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Original Article

Upregulated Expression of Toll-Like Receptor 7 in Peripheral Blood Basophils of Patients With Allergic Rhinitis

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Lihong Wang, MS[1], Mengmeng Zhan, MS[1], Junling Wang, MS[2], Dong Chen, PhD[2], Nan Zhao, MS[2], Ling Wang, MS[1], Wei Wang, MS[2], Xiaowen Zhang, MS[2], Yixia Huang, MS[2], Huiyun Zhang, PhD[1], and Shaoheng He, PhD[1]

Abstract

Background: Recently, it has been reported that Toll-like receptor 7 (TLR7) agonists can improve allergic rhinitis (AR) symptoms by up-regulation of Th1 cytokine release and suppression of Th2 cell functions. However, little is known of the expression of TLR7 in basophils of AR.

Objective: To explore the expression of TLR7 in basophils of AR, and influence of allergens on TLR7 expression.

Methods: The expression levels of TLR7 in basophils of patients with AR were determined by flow cytometry, and the influence of allergens on TLR7 expression was examined by real time (q) PCR.

Results: The percentages of $TLR7^+CCR3^+$ cells ($P < 0.001$ and $P = 0.011$), $TLR7^+CD123^+HLA\text{-}DR^-$ cells ($P = 0$ .016 and $P = 0.042$) and $TLR7^+CCR3^+CD123^+HLA\text{-}DR^-$ cells ($P = 0.046$ and $P = 0.035$) in blood granulocyte and mononucleated cell populations of the patients with AR were increased, respectively compared with HC subjects. TLR7 MFI on $CCR3^+$ cells ($P = 0.050$ and $P = 0.043$), $CD123^+HLA\text{-}DR^-$ cells ($P < 0.001$ and $P = 0.002$) and $CCR3^+CD123^+HLA\text{-}DR^-$ cells ($P < 0.001$ and $P = 0.003$) were enhanced compared with HC subjects. Allergens Der p1 and OVA provoked upregulation of TLR7 expression at both protein and mRNA levels and IL-13 production in KU812 cells. House Dust Mite extract (HDME), Artemisia sieversiana wild allergen extract (ASWE), IL-31, IL-33, IL-37, and TSLP provoked elevation of IL-6 release from KU812 cells following 2 h incubation period.

Conclusions: The percentage of $TLR7^+$ basophils and TLR7 expression intensity in a single basophil are both increased in the blood of patients with AR, indicating that basophils likely contribute to the pathogenesis of AR via TLR7.

Keywords

TLR7, allergen, cytokine, allergic rhinitis, basophil, KU812 cells

Background

AR is considered as an immunoglobulin E-mediated inflammation of the upper airway,[1] which is a common condition with increasing prevalence. The pathogenesis of AR is multi-factorial and complex. There is an infiltration by inflammatory cells, particularly Th2 cells, eosinophils and basophils into nasal mucosal tissue that results in the allergic response.[2] Over the last 20 years, the majority of studies on AR have been focused on proinflammatory cytokines.[3] However, little is known about the expression of Toll-like receptor (TLR)7 in AR.

TLRs are vital elements of the mammalian immune system, bridging innate and adaptive immunity. TLR7, a member of TLR family, is an intracellular receptor expressed on the membrane of endosomes,[4] which recognize single stranded RNA, significantly participate in the amelioration of AR symptoms. For instance, a

[1]Department of Immunology, Translational Medicine Institute, Shenyang Medical College, Shenyang, China
[2]Allergy and Clinical Immunology Research Centre, the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

Corresponding Author:
Shaoheng He, Translational Medicine Institute, Shenyang Medical College, No. 146, Huanghe North Street, Shenyang, Liaoning 110034, China.
Email: shoahenghe@126.com

clinical trial study on the Swedish population with AR revealed that AZD8848 as TLR7 agonist resulted in up-regulation of Th1 cytokines/chemokines (CXCL10, TNFα, IL-6, IFNγ) and suppression of Th2 cell functions in a profile which results in an improvement of AR symptoms.[5] Intranasal application of a TLR7 agonist GSK2245035 was reported to enhance expression of the Th1 cytokines and suppression of AR symptoms.[6] The important roles played by TLR7 may be confirmed by genetic investigations which revealed the significant association between genetic variations in the TLR7 genes and AR.[7] However, little is known of the expression of TLR7 in basophils in AR.

Basophils are known as primary effector cells of allergy,[8] which are the least common granulocytes in peripheral blood of human beings.[9] Apart from their contribution to allergy as initiators of IgE-induced acute reactions,[10] basophils have shown elevated expression of thymic stromal lymphopoietin (TSLP) receptor in patients with AR after allergen stimulation,[11] indicating that basophils are likely to be involved in AR. In the present study, 3 combinations of cell membrane molecules are employed to represent basophils, namely $CD123^+HLA\text{-}DR^-$ cells,[12] $CCR3^+$ cells,[13] and $CCR3^+CD123^+HLA\text{-}DR^-$ cells.

Allergens are the causative factors of allergy. Skin prick tests showed that house dust mite (HDM) is the main allergen for AR in Korea,[14] whereas pollens were more prevalent in Europe.[15] It was reported that Der f and Der p were the most prevalent aeroallergens in China.[16,17] Since HDM major allergens can activate TLRs and upregulate proinflammatory cytokine expression,[16] they may act on TLR7 on basophils.

The aim of the study is to investigate the expression of TLR7 in peripheral blood basophils of patients with AR, and the influence of airborne allergens on TLR7 expression in basophils. We observed that the percentages of $TLR7^+$ basophils and TLR7 expression intensity in a single basophil are both increased in blood of the patients with AR.

Materials and Methods

Reagent

The following reagents were purchased from Biolegend (San Diego, USA): APC/Cy7-conjugated mouse anti-human CCR3, PE/Cy7-conjugated mouse anti-human CD123, PerCP/Cy5.5-conjugated mouse anti-human HLA-DR. PE-conjugated mouse anti-human TLR7, PerCP-conjugated mouse anti-human TLR7 antibodies were obtained from Santa Cruz (Delaware Ave Santa Cruz, CA, USA). Human Fc receptor blocking solution, red blood cell lysis buffer, Zombie Aqua™ fixable viability kit, and all isotype antibodies, Cytofix/Cytoperm™ Fixation/Permeabilization kits were obtained from BD Biosciences Pharmingen (Bedford, MA, USA). Fetal bovine serum (FBS, HyClone) and RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, USA). DSR6434 was purchased from R&D Systems (Minneapolis, MN). Ovalbumin (OVA, grade V), DNase I and Trypan blue dye were purchased from Sigma-Aldrich (St Louis, MO, USA). Human IL-33, and human IL-37 were obtained from Peprotech (London, UK). Human IL-4, human IL-6, and IL-13 ELISA kits were bought form Cayman Chemical (Ann arbor MI, USA). Oligonucleotide primers for real time quantitative PCR (qPCR) were synthesized by Invitrogen Biotechnology Co. (Shanghai, China). Trizol reagent was obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from PANTM Seratech (Aidenbach, Germany). cDNA was synthesized using iScript™ cDNA Synthesis kit. The resultant cDNA was subjected to qPCR that was performed with a LightCycler using a SuperScript III Platinum SYBR Green two-step qPCR kit. RNA was extracted by using a TaKaRa Mini BEST Universal RNA Extraction kit. cDNA was synthesized by using a PrimeScript RT reagent kit. Quantification of mRNA expression level was performed by applying TAKARA SYBR Premix EX Tag kit. *Artemisia sieversiana wild* allergen extract (ASWE) and *Platanus* pollen allergen extract (PPAE) were purchased from Macro Union Pharmaceutical Co. Ltd. (Beijing, China). The recombinant full-length protein of *Dermatophagoides pteronyssinus* allergen 1 (Der p 1) expressed in *E. coli* was obtained from Beijing Protein Innovation Co., Ltd (Beijing, China). *House Dust Mite extract* (HDME) I was supplied by ALKAbellÓ, Inc. (Denmark). Most of the general chemicals, such as salts and buffer components were of analytical grade.

Patients and Samples

A total of 41 patients with AR and 22 healthy control (HC) subjects were recruited in the study (registration number: ChiCTR-BOC-16010279). Sample were obtained at the First Affiliated Hospital of Jinzhou Medical University, China. The informed consent from each volunteer according to the declaration of Helsinki and agreement with the ethical committee of the First Affiliated Hospital of Jinzhou Medical University was obtained. Their general characteristics were summarized in Table 1. The diagnosing criteria for AR in this experiment are in line with the 2015 AR Clinical Practice Guidelines issued by the American Academy of Otorhinolaryngology Head and Neck Surgery[18] and Clinical practice guideline: Allergic rhinitis.[19] Skin prick test was performed by using HDME I, ASWE

Table 1. Characteristics of Subjects.

