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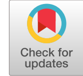
Research Paper

Interleukin (IL)-13 and IL-17A contribute to neo-osteogenesis in chronic rhinosinusitis by inducing RUNX2



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ABSTRACT

Background: There is increasing evidence supporting the impact of neoosteogenesis in the pathophysiology of chronic rhinosinusitis (CRS), especially in the recalcitrant group of patients. Runt-related transcription factor 2 (RUNX2), a member of the RUNX family, controls osteoblast differentiation and bone formation. However, the role and regulation of RUNX2 in CRS patients with neoosteogenesis remain unclear. The aim of the study is to determine the role of RUNX2 in neoosteogenesis of CRS patients.
Methods: Sinonasal bone and overlying mucosa samples were obtained from CRS patients with or without neoosteogenesis ($n = 67$) and healthy controls ($n = 11$). Double immunofluorescence, immunohistochemistry, and immunoblotting were used to evaluate RUNX2 expression in CRS patients with and without neoosteogenesis. In addition, the osteogenic activity of pro-inflammatory cytokines was examined by measuring alkaline phosphatase (ALP) activity and bone mineralisation *in vitro*.
Findings: RUNX2 was highly expressed in osteoblasts of CRS patients with neoosteogenesis compared with tissues from control subjects and those with CRS without neoosteogenesis. Mucosal extracts from CRS patients with neoosteogenesis showed increased RUNX2 expression and ALP activity in C2C12 cells, whereas those from patients without neoosteogenesis did not. Expression of interleukin (IL)-13 and IL-17A was upregulated in CRS patients with neoosteogenesis. ALP activity and Alizarin Red staining showed IL-13 and IL-17A dose-dependent osteoblast differentiation and mineralisation *in vitro*.
Interpretation: These findings suggested that IL-13- or IL-17A-induced RUNX2 contributed to new bone formation in CRS patients through its effect on the activity of osteoblasts. RUNX2 may be a novel target for preventing neoosteogenesis in CRS patients.
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1. Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous and multifactorial disorder of the paranasal sinus mucosa, which might also involve the underlying bone. There is increasing evidence supporting the impact of neoosteogenesis on the pathophysiology of CRS, especially in recalcitrant patients [1–3]. Much effort has been focused on defining and characterizing the condition of neoosteogenesis *via* histopathology and computed tomography (CT) imaging [4]. Neo-osteogenesis patients exhibit greater severity of CRS as evaluated by CT and endoscopy, which is especially prevalent in patients undergoing repeated surgeries [5].

There is controversy regarding whether neoosteogenesis is the cause of disease refractoriness or the result of the refractoriness. Nonetheless, the severity of neoosteogenesis is considered as one of the predictors of postoperative re-stenosis, requiring the Draf III or modified Lothrop procedures, which involve the most challenging frontal sinus surgeries [6]. Notably, there are phenotypic differences in adhesion and mineralisation between osteoblasts in CRS patients when compared with control subjects [7]. These differences suggest that identification

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Research in context

Evidence before this study

Chronic rhinosinusitis (CRS) is a heterogeneous disorder of the paranasal sinus mucosa, which may also involve the underlying bone. RUNX2 (also known as core-binding factor subunit alpha-1) plays a role in the proliferation and differentiation of osteoblasts, which are required for bone formation. However, no study has investigated the role and regulation of RUNX2 in the pathophysiology of CRS with neo-osteogenesis.

Added value of this study

This study shows that RUNX2 was highly expressed in osteoblasts of CRS patients with neo-osteogenesis, and its expression was associated with new bone formation. We found that interleukin (IL)-13 or IL-17A induced osteoblast differentiation in vitro as evidenced by high ALP activity and increased bone mineralization.

Implications of all the available evidence

Our findings suggest that RUNX2 suppression via inhibition of IL-13 or IL-17A may provide new therapeutic target for recalcitrant CRS patients with neo-osteogenesis.

of the immunological and molecular mechanisms of osteoblast activation can be used to help understand neoosteogenesis and treat recalcitrant CRS.

Runt-related transcription factor 2 (RUNX2) is a member of the RUNX family, which is composed of RUNX1, RUNX2, and RUNX3 [8]. RUNX2 (also known as core-binding factor subunit alpha-1) plays a crucial role in the proliferation and differentiation of osteoblasts, which are required for bone formation [9]. It has recently been reported that RUNX2 is upregulated in primary cultured human asthmatic bronchial epithelial cells and in human asthmatic lung tissue, which regulate goblet cell differentiation [10]. The recent study has indicated that RUNX2 expression is increased in idiopathic pulmonary fibrosis [11]. We therefore investigated the expression of RUNX2 in CRS patients with neoosteogenesis, and characterised the relationship between RUNX2 and the Global Osteitis Scoring Scale (GOSS). In addition, we determined the immunological and molecular mechanisms underlying RUNX2 activation using nasal tissue extracts from CRS patients.

2. Methods

2.1. Patients and tissue samples

Sinonasal and polyp tissues were obtained from patients with bilateral CRS during routine functional endoscopic sinus surgery. All participants provided written informed consent for participation in the study. This study was approved (26–2016-57) by the internal review board of Boramae Medical Center. The diagnosis of CRS was based on personal history, physical examination, nasal endoscopy, and CT findings of the sinuses according to the 2012 European position paper on rhinosinusitis and nasal polyp guidelines [12]. Exclusion criteria were the following: 1) < 18 years of age; 2) prior treatment with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs for 4 weeks before surgery; and 3) unilateral rhinosinusitis, antrochoanal polyp, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease. Control tissues were obtained during other rhinological surgeries, such as skull base, lacrimal duct, or orbital decompression surgery, from patients without any sinonasal diseases. Mucosal tissues were obtained from control subjects and from CRS patients without nasal polyps (CRSsNP) or CRS patients with nasal polyps (CRSwNP). The atopic status of study participants was evaluated using the ImmunoCAP® assay (Phadia, Uppsala, Sweden) to detect immunoglobulin E (IgE) antibodies against six mixtures of common aeroallergens (house dust mites, molds, trees, weeds and grass pollen, and animal dander). Participants were considered atopic if the allergen-specific IgE level was >0.35 kU/L to ≥1 of the allergens. A diagnosis of asthma was based on medical history and lung function analysis, including the methacholine challenge test by an allergist. CRSwNP were classified as eosinophilic CRSwNP if eosinophils comprised >10% of the inflammatory cell population and as non-eosinophilic CRSwNP if eosinophils comprised <10% of the inflammatory cells [13,14]. To define the neoosteogenesis score, global osteitis scoring scale (GOSS) were calculated with the CT scans before the surgery [15]. Neo-osteogenesis was classified as not significant (GOSS <5), mild (5 < GOSS <20), and moderate-to-severe (GOSS >20).

Additional information and details of the participants are listed in Table 1. Sinonasal bone and overlying mucosa samples from the uncinate process were obtained from controls (n = 11), CRSsNP (n = 30), and CRSwNP (n = 37). Each sample was divided into two parts; one part was fixed in 10% formaldehyde, decalcified, and embedded in paraffin for histological analysis, and the other part was incubated in 1 mL phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and 1% protease inhibitor cocktail (Sigma-Aldrich) per 0.1 g of tissue. Mucosal extracts from the patients were prepared according to our previously established method [16,17]. In brief, small piece of the mucosal samples were homogenised with a Bullet Blender Blue (Next Advance, Averill Park, NY) and centrifuged at setting 7 or 8 min at 48 °C. After homogenisation, the suspension was centrifuged at 4000 rpm for 20 min at 48 °C, and the supernatants were stored at −80 °C until analysis. The total protein concentrations in each of the extracts were measured by using the Bradford assay. Then we used the extract at the adequate protein concentration (20 μg/ml).

