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Leptin/osteopontin axis contributes to enhanced T helper 17 type responses in allergic rhinitis

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Abstract

Background: Recent studies suggest that T helper 17 (Th17) cell subset, a distinct pro-inflammatory CD4⁺ T cell lineage, may play an important role in the pathophysiology of allergic rhinitis (AR). However, the regulation of Th17 response in allergic disease is not well characterized.

Methods: Thirty AR and 30 healthy children were enrolled. Serum leptin and OPN levels were measured, and their correlation with IL-17 expression was analyzed using enzyme-linked immunosorbent assay (ELISA). Th17 cell differentiation and cytokine production in peripheral blood mononuclear cell (PBMCs) stimulated by leptin and OPN and related inhibitors were analyzed by ELISA. AR mice models were also established to verify the effect of leptin and OPN on Th17 cell regulation. Immunoprecipitation was performed to explore the interaction between OPN and leptin in Th17 cells.

Results: Our results showed that elevated serum leptin and OPN in AR children were correlated with serum IL-17 level ($r = .53$, $P < .01$). The recombinant leptin and OPN enhanced Th17 responses from PBMCs synergistically through nuclear factor κ B (NF- κ B), mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) pathway and β 3 integrin receptor. The AR mice showed as more severe Th17 responses and symptoms compared with control mice. Immunoprecipitation showed that OPN and leptin may interact with each other directly, and this process may be mediated by β 3 integrin.

Conclusions: Our data provide evidence that upregulation of leptin and OPN promotes Th17 responses in AR, and this process may be achieved through NF- κ B, MAPK, and JNK pathway and β 3 integrin.

KEYWORDS

allergic rhinitis, IL-17, leptin, osteopontin, Th17

1 | INTRODUCTION

Allergic rhinitis (AR) is a worldwide common disease of upper airway in children with occurrence of 10%-40%.^{1,2} It negatively affects both quality of life and study of children and often accompanied by asthma.³ Inappropriate CD4⁺ T cell responses, especially the dysregulation

of the T helper 1 and 2 (Th1/Th2) balance leading to excessive Th2 cell activation, have been associated with AR in previous studies.^{4,5} Nevertheless, recent studies suggest that IL-17A and IL-17A-producing T helper 17 (Th17) cell subset, a distinct pro-inflammatory CD4⁺ T cell lineage, are also deeply involved in the pathophysiology of AR.^{6,7} Previous studies also showed that exogenous anti-IL-17 and anti-IL-23

antibodies were able to reduce the symptom severity, Th2 response, and serum IgE level in AR mice induced by ovalbumin, suggesting that Th17 and Th2 responses interacted closely.⁸⁻¹⁰ However, the regulation of Th17 response in AR is not well established.

Osteopontin (OPN) is a pleiotropic protein that is broadly expressed by various cells and involved in a wide range of biological processes, including bone remodeling, inflammation, cancer, and immunity to infectious disease.^{11,12} Binding of OPN to different cell surface receptors, including $\alpha v\beta 3$, $\alpha v\beta 1$, $\alpha 4\beta 1$, $\alpha 8\beta 1$, and $\alpha 9\beta 1$ integrins as well as CD44, induces signaling events to promote cell adhesion, migration, and survival.¹³ OPN was also found to promote the differentiation of Th17 cells and IL-17 production in rheumatoid arthritis, experimental autoimmune encephalomyelitis, and multiple sclerosis (MS).¹⁴⁻¹⁶

Leptin is a 16 kDa non-glycosylated polypeptide encoded by the obese (ob) gene, which regulates energy homeostasis, neuroendocrine function, reproduction, angiogenesis, hematopoiesis, and T cell activation and function.¹⁷ Several studies showed that leptin drives Th17 immune responses by inducing ROR γ t transcription in systemic lupus erythematosus and arthritis.^{18,19}

The similar roles between OPN and leptin in regulation of Th17 response suggested that OPN and leptin may interact with each other in AR. However, the interaction and pathway between OPN and leptin in AR are not well established. Therefore, we aimed to investigate the correlation between leptin and OPN in Th17 response of AR in children in this study.

2 | MATERIALS AND METHODS

2.1 | Patients

Thirty AR children (5-10 years old) were recruited at the Department of Otolaryngology, Guangzhou Women and Children's Medical Center from January 2016 to October 2016. The study protocols were approved by Guangzhou Women and Children's Medical Center's ethics committee boards. Informed consent was obtained from the parents of all subjects. The diagnosis of perennial AR was made according to symptoms, physical examinations, and specific IgE measurement according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guideline (2010).²⁰ The atopic status was evaluated by the detection of IgE specific to common inhalant allergens (dust mites, pets, molds, cockroach, etc.). Specific IgE values greater than or equal to 0.35 kIU/L were considered positive.

