Thompson CF, Homma T, et al. Proton pump
630 inhibitors decrease eotaxin-3/CCL26 expression in patients with chronic rhinosinusitis with
631 nasal polyps: Possible role of the nongastric H,K-ATPase. J Allergy Clin Immunol 2017;
632 139:130-41 e11.
633 35. Li Z, Zeng M, Deng Y, Zhao J, Zhou X, Trudeau JB, et al. 15-Lipoxygenase 1 in nasal polyps
634 promotes CCL26/eotaxin 3 expression through extracellular signal-regulated kinase activation.
635 J Allergy Clin Immunol 2019; 144:1228-41 e9.
636 36. Doran E, Choy DF, Shikotra A, Butler CA, O'Rourke DM, Johnston JA, et al. Reduced epithelial
637 suppressor of cytokine signalling 1 in severe eosinophilic asthma. Eur Respir J 2016;
638 48:715-25.
639 37. Knosp CA, Carroll HP, Elliott J, Saunders SP, Nel HJ, Amu S, et al. SOCS2 regulates T helper type
640 2 differentiation and the generation of type 2 allergic responses. J Exp Med 2011;
641 208:1523-31.
642 38. Seki Y, Inoue H, Nagata N, Hayashi K, Fukuyama S, Matsumoto K, et al. SOCS-3 regulates onset
643 and maintenance of T(H)2-mediated allergic responses. Nat Med 2003; 9:1047-54.
644 39. Stevens WW, Lee RJ, Schleimer RP, Cohen NA. Chronic rhinosinusitis pathogenesis. J Allergy
645 Clin Immunol 2015; 136:1442-53.
646 40. Schleimer RP. Immunopathogenesis of Chronic Rhinosinusitis and Nasal Polyposis. Annu Rev
647 Pathol 2017; 12:331-57.
648 41. Li Y, Wilson HL, Kiss-Toth E. Regulating STING in health and disease. J Inflamm (Lond) 2017;
649 14:11.

42. Lan F, Wang XD, Nauwynck HJ, Holtappels G, Zhang L, Johnston SL, et al. Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1. Rhinology 2016; 54:141-9.
43. Wu J, Chen YJ, Dobbs N, Sakai T, Liou J, Miner JJ, et al. STING-mediated disruption of calcium homeostasis chronically activates ER stress and primes T cell death. J Exp Med 2019; 216:867-83.
44. Sharma S, Campbell AM, Chan J, Schattgen SA, Orlowski GM, Nayar R, et al. Suppression of systemic autoimmunity by the innate immune adaptor STING. Proc Natl Acad Sci U S A 2015; 112:E710-7.
45. Zhao B, Du F, Xu P, Shu C, Sankaran B, Bell SL, et al. A conserved PLPLRT/SD motif of STING mediates the recruitment and activation of TBK1. Nature 2019; 569:718-22.
46. Gries CM, Bruger EL, Moormeier DE, Scherr TD, Waters CM, Kielian T. Cyclic di-AMP Released from Staphylococcus aureus Biofilm Induces a Macrophage Type I Interferon Response. Infect Immun 2016; 84:3564-74.

FIGURE LEGENDS

Figure 1. Decreased expression of STING in eosinophilic nasal polyp tissues. A, The mRNA expression levels of IL-5, IL-13, and STING in sinonasal mucosal samples in different study groups as detected by quantitative RT-PCR. Control, $n = 16$; Eos CRSwNP, $n = 16$; Non-Eos CRSwNP, $n = 16$. **B,** The protein levels of STING in sinonasal mucosal samples in different study groups as detected by western blotting. Control, $n = 15$; Eos CRSwNP, $n = 15$; Non-Eos CRSwNP, $n = 15$. Representative blots are shown, and densitometric analysis of blots was performed. For **A** and **B**, data are presented as median and interquartile ranges and were analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. **C,** STING

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mRNA expression negatively correlated with IL-5 and IL-13 mRNA in nasal polyp tissues in patients with CRSwNP (n = 32). Spearman's correlation was used for correlation analysis. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Figure 2. Reduced expression of STING in epithelial cells in eosinophilic nasal polyps. A, Immunostaining of STING in sinonasal mucosal tissues. Representative photomicrographs are shown (original magnification × 400). Scale bar, 100 μm. The right image shows the higher magnification of the outlined area. The red arrow denotes representative positive basal cells, and the pink arrow denotes representative positive apical cells in the epithelium; yellow arrows denote representative positive cells in the lamina propria. **B,** Dispersed polyp tissue cells (n = 31) were subjected to flow cytometric analysis of STING expression in $CD45^{+}CD326^{-}$ hematopoietic cells and $CD45^{-}CD326^{+}$ epithelial cells. A representative histogram of a noneosinophilic nasal polyp sample is shown. Data were analyzed by the Mann-Whitney U 2-tailed test. **C,** Flow cytometric analysis of STING-positive cells in $CD45^{-}CD326^{+}$ epithelial cells. Representative flow plots of an eosinophilic nasal polyp sample are shown. Control, n = 15; Eos CRSwNP, n = 16; Non-Eos CRSwNP, n = 15. Data are presented as median and interquartile ranges and were analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. ***$P < 0.001$. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

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Figure 3. Type 2 cytokines downregulate STING expression in a STAT6-dependent manner in airway epithelial cells. A and B, HNECs from control subjects were cultured with an air-liquid interface method and stimulated with IL-4 (**A**) or IL-13 (**B**) at the indicated concentration for 24 hours. STING protein levels were measured by western blotting (n = 5). **C-D,** BEAS-2B cells were stimulated with IL-13 (10 ng/mL) with or without pretreatment with the STAT6 inhibitor AS1517499 (100 nM). Cells were collected for western blotting 3 hours after stimulation (**C**) and for PCR assay 6 hours after stimulation (**D**) (n = 5). **E,** BEAS-2B cells were transfected with the STING promoter-luciferase reporter construct and stimulated with IL-13 (10 ng/mL) with or without AS1517499 (100 nM) pretreatment or si-STAT6 (50 nM) pretransfection. Sixteen hours later, cell lysates were harvested for the luciferase assay (n = 6). **F,** BEAS-2B cells were transfected with the indicated promoter reporters and subsequently stimulated with IL-13 for 16 hours. Cell lysates were harvested for the luciferase assay (n = 5). For western blotting, representative blots are shown, and densitometric analysis of blots was performed. Data are presented as the mean and SEM and were analyzed by one-way ANOVA with the Tukey's *post hoc* test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Figure 4. Compromised STING-dependent type I interferon production in nasal epithelial cells in eosinophilic nasal polyps. A, HNECs from different study groups were cultured submerged and transfected with 2'3'-cGAMP (10 μg/mL) or vehicle for 3 hours. Cell lysates were subjected to western blotting (n = 7). Representative blots

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are shown, and densitometric analysis of blots was performed. **B and C,** HNECs from different study groups were submerged cultured and transfected with 2'3'-cGAMP (10 μg/mL) or vehicle. Cells were subjected to quantitative RT-PCR 6 hours after stimulation (n = 8), and cell culture supernatants were subjected to ELISA 24 hours after stimulation (n = 8). Data are presented as the mean and SEM and analyzed by one-way ANOVA with the Tukey's *post hoc* test. *P < 0.05, **P < 0.01, and ***P < 0.001. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Figure 5. Downregulated STING expression augments IL-13 signaling in airway epithelial cells. A, Nasal epithelial cell STING mRNA expression negatively correlated with tissue eosinophilia and nasal epithelial cell CCL26 mRNA expression in patients with CRSwNP. Eos CRSwNP, n = 16; Non-Eos CRSwNP, n = 16. **B,** After si-STING transfection, BEAS-2B cells were treated with IL-13 (10 ng/mL) with or without 2'3'-cGAMP (10 μg/mL) pretreatment for 24 hours. Six hours or 24 hours later, cells or culture supernatants were collected for RT-PCR or ELISA assays, respectively (n = 5). **C,** HNECs from different study groups were cultured submerged and treated with IL-13 (10 ng/mL) for 24 hours. CCL26 levels in cell culture supernatants were measured by ELISA (n = 6 for each group). **D,** HNECs from patients with eosinophilic CRSwNP were cultured submerged and transfected with STING expression or control plasmid for 48 hours and further stimulated with IL-13 (10 ng/mL) for another 24 hours. CCL26 levels in cell culture supernatants were

measured by ELISA (n = 6). **E,** After STING expression or control plasmid transfection, BEAS-2B cells were treated with IL-13 (10 ng/mL) for 3 hours. Cells were subjected to western blotting (n = 5). **F,** After si-STING transfection, BEAS-2B cells were treated with IL-13 (10 ng/mL) for 3 hours. Cells were subjected to western blotting (n = 5). **G,** HNECs from different study groups were cultured submerged and treated with IL-13 (10 ng/mL) for 3 hours. Cells were subjected to western blotting (n = 6 for each group). For western blotting, representative blots are shown, and densitometric analysis of blots was performed. Data are presented as the mean and SEM and analyzed by one-way ANOVA with the Tukey's *post hoc* test. *P < 0.05, **P < 0.01, and ***P < 0.001. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Figure 6. STING induces SOCS1 expression in airway epithelial cells. A and B, BEAS-2B cells were transfected with si-STING or STING expression plasmids for 24 hours and subsequently transfected with 2'3'-cGAMP (10 μg/mL). **(A)** Six hours later, the cells were subjected to quantitative RT-PCR (n = 5). **(B)** Twenty-four hours later, the cells were subjected to western blotting (n = 5). **C and D,** Wild-type (WT) and SOCS1-knockout (KO) BEAS-2B cells were transfected with STING expression plasmids for 48 hours and subsequently stimulated with IL-13 (10 ng/mL). Three hours or 24 hours later, cells or cell culture supernatants were subjected to western blotting **(C)** or ELISA (**D**), respectively (n = 6). For western blotting, representative blots are shown, and densitometric analysis of blots was performed. Data are

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773 presented as the mean and SEM and analyzed by one-way ANOVA with the Tukey's
774 *post hoc* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
775
776 **Figure 7. STING expression positively correlates with SOCS1 expression in nasal**
777 **epithelial cells in patients with CRSwNP. A,** The mRNA expression of SOCS1 in
778 nasal epithelial cells in different study groups as detected by quantitative RT-PCR.
779 SOCS1 mRNA expression positively correlated with STING mRNA expression but
780 negatively correlated with CCL26 mRNA expression in nasal epithelial cells in
781 patients with CRSwNP. Control, $n = 16$; Eos CRSwNP, $n = 16$; Non-Eos CRSwNP, n
782 = 16. **B,** The protein levels of SOCS1 in nasal epithelial cells in different study groups
783 as detected by western blotting. Control, $n = 15$; Eos CRSwNP, $n = 15$; Non-Eos
784 CRSwNP, $n = 15$. Representative blots are shown, and densitometric analysis of blots
785 was performed. Data are presented as median and interquartile ranges and analyzed
786 by Kruskal-Wallis test with the Dunn *post hoc* test. *$P < 0.05$, **$P < 0.01$, and ***$P <$
787 0.001. Spearman's correlation was used for correlation analysis. CRSwNP, chronic
788 rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

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Defective STING expression in epithelial cells potentiates IL-13 signaling in eosinophilic chronic rhinosinusitis with nasal polyps

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METHODS

Subjects

This study was approved by the Ethics Committee of Tongji Hospital of Huazhong University of Science and Technology and was conducted with written informed consent from every patient. For CRSwNP study, 96 control subjects, 91 patients with eosinophilic CRSwNP and 85 patients with noneosinophilic CRSwNP were included in this study. The diagnosis of CRSwNP was made according to the current American and European guidelines.[E1, E2] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of the total infiltrating cells, as reported by our previous study.[3] This cutoff was calculated as twice the standard deviation of the mean eosinophil percentage in control subjects.[E3] Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects. Patients with CRSwNP had bilateral nasal polyps.

Atopic status was evaluated by using skin prick test with a panel of common inhalant allergens and/or by using the ImmunoCAP to detect IgE antibodies against common inhalant allergens (Phadia, Uppsala, Sweden) in our region.[E4, E5] The diagnosis of allergic rhinitis was based on the concordance between the atopic status and typical allergic symptoms. Oral glucocorticoid and intranasal steroid spray were discontinued at least 3 months and 1 month before surgery, respectively. Antileukotrienes were discontinued at least 1 month before inclusion. Patients with an acute upper respiratory tract infection and acute asthma episode within 4 weeks of entering the study, and patients under immunotherapy were excluded. In addition,

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patients who had a history of antrochoanal polyps, cystic fibrosis, primary ciliary dyskinesia, fungal sinusitis, immunodeficiency, or systemic vasculitis were excluded from this study, because these are discrete disorders with unique pathophysiology. None of subjects had a history of aspirin sensitivity. The details of subjects' characteristics in CRSwNP study are summarized in Table E1.

Nasal polyp tissues from CRSwNP patients and inferior turbinate mucosal tissues from control subjects were collected during surgery, respectively. Human nasal epithelial cells (HNECs) were scraped from nasal polyps of CRSwNP patients and from inferior turbinate of control subjects using a sterile Rhino-Pro curette (Arlington Scientific Inc, Springville, UT) as previously described.[E6, E7] Each nasal scraping specimen yielded 1-2 × 10^6 cells, of which more than 95% were epithelial cells based on cytokeratin indirect immunofluorescence staining with anti-pan cytokeratin antibody (Abcam, Cambride, UK). Not all samples were included in every experiment protocol because of limited quantity. The numbers of samples for every experiment were indicated in figures or figure legends.

Histology and immunohistochemistry

Tissue samples were fixed in 4% formaldehyde solution and embedded in paraffin. Paraffin sections (4 μm) were prepared from paraffin-embedded human tissue samples. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin routinely to determine the general pathologic features and to count the number of eosinophils and total inflammatory cells as previously reported.[E3]

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Immunohistochemical and immunofluorescence staining were performed as previously reported.[E3, E8-E11] Sections were subjected to heat-induced epitope retrieval using citric acid repair solution (Guge Biotechnology, Wuhan, China). The 3% hydrogen peroxidase (Boster Biotechnology, Wuhan, China) was used for endogenous peroxidase inhibition and 5% bovine serum albumin (Guge Biotechnology) was used to block nonspecific binding.[E3, E9-E11] Sections were stained with primary antibodies shown in Table E2 overnight at 4°C. For immunohistochemical staining, antigens were detected by using the streptavidin-peroxidase complex method with a histostain-plus kit (Boster Biotechnology) according to the manufacturer's instructions.[E8] Color development was achieved with 3', 3'-diaminobenzidine, which rendered positive cells brown. Sections were counterstained with hematoxylin and mounted. The intensity of positive staining in epithelium detected by immunohistochemistry was analyzed by using image pro-plus 6.0 analysis software (Media Cybernetics, Inc. Silver Spring, MD, USA) and the results were presented as mean optical density value per unit area.[E6, E10, E12] For immunofluorescence staining, fluorescence conjugated secondary antibodies (Table E3) were used. Species- and subtype-matched antibodies were used as negative controls.

Immunofluorescence staining for cultured BEAS-2B cells was performed as previously described.[E13, E14] Briefly, after cultured, BEAS-2B cells were fixed using 4% paraformaldehyde for 30 minutes at room temperature. After washing steps, cells were permeabilized for 20 min using 0.2% Triton X-100 phosphate-buffered saline (PBST). Fixed and permeabilized cells were then blocked with 5% bovine serum

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albumin in PBS for 1 hour at room temperature and incubated with primary antibodies against STING (1:400, Abcam, Cambridge, UK), p-TBK1 (1:400, Cell signaling Technology, Boston, USA), p-IRF3 (1:400, Cell signaling Technology), and p-p65 (1:400, Cell signaling Technology) for 2 hours at room temperature, followed by the incubation with fluorophore-conjugated secondary antibodies (Table E3). The 4,6-diamidino-2-phenylindole was used as the nuclear counterstain. Images were taken under immunofluorescence microscope.

Quantitative RT-PCR

Total RNA was extracted from tissues and cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as mentioned elsewhere.[E3, E9, E10] A total of 1 μg RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China). cDNA equivalent to 50 ng of total RNA was used for PCR assay. Quantitative PCR was performed by using the SYBR Premix Ex Taq kit (TaKaRa Biotechnology) with specific primers (Table E4) on StepOnePlus (Applied Biosystems, Foster City, CA, USA). Amplification was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, specific annealing temperature for 10 sec, and 72 °C for 15 sec. After PCR, a melting curve was constructed by increasing the temperature from 65 to 95 °C with a temperature transition rate of 0.1°C/s. Relative gene expression was calculated by using the 2(-Delta Delta CT) method.[E15] A normal inferior turbinate mucosal sample and a control cell culture sample were used as the calibrator for tissue samples and cell culture samples, respectively.

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Beta-glucuronidase (GUSB) was used as a housekeeping gene for normalization of gene expression. No template sample was used as negative control.

Dispersed nasal tissue cells preparation and flow cytometry

Dispersed nasal tissue cells were prepared from nasal polyp and control tissues by means of mechanical dissociation with GentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA).[E10, E11] The resulting single cell suspension was filtered 2 times through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA) and washed twice with RPMI-1640 medium (HyClone).[E10] Cell pellets were suspended in erythrocyte lysis buffer and incubated for 5 mins. After washing with PBS, cells were resuspended in RPMI-1640 medium. For flow cytometry, cells were stained with fixable viability stain 700 (BD Bioscience, San Jose, CA, USA) to exclude dead cells. Cells were then incubated with APC conjugated mouse monoclonal antibody against human CD326 (1:20, Biolegend, San Diego, CA, USA) and PE-conjugated mouse monoclonal antibody against human CD45 (1:20, Biolegend) for 30 min at 4°C in the dark. For intracellular staining of STING, after CD45 and CD326 staining, cells were fixed and permeabilized using the BD cytofix/cytoperm kit (BD Biosciences) according to manufacturer's protocols, and cells were stained with rabbit polyclonal antibody against human STING (1 μg/mL, Abcam). After incubating for 20 min in the dark at room temperature, cells were incubated with FITC-conjugated F(ab')2 donkey anti-rabbit IgG H&L antibody (1:200, Biolegend, San Diego, CA, USA) for 20 min in dark at room temperature. Species and subtype-matched antibodies were used as

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negative controls. After staining, cells were analyzed by using LSRII flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Western blot analysis

Total cellular protein was extracted from tissues, HNECs and BEAS-2B cells in modified radio-immunoprecipitation assay buffer (Guge Biotechnology, Wuhan, China) containing 2% cocktail of protease inhibitors (Guge Biotechnology) as previously described.[E10] Protein concentrations of the extracts were measured with a BCA protein assay kit (Guge Biotechnology). Samples containing 30 μg of proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinyl difluoride membranes (Guge Biotechnology). The membranes were incubated with 5% fat-free skim milk in Tris-buffered saline with Tween-20 (TBS-T; Guge Biotechnology) for 1 hour at room temperature and then incubated overnight with primary antibodies as listed in Table E5. After washing 3 times in TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, goat anti-mouse, or rabbit anti-goat antibody, 1:4000, Guge Biotechnology) for 1 hour at room temperature. After that, the membranes were processed by using an ECL chemiluminescence reaction kit (Guge Biotechnology) and followed by exposure on chemiluminescent film to visualize the proteins. β-actin quantification was used as an internal standard to correct for variations in total protein loading. Densitometric

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analysis of the blots was performed by using the software AlphaEase FC (Alpha Innotech, Silicon Valley, CA, USA).