2.2. Immunohistochemical analysis

Immunohistochemistry was performed using the Polink-2 plus polymerised horseradish peroxidase broad DAB Detection System (Golden Bridge International Laboratories, Bothell, WA, USA). RUNX2, osteonectin, IL-13, IL-13Rα2, IL-17A, and IL-17RA were immunostained in paraffin sections of sinonasal tissues. The antibodies used are listed in Table E1 (see the online supplement). The numbers of positive cells were determined in five high power fields (400×) by two independent

Table 1
Characteristics of study subjects.

Total no. of subjects	Control	CRSsNP	CRSwNP	
	N = 11	N = 30	Eosinophilic N = 19	Non-eosinophilic N = 18
Tissue used	UP	UP	UP	UP
Age (y), mean (SD)	42.27 (13.64)	48.24 (14.60)	56.78 (7.79)	52.44 (12.31)
Male/Female, n/n	10/1	20/10	15/4	14/4
Allergic rhinitis, n	0	9	11	4
Asthma, n	0	0	4	0
Aspirin sensitivity, n	0	0	0	0
Lund-Mackay CT score, mean (SD)	0 (0)	9.60 (4.43)	12.56 (5.03)	13.33 (5.94)
Global Osteitis Scoring Scale	0 (0)	8.88 (7.01)	12.80 (8.72)	13.25 (10.71)
Methodology used, no				
Tissue IHC	N = 7	N = 25	N = 28	
Tissue extracts	N = 5	N = 5	N = 22	

CRSsNP chronic rhinosinusitis without nasal polyp, CRSwNP chronic rhinosinusitis with nasal polyp, UP uncinated process, CT computed tomography, IHC immunohistochemistry.

observers who were blinded to the group identities. Detailed immunohistochemical procedures are described in the online supplement.

2.3. Cell culture and nasal tissue extract stimulation

The C2C12 (mouse mesenchymal precursor) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in 5% CO_2 using Dulbecco's Modified Eagle's Medium (WelGENE, Gyeongsan, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (WelGENE), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Gaithersburg, MD, USA).

C2C12 cells were grown under 5% CO2/20% O_2 conditions. C2C12 is a multipotent progenitor cell line that has the ability to transdifferentiate into osteoblast. Cells were seeded in 12-well plates at a density of 1×10^4 cells/well. C2C12 cells were then stimulated in fresh medium (supplemented with 10% FBS) containing nasal tissue extract (20 μg/ml) for 48 h. To neutralise IL-13 or IL-17A, cells were cultured in medium containing neutralizing antibody against IL-13 (0.5 μg/ml, AHC0132, Thermo Scientific) and IL-17A (0.5 μg/ml, AF-317-NA, R&D systems). An irrelevant isotype antibody was used as a negative control. C2C12 cells were treated with 100 ng/ml bone morphogenetic protein-2 (BMP-2) used as a positive control.

2.3.1. Cytokine stimulation

Cells were cultured in 12-well plates at a density of 1×10^4 cells/well. Cells were then stimulated in fresh medium containing IL-4, IL-5, IL-13, IL-17A, IFN-γ, TNF-α, IL-25, IL-33, TSLP, IL-1β, IL-7 and TGF-β1 (Peprotech, Rocky Hill, USA) in a concentration dependent manner for 48 h (Table E2).

2.4. Western blot analysis

Cell lysates were separated on 10% SDS/polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Cat. No. IPVH00010, Bedford, MA). The membranes were preincubated in 5% non-fat milk (BD Difco, Cat. No. 232100, Sparks, MD) for 60 min, and then sequentially incubated with a primary antibody (diluted in 5% nonfat milk) overnight at 4 °C and incubated with a secondary antibody conjugated with horseradish peroxidase (diluted 1:3000 in 5% non-fat milk) for 1 h at room temperature (Table E1). The following antibodies were used for western blot analysis at the indicated dilution: anti-RUNX2 (1:1000; D130–3, MBL); anti-SMAD3 (1:1000; #9513,Cell Signaling), anti-phospho-SMAD3 (1:1000; #9520, Cell Signaling); anti-phospho-SMAD2 (1:1000; #3108, Cell Signaling), anti-phospho-SMAD1/5 (1:1000; #9516, Cell Signaling), anti-PCNA (1:1000; sc-25,280, Santa Cruz) and anti-β-tunulin (1:5000, sc-5274, Santa Cruz). We used the following secondary antibodies from Invitrogen: goat anti-rabbit IgG (G21234) and goat anti-mouse IgG (G21040). The membranes were developed using a chemiluminescent reagent (Luminata™ Crescendo; Merck Millipore, Cat. No. WBLUR0500, Billerica, MA) and subsequently exposed to chemiluminescent film to visualise the proteins. Immunoblotting for β-tubulin served as a protein loading control. Densitometric analysis was performed on the scanned images of blots by using ImageJ software program (National Institutes of Health Image processing analysis, http://rsb.info.nih.gov/ij/).

2.5. Immunofluorescence staining and confocal microscopy

C2C12 cells were cultured on cover slips and exposed to IL-13 (100 ng/ml) or IL-17A (100 ng/ml) for 48 h. The cells were fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100. Non-specific protein binding was blocked with 1% normal goat serum, and the cells were incubated with anti-RUNX2 primary antibody (1:100; Novus Biologicals) overnight at 4 °C. After a brief washing, the cells were incubated with Alexa 555-conjugated anti-rabbit antibody (1:300; Invitrogen) diluted in blocking buffer for 1 h at room temperature (Table E1). Detailed procedures are described in the Methods section in the online supplement.

2.6. Alkaline phosphatase and alizarin red S staining

Osteogenic activities were evaluated by alkaline phosphatase (ALP) and alizarin red S staining. For ALP staining, C2C12 cells were cultured in 0.2% gelatin-coated 12-well plates at a density of 1×10^4 cells/well. The cells were fixed with 4% paraformaldehyde (Biosesang, Gyeonggi, Republic of Korea) and stained for ALP using an ALP/TRAP staining kit according to the manufacturer's instructions (Wako Pure Chemicals, Osaka, Japan) and analyzed.

Alizarin red S staining was performed to assess mineral deposition. The cells were cultured in 0.2% gelatin-coated 6-well plates at a density of 1×10^5 cells/well. After fixing in 4% paraformaldehyde for 10 min and rinsing with deionised water, the cells were stained with 2% Alizarin Red S, pH 4.2 (ScienCell Research Laboratories, Carlsbad, CA, USA) for 30 min at room temperature, then rinsed with PBS and photographed. Red staining indicated mineral nodule formation. C2C12 cells were treated with 100 ng/ml bone morphogenetic protein-2 (BMP-2) used as a positive control [18].