For controls, 30 healthy children (5-10 years old) were enrolled. Children with abnormal weight, allergic diseases, or use of drugs within the past 4 weeks were excluded. Children between the 5th and 85th percentiles of body mass index for age were considered to be normal weight and the rest were defined as abnormal weight according to a local population survey.²¹

2.2 | Symptom scores

A total nasal symptom score (TNSS) was recorded for the evaluation of severity in children with the help of parents as described

previously.²² Symptoms including sneezing, rhinorrhea, itchy nose, and nasal congestion were recorded on a 0 to 3 scale (0, none; 1, mild; 2, moderate; and 3, severe) and the TNSS (0-12) was summed. The nasal symptoms were recorded and averaged in an 8-week observation period.

2.3 | Blood sample collection

Venous blood samples were collected between 6 AM and 8 AM after an overnight fast. The serum samples were separated and stored at -80°C for assay after centrifuging at 1000 g for 15 minutes at 4°C .

2.4 | Th17 cell differentiation and cytokine production in peripheral blood mononuclear cells (PBMCs) stimulated by leptin and OPN

Peripheral blood mononuclear cells (PBMCs) from AR and control children were isolated by Lymphoprep density gradient centrifugation from heparinized leukocyte-enriched buffy coats according to the manufacturer's instruction. In brief, the blood was diluted with an equal amount of Dulbecco's phosphate-buffered saline with 2% fetal bovine serum and layered on top of Lymphoprep carefully to minimize mixing of blood with Lymphoprep. Then, the mixture was centrifuged at 800 g for 20 minutes at room temperature, and the upper plasma layer was removed and discarded without disturbing the plasma:Lymphoprep interface. The sorted cells were cultured at 37°C in 5% CO_2 for 3 days in 1 mL medium (2×10^6 cells/mL) with phorbol myristate acetate (PMA) (50 ng/mL) and ionomycin (500 ng/mL) in the presence or absence of house dust mite extract (20 $\mu\text{g/mL}$ HDM, RayBiotech, USA), recombinant leptin and OPN, or other stimulators or inhibitors (R&D, USA). Then, the Th17 cell differentiation was verified by detection of ROR γ t by real-time PCR as described previously²² and analyzing cytokines expression (IL-23, IL-27, and IL-17) in supernatant by enzyme-linked immunosorbent assay (ELISA). The specific inhibitor of p38 mitogen-activated protein kinase (MAPK) (SB-203580), as well as the mitogen-activated protein kinase (MAPK) inhibitors (PD-98059), nuclear factor κB (NF- κB) inhibitor (SN50), c-Jun N-terminal kinase (JNK) inhibitor (SP600125), was purchased from Calbiochem (USA).

2.5 | Animal models

All animal care and experimental protocols were approved by local ethics committee boards (2017010601). Six-week-old male BALB/c mice were housed three per cage on a 12 hours light-dark cycle and fed for 10 weeks with a standard chow diet. A total of 42 mice were randomly divided into the control (12 mice), AR (12 mice), and intervention groups (18 mice).

Ovalbumin (OVA; Sigma, St. Louis, MO, USA)-induced airway inflammation was performed in mice as described previously and summarized in Figure 1.²³ The mice were sensitized with a subcutaneous injection of ovalbumin (100 mg OVA) mixed with 1.6 mg $\text{Al}(\text{OH})_3$ in

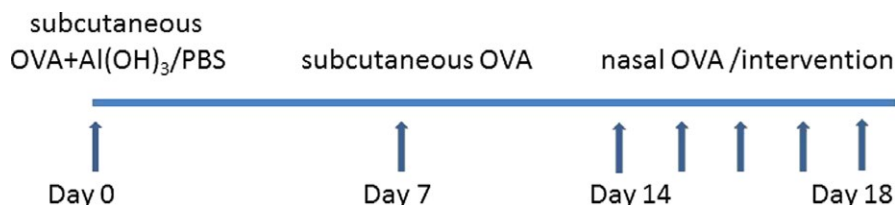


FIGURE 1 Experimental protocol for mouse sensitization and treatment. The mice were sensitized with a subcutaneous injection of ovalbumin (OVA) mixed with Al(OH)₃ on day 0 and day 7. The sensitized mice were challenged nasally by OVA from day 14 to day 18. The control mice received PBS sensitization and challenge instead of OVA. For the intervention group, the 100 µg of recombinant antileptin, anti-OPN protein, or anti-β3 integrin was administered intranasally 2 h before each OVA challenge, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

0.9% NaCl (day 0). One week later (day 7), the mice received a second subcutaneous injection of 100 mg ovalbumin. The sensitized mice were challenged nasally by the administration of 10 mg of OVA in 40 mL of PBS (20 mL per nostril) from day 14 to day 18. For the control groups, the mice received PBS sensitization and challenge instead of OVA. For the intervention group, the 100 µg of recombinant antileptin, anti-OPN protein, or anti-β3 integrin (R&D Systems, Minneapolis, MN, USA) in 40 mL of PBS was administered intranasally 2 hours before each OVA challenge, respectively.