Cell culture

Primary human nasal epithelial cells (HNECs) were cultured with an air-liquid interface (ALI) method as stated elsewhere.[E6, E7, E16, E17] Briefly, the obtained HNECs were washed twice in Dulbecco modified Eagle medium (Thermo Fisher Scientific, Waltham, MA, USA) with penicillin/streptomycin and then grown submerged in bronchial epithelial cell basal medium (BEBM, Lonza, Walkersville, MD, USA) supplemented with SingleQuot Kit Suppl (Lonza) in a 6-well plates coated with rat tail collagen type I (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO_2-humidified atmosphere at 37°C. Upon confluence, the cells were passaged to gel-coated transwell culture inserts (0.4 μm pore size; Becton Dickison, Franklin Lake, NJ, USA) and were grown in bronchial epithelial cell growth medium (Lonza). When they were confluent, medium from the apical side of the culture was removed and medium below the insert was replaced with an air-liquid interface medium consisting of BEBM/Dulbecco's modified Eagle's medium H (50: 50; Invitrogen). HNECs were maintained at ALI culture for 21 days for the differentiation.[E25] After the HNECs were differentiated, they were stimulated with IL-4, IL-6, IL-10, IL-12, IL-13, IL-17A, TNF-α, IL-1β, IFN-γ and IL-22 at 10 ng/mL (R&D systems, Minneapolis, MN, USA), poly (I:C) (dsRNA) at 25 μg/mL (Tocris, Bristol, UK), lipopolysaccharides (LPS) (InvivoGen, San Diego, CA, USA) at 1 μg/mL, staphylococcal enterotoxin B (SEB) (Millipore,

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Merck KGaA, Darmstadt, Germany) at 1 μg/mL, CpG (InvivoGen) at 2 μg/mL, and *Dermatophagoides pteronyssinus* group 1 (Der p1) at 10 μg/mL (Prospec, NJ, USA) for 6 hours. In dose response experiments, HNECs cells were treated with IL-4 (1, 10, and 100 ng/mL, R&D systems), or IL-13 (1, 10, 100 ng/mL, R&D systems) for 6 hours or 24 hours.

Since HNECs may lose *in vivo* disease-specific expression pattern of STING under long-term ALI culture, we cultured HNECs submerged when comparing the differences in HNECs from different study groups. HNECs were cultured submerged in BEBM (Lonza) in 6-well plates coated with rat tail collagen type I (Sigma-Aldrich) in a 5% CO_2-humidified atmosphere at 37 °C as previously described.[E12] When the cells reach 80 to 90% confluence, HNECs were treated with 2'3'-cGAMP (10 μg/mL, R&D systems) or IL-13 (10 ng/mL, R&D systems) for 3, 6 or 24 hours. After stimulation, cells were harvested for quantitative RT-PCR or western blotting assays, and cell culture supernatants were collected for ELISA.

BEAS-2B cells (American Type Culture Collection, Manassas, VA), a human bronchial epithelial cell, were cultured submerged as mentioned elsewhere.[E12] The BEAS-2B cells were cultured with Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) in a 6-well plates at 37 □ with 5% CO_2 in humidified air. When the cells reach 80 to 90% confluence, medium was changed to a serum-free one for further study.

Transfection and functional assay

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Considering the high transfection efficiency and cell viability of cell lines, BEAS-2B cells were used for transfection assays unless specifically stated. Small interfering RNA (siRNA) transfection was performed as previously described.[E12] When reaching 80 to 90% confluence, BEAS-2B cells were transfected with si-STING (50 nM, Thermo Fisher Scientific Waltham, MA, USA), si-STAT6 (50 nM, RiBo biotechnology, Guangzhou, China) or their corresponding negative control siRNA (si-NS) using Lipofectamine 3000 transfection agent (Thermo Fisher Scientific) for 24 hours under the manufacturer's instruction. The sequences are as follows: *STING* siRNA, 5'-GGCCCGGAUUCGAACUUACAAUCAG-3', *STAT6* siRNA, 5'-AGATGAGCCTGCCCTTTGA-3'. In some experiments, cells were further transfected with 2'3'-cGAMP (10 μg/mL, R&D systems) for 24 hours with or without subsequent stimulation with IL-13 (10 ng/mL, R&D systems) for another 6 hours or 24 hours.

For plasmid transfection, when reached 80 to 90% confluence, BEAS-2B cells or submerged cultured HNECs from eosinophilic nasal polyps were transfected with plasmid encoding *TMEM173* (GenBank accession no. NM_198282, 2 μg, Vigene Biosciences, Shandong, China) or empty plasmid (Vigene Biosciences) by using Lipofectamine 3000 transfection agent (Thermo Fisher Scientific) under the manufacturer's instruction for 24 or 48 hours. After transfection, cells were further treated with IL-13 (10 ng/mL, R&D systems) for 3, 6 or 24 hours. After culture, cells and cell culture supernatants were harvested and subjected to quantitative RT-PCR and western blot analysis, and ELISA, respectively.

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237 **Generation of TBK1, IRF3, and SOCS1 knockout BEAS-2B cells**

238 Recombinant lentiviruses carrying single-guide RNA targeting human *TBK1*
239 gene (GenBank accession no. NM_013254, GeneChem, Shanghai, China), *IRF3*
240 (GenBank accession no. NM_001197124, GeneChem) or *SOCS1* (GenBank accession
241 no. NM_003745, GeneChem), or a nonspecific control sequence (GeneChem) were
242 used to infect BEAS-2B cells according to the manufacturer's protocol at a
243 multiplicity of infection of 100 in medium containing polybrene (5 μg/mL,
244 GeneChem). The sequences are as follows: *TBK1* sgRNA, 5'-
245 GAAGAACCTTCTAATGCCTA -3', *IRF3* sgRNA,
246 5'-GGGAGTGGGATTGTCCAAGC-3', *SOCS1* sgRNA,
247 5'-AGTAGAATCCGCAGGCGTCC-3'. GFP-targeting gRNA were used as a
248 negative control: *GFP* sgRNA (control), 5'- CGCTTCCGCGGCCCGTTCAA-3'.
249 Transfection efficiency was identified by observing fluorescence under fluorescence
250 microscope three days after transfection. Infected cells were selected in puromycin (2
251 μg/mL) for two weeks. Knockout efficiency of TBK1, IRF3, and SOCS1 was verified
252 7 days post-transfection by western blot analysis. After transfection, BEAS-2B cells
253 were transfected with a STING expression plasmid as mentioned above. In some
254 experiments, cells were further stimulated with IL-13 (10 ng/mL, R&D systems) for 3
255 or 6 hours. After culture, cells were harvested and subjected to quantitative RT-PCR
256 and western blotting.

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Promoter-luciferase reporter constructs and luciferase assay

DNA fragments containing wild-type and mutated STAT6 binding sites of STING promoter were generated by PCR and were cloned into a firefly luciferase reporter construct (pGL3 vector; Promega, Madison, WI). For luciferase report experiments, BEAS-2B cells were transfected with wild-type or mutated constructs (1 μg per well) together with renilla luciferase plasmids (200 ng per well) by using Lipofectamine 3000 transfection agent (Thermo Fisher Scientific) under the manufacturer's instruction for 24 hours. After transfection, BEAS-2B cells were further treated with IL-13 (10 ng/mL, R&D systems) for 16 hours. In some experiments, transfected cells were treated with AS1517499 (100 nM, Axon Medchem BV, Groningen, Netherlands) or transfected with si-STAT6 (50 nM, Thermo Fisher Scientific) before IL-13 stimulation. AS1517499 was added to culture medium 30 minutes and si-STAT6 were transfected 24 hours before the IL-13 treatment, respectively. Cell lysates were harvested by passive lysis buffer (Promega) and analyzed for luciferase activity using the dual luciferase reporter assay kit (Promega).

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REFERENCES

E1. Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2020. Rhinology 2020; 58:1-464.

E2. Orlandi RR, Kingdom TT, Hwang PH, Smith TL, Alt JA, Baroody FM, et al. International Consensus Statement on Allergy and Rhinology: Rhinosinusitis. Int Forum Allergy Rhinol 2016; 6 Suppl 1:S22-209.

E3. 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. Journal Of Allergy And Clinical Immunology 2009; 124:478-U135.

E4. Wang Y, Chen H, Zhu R, Liu G, Huang N, Li W, et al. Allergic Rhinitis Control Test questionnaire-driven stepwise strategy to improve allergic rhinitis control: a prospective study. Allergy 2016; 71:1612-9.

E5. Qi S, Chen H, Huang N, Li W, Liu G, Wang Y, et al. Early Intervention Improves Clinical Responses to House Dust Mite Immunotherapy in Allergic Rhinitis Patients. Int Arch Allergy Immunol 2016; 171:234-40.

E6. Liao B, Cao PP, Zeng M, Zhen Z, Wang H, Zhang YN, et al. Interaction of thymic stromal lymphopoietin, IL-33, and their receptors in epithelial cells in eosinophilic chronic rhinosinusitis with nasal polyps. Allergy 2015; 70:1169-80.

E7. Zhang XH, Zhang YN, Li HB, Hu CY, Wang N, Cao PP, et al. Overexpression of miR-125b, a novel regulator of innate immunity, in eosinophilic chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med 2012; 185:140-51.

E8. Cao PP, Zhang YN, Liao B, Ma J, Wang BF, Wang H, et al. Increased local IgE production induced by common aeroallergens and phenotypic alteration of mast cells in Chinese eosinophilic, but not non-eosinophilic, chronic rhinosinusitis with nasal polyps. Clin Exp Allergy 2014; 44:690-700.

E9. Song J, Wang H, Zhang YN, Cao PP, Liao B, Wang ZZ, et al. Ectopic lymphoid tissues support local immunoglobulin production in patients with chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2018; 141:927-37.

E10. Wang H, Li ZY, Jiang WX, Liao B, Zhai GT, Wang N, et al. The activation and function of IL-36gamma in neutrophilic inflammation in chronic rhinosinusitis. J Allergy Clin Immunol 2018; 141:1646-58.

E11. Zhang YN, Song J, Wang H, Wang H, Zeng M, Zhai GT, et al. Nasal IL-4(+)CXCR5(+)CD4(+) T follicular helper cell counts correlate with local IgE production in eosinophilic nasal polyps. J Allergy Clin Immunol 2016; 137:462-73.

Journal Pre-proof

E12. Wang BF, Cao PP, Wang ZC, Li ZY, Wang ZZ, Ma J, et al. Interferon-gamma-induced insufficient autophagy contributes to p62-dependent apoptosis of epithelial cells in chronic rhinosinusitis with nasal polyps. Allergy 2017; 72:1384-97.

E13. Xia T, Konno H, Barber GN. Recurrent Loss of STING Signaling in Melanoma Correlates with Susceptibility to Viral Oncolysis. Cancer Res 2016; 76:6747-59.

E14. Jiao J, Wang M, Duan S, Meng Y, Meng N, Li Y, et al. Transforming growth factor-beta1 decreases epithelial tight junction integrity in chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2018; 141:1160-3 e9.

E15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

E16. 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 e10.

E17. de Borja Callejas F, Martinez-Anton A, Alobid I, Fuentes M, Cortijo J, Picado C, et al. Reconstituted human upper airway epithelium as 3-d in vitro model for nasal polyposis. PLoS One 2014; 9:e100537.

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Table E1: The demographic characteristics of subjects.

	Control	Eos CRSwNP	Non-Eos CRSwNP	*P value*
Total subjects enrolled	96	91	85	
Methodology used				
Immunohistochemistry and immunofluorescence				
Subject number	20	23	24	
Gender, male	13 (65%)	16 (70%)	15 (60%)	*0.876*[#]
Age (years)	39 (31, 51)	45 (32, 53)	38 (28, 49)	*0.472*
Patients with atopy	0 (0%)	6 (26%)	2 (8%)	*0.025*[#]
Patients with AR	0 (0)	3 (13%)	1 (4%)	*0.177*[#]
Patients with asthma	0 (0)	3 (13%)	1 (4%)	*0.177*[#]
RT-PCR				
Subject number	32	29	28	
Gender, male	22 (69%)	20 (69%)	19 (68%)	*0.995*[#]
Age (years)	34 (23, 48)	44 (31, 53)	46 (22, 56)	*0.316*
Patients with atopy	1 (3%)	6 (21%)	2 (7%)	*0.062*[#]
Patients with AR	0 (0)	4 (14%)	1 (4%)	*0.056*[#]
Patients with asthma	0 (0)	2 (7%)	0 (0%)	*0.200*[*]
Flow cytometry				
Subject number	15	16	15	

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Gender, male	10 (67%)	12 (75%)	11 (73%)	*0.864*[#]
Age (years)	34 (27, 39)	39 (31, 56)	38 (29, 46)	*0.433*
Patients with atopy	1 (7%)	3 (19%)	1 (7%)	*0.455*[#]
Patients with AR	0 (0)	2 (13%)	0 (0%)	*0.319*[*]
Patients with asthma	0 (0)	1 (7%)	0 (0%)	*0999*[*]
Western blot				
Subject number	15	15	15	
Gender, male	10 (67%)	9 (60%)	8 (53%)	*0.758*[#]
Age (years)	43 (17, 62)	47 (27, 52)	53 (38, 60)	*0.715*
Patients with atopy	0 (0%)	5 (33%)	1 (7%)	*0.018*[#]
Patients with AR	0 (0)	3 (20%)	0 (0%)	*0.096*[*]
Patients with asthma	0 (0)	0 (0%)	0 (0%)	-
Cell culture study				
Subject number	23	20	14	
Gender, male	13 (57%)	15 (75%)	10 (71%)	*0.400*[#]
Age (years)	29 (22, 40)	33 (21, 53)	41 (21, 46)	*0.705*
Patients with atopy	0 (0%)	6 (30%)	1 (7%)	*0.009*[#]
Patients with AR	0 (0%)	2 (10%)	0 (0%)	*0.176*[*]
Patients with asthma	0 (0%)	1 (5%)	0 (0%)	*0.596*[*]

For continuous variables, results are expressed as medians and interquartile ranges. Categorical variables are summarized using percentage.

[#] Data were analyzed by using Chi-square test.

[*] Data were analyzed by using Fisher's exact test.

Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, noneosinophilic chronic rhinosinusitis with nasal polyps.

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Table E2. Primary antibodies used in immunohistochemistry and immunofluorescence.

Antibody	Application	Species	Concentration	Clone ID	Reference	Source
STING	IHC, IF	Rabbit	1:200	EPR13130	Ab181125	Abcam, Cambridge, UK
SOCS1	IF	Goat	1:100	polyclonal	Ab9870	Abcam
p-TBK1	IF	Rabbit	1:50	D52C2	5483	Cell signaling Technology, Boston, USA
p-IRF3	IF	Rabbit	1:50	D601M	29047	Cell signaling Technology
p-P65	IF	Rabbit	1:50	93H1	3033	Cell signaling Technology
CD138	IF	Goat	1:100	polyclonal	AF2780	R&D systems
MBP	IF	Mouse	1:100	BMK13	Ab14462	Abcam
MPO	IF	Mouse	1:100	2C7	Ab25989	Abcam
Elastase	IF	Mouse	1:100	NP57	Ab254178	Abcam
CD3	IF	Mouse	1:100	B1.1	TA320268	Zhongshan Golden Bridge Biotechnology, Beijing, China
CD20	IF	Mouse	1:100	OTI1H4	TA800394	Zhongshan Golden Bridge Biotechnology
CD68	IF	Mouse	1:100	KP1	ZM-0060	Zhongshan Golden Bridge Biotechnology
CD11c	IF	Mouse	1:100	FK24	Ab23602	Abcam

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KRT-5	IF	Mouse	1:100	XM26	Ab17130	Abcam
KRT-8	IF	Mouse	1:100	M20	Ab9023	Abcam
Foxj1	IF	Mouse	1:100	CL3989	Ab220028	Abcam

371 STING, stimulator of interferon genes; SOCS1, suppressor of cytokine signaling 1;
372 p-TBK1, phosphorylated TANK-binding kinase 1; p-IRF3, phosphorylated interferon
373 regulatory factor 3; MBP, major basic protein; MPO, myeloperoxidase; KRT, keratin;
374 Foxj1, forkhead box protein j 1; IHC, immunohistochemistry; IF,
375 immunofluorescence.
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Table E3. Secondary antibodies used in immunofluorescence staining.