Population	Case	Age(y)	Female/ Male	History
Healthy control	22	28 (21–35)	4/18	0
Allergy rhinitis	41	36 (17–64)	20/21	3.1 (1–8)
Artemisia (+)	24	42 (17–64)	14/10	2.5 (1–8)
House dust mite (+)	21	41 (16–63)	6/15	3 (1–8)
Platanu (+)	7	37 (17–64)	5/2	4 (2–8)

and PPAE according to the manufacturer's instruction. After skin prick test, the blood from each patient with AR and HC subject was collected in the outpatient clinic. From each individual, 10 ml of peripheral blood was taken into an EDTA-containing tube before centrifugation at 450 g for 10 min. The cells were used for flow cytometric analysis, and plasma was collected and frozen at −80°C until use.

Flow Cytometry Analysis

To detect expression of TLR7 in human blood basophil cells, blood cells were challenged with or without ASWE, HDME[20,21] or PPAE (all at a concentration of 1.0 μg/ml) for 60 min at 37°C, respectively, and 2 μg/ml brefeldin A was also added into the tube. Cells were then incubated with human Fc receptor blocking solution and a live/dead cell dye (Zombie Green™ Fixable Viability kit for 15 min, and each labelled monoclonal antibody including APC/Cy7-conjugated mouse anti-human CCR3, PE/Cy7-conjugated mouse anti-human CD123, PerCP/Cy5.5-conjugated mouse anti-human HLA-DR was added into the tube. After red blood cells being lysed, resuspended leucocytes were fixed and permeabilized using Cytofix/Cytoperm™ Fixation/Permeabilization kit according to the manufacturer's instructions. This was followed by adding a PE-conjugated anti-human TLR7 antibody into the tube and incubated at 4°C for 30 min.

Finally, cells were resuspended in fluorescence activated cell sorting (FACS)-flow solution and analysed with FACS Verse flow cytometer (BD Biosciences, San Jose, CA). A total of 10,000 events in live cell gate were analysed for each sample. Data were analysed with FlowJo software version 7.0 (Treestar, Ashland, OR, USA). Dead cells and doublets were excluded from analysis by live/dead cell dyes.

Cell Line and Culture

A human basophil cell line, KU812 (ATCC® CRL2099™), was purchased from American Type Culture Collection (ATCC). Cells were cultured in an RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 100 units·ml^{-1} penicillin/streptomycin in 75 cm^2 tissue culture flasks (Falcon) at 37°C in a 5% (v/v) CO_2, water-saturated atmosphere. The procedure for challenging KU812 cells was mainly adopted from a method previously described for P815 cells.[22] Briefly, cultured KU812 cells at a density of 1×10^6 cells·ml^{-1} were incubated with various concentrations of Der p1 (0.03, 0.3, 1.0 and 3.0 μg/ml), OVA (0.03, 0.3, 1.0 and 3.0 μg/ml), DSR6434 (1, 10 and 100 nM), human IL-31 (3 ng/ml), IL-33 (3 ng/ml), IL-37 (3 ng/ml), TSLP (3 ng/ml), plasma from HCs (10%), HDME (3 μg/ml), plasma from perennial AR allergic to dust mite alone (10%) with or without HDME (3 μg/ml), plasma from seasonal AR allergic to *artemisia* alone (10%) with or without ASWE (3 μg/ml), and plasma from seasonal AR allergic to *platanus* pollen alone (10%) with or without PPAE (3 μg/ml) for 30 min, 2 h and 16 h at 37°C, respectively in wells of a 96-well culture plate before being centrifuged at 450 g for 10 min at 4°C. Cell pellets containing approximately 1×10^5 cells were resuspended in PBS for flowcytometry analysis, and 0.9×10^6 cells in trizol for qPCR, and supernatant was collected and frozen at −80°C until use.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of IL-4, IL-6 and IL-13 in KU812 cell culture supernatant were determined by commercially available ELISA kits according to the manufacturer instruction.

RNA Isolation and qPCR Analysis

Total RNA was extracted from collected cells using a TaKaRa Mini BEST Universal RNA Extraction Kit. cDNA was synthesized by using a PrimeScript RT reagent Kit. The resultant cDNA was subjected to qPCR that was performed with a LightCycler System using a SYBR Premix Ex Taq Kit. The amplified product was detected by the presence of an SYBR green fluorescent signal. Each reaction contains 10 μl of 2x SYBR green Master Mix, 300 nM oligonucleotide primers, and 10 μl of the cDNA or plasmid DNA. β-actin cDNA was used as the internal control and the ΔCt for all experimental samples were subtracted by the ΔCt for the control samples (ΔΔCt). The magnitude change of test gene mRNA was expressed as $2^{-\Delta\Delta Ct}$. The primers of TLR7 were forward: cct tga ggc caa caa cat ct and reverse: gta ggg acg gct gtg aca tt.

Statistical Analysis

Statistical analyses were performed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Human peripheral blood basophil data are displayed as a Scatter plot. Where Kruskal-Wallis analysis indicated significant differences between groups, for the pre-planned comparisons of interest, the paired Mann-Whitney U

test was employed. KU812 cell data were expressed as mean ± SEM, and were evaluated by using a Student's *t*-test. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Expression of TLR7 in Peripheral Blood Basophils of Patients With AR

It was reported that intranasal application of TLR7 agonist GSK2245035 suppresses AR symptoms.[6] However, little is known about the expression level of TLR7 on basophils (one of the primary effector cells of allergy) in AR. We therefore examined expression levels of TLR7 in granulocyte populations and mononuclear cell populations of AR, respectively. The results showed that percentages of $TLR7^{+}CCR3^{+}$ cells in blood granulocyte (Figures 1(Ba) and 2(A)) and mononucleated cell (Figures 1(Ca) and 2(B)) populations of the patients with AR were increased by 15.3 (29.5 vs 1.92%, $P < 0.001$) and 5.3-fold (36.9 vs 6.97%, $P = 0.011$), respectively, compared with HC subjects. Percentages of $TLR7^{+}CD123^{+}HLA\text{-}DR^{-}$ cells in blood granulocyte (Figures 1(Bb) and 2(C)) and mononucleated cell (Figures 1(Cb) and 2(D)) populations of the patients