Twenty-four hours after the last challenge, the mice were anaesthetized and exsanguinated and nasal tissues were collected for morphological study, as detailed below.

2.6 | Elisa

The serum or supernatant levels of leptin, OPN, IFN-γ, IL-23, IL-27, and IL-17 were measured in duplicate by ELISA kits (R&D, USA) according to the manufacturer's instructions. The detection limits of the assays were as follows: leptin (22 pg/mL), OPN (312.5 pg/mL), IL-17 (15 pg/mL), IL-23 (16.3 pg/mL), and IL-27 (156 pg/mL).

2.7 | Morphological study

Nasal turbinate sections of 4- to 5-µm thickness were prepared, deparaffinized, and rehydrated. The hematoxylin-eosin (HE) staining was performed. The rabbit polyclonal antibody against OPN (Zhongshan Goldenbridge, Beijing, China) (1:200), rabbit polyclonal antibody against IL-17 (Zhongshan Goldenbridge, Beijing, China) (1:200) or rabbit polyclonal antibody against leptin (1: 200, Thermo Fisher Scientific, Fremont, CA, USA), and secondary biotinylated goat anti-mouse/rabbit IgG antibody (Zhongshan Goldenbridge, Beijing, China) (1:200) were used for immunohistochemistry (IHC). Isotype-matched IgG was used in place of the primary antibody as the negative control.

2.8 | Cell lysis and immunoprecipitation

For Th17 polarization experiments, human naïve CD4⁺ T cells were negatively selected from PBMCs by magnetic activated cell sorting (Miltenyi Biotec). The purity of the CD4⁺ T cells was 97.2% ± 0.9% as assessed by flow cytometric analysis. The cells were seeded with

a density of 1.5×10^6 cells/mL in 24-well plates coated with anti-CD3 and anti-CD28 (1 µg/mL). IL-1β (50 ng/mL), IL-6 (50 ng/mL), IL-23 (40 ng/mL), and TGF-β1 (2 ng/mL) were added at day 0. The supernatants and cells were collected after 96 hours for further experiments.

Cell protein extracts from differentiated Th17 cells were prepared using radioimmunoprecipitation assay buffer (25 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, protease inhibitor). Immunoprecipitation was conducted using rabbit antihuman leptin or rabbit antihuman OPN antibody (Santa Cruz Biotechnology) for co-immunoprecipitation assay and analyzed by SDS-PAGE (Invitrogen, Paisley, UK) and detected with Western blot analysis by chemiluminescence (ECL Plus; GE Healthcare, Chalfont St. Giles, UK) using mouse anti-rabbit HRP-conjugated secondary antibodies.

2.9 | Statistical analysis

All data are expressed as the medians and interquartile ranges and analyzed using the Kruskal-Wallis H test or the nonparametric Mann-Whitney U test except additional note. Correlations between the various parameters were assessed by the Spearman rank correlation analysis. A *P* value of less than .05 was defined as statistically significant.

3 | RESULTS

3.1 | Elevated serum leptin and OPN in AR children and its relationship with Th17 cytokines and disease severity

The characteristics of participants are summarized in Table 1. The subgroups had comparable age, sex ratio, and age (*P* > .05). The expression of IL-17 and IL-23 was significantly higher in AR children compared with controls, while the expression of IL-27 was significantly higher in controls compared with AR children (*P* < .05). Our results suggested that serum leptin and OPN concentrations were significantly increased in children with AR compared with normal controls (*P* < .05) (Figure 2). The elevated leptin and OPN levels were also significantly correlated with total nasal symptom score in children with AR (*r* = .52, *P* < .01; *r* = .59, *P* < .01), suggesting that leptin

and OPN levels were related to disease severity (Table 2) ($P < .05$) (Figure 2). We also found that the elevated serum leptin concentration was significantly correlated with serum IL-17 and IL-23 in AR patients ($r = .53$, $P < .01$; $r = .51$, $P < .01$), while serum OPN concentration was significantly correlated with serum IL-17 and IL-27 ($r = .55$, $P < .01$; $r = -.65$, $P < .01$) (Figure 2).