2.7. Statistical analyses

Statistical analyses used in this study include the Mann-Whitney *U* test with the 2-tailed test for unpaired comparisons, the Wilcoxon signed rank test, the Pearson correlation test, and linear regression, which were performed with IBM SPSS 21 software (SPSS, Chicago, Ill) and GraphPad Prism software (GraphPad, La Jolla, CA, USA). Expression results are presented in dot plots. Symbols represent individual samples, horizontal bars represent the mean values, and the upper and lower bars represent the standard deviations. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. RUNX2 is highly expressed in osteoblasts of CRS patients with neoosteogenesis

First, we evaluated the expression levels of RUNX2 in sinonasal bone and overlying mucosa, which were collected from control ($n = 7$), CRSsNP ($n = 25$), CRSwNP ($n = 28$) subjects. The demographic data for each group are presented in Table 1. CRSsNP with neoosteogenesis showed a significantly higher number of RUNX2-positive cells than control subjects and those without neoosteogenesis ($P < .01$) (Fig. 1a and c). Moreover, our immunohistochemical data confirmed that there were more RUNX2-positive cells in CRSwNP with neoosteogenesis than those without neoosteogenesis ($P < .01$). RUNX2 expression was detected in cells around new bone surfaces in CRS sinonasal specimens. To identify the cellular source of RUNX2, immunohistochemical staining was performed using osteonectin as a marker for osteoblasts (Fig. 1b; Fig. E1a). RUNX2 immunoreactivity was mainly localised in the nuclei, whereas positive staining of anti-osteonectin was generally found in the cytoplasm, as detected by immunofluorescence staining. A significant positive correlation was found between GOSS and RUNX2-positive cells ($r = 0.502$; $P < .001$) (Fig. 1d). Together, the results showed that RUNX2 was more highly expressed in osteoblasts of CRS patients with neoosteogenesis, and that this increased expression was possibly associated with new bone formation.

3.2. RUNX2 expression induced by nasal tissue extracts from CRS patients with neoosteogenesis

To determine how RUNX2 was activated, we examined the effect of nasal tissue extracts from control ($n = 5$), CRSsNP (n = 5), and CRSwNP ($n = 22$) subjects on RUNX2 expression in C2C12 cells. The detailed

Fig. 1. RUNX2 is increased in chronic rhinosinusitis patients with neo-osteogenesis. (a) Control UP mucosa from patients without nasal disease ($n = 7$), UPs from patients with CRSsNP ($n = 25$) and CRSwNP ($n = 28$) with and without evidence of neo-osteogenesis were immunostained with RUNX2 antibody. RUNX2 was detected in osteoblast lining around new bone surfaces in CRS patients with neo-osteogenesis (400× magnification). The dark arrowheads showed the representative positive staining of RUNX2. Inset shows a higher magnification of the outlined area. Scale bar = 50 μm. (b) Double immunofluorescence staining for RUNX2 and osteonectin were performed. Arrowheads indicate RUNX2 and osteonectin double positive cells. Scale bar = 20 μm. (c) Average number of RUNX2-positive cells per high-powered field (HPF) was quantified. Detailed description for counting of immunopositive cells are described in the Methods section in this article's Online Repository. Data are expressed as mean ± SD. $^{*}P < .05$, $^{**}P < .01$. Mann-Whitney U test. (d) Relationship between RUNX2-positive cells and Global Osteitis Scoring Scale. The Pearson correlation test was used.

protocol and concentrations used are described in the Methods section in the online supplement for this study. First, C2C12 cells were treated with different nasal tissue extracts for 48 h, and RUNX2 expression was measured (Fig. 2a–c). Mucosal extracts from CRSwNPs with GOSS >20 showed increased RUNX2 expression in C2C12 cells, regardless of tissue eosinophilia, whereas those from patients with GOSS <5 did not show an increase. We then determined whether nasal tissue extracts influenced ALP activity and calcium deposition. The level of ALP activity and bone mineralisation increased in response to treatment with tissue extracts from non-eosinophilic and eosinophilic CRSwNP with high GOSS (Fig. 2d). RUNX2 levels, ALP activity, and calcium deposition were not increased in response to treatment with tissue extracts from

CRSsNP and control subjects (Figs. E2a–c). To confirm the effect of nasal extracts on the proliferation of C2C12 cells, we measured PCNA by Western blotting. PCNA expression level decreased in nasal extract-treated groups, but there was little difference among the nasal extract groups (Supplementary Fig. E7).

3.3. Interleukin (IL)-13 and IL-17A induced RUNX2 expression, ALP activity, and calcium deposition in vitro

The above findings prompted us to determine the effects of pro-inflammatory cytokines on RUNX2 expression in C2C12 cells (Table E2). First, we screened major inflammatory cytokines including IL-4, IL-5, IL-13, IL-17A, IFN-γ, TNF-α, IL-25, IL-33, TSLP, IL-1β, IL-7, which were known to be associated with the pathogenesis of CRS and to exist in nasal extracts. Among them, recombinant cytokine IL-13 or IL-17A significantly increased the expression of RUNX2 at the protein level (Fig. E3). Considering that transforming growth factor (TGF)-β1 is a potent RUNX2 activator [19], we tested whether it could increase RUNX2 in C2C12 cells treated with nasal extracts from CRS patients with high GOSS (GOSS >20). TGF-β1 treatment significantly upregulated RUNX2 (Fig. E4a). However, the phosphorylation of SMAD2/3, involved in downstream TGF-β1 signaling, was not changed by the nasal extracts from non-eosinophilic and eosinophilic CRSwNPs with high GOSS, which could increase RUNX2 expression (Fig. E4b).

We then evaluated the ability of IL-13 and IL-17A to induce osteoblast differentiation. RUNX2 was upregulated in a dose-dependent manner and detected in the nucleus after IL-13 and IL-17A treatment (Figs. 3a-c). IL-13 or IL-17A induced osteoblast differentiation in C2C12 cells as evidenced by high ALP activity and increased bone mineralisation (Fig. 3d, e, E8a and E8b). Together, these results

