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## M2 macrophages correlated with symptom severity and promote type 2 inflammation in allergic rhinitis

To the editor,

Macrophages play a central role in the balance of the immune response by switching phenotype and function according to a delicate balance between M1 and M2 profiles.<sup>1,2</sup> While M1 macrophages may be derived from M0 macrophages by exposure to interferon- $\gamma$ , M2 macrophages are further classified into M2a, M2b, and M2c, based on the polarization stimulus.<sup>1,2</sup> It is likely that cytokines and chemokines derived from M2 macrophages may facilitate Th2 cell differentiation/recruitment and eosinophil infiltration.<sup>3,4</sup>

M2 polarization of macrophages has been implicated in the development, persistence, and progression of asthma.<sup>5,6</sup> We hypothesized that alternatively activated M2 macrophages play a key role in the pathogenesis of allergic rhinitis (AR). Thus, we have comprehensively investigated local and circulating M2 macrophages, as well as in vitro stimulated macrophages, in AR patients, compared with control subjects.

Fifty-five AR patients and 30 healthy controls were recruited to this study. Nasal symptom scores were recorded. Peripheral blood and nasal mucosa were collected. M1 and M2 macrophages in inferior turbinate were detected by immunofluorescence staining. CD14+ primary monocytes from *Dermatophagoides pteronyssinus* (*Der p*)-sensitized AR patients and healthy controls were stimulated with *Der p*. Circulating CD14+CD16-CD206- and CD14+CD16+CD206+ cells and in vitro cultured macrophage subsets were analyzed according to cell surface markers, gene expression, and M1/M2-associated cytokine/chemokine release. Details of study methods are described in Appendix S1 repository for this manuscript.

Local CD68+CD206+ M2 macrophages in inferior turbinate from AR patients were significantly higher than controls ( $29.1 \pm 14.9$  cells/HPF vs  $2.7 \pm 2.6$  cells/HPF,  $P < 0.0001$ ) (Figure 1A,B, Figure S1). However, local CD68+IRF5+ M1 macrophage infiltration in inferior turbinate was similar between the two groups. Additionally, local M2 macrophages were positively correlated with total nasal symptom score (TNSS) ( $r = 0.6157$ ,  $P = 0.0145$ ) and itching score ( $r = 0.5457$ ,  $P = 0.0354$ ). Circulating CD14+CD16+CD206+ cells were significantly higher in symptomatic AR patients compared to nonallergic controls (Table S1). Although circulating CD14+CD16+CD206+ cells were not significantly different between *Der p*- and pollen-sensitized symptomatic patients (Figure 1C), these cells were significantly increased with the onset of AR symptoms compared to asymptomatic phase (Figure 1D). Furthermore, in AR patients, the percentage of circulating CD14+CD16+CD206+ cells were significantly correlated with both the level of YKL-40 in serum ( $r = 0.7296$ ,  $P < 0.0001$ , Figure 1E), TNSS ( $r = 0.6178$ ,  $P < 0.0001$ , Figure 1F), and itching score ( $r = 0.7721$ ,  $P < 0.0001$ ). However, these findings need to be confirmed in larger numbers of patients and samples in future studies.

Comparison of macrophage polarization from *Der p* mono-sensitized AR patients and healthy controls showed that *Der p*-stimulated M2 macrophage polarization was significantly higher in the AR group compared with control group ( $P = 0.004$ ) (Figure 2A). M2 macrophages were significantly increased in the *Der p*-sensitized AR group ( $P = 0.001$ , Figure 2A). Indeed, *Der p* did not appear to promote M2 polarization in nonallergic control group, as indicated by a lack of significant difference between *Der p*-treated and untreated cells ( $P = 0.1013$ , Figure 2A). However, M1 macrophage polarization was comparable ( $P = 0.0878$ ) and unaltered in the two groups (Figure 2B).

Expression gene profiles of in vitro cultured macrophages demonstrated that M2a-associated genes, including CD206, CD209, and YKL-40, were expressed significantly higher in the AR group compared to the nonallergic control group (Figure S2). However, neither of M2c-related CD163, C-C motif chemokine ligand (CCL)-24 and interleukin (IL)-10 genes and M1-associated CD86 and tumor necrosis factor- $\alpha$  genes showed significant differences between two groups (Figure S2).

Assessment of cytokines/chemokines released from different macrophage subsets in AR patients and nonallergic control groups showed that M2a-associated YKL-40, CCL17, CCL18, and CCL26, but not M2c-associated chemokines CCL22 and CCL24, or the M1-associated cytokine IL-8, were significantly higher in serum of AR patients compared to the controls (Figure S3). Similarly, analysis of relevant cytokines/chemokines in culture supernatants collected from *Der p*-stimulated macrophages demonstrated a similar inflammatory profile (Figure S4), as observed in the serum of AR patients (Figure S3).

To our knowledge, the current study is the most comprehensive investigation of M2 macrophage biomarkers in AR. Our study demonstrated increased alternative activation of macrophages in nasal mucosa and peripheral blood of AR patients relative to healthy control subjects. Consistently, significantly higher levels of YKL40, CCL17, CCL18, and CCL26, which were associated with M2a subset,<sup>1,2</sup> were observed in AR compared to healthy controls. Additionally, the percentage of local M2 macrophages and circulating CD14+CD16+CD206+ cells were found to positively correlate with severity of symptoms in AR, further supporting the importance of M2 macrophages in the etiology of AR.

Importantly, our study is the first to demonstrate the presence of alternative activation program in macrophages when exposed to a sensitizing allergen in vitro, as evidenced by significantly increased expression of M2 macrophage genes YKL-40, CD206, CD209, and secretion of the M2 macrophage chemokines CCL17, CCL18 and CCL26, thus suggesting that M2 macrophages may play an important role in natural immunoregulatory processes