TABLE 1 Demographic characteristic of AR children and normal controls

Groups	AR group	Control
Number	30	30
Sex (Male:Female)	17:13	15:15
Age (mo)	85.6 ± 23.1	83.2 ± 26.1
TlgE	132.1 (15.6-569.3)*	22.6 (7.1-67.8)
Sensitization		
D1 (n)	21	-
D2 (n)	18	-
Cockroach (n)	8	-
Cat (n)	12	-
Dog (n)	7	-
Others (n)	3	-
TNSS score	8.7 ± 2.5	-
IL-4 (pg/mL)	13.5 ± 4.1*	3.9 ± 1.1
IL-13 (pg/mL)	123 ± 29*	87 ± 18
IL-17 (pg/mL)	98 ± 35*	32 ± 21
IL-23 (pg/mL)	81 ± 19*	28 ± 19
IL-27 (pg/mL)	521 ± 271*	856 ± 332

TNSS, total nasal symptom score.

D1, *Dermatophagoides pteronyssinus*. D2, *Dermatophagoides farinae*.

*Compared with the control group, $P < .05$.

3.2 | Th17 cell differentiation and cytokine expression by PBMCs stimulated by leptin/OPN and related signal pathway

Peripheral blood mononuclear cells from the healthy control group did not produce IL-17 in response to HDM. Our results showed that both leptin (6.25 ± 1.23 , $P < .05$) and OPN (6.08 ± 1.05 , $P < .05$) promote significant elevated relative expression of ROR γ t compared with control, especially when leptin and OPN (9.38 ± 2.45 , $P < .05$) were added together. Moreover, PBMCs from AR children produced significant upregulation of IL-17 and IL-23 protein after stimulation with leptin, while OPN promotes upregulation of IL-17 and inhibits IL-27 expression ($P < .01$). We also found that IL-17 production was enhanced when leptin and OPN were given together. Besides, leptin- or OPN-mediated IL-17 production may be inhibited by NF- κ B, MAPK, and JNK inhibitors and anti- β 3 integrin (Figure 3). However, the p38 pathway was not changed during the IL-17 production (data not shown).

Additionally, leptin also induces OPN expression in PBMCs dose-dependently and this process can be inhibited by phosphatidylinositol 3-kinase (PI3K) and serine/threonine protein kinases (AKT) inhibitors (Figure 3), whereas OPN has no direct effect on leptin production by PBMCs (data not shown).

Next, we also detected the effect of Th17 cytokines on the expression of leptin or OPN. Our results showed that IL-17 is able to inhibit expression of OPN and leptin, while IL-23 promotes leptin expression and IL-27 suppresses OPN expression (Figure 3).

3.3 | Leptin/OPN augments Th17 responses in mice

The OVA-specific IgE, the times of nasal rubbing and sneezing, the count of lymphocytes, and Th17 cells were significantly higher in AR mice than that of control mice (Figure 4). Our results also showed that the antileptin, anti-OPN, or anti- β 3 integrin attenuate

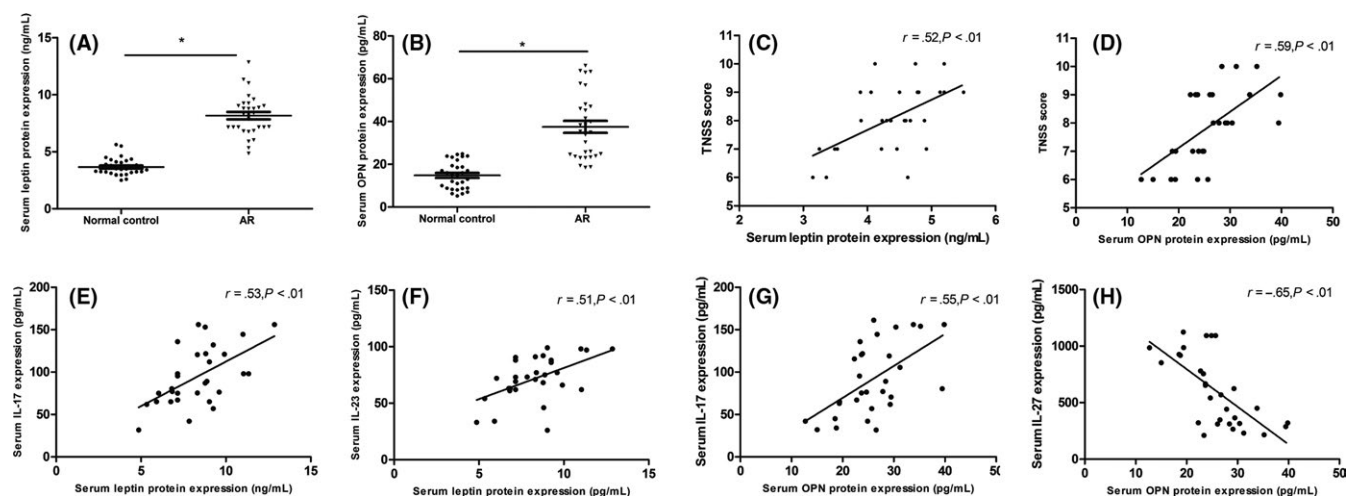


FIGURE 2 Elevated serum leptin (A) and Osteopontin (OPN) (B) expression in AR children and the association between serum leptin and TNSS score (C), IL-17 (E), and IL-23 (F) levels as well as the association between serum OPN and TNSS score (D), IL-17 (G), and IL-27 (H) levels in patients with AR. * $P < .05$. TNSS, total nasal symptom score