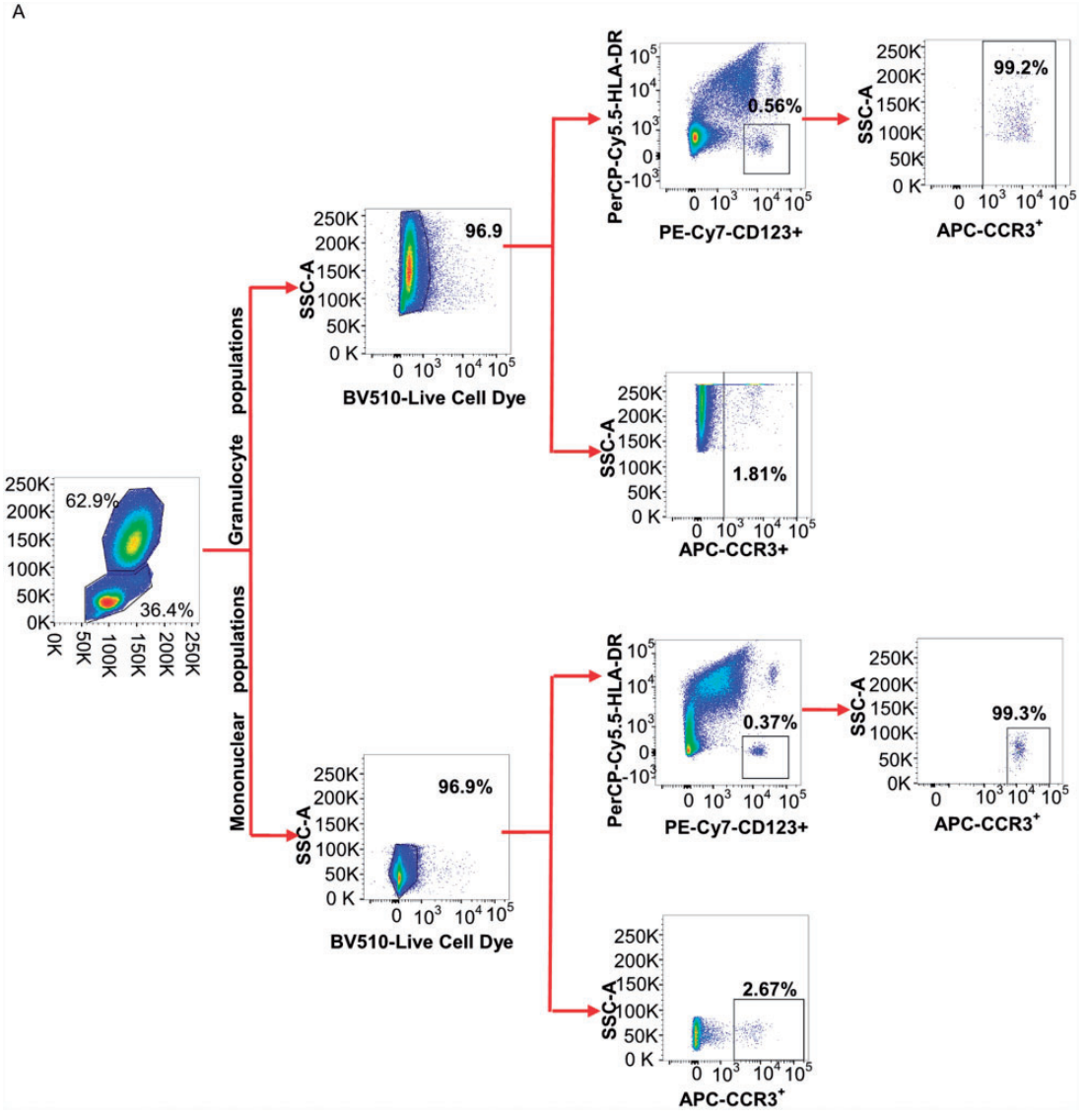
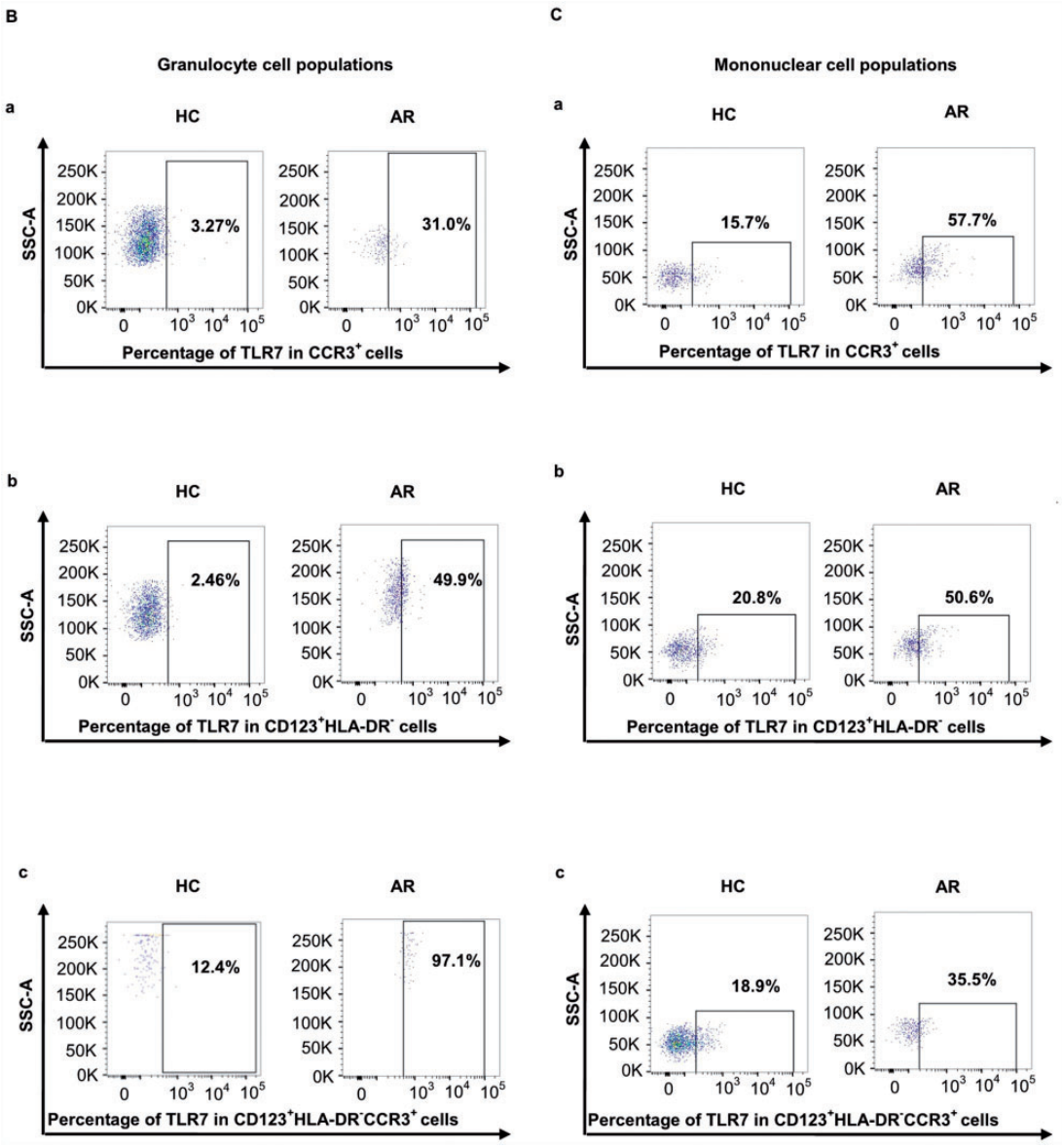


Figure 1. Flow cytometry analysis of the expression of TLR7 in peripheral blood basophils from allergic rhinitis (AR) and healthy control (HC) subjects. A, Representative graphs of the gating strategies of basophils. B, C, Representative graphs of the gating strategies of $TLR7^{+}$ basophils in mononuclear cell populations and granulocyte populations, respectively. D, Representative flow cytometric figures of mean fluorescent intensity (MFI) of TLR7 on $CCR3^{+}$, $CD123^{+}HLA\text{-}DR^{-}$ and $CD123^{+}HLA\text{-}DR^{-}CCR3^{+}$ cells of granulocyte populations. E, Representative flow cytometric figures of MFI of TLR7 in the mononuclear cell populations.



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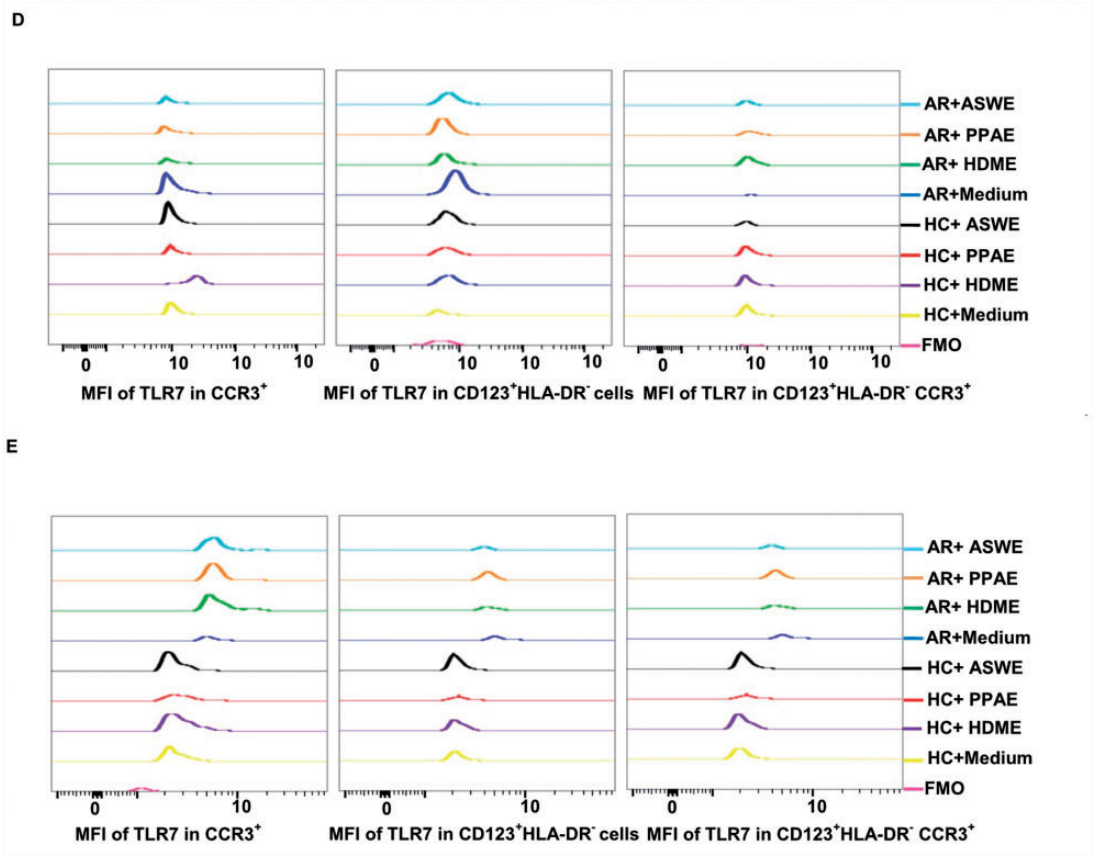
with AR were increased by 6.9 (43.1 vs 6.21%, $P = 0.016$) and 2.2-fold (47.8 vs 21.5%, $P = 0.042$) compared with HC subjects. Similarly, proportion of $TLR7^{+}CCR3^{+}CD123^{+}HLA\text{-}DR^{-}$ cells in blood granulocyte (Figures 1(Bc) and 2(E)), and mononucleated cell (Figures 1(Cc) and 2(F)) populations of the patients with AR were increased by 4.9 (41.1 vs 8.32%, $P = 0.046$) and 1.8-fold (43.4 vs 23.7%, $P = 0.035$) compared with HC subjects.