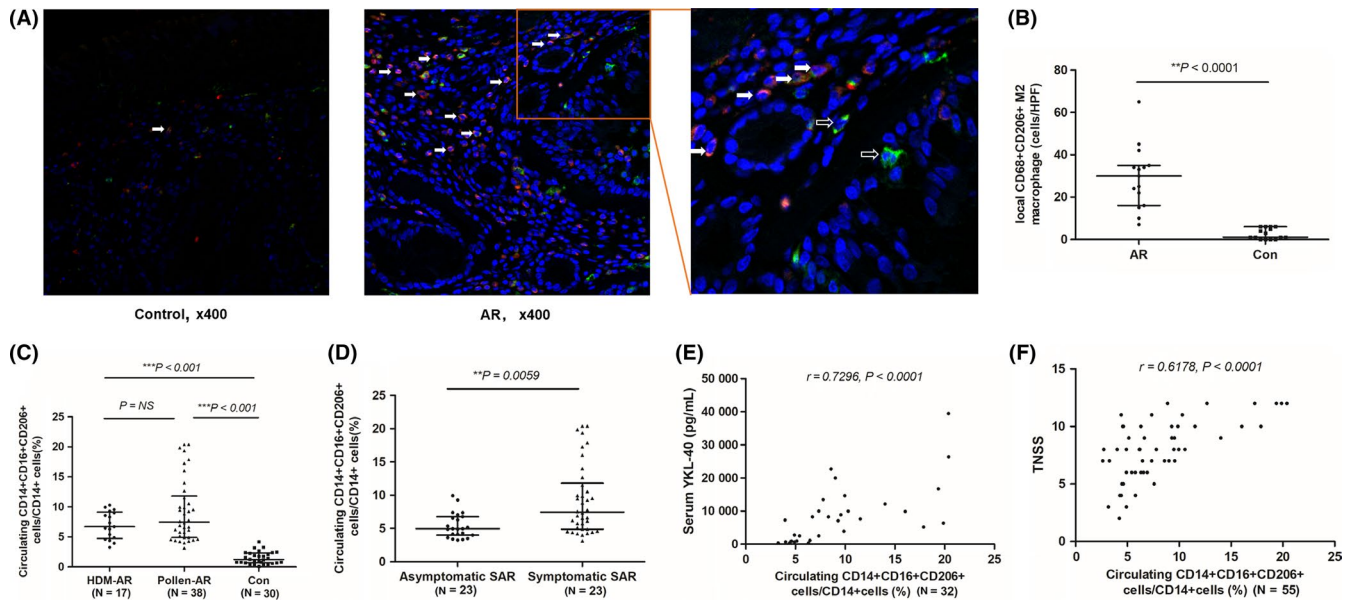


**FIGURE 1** Local M2 macrophage and circulating CD14+CD16+CD206+ cells in allergic rhinitis (AR) patients and healthy controls. A, Immunofluorescence staining of CD68+CD206+ M2 macrophages in inferior turbinates (CD68, green stain; CD206, red stain. Solid arrow, M2 macrophage; blank arrow, CD68+CD206- macrophage). B, Comparison of local M2 macrophages in AR and control. C, Comparison of circulating CD14+CD16+CD206+ cells in symptomatic AR and control subjects. D, Comparison of circulating CD14+CD16+CD206+ cells in seasonal AR (SAR) patients with and without onset of symptoms. E, Correlation of peripheral CD14+CD16+CD206+ cells with serum YKL-40. F, Association between peripheral CD14+CD16+CD206+ cells and total nasal symptom score (TNSS). HDM, house dust mite

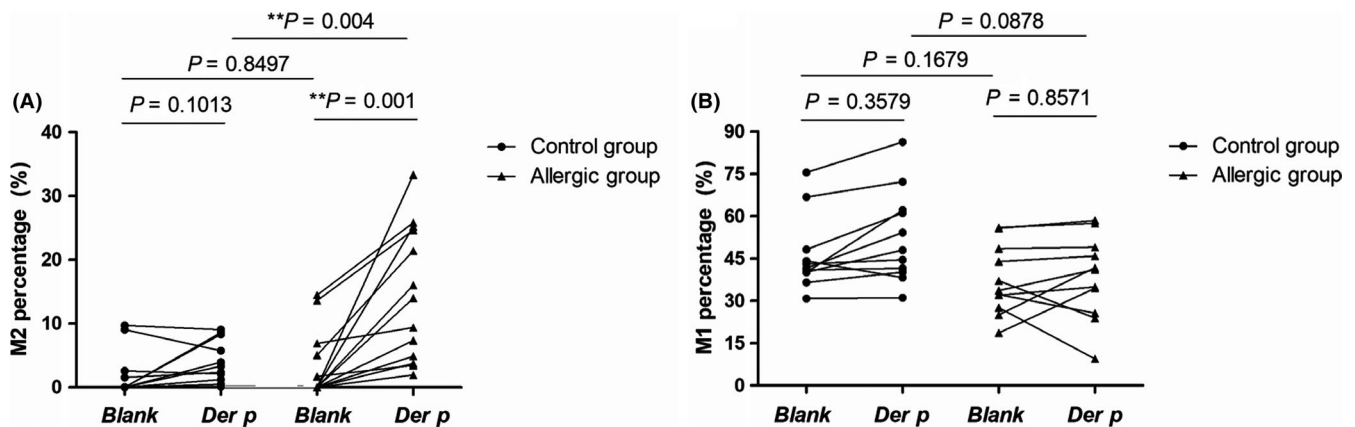


**FIGURE 2** Macrophage polarization profiles following stimulation with or without *Dermatophagoides pteronyssinus* (Der p) (A and B), in vitro. N = 13 patients who were mono-allergic to Der p and 11 healthy controls

in AR. Furthermore, the demonstration that the M2a-associated biomarkers, but not M2c- or M1-associated biomarkers, were increased significantly in both serum and cultured supernatants from AR patients compared to nonallergic controls suggests that M0 macrophage predominantly polarizes toward the M2a phenotype in AR.

Although Th2 cytokines enhance the differentiation of M2 macrophages,<sup>1,2</sup> the M2 macrophages are not only bystander cells responding to Th2 cytokines, but may also actively contribute in orchestrating Th2-driven inflammation.<sup>7,8</sup> This is further supported by the finding of the present study that the CD14+CD16+CD206+ cells derived from peripheral blood monocytes of AR patients

released large amounts of YKL-40, CCL17, CCL18, and CCL26, which have been shown to influence the activity of Th2 cells and eosinophils.<sup>3,4,7-9</sup>

In summary, the present study showed a predominantly M2 predisposition in local nasal mucosa and peripheral blood of patients with AR, with M2 macrophage numbers being positively correlated with AR symptom severity, and possibly augmenting type 2 inflammation directly by releasing YKL-40 and a distinct set of chemokines including CCL17, CCL18, and CCL26 in AR. These findings suggest that M2 macrophage plays an important role in the pathophysiology of AR and may be useful as a potential biomarker of AR severity and targeted treatment.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## FUNDING INFORMATION

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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# Immune microenvironment controls the outcome of PD-1 blockade in cutaneous immune response

To the Editor

The introduction of immune checkpoint inhibitors (ICI) targeting the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1)

pathway or cytotoxic T-lymphocyte antigen 4 (CTLA-4) has brought about a revolution in anticancer therapy by embodying the concept that the host's exhausted pre-existing antitumor immunity can be re-activated. The frequency of ICI treatment-associated skin toxicities

Ogawa and Ishitsuka have equal contribution.