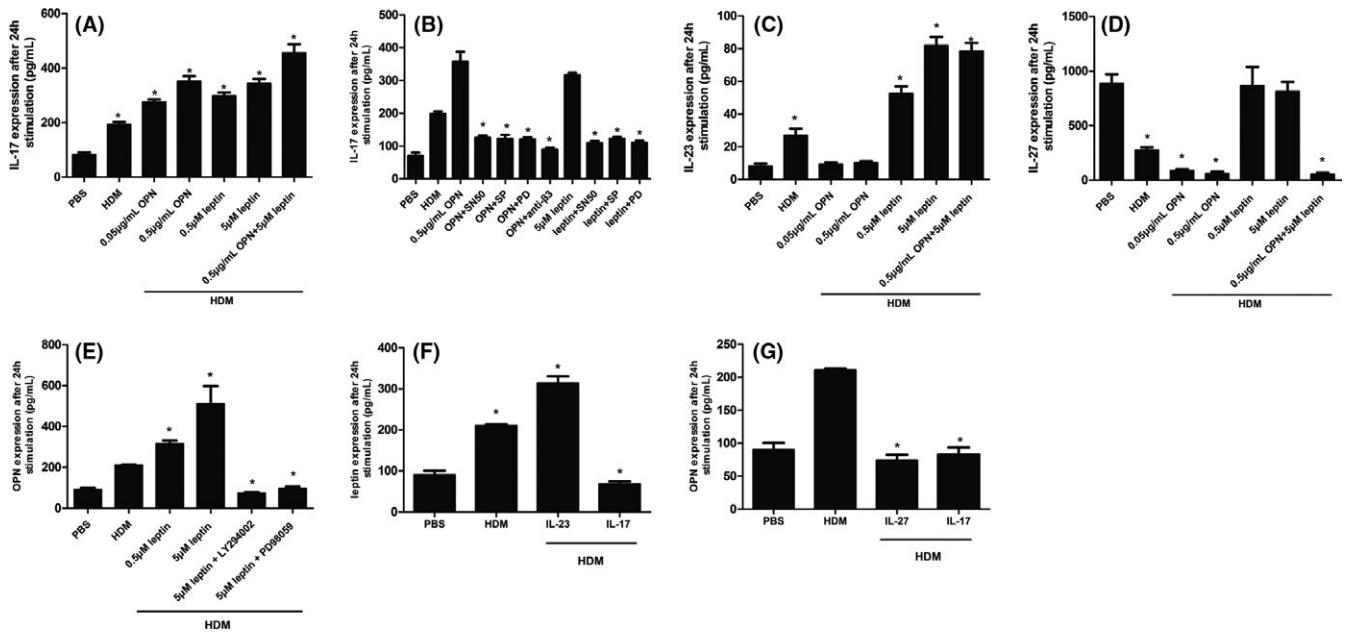


FIGURE 3 The expression of IL-17 (A, B), IL-23 (C), IL-27 (D), and Osteopontin (OPN) (E) by CD4⁺ T cells from AR children after leptin and/or OPN stimulation. The effect of IL-17 and IL-23 on the expression of leptin and OPN is shown in Figure F and G. HDM, house dust mite, PD, MAPK inhibitors, SN50, nuclear factor κ B inhibitor, SP, JNK inhibitor. **P* < .05