Allergen (ASWE, HDME or PPAE at the concentration of 1.0 μg/ml) stimulation did enhance TLR7 expression in HC, while no effect was noticed on AR patients, nevertheless, the allergen enhanced levels in HC are still well below the values in AR patients. This stimulation effect in HC was not noticed in $CD123^{+}HLA\text{-}DR^{-}$ cells and in $CCR3^{+}CD123^{+}HLA\text{-}DR^{-}$ cells, then the effect is limited only to $CCR3^{+}$ cells (Figure 2(A) to (E)).

In terms of MFI, TLR7 MFI on $CCR3^{+}$ cells were enhanced by 1.3 (2787 vs 2186, $P = 0.05$) and 1.4-fold (819 vs 604, $P = 0.043$) in blood granulocyte (Figures 1 (D) and 2(G)) and mononucleated cell (Figures 1(E) and 2(H)) populations of the patients with AR, respectively, compared with HC subjects. Similarly, TLR7 MFI on $CD123^{+}HLA\text{-}DR^{-}$ cells were elevated by 2.4 (969 vs 408, $P < 0.001$) and 1.7-fold (691 vs 395, $P = 0.002$) in blood granulocyte (Figures 1(D) and 2(I)) and mononucleated cell (Figures 1(E) and 2(J)) populations, and on $CCR3^{+}CD123^{+}HLA\text{-}DR^{-}$ cells were increased by 2.1 (1001 vs 479, $P < 0.001$) and 1.8 (721 vs 408, $P = 0.003$) -fold in blood granulocyte (Figures 1(D)



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and 2(K)) and mononucleated cell (Figures 1(E) and 2 (L)) populations of the patients with AR, respectively, compared with HC subjects. Allergens ASWE, HDME or PPAE at the concentration of 1.0 µg/ml had little effect on MFI of TLR7 on $CCR3^+$ cells (Figures 1(D) and (E) and 2(G) and (H)), $CD123^+HLA\text{-}DR^-$ cells (Figures 1(D) and € and 2(I) and (J)) and $CCR3^+$ $CD123^+HLA\text{-}DR^-$ cells (Figures 1(D) and (E) and 2 (K) and (L)) of HC subjects and the patients with AR.

Flow Cytometry Analysis of the Expression of TLR7 in KU812 Cells

In order to understand influence of allergens on TLR7 expression in basophils, we investigated actions of Der p1, OVA, HDME and ASWE in KU812 cells. The results showed that only less than 3% of KU812 cells expressed TLR7 (Figure 3), and OVA at 1.0 µg/ml as well as Der p1 at 0.3 µg/ml induced upregulation of TLR7 expression in KU812 cells at 16 h following incubation (Figure 3(C)). Cytokines IL-33 at 3 ng/ml and IL-37 at 3 ng/ml, but not IL-31 and TSLP also provoked up to 2.02 and 2.01-fold increases in TLR7 expression in KU812 cells at 2 h and 16 h following incubation. Unexpectedly, DSR6434, a potent agonist of TLR7[23] inhibited expression of TLR7 in KU812 cells at 2 h and 16 h following incubation (Figure 3(A) and (B)). IL-31, IL-33, IL-37, TSLP, OVA, HDME, ASWE, plasma from HC, plasma from seasonal AR allergic to *Artemisia* alone with or without ASWE (3 µg/ml), plasma from seasonal AR allergic to *Platanus* pollen alone (10%) with or without PPAE, plasma from perennial AR allergic to dust mite alone (10%) with or without HDME (3 µg/ml) at the concentrations tested failed to alter expression of TLR7 in KU812 cells following a 30 min incubation period (data not shown).

qPCR Analysis of TLR7 mRNA Expression in KU812 Cells

To confirm effect of allergens on TLR7 expression in KU812 cells, the expression of TLR7 mRNA in KU812 cells was examined. It was found that OVA at 3.0 µg/ml and Der p1 at 1.0 µg/ml induced up to 2.1 and 2.9-fold increases in the expression of TLR7 mRNA over baseline control, respectively at 30 min following incubation (Figure 4). DSR6434 failed to induce significant TLR7 mRNA expression in KU812 cells (Figure 4). Apart from IL-37 (3 ng/ml) at 16 h following incubation, IL-31, IL-33 and TSLP all at 3 ng/ml,