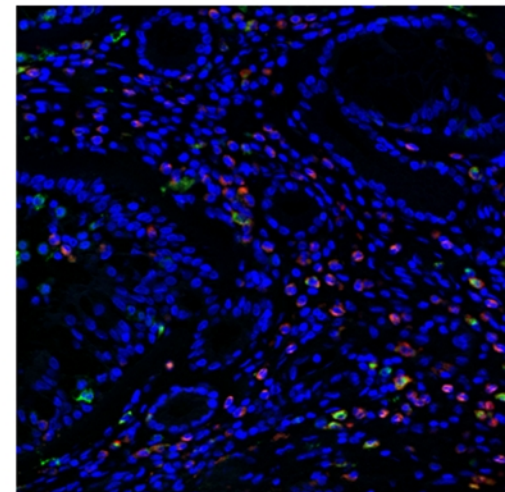
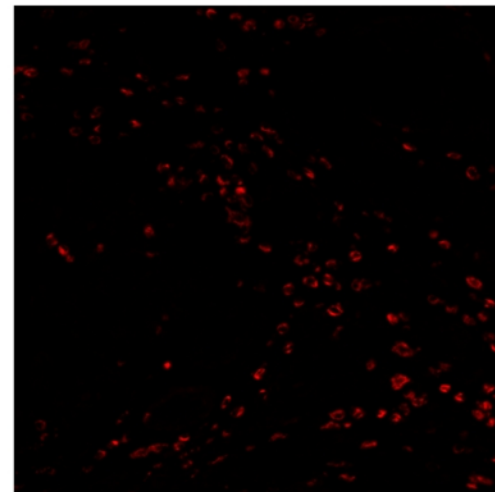
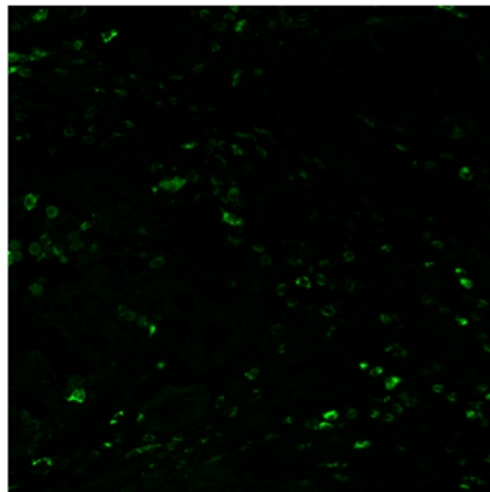
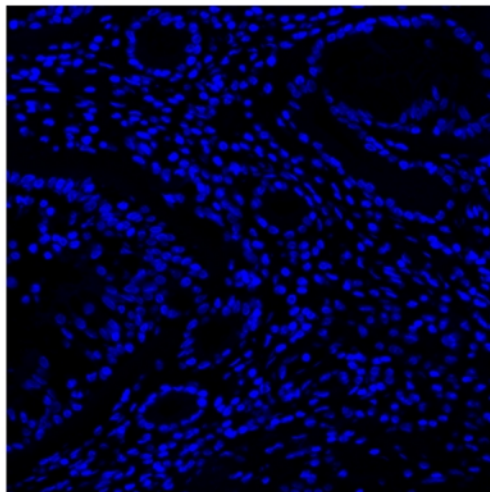
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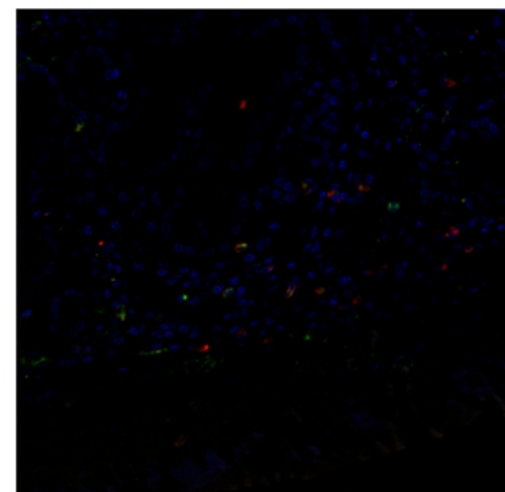
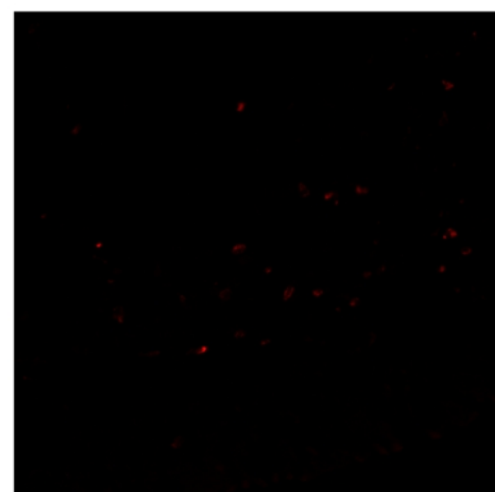
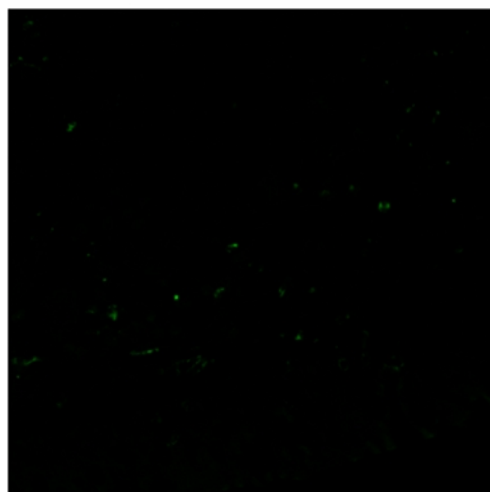
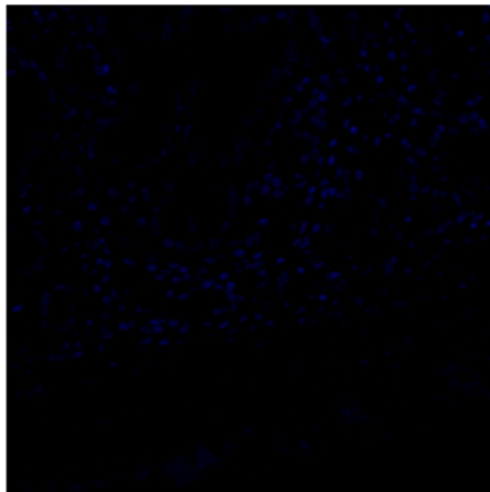
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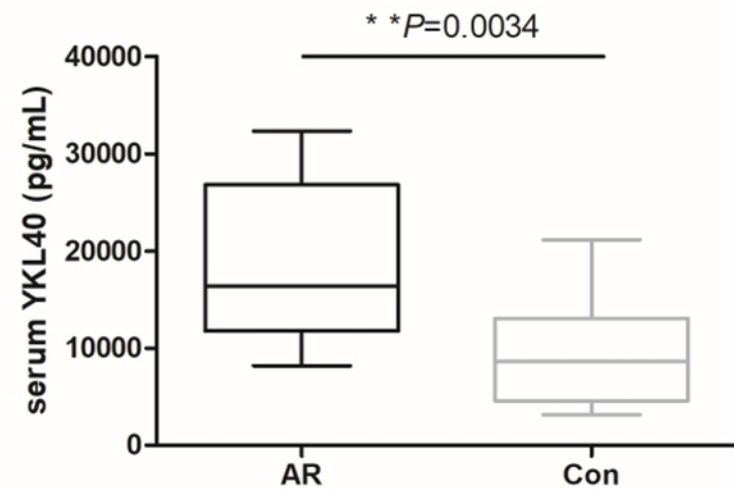
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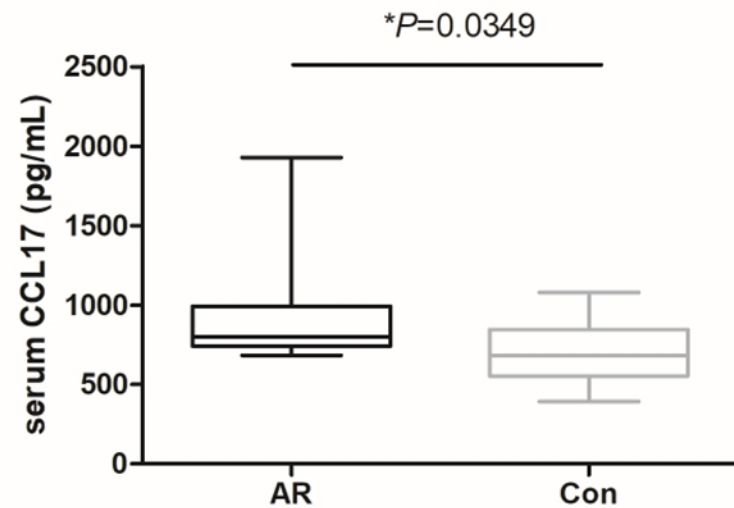
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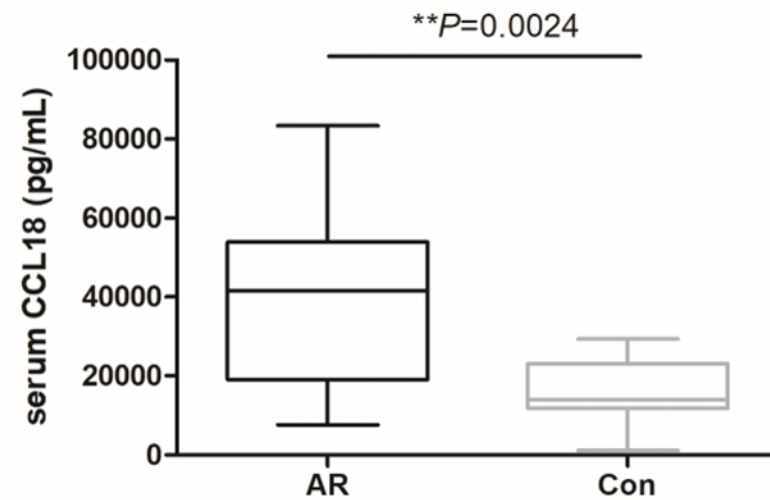
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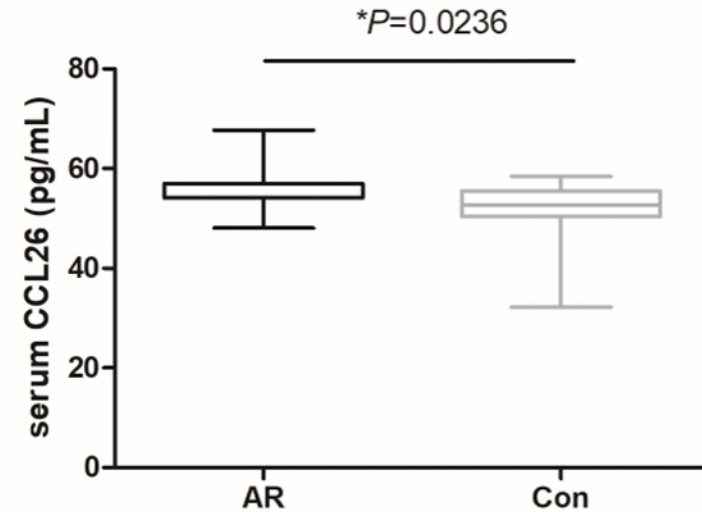