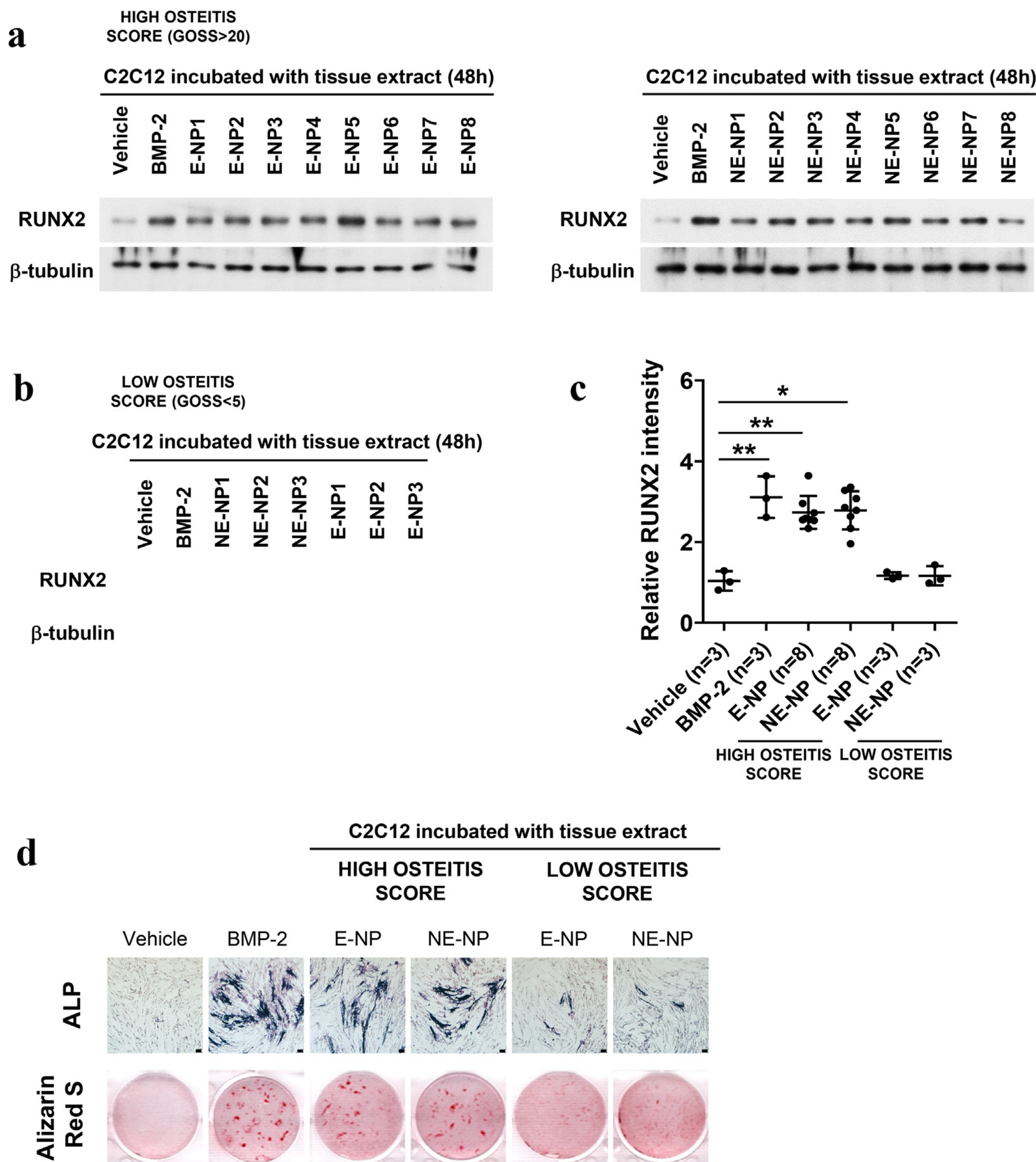

Fig. 2. Increased expression of RUNX2 in C2C12 cells treated with tissue extracts from non-eosinophilic and eosinophilic CRSwNP patients with neo-osteogenesis. (a-b) RUNX2 expression in C2C12 cells treated with vehicle, BMP-2, and tissue extracts from non-eosinophilic and eosinophilic CRSwNP ($n = 22$) patients. RUNX2 protein level was increased by tissue extracts from non-eosinophilic and eosinophilic CRSwNP patients with high osteitis score (GOSS>20) in C2C12 cells (a), whereas tissue extracts with low osteitis score (GOSS<5) did not influence RUNX2 expression (b). (c) The relative band intensity was determined by densitometry of RUNX2 band intensity, normalised to β-tubulin band intensity. (d) C2C12 cells differentiate to osteoblasts upon tissue extract stimulation. Osteoblast differentiation was monitored by staining alkaline phosphatase (ALP) and by measuring its activity at day 7. Mineralised cell nodule formation detected by alizarin red S staining at day 21. The recombinant BMP-2 protein (100 ng/ml) was used as a positive control. Data are expressed as mean ± SD. $^{*}P < .05$. $^{**}P < .01$. Scale bar = 200 μm.

indicated that IL-13 and IL-17A could promote osteoblastic differentiation by enhancing RUNX2 expression in C2C12 cells.

3.4. Neutralizing antibodies against IL-13 and IL-17A reversed RUNX2 expression, ALP activity, and calcium deposition in vitro

We next investigated the effects of neutralizing antibodies against IL-13 and IL-17A on the ability of osteogenic activities. Considering that IL-13 and IL-17A are signature cytokines in eosinophilic CRS [20] and non-eosinophilic CRS [21–24], respectively, we used the mucosa extracts from eosinophilic and non-eosinophilic NP patients. Neutralizing IL-13 downregulated RUNX2 expression in C2C12 cells treated with extracts from eosinophilic CRS patients, whereas neutralizing IL-17A did not (Fig. 4a–c). In addition, Fig. 5a–c shows that blocking IL-17A prominently suppressed RUNX2 levels of C2C12 cells treated with mucosal extracts of non-eosinophilic participants, whereas blocking IL-13 did