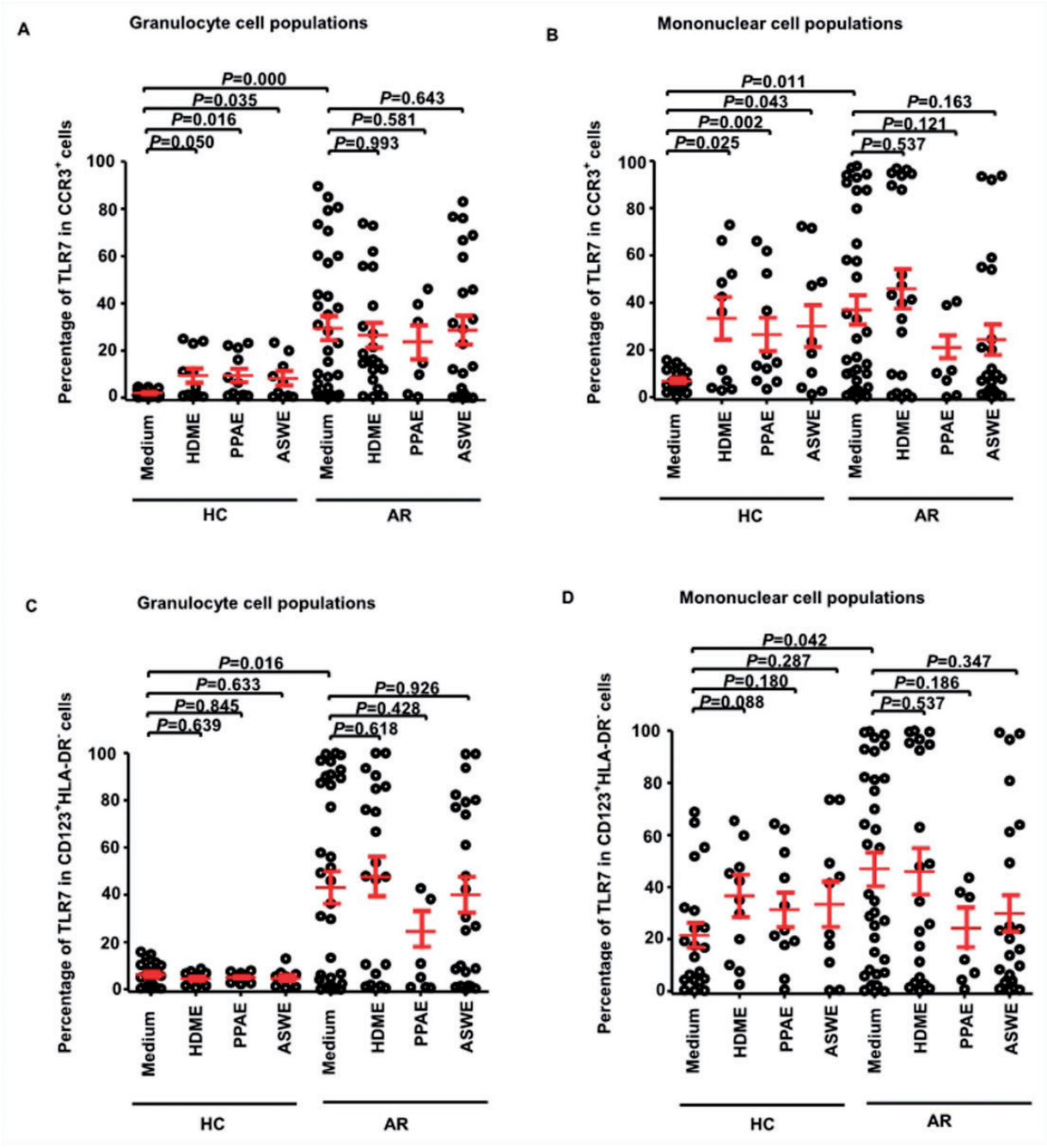
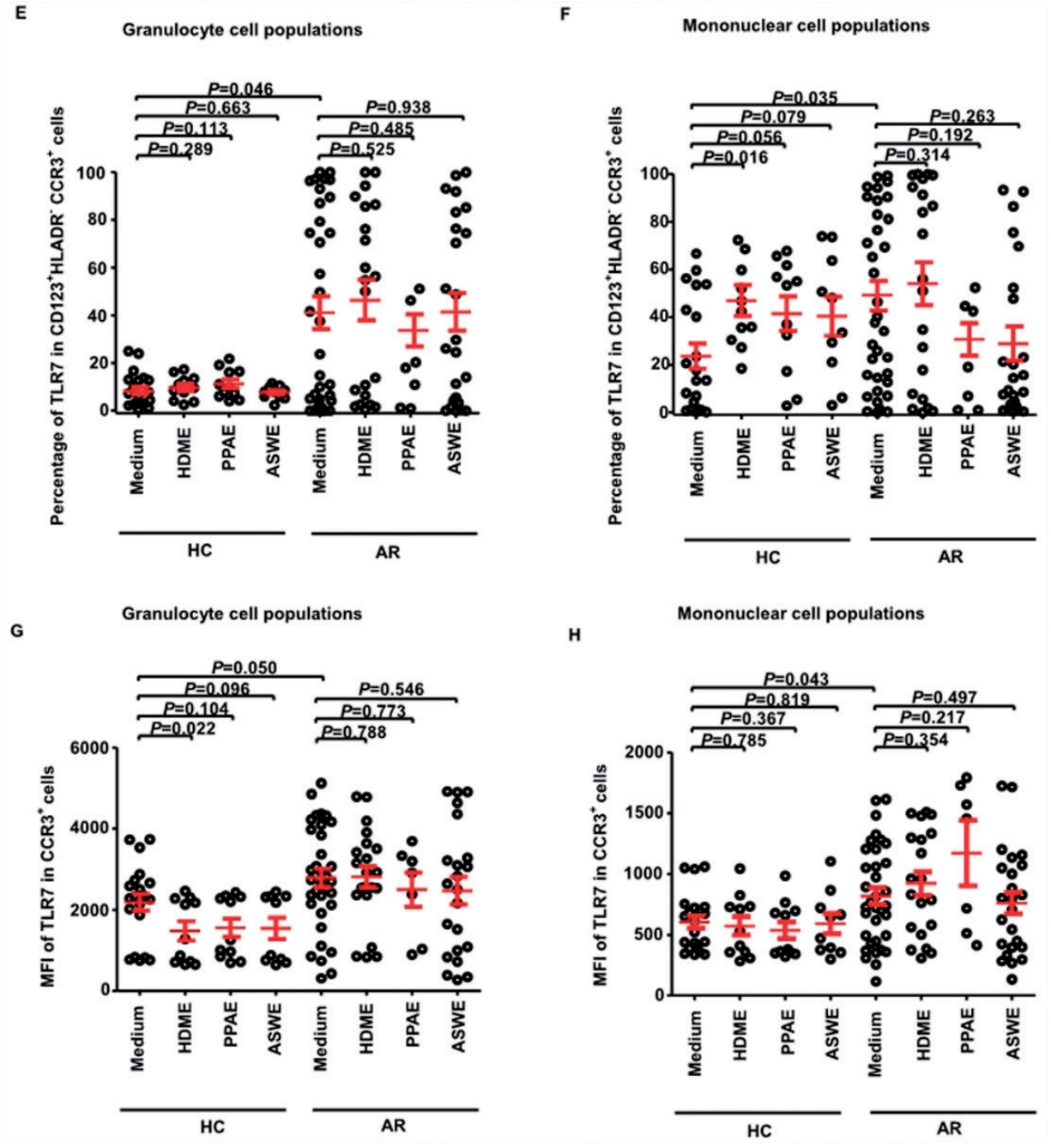


Figure 2. Flow cytometry analysis of expression of TLR7 in the subpopulations of peripheral blood leukocytes of patients with allergic rhinitis (AR) and healthy control (HC) subjects after allergen challenge. (A), (C) and (E) represent the percentages of $TLR7^+$ cells in $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CD123^+HLA\text{-}DR^-CCR3^+$ cell populations, respectively of granulocytes in AR and HC subjects. (B), (D) and (F) represent the percentage of $TLR7^+$ cells in $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CD123^+HLA\text{-}DR^-CCR3^+$ cell populations, respectively of mononuclear cells in AR and HC subjects. (G), (I) and (K) represent the MFI of TLR7 in $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CD123^+HLA\text{-}DR^-CCR3^+$ cells of granulocytes in AR and HC subjects, respectively. (H), (J) and (L) represent the MFI of TLR7 in $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CD123^+HLA\text{-}DR^-CCR3^+$ cells of mononuclear cell population of AR and HC subjects, respectively. Each symbol represents the value from one subject. The median value is indicated by a horizontal line. $P < 0.05$ was taken as statistically significant. *House Dust Mite extract* (HDME, 1 μg/ml), *Artemisia sieversiana wild* allergen extract (ASWAE, 1 μg/ml) or *Platanus* pollen allergen extract (PPAE, 1 μg/ml).



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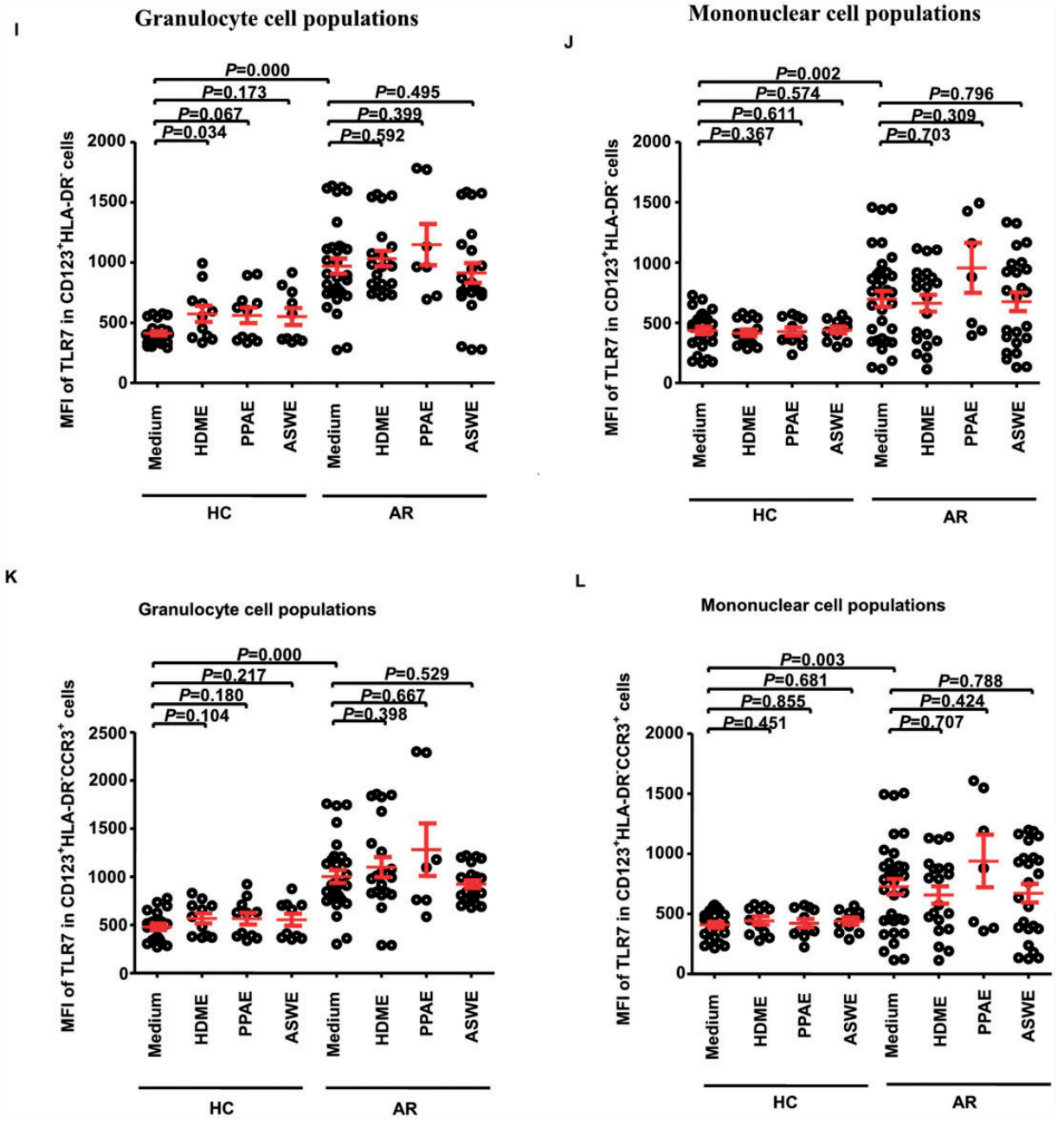
allergens HDME and ASWE both at 3 μg/ml did not induce significant increases in expression of TLR7 mRNA in KU812 cells following 30 min, 2 h and 16 h incubation periods (Figure 4).