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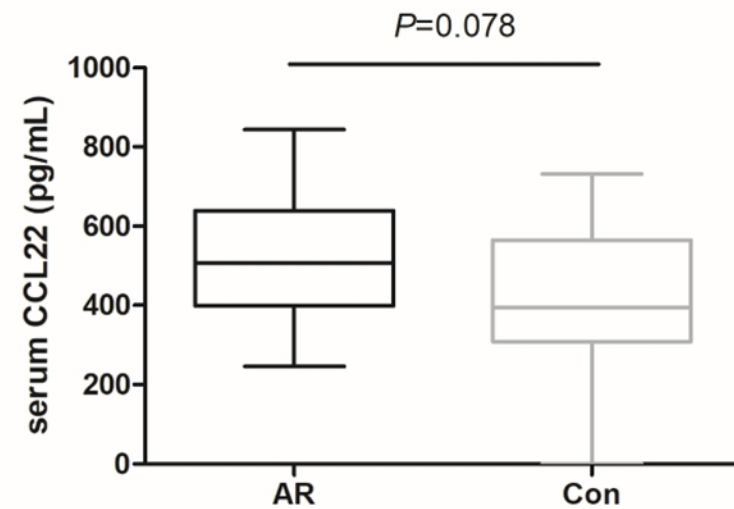
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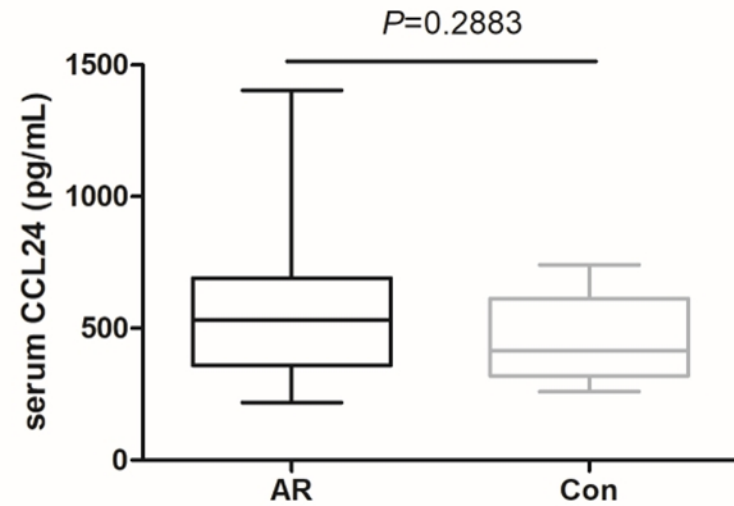
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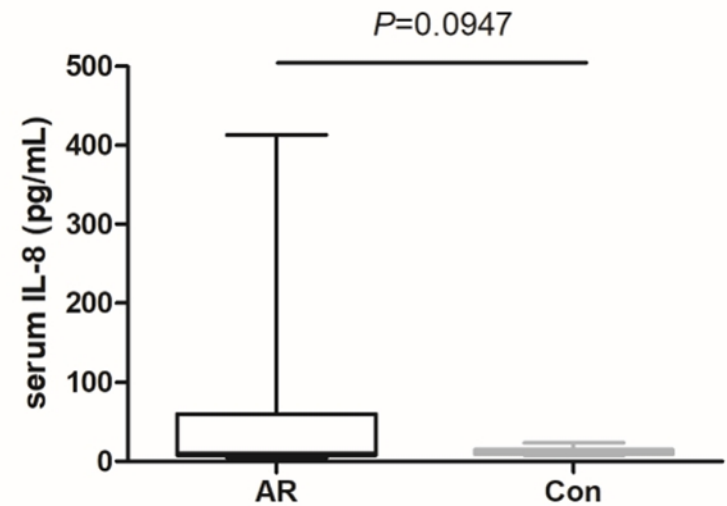
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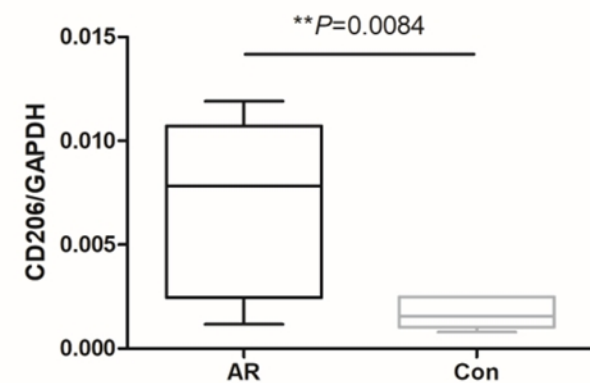
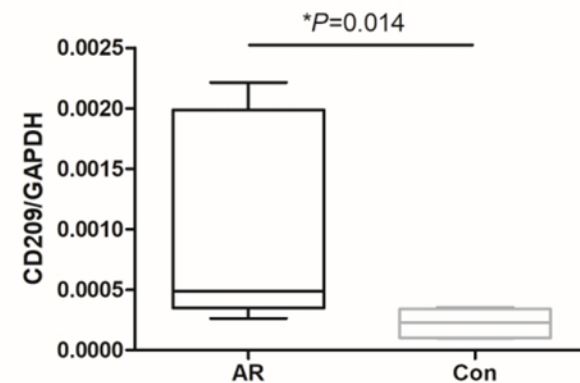
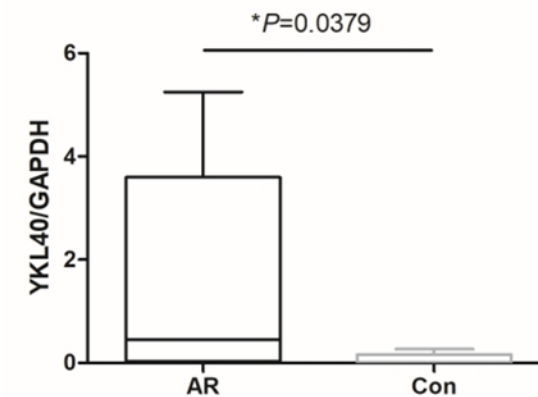
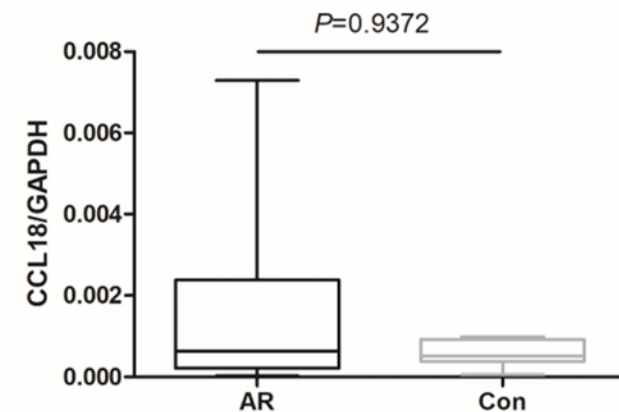
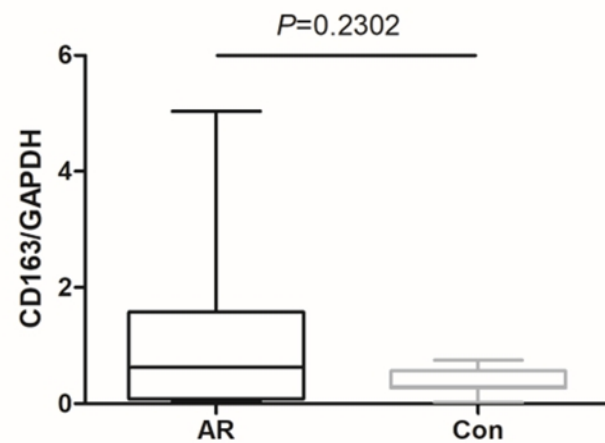
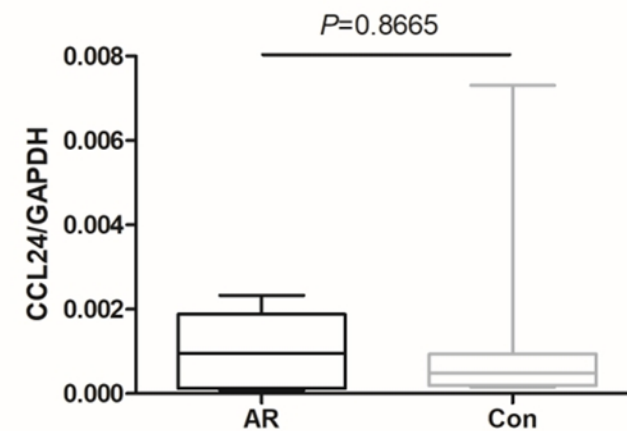
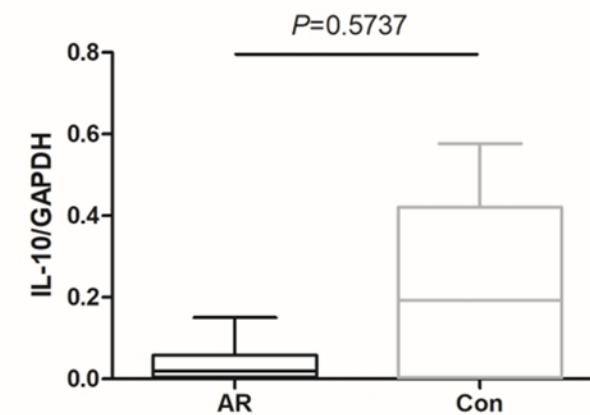
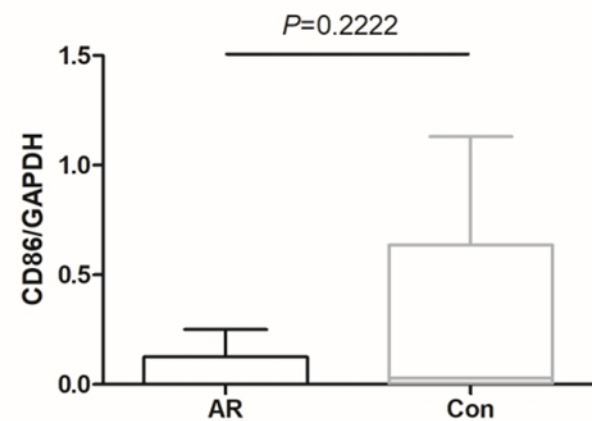
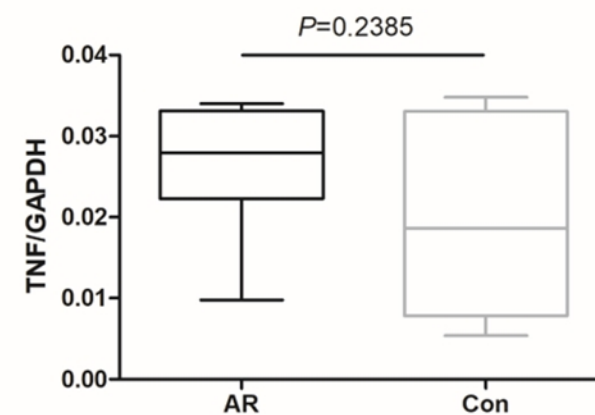