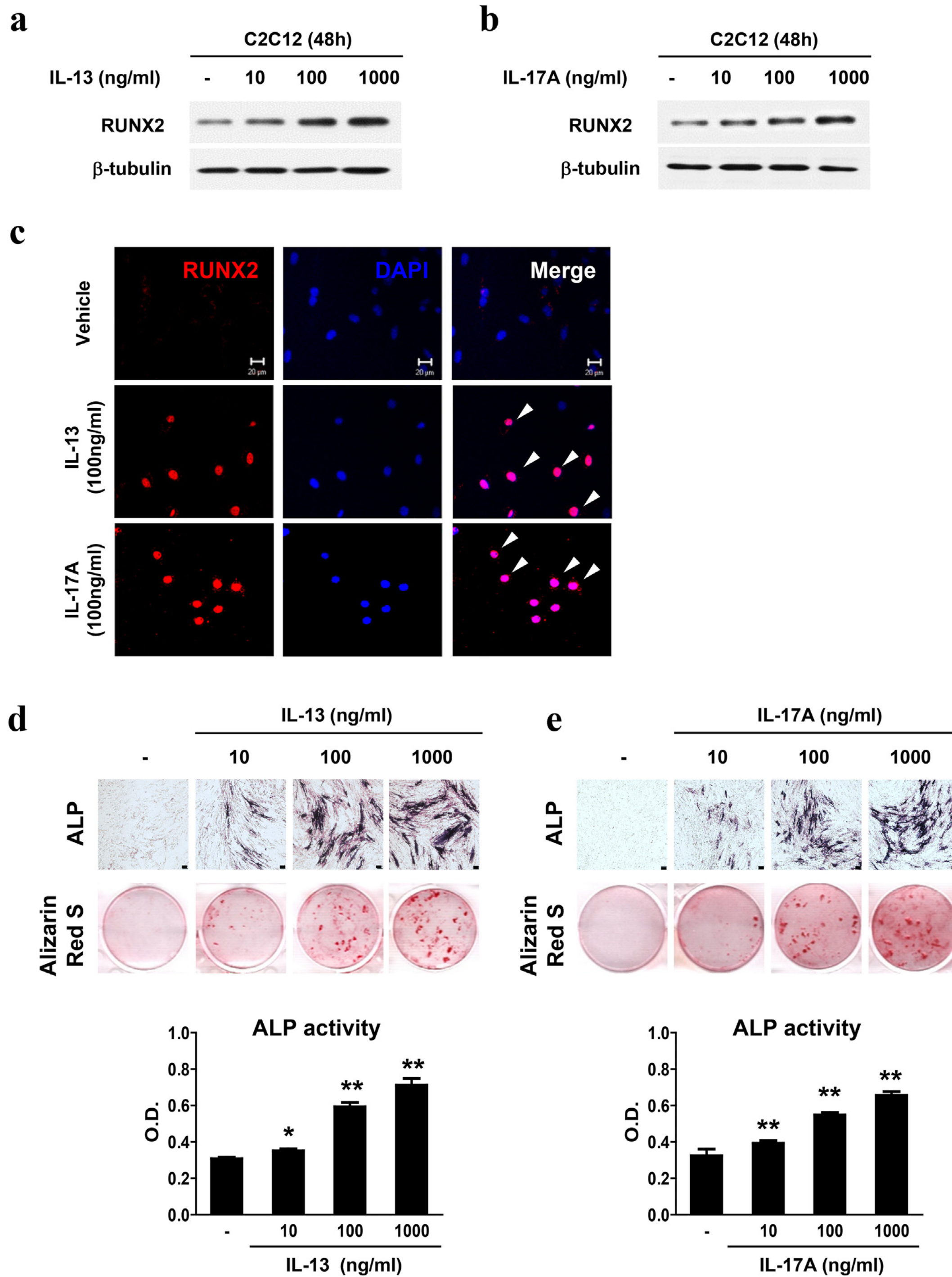

Fig. 3. Interleukin (IL)-13 and IL-17A induced RUNX2 expression, ALP activity, and calcium deposition in C2C12 cells. (a-b) C2C12 cell were incubated with IL-13 (10–1000 ng/ml) and IL-17A (10–1000 ng/ml) for the indicated time. The results showed that treatment with IL-13 or IL-17A increased the expression of RUNX2 in a dose-dependent manner. (c) C2C12 cells were treated IL-13 or IL-17A (100 ng/ml) and subjected to immunocytochemical analysis with anti-RUNX2 antibody. RUNX2 expression was detected in the cell nucleus (arrowhead). Scale bar = 20 μm. (d-e) C2C12 cells differentiate to osteoblasts upon IL-13 (d) or IL-17A (e) stimulation. Osteoblast differentiation was monitored by staining alkaline phosphatase (ALP) and by measuring its activity at day 7. Alizarin red S staining was performed to assess calcium deposition at day 21. All experiments were performed three times and data are expressed as mean ± SD. $^{*}P < .05$. $^{**}P < .01$. Mann-Whitney *U* test. Scale bar = 200 μm.

not. In addition, neutralizing antibodies to IL-13 and IL-17A reduced ALP activity and mineral deposition *in vitro* when they were treated in tissue extracts from eosinophilic and non-eosinophilic NP patients, respectively (Figs. 4d, 5d, E8c and E8d).

3.5. Increased expression of IL-13 and IL-17A in CRS patients with neoosteogenesis

We then investigated whether IL-13 and IL-17A were present in nasal tissues from CRS patients with neoosteogenesis. As shown in Fig. 6a and b, we determined the immunolocalisation of IL-13 and IL-17A in tissue sections from representative patient groups. IL-13- and IL-17A-positive immune cells were found more frequently in CRSwNP with neoosteogenesis when compared with those from CRSwNP without neoosteogenesis (Fig. 6c and d). In addition, the intense IL-13- and IL-17A-immunoreactive cells were found near bony surfaces (Fig. 6a and b). IL-13-positive cells were more frequently observed in eosinophilic CRS with neoosteogenesis than in non-eosinophilic CRS with neoosteogenesis (Fig. 6c). On contrary, it seemed that IL-17-expressed cells were relatively higher in non-eosinophilic CRS with neoosteogenesis than eosinophilic CRS (Fig. 6d). Furthermore, IL-13- or IL-17A-positive cells were correlated with RUNX2-positive cells as well as GOSS (Fig. E6).

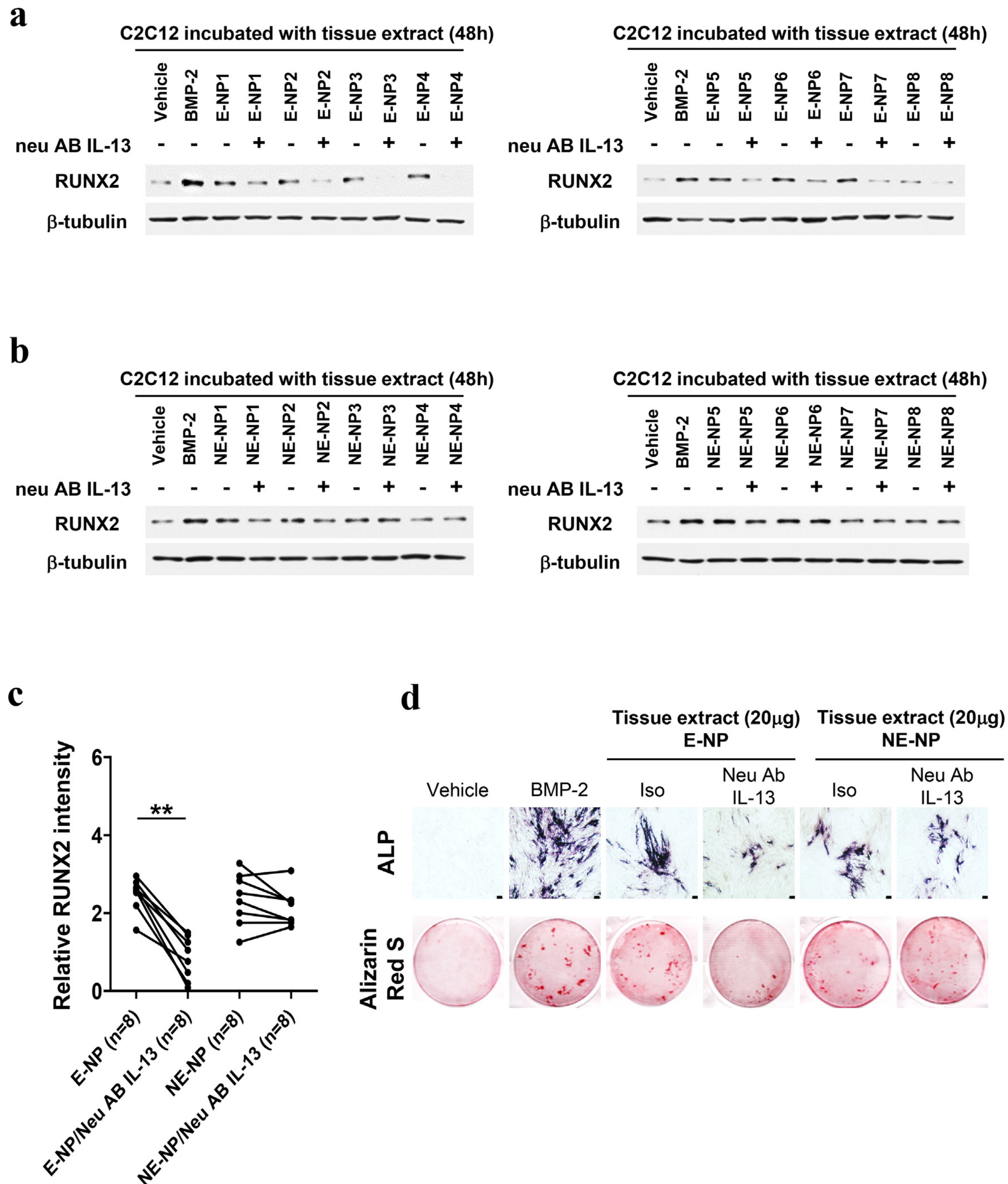

Fig. 4. The effect of IL-13 neutralization on RUNX2 expression in C2C12 cells. (a-c) Blocking IL-13 activity with a neutralizing antibody (0.5 μg/ml) reduced RUNX2 protein level in C2C12 cells treated with tissue extracts from eosinophilic CRSwNP. Whole cell extracts were immunoblotted with the indicated antibodies. (d) Osteoblast differentiation was monitored by staining ALP and by measuring its activity at day 7. Calcium deposition was assessed by staining with alizarin red S at day 21. Data are expressed as mean ± SD. *$P < .05$, **$P < .01$. Scale bar = 200 μm.