Antibody	Concentration	Clone ID	Reference	Source
IFKine™ Green donkey anti-Rabbit IgG	1:100	polyclonal	A24221	Abbkine Scientific Company (Wuhan, China)
IFKine™ Red donkey anti-mouse IgG	1:100	polyclonal	A24411	Abbkine
IFKine™ Red donkey anti-Goat IgG	1:100	polyclonal	A24431	Abbkine

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412 **Table E4. Primers used for quantitative RT-PCR analysis.**

Primer	Sequence	Annealing temperature (□)	Expect product size (bp)
STING	(F)5'-CACTTGGATGCTTGCCCTC-3' (R)5'-GCCACGTTGAAATTCCCTTTTT-3'	60	117
SOCS1	(F)5'-TTTTCGCCCTTAGCGTGAAGA-3' (R)5'-GAGGCAGTCGAAGCTCTCG-3'	60	107
SOCS2	(F)5'-TTAAAAGAGGCACCAGAAGGAAC-3' (R)5'-AGTCGATCAGATGAACCACACT-3'	60	199
SOCS3	(F)5'-CCTGCGCCTCAAGACCTTC-3' (R)5'-GTCACTGCGCTCCAGTAGAA-3'	60	99
IFN-α	(F)5'-CAGAGTCACCCATCTCAGCA-3' (R)5'-CACCACCAGGACCATCAGTA-3'	60	86
IFN-β	(F)5'-GCGACACTGTTCGTGTTGTCA-3' (R)5'-CCAAGCAAGTTGTAGCTCATGGA-3'	60	106
IL-5	(F)5'-CCCACAAGTGCATTGGTGAA -3' (R)5'-CCTCAGAGTCTCATTGGCTATCAG -3'	56	81
IL-13	(F)5'-CCTCATGGCGCTTTTGTTGAC-3' (R)5'-TCTGGTTCTGGGTGATGTTGA-3'	62	134
IL-17A	(F)5'-CAAGACTGAACACCGACTAAG-3' (R)5'-TCTCCAAAGGAAGCCTGA-3'	60	230
IFN-γ	(F)5'-TCAGCTCTGCATCGTTTTGG -3' (R)5'-GTTCCATTATCCGCTACATCTGAA -3'	55	120
CCL11	(F)5'-CCCCTTCAGCGACTAGAGAG -3' (R)5'-TCTTGGGGTCGGCACAGAT -3'	60	109
CCL24	(F)5'-GGAGTGGGTCCAGAGGTACAT -3' (R)5'-CAGGTGGTTTGGTTGCCAG -3'	60	120
CCL26	(F)5'-GGAACTGCCACACGTGGGAGTGAC-3' (R)5'-CTCTGGGAGGAAACACCCTCTCC-3'	60	354
GUSB	(F)5'-GACACGCTAGAGCATGAGGG-3' (R)5'-GGGTGAGTGTGTTGTTGATGG-3'	60	121

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413 STING, stimulator of interferon genes; SOCS, suppressor of cytokine signaling; IFN,
414 interferon; BAFF, B cell-activating factor of the tumor necrosis factor family; CXCL,
415 chemokine (C-X-C motif) ligand; IL, interleukin; IFN-γ, interferon-γ; CCL,
416 chemokine (C-C motif) ligand; STAT, signal transducer and activator of transcription;
417 GUSB, beta-glucuronidase

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419 **Table E5. Primary antibodies used in western blotting**

Antibody	Species	Concentration	Clone ID	Reference	Source
STING	Rabbit	1:4000	EPR13130	Ab181125	Abcam, Cambridge, UK
SOCS1	Goat	1:1000	polyclonal	Ab9870	Abcam
SOCS2	Rabbit	1:1000	EPR2588	Ab109245	Abcam
SOCS3	Rabbit	1:1000	polyclonal	Ab16030	Abcam
p-TBK1	Rabbit	1:1000	D52C2	5483	Cell signaling Technology, Boston, USA
TBK1	Rabbit	1:1000	D1B4	3504	Cell signaling Technology
p-IRF3	Rabbit	1:1000	D601M	29047	Cell signaling Technology
IRF3	Rabbit	1:1000	D6I4C	11904	Cell signaling Technology
p-P65	Rabbit	1:1000	93H1	3033	Cell signaling Technology
P65	Rabbit	1:1000	D14E12	8242	Cell signaling Technology
STAT6	Rabbit	1:1000	D3H4	5397	Cell signaling Technology
p-STAT6	Rabbit	1:1000	D8S9Y	56554	Cell signaling Technology

420 STING, stimulator of interferon genes; SOCS, suppressor of cytokine signaling;
421 TBK1, TANK binding kinase 1; p-TBK1, phosphorylated TANK-binding kinase 1;

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IRF3, interferon regulatory factor 3; p-IRF3, phosphorylated interferon regulatory factor 3; STAT6, signal transducer and activator of transcription 6; p-STAT6, phosphorylated signal transducer and activator of transcription 6.

FIGURE LEGENDS

Fig E1. Mucosal type 1/type 17 cytokine profiles and their association with STING expression in patients with CRSwNP. A, The mRNA expression levels of IL-17A and IFN-γ in sinonasal mucosal samples in different study groups as detected by quantitative RT-PCR. Data are presented as median and interquartile ranges and analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. Control, $n = 16$; Eos CRSwNP, $n = 16$; Non-Eos CRSwNP, $n = 16$. $^{*}P < 0.05$ and $^{***}P < 0.001$. **B,** No correlation between STING mRNA expression and IL-17A or IFN-γ mRNA expression in patients with CRSwNP ($n = 32$). Spearman's correlation was used for correlation analysis. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Fig E2. STING expression in infiltrating immune cells in nasal polyps. Double immunofluorescence staining of nasal polyp tissue sections demonstrates that STING is expressed by $CD3^+$ T cells, $CD20^+$ B cells, $CD11c^+$ dendritic cells, $CD68^+$ macrophages, $CD138^+$ plasma cells, major basic protein (MBP) positive eosinophils, and myeloperoxidase (MPO) and elastase positive neutrophils in nasal polyps.

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Representative photomicrographs are shown (original magnification × 400). Scale bar, 100 μm. Arrows indicate the representative positive cells. For $CD3^+$ T cells, $CD20^+$ B cells, $CD68^+$ macrophages, $CD138^+$ plasma cells, and major basic protein (MBP) positive eosinophils, representative photomicrographs of tissue sections from patients with eosinophilic CRSwNP are shown; for $CD11c^+$ dendritic cells, MPO^+ neutrophils, and $elastase^+$ neutrophils, representative photomicrographs of tissue sections from patients with noneosinophilic CRSwNP are shown.

Fig E3. Immunofluorescence staining (left) and quantification (right) of CD3 (**A**), CD4 (**B**), CD8 (**C**), CD11c (**D**), CD20 (**E**), CD68 (**F**), MBP (**G**), elastase (**H**), and MPO (**I**) positive cells in the sinonasal mucosa (original magnification: ×400). Scale bar, 100 μm. Data are presented as median and interquartile ranges and analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. $*P < 0.05$ and $***P < 0.001$. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic; MBP, major basic protein; MPO, myeloperoxidase.

Fig E4. A) Representative immunofluorescence staining of nasal polyp tissue sections demonstrates that STING is expressed by $KRT\text{-}5^+$ basal cells (original magnification × 400). Scale bar, 100 μm. Arrows indicate representative $KRT\text{-}5^+$ basal cells. **B**) Representative immunofluorescence staining of nasal polyp tissue sections demonstrates that STING is expressed by $KRT\text{-}8^+$ apical cells (original magnification × 400). Scale bar, 100 μm. Arrows indicate representative $KRT\text{-}8^+$ apical cells. **C**)

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Representative immunofluorescence staining of nasal polyp tissue sections demonstrates that STING is expressed by $Foxj1^+$ ciliated cells (original magnification × 400). Scale bar, 100 μm. Arrows indicate representative $Foxj1^+$ ciliated cells. For **A** and **B**, representative photomicrographs of tissue sections from patients with eosinophilic CRSwNP are shown; for **C**, representative photomicrographs of tissue sections from patients with noneosinophilic CRSwNP are shown. Foxj1, forkhead box protein j 1; KRT, keratin.

Fig E5. The immunoreactivity of STING in sinonasal mucosal tissues. The representative photomicrographs are shown (original magnification × 400). The staining intensity in epithelium was quantified and the results were presented as average optical density value per unit area. Scale bar, 100 μm. Control, n = 12; Eos CRSwNP, n = 16; Non-Eos CRSwNP, n = 16. Data are presented as median and interquartile ranges and analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. $^{**}P < 0.01$ and $^{***}P < 0.001$. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Fig E6. **Regulation of mRNA expression of STING in human nasal epithelial cells.** Human nasal epithelial cells (HNECs) scraped from inferior turbinate mucosa of control subjects were cultured with an air-liquid interface method. **A,** After differentiation, HNECs were stimulated with *Dermatophagoides pteronyssinus* group 1 (Der p1) at 10 μg/mL, poly (I:C) (dsRNA) at 25 μg/mL, staphylococcal enterotoxin

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B (SEB) at 1 μg/mL, lipopolysaccharides (LPS) at 1 μg/mL, CpG at 2 μg/mL, and IL-1β, TNF-α, IL-4, IL-6, IL-12, IL-13, IFN-γ, IL-17A, IL-22, and IL-10 at 10 ng/mL for 6 hours (n = 4-5). **B,** HNECs were stimulated with IL-4 or IL-13 at the indicated concentrations for 6 hours. After stimulation, cells were harvested for quantitative RT-PCR assay (n = 5). Data are presented as mean and SEM, and analyzed by unpaired Student's t test. *$P < 0.05$ and ***$P < 0.001$, compared with controls.

Fig E7. The transfection efficiency of si-STAT6 in BEAS-2B cells. BEAS-2B cells were transfected with si-STAT6 (50 nM) or control siRNA for 24 hours. The transfection efficiency was confirmed at mRNA (**A**) and protein (**B**) expression level by RT-PCR and western blotting, respectively. Representative blots are shown. *$P <$ 0.05. Data of 5 independent experiments are presented as mean and SEM and analyzed by unpaired Student's t test.

Fig E8. 2'3-cGAMP activates canonical STING pathway and upregulates IFN-α and IFN-β mRNA expression in BEAS-2B cells. A, Immunofluorescence microscopy analysis of STING, p-TBK1, p-IRF3 and p-p65 in BEAS-2B cells transfected with 2'3'-cGAMP (10 μg/mL) or vehicle for 3 hours. The representative photomicrographs are shown (original magnification × 400). Scale bar, 20 μm. **B,** BEAS-2B cells were transfected with 10 μg/mL 2'3-cGAMP for 6 hours. Cells were then harvested for quantitative RT-PCR assay of IFN-α and IFN-β mRNA expression (n = 6). Data are presented as mean and SEM and analyzed by unpaired Student's t

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test. $^{**}P < 0.01$.

Fig E9. CCL11, CCL24 and CCL26 expression in nasal epithelial cells and their associations with tissue eosinophilia in patients with CRSwNP. A, The mRNA expression of CCL11, CCL24, and CCL26 in nasal epithelial cells in different study groups as detected by quantitative RT-PCR. Control, $n = 16$; Eos CRSwNP, $n = 16$; Non-Eos CRSwNP, $n = 16$. Data are presented as median and interquartile ranges and analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. **B,** The correlations between CCL11, CCL24, and CCL26 mRNA expression in nasal epithelial cells and tissue eosinophilia in patients with CRSwNP. Spearman's correlation was used for correlation analysis. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

Fig E10. The transfection efficiency of si-STING and STING expression plasmid in BEAS-2B cells. BEAS-2B cells were transfected with si-STING (50 nM) **(A and B)**, STING expression plasmid (2 μg) **(C and D)** or their corresponding negative control using Lipofectamine 3000 transfection agent for 24 hours. The transfection efficiency of si-STING was confirmed at mRNA (**A**) and protein (**B**) expression level by RT-PCR and western blotting, respectively. The transfection efficiency of STING expression plasmid was also confirmed at mRNA (**C**) and protein (**D**) expression level by RT-PCR and western blotting, respectively. Representative blots are shown. Data of 5 independent experiments are presented as mean and SEM and analyzed by

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unpaired Student's t test. *$P < 0.05$.

Fig E11. STING overexpression is unable to inhibit CCL26 mRNA expression in SOCS1-KO BEAS-2B cells. Wild-type (WT) and SOCS1-knockout (KO) BEAS-2B cells were transfected with STING expression or control plasmid for 48 hours and subsequently stimulated with IL-13 (10 ng/mL) for another 6 hours. Cells were then harvested for quantitative RT-PCR assay of CCL26 mRNA expression ($n = 6$). Data are presented as mean and SEM and analyzed by one-way ANOVA with the Tukey's *post hoc* test. *$P < 0.05$ and **$P < 0.01$.

Fig E12. Representative western blot films of SOCS1 protein expression in wild-type (WT) and SOCS1-knockout (KO) BEAS-2B cells.

Fig E13. Representative western blot films of TBK1 (**A**) and IRF3 (**B**) protein expression in wild-type (WT) and TBK1- and IRF3-knockout (KO) BEAS-2B cells.

Fig E14. TBK1 and IRF3 knockout impair the mRNA production of IFN-α and IFN-β induced by 2'3-cGAMP in BEAS-2B cells. A, Wild-type (WT) and TBK1-knockout (KO) BEAS-2B cells were transfected with 10 μg/mL 2'3-cGAMP for 6 hours. Cells were then harvested for quantitative RT-PCR assay of IFN-α and IFN-β ($n = 6$). **B,** WT and IRF3-KO BEAS-2B cells were transfected with 10 μg/mL 2'3-cGAMP for 6 hours. Cells were then harvested for quantitative RT-PCR assay of

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IFN-α and IFN-β (n = 6). Data are presented as mean and SEM and analyzed by one-way ANOVA with the Tukey's *post hoc* test. ***$P < 0.001$.

Fig E15. STING-induced SOCS1 expression is unrelated to canonical TBK-IFR3-type I IFN pathway. A, Wild-type (WT) and TBK1-knockout (KO) BEAS-2B cells were transfected with STING expression or control plasmid for 48 hours. Then the cells were subjected to western blotting (n = 5). **B,** WT and IRF3-KO BEAS-2B cells were transfected with STING expression or control plasmid for 48 hours. Then the cells were subjected to western blotting (n = 5). **C,** BEAS-2B cells were stimulated with IFN-β (10 ng/mL) for 6 hours. Cells were harvested for quantitative RT-PCR assay of SOCS1, BAFF, CXCL10 and CXCL11 expression (n = 6). For western blotting, representative blots are shown and densitometric analysis of blots was performed. Data are presented as mean and SEM and analyzed by unpaired Student's t test. ***$P < 0.001$.

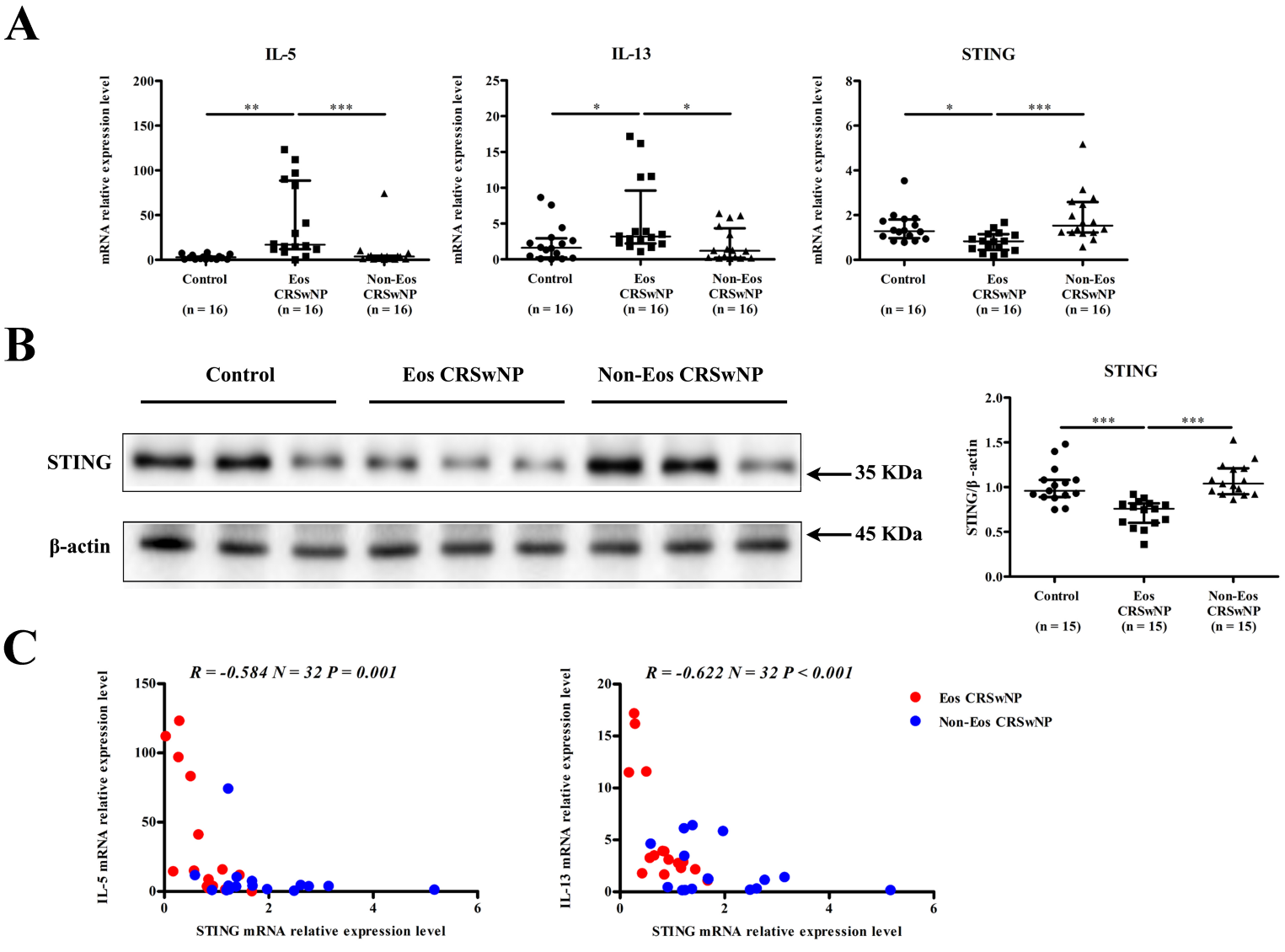
Fig E16. Expression of SOCS1 in nasal epithelial cells as detected by immunofluorescence staining. Representative photomicrographs from patients with noneosinophilic CRSwNP are shown (original magnification × 400). Scale bar, 100 μm.