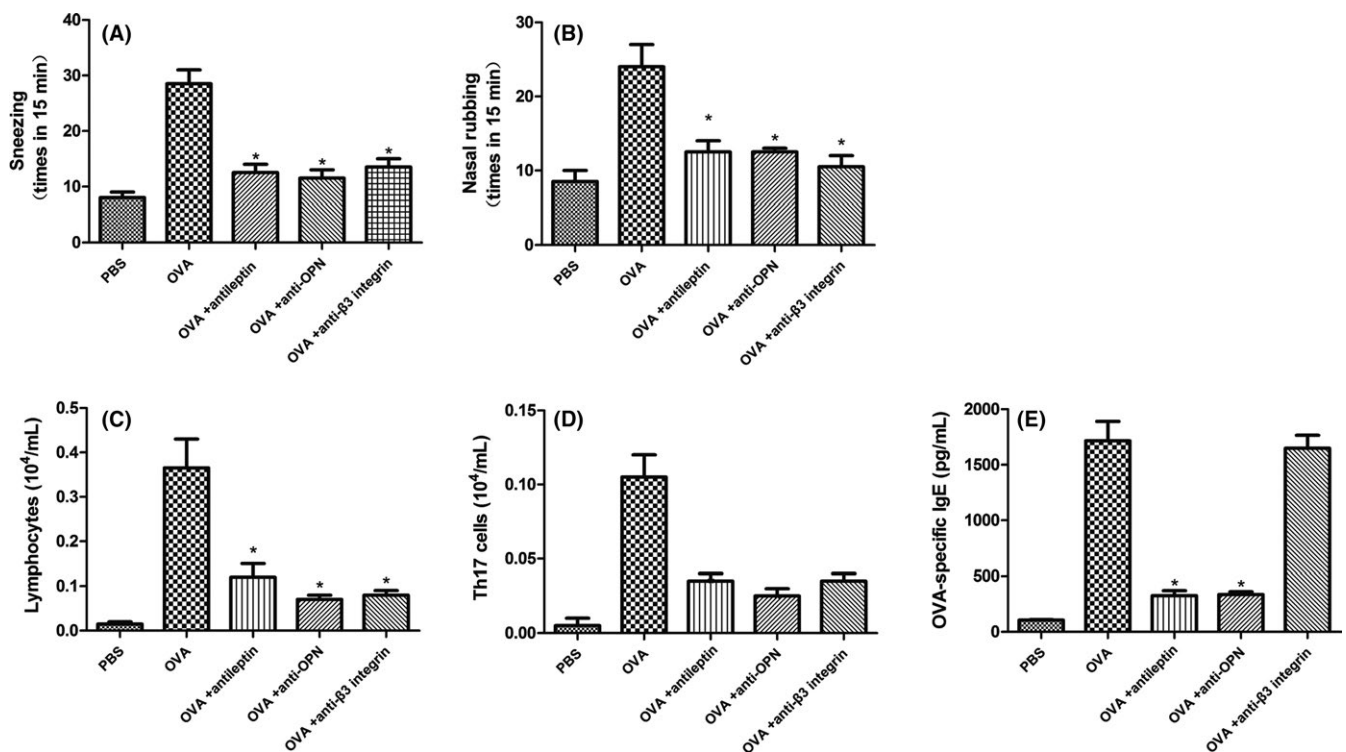


FIGURE 4 The times of nasal rubbing and sneezing (A,B), the count of lymphocytes (C), Th17 cells (D), and OVA-specific IgE (E) were significantly higher in AR mice, and the antileptin, anti-OPN, or anti- β 3 integrin may attenuate inflammation in AR mice. **P* < .05

Th17 responses in AR mice, manifested as lower symptom score and less Th17 cell infiltration (*P* < .05). In inferior turbinate mucosal tissues from OVA-challenged mice, epithelial cells, interstitial cells, glandular cells, and endothelial cells were positive for leptin and OPN, IL-17 staining and the number of OPN, leptin and

IL-17 positive cells per high power field (HPF, 400 $\times$) were significantly higher than that of normal controls (*P* < .05) (Figure 5). We also found that the leptin (*r* = .67, *P* < .05) and OPN (*r* = .59, *P* < .05) concentrations of inferior turbinate mucosal tissues were significantly correlated with upregulated IL-17 levels.