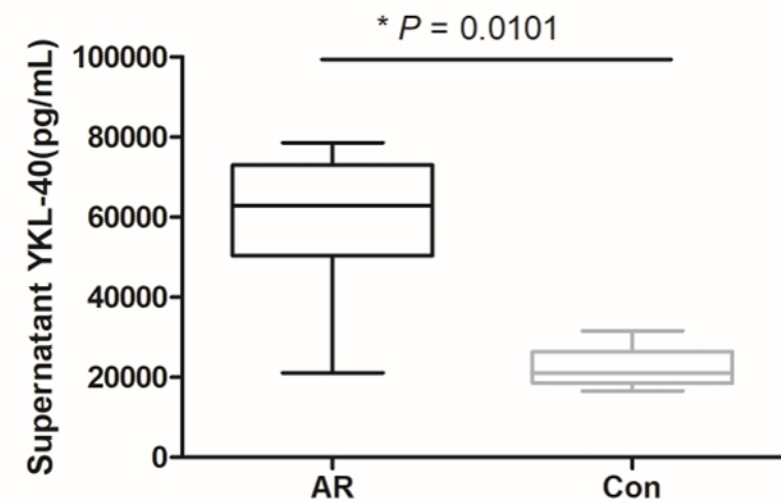
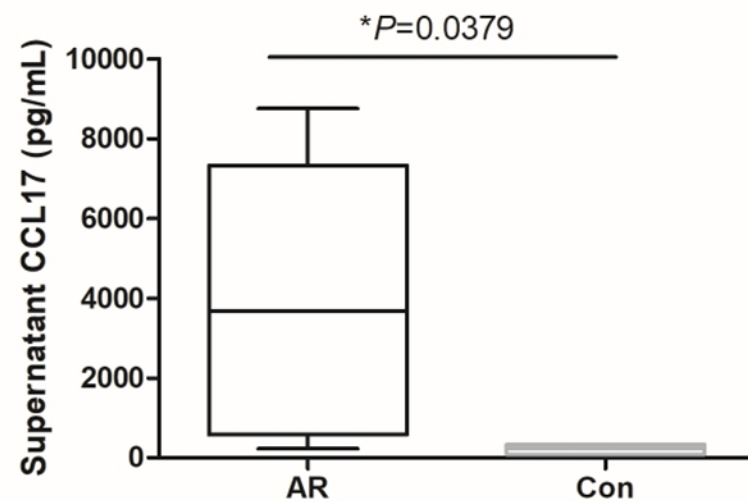
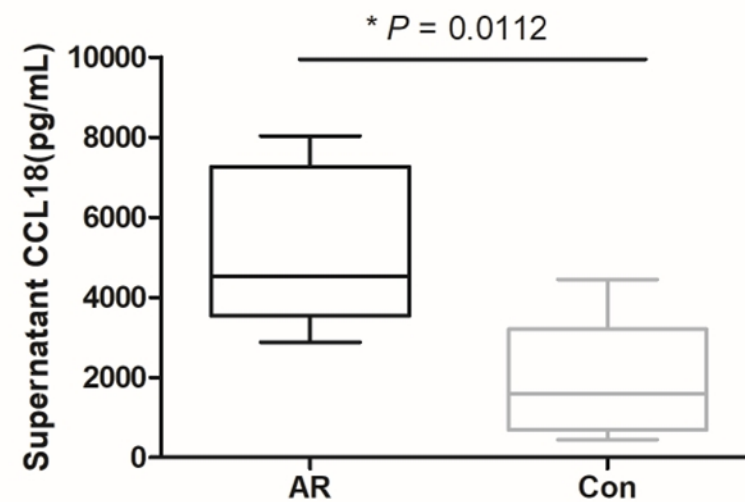
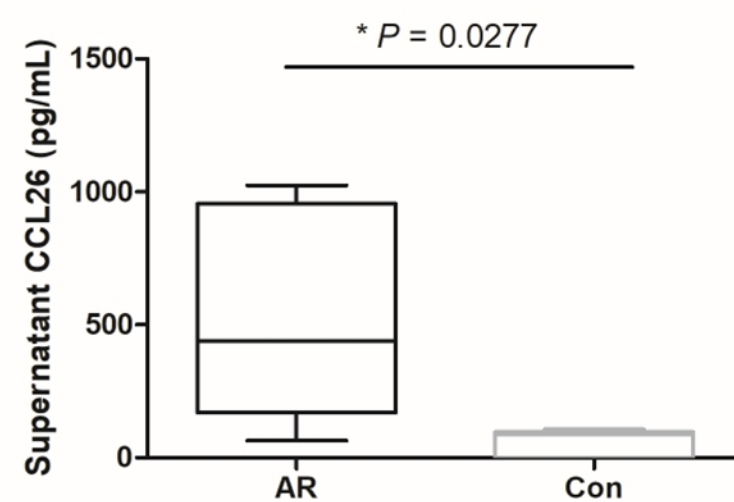
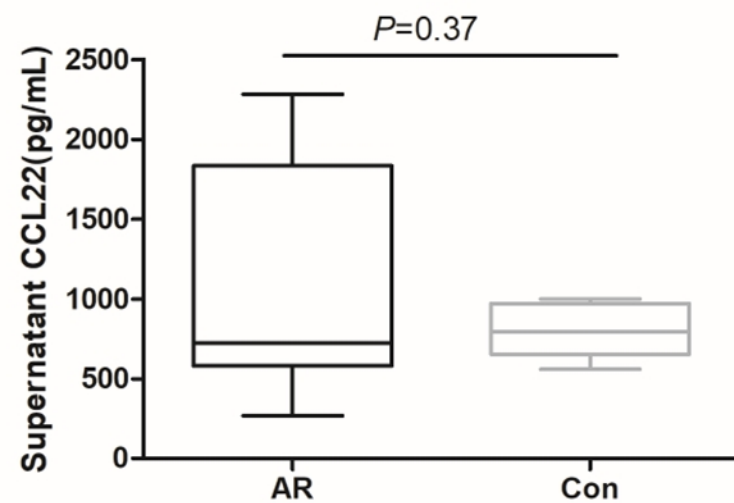
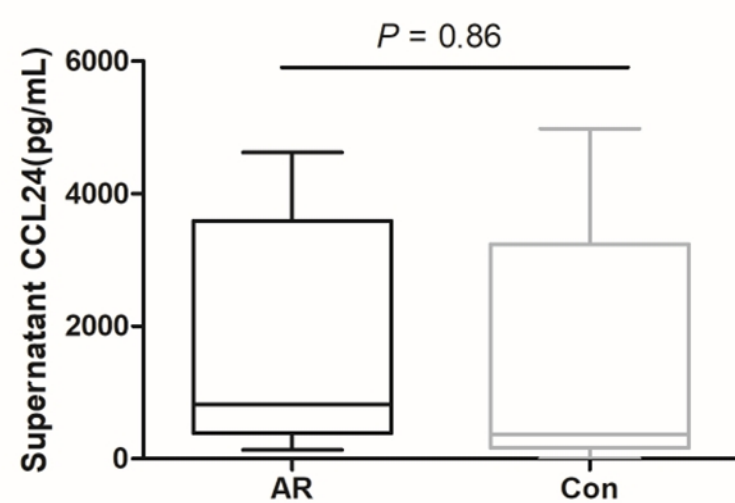
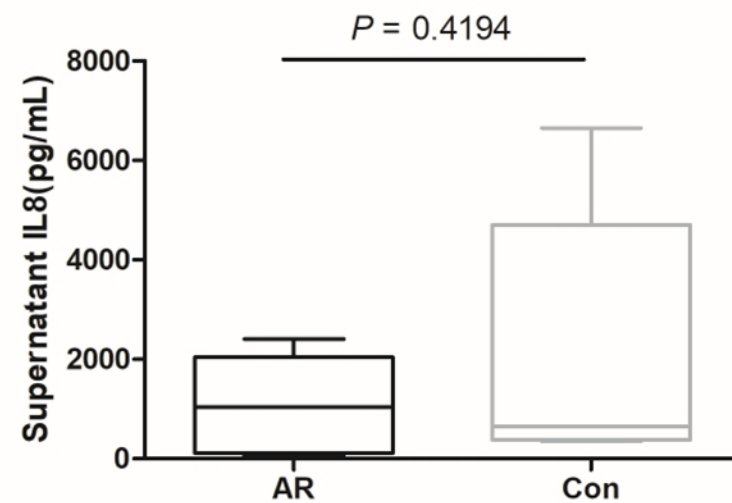
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**Hongfei Lou et al - M2 macrophages correlated with symptom severity and promote type 2 inflammation in allergic rhinitis**