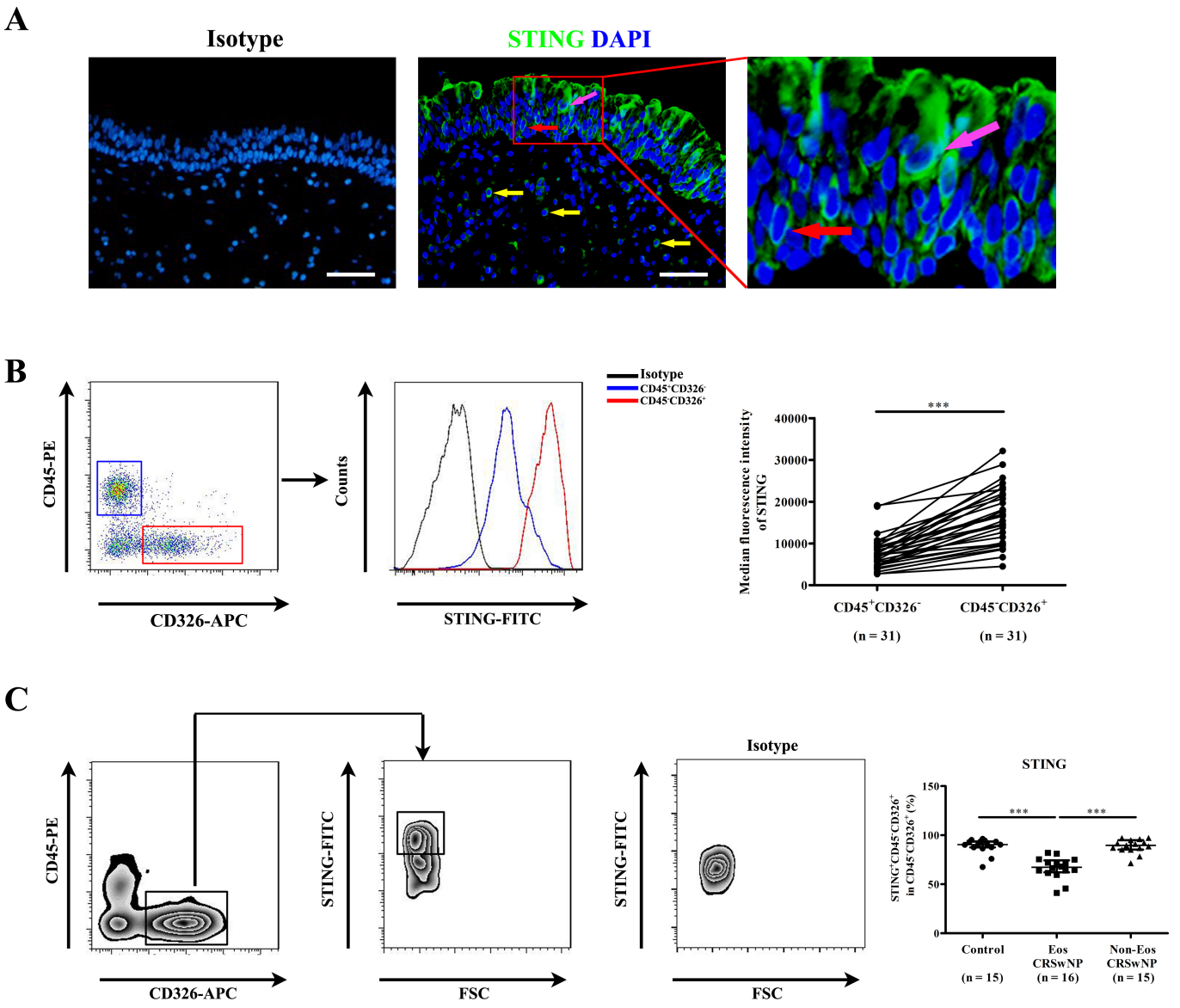
Fig E17. Assessment of SOCS2 and SOCS3 mRNA expression in nasal epithelial cells and their associations with STING expression in patients with CRSwNP. A,

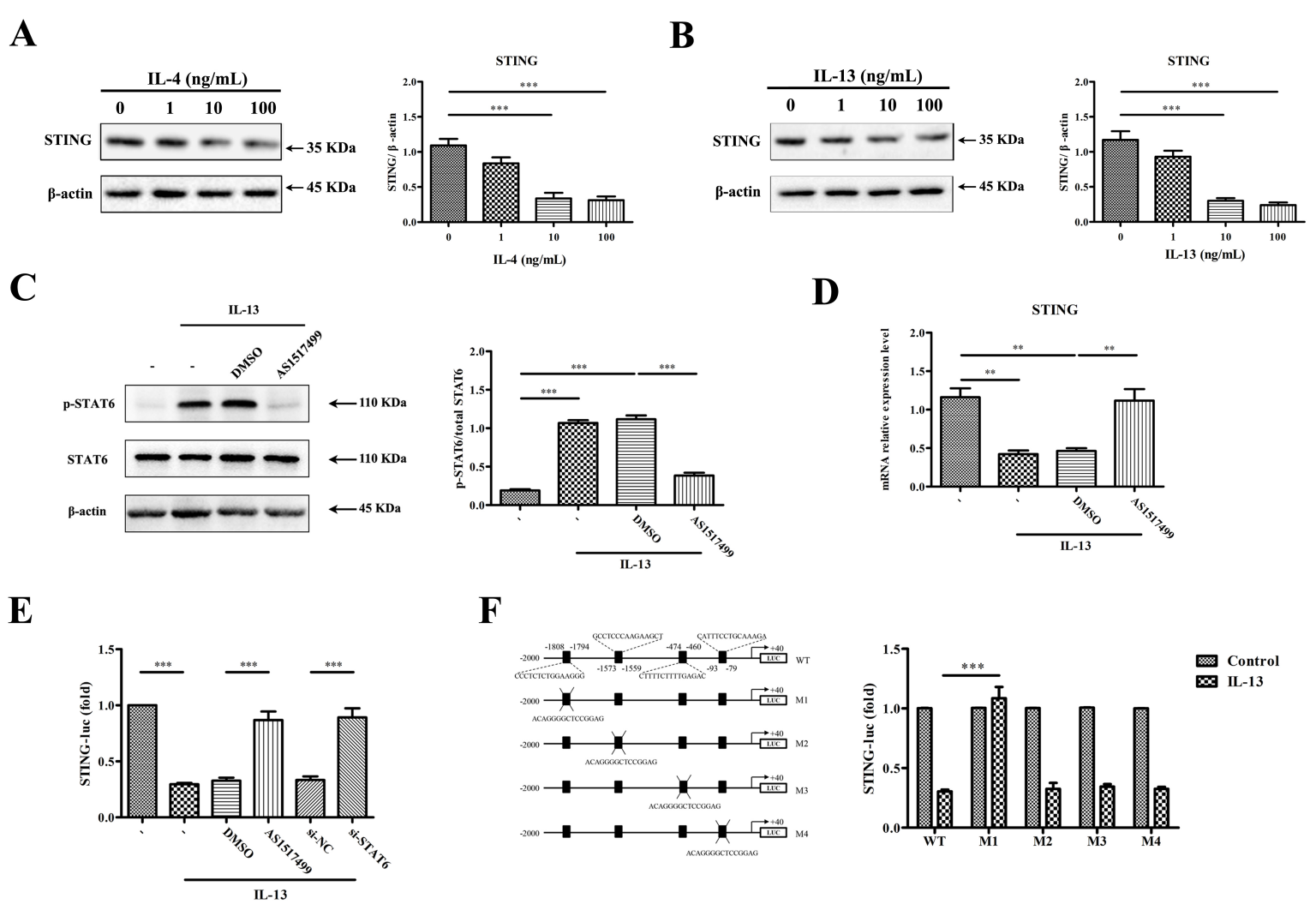
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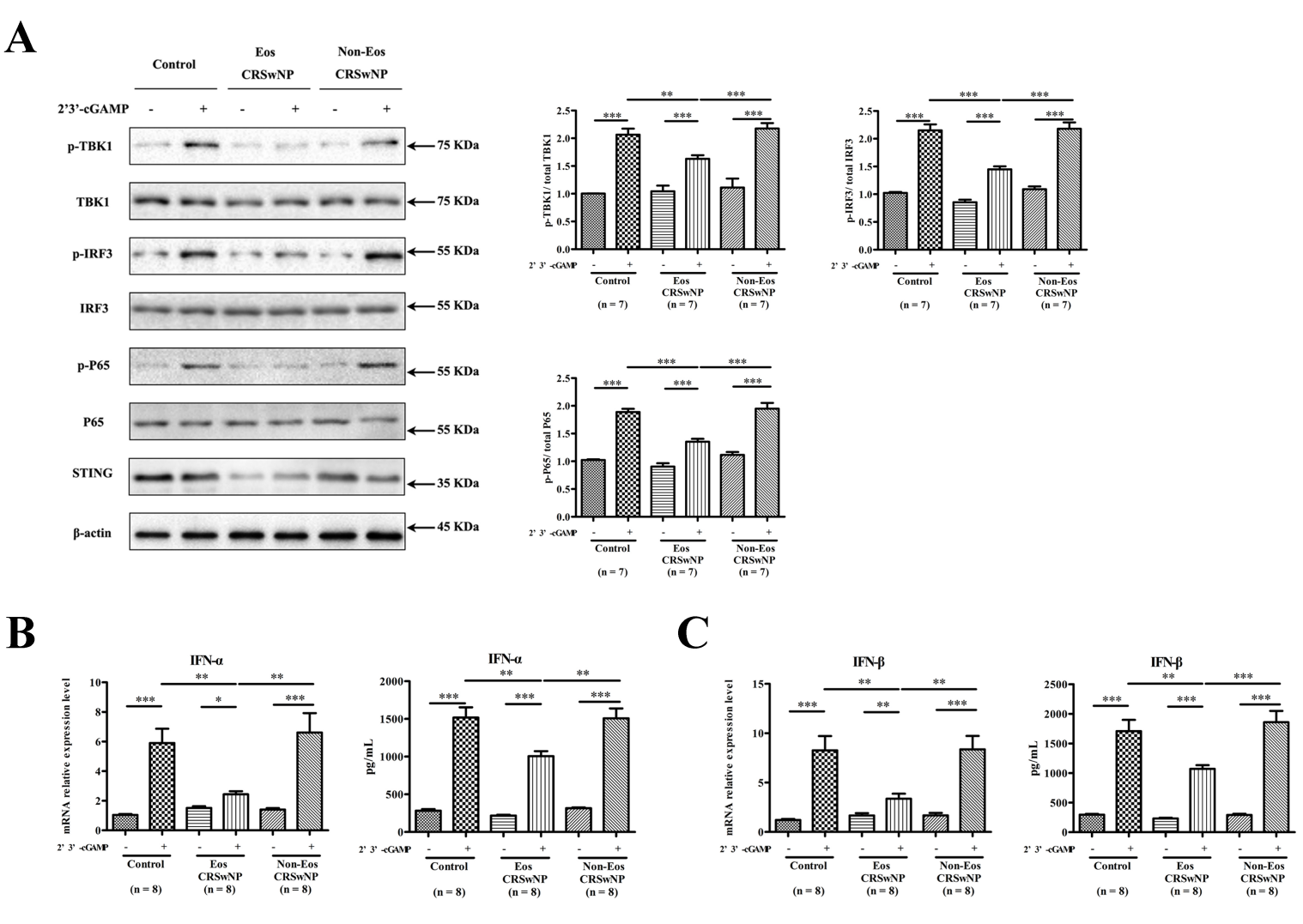
The mRNA expression of SOCS2 and SOCS3 in nasal epithelial cells in different study groups as assessed by RT-PCR. Control, *n* = 16; Eos CRSwNP, *n* = 16; Non-Eos CRSwNP, *n* = 16. Data were presented as median and interquartile ranges and analyzed by Kruskal-Wallis test with the Dunn *post hoc* test. **B,** No correlation of STING mRNA expression with SOCS2 or SOCS3 mRNA expression in nasal epithelial cells in patients with CRSwNP (*n* = 32). Spearman's correlation was used for correlation analysis. CRSwNP, chronic rhinosinusitis with nasal polyps; Eos, eosinophilic; Non-Eos, noneosinophilic.

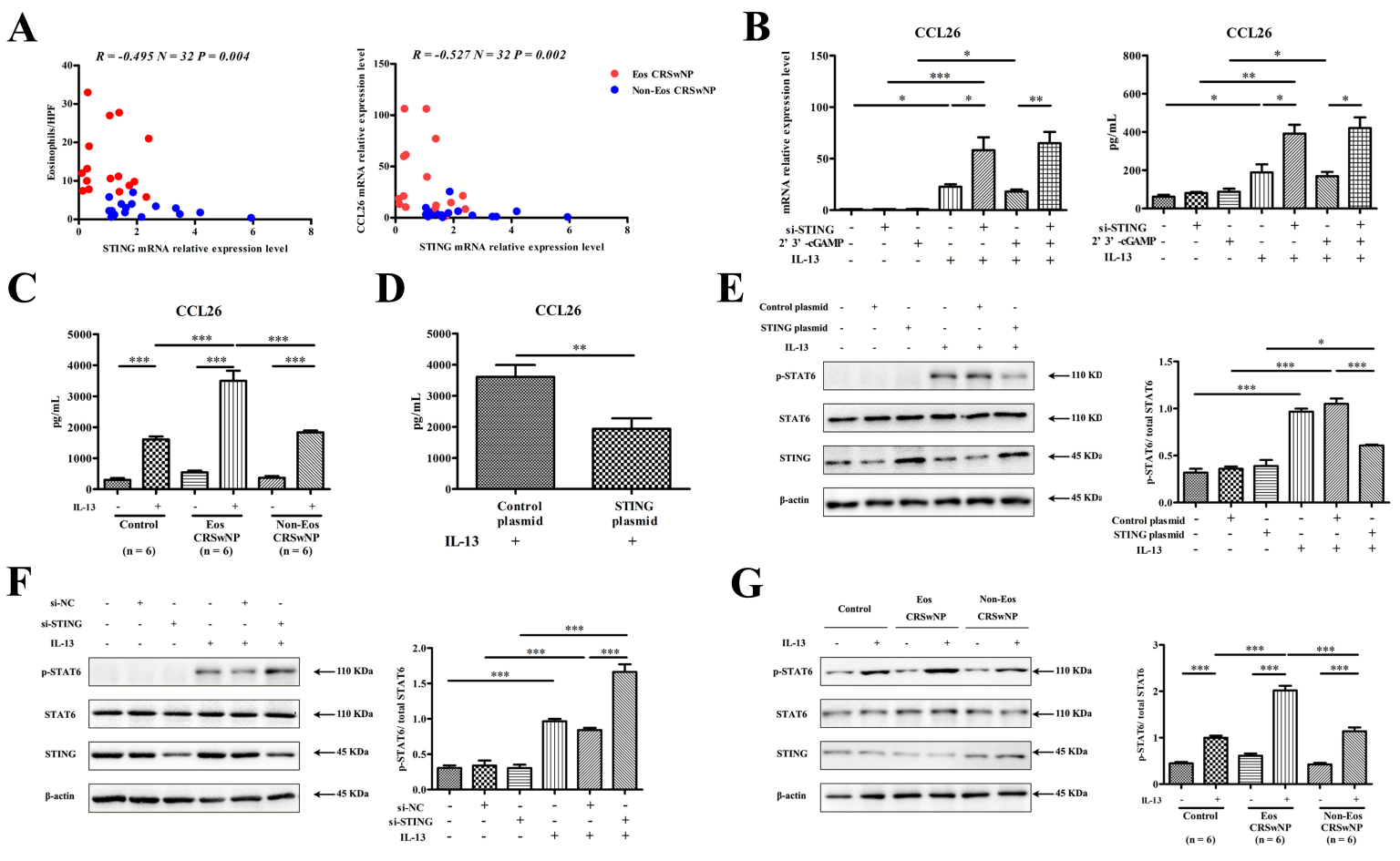
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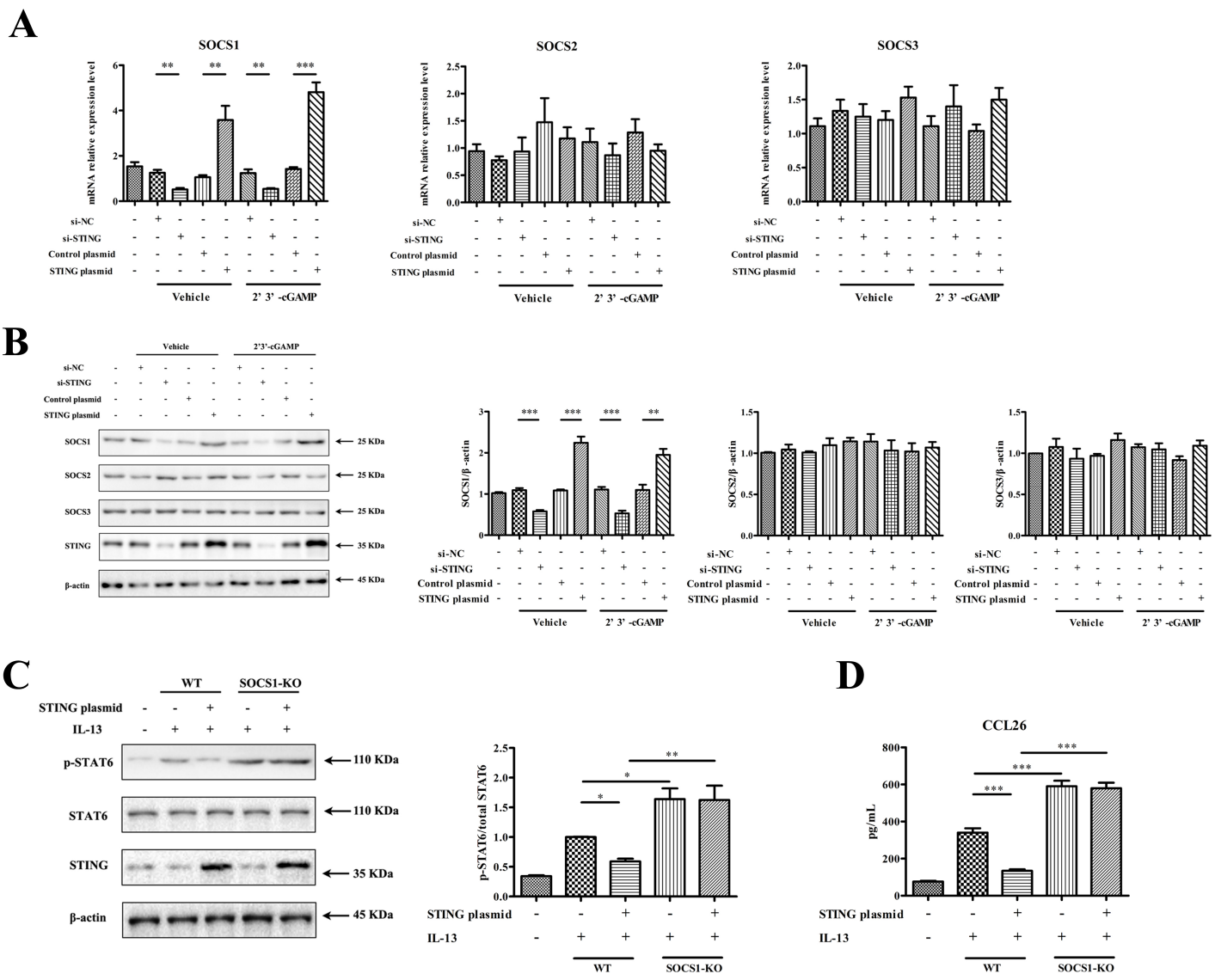