Levels of IL-4, IL-6 and IL-13 in the Culture Supernatant of KU812 Cells

In order to examine the actions of allergens, cytokines and TLR7 agonist in induction of cytokine release from KU812 cells, IL-4, IL-6 and IL-13 release from KU812 cells was investigated. The results showed that HDME and ASWE both at 3 μg/ml and plasma from HC provoked 2.13, 1.67 and 1.72-fold elevations of IL-6 release from KU812 cells following 2 h (Figure 5(B)), but not 30 min (Figure 5(A)) and 16 h (data not shown) incubation periods. Similarly, IL-31, IL-33, IL-37, TSLP all at 3 ng/ml stimulated 1.62, 1.94, 1.71 and 1.51-fold increases in IL-6 release from KU812 cells following 2 h (Figure 5(B)). OVA, Der p1 and DSR6434 at the



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concentrations tested had little effect on IL-6 release from KU812 cells following 30 min (Figure 5(A)), 2 h (Figure 5(B)) and 16 h (data not shown) incubation periods.

At 2 h following incubation, OVA at 0.3 µg/ml and Der p1 at 1.0 µg/ml provoked 1.48 and 1.47-fold increases in IL-13 release (Figure 5(D)). However, at 30 min following incubation, only OVA at 0.3 µg/ml, but not Der p1, stimulates 1.88-fold IL-13 release from KU812 cells (Figure 5(C)). HDME and ASWE, plasma from HC, IL-31, IL-33, IL-37, TSLP and DSR6434 at the concentrations tested had little effect on IL-13 release from KU812 cells following 30 min (Figure 5 (C)), 2 h (Figure 5(D)) and 16 h (data not shown) incubation periods.

Plasma from perennial AR allergic to dust mite alone (10%) with or without HDME (3 µg/ml), plasma from seasonal AR allergic to artemisia alone (10%) with or without ASWE (3 µg/ml), plasma from seasonal AR allergic to *Platanus* pollen alone (10%) with or without PPAE (3 µg/ml) failed to alter IL-6 and IL-13 release from KU812 cells following 30 min, 2 h and 16 h

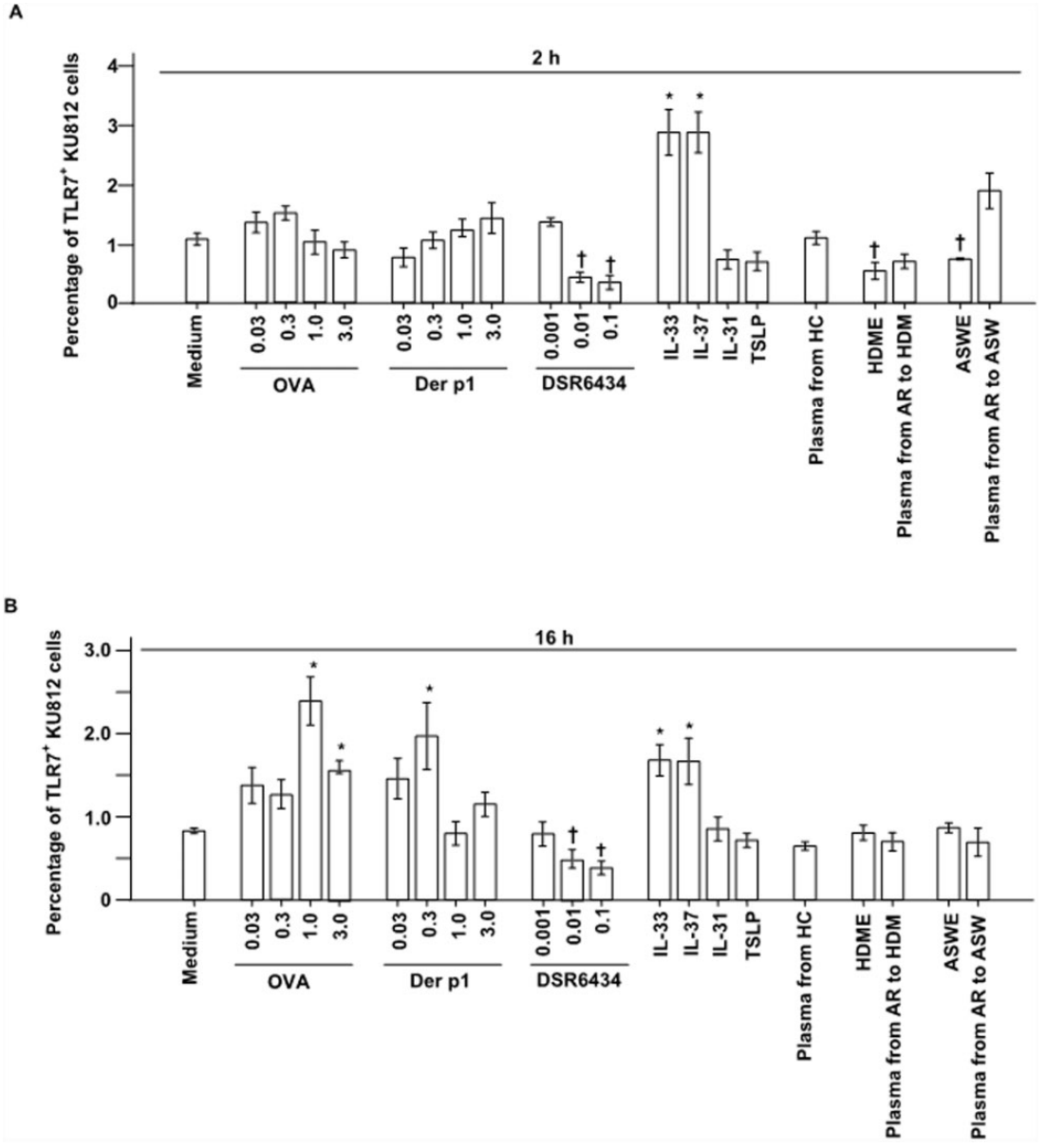

Figure 3. Flow cytometry analysis of expression of TLR7 in KU812 cells. Cells were challenged by various concentrations of OVA (0.03, 0.3, 1.0 and 3.0 μg/ml), Der p1 (0.03, 0.3, 1.0 and 3.0 μg/ml), DSR6434 (0.001, 0.01, 0.1 μg/ml), IL-31 at 3 ng/ml, IL-33 at 3 ng/ml, IL-37 at 3 ng/ml, TSLP at 3 ng/ml, plasma from healthy control (HC, 10%) subjects, *House Dust Mite extract* (HDME, 3 μg/ml), plasma from allergic rhinitis (AR) sensitive to mite alone (10%), *Artemisia sieversiana wild* allergen extract (ASWAE, 3 μg/ml), plasma from seasonal AR sensitive to *artemisia* alone (10%) at 37°C for 2 h (A) and 16 h (B) before cells being collected. The expression of TLR7 was analyzed by flow cytometry analysis. The data were expressed as mean ± SE for five separate experiments performed in duplicate. $P < 0.05$ compared with the response to corresponding medium alone control.

incubation periods (data not shown). IL-4 is not detectable in the culture supernatant of KU812 cells (assay sensitivity: 2 pg/ml).