**Online Supplementary information**

**METHODS**

**Subject recruitment**

Forty-four males and forty-one females, aged 18-57 years, were recruited from Beijing TongRen Hospital, between January 2015 and November 2018(Table S1).

These comprised 55 symptomatic allergic rhinitis (AR) patients and 30 healthy control subjects, who completed a questionnaire, which included demographic data and symptom scores for AR. The severity of each nasal symptom of AR; including sneezing, nasal itching, rhinorrhea and nasal obstruction; was evaluated on a visual analogue scale (VAS) between 0 and 10 (1-3, mild; 4-6, moderate; 7-10, severe). Total nasal symptom score (TNSS) was calculated as the sum of a 4-point scale for these four symptom scores (0=absent, 1=mild, 2=moderate, 3=severe), ranging from 0 to 12. Peripheral blood samples were obtained and serum total IgE was measured. Additionally, peripheral blood samples were collected from 23/55 AR patients when they were asymptomatic during the period without pollen exposure, to compare with their samples collected during the pollen season. Nasal mucosa from inferior turbinate was collected from 15 participants in each group for immunofluorescence analysis. Exclusion criteria included asthma, smoking history, active upper respiratory

tract infection within 3 weeks, utilization of nasal or systemic medication for AR within 4 weeks, and acute sinus disease requiring antibiotic treatment within one month before recruitment.

The patients who had AR symptoms with positive skin prick test ( $\geq 2+$ ) or allergen specific IgE ( $> 0.7\text{kU/mL}$ ) were considered to be allergic. Those who did not have AR symptoms and had negative phadiatop were considered to be nonallergic controls. Sensitivity was determined to the most common aeroallergens in the region; including house dust mites (HDM), mugwort, and ragweed.

A written informed consent was obtained from each participant and the study was approved by the Medical Ethics Committee of Beijing TongRen Hospital.

### **Immunofluorescence analysis**

M1 and M2 macrophages infiltration in local tissue was quantified by immunofluorescence analysis. Nasal mucosa was fixed, embedded, and sectioned at  $5\mu\text{m}$  thickness. 15 samples from inferior turbinate in each group were assessed by fluorescent immunohistochemistry, using mouse-anti-CD68 (Zhongshan Golden Bridge Biotechnology, Beijing, China), rabbit-anti-IRF5 (Abcam, Cambridge, UK) and mouse-anti-CD206 (Biolegend, San Diego, USA) to identify macrophages. The stained sections were observed by two independent physicians, blinded to the clinical diagnosis of the patients, using a fluorescence microscope. All samples were assessed at  $\times 400$  magnification and double-stained cells IRF5+CD68+ M1 and CD206+CD68+ M2 macrophages were counted in 10 randomly selected fields per sample. The results were expressed as the mean number in 10 fields.