A
IL-5
IL-13
STING
mRNA relative expression level
Control (n = 16)
Eos CRSwNP (n = 16)
Non-Eos CRSwNP (n = 16)
B
Control
Eos CRSwNP
Non-Eos CRSwNP
STING
β-actin
35 KDa
45 KDa
STING/β -actin
Control (n = 15)
Eos CRSwNP (n = 15)
Non-Eos CRSwNP (n = 15)
C
R = -0.584 N = 32 P = 0.001
R = -0.622 N = 32 P < 0.001
IL-5 mRNA relative expression level
IL-13 mRNA relative expression level
STING mRNA relative expression level
Eos CRSwNP
Non-Eos CRSwNP

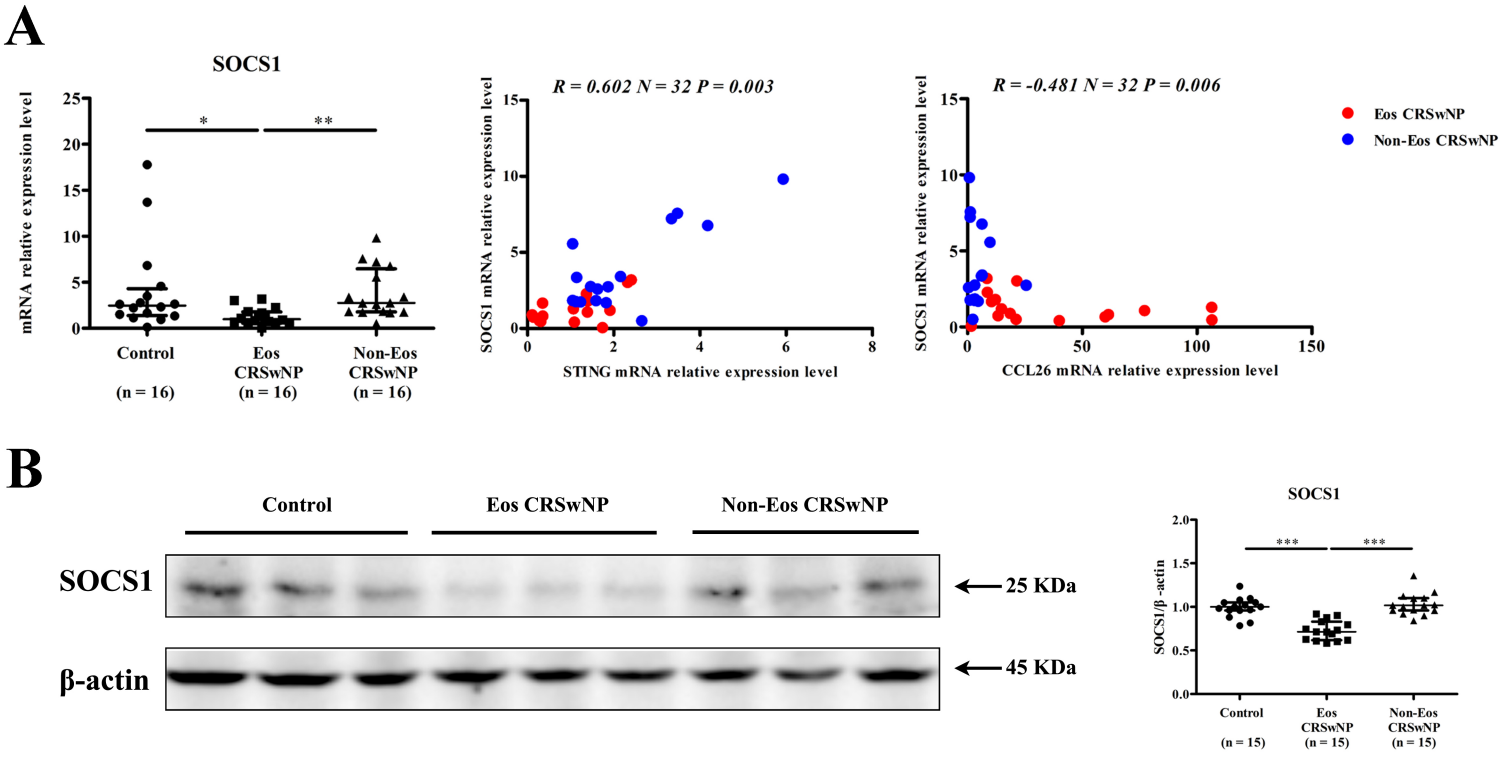

A
Isotype
STING DAPI
B
CD45-PE
CD326-APC
Counts
STING-FITC
Isotype
CD45-CD326-
CD45-CD326+
Median fluorescence intensity of STING
***
40000
30000
20000
10000
0
CD45+CD326-
(n = 31)
CD45-CD326+
(n = 31)
C
CD45-PE
CD326-APC
STING-FITC
FSC
Isotype
STING-FITC
FSC
STING
STING+CD45-CD326+ in CD45-CD326+ (%)
150
100
50
0
***
***
Control
(n = 15)
Eos CRSwNP
(n = 16)
Non-Eos CRSwNP
(n = 15)

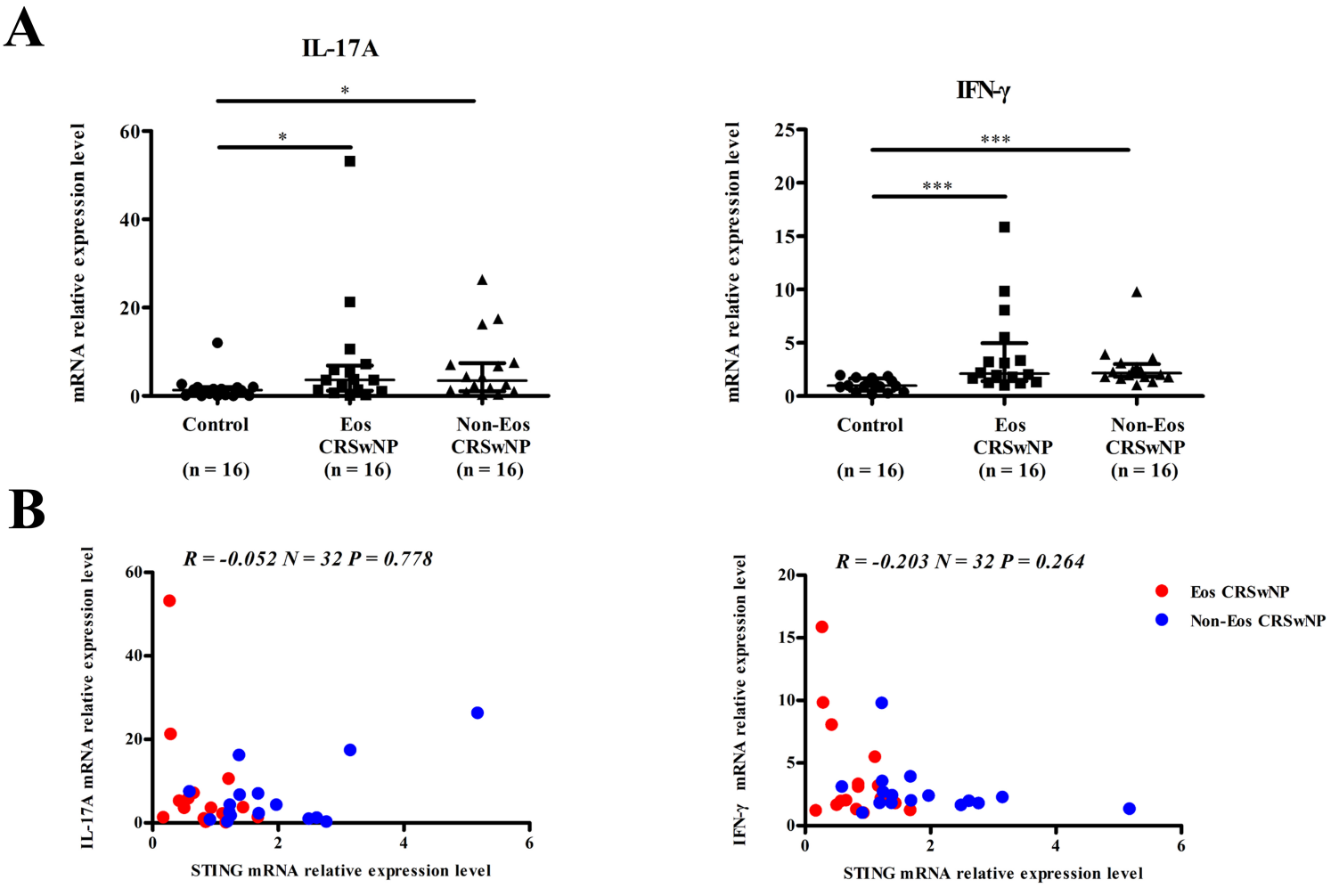

A
IL-4 (ng/mL)
0
1
10
100
STING
β-actin
35 KDa
45 KDa
STING
STING/ β -actin
IL-4 (ng/mL)
B
IL-13 (ng/mL)
STING/ β -actin
IL-13 (ng/mL)
C
IL-13
DMSO
AS1517499
p-STAT6
STAT6
110 KDa
p-STAT6/total STAT6
D
STING
mRNA relative expression level
E
STING-luc (fold)
si-NC
si-STAT6
F
WT
M1
M2
M3
M4
Control
IL-13

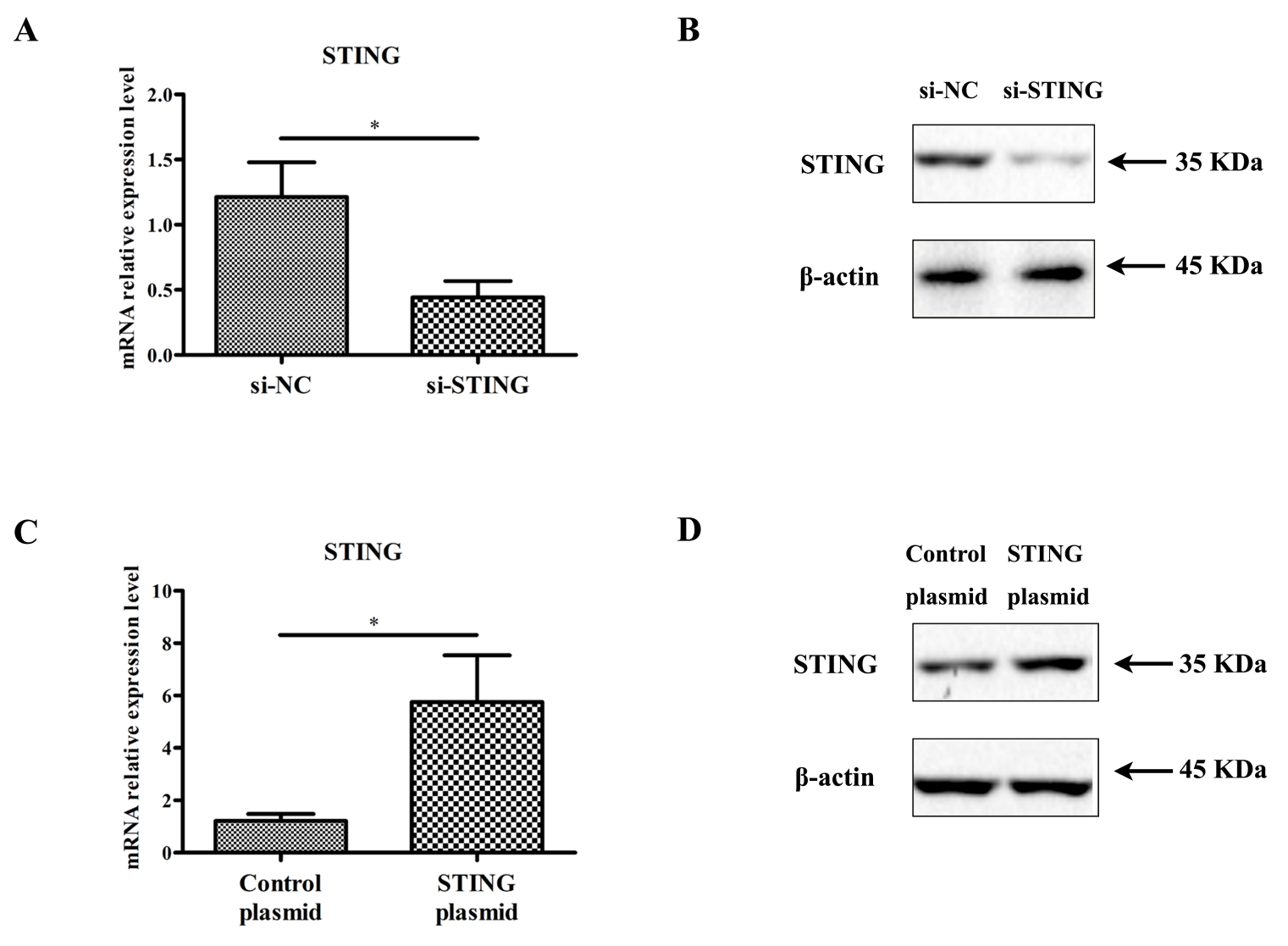

A
Control
Eos CRSwNP
Non-Eos CRSwNP
2'3'-cGAMP
p-TBK1
TBK1
p-IRF3
IRF3
p-P65
P65
STING
β-actin
75 KDa
75 KDa
55 KDa
55 KDa
55 KDa
55 KDa
35 KDa
45 KDa
p-TBK1/ total TBK1
p-IRF3/ total IRF3
p-P65/ total P65
2' 3' -cGAMP
Control (n = 7)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 7)
B
IFN-α
mRNA relative expression level
pg/mL
Control (n = 8)
Eos CRSwNP (n = 8)
Non-Eos CRSwNP (n = 8)
C
IFN-β
mRNA relative expression level
pg/mL
Control (n = 8)
Eos CRSwNP (n = 8)
Non-Eos CRSwNP (n = 8)

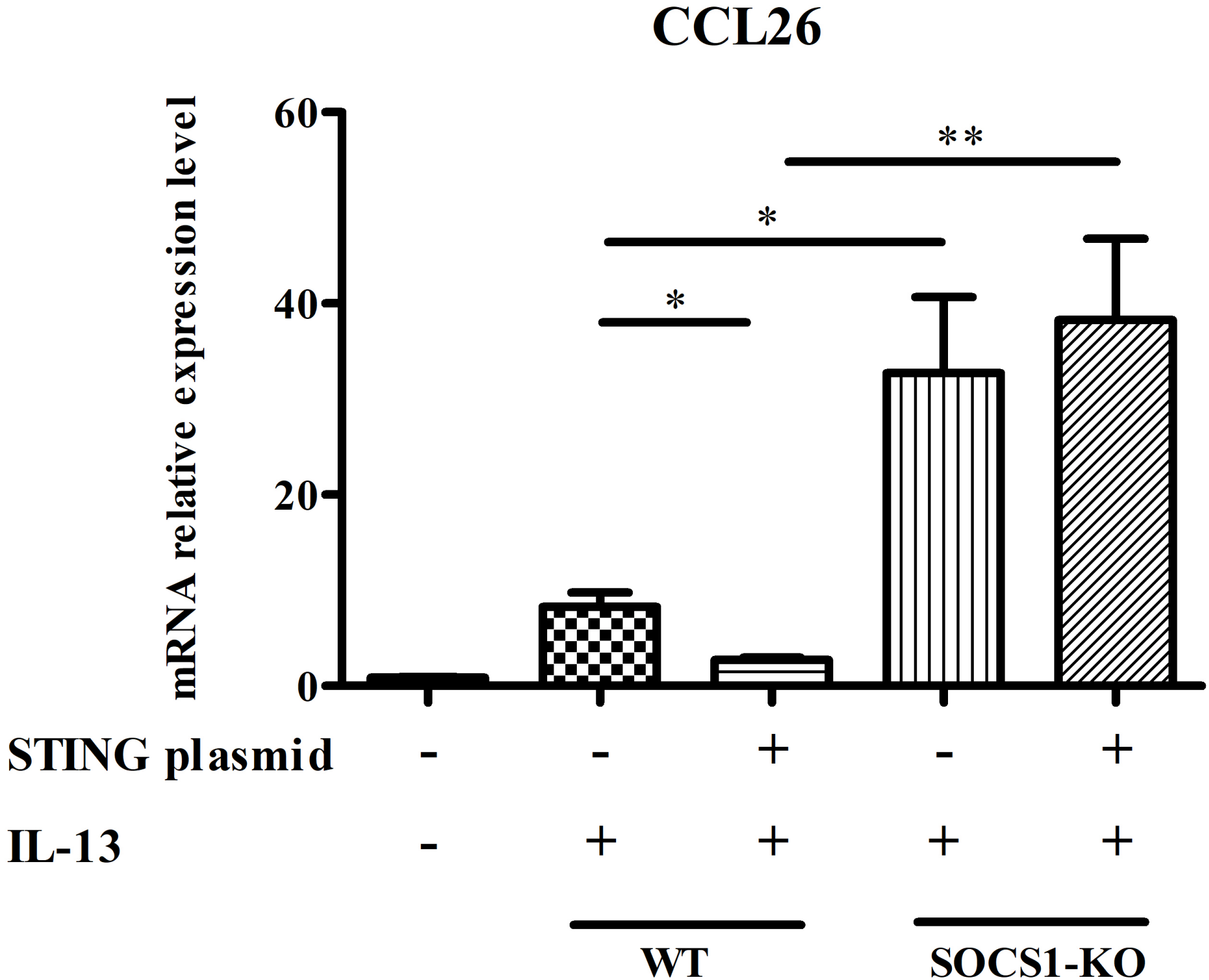

A
R = -0.495 N = 32 P = 0.004
Eosinophils/HPF
STING mRNA relative expression level
R = -0.527 N = 32 P = 0.002
Eos CRSwNP
Non-Eos CRSwNP
CCL26 mRNA relative expression level
STING mRNA relative expression level
B
CCL26
mRNA relative expression level
si-STING
2' 3' -cGAMP
IL-13
CCL26
pg/mL
si-STING
2' 3' -cGAMP
IL-13
C
CCL26
pg/mL
IL-13
Control
(n = 6)
Eos CRSwNP
(n = 6)
Non-Eos CRSwNP
(n = 6)
D
CCL26
pg/mL
Control plasmid
STING plasmid
IL-13
E
Control plasmid
STING plasmid
IL-13
p-STAT6
STAT6
STING
β-actin
110 KD
110 KD
45 KDa
45 KDa
p-STAT6/ total STAT6
Control plasmid
STING plasmid
IL-13
F
si-NC
si-STING
IL-13
p-STAT6
STAT6
STING
β-actin
110 KDa
110 KDa
45 KDa
45 KDa
p-STAT6/ total STAT6
si-NC
si-STING
IL-13
G
Control
Eos CRSwNP
Non-Eos CRSwNP
IL-13
p-STAT6
STAT6
STING
β-actin
110 KDa
110 KDa
45 KDa
45 KDa
p-STAT6/ total STAT6
IL-13
Control
(n = 6)
Eos CRSwNP
(n = 6)
Non-Eos CRSwNP
(n = 6)