Discussion

It is found for the first time that the percentages of $TLR7^+$ cells are increased in blood $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CCR3^+CD123^+HLA\text{-}DR^-$ granulocyte and mononucleated cell populations of the patients with AR, indicating that basophils likely contribute to the pathogenesis of AR via TLR7. However, CCR3 is not a specific marker of basophils in granulocyte population, and a large proportion of eosinophils also express CCR3,[24] suggesting that eosinophils may also be involved in AR through TLR7. This

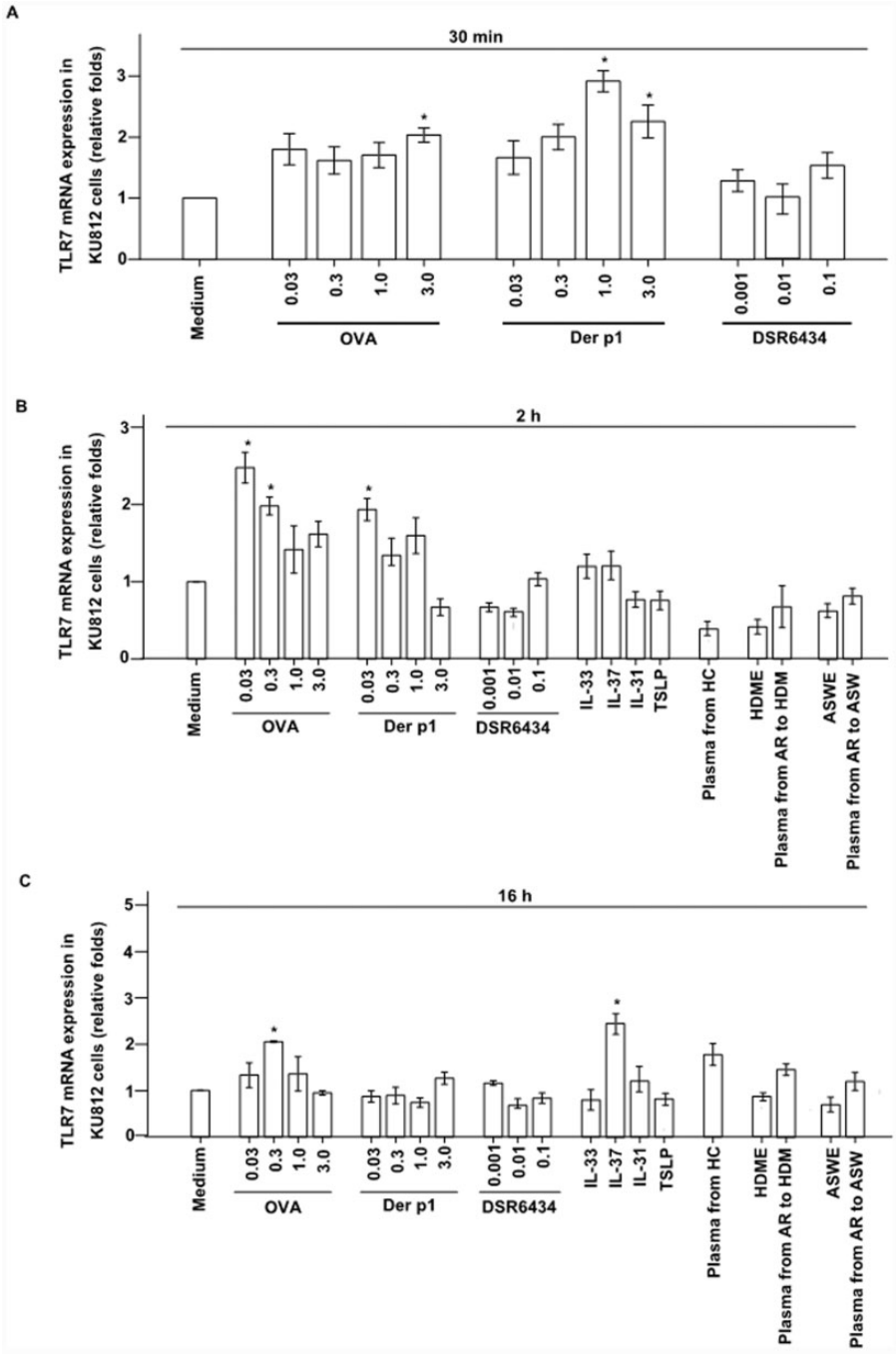

Figure 4. Quantitative real-time PCR (qPCR) analysis of expression of TLR7 mRNA in KU812 cells. Cells were challenged by various concentrations of OVA (0.03, 0.3, 1.0 and 3.0 μg/ml), Der p1 (0.03, 0.3, 1.0 and 3.0 μg/ml), DSR6434 (0.001, 0.01, 0.1 μg/ml), IL-31 at 3 ng/ml, IL-33 at 3 ng/ml, IL-37 at 3 ng/ml, TSLP at 3 ng/ml, plasma from healthy control (HC, 10%) subjects, *House Dust Mite extract* (HDME, 3 μg/ml), plasma from allergic rhinitis (AR) sensitive to mite alone (10%), *Artemisia sieversiana wild* allergen extract (ASWAE, 3 μg/ml), plasma from seasonal AR sensitive to *artemisia* alone (10%) at 37°C for 30 min (A), 2 h (B) and 16 h (C) before cells being collected. The expression of TLR7 mRNAs was analyzed by qPCR. The data were expressed as mean ± SE for five separate experiments performed in duplicate. $P < 0.05$ compared with the response to corresponding medium alone control.

may explain the observation that percentage of $TLR7^+CCR3^+$ cells in blood granulocyte increased by 15.4-fold, whereas $TLR7^+CD123^+HLA\text{-}DR^-$ and $TLR7^+CCR3^+CD123^+HLA\text{-}DR^-$ cells enhanced only 6.9 and 4.9-fold, respectively in the patients with AR. In mononucleated cell population, apart from basophils, mononuclear phagocytes[25] and CD34+ hematopoietic progenitor cells[26] express CCR3, which may account