## 45    **Macrophage polarization experiments *in vitro***

46    Human macrophage polarization was studied in primary monocyte (peripheral blood  
47    monocyte, PBMC)-derived macrophages. Briefly, PBMCs were separated from 30  
48    mL heparinized blood samples of 13 AR patients who were mono-allergic to  
49    *Dermatophagoides pteronyssinus* (*Der p*) and 11 healthy controls, by means of  
50    Ficoll-Hypaque Plus density gradient centrifugation (Amersham Biosciences, NJ,  
51    USA). The PBMC layer was washed three times with sterile phosphate-buffered  
52    saline and monocytes were isolated using CD14<sup>+</sup> magnetic microbeads (Milteny  
53    Biotech, Bergisch Gladbach, Germany). CD14<sup>+</sup> monocytes of >95% purity were  
54    cultured at a concentration of  $1 \times 10^6$ /mL in RPMI 1640 medium (Sigma-Aldrich Co.,  
55    Steinheim, Germany), supplemented with recombinant human macrophage colony  
56    stimulating factor (50ng/ml) (Invitrogen, Carlsbad, CA), 10% foetal bovine serum,  
57    100U/mL penicillin, 100 µg/mL streptomycin and 2mM L-glutamine, for 7 days at 37°  
58    in 5% CO<sub>2</sub> atmosphere; by which time they had differentiated into uncommitted  
59    macrophages (M0). The freshly cultured M0 macrophages harvested and then either  
60    left unstimulated (blank control) or stimulated by incubation with *Der p* (10ng/mL)  
61    (ProSpec-Tany TechnoGene, Rehovot, Israel) for 2 days in serum-free RPMI 1640  
62    medium (Sigma-Aldrich Co., Steinheim, Germany). At the end of this incubation, the  
63    polarized macrophages were harvested by centrifugation for confirmation of the  
64    macrophage phenotype and the cell-free supernatants were collected for analysis of  
65    YKL-40, interleukin (IL)-8, C-C chemokine motif ligand (CCL)17, CCL18, CCL22,  
66    CCL24, and CCL26 levels, using Luminex-based multiplex assay (Luminex 100

System, Austin, Tex, USA); whilst the cells were resuspended in medium and analyzed for a panel of macrophage biomarkers, using a combination of flow cytometry and quantitative real time polymerase chain reaction (RT-PCR).

#### **Flow cytometry analysis**

Macrophage subsets were quantified by flow cytometry (FACSAria flow cytometer, BD Biosciences, NJ, USA) according to manufacturer's instructions. The cells were labeled with specific monoclonal antibodies; including CD14 (eBioscience, San Diego, CA, USA), CD16 (BioLegend, San Diego, CA, USA) and CD206 (BD Bioscience Immunocytometry Systems, CA, USA) for macrophage phenotyping. Macrophages subsets were selected as follows: (1) M1 macrophages: CD14+CD16-CD206- cells; (2) M2 macrophages: CD14+CD16+CD206+ cells. A minimum of  $10^6$  cells per staining was required with at least  $10^5$  events being measured.

#### **Quantitative RT-PCR**

Total RNA was extracted from *Der p*-stimulated polarized macrophages, using RNAiso Blood (Takara, Otsu, Japan), and cDNA was synthesized with PrimeScript<sup>TM</sup> RT Master Mix (Takara, Otsu, Japan) according to the manufacturers' instructions. Specific primers for M1-associated genes (CD86 and tumor necrosis factor- $\alpha$ ), M2a-associated genes (CD206, CD209, CCL18 and YKL-40) and M2c-associated genes (CD163, CCL24, IL-10) were purchased from Shanghai Sangon Biotech. Quantitative RT-PCR was performed with SYBR Premix Ex Taq with ROX reference dye II (Takara, Otsu, Japan) and a 7900HT PCR system (Applied Biosystems).

89 Respective mRNA levels were analyzed based on the  $2^{-\Delta Ct}$  method, using  
90 housekeeping gene GAPDH as reference.

#### 91 **Luminex-based multiplex assay**

92 Macrophage-associated cytokines and chemokines in serum and cell culture  
93 supernatants from *in vitro* macrophage polarization experiments were assessed to  
94 define M1 (IL-8) and M2 (CCL17, CCL18, CCL22, CCL24, CCL26, and YKL-40)  
95 phenotypes. All cytokines/chemokines were determined using Fluorokine MAP  
96 Multiplex Kits (R&D Systems, Minneapolis, Minn, USA) and analyzed on a Luminex  
97 100 analyzer (Luminex 100 System, Austin, Tex, USA).

#### 98 **Statistical analysis**

99 SPSS version 22.0 software package (IBM Corp., Armonk, NY, USA) was used for  
100 statistical analysis. Data were expressed as medians and interquartile range, unless  
101 otherwise specified. Continuous variables differences were analyzed by Student's  
102 *t*-test or non-parametric tests according to the distribution of independent data sets.  
103 Differences of categorical variables were assessed by *Chi*-square test. Correlation  
104 analysis was conducted among M2 frequency, YKL-40 and AR symptom scores. *P*  
105 values  $\leq 0.05$  were considered statistically significant.

106

**Figures and Tables**

**Figure S1.** Immunofluorescence staining of local CD68+CD206+ M2 macrophages in inferior turbinate from allergic rhinitis (AR) patients and healthy controls.

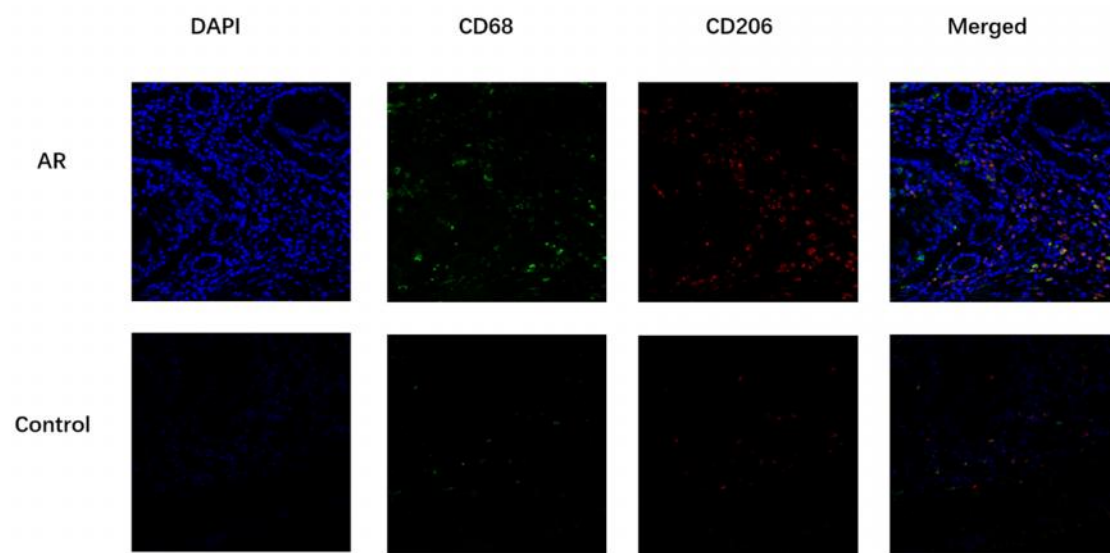


**Figure S2.** Gene expression profiles of polarized macrophages stimulated with *Der p*,  
*in vitro*. (N=8 in each group.)