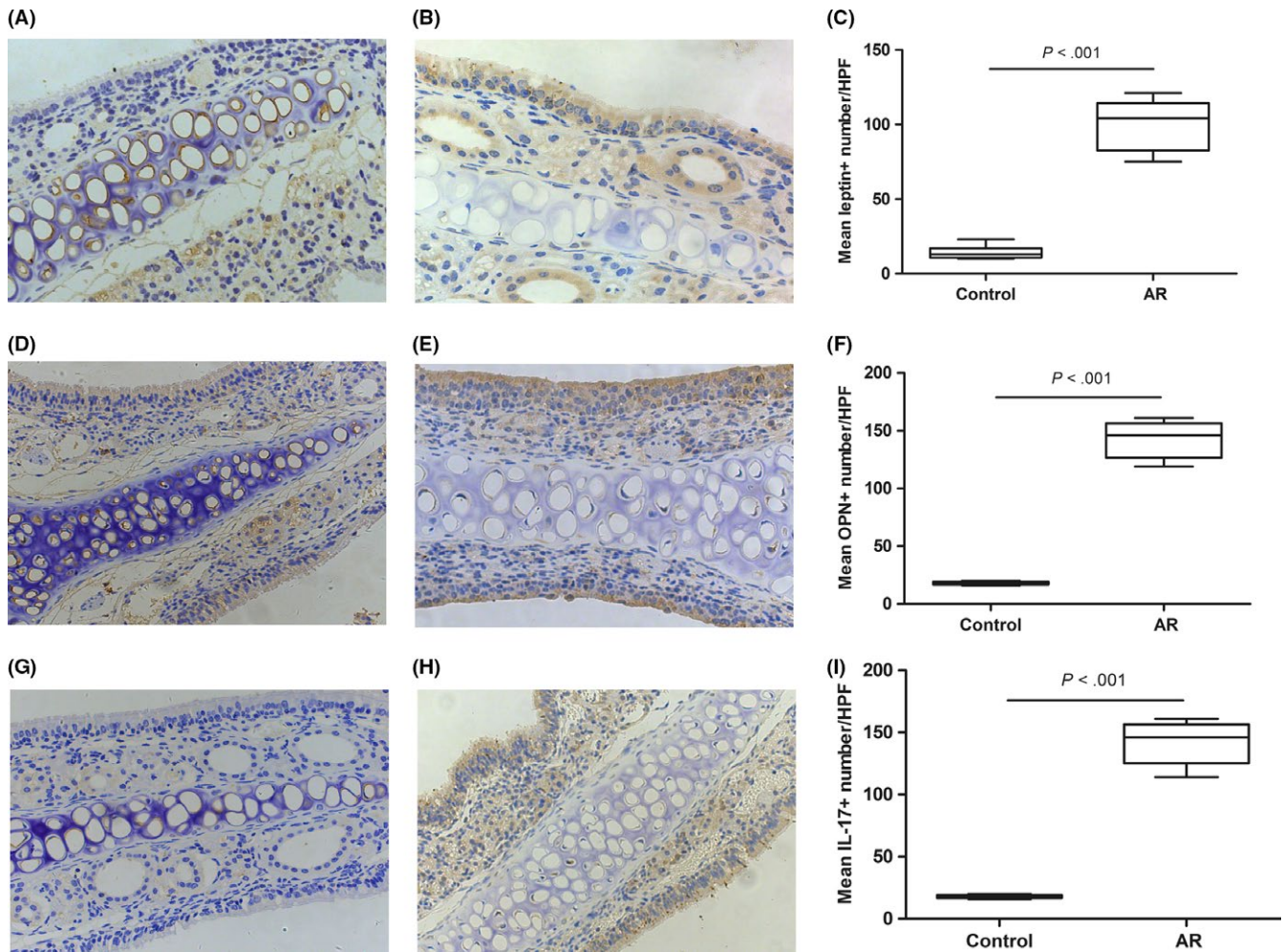


FIGURE 5 The expression and cellular sources of leptin, Osteopontin (OPN), and IL-17 in the nasal mucosa of OVA-challenged mice (B,E,H) were significantly higher than in control mice (A,D,G). The mean number of leptin-positive cells (C), OPN-positive cells (F), and IL-17 positive cells (I) in nasal tissues and normal control

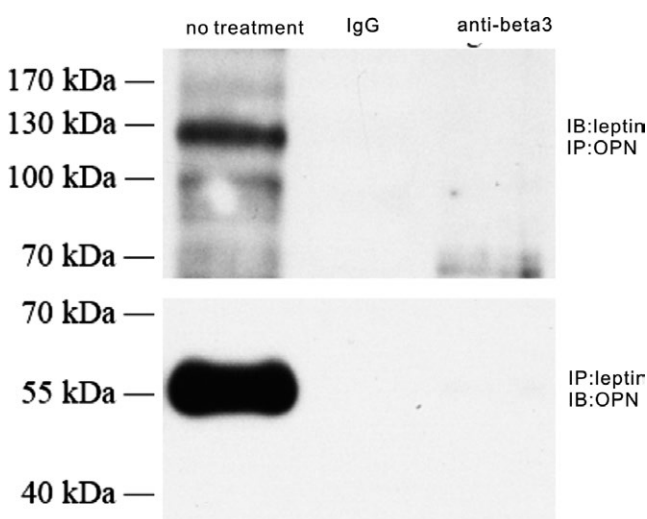


FIGURE 6 Interaction between leptin and Osteopontin (OPN) by co-immunoprecipitation in differentiated Th17 cells. The anti-OPN, antileptin, or anti- $\beta 3$ integrin treatment may block the interaction between OPN and leptin

3.4 | Co-immunoprecipitation

Whole protein was extracted from differentiated Th17 cells, and leptin was co-immunoprecipitated with OPN. Our results showed that leptin was detected in OPN immunoprecipitates, suggesting that leptin and OPN were in the same complex. In the same way, co-immunoprecipitation with leptin or OPN was conducted at 48 hours after anti- $\beta 3$ integrin treatments and the results suggested that all these antibodies can block the interaction between leptin and OPN (Figure 6).