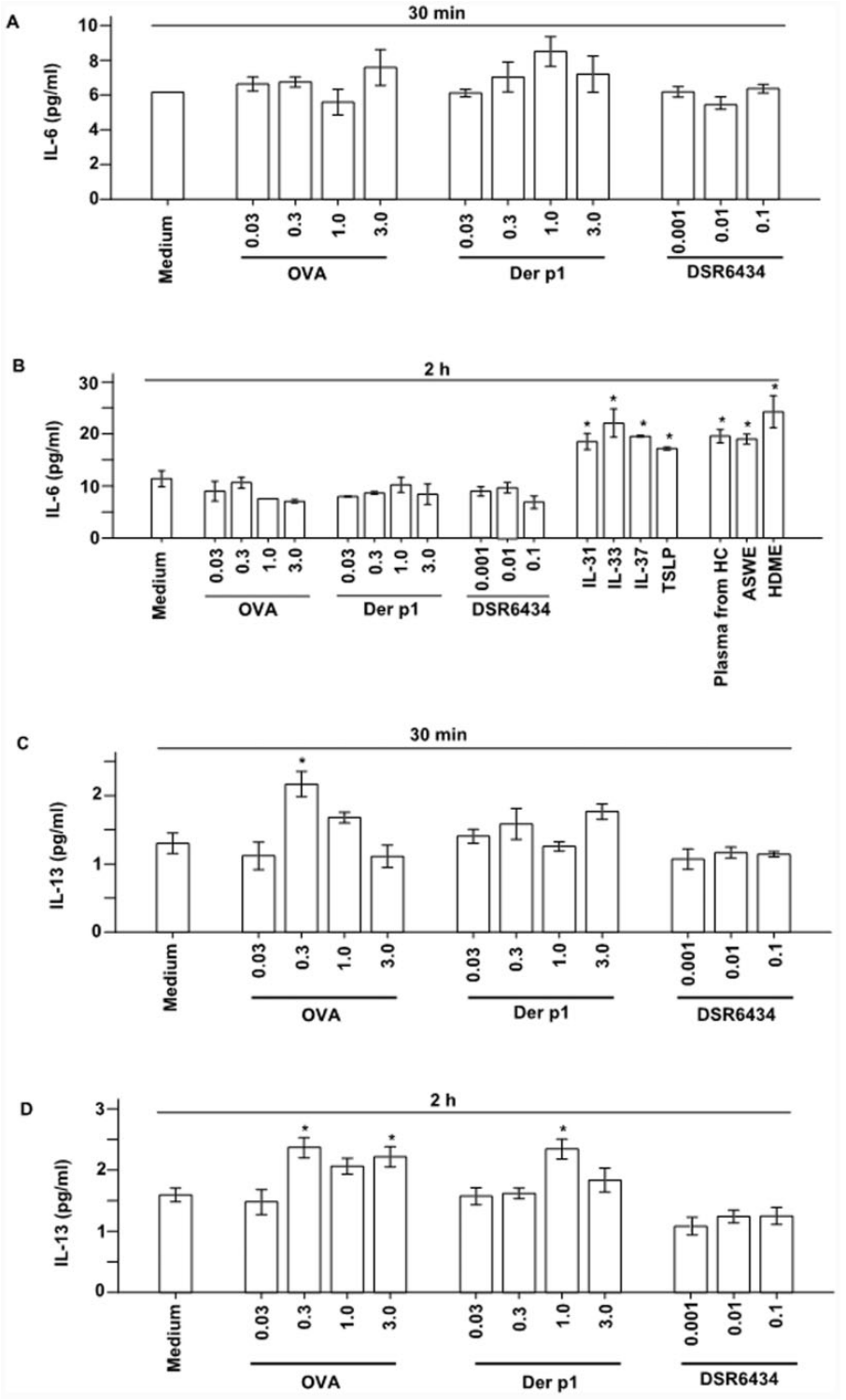

Figure 5. IL-6 and IL-13 release from KU812 cells. KU812 cells were incubated with various concentrations of OVA (0.03, 0.3, 1.0 and 3.0 μg/ml), Der p1 (0.03, 0.3, 1.0 and 3.0 μg/ml), DSR6434 (0.001, 0.01, 0.1 μg/ml), IL-31 at 3 ng/ml, IL-33 at 3 ng/ml, IL-37 at 3 ng/ml, TSLP at 3 ng/ml, plasma from healthy control (HC, 10%) subjects, *House Dust Mite extract* (HDME, 3 μg/ml), *Artemisia sieversiana wild* allergen extract (ASWAE, 3 μg/ml) at 37°C for 30 min (A), 2 h (B) before culture supernatant being collected and IL-6 was measured. (C) and (D) demonstrate levels of IL-13 at 30 min and 2 h following incubation, respectively.

for the finding that percentage of $TLR7^+CCR3^+$ cells in blood mononuclear cells increased more than percentage of $TLR7^+CD123^+HLA\text{-}DR^-$ and $TLR7^+CCR3^+$ $CD123^+HLA\text{-}DR^-$ cells in the patients with AR. Similar degree of increases of $TLR7^+CD123^+HLA\text{-}DR^-$ and $TLR7^+CCR3^+CD123^+HLA\text{-}DR^-$ cells in mononucleated cell population of the patients with AR implicate that basophils in mononucleated cell population may also contribute to AR through TLR7. Moreover, the fact that TLR7 expression intensity of a single $CCR3^+$, $CD123^+HLA\text{-}DR^-$ and $CCR3^+$ $CD123^+HLA\text{-}DR^-$ granulocyte or mononucleated cell increased in blood of the patients with AR also supports the view that basophils likely contribute to AR through TLR7. Since TLR7 is a therapeutic candidate target for allergic disorders, administration of TLR7 inhibitor AZD8848 can effectively improve the respiratory symptoms in patients with allergic asthma,[27] our study provides important targeting cell basophil that TLR7 antagonists may act upon. Since basophils are well known primary effector cells in allergy,[8] the current study may aid understand of the mechanism through which basophils are involved in the pathogenesis of AR.

It is not surprising that allergen extracts ASWE, HDME or PPAE have shown limited effects on TLR7 expression in basophils as these allergens normally uneasy to enter in blood and act directly on basophils. The report that HDM major allergens can activate TLRs on airway epithelium cells and upregulate proinflammatory cytokine IL-6 and IL-8 expression[28] may support our idea to look into effects of allergen extracts on TLR7 expression in basophils. Allergen stimulation did enhance percentage of $TLR7^+$ $CCR3^+$ cells in HC but did not show any significant effect on TLR7 MFI on $CCR3^+$ cells. This implicates that these allergens can increase proportion of TLR7 expression $CCR3^+$ cells in blood, but cannot enhance the density of TLR7 expression on a single $CCR3^+$ cell.

It is rather difficult to obtain large amount of highly purified inactive basophils, therefore a human basophil cell line, KU812 cells were employed to investigate the direct effect of allergens on basophils in the present study. Since KU812 cells have been shown to be a suitable model for studying the activation and degranulation of human basophils,[29] we thought that these cells may be useful for study TLR7 expression and basophil activation. However, it was found that only up to 3% of KU812 cells expressing TLR7. Nevertheless, allergens Der p1 and OVA induced upregulation of TLR7 expression in KU812 cells at both protein and mRNA levels. The observation that TLR7 mRNA expression was enhanced by OVA at 2 h and Der p 1 at 30 min following incubation, and upregulated TLR7 protein expression provoked by them occurred at 16 h suggests that the transcription of OVA- and Der p1-induced TLR7 production is a relatively slow process in KU812 cells. As for TLR7 expression in primary blood basophils, KU812 cells showed little response to allergen HDME and ASWE challenge following up to 16 h incubation. These results may suggest that only certain types of allergens can alter TLR7 expression in basophils without relying on antigen-presenting cells.

Reduction of expression of TLR7 in KU812 cells by DSR6434 a potent agonist of TLR7[23] is an unexpected result. Nevertheless, a report that treatment of bone marrow-derived plasmacytoid dendritic cells with DSR6434 led to downregulation of TLR7 expression[30] does suggest that DSR6434 can eliminate TLR7 expression on cells. Unexpectedly, IL-33 and IL-37 induced significant upregulation of TLR7 protein expression, but not significant TLR7 mRNA expression. It could result from an efficient transcription process of TLR7 protein in KU812 cells provoked by these two cytokines. Regulation of TLR7 expression by cytokines has been observed previously,[31,32] which may support our current observation.

Induction of IL-6, but not IL-13 release from KU812 cells by HDME and ASWE, provocation of IL-13, but not IL-6 release from KU812 cells by OVA and Der p1 are interesting results. Since IL-6 release and IL-13 secretion by KU812 cells are very likely through different signal transduction pathways,[31,32] our results way suggest that certain allergen may selectively alter cytokine release from KU812 cells via different cellular mechanisms. Similarly, it was observed that IL-31, IL-33, IL-37, TSLP stimulated IL-6 release, but not IL-13 release from KU812 cells, which implicates that these cytokines are able to selectively activate IL-6 release signaling pathway. Since IL-13 is a classical Th2 cytokine,[33] and allergens can induce IL-6 and IL-13 release from KU812 cells, it is possible that allergens may contribute to allergy via upregulation of expression of TLR7 and enhancement of IL-6 or IL-13 production in basophils. The findings that birch pollen allergen stimulated PBMC cultures from asthmatic patients produced elevated levels of IL-5 and IL-13,[34] *Dermatophagoides farinae* or house dust stimulated increase in IL-13 production from PBMC cultures from patients with atopic dermatitis,[35] and Der p extracts, TNF-α, IL-4, or IL-13 enhanced GM-CSF and IL-8 release from airway epithelial cultures[36] may emphasize the effect of allergens on the release of Th2 cytokines.

Conclusion

In conclusion, the percentages of $TLR7^+$ basophils and TLR7 expression intensity in a single basophil are both increased in blood of the patients with AR, indicating that basophils likely contribute to the pathogenesis of AR via TLR7. The enhanced expression of TLR7 on KU812 cells, induced by allergens Der p1 and OVA at

both protein and mRNA levels suggested that allergens may be capable of upregulating TLR7 expression on basophils.

Abbreviations

AR: allergic rhinitis. TLR7: Toll like receptor 7. ASWE: *Artemisia sieversiana* willd allergen extract. HDME: House dust mite extract. PPAE: *Platanus* pollen allergen extract. KU812: Human peripheral blood basophilic leukemia cells. MFI: Mean Fluorescence Intensity. ELISA: enzyme-linked immunosorbent assay. TSLP: thymic stromal lymphopoietin. qPCR: real time quantitative Polymerase Chain Reaction. DSR6434: a TLR7 agonist. CCR3: C-C motif chemokine receptor 3. TNFα: Tumor Necrosis Factor alpha. INF-γ: Interferon gamma. CD123: Cluster of differentiation 123. HLA-DR: Human Leukocyte Antigen-DR. IL: Interleukin. OVA: Ovalbumin. Der p1: Dermatophagoides Pteronyssinus 1.

Author Contributions

L. W. carried out most experiments, generated the majority of the data, and wrote large part of the first draft of the manuscript. M. Z. and J. W. took part in flow cytometry, cell culture and challenge test and wrote a part of the first draft of the manuscript. D. C. carried out the clinical study and participated in data analysis. N. Z. and L. W. took part in ELISA and qPCR and wrote a part of the first draft of the manuscript. W. W., X. Z. and Y. H. participated in animal experiment and challenge test. H. Z. and S. H. designed and conducted the study, analyzed the data, and wrote the second and final drafts of the paper. All authors read and approved the final paper.

Declaration of Conflicting Interests

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ORCID iD

Shaoheng He https://orcid.org/0000-0002-4230-7639

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