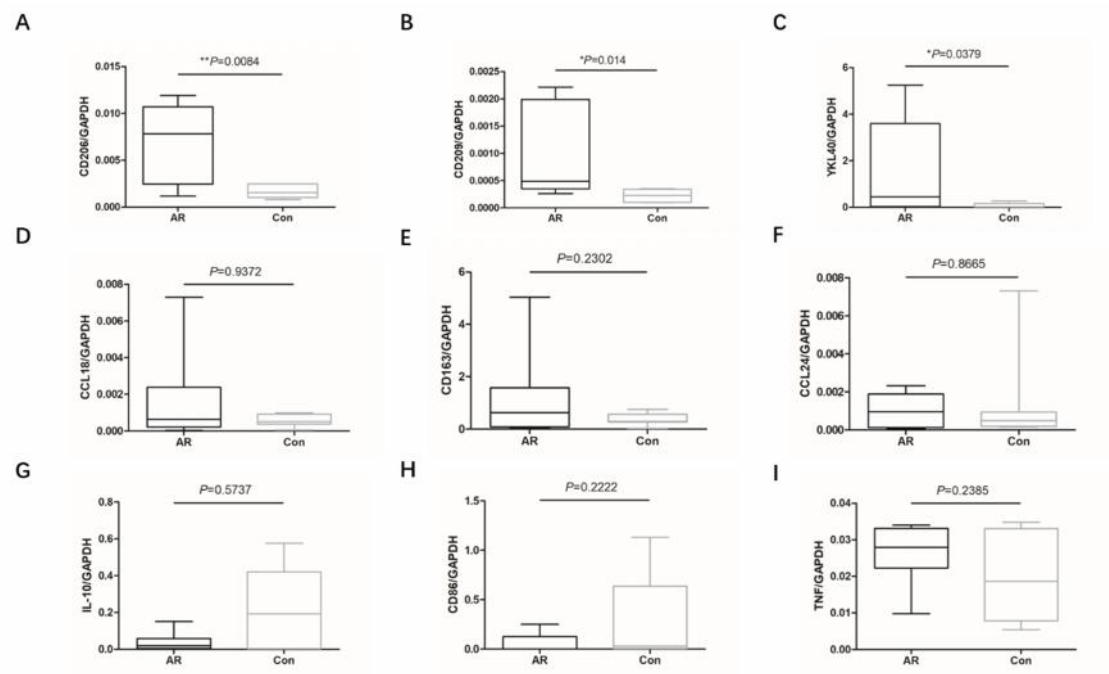


**Figure S3.** Macrophage-associated cytokines/chemokines released in serum of AR patients and healthy controls. N=32 in allergic rhinitis (AR) group, and N=30 in nonallergic control group.

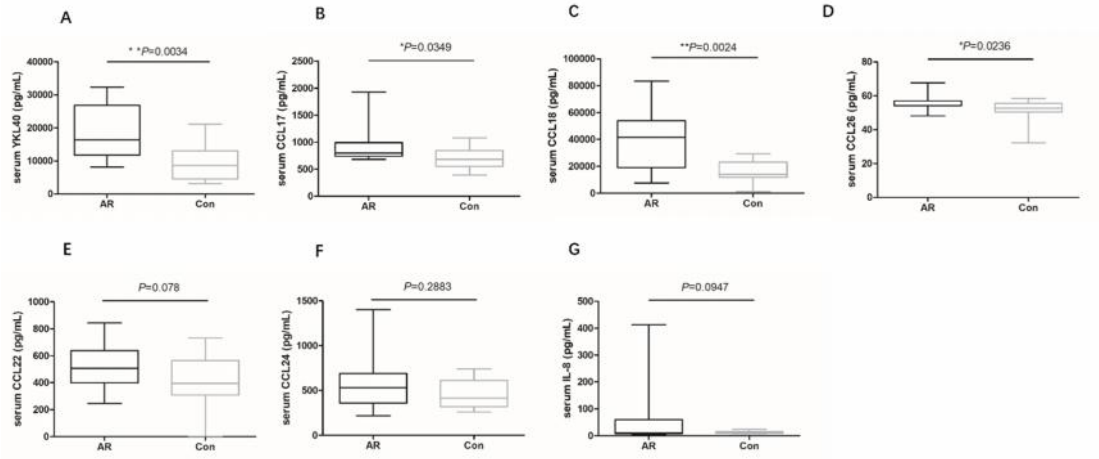
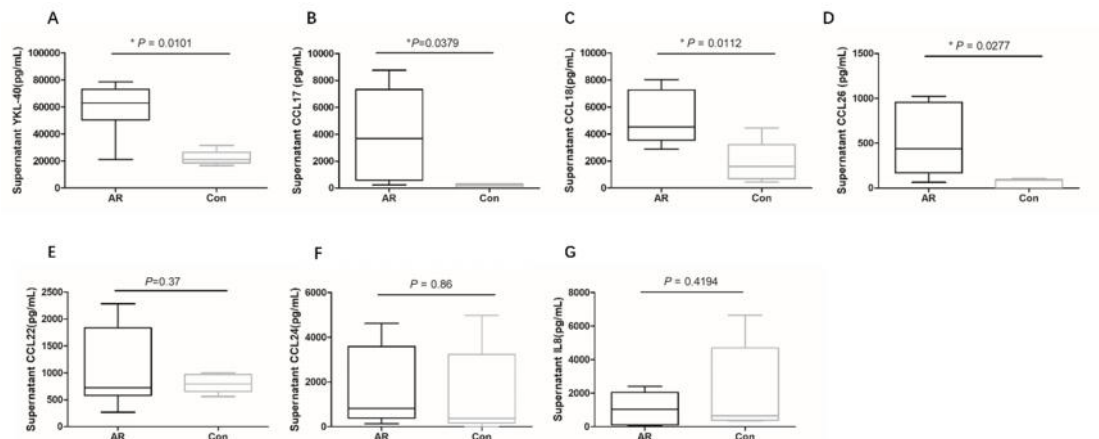


**Figure S4.** Inflammatory mediators released in culture supernatant collected from *Der p*-activated macrophages.



126 **TABLES**

127 **Table S1.** Characteristics of the study population with onset of allergic rhinitis (AR).

| Variables                                              | AR group<br>(n = 55) | Control group<br>(n = 30) | <i>P</i> value |
|--------------------------------------------------------|----------------------|---------------------------|----------------|
| Age (yrs)                                              | 36.6±8.1 #           | 36.7±8.2 #                | 0.93           |
| Sex(M/F) <sup>\$</sup>                                 | 29/26                | 15/15                     | 0.81           |
| Serum Total IgE(kU/mL)                                 | 139.0(123.0-187.0)   | 35.5(27.8-45.8)           | <0.001*        |
| Positive specific IgE<br>(>0.7kU/mL) (n)               |                      |                           | /              |
| HDM                                                    | 17                   | 0                         |                |
| Mugwort                                                | 27                   | 0                         |                |
| Ragweed                                                | 11                   | 0                         |                |
| TNSS                                                   | 8.0±2.6 #            | 0                         | /              |
| Sneezing score                                         | 4(2-6)               | 0                         | /              |
| Rhinorrhea score                                       | 4(2-6.5)             | 0                         | /              |
| Itching score                                          | 4(2-6)               | 0                         | /              |
| Obstruction score                                      | 4(2-6.5)             | 0                         | /              |
| circulating<br>CD14+CD16-CD206-cells/CD14+<br>cell (%) | 75.7(69.1-81.1)      | 74.7(65.8-85.0)           | 0.48           |
| circulating CD14+CD16+CD206+<br>cells/CD14+ cell (%)   | 7.3(4.9-9.9)         | 1.3(0.7-2.3)              | <0.001*        |

128 AR, allergic rhinitis; M, male; F, female; HDM, house dust mite; TNSS, total nasal  
129 symptom score.

130 #Data were presented as mean ± standard deviation and analyzed by student's t-test.

131 \**P* < 0.05.

- 132   <sup>\$</sup>Chi-square test was applied in this categorical variable.
- 133   Non-parametric tests were employed to other variables.