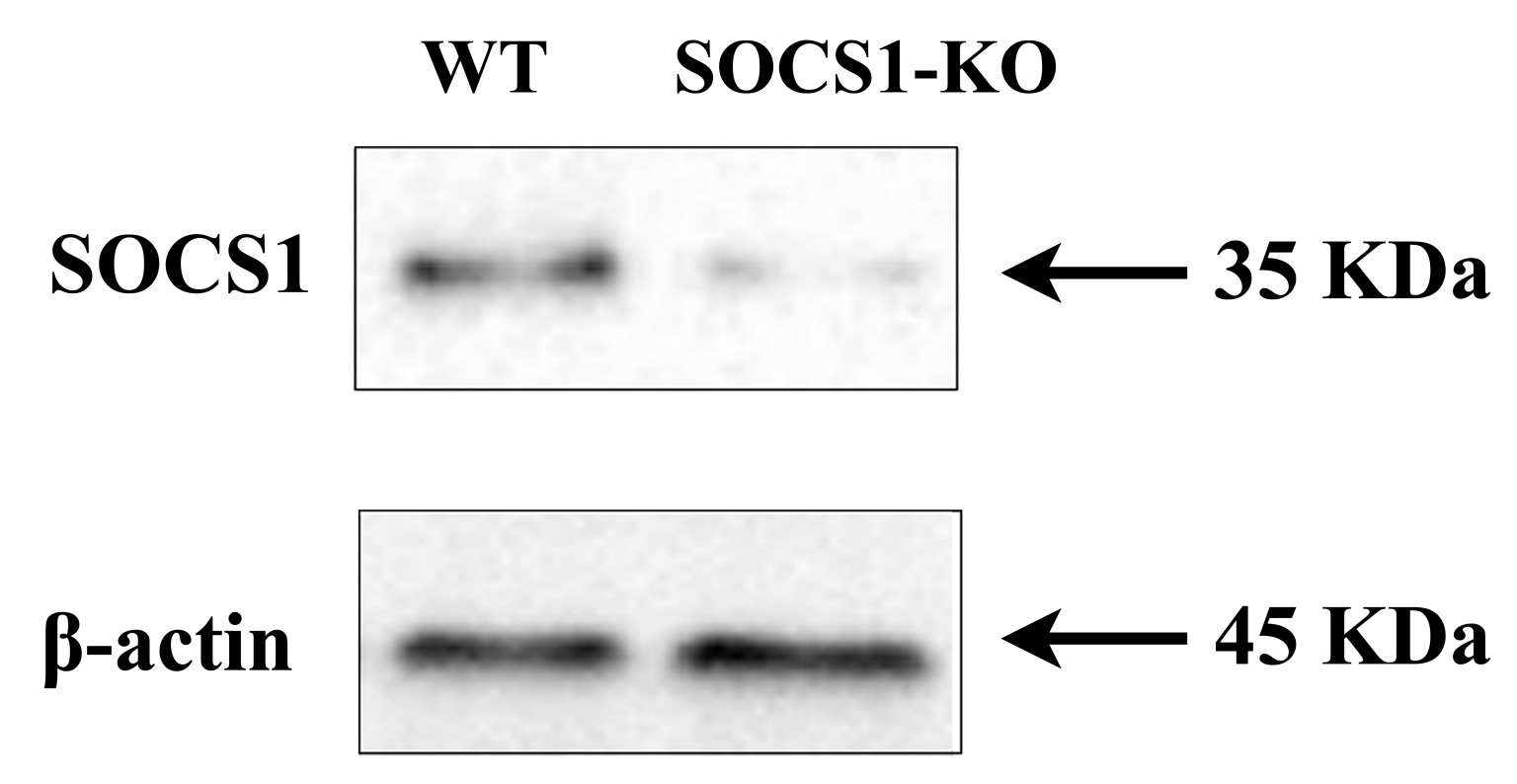

A
SOCS1
SOCS2
SOCS3
mRNA relative expression level
si-NC
si-STING
Control plasmid
STING plasmid
Vehicle
2' 3' -cGAMP
B
Vehicle
2'3'-cGAMP
SOCS1
SOCS2
SOCS3
STING
β-actin
25 KDa
35 KDa
45 KDa
SOCS1/β-actin
SOCS2/β-actin
SOCS3/β-actin
C
WT
SOCS1-KO
STING plasmid
IL-13
p-STAT6
STAT6
110 KDa
p-STAT6/total STAT6
D
CCL26
pg/mL

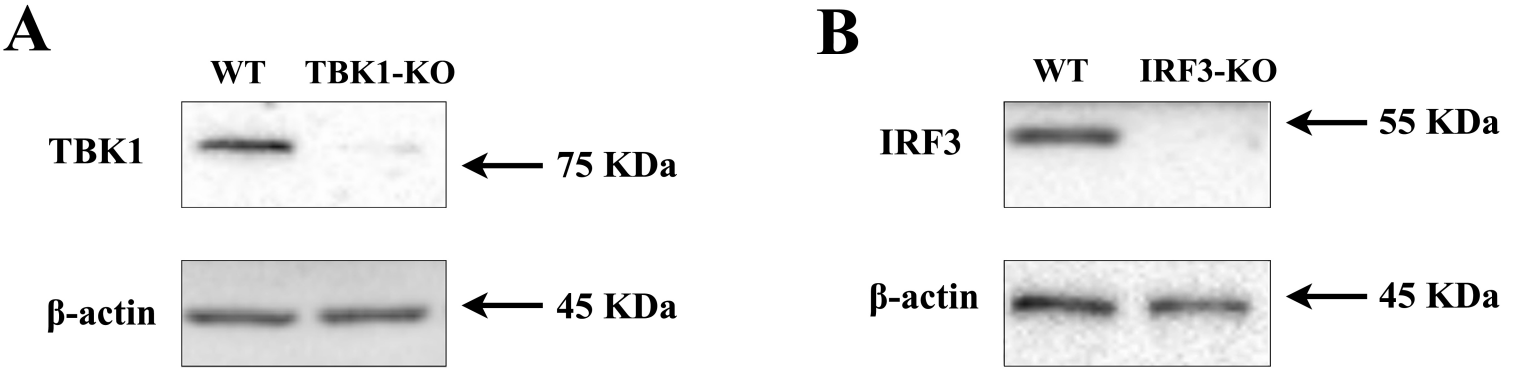

A
SOCS1
mRNA relative expression level
25
20
15
10
5
0
*
**
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
R = 0.602 N = 32 P = 0.003
SOCS1 mRNA relative expression level
STING mRNA relative expression level
0
2
4
6
8
R = -0.481 N = 32 P = 0.006
CCL26 mRNA relative expression level
50
100
150
Eos CRSwNP
Non-Eos CRSwNP
B
Control
Eos CRSwNP
Non-Eos CRSwNP
SOCS1
β-actin
25 KDa
45 KDa
SOCS1
SOCS1/β-actin
2.0
1.5
1.0
0.5
0.0
***
***
Control
(n = 15)
Eos
CRSwNP
(n = 15)
Non-Eos
CRSwNP
(n = 15)

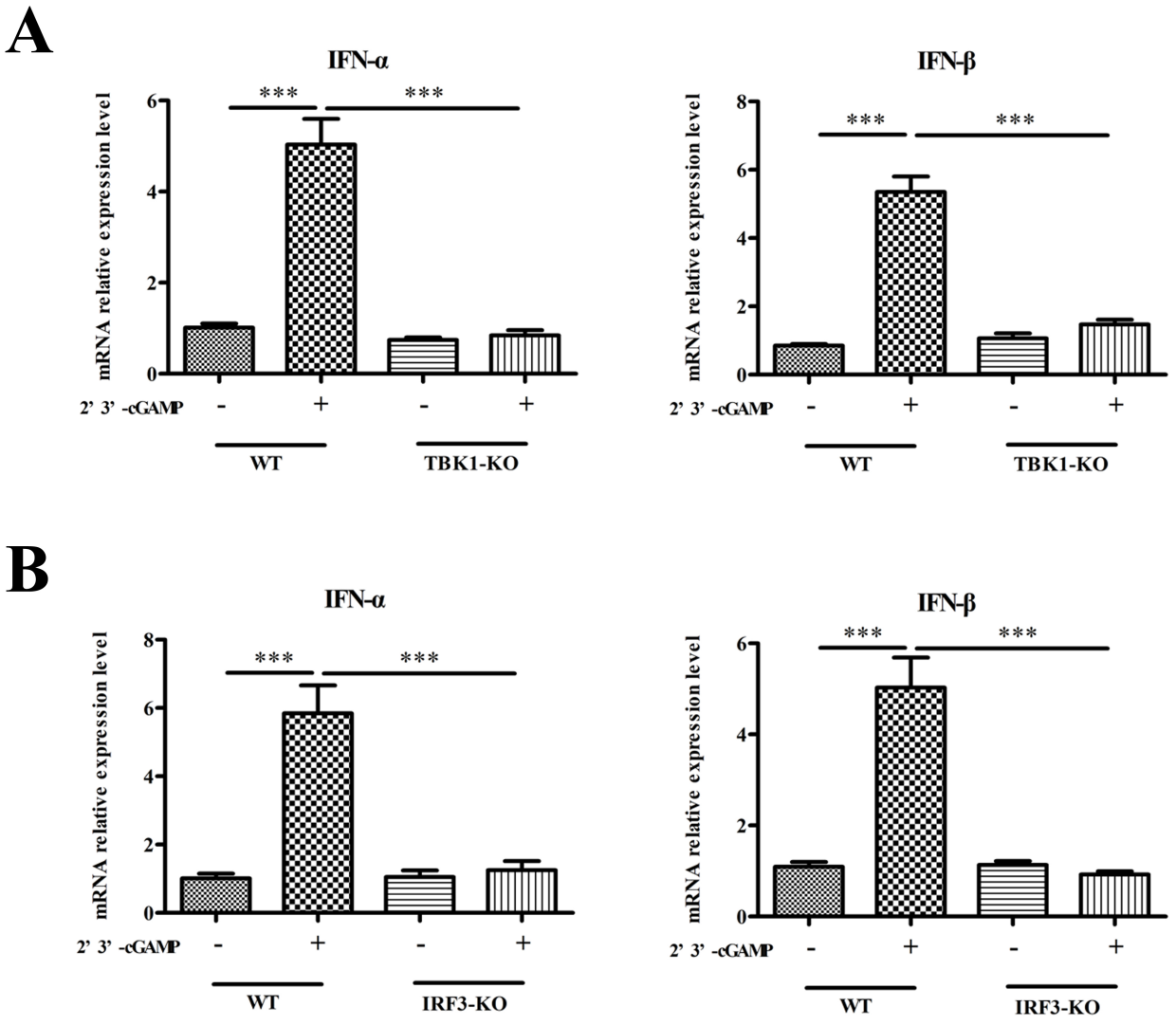

A
IL-17A
mRNA relative expression level
60
40
20
0
*
*
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
IFN-γ
mRNA relative expression level
25
20
15
10
5
0
***
***
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
B
R = -0.052 N = 32 P = 0.778
IL-17A mRNA relative expression level
60
40
20
0
0
2
4
6
STING mRNA relative expression level
R = -0.203 N = 32 P = 0.264
IFN-γ mRNA relative expression level
20
15
10
5
0
0
2
4
6
STING mRNA relative expression level
Eos CRSwNP
Non-Eos CRSwNP

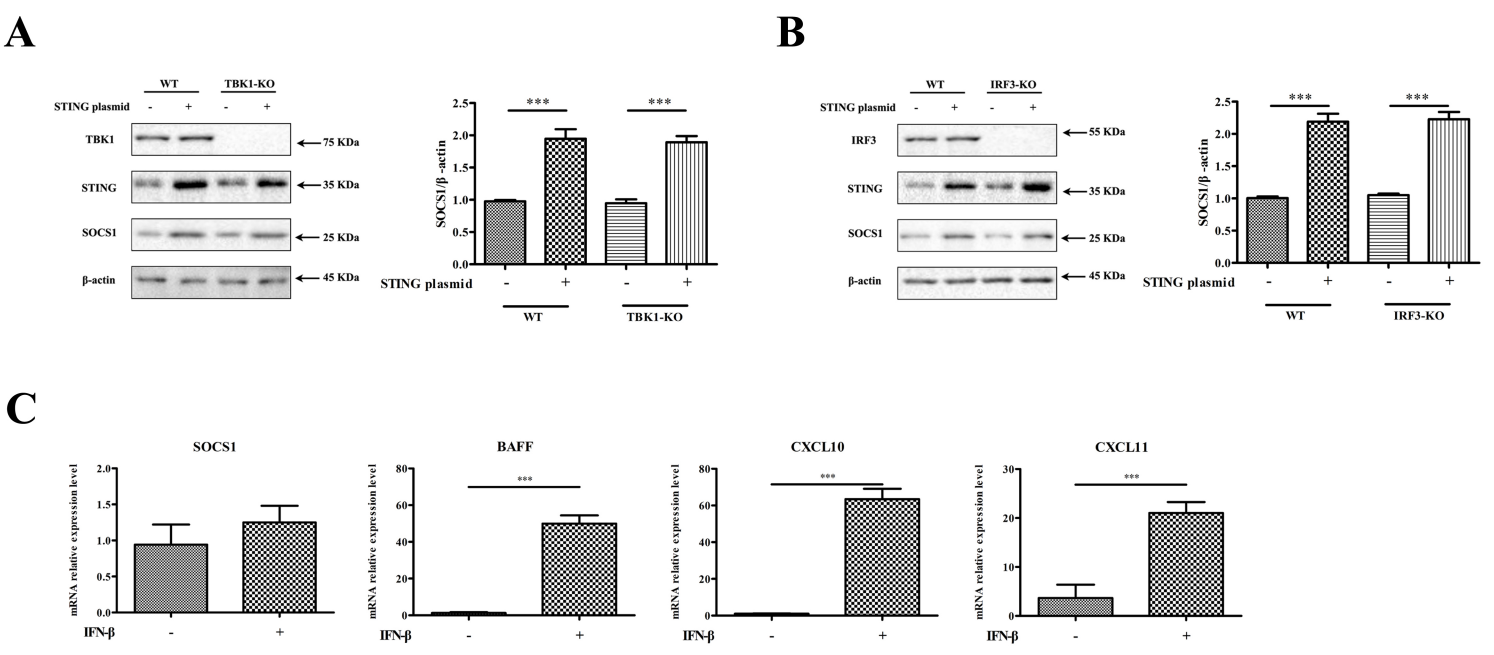

A
STING
mRNA relative expression level
2.0
1.5
1.0
0.5
0.0
*
si-NC
si-STING
B
si-NC
si-STING
STING
35 KDa
β-actin
45 KDa
C
STING
mRNA relative expression level
10
8
6
4
2
0
*
Control plasmid
STING plasmid
D
Control plasmid
STING plasmid
STING
35 KDa
β-actin
45 KDa

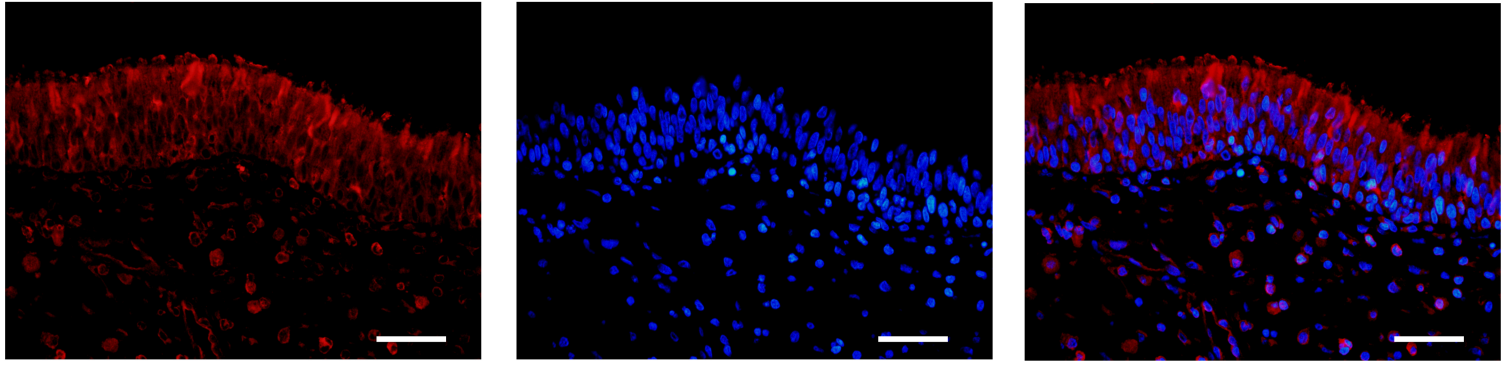

CCL26
mRNA relative expression level
60
40
20
0
**
*
*
STING plasmid
-
-
+
-
+
IL-13
-
+
+
+
+
WT
SOCS1-KO