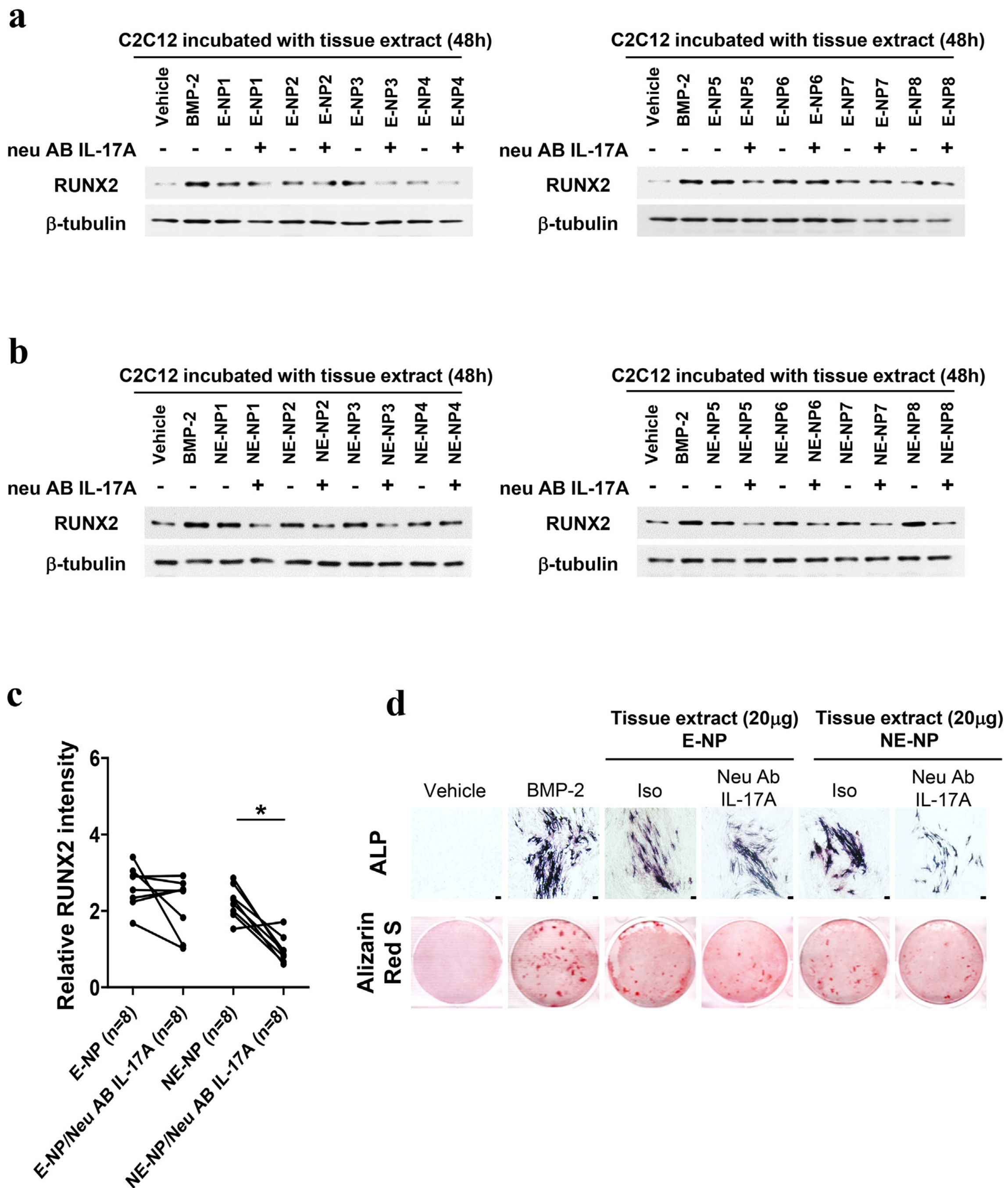

Fig. 5. The effect of IL-17A neutralization on RUNX2 expression in C2C12 cells. (a-c) Blocking IL-17A activity with a neutralizing antibody (0.5 μg/ml) reduced RUNX2 protein level in C2C12 cells treated with non-eosinophilic tissue extracts. Blocking IL-17A activity in tissue extracts from eosinophilic CRSwNP had no effect on RUNX2 level. Whole cell extracts were immunoblotted with the indicated antibodies. (d) Osteoblast differentiation was monitored by staining ALP and by measuring its activity at day 7. Calcium deposition was assessed by staining with alizarin red S at day 21. Data are expressed as mean ± SD. *$P < .05$, **$P < .01$. Scale bar = 200 μm.

3.6. IL-13Rα2 and IL-17RA expression in CRS patients with neoosteogenesis

Cytokines mediate their biological functions *via* surface receptors on target cells. IL-13 was recently reported to signal through the IL-13 receptor alpha 2 (IL-13Rα2) [25–27]. In addition, IL-17A was previously reported to bind and signal through the IL-17 receptor A (IL-17RA), a member of the IL-17A family of cytokine receptors [28]. Based on these, we examined the presence of IL-13Rα2 and IL-17RA around new bone surfaces on tissue sections from representative patient groups (Fig. 7a and b). Moreover, we found that the numbers of IL-13Rα2- and IL-17RA-positive cells were greater in patients with CRS with neoosteogenesis compared to healthy controls (Fig. 7c and d). Similar to the IL-13-positive cells, IL-13Rα2-positive cells were relatively higher in eosinophilic CRS with neoosteogenesis than in non-eosinophilic CRS with neoosteogenesis (Fig. 7c).

4. Discussion

Here we showed that CRS patients with neoosteogenesis showed the elevated RUNX2 expression, which was positively correlated with