4 | DISCUSSION

In this study, our results suggested that upregulation of leptin and OPN was correlated with Th17 responses in AR. We provide evidence that leptin amplifies Th17 responses through upregulation of IL-23, while OPN promotes Th17 responses through downregulation of IL-27. Those above regulation between leptin and OPN may be through NF- κ B, MAPK, and JNK pathways and $\beta 3$ integrin. The

Subgroups	IL-17 (pg/mL)	IL-23 (pg/mL)	IL-27 (pg/mL)
Sensitization			
Mono-allergens	84 ± 31	72 ± 16	537 ± 189
Poly-allergens	99 ± 27	85 ± 21	496 ± 195
Disease duration			
<2 y	82 ± 32	79 ± 23	511 ± 173
>2 y	93 ± 38	89 ± 19	478 ± 188
TNSS score			
≤8	65 ± 22	61 ± 14	589 ± 163
>8	101 ± 31*	92 ± 19*	376 ± 144*

TNSS, total nasal symptom score.

*Comparison between subgroup, $P < .05$.

TABLE 2 Comparison of Th17 cytokines among different subgroups of AR children

interaction between leptin and OPN and Th17 regulation was also proved in differentiated Th17 cells in our study. These findings suggested leptin and OPN may be regarded as a promising therapeutic target for the management of AR patients.

To address a direct role of leptin and OPN in modulating Th17 cells, we performed in vitro studies on PBMCs. Our data showed that leptin promotes Th17 responses in a dose-dependent manner and this effect may be blocked by NF- κ B, MAPK, and JNK pathway inhibitors. Consistent with our findings, Deng et al reported increased Th17-cell immune responses when leptin was injected intra-articularly.¹⁹ Yu's study also found that leptin-induced ROR gamma expression in CD4+ T cells promotes Th17 responses in systemic lupus erythematosus.¹⁸ Several recent studies also reported the positive role of leptin or leptin receptor in Th17 differentiation.²⁴⁻²⁶ However, Moraes's study found that leptin decreases CD4+ Foxp3+ and CD4 + IL-17+ cell frequency in the draining lymph nodes of immunized mice but increases subsequent delayed-type hypersensitivity responses.²⁷ This discrepancy may arise from different experimental condition and definition of CD4+ T cells in different studies. Therefore, further investigation is needed to better understand how leptin might differentially influence the priming and the effector phases in vivo.

Osteopontin is known to function both as an extracellular matrix molecule and as a cytokine that contributes to Th17-skewed diseases, such as acute coronary syndrome, hepatitis, and so on.^{28,29} Consistently, we found that OPN promotes Th17 responses in a dose-dependent manner, and this effect may be blocked by NF- κ B, MAPK, and JNK pathway inhibitors and anti- β 3 integrin.

In this study, we provided first evidence that leptin and OPN contribute to enhanced Th17 responses synergistically. On the other side, IL-17 inhibited leptin and OPN expression negatively. Our data showed that leptin promotes IL-23 expression, which maintains the survival of differentiated Th17 cells. The upregulated IL-23 further promotes leptin expression, forming a positive feedback loop. Similarly, Goto's study showed that leptin deficiency impairs the secondary immune response against nephrotoxic serum and downregulates IL-23 production and Th17 responses in the nephritis kidney.³⁰ We also found that OPN suppresses the expression of IL-27 and vice versa, which is known to inhibit the development of Th17 cells.³¹

Our results showed that leptin promotes OPN expression by PBMCs, while OPN and HDM-activated PBMCs did not express elevated leptin, suggesting the interaction between leptin and OPN may be one-way. Our results also showed that leptin exerts its action on OPN through the PI3K and AKT signaling pathways. However, our results suggested that OPN does not have a direct effect on leptin production, suggesting that OPN may be a downstream effector of PI3K/AKT, and the activation of leptin was not related to OPN despite that the detailed mechanism needed further exploration.

To prove the effect of leptin and OPN on Th17 responses in vivo, we established OVA-challenged mice model to prove our hypothesis. Similarly, we found that enhanced Th17 responses and disease severity in OVA-challenged mice compared with control mice. However, after treated with antileptin or anti-OPN or anti- β 3 integrin, nasal inflammation reduced significantly, suggesting that leptin and OPN mediated Th17 responses through β 3 integrin receptor on OPN. As α v β 3 is the receptor of OPN, the anti- β 3 antibody mediated blocking of Th17 responses suggesting that OPN may inhibit Th17 responses through α v β 3 receptor on Th17 cells.

To prove the direct interaction between leptin and OPN, leptin was co-immunoprecipitated with OPN in differentiated Th17 cells. Our results suggested that leptin and OPN may interact with each other directly through β 3 integrin, which provides strong evidence of leptin/OPN axis in Th17 responses.

In sum, our data provide evidence that leptin-mediated OPN upregulation regulates Th17 responses, and this process may be achieved through NF- κ B, MAPK, and JNK pathways and β 3 integrin pathways through in vivo and in vitro studies.

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CONFLICT OF INTEREST

The authors declare that they have no relevant conflict of interests.

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