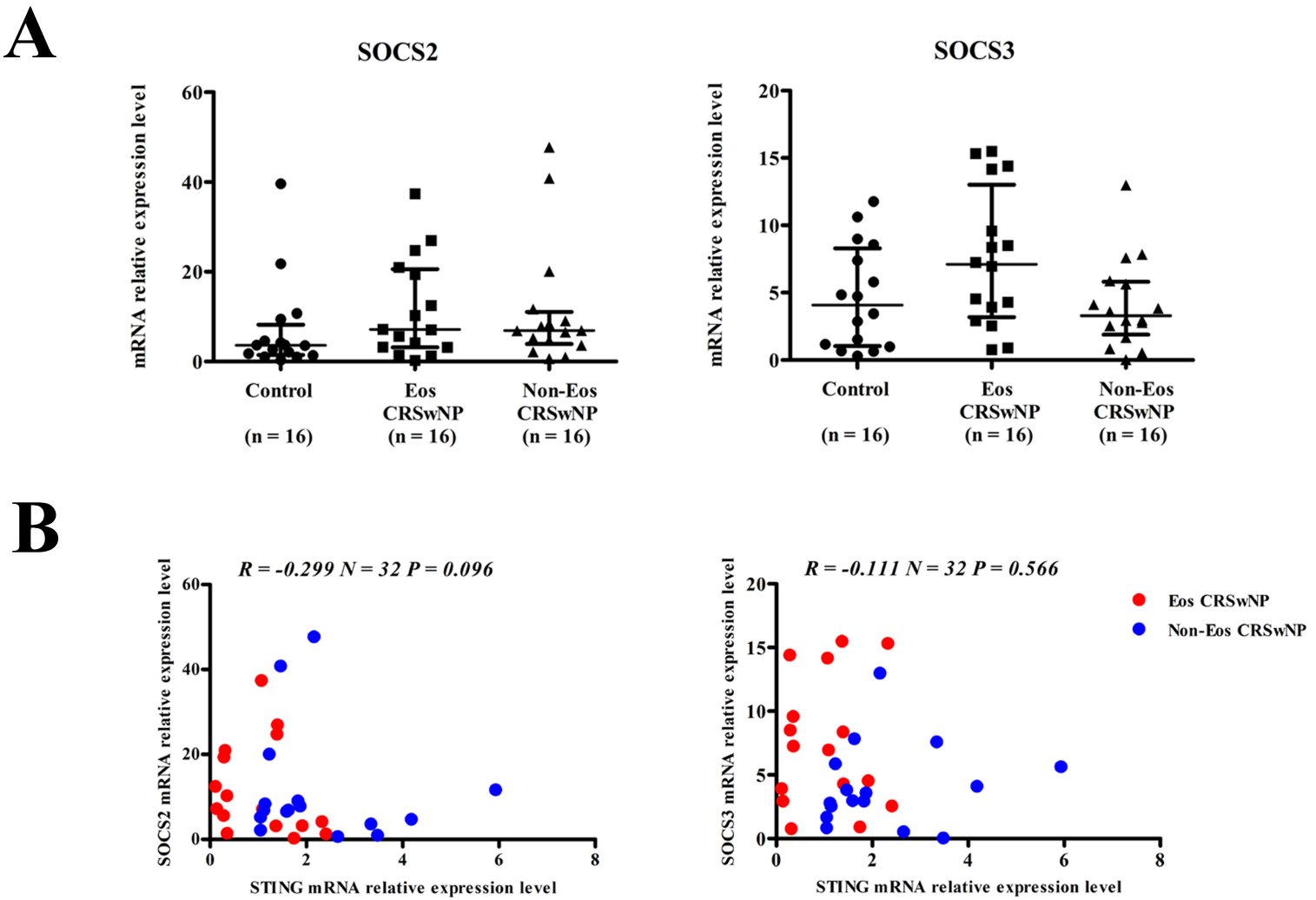

WT
SOCS1-KO
SOCS1
35 KDa
β-actin
45 KDa

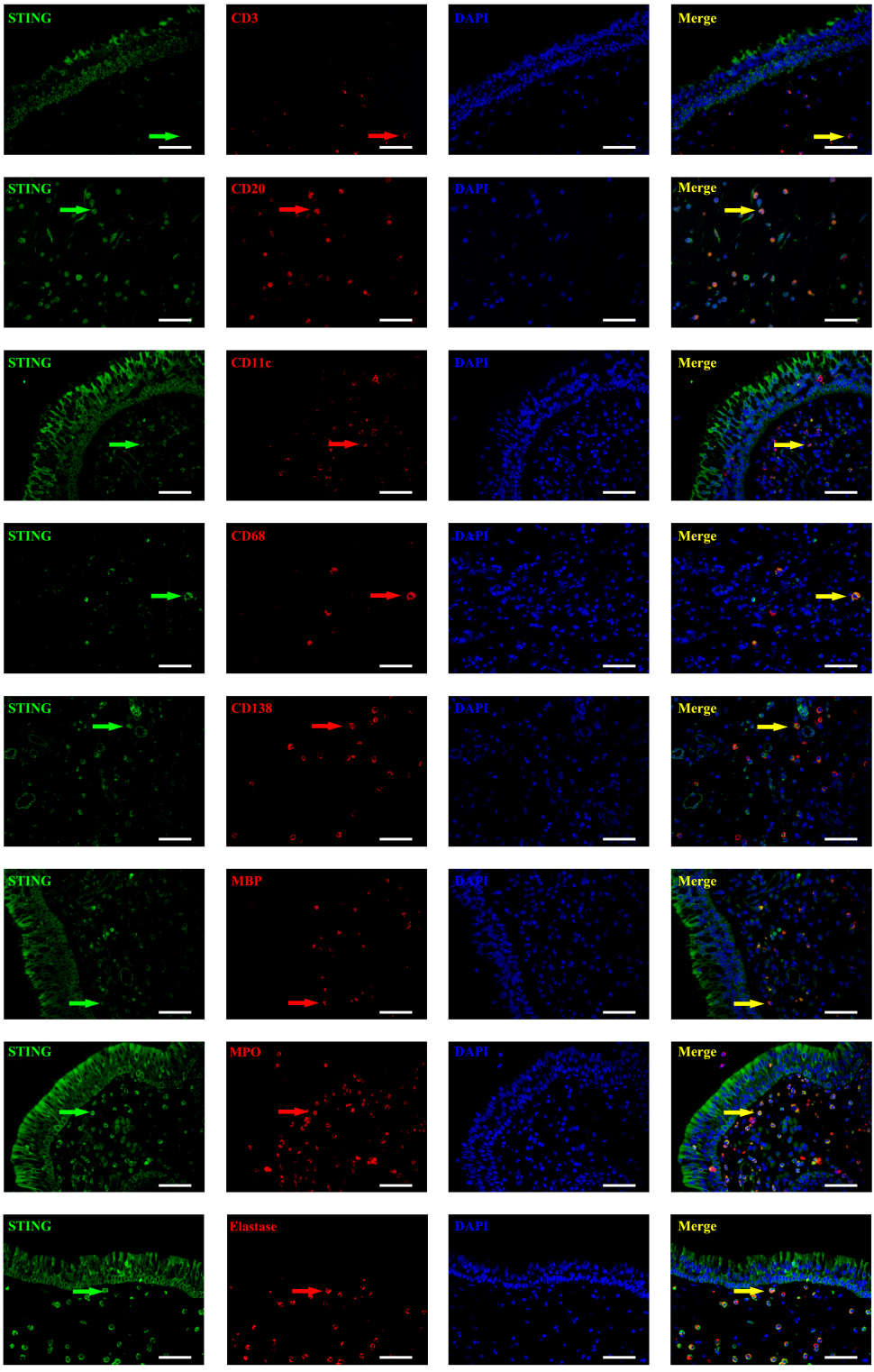

A
WT
TBK1-KO
TBK1
75 KDa
β-actin
45 KDa
B
WT
IRF3-KO
IRF3
55 KDa
β-actin
45 KDa

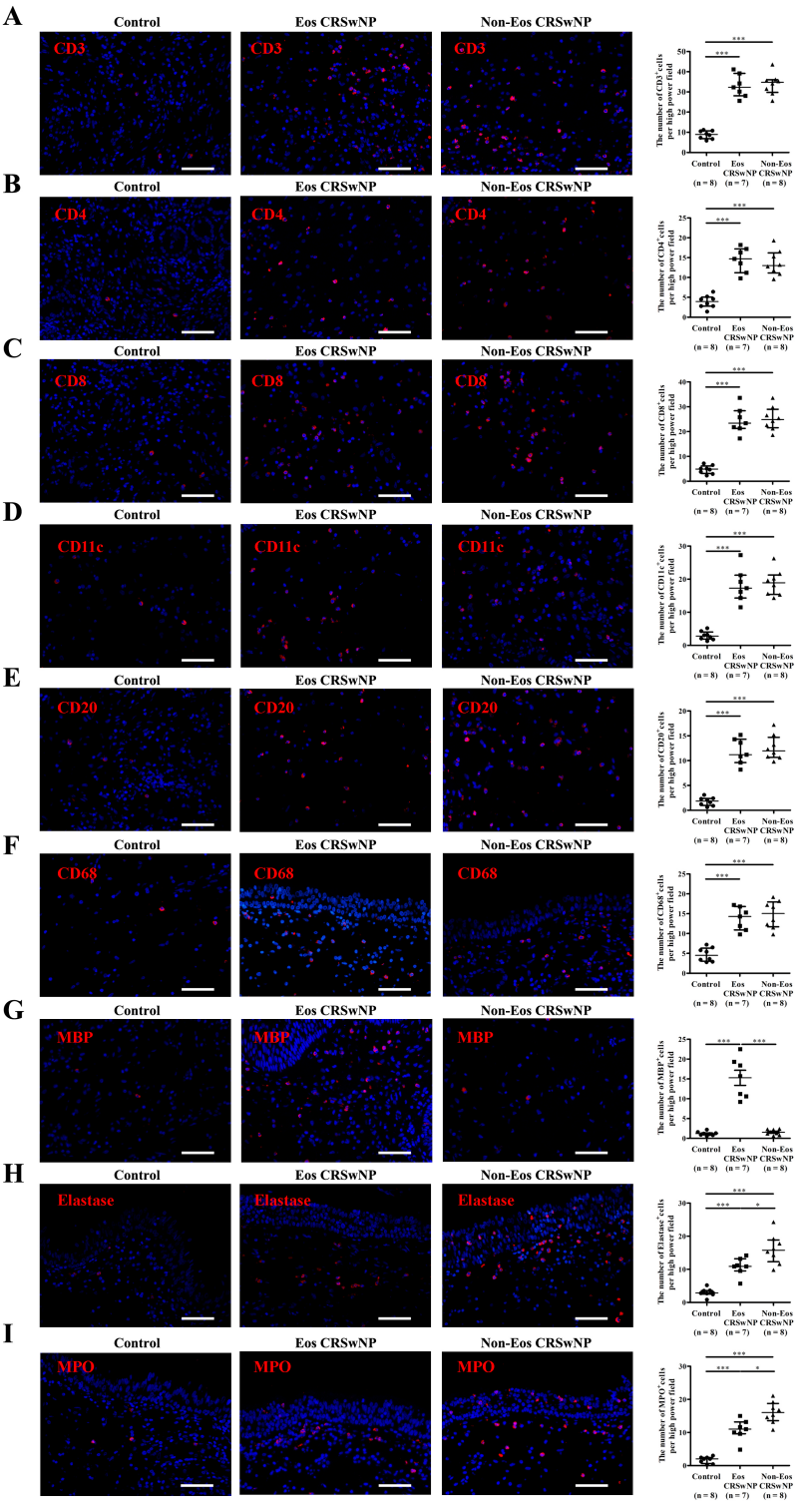

A
IFN-α
mRNA relative expression level
6
4
2
0
***
***
2' 3' -cGAMP
-
+
-
+
WT
TBK1-KO
IFN-β
mRNA relative expression level
8
6
4
2
0
***
***
2' 3' -cGAMP
-
+
-
+
WT
TBK1-KO
B
IFN-α
mRNA relative expression level
8
6
4
2
0
***
***
2' 3' -cGAMP
-
+
-
+
WT
IRF3-KO
IFN-β
mRNA relative expression level
6
4
2
0
***
***
2' 3' -cGAMP
-
+
-
+
WT
IRF3-KO

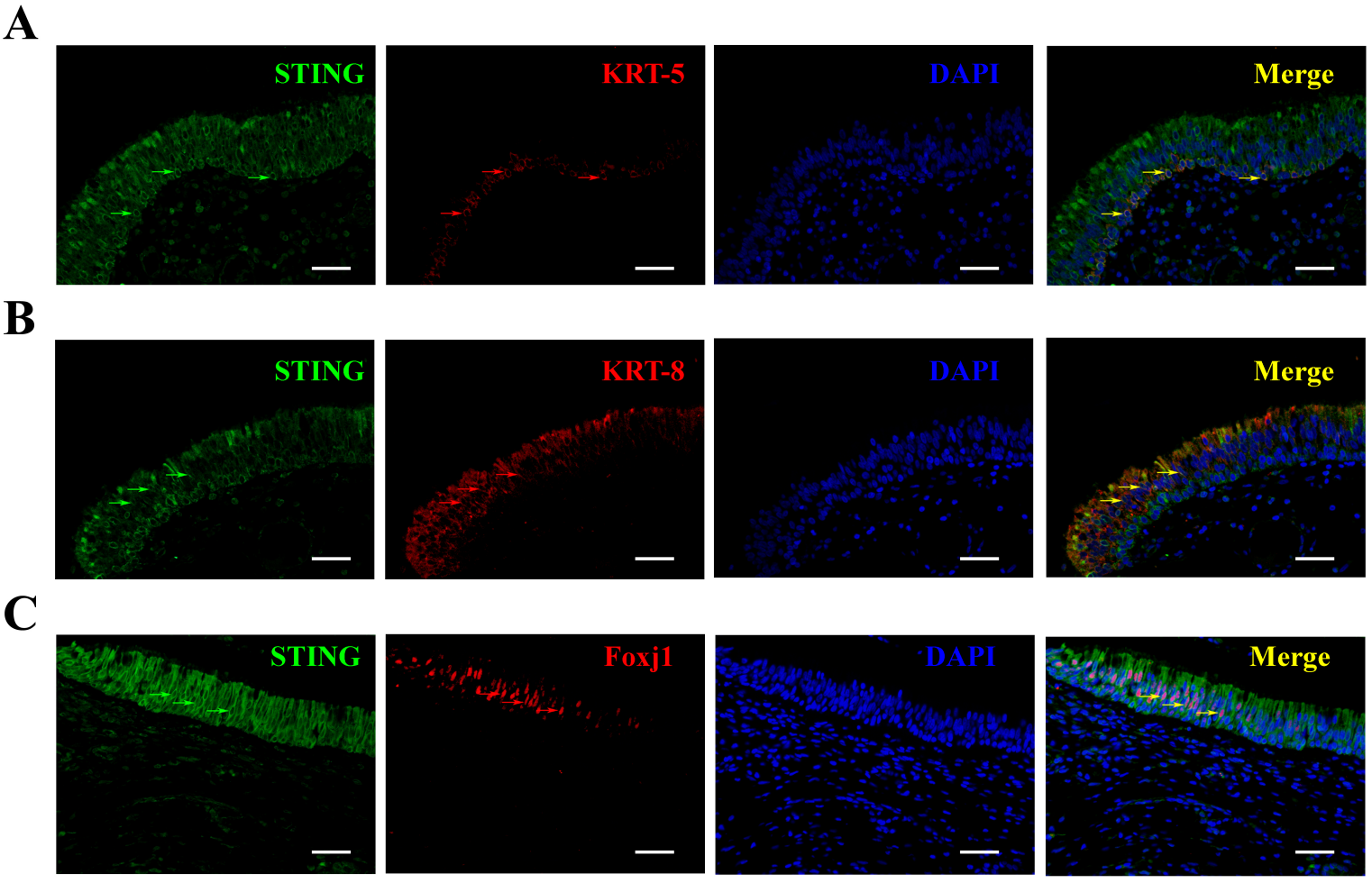

A
WT
TBK1-KO
STING plasmid
TBK1
STING
SOCS1
β-actin
75 KDa
35 KDa
25 KDa
45 KDa
SOCS1/β -actin
***
WT
TBK1-KO
B
IRF3-KO
IRF3
55 KDa
IRF3-KO
C
SOCS1
BAFF
CXCL10
CXCL11
mRNA relative expression level
IFN-β

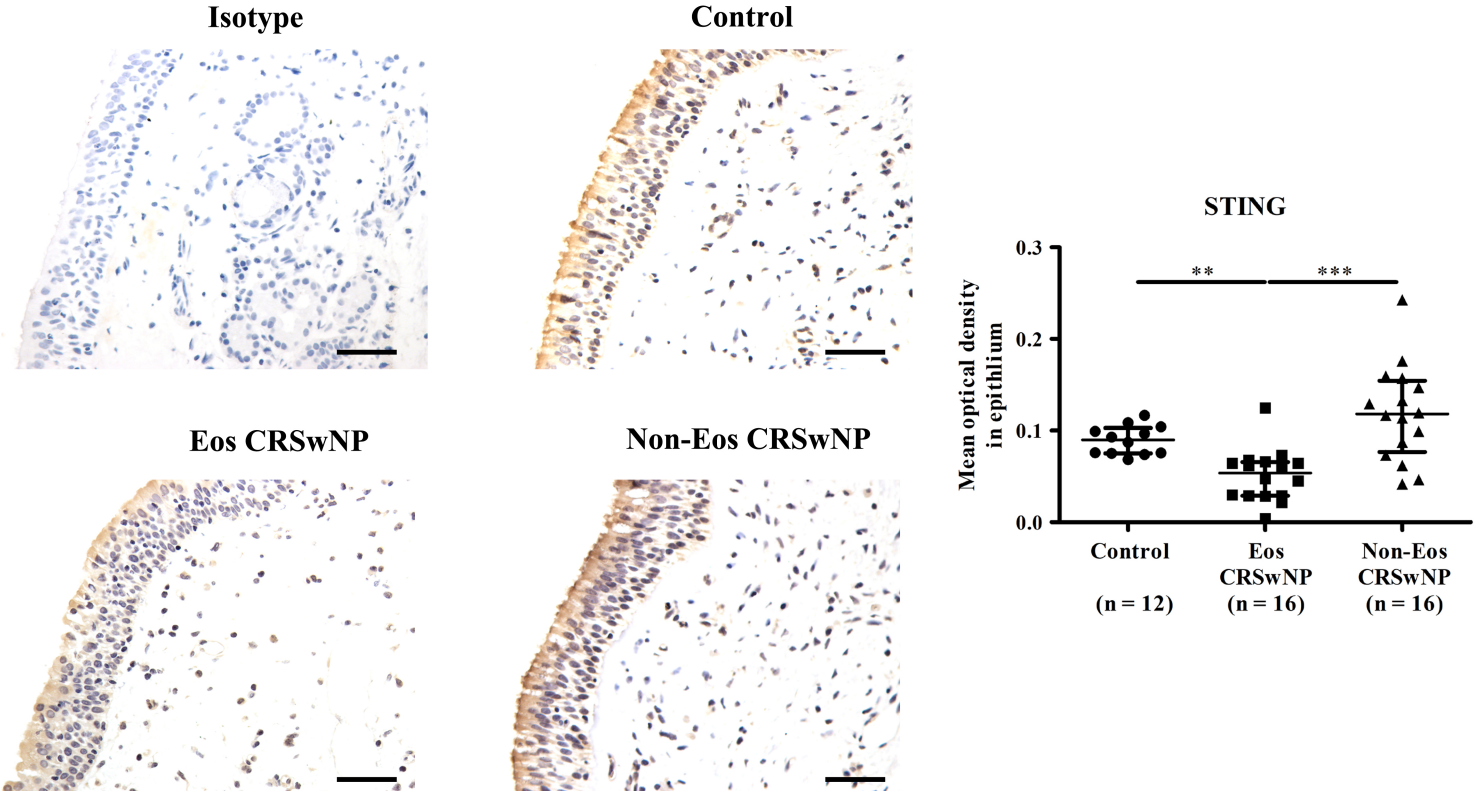

SOCS1
DAPI
Merge


A
SOCS2
mRNA relative expression level
60
40
20
0
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
SOCS3
mRNA relative expression level
20
15
10
5
0
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
B
R = -0.299 N = 32 P = 0.096
SOCS2 mRNA relative expression level
60
40
20
0
0
2
4
6
8
STING mRNA relative expression level
R = -0.111 N = 32 P = 0.566
SOCS3 mRNA relative expression level
20
15
10
5
0
0
2
4
6
8
STING mRNA relative expression level
Eos CRSwNP
Non-Eos CRSwNP