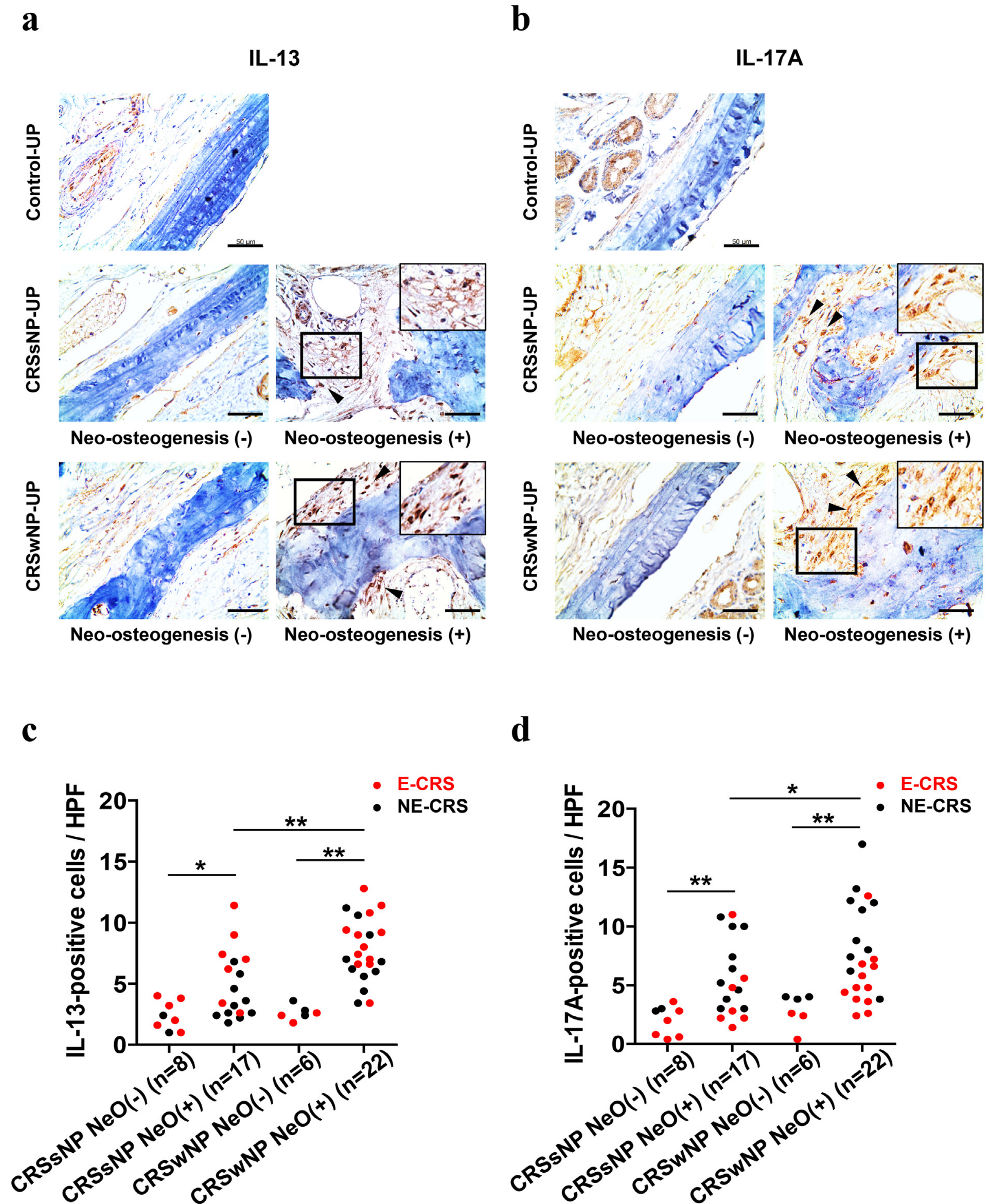

Fig. 6. Increased expression of IL-13 and IL-17A in chronic rhinosinusitis patients with neo-osteogenesis. (a-b) Representative images of IL-13 (a) and IL-17A (b) expressions on tissue sections from representative patients groups. IL-13 and IL-17A-producing immune cells were found at high frequency around bone tissue in CRS with neo-osteogenesis group. Arrowheads denote positive cells. Inset shows a higher magnification of the outlined area. Expression levels of IL-13 (c) and IL-17A (d) were reviewed under the HPF (magnification x400). The final score for each sample is presented as the average of scores from the 5 areas (HPF; magnification x400). Data are expressed as mean ± SD. $^{*}P < .05$, $^{**}P < .01$. Mann-Whitney *U* test. Scale bar = 50 μm.

GOSS. In addition, the pro-inflammatory cytokines IL-13 and IL-17A were found to augment osteoblast differentiation by activating RUNX2 in eosinophilic and non-eosinophilic CRS, respectively. RUNX2 induction by nasal extracts from CRS patients with high GOSS was reversed following treatment with anti-IL-13 or anti-IL-17A neutralizing antibodies. These findings implicated that IL-13- and IL-17A-induced RUNX2 could play a role in the progression of CRS into the severe status with neoosteogenesis.

New bone formation is frequently found in the severe form of CRS with or without NP [29]. Neo-osteogenesis in CRS involves structural changes in the underlying bone that lead to persistence of disease. There has been strong evidence for the positive correlation of osteitic

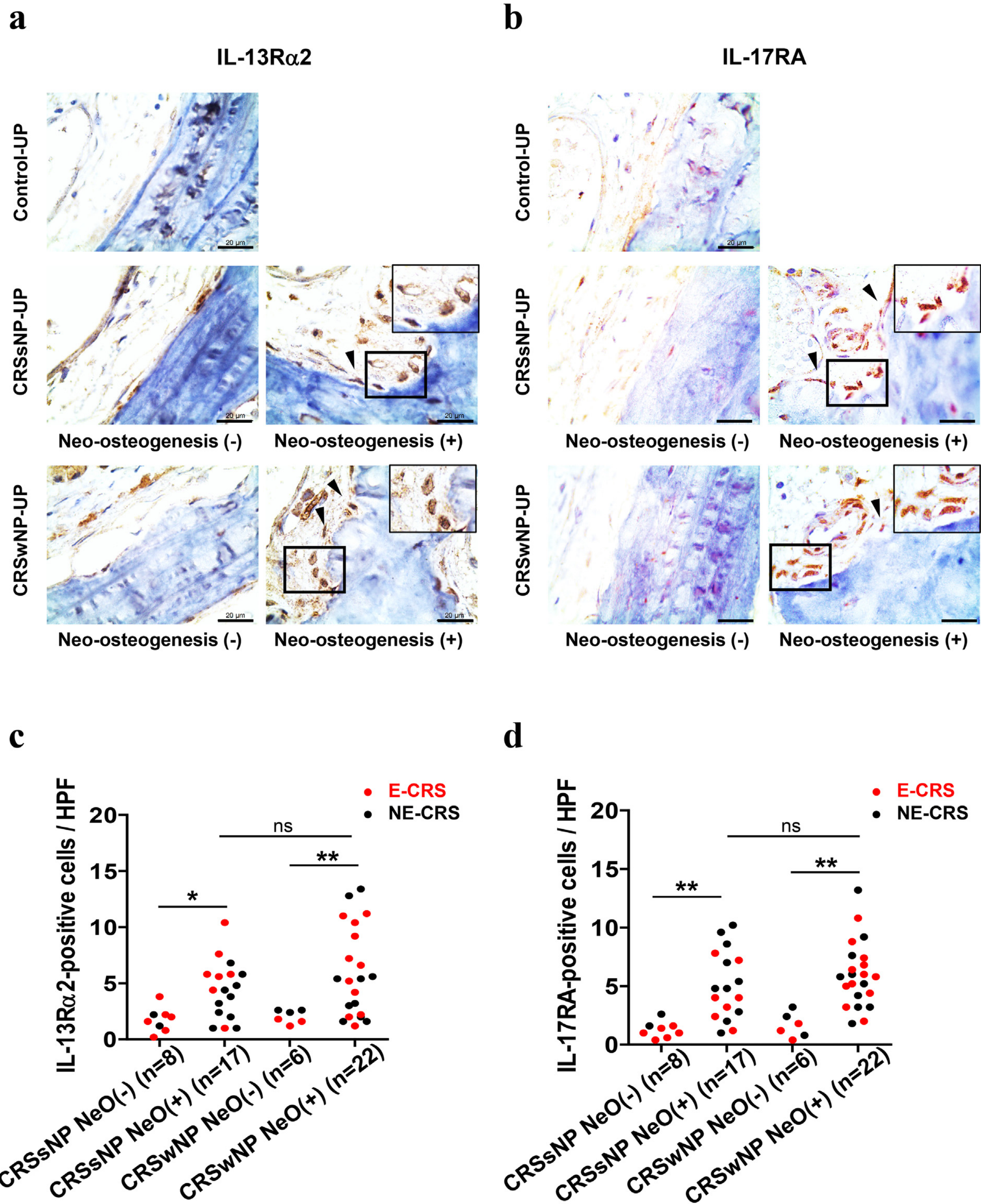

Fig. 7. IL-13Rα2 and IL-17RA expression in chronic rhinosinusitis patients with neo-osteogenesis. (a-b) Representative images of immunohistochemical staining for IL-13Rα2 (a) and IL-17RA (b) were shown from patients in the control and CRS groups. Arrowheads denote positive cells. Inset shows a higher magnification of the outlined area. Comparison of IL-13Rα2 (c) and IL-17RA (d) expression on tissue sections from representative patients groups. The final score for each sample is presented as the average of scores from the 5 areas (HPF; magnification x1000). Data are expressed as mean ± SD. $^{*}P < .05$. $^{**}P < .01$. Mann-Whitney U test. Scale bar = 20 μm.

changes to the severity of CRS. The bony changes have been identified in 33% of CRS patients with primary surgery [15]. The prevalence of bony changes was reported as around 76% in patients with previous sinus surgery [30]. Studies conducted by Georgalas et al. [15] and Huang et al. [31] confirmed the statistically significant association of higher osteitic scores with greater severity of disease. The impact of neoosteogenesis has been proposed as a prognostic factor for poor-operative outcome in a study by Bahadakar et al. [32] However, the etiology and pathogenesis of neoosteogenesis in CRS patients remain undefined.

The presence and acceleration of bone remodeling depends on the interaction of various inflammatory factors. Here we revealed that IL-13 and IL-17A within the mucosal environments from CRS patients with high osteitis scores could enhance RUNX2 as well as osteogenic

activities contributing on CRS progression. Actually there were several reports supporting our findings. Previously, Oue et al. investigated genetic and cellular changes in tissue associated with neoosteogenesis in CRS patients [33]. In their study, expression of IL-13, CCL13, colony-stimulating factor 3, integrin alpha M, tumor necrosis factor-α, and ALP liver/bone/kidney were significantly upregulated in CRS patients with neoosteogenesis when compared with the control and CRS patients without neoosteogenesis. Moreover, a positive correlation was found between IL-13 concentrations and the degree of mineralisation in primary osteoblasts isolated from CRS patients. Another study by Silfversward et al. reported that IL-4 and IL-13 enhanced markers of differentiated osteoblastic activity, including stimulation of collagen secretion, ALP expression, and mineralisation [34]. On contrary, the mechanism or molecular target underlying osteoblastic activities has not been revealed much. One paper mentioned that RUNX2 protein level was induced by IL-13 (50 ng/ml) *in vitro* in bronchial epithelial cells [10]. However, this was not proven in osteoblasts or similar cellular conditions.

In addition to IL-13, several cytokines were also found in the sinonasal tissue of neoosteogenesis. Tuszynska et al. reported significant upregulation of IL-6, IL-11, and TNF-α expression levels in bone tissue in CRS patients [35]. More recently, Wang et al. reported that the TGF-β/Smads signaling pathway contributes to osteitis in CRS patients [36]. However, we did not detect TGF-β activation here. Similar to our finding, Katarigi et al. reported that C2C12 myoblasts treated with TGF-β did not induce ALP activity and osteocalcin production [18]. In addition, we screened other Type 2 (IL-5, periostin, eotaxin-1, −2) and Type 17 cytokines (IL-22, IL-23, and IL-6) and type 1 cytokine (IFN-ɤ) using multiplex cytokine assay and investigated associations between these cytokines and neoosteogenesis based on GOSS. There was no correlation between these cytokines and neoosteogenesis (data not shown).

We also found that IL-17A and its receptor were highly expressed in CRS with neoosteogenesis. Especially, anti-IL-17A neutralizing antibody suppressed RUNX2 levels of C2C12 cells treated with mucosal extracts of non-eosinophilic participants. IL-17A (formerly called IL-17) is a member of the IL-17 cytokine family, which consists of six cytokines, termed IL-17A-F [37]. Th17 cells, innate lymphoid cells, neutrophils, and macrophages are known to as the major sources of IL-17A production [38]. IL-17A mainly mediates its immune regulatory function by promoting the generation of pro-inflammatory cytokines and chemokines, but recent studies have examined the possible role of IL-17A in osteogenic differentiation. Kocic et al. reported that IL-17 (100 ng/ml) promotes osteogenic differentiation of C2C12 myoblasts by activating Erk1/2 [39]. In addition, Yu et al. presented that IL-17A treatment (10 ng/ml) increased the expression of osteogenic proteins (RUNX2, osteocalcin, and ALP) and calcium module formation in human dental pulp stem cells when compared to the untreated control group [40]. Furthermore, Ono et al. showed that IL-17A promoted bone formation in injury-associated mesenchymal cells through the stimulation of both osteoblast proliferation and differentiation [41]. A previous study by Jo et al. reported that IL-17A promoted osteoblastic differentiation by upregulating the expression of JAK2/STAT3, C/EBPβ, and RUNX2 in primary bone-derived cells [42]. Osta et al. reported that a high level (50 ng/ml) of recombinant human IL-17A increased osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by inhibiting Dickkopf-1 mRNA expression [43]. Moreover, IL-17A induced osteogenic differentiation in human exfoliated deciduous teeth as evidenced by high ALP activity, increased matrix mineralisation, and upregulation of the mRNA expression of osteogenic markers [44]. Thus we thought that IL-17A could play a critical role in the new bone formation in CRS and NP, especially non-eosinophilic and Asian CRS, where IL-17A has been shown as higher rather than eosinophilic CRS.

Eosinophilic CRSwNP was characterised by ethmoidal dominance, whereas non-eosinophilic CRSwNP showed maxillary dominance based on CT findings [45]. Considering that neoosteogenesis is observed in the center of inflammation, there seems to be differential location of neoosteogenesis according to CRS subtype. In addition, eosinophilic CRSwNP is driven by Th2 cytokines such as IL-13 and non-eosinophilic CRSwNP has signature cytokines such as IL-17A [46]. Like these, we also found that IL-13 and IL-17A activated osteoblasts in eosinophilic and non-eosinophilic CRSwNP here. Furthermore, the pattern of bone remodeling might be different in each subtype of CRS. There is an evidence that IL-17, a signature cytokine in non-eosinophilic CRS, induces RANKL on osteoblast, resulting in bone destruction and inflammation by activation of osteoclasts *via* RANK/RANKL axis in other chronic inflammatory diseases such as rheumatoid arthritis [47] and in periodontitis [48]. Therefore, neoosteogenesis and osteolysis might be mixed in non-eosinophilic CRSwNP. To characterise each subtype of CRSwNP is also one of major topics in CRS osteitis and needed in further study.

We proved that the signature cytokine of each subtype of CRSwNP could activate osteoblasts resulting in neoosteogenesis which may be the result of refractory CRS. Given that neoosteogenesis is a characteristic finding of revision surgeries and a prognostic factor of poor surgical outcomes, neoosteogenesis may exacerbate inflammation. One explanation is that activated osteoblasts can produce multiple inflammatory mediators such as receptor activator of nuclear factor κB ligand (RANKL) [49], oncostatin M (OSM) [50] and bone morphogenetic protein (BMP)-2 [51] which influence surrounding microenvironments. RANKL can be involved in B cell differentiation and lymphoid organogenesis in airway as well as osteoclastogensis in bone [52]. Recently, it was reported that ectopic lymphoid follicle formation is one of signature findings of refractory CRSwNP [53]. Recent studies showed OSM upregulation in CRSwNP which disrupted epithelial barriers [54]. BMP-2 is one of strong inducer of neoosteogenesis. Although it has not been fully investigated in nasal polyposis, BMP-2 can induce epithelial-mesenchymal transition in epithelial cells [55], which is one of signature findings of CRSwNP [56].

Taken together, we found that RUNX2 was highly expressed in CRS patients with neoosteogenesis, and its expression level correlated with GOSS. Our findings strongly suggest that RUNX2 plays a crucial role in the pathogenesis of neoosteogenesis in CRS patients, and that inhibition of pro-inflammatory cytokines including IL-13 and IL-17A with osteogenic function may provide a new treatment strategy for patients with severe CRS.

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Author contributions

Conception and design: H.-W.S. and D.W·K.; acquisition of data: R.K. and D.W.K; analysis and interpretation of data: R.K., H.-W.S., D.W.K. and J.-W.P.; drafting the article or revising it critically for important intellectual content: R.H., H.-W.S. and D.W.K.; supervision: H.-W.S. and D.W.K.

Declaration of Competing Interest

The authors have declared that no competing interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.035.

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