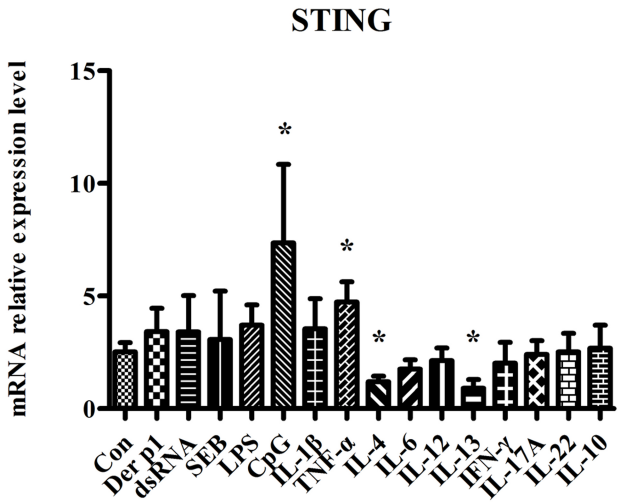

STING
CD3
DAPI
Merge
STING
CD20
DAPI
Merge
STING
CD11c
DAPI
Merge
STING
CD68
DAPI
Merge
STING
CD138
DAPI
Merge
STING
MBP
DAPI
Merge
STING
MPO
DAPI
Merge
STING
Elastase
DAPI
Merge


A
Control
Eos CRSwNP
Non-Eos CRSwNP
CD3
CD3
GD3
The number of CD3+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
B
Control
Eos CRSwNP
Non-Eos CRSwNP
CD4
CD4
CD4
The number of CD4+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
C
Control
Eos CRSwNP
Non-Eos CRSwNP
CD8
CD8
CD8
The number of CD8+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
D
Control
Eos CRSwNP
Non-Eos CRSwNP
CD11c
CD11c
CD11c
The number of CD11c+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
E
Control
Eos CRSwNP
Non-Eos CRSwNP
CD20
CD20
CD20
The number of CD20+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
F
Control
Eos CRSwNP
Non-Eos CRSwNP
CD68
CD68
CD68
The number of CD68+ cells per high power field
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
G
Control
Eos CRSwNP
Non-Eos CRSwNP
MBP
MBP
MBP
The number of MBP+ cells per high power field
***
***
***
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
H
Control
Eos CRSwNP
Non-Eos CRSwNP
Elastase
Elastase
Elastase
The number of Elastase+ cells per high power field
***
***
*
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)
I
Control
Eos CRSwNP
Non-Eos CRSwNP
MPO
MPO
MPO
The number of MPO+ cells per high power field
***
***
*
Control (n = 8)
Eos CRSwNP (n = 7)
Non-Eos CRSwNP (n = 8)

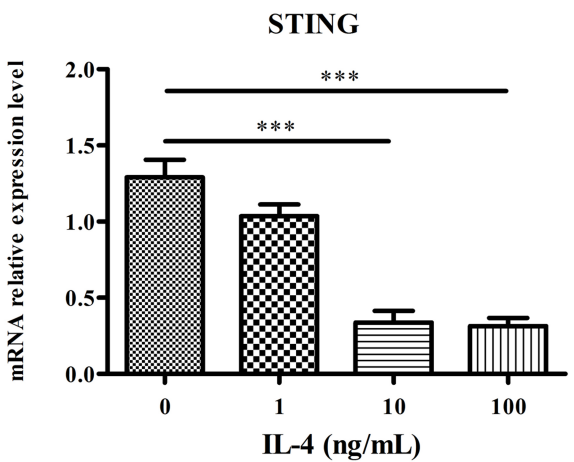

A
STING
KRT-5
DAPI
Merge
B
STING
KRT-8
DAPI
Merge
C
STING
Foxj1
DAPI
Merge

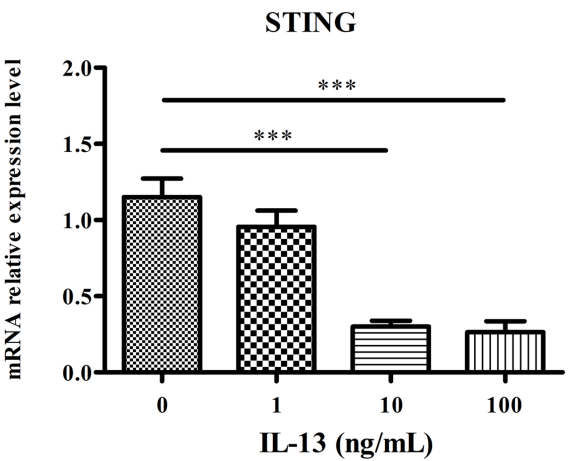

Isotype
Control
Eos CRSwNP
Non-Eos CRSwNP
STING
Mean optical density in epithlium
0.3
0.2
0.1
0.0
**
***
Control (n = 12)
Eos CRSwNP (n = 16)
Non-Eos CRSwNP (n = 16)

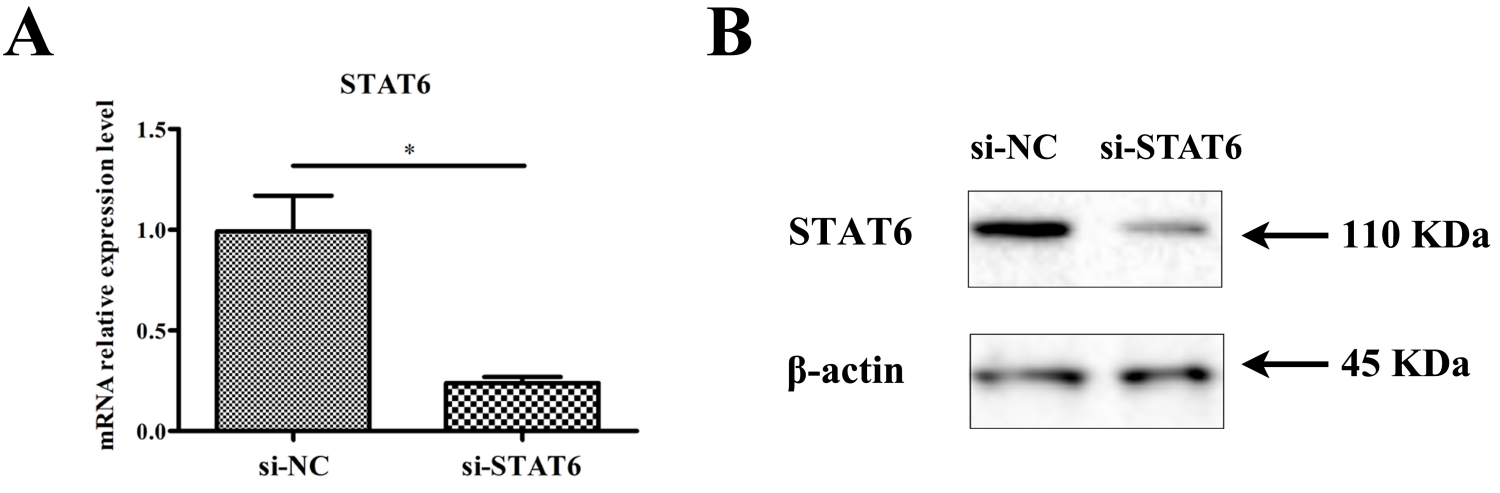
A

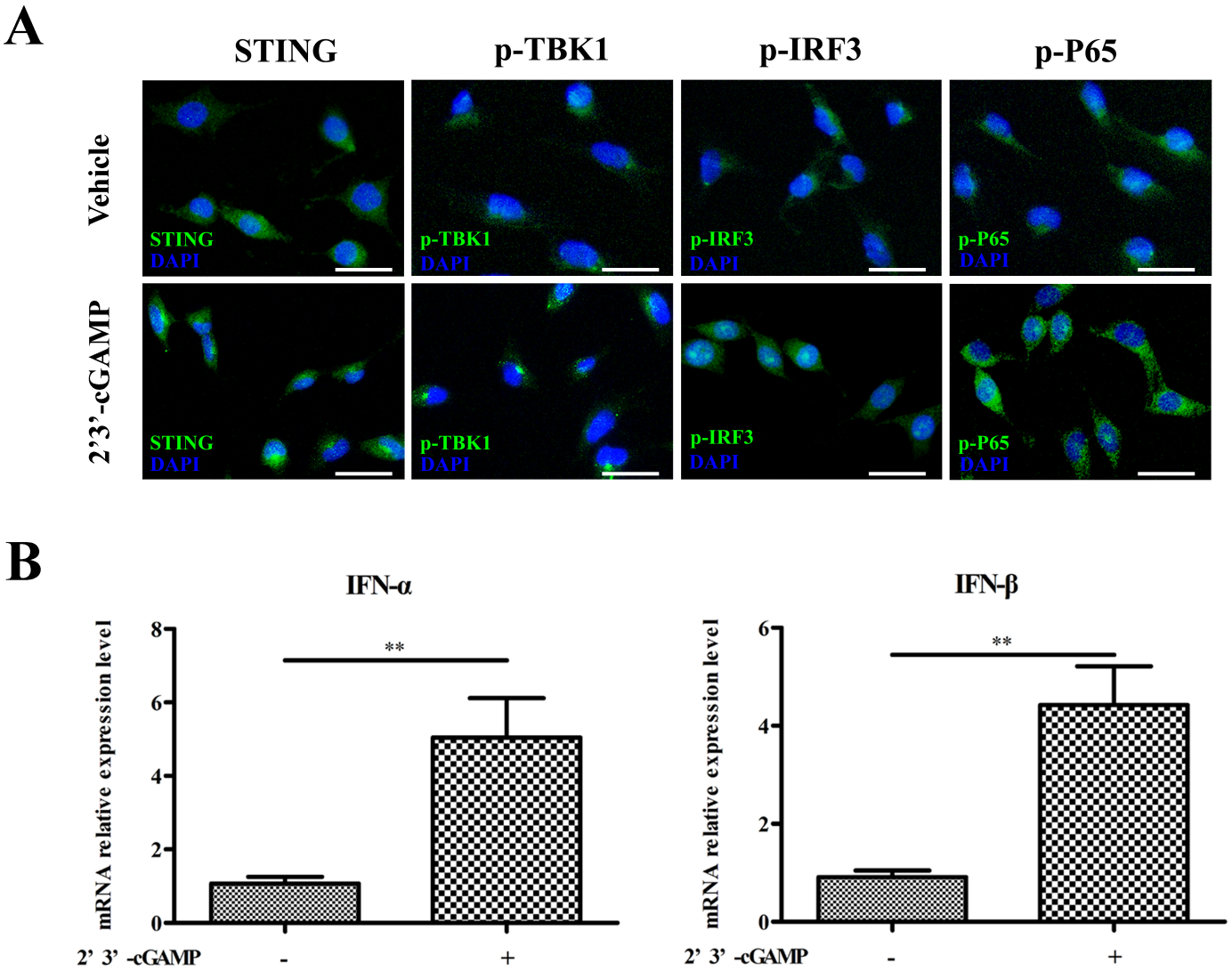

STING
mRNA relative expression level
15
10
5
0
*
*
*
*
Con
Der p1
dsRNA
SEB
LPS
CpG
IL-1β
TNF-α
IL-4
IL-6
IL-12
IL-13
IFN-γ
IL-17A
IL-22
IL-10

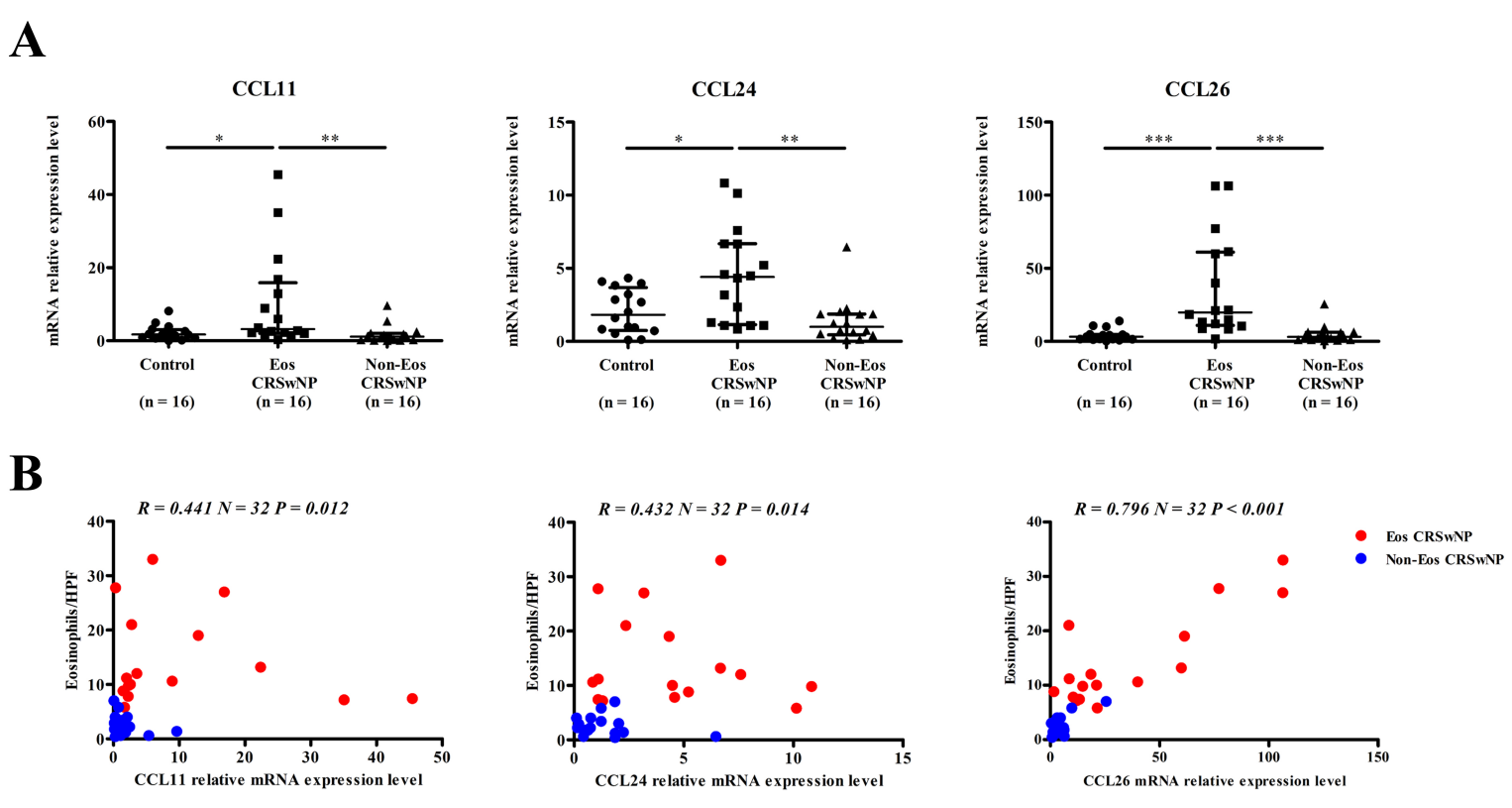
B


STING
mRNA relative expression level
2.0
1.5
1.0
0.5
0.0
***
***
0
1
10
100
IL-4 (ng/mL)


STING
mRNA relative expression level
2.0
1.5
1.0
0.5
0.0
***
***
0
1
10
100
IL-13 (ng/mL)


A
STAT6
mRNA relative expression level
1.5
1.0
0.5
0.0
*
si-NC
si-STAT6
B
si-NC
si-STAT6
STAT6
110 KDa
β-actin
45 KDa


A
STING
p-TBK1
p-IRF3
p-P65
Vehicle
2'3'-cGAMP
STING
DAPI
p-TBK1
DAPI
p-IRF3
DAPI
p-P65
DAPI
B
IFN-α
IFN-β
mRNA relative expression level
**
2' 3' -cGAMP
-
+


A
CCL11
CCL24
CCL26
mRNA relative expression level
Control
(n = 16)
Eos
CRSwNP
(n = 16)
Non-Eos
CRSwNP
(n = 16)
B
R = 0.441 N = 32 P = 0.012
R = 0.432 N = 32 P = 0.014
R = 0.796 N = 32 P < 0.001
Eosinophils/HPF
CCL11 relative mRNA expression level
CCL24 relative mRNA expression level
CCL26 mRNA relative expression level
Eos CRSwNP
Non-Eos CRSwNP